Bacilli in Climate Resilient Agriculture and Bioprospecting

M. Tofazzal Islam
Mahfuz Rahman
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Bacilli in Agrobiotechnology

Plant Stress Tolerance, Bioremediation, and Bioprospecting

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Microorganisms play an important role due to their presence all over the universe, and regulating biogeochemical systems in virtually all of our planet's environments. Among all microbes especially in prokaryotic genera, *Bacillus* spp. represents one of the most important unmapped pools of biodiversity with immense potential of applications in agriculture, industry and medicine. These Gram-positive spore forming bacteria are some of the most dominant groups that exist in various ecological niches on the earth due to their survivability in adverse environmental conditions by producing endospores. Recognizing the enormous potential of the bacteria in this genus, scientists all over the world have directed significant research towards selection and commercialization of the best organisms that may provide protection of plants from harmful microbes and/or enhance plant growth, produce industrially important enzymes, antibiotics, probiotics and other biochemicals. Innovative approaches are also being explored utilizing *Bacillus* mediated bioremediation of environmental pollutants such as pesticides, explosive wastes, dyes and polycyclic aromatic hydrocarbons. Discovery of insecticidal toxin from *Bacillus thuringiensis* (Bt) revolutionized insect pest management in many economically important crops by developing resistant crop varieties. Agricultural biotechnology that involves a wide range of insecticidal toxin producing genes from Bt has dominated the pest management research for last few decades. The emergence of resistant insect pests to Bt-based bioinsecticides and Bt-crops has created new challenges invoking more research on stacking resistant genes and further modifications of Bt toxin chemistries. While Bt transgenic crops are being released, questions on ecological aspects are getting louder provoking more research in this area. Genetics and genomics research on allied species of *Bacillus* has grown at a bewildering pace in the last decade.

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Chapter 1
Heavy Metal Removal by *Bacillus* for Sustainable Agriculture

Sougata Ghosh, Joorie Bhattacharya, Rahul Nitnavare, and Thomas J. Webster

Abstract Microbial biosorbents are widely used for the removal of various toxic metals which pose a significant threat to agriculture. Metals like cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, nickel, palladium, platinum, and zinc are the predominant metal contaminants in our soils and water which call for instantaneous action to design microbiological techniques for effective bioremediation. The associated anthropogenic activities lead to a significant release of toxic metals into the environment purposely. Various industries related to mining, surface finishing, energy and fuel producing, fertilizer, pesticide, metallurgy, iron and steel, electroplating, electrolysis, paints and ceramic discharge metal laden effluents result in severe environmental pollution and health hazards. An indefinite persistence of heavy metals in the environment is a potential health hazard as it leads to bioaccumulation of toxic metals in the crops that eventually leads to biomagnification upon entering the food chain. This chapter highlights the promises of *Bacillus* as a potential biosorbent for the effective removal of toxic heavy metals from the environment. Numerous members of the genus *Bacillus*, like *B. subtilis, B. thuringiensis, B. sterothermophilus, B. megaterium, B. cereus*, *B. pumilus, B. licheniformis*, and *B. jeotgali* have been reported to remove heavy metals most effectively. Diverse functional groups like carboxyl, amino, amide, phosphate, and hydroxyl groups

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associated to bacterial cell walls which attribute to biosorption capacity have been described herein. Numerous contributing factors like time, temperature, pH, cell density, and agitation are also discussed. *Bacillus*-mediated biosorption and bioaccumulation is a powerful strategy for the removal of toxic heavy metal stress in order to ensure sustainable agriculture.

Keywords *Bacillus* · Heavy metal toxicity · Bioremediation · Biosorption · Bioaccumulation · Detoxification

1.1 Introduction

Heavy metals are nondegradable, environmentally hazardous, and toxic. Various adverse effects of heavy metals include immune suppression, cancer, and neurotoxicity. Critical enzymes involved in vital metabolic processes may be inhibited by heavy metals. Numerous industrial activities such as mining minerals, smelting, and tannery result in the release of toxic metals such as cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, nickel, palladium, platinum, and zinc. These metals can accumulate within plants from contaminated soils or water leading to biomagnification. Consumption of metal-contaminated crops can cause damage to the lung, kidney, liver, and pancreas as well as lead to nervous disorders (Arivalagan et al. 2014). The indiscriminate use of excessive pesticides, weedicides, herbicides, and chemical fertilizers has contaminated soil and irrigational water with toxic elements metals (Ramirez et al. 2019).

Although heavy metals are found in trace amounts in the environment, upon bioaccumulation and biomagnification, their levels in the organism may be at different levels that can cause severe toxicity and impairment of regular metabolic activities. Conventional chemical and physical methods of metal removal like ion exchange, photo catalytic reduction, precipitation, ultrafiltration, and reverse osmosis are costly, time-consuming, inadequate, and energy-consuming. Thus, economical, environmentally benign, and efficient biological techniques are being designed with high heavy metal removing efficiency. Bacteria, yeast, fungi, and algae are capable of binding to and adsorbing metal ions. Many organisms dwelling in metal-contaminated ecological niches have developed chromosomally or extrachromosomally controlled detoxification mechanisms to overcome the detrimental effects of toxic heavy metals. Metal tolerance can be attributed to mechanisms such as extracellular precipitation and exclusion, binding to the cell surface, and intracellular sequestration. The most significant mode of bioremediation is binding of metal cations onto the negatively charged outer surface of bacterial cells, which can be later recovered as well (El-Helow et al. 2000). Thus, recently, bacterial biomass is considered as the most efficient biomaterial for removing metals. Specific metabolic processes and diverse active functional groups and proteins on bacterial cell walls, such as sulfate, amine, hydroxyl, and phosphates, enable microbes to interact and accumulate heavy metal ions on their surface (Dadrasnia et al. 2015).

In this chapter we have highlighted the potential of *Bacillus* as one of the most efficient heavy metal removing agents that can be beneficial for sustainable agriculture.

1.2 Heavy Metal Toxicity

Heavy metals, like cadmium, are extremely toxic pollutants which are widely used in rechargeable nickel–cadmium batteries, fertilizers, pesticides, pigments, metal refineries, electroplating, mining, etc. (Arivalagan et al. 2014). Its harmful effects include "itai-itai" disease, renal damage, emphysema, hypertension, testicular atrophy, atherosclerosis, and cancer (Ganguly et al. 2011). Chromium (Cr) is considered as a highly hazardous heavy metal as it does not easily degrade and is mostly released from textile industries, petroleum refining, and chemical and electronic manufacturing, as well as agriculture and mining activities. High levels of chromium consumption cause headache, nausea, vomiting, as well as skin, throat, and lung cancers. In nature, chromium exists as Cr (VI) or Cr(III), which differ in biochemical reactions and physicochemical properties. Cr (VI) is found as chromate (CrO_4^{2-}) which is soluble, mobile, toxic, and carcinogenic with a potential health hazard while Cr(III) is immobile, less toxic, and readily precipitated as $Cr(OH)_3$. Cr (III) is also an essential element in the metabolism of lipids and carbohydrates (Dadrasnia et al. 2015). Cobalt (Co) is radioactive cobalt ($^{58}Co/^{60}Co$) and has diverse applications in alloy production for orthopedic use, as a cold pasteurization agent in the food industry and radiotherapy (Paraneeiswaran et al. 2015). Iron (Fe) comprises 5% of the earth's outer crust and is also present in trace amounts in the human body. Anthropogenic activities greatly alter the natural biogeochemical composition of the soil and ecosystem. Iron in the form Fe(III)-O-arsenite is the most toxic (Huang et al. 1994). Lead (Pb) is highly toxic to the environment as well as humans. It is also known to cause neuronal disorders with long-term effects (Varghese et al. 2012). Manganese (Mn) is a trace element and is required by phytoplanktons and other higher plants for photosynthesis (Dismukes 1986). Higher concentrations Mn can be toxic to microorganisms when it is released in the environment from the effluent of industries associated with steel and alloys, paint, batteries, and metal coatings (Pakarinen and Paatero 2011). High doses of Mn have been recorded to be neurotoxic and are known to cause neurodegenerative diseases such as Parkinson's (Olanow 2004; Hussain et al. 2006). Bioaccumulation of Mn in the food chain causes grave consequences to human and animal health as well as the ecosystem. Mercury (Hg) in the form of mercuric chloride is toxic both to animals and the environment. They can penetrate the cell membrane causing an imbalance in cellular enzymatic regulation (Glendinning et al. 2005). Nickel (Ni) is one of the major constituents of the earth's crust and is used in several industries such as mineral processing, electroplating, power plants, paint, and porcelain manufacturing factories (Patterson 1985). Palladium (Pd) is used in industries having a greater operation in crystal engineering, photo-electrochemistry and catalysis which intend to use Pd

in larger quantities. The automobile industry is the highest (50%) among the industries that use Pd (Matthey 2002). Extraction of Pd from waste and contaminated areas have previously been done through chemical methods and pyrometallurgical processes; however, these methods have proven to be highly labor- and time-intensive, as well as expensive (Jacobsen 2005). Platinum (Pt) is a noble element that forms an important chunk of jewellery and is present in the chemical, electrical, glass, and petroleum industries. A huge proportion of Pt is involved in the automobile industry as a vehicle exhaust catalyst (VEC) with emissions as high as 0.5–1.4 tons/year (Ravindra et al. 2004). Zinc (Zn) is a natural element and is found in the earth's crust imbibed in rocks and minerals. Years of weathering leads to demineralization of zinc and its subsequent release in water bodies and groundwater. Zinc is also an important industrial raw material used in batteries, electrical equipment, paint, rubber as well as in pharmaceuticals. In the human body, zinc exists as an inorganic metal and is vital for certain metabolic and redox reactions (Igiri et al. 2018). In dietary terms, the excessive intake of zinc has proven to be toxic leading to several gastrointestinal complications. The presence of high concentrations of zinc in aquatic ecosystems can result in subsequent accumulation in the food chain giving rise to the urge for bioremediation techniques for its efficient removal.

1.3 Heavy Metal Stress in Agriculture

Geologic and anthropogenic activities increase heavy metal concentration in soil. The rapid accumulation of heavy metals in agricultural soil, irrigational water, and living systems causes excessive harm in amounts that are harmful to crops. High concentrations of heavy metals in the soil result in their absorption and accumulation by plants, which ultimately pass into humans via the food chain. The absorption of toxic metals by aerial and underground plant organs directly or indirectly affect plant health and subsequently the yield of crops. Direct consequences include inhibition of cytoplasmic enzymes and damage to cell structures due to oxidative stress. The generation of reactive oxygen species (ROS) and cytotoxic compounds like methylglyoxal (MG) impair the equilibrium of ionic homeostasis within plant cells. Indirect oxidative stress includes multiple mechanisms such as glutathione depletion, binding to sulfhydryl groups of proteins, inhibiting antioxidative enzymes, or inducing ROS producing enzymes like NADPH oxidases. The cumulative effect of direct and indirect heavy metal toxicity leads to a reduction or even complete cessation of all metabolic activities of the plants. Beyond a certain limit of heavy metal concentration, defense strategies from plants to avoid or tolerate heavy metal intoxication fail protect it and the survival of the plant is jeopardized. Hence removal of heavy metals in order to ensure plant health and superior crop yield is most important, and therefore, the microbial approaches, which are cost effective, eco-friendly, and rapid, have been preferred over conventional strategies (Mishra et al. 2017).

1.4 *Bacillus* and Bioremediation

Several microorganisms can adapt to metal-polluted environments owing to their ability to protect themselves from various mechanisms that include adsorption, uptake, methylation, oxidation, and reduction. Bacteria like *Acinetobacter* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Serratia marcescens*, *Ochrobactrum* sp., *Desulfovibrio vulgaris*, *Cellulomonas* sp., and *Bacillus* sp. are known for their significant role in the bioremediation of heavy metal-contaminated soils (Ramirez et al. 2019). Several species of *Bacillus* can detoxify and/or degrade the pollutants in the soil. These microbes play a crucial role in nutrient recycling in ecosystems, and especially can survive at toxic concentrations of metals as they develop a variety of resistance mechanisms to neutralize heavy metal stresses (Arivalagan et al. 2014). The *B. thuringiensis* strain DM55 expresses a variety of extracellular degradative enzymes such as amylase, mannanase, lichenase, and caseinase, and it is reported that their resistance to toxic metals like cadmium is not a plasmid-controlled character. Similarly, as the most characterized Gram-positive bacterium, *B. subtilis* 168 is a widely used model to investigate the influence of a histidine kinase, KinA, on the negative charge of the outer cell surface in *Bacillus* cells. Surface properties of *Bacillus* are very significant determinants of their metal binding capacity (El-Helow et al. 2000). *Bacillus* can adopt two ways for bioremediation; bioaccumulation, where the metal is taken up inside the cell, and biosorption, where the metals remain attached to the cell surface. Both dead and live cells can potentially exhibit biosorption; however, the percentage removal may vary. Dead bacterial cells as bioremediators are more beneficial over live cells as any external nutrient medium is not required for maintenance, and is also stored comparatively easily (Velásquez and Dussan 2009). The microbial systems according to their adaptive and physiological mechanisms can detoxify the metal by: (i) effluxing it out, (ii) accumulating in the cytoplasm, or by (iii) converting it into a less-toxic form.

1.5 Heavy Metal Removal by *Bacillus*

1.5.1 Cadmium

B. cereus (strain KTSMBNL 43) isolated from the electroplating industry-contaminated soil at Coimbatore, Tamilnadu, India exhibited Cd^{2+} tolerance. Isolation of cadmium-resistant bacteria was carried out on LB medium amended with 10, 50, 100, 200, and 300 mg L^{-1} of Cd^{2+}. The effect of different parameters like pH, temperature, initial metal concentration, and contact time on Cd^{2+} biosorption was evaluated using atomic absorption spectroscopy (AAS) and the biomass was determined using a UV-spectrophotometer at 595 nm. The maximum biosorption capacity (82%) was observed at pH 6.0 and at 35 °C with an initial metal concentration of 200 mg L^{-1} Cd^{2+} (Fig. 1.1). FTIR spectra of before and after Cd^{2+}

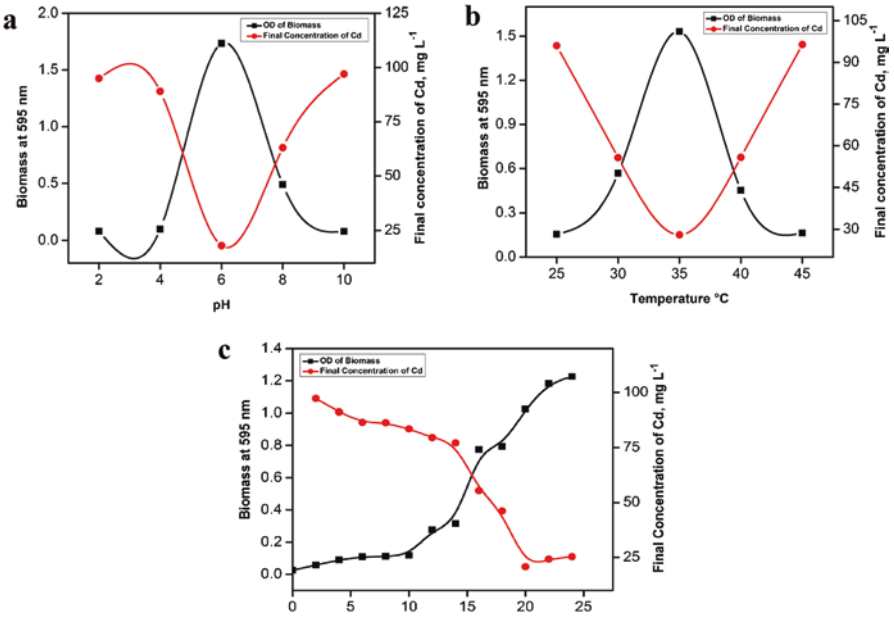

Fig. 1.1 Effect of various parameters on Cd^{2+} biosorption: (**a**) Effect of pH on Cd^{2+} biosorption by *B. cereus*. (Initial Cd^{2+}concentration—200 mg L^{-1}; temperature—35 °C; contact time—24 h; agitation speed—120 rpm); (**b**) Effect of temperature on Cd^{2+} biosorption by *B. cereus*. (Initial Cd^{2+} concentration—200 mg L^{-1}; pH —6.0; contact time—24 h; agitations peed—120 rpm); (**c**) Effect of contact time on Cd^{2+} biosorption by *B. cereus*. (Initial Cd^{2+} concentration—200 mg L^{-1}; pH —6.0; temperature—35 °C; agitation speed—120 rpm). (Reprinted with permission from Arivalagan et al. 2014. Copyright © 2014 Elsevier B.V)

biosorption revealed the cell surface-associated functional groups responsible for biosorption and thereby metal removal. A broad spectra of bands at 3364 and 3422 cm^{-1} corresponding to the presence of a hydrogen bond OH stretch was visible apart from the C-H and the C=O stretching at 3005 and 1729 cm^{-1}, respectively, in the Cd^{2+}-loaded biomass (Table 1.1). The alteration in peaks observed between 1244 and 1283 cm^{-1} might be attributed to the stretching vibration of SO_3 groups in the adsorption of Cd^{2+}. Hence, *B. cereus* may serve as an interesting low-cost, eco-friendly alternative for Cd^{2+} removal from polluted environments (Arivalagan et al. 2014). In another study, about 75% and 88% of the cadmium was removed by growing and nongrowing cells of *B. cereus* $M^1{}_{16}$ from its aqueous solution at pH 6.8 ± 0.2, temperature 30 ± 1 °C and 120 rpm shaking speed. For growing cells, an inoculum size (2%) and medium volume (50 mL) was optimum. It is important to note that the effective desorption of cadmium was achieved using mineral acids such as HNO_3, H_2SO_4, HCl, and EDTA, which resulted in 98%, 93.5%, 90%, and 88.9% Cd(II) recovery from the loaded biomass, respectively. A greater affinity of the functional groups towards the H^+ ions might have attributed to this efficient metal recovery (Ganguly et al. 2011).

Table 1.1 Main functional groups on the surface of *B. cereus* as observed from FTIR spectroscopy

FTIR spectra range (cm^{-1})	Wavenumber range (cm^{-1})		Functional group assignment
	Before Cd biosorption	After Cd biosorption	
3600–3100	3364	3422	Bonded -OH stretch
3000–2800	Not observed	3005	C-H stretch
1730–1650	Not observed	1729	C=O stretch (it may represent carboxylic acids, ketones, and aldehydes)
1670–1640	1653	1652	C=O stretching (carboxylic groups)
1600–1500	1545	1545	N=O stretch (nitro groups)
1500–1440	1451	1448	H-C-H bend (alkanes)
1600–1500	1396	Not observed	COO- stretch (fatty acids)
1600–1500	1308	Not observed	N=O bend (nitro groups)
1300–1000	1244	1283	$-SO_3$ stretch
1300–1000	1082	1058	C-O stretch (alcohol groups)
600–500	539	559	C-I stretch (aliphatic iodo compounds)

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Cadmium uptake by the metal-resistant bacterium *B. circulans* strain EB1 was checked by enriching 50 mL of Mueller–Hinton broth with cadmium (28.1 mg/L). Before inoculation with 1 mL of mid-logarithmic culture, the bacterial cells were preconditioned with 11.2 mg/L cadmium, *B. circulans* exhibited a specific biosorption capacity of 5.8 mg Cd/g dry wt biomass in the first 8 h. When preconditioning the cells with low concentrations of cadmium, the uptake was enhanced to 6.7 mg Cd/g dry weight biomass. The maximum uptake of cadmium was observed during the mid-logarithmic phase of growth. The specific biosorption capacities of wet and dry biomasses were 9.8 and 26.5 mg Cd/g dry weight biomass, respectively, at pH 7.0. Resting cells remarkably removed more cadmium compared to the growing cells (Yilmaz and Ensari 2005).

The biosorption of Cd(II) from aqueous solutions was studied in a batch method by using dead bacteria *B. licheniformis* sp. extracted from the soil in the area of the Tigris River. Initially, the bacterium was inoculated in liquid nutrient broth and the recovered biomass was washed, dried, and sieved to select particles with 180-μm size. The *B. licheniformis* sp. biomass was added to 50 mL of the Cd(II) solution. The monolayer adsorption capacity of *B. licheniformis* was 24.51 mg/g while the activation energy was determined as 23.24 kJ/mol for Cd(II) biosorption, indicating that the biosorption of metal ions onto the *B. licheniformis* biomass might be attributed to physical adsorption (Baran and Duz 2019).

Vijayakumar et al. (2011) reported the removal of Cd^{2+} ions from an aqueous solution using live and dead *B. subtilis*. An increase in initial concentration of Cd^{2+} leads to an increase in uptake capacity of the *B. subtilis* owing to the interactions between metal ions and different functional groups of the biomass. The percentage removal of Cd^{2+} increased from 33 to 60% with an increase in pH from 2.0 to 6.0

and a decrease to 29.78% at pH 10 for the sorption of live *B. subtilis* and dead *B. subtilis*, respectively. It was speculated that the adsorption of Cd^{2+} ions on the adsorbent was dependent on the nature of the microbial surface and the species distribution of the metal cations.

A marine bacterial strain, *B. thuringiensis* strain DM55, with multiple heavy metal resistance and biosorption phenotypes, exhibited considerably high cadmium resistance. The successive transfers of DM55 cells into media containing up to 0.25 mM $CdCl_2$ helped to induce cadmium resistance. Cd^{2+}-amended culture exhibited a lag phase of 3.5 h with about a 1.85-fold increase in doubling time and a two-fold decrease in viable count when compared to the untreated control. Potassium phosphates and peptone were the most significant variables in cadmium removal. DM55 cells grew in the presence of 0.25 mM $CdCl_2$ to remove about 79% of the metal ions within 24 h with a specific biosorption capacity of 21.57 mg g^{-1} of biomass. Both fresh and dry cells of DM55 were able to remove Cd^{2+} under the influence of phosphate in the medium. Moreover KinA, a major phosphate provider in the phosphorelay of *Bacillus* cells, also regulated the magnitude of cell surface affinity for cadmium ions (El-Helow et al. 2000).

1.5.2 Chromium

Live and dead cells of *B. salmalaya* isolated from agricultural soil in Malaysia exhibited excellent chromium biosorption capacity. Initially, the *B. salmalaya* strain 139SI was inoculated into BHI medium and the cells in late exponential phase and live cells were harvested. The dead cell biomass was prepared by autoclaving. Live and dead pellets were mixed with an initial Cr(VI) concentration of 50 ppm (prepared from potassium dichromate). The maximum biosorption capacity was 20.35 mg/g at 25 °C, with pH 3 and a contact time of 50 min with a pseudo second-order mechanism. Alteration of the cellular morphology was observed. Cells of dead biomass that were long, thin, and rod-shaped before chromium adsorption became fat, spongy, and plumped after adsorption of chromium ions onto the cell surface (Fig. 1.2). Chromium accumulation occurred in the cell wall of *B. salmalaya* 139SI rather than intracellularly. High desorption efficiency up to 92% and 70% using dead and live cells, respectively, indicated the reusability and recyclability of *B. salmalaya* 139SI (Dadrasnia et al. 2015).

Bacillus sp. MH778713, close to the *B. cereus* group, was isolated from the nodules of mesquite trees (*Prosopis laevigata*) growing in aluminum, titanium, chromium, and zirconium-polluted soils of a semi-arid region in Nexapa River Chietla, Puebla, Mexico. This bacteria was the most resistant strain, which could tolerate up to 15,000 mg/L Cr (VI) and 10,000 mg/L of Al. *Bacillus* sp. MH778713 accumulated up to 100 mg Cr(VI)/g of cells when it was exposed to 1474 mg/L of Cr (VI). *Bacillus* sp. MH778713 further assisted *P. laevigata*-mediated phytoremediation when plants were inoculated with *Bacillus* sp. MH778713 and grown in a nitrogen-free Jensen's medium added with 0, 10, and 25 mg/L of Cr(VI). The growth of

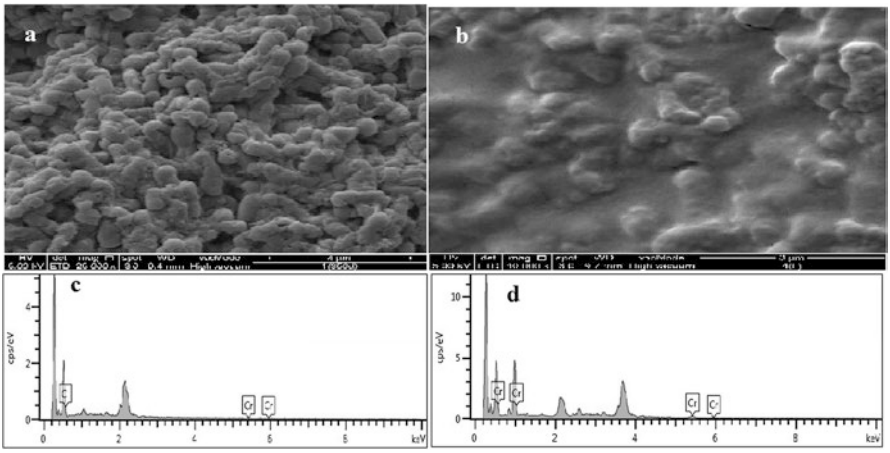

Fig. 1.2 SEM analysis of dead cells (**a**) before adsorption; (**b**) after adsorption; (**c**) EDX analysis of **a**; and (**d**) EDX analysis of **b**. (Reprinted from Dadrasnia et al. 2015. (Open access))

plants inoculated with *Bacillus* sp. in the presence of chromium indicated combinations of *P. laevigata* and *Bacillus* spp. as ideal candidates for soil restoration of chromium-contaminated arid and semiarid sites (Ramirez et al. 2019).

Chromium(VI)-resistant *Bacillus* sp. isolated from Cr-electroplating sludge from Daxing Electroplate Factory in Changsha, China, were grown with Cr(VI) (60 mg/L). The biosorbent was prepared by harvesting cells followed by washing. The live cells were freeze-dried for 12 h and used for biosorption. Living and freeze-dried biomasses are capable of absorbing up to 34.5 mg/g and 17.8 mg/g of chromium (VI) using an initial concentration of 60 mg/L, respectively, that followed pseudo-second order kinetics. The maximum metal uptake of 25.5 mg/g (living cells) and 18.3 mg/g (freeze-dried cells) were observed at pH 2.0. As the initial Cr (VI) concentration increased from 40 mg/L to 400 mg/L, the sorption capacity increased from 22.3 mg/g to 79.5 mg/g for a living biomass and 14.9 mg/g to 47.5 mg/g for freeze-dried biomass. A high efficiency for Cr(VI) removal was evident as a sorption capacity of 45 mg/g and 28.21 mg/g was achieved when the mass of cells used was just 0.01 g (dry mass) for living and freeze-dried cells, respectively (Yun-guo et al. 2008).

Sukumar et al. (2017) reported a fixed-bed column for using co-immobilized (activated carbon and *B. subtilis*) beads to remove Cr(VI) from aqueous solution. Various parameters like initial Cr(VI) concentration, flow rate, and bed depth were optimized for maximum Cr(VI) adsorption onto co-immobilized beads. The duration to reach the breakthrough (t_b) decreased from 150 to 25 min upon increasing the flow rate from 3 to 15 mL/min. Further, the Cr(VI) removal decreased from 57.8 to 10.8% as the flow rate increased to 15 mL/min. However, superior adsorption (57.8%) was observed at a lower flow rate (3 mL/min) which might be attributed to the availability of more residential time for the effective binding of Cr(VI) to co-immobilized beads. Significant elution (90%) was achieved using 0.1 M NaOH in

all the six cycles emphasizing the potential of co-immobilized beads as effective agents for the removal of Cr(VI) from an aqueous solution.

1.5.3 Cobalt

Paraneeiswaran et al. (2015) reported the reduction of [Co(III)–EDTA] − to [Co(II)–EDTA]$^{2-}$ by bacterium *B. licheniformis* SPB-2 from solar salt pan locations of solar salt pan at Marakkanam, the Southern coast of the Bay of Bengal, Pondicherry, India. The bacterial colonies were anaerobically transferred to Tris–G broth supplemented with 1, 3 and 5 mM [Co(III)–EDTA] − and were incubated at 30 °C. *B. licheniformis* SPB-2 might have gained [Co(III)–EDTA] − resistance either by reducing the oxidation state of Co(III) to Co(II) or by physical adsorption. Moreover, *B. licheniformis* SPB-2 reduced 1 mM [Co(III)–EDTA] − in 14 days when grown under batch mode with a significant increase in the reduction activity up to four cycles (Fig. 1.3). It is interesting to note that [Co(III)–EDTA] − induced spore germination and the resulting vegetative cells could successfully reduce [Co(III)–EDTA]−. After reduction, the resulting [Co(II)–EDTA]$^{2-}$ complex could be effectively removed by *B. licheniformis* SPB-2-mediated biosorption. *B. licheniformis* SPB-2 showed a D_{10} value (radiation dose required to kill 90% cells) of- 250 Gray (Gy) indicating its potential for bioremediation of moderately active nuclear waste.

Lee and Tebo (1994) reported cobalt (II) oxidation by a marine manganese (II)-oxidizing *Bacillus* sp. strain SG-1. This bacteria could oxidize Mn(II) by its mature spores which was attributed to a spore coat-associated protein. Similarly, SG-1 spores also oxidized Co(II) optimally around pH 8 and between 55 and 65 °C. SG-1 spores oxidized Co(II) at all Co(II) concentrations tested from the trace levels found in seawater to 100 mM. A kinetics study using an Eadie-Hofstee plot indicated that SG-1 spores exhibited two oxidation systems, a high-affinity-low-rate system (K_m, 3.3×10^{-8} M; V_{max}, 1.7×10^{-15} M .spore^{-1}. h^{-1}) and a low-affinity-high-rate system (K_m 5.2×10^{-6} M; V_{max}, 8.9×10^{-15} M .spore^{-1}. h^{-1}). SG-1 spores failed to oxidize Co(II) in the absence of oxygen, which proved the fact that oxidation was a biological associated with the preformed Mn (III,IV) oxide surface. This process may have potential applications in metal removal, recovery, and immobilization processes.

1.5.4 Copper

Copper was reported from an aqueous solution employing growing cells and washed cells of *B. cereus* $M^{1}{}_{16}$. Initially, the bacteria was grown in 50 mL medium (beef extract: 1.0; yeast extract: 2.0; peptone: 5.0; NaCl: 5.0 g/L; and pH: 6.0) containing 50 mg/L Cu (II) ions. Under optimum conditions, growing cells could remove 73.3% of Cu (II) ions from a 50 mL aqueous solution at 30 °C, pH 6.0, and after

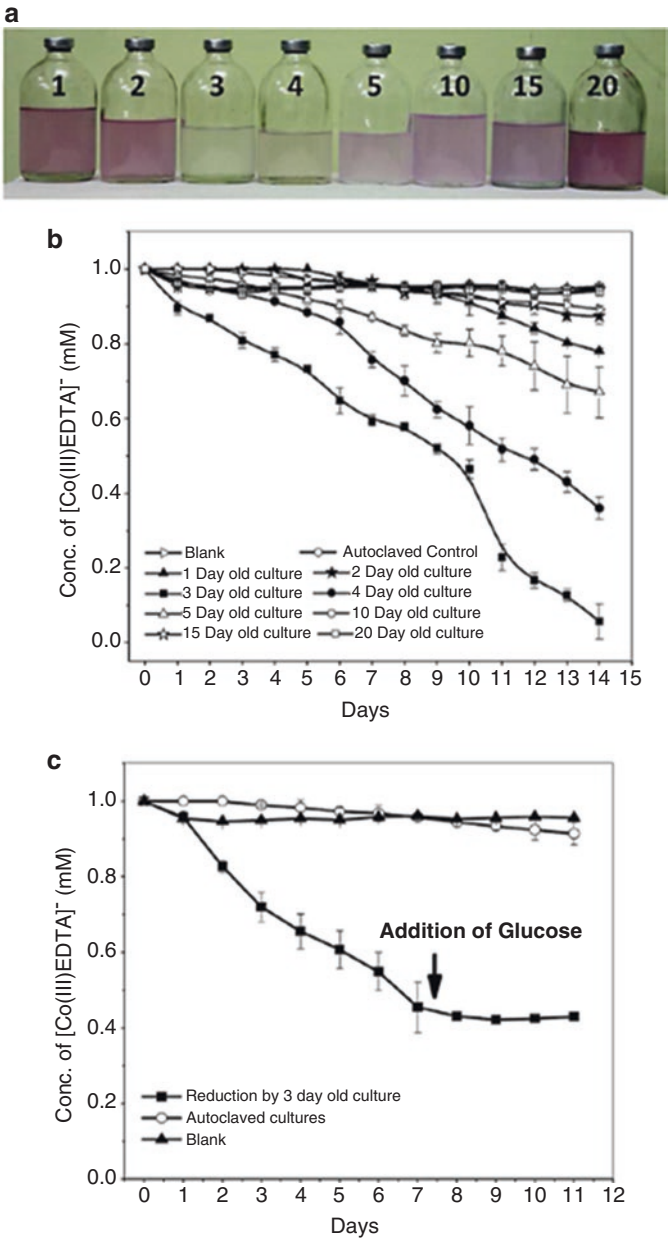

Fig. 1.3 (**a**) Image showing [Co(III)–EDTA] − reduction (decolouration) in serum bottles starting with different days old *B. licheniformis* SPB-2 cultures after one month of incubation. (**b**) Day-wise reduction of [Co(III)–EDTA] − by different days old *B. licheniformis* SPB-2 culture in Tris–G. (**c**) [Co(III)–EDTA] − reduction by *Bacillus licheniformis* SPB-2 during the addition of 0.2% glucose in the middle of the experiment. Each data point indicates the mean ± SD of triplicates. (Reprinted with permission from Paraneeiswaran et al. 2015. Copyright © 2014 Elsevier B.V)

24 h with an inoculum size (24 h cell growth) of 3% and initial metal ion concentration of 75 mg/L. Biosorption employing washed bacterial cells attained equilibrium within 180 min at 30 °C, pH 3.5 and with an initial metal ion concentration of 100 mg/L using 8.18 g/L biomass (dry weight) in a 50 mL reaction volume (Bairagi et al. 2010).

Copper(II)-resistant *B. cereus* KTSMBNL 81 was isolated from soil samples collected around an electroplating industry in Coimbatore, Tamil Nadu, India. Initial Cu(II) ion concentrations, pH of the solution, temperatures, and contact times played an important role in the biosorption of Cu(II) using *B. cereus*. Initial Cu(II) concentration was inversely proportional to the biosorption capacity. Saturation of binding sites due to the more number of Cu(II) ions might be the reason for reduced biosorption capacity. Although the removal capacity of *B. cereus* was low (3%) at pH 2.0, it increased up to 9% at pH 4.0. Maximum removal of Cu(II) (89%) was seen at pH 6.0. The highest removal of Cu(II) (86%) was observed at 35 °C. An enhancement in biosorption with a rise in temperature from 25 to 35 °C might be due to enlargement of pores that increased the surface area facilitating high sorption, diffusion, and penetration of Cu(II) into the biomass. Cu(II) sorption increased and a maximum (88%) was achieved with a contact time of 24 h. The FTIR spectrum of the biomass revealed the involvement of carboxyl, hydroxyl, and amino groups for the biosorption of Cu(II). As evident from Fig. 1.4, after biosorption of Cu(II), the cell surface morphology changed to rough with severe damage of the cell wall due to the toxicity of the heavy metals (Pugazhendhi et al. 2018).

Immobilized *B. subtilis* cells in chitosan beads (BICB) showed efficient removal of Cu(II) from aqueous solutions. The stable bacteria immobilized beads were filtered and washed with sterile water. A pH of 6, biosorbent dosage of 3% (w/v) and 37 °C temperature were optimum for maximum biosorption. Low Cu(II) concentration of 10 mg/L resulted in the removal efficiency of 98.4%. A desorption ratio of Cu(II) with EDTA and HCl was much lower than that with NaOH with a desorption rate of 76.02% even after 5 cycles (Liu et al. 2013).

A metal biosorbing *B. thuringiensis* strain OSM29 was isolated from rhizosphere of cauliflower grown in soil irrigated consistently with metal contaminating industrial effluents near Aligarh, North India. A dry biomass of *B. thuringiensis* OSM29 showed 91.8% of copper at pH 6 and 30 ± 2 °C within 30 min. FTIR analysis revealed that amino, carboxyl, hydroxyl, and carbonyl groups were involved in the biosorption (Oves et al. 2013).

1.5.5 Iron

Bioremediation offers a viable option for the safe removal of iron from the environment in an effective and efficient manner. A strain of *B. subtilis* D_{215} was tested for its potential in iron removal from effluents. Both live and dead cells were taken for a study at different pH, temperatures, and contact time. It was found that the above factors played an essential role in governing bioremoval capacity. With 600 µL of an

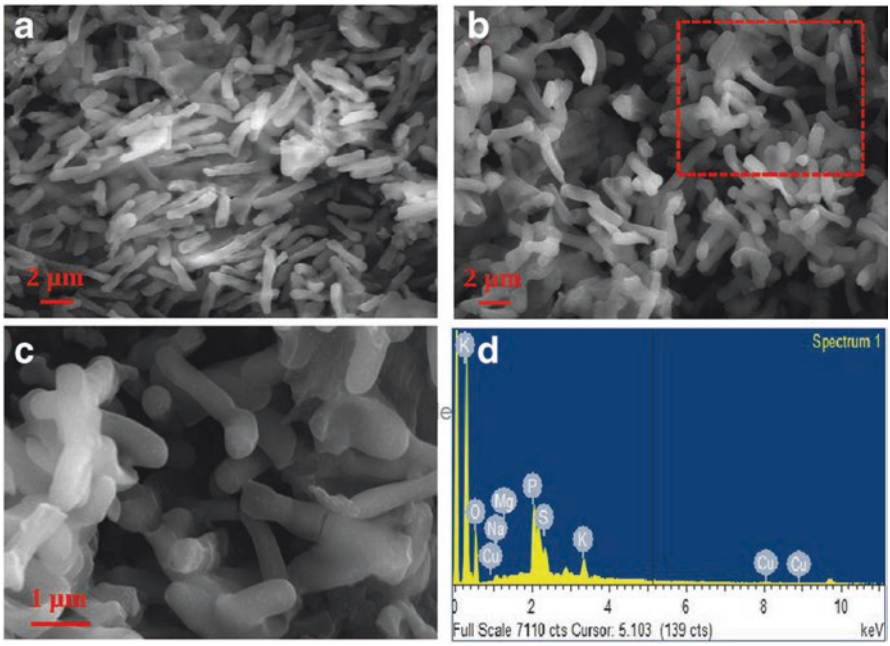

Fig. 1.4 Scanning electron micrographs and EDXA spectra of *B. cereus* KTSMBNL 81. (**a**) SEM image of Cu(II) unloaded; (**b**) SEM image of Cu(II) loaded; (**c**) SEM image of Cu(II) loaded (enlarged view); and (**d**) EDXA spectra of Cu(II) loaded. (Reprinted with permission from Pugazhendhi et al. 2018. Copyright © 2018 Springer International Publishing AG, part of Springer Nature 2018)

inoculum, an average of 70.35% of Fe^{2+} was removed at a pH 7, temperature of 30 °C, and contact time of 48 h. On the other hand, dead *B. subtilis* cells removed 21.30% of the heavy metal in the exact same biophysical conditions. Further, genetic studies of the bacterial genome has led to the discovery of several metalloregulatory proteins with Fur and DTxR homologues which a function of both metal and DNA binding sites, thereby facilitating metal uptake (Sabae et al. 2006). Further exploration of the genetic composition of *Bacillus* would help in highlighting genes responsible for Fe^{2+}tolerance and resistance in *Bacillus.*

The biosorption capacity of nine different *B. sphaericus* strains, isolated from beetle larvae and oaks forest (Lozano 1998), was studied in live conditions. These were: 2362, OT4b25, OT4b31, OT4b48, OT4b51, OT4b56, III(2)3, IV(1)8, and IV(4)10. The cells were grown at 30 °C for 24 h. Out of these, OT4b31 and IV(1)8 showed the highest biosorption capacity for Fe^{2+} at 2.1 mg/L and 2.5 mg/L, respectively. The presence of an S-layer was also studied by running it over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization (MALDI) (Velásquez and Dussan 2009). The S-layer of the bacterial cell wall is present on the cell surface as a paracrystalline layer with a high porosity between 30–70% (Sleytr and Beveridge 1999) which aids

in metal binding. The studies strongly rationalize the introduction of S-layer proteins into high metal-contaminated environments for effective bioremediation.

Hyper-alkaline and hypersaline aqueous environments are often developed due to contamination with heavy metals. One such region with high iron contamination was studied to understand the abundance of tolerant species surviving in such extreme conditions. 16S rRNA sequencing and terminal restriction fragment length polymorphism (T-RFLP) indicated *B. alkalinitrilicus* as the most copiously living microorganism in those conditions. At a pH above 9 and salinity of over 100 PSU, *Bacillus* sp. was previously reported to thrive in haloalkaline conditions (Sorokin et al. 2008).

Living cells of *B. licheniformis* were isolated for experiments regarding iron removal from sewage water. A high percentage removal of 95% (w/v) was observed for iron at pH 3.5, 28 °C and for 48 h with a maximum tolerable iron concentration (MTC) of 1500 mg/L. The *B. licheniformis* cell wall is composed of teichoic and teichouronic acid which are responsible for effective metal ion binding (Beveridge et al. 1982). Additionally, the biosorption rate was determined with dead *Bacillus* cells as well. However, in the case of bioremoval of iron with dead cells of *B. licheniformis*, the bioremoval percentage of only 60% implied greater efficiency of living cells (Samarth et al. 2012).

A reduction of ferric ions is essential to remove the toxic ferric oxyhydroxide forms from iron-contaminated soils. Several bacterial species have been described which possess the ability to reduce ferric ions (Lovely 1987). The *Bacillus* sp. SO-10 was isolated due to its metal-degrading ability and resistance. The *Bacillus* sp. was studied for various concentrations of iron from 100–1000 mg/L at 30 °C for 72 h. Maximum solubilization of iron was observed at pH 7; however, the pH was not a determining factor of solubilization rate. The study also concluded that an additional carbon source of 1% glucose enhanced the solubilization rate of iron by SO-10 (Sivakami et al. 2012).

In a rather elaborate study, 183 bacterial isolates growing in iron rich soil were isolated and studied. 12 bacterial strains were found to have notable growth in the presence of 1 g/L of iron. *B. subtilis* UFLA SCF590 showed the maximum bioremoval efficiency of 100% at 0.77 g/L at an acidic pH of 3.5 (Kelly et al. 2014).

Agricultural biowaste in the form of soil contaminants due to a high level of heavy metals present in fertilizers is a matter of great concern. One such fertilizer is phosphogypsum (PG) which contains metals such as cadmium, cobalt, chromium, copper, zinc, manganese, and iron. The concentration of iron in PG is the highest with 1199 mg/kg. The gradual bioaccumulation of PG in soil leads to an increase in soil metal concentration which poses an environmental threat as well as animal/human health hazard (Blaudez et al. 2000). The *B. megaterium* BM_{30} strain is a known remover of metal waste and is also a part of the biogeochemical cycle (Kumar and Achyuthan 2007; Shazia et al. 2002). This bacteria immobilizes metals through sorption and converts them into less toxic forms such as oxalates and phosphates. The bioaccumulation rate is directly related to the biomass. An increase in sporulation of *Bacillus megaterium* leads to an enhancement of biomass accumulation and subsequently increasing the bioremediation of metals. In this study, the pH

was maintained at slightly alkaline conditions between 7.4–7.6 and a temperature of 30 °C. Optimal iron bioaccumulation was observed at 18 h when the bacteria were in the stationary phase. At varying concentrations of PG, 2 g/L, 6 g/L and 10 g/L, the maximum iron bioaccumulation was observed as 1 mg/L, 7 mg/L, and 9 mg/L, respectively. This study shows promising results for the bioremoval of iron using a new species of *Bacillus* (Stefanescu 2015).

Another agro-based industry includes the distilleries, which produce waste in the form of aromatic amines, organic and inorganic compounds as well as metal ions such as iron. Microbes isolated from a nearby soil source of such distilleries was identified as *B. subtilis.* The treatment of distillery effluents with *B. subtilis* saw a gradual decrease in iron concentration. In an experiment performed at an interval of 5 days, the level of iron was observed as 3.25 mg/L at the fifth day, 2.18 mg/L at the tenth day and 1.16 mg/L at the 15th day indicating a decrease with time. An overall percentage reduction of 80% was observed at a pH range of 7.4–7.8. This can be applied for water treatment due to severe metal contamination. Further, time based studies allow for the opening of newer avenues of research to comprehend and determine the minimum time interval required for the maximum reduction of heavy metals in optimized biophysical environmental conditions (Deborah and Sebastin Raj 2016).

1.5.6 Lead

Extracellular polysaccharides (EPS) from bacteria can be used for biosorption. The EPS produced by *B. firmus* MS-102 was studied to analyze the potential of the biosorption of lead. The biosorption capacity was found to be dependent on a wide array of factors such as pH, initial polysaccharide concentration, and the initial metal concentration. At neutral pH, the absorption was observed to be 55.6 mg/g. However, at higher pH levels the uptake increased. The optimal pH was found to be 4.5, while at neutral pH, 43.6% biosorption was achieved. This may be due to the interaction between acidic groups of polysaccharides with cations. Further studies with metal concentrations demonstrated that above 1000 mg/L, metal saturation occurred and there was a subsequent decrease in metal uptake. Additionally, the optimal temperature was found to be 25 °C in this study (Salehizadeh and Shojaosadati 2003).

In another study, *Bacillus* species isolated from animal excreta were exposed to increasing concentrations of industrial lead. It was found that *Bacillus* was able to survive and resist even high concentrations of lead up to 500 µg/mL (Akujobi et al. 2012). However, it was evident from the growth curve of the organism that with increasing dosages of lead, the rate of survival decreased (Akujobi et al. 2012). The inherent cellular resistance of bacteria to toxic heavy metals is attributed to several factors such as enzymatic conversion of the metal ions, decrease in sensitivity, and removal of ions by a cellular permeable barrier (Brewer and Taylor 1997).

Studies have demonstrated the biosorption capacity of Gram-positive bacteria, *B. subtilis* for lead (IV). Conditions like pH, temperature, and initial biomass play an important role in efficient bioremoval of toxic metals. In the case of *B. subtilis*, the maximum biosorption was observed to be 97.68% (w/w) at pH 4.5 and temperature 40 °C within 48 h at an initial load of 700 ppm (Hossain and Anantharaman 2006).

The presence of lead is diverse and can be seen in soil, water as well as sediment. A study performed by Varghese et al. (2012) confirmed *Bacillus* sp. as an excellent agent for the bioaccumulation of lead ions. A total of 164 isolates were collected from varying regions of wetlands, canals, and river water. 45% of the isolates were found to have very high tolerance to lead up to 6000 μg/mL, exhibiting a 50% lead reduction in in vitro conditions. Similar to previous results, an average of pH 5 was found to be the optimal condition. The increase in biomass of the bacteria corresponded with an increase in adsorption. This may be due to the associated increase in surface area of the microorganism (Bai et al. 2002). The cell wall is composed of different functional groups, such as amine, phosphates, and carboxyl groups. These may serve as scavengers for cations and hence increase the reduction of metal ions (Varghese et al. 2012).

Mines are an important source of lead contamination of nearby agricultural fields as well as water bodies through leaching. Biomagnification of the heavy metals is observed and different methods such as reduction, precipitation, and membrane processes have been adopted to manage the damage cause by lead (Wang and Chen 2009). *Bacillus* sp. KK1 exhibited maximum tolerance to lead (300 mg/L). Reduction capacity of the microorganism into nontoxic forms is an important attribute of microbes used for bioremediation. *Bacillus* sp. KK1 reduced lead nitrates into lead sulfides and lead silicone oxides (Govarthanan et al. 2013). Isolation of *B. licheniformis* from mining sites demonstrated 86 ± 5% removal of lead at pH 6 after 192 h at a flow rate of 10 mL/min. This study validated the role of biomass in the effective adsorption of heavy metals from contaminated soil (Akshatha Jain et al. 2017).

A lead-tolerant bacterium, *B. thuringiensis* strain OSM29, was isolated from the rhizosphere of cauliflower growing in an agriculture field irrigated with factory waste. It was observed that after a certain time period, the absorption rate decreases with an increase in metal ions which might be due to saturation of the adsorption sites (Oves et al. 2013). Bai et al. (2002) showed an increase in metal adsorption with an increase in biomass. Maximum biosorption was achieved at an optimal pH of 6 at a temperature of 30 ± 2 °C within 30 min of contact time. Fourier transform infrared spectroscopy (FTIR) revealed that the main functional groups like amino, hydroxyl, carboxyl, and carbonyl were responsible for heavy metal biosorption. An average of 25 ± 2 mg/g bioremoval of lead was observed at optimal conditions. Conclusive results deduced the efficient use of this strain for reliable bioremoval of Pb in aqueous conditions (Oves et al. 2013).

Govarthanan et al. (2015) reported *Bacillus* sp. SKK11 from a brackish environment that was tolerant to about 750 mg/L of lead and could convert lead nitrates to lead sulfides either intracellularly or extracellularly. Subsequently, enzymes like urease, amylase, cellulose, dehydrogenase, phosphatase, and invertase resulted in

enhanced bioremediation of lead-contaminated soil. The conversion of lead into carbonate-bound lead is a phenomenon observed previously in another bacterium. This reduces the prevalence of free lead in the soil therefore reducing soil stress. Govarthanan et al. (2013) observed a 38% increase in carbonate-bound lead in soil treated with KK1. On similar lines, SKK1-treated soil also demonstrated an increase in 15.3% carbonate fraction (Govarthanan et al. 2015).

Bioremediation using *Bacillus* as a probiotic was also explored. Belapurkar et al. 2016, demonstrated *B. coagulans*-mediated reduction in the concentration of lead. *Bacillus* sp. is known to have a greater adsorptive nature due to the presence of teichoic acid and peptidoglycan in its cell wall (Gavrilescu 2004). *B. coagulans* reduced lead (II) up to 86% within 72 h in the gut. These studies can be further explored for other species of *Bacillus* in the development of similar probiotics and effective bioremediation.

B. badius AK isolated from water hyacinth green compost showed maximum removal of lead(II) with a pH of 4–4.5 with inoculums at a density of 1.1×10^{11} CFU/mL at 30 ° C. Results showed that at optimal environmental conditions, *B. badius* was able to remove 100 mg/L of lead within 24 h with an initial bacterial biomass volume of 20 mL having a 1.7×10^{16} CFU/mL value (Vishan et al. 2017).

Studies based on the transformation of lead by rhizospheric bacteria showed that *Bacillus* inoculated in fulvic and humic soils can be a significant strategy. Increased mobilization of lead was observed due to a decrease in humic acids and an increase in fulvic acids. Technogenic soil facilitated the immobilization of Fe-hydroxide-bound, chelate-bound lead in the rhizosphere and in roots resulting in decreased lead uptake by the upper parts of plants (Belogolova et al. 2020).

1.5.7 Manganese

An isolate of *Bacillus* was found to have the capacity to generate manganese oxide nanoparticles. Nanoparticles of this nature are known to have tremendous potential in the pharmaceutical industry for drug delivery, as biosensors and also as magnetic storage media. The heavy metal-resistant strain of *Bacillus* was identified as MTCC10650 which reacted with a filter sterilized $MnCl_2 \cdot 4H_2O$ to form manganese oxide nanoparticles. Very few, small-sized, and randomly dispersed nanoparticles were found on the cell wall at 24 h (Fig. 1.5a). However, enhanced accumulation of manganese in the cytoplasm was observed after 48 h (Fig. 1.5b) and 72 h (Fig. 1.5c) of growing the cells. The accumulated particles were mostly spherical, lesser than 6 nm in diameter and uniformly dispersed into the cytoplasm. The average size of the bacteriogenic nanoparticles was 4.6 ± 0.14 nm, with some particles having 6–8 nm size and a very small percentage having a diameter greater than 10 nm (Sinha et al. 2011). This study implied two important conclusions: the production of nanoparticles for industrial purposes as well as the potential of *Bacillus* sp. in manganese bioremediation of industrial discharge.

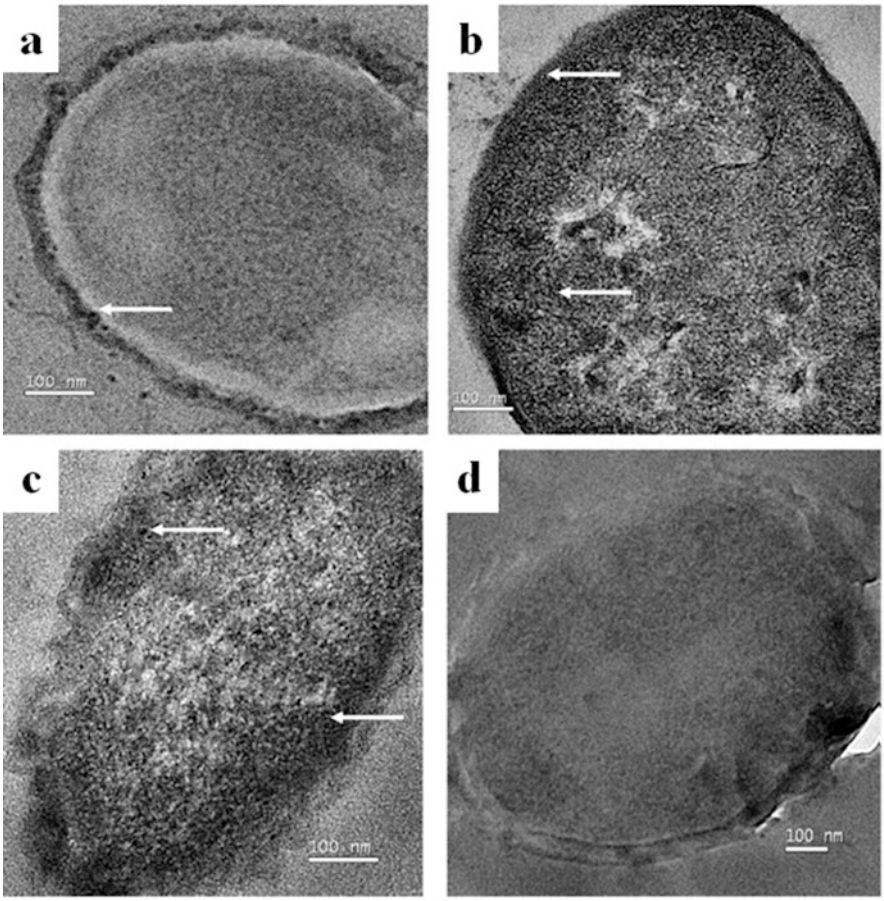

Fig. 1.5 Transmission electron micrograph (TEM) of *Bacillus* sp. cells taken at different times of growth. Cells grown in manganese for (**a**) 24 h; (**b**) 48 h; (**c**) 72 h; and (**d**) control, cells grown in the absence of manganese for 72 h. Arrow heads show manganese oxide accumulation. Bar scale 100 nm. (Reprinted with permission from Sinha et al. 2011. Copyright © 2011 Elsevier B.V)

Agro-based industries are also known to generate a copious amount of organic waste which contributes to contamination of water bodies (Govarthanan et al. 2013). In this regard, a consortium of immobilized bacteria with *B. subtilis* sp. and two other genus were used for the bioremediation of sugar mill effluents. Along with having high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and dissolved solids, the discharge consists of a number of heavy metals, with toxic manganese being one of them. Treatment with a consortium of bacteria leads to a reduction in concentration to manganese below detection levels (Saranraj and Stella 2012). Agro-based products such as fertilizers like phosphogypsum (PG) also contribute to soil pollution to a certain extent due to the presence of heavy metals. PG contains manganese which leads to toxicity as a result of biomagnification.

B. megaterium, $BM_{30,}$ is known for its ability to bioaccumulate heavy metals through sorption and conversion into compounds such as sulfites and phosphates. An increasing trend of bioaccumulation of manganese was seen with increasing levels of PG in contaminated soil in the presence of Bm_{30}. Therefore, bioremediation using BM_{30} bioaccumulates manganese as monitored in cell biomass which was about 80% (Stefanescu 2015).

Although several bacterial species can biosorb manganese, *Bacillus* sp. is the most efficient. *Bacillus* sp. HAH1 was able to biosorp 13.2 mg/g MnII at a pH range of 5–8. With an increase in pH, there was also an increase in biosorption (Hasan et al. 2010). In an experiment carried out in contaminated water at the laboratory scale, a *Bacillus* sp. was able to biosorp 43.5 mg/g Mn(II). The functional groups, such as carboxyl, carbonyl, and amide, were found to be responsible for effective biosorption (Hasan et al. 2012). This study formed the basis for the biosorption for drinking water treatment.

Regulators inherently present in bacteria enable them to modulate the tolerance levels to manganese toxicity at varying levels. This is achieved through metabolic mechanisms of the bacteria such as extracellular polysaccharides and cell walls as barriers, reduced intake or increased efflux and enzymatic regulation. Manganese in the II oxidation state is the most abundantly found compound which contributes to brackish water of water bodies. It is necessary to convert it into its oxidized form, MnO_2, which can be easily removed by filtration, absorption, or clarification processes (Barboza et al. 2016). Enzymatic reactions in various regions of the cell contribute to this oxidation process. This matrix is found in the exosporium in *Bacillus*. Maintenance of homeostasis of $Mn^{2+}$is achieved through regulation of the intake and efflux of the ion. Pumps present in the cytoplasm facilitate this in *B. subtilis*. Natural resistance-associated macrophage protein (Nramp) acts on various transporter uptake systems existing in the bacterial cell such as *mntH, mntA,* and *mntABCD* which are metal ion transporters (Que and Helmann 2000). During high levels of Mn^{2+}, a protein repressor *MntR* acts on *mntH* such that the Nramp is repressed; whereas during low Mn^{2+}, it activates the mntABCD transporter which in turn activates the ABC transporters (Que and Helmann 2000).

In seawater, bacteria are able to convert manganese into its oxide form with the help of multicopper oxidases (MCOs). Gene encoding for these enzymes have been identified as *mnxG.* Spores produced by the *Bacillus* sp. strain SG-1 formed amorphous MnO_2. Few Mn II- oxidizing proteins required during processes, like post-translational modifications, were also found in this species (Francist and Tebo 2002; Dick et al. 2008). This ability of the *Bacillus* sp. strain SG-1 could be used for effective bioremediation of manganese in toxic metal-contaminated saltwater.

The *B. cereus* strain HM-5 obtained from manganese ore was studied for biosorption capacity. Similar to most species of *Bacillus*, biosorption was dependent on factors such as pH, temperature, contact time, initial microbial biomass as well as the metal ion concentration. The most optimal pH and temperature for this strain was found to be 6 and 35 °C with a metal ion concentration of 600 mg/L. *B. cereus* reached a capacity of 98.9% with a contact time of 5 days. Fourier transform infrared (FTIR) analysis revealed the essential functional groups of this bacteria to be

hydroxyl, amide, and phosphoryl groups for biosorption. Morphological analysis showed that without manganese bioabsorption, the cells were smooth, while post biosorption, the cells appeared wrinkled and damaged. This suggests that metal ions were trapped in the polymer matrix of the cell causing the observed deformation (Zhenggang et al. 2019). These observations indicated that *B. cereus* can be a viable option for the removal and recovery of toxic manganese (II) from polluted environments.

1.5.8 Mercury

The first incidence of mercury (Hg) resistance observed in *Bacillus* sp. was in the strain RC607 (Wang et al. 1989). No such occurrence of *Bacillus* sp. resistance was seen in extreme conditions until thermophilic bacteria, *Bacillus* sp. RC607, at temperatures as high as 62 °C was reported. *Bacillus* sp. RC607 had a minimum inhibitory concentration (MIC) of 60 μg/mL for $HgCl_2$ (Glendinning et al. 2005). The MIC values for the isolated *B. pallidus* and *Ureibacillus thermosphaericus* were 80 μg/mL and 60 μg/mL at 62 °C. Mercury resistance is an induced event due to genetic modification while on the other hand, tolerance is a detoxification process due to regular metabolism (Baldi 1997). In usual circumstances, the reduction of mercury results in the formation of sulfides like H_2S. However, neither of the two isolates demonstrated reductase activity, in spite of showing removal of mercury. This opens newer aspects of mercury removal without the production of toxic sulfides and possibly volatile thiol species (Glendinning et al. 2005).

The removal of mercuric ions from sludge wastewater of a chlor-alkali industry was studied by the introduction of a new bacterial strain, *B. cereus* JUBT1. The successful removal of Hg^{2+} was observed in both free and attached forms. Mercury removal using immobilized bacteria was studied with *B. megaterium* MB1. This species is nonpathogenic and possesses high mercury tolerance. Bacterium immobilized on alginate beads converted mercury into its volatile form. Also, it was able to trap a substantial amount of mercury in the beads which could be later treated for recovery. Alginate gel immobilized *B. megaterium* MB1 cells efficiently removed 80% of mercury from the solution containing 10 mg/L mercuric chloride within 24 h. The efficiency of the beads was not compromised by repeated exposure of the same beads to mercuric ions. 73.3% was seen to be converted into metallic mercury and out of which 44% was entrapped in the alginate beads (Chien et al. 2012).

On similar lines, immobilized *B. cereus* was used for the bioremediation of mercury with synthetic effluents. The experiment saw a biosorption concentration of 104.1 mg/g at biophysical conditions such as pH 7, temperature of 30 °C and contact time of 72 h. The initial biomass was 0.02 g/L. The immobilized beads had a removal capacity of 10 g/L of Hg^{2+} at a continuous pace of 11 h (Sinha et al. 2012).

Most of the mercury reducing bacteria consist of a *mer* operon which helps in the conversion of mercury into a nontoxic form and often encodes for reductase enzymes. The *mer* operon consists of specific functional genes along with a

promoter, regulator, and operator. The most common functional genes are *mer*A and *mer*B, which code for mercuric ion reductase and organomercurial lyase, respectively. The lyase is responsible for reducing highly toxic organomercurial compounds, such as methylmercury and phenyl mercuric acetate, into almost nontoxic volatile elemental mercury with the help of the enzyme reductase (Kannan et al. 2006; Dash and Das 2012).

The occurrence of toxic mercury in marine environments is a common occurrence due to various anthropogenic activities. Several marine bacteria have therefore been studied for the bioremoval of toxic metals from seawater (Naik et al. 2012). A strain of *B. thuringiensis*, PW-05, was able to remove about 50 ppm of $HgCl_2$ from marine water. The isolate was found to possess the *merA* operon. The functional groups present in the cell wall of the bacteria, namely sulfhydryl and carboxyl, are responsible for the binding of mercury. Due to their high tolerance, *Bacillus* found in extreme conditions are highly beneficial for the bioremediation in saltwater. *B. thuringiensis* also formed biofilms that enhanced the bioremediation potential. This can be attributed to the secretion of biosurfactants and polysaccharides. The strain showed >90% volatilization of mercury and this ability can be further explored for bioremediation of inorganic mercury in extreme geophysical conditions (Dash et al. 2014).

Electroplating industries generate a mercury-laden effluent from which *B. cereus* MRS-1 was isolated that could convert mercury chloride into mercury sulfide nanoparticles (HgSNPs) as confirmed by transmission electron microscopy (TEM). The resulting HgSNPs were 10–100 nm in size with spherical morphology. Adsorbed nanoparticles on the bacterial cell wall indicated that reduction was the underlying mechanism of synthesis (Sathyavathi et al. 2013).

Another metal tolerating bacteria, *B. subtilis,* was able to remove 29.9% of Hg^{2+} in 5 days with an initial concentration of 500 ppm (Imam et al. 2016). Even though the bacteria were not able to bioaccumulate as much Hg^{2+} as its other counterparts, it still forms a basis for further studies of this species under differing environmental conditions to generate optimal results. As emphasized previously, environmental conditions play a significant role in the level of bacteria-mediated bioremediation and hence an optimum value is necessary for effective bioaccumulation. Along these lines, the ability of *B. licheniformis* for Hg^{2+} biosorption with respect to biophysical factors such as pH, temperature, contact time, and initial concentration of metal was studied. At pH 7, with initial bacterial biomass of 25 mg for 1 h, 70% bioremoval of mercury was observed. An increase in biosorbent concentration resulted in an enhancement of mercury biosorption (Upadhyay et al. 2017). The availability of metal binding sites and concentration of metal are inversely proportional to each other and therefore may limit the effectiveness of uptake of metal ions (Al-Homaidan et al. 2014). The role of extracellular polymeric substance (EPS) producing ability in *B. licheniformis* might have attributed to its efficient biosorption of mercury (200 μg/mg). Due to its extremely high efficacy, *B. licheniformis* is a prospective candidate for the bioremediation of Hg from contaminated water.

1.5.9 Nickel

Bacillus sp. KL1 isolated from municipal waste-contaminated sites in the Kermanshah province of Iran showed efficient nickel (Ni) removal, which was dependent on pH, temperature, and contact time of bacteria. It was concluded that the initial concentration of bacteria and biomass was the main contributing factor to the percentage of bioremoval. The highest bioremediation of nickel (II) was observed as 55.06% after 24 h at 30 °C, pH 7, and 100 ppm concentration. Among various interacting pairs of factors, time and concentration were found to be the most efficient (Taran et al. 2015).

The biosorption capacity of *B. thuringiensis* was studied, wherein, both its vegetative and sporulating stages were considered. Due to its unique ability to form a crystal protein during sporulation, which serves as a biopesticide to a wide range of insects, both the stages were taken into account. Both these forms were found to be efficient adsorbents for nickel(II)ion. The optimal temperature for maximum biosorption was 35 °C and pH was 6 for both stages, in which spore stages had a bioremoval capacity of 45.87 mg nickel (II)/g dry biomass while the vegetative cell was 35.46 mg nickel (II)/g dry biomass. At a 250 mg/L nickel (II) concentration, the adsorption percentage was 15.7% for spore-crystal cells and 10% for vegetative cells (Öztürk 2007). Another strain of *B. thuringiensis* KUNi1, isolated from industrial waste-contaminated soil, exhibited extremely high potential for nickel removal. It was able to remove 82% nickel from the culture tolerating a maximum of 7.5-10 mM nickel concentration within a pH range of 5–9. Further, dead cell biomass had no effect on the bioremoval percentage by KUNi1 (Das et al. 2014).

Thermophilic *Bacillus* sp. resistant to heavy metals have been used as living, immobilized, and dead cells to study their effects on the bioremoval of nickel. Four species were taken up for studies, namely, *B. sphaericus, B. pumilus, Panibacillus alvae*, and *Geobacillus sterothermophilus.* All of these species exhibited tolerance to nickel (II) at a concentration of 100 mg/L. Bioremoval in all the species appeared to be dependent on temperature and optimal results were obtained in the range of 37–70 °C. Dead cells had a higher adsorption capacity as compared to live cells due to the presence of functional groups in the cell wall and a lack of protons generated during metabolism (Pardo et al. 2003). The mean sorption efficiency percentage was observed as follows amongst the four species: immobilized cells 59–73%, dead cells 55–71%, and living cells 39–55% (Al-Daghistani 2012). An increase in adsorption capacity was observed at higher temperatures across all four species. This was attributed to an increase in diffusion rate through the adsorbate pore causing a reduction in viscosity of the liquid. However, at higher temperatures above 40 °C, the efficiency was also seen to decrease in *B. sphaericus*. This may be attributed to a decrease in surface activity of the microorganism (Sari et al. 2008). Further, the role of functional groups in the cell wall of the microorganisms for removal has been attributed to carboxyl and amino groups. Esterification of the carboxyl and methylation of amino groups occurred due to the treatment with sodium azide and

formaldehyde resulting in a reduction in nickel removal in *B. subtilis* (Abdel-Monem et al. 2010).

Heavy metal-resistant *Bacillus* sp. EB1, isolated from contaminated soil, showed a considerable amount of nickel bioremoval percentage in aerobic conditions. EB1 was capable of removing 45% nickel during the active log phase with a biosorption capacity of 13 mg/L (Yilmaz 2003).

A probiotic species of *Bacillus* was used effectively for the removal of nickel as well. The activity of *B. clausii* and *B. coagulans* was demonstrated in which it exhibited both the properties of probiotics and also bioremediation potential (Belapurkar et al. 2016). Correspondingly, dead cells of *B. coagulans* were found to be able to reduce nickel at 68.4 mg/g of biomass (Lei et al. 2014). This property of certain bacterial species has been ascribed to the biochemistry of the cell wall associated with EPS (extracellular polysaccharides), techoic acid and lipotechoic acid.

Flagellin protein was produced more under metal stress. This protein facilitated the movement of the bacterium from a region of high metal concentration to that of a lower one. This study was carried out in *B. cereus* CMG2K4 demonstrating the role of genetics in bioremediation (Ottemann and Miller 1997).

Most of the studies conducted till date on the bioremoval of nickel with bacteria showed optimal activity at high temperatures. However, it is essential to note that bacteria which are functionally active in colder temperatures can remove nickel as well. Psychrotolerant bacteria are able to survive at temperatures at a maximum of 20 °C. *B. cereus* D2 was isolated from a nickel mining area in China that grew well in harsh environmental conditions at a temperature of 10 °C, and at a nickel (II) concentration up to 400 mg/L. Under a low temperature (10 °C), *B. cereus* D2 induced carbonate precipitation ($Ni_2CO_3(OH)_2{\cdot}H_2O$) through biomineralization for removing the high efficiency of nickel ions (73.47%) from the culture solution. *B. cereus* D2 was found to be able to remove nickel at 10 °C. It demonstrated urease enzyme activity wherein, during urea hydrolysis, it produced ammonia, carbonate, and hydroxyl ions which consequently altered the pH of the solution. The optimum pH for the growth of the bacterium was well within a range of 6–9 at 10 °C implying its peak activity at mostly alkaline conditions. However, at alkaline conditions above 9, there was a reduction in bacterial activity and growth (Do et al. 2020).

1.5.10 Other Metals

The reduction of palladium (II) into zero valent, nontoxic metallic forms requires microbial reductases (De Corte et al. 2012). *B. sphaericus* JG-A12, a Gram-positive bacteria, accumulated high concentrations of palladium on the surface. This is attributed to the S-layer on the bacterial cell wall which has a unique paracrystalline proteinaceous nature. In this study, a sol-gel technique was adopted that rendered high mechanical and photochemical stability along with biological inactivity and stable porosity. The *B. sphaericus* JG-A12 cells were encapsulated on a silica gel forming a biological matrix called a biocer or bioceramic. Effective and high metal

binding on this surface was observed which was also found to be reversible. Due to its flexibility, stability, specificity, and ability of easy recovery of immobilized metal on a surface, biocers are a suitable option for bioremediation (Pollmann et al. 2006). Understanding the biochemical composition of the S-layer could open newer avenues into the synthesis of stable bionanoparticles. The S-layer of bacteria is composed of functional groups such as sulfhydryl, carboxyl, phosphates, amino and hydroxyl which serve as a template for nanoparticle generation (Dieluweit et al. 1998). Palladium nanoparticles synthesized by irradiation of the S-layer of *B. sphaericus* NCTC 9602 was achieved. The accumulation of palladium is a two-step process consisting of biosorption of the metal onto the S-layer surface and then the reduction of the metal by the addition of an electron donor, such as H_2 (Pollmann et al. 2005; Pollmann et al. 2006). X-ray absorption fine structure (EXAFS) spectroscopy and attenuated total reflectance Fourier transform-infrared-spectroscopy (ATR-FT-IR-spectroscopy) studies demonstrated the role of carboxyl functional groups in the formation of palladium nanostructures which in turn keeps the S-layer stable even in extremely acidic conditions (pH 0.8) (Pollmann et al. 2005). Thus, the *Bacillus*-mediated recovery of metals from waste water and the synthesis of nanoparticles can be further used as catalysts.

Platinum is an essential metal emerging in the catalysis and electronics industry. Therefore, their recovery from industrial wastes is of immense significance. Similar to palladium, the recovery of platinum also has a bioremediation perspective through the synthesis of nanoparticles. The significance of the S-layer is emphasized in the synthesis of platinum nanoparticles by irradiating *B. sphaericus* NCTC 9602 in the presence of K_2PtCl_4. The S-layer allows for the formation of an organized template which facilitates the synthesis of inorganic nanoparticles (Wahl et al. 2001).

Bacillus is a potent biosorbent for many metals including zinc. The *B. jeotgali* strain U3 was found to actively remove zinc. The bacteria was isolated from the sediment collected from the Urıas coastal lagoon, Sinaloa, Mexico. Maximum zinc removal was observed at pH 7, salinity of 0 (freshwater), and at 30 °C (Green-Ruiz et al. 2008).

Conventional methods such as coagulation, ion exchange, and filtration have many limitations which are now being replaced by more efficient and safe alternatives employing the use of extracellular polysaccharides (EPS) extracted from bacteria, yeast, and fungi (Bender et al. 1994; Volesky 1994; Salehizadeh and Shojaosadati 2001). An acidic EPS produced by the strain, *B. firmus*, could remove zinc metal from solutions. The maximum biosorption took place at a pH of 6 and a temperature of 25 °C. The sorption rate was found to increase as the solution moved towards more acidic. This may be due to the presence of negative polysaccharides and its interaction with the cations in the acidic solution (Salehizadeh and Shojaosadati 2003).

Living cells of the *B. subtilis* D_{215} strain was also used where bioremoval percentage up to 63.73 was achieved when incubating at 30 °C at a pH of 7. The incubation period also is an important factor governing the bioremediation process. In this case, the incubation of the bacterial strain with the metal solution was kept for 48 h.

On the other hand, zinc bioremoval with dead *B. subtilis* was 26.83% (Sabae et al. 2006).

Other strains such as *B. licheniformis* also demonstrated a significant reduction in zinc concentration from 21.32 to 5.31 mg/L. The mechanism of bioremoval was an interaction between the metal ions and cell wall through weak interactions such as Van der Waals forces and covalent bonding (Blencowe and Morby 2003). In the above study, the bacterial strain was isolated from textile dye effluents which contributes to a vast chunk of the water toxicity due to metal pollution (Basha and Rajaganesh 2014).

A *B. thuringiensis* strain, ISI, was studied in agricultural effluents contaminated with heavy metals and substantial removal of zinc was observed (54%). However, after the fourth day, degradation efficiency reduced to 31%. Even though the results do not indicate a robust system of bioremediation using this strain, nevertheless, it indicates its potential use from an agricultural perspective (Kumar et al. 2015).

1.6 Conclusions and Future Perspectives

Several strains of *Bacillus* are routinely isolated and tested for their potential to remove heavy metals from soils and effluents from large-scale industries. It is important to note that various isolates of *Bacillus* vary in their extent of metal removal, detoxification and recovery. Chemical modification methods can increase/activate the binding sites on the surface of the biomass that can be achieved by pretreatment, binding site enhancement, binding site modification, and polymerization. Functionalization of long polymer chains onto the bacterial surface either by direct grafting or polymerization of a monomer can introduce functional groups. Modification by graft polymerization of acrylic acid (AAc) on the surface of ozone-pretreated biomass can be a powerful strategy for enhancing metal binding. Adsorption of metals like copper and cadmium can be significantly enhanced when the carboxylic acid group on the biomass surfaces is converted to carboxylate ions using NaOH treatment.

Further, genetic manipulation can also be employed to develop novel recombinant *Bacillus* based biosorbents. This strategy can improve or empower microorganisms with enhanced selectivity as well as the bioaccumulating properties of the cells. Simultaneously, elucidation of the underlying molecular mechanism using biotechnology will enable one to design an optimized process for bioreactors where genetically engineered *Bacillus* can be used for effective metal removal. Understanding of metal uptake mechanisms, engineered technologies, including the cell surface display technology, and a further improvement in the metal removal performance of biomass from an aqueous solution can be achieved.

Metalloregulatory protein, MerR, exhibits a high affinity and selectivity toward mercury, and can be exploited for the construction of microbial biosorbents specific for mercury removal. Whole-cell biosorbents can be constructed with MerR genetically engineered onto the surface of *Bacillus* cells by using an ice nucleation protein

anchor. This strategy for construction of surface-exposed MerR on the engineered strains can lead to a many-fold higher biosorption capacity compared to wild types of *Bacillus*. A variety of investigations have demonstrated that heavy metal removal using *Bacillus* is a useful alternative to conventional systems that can be significant for sustainable agriculture. Further investigation is required towards modelling, immobilization, and treatment of irrigational water in order to develop microbial processes for heavy metal bioremediation in agricultural fields.

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Chapter 2
Peptide Antibiotics Produced by *Bacillus* Species: First Line of Attack in the Biocontrol of Plant Diseases

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Abstract *Bacillus* spp. contain a wide arsenal of weapons against plant pathogens such as bacteria, fungi, and oomycetes, including antibiotic peptides as part of the first line of attack and plant protection. In general, such peptides, based on their biosynthetic pathways, can be of ribosomal or non-ribosomal origin. In both cases, a complex genetic structure is required that codes for different enzyme complexes. Here, we review the capabilities and advantages of *Bacillus* species to antagonize and control diseases of different crops through the production of peptide antibiotics. Finally, this chapter discusses the potential of engineered microbes and different types and mechanisms of action of non-ribosomally or ribosomally synthesized peptide antibiotics in *Bacillus* spp., with the final goal of reducing the use of toxic chemicals in agriculture.

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2.1 Introduction

Plant pathogens cause serious plant diseases that lead to great economic losses in conventional agricultural practices. The use of polluting agrochemicals is the immediate solution to control plant pathogens (Adesemoye and Kloepper 2009); however, the damage that these agrochemicals can cause to the environment, and human and animal health has been widely documented. As a result, there is a global trend to eliminate, or at least reduce, the use of agrochemicals in agriculture.

Organic agricultural production is a viable and sustainable alternative to conventional agriculture. These practices have allowed the production of grains, fruits, and vegetables in general, with reduced environmental damage (Bacenetti et al. 2016). On the other hand, the costs of the organic products are usually higher than those produced by conventional agriculture. In countries where per capita economic income is lower, such as Latin American countries, the increased cost of organic products limits their acceptance and the development of organic farming practices. Ultimately, novel solutions are necessary in order to decrease the production costs of sustainable organic agriculture.

Other options to control plant diseases in an eco-friendly way, and which have been suggested for decades, include the use of biological control agents such as beneficial fungi (i.e. *Trichoderma* spp.) and plant-growth-promoting bacteria (PGPB) such as *Bacillus spp.* (Santoyo et al. 2019).

PGPB can stimulate plant growth directly through the synthesis of phytohormones or substances that improve nutrition or nutrient uptake by the plant, or, indirectly, which involves antagonistic action towards potential pathogens of vegetable crops (Ghazanfar et al. 2018).

PGPB are part of the associated plant microbiome and are usually selected by the plant. PGPB can inhabit in environments such as the rhizosphere, which is defined as the part of the soil that surrounds the root system and is influenced by exudates from the roots or can colonize internal plant tissues without causing harm. The PGPB found in the rhizosphere are called rhizobacteria or plant-growth-promoting rhizobacteria (PGPR) (Vacheron et al. 2013). Likewise, PGPB found colonizing internal plant tissues are known as endophytic bacteria or plant-growth-promoting bacterial endophytes (PGPBE) (Santoyo et al. 2016).

Various PGPR and PGPBE have been studied for their direct or indirect abilities to promote plant growth and health. These include members of the Firmicutes such as, *Bacillus* spp. and *Paenibacillus* spp.; Actinobacteria such as *Streptomyces* spp. *and Arthrobacter* spp.; and several members of the Proteobacteria such as

Rhizobium spp., *Paraburkholderia* spp., *Pseudomonas* spp., *Pantoea* spp., and *Serratia* spp., to mention but a few (Glick 2012). The application of these PGPB have improved the yield of crops such as lettuce, cucumber, tomato, canola, wheat, maize, tomato, soybean, bean, sunflower, rice, cotton, beet, watermelon, sugar beet, blueberry, husk tomato, as well as various tree species like beech, scotch pine, black spruce, jack pine, white spruce, pine, mangrove, mango, apple, and spruce (Lucy et al. 2004).

The antagonistic effects of PGPB against plant pathogens can occur through a wide variety of mechanisms. For instance, Biocontrol PGPB can synthesize multiple volatile (i.e. hydrogen cyanide) and diffusible compounds (i.e. pyoluteorin, phenazines, pyrrolnitrin, siderophores, 2,4-diacetylphloroglucinol), lytic enzymes (i.e. proteases, cellulases, chitinases, and β-glucanases) as well as other compounds (i.e. bacteriocins, antibiotics) that inhibit the growth of potential plant pathogens, including bacteria, fungi, and oomycetes (Ongena and Jacques 2008).

In this chapter, we focus on the genus *Bacillus* and its different species with capacities to synthesize a group of peptides, of ribosomal and non-ribosomal origin, with antagonistic activity against fungal and bacterial pathogens of various plant crops.

2.2 Generalities of Antagonistic *Bacillus* Species

Some generalities of beneficial bacteria of the *Bacillus* genus have been described recently by Santoyo et al. (2012, 2019), as well as in some other chapters included in this book. Briefly, it is the genus *Bacillus* that was first reported by Cohn in 1872 (Cited in Santoyo et al. 2019). It was originally described as a species of bacteria that tolerated heat and produced endospores. Currently, just over 300 species have been described. *Bacillus* are ubiquitous and can be found in a wide variety of environments, including bulk and rhizospheric soil, where they are particularly abundant among the plant microbiome (Alcaraz et al. 2010; Bazinet 2017).

Each of the PGPB species or genera have different beneficial capacities, as well as advantages versus other genera. In the case of *Bacillus*, the ability to form spores allows them to be excellent candidates to be biological agents of commercial inoculants. It has been observed that *Bacillus* spores can be stored for a long time, remaining viable until inoculation in the field, and can survive even in adverse conditions such as saline or abiotic stress (Villarreal-Delgado et al. 2018). In fact, for several years, species of the genus *Bacillus*, such as *B. pumilus*, *B. subtilis*, *B. circulans*, *B. thuringiensis*, *B. megaterium*, *B. coagulans, B. amyloliquefaciens*, *B. licheniformis*, *B. methyltrophicus* or *B. toyonensis*, have stood out as effective bioinoculants (either as biofertilizers, biostimulants and biocides) due to their consistent results (Flores et al. 2020; Glick and Skof 1986; Lucy et al. 2004; Rojas-Solis et al. 2020).

2.3 *Bacillus* Peptide Antibiotics

Several species of the genus *Bacillus* are able to synthesize a wide range of antimicrobial peptides and therefore are viewed as a promising candidates for biotechnological applications and precursors in the search for new inhibitory compounds (Chen et al. 2019). These species have been the object of particular interest because of their safety, their ubiquity, and their ability to survive adverse environmental conditions (Al-Thubiani et al. 2018; Seel et al. 2018) (Fig. 2.1 and Table 2.1).

Bacillus antimicrobial compounds constitute a large collection of significant molecules that present an important ecological role and impact in the microbial community (Andersson and Hughes 2014). Their sub-inhibitory concentrations can trigger nonlethal physiological responses like induction of quorum sensing, antibiosis, defense responses in the host plants, production of virulence factors or biofilm formation, and competition for nutrient sources and space (Xu et al. 2019). Implication of peptide antibiotics by *Bacillus* species in biocontrol of phytopathogens causing aerial, soil, and post-harvest diseases in turn results in improved fitness in plant environment, thus ensuring sustainable agriculture. Among *Bacillus*, the production of peptide antibiotics have been recognized for *Bacillus subtilis*, *B. amyloliquefaciens, B. thuringiensis, B. cereus,* and *B. licheniformis,* but they were also reported in other Bacillus species (Saxena et al. 2019). These peptides fall into two categories, non-ribosomal peptides (NRP) (i.e. gramicidin, tyrocidine, bacitracin, glycopeptides, lipopeptides) and ribosomal peptides or natural peptides, including bacteriocins, and can be classified according to peptide biosynthesis, structure, and molecular weight. We will briefly summarize here with emphasis on three main families (surfactin, iturins, fengycins) and bacteriocins in detail.

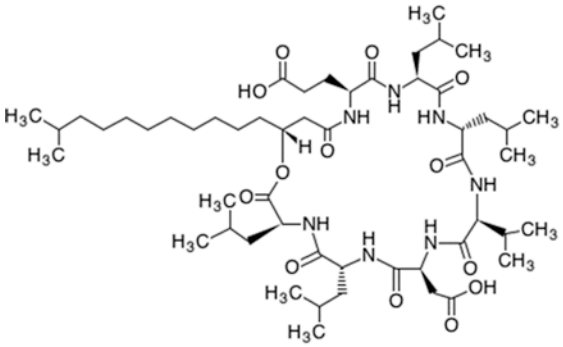
2.4 Biosynthesis and Characteristics of *Bacillus* Peptide Antibiotics

Peptide antibiotics produced by *Bacillus* species are divided into two subgroups as mentioned above on the basis of synthesis pathway (Horwood et al. 2004; Myo et al. 2019). One of these subgroups includes small microbial peptides which are non-ribosomally produced by large enzymatic complexes, while the second subgroup constitutes ribosomally synthesized peptide (Fira et al. 2018).

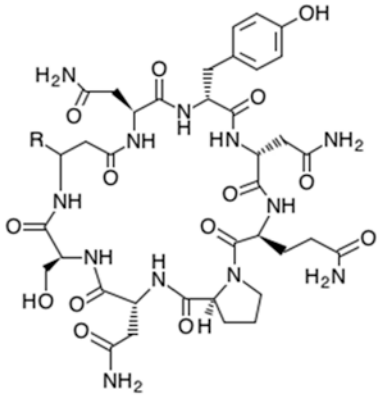
2.4.1 Non-ribosomal Peptide Antibiotics

Bacillus antibiotics include non-ribosomal peptides (NRPs) such as gramicidin, tyrocidine, bacitracin, or lipopeptides such as surfactin, iturins, and fengycins, which are synthesized by non-ribosomal peptide synthetases via multistep

Surfactin structure from *Bacillus amyloliquefaciens*



Iturin A structure from *Bacillus subtilis*



Fengycin structure from *Bacillus amyloliquefaciens*

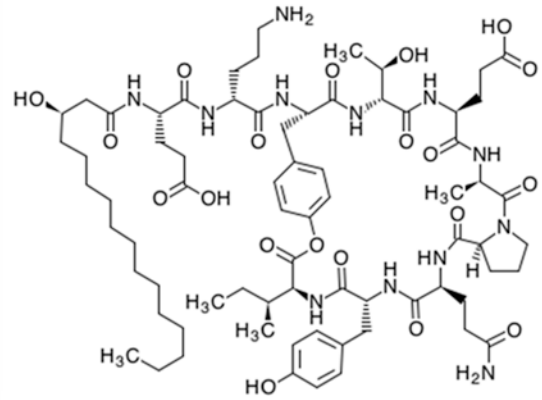


Fig. 2.1 Structures of surfactin, fengycin, and iturin A from *Bacillus* spp. Chemical structures were retrieved from KEGG COMPOUND Database (https://www.genome.jp/kegg/compound/)

Table 2.1 *Bacillus*-derived non-ribosomally or ribosomally synthesized peptide antibiotics. General description, types, and species of *Bacillus* that produces the antibiotic peptide

Surfactin family			
Description	Types	*Bacillus* spp.	References
Surfactin family is composed of heptapeptides with chiral carbon central sequence interlinked by *b*-hydroxy fatty acid to form cyclic lactone ring structure. Displays antiviral, antimycoplasma, antifungal, and antibacterial activities.	Surfactin, esperin, Hallobacillin, pumilacidin, linchenysin WH1fungin	*Bacillus subtilis, B. Amyloliquefaciens B. pumilus B. Thuringiensis B. Licheniformis B. firmus*	Hu et al. (2020) Penha et al. (2020) Kim et al. (2010) Roongsawang et al. (2011) Kakinuma et al. (1969)
Iturin family			
Description	Types	*Bacillus* spp.	References
Cyclic lipopetides contain a heptapeptide (L- AsnD-Tyr-D-Asn-L-Gln-L-pro-D-Asn-L-Ser) cyclized with a fatty amino acid, displaying a strong antimicrobial activity. Their efficiency against various phytopathogens is similar to available chemical pesticides. Iturin family has a wide spectrum of antibiotic activity, confers low toxicity, low allergic effects, high biodegradability, and are best candidates as environmentally safe biological pesticides	Iturin A, iturin C, Iturin D, iturin E, Bacillomycin D, bacillomycin F, bacillomycin L, bacillomycin Lc, mycosubtilin.	*Bacillus subtilis B. Amyloliquefaciens B. cereus B. Methyltrophicus B. Licheniformis*	Dang et al. (2019) Mizumoto et al. (2007) Shi et al. (2018) Yuan et al. (2012) Béchet et al. (2013)
Fengycin family			
Description	Types	*Bacillus* spp.	References
Peptides are synthesized by five synthetases (*FenC, FenD, FenE, FenA, and FenB)* that interlock to form a chain. Exhibit moderate surfactant activities. Regulation of fengycin genes is controlled by DegQ (enhancer of extracellular protease production), which strongly inhibits the growth of filamentous fungi	Fengycin, plipastatin, agrastatin1	*B. Amyloliquefaciens Bacillus subtilis B. Atrophaeus B. circulans*	Guo et al. (2014a) Roongsawang et al. (2011) Romero et al. (2007)
Bacteriocins			
Description	Types	*Bacillus* spp.	References
Heterologous group of proteinaceous antimicrobial substances produced by bacteria from every major lineage. They display a high degree of target specificity and are Ribosomally synthesized.	Lantibiotics Subclasses: Mersacidin, Sublancin Paenibacillin Haloduracin Lichenicidin Subtilosin A	*Bacillus subtilis Bacillus circulans B. Thuringiensis B. Megaterium B. coagulans*	Riley and Wertz (2002) Jack et al. (1995) Abriouel et al. (2011)

mechanisms that involve the selection and condensation of amino acid residues (Leães et al. 2016)(Tan et al. 2020). NRPs are assembled from more than 300 precursors and may assume linear or cyclic structures containing hydroxyl groups, *L*-amino acids, or *D*-amino acids. These peptides can be further modified by N-methylation, acylation, glycosylation, or heterocyclic ring formation (Tajbakhsh et al. 2017).

Bacilli NR lipopeptides are represented by three major families, namely surfactins, iturins, and fengycins (Dunlap et al. 2011). The surfactin family encompasses several structural variants (e.g. esperin, lichenysin, pumilacidin) but all members are heptapeptides associated with a *b*-hydroxy fatty acid and present a cyclic lactone ring structure (Tsuge et al. 1999).

Surfactins were the first members among the cyclic lipopeptides to be identified and characterized (Nair et al. 2016). These are stable in extreme environments such as high salinity, heat stress, and extreme pH conditions (Liu et al. 2015) and are involved in *Bacillus* motility and biocontrol functions in the rhizosphere (Sabaté et al. 2020). It was reported that surfactins are essential not only for efficient root colonization but also for reduction of infection caused by *Pseudomonas* species *(Pseudomonas syringae)* on *Arabidopsis* plants (Bais et al. 2004).

Iturins, which consist of iturin A-E, bacillomycin D, F, L, and mycosubtilin, are a group of antifungal cyclic heptapeptides mainly produced by *Bacillus* species that present a wide range of antagonistic effects against phytopathogens (Romero et al. 2007). Iturin A possesses potential antagonistic activity against several plant pathogens and is also used in the treatment of human and animal mycoses because of its low toxicity and lack of allergic effects on the host (Jin et al. 2014; Peng et al. 2014). It is synthesized by non-ribosomal peptide synthetases and its biosynthetic operon constitutes four genes, *ituD*, *ituA*, *ituB*, and *ituC* (Tsuge et al. 2001). The *ituD* gene plays a central role in the synthesis of Iturin A since it encodes a malonyl coenzyme A transacylase. A mutation in the *B. subtilis* RB14 *ituD* gene carries a phenotype where iturin A synthesis is lacking. With respect to the other genes, *ituA* encodes for a 449-kDA protein with three functional domains with high homology to fatty acid synthetase, amino acid transferase, and peptide synthetase. The other two genes, *ituB* and *ituC*, both encode for peptide synthetases with sizes of 609- and 297-kDa, respectively (Tsuge et al. 2001; Yao et al. 2003).

Work carried out by Yao and colleagues in 2003 characterized the role of the operon in the synthesis of iturin A, based on analyses of biocontrol strains of *Bacillus subtilis*. For example, *B. subtilis* strain B3 exhibits antagonism against pathogens like *Fusarium graminearum*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, and *Pyricularia grisea* (Yao et al. 2003).

In the case of the *B. subtilis* RB14 strain, antagonism towards *Rhizoctonia solani* has been observed, being the production of iturin A and mycosubtilin the underlying mechanisms of inhibiting the growth of this pathogen causing the damping off

disease in tomato (Asaka and Shoda. 1996). Iturin A and mycosubtilin are both members of the iturin group, whose structures are very similar (Ongena and Jacques 2008; Stein 2005). Overproduction of mycosubtilin by the biocontrol strain *Bacillus subtilis* BBG100 enhances hemolytic activity compared with that of the wild-type strain; however, this overexpression is also associated with an enhanced antifungal activity (Leclère et al. 2005). Iturin production has been reported in other several *Bacillus* species such as *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, and *B. methyltrophicus* (Kupper et al. 2020; Li et al. 2020a; Roslan et al. 2020; Yao et al. 2003).

Fengycin family contains B-hydroxy fatty acid that has a side chain length of 16–19 carbon atoms and is synthesized by five biosynthetic genes; *ppsA*, *ppsB*, *ppsC*, *ppsD*, and *ppsE* (Batool et al. 2011). It is composed of two isoforms, which differ in their amino acid residue at position 6, mainly fengycin A and fengycin B (Falardeau et al. 2013). Numerous Bacillus species are able to produce both isomeric forms of fengycin i.e. A and B (Wang et al. 2004). In *Bacillus amyloliquefaciens* Q426, fengycin A was more abundant than B, while, *B. subtilis* F29–3 generally produces twice as much fengycin B as A (Shu et al. 2002; Zhao et al. 2014). Fengycin reportedly has strong antifungal activity, particularly against filamentous fungal species like *Rhizoctonia solani*, *Monolinia laxa*, and *M. fruticola* (Alvarez et al. 2012; Guo et al. 2014b). (Deleu et al. 2005). Moreover, they play a functionally redundant role in defense against the inhibition of *R. solanacearum* as well as banana *Fusarium* wilt caused by *Fusarium* species (Cao et al. 2018).

2.4.2 *General Mechanism of Action of* Bacillus *Lipopeptides*

Due to their lipophilic nature, *Bacillus* lipopeptides present an increased affinity for biological membranes and their lipidic layers. As a result, *Bacillus* lipopeptides bind to the membranes of competing microbes and act as antimicrobials by disrupting membrane functions (Jourdan et al. 2009).

When present at increased concentrations, surfactins extensively bind to the membrane lipid layers (through its fatty acid moiety) inducing an increased membrane permeabilization and the formation of irreversible pores and mixed micelles. These effects ultimately lead to the disruption and solubilization of the membrane lipid bilayer. Curiously, the disruption and solubilizing effects of surfactins are decreased when sterols are present in the membrane (a trait mostly found in fungi), which may explain its low fungicidal activity (Ongena et al. 2007). Like many other natural antimicrobial peptides, its physicochemical characteristics, rather than its specific amino acid sequence, have been proposed to be the key to its antimicrobial activity (Findlay et al. 2010; Heerklotz et al. 2004).

Iturins and fengycins also bind to the membrane lipidic fraction; however, contrarily to surfactins, these seem to be unaffected by sterol components and present an increased fungitoxicity. Despite binding to membrane lipids, the action of iturins

and fengycins results from a mechanism of action that does not solely rely on membrane solubilization. For example, Patel and colleagues (2011) observed that fengycins possessed a decreased ability to form micelles and solubilize membranes; however, they presented a strong pore-forming ability even at very low concentrations. Ultimately, the powerful pore-forming abilities of iturins and fengycins may impact the osmotic balance as well as the acquisition/maintenance of important ions/nutrients by cells, therefore, leading to an abnormal cell function and cell death (Ongena et al. 2007).

Bacillus lipopeptides can also induce plant defense responses; however, these do not seem to be caused by strong modifications in plant membrane functions (Ongena et al. 2007; Jourdan et al. 2009; Cawoy et al. 2014). Possibly, this occurs due to the fact that plants possess different sterols (phytosterols) in their membranes, as well as strong cell walls that limit the entrance of *Bacillus* lipopeptides. Still, lipopeptides may bind to specific receptors present in the plant cell and induce the activation of signaling cascades involved in plant defense. If so, *Bacillus* lipopeptides can be considered as Microbial Associated Molecular Patterns (MAMPs). Nevertheless, these molecular interactions remain to be conclusively proven.

2.4.3 Ribosomally Synthesized Peptide Antibiotics

Bacteriocins are microbicidal (or microbiostatic peptides), produced by a wide range of bacterial species, including *Bacillus* species (Abriouel et al. 2011). Bacteriocins have a wide variety of characteristics, such as a range of molecular masses (contain between 12 and 50 amino acid residues), heat stability, posttranslational modifications, and are typically cationic with greater structural diversity being largely distributed in nature (Marx et al. 2001). Bacteriocins belong to the group of peptides which are small, heat stable, amphiphilic protein components that act against target cells mainly via interaction with cell envelope (Papagianni 2003). *Bacillus*-originated bacteriocins like subtilosin A, plantozolicin, and subtilomycin are well studied as antibiotics (Li et al. 2020c; Nikiforova et al. 2016). They are classified into four major classes; Class I contains small ribosomally produced and posttranscriptionally modified peptides (lantibiotics); Class II contains heat-stable unmodified peptides; Class III contains large heat-labile antimicrobial peptides; and Class IV contains complex bacteriocins carrying lipids or carbohydrates moieties (Cotter et al. 2013; Zhao and Kuipers 2016). Bacteriocin production have been reported in several *Bacillus* species such as *B. pumilus*, *B. velezensis, B. altitudinis*, and *B. laterosporus* (Li et al. 2020b).

Most bacteriocins have been studied for their antibiotic activity against animal and human pathogens in lactic acid bacteria (Abriouel et al. 2011); however, there are some examples of bacteriocins isolated from *Bacillus* strains of rhizospheric soil

origin. For example, *Bacillus subtilis* strain 14B was isolated from the rhizosphere of healthy bitter almond plants. Such strain, 14B, produces a bacteriocin-like inhibitory substance (BLIS) active against the tumor-producing *Agrobacterium tumefaciens*, reducing the percentage of infected plants (Hammami et al. 2009).

Thuricin 17 is a peptide belonging to the Class II bacteriocins produced by some strains of *B. thuringiensis* and *B. cereus* (Lee et al. 2009). Thuricin 17 has been characterized from the plant growth-promoting rhizobacterium *B. thuringiensis* NEB17, a strain isolated from soybean root nodules. Thuricin 17 is encoded by three copies in tandem of the same structural gene in the NEB17 genome and has a 3160-Da protease-sensitive peptide stable at a high temperature and within the pH range of 1.5–9.0 (Gray et al. 2006). Application of this bacteriocin to leaves or roots directly stimulated the growth of soybean and corn plants. Interestingly, the beneficial effect of Thuricin 17 was similar to the application of Nod factors in plants. (Lee et al. 2009).

Pseudomonas syringae is a bacterial plant pathogen that causes serious damages in diverse agronomically important plant crops, such as tomato, kiwifruit, pepper, olive, and soybean (Lamichhane et al. 2015). Recently, Rooney and colleagues (2019) demonstrated that one bacteriocin, putidacin L1, which was expressed at high levels in Arabidopsis and in *Nicotiana benthamiana in planta*, provided an effective resistance against multiple pathovars of the pathogen *Pseudomonas syringae.* The authors concluded that the expression of bacteriocins *in planta* might be an effective tool for managing bacterial diseases in crops.

2.4.3.1 General Mechanisms of Action of Bacteriocins

It has been proposed that different classes of bacteriocins exert their activity dependent on the target bacteria, if it is against Gram-positive or Gram-negative bacteria. Although it must be said that, in general, the action is mainly due to damage to the integrity of the cell wall. Also, it has been observed that there is an antibiotic action through the inhibition of DNA or protein synthesis. Additionally, it can be mentioned that bacteriocins are able to bind to different components of the cell wall (i.e. phospholipids). Likewise, bacteriocins bind to specific or nonspecific receptors, allowing the formation of pores. This leads to a loss of the membrane structure or cell lysis (Kumariya et al. 2019).

Moreover, they specifically target a particular subset of pathogenic strains through the formation of pores leading to the dissipation of membrane potential and the efflux of small metabolites from the sensitive cells (Lv et al. 2020). Antibiotic, antifungal, and anticancer activities have also been described for bacteriocins production by *Bacillus thuringiensis* strains (Raddadi et al. 2009).

2.5 Engineering of Peptide Antibiotics

Modifying what nature has built has always been attractive to science. Therefore, engineering or genetic modification of coding elements for non-ribosomal antibiotics is part of any biotechnological strategy. The objective may differ, but in general it is about improving the capacities or action spectra of such antibiotic peptides. Likewise, the genomic modification of the microorganisms that produce them is also part of a strategy to reduce production or purification costs at the industrial level, mainly. Exploiting the potential of *Bacillus* spp. to synthesize diverse antibiotic peptides with antibacterial and/or antifungal activity has been always important to control plant pathogens (Moyne et al. 2001). Unfortunately, efforts to engineer or modify *Bacillus* species or the production of non-ribosomal peptides are scarce. One possible explanation is the number of elements involved in the synthesis of such peptides, which requires several enzyme functions. However, some strategies have been proposed to modify such *Bacillus* cell factories. For example, the environmental strain widely studied as a pathogen biocontrol agent, *Bacillus amyloliquefaciens* FZB42, exhibits a broad range of action against phytopathogenic bacteria, fungi, and nematodes, through the production of several non-ribosomal cyclic lipopeptides and polyketides. Therefore, and although it has not been done, the modification of a global regulatory gene, *degU*, which controls the synthesis of bacillomycin D and bacilysin in the strain FZB42, has been proposed (Qiao et al. 2014).

Bioengineered bacteriocins have also been recommended as promising alternatives to already existing antibiotics due to their effectiveness and nontoxicity in both animals and humans. In part, because of the availability of narrow- and broad-spectrum peptides, their possibility in model membrane systems and their potential cytotoxicity reported against eukaryotes and erythrocytes (Abriouel et al. 2011).

Some genetic engineering methods have been listed by Ahmad and colleagues (2017), including mutagenesis, gene fusion, and/or specific amino acid inserts, in order to design or improve some functions of bacteriocins. For example, solubility at neutral pH has been improved by incorporating lysine residues or the stability and spectrum of some bacteriocins such as pyocin S-35 and microcin V have also been improved. Other molecular tools such as gene fusion have also achieved modifications in the spectrum of bacteriocins.

2.6 Conclusion

The antibiotic potential of peptides produced by bacteria of the *Bacillus* genus is broad and promising. However, more research is needed in the area of peptide stability for efficient action against pathogens prevalent in the field, where diverse environmental stresses affect the activity of such peptide antibiotics. Likewise, it is required to further explore its potential based on genetic modification techniques using *Bacillus* spp. like microbial factories. Lipopeptide extraction and purification

techniques are important to obtain a product as pure as possible, which will result in rapid commercialization and effective application in different agricultural systems. Techniques such as acid precipitation, ultrafiltration, solid phase extraction, and chromatography are some of the tools most used in the study of lipopeptides, mainly those characterized and isolated from *Bacillus* strains (Valenzuela-Ruiz et al. 2020). The study and optimization of the aforementioned processes will allow increasing the field application of the different peptides with antimicrobial activity, reducing the use of agrochemicals and taking agriculture to terms of sustainability.

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Chapter 3
Discovery of Bioactive Natural Products from *Bacillus* Species: Chemistry, Biosynthesis and Biological Activities

Musrat Zahan Surovy, Shahinoor Rahman, Zerihun T. Dame, and Tofazzal Islam

Abstract *Bacillus* is one of the most widely distributed genera of bacteria. It produces various groups of bioactive compounds such as cyclic lipopeptides, surfactins, iturins, fengycins, various polyketide-derived nonribosomal peptides, linear lipopeptides, enzymes, and δ-endotoxins with strong surfactant, antimicrobial, and insecticidal properties. Biosynthesis and chemistry of these bioactive natural products are largely been known. Genes involved in the biosynthesis of some of the bioactive compounds produced by *Bacillus* spp. are used in the genetic engineering of plants and microorganisms. Among them, genetically engineered Bt-crops significantly improved yield and quality of several crops. Recently developed CRISPR-Cas genome editing technology would facilitates the large-scale application of new genes of *Bacillus* involved in biosynthesis of bioactive compounds for promoting crop production and drug industry. This chapter comprehensively reviews chemistry, biosynthetic pathways, and biological activities of natural products discovered

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in various strains of *Bacillus* spp., and discusses their application in biocontrol of plant diseases, medicines, and bioprospecting.

Keywords *Bacillus* spp. · Linear lipopeptides · Biocontrol agent · Bioremediation · CRISPR-Cas genome editing

3.1 Introduction

Microbes are the prospective source of bioactive natural compounds (Bhardwaj et al. 2017; Islam and Tahara 2005; Islam et al. 2005; Islam 2011; Tareq et al. 2015; Chakraborty et al. 2020a, b). The discovery of bioactive natural products from microbes has been an important scientific discovery of the early twentieth century (Fleming 1941; Stoica et al. 2019). The production of microbial secondary metabolites was regulated by the specific genetic constitution and growing conditions of microbes. *Bacillus* species are one of the leading sources of bioactive natural products (Matloub et al. 2019; Mondol et al. 2013). This genus of bacteria is ubiquitous as they can tolerate a wide range of environmental conditions (Rampelotto 2010; Mondol et al. 2013). The higher capability of biofilm formation and the quorum sensing mechanisms help them to survive even in extreme environmental conditions. Cohn first proposed the genus *Bacillus* in 1872 (Feng et al. 2016). From 2008 to 2018, 167 new species of *Bacillus* were described globally (https://www.bacterio.net/bacillus.html). However, a vast majority of the *Bacillus* species remain undiscovered. Many of the *Bacillus* spp. are used as model organisms in laboratory studies for many years. Biotechnological industries have employed these bacteria for synthesizing a broad range of biologically active compounds and enzymes. Due to the lack of outer membrane and natural competence, genetic manipulation of *Bacillus* is faster than many other bacteria (Drejer et al. 2018). The members of the *Bacillus* genus are known for their biological control efficiency. They are strong biocontrol agents against many notorious phytopathogens, promote plant growth by phytohormone production, and induce systemic resistance in the host plants (Islam et al. 2016a, b, 2019a, b; Rahman et al. 2018; Ansary et al. 2018; Tareq et al. 2015; Chakraborty et al. 2020a). Strains of *Bacillus* produce diverse metabolites such as lipopeptide, peptides, and polyene substances (surfactin, iturin, and fengycin) (Surovy et al. 2019). These metabolites suppress the growth and development of phytopathogens and thus are useful as a biopesticides (Kefi et al. 2015; Qiu et al. 2017). Bacteriocins and bacteriocin-like inhibitory substances, nonribosomal biosynthesized peptides, and nonpeptide-based antibiotics are also produced by the *Bacillus* species. Subtilin, ericin S (active against *Clavibacter michiganensis*), ericin A, subtilosin A, sublancin 168, and mersacidin were also isolated from this genus of bacteria. Extracellular polysaccharides with antioxidant capacity (Qiu et al. 2017) from *Bacillus* sp. protect them from starvation and drastic

environmental conditions (Osta Oliveira et al. 2017). These polysaccharides are used as bio-thickener, texture stabilizers, or gelling agents to improve food texture and quality (Matloub et al. 2019).

Indiscriminate use of pesticides for crop production and toxic industrial waste results in the deposition of toxic heavy metals (Pd, Cu, Cd, Cr, Hg, Co, etc.) in the soil and water bodies. Detoxification of a trace amount of these heavy metals requires a huge amount of energy and biochemical activities. Interestingly, many strains of *Bacillus* spp. have the capacity to remediate toxic heavy metals and radionuclides from wastewater and natural waters (Bernard et al. 2018). They reduce the toxic heavy metals from the environment through accumulation, complexation, and reduction processes (Mondol et al. 2013).

The Bacilli are a promising group of bacteria producing diversified biologically active secondary metabolites with a novel mechanisms of action (Islam et al. 2016a, b, 2019a, b; Surovy et al. 2019; Dame and Islam 2019) (Fig. 3.1). This group of bacteria are also widely used in agriculture in controlling insect pests and pathogens. They can produce crystal proteins (δ-endotoxins) with specific activity against certain insect species, nematodes, mites, and protozoa. Moreover, they produce a good number of extracellular compounds including- chitinase, phospholipases, proteases, insecticidal proteins, and antibiotic compounds with antifungal activity (Nkiru and Franklin 2020; Rong et al. 2020). Intensive studies on the relationship between biological activities and molecular structures would help their industrial production as novel biopesticides, biofungicides, and drugs (Penha et al. 2020). The

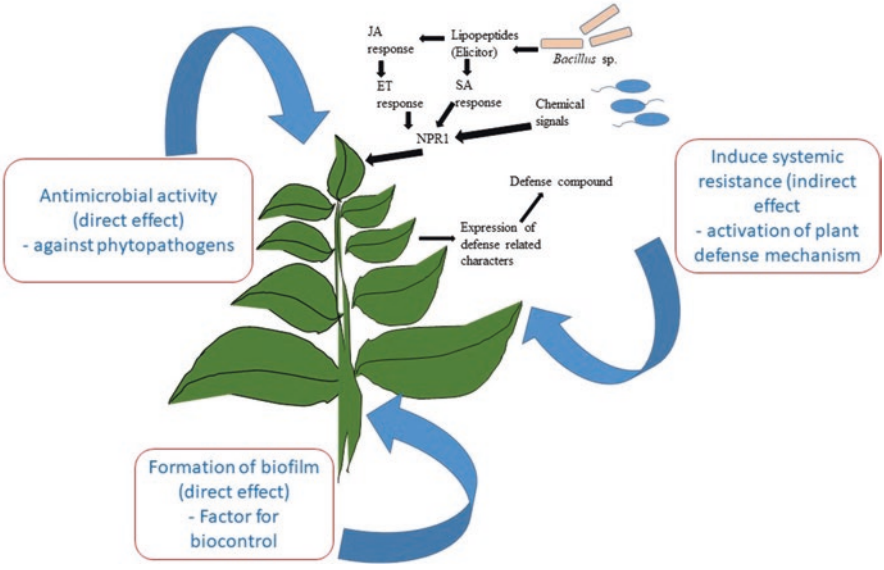

Fig. 3.1 Relationship between bioactive compounds from *Bacillus* species and their effects on plant. (JA jasmonic acid, ET ethylene, SA salicylic acid, NPR1 nonexpressor of pathogenesis-related genes 1)

molecular mechanisms of biosynthesis of bioactive metabolites from metabolites by *Bacillus* spp. are already been known and some of the responsible genes or biosynthesis pathways are exploited in the development of insect-resistant crop plants (like- corn, cotton, and brinjal) by genetic engineering. Further genomics and post-genomics studies are needed for better understanding the underlying molecular mechanisms involved in the production of diverse bioactive metabolites produced by *Bacillus* species. Recently developed CRISPR-Cas toolkits would facilitate to apply the genetic information in the practical field of crop improvement and industrial production of medicinally important compounds.

The application of Bacilli in agrobiotechnology and industry has been discussed in several books and reviews (Islam et al. 2016a, b, 2019a, b; Surovy et al. 2019; Dame and Islam 2019). A large body of literature describes the chemistry, biosynthesis, and biological activities of metabolites produced by *Bacillus* species (Mondol et al. 2013). This chapter comprehensively summarizes the biosynthesis, chemistry, and activities of diverse metabolites discovered from *Bacillus* species and discusses the application of this new knowledge in promoting sustainable agriculture and bioprospecting.

3.2 Isolation and Taxonomic Diversity of Bacilli

There are numerous protocols established for the isolation of *Bacillus* species from the environmental samples. Polanczyk and Alves (2004) proposed an effective modified isolation method for *Bacillus* species. According to their proposal, there are two steps in the isolation procedure *of Bacillus* - (i) bacterial growth in solid medium, and (ii) sporulation of bacteria in liquid medium supplemented with antibiotics under 12–48-h incubation period. This isolation method seems more efficient than the previously described World Health Organization developed method (World Health Organization (WHO) 1985).

The genus, *Bacillus* is one of the largest groups of bacteria under the phylum Firmicutes. More than 370 species and 8 subspecies with validly published names have been reported (www. Bacterio. net/ bacillus. Html, accessed: August 3, 2020). Bacilli are aerobic, Gram-positive, and endospore-producing rod-shaped bacteria (Cutting 2011; Cote et al. 2015). They are widespread in the environment and found in dust, soil, water, air, plants, and even in extreme environments. They have been isolated from a wide range of environments such as desert soil, forest soil, mineral pool, soda lake, alcohol fermentation pit mud, seawater, sediments, rice paddies, dried foods, milk, and honey (Zheng et al. 2020; Lee et al. 2016; Al-Thubiani et al. 2018). The endospores of *Bacillus* are generally oval, round, or cylindrical in shape. The exposure to air is not necessary for their sporulation (Bergey et al. 1975). Based on the morphology of the spore and sporangium, *Bacillus* sp. Are broadly divided into three subgroups (Savini 2016; Gillespie and Hawkey 2006). Group 1: Gram positive, produce central or terminal, cylindrical or ellipsoidal spore without distend sporangium. This group consists two subgroups. *B. anthracis, B. cereus,*

B. mycoides, B. thuringiensis, and *B. megaterium* under the large cell subgroup and *B. pumilus, B. subtilis*, and *B. licheniformis* under small cell subgroup. Group 2: Central or ellipsoidal spores with gram variable swollen sporangia. *B. circulans, B. coagulans, B. alvei, B. brevis,* and *B. marcerans* are under this group. Group 3: Gram variable, swollen sporangia with terminal or subterminal spores. *B. sphaericus* belongs this group. In recent years, the taxonomy of two selected groups of *Bacillus* species has been developed. They are *B. subtilis* group (*B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, *B. mojavensis, B. vallismortis, B. clausii, B. atrophaeus, B. amyloliquefaciens, B. licheniformis, B. sonorensis, B. firmus, B. lentus*, and *B. sporothermodurans*) and *B. cereus* group (*B. anthracis, B. cereus, B. mycoides, B. pseudomycoides B. thuringiensis*, and *B. weihenstephanensis*) (Fritze 2004; PHEBacteriology-Identification 2018). Detailed phylogeny and taxonomy of agriculturally important *Bacillus* species have recently been reviewed by Dunlap (2019).

3.3 Purification and Identification of Bioactive Compounds

Bacillus species is one of the most studied species for the production of bioactive compounds (Table 3.1). Till date *B. subtilis* and *B. amyloliquefaciens* are the commonly studied groups of bacteria (Cawoy et al. 2015; Penha et al. 2020). Different media like brain heart infusion broth (Perez et al. 2017), nutrient broth (Soares et al. 2016), landy medium (Chen et al. 2016), and YPG medium (Gong et al. 2015) are used for culture and extraction of metabolites produced by *Bacillus* species. During fermentation, *Bacillus* sp. produce a huge amount of foam due to their surfactant nature. This intense foam production is the main challenge in the production of bioactive compounds that affect continuous recovery and identification of the compounds (Coutte et al. 2013). The use of rotating disk bioreactors can perform fermentation without producing foam and help in the industrial production of bioactive compounds from the strains of *Bacillus* species. Bubble-less membrane bioreactor, solid-state fermentation reactors, and biofilm reactors are also used for recovery of compounds but rotating disk bioreactors perform the best. The yield from the rotating disk bioreactor is significantly higher than any other reactor (Penha et al. 2020). The use of a bioreactor with continuous removal of foam from the fermentation chamber is good for the productivity of bioactive compounds (Barros et al. 2008).

Extraction of bioactive compounds from *Bacillus* sp. is generally performed by two methods. Firstly, adding ethyl acetate to the cell-free supernatant in addition with NaCl (30 g/L). The ethylacetate fraction is concentrated from the homogenized samples using a rotary evaporator (Dimkić et al. 2017). Secondly, use of acid precipitation method. In this method, the cell-free supernatant is adjusted in pH 2.0 with HCl. The precipitate from the suspension is resuspended in methanol. Methanol is more suitable to dissolve bioactive compounds (Asari et al. 2017; Dimkić et al. 2017). After the successful extraction of compounds, it is important to perform a finer purification process. A finer purification of extracts is essential

Table 3.1 List of bioactive compounds, their identification method, and biological activities from various strains of *Bacillus* spp

Species and strain	Bioactive compounds	Identification method	Inhibited pathogenic organisms	References
Bacillus methylotrophicus DR 08	Difficidin and oxydifficidin	Bioassay-guided chromatographic fractionation and spectroscopic analyses	*Ralstonia solanacearum*	Im et al. (2020)
B. amyloliquefaciens EZ1509	Surfactin, iturin, fengycin, and bacilysin	MALDI-TOF-MS analysis	*Sclerotinia sclerotiorum*	Farzand et al. (2020)
B. amyloliquefaciens	Fengycin	LC–MS.MS analysis	Phytopathogenic fungi	Kaki et al. (2020)
Bacillus sp.	Kurstakins, iturins, surfactins, and fengycins	HPTLC and MALDI-TOF MS analyses	*Xanthomonas arboricola* and *Pseudomonas syringae*	Dimkić et al. (2017)
B. subtilis DSM 10^{T}, *B. amyloliquefaciens* DSM 7^{T} and *B. methylotrophicus* DSM 23117	Iturin, surfactin, and fengycin	HPTLC (silica gel 60 plates—Merck)	Phytopathogens	Geissler et al. (2017)
B. amyloliquefaciens DSM 23117	Surfactin	HPLC (Luna C18 reversed phase column, equipped with a Luna C18 pre-column)	*Botrytis cinerea*	Pretorius et al. (2015)

for the identification of a bioactive compound. Thin layer chromatography, high performance thin layer chromatography, liquid column chromatography, and high pressure liquid chromatography are extensively been used for the purification of the compounds. Complete identification of compounds requires several spectroscopic methods such as various mass spectroscopy and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Penha et al. 2020) (Fig. 3.2).

3.4 Bioactive Compounds Isolated from *Bacillus* spp.

3.4.1 Lipopeptides

Lipopeptides are small secondary metabolites that contain 7–10 cyclic amino acids (including 2–4 D amino acids) and 13–19 C atoms of beta-hydroxy fatty acid (Zhao et al. 2017). They are classified into two groups: cyclic and linear lipopeptides. Surfactin, iturin, fengycin, lacilomycin, and bacilotetrins are cyclic lipopeptides

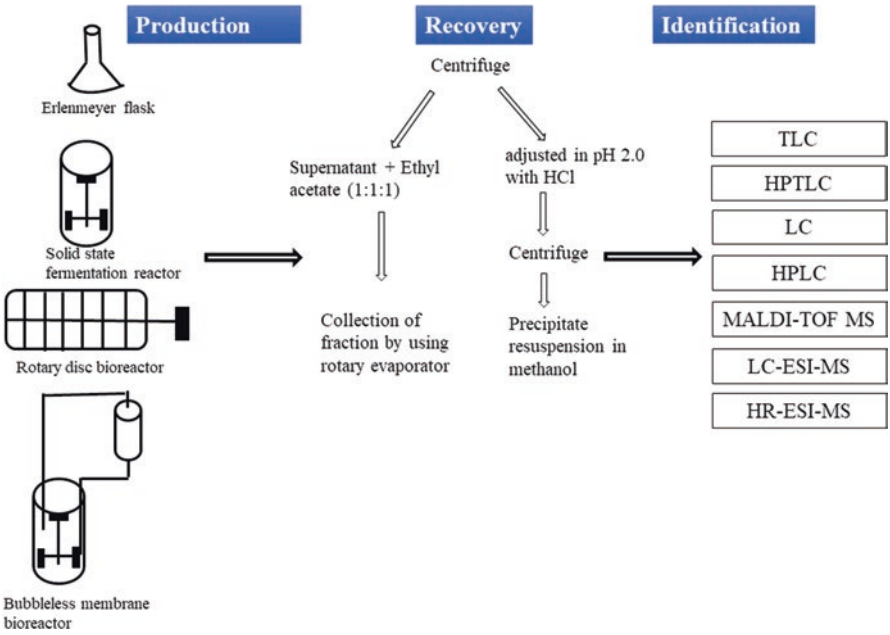

Fig. 3.2 Overview of production, recovery, and identification of bioactive compounds from *Bacillus* sp. (Adapted from Penha et al. 2020. *TLC Thin Layer Chromatography, HPTLC High Performance Thin Layer Chromatography, LC Liquid Chromatography, HPLC High performance Liquid Cromatography, MALDI-TOF MS Matrix Assistierte Laser Desorption/Ionisierung (MALDI) with the flight analysis (Time of Flight, TOF) released ions to mass spectrometry, LC-ESI-MS Liquid Chromatography/Electrospray Ionisation Tandem Mass Spectrometry, HR-ESI-MS High Resolution/ Electrospray Ionisation Tandem Mass Spectrometry

derived from *Bacillus* sp. On the other hand, gageotetrins, gagoepeptides, isocoumarins, amicoumacin, bacilosarcins, damxungmacin, hetiamacin, clorotetain, rhizocticins etc., are under linear polypeptides group. Lipopeptides directly act against fungi and bacteria and can induce systemic resistance (ISR) in plants against phytopathogens (Ongena et al. 2007) (Table 3.2). For example, *B. velezensis* SQR9 triggers ISR against *P. syringae* pv. tomato (Pst DC3000) and *Botrytis cinerea* phytopathogens in *Arabidopsis* plantlets (Wu et al. 2018). The surfactins, iturins, fengycins or plipastatins, and the kurstakins are the major families of lipopeptides produced by *Bacillus* species (Jacques 2011; Dimkić et al. 2017).

Surfactin (Fig. 3.3a) is a heptapeptide with LLDLLDL chiral central sequence which is internally linked by a β-hydroxy fatty acid. Surfactin, esperin, halobacillin, and pumilacidin are the main members of surfactin family (Penha et al. 2020). Surfactins are widely studied for their biological activities. They have antiviral, antimycoplasma, and antibacterial activities (Ongena and Jacques 2008). Amphiphilic structure of surfactin has led to its broad range of antibiotic treatment, cancer therapy, oil recovery, and biomedical applications. Under optimum conditions, wild type of *Bacillus* sp. can yield a large scale of surfactins for industrial

Table 3.2 List of lipopeptides produced by *Bacillus* sp. and their biological activities

Species and strain	Lipopeptide	Biological activity	Reference
Bacillus amyloliquefaciens FZB42	Surfactin	Support colonization and acquisition of nutrients through surface-wetting and detergent properties	Borriss (2020)
B. velezensis	Surfactin	Biofilm formation	Chen et al. (2007)
B. subtilis	Iturin	Antibiotic production	Dunlap et al. (2019)
B. safensis B21	Iturin A and Iturin B	Affect hyphal membrane permeability of blast fungus, *Magnaporthe oryzae*	Rong et al. (2020)
B. amyloliquefaciens HAB-2	Bacillomycin D	Roughening and damage of cell membrane of *Burkholderia pseudomallei*	Rajaofera et al. (2020)
B. megaterium (KC246043.1)	Bacitracin	Antimicrobial activities against *Micrococcus luteus, S. typhi*, *E. coli, P. aeruginosa*, and *S. aureus*	Al-Thubiani et al. (2018)
Bacillus sp. CND-914	Halobacillin	Anticancer activity	Trischman et al. (1994)
Bacillus sp.	Loloatin B	Antibacterial activity	Mondol et al. (2013)
B. laterosporus	Basiliskamides A and B	Antifungal activity	Barsby et al. (2002)
B. subtilis	Gageopeptides A-D (1–4) and Gageotetrin B (5)	Antifungal activity	Chakraborty et al. (2020a) and Mondol et al. (2013)

purposes. Biofilm produced by *Salmonella enterica* is destroyed by the lytic activity of *Bacillus* sp. (Kaspar et al. 2019).

Iturins (Fig. 3.3b) are also an important class of lipopeptides. They are monomeric or neutral lipopeptides with LDDLLDL chiral sequence with a restricted number of *Asx, Glx, Pro, Ser, Thr, Tyr* residues (Bonmatin et al. 2003). Iturin A, iturin C, bacillomycin D, bacillomycin F, bacillomycin L, bacillopeptin, and mycosubtilin belong to the iturin family (Penha et al. 2020). Iturin A composed of the heptapeptide NYNQPNS is linked to a β-amino fatty acid (Hiradate et al. 2002). They are widely studied for their antibiotic activities and are mostly produced by *B. subtilis* group (Ongena and Jacques 2008). Dunlap et al. (2019) found 330 iturinic lipopeptide clusters in *B. subtilis*. *B. amyloliquefaciens, B. siamensis,* and *B. velezensis*. Each isolate produces more than one lipopeptide (Dunlap et al. 2019). Cyclic lipopeptides (Iturin and surfactin) from *Bacillus* sp. have a broad range of antimicrobial activity against fungi (Phae et al. 1990). Both lipopeptides have the capacity to elicit induced systemic resistance (ISR) in the host plants (García-Gutiérrez et al. 2013; Jourdan et al. 2009; Kawagoe et al. 2015). They play an important role in protecting strawberry plant from the pathogens. They trigger plant

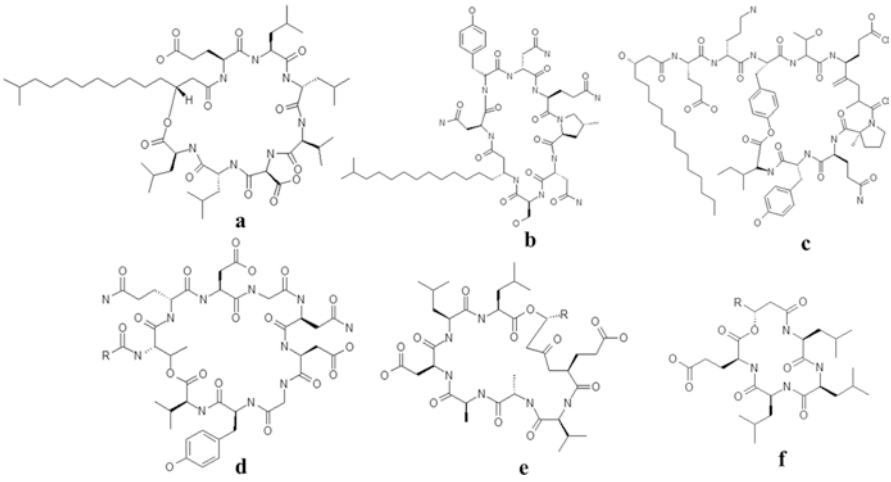

Fig. 3.3 Chemical structure of some lipopeptides (**a**) surfactin; (**b**) iturin; (**c**) fengycin; (**d**) locillomycin A (n =10), B (n =11) and C (n =12); (**e**) bacilotetrin; (**f**) gageopeptin

defense response against *Colletotrichum gloeosporioides* (Yamamoto et al. 2015). *Bacillus* LPs also induce defense responses in rice (Chandler et al. 2015), *Arabidopsis* (Kawagoe et al. 2015), tomato (Abdallah et al. 2017), and maize (Gond et al. 2015).

Nonribosomal peptides are a class of peptide secondary metabolites. Ten percent (10%) of *B. amyloliquefaciens* FZB42 genome possesses genes for NPRS. It produces antifungal lipopeptide bacillomycin D and the antibacterial polyketide difficidin, which are important for plant protection (Borriss 2020). Fengicin (Fig. 3.3c) was first discovered by Vanittanakom et al. in 1986. Fengycin A, fengycin B, plipastin A, and plipastin B are the main members of the fengycin family (Penha et al. 2020). This group is mainly responsible for antimicrobial activity against a wide range of filamentous fungi and yeasts (Zihalirwa et al. 2017). Fengycins A and B differ only in one amino acid residue. Fengycin A contains a D-Ala ring and Fengycin B contains a D-Val ring in their structure. They are less toxic to plants but have selective activity against filamentous fungus (Kaspar et al. 2019).

The plipastatins from *B. cereus* are related to fengycins. These are inhibitors of phospholipase A2 of *B. cereus* (Volpon et al. 2000). The structures of plipastatins are almost identical to fengycin counterparts, the only difference is the two *Tyr* stereocenters. Locillomycins (Fig. 3.3d) are cyclic lipopeptides isolated from a Chinese *B. subtilis* 916 strain. Gene cluster of *Bacillus* sp. encodes for *locA-locD* genes. This is potential in controlling *S. aureus* and *X. oryzae* but commercial applications are very limited due to their low fermentation yields (Luo et al. 2015).

Marine *B. subtilis* strain 109GGC020 produces bacilotetrin (Fig. 3.3e) and gageopeptin (Fig. 3.3f) (Tareq and Shin 2017; Tareq et al. 2015). They are moderately antibacterial and antifungal against *Colletotrichum acutatum, Botrytis cinerea, Staphylococcus aureus*, and *Pseudomonas aeruginosa* in broth dilution assay. Gageopetin A strongly inhibits the growth of *P. capsici* (Tareq et al. 2015).

Wheat blast is a destructive fungal disease caused by *Magnaporthe oryzae Triticum* (*MoT*) pathotype. It poses a serious threat to food security of South America, South Asia, and Africa (Islam et al. 2016a, b, 2019a, b, 2020). Recently, Chakraborty et al. (2020a) demonstrated that gageopeptides A–D and gageotetrin B isolated from a marine *B. subtilis* strain 109GGC020 inhibited the growth of *MoT* mycelia in a dose-dependent manner. Application of these natural compounds has also completely blocked formation of conidia in the *MoT* fungal mycelia in the agar medium. Further bioassay revealed that these compounds inhibited the germination of *MoT* conidia and, if germinated, induced deformation of germ tube and/or abnormal appressoria. Interestingly, application of these linear lipopeptides from strain 109GGC020 significantly suppressed wheat blast disease on detached wheat leaves. A further study is warranted to test the efficacy of wheat blast control at field conditions, and also to evaluate the mode of action of these natural compounds for considering them as biopesticides for managing this notorious cereal killer.

3.4.2 Polyketides/Lipoamides

Polyketides are a large association of multifunctional polypeptides with a series of catalytic domains (ketosynthase, KT; acyl transferase, AT; acyl carrier protein, ACP). They are an essential group of secondary metabolites, generally synthesized through decarboxylative condensation of carboxylic acids by polyketide synthases (PKSs) (Borriss 2020). *B. amyloliquefaciens* FZB42 has a special class of PKSs, which require a discrete AT enzyme and lack the cognate AT domain (Shen 2003). Type III polyketides catalyze the *B. amyloliquefaciens* FZB42 for priming, extension, and cyclization reactions to customize a huge array of various polyketide products (Yu et al. 2012). The *bspA* and *bspB* operons of *B. subtilis* are involved in the synthesis of triketide pyrones. The *bspA* synthesizes alkylpyrone and *bspB* operon acts on the alkylpyrones to produce alkylpyrone methyl ethers (Nakano et al. 2009). Aurantinin B (Fig. 3.4a), an antibacterial polyketide, was originally isolated from

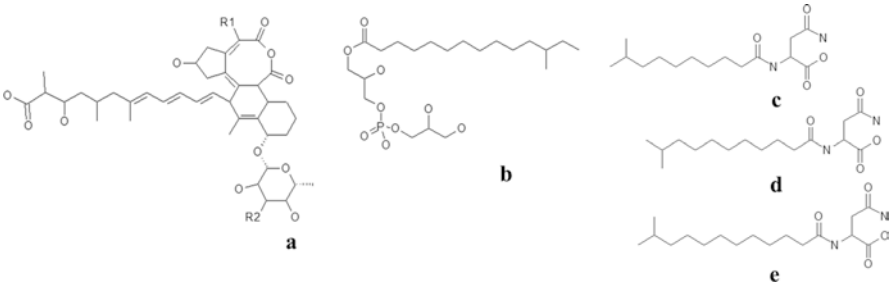

Fig. 3.4 Chemical structure of polyketides (**a**, **b**) and lipoamides (**c**, **d** and **e**) (**a**) aurantinin B (R1 = Me; R2 = O=), C (R1 = Me; R2 = OH), and D (R1 = H; R2 = O=); (**b**) bacilysocin; (**c**) lipoamide A (N = 5); (**d**) lipoamide B (N = 6); (**e**) lipoamide C (N = 7))

B. aurantinus (Kaspar et al. 2019). Aurantinins C and D (Fig. 3.4a) are two new analogs of aurantinin that are isolated from *B. subtilis* Fmb60. These two aurantinins possess very strong antibacterial activity against *M. luteus* and *B. pumilis*. These compounds disrupt cell wall and cell membrane with a highly selective mode of action (Yang et al. 2016).

Bacilysocin (Fig. 3.4b) is a unique example of modified phospholipid, which does not originate from non-ribosomal peptide synthetase or polyketide pathway of *B. subtilis*. This is very weak in controlling Gram-positive bacteria and its mode of action is still unrevealed (Tamehiro et al. 2002).

Furanoids and pyranoids were also isolated from a marine alga-associated *B. subtilis* (Chakraborty et al. 2017; Chakraborty et al. 2016). They are biosynthsized via hitherto polyketide synthase like pathways and moderately active against *Vibrio parahaemolyticus* (Chakraborty et al. 2017). Lipoamides A-C (Fig. 3.4c, d and e) were isolated from culture broth of *B. pumilus*. Lipoamide A is a weak antibacterial compound against *P. aeruginosa* and *S. aureus* with minimum inhibitory concentration >100 μg/mL (Mondol et al. 2013).

3.4.3 Isocoumarins

The isocoumarins comprise of a large diverse class of natural compounds (Saeed 2016). The first reported isocoumarins from *Bacillus* were amicoumacins A-C (Fig. 3.5a, b) and AI-77-F (Fig. 3.5c) (Shimojima et al. 1984; Kaspar et al. 2019). The *amiA-O* genes of *B. subtilis* utilize a genomic capture and with the help of expression vector enable to produce amicoumacins A-C in heterologous hosts. They are potential for drug development due to their potential antibacterial and anti-inflammatory properties (Li et al. 2015).

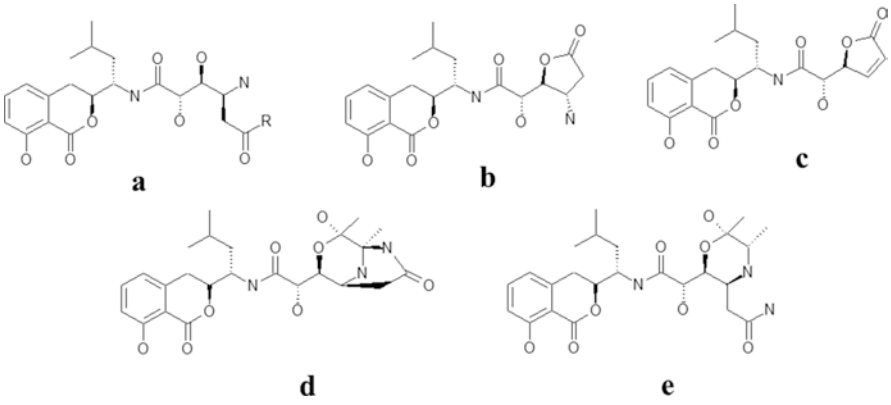

Fig. 3.5 Chemical structures of isocoumarins (**a**) amicoumacin A (R = NH_2), B (R = OH); (**b**) amicoumin C; (**c**) AI-7-F; (**d**) bacilosarcin A; (**e**) bacilosarcin B

Bacilosarcins A and B (Fig. 3.5d and e) are marine-derived bioactive compounds from *B. subtilis* TP-BO611. They showed herbicidal activity (Azumi et al. 2008).

The *B. subtilis* subsp. *subtilis* produces psoralen, 4, 6-dimethyl-3 coumarin, and xanthotoxin (all are member of coumarin group). The bioautophagy test showed that these compounds are capable of inhibiting the growth of *P. oryzae* race 173 (Wiraswati et al. 2020).

3.4.4 Fatty Acids

For synthesis of fatty acid, *Bacillus* sp. uses fatty acid synthase pathway (Mondol et al. 2013). Haplophilic *Bacillus* spp. produces iso-anteiso fatty acids (Fickers 2012; Li et al. 2011) with C_{11} to C_{19} but their biological activity still unknown (Carballeira et al. 2001). Ethanoic extract of marine *Bacillus* sp. 09ID194 contains ieodomycins A (Fig. 3.6a), B, C, and D (Tareq et al. 2013), the unsaturated hydroxyl fatty acids. These fatty acids are antimicrobial against *E. coli* and *S. cerevisiae* (Mondol et al. 2013).

3.4.5 Macrolactins

Polyene macrolactones with 24-membered rings of lactones are attached to glucose β-pyranoside. *B. amyloliquefaciens* FZB42 produces difficidin (59) (Fig. 3.6b) and oxydifficidin (60) (Fig. 3.6b). Difficidin inhibits the protein synthesis of *Erwinia amylovara.* This fungus causes blight diseases in apple, pear, and other rosaceous fruits (Mondol et al. 2013).

Macrolactin A is a potential compound to inhibit murine melanoma cancer cell and Herpes simplex virus *in vitro* (Mondol et al. 2013). Macrolactin G from marine *Bacillus* sp. PP19-H3 is active against *S. aureus,* whereas keto group (C=O) containing macrolactin K showed weak antibacterial activity (Nagao et al. 2001). *B. marinus* produces macrolactin T and macrolactin U. Macrolactin T exhibited

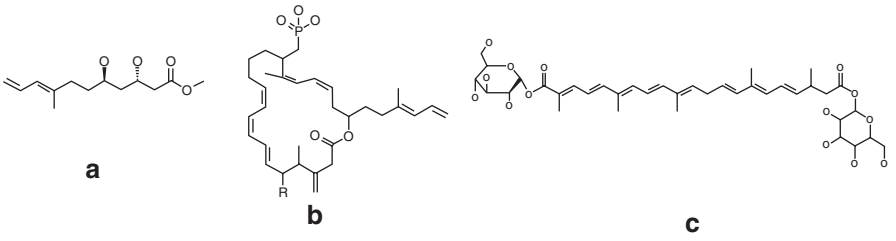

Fig. 3.6 Chemical structures of ieodomycin A (**a**), macrolactins (**b**) difficidin (R = H) and oxydifficidin (R = OH), and glycosylated carotenoid (**c**)

weak inhibitory activity towards *A. solani, P. oryzae*, and *S. aureus* (Xue et al. 2008). *B. amyloliquefaciens* SCSIO 00856 produces macrolactin V, a potential antibacterial agent against *E. coli* and *S. aureus* (Gao et al. 2010). Macrolactin W, X, Y, and Z were also isolated from *Bacillus* sp. 09ID194 in low saline medium (Mondol et al. 2011). Marine *Bacillus* sp. Sc026 produces macrolactin A, 7-0-succinyl macrolactin F and 7-0-succinyl macrolactin A. These compounds show antibacterial activity against *B. subtilis* and *S. aureus* (Jaruchoktaweechai et al. 2000).

3.4.6 Enzymes

Bacillus spp. produce different types of industrially important enzymes (Table 3.3), such as amylases, glucanases, and proteases. The *B. siamensis* also produces enzymes used in various Asian foods (Borriss 2020). *Bacillus* sp. C-4 produces serine protease, which is helpful in the degumming process in the silk industry (Prathumpai et al. 2020). Extracellular cellulase from *B. subtilis* helps in bio-refining (Reddy et al. 2016). Amylase production by *Bacillus* sp. is enhanced by the addition of ammonium nitrate in the culture medium. *B. amyloliquefaciens* produces amylase at pH 7.0 (Tanyildizi et al. 2007). *Bacillus* sp. FPF-1 is a potential source of keratinase. This enzyme is useful for degradation of chicken feathers and very important for agro chemical sectors (Nnolim et al. 2020). Some strains of *Bacillus* spp. are also capable of producing laccases enzymes from agro-waste. The laccases are important enzymes for bio-remediation, bio-pulping, and food industry (Kumar et al. 2020).

3.5 Detoxification of Heavy Metals

Heavy metal-resistant bacteria are gaining importance because of their prospective use in bioremediation. *Bacillus* spp. are one of the promising bacteria that are potential in detoxification of heavy toxic metals from soil and water bodies (Table 3.4). The negatively charged carbohydrates and protein moiety of the extracellular polymeric substances (EPS) of *B. licheniformis* strain KX657843 are capable of binding with heavy metals [Cu (II) and Zn (II)] through electrostatic interactions. This bacterial EPS can perform better at high pH. It is documented that, at pH 8, the EPS was able to remove 86 and 81% Cu (II) and Zn (II), respectively, from a metal solution (Biswas et al. 2020). Zheng et al. (2008) reported that *B. megaterium* strain causes the highest flocculation at pH 9, and the pH range for flocculation is 7–12. The *Bacillus* sp. S3 considerably enhances the adsorption and detoxification capacity of heavy metals [Sb (III), Cu (II), Cr (VI), Cd (II), Cr (VI) and Cu (II)] by the secretion of EPS (Zeng et al. 2019). *B. cereus* AJK3 is a potential natural renewable source for bacteriogenic synthesis of nanoparticle. This nanoparticle reduced selenite

Table 3.3 List of enzymes produced from *Bacillus* sp. and their role

Species and strain	Produced enzymes	Activities of the enzymes	References
Bacillus amyloliquefaciens and *B. subtilis*	Esterase, endoglucanase, β-glucanase, pectatelyase	Degrade fungal cell wall	Dutta et al. (2018)
Bacillus sp. VITL8	Lipase	Pretreatment of food industry effluents	Balaji et al. (2020)
B. australimaris P5	Xylanase	Prebleaching of bamboo pulp fiber treatment	Dutta et al. (2020)
Bacillus sp. MD-5	Chitosanase (Csn-BAC)	Good candidate for oligosaccharide production-based industries	Yang et al. (2020a)
Bacillus sp.	Xylanase	Degrade xylan into usable products such as xylooligosaccharides, xylotetrose, xylotriose, xylobiose, and xylose	Rosli et al. (2020)
Bacillus sp. Khoz1	Protease	Act as detergents, cheese-making, baking, and meat tenderization beside medical applications	Far et al. (2020)
Bacillus sp.	Protease, amylase and cellulase	Degrade alfatoxin	Pereyra et al. (2020)
Bacillus sp. CSB55	β-glucanase	Potential bio-industrial catalyst	Regmi et al. (2019)
Bacillussubtilis US191	β-mannanase	Potential candidate for poultry feed supplement	Blibech et al. (2020)
Bacillus sp. RN1	Pectate lyases	Effective in degumming ramie fibers	Zheng et al. (2020)
Bacillus sp. NR5 UPM	β-cyclodextrin glycosyltransferase	Catalyzes transglycosylation reactions, useful for food, agriculture and pharmaceutical industries	Nik-Pa et al. (2020)
Bacillus sp.	Mannanase	Degradation of mannan, important for shrimp farming	Sumardi et al. (2020)
Bacillus sp.	Keratinolytic enzymes	Chicken feather degradation	Nnolim et al. (2020)

accumulation and remove selenite from the contaminated environment through bioremediation process (Kora 2018). Li et al. (2020) found that exogenous sulphur stress increases the chemical composition of EPS of *B. vallismortis* sp. and it doubled at 20 mg/L concentration of Na_2S. This increased EPS concentration improves the adsorption capacity of Cu (II) (Li et al. 2020). The lipopeptides of *B. subtilis* form complexes and chelate with Pb^{2+} through -OH, C-O, O=C-O, and -NH- bonding. It follows pseudo-first-order model and adsorbs Pb^{2+} from water (Zhao et al. 2020). *Bacillus* sp. TZ5 immobilizes cadmium from the plant and reduces cadmium concentration in the plant. Some of the *Bacillus* spp. also reduces cadmium from the soil, increase microbial count and enzymatic activities. Therefore, they can improve soil health significantly (Ma et al. 2020).

Table 3.4 List of heavy metal detoxifying *Bacillus* spp. with their mode of action

Species and strain	Source of isolation	Contaminant heavy metal	Mode of action	Reference
B. subtilis	Brassica juncea (Indian Mustard)	Ni	Ni accumulation	Abou-Shanab et al. (2018); Chaudhary and Shukla (2019)
B. licheniformis strain KX657843	Earthworm	Cu (II) and Zn (II)	Sorption of Cu (II) and Zn (II) and in flocculation	Biswas et al. (2020)
Bacillus sp. S3	Soil from Xikuangshan, China	Sb (III), Cu (II), Cr (VI), Cd (II), Cr (VI) and Cu (II)	Stimulate secretion of EPS	Zeng et al. (2019)
B. cereus RC-1	–	Cd (II)	Absorb Cd (II) by bacterial cell membrane	Huang et al. (2013)
Bacillus sp.	Eloor-Edayar industrial area, Kerala, India	Pb	Bioaccumulation of Pb	Varghese et al. (2012)
Bacillus sp.	Eloor-Edayar industrial area, Kerala, India	Zn	Accumulation of Zn	Krishna et al. (2012)
B. cereus AJK3	Pedda cheruvu, India	Selenite	Bioremediation of selenite	Kora (2018)
B. cereus	Gut of C. gibelio	Cadmium	Reduce cadmium toxicity	Wang et al. (2020a)
B. vallismortis	Lijiao Sewage Treatment Plant, Guangzhou	Cu (II)	Adsorb Cu (II)	Li et al. (2020)
Bacillus sp. TZ5	Agricultural soil, Guangyuan, China	Cd	Immobilize cadmium	Ma et al. (2020)
B. licheniformis	–	Cr (VI)	Reduce Chromium toxicity	Kavitha et al. (2011)
Bacillus sp. strain SG1	–	Mn (II)	Oxidize Mn (II) to MnO_2	Mondol et al. (2013)

3.6 *Bacillus* Strains as a Source of Bioactive Compounds

3.6.1 Antimicrobial Compounds

Bacillus species are one of the largest sources of bioactive compounds, which exert a wide range of antimicrobial activities (Table 3.5). They produce low molecular weight polypeptides by non-ribosomal or ribosomal synthetic mechanisms. Bacteriocins and bacteriocin-like inhibitory substances (BLIS) are ribosomally synthesized peptides produced by *B. thuringiensis, B. subtilis, B. stearothermophilus,*

Table 3.5 List of antimicrobial activities shown by *Bacillus* spp. metabolites

Species and strain	Source of isolation	Compound(s) produced	Tested pathogenic organism	Reference
Bacillus amyloliquefaciens and *B. subtilis*	Rice seeds	Bacillaene, difficidin, macrolactin, bacilysin, plipastatin, bacillibactin	*Magnaporthe oryzae*	Dutta et al. (2018) and Surovy et al. (2017)
B. subtilis	Gageocho reef, Republic of Korea	Linear lipopeptides	*M. oryzae*	Chakraborty et al. (2020a)
B. velezensis	Aquaculture system	Anti-vibrio substances	*Vibrio* sp.	Gao et al. (2017)
B. licheniformis	Mussel	Anti-microbial compounds	Distrupt initial adhesion of *V. harveyi* and *P. aeruginosa*	Hamza et al. (2015)
B. pumilus	Sponge	Nonribosomal peptides	Antagonistic against *B. cereus*	Matobole et al. (2017)
B. pumilus	Collection of marine bacteria	Pumilacidin and surfactin	Antagonistic against *Listeria monocytogenes* and *S. aureus*	Saggese et al. (2018)
B. velezensis HC6	Intestinal tract of healthy piglet	Produce iturin, fengycin, and surfactin	Multiple pathogenic fungus of maize and reduce aflatoxin, ochratoxin	Liu et al. (2019)
B. amyloliquefaciens MTCC 12716	Gulf of Mannar region, India	Polyketides panned macrolides	Bactericidal activity against *Staphylococcus aureus, Enterococcus faecalis, P. aeruginosa and Klebsiella pneumoniae*	Kizhakkekalam et al. (2020)
Bacillus subsp. *subtilis*. strain A52	Marine sediment	Sublancin, surfactin	*Candida* sp.	Sharma et al. (2020)

Bacillus strain Bam22	Rhizosphere of oilseed rape	Cyclic lipopeptide antibiotics	*S. sclerotiorum*	Yang et al. (2020b)
B. velezensis BM21	Corn rhizosphere soil	Cyclic lipopeptide	*F. graminearum* strain YJH2	Wang et al. (2020b)
B. licheniformis W10	Plant rhizosphere	Serine protease	*B. cinerae*	Ji et al. (2020)
B. siamensis CBSMS07	Kimchi	Antimicrobial peptide	*E. coli, Alcaligenes faecalis*, and *P. aeruginosa*	Khan et al. (2020a)
B. velezensis strain AL7	Cotton soil	Antifungal antibiotics	*Verticillium dahlia* Kleb	Liu et al. (2020a)
B. subtilis	Rhizosphere soil	Surfactin	*Mucor* sp. and *Aspergillus niger*	Meena et al. (2020)
B. velezensis Ll-9	Bulbs of *Lilium leucanthum*	Diketopiperazines, cyclo-peptides, linear peptides, latrunculin A, 5α-hydroxy-6-ketocholesterol, (R)-S-lactoylglutathione, triamterene, rubiadin, moxifloxacin, 9-hydroxy-5Z, 7E, 11Z, 14Z-eicosatetraenoic acid, D-erythro-C18-sphingosine, citrinin, and 2-arachidonoyllysophosphatidylcholine.	*Botryosphaeria dothidea, F. oxysporum, B. cinerea* and *F. fujikuroi*	Khan et al. (2020b)
B. subtilis (SSL2)	Root of *S. surattense*	Citrulline, chloramphenicol and carnitine	*R. solanacearum* and *F. oxysporum*	Jinal and Amaresan (2019)
B. amyloliquefaciens	–	Cis-cyclo	*Candida albicans*	Adibi et al. (2017)
Bacillus sp. Sh10	–	Bacteriocin	*Proteus mirabilis*	Shayesteh et al. (2020)
B. tequilensis SDS21	Endo-rhizosphere of *Parthenium hysterophorus*	Surfactin	Planktonic cells	Singh and Sharma (2020)
B. subtilis	Gut of *Penaeus indicus*	Bacteriocin	*Pseudomonas* sp.	Kim et al. (2020)
B. safensis B21	*Osmanthus fragrans* Lour. Fruits	Iturin A2 and A6	*Magnaporthe oryzae*	Rong et al. (2020)

B lichenifirmis, B. megaterium, and *B. cereus*. Lanthionine and β-methyl lanthionine residues are post-translationally modified peptides. The lantibiotics and subtilin are single-peptide antibiotics with strong antibacterial activity, also produced by *Bacillus* species. Subtilin is a type of bacteriocin which is structurally similar to nisin. Subtilin is used as food preservatives and capable of inhibiting a broad range of Gram-positive bacteria (Gálvez et al. 2007). Fuchs et al. (2011) isolated entianin from *B. subtilis* subsp. *spizizenii* DSM 15029, which is potential in controlling *S. aureus, E. faecalis*, and other Gram-positive bacterial pathogens. The spore-forming *B. amyloliquefaciens* subsp. *plantarum* is able to enhance the yield of crop plants (plant growth promotion) by suppressing plant pathogens (biocontrol activity) (Borriss et al. 2011). Gene expression profiling of *B. subtilis* CBR05 induced vitamin B6 (VitB6) biosynthesis and salvage pathway against *Xanthomonas campestris* pv. *Vesicatoria* in tomato (Chandrasekaran et al. 2019). The *Bacillus* sp. Fcl1 inhibits a wide range of phytopathogens by producing iturin A and a blend of surfactin compounds (Jayakumar et al. 2018). *B. velezensis* F21 helps in the expression of numerous transcriptomic factors triggering ISR in watermelon plants and reduces the incidence of Fusarium wilt in watermelon (Jiang et al. 2019). *B. subtilis* NCIB 3610 shows antiviral, antimicrobial, antifungal, and cytotoxic activities. The 2, 6-di-t-butyl-4-methyl phenol, intracellular polysaccharide fraction (FC1), and 14-methyl hexadecanoic methyl ester are produced by this bacterium, and its intracellular polysaccharide (FC1) displays a promising antibacterial activity against *S. aureus* and *S. pneumoniae* (Matloub et al. 2019). A consortium of *B. subtilis* QST-713 and *B. pumilus* is effective against Asian soybean rust. It reduced 23% disease severity in field conditions (Dorighello et al. 2015). A cyclic polypeptide characterized from n-butanol extract of *B. megaterium* (KC246043.1) shows a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria. The lowest inhibitory concentrations were 0.25, 0.5, 1.0, 3.125, and 6.25 μg/ml against *M. luteus* ATCC10240, *S. typhi* ATCC19430, *E. coli* ATCC35218, *P. aeruginosa* ATCC27853, and *S. aureus* ATCC25923, respectively (Al-Thubiani et al. 2018). The *B. methylotrophicus* DR-08 also exhibits strong antibacterial activity against *R. solanacearum*. It produces two antibacterial metabolites: difficidin and oxydifficidin (Im et al. 2020). *B. amyloliquifaciens* EZ1509 produces surfactin, iturin, fenzycin, and bacilycin. These metabolites strongly inhibit the growth of *S. sclerotiorum*. These lipopeptides modulate the cell integration and cause shrinkage, plasmolysis, and breakdown of fungal hyphae through downregulation of endopolygalacturonase-3, oxalic acid hydrolase, and endopolygalacturonase-6 resistance genes of *S. sclerotiorum* (Farzand et al. 2020). *B. amyloliquifaciens* EZ1509 also secretes potential antibacterial material ß-1, 3–1, 4-glucanase and contains genes encoding *LCI* (47 residues cationic antimicrobial peptide), yellowfin tuna GAPDH-related antimicrobial peptide, and human GAPDH-related AMPs (Zhang et al. 2020).

3.6.2 Insecticidal Compounds

The use of bacterial agents to control insect pests is considered as an eco-friendly and safe approach to increase crop production (Dihazi et al. 2012). Application of plant-beneficial *Bacillus* spp. enhances the sustainable production of diverse crops in modern agriculture with minimum use of chemical fertilizers and pesticides (Myresiotis et al. 2014). Some strains of *Bacillus* spp. can kill the larvae of the insect pest and also enhance induced systemic resistance (ISR) in crop plants (Table 3.6). Maize root colonized with *B. subtilis* can be able to uptake insecticide (thiamethoxam) and control pest infestations (Myresiotis et al. 2014). However, the mechanism of *Bacillus*-induced insect control in crops varies with insect species as well as the crop varieties (Navon 2000; Paramasiva et al. 2014; Mnif and Ghribi 2015; Wielkopolan and Obrępalska-Stęplowska 2016). Generally, they colonize in plant organs, including the phyllosphere, and the larvae and/or adult insects are exposed to bacteria during ingestion of the *Bacillus*-colonized plant tissues during feeding. A well-known bio-insecticide, *B. thuringiensis*, can control a broad range of diverse lepidopteran insects and thus useful for pest management in the agricultural field (Navon 2000) without affecting other microbial organisms within the phyllosphere (Wang et al. 2014). Generally, the primary site of bacterial antagonism begins with a severe damage to the larval midgut epithelium by the bacterial crystal proteins, which interact with chitin and peritrophic membranes (Vachon et al. 2012; Feng et al. 2015). In later stages, the crystal protein endotoxin, lipopeptides, and polyketides (fengycin, macrolactin, iturin, surfactin, bacillomycin, bacillaene, and difficidin) modify the vacuolization of the cytoplasm, induce vesicle formation, lyse brush border membrane, and degenerate apical membranes, leading to damage of microvilli and finally resulting in the death of insect larvae (Khedher et al. 2015; Boukedi et al. 2016). Surfactin attaches to the Ca^{2+} receptor site and changes the peptide composition in the cellular phospholipid bilayer (Maget-Dana and Ptak 1995), while iturin increases cell membrane permeability via the formation of ion-conducting pores (Maget-Dana and Peypoux 1994). *Bacillus* spp. trigger the expression of the jasmonic acid (JA) pathway-related genes and simultaneously increase the gene expression for other secondary metabolites (allelochemicals, which inhibit the insect larval growth) in plants to defend against insects (Zebelo et al. 2016). A large body of literature suggests that *Bacillus* sp. controls the larval population of insects and triggers the ISR mechanism and allelochemicals in plants to prevent insect damage.

A protein elicitor, AMEP412, from *B. subtilis* can trigger plant defense and induce acquired systemic resistance. When AMEP412 protein is ingested by white fly (*Bemisia tabaci*), it gets digested by gut proteases and releases hydrophobic peptides in the white fly guts. This causes pores in guts and ultimately causes insect death (Liu et al. 2020b). Recombinant *B. subtilis* 26DCryChS showed aphicidal activity against green bug (*Schizaphis graminum*). Insertion of *Btcry11a* gene in *B. subtilis* 26D genome contributed higher aphicidal activity compared to wild-type *B. thuringiensis* B-5351 and *B. subtilis* 26D. These mutant *Bacillus* species

Table 3.6 List of insecticidal *Bacillus* sp. with their mode of action

Species and strain	Tested insect	LC_{50}/LD_{50} /Doses	Mode of action	Reference
B. thuringiensis	Larvae of mosquito	Highest mortality was found after 4 h exposure of 5 ml suspension of *Bt.*	Secretion of parasporal crystals with high polypeptide toxin	Nkiru and Franklin (2020)
B. thuringiensis var. kurstaki	Larvae of *Drosophila melanogaster*	5×10^7 to 1×10^9 Colony Forming Units (CFU) of *Btk.* spores/g of fly food medium	Disturb homeostasis of the larval intestine	Nawrot-Esposito et al. (2020)
B. subtilis, *B. amyloliquefaciens* and *B. thuringiensis var. kurstaki*	Tobacco whitefly (*Bemisia tabaci*)	–	Plant protection by antibiosis and/or ISR elicitation, parasporal inclusions produced during sporulation	Arabiat et al. (2018)
B. subtilis	Whitefly (Bemisia tabaci)	15.57 µg/ml AMEP412 protein	Causes gut cell lysis	Liu et al. (2020b)
B. subtilis 26DCryChS	*S. graminum*	Stimulate *TapI*, *TaChiI* genes and induce *Tapr1*, *TaPrx* genes	Inhibit the growth of green bug and aphid in wheat plants	Maksimov et al. (2020)
B. subtilis V26	*Tuta absoluta* larvae	278.78 ng/cm^2 surfactin	Causes histological damage of the larval midgut	Khedher et al. (2020)
B. amyloliquefaciens	*Tuta absoluta* larvae	180 ng/cm^2 surfactin	Damage larval midgut	Ben Khedher et al. (2015)
B. thuringiensis subsp. *israelensis*	Mosquito larvae	–	Damage larval gut	Dahmana et al. (2020)
B. clausii DTM1	Aphid and mosquito larvae	–	Causes cell lysis	Guo et al. (2015)
B. clausii BS02	Pulse beetle and mealybug	–	Causes cell lysis	Hazra et al. (2015)
B. amyloliquefaciens, *B. subtilis*, and *B. pumilus*	Silver whitefly (*Bremisia argentifolii*)	1×10^7 CFU/g of seed or planting medium	–	Murphy et al. (2000)
B. thuringiensis	*Spodoptera frugiperda* and *S. exigua*	–	Binding of *B. thuringiensis* toxins to the *Spodoptera* midgut	Herrero et al. (2016)

(continued)

Table 3.6 (continued)

Species and strain	Tested insect	LC_{50}/LD_{50} /Doses	Mode of action	Reference
B. thuringiensis and *B. sphaericus*	Mosquito larvae (*Culex pipiens* L.)	LC_{50} = for *B. thuringiensis* 5.49×10^6 spore/ml. and for *B. sphaericus* 6.70×10^6 spore/ml	–	Kelada and Shaker (1988)
B. cereus, B. subtilis, and *B. amyloliquefaciens*	Phloem-feeding aphid (*Brevicoryne brassicae*)	1×10^8 cfu/ml suspension	–	Gadhave and Gange (2015)
B. thuringiensis	Spotted stem borer (*Chilo partellus*)	2×10^7 spores/ml	–	Brownbridge (2001)
B. thuringiensis	Rice water weevil (*Oryzophagus oryzae*) and fall armyworm (*Spodoptera frugiperda*)	LC_{50} = for *O. oryzae* 5.40 µg/mL and for *S. frugiperda* 1×10^{10} CFU/mL resulting 100% mortality	–	Berlitz et al. (2012)
B. thuringiensis subsp. *kurstaki*	Second instar larvae of *Spodoptera littoralis*	*B. thuringiensis* spore-crystal mixture corresponding to 100 mg of delta-endotoxin	Delta-endotoxins that form the crystal of *B. thuringiensis kurstaki* in addition to *Cry1Aa* and *Cry2Aa*	Benfarhat-Touzri et al. (2013)
B. thuringiensis	Larvae of (*Helicoverpa armigera*)	Concentrations 10^9–10^{11} occlusion bodies (OBs) L^{-1} of *H. armigera* NPV resulting in 89–100% mortality	–	Arrizubieta et al. (2015)

synthesize surfactin and can attack green bug. Treatment of wheat seeds with this recombinant *Bacillus* species also promoted greater resistance against aphid in wheat plants. It stimulates the transcription of jasmonate-sensitive genes (*TapI* and *TaChiI*) and induces salicylate-dependent genes (*Tapr1* and *TaPrx*) (Maksimov et al. 2020). A complex of *B. cereus* contains *P19* and *P20* genes that are able to control mosquito larvae (Dahmana et al. 2020). African catfish diet supplemented with *Thymus vulgaris* (TV) essential oil and *B. subtilis* reduces the possible risk of thiamethoxam (TMX) insecticide. When this insecticide is applied in a waterbody to control fish insects, it causes gill, liver, head, and kidney necrosis of the African catfish (*Clarias garipenus*). Feeding with TV essential oil and *B. subtilis* significantly decrease splenocyte proliferation and percentage of melanomacrophage centers as well as increase the caspase-3 immunopositive cells. *B. subtilis* reduces lead

toxicity by bioaccumulation of lead and increases the immunity of catfish (Euony et al. 2020). *Bacillus* spp. are potential alternatives to synthetic insecticides for controlling pests through an integrated pest management system.

3.6.3 Antinematicidal Compounds

Use of *Bacillus* spp. as biocontrol agents are safer for the environment and non-target organisms (humans, animals and other soil-dwelling organisms) (Gao et al. 2016). Under aerobic conditions, they form highly resistant endospores that are capable to survive in the environment for a longer period (Ongena and Jacques 2008; Padgham and Sikora 2007; Sansinenea and Ortiz 2011). These dormant spores have elevated levels of tolerance against extreme environmental conditions, such as high temperatures, nutrient and water deficiencies and unfavorable pH levels (Cawoy et al. 2011). They can colonize into plant roots (Jamal et al. 2017) and ubiquitous within rhizosphere (Sansinenea and Ortiz 2011). They promote plant growth (Abbasi et al. 2013; Lee and Kim 2015) and possess distinctive attributes as to control plant pathogenic nematodes (Table 3.7). They produce biologically active molecules (Moghaddam et al. 2014b) such as antibiotics, enzymes, exotoxins and other metabolites with nematicidal activity (Sansinenea and Ortiz 2011; Abbasi et al. 2013). From a technological viewpoint, the ability of *Bacillus* to produce heat and desiccation-resistant endospores is highly advantageous for the formulation of a stable product (Emmert and Handelsman 1999; Ongena and Jacques 2008) which has been used commercially under natural field conditions (Padgham and Sikora 2007). The endospores of nematicidal *Bacillus* spp. also contribute to the stability of bio-nematicides during storage (Rosas-Garcia 2009).

B. firmus is widely used as a bionematicidal agent (Tian et al. 2007; Wilson and Jackson 2013). It causes paralysis and mortality in the plant pathogenic nematode, *M. incognita*. It also controls burrowing nematode (*Radopholus similis*) and stem nematode (*Ditylenchus dipsaci*) (Mendoza et al. 2008). *B. firmus* reduces the infestation of sting nematode (*Belonolaimus longicaudatus*) on Bermuda grass (Crow 2014). Extracellular protease of *B. cereus* BCM2 has significant nematicidal activity against second-stage juveniles (J2) of *M. incognita.* It absorbs by the nematode body and degrade the body wall. The bacterial colonization into the plant induces systemic resistance in plants and expresses nematode defense-related genes (Hu et al. 2020a). Serine proteases and neutral protease from *B. nematocida* directly degrade the main chemical compositions of nematode cuticles and eggshell (Niu et al. 2006, 2011). However, the safety of using *Bacillus* spp. in bio-nematicides is evidenced by the US Food and Drug Administration (USFDA) granting *Bacillus* sp. the status of "generally regarded as safe" (Usta 2013). Therefore, *Bacillus* spp. are ideal bacteria for the biocontrol of nematodes. Discovery of novel species and strains of *Bacillus* spp. from diverse environments should be considered as a promising research approach for finding nematicidal bacteria with a novel mode of action.

Table 3.7 List of various nematicidal *Bacillus* spp. with their mechanisms of action

Species and strain	Tested nematode	Mode of action	Reference
B. cereus	*Meloidogyne incognita*	Produces extra cellular protease, sphingosine (a nematicidal toxin) and helps the metabolites	Hu et al. (2020a), Gao et al. (2016) and Xiao et al. (2018)
B. pumilus and *B. cereus*	*M. javanica*	Production of proteolytic enzyme	Moghaddam et al. (2014a, b)
B. firmus	*M. incognita*	Production of bioactive secondary metabolites	Jansen-Girgan et al. (2016) and Xiong et al. (2015)
B. *subtilis, B. firmus* and *B. coagulans*	*M. javanica*	Increases the enzymatic activity like superoxide dismutase, ascorbate peroxide, Guaiacol peroxidase, polyphenol oxidase etc.	Abbasi et al. (2013)
B. velezensis and *B. mojavensis*	*M. incognita*	–	Xiang et al. (2017)
B. nematocida	*Panagrellus redivius* and *Bursaphelenchus xylophilus*	Combined effect of two proteolytic enzyme, neutral protease and serine protease	Niu et al. (2006)
B. thuringiensis	*M. javanica*	Form pores in the cell membrane of gut epithelial cells	Ravari and Moghaddam (2016)
B. megaterium	*M. incognita*	Nematicidal volatiles (benzeneacetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide) produced by the bacterium	Huang et al. (2009)
B. megaterium	*M. graminicola*	Production of secondary metabolites	Padgham and Sikora (2007)
B. pumilus	*M. arenaria*	Production of crude enzymes	Lee and Kim (2015)
B. coagulans and *B. cereus*	*M. javanica*	Production of secondary metabolites	Abbasi et al. (2017)
B. methylotrophicus	*M. incognita*	–	Zhou et al. (2016)
B. amyloliquefaciens	*M. incognita*	The dipeptide cyclo (d-pro-l-Leu) act as nematocidal activity	Jamal et al. (2017)
B. cereus and *B. pumilus*	*M. javanica*	Produces extracellular protease	Moghaddam et al. (2014a)
B. licheniformis	*M. incognita*	Degrade the nematode cuticle and/or to produce toxic metabolites	Colagiero et al. (2018)

(continued)

Table 3.7 (continued)

Species and strain	Tested nematode	Mode of action	Reference
B. subtilis, B. amyloliquefaciens, *B. cereus*, *B. pumilus*	*M. incognita*	–	Sarangi et al. (2014)
B. subtilis	*M. incognita*	Production of certain enzymes i.e. protease, chitinase and gelatinase	Metwally et al. (2015)
Bacillus spp.	M. *javanica* and *Ditylenchus* spp.	–	Turatto et al. (2018)
B. firmus	*Radopholus similis, M. incognita, D. dipsaci*	Production of bioactive compounds of secondary metabolites	Mendoza et al. (2008)

3.7 *Bacillus* Isolates as a Potential Source of Natural Carotenoids

Carotenoids are widely distributed in photosynthetic microorganisms (bacteria, fungi etc.) and plants. They are usually red, orange or yellow pigments that contain a poly C_{40} carbon skeleton with one or both ends having acyclic or cyclic group backbone (Nelis and Leenheer 1991; Mondol et al. 2013). Generally, plants, fungi and algae contain C_{40} carbon skeleton and bacteria produce C_{40} or C_{30} carbon skeleton. Carotenoids from natural sources are entirely "*trans"* form. They are highly recognized for their antioxidant, pro-vitamin A and other food or feed additives properties (Mondol et al. 2013). Duc et al. (2006) provides one of the first detailed reports of pigmented marine *Bacillus* species. The most commonly found pigments were yellow, orange and pink. The most related carotenoid producing species are *B. marisflavi, B. indicus, B. firmus, B. altitudinis* and *B. safensis.* The intensity of the colour varied depending on the strain (Khaneja et al. 2009). The presence of the pink carotenoids with the main absorbance maximum at 492 nm seems to be species specific, to the *B. firmus*-related isolates. Based on reference spectra in the literature, the predominant *Bacillus* carotenoids were putatively identified to be acyclic-carotenoids and potentially monocyclic, while other reports have stated the presence of astaxanthin (Pane et al. 1996). Other studies with the coat-associated melanin of *B. subtilis* spores have shown that in absence of the spore outer coat, the resistance to UV-C was increased (Riesenman and Nicholson 2000). *B. indicus* HU36 and *B. firmus* GB1 produce useful carotenoids (Fig. 3.6c) for humans. They produce glycosylated carotenoids, which is ca. 4.5 times more bioavailable than pure β-carotene (Duc et al. 2006).

3.8 Genetics and Biosynthesis Pathways

Bacillus spp. produce diverse bioactive compounds with different biosynthetic pathways. Surfactin genes in *Bacillus* species expressed with an increase of cell number. Fatty acid synthesis II system regulates fatty acid degradation or efficiency of fatty acid according to the requirement of *Bacillus* vegetative cells (Diomandé et al. 2015). Whereas biosynthesis of fengycins and iturins occur at the late stationary growth phase. Surfactin gene determinants are solely linked to the srf operon. Posttranscriptional activation of the Srf synthetase mainly depends on Spf transferease, which is encoded by *sfp* gene situated downstream of srfABCD (Steller et al. 2004; Raaijmakers et al. 2010). The surfactin and iturin of *Bacillus* spp. played a key role in the expression of chitinase and β-1, 3-glucanase in strawberry leaves (Yamamoto et al. 2015). For fengycin biosynthesis, degQ regulates ppsABCDE plipastatin operon (Tsuge et al. 2007). *Bacillus* spp. produce lipopeptides and regulate defense-related gene expression. Higher expression of the *PDF 1.2* bacterial gene encodes for defensin and induces host defense system (Chowdhury et al. 2015). Gene expression analysis also proposes that mycosubtilin (iturin family) triggered the SA and JA signaling pathways, and surfactin prompted a SA-regulated pathway in grapevine. As a result, grapevine leaves are more tolerant against *B. cinerea* (Farace et al. 2015). The expression of mycosubtilin gene in *B. subtilis* is influenced by *AbrB* transition state regulator (Duitman et al. 2007). *B. amyloliquefaciens* produces surfactin, iturin, and fengycin, which inhibit the growth of *Clostridium difficile*. The *srfABCD*, *ituABCD*, *fenABCDE*, *bmyABC*, *baeEDLMNJRS*, *difABCDEFGHIJ*, and *mlnABCDEFGHI* gene clusters of *B. amyloliquefaciens* contribute synergistically to control *C. difficile* (Lv et al. 2020). *B. amyloliquefaciens* FZB42 produces bacillomycin D. The *DegU* gene of *Bacillus* FZB42 regulates the cellular process of *Bacillus* and *bmy* operon helps in interacting with *DegU* regulator to produce bacillomycin D (Koumoutsi et al. 2007). It also contains three PKS operons (*pks1*, *pks2*, and *pks3*). These three gene clusters organize the type I PKS system. The *Pks1* and *Pks3* gene clusters produce difficidin and oxydifficide, respectively. On the other hand, *pks2* gene cluster is involved in macrolactin biosynthesis (Chen et al. 2006; Schneider et al. 2007). The production of surfactin by the *B. amyloliquefaciens* is enhanced with addition of Fe nanoparticle in the growth medium. It stimulates the key surfactin biosynthetic pathway (Yang et al. 2020c). By using genetic recombination of *B. subtilis* Symmank et al. (2002) produced lipohexapeptide for surfactin biosynthesis. In this process, deletion of individual A and T domain creates three novel plipastatin derivatives. Recombinant [Δ*Leu3*] and [Δ*Leu6*] surfactin offer reduced toxicity, and [Δ*Asp5*] surfactin displayed higher inhibition in comparison with intrinsic surfactin against *B. pumilus* and *M. luteus* (Jiang et al. 2016). The *bpsA-ypbQ* operon encodes type III polyketide synthases (PKSs) and a methyltransferase. The *bpsA* catalyzing the synthesis of acyl-coenzyme A (CoA) thioesters and malonyl-CoA and *ypbQ* involve in the biosynthesis of aliphatic polyketides (Nakano et al. 2009). The combination of *crtM* and *crtN* genes and *pHCMC04G* plasmid of *B. subtilis* carrying synthetic operon for MEP pathway

produces carotenoids, diaponeurosporene, and diapolycopene (Xue et al. 2015). The hybrid PKS/NRPS gene cluster in marine *B. subtilis* TP-B0611 is responsible for the biosynthesis of amiocoumacins and bacilosarcins (Komaki et al. 2016). Surfactin A produced by *B. subtilis* upregulates the hypoxia-inducible factor-1α and vascular endothelial growth factor. These two factors accelerate keratinocyte signaling and regulate the pro-inflammatory cytokines and macrophage phenotypic switch of human body. As a result, it is used as a wound healing agent in medical science (Yan et al. 2020). Genetic engineering of *B. thuringiensis* overexpresses the *krsE* gene and produces kurskatin, which shows antimicrobial activity against *Galactomyces geotrichum* and *B. cinera* (Lereclus et al. 2017). Genetic engineering of lipopeptides synthetase polypeptide also produces higher amounts of iturin and surfactin, which effectively control insects and increase crop yield (Keenan et al. 2008). Ginsenoside *Rg3* is a bioactive compound that has notable biological properties. The final biosynthetic step of ginsenoside *Rg3* is glycosylation and glycosylation catalyzed by uridine diphosphate-dependent glycosyltransferase (*UGT*). A promiscuous *UGT Bs-YjiC* discovered from *B. subtilis* 168 and the C12 hydroxyl group of ginsenoside *Rg3* to unnatural ginsenoside *Rd12* selectively glycosylate by *Bs-Yjic* cloned from *B. subtilis* 168. Ginsenoside *Rd12* is effective against diverse cancer cell lines and might be a potential anticancer drug for cancer cell recovery (Hu et al. 2020b).

3.9 Conclusions and Future Perspectives

Bacillus species is a large group of bacteria that produces diverse classes of bioactive secondary metabolites (lipopeptides, polyketides, isocoumarins, carotenoids, fatty acids, lipoamides, etc.). These properties of *Bacillus* spp. make them an excellent option for using them as antimicrobial and biocontrol agents in agriculture. They also detoxify toxic heavy metals from the environment. Production of bioactive molecules from *Bacillus* sp. inexorably contribute for a clean and sustainable eco-friendly environment. The mechanisms of the biosynthesis of bioactive secondary metabolites, enzymes, and endotoxins by various species and strains of *Bacillus* are largely been known. Some of the genes involved in biosynthesis of these bioactive compounds are used in genetic engineering. Among them, genetically engineered Bt-crops such as cotton, corn, and brinjal not only increased productivity of these crops but also promoted green agriculture (reduced insecticide application). Discovery of the new biologically active strains of *Bacillus* spp. from diverse environment might lead to the development of new biologicals for promoting agriculture and industry. Recently developed CRISPR-Cas genome editing technology should facilitate to design commercially important microbes and plants using important genes discovered in the genomes of *Bacillus* species. To utilize the genome editing toolkit, further genomics and post-genomics studies are needed to dissect the underlying molecular mechanisms of biosynthesis of diverse bioactive compounds by the *Bacillus* species.

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Chapter 4
The Industrially Important Enzymes from *Bacillus* Species

Aurelio Ortiz and Estibaliz Sansinenea

Abstract *Bacillus* sp. genus produces several interesting extracellular enzymes, which have been used in a commercial way for detergent, textile, food, feed, and beverage industries. The improvement of strains and production strategies has recently been influenced or facilitated by the application of molecular biology techniques. *Bacillus* sp. species are attractive industrial organisms due to their short growth time, capacity to secrete proteins, and safety for humans. The enzymes that are secreted by many species of *Bacillus* can be used in several industrial processes. In this chapter, we revise the most important enzymes produced by *Bacillus* spp. species that are applied in industry reviewing the current status of their applications.

Keywords Enzymes · Industrial applications · *Bacillus* sp.

4.1 Introduction

Bacillus species have been used worldwide in several fields such as agriculture and biotechnology due to the production of antipathogenic chemicals, or triggering the host defense mechanism and predation against enemies and pathogens. *Bacillus* species have the ability to replicate rapidly, to generate spores when they do not find enough nutrients, and they have biocontrol ability, making them versatile and competitive weapons for pathogens.

They have been applied in several industries such as, textile, food, detergent, and beverages industries because they secrete extracellular proteins and enzymes, which are completely dissociated from the cell and found free in the surrounding medium. Genetic engineering has been the tool that has allowed the strain development to apply these biopesticides. Since *Bacillus* species have many advantages, including that they are classified as safe organisms, they are widely employed in biotechnology and industry. Besides, as they produce many compounds including enzymes, which have been applied in several industries, they are often considered microbial

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factories (Ortiz and Sansinenea 2019). This capacity allows a variety of industrial applications of this genus.

The industrial enzymes world market is 1.6 billion $US, (Schallmey et al. 2004); *Bacillus* spp. enzymes being the half of the total enzyme market. The enzymes produced by this genus have several applications in many industries. In this chapter we summarize the industrially important enzymes produced by *Bacillus* spp reviewing the current status of their applications.

4.2 Proteases

Proteolytic enzymes are one of the most interesting enzymes within the world market. These enzymes decompose proteins by hydrolyzing their peptide bonds having many applications in industrial processes, highlighting the search for new proteases as a research topic that it has been developing (Sharma et al. 2017). In this sense, the genus *Bacillus* produces neutral and alkaline proteolytic enzymes in high yields, with remarkable properties, such as their high stability to wide range of pHs and temperatures and their molecular weight between 27 and 71 KDa (Contesini et al. 2017). For example, some *Bacillus* species produce alkaline serine proteases which are applied as household detergents, while neutral zinc metalloproteinases are used in milk protein modification among others (Schallmey et al. 2004).

It is important to reduce their production cost, and the use of some agro-industrial wastes can be an alternative. However, the modern techniques such as genetic engineering have been studied and applied to produce proteases in good yields (Jaouadi et al. 2012).

One of the major applications of proteases is in the detergent industry to remove stains from fabrics. However, there are some features of proteases that are important to take into account, such as several compounds present in detergents that can inactivate the proteases (Annamalai et al. 2013). Besides, due to their stability in organic solvents they can be used in organic syntheses (Caille et al. 2002).

Proteases can be classified into four main groups such as serine-proteinase, cysteine proteinases, the aspartic-, carboxyl-, or acidic proteinases, and metalloproteases. Most extracellular proteases produced by *Bacillus* are serine proteases (Adinarayana et al. 2003), cysteine proteases (Rozs et al. 2001) and metalloproteases (Sookkheo et al. 2000).

For protease isolation and purification, they can be isolated using organic solvents or ammonium sulfate followed by chromatography purification (Anbu 2013). Their tertiary structure can be elucidated using X-ray diffraction of proteins from their crystal structure (Nonaka et al. 2004).

The production of these proteolytic enzymes should take into account the physiology of the producing microorganism and the genetic engineering to increase the production yield. Besides, some steps at industrial scale are very expensive therefore the cost of the fermentation process should be considered (Kirk et al. 2002). Efficient protease production also depends on the substrate concentration,

physicochemical parameters, the initial pH, agitation, incubation time, and incubation temperature (Gupta et al. 2003). Genetic engineering can optimize the production by two ways: the improvement of biochemical and catalytic properties of these enzymes, altering specific amino acids in the protein structure, and the improvement of the proteases production enhancing secretion of homologous proteases.

Proteolytic enzymes and other enzymes such as lipases and amylases are used in detergent industry to remove proteinaceous stains. In food industry the proteases can generate bioactive peptides producing hydrolyzed peptides with important biological activities. Besides, *Bacillus* proteases have been applied on the food maturation process. In pharmaceutical industry the proteases are applied to some organic syntheses to obtain compounds with good yields reducing the use of organic solvents. In textile and leather industries, the proteases have been applied for hydrolyzing keratin, an insoluble fibrous protein (Contesini et al. 2017).

4.3 Amylases

This enzyme breaks down the starch into dextrin, maltose, and finally glucose units. They are applied in starch liquefaction, paper, desizing of textile fabrics, and preparing starch coatings of paints among others (Tanyildizi et al. 2005); therefore, the current amylase production implies 65% of enzyme market. Microbial amylase can be highly and easily produced by several microorganisms. *Bacillus* spp. amylases are applied in many industrial processes, such as the food, fermentation, textile, and paper industries (Pandey et al. 2000). α-Amylase (Fig. 4.1) from *Bacillus* spp. cleaves internal α-1,4-linkages, has a certain thermostability, and operates at 95 °C; therefore it is applied to liquefy starches, especially cornstarch, which only gelatinizes at 100 °C. On the other hand, *Bacillus* β-Amylases operate to remove maltose units from starch.

To improve the enzyme production the culture conditions can be optimized. Utilization of waste materials provides low-cost media and solves the environmental pollution problem (Kalyani and Rajesh 2018). Moreover, crude amylase can be applied in detergent industry and in starchy materials saccharification (Simair et al. 2017).

4.4 Galactosidases

α-Galactosidase hydrolyzes α-1,6-linked galactoside residues from simple oligosaccharides and the polysaccharides of galactomannans. It also catalyzes transglycosylation reactions (Carneiro et al. 2018) being applied in the beet sugar industry, pulp and paper industry, soya food processing, and animal feed processing. It is applied to remove oligosaccharides from soy-based foods avoiding the production of flatulence in the human small intestine (Patil et al. 2010). It is also used in blood

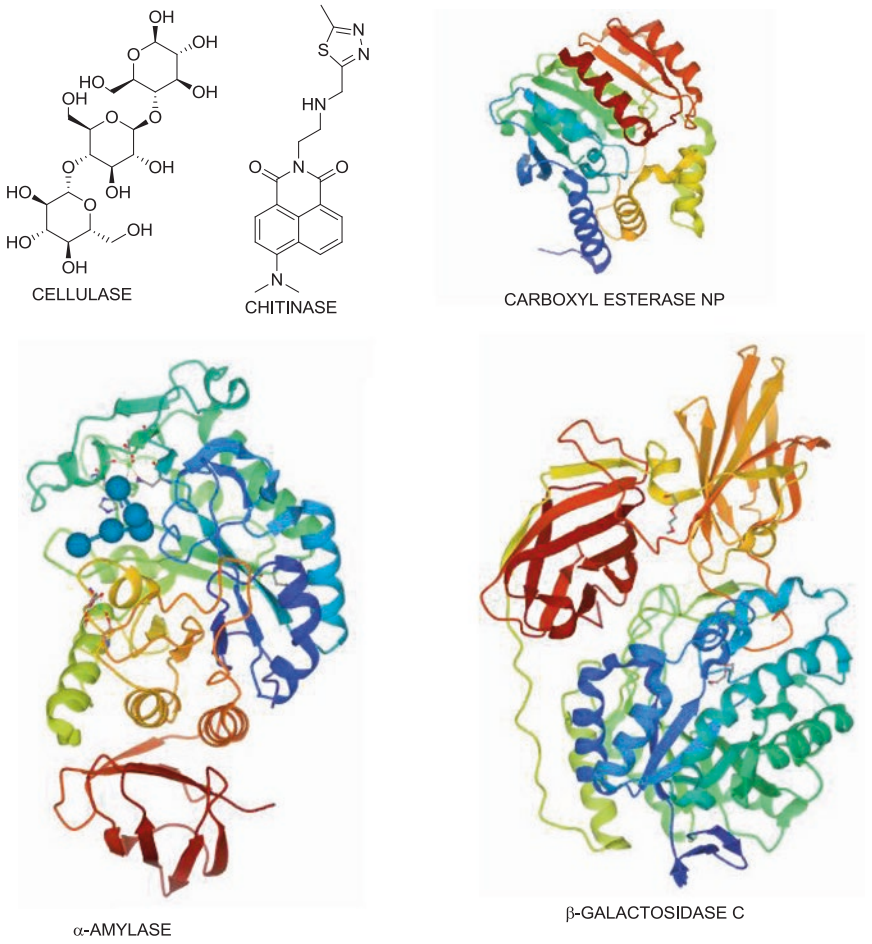

Fig. 4.1 Chemical crystal structures of some reviewed enzymes

group transformation, the treatment of Fabray's disease, and xenotransplantation (Fuller et al. 2004). New α-galactosidases have been described from *B. megaterium* in the last years (Xu et al. 2014; Huang et al. 2018).

On the other hand, the enzyme β-galactosidase (Fig. 4.1), found in microorganisms, plants, and animals, hydrolyzes β(1–3) and β(1–4) galactosyl bonds in oligosaccharides (Oliveira et al. 2011). In the food industry, it is used for the production of lactose-free milk for lactose-intolerant individuals (Ishikawa et al. 2015). Since lactose intolerance is a common problem, which affects over 70% of the world's adult population and causes symptoms such as abdominal pain, gas, nausea, and diarrhea, the market for lactose-free milk and dairy products is currently growing rapidly. This can be obtained by enzymatic hydrolysis using β-galactosidases.

4.5 Pullulanases

Bacillus spp. pullulanases, important debranching enzymes, hydrolyze the α-1,6 glucosidic linkages in starch, and related oligosaccharides, which convert the branched polysaccharides into small fermentable sugars (Hii et al. 2012). In the saccharification process pullulanase has been used to increase the final glucose concentration. To date, five groups of pullulanase enzymes have been reported: (1) pullulanase type I, (2) amylopullulanase, (3) neopullulanase, (4) isopullulanase, and (5) pullulan hydrolase type III. Some *Bacillus* sp. are pullulanase producers such as *Bacillus acidopullulyticus*, *Bacillus deramificans*, thermophilic *Bacillus* sp. AN-7 (Kunamneni and Singh 2006), *Bacillus cereus* FDTA-13 (Nair et al. 2007), and *Geobacillus stearothermophilus* (Zareian et al. 2010). However, pullulanases with appropriate features to industrially employ them is currently limited (Nisha and Satyanarayana 2016). However, molecular biotechnology may provide solutions to improve enzymatic properties for industrial application (Wang et al. 2019). *B. subtilis* is appropriate to produce pullulanase since this organism is recognized as safe.

4.6 β-glucanases

Glucanases break down the glucan polysaccharide hydrolyzing the glucosidic bond. They are used during the aging process of wine in enological practices improving the aroma of wines. Macerating enzymes also improve press-ability, settling, and juice yields of grapes used for wine fermentation.

It is used to reduce barley mash viscosity helping with the filtration process and improving wort yield. The *B. subtilis* enzyme is commercially available and used in the brewing industry (Niu et al. 2018), and hybrid enzymes have been developed using *B. macerans* and *B. amyloliquefaciens* enzymes (Borriss et al. 1988, 1989). The β-1,3-glucanase hydrolyzes the nonreducing end of the glucan, resulting in the formation of oligosaccharides and glucose. It has been seen that this enzyme delays the pathogenic fungi growth decreasing the damage caused by these fungi in fruits (Confortin et al. 2019).

4.7 Xylanases

Xylanase degrades the linear polysaccharide xylan into xylose (Beg et al. 2001), breaking down hemicellulose, one of the major components of plant cell walls. Thus, xylanase is applied for the chlorine-free bleaching of wood pulp prior to the papermaking process. They are also applied as food additives to poultry, in wheat flour for improving dough handling and quality of baked products (Ammoneh et al. 2014) among others (Subramaniyan and Prema 2002). These enzymes are widely

produced by *Bacillus* spp. and applied for hydrolyzing cellulosic material such as cellulases and xylanases (Aizawa et al. 2010), for example, *B. pumilus* can produce a high quantity of xylanase (Nagar et al. 2011).

4.8 Cellulases

Microbial cellulases catalyze the bioconversion of cellulose to soluble sugars and glucose (Fig. 4.1) whose demand has grown rapidly (Li et al. 2009). Even though a variety of *Bacillus* species secrete cellulases, the study of these enzymes in this organism has remained behind (Kim et al. 2012). The explanation of this situation is due to that most *Bacillus* cellulases hydrolyze synthetic carboxymethyl cellulose, but barely hydrolyze the crystalline form of cellulose (Balasubramanian and Simões 2014). Even to improve the production cost of these enzymes *Bacillus* sp. can utilize the organic matter from several wastes (Ladeira et al. 2015). The cellulases has been applied in various industrial processes (Karmakar and Ray 2011; Kuhad et al. 2011), for example, they are used for deinking the paper wastes since they can remove the ink from the fiber surface hydrolyzing carbohydrate molecules. Cellulases are also applied for jeans biostoning and cotton biopolishing. The most popular application that is being investigated is enzymatic saccharification of lignocellulosic materials by cellulases for biofuel production (Sukumaran et al. 2005).

4.9 Chitinases

Chitin, an insoluble polysaccharide composed of linear chains of β-1,4-N-acetylglucosamine (GlcNAc), is abundant in crab, shrimp, and lobster shells. Each year, a vast amount of chitin waste material is released into the environment, which creates a serious environmental problem (Yan and Chen 2015). Therefore, a successful method for chitin recycling is very convenient for the ecosystem. Chitinase (Fig. 4.1) is the key enzyme in chitin degradation, since it hydrolyzes chitin polymer. The applications of chitinase includes bioconversion of chitin to useful products such as fertilizer and production of non-allergenic, non-toxic, biocompatible, and biodegradable materials (Hamid et al. 2013). Many *Bacillus* bacteria, such as *B. amyloliquefaciens, B. cereus, B. circulans, B. thuringiensis*, and *B. subtilis*, degrade chitin avoiding waste material accumulation (Wang et al. 2018). Many works have been done about chitinases including molecular engineering of chitinases (Pan et al. 2019).

B. thuringiensis produces small quantities of chitinases which are classified in four groups based on amino acid sequence alignments. Chitinases contain a catalytic domain and a putative chitin-binding domain, typical structure of enzymes that degrade biopolymers (Barboza-Corona et al. 2012). Chitinases have been employed for different environment purposes such as degradation of shrimp wastes,

improving insecticidal activity, or controlling phytopathogenic fungi. Shrimps are part of traditional food in seafood restaurants. The wastes of them, which include heads and exoskeleton, represent more than the half of the volume. These wastes are composed of chitin, therefore chitinases can be employed to reduce shrimp wastes. On the other hand, chitinases destroy peritrophic membrane of the insects, increasing insecticidal activity of Cry proteins. Also, chitin is part of the cell wall of fungi, therefore, chitinases have activity against them (Barboza-Corona et al. 2012).

4.10 Esterases and Lipases

Esterases catalyze the cleavage and formation of ester bonds. Carboxyl ester hydrolases catalyze the cleavage or formation of carboxyl ester bonds, being often classified as esterases and lipases (De Vitis et al. 2018), which are the two major classes of hydrolases. Lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze "simple" esters (e.g. ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C6 (Bornscheuer 2002). They are used in the organic synthesis of optically pure compounds since they have high enantioselectivity, therefore they have been applied in the synthesis of chiral drugs employing them in reactions where chemo-or regioselectivity is an important matter. Probably the best studied enzyme is the so-called carboxyl esterase NP (Fig. 4.1) (NP from naproxen, a non-steroidal anti-inflammatory drug) originating from *B. subtilis* (Quax and Broekhuizen 1994). They can also be used in the mild removal of protecting groups. Esterases are employed in dairies and to produce wine, fruit juices, beer, and alcohol, besides they are used as trans-esterification catalysts to transform low value fats and oils into more valuable ones (Panda and Gowrishankar 2005).

4.11 Levansucroses

Levansucroses are the enzymes which catalyze transfer of the fructose moiety from sucrose to polyfructose (β-(2-6)-levan), releasing D-glucose. Levan-type fructooligosaccharides (FOSs) and β-(2-6)-levan selectively support the intestinal health having potential health benefits, besides they are applied in food and pharmaceutical fields. Levansucroses from microbial sources exhibit different oligomerization (FOSs) vs. polymerization (levans) ratios. In this sense, levansucroses from *Bacillus megaterium* and *Bacillus subtilis* were found to catalyze dominantly the synthesis of levan (Tian et al. 2011). Levansucroses effectivity is hindered by its hydrolytic activity, resulting in the release of glucose and fructose instead of desirable FOSs and levan. For this reason, the search of new levansucrase enzymes with improved catalytic activity to produce levan is desirable (Hill et al. 2019).

4.12 Keratinases

Keratinases are proteolytic enzymes that digest keratin, a strongly cross-linked structural polypeptide. Keratin wastes are increasingly accumulating in the environment mainly in the form of feathers or hair generated from various industries (Verma et al. 2017), for example in the leather industry dehairing process serves to eliminate the hair from the skin. The keratinases produced from hair degradation might be significantly applied due to their specificity (Hassan et al. 2020). Therefore, keratinases have many applications in several industries (Gupta and Ramnani, 2006). In the textile industry, keratinases serve to smooth the raw wool without damaging the internal parts of wool fibers, replacing the chemical methods (Ghaffar et al. 2018). The dermatologists prescribe various formulated drugs based on keratinases to treat hyperkeratosis which forms corns and calluses. For instance, Keratoclean®Hydra PB and Pure100 Keratinase have been produced by Proteos Biotech Company to treat the skin suffering from hyperkeratosis corns and calluses (Hassan et al. 2020). Keratinases are also used as fertilizers in organic farming to suppress nematodes and in Prion protein management (Verma et al. 2017). Therefore, these enzymes have multiple applications opening a new perspective to cover the unsatisfied demands in the pharmaceutical industries.

4.13 Conclusions and Perspectives

Bacillus enzymes are varied and with wide applications. This fact leads the great interest of industry to these metabolites. As we have seen, there are many enzymes produced by different strains of *Bacillus* with different industrial applications. In many of them, the major industrial application is oriented to green ecology minimizing the wastes produced by the human beings to avoid environment contamination. Besides, the enzymes produced by *Bacillus* have been applied to several industries and activities such as food, textile, detergents, and pharmaceutical. This chapter describes the enzymes produced by *Bacillus* species used in industries and their applications. The advances in recombinant DNA technologies can be highlighted, which help in improving enzymes production. By improving the production of the enzymes, they can be better applied in the industry. We expect that this review opens the door to new discoveries about these interesting enzymes which give the opportunity to approach the research to this topic. There is a need for a better production in almost all cases to take advantage and make better use of these amazing secondary metabolites from *Bacillus*.

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Chapter 5
Bacillus Species and Their Invaluable Roles in Petroleum Hydrocarbon Bioremediation

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Abstract Exploration, production activities as well as improper waste disposal practices have led to major contamination of both aquatic and terrestrial ecosystems. Oil spills in particular have proven to be a global problem, with oil spills in the Gulf of Mexico, the coasts of Cape Town (South Africa) and Montara (CL, USA) being some of the landmarks. Efficient biodegradation of petroleum hydrocarbon (H-C) necessitates the presence of a stable biosurfactant and a microbe that can use the petroleum H-C as a sole carbon source. In light of this fact, *Bacillus* spp. have been reported to both produce a stable biosurfactant and tolerate high crude oil concentration and degrade aliphatics, monoaromatics and polyaromatics, which are the major constituents of crude petroleum. Such peculiarity makes the species the major source of potential candidates for H-C degradation. However, most of the oil-spill bioremediation products are presented as a magic cocktail of microorganisms, even though *Bacillus* spp. is the central genus if not the only one. Hence, this chapter aims to highlight the central and significant roles played by *Bacillus* spp. in oil-spill bioremediation and the mechanisms therein. Most importantly, highlighting the central roles played by *Bacillus* spp. and the mechanisms involved will contribute to targeted development of microbial and/or enzyme cocktail products and metabolic engineering to design next-generation oil-eaters in the future. Therefore, details of the major metabolic pathways, enzymes and biosurfactants deployed by *Bacillus* spp. during H-C biodegradation are addressed at possible depth.

Keywords *Bacillus* species · Biosurfactant · Enzyme cocktail · Hydrocarbon biodegradation · Oil-spill bioremediation · Petroleum

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5.1 Introduction

Our planet suffers greatly from various pollution problems, which include water, soil and air pollutions. Petroleum hydrocarbons are amongst the most toxic compounds released into oceans and spilled onto soil, thereby making them some of the major contributing factors to water and soil pollutions. Oil is introduced into the environment through natural seepage and human activities, which include pipeline and tanker leaks and spills. For instance, the British Petroleum (BP) /Deepwater Horizon discharge in 2010 stands out as the largest marine open water hydrocarbon discharge to date. The landmark incident caused a discharge of five million barrels of oil and at least 250, 000 metric tonnes of natural gas into the Gulf of Mexico (Joye et al. 2016).

The most important classes of organic pollutants in the environment include the mineral oil constituents and the halogenated products of petrochemicals (Chandra et al. 2013). Most petroleum compounds are reported to have carcinogenic properties (Souza et al. 2014). These compounds have a wide distribution and high toxicity hence hydrocarbons are considered as the principal organic markers of the anthropogenic activity in the ecosystems (Koshlaf and Ball 2017). Petroleum is a heterogeneous mixture of hydrocarbons (organic compounds containing carbon and hydrogen), which includes aliphatic (n-alkanes), alicyclic and aromatic hydrocarbons (polycyclic aromatic hydrocarbons), which are insoluble in water (Joye et al. 2016). Therefore there is an increasing need to develop a green technology to remove or transform hydrocarbons to a non-toxic or less-toxic version of the compound through application of hydrocarbon-degrading microbial or enzyme cocktails.

Bioremediation is a less-invasive and less-expensive process as compared to the classical decontamination methods (Zafra et al. 2016). Some microbes have been documented to inhabit and proliferate in hydrocarbon-contaminated environments, thus, using hydrocarbon as sole carbon and energy sources (Chebbi et al. 2017). The characterisation of certain metabolic pathways indicates that high enzymatic capacity allows the microbial communities to degrade complex hydrocarbons, and this therefore promotes the importance of enzymes in the bioremediation process (Peixoto et al. 2011). Bioremediation can be carried out through bioattenuation, biostimulation and/or bioaugmentation (Adams et al. 2015). The classical bioremediation approach that has been in use is bioaugmentation, which is application of a consortium of *in vitro*-cultivated inocula into the polluted site. However, there are multiple factors that lessen the effectiveness of this technique. This includes biotic factors such as predation and niche-exclusion, which could in turn affect the activity of the introduced microorganisms and result in the decline of the introduced populations. Moreover, bioaugmentation poses a potential risk with regard to the addition of microorganisms, in some cases a consortium that contains genetically modified ones, to the environment, which could be difficult to monitor and contain in the natural environment. Besides, relatively the time required for the microorganism to grow and be able to produce the enzyme required to biodegrade the hydrocarbons has negative implications on the rate of degradation (Adams et al. 2015).

There are a multitude of extreme environments on earth which are colonised by microorganisms called extremophiles, which can thrive under diverse harsh conditions (Mirete et al. 2016). Particularly those microorganisms with the evolved ability to grow in petroleum hydrocarbon-contaminated soils and water as highlighted in this chapter. Many technologies already developed through the years for remediation, rehabilitation and restoration of contaminated environments exploit the potential of microbial biological systems to use toxic compounds as substrates for growth and convert them into harmless by-products. Hence, the vast amount of research conducted to date concentrate on the capabilities of a single or a consortia of microbes exhibiting robust and effective hydrocarbon degradative properties (Mukherjee et al. 2017). The *Bacillus* spp. has been put in the spot light over the years for being an extremophile and has been studied in great extent over the years (Parthipan et al. 2017; Elumalai et al. 2019; Jin et al. 2019; Wang et al. 2019). More particularly, they have been found in hydrocarbon-polluted environments (Al-Dhabaan 2019; Christova et al. 2019; Lima et al. 2020) This provides promising potential for *Bacillus* spp. independent as well as in co-culture application for hydrocarbon bioremediation as reviewed extensively in this chapter.

5.2 The Chemistry of Hydrocarbons in Petroleum Crude Oil

Hydrocarbons are compounds that consist exclusively of carbon and hydrogen and lack functional groups. The lack of functional groups make hydrocarbons exhibit apolar properties, low chemical reactivity at room temperature and low water solubility in long-chain hydrocarbons. Hydrocarbons can be classified into the following groups: (a) aliphatic groups which include straight-chain (n-alkanes), branched-chain and cyclic compounds and (b) aromatic groups which include mono- or polycyclic hydrocarbons and many important compounds which contain aliphatic hydrocarbon chains (e.g. alkylbenzenes) (Koshlaf and Ball 2017). Petroleum crude oil is a complex mixture of low and high molecular weight hydrocarbons like alkanes, aromatics, asphaltenes and resins associated with other organic compounds containing sulphur, nitrogen and oxygen (Fig. 5.1). Among these hydrocarbons mentioned, aromatic hydrocarbons constitute a major fraction (Arun et al. 2011). The structural complexity of the various hydrocarbon constituents from petroleum makes the process of degradation highly difficult.

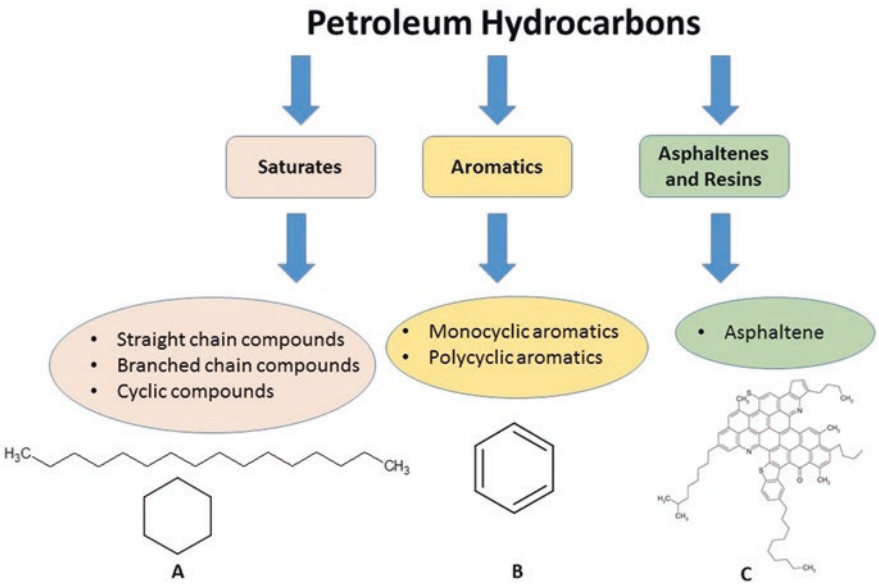

Fig. 5.1 The different classes of hydrocarbons that comprise crude oil are (**a**) saturated hydrocarbon; (**b**) aromatics and (**c**) asphaltenes and resins

5.2.1 Toxicity and Fate of Petroleum Hydrocarbons in the Environment

It is of utmost importance to expand our knowledge about the fate of hydrocarbons in the environment in order to have some level of pollution control. Due to their persistence and toxicity they cause permanent damage to the ecosystem (Atlas et al. 2015). The toxicity of the petroleum hydrocarbons depends on the solubility and bioavailability of the hydrocarbons. The water soluble fractions of the aromatics and polyaromatics are considered to be the most toxic (mutagenic, teratogenic and carcinogenic), though it is generally reported that most petroleum hydrocarbons have carcinogenic properties (Pandey et al. 2016). As a result of the reported toxicity, several hydrocarbons are classified by the Agency of Environmental Protection, the World Health Organisation and the European Union as top priority pollutants (Koshlaf and Ball 2017). Anthropogenic hydrocarbon contamination of soil and water is a global issue throughout the industrialised world (Umeh et al. 2017). Anthropogenic activity in different ecosystems are based on hydrocarbons as the principal organic markers due to their wide distribution and their high toxicity (Maikudi Usman et al. 2016).

Soil acts as a repository for many hydrocarbons; this poses a great concern due to the adverse effects on human health and the high levels of accumulation in the environment over long periods of time (Mukherjee et al. 2017). Throughout history there have been major global oil spills which have had catastrophic implications on

the environment (Zampolli et al. 2014; Joye et al. 2016). The Deepwater Horizon oil spill also referred to as the Gulf of Mexico oil spill in 2010 was classified as a major oil spill . The US Government estimated the total discharge at 4.9 million barrels (210 million US gal; 780,000 m^3) (On Scene Coordinator Report on Deepwater Horizon Oil Spill 2011). In addition, in 2011, an Exxon Mobil pipeline spilled 63,000 gallons of crude oil into the Yellowstone River near Laurel, Montana. Following this, in January 2015 a ruptured oil pipeline leaked up to 50,000 gallons of crude oil into the Yellowstone River in Montana. Days after the spill, officials detected benzene, a cancer-causing agent, in the water supply downstream of the river (CBS news 2015). These are but a few of the major oil spills which have affected land and water ecosystems. Therefore, there is a great need for bioremediation methods and preventative measures to be put in place to bioremediate and prevent future incidences.

5.3 Bioremediation

Technologies commonly used for soil remediation of petroleum hydrocarbons include mechanical burying, evaporation, dispersion and washing. However, these measures for remediation are very costly, time-consuming and not effective in the long run. Bioremediation is the preferred method since it is cost-effective, efficient and eco-friendly as compared to other treatments (Varjani 2017). Bioremediation is applied after the use of physical and chemical methods and natural attenuation methods have not been successful (Roy et al. 2018). Bioremediation is the preferred method since it is cost effective, efficient and eco-friendly as compared to other treatments (Varjani 2017). Various species of archaea, bacteria, algae and fungi have been reported to play a significant role in treatment of oil spills in different environments (Sharma et al. 2018a). The details of different bioremediation approaches in general and of oil spills in particular are discussed in the following sections.

5.3.1 Biostimulation and Bioaugmentation Methods for Bioremediation

There are two main types of bioremediation methods, these include biostimulation and bioaugmentation (Wu et al. 2016). Many reports have shown that both methods have greatly enhanced the biodegradation of hydrocarbons in oil-polluted soils. The effects of both methods are case-specific and results could be inconsistent. Hence, the use of bioaugmentation and biostimulation needs to be carefully planned and studied for each type of contaminant and environmental conditions (Roy et al. 2018).

Biostimulation involves the modification of natural conditions of a site to increase the rates of biological degradation by stimulating indigenous microbial community

endowed with catabolic pathways for hydrocarbon degradation. The environmental conditions can be modified through the addition of surfactants, nutrients, water and chemical species acting as donors or acceptors of electrons (Zrafi-Nouira and Saidane-Mosbahi 2012). Perfumo et al. (2007) added nitrogen, phosphate, inorganic potassium and surfactant on the bioremediation of a ground polluted with hexadecanes. Following treatment of the ground, there was a 10% increase in the degradation. Bragg et al. (1994) used fertilisation of the marine environment to stimulate the natural biomass in areas of Alaska following the accident of the Exxon Valdez. Recently Wu et al. (2016) compared the efficiency of bioaugmentation and biostimulation approaches for hydrocarbon biodegradation. Accordingly, after six weeks of treatment they reported that 60% and 34% of the total petroleum hydrocarbons biodegradation, respectively were achieved.

In some cases, the indigenous microbial populations of polluted ecosystems may not contain the metabolic tools to carry out complete degradation of the pollutants (Wu et al. 2013). In such cases bioaugmentation can be used to overcome discrepancy by incorporating microorganisms (*in vitro*-cultivated inoculum) with adapted metabolism for hydrocarbon degradation in contaminated sites (Zrafi-Nouira and Saidane-Mosbahi 2012). For instance, Da Silva et al. (2005) showed an improvement of anaerobic degradation of a mixture composed of benzene, toluene, ethylbenzene, xylene and ethanol after the addition of methanogen consortia.

It is important to emphasise that the success of the implementation of bioaugmentation, for example, depends on competitiveness of the inoculated strains in different environments (Adams et al. 2015). Genetically modified organisms can also prove to be beneficial in the bioremediation process but there are some limitations to their applications such as problems with government legislation and monitoring, and long-term assessment is therefore very difficult. In either case of introduction of genetically modified or wild-type strains, the potential impacts of introducing degrading microorganisms in the presence of indigenous microbes must be evaluated and monitored (Lim et al. 2016). Some examples of genetically modified *Bacillus* used for bioremediation studies include that of *Bacillus subtilis* developed for bioremediation of arsenic-contaminated organic waste (Huang et al. 2015).

Recent studies by Roy et al. (2018) demonstrated an effective strategy for the bioremediation of petroleum refinery sludge using a combined biostimulation–bioaugmentation approach. This nutrient amendment to the sludge resulted in a native microbial shift that promoted hydrocarbon degrading populations within the sludge microbiome. It resulted in 57–75% total petroleum hydrocarbon reduction in the sludge. Therefore, nutrient(s)-induced community dynamics and metabolic interplay might be involved in accelerating bioremediation applications.

5.3.2 The Potential of Microorganisms for Hydrocarbon Bioremediation

Microorganisms have the amazing ability to metabolise many organic contaminants, using them as an energy source or converting them to non-toxic products (carbon dioxide, water and biomass). Many petroleum-degrading strains are being characterised specifically for their metabolic pathways involved in biodegradation of petroleum. This natural biodegradation by microorganisms prevents the dispersion of petroleum hydrocarbons in the soil and water and minimises the pollution levels to some degree (Koshlaf and Ball 2017). Numerous microorganisms, such as bacteria, cyanobacteria, green algae and fungi, are capable of either degrading or producing hydrocarbons; this completely depends on the metabolic pathways involved and the different environmental conditions (e.g. aerobic, anaerobic, varied pHs and salinities (Varjani 2017). The use of microbes and their enzymes for the removal of pollutants has proven to be an effective, safe and a less-expensive method. Native microbes growing in a polluted environment are expected to be more robust in degradation than non-native species since they produce certain degrading metabolites, allowing them to out-perform non-native species (Parthipan et al. 2017). Alternatively, the method of co-cultivation of native microbes with efficient pollutant-degrading microbes is believed to be a valuable strategy to increasing contaminant removal in a short period of time (Patowary et al. 2016).

A large number of bacterial species have been classified as hydrocarbonoclastic bacteria and play key roles in the removal of hydrocarbons from polluted environments (Parthipan et al. 2017; Sharma et al. 2018a). *Bacillus* spp. are one such example of hydrocarbonoclastic alkane degrading bacteria found in literature (Freitas de Oliveira et al. 2013; Borah and Yadav 2014a; Liu et al. 2016; Parthipan et al. 2017). Some microorganisms have the ability to degrade aliphatics, monoaromatics or polyaromatics while others are involved in the degradation of resins (Varjani 2017). Some examples of *Bacillus* spp. involved in petroleum hydrocarbon compounds are listed in Table 5.1.

Although many bacteria are able to metabolise organic pollutants, a single bacterium is not capable of degrading all or even most of the compounds in polluted soils. The main reason for this stems from the fact that a single bacterium does not possess the enzymatic capability to complete such a task. Therefore, mixed microbial communities are most effective in degrading complex mixtures of hydrocarbons present in contaminated areas since the genetic information from all the microorganisms is present (Dombrowski et al. 2016). This process occurs gradually by sequential metabolism of its compounds. The genes involved in this degradation process can be located either on chromosomal or plasmid DNA. Therefore, it is crucial to assess biodegradation as a multi-domain community for one to completely appreciate the metabolic potential of the indigenous microbial community (Varjani 2017).

The importance of efficient bacterial consortiums for potential hydrocarbon remediation was reinforced by Patowary et al. (2016). Fourteen different bacterial

Table 5.1 *Bacillus* spp. involved in the degradation of specific classes of petroleum hydrocarbon compounds

Petroleum hydrocarbon	Microorganism responsible for biodegradation of the H-C	References
Aliphatics	*Bacillus thermoleovorans* *Bacillus subtilis* *Bacillus licheniformis Y-1* *Bacillus sp.* L26 *and* *Bacillus sp.* L30	(Ahmed et al. 2010, Darsa and Thatheyus 2014, Liu et al. 2016, Parthipan et al. 2017), (Lu et al. 2020), (Lima et al. 2020)
Monoaromatics	*Bacillus lentus* strain LP32 *Bacillus pumilus* MVSV3 *Bacillus amyloliquefaciens*	(Opere et al. 2013), (Surendra et al. 2017), (Wongbunmak et al. 2020)
Polyaromatics	*Bacillus cereus* Strain DRDU1 *Bacillus subtilis* 3KP *Bacillus subtilis* BL-27 *Bacillus cereus Strain* JMG-01	(Mittal and Singh 2009, Borah and Yadav 2014a, 2014b), (Vinothini et al. 2015), (Ni'Matuzahroh et al. 2017), (Wang et al. 2019), (Das et al. 2017)

consortia were designed involving both biosurfactant producing and non-producing isolates. The results obtained from this study showed that a consortium comprising two *Bacillus* stains namely, *B. pumilus* KS2 and *B cereus* R2, displayed the best result in the desired degradation of crude oil. This consortium showed degradation of up to 84.15% of TPH after 5 weeks of incubation using gravimetric analysis, further cementing the central and dominant role played by *Bacillus* spp. Fourier transform infrared (FTIR) and Gas chromatography-mass spectrometer (GC-MS) analyses correlated with the gravimetric results revealed that the consortium had removed a wide range of petroleum hydrocarbons including both aliphatic and aromatic hydrocarbons. Furthermore, analysis carried out by Dombrowski et al. (2016) through reconstruction of metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill further supports the complexity of oil-degrading community and the co-ordination of its metabolic pathways to ensure complete degradation of petroleum hydrocarbons in the environment.

The enzymatic mechanisms are also crucial to the success of microbial degradation of hydrocarbons. Different microbial electron acceptors such as oxygen, nitrate, manganese, iron and sulphate are all involved in the biotransformation of aliphatic and aromatic hydrocarbons. For example, the breakdown of n-alkanes are catalysed by alkane activating enzymes, which are monooxygenases that lower chemical reactivity of the hydrocarbon by generating reactive oxygen species. The best alkane-degrading pathway identified is encoded by the OCT plasmid of *P. putida* GPo1 (van Beilen et al. 2001). The first enzyme in this pathway is an integral-membrane non-haem diiron monooxygenase (AlkB) that hydroxylates alkanes at the terminal position. This produces a primary alcohol which is further oxidised by alcohol and aldehyde dehydrogenases to form the corresponding aldehyde. The

product is finally converted to fatty acid via oxidation, which is channelled into the β-oxidation pathway in the form of acetyl-CoA.

5.3.3 *The Mechanisms Employed by* Bacillus *spp. for Bioremediation of Hydrocarbons*

A number of bacterial species belonging to *Bacillus* have been identified as petroleum hydrocarbon degraders and have specifically been involved in naphthalene and pyrene degradation (Annweiler et al. 2000). Thamer et al. (2013) conducted studies showing that *Bacillus thuringiensis* has great capacity for the biodegradation of crude oil. This bacteria exhibited the ability to break down hydrocarbon compounds by 80% and total biomass reaches 5g/l, while the amount of emulsion reaches 2.3 g/l. Numerous studies have supported the implementation of *Bacillus* spp. in bioremediation activities. Sakthi Priya et al. (2015) showed that *Bacillus subtilis* isolated from a polymer dump site in India was used for degradation of crude oil. It was noted that the crude oil degradation and viscosity reduction was observed to be 80% and 60% respectively within a period of 10 days. It was concluded that high microbial adherence, surface tension reduction, emulsification activity, quantity of biosurfactant produced and stability provide a true indication that *Bacillus subtilis* is a potential microorganism for oil spill treatment. These results are compared with the other studies conducted (Borah and Yadav 2014a; Darsa and Thatheyus 2014; Jabeen et al. 2015; Vinothini et al. 2015; Parthipan et al. 2017; Tao et al. 2017).

Work done by Bujang et al. (2013) demonstrated a very good biodegradation capability of oily wastewater by *Bacillus cereus* from three different automotive workshops. Oily waste water has a complex composition of hydrocarbons and it was found that the degradation was up to 91% after 5 days of incubation. This finding was further supported by studies conducted (Borah and Yadav 2014b, 2017) on *Bacillus cereus* DRDU1 which was found to efficiently degrade 96% of kerosene. The results obtained for this biosurfactants emulsification index for kerosene, crude oil and used engine oil were in a good range thus making it an attractive future application for MEOR process. Other species of *Bacillus* recently found to have hydrocarbon degradation potential include *Bacillus thermoleovorans* (Annweiler et al. 2000), *Bacillus amyloliquefaciens* An6 (Ayed et al. 2015), *Bacillus licheniformis* Y-1 (Liu et al. 2016), *Bacillus pumilus* (Patowary et al. 2016) and *Bacillus methylotrophicus* USTBa (Chandankere et al. 2014).

5.3.3.1 Surfactants and Biosurfactants for Bioavailability of Pollutants

Surfactants are surface active substances which consist of hydrophilic (polar) and hydrophobic (nonpolar) portions on their molecules (Mulligan 2005). The polar part of the biosurfactant can be an amino acid, a carbohydrate and/or a phosphate group.

The long chain fatty acid makes up the nonpolar portion. Synthetic surfactants are widely used to treat oil spills by dispersing them and accelerating their mineralisation (Fernandes et al. 2016). The mechanism is the ability to reduce surface and interface tensions between liquids, solids and gases, allowing hydrocarbons to readily emulsify in water by forming aggregates called micelles (Maikudi Usman et al. 2016). Knowing the number of bacteria involved with biosurfactant production will enhance hydrocarbon degradation of hydrocarbons in two ways, the first is a direct method which favours the solubility of hydrophobic compounds and the second is by indirectly increasing the availability of hydrophobic compounds to the native microorganisms (Noparat et al. 2014). This bioavailability of petroleum crude pollutants to microbes is crucial for the process of bioremediation (Varjani and Upasani 2017a).

The demand for surfactants for many years has been met by those derived from petroleum which proved to be toxic to the environment and non-biodegradable. This has therefore led to using biosurfactants which serve the same purpose but are produced extracellularly or as part of the cell membrane by bacteria, fungi and yeast and utilise different substrates like simple sugars, oils and hydrocarbons from contaminated environments. Biosurfactants have advantages over their chemical counterparts with regard to their effective response to extreme environmental conditions, biodegradability, lower toxicity and the ability to be produced from cheap organic sources, which facilitate commercialisation (Díaz De Rienzo et al. 2016). Biosurfactants are produced extracellularly by a few microorganisms including bacteria, fungi and yeast. *Bacillus* spp. (Bezza and Chirwa 2015), *Streptomyce*s spp. (Ferradji et al. 2014) and *Sphingobacterium* spp. (Noparat et al. 2014) are but a few examples of biosurfactant producing bacteria. The addition of biosurfactant producing bacteria such as those mentioned above can resolve the problem of unavailability of hydrocarbons, which is due to the pollutants' lack of solubility (Varjani and Upasani 2016, 2017b).

The quality, quantity and type of biosurfactant produced are influenced by the concentration of carbon, nitrogen, iron and phosphorous iron sources. Environmental factors such as growth conditions (pH, agitation, temperature and oxygen accessibility) are also a major contributor and are also valuable in assessing biosurfactant production (Maikudi Usman et al. 2016). This was clearly demonstrated by Khan et al. (Ali Khan et al. 2017) with regard to the role of nutrients in bacterial biosurfactant production by four bacterial strains (*Pseudomonas poae* BA1, *Acinetobacter bouvetti* BP18, *Bacillus thuringiensis* BG3 and *Stenotrophomonas rhizophila* BG32). Further, pH and temperature were shown to influence biosurfactant production by *Pseudomonas* spp. as concluded by Müller et al. (2011). Kinetics of nutrient-enhanced crude oil degradation was also carried out by Chettri et al. (2016) for *Bacillus* spp. AKS2 isolated from a refinery in Guwahati, and further support work was done by Khan et al. (Ali Khan et al. 2017). The results showed a seven-fold increase in biodegradation due to nutrient enhancement.

Many recent reports have shown the application of biosurfactant producing microbes (Bezza and Chirwa 2015; Díaz De Rienzo et al. 2016; Joy et al. 2017). The remediation of petroleum-contaminated soils and water in the presence of

microorganisms can be enhanced by the production of biosurfactants (Ferradji et al. 2014). It is therefore important to characterise biosurfactant producing bacteria. A vast amount of research have concluded that the *Bacillus* spp. are potential biosurfactant producers (Chandankere et al. 2013; Ferradji et al. 2014; Díaz De Rienzo et al. 2016; Maikudi Usman et al. 2016; Parthipan et al. 2017), biodegrading microbes and have been extensively used in microbial enhanced oil recovery (MEOR) (Gudiña et al. 2013; Youssef et al. 2013; Al-Wahaibi et al. 2014), bioremediation purposes (Bezza and Chirwa 2015; Liu et al. 2016) and biodegradation (Bujang et al. 2013; Thamer et al. 2013; Darsa and Thatheyus 2014).

There are pathways for biosurfactants synthesis that are well described by many studies for the *Bacillus* genera for the production of surfactin linked to the following genes (*spf, srfAA, srfAB, srfAC* and *srfAD* among others). In particular, two novel surfactin molecules isolated from cell-free cultures of *Bacillus subtilis* HSO121 were presented by Liu et al. (2016). Surfactins have the ability to form sphere-like micelles; their amphipathic and surface properties play a role in the minor polar and major hydrophobic domains of a surfactin molecule (Liu et al. 2015). Currently, a vast amount of research has been focused on the discovery of a novel hydrocarbon-degrading biosurfactant that would enhance biodegradation in hydrocarbon-polluted environments. Ayed et al. (2015) had successfully produced a biosurfactant from a bacterial strain *Bacillus amyloliquefaciens* An6. They reported that this biosurfactant displayed good stability over a wide range of temperatures, pH and salinity. The solubility of diesel was enhanced by up to 10% as compared to SDS or Tween 80 respectively.

Comparative studies have also been carried out between *B. thailandensis* E264 and *P. aeruginosa* ATCC 9027 with regard to rhamnolipid biosurfactant production (Díaz De Rienzo et al. 2016). Furthermore, Chandankere et al. (2014) carried out work to show the properties and characterise the biosurfactant in crude oil biodegradation by *Bacillus methylotrophicus* USTBa. The biosurfactant produced during the course of hydrocarbon degradation in this study was monitored by surface tension and cell hydrophobicity measurements. The results showed the ability of this biosurfactant to decrease the surface tension of water from 72 to 28 mN/m, with a critical micelle concentration of 35 mg/L. It exhibited 90% emulsification activity on the crude oil. The combination of these results made this biosurfactant an appropriate candidate for bioremediation of crude oil contaminants.

5.3.3.2 Bacterial Chemotaxis, Flagellar Motility and Biofilm Formation

The exposure of bacteria to pollutants such as hydrocarbon contaminants induces chemoattraction or chemorepellent reactions. Recent research suggests that the capacity to degrade harmful compounds have co-evolved in bacteria by reacting chemotactically to the pollutant therefore increasing their bioavailability which directly enhances biodegradation rates (Krell et al. 2013). Biosurfactant synthesis can be related to chemotaxis, flagellar motility and biofilm production (Chrzanowski et al. 2012). Structures used by microorganisms for locomotion such as flagella and

pili have important roles in cell motility and chemotaxis. This helps the bacteria to move to relatively better niches as well as to attach to surfaces so as to start the biofilm formation through the complex cell-to-cell signalling. In hydrocarbon-polluted niches, the process of alkane degradation will take place in the oil–water interfaces. This makes more carbon available for growth, hence increasing cell density leading to the biosynthesis of biosurfactants which can disperse hydrophobic compounds and promote their bioavailability (Nie et al. 2012).

Research recently carried out by Vasconcellos et al. (2017) in which 16 genes associated with chemotaxis and flagellar motility in prokaryotes were found in fosmid clones from a constructed fosmid library using metagenomic DNA from a petroleum reservoir in Northeast Brazil, thus showing the link between hydrocarbon degradation and chemotaxis. Anthracene degradation studies carried out by Das et al. (2017) using *B. cereus* strain JMG-01 isolated from hydrocarbon-contaminated soils showed that the presence of anthracene and the process of degradation lead to the modification of the cell surface morphology and the formation of an exopolymeric matrix. The filamentous growth of the biomass in the form of biofilm reveals the chemotaxis behaviour of this *Bacillus* strain in enhanced anthracene degradation.

Further research by Dombrowski et al. (2016) showed the presence of major genes for motility and for use of scarce nutrients, which could suggest that microorganisms are well adapted for chemotactic motility towards a hydrocarbon substrate. These genes might be of great importance for both the survival and growth of these oil-degrading organisms found in the Gulf of Mexico.

5.3.3.3 Uptake and Trans-membrane Transport of Hydrocarbons

The uptake of hydrocarbons have been the subject of many studies through the years. In particular, biosurfactants discussed previously have received a lot of the limelight. However, the transport mechanisms by which hydrocarbons cross the cell membrane have received little attention. The membrane of bacterial cells is hydrophobic. This causes an issue with biodegradation due to the decreased availability of hydrocarbons for uptake by the cells. Most substrates need to undergo cellular attachment to become accessible. Bacteria need to have access to the substrate, hence they need to be either dissolved in the aqueous phase or the bacteria have to directly adhere to the hydrocarbon. Hydrocarbons that are adsorbed on the surface of the cell are transported across the membrane into the interior of the microorganism. These hydrocarbons are degraded in the presence of enzymes, which is a quick response (Hua and Wang 2014).

Following the physical interaction between bacterial cells and hydrocarbons, the next step is trans-membrane transport. Microbial cells have resources to physically access soluble, emulsified hydrocarbons and large oil droplets and transport these substrates across cell membranes and form inclusions before the hydrocarbons are metabolised (Alvarez et al. 1997; Bouchez-Naïtali and Vandecasteele 2008). Studies have been conducted on trans-membrane transport of phenanthrene, naphthalene

and *n*-hexadecane (Kim et al. 2002; Kallimanis et al. 2007). For Gram-negative bacteria, outer membrane proteins have been reported to be involved in the transport of hydrocarbons across the cell membrane. Majority of the research has been carried out on *E. coli* with respect to long chain fatty acid transporter, FadL (Wiener and Horanyi 2011). This mechanism of trans-membrane transport is still poorly understood and needs to be further investigated to close the knowledge gap. However, it can be concluded that it is a huge player in the success for the ability of microorganisms to degrade petroleum hydrocarbons.

5.3.4 *Enzymatic Approach for Bioremediation of Hydrocarbons*

Enzymes are complex biological molecules with the sole purpose of catalysing a number of biochemical reactions involved in the degradation of hydrocarbons (Kalogerakis et al. 2017). Enzyme engineering has also been employed to improve the catalytic activity of isolated enzymes in different environments (Fig. 5.2). This is done by changing or modifying the basic amino acid structure of the enzymes

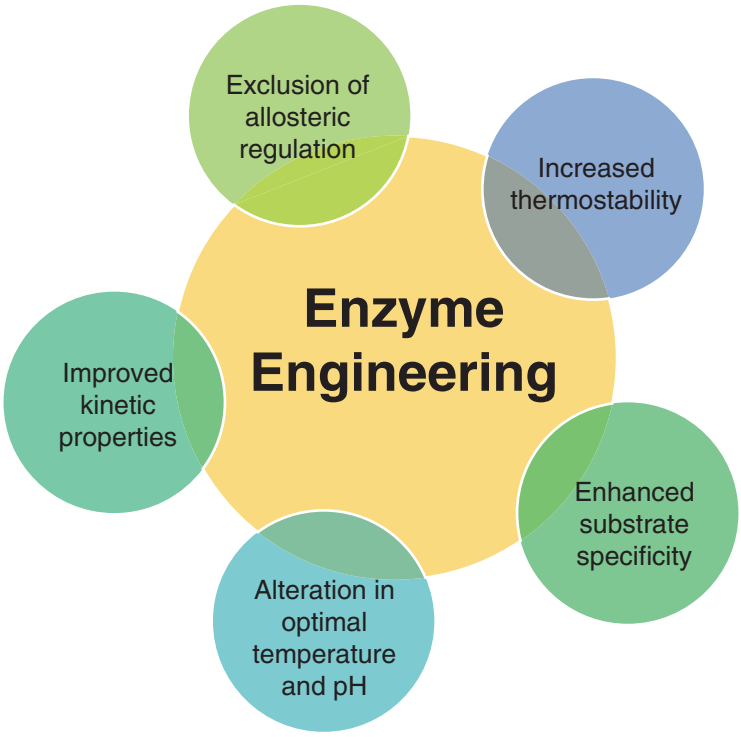

Fig. 5.2 Enzyme engineering to improve enzymatic properties for bioremediation

(Sharma et al. 2018a). Reseach carried out by Parthipan et al. (2017) showed that *Bacillus subtilis* A1 is able to produce degradative enzymes such as alkane hydroxylase and alcohol dehydrogenase. Recently Lu et al. (2020) showed the co-expression of alcohol dehydrogenase and aldehyde dehydrogenase in *Bacillus subtilis* for alcohol detoxification. Hence, these enzymes identified in *Bacillus* have the potential to be used in bioremediation applications.

Biodegradation of hydrocarbons including both aliphatic and aromatic compounds may occur under aerobic or anaerobic conditions (Koshlaf and Ball 2017). When considering aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons (monooxygenases introduce one oxygen atom to a substrate while dioxygenases introduce two (Parthipan et al. 2017). The most rapid and complete degradation of the majority of pollutants is brought about under aerobic conditions. Figure 5.3 shows the processes involved during the microbial degradation of hydrocarbons as a sole carbon source under aerobic conditions. Anaerobic degradation is catalysed by anaerobic bacteria; these include sulphate-reducing bacteria that use terminal electron acceptors (Van Hamme et al. 2003). The catabolism of hydrocarbons under aerobic conditions is faster than under anaerobic conditions due to the availability of oxygen as an electron acceptor (Cao et al. 2009).

There are several enzymes involved in the hydrocarbon bioremediation process which have been identified. These include oxidoreductases (laccases, oxygenases and peroxidases) and hydrolases, which are depicted in Table 5.2 with their catalytic function (Kadri et al. 2017).

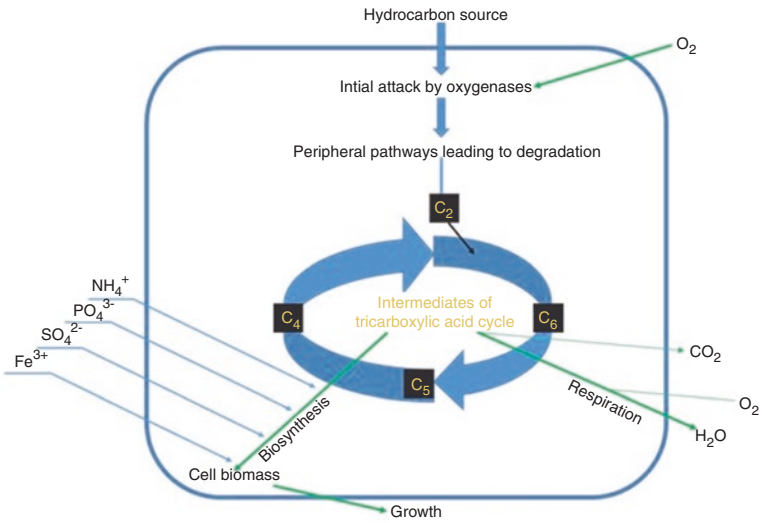

Fig. 5.3 Processes involved during the microbial degradation of hydrocarbons as a sole carbon source under aerobic conditions modified from (Fritsche and Hofrichter 2008)

Table 5.2 Enzymes involved in the bioremediation of hydrocarbons and their functions

Enzyme family	Examples	Functions	Reference
Oxidoreductases	Oxygenases	Catalyses the cleavage of the ring in aromatic compounds by adding one or two molecules of oxygen.	(Thomas et al. 2016, Sharma et al. 2018b)
	Laccases	Catalyses the oxidation of phenolic and aromatic compounds.	(Shekher et al. 2011)
	Peroxidases	Catalyses reduction reactions in the presence of hydrogen peroxide and generate reactive free radicals after oxidation of organic compounds.	(Sharma et al. 2018a)
Hydrolases	Haloalkane Dehalogenase	Catalyses the biodegradation of halogenated aliphatic compounds.	(Nagata et al. 2015)

5.3.4.1 Enzymatic Degradation of Aliphatic Hydrocarbons

When saturated aliphatic hydrocarbons are oxidised, the final product is acetyl-CoA, which is catabolised in the citric acid cycle as well as the production of electrons in the electron transport chain. This chain of events is repeated to further degrade the aliphatic hydrocarbons, which are then oxidised to carbon dioxide (Peixoto et al. 2011). The *n*-Alkanes are the main constituents of mineral oil contamination (Varjani 2017). Alkane metabolism is not a straightforward method since alkanes are very hydrophobic and less soluble in water (Rojo 2009). This poses a great challenge with regard to their uptake and hence facilitates their accumulation in the cytoplasmic membrane. Such accumulation could be detrimental as it might alter the membranes' fluidity. Despite such problems, many microorganisms have acquired the ability to degrade alkanes and use them as a carbon source (Wentzel et al. 2007). Four pathways have been identified for the initial attack on *n*-alkanes as shown in Fig. 5.4.

First is the terminal oxidation pathway, which has been well studied in *Geobacillus thermodenitrificans* NG80-2 (Li et al. 2008). In this pathway the alkanes are first attacked at their terminal methyl group to yield corresponding primary alcohols. These alcohols are further oxidised by alcohol dehydrogenases and aldehyde dehydrogenases to yield fatty acids which enter the β-oxidation pathway. The second pathway is called the biterminal oxidation, in which the termini of the *n*-alkane undergo oxidation to the corresponding fatty acid without rupturing of the carbon chain. The fatty acid produced undergoes ω-hydroxylation at the terminal methyl group. This yields a ω-hydroxy fatty acid that is further converted to a dicarboxylic acid which enters β-oxidation (Coon 2005; Li et al. 2008). Subterminal oxidation has also been identified in a number of microorganisms and takes place when alkanes are oxidised at the subterminal position to form a primary alcohol and a secondary alcohol with the same chain length as the substrate.

Microorganisms degrading short chain alkanes (C_2-C_4) have enzymes related to methane monooxygenase (Wang et al. 2017a). Those that degrade medium chain alkanes (C_5-C_{11}) or long chain alkanes (>C_{12}), contain integral membrane non-haem

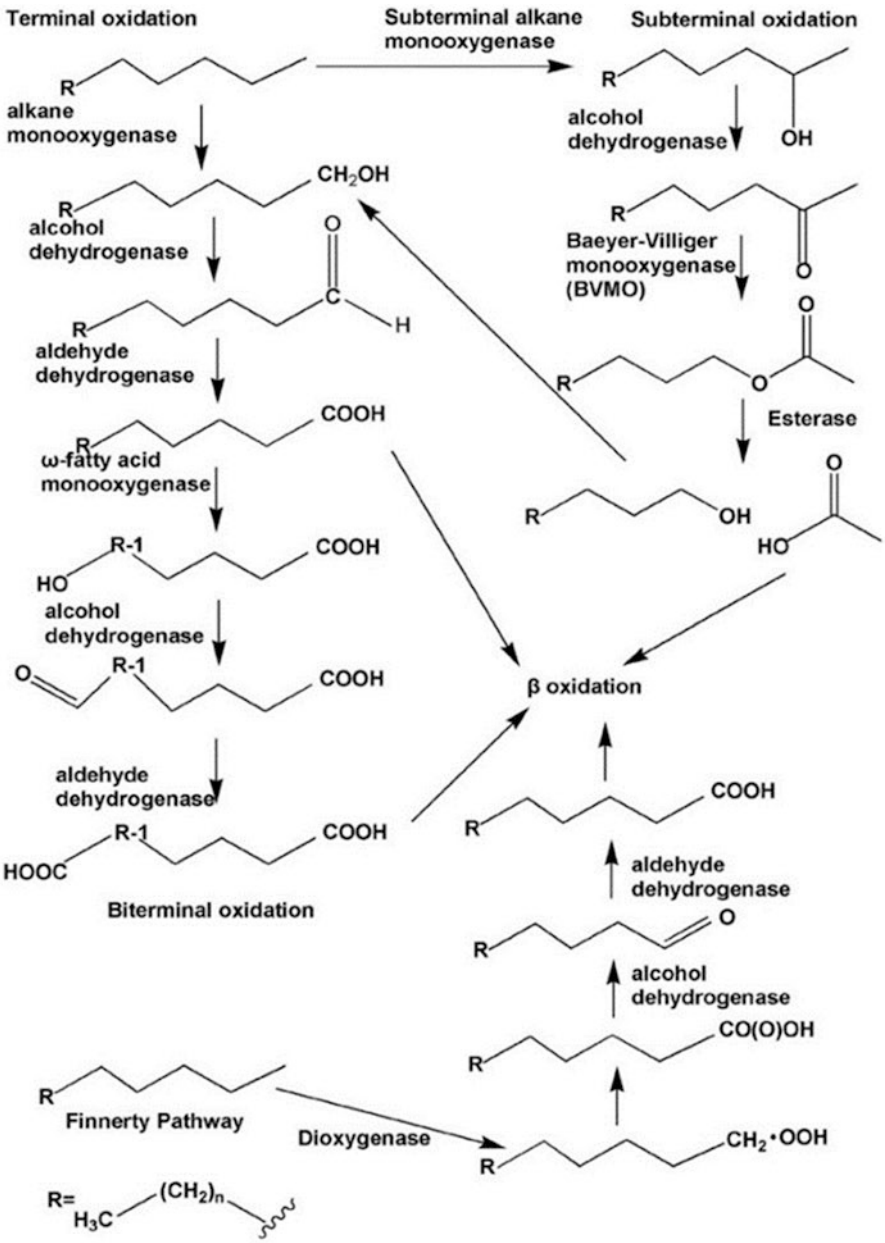

Fig. 5.4 Metabolic pathway for alkane degradation showing enzymes involved at different stages of degradation for both terminal oxidation and sub-terminal oxidation (Ji et al. 2013)

iron monooxygenases related to a well-characterised *Pseudomonas putida* GPo1 AlkB alkane hydroxylase (Rojo 2009). Several strains that assimilate alkanes

greater than 18 carbon atoms contain alkane hydroxylases that are apparently unrelated to the former ones and have only recently been characterised (Wang et al. 2017b) Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, yeast, fungi and algae (Van Beilen and Funhoff 2007). The most extensively studied alkane degradation pathway is that for *Pseudomonas putida* GPo1, encoded by the OCT plasmid (van Beilen et al. 1994) and *Gordonia* sp. TF6 (FUJII et al. 2004). This pathway describes the conversion of an alkane into an alcohol using membrane monooxygenases, soluble rubredoxin and rubredoxin reductase (Van Hamme et al. 2003).

5.3.4.2 Enzymatic Degradation of Aromatic Hydrocarbons

Cyclic alkanes represent minor components of mineral oil and have been found to be relatively resistant to microbial attack. Only a few species are capable of using cyclohexane as sole carbon source. Alkyl side chains of cycloalkanes facilitate their enzymatic degradation. With regard to aromatic hydrocarbons e.g. benzene, toluene, ethylbenzene, xylenes and naphthalene, they belong to the large volume of petrochemicals which are used as fuels and industrial solvents. Many microorganisms have evolved catabolic pathways to degrade aromatic compounds. The compounds can be enzymatically converted to natural intermediates of the degradation: catechol and protecatechuate (Fritsche and Hofrichter 2008) (Fig. 5.5). Numerous studies have shown enzymes identified from *Bacillus* with the ability to degrade aromatic hydrocarbons (Bello-Akinosho et al. 2016; Das et al. 2017; Lu et al. 2020; Wongbunmak et al. 2020)

Benzene and related compounds are much more thermodynamically stable than aliphatics. They are very problematic since they are highly water soluble and toxic (Bello-Akinosho et al. 2016). Only a few reports on bacteria capable of degrading benzene have been reported but great progress has been made in this area through the years (Smith 1990; Vogt et al. 2011; Ren et al. 2015). The class of enzymes involved in the degradation of aerobic aromatic hydrocarbons is catechol dioxygenases, which are bacterial iron-containing enzymes. These enzymes are involved in aromatic ring cleavage since they are able to catalyse the addition of molecular oxygen atoms (Van Hamme et al. 2003).

Enzyme Bioprospecting Through Metagenomics

Over the past few years, the field of metagenomics has proven to be a very valuable tool for accessing the biosynthetic machinery of uncultured microbiota, that constitute for at least 80–99% of the total population, as opposed to the conventional culture-based method (Lam et al. 2015). Novel biocatalysts and small molecule biosynthetic genes can be identified in a very short span of time as compared to the conventional molecular biology methods (Duarte et al. 2017). The two complementary parallel approaches of metagenomic strategies for mining novel enzyme are as illustrated in Fig. 5.6.

Accordingly, there have been a great number of successes over the past few years with regard to the identification of various novel enzymes involved in hydrocarbon

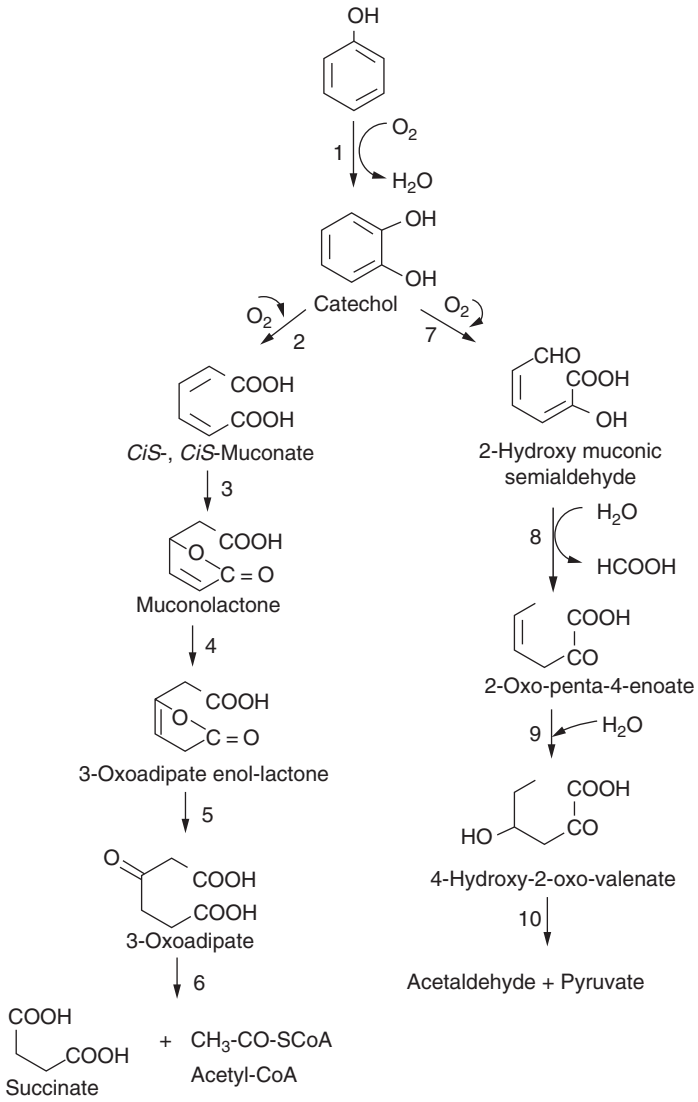

Fig. 5.5 Aerobic degradation pathway of aromatic compounds showing *o*- and *m*-cleavage (Rojo 2009)

degradation using metagenomic approaches. Recently, works carried out by Duarte et al. (2017) involving a metagenomic function-based screening approach were used to assess the microbial catabolome for polycyclic aromatic hydrocarbons from soils subjected to 12 years of *in situ* bioremediation. A total of 422 750 fosmid clones were screened for aromatic ring-cleavage activities using 2,3 dihydroxybiphenyl as a substrate. The positive fosmid clones that were sequenced showed that

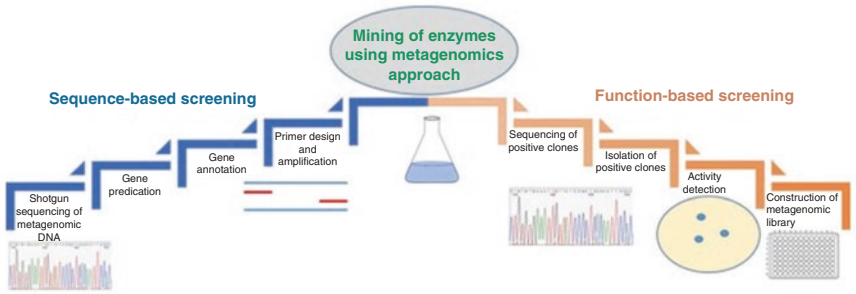

Fig. 5.6 Schematic diagram describing the general process of metagenomic strategies for mining novel genes

nearly 200 extradiol dioxygenase encoding genes of three different superfamilies were identified. Other genes including oxygenases were also identified and provided detailed information on enzymes activating aromatic compounds, thus providing insights into the complex microbial enzyme network. This was also shown by Dombrowski et al. (2016) working on reconstructing metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill, where a broad gene set for degrading hydrocarbons was identified and found to work as a co-ordinated network for polycyclic aromatic degradation.

Moreover, in an ongoing experiment, Baburam and Feto (unpublished data) also used a function-driven metagenomic approach to identify diverse and potentially novel hydrocarbon biodegraders from petroleum-contaminated soils. A fosmid library was constructed using metagenomic DNA from contaminated soil samples. Hexadecane, octadecene and cyclohexane were used to screen the library for hydrocarbon-degrading fosmid clones. The growth of clones over a period of 5 days and the size of the fosmid clones on the different substrates (hexadecane, octadecene and cyclohexane) were an indication of positive clones with enzymatic-degrading capabilities for a specific hydrocarbon. Unpublished data from this research study showed a large number of clones with the ability to grow on multiple substrates. The ability of the positive fosmid clones to use the difficult-to-degrade substrate as a sole carbon source indicates the presence of the enzymatic machinery and the necessary catabolic pathways to degrade the candidate hydrocarbons. Therefore, the preliminary data show the potential of metagenomics approach to mine potent enzymes that could be part of an enzyme cocktail with a potential for an efficient bioremediation of oil spills. Most importantly, unravelling the role of an enzyme cocktail as an efficient oil-eater could lay solid ground for future development of next-generation oil-eaters through metabolic engineering.

5.4 Conclusion

Natural oil seepages and anthropogenic oil discharges continue to plague the water body including its flora and fauna. Some microorganisms have been found to be efficient in degrading hydrocarbons as they are capable of surviving in hydrocarbon-contaminated waters and soils across the world. Developing a deeper understanding of the regulation and capacity for microbial hydrocarbon remediation in various environments is critical. While much has been learned over the past few decades, there is still more that needs to be understood. From the review it is apparent that data and findings observed by various researchers in the field, the central roles of the *Bacillus* spp. cannot be denied and its future uses in the environment, be it natural or modified, will be of great value for bioremediation advances in the future. Research further points to the benefit of mining enzymes especially novel enzymes with hydrocarbon degrading characteristics from *Bacillus* spp. As a matter of fact the majority of the biomolecules discovered to date are developed using the culture-based approach, which accounts only for 1–10% of the microbial community. However, with the onset of the metagenomics approach, which literally deal with 100% of the population, scientists have been unravelling a number of novel biocatalysts with outstanding physiochemical as well as catalytic activities. Such approach will definitely contribute to discover novel biocatalysts with a potential to turn the tide against the oil spills across the globe. Nonetheless, the field is yet to develop, and thus, there are a number of limitations that should be addressed in the future research endeavour, from handling of huge data generated through Next Generation Sequencing (NGS) of metagenome, NGS-sequence analysis, accurate annotation, robust heterologous expression to a lack of modular high-throughput (HTP) screening techniques.

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Chapter 6
Current Understanding and Future Directions of Biocontrol of Plant Diseases by *Bacillus* spp., with Special Reference to Induced Systemic Resistance

Sunita Mahapatra, Sunanda Chakraborty, Malay Samanta, Srikanta Das, and Tofazzal Islam

Abstract Plant pathogens represent one of the prime threats to sustainable crop production. Till date, synthetic agrochemicals are considered as effective tools for the management of various biotic stresses such as pathogenic microorganisms and insects in plants. Unfortunately, their injudicious and intensive usages in agriculture pose a serious threat to the environment and all living beings dwelling on the earth. Under such circumstances, the application of beneficial *Bacillus*-mediated management of plant pathogens has emerged as one of the most benevolent and sustainable options. A large number of *Bacillus* species has been identified as promising candidates for managing a number of plant pathogens through induction of systemic resistance in plants. Significant research progress has been attained in the characterisation and understanding of the role of *Bacillus*-induced systemic resistance (ISR) against a wide range of pathogens of crop plants. In this chapter, we aim to provide an overview of the mechanisms of *Bacillus*-induced ISR for instance, elici-

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tors, phytoalexins, lipopeptides, antibiotics, hormones and enzymes to protect plants from various pests. Additionally, glimpses of the research progress in the identification of different *Bacillus* strains and their evaluation as a potential biocontrol agent have also been presented.

Keywords *Bacillus* · Induced resistance · Bio-control · Lipopeptides · Antibiotics

6.1 Introduction

Steady increase in crop yield to feed the ever-increasing population is the greatest challenge faced by the agriculturists worldwide. Apart from factors such as nutrient supply, soil conditions, moisture, degrading cultivable land, and cultivar quality, biotic stresses pose a huge threat to the food security of the ever-increasing global population (Wulff et al. 2011; Islam et al. 2016a, b, 2019a, 2020). Although practices like crop rotation, use of chemical pesticides, and development of better cultivar have been utilised for the management of emerging diseases, these approaches are no longer ecologically and economically sustainable. Thus, application of beneficial microorganisms have emerged as a suitable alternative to the pre-existing traditional approaches (Hardoim et al. 2015). Coordination between microbiota and root endodermis supports plant mineral nutrient homeostasis (Salas-González et al. 2020). Among the beneficial microorganisms studied so far, *Bacillus* spp. have been observed to perform especially well not only in managing diseases but also in increasing nutrient availability, enhancing plant growth, improving beneficial microbial community in the rhizosphere, and inducing sustained defence reactions in the plant (Islam et al. 2016a, b, 2019b).

Bacillus is a genus of Gram-positive and endospore-forming bacteria, which is cosmopolitan in nature and widely spread across a diverse range of environments. They occur naturally in crop fields and aid in crop productivity both directly and indirectly. Inherent traits like durable cell wall, formation of endospores, peptide and antibiotic secretion, and production of antimicrobial enzymes play a significant role in the survival of these ubiquitous organisms even in a highly adverse situation.

A large body of research on the *Bacillus* species has attributed its success in the production of antibiotics and lipopeptides, quorum quenching, lysis of pathogen hyphae, competition for space and nutrients, and induced systemic resistance (Li et al. 2013; Islam et al. 2016a, b, 2019a, b; Surovy et al. 2019; Rayanoothala et al. 2020). Furthermore, *Bacillus* spp. increase nutrient uptake, siderophore production, and promote plant growth. For example, *B. subtilis* increases N uptake, phosphate solubilisation, and siderophore and phytohormone production, and promotes plant growth. *Bacillus* species has also been observed to alter the microbial community in the rhizosphere which facilitated disease suppression (You et al. 2016; Mahapatra et al. 2020).

This chapter aims to review the current understanding of the induction of resistance mechanism in host plant by *Bacillus* species. It deals with the various mechanisms employed by *Bacillus* species to suppress the phytopathogens in the plant rhizosphere and phyllosphere. It also discusses the genomic and molecular bases of disease resistance in plants imparted by the *Bacillus* species. Finally, it summarises the field application of *Bacillus*-based formulation, its prospects and challenges. This report also tries to bridge the knowledge gap and help to develop *Bacillus* species as a reliable and effective strategy for the management of emerging pests and diseases.

6.2 *Bacillus* Diversity and Antagonism

The type of soil plays a crucial role in the management of phytopathogens. Suppressive soils contain a large number of beneficial microbes that are able to inhibit the growth of bacterial and fungal pathogens. Among the biocontrol agents, the *Pseudomonas* genus has been most extensively studied for its anti-fungal metabolites (AFMs) such as pyrrolnitrin, phenazines, pyoluteorin, 2,4-diacetylphloroglucinol (DAPG), and viscosinamide (Nielsen et al. 1999; Nielsen et al. 2000). However, *Bacillus* species has gained popularity in the recent years due to its production of a wide array of antimicrobial substances and the ability of certain strains to promote plant growth (Choudhary and Johri, 2008). A large number of cultivable strains of *B. cereus* and *B. subtilis* have been identified in different studies (Vargas-Ayala et al. 2000), while cultivation-independent studies have demonstrated the existence of a much more number of uncultivable strains of *Bacillus* spp. (McSpadden Gardener 2004).

Some studies have found *B. megatarium* to be the most abundantly available species, while some researchers observed *Paenibacillus*, previously known as *Bacillus polymyxa*, to dominate a variety of soils. *Paenibacillus* has the ability to fix atmospheric nitrogen and thus are helpful in meeting the N requirement of a large number of crop plants like Canadian wheat (Priest 1993). *Brevibacillus*, previously known as *Bacillus brevis*, is terrestrial and aquatic in nature (Panda et al. 2014). A member of the *Bacillus* species, *B. sphaericus* is a notable entomopathogen and thus thrives in the habitats of insect larvae like pools, ditches, and lakes (El-Bendary 2006).

Spatiotemporal analysis of the microbial communities in soil, rhizoplane, and rhizosphere revealed that soil type had greater effect than plant type in determining the microbial population diversity (Wieland et al. 2001). Arias et al. (1999) studied the distribution and diversity of *Bacillus* spp. in the soybean phylloplane. Their study revealed that *B. pumilus* is the most widely distributed species in the soybean phylloplane. Although other bacterial strains like *B. brevis*, *B. subtilis*, *B. circulans*, and *B. firmus* were also observed in the phylloplane, their population continued to decline with the growth of the crop and became completely undetectable at 85 days

of the cropping season. As mentioned earlier, *Paenibacillus* genus contains many nitrogen fixing bacterial species, like *P. azotofixans*, *P. polymyxa*, and *P. macerans* (Ash et al. 1993). But amongst all the reported species, *P. azotofixans* has been found to be the most efficient, and hence, is abundant in the rhizosphere of wheat, maize, sorghum, banana, sugarcane, and some forage crops (Seldin 1992). *P. azotofixans* is more abundant in bulk soil than the rhizosphere, while its population varied largely with the soil type in the same crop rhizosphere (Rosado et al. 1998, Seldin et al. 1998).

The distribution and diversity of the *Bacillus* species is largely dependent on the interaction between the soil and the plant. The exudates from the plant roots, which play a significant role in determining the microbial distribution, are specific to the plant and are also correlated to a particular soil habitat (Crowley and Rengel 1999; Duineveld et al. 2001). The exudates vary with the stage of the crop as well, which is reflected in the findings of Jaegar and co-workers. The growth stage of the crop could be a crucial factor in determining the rhizobacterial community, as observed by Van Overbreek and van Elsas (2008) in potato. It has been observed that plants encourage the growth of specific microorganisms, from the indefinite microdiversity based on the chemical influences in the rhizosphere. Thus the manipulation of the chemical constituents of the rhizosphere could be done to encourage the growth of promising *Bacillus* strains among the rhizospheric community of microorganisms.

6.3 Mechanism of Induction of Resistance Against Plant Disease by *Bacillus* spp.

When *Bacillus* is introduced in the host-pathogen-environment system, it interacts with all the components in a number of complex mechanisms that affect the growth of the phytopathogen directly or indirectly. These interactions may lead to plant growth promotion, biofilm formation, induced systemic resistance (ISR), competition for nutrients, production of antibiotics, and cell lysis (Fig. 6.1). Strains of *Bacillus* spp. have been recorded to exhibit one or more of these traits that work synergistically with the plant and the environment in suppressing the phytopathogens. Furthermore, these interactions may lead to increased plant growth, vigour, and a shift in the rhizospheric microbial community, which would indirectly reduce the impact of the plant pathogens on the host plant.

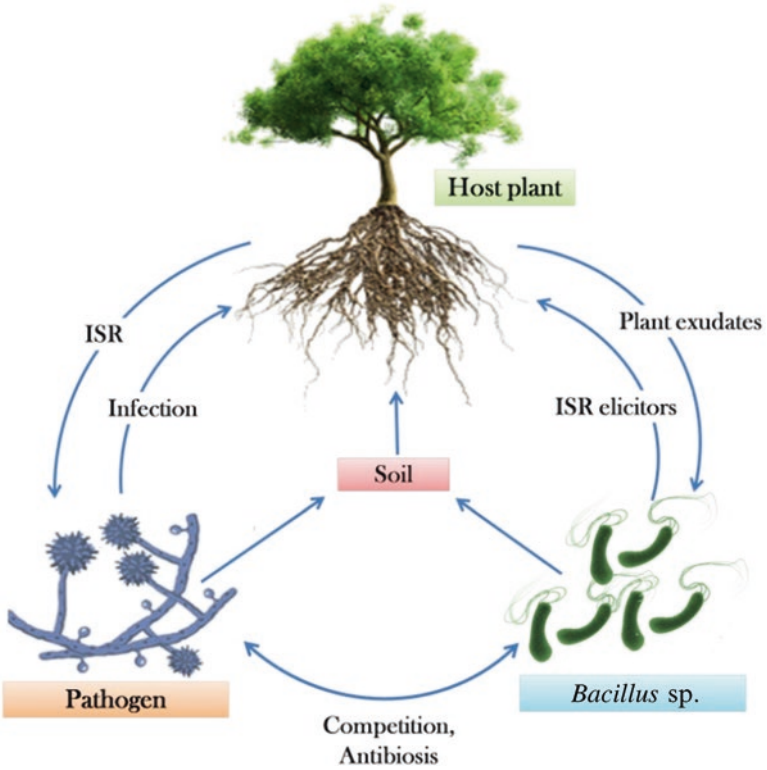

Fig. 6.1 Interactions between *Bacillus* spp., the pathogen and the host plant

6.3.1 Competition for Nutrients

Nutrients are always limiting factors for the growth and development of microorganisms and plants in the any crop fields. Biological control aims at utilizing this competition for nutrient and space to reduce the growth and productiveness of the pathogen by the non-pathogenic organisms. Generally, soil-borne pathogens like *Fusarium* and *Pythium* infect through mycelial contact, which makes them more vulnerable to competition from the non-pathogenic organisms. Micronutrients, such as iron, are extremely essential but its availability is limited and largely dependent on the soil conditions. In such situations, microbes produce siderophores, which are capable of attracting iron from the rhizosphere, thus meeting the iron requirement of the microorganism (Chakraborty et al. 2020a, b). In many instances, *Bacillus* has been found to compete with pathogenic microbes, such as *Erwinia carotovora*, by a similar mechanism, hence, restricting the growth and the development of the pathogen (Kloepper et al. 2004a, b). Studies also revealed that the inoculation of *Bacillus* sp. in the rhizosphere resulted in a shift of the pathogenic microflora from the site of infection. *Bacillus* species was reported to cause rapid colonisation in the tissues

Table 6.1 List of important *Bacillus* strains in respect to their competition, target pathogens, and host plants

Bacteria	Strain	Host	Target pathogen	References
Bacillus subtilis	HU5	Cotton	*Verticillium dahlia*	Li et al. (2013)
B. subtilis	SQR9	Cucumber	*Fusarium oxysporum*	Cao et al. (2011)
B. subtilis	E1R-j	Wheat	*Ustilago tritici*	Baker et al. (1983)
B. subtilis	SB24	Tomato	*Sclerotinia sclerotiorum*	Clayton and Hudelson (1991)
B. Amyloliquefaciens	CM-2 and T-5	Tomato	*Ralastonia solanacearum*	Tong-Jian et al. (2013)
B. Amyloliquefaciens	54	Watermelon	*Acidovorax avenae* subsp. *citrulli*	Jiang et al. (2015)
B. Megaterium	A6	Oilseed rape	*Sclerotinia sclerotiorum*	Hu et al. (2013)
B. Megaterium	B153-2-2	Soybean	*Rhizoctonia solani*	Zheng and Sinclair (2000)
B. pumilus	SE34	Pea	*F. oxysporum f.* sp. *pisi*	Benhamou et al. (1996)
B. cereus	UW 85	Tobacco	*Phytophthora parasitica*	Blagoeva-Nikolaeva et al. (1995)

of tomato plant, which reduced the disease severity and wilting index of *Fusarium* sp. in the host plant (Jangir et al. 2018). Similar results were obtained by Zhang and co-workers (2011) when working with *Fusarium* wilt of banana. They found that *Bacillus* was a rapid coloniser under hydroponic, sand and soil conditions, which inhibited the development of *Fusarium* sp. in the treated banana plant. Many studies have been conducted on the role of competition and colonisation in establishing *Bacillus* species as successful bio-control agents (Table 6.1).

6.3.2 Synthesis and Excretion of Lytic Enzymes

Aside from the production of lipoproteins and antibiotics, *Bacillus* has been known to produce a number of defense-related oxidative enzymes like peroxidase (PO) and polyphenol oxidase (PPO), which bring about structural changes in the host cell wall, thus strengthening the defense barriers against the invading pathogen. Several studies conducted so far have revealed that *Bacillus* synthesizes a large number of phytosanitary enzymes, which are enlisted in Table 6.2. Phenol oxidase enzymes produce quinones and release hydrogen peroxide. These enzymes trigger the release of toxic free radicals and polymerise the phenolic compounds into lignin like substances. The resulted metabolites are then deposited in the host cell wall, which

Table 6.2 List of enzymes activated by Bacillus species, along with the target pathogen and host plant

Bacteria	Strain	Host plant	Enzyme activated	Target pathogen	References
Bacillus subtilis	174	Tomato	PO,PPO, PAL	*Fusarium oxysporum*	Akram and Anjum (2011)
B. subtilis	AUBS1	Rice	PAL, PO and PR proteins	*Rhizoctonia solani*	Jayaraj et al. (2004)
B. subtilis	PTA-271	Grapevine	Lipoxygenase, PAL and chitinase	*Botrytis cinerea*	Trotel-Aziz et al. (2008)
B. subtilis	B4	Cucumber	Indole acetic acid	*Colletotrichum orbiculare*	Park et al. (2013a, b)
B. subtilis	SE34 and GBO3	Rice	PO and PAL, PPO	*Xanthomonas oryzae pv. oryzae*	Udayashankar et al. (2011)
B. pumilus	SE34	Pea	Phenolic compounds	*F. oxysporum f.* sp. *pisi*	Benhamou et al. (1996)
B. vallismortis	BS07	Chili pepper	Salicylic acid (SA)	*Phytophthora capsici and Colletotrichum acutatum*	Park et al. (2013a, b)
B. mycoides	Bac J	Sugar beet	Chitinase, b-1,3-glucanase and peroxidase	*Cercospora beticola*	Bargabus et al. (2002)
B. cereus	AR156	Loquat	PAL, PO, chitinase, β-1,3-glucanase, polyphenoloxidase and promoted accumulation of H_2O_2	*Colletotrichum acutatum*	Wang et al. (2014)
B. subtilis	BBG111	Rice	Jasmonic acid (JA) and ethylene (ET) as well as abscisic acid (ABA) and auxin signalling	*Rhizoctonia solani*	Chandler et al. (2015)

affect the growth of the pathogen. In many instances, *Bacillus* spp. have been found to be highly effective in the production of these oxidative enzymes. According to a study by Ramyabharathi et al. (2012), the liquid formulation of *B. subtilis* EPCO16 was capable of eliciting defense related enzymes like catalase and phenylalanine ammonia lyase (PAL) in tomato plants infected with *F. oxysporum* f. sp. *lycopersici.* Spectrophotometric analysis of plants revealed the activity of defense enzymes at their highest on the seventh day after inoculation by the pathogen.

Chitin is an important polysaccharide, which imparts structural rigidity and integrity to the fungal cell wall. But, the presence of hydrolysing enzymes like chitinase and glucanase breaks down the glycosidic bonds holding the constituting polysaccharides together, that leads to cell leakage and cell lysis. Many species of the *Bacillus* spp. are known to produce chitinolytic enzymes and suppress the

phytopathogens (Podile and Prakash 1996). Wang et al. (2004) observed that two types of chitinase were produced by *B. amyloliquefaciens* V656, which were highly effective in inhibiting the growth of *F. oxysporum* growth. *B. thuringiensis* is known to inhibit the growth of *Sclerotium rolfsii* in soybean plant by a similar mechanism. Studies by Liu et al. (2010) revealed the production of chitinase from *B. thuringiensis* sub sp. *colmeri,* which was instrumental in preventing spore germination of various phytopathogenic fungi. Generally, bacteria possess a variety of chitinases for breaking down the wide array of chitin molecules that naturally exist in nature. Ruiz-Sanchez (2007) observed that five different types of chitinase (with 42, 49, 53, 62, and 66 kDa) were synthesised by *B. licheniformis*.

6.3.3 Production of Lipopeptides and Antibiotics

Bacillus spp. are considered to be the producers of a vast array of antimicrobial compounds such as bacillomycin and zwittermycin A (Mondol et al. 2013). According to studies, 4-5% of the *Bacillus* genome is devoted for the production of antimicrobial compounds (Stein 2005). Among these compounds, lipopepetides of the surfactins, iturins, and fengycin group are most widely studied. Surfactins are antiviral, antibacterial agents, while iturins and fengycins, also known as plipastatins, exhibit highly effective antifungal activities. The lipopeptides (LPs) interact with the each other in a synergistic way, and bring about an alteration of the cell membrane permeability of the microorganisms. The LPs produced by *Bacillus* are non-ribosomal in nature and are produced by non-ribosomal peptide synthetases (NRPSs) or hybrid polyketide synthases and non-ribosomal peptide synthetases (PKSs/NRPSs). The efficacy of biocontrol of plant diseases by lipopeptides produced by *Bacillus* spp. is well established (Chakraborty et al. 2020a, b).

The surfactins are excellent biosurfactants, with notable foaming ability. They attach themselves to the lipid layers, and thus hamper the integrity of the microbial membranes. The surfactins induce pore formation, which is followed by complete solubilisation and disruption of the membranes. Studies also reveal that the surfactins are unable to function in the presence of cholesterol in the phospholipid bilayer, which explains why they are inactive as antifungal compounds (Meena and Kanwar 2015).

Iturins are a group of heptapepetides linked to a β-amino fatty acid chain. They are named so after their place of discovery, Ituri, in the Democratic Republic of the Congo. The family of iturins mainly include iturins A, C, D, and E; bacillopeptin; bacillomycins D, F, L and LC; and mycosubtilin (Mnif and Ghribi 2015). They are highly fungitoxic in nature but with limited antiviral and antibacterial activity. The iturins are known to form ion-conducting pores, which lead to the permeablilisation of the membranes, leading to fungal toxicity.

The fengycins, also known as plipastatins, are a group of lipodecapeptides attached to a β hydroxy fatty acid chain (Wang et al. 2015). Fengycins are highly fungitoxic in nature, especially against filamentous fungi; however, they are less

effective as compared to surfactins and iturins. The mechanism of fungitoxicity of the fengycins is elusive so far. However, these natural compounds have been shown to induce structural imbalance in the microbial membrane (Ongena and Jacques, 2008). Other antibiotics produced by *Bacillus* species include bacteriocins, bacillaene, difficidin, oxidifficidin, sporulenes A–C, bacisubin, and bacilysocin. All these antibiotics exhibit antifungal and antibacterial activity at varying levels of concentrations.

The antibiotics produced by *Bacillus* have a crucial role in its biocontrol activities. The LPs are attributed to biofilm formation by the bacteria in the soil and water surfaces. They bring about the flagella-driven motility of the bacteria, leading to biofilm spread over the plant and soil surfaces. They are also responsible for reducing the surface tension, which helps in further spread of the bacteria. Bais and collaborators (2004) reported that *B subtilis* strain 6051 produced surfactin molecules that resulted in biofilm formation in the roots of *Arabidopsis* plant.

It has been reported by Asaka and his co-workers (1996) that *B subtilis* strain RB14 produces iturin A, which is instrumental in inhibiting the growth of *Rhizoctonia solani* in tomato. Leclere and co-workers (2005) found that mycosubtillin, produced by *B. subtilis* ATCC, helps in the reduction of *Pythium aphanidermatum* infection. The combined role of fengycin and iturin in inhibiting the growth of *Podosphaera fusca* in melon leaves was demonstrated by Romero and co-workers (2007). Ongena and Jacques (2008) observed that *B. subtilis* S499 produces all three families of LPs that help in the management of plant diseases by this strain. This strain also inhibits the growth of *Botrytis cinerea* in wounded apple fruits by the production of lipopeptide-enriched extracts, which contained high doses of fengycins in it.

The production of antibiotics was also linked to the induction of defense reactions in a number of treated plants. In tomato and bean, fengycins and surfactins were directly attributed to induce ISR. *B subtilis* S499 inhibited the growth of *Colletotrichum lagenarium* in cucumber plants by the stimulation of systemic resistance. Potato tubers exhibited an accumulation of phenolics when being treated with purified fengycins, which activate the phenylpropanoid pathway. The activation of phenylpropanoid pathway leads to the accumulation of mRNAs, encoding phenylalanine ammonia lyase (PAL).

Although the antibiotics are highly potent in inhibiting the growth of harmful microorganisms, they have not been reported to produce any phytotoxicity in the host plants. These compounds are able to induce a cascade of biochemical reactions that stimulated the defense system of the plant against the pathogen, without causing any adverse effects on the cellular integrity of the plant. Researchers believed that the differing compositions of phytosterol from that of bacterial and fungal compositions are mainly responsible for the attenuation of the disruption of plant membranes.

6.4 Induction of Resistance in Plants

Several lines of evidence suggest that strains of *Bacillus* spp. elicit systemic resistance in the plants. Induced systemic resistance (ISR) was elicited in plants by *Bacillus* spp. in response to a number of biotic stresses like fungi, bacteria, viruses, nematodes, and insects. *Bacillus* spp. produce LPs which are considered to be the key components in ISR elicitation in plants (Rahman et al. 2015). The ISR modulates a number of cytological and biochemical processes in the host plant such as lignin deposition in the plant cell wall, production of phytoalexins, and synthesis of other antimicrobial substances like peroxidases and β-1, 3-glucanases (GarcíaGutiérrez et al. 2013). Among the LPs produced, surfactin is essential for ISR elicitation as observed by Chowdhury and his collaborators in 2015. As discussed earlier, the iturin and surfactin family of lipopepetides are highly effective in suppressing phytopathogens. After analysis of gene expression, it was observed that surfactin activated salicylic acid (SA) regulated pathway, while mycosubtillin of the iturin family, had the ability to activate both jasmonic acid (JA) and salicylic acid (SA) pathways in grapevine (Farace et al. 2015). Similar results were observed in strawberry, which helped in the successful inhibition of *Colletotrichum gleosporioides* (Yamamoto et al. 2015). The role of LPs was also demonstrated in maize (Gond et al. 2015), rice (Chandler et al. 2015), and tomato (Abdallah et al. 2017). The ISR generally leads to plant growth promotion as seen in strain *B. subtilis* 21-1. Lee and his co-workers (2014) observed that *B. subtilis* 21-1 helps in disease suppression of vegetable crops, by activating the plant defense system, as well as helps in plant growth promotion. It can be concluded that *B. subtilis* elicited plant defense system and growth promotion of the host, which collaborates to inhibit the phytopathogens.

The phytopathogens differ considerably in their morphology and cellular compositions. Hence, *Bacillus* employs different mechanisms against these phytopathogens (Fig. 6.2). It synthesises lipopeptides against some, while it produces enzymes for managing other pathogens. A brief description of the mechanisms used by *Bacillus* against phytopathogens is provided in the following section.

6.4.1 Bacillus *Against Fungi*

Extensive research has been conducted on the mechanism of *Bacillus* for the inhibition of fungal growth. Chitin forms a major constituent of the cell wall of all pathogenic fungi; hence, biocontrol agents such as *Trichoderma, Alteromonas,* and *Serratia* produce different type of chitinases (Elad et al. 1982). *Bacillus* has also been reported to produce chitinolytic enzymes for lysing the fungal cell wall (Mitchell and Alexander, 1962). *Bacillus* species was observed to reduce the radial growth *of Penicillium* sp. (72%), *Aspergillus niger* (64.58%), *Aspergillus*

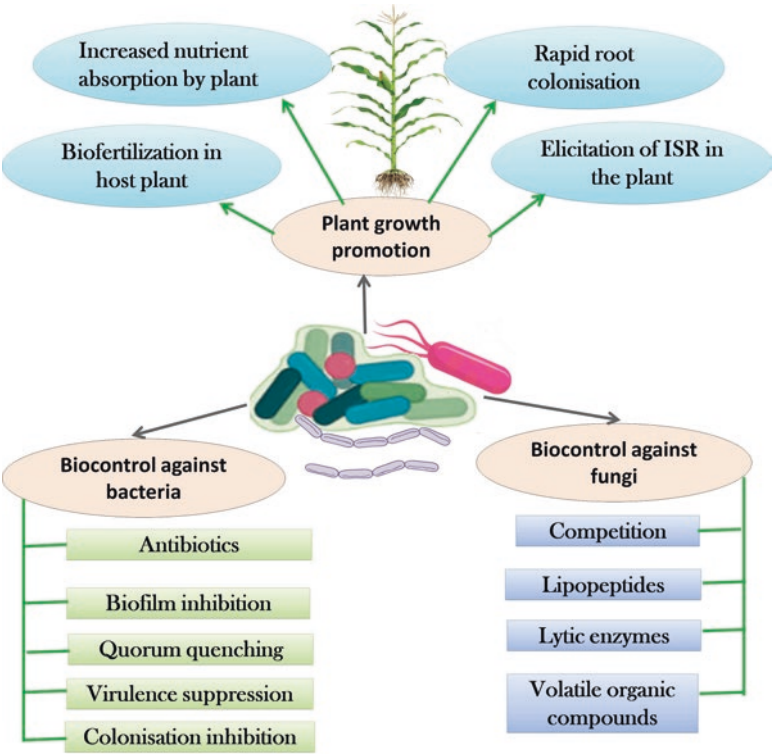

Fig. 6.2 Mechanisms used by *Bacillus* sp. as a growth promoter and biocontrol agent

fumigatus (51.61%), *Fusarium* sp. (39.13%), *Curvularia* sp. (37.50%), *Aspergillus flavus* (35.43%), and *Alternaria* sp. (31.82%), after 6 days of incubation. The inhibitory action was attributed to chitinolytic activity and hyperparasitism as concluded by Basha and Ulaganathan (2002). It was observed by Bargabus et al. (2002, 2004) that *B. mycoides* isolate BacJ and *B. pumilus* isolate 203–7 were highly effective in controlling the *Cercospora* leaf spot of sugar beet to a considerable extent.

Studies revealed that many *Bacillus* strains such as *B. lentimorbus* and *B. cereus* are able to inhibit the development of coffee rust. It has been observed that the bacterium prevented the pathogen from developing uredospores, which resulted in suppression of the pathogen up to 50% (Shiomi et al. 2006). The wide array of volatile compounds produced by *B. velezensis* ZSY-1 was studied by Gao et al. in 2017. They observed that these compounds possessed significant antifungal activities. *Bacillus* was able to inhibit the growth of *B. cinerea, Monilinia fructicola, Colletotrichum lindemuthianum, Alternaria solani,* and *F. oxysporum* f. sp. *capsicum*. It was also observed that benzothiazole, pyrazine, and phenolic compounds

played a crucial role in inhibiting fungal diseases in tomato such as grey mould and early blight.

6.4.2 Bacillus *Against Nematodes*

Limited studies have been conducted on the interaction between *Bacillus* and plant pathogenic nematodes. In some instances, the involvement of LPs has been correlated with the reduction in nematode infestation (Hallmann 2001). Hallmann (2001) observed that bacterial endophytes successfully inhibit the infestation of *Meloidogyne incognita* through extensive root colonisation and elicitation of ISR in the host plant. Reitz et al. (2000) observed that the bacterial endophytes, including *Bacillus*, produced a number of lipopeptides, which successfully controlled potato cyst nematode, *Globodera pallida*. Hallmann and his co-workers (1997) studied the role of endophytic bacteria in inhibiting root-knot nematode, *Meloidogyne incognita*, in cotton roots. They also observed that the nematodes helped the endophytic bacteria in entering the host plant which aided in successful colonisation of the plant roots. Mendoza and Sikora (2009) observed a significant reduction in *Radopholus similis* infestation in banana plants, when a combined treatment of *B. firmus* and *F. oxysporum* was applied on the banana plant.

6.4.3 Bacillus *Against Bacterial Pathogens*

Phytopathogenic bacteria communicate among each other by the production of density-dependent autoinducers (AI), which is known as quorum sensing. Quorum sensing plays a crucial role in establishing the virulence factors in bacteria, which determines the disease severity. In the case of Gram-negative bacteria, *N*-acyl homoserine lactones (AHL) are mostly used as signalling molecules during quorum sensing. *Bacillus* species, however, have the ability to produce quorum quenching molecules that modify the AIs and hinder the bacterial signalling, thus affecting the virulence of the pathogen. *Bacillus* possesses *aiiA* gene encoding N-acyl homoserine lactonase (AiiA) enzyme, which brings about the hydrolysis of AHLs. *Bacillus* species, such as *B. cereus, B. subtilis, B. firmus,* and *B. toyonensis*, have been known to suppress a number of plant pathogenic bacteria by inhibiting quorum sensing, such as *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, and *Pectobacterium carotovorum (Erwinia carotovora).* In addition to directly using the AiiA-producing *Bacillus* species to suppress the virulence of phytopathogenic bacteria (Dong et al. 2004), *aiiA* can be heterologously expressed in other bacterial strains (Molina et al. 2003) or in genetically modified plants (Ouyang and Li 2016).

6.4.4 Bacillus *Against Viral Phytopathogens*

Bacillus species has been found to provide resistance in plants against many viruses, such as Tomato Mottle Virus (ToMov) and cucumber mosaic virus. The application of *Bacillus* leads to the reduction in visible symptoms as well as reduced viral accumulation as is evident from ELISA analysis (Murphy et al. 2000). Some studies indicate that the *Bacillus* species results in an accumulation of IAA, leading to growth enhancement in the plants, that results in systemic protection against the virus as observed by Murphy and his co-workers (2000), in tomato against CMV. However, according to some researchers, *Bacillus* elicits ISR in the virus affected plants, that triggers the expression of PR genes, leading to an accumulation of PR proteins like chitinase and β 1,3-glucanase, as observed by Wang et al. (2009) in tobacco plant against TMV. It is known that NPR1 regulates SAR and ISR resistance pathways while Coi1 regulated ISR pathway. *Bacillus* species has been observed to induce the expression of both *NPR1* and *Coi1* genes in the TMV-resistant tobacco plants, indicating a correlation between the gene expression and disease resistance.

6.5 Genomics and Molecular Basis of Induction Resistance by *Bacillus* on Plant

Bacillus has been known to produce a number of antimicrobial compounds that aid in plant disease management. *B. subtilis* has been considered to be a model microorganism for the analysis of gene functions, as it encodes for a number of antibiotics and lytic enzymes in order to manage phytopathogens. Research on the genomics and molecular basis of resistance induction may help in the development of biologically feasible weapons against plant diseases. Fungal cell walls are made of chitin that provides mechanical strength to the fungus. According to a study, *B. subtilis* CHU26, isolated from a potato field in Taiwan, exhibited strong chitinase activity in vitro, that was successful in inhibiting the growth of *Rhizoctonia solani.* Further investigations revealed the presence of chitinase encoding gene *chi18* that was responsible for this antimicrobial activity (Yang et al. 2009).

Hydrogen peroxide is known to exhibit cytotoxic activity in organisms by the production of hydroxyl radicals that react to lipids, proteins, and nucleic acids. However, *B. subtilis* has been able to mitigate the H_2O_2 stress by the production of enzymes catalase and an alkyl hydroperoxide reductase, encoded by genes *katA* and *ahpC*, respectively (Broden et al. 2016).

For evaluating the role of *Bacillus* in stimulation of ISR, Lee and co-workers (2015) studied strain HK34 of *B. amyloliquefaciens* against *Phytophthora cactorum* by applying *Bacillus* on the leaves and roots of *Panax ginseng*. It was observed that the bacterium was able to reduce the pathogen growth up to 99.1%. The inhibition of the pathogen was found to correlate with the expression of *PgCAT*, *PgPR5*, and *PgPR10* genes. These outcomes showed the ISR-eliciting potential of strain HK34.

Table 6.3 List of genes of *Bacillus* species encoded for the production of various antimicrobial metabolites that help them in biocontrol of phytopathogens

Strain	Gene cluster	Products/function	Reference
Bacillus sp.	*srf*A	Surfactin	Hsieh et al. (2004)
Bacillus sp.	*fen*B	Fengycin	Ramarathnam et al. (2007)
Bacillus sp.	*bac*A	Bacilysin	Mora et al. (2011)
B. subtilis	*Eps* A-O operon	Synthesis of biofilm matrix	Vlamakis et al. (2013)
*B. subtilis*168	*deg*Q	Secretion of degradative enzymes	Parashar et al. (2013)
B. subtilis	*Pks* gene cluster	Bacillaene	Muller et al. (2014)
B. subtilis 916	locA, locB, locC and locD, gene cluster	Locillomycins	Luo et al. (2015)
B. subtilis	*tyr*Z	Growth and biofilm formation	Williams-Wagner et al. (2015)
B. subtilis	*tap*A	Surfactin synthesis	van Gestel et al. (2015)
B. subtilis	*Apr*E and *Npr*E	Extracellular proteases	Barbieri et al. (2016)
B. subtilis	*lta*S	Lipoteichoic acid (LTA) synthase	Kasahara et al. (2016)
B. subtilis	SWR01 *min*J	Swarming	Gao et al. (2016)

Ryu et al. (2004) worked on the production of volatile compounds, produced by *Bacillus* species. They observed that *B. subtilis* GB03 synthesised 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) in abundance, which helped in the induction of ISR. Furthermore, the volatiles produced by the bacteria were controlled by the expression of genes *CHIB*, *GST2,* and *ERF1*, which were known for the biosynthesis of ethylene. Recent studies have been able to discover a number of gene clusters in *Bacillus* species, which are shown in Table 6.3.

6.6 Commercial Applications of *Bacillus* Species

Bacillus strains have been utilised commercially for the generation of a variety of products. It is used not only as a potent host for genetic modifications but also as a source of naturally obtained biocontrol compounds (Bunk et al. 2010). Furthermore, *Bacillus* species produces stress-resistant endospores that contribute towards better environmental stability and longer shelf-life of the products. Among the wide array of *Bacillus* species discovered so far, *B. thuringiensis* is the most widely exploited with more than 70% of the market share (Ongena and Jacques 2008). Different companies utilise different mechanisms of the biocontrol agent in developing its products. For example, Bio-Yield, produced by 3Bar Biologics Inc., USA, contains a combination of two *Bacillus* species, *B. amyloliquefaciens* GB99 which is responsible for the elicitation of ISR. The *B. subtilis* GB122 inhibits soil-borne pathogens by the production of lipopeptide iturin. Also the product contains chitosan, which is effective against insects and nematodes (Kloepper et al. 2004a, b). Another product,

Yield Shield produced by Bayer CropScience Inc., USA, is composed of *B. pumilus* GB34, which activates the plant defense system and promotes plant growth as well (Jeong et al. 2014). Similarly, many other *Bacillus*-based products have been developed commercially that can be utilised for the successful management of phytopathogens (Table 6.4).

Table 6.4 Commercial phytosanitary products obtained from *Bacillus*

Name of the product	*Bacillus* strains	Target pathogen	Crop	Company
Bio-yield	*B. subtilis* GB122, *B. Amyloliquefaciens* GB99	*Fusarium, Rhizoctonia, Pythium*,	Bedding plants	3Bar biologics, USA
Nacillus	*B. subtilis Antumávida, B. subtilis Vilcún, B. licheniformis Mallerauco Brevi, Bacillus brevis Maguellines Brevi, Bacillus brevis Maguellines I*	*Clavibacter pseudomonas, Xanthomonas, Acetobacter*	Vegetables, blueberry Cherry, hazelnut tree, tomato, pear, kiwi tree,	Bio Insumos Nativa, Chile
Yield shield	*B. pumilus* GB34	*Fusarium sp., Rhizoctonia solani,*	Soybean	Bayer CropScience, USA
Votivo	*B. firmus* I-1582	*Pratylenchus, Meloidogyne*	Corn, cotton, soybean,	Bayer CropScience, USA
Dipel	*B. thuringiensis* subsp. *kurstaki* HD-1	*Helicoverpa, Diatraea saccharalis, Grapholita molesta, Diaphania nitidalis, Plutella xylostella, Argyrotaenia sphaleropa*	Citrus, pineapple, tomato, cotton, apple tree, sugarcane, melon, cabbage,	Valent BioSciences, USA
AvoGreen	*B. subtilis*	*Colletotrichum gloeosporioides, Cercospora sp.*	Avocado	Ocean agriculture, South Africa
Ecoshot	*B. subtilis*	*Botrytis cinerea*	Citrus, legumes, vegetables, grape	Kumiai chemical industry, Japan
HiStick N/T, Subtilex	*B. subtilis* MB 1600	*Fusarium sp., aspergillus sp., Rhizoctonia sp., Pythium sp.*	Vegetables, ornamentals	Becker underwood, Ames, IA, USA
Kodiak	*B. subtilis* GB03	*Fusarium sp., aspergillus sp., Rhizoctonia sp., Alternaria sp.*	Legumes	Gustafson Inc., Dallas, Texas, USA
Biosafe	*B. subtilis*	*Xanthomonas axonopodis pv. Phaseoli*	Bean	Laboratorio de Biocontrole Farroupilha, Brazil

6.7 Mode of Application of *Bacillus* Species

The method of application of the biocontrol agent depends on its mode of action. Among the different methods of application, seed coating is the most extensively used, as it is easy to use and can be applied efficiently with a little quantity of inoculum. When the microbes are introduced as granular applications, the biocontrol agent is applied with a mixture of marble, peat, perlite, charcoal, and soil, with an aim of enhancing the contact between the plant roots and the biocontrol agent.

In case of *Bacillus*, both liquid and powdered formulations are available commercially, which are applied in the form of pellets, as well as soil drench, foliar spray, or as seed dressing. When *Bacillus* was applied as liquid formulation on tomato plant, it was able to successfully reduce the symptoms of wilt, as well as increase the shelf-life of *Bacillus* (Ramyabharti et al. 2016). Gao and his co-workers (2015) researched upon the most successful method of *B. subtilis* application against *Blumeria graminis* in wheat. They observed that all the preparations, i.e., cell-free culture, non-protein fermentation liquid, and crude proteins, were able to reduce the infection to some extent. However, fermentation liquid formulation was the most effective among all the preparations studied. Hsieh and his collaborators (2009) observed that talc-based formulation of *B. subtilis* and *P. fluorescens* was able to reduce the Banana Bunchy Top Virus incidence up to about 52% and helped in growth promotion of the plant under field conditions.

According to the studies conducted by Selim and his co-workers (2017), the application of endophytic bacteria as both soil drench and talc-based formulations was able to manage *Rhizoctonia solani* in cotton. But among the two formulations studied, soil drench was found to be more effective. However, Yamamoto and co-workers (2015) found that *Bacillus amyloliquefaciens* S13–3 was highly efficient in managing anthracnose in strawberry when applied as foliar spray. Spraying of fermentation liquid formulation of *Bacillus* sp. significantly improves growth, yield, and content of antioxidants in strawberry fruit (Rahman et al. 2015).

After the analysis of each method, Hallman and his co-workers (1997) observed that every method had its pros and cons. However, seed treatment was found to be the most efficient when ease of application, economic feasibility, and environmental stability was taken into consideration. They further stated that the combined application as seed treatment, soil drench, and foliar spray would be able to enhance the colonisation of the bacteria and also increase the benefits manifold.

6.8 Conclusion

Bacillus sp. is considered as the new tools for the promotion of sustainable agricultural system. A lot of information on biocontrol of plant diseases by *Bacillus* spp. has been generated so far by different researchers throughout the world. In this chapter, we summarised the details of the recent advances on mode of action,

molecular basis of pathogenic antagonism, application in field level and formulations with their stability potential of *Bacillus* spp. In these aspects, this chapter is unique, informative and a good source of molecular insights for the future researchers to deal with the *Bacillus* biocontrol agent in the crop field without harming the nature.

In this chapter, we summarised the mode of actions involved in successful antagonism and the PGPR activities that include – secretion of lytic enzymes, production of lipoproteins, and antibiotics that are explained with examples. The mechanism of induced systemic resistance against plant pathogens depends on interactions of host-pathogen and environment. One or more *Bacillus* strains can work synergistically with plant and environment in suppressing the phytopathogens. The mechanism is varied according to the application of *Bacillus* sp. against fungus, bacteria, virus and nematodes. The most important part of this chapter is the genomics and molecular basis of induction of systemic resistance by the *Bacillus* spp. *Bacillus* is known to produce a number of antimicrobial compounds that deal with plant diseases resistance mechanism. In this case, the spore producing ability and genetically modified host with *Bacillus* genes may provide an effective solution in their colonisation simultaneously along with other beneficial microbes. But for successful colonisation, understanding ecological requirements are mandatory.

Among the wide functional assay of *Bacillus* sp. so far, *B. thuringiensis* is the most widely exploited even more than 70% of the market shares. But there are lots of conflicts on its commercialisation and acceptation country to country. So, successful application and commercialisation depends on the practical understanding on mode of action, methods of application, ecological distribution and interacting environment. But for more popularity among the farmers, a perfect demonstration of benefit:cost ratio would be mostly required.

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Chapter 7
Enhanced Root Morphogenesis in Non-legumes as Induced by Rhizobacteria *Bacillus* spp.

M. A. Baset Mia

Abstract Rhizobacteria exert tremendous beneficial effects on various crop plants especially the non-legumes through multidimensional approaches namely biofertilizing, bioenhancing and biocontrolling activities. They create a conducive environment in the rhizosphere, endorhizosphere, apoplastic areas of tissue in the roots as well as stem, and form new organs by producing and secreting various types of phytohormones. Among the bacteria, species of the genera *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Herbaspirillum, Klebsiella, Pseudomonas, Rhizobium, Bradyrhizobium, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas* and *Serratia* are main candidates for the beneficial effects on plants. Among them *Bacillus* spp. are gaining prominence; application of *B. sphaerichus* strain UPMB10 showed beneficial effects on banana, oil palm, rice, sweet potato and other non-legumes. The bacteria are also endophytic, and found in the apoplastic area of banana roots as have been observed by transmission electron micrography (TEM). Certain rhizobacteria isolated from rice seeds have shown beneficial effects on rice under field and laboratory conditions. Proliferation of root hairs in rice due to the application of *Rhizobium* and *Bacillus* has been documented in *Indica* type of rice under humid tropic condition. Plant juvenile hormones like auxin and gibberellin have been isolated from *Bacillus* spp.-inoculated roots of rice, banana and oil palm, which triggered induction of meristem organization in pericycle in the young roots and consequently forming the lateral roots as documented by various researchers. Inoculation process by *Bacillus* spp. resulted in more root hairs and seedling vigor index of rice and enhanced root growth in banana tissue-cultured plantlets under hydroponic condition.

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7.1 Introduction

Roots are the vital organ of higher plants for anchorage and absorption of nutrients. In dicotyledons, there are two types of roots, namely primary roots, which are produced from the embryo during germination phase, and secondary roots, which originate from the primary roots and also known as lateral roots. However, in monocotyledons, the embryonic roots are usually small, short-lived and only important at the early stage of seedling development. The primary roots are destroyed after few days of germination and new roots are formed from the base of the stem which are known as adventitious or fibrous roots (Bellini et al. 2014). It is interesting to note that root growth is continued until reproductive stage that ceases upon flowering of the plant. The architecture of roots reveals the spatial arrangement which are influenced by the growth and development of plant. The primary root produces the lateral (secondary) roots, and the ratio of both root types changes throughout the life of a plant (Waidmann et al. 2020). The growth, morphogenesis and development of roots are greatly influenced by various biotic and abiotic factors. The rhizobacteria are the most influential biotic factors affecting growth and development of roots by secreting various types of phytohormones. Biotic and abiotic stresses may stimulate morphogenic changes in roots viz. increased or decreased length, abnormal branching and hair formation. Some of these morphological changes of roots are correlated with altered auxin distribution (Potters et al. 2007; Péret et al. 2009). On the other hand, legumes have a strong symbiotic association between bacteria and roots by forming nodule for establishing a strong beneficial association. Non-legumes are potentially capable of establishing an association with diazotrophic bacteria consequently getting beneficial effects through diverse mechanisms (Mia and Shamsuddin 2013). They provide multiple beneficial effects to the host plant through enhanced root formation, acting as biological N_2 fixer (BNF), greater nutrient accumulation, rhizoremediation, serving as crop enhancer and functioning as biopesticides (Levanony and Bashan 1989; Mia and Shamsuddin 2013). They belong to various genera of bacteria like *Agrobacterium*, *Arthrobacter*, *Azotobacter, Azospirillum, Bacillus, Borkholderia, Herbaspirillum, Klebsiella, Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas,* and *Serratia* (Bhattacharyya and Jha 2012). The bioenhancing vis-à-vis crop-enhancing activities is the most important avenue, which is mediated via morphological and physiological changes and enhancement of water and mineral nutrient uptake as observed in inoculated sorghum, banana, rice and oil palm roots (Sarig et al. 1988; Mia et al. 2010a, b; Amir et al. 2001). Murty and Ladha (1988) reported that inoculation by *A. lipoferum* resulted in reduced root length without showing any effect on root surface area in

rice. These changes were attributed to the effect of auxin produced by the root-colonized bacterial strains of *B. subtilis*, which enhanced additional rooting in plants. Roots are the most sensitive organs to alter the IAA levels, resulting in the elongation of primary roots and forming the adventitious and lateral roots. The hormone produced by the bacteria can be used as a relation maker between roots and bacteria. The fact that *B. subtilis* improved rooting response in legumes like mung bean (*Vigna radiata* (L.) Wilczek) has been documented by various researchers. Recently, among the rhizobacteria, the genus *Bacillus* are gaining importance as bioenhancers and biofertilizers for non-legumes throughout the world.

The bacteria of genus *Bacillus*, are rod-shaped, Gram-positive, and facultative Gram-negative properties. They belong to the phylum Firmicutes containing 266 known species. They are motile, endospore-forming and have been found to be colonized in sugarcane roots, employing beneficial effects in association with VAM fungi, which has been documented by Bellone et al. (1997) in Argentina.

Inoculation of *B. licheniformis* along with other PGPR has been shown to enhance plant growth and nutrient uptake in *Salicornia bigelovii* (Bashan et al. 2000), and some species of *Bacillus* namely *B. macerans, B. polymyxa / Paenibacillus azotofixans* can fix N_2 and are also good as P-solubilizing agents, thus recognized as potential biofertilizers (Freitas et al. 1997). *B. azotofixans* are nitrate dependent for growth and are capable of fixing N_2 in the presence of combined N in the media (Subba Rao 1995). Wei (1997) reported that these bacteria can fix N_2 in association with different non-legumes; where inoculated plants had 15% higher yield compared to uninoculated control. Combined inoculation of *B. licheniformis* and *Phyllobacterium* sp. increased plant height and dry weight and consequently more P absorption and lipid content in *Salicornia bigelovii* Torr. (Bashan et al. 2000). Among the bioenhancing beneficial activities on non-legumes, the enhanced root stimulation is the champion by which numerous species of *Bacillus* showed significant effect as shown in the Table 7.1.

Table 7.1 List of *Bacillus* spp. capable of promoting root morphogenesis in different plant species

Bacillus species	Growth of roots	References
Bacillus amyloliquefaciens subsp. *plantarum* UCMB5113	Increases root growth, especially lateral root outgrowth and elongation and root-hair formation	Asari et al. (2017)
B. insolitus, B. subtilis, B. methylotrophicus	Increase root growth	Ashraf et al. (2004), Barnawal et al. (2013), and Radhakrishnan and Lee (2013)
B. subtilis, B. methylotrophicus	Synthesis of plant growth hormones (IAA, GAs, cytokinins and spermidines) trigger plant growth	Arkhipova et al. (2005), Xie et al. (2014), and Radhakrishnan and Lee (2013)
B. sphaericus strain UPMB10	Increases root growth	Mia et al. 2010b, and Amir et al. (2001)
B. Seropedicae	Increases root growth	Weber et al. (1999)

The contrivances of root stimulation have been documented sporadically by a large body of researches throughout the globe. However, their chronological achievements by a thorough review and unravelling precise mechanisms are yet to be done. Keeping these views in mind, this chapter compiles available information and updates our knowledge on the root morphogenesis in non-legumes by rhizobacteria and their mechanism (s) in root morphogenesis.

7.2 Mode and Mechanism of Beneficial Effects

7.2.1 Colonization of Bacteria

Root colonization is recognized as a major factor in establishing a successful beneficial association between bacteria involving four phases, initial phase i.e. movement of microbes to the plant root surface, which can be passive, via soil water fluxes, or active, via specific induction of flagella activity by chemotaxis (Suslow 1982; Mia et al. 1999, 2010a, b). The second phase in colonization is the adsorption to the root surface followed by anchoring, specific and/or complex interaction between the bacterium and the host by ensuring of induction for bacterial gene expression (Brimecombe et al. 2001). It is an established phenomenon that PGPR could colonize the roots both ecto- and endophytically, which has been shown by scanning electron micrography (SEM) and transmission electron micrography (TEM) (Fig. 7.1) (Del Gallo and Fendrik 1994; Mia et al. 1999).

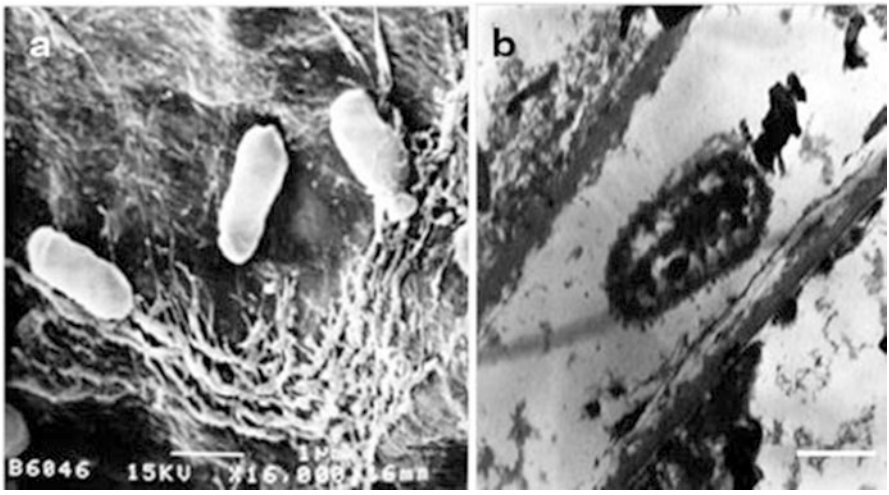

Fig. 7.1 (**a**) Scanning electron micrograph of banana roots shows bacterial colonization by *Azospirillum brasilense* strain Sp7; (**b**) Transmission electron micrograph (TEM) of banana roots shows bacterial colonization by *A. brasilense* strain Sp7as seen in the apoplastic area of root (horizontal bar shows 1 μm). (The images are adopted from Mia et al. 2013)

The root colonization study using *A. brasilense* and *B. sphareicus* has clearly demonstrated that bacterial colonization occurred mainly on root surface area and more cells were observed in the root hair proliferation zone of banana tissue-cultured plantlets. The latter might be due to the presence or availability of root exudates in this area.

Anchoring of bacterial cells on the root surface through a network of fibrillar materials was seen in Sp7 while fibrillar matrices were not observed in UPMB10 (Mia et al. 1999, 2010a, b). The formation of fibrillar matrix is a physiological process during the growth of Sp7 (Bashan and Levanony 1988). More number of Sp7 cells in the root hair proliferation zone is due to presence of fibrillar matrices, which restricted the movement of the bacterial cells to this zone.

The assured and smart attachment of rhizobacteria is essential for a sustainable long-term association with the host roots for having the following reasons:i) if the bacteria are not well attached to root epidermal cells, substances excreted by the bacteria will diffuse into the rhizosphere and are consumed by nutritionally versatile microorganisms before reaching the roots, ii) the bacteria might be washed out from the root zone to succumb to the surrounding soil and iii) association sites on roots with no attached PGPR are vulnerable to other virulent non-beneficial and pathogenic microbes (Bashan and Holguin 1997). The bacteria sometime or even frequently form a biofilm on the rhizoplane which interact between the root surface and the microbe consequently serve as a diffusion barrier of both nutrients and water (Vlamakis et al. 2013).

Bacillus and other rhizobacteria like *Azospirillum* are smart root colonizers, non-plant specific and able to colonize a wide array of plant species (Bashan and Holguin 1997; Mia et al. 2009a). They are also recognized as good colonizers of non-legumes (Bashan et al. 1986; Bashan et al. 1987; Murty and Ladha 1987), vegetables (Bashan et al. 1989), oil palm (Amir et al. 2001), banana (Mia et al. 1999, 2010a), and mangrove plants (Puente et al. 1999). However, the mode of colonization vary greatly among strains (Bashan et al. 1990) where a high level of bacterial colonization is found in the middle lamellae, an area of longitudinal contact between epidermal cells of the roots (Bowen and Rovira 1999), and generally, root caps have less or no bacterial colonizing capacity (Foster and Bowen 1982). However, the preferred site for colonization is the elongation and hairy zone forming an aggregated type of colonization supported by massive fibril material, and colonization sites in non-legumes correspond to the areas where root mucilage is present. The area around the point of emergence of lateral roots usually shows high colonization (Mia et al. 1999, 2010a, b; Bashan and Levanony 1990).

Recently, endophytic plant growth promoting bacteria have been introduced; these colonize roots, stems and leaves of cereals in the apoplastic area of tissue where they probably suffer less competition from other microorganisms for carbon substrates than rhizosphere bacteria, and possibly excrete part of their fixed N_2 and plant growth regulator directly into the host tissue (Baldani et al. 2000; Mia et al. 2007). It is clearly understood that rhizobacteria, especially the species of *Bacillus*, could form colonies on the roots both externally and endophytically i.e. in the apoplastic areas of the root cortical region.

Table 7.2 Root growth of banana plantlets inoculated with PGPR strains Sp7 and UPMB10 grown under hydroponic condition for 45 days (under N-free nutrient solution)

Treatments	Total no. of 1^0 roots (plant^{-1})	Total length of 1^0 roots (cm)	Root volume (cm^3 plant^{-1})	Root dry weight (g plant^{-1})
-PGPR	6.0 a	236 b	6.2 c	0.27 b
+Sp7	6.3 a	314 a	10.9 b	0.65 a
+UPMB10	7.0 a	341 a	16.6 a	0.64 a

Means having same letter (s) in a column do not differ significantly at 0.05 level by DMRT

7.2.2 Enhanced Root Growth and Development

Application of *A. brasilense* strain Sp7 and *B. spharecus* strain UPMB10 enhanced root growth in multiple facet in dessert-type banana under soilless culture. The pronounced effects have been found in root hair formation. Additionally, inoculation process greatly increased the production of primary, secondary and tertiary roots and total primary root length by 33-44% over un-inoculated control by inoculation process. Root volume was also significantly increased and effect of UPMB10 produced a greater volume than Sp7, an increase of 52%. The root dry weights were increased through inoculation by 137-141%. Strong positive results on root stimulation are shown in bananas under soilless culture condition (Table 7.2).

Results of a study on the application of rhizobacteria on tissue-cultured plantlets under hydroponics indicated that PGPR inoculation could increase root growth (root mass (137-141%), volume (76-168%) and length (33-44%)) with a consequent increase in N yield and higher growth of plant tops viz. plant height (42-50%), leaf area (128-134%) and dry matter (129-176%). It was demonstrated by scanning electron micrography (SEM) that *A. brasilense* strain Sp7 and *B. sphaericus* strain UPMB10 successfully formed colonies on the banana roots. Bacterial colonization by Sp7 and UPMB10 occurred mainly on the root surface and more cells were found in the root hair proliferation zone while the root hair per se is devoid of bacteria where Sp7 colonized mainly the root hair proliferation zone with a few cells found on the root elongation and root cap zone. Occurrence of more cells of Sp7 in the root hair zone was due to the presence of fibrillar matrix which aided in the attachment of bacterial cells on the root surface. But strain UPMB 10 performed better i.e. efficiently colonized all the three root zones viz. cap, elongation and hair proliferation areas. The presence of more UPMB10 cells throughout the whole roots indicated that this strain was able to utilize exudates produced by the three zones (Mia et al. 2010a, b). Similar fashions of root colonization have been documented by several researchers (Assmus et al. 1995; Bashan and Holguin 1997) who concluded that colonization of rhizobacteria corresponded to the areas where root mucilage was present. *A. brasilense* strain Sp7 preferred to colonize the root hair zone, and inoculation with different strains of *A. brasilense* to young wheat plants showed colonization both in rhizoplane and endorhizosphere and stimulated root and shoot growth. (Levanony et al. 1989; Saubidet and Barneix 1998) (Table 7.3).

Table 7.3 Root growth of banana plantlets inoculated with PGPR strains Sp7 and UPMB10 grown under low fertilizer-N regimes (33% of the requirement)

Treatments	No. of 1^0 roots plant^{-1}	Total 1^0 root lengths (cm)	Base diameter of 1^0 root (mm)		Root volume (cm^3 plant^{-1})	Root dry wt. (g plant^{-1})
			Feeder	Pioneer		
N_0-PGPR	6.0 b	236 b	0.13 b	0.25 c	11.3 d	0.27 c
N_0 + Sp 7	8.3 ab	314 ab	0.17 ab	0.27 bc	18.0 c	0.65 b
N_0 + UPMB 10	7.0 ab	341 a	0.23 a	0.35 abc	27.3 b	0.61 b
$N_{33\%}$ + Sp 7	9.6 a	308 ab	0.21 ab	0.38 b	55.0 a	1.02 a
$N_{33\%}$ + UPMB 10	9.6 a	355 a	0.19 ab	0.42 a	59.0 a	0.99 ab
$N_{100\%}$-PGPR	8.6 ab	372 a	0.15 ab	0.42 a	50.0 a	0.69 b

Means having same letter (s) in a column do not differ significantly at 0.05 level by DMRT

The growth enhancing effects by rhizobacteria are mainly derived from morpho-physiological attributes in roots of inoculated plant (Okon et al. 1988; Sarig et al. 1988), and the increased root growth occurred almost in all dimensions namely production of primary (1^0) and secondary (2^0) roots, longer roots (42%), and greater volume (48%) and mass (43%). Primary root elongation rate and 2^0 root initiation capacity of inoculated plant were increased due to the synergy of PGPR inoculation and minimal fertilizer-N application. Plants inoculated with strain UPMB10 and supplied with fertilizer-N showed more 1^0 root elongation while those with Sp7 and fertilizer-N showed greater 2^0 root initiation. The primary root elongation of UPMB10-inoculated plants might be due to more number of bacterial cells present throughout the whole root system which resulted in the root elongation from all the root zones, whereas the presence of more cells of Sp7 in the root hair zone stimulated the initiation of 2^0 roots. The higher 2^0 root initiation in Sp7-inoculated plants is due to the presence of more bacterial cells and their beneficial interaction in the hair proliferation zone *vis-a-vis* the zone of secondary root initiation in bananas.

It is interesting to note that as monocotyledon, banana roots are adventitious, arise in a group, white and fleshy when young, but corky when old. Most of the banana roots are not deep-statured, mainly present in the topsoil and grow horizontally (Sioussaram 1968). Swennen et al. (1986) classified banana roots as primary, secondary and tertiary roots which arise from the corm, primary roots and the secondary roots, respectively. The primary roots can further be classified into two groups namely feeders and pioneers. Feeders are significantly longer but thinner than pioneers and have a higher density of secondary roots. Every root has three different zones, namely root cap, elongation and hairy zone (Fig. 7.2).

Root surface area is considered as one of the important arena for absorption of nutrients through roots. The surface areas have been increased in maize by the inoculation of *Azospirillum*, which is reported by Jacoud et al. (1999). The surface area of inoculated plants were always greater than non-inoculated control plants. The positive results were remarkable during the early stage of the plants i.e. germination to seedling stage.

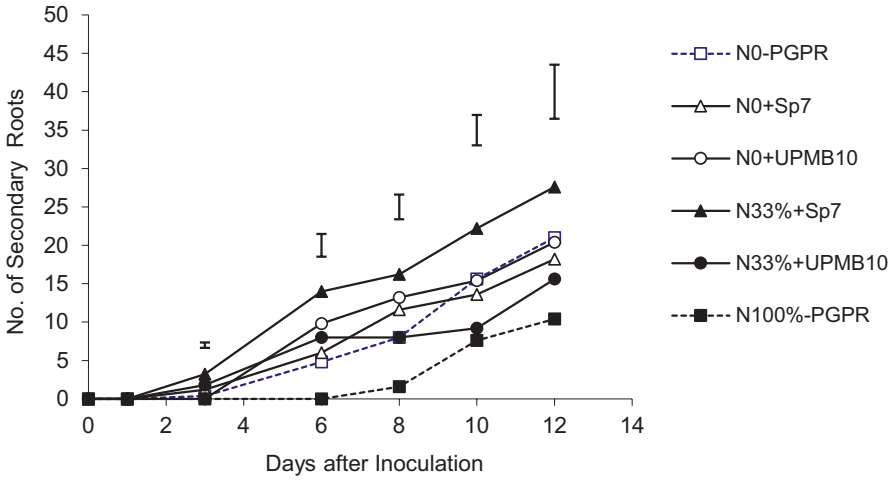

Fig. 7.2 Secondary root initiation of banana plantlets inoculated with PGPR strains Sp7 and UPMB10 (vertical bar represents LSD at 0.05 significant level) (Adopted from Mia et al. 2009b)

7.2.3 Root Hair

The epiblema generates distinctive outgrowths known as hair, which greatly increased the surface area of roots, consequently improving water and nutrient absorption. Generally, root hairs located 1-2 cm behind from the root cap, or tip, and hairs live for only a few days and never develop into multi-cell roots. Because of their short life, roots need to grow continually. Its development is particularly sensitive to both biotic and abiotic stimuli and is a useful marker of differentiation processes that take place in the root (López-Bucio et al. 2003). They perform significant roles in absorption of nutrients and water by increasing the surface area of root system. The formation of root hairs follows three distinct phases, namely selection of site, bulge formation and elongation phase, where the initiation phase is regulated by transcription factors and activity of certain enzymes, namely GTPase and wall modifier enzymes (Datta et al. 2011; Dittmer 1937).

Rhizosphere microorganisms contributed remarkable effects on the productivity of crop plants in agricultural and marginal soils by influencing growth and development of roots and improving nutrient availability in the rhizosphere (Mia 2015). These organisms have been recognized from a wide range of plant species of both dicot and monocot like sweet potato, tomato, chili, sugarcane, maize, wheat, rice, barley, canola, banana and bean. Their contribution can be exerted through direct and indirect methods. The direct methods include those by which bacteria directly influence root-system architecture and shoot development by secretion of plant growth-promoting substances such as auxins and cytokinins (Persello-Cartieaux et al. 2001).

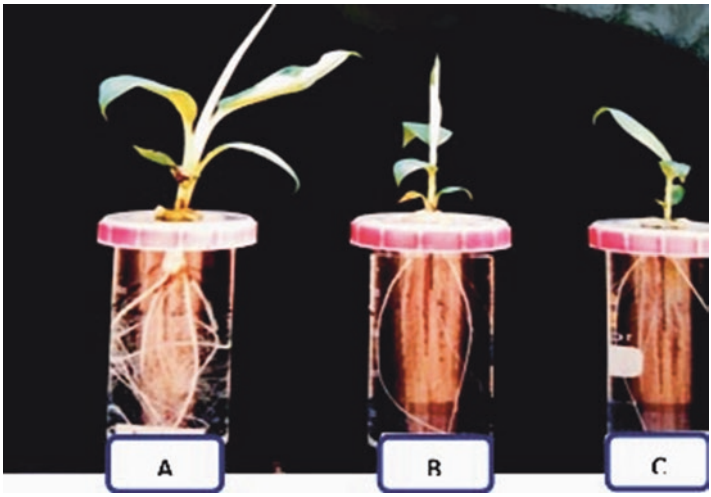

Fig. 7.3 Increased root hairs of inoculated banana roots showed proliferated root hairs by the inoculation of (**a**) *A. brasilense* strain Sp7; (**b**) *B. sphaericus* strain UPMB10; (**c**) Control

Similarly, inoculation of *Bacillus* spp. influences root hair formation in banana (Fig. 7.3), rice, wheat, maize and oil palm, which has been observed by various researchers (Mia et al. 2013, 2016). Additionally, inoculation process of rhizobacteria on germinated low-land *Indica*-type rice showed profuse hair, elongated roots and consequently greater root mass production, which has been documented by Mia and Shamsuddin (2009).

The main mechanism of enhanced root hair formation is due to the production of phytohormone especially auxin and gibberellin, as they enable dynamic hormone-driven changes resulting in altered root hair growth, density, length and morphology (Vissenberg and Gonzalez 2020). The rhizobacteria influences on proliferation of hairs by secreting growth hormone like auxin and gibberellin, which has been documented by various researchers in both legumes and non-legumes. Similarly, production of plant growth regulators by the application of *Bacillus* spp. has been recognized by a large body of research findings in legumes and non-legumes (Hashem et al. 2019; Naher et al. 2013). Among the growth regulators, auxin is predominantly the secondary plant-friendly metabolite secreted by the bacteria, contributing in the enhancement of root hair in epiblema. This is a class of essential hormones which controls the positioning of the hair initiation where the alteration of density, length and morphology of hairs are governed by hormonal signalling interconnected with the hair signalling and the degree of morphological changes (Datta et al. 2011; Ishida et al. 2008; Péret et al. 2009).

7.2.4 Lateral Roots Formation

The roots branching out from the primary root are called secondary or lateral roots. In contrast to the taproot system, the majority of monocot roots form a fibrous 'homorhizic' root system, which is characterized by the development of many adventitious roots (Osmont et al. 2007; Bellini et al. 2014; Levanony and Bashan 1989). These are very important in absorbing nutrients, and at the tip of the root, there is an area where new cells develop, called the apical meristem. Root cap over the top of roots protect the meristem from damage when it grows quickly through soil particles, and the surface of the root is covered with a layer of cells called the epidermis which is devoid of cuticle. The rhizobacteria influence the stimulation of lateral roots and enhance proliferating of hairs by secreting growth hormones like auxin and gibberellin and have been documented by various researchers both legumes and non-legumes. As it is known that there are three parts of roots, namely root tip, root elongation and root hairy zones which are responsible for varied levels of nutrients absorption. Nevertheless, rhizosphere, an adjacent layer which is very close and outside of the surface and influenced by the root activity, is the hub of nutrients which are taken up by the epiblema of roots. The epiblema contains root hair, the extension of the epidermal cell is directly involved in nutrient absorption. The increased number of lateral roots along with root hair formation have been observed in bean by the inoculation of *B. megaterium*. However, primary-root growth was inhibited by the process of inoculation which might be happened due to reduction in cell elongation and by reduction of cell proliferation in the root meristem. Nevertheless, lateral roots originate from the pericycle beneath the endodermis. These tissues are very important to initiate lateral or secondary root formation, and they are located at the periphery of vascular cylinder. The layer of those tissues are located just beneath the endodermis in front of vascular tissue especially the phloem, composed of parenchymatous cell and surrounds the stele, and are heterogeneous in nature (Beeckman et al. 2001; Himanen et al. 2004; Laplaze et al. 2005; Mähönen et al. 2006; Parizot et al. 2008). The pericycle may be dedifferentiated into meristem by the activity of certain growth regulators. The inoculated roots resulted in the production of more lateral roots, which is mediated by the secretion of phytohormone by the PGPR. The lateral roots are initiated by the induction of hormone, especially auxin, which is accumulated in the pericycle, and it triggers the asymmetric meristematic activity. The endodermis plays a significant role in supplying auxin to the pericycle (Marhavy et al. 2012), and this accumulation of auxin in a selective number of pericycle cells is one of the earliest events during founder cell specification that precedes lateral roots initiation (Dubrovsky et al. 2008), although the molecular mechanism of auxin-mediated lateral root formation is not clear (Fukaki et al. 2007). However, it is recognized that the process progress is mediated by at least four recognizable phases like priming, initiating, patterning, and emerging (Malamy 2009; Péret et al. 2009; Joseph et al. 2012), and each of these phases is either controlled or at least influenced by auxin. The steps have been shown in the schematic diagram below:

The IAA can initiate the developmental program for lateral roots formation via a model where it requires at least two steps viz. initiation of cell division in the pericycle and promote cell division and maintain cell viability in the developing lateral roots (Celenza et al. 1995). The overall mechanism has been shown in the schematic diagram below (Figs. 7.4 and 7.5):

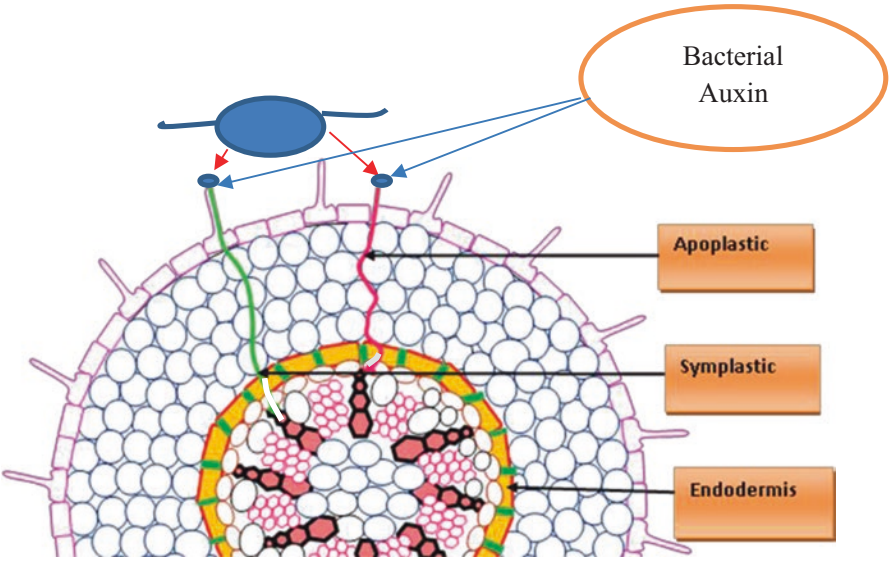

Fig. 7.4 Diagram shows movement of bacterial auxin to the endodermis through symplastic and apoplastic pathways in the root (Adapted from Mia 2015)

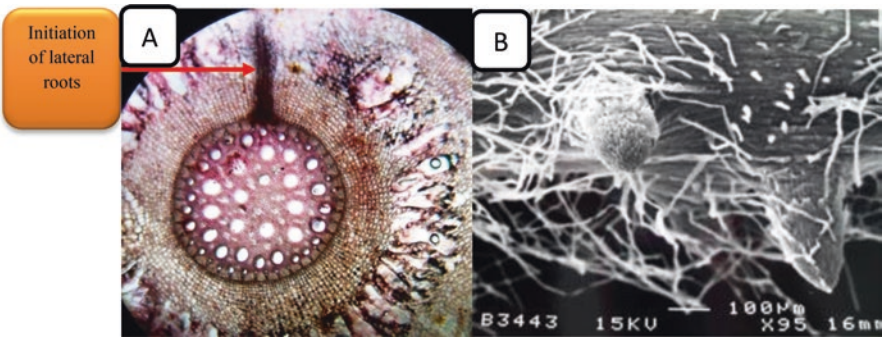

Fig. 7.5 (**a**) Transverse section of banana roots showing initiation of lateral roots from pericycle observed by compound light microscope (X-100); (**b**) Emerging of lateral roots as shown by electron micrography (SEM) of banana roots (horizontal bar shows the micrometre)

7.2.5 Role of Auxin in Lateral Root Initiation

Root systems show variable architectures that contribute to functionality of plants, and the phytohormone auxin fulfils a large body of roles throughout lateral root development. The recent advancement clarified four phases of lateral root formation: (i) positioning of lateral roots, which determines the spatial distribution of lateral root primordia and lateral roots along primary roots; (ii) lateral root initiation, encompassing the activation of nuclear migration in specified lateral root founder cells (LRFCs) up to the first asymmetric cell division; (iii) lateral root outgrowth, the 'primordium-intrinsic' patterning of de novo organ tissues and a meristem; and (iv) lateral root emergence, an interaction between lateral root primordia and draping tissues for allowing passage through cell layers. The auxin plays a significant role through signalling and changing the developmental cortex for completing all the phases (Bao et al. 2014).

The phytohormone auxin is a key regulator of various features in the growth and development of plant including cell division and elongation, differentiation, tropisms, apical dominance, senescence, abscission and flowering (Woodward and Bartel 2005; Teale et al. 2006), where Naxillan, a non-auxin compound, enhances the conversion of auxin precursor IBA (indole-3-butyric acid) to the active auxin-like IAA (indole-3-acetic acid) (De Rybel et al. 2012). The root cap turnover dynamic may co-ordinate primary root growth with lateral root formation in response to environmental stimuli, namely gravity, water and nutrients, and this highlights possible interaction sites in plants to transform extrinsic environmental signals into intrinsic developmental strategies where rhizobacterial influence is yet to be elucidated (Xuan et al. 2016).

The plant growth regulators like auxin and cytokinin are involved in several stages of plant growth and development, namely division and elongation of cell, tissue differentiation and apical dominance. However, the biosynthesis and the underlying mechanism of auxin action on root formation need to be continued. The genetic mechanism of auxin biosynthesis and regulation by *Pseudomonas, Agrobacterium*, *Rhizobium*, *Bradyrhizobium* and *Azospirillum* are well studied; in these bacteria several physiological effects have been correlated to the bacterial phytohormones biosynthesis. However, they do also follow chromosomally encoded indole-3-pyruvic acid pathway; additionally, they have genes that can conjugate free auxins or hydrolyze conjugated forms of auxins and cytokinins. Several genes are located near the auxin and cytokinin biosynthetic genes which are involved in the regulation of auxins and cytokinins sensibility of the transformed plant tissue in *Agrobacterium*. On the other hand, symbiotic bacteria viz. *Rhizobium* and *Bradyrhizobium* synthesize IAA via indole-3-pyruvic acid; also the genetic determinants for the indole-3-acetamide pathway have been detected, but their activity has not been demonstrated. In the plant growth-promoting bacterium *Azospirillum*, as in *Agrobacterium* and *Pseudomonas*, both the indole-3-pyruvic acid and the indole-3-acetamide pathways are present, although in *Azospirillum* the indole-3-pyruvic acid pathway is of major significance.

In case of *Bacillus* strains *B. cereus* (So3II) and *B. subtilis*(Mt3b) showed variable pathways for the production of IAA under different set of growth and environmental conditions where the IAA production potential can be enhanced by affecting optimum growth conditions (Wagi and Ahmed 2019).

7.2.6 Secretion of Hormone and Translocation Through Root System

Plant growth hormones are biochemical substances which are produced in one place of a plant, translocate to another site and perform distinct functions on growth and development. They are produced in the shoot of the plant and translocate basipetally up to the base of the stem, thereafter moving acropetally in the root system (Skoog 1938). Most of the beneficial rhizobacteria, endophytic and phyllospheric bacteria are able to synthesize IAA, and it is a potent signaling molecule essential for plant–microbe interactions and improving plant growth directly (Matsuda et al. 2018).

Nearly 80% of the rhizobacteria are capable of synthesizing IAA, and the majority of the IAA produced from tryptophan is released by root exudates (Khalid et al. 2009). It is proved that higher amount of IAA could be produced by applying tryptophan in the culture media. In the symbiotic bacteria *Rhizobium fredii* and *Bradyrhizobium japonicum*, the production of IAA via IAM pathway was suggested, as bacterial cultures can convert an IAM analog. A gene (*bam*) with sequence similarity to *iaaH* could be isolated from *B. japonicum* (Peyush et al. 2000). Later, the presence of the IAM pathway was also confirmed for *Rhizobium* sp. strain NGR234 by LC-MS detection of intermediates (Theunis 2004).

7.2.7 Translocation of Phytohormone Through Root System

Whatever the production of hormone via various pathways, bacteria secrete these in the epiblema or in the apoplastic area of root cortical regions. The secreted hormones are translocated through apoplastic or symplastic movement most probably by the diffusion process. However, endogenous IAA in the root cap could move to the growing zone and cause a unilateral inhibition of growth. In the case of exogenously applied IAA, the pathway of transport pool is also similar.

Nevertheless, both rhizospheric and endophytic bacterium-released IAA change the plant auxin pool to either optimal or supraoptimal levels and induce root stimulation (Iqbal et al. 2017). The IAA is a signalling molecule comprising heterogeneous carboxylic acid group responsible for controlling diversified physiological processes especially the additional root formation in the plants. Generally, it is produced in aerial parts like shoot apex and is transported through phloem to sub-aerial parts of the plants actively by utilizing two common transportation pathways towards the root via non-directional passive pathway or via cell to bi-directional active pathway known as polar auxin transport (Park et al. 2017).

Anyway, IAA production by bacteria is one of the most important direct mechanisms utilized by plant growth-promoting bacteria (PGPB) for the betterment of plants naturally because auxin is a plant-friendly secondary metabolite synthesized naturally by bacteria, and hence improves the growth of associated plants. Bacterial IAA changes the auxin pool to either supraoptimal or optimal, thereby improving root growth, especially the development of lateral roots resulting in greater surface area that promotes plant nutrient uptake (Ahmed and Hasnain 2010).

7.3 Conclusions and Future Perspective

The application of plant beneficial bacteria is increasing tremendously throughout the world for its environment-friendly uses. They provide advantages in counteracting adverse soil condition where enhanced root growth is required for better productivity. The use of these bacteria for increasing productivity is gaining eminence. Keeping the plants in juvenility is important, as it is vital for proper morphogenesis of plant organ which is mediated by the action of phytohormone like IAA. Plant beneficial microbes viz. rhizobacteria, endophytic, and phyllosphere, provide significant contribution by producing hormone and subsequently secreting to the host plant for providing beneficial effects. Root morphogenesis through hair and lateral roots formation is mediated by this hormone, and the mechanism has been discussed here comprehensively. The mechanism of root morphogenesis by rhizobacteria especially the *Bacillus* involves the following steps: synthesis of IAA by using tryptophan, release of IAA to the epiblema or apoplastic region of root cortical zone of root, translocation to the endodermis and release to pericycle cells for induction, forming founder cell followed by lateral root primordia development and finally emerging the lateral roots. In addition, this root hair is formed by the induction of IAA through differentiation of epiblema to epiblemal outgrowth formation. However, the molecular mechanism for bacterial synthesis of hormones, their secretion and utilization by the root tissue were not investigated comprehensively, which needs to be elucidated in future studies.

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Chapter 8
Mechanisms Involved with Bacilli-Mediated Biotic and Abiotic Stress Tolerance in Plants

Mahfuz Rahman, Md Nur Alam Miah, and Whitney Dudding

Abstract Due to increased demand for food and feed, plants are being grown in marginal lands dominated by abiotic stresses. These abiotic stresses predispose plants to biotic stresses compromising the yield and quality. Mitigation efforts of these stresses with synthetic chemicals further complicated the situation. However, use of beneficial microbes opened a new horizon for managing these stresses in the agricultural ecosystem. To date, an appreciable amount of research elucidated the underlying mechanisms how these microbes, especially numerous species belonging to the genus *Bacillus*, play a positive role in mitigating these stresses. Colonization of plant rhizosphere or phyllosphere by these microbes contributes to alleviating these stresses through up- or downregulation of major metabolic pathways in plants. Regulation of metabolic pathway helps in reducing/neutralizing the level of stressors or inducing plants to overproduce stress-mitigating biochemicals. This chapter compiles all the major mechanisms pertaining to biotic and abiotic stress alleviation in plants by *Bacilli* to aid in elucidating more complex mechanisms by future research endeavors.

Keywords Stress alleviation · Metabolic pathway · Molecular mechanisms · Gene expression · Peptides · Climate change · Biotic factors · Agro-ecosystem · Plant health · Toxic effluent · Innate immune system · *Bacillus* sp.

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8.1 Introduction

Crop productivity is influenced by a combination of environmental and genetic factors (Kleinwechter et al. 2016). Genetic potential of many crop varieties is not achieved since crops in most cases are grown in stressful or less-than ideal growing conditions, which include unfavorable edaphic and climatic environments. It is universally proven that various stress factors adversely influence the physiology of a plant starting from germination through growth and yield. These stresses are primarily classified into two major groups: abiotic and biotic. Our agro-ecosystem is subjected to continuous exposure from a series of ever-changing abiotic and biotic factors, many of which eventually create an unfavorable environment for soil health, fertility, crop growth, and productivity. It is now well documented that these abiotic and biotic stresses can affect all aspects of plant growth including biomass production and intended yield (Pandey et al. 2017). While all biotic stresses are of biological origin like diseases, insects, and weeds; abiotic stresses are generally physical or chemical, and imposed on plants by their environment. Disease-causing organisms include fungi, bacteria, viruses, nematodes, and phytoplasmas. Abiotic stresses on plants are on the rise due to human activities that drive global warming and climate change, releasing toxic effluent to arable land. Despite the consequences, people attempt to grow crops in saline or nutrient-deficient, unsuitable land, due to the increased demand for food and feed worldwide. Exposure of plants to abiotic stresses predispose them to biotic stresses such as pathogens, insects, and may also reduce their ability to compete with weeds. Combined abiotic and biotic stresses can severely affect crop productivity. Even if these stresses occur separately, 50% and 30% of worldwide agricultural productivity could be lost to abiotic and biotic stresses, respectively. However, plants have evolved a sophisticated defense network, also called innate immune system, in response to fluctuating environmental conditions that provide protection to some extent against these stress factors. There are no simple solutions to fully counteract these stresses with modern agricultural technologies discovered over the last few centuries. However, many plant-beneficial microbes living in the soil and microbes existing on or inside plants as endophytes or epiphytes have been found in several studies to trigger a plant's innate defense system, thereby promoting growth and protecting plants from biotic and abiotic stress factors (Glick 1995; Radhakrishnan et al. 2014, 2017; Tonelli et al. 2010). These beneficial microbes contributed to boosting crop yield by minimizing either abiotic or biotic or both types of stresses. Among the microbes, *Bacillus* and *Pseudomonas* species are the major plant stress mitigators and growth-promoting bacteria (Kang et al. 2015a). However, the ability of *Bacilli* to form spores distinguishes them from that of *Pseudomonas*. The survival capacity of the members of this genus for a long time under unfavorable environmental conditions make them suitable to be used for prevention of both biotic and abiotic stresses encountered by plants. That is why *Bacillus* spp. is ranked at the top as a microbial agent for mitigating both abiotic and biotic plant stresses due to their unparalleled genetic fitness. Dihazi et al. (2012) noted that organic and sustainable farming practices prefer

application of *Bacillus* or other bacterial biocontrol agents as an eco-friendly option to enhance disease resistance in crops, which is also considered as a safer method of increasing crop productivity. Use of synthetic pesticides can be reduced in modern agriculture by utilization of *Bacillus*-derived formulations or products due to their proven efficacy (Myresiotis et al. 2015). In addition, an appreciable number of studies have been directed towards understanding *Bacillus*-mediated protection of crop against adverse biotic and environmental stresses at the physiological, biochemical, and molecular levels. Similar advancements through numerous studies have also taken place in the area of *Bacillus*-based remediation or detoxification of metals and other pollutants from the edaphic environments. As a result, remarkable development has occurred in elucidating the underlying mechanisms pertaining to biotic stress tolerance in plants by the use of *Bacillus* (Choudhary and Johri 2009; Ongena and Jacques 2008). However, information on *Bacillus*-mediated abiotic stress tolerance mechanisms are still very inadequate (Arkhipova et al. 2007; Wolter and Schroeder 2012) as most of the studies on *Bacillus*-based abiotic stress tolerance focused on evaluating plant growth promoting effects rather than unraveling specific mechanisms (Dimkpa et al. 2009). *Bacillus*-mediated stress mitigation mechanisms have been explored by many researchers to improve efficacy by using a consortium of beneficial microbes and combining them for diverse multiple modes of action. Many potential stress mitigation mechanisms that have been observed indicated that *Bacillus* spp.-induced actions on the adverse environmental factors and host plants are diverse, numerous, and may be indirect or direct (Berg 2009; Numan et al. 2018; Perez-Garcia et al. 2011; Turan et al. 2012). It is not uncommon for some species of *Bacillus* to mitigate multiple stresses if present at the right time in ample populations. This chapter highlights the major *Bacillus*-mediated plant abiotic and biotic stress tolerance mechanisms by comparing with plants' innate responses to stresses.

8.2 Major Abiotic Stresses and Their Impacts on Crop Growth and Yield

Abiotic stressors are a major cause that puts obstacle against enhancement of worldwide crop production (Bray et al. 2000; Wang et al. 2003). Crop plants have to cope with adverse environmental and edaphic factors with biological mechanisms that are intrinsic to them like hormonal signaling (Nguyen et al. 2016) and interaction with beneficial microbes (Numan et al. 2018). Plant growth, development, and productivity can suffer immensely if they fail to counteract those stresses (Pereira 2016). As more arable land is lost to urbanization and non-agricultural use, crops are being grown in less-suitable areas where abiotic stresses are common. As a result, 50–82% of major crops encounter losses that pose a serious threat to agriculture and food security due to adverse environmental conditions like drought, salinity, extreme temperature, UV radiation, heavy metals, or various oxidative stresses

(Benedetto et al. 2017; FAO 2016). By impairing biochemical/physiological and molecular processes, these stress factors (individually or combined) may induce numerous hostile effects in plants, eventually reducing plant growth, development, and productivity. Stresses from abiotic factors are now considered as one of the biggest potential threats all over the world to agricultural productivity that may affect yields up to 70% for staple food crops as projected by many studies (Kaur et al. 2008; Mantri et al. 2012). Among many different types of abiotic stresses, accumulation of various heavy metals in agricultural soil has created a major concern to the agriculture system and health of many millions of people in countries such as Zambia, Ukraine, Russia, Peru, China, and India (ENS 2006). Wang et al. (2003) estimated that 30% arable land may be lost by the end of 2028, and at this rate it may reach 50% by mid-twenty-first century due to high salinity. Other environmental factors such as the projected increase of mean temperature by 3 °C due to the rise in CO_2 concentration by the end of twenty-first century by about 500–1000 ppm, which will cause heat stress to crops (Khan et al. 2013). Thus, salinity of arable land, nutrient deficiency, drought, metal toxicity, and unforeseen impact of climate change are likely to significantly worsen the problem (Anjum et al. 2014). The combined effect of these stresses may result in losses of soil microbial diversity, soil fertility, and availability of nutrients (Chodak et al. 2015). Plants require a favorable growing site for their physiological and developmental processes. Less than favorable growing condition induced abiotic stress factors can also predispose plants to biotic stresses that interfere with normal growth and development thereby reducing productivity. Plants use their intrinsic recognition mechanism to detect and respond to stresses to some extent by activating the defense pathways to support their nourishment (Jiang et al. 2016; Ahmad et al. 2015; Crane et al. 2011). However, this response may not be enough to overcome stresses if stress levels exceed a certain threshold. When beneficial microbes such as *Bacilli* are present in or on plants within a stressful environment, they modulate the environment and provide enhanced stress tolerance capacity to plants through several procedures described below.

8.2.1 Mechanisms of Abiotic Stress Alleviation

Plants have developed an efficient innate defense network that is turned on by external stimuli representing abiotic (environmental) and biotic stresses. Depending on the genetic makeup of plants, they can employ various self-defense mechanisms to prevent these stresses through up- or downregulation of major metabolic pathways, such as TCA cycle, photosynthesis, accumulation of secondary metabolites, and specific sugars or amino acids (Lotfi et al. 2010; Rai 2002; Lewis et al. 2001). This wide array of different metabolites may play a significant role in plant stress tolerance. For example, phytohormones profoundly influence control of specific molecular mechanisms in plants, and thus optimizing plant responses against stresses of abiotic nature (Nguyen et al. 2016). However, presence of plant beneficial microbes such as *Bacillus* alleviate these stress factors by either reducing/neutralizing the

level of stressors or inducing plants to overproduce stress-mitigating biochemicals. During low water availability, salt and heavy metals can accumulate on top soil layers. Heavy metal accumulation in agricultural soil has also been increasing due to the release of industrial effluent and spread with water. *Bacillus* spp. if present in an environment can produce ample amount of exopolysaccharides and siderophores to bind Fe, which usually prevent the movement of toxic ions and adjust the ionic balance and water transport in plant tissues. Plants can also rapidly sense changing environmental conditions and defend themselves with their innate defense mechanism. Plant responses to abiotic stress involve an induced metabolic cross talk within various biosynthetic pathways. Root system can also sense abiotic stress signals and respond accordingly to the stresses originating from soils (khan et al. 2016). These responses are results of an intricate mechanism that involves changes at genetic, cellular, metabolic, and physiological levels (Atkinson and Urwin 2012). Major impact of abiotic stress is water-deficient conditions created within cells, which is followed by a series of biochemical, molecular, and phenotypic defense action (Xu and Zhou 2006; Almoguera et al. 1995). As the number of stressors experienced by a plant increases, so does the complexity of their responses when compared to plants with a single stress. The complex nature of such responses is due to the activation of a specific gene along with metabolic programming in cells against individual stresses in a specific growing condition. Stress tolerance is a vital phenomenon that may vary with different stages of plant development. Abiotic stress responses may reduce or increase the susceptibility of plants toward biotic stress caused by pests or pathogens (Rizhsky et al. 2004). This becomes more important in agricultural crops because, in various agricultural systems, most crops grow in unfavorable environmental conditions that restrict genetic growth and development potential of plants (Bray et al. 2000). Metabolic regulations including wide changes in the composition, concentration, and distribution of secondary and primary metabolites are among the common responses of plants to abiotic stresses. While secondary metabolites such as alkaloids and flavonoids produced by plants in response to stresses are utilized mostly for their defense, primary metabolites such as carbohydrates and amino acids play a crucial role in the plant's growth and development. Major *Bacillus*-mediated abiotic stress mitigation mechanisms are presented below:

8.2.1.1 Mechanisms to Mitigate Drought Stress on Plants

Global climate change is arguably one of the most pressing ecological concerns of our lifetime. Future changes in water availability and increasing drought stress will likely alter the crop production system, ultimately leading to exploring sustainable mitigation measures and necessary modifications to the system. Drought was identified as a key factor that limits crop productivity among various abiotic stresses plants encounter in noncontrolled environments or vast crop growing environments that lack irrigation facilities. Incidence and effect of drought is predicted to get worse in the coming years as a result of global warming and adverse climate change.

Crop breeders continue their efforts to overcome drought problems with resistant varieties (Araus et al. 2002). However, it is true that many of these varieties can only tolerate and survive under drought stress for a short period of time. As such, any other natural agent such as *Bacillus* spp. capable of alleviating drought stress is considered one of the significant agricultural inputs as it relates to sustainable agriculture. Lack of water availability primarily restricts nutrient uptake and photosynthesis as plants curtail water loss by increased diffusive resistance and make morpho-physiological adaptations, such as closing their stomates. As a result, plants lose turgidity, and leaf size expansion, stem extension, and root proliferation are halted. Overall, innate plant adaptation strategies against drought stress include either or all of physiological, biochemical (increased production of multiple phytohormones, accumulation of compatible osmolytes, antioxidant and other secondary metabolites or signaling molecules), and molecular (upregulation of multiple drought stress related genes) mechanisms (Chaves et al. 2003; Krasensky and Jonak 2012). These changes help plants maintaining turgor pressure of cells, protect cell membranes, macromolecules and enzymes from oxidative injury (Krasensky and Jonak 2012; Gill and Tuteja 2010). Unfavorable environmental stimuli such as drought can affect normal plant metabolism leading to suppression of crop growth and yield. Association of *Bacillus* with plant rhizosphere or phyllosphere stimulates plant immunity against drought stress by altering stress-responsive genes that optimize production of proteins, phytohormones, and other related metabolites.

8.2.1.2 *Bacillus*-Mediated Mechanisms of Mitigating Drought Stress

As most of the studies conducted to evaluate efficacy of *Bacillus* in providing abiotic stress tolerance and unraveling the associated mechanisms had to exert drought stress on plants, effect of *Bacillus* could be confounded with innate drought response of plants. However, additive effects from *Bacillus* application and quantitative expression of responses were indicative of the role of beneficial microbes. For example, exposure of *Bacillus* inoculated timothy (*Phleum pratense* L.) plants to drought stress for 8 weeks provided enhanced shoot (26.6%) and root (63.8%) biomass, stomatal conductance (214.9%), and photosynthetic activity (55.2%) compared to plants grown under similar drought stress conditions without inoculation (Gagne-Bourque et al. 2016). Underlying mechanisms that supported increased stress tolerance in this study by *Bacillus* included enhanced accumulation of osmolytes, such as many different types of amino acids and sugars in roots and shoots. Another documented mechanism of drought stress avoidance in wheat by *Bacillus* is production of growth-promoting phytohormones such as indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate deaminase (ACCD) to counteract the stress-induced increase of abscisic acid (Barnawal et al. 2017). *Bacillus* spp. treatment of plants exposed to drought were found to increase the uptake of water and macronutrients like N, P, and K (Barnawal et al. 2013), which is the direct influence of increased root growth by enhanced IAA production. A higher production of scavenging enzymes of reactive oxygen species (ROS) was observed by Saikia et al.

(2018) when mung bean was inoculated with a group of plant growth enhancing bacteria including *Bacillus subtilis* RJ46 followed by exposure to drought stress. Quantity of ROS scavenging enzymes (ascorbate peroxidase, SOD, PO, CAT, glutathione reductase) and relevant compounds (glutathione, ascorbic acid, and cysteine) that prevent buildup of ROS is usually found to be higher in *Bacillus*-inoculated plants, and they lessen the oxidative damage that occurs at the time of drought stress (Kaushal and Wani 2015). Thus, production of antioxidants is the most important known mechanism of *Bacillus*-mediated drought stress management.

8.2.1.3 Extent and Impact of salinity Stress on Plants

Climate change and global warming has been affecting regular rainfall and distribution over recent decades. More specifically, productivity of agricultural land has been worsening worldwide due to accumulation of salt that resulted from low rainfall, improper irrigation practices and high water evaporation rates due to dry weather condition (Al-Karaki 2006). The high concentration of salts in saline soils cause oxidative, osmotic, ionic, and water stress in plants. Accumulation of salt in upper soil layers eventually affects nutrient and water uptake by plant roots due to reduced soil water potential (Porcel et al. 2012). This mimics a drought stress situation that induces plant response to accumulate compounds with osmo-protective properties such as special types of sugars, amino acids, and other secondary metabolites that were previously absent or present in lower quantity. Under conditions of salinity, plants are subjected to nutrient imbalance like K^+ deficiency and Na^+ toxicity and may also suffer from oxidative stress. Plants may eventually face metabolic pathway disorders related to respiration, photosynthesis, homeostasis in redox system, and phytohormone regulation. As a result, carbohydrate and amino acid syntheses are affected leading to reduced seed germination, plant vigor, growth, and yield (Radhakrishnan and Lee 2013, 2014; Rady 2011; Munns and Tester 2008). However, induction of osmoregulators may counteract the above disorders as well as protect structures of different cellular organelles including membranes (Hare et al. 1998). These osmoregulators also work as scavengers of free radicals to prevent their damaging effects to DNA (Ashraf and Foolad 2007).

8.2.1.4 *Bacillus*-Based Mechanism of Salinity Stress Tolerance

Inoculation of plants with beneficial microbes including *Bacillus* spp. enhances plant growth and development during salt stress compared with non-inoculated. This is considered an eco-friendly approach to counteract abiotic stresses and make agriculture more sustainable (Hashem et al. 2015, 2016a, b; Radhakrishnan et al. 2014). Production of growth hormones such as IAA has been found by Bochow et al. (2001) as one of the mechanisms to compensate salt stress-induced growth and yield loss. Authors found significant yield increase in pepper and eggplant due to inoculation with *B. subtilis* FZB24 compared to the nontreated, despite irrigating

the plots with saline groundwater. For further investigation to unravel the mode of action, pepper seedlings were pretreated with millimolar amounts of auxin precursors (indole-3-acetic aldehyde or indole-3-pyruvic acid, tryptophan) followed by exposure to saline water. Results revealed that auxin precursors treatment had compensated 75% growth loss of seedlings after 1 week that would occur due to salt stress. This finding further supports that one of the *Bacillus*-based modes of action of alleviating salinity-induced stress in plants is production of IAA. However, other investigators found that growth hormones like gibberellins were also produced by *Bacillus* and played a role in addition with enhanced uptake of N, P, K^+, $Mg2^+$, and $Ca2^+$(Mohamed and Gomaa 2012). They also reported decreased abscisic acid and Na^+ and Cl^- content compared to the non-inoculated, which may have occurred due to the relatively high uptake of required nutrients as facilitated by *Bacillus* to alleviate stress induced by salinity. Egamberdieva et al. (2017) also reported improved uptake of N, P, K, and Mg by chickpea, grown under saline soil conditions that were inoculated with a consortium of *B. subtilis* and *Mesorhizobium ciceri* compared with non-inoculated plants. Salinity stress significantly increases reactive oxygen species level and H_2O_2 in plants resulting in peroxidation of membrane lipid (Yazici et al. 2007; Koca et al. 2007). Thus, reduction of membrane lipid peroxidation or oxidation of other cellular organelles due to *Bacillus* inoculation under salinity is a proof of *Bacillus*-based stress mitigation. Lastochkina et al. (2017) reported reduction of stress-induced lipid peroxidation (MDA) in wheat due to inoculation of plants by *B. subtilis* 10–4 followed by exposure to water containing 2% NaCl salt compared with non-inoculated plants. If *Bacilli* colonize plants under salt stress, they trigger general antioxidant defense process resulting in synthesis of various anti-oxidant enzymes namely peroxidase, catalase (CAT), nitrate reductase (NR), superoxide dismutase (SOD), polyphenol oxidase (PO), glutathione reductase (GR), and guaiacol peroxidase (GP) that may vary with plant species (Chawla et al. 2013). However, halotolerant (HT) PGPR *Bacillus licheniformis* A2 recovered from saline soil showed higher growth promoting effect when plants were inoculated with the organism and grown in saline soil. Although several plant growth-promoting features such as production of IAA, phosphate solubilization, and siderophore production were considered as potential *Bacillus*-based mechanisms in alleviating the salt stress-induced detrimental effects and increasing plant growth of stressed peanuts (Goswami et al. 2014), study conducted by Zhang et al. (2008) with Arabidopsis in salt-affected soil revealed that HT *Bacillus subtilis* reduced the uptake of Na^+ by roots that involved downregulation of high-affinity potassium transporter (HKT1). However, IAA synthesis being transcriptionally related to ethylene production in plants via the expression of ACC synthase gene can halt their growth and development despite ethylene's capacity of providing salt tolerance to plants. Thus, after the initial ethylene production step, HT Bacilli start synthesizing ACC deaminase (ACCD) enzyme to stop ACC and ethylene production, supporting resumption of growth of stressed plants (Nabti et al. 2015). In addition, *Bacillus* can excrete exopolysaccharides (EPS) that facilitate binding of Na^+ in root cells thereby preventing their translocation to foliage. Similar binding of Na^+ by EPS may take place in the rhizosphere by *Bacillus* excreted EPS to prevent its absorption like a physical

barrier around the roots (Arora et al. 2020). Thus, association of *Bacillus* spp. with salt-stressed plants has the potential to alter their metabolism to sustain or promote plant growth. Halotolerant *Bacillus*-mediated expression of genes to alleviate salinity stress is presented in Table 8.1.

Table 8.1 Bacillus-mediated gene expression in plants that were identified to be responsible for salinity stress amelioration

Plant species	*Bacillus* species included in the study	Genes responding to stresses	Role/Results
Triticum aestivum	*Bacillus safensis* W10	Upregulation of multiple genes including sulfur-rich thionin, S adenosylmethionine decarboxylase precursor, expansins, endotransglucosylase/ hydrolase and metallothionines Downregulation of protein phosphatases, flavonones hydroxylases, oxalates, and oxidases	Mitigation of salt stress
Solanum lycopersicum	*Bacillus megaterium*	Expression of MT2 receptor and gamma response (GR1)	Synthesis of metallothionin and glutathione reductase enzyme
Glycine max	*Bacillus firmus* SW5	Upregulation of salt tolerance genes GmVSP, CHS, GmPHD2, GmbZIP62, GmOLPb, and GmWRKY54 Expression of antioxidant enzyme-encoding APX, Fe-SOD, CAT and POD	Production of antioxidant enzyme, salinity tolerance, flavonoid biosynthesis
Zea mays	*Bacillus amyloliquefaciens* SQR9	Upregulation of several genes including NHX1, NHX2, RBCS, RBCL, H + -PPase, and NHX3	Enhanced photosynthesis, Na^+ sequestration, and export
Puccinellia tenuiflora	*Bacillus subtilis (GB3)*	Upregulation of *PtSOS1* and *PtHKT1;5* Downregulation of NCED	Modulation of Na^+ homeostasis
Oryza sativa	*B. Amyloliquefaciens* SN13	Upregulation of EREBP, *NADP-Me2, SOSI, SERK1*, and *BADH* Suppression of *GIG* and *SAPK4*	Na^+/H^+ reverse porter system, ion homeostasis; abiotic stress response and oxidative decarboxylation of L-malate
Arabidopsis thaliana	*Bacillus subtilis* (GB03)	Expression of HKT1	Na^+ transport in roots

Adapted from Arora et al. (2020)

8.2.1.5 Impact of Heavy Metal Stress on Plants

Due to unplanned industrialization all over the world, arable lands are inadvertently getting contaminated with metal toxicants that are part of industrial effluents released. These contaminants are affecting ecology of food chain by altering microbial communities and crop cultivation (Hu et al. 2009; Ashraf et al. 2017). Accumulation of Cu, Mn, Zn, Pb, Cr, and other heavy metals are listed as major pollutants in soil and water that are not degraded into harmless substances easily (Ma et al. 2009; Arthur et al. 2012). These metal-contaminated soil and water are not only toxic to the flora and fauna in a certain area but also create a huge risk to human health if contaminated soils are used for crop production and metal is taken up and transferred into food chain at higher-than-acceptable concentrations (Oves et al. 2016). Heavy metal content can affect activities of microorganisms, such as respiration and metabolism (metabolic entropy response), thereby affecting soil respiration (Blagodatskaya et al. 2006). Thus, microbes such as *Bacillus* that can survive these harsh conditions are more suitable for heavy metal remediation. Traditionally, chelators have been used to reduce metal toxicity from soil; however, chelators can be harmful to organisms in the edaphic environment (Tandy et al. 2006). In contrast, beneficial microorganisms like *Bacillus* spp. solubilize and change toxic metals to nontoxic forms. This method can be utilized in management of heavy metal phytoremediation when used in the integrated approach with hyperaccumulator plants (Kang et al. 2015c, Bosecker 1997). However, this remediation method of heavy metals is also known as bioremediation when microbes are used solely, which is considered the most sustainable, environmentally friendly, and cost-effective without any adverse effect to any component of the environment (Dixit et al. 2015). The release of *Bacillus* spp. into soil contaminated with heavy metals can enhance reduction of toxic effects of these metals on plant growth when combined in a phytoremediation effort. Brunetti et al. (2012) found that *B. licheniformis* enhanced Cu, Zn, Cd, Pb, especially Cr accumulation in *Brassica* plants that were grown to test their capacity as hyperaccumulators of metals for phytoremediation of heavy metal-contaminated soil. This eventually led to reduced levels of toxic metals in soil compared to nontreated plants. However, due to the low bioconcentration factors (>1), investigators could not conclude the suitability of the species for the phytoextraction of toxic metals from polluted soils. However, these species can be utilized successfully for low metal polluted soil. Besides accumulation and uptake of heavy metals or supporting enhanced uptake by plants, *Bacillus* and similar microbes mitigate plant stress from heavy metal through other mechanisms described below.

8.2.1.6 *Bacillus*-Based Heavy Metal Stress Alleviation in Plants

Rhizospheric and endophytic bacteria including *Bacillus* enhance growth and development of plants in metal polluted soils by two major methods: i) These microbes can remove heavy metals from soil or modify capacity of metal accumulation

through efflux of ions outside the cells, transformation of metal ions to lower forms of toxicity, sequestration of metal ions on the cell surface or in polymers inside cells, and biomethylation, precipitation, adsorption or desorption; ii) these microbes can also alleviate heavy metal-induced plant stress through production of beneficial plant growth enhancing substances that may include solubilization/transformation of mineral nutrients, such as phosphate, nitrogen, and potassium, and production of plant beneficial enzymes, siderophores, and phytohormones (Ma et al. 2011).

8.2.1.6.1 Adsorption and Absorption of Heavy Metal by *Bacillus*

Bacillus can remove large amount of soil heavy metals by both adsorption and absorption, although Wang et al. (2013, 2001) reported that the principal mechanism of heavy metal ion accumulation is adsorption, which normally is independent of energy metabolism. However, absorption, a closely related mechanism depends largely on energy metabolism and occurs mostly in living cells. Active export of heavy metals via an ATPase efflux P-type pump was found by Shin et al. (2012) in an endophytic bacterial strain *Bacillus* sp. MN3–4. *Bacillus* has evolved a well-defined metal-resistance mechanism, which is capable of transporting metal ions against the concentration gradient across cell membranes to enhance hyperaccumulator plants' capacity of removing heavy metals from soil. This process uses ATP hydrolysis-released energy. In this regard, Wang et al. (2001) found that *Bacillus* could saturate 60% of its Cu^{2+} adsorption capacity within the first minute and reach an equilibrium as early as within 10 min. However, Wierzba (2015) found that addition of ethylenediaminetetraacetic acid (EDTA) and lemon oil in the media could improve removal rates by 31.5 and 26.3%, respectively.

8.2.1.6.2 Bioleaching

The process of extracting metals from waste or ores by using microorganisms is defined as bioleaching as microbes oxidize the metals and produce soluble compounds in the form of organic acids. Thus, production of organic acids and chelating or complexing compounds that are excreted into the environment are used for metal extraction. Bacteria belonging to the genus *Thiobacilli* are considered the most active group in bioleaching of metal ions; they generally grow under aerobic conditions. It was shown that bacteria leach a higher amount of heavy metals from sludge deposited when elementary sulfur was added as a stimulant for the activity of thionic acidophilic *Bacilli*. It took place by way of bacterial oxidation of elementary sulfur releasing sulfuric acid (Marchenko et al. 2015). As a result, the pH of sludge deposits goes down, which is an important factor for effective leaching of heavy metal.

8.2.1.6.3 Other Mechanisms of *Bacillus*-Based Heavy Metal Remediation

Microbial cells are capable of converting metals from one oxidation state to another, which can reduce their toxicity. Microbial enzymes and other secretions from their metabolic activities can dissolve heavy metals that are stuck in soil particles. Thus, precipitation, biosorption, and enzymatic transformation are the processes used by *Bacillus* and similar microbes to degrade, detoxify, or transform heavy metals to more stable, less mobile, or inert forms (Kumar and Bharadvaja 2020). *B. subtilis* inoculation of rice under Cd stress showed that roots and shoots at 45 days after inoculation (DAI) and grains at 120 DAI had lower Cd accumulation compared with non-inoculated (Treesubsuntorn et al. 2017). Authors hypothesized that the mechanism by which *B. subtilis* reduced Cd accumulation was its capacity to effectively absorb Cd from the medium. Their findings also suggested that *B. subtilis* was more effective in absorbing Cd compared to *B. cereus*. Ahmad et al. (2014) showed that *Bacillus* and a few other bacterial genera such as *Klebsiella*, *Stenotrophomonas*, and *Serratia* had supported plant growth under Cd stress by increasing water uptake and reducing electrolyte leakage in maize and wheat. As *Bacillus* increases availability and uptake of essential nutrients, heavy metal uptake by plants is reduced due to competitive exclusion. For example, Naseem et al. (2016) found that *Bacillus* sp. AMP2-inoculated wheat seedlings took up less Cr of different chromium salts (CrCl3, K2CrO4, K2Cr2O7) both at 10 and 20 ppm concentrations than control. Similar to other *Bacillus*-based abiotic stress mitigation mechanisms, when *Bacillus*-inoculated plants were grown in high concentration of heavy metal containing medium, it was found that tissues increased antioxidant enzyme activity including CAT, PA, APX, and SOD and concurrent decrease of metal concentration in the roots compared to non-inoculated plants (Hao et al. 2015). Inoculation of *B. subtilis* could also increase dry weight of rice plant as well as protect them from Cd stress. Mechanisms included ability of *B. subtilis* producing IAA, solubilizing phosphate, and controlling ethylene levels by the activity of ACC deaminase (Treesubsuntorn et al. 2017).

8.2.1.7 Mechanism of Nutrient Stress Alleviation by *Bacillus*

Multiple species of *Bacillus* were found to improve plant uptake of P and K by solubilizing fixed soil nutrients (Chen et al. 2006). For example, use of insoluble K sources together with *Bacillus edaphicus* NBT strain to treat soil for growing cotton and rape could increase K content in plants by 30% and improve growth compared with no *Bacillus* inoculation (Sheng and He 2006). Plant growth promotion in this study was attributed to K solubilization by *B. edaphicus* strain. In a similar study, Wu et al. (2005) found improved N, P, and K assimilation in maize due to *B. megaterium* and *B. mucilaginous* inoculation. Several other investigators also reported similar mechanism of nutrient stress alleviation such as solubilization of tricalcium

phosphate (Calvo et al. 2010; Almoneafy et al. 2012) and zinc (Ajilogba and Babalola 2013) by *B. amyloliquefaciens* isolated from potato. Phosphate solubilization abilities of multiple *Bacillus* species, *B. thuringiensis*, *B. sphaericus*, and *B. megaterium*, were found by Akgul and Mirik (2008).

8.3 Biotic Stress Mitigation in Plants by *Bacillus* spp.

8.3.1 Extent and Impact of Biotic Stress on Crop Growth and Productivity

Like abiotic stresses, plants encounter many different types of biotic agents such as fungi, viruses, bacteria, nematodes, and insects during their life cycle. These entities may exert biotic stresses when they invade plants to use them as their host resulting in disruption of plant's normal metabolism. This interrupts plant growth, and in some cases, biotic stresses become the cause of plant mortality (Hashem et al. 2019). Many of these biotic agents can also cause post-harvest losses of crop yield (Singla and Krattinger 2016). However, in most cases plants do well even under these stressful situations because some beneficial microbes, if present in the microenvironment, interact with their host plants symbiotically or synergistically to counteract harmful effects from pathogenic microbes. These beneficial microbes can play similar role as synthetic fertilizers or pesticides to minimize adverse effect from biotic stresses and promote plant growth. Due to their significant role in plant growth enhancement they are often termed as plant growth promoting rhizobacteria (PGPR), where *Bacillus* is considered a prominent member. PGPR, including *Bacillus,* have been used in agricultural, especially crop production, systems to alleviate biotic stresses and develop environmentally friendly, sustainable management options (Grover et al. 2011, Vejan et al. 2016) for a long time, which is now gaining momentum. Application of beneficial microbes to the rhizosphere in an augmentative approach can be utilized for improving biotic stress tolerance. A few *Bacillus* species including *B. megaterium*, *B. subtilis*, and *B. cereus* were found by GroBkinsky et al. (2016) to produce cytokinins and other growth hormones. Synergism is another known mechanism of effect of Bacilli when used with other beneficial organisms for plant growth and development. Alam et al. (2011) applied *B. subtilis* together with arbuscular mycorrhizal (AM) fungi to rose-scented geranium. Results indicated a synergistic effect of *B. subtilis* that increased >10% yield (59.5%) compared to AM fungi alone (49.4%). Total oil yield from the harvested biomass was increased significantly as biomass production went up, although oil content percentage did not increase on a dry weight basis. Underlying mechanisms of *B. subtilis* providing synergistic effect to greater promotion of plant growth included increased growth supporting enzyme production, higher antioxidants and P solubilization, root nodulation, nitrogen fixation, and biocontrol activity.

8.3.2 *Mechanism of Biotic stress Mitigation in Plants by* Bacilli

Members of the genus *Bacillus* alleviate biotic stress of plants through a variety of mechanisms that include depriving biotic agents by outcompeting them for nutrients and space on plant rhizosphere or phyllosphere; inhibition of biotic agents by producing a variety of inhibitory biochemicals; induction of plant resistance; and facilitating plant growth by producing hormones, so that plant pathogens fail to infect plants. These can further be divided into two groups: direct and indirect mechanisms. The direct mechanism refers to production of biochemicals by cells of *Bacillus* spp. such as synthesis of several secondary metabolites (antibiotics, cell wall-degrading enzymes, hormones, antioxidants) that stimulate plant growth and assist plants to defend against pathogenic attack (Goswami et al. 2016). Stimulation of plant growth and induction of acquired systemic resistance are among the major indirect mechanisms involved with *Bacillus*-based biotic stress mitigation in plants.

8.3.2.1 Depriving Biotic Agents by Outcompeting them for Nutrients and Space

Early colonization of plant roots by *Bacillus* can provide a barrier against pathogenic microorganism invasion (Bais et al. 2004). Root exudates provide required nutrients to rhizosphere microbes to thrive. That is why microbial density in proximity of roots is higher compared to areas away from them, and there is always a competition for space on the root surface. Due to the competitive advantage of *Bacilli* for nutrients and space in plant rhizosphere, they can suppress pathogen proliferation, which is an important feature of the mode of action of *Bacillus*-based biotic stress alleviation in plants (Cawoy et al. 2011). It was found that many members of *Bacilli* can form biofilms, a densely packed surface or multicellular interface of associated aggregates under unfavorable environmental factors (Morikawa 2006) occupying most of the root surface. Formation of a biofilm by *Bacillus* on plant root can be very quick, requiring only a few hours (Allard-Massicotte et al. 2016), thus preventing attachment of pathogenic microbes on roots (Morikawa 2006). In general, bacteria utilize chemotaxis for finding root to colonize at early stages of plant growth (Allard-Massicotte et al. 2016). Due to the presence of many chemoreceptors involved in root colonization, *Bacilli* can efficiently colonize root system in soil (Allard-Massicotte et al. 2016). In the case of lack of availability of nutrients to *Bacilli*, major competition occurs for carbon, and is considered an important factor in biological control. *Bacillus* also competes for several micronutrients with other biotic disease-causing biotic agents that include copper, manganese, iron, and zinc. Among these elements, iron is highly important because of its limited presence in available form in soil (Loper and Henkels 1997). Due to their ability of producing siderophores that have a strong affinity for iron, *Bacillus* can solubilize and acquire ferric ions efficiently. Thus, depriving other soil microbes, including pathogens,

from acquiring iron (Cawoy et al. 2011; Haas and Defago 2005; Loper and Henkels 1997).

8.3.2.2 Production of Inhibitory Biochemicals by *Bacillus*

Production of compounds of low-molecular-weight including antibiotics is common to many *Bacillus* species (B. subtilis, *B. megaterium*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. mycoides*, and *B. pumilus*) that directly affect other microorganisms through a process known as antibiosis (Weller and Thomashow 1993; Handelsman and Stabb 1996; Weller 1988). Some of these species can produce multiple antibiotics. For example, *B. subtilis* can produce more than two dozens of antimicrobial compounds with diverse structures (Stein 2005). These include polymyxin, subtilin, difficidin, and mycobacillin usually possessing broad-spectrum antimicrobial activity. *Bacilli* can produce antibiotic molecules during most of their life cycle, but production reaches very high levels during sporulation. Most of these compounds belong to the peptide class despite very high diversity in their sizes and structures. These antibiotics mostly contain amino acids and, in some cases, other residues that help them forming linear, cyclic or basic aminoglycoside type antibiotics (Stein 2005). Almost all the antimicrobial cyclic peptides can directly affect the integrity of fungal cell membranes through lysis and change their structure, thereby inhibiting their growth and development. *Bacillus*-produced antimicrobial peptides can reliably be used as fungicides due to their direct interaction with fungal cell membrane or interference in biosynthesis of chitin, glucan, and sphingolipid that are essential components of fungal cell wall and membrane. In this regard, positive results have been obtained by investigators from numerous studies. For example, two *B. brevis*- and *B. polymyxa*-produced peptide antibiotics gramicidin S and polymyxin B, respectively, inhibited gray mold-causing fungus *Botrytis cinerea* both in vitro and in vivo (Haggag 2008). The three main *Bacillus* spp.-produced families of cyclic lipopeptides (CLPs) are surfactins, iturins, and fengycins. It was reported that all three may although be present in bacterial secretion/culture filtrate but may not be needed for antimicrobial activity. In a study by Waewthongrak et al. (2015) on the inhibitory effect of all three CLPs, they found that growth of *Penicillium digitatum* was inhibited by iturin A and fengycin to control green mold on mandarin, but surfactins had no direct effect. However, Surfactin likely supports bacterial colonization of root tissues and establishment in the rhizosphere of plants through biofilm layer formation (Mihalache et al. 2017; Cawoy et al. 2014), and is considered essential component of the mechanisms by which *Bacilli* become successful in alleviating biotic stresses in plants. Investigators from different studies with different strains of *Bacilli* found diverse peptide antibiotics such as bacillomycin D, subtilisin, and xanthobaccin. However, due to the consistency and frequency of its occurrence, bacilysin is regarded taxonomically the most related and significant for the genus *Bacillus* (Loeffler et al. 1990). In addition, chitinase, amylase, protease, cellulase, pectinase, glucanase, and similar cell wall-degrading enzymes or substances like HCN can damage pathogens and pests to lower their population on plants.

8.3.2.2.1 Specific Mechanism Associated with Activity of Cyclic Peptides

Some cyclic peptide molecules disrupt membrane structure by binding only to the surface without passing through. However, other cyclic peptides can bind with specific membrane-associated structures such as ion channels, transporters and different types of receptors by traversing membranes. After successful binding, cyclic peptide molecules aggregate in a specific site on membrane to form variable size aqueous pores. Ions and other solutes pass through the channels of these pores in an uncontrolled way that ultimately leads to cell death. Debono and Gordee (1994) found that impairment of biosynthesis microbial cell wall macromolecular components such as glucan, chitin, and mannoproteins was the underlying mechanism of antifungal activity of several cyclic peptides. These cyclic peptides have the capacity to form a complex with the precursor molecule of microbial cell wall macromolecules that eventually promote pore formation and cell wall disruption. While pore formation on membrane or degradation of cell walls is the major mode of action, alternation of ribosome function is also considered a potential mechanism to suppress growth of pathogenic microbes. For example, Katz and Demain (1977) found that bacitracin A produced by *B. licheniformis* inhibited cell wall synthesis but the butirosin complex belonging to amino glycosides produced by *B. circulans* altered ribosome function of diverse microbial groups (Defuria and Claridge 1976).

8.3.2.3 Induction of Host Defense against Biotic Stress

In addition, with above-mentioned mechanisms, some strains of *Bacilli* activate defense systems in host plants that results in an enhanced level of resistance against pathogen attack (Conrath et al. 2006). This can be explained as supporting plant's immune defense arsenal through sensitization and priming to defeat invading pathogens. This process initiated by biotic stimuli helps in scaling up expression of a plant's defense-related genes to accumulate antifungal biochemicals. Strains of *Bacilli* or their metabolites can turn on plant's defense system when pathogens attack host plants or can be triggered by pre-inoculation (Schonbeck et al. 1993) that results in an enhanced resistance level (Conrath et al. 2006). Higher level of induced resistance in plants is superior over other modes of action of *Bacilli* such as antibiosis or competition, as it provides protection to plants usually for a long period of time even when bacterial population subsides. This happens as the response has already transduced to the distal organ of the plant from the point of instigation. Induction of systemic resistance occurs through a well-orchestrated sequence of biological events. Beneficial rhizobacteria such as *Bacilli* can trigger a defense response by stimulating the plant through activation of a variety of cellular defense responses in a well-coordinated manner. These responses result in oxidative burst, defense-related enzymes buildup (Rahman et al. 2015), cell-wall strengthening (Heil and Bostock 2002), and production of secondary metabolites (Yedidia et al. 2003). The presence of the microbe is detected by pattern recognition receptors (PRRs) of a plant cell membrane through microbe-associated molecular patterns (MAMPs), which may include lipopolysaccharides, flagellin, glycoproteins, and

chitin (Jones and Dangl 2006). These are also known as elicitors. *Bacillus* spp. are also known to produce lipopeptides and volatile compounds, which can play similar role as elicitors for inducing systemic resistance in plants. Surfactin produced by *B. subtilis* strain S499 was found to induce systemic resistance (Ongena et al. 2007). Complex interaction between PRRs and MAMPs (elicitors) subsequently results in immunity (PTI) through defense-related gene expression, oxidative burst, and callose deposition (Schwessinger and Ronald 2012; Altenbach and Robatzek 2007) from ISR-type defense signaling. Different *Bacillus* spp. may induce different but relevant signaling pathways. For example, ISR mediated by *Bacillus cereus* AR156 required both SA and JA/ET signaling pathways together with NPR1 (Niu et al. 2011). Defense response against pathogens involve several molecules such as phytoalexins, pathogenesis-related proteins (proteinase inhibitors, chitinases, β-1,3-glucanases), and lignin (Van Loon 2007). Fungal hyphal growth during infection process is prevented by thickened cell wall in combination with PR proteins (Lugtenberg et al. 2001).

8.3.3 *Mechanism of Bacterial Disease Prevention by* Bacillus *Spp.*

Together with fungi, viruses, and nematodes, plant disease-causing pathogenic bacteria pose major challenges to plant health and yield in agricultural production systems (Hussey and McGuire 1987; Guo et al. 2013; Narasimhan and Shivakumar 2015). Major pathogenic bacteria that are known to infect plant with detrimental effect on plant growth and development include but not limited to *Pseudomonas, Xanthomonas, Erwinia,* and *Ralstonia*. Although *Bacillus* spp. belong to microbial group bacteria, upon inoculation of plant or plant growing media they not only counteract and suppress pathogen growth but also promote plant growth (Krid et al. 2012; Yi et al. 2013). Mechanisms of *Bacillus*-based protection of plant from bacterial infections include biofilm formation around the root surface and their secretion of several toxins such as surfactin, iturin, macrolactin, bacillomycin, and fengycin that destroy pathogenic populations of bacteria resulting in plant disease control (Hinarejos et al. 2016; Chen et al. 2013; Huang et al. 2014; Elshakh et al. 2016). Pathogenic bacterial cell walls can be degraded quickly by the secretions of *Bacillus* spp., eventually killing the pathogen (Elshakh et al. 2016).

8.3.4 *Mechanism of Fungal Disease Control*

Mycelial growth of many different fungi is inhibited by the antagonistic activity of *Bacillus* spp. to control fungal diseases (Handelsmann and Stabb 1996; Aydi-Ben-Abdallah et al. 2016; Abdalla 2015; Chowdhury et al. 2015a; Akram et al. 2016;), thereby enhancing plant growth and yield (Narasimhan and Shivakumar 2015).

Underlying mechanism of *Bacillus* spp.-mediated fungal disease control include either or all of the events and interactions occur when *Bacillus* come in contact with fungal pathogens. Immediately after attachment of *Bacillus* to the mycelial cell walls, production of fungal cell wall-degrading enzymes (chitosanase, protease, glucanase, cellulase siderophores) and HCN takes place from the bacteria, which crack and deform the hyphae leading to altered cell functions and structures such as protoplast leakage and vacuolation (Ben-Khedher et al. 2015; Han et al. 2015). Mitigation of pathogen-induced biotic stress may also occur via *Bacillus* spp.-mediated physiological changes in plants. These include alteration of respiratory and photosynthetic pathways in diseased plants together with regulation of phenyl-propanoid, carbohydrates, defense-related proteins, and nitrogen metabolism (Jain et al. 2015). Plant beneficial *Bacillus* spp. increase antioxidant enzymes (APX, CAT, GR, GPX, POD, PPO) and reduce lipid peroxidation in plants. They also enhance production of other defense enzymes such as PAL, β-1,3-glucanase, chitinase, and phenolic acids that lessen the hostile effects of plant infection by pathogens (Chowdappa et al. 2013; Jain et al. 2013; Solanki et al. 2012).

8.3.5 Mechanism of Nematode and Virus Disease Control by Bacillus

Viruses, the second largest group of plant pathogens cause most plant diseases after fungi. Many *Bacillus* spp., produce antiviral compounds that minimize the adverse effects of these pathogens on plants (Esawy et al. 2011). Prevention of viral diseases by *Bacillus* spp. has been reported in a few cases. However, in most cases it was due to reduction of disease rate as a consequence of *Bacillus* spp. induced systemic resistance (ISR) in plants. Zhang et al. (2004) reported enhanced plant growth of cucumber during cucumber mosaic virus infection when plants were inoculated with *Bacillus* compared to non-*Bacillus* treated. Formation of biofilm and surfactin production by *B. amyloliquefaciens plantarum* was found to subvert the viral disease in plants by Chowdhury et al. (2015b). Underlying mechanism in this case was due to triggering of ISR machinery. Similarly, viral disease caused by tobacco mosaic virus was suppressed by *Bacillus* spp. because of inhibition of viral coat protein synthesis from induced systemic resistance. Additional mechanisms involved were increased expression of plant defense genes (PR-1a and PR-1b), disease-resistant signaling genes (*Coil* and *NPR1*), and cell wall expansion (*NtEXP2* and *NtEXP6*) genes (Wang 2009).

Nematodes, which are known as microscopic worms, can also damage plants by being parasitic and feeding on roots. Among many plant parasitic nematodes, root-knot causing ones are most damaging worldwide. Nearly 5500 plant species are within the host range of this nematode (Trudgill and Blok 2001). Prevention of root-knot nematode infection in crops by *Bacillus* spp. includes resistance development and reduction of gall and egg masses (Adam et al. 2014). Chowdhury et al. (2015b)

reported that *Bacillus* spp. synthesized antimicrobial peptides such as bacteriocins that inhibit pathogenic nematode growth. Genes encoding nematicidal activity were identified by Liu et al. (2013) as PZN gene cluster in *B. amyloliquefaciens*. *Bacillus* spp. secreted crystal proteins (Cry5B and Cry6A) were found to control the growth of plant-parasitic and free-living nematodes *Meloidogyne hapla* and *Caenorhabditis elegans*, respectively (Yu et al. 2015).

8.4 Mechanism of Insect Stress Alleviation in Plants by *Bacillus*

A broad range of insect control in plants is provided by *Bacillus thuringiensis* (Bt)-produced Bt toxin (Navon 2000). In addition, Bt was also found to inhibit the growth of insect larvae, thereby decreasing plant damage and increasing growth (Boukedi et al. 2016; Arrizubieta et al. 2014). Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins also known as crystal proteins or Cry proteins (Roh et al. 2007). In most strains of *Bacillus*, genes located on a plasmid instead of a chromosome that are known as cry genes encode these toxins. Cry toxins show precise activities against insect species belonging to several orders such as Coleoptera (beetles), Diptera (flies and mosquitoes), Lepidoptera (moths and butterflies), and Hymenoptera (wasps, bees, ants and sawflies) (Schnepf et al. 1998). Upon ingestion of toxin crystals by insects, insoluble crystals get denatured in their alkaline digestive tracts. Proteases from the insect gut then cut these soluble crystals to liberate the toxin. Inside the insect gut, the Cry toxin at this stage is injected into the cell membranes, which paralyzes the digestive tract including formation of pores. The insect starves to death as it stops eating. Live Bt bacteria from the environment may also colonize the insect causing death. Additional findings on the mechanism suggested that bacteria in the midgut of susceptible larvae stimulate insecticidal activity of *B. thuringiensis* (Broderick et al. 2006). Some relevant studies suggested that due to its insecticidal and plant growth promotion properties, *B. thuringiensis* could be used as a biological control agent (Compant et al. 2005).

8.5 Conclusion and Future Perspectives

Field grown plants are constantly exposed, either sequentially or simultaneously, to many abiotic or biotic stresses. These biotic and abiotic stresses significantly affect crop yield, food quality and ultimately global food security. Plants must cope with these stressful conditions to thrive and complete their life cycle. Among many beneficial microbes, *Bacillus* species are exceptional, as members of this group can form endospores that are extremely robust under harsh environmental conditions, suppress harmful microbes, and can also secrete secondary metabolites that

stimulate plant growth. Thus, beneficial microbes such as *Bacillus* provide a model for enhancing stress tolerance upon successful application in plant growing environment. *Bacillus* spp. were found in numerous studies to alleviate biotic and abiotic stresses and improve plant growth either directly or indirectly. Both biotic and abiotic stresses can cause physiological, biochemical, and molecular changes in plants affecting normal growth and development. *Bacillus* spp. either prevent or counteract these negative changes through a series of biological and biochemical mechanisms that have been interpreted from an appreciable number of studies. *Bacilli*-mediated abiotic and biotic stress tolerance in plants includes biological, physiological, biochemical, metabolic, and molecular mechanisms triggered in response to stresses. Promotion of plant growth by these underlying mechanisms usually involve regulation of plant hormones, improved nutrition acquisition, siderophore production, and enhanced antioxidant activity. Secretion of exopolysaccharides and siderophores by *Bacillus* spp. inhibit or stop movement of toxic ions and assist in uptaking water by roots as well as maintaining ionic balance. These compounds were also found to inhibit pathogenic microbial populations through multiple mechanisms that have been unraveled by numerous studies. However, many of the possible mechanisms either remain unclear or not compiled in a systematic method to make them available to researchers of *Bacillus* spp. This review compiled most of the processes unraveled by various studies to date and provides a reliable source of information for designing relevant research plan to further explore the mechanisms that have not been fully elucidated to the molecular level. As new and more effective strains of *Bacilli* to counteract plant abiotic and biotic stresses are discovered on a regular basis, future studies should focus on discerning mechanisms associated with enhanced efficacy.

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Chapter 9
Amelioration of Salinity Stress by *Bacillus* Species as Promoters of Plant Growth in Saline Soil

Mohammad Tofajjal Hossain and Tofazzal Islam

Abstract Salinity is a major factor of osmotic stress of the high salt in the rhizosphere, which limits plant growth and development. The uptake of salt inhibits the physiological and metabolic processes of plants and severely impacts agricultural productivity. About 62 million hectares (20%) of the world's irrigated land are affected by salinity. *In vitro* and *in vivo* bioassays reveal that some salt-tolerant plant-associated *Bacillus* spp. enhance plant tolerance to salinity. The roots and fresh weight of plant seedlings showed better growth promotion significantly by the application of *Bacillus* species in the saline environment. However, the precise mechanism of higher tolerance of plants to salinity by *Bacillus* spp. is still poorly understood. Salt-tolerant plant growth-promoting *Bacillus* species, viz., *B. oryzicola* YC7007, *B. velezensis*, *B. licheniformis* SA03, *B. safensis* ZY16, *B. megaterium*, *B. pumilus*, *B. firmus* SW5, *B. subtilis* SU-12, *B. amyloliquefaciens*, *B. aryabhattai* MS3, *B. cereus*, and *B. aquimaris* DY-3 enhance salinity tolerance of plants through inducing plant gene/protein/pattern recognition receptors against salt stress. Out of different genera of salt-tolerant bacteria such as *Pseudomonas*, *Enterobacter*, *Agrobacterium*, *Streptomyces*, *Klebsiella*, and *Ochromobacter*, *Bacillus* show the highest performances in enhancing plant tolerance to salinity. This chapter summarizes current knowledge on the amelioration of salinity stress in plants by plant growth-promoting *Bacillus* species.

Keywords Abiotic stress tolerance · Climate change · Food security · PGPR · Genomics

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9.1 Introduction

Plants face various abiotic stresses that significantly impact crop production (Bao et al. 2009; Kumar et al. 2019; Mahajan and Tuteja 2005). Abiotic stresses such as salinity, extreme temperature, heavy metals, heat, and cold stresses can cause up to 80% of yield reduction in crops (Bharti and Barnawal 2019). Among these stresses, salt adversely affects plant health, which is a serious threat to the reduction of yield losses between 20 and 50% (Shrivastava and Kumar 2015; Vij and Tyagi 2007). In plants, salinity induces oxidative stresses and causes a deficiency of essential nutrients, such as K^+, which enhances the toxicity of Na^+ inside the plants, which subsequently hinders plant photosynthesis, lipid metabolism, protein synthesis, and biomass accumulation (Munns and Tester 2008). It also causes water deficit, osmotic stress, stomatal closer, and reduction of leaf expansion. The salinity is a serious threat to crop production in coastal areas worldwide. About 62 million hectares (20%) of the world's irrigated land is affected by salinity. The coastal region of Bangladesh also covers almost 29,000 Km^2 or about 20% of the country that is almost 30% of the cultivable land area (Chinnusamy et al. 2006; Egamberdieva et al. 2019; Haque 2006; Zhang et al. 2008). To avoid salinity, now, it is challenging how it should be controlled over the world. In these circumstances, to mitigate the salinity from the soil, many strategies have been imposed already, and one of the promising ways is the use of plant growth-promoting rhizobacteria (PGPR) (Gray and Smith 2005). The PGPR are naturally soil microorganisms that colonize roots and stimulate plant growth. More than 33,000 bacterial species under proteobacteria, firmicutes, and actinobacteria are present out of one million microbes in one gram of bulk soils in plant rhizosphere which was discovered by phyloChip analysis. To influence bacterial diversity and activities, root exudates contain about 21% photosynthetically fixed carbon in the root-soil interface (Lynch et al. 2012; Mendes et al. 2011). These types of beneficial bacteria can be either free-living in the soil or colonized in the rhizosphere, phyllosphere, or plant tissue interior (endophytes). They enhance plant growth and induce systemic resistance in host plants to a broad spectrum of pathogens (Chung et al. 2015; Hossain et al. 2016; Kloepper et al. 2004; Khan et al. 2016a) and also increase tolerance of plants to abiotic stresses including salinity (Baek et al. 2020; Chinnusamy et al. 2006). The PGPR include the strains in the genera *Bacillus*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Erwinia*, *Enterobacter*, *Rhizobium*, *Flavobacterium*, and *Serratia* (Bashan and de-Bashan 2005). Out of these PGPR, *Bacillus* consortium has been utilized since long with an eco-friendly manner for promoting sustainable agriculture (Rahman et al. 2018; Dame et al. 2021). Application of salt-tolerant *Bacillus* spp. to alleviate salinity stress in crop production is a new approach in crop production. The beneficial *Bacillus* species play a prominent role in the plant's development in many ways. Among them, resistance induction in host plants (Niu et al. 2016) against various biotic and abiotic stress including soil salinity is prominent. *Bacillus subtilis* GB03 and *B. oryzicola* YC7007 are soil-borne plant symbionts that promote plant growth promotion and

protect host plants from salinity stress (Baek et al. 2020; Hossain et al. 2016). *Bacillus* species confer plant tolerance by tissue-specific regulation of the sodium transporter *HKT1* (Zhang et al. 2008). *Bacillus subtilis* GBO3 induces growth promotion and enhances tolerance in the halophyte grass *Puccinellia tenuiflora* to salinity (Han et al. 2014). Furthermore, *Bacillus* species induce physical and chemical changes that increase tolerance against abiotic stresses especially salinity (Yang et al. 2009). Although improvement of salinity tolerance in plants by various plant growth-promoting bacteria has been reviewed (Numan et al. 2018). No review has so far been published on salinity tolerance by *Bacillus* species (Paul and Lade 2014). This chapter describes the potential of *Bacillus* strains to increase plant tolerance to salt stress and discusses the utilization of these beneficial bacteria in the promotion of sustainable agriculture.

9.2 Diversity of Salt-Tolerant PGPR Bacteria

Bacteria from diverse taxonomic groups show tolerance to salinity stress. A large body of literature describes the tolerance of plant growth-promoting rhizobacteria (PGPR) to salinity. Since the discovery of salt-tolerant PGPR in high-salt environments, many researchers explore novel strains of bacteria that enhance plant growth under soil salinity. The PGPR belonging to different genera such as *Bacillus*, *Microbacterium*, *Pantoea*, *Achromobacter*, *Rhizobium*, *Pseudomonas*, *Paenibacillus*, *Enterobacter*, *Burkholderia*, *Methylobacterium*, *Azospirillum*, *Variovorax*, etc. induce tolerance in host plants to abiotic stresses, including salinity (Vacheron et al. 2013). During the last few decades, the beneficial effects of these bacterial genera have been shown to enhance the tolerance of crop plants (e.g., rice, wheat, maize, cotton, lettuce, tomato, and pepper) to drought, salinity, heat stress, and chilling injury (Jha and Subramanian 2014). Based on plant growth-promoting traits, seed germination and seedling vigor index of plants in salt-affected soils were analyzed to identify the salt-tolerant PGPR strains. NaCl uptake and tolerance at the different concentrations of 0–20% were also examined in different studies (Upadhyay et al. 2009; Zhang et al. 2008). Diversity of salt-tolerant PGPR bacteria representative cluster groups based on taxonomic and phylogenetic placement was investigated by analysis of the 16S rRNA gene sequencing (Bibi et al. 2012). Out of the different strains, the findings belong to three phyla (Proteobacteria, Firmicutes, Actinobacteria), four orders (Bacillales, Actinomycetales, Rhizobiales, Oceanospirillales), nine families, eighteen genera, and thirty-five species. The 18 genera were as follows (number of strains in parentheses): *Bacillus* (39), *Streptomyces* (9), *Fictibacillus* (5), *Halomonas* (3), *Nocardia* (3), *Microbacterium* (2), *Nesterenkonia* (2), *Oceanobacillus* (1), *Arthrobacter* (1), *Rhizobium* (1), *Jeotgalibacillus* (1), *Bhargavaea* (1), *Chryseomicrobium* (1), *Paenisporosarcina* (1), *Rhodococcus* (1), *Brevibacterium* (1), *Arsenicicoccus* (1), and *Solibacillus* (1). Bacillales, Rhizobiales, and Oceanospirillales were 88%, and out of them, Bacillales belonged 80% which increased shoot and root lengths 63% and 87%, respectively

(Zhang et al. 2018). The novel PGPR strain *Bacillus* species of the Firmicutes group display various beneficial effects in the host plants including abiotic stress tolerance. Among the endophytic bacteria, *Bacillus* spp. exhibit the highest abundance of 52.7% of the strains followed by *Streptomyces* and *Fictibacillus* of 12.2% and 6.8%, respectively (Damodaran et al. 2019; Upadhyay et al. 2009). *Bacillus pumilus* (Jha and Subramanian 2013), *B. amyloliquefaciens* (Nautiyal et al. 2013), *B. thuringiensis* (Raheem and Ali 2015), and *B. oryzicola* YC7007 (Baek et al. 2020) are most effective in enhancing plant growth in the saline environment. Chen et al. (2010) screened 114 strains of halophiles and salt-tolerant bacteria from orchards, paddy, sandy soil, and forest soils using five media and different NaCl concentrations (5–20%). Bacillaceae (33 strains; 54.1%) was the most frequently occurring family that was the most functional to salt stress. Similarly, Echigo et al. (2005) isolated 176 strains of salt-tolerant bacteria from orchards, lawn, pasture, and woodlands around Tokyo, Japan, using one medium (20% NaCl concentration). From all together of our studies, salt-tolerant PGPR are *Bacillus* species (80%) in the saline environment; therefore, we should focus salt-tolerant *Bacillus* species to salt stress.

9.3 The Genus *Bacillus* Is a Good Source for Making a Green Revolution in the Saline Area

Considering the importance of PGPR for mitigating the salinity in plants, we reviewed literature mainly related to the *Bacillus* species that play a key role in the saline environment. The genus *Bacillus* was first described by Cohn in 1872 (Claus and Berkeley 1986), and since then, many scientific works have been conducted on many aspects of this genus in relation to agriculture (McSpadden Gardener 2010). Numerous *Bacillus* species have been reported as biocontrol agents against phytopathogens. Some of them enhance plant tolerance to various abiotic stresses including salinity. Recently, an endophytic *B. oryzicola* YC7007 has been well-versed as a novel species, which successfully controlled the rice bakanae and bacterial blast diseases and mitigated salinity by inhibiting malondialdehyde and Na^+ in the dicot model plant *Arabidopsis thaliana,* cabbage, and radish (Baek et al. 2020; Chung et al. 2015; Hossain et al. 2016). A strain of another species, *B. subtilis* GB03, effectively enhances salinity stress tolerance in *A. thaliana* (Zhang et al. 2008). The cocultivation of wheat seeds with *B. amiloliquefaciens* BcA12 and *B. laevolactivus* BcL28 increases root and shoot growth (15–50%) in the saline environment compared with untreated plants (Egamberdiyeva and Höflich 2003). In another study, *Bacillus* strains BcP26 and BcM33 increase root and shoot growth of pea (18%) and maize (27%) and increase the uptake of N and P uptake (55%) under arid saline soil conditions compared with untreated plant (Egamberdiyeva and Höflich 2004). *Bacillus aryabhattai* RS341 increases up to 40% in root elongation and dry weight compared with untreated salt-stressed canola seedlings (Siddikee et al. 2010). Application of the rhizobacterium *B. subtilis* FZB24 at the dose of 1×10^8 CFU/ml

has been increased 6.5 and 5.3 times more yield in the eggplant and pepper plant, respectively, compared to untreated control under the saline environments. The bacterization by the strain FZB24 resulted in 50% and 25% reduction in salinity effects on the yield of eggplant and pepper plant, respectively (Bochow et al. 2001). *Bacillus pumilus* stimulates plant growth and enhances stress tolerance of rice under salt stress by inducing the antioxidative enzymes and lowering Na^+ accumulation in the leaves (Khan et al. 2016a, b). Under saline soils, *B. subtilis* significantly increases in fresh and dry masses of roots and leaves, photosynthetic pigments, proline, total free amino acids, crude protein, and N, P, K^+, Ca^{2+}, and Mg^{2+} uptake compared to untreated radish plants (Mohamed and Gomaa 2012). In fact, *B. megaterium* is an absolute majority of the Bacillaceae family. *Bacillus megaterium* was found to be the most dominant phosphate solubilizer in saline conditions (Nakade 2020). *Bacillus megaterium* F-58 exhibited significant improvement in fresh weight 42% of plants compared to untreated control under the saline soil at 200 mM concentration (Aslam and Ali 2018). *Bacillus subtilis* EY2, *B. atrophaeus* EY6, and *B. sphaericus* enhance plant growth and ultimately increase yield in radish and strawberry plants in saline conditions (Karlidag et al. 2010). These findings indicate that *Bacillus* species is an ideal candidate for plant growth promotion under saline soils.

9.4 Induction of Salt Tolerance in Plant by the *Bacillus* Species

Plants have their own mechanisms to bear salinity stress at a certain level depending on species and genotypes. Most of the plants release different hormones, activate antioxidant enzymes, and regulate their ion uptake and their metabolic and genetic pathways to grow under harsh saline conditions (Perez-Alfocea et al. 2010). The soil biota may improve salt tolerance efficiency in crop plants through altering hormonal root-shoot signaling, various classes of plant hormones that respond to changes in salinity. Plant growth-promoting bacteria (PGPB), especially *Bacillus* species are playing an important role in mitigating salinity by triggering biosynthesis of various plant growth hormones such as auxin, cytokinin, and gibberellin and by inducing the pattern recognition receptors (PRRs) or volatile organic compounds (VOCs) (Arkhipova et al. 2005; Ortiz-Castro et al. 2008; Hossain et al. 2016). The colonization of plants by *Bacillus* spp. also triggers the accumulation of abscisic acid (ABA) and antioxidants in the plant cells. Antioxidant defense enzymes such as ascorbate peroxidase (APOX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) have been induced by the *Bacillus* and other PGPR to detoxify reactive oxygen species (ROS) (Chiquito-Contreras et al. 2019; Hossain et al. 2016: Zhao et al. 2019). These PGPR *Bacillus* species also produce siderophores to enhance plants' iron uptake and also promote atmospheric nitrogen fixation and solubilization of organic and inorganic phosphates in the soils. The elite strains of *Bacillus* spp. might be used as an effective measure for inducing salinity tolerance

genes in the plant that could help the plant to survive in a saline environment. Several studies have been reported that stress-tolerant genes or proteins or transcription factor or pattern recognition receptors in plants are induced by these beneficial bacteria through various mechanisms (Munns and Tester 2008). Somewhat, they produce indole acetic acid and some unidentified elements that increase root architecture, viz., root length, lateral roots, and root tips, and most importantly enhance nutrient content, by improving the health of plants under salt stress conditions (Idris et al. 2007: Khan et al. 2016a, b; Sayyed 2019). The ACC (1-aminocyclopropane-1-carboxylic acid) deaminase produced by bacteria degrades plant ACC for acquiring energy. Moreover, it also decreases the harmful impact of ethylene, by enhancing stress tolerance and promoting the growth of plant. Some *Bacillus* spp. also produce exo-poly saccharides (EPS) that mitigate the salinity of soil. Inoculations of specific Bacilli PGPR help to enhance salt stress tolerance in plants via induced systemic tolerance (IST), which causes many functional and biochemical changes (Yang et al. 2009). Recently, an endophytic *B. oryzicola* YC7007 has been found to mitigate salinity stress by inducting *SOS1* in the dicot model plant *A. thaliana* (Baek et al. 2020; Rodríguez et al. 2005). The salinity of soils can be attributed to cations such as magnesium (Mg^{2+}), calcium (Ca^{2+}), and sodium (Na^{+}) and also anions like bicarbonate ($HCO3^{-}$), sulfate ($SO4^{2-}$), and chloride (Cl^{-}) (Tester and Davenport 2003). However, under stressed conditions, K^{+} and Na^{+} are two major contributors of osmotic pressure and ionic strength. Salinity increases the uptake of sodium ion (Na^{+}), which eventually results in decrease in the uptake of calcium (Ca^{2+}) and potassium (K^{+}) ions (Yildirim et al. 2006). The PGPB improves plant growth under salt stress by maintaining a favorable K^{+}/Na^{+} ratio, by enhancing the production of certain osmolytes such as proline, total soluble sugar, and total protein content, or by both these methods (Singh and Jha 2016). Another strain *B. subtilis* GB03 induces tissue-specific regulation called *HKT1* (High-affinity K^{+} transporter) that controls salinity in *A. thaliana* (Zhang et al. 2008). The *AVP1* (*Arabidopsis Vacuolar* H^{+}*-pyrophosphatase*) gene transformed into alfalfa (*Medicago sativa*) enhanced salt tolerance in the alfalfa up to 200 mM NaCl (Bao et al. 2009). *Pisum sativum* calcineurin B-like protein (*PsCBL*) and *P. sativum* calcineurin B-like (CBL)-interacting protein kinase (*PsCIPK*) genes into rice (*Oryza sativa* L.) have been transformed, and therefore, rice was able to survive at 150 mM NaCl (Sikdar et al. 2015). Research would be focused on how to induce salt tolerance genes in the plant by the application of PGPR *Bacillus* species.

9.5 Conclusion and Future Trends

Bacillus spp. plays an important role in regulating the PGP traits, tolerance to salt stress which enhances agricultural crop production. They could induce tissue-specific regulation of *HKT1* and *SOS* signaling in the plant system to mitigate the salinity stress. The application of *Bacillus* spp. enhance the accumulation of abscisic acid (ABA) and antioxidant defense enzymes such as ascorbate peroxidase (APOX),

superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) that detoxify ROS and protect the host plant from salt stress toxicity. Endophytic *Bacillus* species are rich in bioactive compounds that induce systemic resistance in host plants to tolerate harsh environments. The good symbiotic relationship of Bacilli with the plant host could be utilized for increasing crop yield under various abiotic stresses including salinity. However, underlying molecular mechanisms of beneficial effects of various strains *Bacillus* spp. on plants are largely unknown. The advancement of genomics and post-genomics analytical methods and recently developed CRISPR-Cas genome editing technology would be helpful for dissecting molecular cross-talks between *Bacillus* spp. and their plant hosts during exerting their beneficial effects. A better understanding of molecular plant-Bacilli interactions would immensely promote the development of *Bacillus*-based technology for alleviating salinity and other abiotic stresses for promoting sustainable crop production under the changing climate of the world.

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Chapter 10
Bacillus spp. of Ruminant Origin as Major Sources of Potential Industrial Amylases

Kgodiso Judith Rabapane, Alfred Mitema, Karen Nelson, and Naser Aliye Feto

Abstract The microbiome of ruminant's gastrointestinal tract origin is dominated by two major bacterial phyla *Firmicutes* and *Bacteroidetes*. Meanwhile, *Bacillus* spp. belonging to *Firmicutes*, have been reported to be potent producers of thermostable amylases and other biocatalysts that are of considerable commercial interest. Hence, they are primarily exploited as a significant source of thermostable biomolecules. Among some industrial enzymes, amylases are highly efficient commercial biocatalysts with an extensive range of utilization in textile, paper and pulp, detergent, leather, waste management, biofuel production and pharmaceutical and food industry. The ruminant's GIT microbiome is a relatively less exploited niche despite being the potentially rich source of biocatalysts of therapeutic, environmental and industrial importance. Therefore, in this chapter, we highlight the need to unravel the ruminant's GIT microbiome with particular emphasis on exploiting *Bacillus* spp. of ruminant origin as potential producers of amylases for utilization on a commercial level.

Keywords *Bacillus* spp. · Amylases · Ruminant · GIT · Microbiome

10.1 Introduction

The use of chemical catalysts to drive several processes in a wide range of sectors has been a significant contributor to environmental pollution. Besides the harmful effects they pose to the environment, chemical catalysts are carried in adverse

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reaction conditions, thus leading to elevated costs due to high energy consumptions (Singh et al. 2016a). Microorganisms are the preferred source of many industrial enzymes because of their generation time, ease for genetic manipulation towards desired traits, high yield at low production cost and the relatively stable enzymes they produce, thus resulting in less energy being consumed during industrial applications. In addition, microorganisms can be reproducible within a reasonable time frame and can produce more than one enzyme when used as whole cells during biocatalysis and bioprocessing of biomass.

Among major microbial enzymes, amylases are highly efficient commercial biocatalysts that are in high demand due to their broad range of usage from textile, paper and pulp, detergent, leather, waste management, biofuel production and pharmaceutical to food industry (Gopinath et al. 2017). Although amylases from fungal strains are employed extensively in the starch processing industry, most of the amylases that are applied in the industry have been reported to be produced by *Bacillus* species (Gopinath et al. 2017; Schallmey et al. 2004). *Bacillus* spp. are renowned as bacterial workhorses in microbial fermentation, and their proficiency of certain strains to produce large quantities (20–25 g/l) of extracellular enzymes and to withstand harsh environmental conditions such as various pH and temperature ranges among others has rated them among the most industrial enzyme producers (Schallmey et al. 2004). The ruminant's GIT is a potentially rich, under-exploited source of consortia of microorganisms including *Bacillus* spp., and their presence in the GIT can contribute to our knowledge of microbial responses to environmental stress and their function in complex and fastidious microbial ecologies. *Bacillus* is a broad and diverse genus of bacteria originating from the family of *Bacillaceae*. *Bacillus subtilis* and *B. mesentericus* were the first species to be used for the production of amylase on a commercial scale by Boidin and Effront during the early nineteenth century (Boidin and Effront 1917). This elucidation paved a way for other *Bacillus* and bacterial species and have since been used for the production of enzymes on a commercial scale. Among the primary enzymes produced by *Bacillus* spp. are amylases (Gopinath et al. 2017). Amylase enzymes are required for the complete hydrolysis of starch into fermentable sugars such as glucose and maltose (Liu and Kokare 2017; Sundarram and Murthy 2014). The breakdown process of starch and starch-related polymers by amylase enzymes in food industries is carried out under harsh conditions at very low or high pH and temperature, which involves a two-step process, liquefaction and saccharification that run at 50–60 °C, and thus, the process requires stable amylases that can also tolerate these conditions (Jiang et al. 2015). Furthermore, the detergent industry requires amylases which are stable under alkaline and oxidative, consistent and active at a broad range of temperatures (Fitter 2005). Therefore, there is a high demand to develop enzymes with improved or enhanced operational parameters such as pH and temperature among others that will meet the requirements set by specific applications.

Accordingly, the potential use of microbial enzymes on a commercial scale continues to encourage the development of novel extracellular enzymes with enhanced characteristics from unusual sources of microorganisms (Gopinath et al. 2017). The ruminant's GIT is among one of the sources of microbiomes that haven't been

explored comprehensively for industrial use due to certain limitations; hence, in this chapter, we highlight the importance of unravelling the GIT microbiome with particular emphasis on exploiting *Bacillus* spp. of ruminant GIT origin as a potential source of industrial amylases.

10.2 Mechanism of Action and Structure Variation of Amylases

As mentioned in the previous section, among many industrial enzymes, amylases are highly efficient commercial biocatalysts that are in high demand globally. This class of enzymes is responsible for the degradation of starch and other related polymers into simple fermentable sugars (Sundarram and Murthy 2014). The monomeric sugars in starch are held together by glycosidic bonds, which are targeted by amylase during the breakdown of the polymer. Consequently, the manner in which various amylases act on the glycosidic bonds assists in their classification (Liu and Kokare 2017).

In order to fully understand how amylases act on the glycosidic bonds mentioned briefly in the previous section, it is crucial to first elaborate on the metabolism of their primary substrate, starch, a glucose polymer that serves as a major component of food as well as feed, providing a source of energy and carbon to living organisms. This polysaccharide is composed of glucose molecules that are linked together through glucosidic bonds which are required to be hydrolyzed in order for the glucose monomers to be assimilated as fuel. Amylases are responsible for the breakdown down of these glucosidic bonds to produce glucose monomers, maltodextrin, modified starches or glucose syrups (El-Fallal et al. 2012).

Furthermore, starch represents the most abundant form of polysaccharide stored in plants and next to cellulose is the most abundant polysaccharide on earth (Walsh 2014). The two significant constituents that contribute to the complexity of starch are amylose which is a linear molecule and the branched amylopectin (Fig. 10.1). Frequently, amylose makes up approximately 20–30% of the starch, while amylopectin makes 70–80% of the starch. The structure and composition of these molecules depend highly on the source of the carbohydrate. Examples include amylo-maizes which contain over 50% of amylose whereas ‘waxy’ maize has almost none (~3%) (JJM 1985; Singh et al. 2003).

Starch and starch-containing substrates are utilized in food, textiles, alcohol and other applications; hence, its industry has a significant stake in the market. These substrates are widely available from cheap plant sources, rendering the potential uses of the enzyme more plentiful regarding costs (Gopinath et al. 2017). According to market research future®, the global industrial starch market is projected to amass a revenue of $112 billion by 2024 with a compound annual growth rate of approximately 5.9%. These projections ultimately stimulate the demand for amylase enzymes (Gopinath et al. 2017). The complete breakdown of starch requires a consortium of amylolytic enzymes which break down the $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glycosidic linkages into glucose.

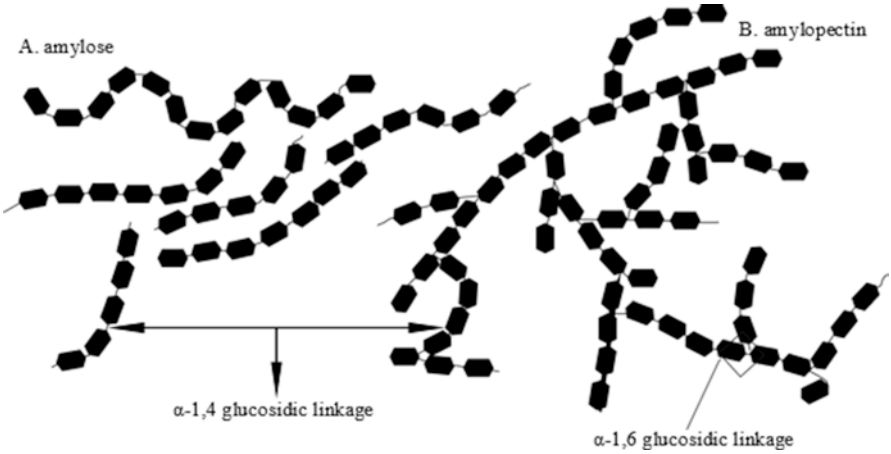

Fig. 10.1 Starch consists of two polymers. (**a**) Amylose is a linear polymer that comprises approximately 6000 glucose units (Gopinath et al. 2017) that are attached by α1 → 4 glycosidic linkages and (**b**). Successive glucose residues are linked via α1 → 4 glycosidic linkages along the linear portion of the molecule, with branch points consisting of α1 → 6 glycosidic linkages. Amylopectin has short α1 → 4 bonded to linear chains of 10–60 glucose units that are branched by α1 → 6 tied to side chains with 15–45 glucose units

Consequently, they are broadly classified into four distinct groups based on the manner in which they act on the substrate, mainly as endoamylases, exoamylases, debranching amylases and glucotransferases. Accordingly, their mechanism of action and structural variations are outlined in the following subsection outlines.

10.2.1 Endoamylases

Endo-acting amylases such as α-amylase (EC 3.2.1.1), also known as endo-1,4-α-D-glucan glucohydrolase, cleave the α-1,4 glycosidic linkages in the internal part of the linear amylose and branched amylopectin chain to yield oligosaccharides with varying lengths and α-limit dextrins, respectively, that ultimately contributes to the branched oligosaccharides (Fig. 10.2) (Walsh 2014; Aiyer 2005). It is also crucial to note that generally, endo-acting amylases cannot cleave the α-1 → 6 of amylopectin, one exception being the α-amylase produced by *Thermoactinomyces vulgaris*, which has been reported to hydrolyze both α-1 → 6 and α-1 → 4 glycosidic linkages in trisaccharides (Sakano et al. 1983). Most α-amylases are metalloenzymes and thus require a calcium ion for their function and stability. Compared to the other classes of amylases, α-amylases are faster rate as they degrade the starch at random locations (Tiwari et al. 2015b; Sarian 2016).

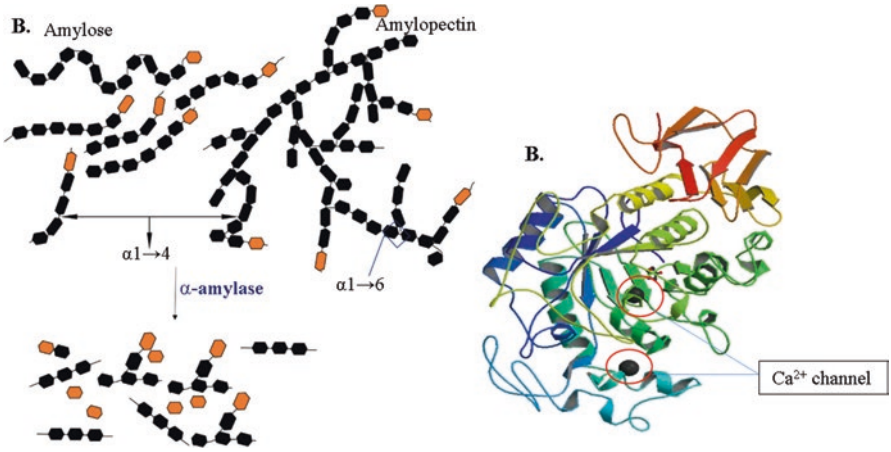

Fig. 10.2 (**q**) Schematic diagram of the action of alpha amylase on starch and (**b**) the three-dimensional structure of α-amylase with a Ca^{2+} in the middle (Ramasubbu et al. 1996)

10.2.2 Exoamylases

The second group of amylases act on the substrate from the non-reducing end to generate small oligo- and monosaccharides such as maltose by β-amylase or the smallest monomer glucose by glucoamylase. There are three well-known types of exo-acting amylases in the starch degradation process. (**1**) β-amylase (EC 3.2.1.2) exclusively acts on the α-1 → 4 glycosidic bonds at non-reducing ends of the substrate to ultimately generate maltose and β-limit. Animal tissues do not contain this type of amylase; however, it has been reported to be present in microbes inhabiting the digestive tract (Gurung et al. 2013), further cementing the potential of the digestive tract of animals as a source of potent enzymes. (**2**) Glucoamylase (3.2.1.3) hydrolyzes the α-1 → 4 glucosidic bonds at the non-reducing end of amylose to generate glucose monomers in the β-configuration and even though, at a slower rate, it also acts on the α-1 → 6 glucosidic bonds at the branch points of amylopectin to yield glucose molecules (Fig. 10.3). Glucoamylases are capable of reversing hydrolysis reactions to produce maltose and isomaltose, which in turn has a great significance in the industrial process where the sugar content is present (Negi and Vibha 2017). The most distinguishing feature of glucoamylase is its ability to operate under acidic conditions (active around pH <3). The third type of amylase in the exo-acting group is (**3**) α-glucosidase (3.2.1.20); this type of amylase acts on the external glucose residues of either amylose or amylopectin and both to ultimately yield glucose (Sarian 2016).

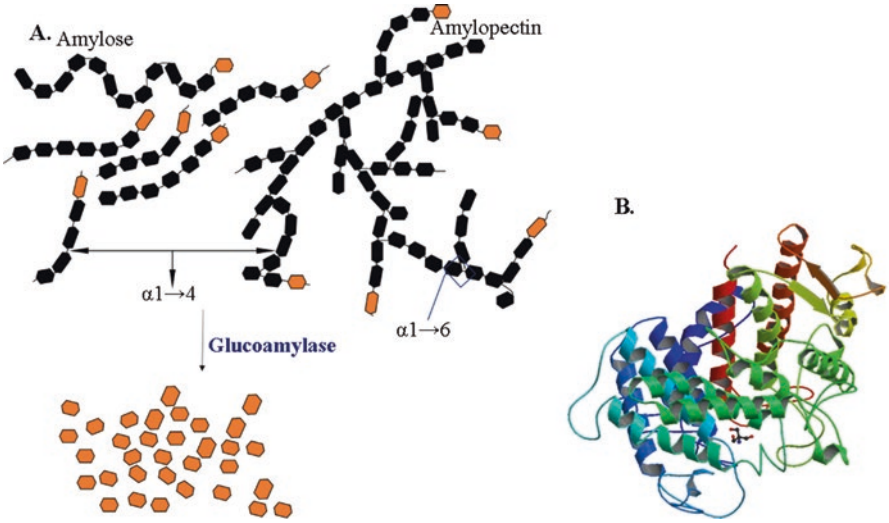

Fig. 10.3 (**a**) Schematic diagram of the action of glucoamylase on starch and (**b**) the three-dimensional structure of glucoamylase from *Saccharomycopsis fibuligera* (Sevcik et al. 1998)

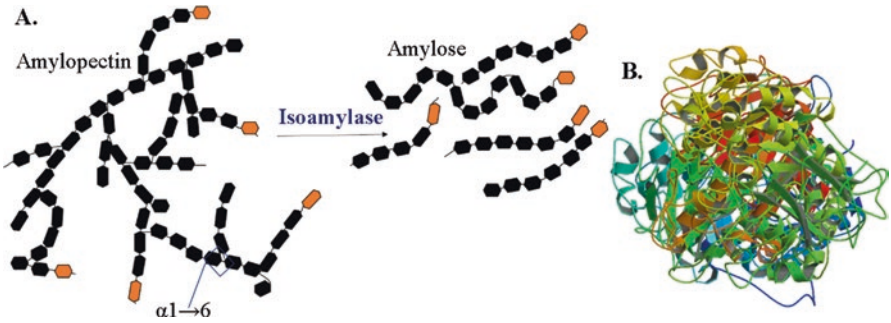

Fig. 10.4 (**a**) Schematic diagram of the action of isoamylase on starch and (**b**) the three-dimensional structure of isoamylase 1 from *Chlamydomonas reinhardtii* (Sim et al. 2014)

10.2.3 Debranching Enzymes

This group of amylases catalyzes the hydrolysis of α1 → 6 glucosidic bonds in amylopectin and or glycogen and other related polymers to yield linear molecules (Fig. 10.4). An example of this group is isoamylase (EC 3.2.1.68).

10.2.4 Glucotransferases

The last group of amylases cleaves the α-1 → 4 glycosidic bond of the donor molecule and transfer a portion of the donor to a glycosidic acceptor with the formation of the new glycosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and

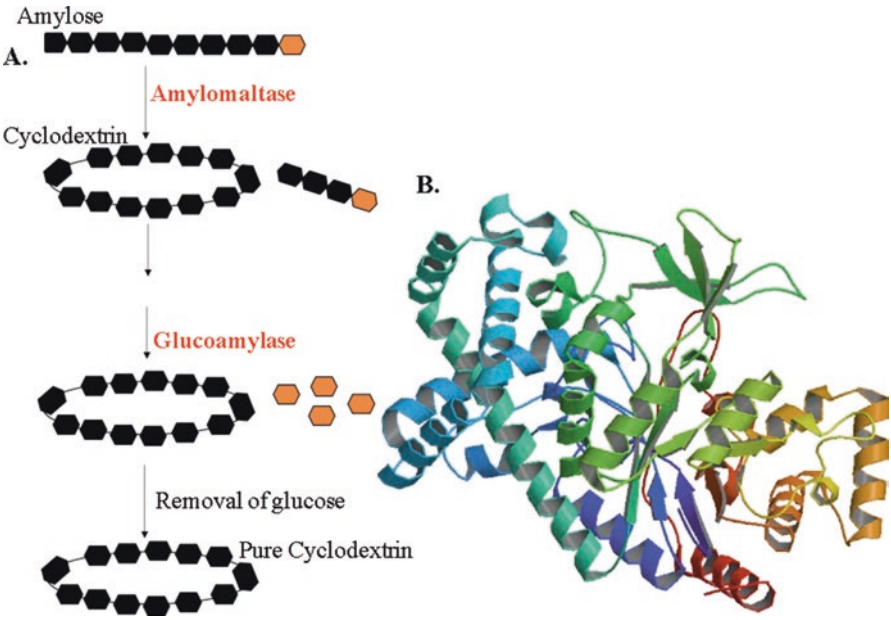

Fig. 10.5 (**a**) Schematic diagram of the effect of amylomaltase on starch (Fujii et al. 1998) and (**b**) the three-dimensional structure of amylomaltase from *Thermus aquaticus* (Przylas et al. 2000)

cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new α-1 → 4 glycosidic bond while branching enzyme (EC. 2.4.1.18) establishes a new α1 → 6 glycosidic bond (Van Der Maarl et al. 2002). A unique feature of amylomaltase is its ability to catalyze the cyclization reaction of cyclic amylose to generate cyclodextrins (Rachadech et al. 2015). Once the cyclodextrins have been created, the amylomaltase is then inactivated, and glucoamylase is introduced to catalyze the hydrolysis of the linear oligosaccharides to produce glucose monomers, Fig. 10.5 shows the action of amylomaltase.

10.3 Source of Microbial Amylases

The breakdown of the glycosidic bonds by amylases is a very crucial process that occurs within biological systems to produce and store energy. Thus, amylases are diversified among various sources ranging from plant, to animal, to microbial kingdoms where they play a crucial role in carbohydrate metabolism (Gopinath et al. 2017). Though plants and animals produce amylases, amylases from both sources don't meet industrial demands, mainly because plant sources are seasonal, and therefore, inconsistencies in enzyme production can be experienced, and yield may be less (Belorkar n.d.). Also, enzymes from plant sources are not stable under adverse conditions. On the other hand, using animal cells to produce enzymes involves sacrificing animals in the process, and the overall operation of animal cell

culture makes the production of enzymes from animal sources uneconomical (Belorkar n.d.; Mahalakshmi and Jayalakshmi 2016). As a result, microbial sources are generally dominating driving forces for industrial applications due to enzyme yield, stability and specificity as these attributes in conjunction with additional benefits such as cost-effectiveness, consistency, less time and space required for production, as well as ease of genetic manipulation and optimization (El-Fallal et al. 2012; Pandey et al. 2000), which continue to be significant advantages or requirements to meet industrial demands. Thus, microbial sources are preferred over plant and animal sources.

Although microbial sources include yeast, bacteria and fungi, majority of industrial applications utilize fungal and bacterial strains. Among fungal strains that have been reported to produce amylases are those of the genus *Aspergillus* and have been employed for the preparation of Asian foods (Tiwari et al. 2015a). Furthermore, filamentous fungi, namely, *Rhizopus oryzae*, *Aspergillus awamori* and *A. niger*, have also been reported to be the major producers of industrial glucoamylases which operate at a temperature range of 55–60 °C (Negi and Vibha 2017). Generally, *Bacillus* spp. are renowned to have enhanced attributes when compared to other microbial strains because they are capable of growing in extreme environmental conditions, and therefore, it is expected that they will produce enzymes which are stable in adverse conditions (Feto 2016). Accordingly, some of the species that have been reported to overproduce amylases include *B. cereus (*Sundarram and Murthy 2014*)*, *B. circulans (*Singh and Rani 2014*)*, *B.subtilis* (El-Banna et al. 2007) and others (Gopinath et al. 2017). *Bacillus* spp. including *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* are reported to be prolific producers of different types of amylase particularly α-amylases (Liu and Kokare 2017; Pandey et al. 2000; Saini et al. 2017) and also have been widely exploited for the production of many other industrial enzymes due to the inheritance of these thermostable attributes (Schallmey et al. 2004; Tiwari et al. 2015b). *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* are reported to provide approximately 60% of commercially available enzymes (Dash et al. 2015). Moreover, *Bacillus* spp. have been approved as Generally Recognized As Safe (GRAS) by the Food Drug and Administration (FDA) (Irajie et al. 2016).

Emerging research studies are continuously focusing on developing *Bacillus* spp. for the production of amylases with improved characteristics to meet industrial demands. Li et al. (2013) purified, characterized and cloned a thermotolerant isoamylase produced from *Bacillus* sp. CICIM 304. This isoamylase displayed its optimal activity at a remarkably high temperature of 70 °C and pH 6.0, with thermostability between 30 °C and 70 °C and an alkaline pH range from 5.5 to 9.0 (Li et al. 2013). Gurumurthy and Neelagund (year) characterized *Geobacillus* sp. iso5, an industrially viable extreme thermostable novel alpha-amylase. The strain was isolated from the thermal water of a geothermal spring for industrial application. This bacterium showed attributes of thermotolerance and alkali-resistance, and it is reported as novel due to its ability to withstand an optimum activity of temperature 90 °C and pH 8.0 (Gurumurthy and Neelagund 2012). The primary interest in exploring thermophilic amylases from unusual sources is their use in elevated

temperatures as this increases the reaction rate and thus accelerates the overall process. Moreover, higher temperatures are beneficial because they reduce the risk of microbial contamination and also reduce the viscosity of the reaction mixture, thus providing a considerable energy saving process (Sarian 2016; Pandey et al. 2000). For instance, in the production of glucose from starch, the liquefaction process of starch is carried out by a thermostable α-amylase produced by *B. licheniformis*, which is then followed by a saccharification process achieved by the use of a fungal glucoamylase. The liquefaction process is completed faster because it is carried out by an α-amylase that can operate at 95–105 °C (Sundarram and Murthy 2014; Sarian 2016; Ward 1991). However, because fungal glucoamylases are stable generally up to a temperature range of 55–60 °C (Pavezzi et al. 2008), they delay the overall process because the reaction mixture must be cooled to a temperature that is within that range. Therefore, potential thermostable glucoamylases from thermophilic bacteria can be used at a higher temperature for saccharification processes and thereby reduce the production cost of glucose from the high energy required for cooling (Negi and Vibha 2017).

A suitable organism is crucial for the production of amylases that will meet industrial demands. Depending on the type of application, thermotolerance is a desirable trait for some amylase enzymes and other types of enzymes to meet industrial needs (Gurung et al. 2013). Table 10.1 shows some of the enzymes and parameters produced by fungal and bacterial strains. From these findings, it is clear that *Bacillus* spp. sources have a higher thermostability and higher pH range, and this, among other attributes, has resulted in their utilization in industrial applications that are carried out at higher temperatures and alkaline conditions. Hence, there is a continuous need to explore bacterial species with enhanced characteristics, in particular those of *Bacillus*, for the production of amylases for use on a commercial scale.

A suitable organism is crucial for the production of amylases that will meet industrial demands. For an amylase enzyme and other types of enzymes to meet

Table 10.1 Optimum pH and temperature of enzymes produced by fungal and bacterial strains

Microbe	Enzyme	pH	Temperature °C	References
Bacillus flavocaldarius	Type I pullulanase	7.0	75–80	Suzuki et al. (1991)
Bacillus subtilis	α-Amylase	7.0	135	Asgher et al. (2007)
Halomonas meridian	α-Amylase	7.0	37	Coronado et al. (2000)
Aspergillus flavus	α-Amylase	6.0	55	Khoo et al. (1994)
Aspergillus awamori	Glucoamylase	4.5	60	Yamasaki et al. (1977)
Clostridium	Glucoamylase	4.5	65	Ohnishi et al. (1991)
Thermomyces	Glucoamylase	–	70	Rao et al. (1981)
Bacillus clausii	Protease	11.5	60	Horikoshi (1971)
Aspergillus awamori	Glucoamylase	5–7	55	Pavezzi et al. (2008)
Geobacillus sp. Iso5	α-Amylase	8.0	90	Gurumurthy and Neelagund (2012)

industrial needs, it has to be thermotolerant because thermotolerance is a desirable attribute for significant groups of enzymes that are exploited at the industrial level (Gurung et al. 2013). Table 10.1 shows some of the enzymes and parameters produced by fungal and bacterial strains. From these findings, it is clear that *Bacillus* spp. sources have a higher thermostability and higher pH range, and this, among other attributes, has resulted in their utilization in industrial applications that are carried out at higher temperatures and various pH ranges. Hence, there is a continuous need to explore bacterial strains with enhanced characteristics more especially those of *Bacillus* for the production of amylases for use on a commercial scale.

10.4 Amylase Enzyme Production by *Bacillus* spp. in the Ruminant's GIT

The digestive system in ruminants is represented by a four-stomach compartment that is divided into the paunch (rumen), reticulum, omasum and abomasum (Fig. 10.6) (Hungate 1947). This digestive system is uniquely designed to process plant-based feed such as phytate and lignocellulosic material, which sets them apart from monogastric mammals. Unlike monogastrics, which have specialized enzymes, ruminants depend on consortia of microorganisms within their GIT to bring about feed conversion through a fermentation process (Ribeiro et al. 2016). Thus, the ruminant's GIT is an anaerobic ecosystem that is inhabited by an internetwork of various microorganisms working in synergy to bring about feed conversion. Feed intake by ruminants triggers the release of large volumes of saliva which facilitate the mechanical pretreatment of feed from the oesophagus to the rumen, reticulum and/or omasum until it reaches the abomasum which is also called the true stomach because it is similar to that of monogastrics (Moran 2005). The actual microbial fermentation of feed starts in the rumen, and the rumino-reticulum permits regurgitation of partially fermented ingesta (cud) back to the mouth for rechewing (rumination) which further breaks down the ingested feed into a small particle size that is

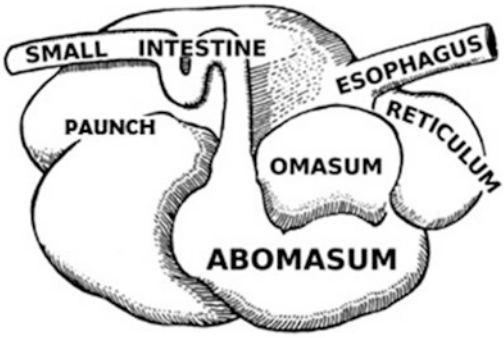

Fig. 10.6 A digestive system of a ruminant animal (Ruminant n.d.)

able to pass through the other compartments for complete digestion (Moran 2005) and further goes processed through the intestines as faecal matter.

The secreted saliva also releases salts such as bicarbonate and ultimately acts as a buffering agent regulating the physiological pH range between 5.5 and 6.9 (Choudhury et al. 2015). In addition to the maintained pH, the temperature range in the ruminant's GIT is between 38 °C and 42 °C (Hill et al. 2016), creating a conducive environment which potentially allows a vast array of anaerobic and facultative anaerobic microorganisms to grow. Accordingly, the microorganisms in the GIT include bacteria (10^{11} cells/mL), methanogenic archaea (10^{6} cells/mL), protozoa (10^{4}–10^{6} cells/mL), fungi (10^{3}–10^{6} zoospore/mL) and viruses (10^{9} particles/mL), with bacteria being the most abundant and extensively studied [54, 59]. Although diverse and operate synergistically to facilitate feed conversion sequentially, anaerobic fungi being the most efficient fibre degraders by acting on the cuticle of lignocellulosic material which then becomes easier for bacteria and ciliate protozoa to gradually act upon. With a biomass turnover of approximately 50%, bacteria in the GIT are also actively involved in both the degradation of complex and simple carbohydrates. The protozoa make use of the complex and simple carbohydrates by means of engulfment to bring about end products including ammonia and amino acids from microbial protein as they also engulf bacteria in addition to feed (Kamra 2005). Ultimately, the interaction between bacteria, fungi and protozoa results in the production of gases such as carbon dioxide (CO_2) and hydrogen (H_2) which are then utilized by methanogens to produce methane gas (CH_4). Hence, methanogens are accountable for CH_4 belching in ruminant animals as they actively reduce the buildup of H_2, which would interfere with bacterial dehydrogenases in the GIT, consequently reducing the fermentation rate, digestibility and eventually feed intake (Patra et al. 2017; Cieslak et al. 2013). Meanwhile, the viruses in the GIT are reported to be responsible for the biomass turnover through cell lysis which leads to direct availability of amino acids from microbial protein for the ruminants as a host (Kamra 2005), and some of these phages are under investigation for the abatement of methane in the GIT (Leahy et al. 2010).

Overall, the microorganisms in the GIT all play unique roles including pectinolytic (Paster and Canale-Parola 1985), cellulolytic (Fujimoto et al. 2011), amylolytic (Hungate et al. 1952), xylanolytic (Seo et al. 2013), proteolytic (Wallace 1996), lipolytic (Hobson and Mann 1961) and hemicellulolytic (Seo et al. 2013; Nyonyo et al. 2014; Šimůnek et al. 2018), among others, during feed fermentation which results in the production of VFAs, amino acids and various gases among other by-products, thus making the ruminant's GIT a potentially rich source of not only amylase enzymes but also other biocatalysts for industrial applications, such as cellulase, protease, lipase and xylanase. Although the GIT microbiome is well adapted to convert various ingested feed into nutrients, it is still mostly unknown and represents an untapped microbial consortium. This limitation is supported by the fact that the GIT is a fastidious environment in that it is anoxic, the temperature and pH are naturally maintained and mimicking this type of environment in the lab remains a challenge (Zehavi et al. 2018). Accordingly, conventional culture techniques have resulted as a major limitation as they have been reported to cover about 11%

(Zehavi et al. 2018) of the representative sample which ultimately results in insufficient functional genomic studies on the ruminant's GIT species.

Bacteriodetes, *Firmicutes*, *Proteobacteria* and *Actinobacteria* are the most reported dominant phyla in the GIT (Patel et al. 2014; Firkins and Yu 2006). The population of the GIT microbiome is highly dependent on the host species, age, feeding habits (diet) and geographical localizations with some being common among ruminants worldwide (Ogata et al. 2019; Kim et al. 2018; Henderson et al. 2015). Cunha et al. (2011) reported the characterization of the bacterial and archaeal community structure in the rumen microbiome of goats (*Capra hircus*) from the semiarid region of Brazil, and accordingly, sequences of the bacteria from the phyla *Bacteroidetes* and *Firmicutes* were predominant (Cunha et al. 2011). Patel et al. (2014) explored the buffalo rumen metagenome where they outlined carbohydrate-active enzymes (CAZymes) and provided insight on the abundance of other enzyme families and the alteration of microbial diversity in response to variation in the diet (Patel et al. 2014). They described the analysis of (CAZymes) from 3.5 gigabase sequences of metagenomic data from rumen samples of Mehsani buffaloes fed on different proportions of green or dry roughages to concentrate ration. A total of 2597 contigs encoding putative CAZymes were identified, and the phylogenetic analysis of these contigs by MG-RAST revealed the predominance of *Bacteroidetes*, followed by *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla (Patel et al. 2014). Consequently, with *Firmicutes* being among the dominant phyla in the GIT, chances of obtaining *Bacillus* spp. are potentially higher. This, however, stands to be supported by research that is focused on the objective of exploring *Bacillus* spp. of which is currently insufficient in this niche due to the primary limitation mentioned previously. Thus, although the sequence and structural community studies reveal knowledgeable insight on the abundance of microbial population in the GIT, this has resulted in limited information on the individual species because there is no link between the individual species and the predicted genes.

Interestingly, there are developments on the improvement of the reference databases such as the Hungate 1000 project which aims to increase the microbial culture collection of rumen origin and to culture as-yet uncultured microorganisms from the ruminant's GIT in order to fully understand how the GIT system operates (Seshadri et al. 2018). In a study by Seo et al. (2013) (Seo et al. 2013), a facultative anaerobic bacteria *Bacillus licheniformis* JK7 was isolated and was reported to be capable of secreting endoglucanase, β-glucosidase and xylanase in the rumen of a native Korean goat, which can survive on harsh condition, such as provision of low-quality roughage as a sole feed source. According to the authors, the isolation of cellulolytic and xylanolytic *Bacillus* sp. from the rumen of goats has not previously been reported. Among the Hungate 1000 project is a study by Nathani et al. (2014) (Nathani et al. 2014), which reported the whole genome sequencing and functional annotation of amylolytic anaerobic spore-forming *Bacillus nealsonii strain* AAU1 isolated from the rumen of Surti buffalo. The optimal growth of *B. nealsonii strain* AAU1 was observed at 40 °C (range: 30 °C to 45 °C) and pH 6.5 (range: 5.5 to 7.5) under anaerobic conditions. Three unpublished strains *B. cereus* KPR-7A and *Bacillus* sp. MB2021 from cow rumen as well as *B. licheniformis* VTM3R78 from

moose rumen are also reported (Seshadri et al. 2018). Among these three strains, only the latter is capable of utilizing starch as a substrate. According to literature reviews, research on the production of amylase enzymes by *Bacillus* spp. of ruminant's GIT origin is insufficient and remains a field to be explored in depth.

10.5 Industrial Application of Amylases

The industrial enzyme market is estimated to reach US $ 6.2 billion by 2020 (Mehta and Satyanarayana 2016). One of the significant reasons for the continuous increase in the global sales of microbial enzymes is the rise in demand for consumer goods and biofuels. The use of amylase enzymes has replaced acid hydrolysis of starch, and currently, amylases have a great significance in present-day biotechnology having approximately 25–30% of the world enzyme market to date (Dash et al. 2015). These vast potentials of amylases to be employed in a wide range of industries have placed a more significant challenge for researchers. As a result, there is a continuous exploration for more active amylase producing *Bacillus* strains with enhanced potential characteristics for industrial utilization (Table 10.2).

As mentioned previously, amylases are one of the primary enzymes that are exploited in the industry. The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat bran koji culture of *Aspergillus oryzae* in 1894.

Table 10.2 Industrial applications of amylases from *Bacillus* spp.

Industry	Enzyme	Function	Source	References
Baking	Amylase Maltogenic α-Amylase	Flour adjustment Enhance the shelf life of bread	*Bacillus* sp. *Bacillus stearothermophilus*	Singh et al. (2016a)
Beverage	α-Amylase β-Amylase	Starch hydrolysis Starch hydrolysis	*Bacillus* sp. *Bacillus* sp.	Singh et al. (2016a) and Rejzek et al. (2011)
Paper and pulp	Amylase	Deinking, drainage improvement	*Bacillus licheniformis*	Mojsov (2012)
Detergent	Amylase	Carbohydrate strain removal	*Bacillus subtilis*	Schallmey et al. (2004)
Leather	Amylase	Fibre splitting	*Bacillus subtilis*	Singh et al. (2016a)
Waste management	Amylase	Bioremediation of vegetable wastes	*Bacillus licheniformis*	Singh et al. (2016a)
Textile	Amylase	Desizing	*Bacillus* sp., *B. licheniformis*	Schallmey et al. (2004)
Therapeutic	α-Amylase	As a digestive disorder treatment	*Bacillus* spp.	Singh et al. (2016a)
Ethanol production	α-Amylase	Starch hydrolysis	*Bacillus licheniformis*	Gurung et al. (2013)

Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase then began in 1959. From then on, amylases are being utilized for various purposes (Aiyer 2005). Moreover, amylases have recently found their use in other applications such as fuel production, removal of starch sizer from textile, detergent industry, paper and pulp, brewing and pharmaceutical industry among others (Saini et al. 2017).

10.5.1 Biofuel Production

The focus on biofuels to secure future energy solutions promotes a high demand for enzymes that are able to produce biofuels in an eco-friendly manner while proficiently utilizing abundant polysaccharides such as lignocellulosic materials and starch polymers, for instance, ethanol, which is among the most utilized liquid biofuel that can be derived from renewable resources such as waste generated from crops and by-products. Accordingly, starch is the most used substrate for the production of bioethanol due to its low price and readily available raw material in most regions of the world (Gopinath et al. 2017). Amylases, in particular alpha amylases and glucoamylases, are crucial to producing fermentable sugars for the production of ethanol (Saini et al. 2017).

10.5.2 Therapeutic

Mayo Clinic refers to malabsorption syndrome as many disorders in which the small intestine can't absorb enough specific nutrients and fluids. As a result of this syndrome, individuals can experience symptoms such as weight loss, bloating and diarrhoea, and in most cases, this condition can eventually affect the brain, nervous system, bones, liver and other organs. One of these disorders caused by malabsorption is a digestive disorder which generally occurs due to lack of or insufficient digestive enzymes in the body. In cases like these, amylases are used as therapeutic drugs in health issues related to enzymatic deficiency and gastrointestinal disorders (Singh et al. 2016a; Mane and Tale 2015).

10.5.3 Brewing

In the brewing industry, once the starch has been solubilized, it is subjected to two enzymatic steps including saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as glucoamylase and α-amylase to obtain fermentable sugars, followed by fermentation, where sugar is

converted into ethanol using an ethanol fermenting organism such as yeast *Saccharomyces cerevisiae (*Tiwari et al. 2015a*;* Mobini-Dehkordi and Javan 2012*)*. Accordingly, in the conversion of crushed starch to fermentable sugars, such as maltose, amylase enzymes are used to improve malting and mashing as well as enhancing the aroma and quality of beer together with the primary fermentation (Kuhad et al. 2011).

10.5.4 Paper and Pulp

The paper and pulp industry is among the practices that utilize a large amount of resources such as water, wood and energy. The activities in this industry result in a significant amount of solid wastes and waste water that require extensive treatment. Thus, the increasing awareness of sustainability concerns the use of microbial amylases in this industry has grown steadily to minimize the adverse effect on the environment. The use of amylase enzymes improves draining and enzymatic deinking which then result in reduced energy and chlorine requirement. Furthermore, the paper and pulp use amylases for the reduction of starch viscosity to achieve the appropriate coating of paper for application to fibres.

10.5.5 Textile

Starch is incorporated into the yarn before fabric production for a fast and weaving process where amylase is then employed to hydrolyze and solubilize starch, which then washes out the cloth increasing the stiffness of the finished material. Fabrics are further sized with starch, and alpha-amylase is then used as a desizing agent for removing starch from the grey cloth before it is further processed in bleach and dye (Saini et al. 2017).

10.5.6 Bioremediation

The execution of strict protocols for waste disposal into the environment has compelled the urge to reinforce research that is based on finding alternative processes in particular enzymes for waste water treatment. Starch occurs extensively in waste materials produced from the processing of plant raw material. Starch processing waste is produced in large quantities, and this results in environmental pollution. A cocktail of microbial amylases or a consortium of microbes producing amylases are then used for bioremediation of starch pollutant materials (Tiwari et al. 2015a; Mobini-Dehkordi and Javan 2012).

10.5.7 Detergent

The detergent industry is among the primary consumers of enzymes as it relates to both volume and value. It dates back to 1913 when the pancreatic extract was used for the first time in the enzyme-detergent development, and then later, a microbial enzyme under the trade name BIO-40 was commercialized. Amylases are the second group of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid soaps contain these enzymes. They catalyze the hydrolysis of glucosidic linkages in starch stains, commonly found in foods such as pasta, fruit, chocolate, baby food and barbecue sauce, as well as gravy, and consequently provide an effective cleaning action without damaging fibres while making the detergent environmentally safe. As coloured stains, their removal is of interest in both detergent and dishwashing contexts. Amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus (*Sahni and Goel 2015*;* Mobini-Dehkordi and Javan 2012*)*.

10.5.8 Medicine and Analytical

Enzyme utilization in medicine is extensive as in the industry and is overgrowing. Plasma serum amylase is measured for medical diagnosis. A standard concentration of amylase for adults in the blood is in the range of 21–101 U/L. Higher than normal levels may speculate one of several medical conditions including acute inflammation of the pancreas, perforated peptic ulcer, strangulation of ileus in humans, torsion of an ovarian cyst, macroamylasemia and mumps. Amylase concentration may be measured in other body fluids, including urine and peritoneal fluid (Tiwari et al. 2015a).

In recombinant DNA technology, the presence of amylase serves as an additional approach for selecting successful recombinants of a reporter construct in addition to antibiotic resistance. As homologous regions of the structural gene flank reporter genes for amylase, successful integration will disrupt the amylase gene and prevent starch degradation, which is easy to detect when stained with iodine (Singh et al. 2011).

10.5.9 Baking

The global market for baking enzymes is estimated at US $754.1 million for the current year, 2020, and is projected to reach a revised size of US $1 Billion by 2027, growing at a compound annual growth rate of 4.7%. Among the major baking enzymes, amylases play a pivotal role in the hydrolysis of starch and produce small dextrins for the yeast to act upon. Additionally, amylases also degrade the damaged starch in the wheat flour to produce small dextrins, which allows the yeast to be

continually active during dough fermentation, resulting in improved bread volume and crumb texture (Saini et al. 2017). Although amylases can be produced by these yeast cells, it takes a while for the yeast to produce enough of these amylases to hydrolyze substantial amounts of starch in the bread. Hence, amylases are included in the bread improver to accelerate the process, thereby making the bread-making process economical. Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry (Mobini-Dehkordi and Javan 2012).

10.5.10 Other Applications

The starch hydrolysis industry has the most widespread applications of amylases that are used during starch hydrolysis in the starch liquefaction process which converts starch into fructose and glucose syrups (Nielsen and Borchert 2000). In the therapeutic industry, amylases are utilized as therapeutic drugs in health issues related to enzymatic deficiency and gastrointestinal disorders (Mane and Tale 2015; Singh et al. 2016b) such as malabsorption, a digestive disorder which generally occurs due to lack of or insufficient digestive enzymes in the body (Singh et al. 2016a; Mane and Tale 2015). In the candy industry, amylases are used for the production of desired softness in candy. They are also used for partial or total hydrolysis of cornstarch to produce large quantities of sweeteners in the glucose and syrup industry. Moreover, they are applied for hydrolysis of starch causing turbidity due to insolubility in the fruit juice industry (Saini et al. 2017).

10.6 Future Research Outlooks

1. Evaluate the cultural possibilities of capturing more *Bacillus* strains including other microorganisms from the ruminant's GIT by tampering with the culturing conditions and exploring the depth of OMICS technology, in particular, culturomics and functional genomics, among others, in order to bridge the gap between potential *Bacillus* spp. and the predicted sequences.
2. It may also be advisable to conduct more studies on ruminants that are free grazing as this will potentially increase the chances of obtaining more strains as compared to studies whereby animals are under a specified diet due to a constant evolution of microorganisms adapting to various feed.
3. Although the utilization of amylases, in starch processing, has been playing a major role for decades, the application of amylases or enzymes in general as pure extracts are not competent as industrial biocatalysts in their natural state because their catalytic efficiency is low, and they are prone to denaturation, thus affecting their performance and shelf-life. Furthermore, even when they are used as whole microbial cells for bioprocessing of biomass, overproduction or high

titer values are near impossible to obtain in the native microorganism due to feedback inhibition because the organism can only produce the required amount of enzyme for its survival. Therefore, it is advisable to overcome these limitations with molecular techniques such as genetic engineering and recombinant DNA technology that improve or enhance the attributes of the potential *Bacillus* strains once obtained.

4. Another alternative and sustainable approach which contributes towards the economic use of enzymes and to enhance operational activity and continuous usage of enzymes is immobilization, which involves physical adsorption (an enzyme can be attached to an inert, organic or inorganic or insoluble support), entrapment, covalent bonding and cross-linking as some of the most used methods to facilitate enzyme immobilization. This technique should be employed to ensure specificity and stability of the biocatalysts for continuous applications on a large scale under a wide range of adverse conditions, such as pH and temperature. Additionally, with the advent of frontiers, it may also be worthy to explore the use of nanomaterials to immobilize and stabilize amylases.

10.7 Concluding Remarks

When considering all the research that has been done so far, it can be concluded that the starch industry is on a continuous rise due to its high demand in different industries from food, to detergent, to textile industries, which in turn implies the increased demand for amylolytic enzymes. Amylases continue to prove their importance in the industrial and environmental sectors and for medicinal purposes. To this effect, efforts are being made to develop amylases with an extreme range of pH, temperature and better catalytic efficiency to meet the industrial requirements. As far as the literature reviewed is concerned, biomolecules of *Bacillus* spp. largely fulfil such requirements, further affirming the central role of the genus as potential microbial cell factories. The utilization of the technique progression can also contribute in many significant ways towards the improvement and enhancement of amylase enzymes for a constant supply to the high demand and to meet future challenges. Despite the potential of ruminal microorganisms as a source of industrial enzymes, knowledge about these microbial diversity remains relatively unknown.

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Chapter 11
Bacilli and Sustainable *Jhum* Agrobiotechnology

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Abstract *Bacillus,* the name that rekindles a lot of inquisitiveness in agricultural research, has been known for its multifaceted impacts on microbial processes. Mostly soil-dwelling, the genera of *Bacillus* has been studied for various biotechnological usage in the environment and industry. This group of eubacteria undergo various external environmental influences which helped them develop adaptive mechanisms at the genetic level related to survival strategies, making them tough performers in biotechnological applications.

In the northeastern region of India, *Jhumming,* a primitive form of agriculture, is still in practice where the natural environmental set-up is taken under the control of man, employing several tools including different stressors, fire being the main. The *jhum* fields in their coordinated cropping and fallow cycles involving slash and burn undergo a net change in nutrients along with other important components which results in a competitive environment for the soil micro-dwellers. Eco-restoration and making the entire *jhum* system sustainable is a key goal of many research strategies. Organic and environmental-friendly farming being the trend of modern-day agriculture, the usage of Bacilli in the rehabilitation of degraded ecosystems look promising to be exploited for eco-restoration goals. The well-documented roles of *Bacillus* spp. in plant protection and plant growth-promoting (PGP) properties along with other beneficial parameters is presented in this chapter, with reference to their usage in *jhum* agroecosystems prevalent in the north-eastern part of India.

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11.1 Introduction

Soil, an excellent culture medium pulsating with life, harbours an enormous population of micro dwellers and actively contributes to the physico-chemical equilibriums of the earth. Microorganisms, the reservoir of resources, represent by far the richest array of molecular and chemical diversity in nature, providing huge space for innovative research useful to man (Riesenfeld et al. 2004). In this dynamic environment, *Bacillus* has been one of the major micro-workers with a long history of importance, that is much thoroughly studied to date, both from an economic point of view and as a source of investigational microbes. Mostly occurring as spores, *Bacillus* was reported by Waksman as 'saprophytic organisms whose natural habitat is the soil' (Vilain et al. 2006).

The major species of *Bacillus* belong to *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. thuringiensis* (Jensen et al. 2003; Priest et al. 2004; Rasko et al. 2005). Although these species of *Bacillus* report high genetic closeness, their phylogenetic and taxonomic relationships are still argued (Helgason et al. 2004). *B. cereus* is one of the widely reported soil *Bacillus* groups (Guinebretière et al. 2008; Liu et al. 2015). This genus is characterised by the intricate cell wall structure, stress-resistant endospores, synthesis of secondary metabolites like peptide antibiotics, various peptide signal molecules, and extracellular enzymes, with the ability to enrich plant growth, suppression of diseases of the rhizosphere, and perform a plethora of beneficial functions important to natural life (Gardener 2004; Beneduzi and Passaglia 2011; Bahadir et al. 2018). While *Bacillus* remains most studied and reported bacteria isolated from soils, our knowledge of the ecology of these *Bacillus* species in soil remains rudimentary and far from complete.

11.1.1 Bacillus *and Its Distribution in Soil*

This genus of aerobic gram-positive spore-formers comprising of almost 200–300 bacterial species are essentially ubiquitous in nature with their habitats ranging from the extreme hot springs (Pandey et al. 2015; Mohammad et al. 2017) to the polar deep sea sediments (Rűger et al. 2000; da Silva et al. 2013) existing in composts like *Bacillus composti* and *Bacillus thermophilus* (Yang et al. 2013) and high pH conditions like *Bacillus firmus* (Pikuta et al. 2007) apart from residing in its regular environments like soil, water, and plant surfaces (Ichimatsu et al. 2001; Motta et al. 2004). The aerobic spore-forming members of the genera *Bacillus* have been isolated from many soil types depending on depths and altitudes (Wang et al. 2006; Lyngwi et al. 2013), with the population varying to different extents

depending on the soil organic content, with strains being isolated from the extremes of the desert and Arctic or tundra type climatic conditions (Stabb et al. 1994; Garbeva et al. 2003; Matsui et al. 2016; Yadav et al. 2011) (Table 11.1). Culture-based isolation methods from soil samples recorded a CFU (colony forming unit) range from log 3 to log 6 cells with multiple incidences of related species of *B. subtilis* and *B. cereus* (Vargas-Ayala et al. 2000), while the CFU counts characteristically exceeding in the case of rhizospheric soil (Mahaffee and Kloepper 1997; Seldin et al. 1998). Soils with low organic content are normally dominated by *B. subtilis*, *B. licheniformis*, and *B. cereus* but with change in the nutrient input, a wide-ranging population emerge (Ranjard and Richaume 2001; Yang et al. 2014). Microbes are the key components in soil that determine life support functions, but the functional redundancy in the microbiota of most soils has long been thought to engulf microbial diversity-function relationships (van Elsas et al. 2012).

The significant soil-microbe-plant communications are the underlying aspects behind plant health and soil fertility (Glick 2012), and these microbes tend to play a central role in the biogeochemical cycling of both organic and inorganic nutrients in the soil, maintaining its health and quality (Jeffries et al. 2003; Madsen 2011). Soil is considered to be biologically unhealthy with little or no microbial growth in it, which in turn makes it not fertile to support the growth of plants. Several species of *Bacillus* along with the gram-negative *Pseudomonas* fall in the group of potent growth-promoting bacteria (Tilak et al. 2005; Islam et al. 2016). Many species have been studied for their characteristic phosphate solubilisation, phytohormone production, and antagonistic properties with respect to their possible use for plant growth and biocontrol (Lugtenberg and Kamilova 2009; Banerjee et al. 2017). In sight of the ecological specificity, isolation, identification, and characterisation of these native beneficial microbial organisms from diverse environmental habitats has become predominantly essential (Wang et al. 2007). Most farm fields have been found to be inhabited by different *Bacillus* spp., associated with a variety of crop plants playing a significant role in enhancing crop productivity by its direct or indirect mechanisms (Grayston et al. 1998; Bais et al. 2006; Berendsen et al. 2012). The rhizosphere region of soil has been one of the most favourable habitats for a number of *Bacillus* spp., attracting a huge number of microbiologists to study their role in plant growth promotion and biocontrol (Ramani and Patel 2011; Tallapragada and Seshachala 2012). The plant-microbe exchanges in the rhizosphere carry out crucial roles in the transformation, mobilisation, and solubilisation of nutrients from a confined pool in soil that is uptaken by the crop plants. Usage of PGPRs in agriculture is gradually increasing which offers a potential way of replacing the unfavourable chemicals in fertilizers and pesticides (Dey et al. 2004; Herman et al. 2008; Minorsky 2008).

As the genus *Bacillus* sp. exhibits diverse physiological and biochemical properties, such as the ability to produce enzymes like cellulase (Ray et al. 2012; Nair et al. 2018), phytase (Choi et al. 1999), tannase (Mondal et al. 2001), chitinase (Chang et al. 2003), xylanase (Sharma et al. 2015), protease (Liu et al. 2009), and lipase (Ghosh et al. 2017) as well as certain bacteriocins and peptide and lipopeptide antibiotics (Stein 2005; Abriouel et al. 2011), they are at the growing stage for bioprospecting.

Table 11.1 Recent reports on *Bacillus* from soil and unusual terrains with its physiological features

Species of *Bacillus*	Sampling site	Sampled source	Features	References
Bacillus subtilis	Assam, India	Petroleum contaminated soil	Capable of degrading petroleum	Das and Mukherjee (2007)
Geobacillus thermodenitrificans.	Northern China	Soil from depths of Subterranean oil reservoir	Isolate capable of degrading long-chain n-alkanes	Wang et al. (2006)
Geobacillus stearothermophilus, Geobacillus stearothermophilus, Bacillus oceanisediminis, Bacillus pichinotyi, Bacillus alcaliinulinus, Bacillus firmus, Bacillus sporothermodurans, Geobacillus subterraneus and *Bacillus licheniformis*	Panarea Island, Italy	Hydrothermal vents		Gugliandolo et al. (2012)
Bacillus, Oceanobacillus, Paenibacillus	Eastern South Atlantic Ocean	Deep sea sediment soil		da Silva et al. (2013)
Bacillus ectoiniformans	South China Sea	Deep sea sediment soil	Halotolerant	Zhu et al. (2016)
Cohnella kolymensis sp. nov.	Siberian permafrost	Deep sea sediment soil	Novel *Bacillus*	Kudryashova et al. (2018)
Paenibacillus borealis, P. donghaensis, P. macquariensis subsp. *macquariensis* and *B. circulans*	Arctic tundra	Arctic soil	could grow at 10°C- 20°C	Kim et al. (2013)
Bacillus licheniformis B. altitudinis, B. cereus, B. halmapalus, B. thuringiensis, B. isronensis, B. pseudomycoides, Lysinibacillus xylanilyticus, L. fusiformis, L. sphaericus Paenibacillus taichungensis, P. nanensis, P. amylolyticus, P. alkaliterrae and *P. pabuli*	Domiasiat, Meghalaya	Uranium ore deposited soil	Uranium Metal tolerant bacteria	Kumar et al. (2013)
Bacillus sp.	Tarbalo hot springs, Odisha, India	Muddy soil of hot spring		Panda et al. (2013)

(continued)

Table 11.1 (continued)

Species of *Bacillus*	Sampling site	Sampled source	Features	References
B. licheniformis, B. sonorensis and *B. tequilensis, Paenibacillus ehimensis,*	Ringigad hot springs, Uttarakhand, India	Soil from hot spring	(80 °C)	Pandey et al. (2015)
Bacillus sp.	Ganeshpuri, Maharashtra, India.	Soil from hot spring	Extracellular enzymatic activities	Lele and Deshmukh, (2016)
Bacillus licheniformis	5 thermal hot springs in Jordan (Hammamat Ma'in, Zara Dead Sea, Hammamat Afra, Al-Burbita, and Al-Hemma)	Thermal hot springs		Mohammad et al. (2017)
Bacillus zhangzhouensis sp. nov. and *Bacillus australimaris* sp. nov.	South China sea, China	Soil from surface sediment		Liu et al. (2016)
Bacillus thuringiensis, B. marisflavi, B. aryabhattai, B. psychrosaccharolyticus, B. weihenstephanensis, L. xylanilyticus, L. parviboronicapiens, Viridibacillus arenosi, V. arvi, P. taichungiensis and *P. tylopili*	Meghalaya, India	Pristine soils of sacred groves	Uninhabited soil source potential for bioprospection	Lyngwi et al. (2016)
Bacillus clausii, B. licheniformis, B. megaterium, B. subtilis, and *B. thuringiensis*	Itanagar, Arunachal Pradesh, India	'Fired Plots' Under Shifting Cultivation in Lobi of Papumpare	Plant growth promoters and biocontrol agents	Pandey et al. (2011)
Bacillus thuringiensis (Bt) strains	Mizoram, India	*Jhum* agroforestry fallow soil samples	Novel Bt entomopathogens may help in regulating mosquito vectors	Zothansanga et al. (2016)
Bacillus cereus and *B methylotrophicus*	Northeast India	Fallow soil samples of shifting cultivation fields	Plant growth promotion enhancing rice	Banerjee et al. (2017)

11.2 *Bacillus* as a Potential PGPR for Agriculture: Roles and Reports

Soil *Bacillus* plays a significant role in improving soil health in both cultivated and natural environments. Bacterial populations colonizing the root region in the rhizosphere improving and sustaining plant growth are designated as plant growth-promoting rhizobacteria (PGPR) (Prashar et al. 2014). The rhizo-microbiome is a diverse community of microorganisms, (Hinsinger et al. 2009; Chaparro et al. 2013) influenced by the plant root exudates released in the rhizosphere, which act as chemical signals (Venturi and Keel 2016), allowing microorganisms to thrive and flourish (Kaul et al. 2018). *Bacillus* and its relative genera are important performers in the most studied PGPRs (Kumar et al. 2011; Sivasakthi et al. 2014). Phosphate solubilisation, auxin synthesis, siderophore production, synthesis of antagonistic substances, and suppression of fungal pathogens are some of the important traits of *Bacillus* (Mehta et al. 2010; Kumar et al. 2011; Goswami et al. 2014;). They exhibit an extensive interaction with plant roots (Sharma et al. 2014) delivering the inaccessible nutrients available to the plants affecting plant growth and also reducing both biotic and abiotic stresses (Grover et al. 2011; Shameer and Prasad 2018).

11.2.1 Bacillus*, a PGPR*

The PGP role of *Bacillus* has been studied to positively stimulate plant growth by two distinguished behaviours: directly or indirectly, forming long-lasting, stress-tolerant spores, secreting metabolites that stimulate plant growth, and aid in crop productivity (Radhakrishnan et al. 2017). The soil nutrients, utilised by the saprophytic *Bacillus* and converted to molecules directly utilized by the plants, help to alleviate stress and entail the direct mode of plant growth promotion (Goswami et al. 2016). Solubilisation of inorganic phosphorus and mineralisation of organic phosphates, increasing phosphorus availability to plants (Zaidi et al. 2009); production of phytohormones, such as IAA, GAA (Idris et al. 2007), fixation of atmospheric N_2 (Hernandez et al. 2009); reduction of root membrane potential (Choudhary and Johri 2009); and synthesis of ACC-deaminase, which modulates the level of plant hormone (Penrose and Glick 2003), are a few of the direct roles exerted by *Bacillus* and other PGPRs. The indirect mode of the action takes place when bacteria reduce or prevent some toxic effect of a phytopathogenic organism by synthesizing antibiotics (Cazorla et al. 2007; Kumar et al. 2011), produce induced systemic resistance against the number of plant diseases (Tripathi et al. 2012; Pingping et al. 2017; Wang et al. 2018), and by siderophores (Gray and Smith 2005; Hashem et al. 2019). Application of these identified PGPR *Bacillus* possessing exclusive features, diversity, and relationship to plants could be used to improve various biotic and abiotic stress tolerance encountered by plants. These bacilli could be deployed in agricultural production systems as environmentally-friendly

management tools to advance sustainable forms of agriculture (Grover et al. 2011; Vejan et al. 2016).

11.2.1.1 *Bacillus* as a P Solubiliser

Most soils have an insufficient amount of total available phosphorus required for a significant increase in plant growth and also have limited numbers of phosphate solubilising organisms in soil which fail to compete with the existing bacterial population thriving in the rhizosphere. Hence, inoculating the phosphate solubilizing bacteria (PSB) in the soil at a higher concentration than ordinarily found is essential for eliciting plant growth promotion. A number of studies reported bacterial plant growth promotion by solubilising in organic and/or organic phosphorus after being inoculated in soil or plant seeds (Chaiharn and Lumyong 2011; Qureshi et al. 2012). Synthesis of plant favourable metabolites such as phytohormones, antibiotics, or siderophores (Kloepper et al. 1989), among the many roles of PGPRS, the specific contribution of phosphate solubilizing PGPRs in plant stimulation is still under research (Glick 2012). The fundamental contribution of PSBs to plant nutrition suggests that phosphorus uptake by plants is a limited step in plant nourishment (Rengel and Marschner 2005), hence, highlighting the profound contribution of PSBs to plant nutrition and, consequently, in enhancing plant growth (Beneduzi et al. 2012). Considering phosphorus availability as a limiting step in plant nutrition (Rengel and Marschner 2005), suggests a fundamental contribution of PSBs to plant nutrition and consequently, in enhancing plant growth (Beneduzi et al. 2012). Phosphate solubilising bacteria is always under some environmental stress with reports on *Bacillus* sp. isolates present in tomato rhizosphere able to solubilise higher amounts of $AlPO_4$ and $FePO_4$ (Banerjee et al. 2010). *Bacillus* has been found in the *Zea mays* (Szilagyi-Zecchin et al. 2014), rice (Chaiharn and Lumyong 2009), wheat (Ahmad et al. 2008), soybean (Wahyudi et al. 2011), mustard (Kang et al. 2014), aubergine, and chilli (Pena-Yam et al. 2016) rhizosphere enhancing the respective plant growth. Tricalcium phosphate solubilizing *B. cereus* and *B. methylotrophicus* from acidic soil environments of a shifting cultivated field have shown to enhance rice growth *in vitro* (Banerjee et al. 2017).

Bacillus brings about the phosphate solubilisation by the production of various organic acids which in one study showed to improve with an increase in the presence of acids like malic acid or quinic acid resulting in increased chlorophyll, sucrose, glucose, fructose, and amino acids in the treated plant (Kang et al. 2014). Mineral phosphate solubilisation is well documented in many gram-negative bacteria (*Pseudomonas* sp. *Acinetobacter* sp.) with active participation in direct oxidation pathways (Sashidhar and Podile 2010) which is lacking in the case of the gram-positive bacilli, but enzymes like phytase and phosphatase have been reported in many plant growth-promoting *Bacillus* carrying out phosphate solubilisation (Yip et al. 2003; Matos et al. 2017). Detailed and systematic studies on the mode of action of phosphate solubilisation in the gram-positive *Bacillus* to better understand the biological process and optimisation of the nutrient cycling is way scarce, and the

genetic studies to better improve the trait is required for developing the strains as effective biological tools for increasing crop productivity.

11.2.1.2 Role in IAA

Phyto stimulation by most *Bacillus* PGPRs occurs due to the biochemical synthesis of bacterial growth regulators such as indole-3-acetic acid (IAA) (Idris et al. 2007), gibberellins (Bottini et al. 2004), and cytokinins (Bloemberg and Lugtenberg 2001; Karadeniz et al. 2006). Different species of *Bacillus* have been tested to produce IAA *in vitro* but reports on the gram-positive soil-living bacteria are only a few. IAA production by *B. amyloliquefaciens* utilising tryptophan dependant pathways was confirmed by Idris et al. (2007) and *B. pumilus* and *B. licheniformis* produced high amounts of gibberellins in the alder rhizosphere (Gutiérrez-Mañero et al. 2001). The different phytohormones required and produced by plants to control their metabolism and growth in very minute concentrations are also secreted by the PGPRs as metabolic end products in various biochemical pathways (Glick 2010; Müller and Munné-Bosch 2011). Tryptophan is the major precursor molecule for the biosynthesis of IAA in bacteria which helps in root development, tissue differentiation, lateral root development, root anchoring, and root hair positioning (Lewis et al. 2013). These secreted molecules act as mechanical signals, transducing information among bacteria, thus, synchronizing their activities in the rhizosphere (Spaepen et al. 2007). *B. megaterium* and *B. cereus* along with other gram-negative microorganisms was reported to produce more than one phytohormone (auxin, gibberellin, cytokinin and abscisic acid) simultaneously (Karadeniz et al. 2006); however, this can occur as the production of one hormone and degradation of the other, which may act as precursor in the chain of molecular pathways (Boiero et al. 2007; Leveau and Lindow 2005). Multifaceted *Bacillus* sp. PGPRs like *B. methylotrophicus* have been shown to increase seed germination, shoot, and root elongation that are potent IAA producers (Nain et al. 2012; Banerjee et al. 2017).

11.2.1.3 *Bacillus* in the Indirect Mechanism of Plant Growth Promotion

Several literatures target mostly a single trait in the indirect mechanism of action by the plant growth promoters which mainly include the biocontrol ability by siderophore production, ammonia production, secretion of hydrolytic enzymes, antibiosis, induced systemic resistance (ISR) etc. (Choudhary and Johri 2009; Kumar et al. 2011). The indirect ways of bringing about plant growth by bacteria is a composite action of the many interlinked biochemical pathways carried out by the cells that result in stress reduction or provide local and systemic host resistance. *Bacillus* is reported in numerous studies as a biocontrol agent along with *Trichoderma* and *Pseudomonas* (McSpadden Gardener and Driks 2004). The biocontrol ability of *Bacillus* is mostly attributed to antibiosis, quorum quenching, siderophore production, and ISR (Ryu et al. 2004) and this has been well portrayed for *Bacillus* in the

review by Kumar et al. (2011). Results of extensive research and field testing of different species of *Bacillus* biocontrol agents made it widely available as formulations or immobilised cakes for *in situ* usage, thus making *Bacillus* a promising candidate as a biocontrol agent in agricultural fields (Borriss 2011; Pérez-García et al. 2011; Yánez-Mendizábal et al. 2012).

11.2.2 Bacillus *in Agricultural Use*

Bacillus, as described in the above sections, has been identified as a species with abundant benefits and very few drawbacks, finds ample use in agriculture mainly due to the many growth-promoting properties, and disease resistance in plants. The most researched *Bacillus thuringiensis*, a proven biopesticide, whose formulations of spores and crystals have been conventionally used for integrated insect and pest management in several agricultural and horticultural crops (George and Crickmore 2012). The setback in the use of the same has led to further research towards improving their efficacy with nanoscience in crop protection, opening new boundaries in the field of nanobiotechnology (Mahadeva Swamy and Asokan 2013; Vimala Devi et al. 2019). Many studies have targeted to enhance the growth of various plants using a single strain of *Bacillus* or in consortia. In one study, 12 strains of *Bacillus* from wheat rhizospheres were evaluated for their effects on the growth of staple crops like corn, soybean, and wheat. *Bacillus megaterium*, *B. safensis*, *B. simplex*, and *Paenibacillus graminis* isolates simultaneously increased the growth in pot experiments but no single PGPR trait could be significantly predicted for growth promotion efficacy (Akinrinlola et al. 2018). In field studies, with an isolated strain of *B. methylotrophicus,* increased plant growth and height was observed which showed potential commercial application as a biofertilizer or biocontrol agent (Ge et al. 2016). Trials using consortia of *Bacillus cereus*, *Bacillus subtilis*, and *Serratia* sp. application significantly reduced the incidence of *Phytophthora* blight disease, improving fruit quality and soil properties in sweet pepper as compared to the control. Interestingly, soil microflora transformed with the dominance of species like *Comamonas, Burkholderia* and *Ramlibacter*, which are negatively associated with disease severity (Guo et al. 2019). Thus, the consortium of *Bacillus* treatment-controlled soil-borne disease and improved the soil chemical properties (Guo et al. 2019). A very recent study reported a consortium of indigenous *Bacillus* sp., consisting of 2–3 well-suited species, which were able to increase field emergence in chilli seeds compared to the controls. Increased growth of chilli both in the nursery stage and after planting was noted while simultaneous controlling of wilting was found to be effective (Yanti et al. 2020).

Due to its multipurpose use and ubiquitous nature, research in *Bacillus* has received ardent interest from the scientific community with more work focussing on its advancement and application. Govindasamy et al. (2010) highlighted a considerable amount of work on *Bacillus* and *Paenibacillus spp.* and their potential PGPR for sustainable agriculture. A review on the secondary metabolites of the soil

Bacillus of agricultural and industrial importance, mostly of *B. thuringiensis*, help in describing the role of *Bacillus* spp. in the soil ecosystem (Sansinenea and Ortiz Sansinenea and Ortiz 2011). Plant-associated *Bacillus* strains used as biocontrol agents and biofertilizers in agriculture was a cumbersome review work published by Borriss (2011). This article aims to disseminate the environmental views and the importance of the PGPR *Bacillus* species with emphasis and relevance to its role in shifting cultivated (*Jhum*) fields of north-eastern India and its possible implications in constructing a sustainable *jhum* agrobiotechnology. Some published and unpublished work has been summarised in this chapter to highlight the role of *Bacillus* as a PGPR and soil inoculant.

11.3 *Bacillus* Diversity in a *Jhum* System of Farming

Jhum, a primitive shifting cultivation practice predominant in the most biologically diverse northeastern part of India, is a source of livelihood in several parts of the Indian subcontinent concentrated in the states of Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, and Nagaland in the north-east and some belts in Andhra Pradesh, Madhya Pradesh, Kerala, and Orissa (Jha 1997; Freeman 1999). The *jhum* system, as reported by Ramakrishnan and Toky (1981) in the lower altitude of Meghalaya, is characteristic of that practised in several parts of the north-eastern region. The *jhum* cycle varies from 4–5 years to 10–30 years, depending on the length of the fallow period between two successive croppings. The agrarian practices of shifting cultivation and horticulture significantly impact temporal and spatial changes in microbial biomass and several enzyme activities in soil (dehydrogenase and urease). These factors were reported to be greater in the soils of the undisturbed forest ecosystem than in the profoundly affected *jhum* soils (Ralte et al. 2005). Recurrent slashing and burning processes on the hilly slopes accompanied by heavy rainfall result in the loss of soluble organic matter and nutrients in the topsoil. This appears to be responsible for low enzyme activity in the *jhum* fallows (Maithani et al. 1998; Reza et al. 2014). Low input of detrital matter and nutrients through litter-fall in the *jhum* field results in decreased microbial biomass nitrogen and enzyme activity (Saplalrinliana et al. 2016). With increasing age of the plant growth on the *jhum,* fallows result in increased microbial biomass carbon and nitrogen, which may be attributed to greater input of plant detritus that is eventually incorporated into the soil, improving the nutrient pool (Arunachalam et al. 1996; Maithani et al. 1998; Saplalrinliana et al. 2016).

In the past few years, research has been directed towards the PGPR efficiency of *Bacillus*, but only recently, the importance of the application of a consortium of bacteria has been realised. Studies on microbes and microbial diversity with additional emphasis on *Bacillus* sp. from this ancient type of agriculture is rare. Shifting agriculture has its own biological merits and demerits. The uncultivated or fallow stages in *jhum* help in regeneration and enrichment of diverse soil properties along with increased soil microbial biomass and diversity (Pandey et al. 2011). The

survival of the plant growth-promoting microbes under such stressful environmental conditions and their regeneration during the fallow period is essential for the health and yield of the cultivated crops. Depleting nutrients like reduction in organic carbon and nitrogen content (Ramakrishnan and Toky 1981) with a decrease in soil phosphatase activity, and β-glucosidase activity is the characteristic of *jhum* soils observed after burning (Boerner et al. 2000). In context to such degrading soil conditions, it has become immensely vital to study the existence of PGPRs inherent to these soils and their function in improving the different features of soil conditions, which in turn will enhance the plant growth, making the system a sustainable one (Fig. 11.1).

The species of *Bacillus* isolated from the various shifting cultivated fired plots or fallows of north-east India are provided in Table 11.1. In a study conducted by Pandey et al. (2011), bacterial species of *Bacillus* and *Pseudomonas* were recovered from the 'fired plots' soil samples in Arunachal Pradesh which revealed lower amounts of total organic carbon and nitrogen as compared to the fallowed soils. A report on *B. thuringiensis* (Bt) strains characterised by diverse endotoxin genes and crystal proteins, isolated by acetate selection method from *jhum* fallow soil of Mizoram suggested it to be novel species capable of controlling mosquito vectors (Zothansanga et al. Zothansanga et al. 2016). In an investigation for microbial isolates with combined PGP properties from *jhum* fallows, an inadequate number of isolates were obtained but the isolated organisms present were able to enhance plant growth. This work reported two isolates of *Bacillus* sp. out of a number of bacteria and fungi studied. The nearest homologs of bacterial isolates reported were *B. cereus* and *B. methylotrophicus* (Banerjee et al. 2017). In present days, with increased population, the span of fallows has considerably decreased (up to 3 years) which has led to severe problems including reduced soil fertility, crop productivity, and increased soil erosion. In this scenario, plant growth-promoting bacteria can be utilised in the nutrient-depleted soils of the *jhum* fields This expands the need for study in the field of isolation of varied *Bacillus* species concentrated in *jhum* fallows for their unique properties to be exploited in bioprospection and sustainable agriculture.

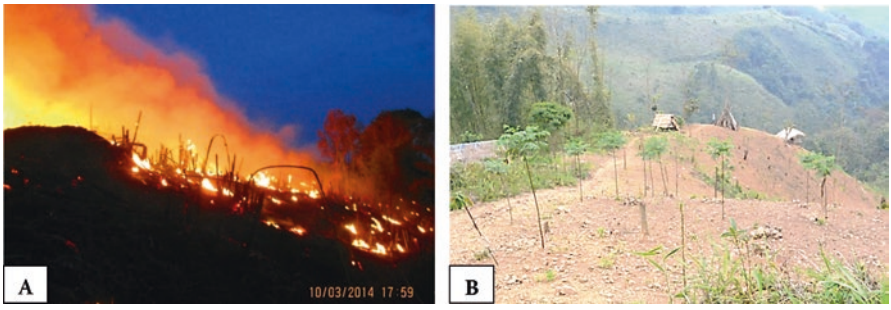

Fig. 11.1 (**a**) Fired plot in a *Jhum* field in Mizoram; (**b**) fallow land in Nagaland

11.4 *Bacillus* and Their PGP Role in *Jhum* Agroecosystem

The use of beneficial microbes is a rising trend in the field of agriculture and is required for maintaining its sustainability. Uneven distribution of microbial inhabitants has been demonstrated in soil with reports suggesting that shifting cultivated fields depict a significant diminution in the microbial population, along with loss of certain species of bacteria and fungi, as compared to that of uncultivated forestlands (Miah et al. 2010). Characterisation of soil microbial communities is difficult due to their vast phenotypic and genotypic diversity. This is applicable to different ecosystems, populations, and even to different micro-individuals (Fierer and Jackson 2006). The soil bacterial community ratio, composition, density, and activity, with respect to the wide range of organic root exudates, vary with the stage of the aboveground plant vegetation and an improved understanding of facts affecting the ability of bacterial colonisation the associated plant population has to be taken into account. The study of microbes from agricultural soils is not new. However, a study on the effect of the activities of *jhum,* mainly on the soil bacterial community, the differences in the ages of fallow phases and their effect on soil restoration would help to assess the diversity of bacteria associated with these soils. In the different states of north-east India, shifting cultivation is practised in their own indigenous way where the biological diversity of ecosystems is used and preserved by ethnic communities through numerous informal institutions using traditional knowledge. The practice varies in terms of principles, actions, culture and types of crops grown among *jhum* fields of the lower altitudes of Assam, Mizoram, Meghalaya, and Nagaland.

The cultivation-based methods shown in Fig. 11.2 used to study the gram-positive rod-shaped cells (Fig. 11.3) of the aerobic chemoheterotrophic bacteria from different soil samples from fallows of *jhum* revealed the occurrence of *Bacillus* isolates (Figs. 11.2, 11.3, and 11.4), identified and accession numbers were received for the sequences of marker gene from GenBank. Biochemical characters along with the plant growth-promoting properties and molecular identification with 16S rRNA gene sequences of these isolates revealed their phylogenetic relationship with 12 different genera. *Bacillus* and *Actinobacteria* being the most populous (Banerjee et al. unpublished) (Fig. 11.5).

11.5 *Bacillus* Bioinoculants in *Jhum* Fields: *In Vitro* and *In Situ* Applications

Jhum cultivation is based on a two-step cropping system i.e. sequential cropping and intercropping. The rich agro-biodiversity results in a sustainable cropping pattern. The environmental load on using the recalcitrant chemical fertilizers have become intertwined with the food chain with the phenomenon of biomagnification or biological amplification. Cutting down usages of these chemicals replacing with biofertilizers can be considered an important strategy for ecological management

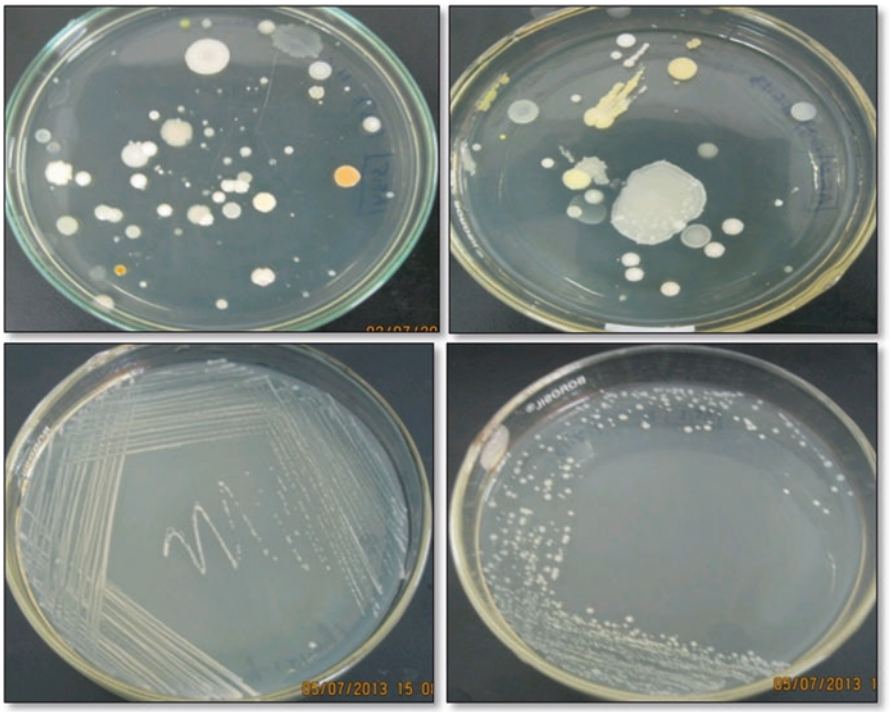

Fig. 11.2 Isolated bacteria from the fallow soil samples by spread plating and a pure culture grown in Agar plates

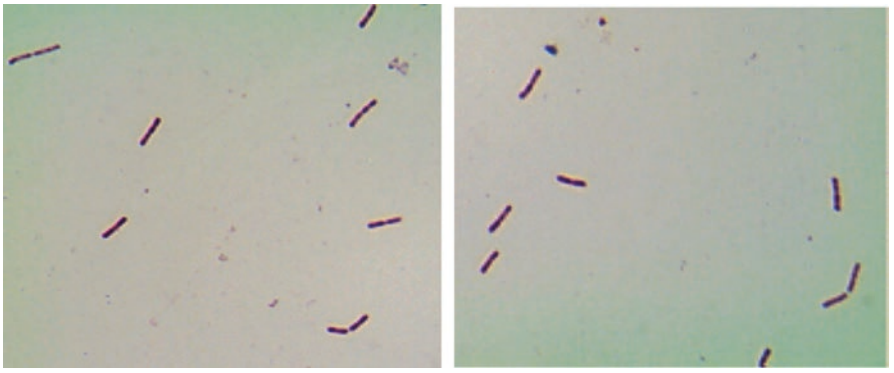

Fig. 11.3 Gram-stained rod-shaped cells of *Bacillus* sp. isolated from the fallow fields of north-east India observed in 100X OIL (Oil Immersion Lens)

and for reducing environmental hazards (Balasubramanian et al. 2004; Hungria et al. 2010, 2013). Bacterial inoculants parameterised by their PGPR traits determine the ability to be used as successful inoculants. The effectiveness and usage of

Fig. 11.4 Biochemical properties of the *Bacillus* isolated from the *jhum* fallows in northeast India. (**a**) Lipase assay; (**b**) Amylase assay; (**c**) Cellulase assay

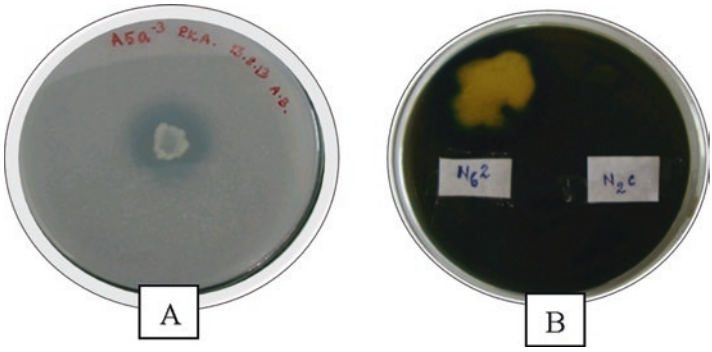

Fig. 11.5 Plant growth-promoting characters of the *Bacillus* isolated from the *jhum* fallows in north-east India. (**a**) Phosphate solubilizing test; (**b**) Siderphore production

these PGPR microbes inoculants rest on the ability of these bacteria to interact in the rhizosphere rich in root exudates and to inhabit and colonise the plant roots (Meneses et al. 2011; Alquéres et al. 2013; Beauregard et al. 2013). The plant-root secretions influence the bacteria-root surface interaction which changes with varying environmental conditions, affecting the potentiality of the inoculants (Cai et al. 2012; Carvalhais et al. 2013). Their role in nutrient cycling and ability to boost up plant growth, make them a dynamic tool in the sustainable form of modern-day agriculture (Rengel and Marschner 2005). Phosphate solubilizing bacteria have been in use as bioinoculants since the 90's and in the Soviet Union 'phosphobacterin' was prepared and commercialised for agricultural applications (Khan et al. 2006). This contained the isolate of *B. megaterium* var. *phosphaticum* which was later introduced in India.

An *in vitro* study in the primitive phase was conducted to isolate and identify the native PGPRs inherent to the shifting cultivated fields of north-eastern India. The traits of phosphate solubilisation, IAA production, along with siderophore, ammonia, and catalase production tests for the isolates were used to determine the PGPR ability out of the 55 bacterial isolates and 32 fungal isolates obtained from the different fallows of *jhum*. Three bacterial isolates reported, showed plant growth

promotion in the local upland variety of rice along with its fungal counterparts. The rice seeds treated with the microbial inoculum exhibited positive plant growth-promoting properties in the plate assay (Fig. 11.6a). Early induced seed germination was observed in the plate assay by the bacterial isolates SB3, SB5, and SB9 than that in the control. Pot experiments with the microbial isolates exhibiting PGP abilities under greenhouse conditions were carried out with a more definitive outcome (Fig. 11.6b). The rice seeds treated with SB3 induced the formation of the longest roots as it was the highest IAA producer while the isolate SB9 induced the highest growth as it was both phosphate solubiliser and a moderate IAA producer. The closest homologs of bacterial isolates were identified as *B. cereus*, *B. methylotrophicus*, and *Curtobacterium oceanosedimentum* (Banerjee et al. 2017).

These were considered as potent bioinoculants which was further tested in field applications in a similar environment of the *jhum* cultivation fields (Fig. 11.7). The same variety of rice was used which was treated with the bacterial inoculum (*Bacillus* sp was used) and sowed intermittently with each isolate treatment divided into plots by permutation and combination. Weekly weeding and watering of the seeds were performed until the onset of the rains. Results showed increased seed germination rate and harvesting index was high. The potent isolates when tested in actual field trials gave satisfactory outcomes in accordance with the pot

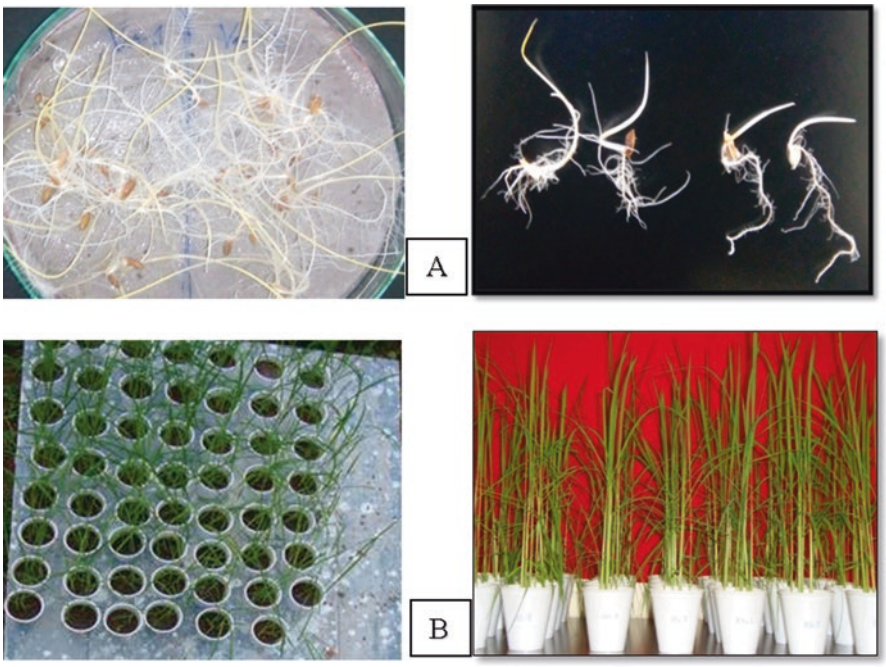

Fig. 11.6 *In vitro* study of the upland variety rice seed germination by microbial isolates from *Jhum* fields. (**a**) plate assay; (**b**) pot experiments under greenhouse conditions

Fig. 11.7 *In situ* application of the microbial isolates under field conditions showing germination of rice and full-grown rice plant

Fig. 11.8 Field sites of *Jhum* in Nagaland (**a**) and Meghalaya(**b**)

experiments. There was significant variation in rice growth from the treated to that of control (unpublished) (Fig. 11.8).

11.6 Conclusion

In order to achieve efficient PGPR strains using *Bacillus* and its consortia, with utmost growth-promoting association between the microbe and the plant root, it is important to identify the various factors affecting the interaction and also the presence of other microbes (Bent et al. 2001). The possible way to find efficient strains is to carry out a diversity study on the inherent soil microbes that are native to the particular soil conditions and can perform amidst the stresses present in the soil environment. In bioinoculant production, formulating a competent microbial strain depending on its performance is crucial in determining the achievement of the biological agent (Bashan 1998). Often an isolate with optimised *in vitro* reports deliberately fail in field trials. Thus, research is still in its budding stage to provide an optimal efficient performing bioinoculant PGPR.

Jhum has been a different situation with its complicated system of cropping intricated to the cultural practices native to the various ethnic human populations of north-eastern India which still perform this indigenous type of farming. The use of modern-day agricultural practices is not prominent in *jhum* type of farming. Hence, the application of native PGPs for plant growth and yield enhancement is a way of supporting the eco-restoration and maintaining the sustainability of an already degraded *jhum* ecosystem. The study carried out with the *Bacillus* sp. isolates obtained from *jhum* fields was the first report of its kind showcasing phosphate solubilizing and IAA producing PGPRs from the natural *jhum* conditions which possibly will aid in plant growth promotion in the nutrient-depleted soils of the *jhum* fields. This will provide a better understanding of the soil microbiota while enabling the *jhum* farmers to become technologically enriched with emphasis on the use of the biological tools to optimise nutrient cycling and crop productivity and simultaneously, in eco-restoration of the *jhum* fallows.

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Chapter 12
Bacillus Species of Ruminant Origin as a Major Potential Sources of Diverse Lipolytic Enzymes for Industrial and Therapeutic Applications

Grace Mujinga Mukendi, Alfred Mitema, Karen Nelson, and Naser Aliye Feto

Abstract Lipases are biocatalysts that catalyze a wide range of reactions such as the hydrolysis of triglycerides or lipids and esterification of fatty acids in a nonaqueous medium. This versatility renders lipases to be potential biocatalysts for the food, detergent, paper and pulp, leather and textile industries, biodiesel production, and therapeutic applications. Lipases are naturally sourced from either plants, animals, or microorganisms. Microbial lipases are quite stable, selective, substrate-specific, and, thus, classified as one of the most extensively used industrial enzymes. The rumen is a four-chamber stomach of the ruminant animal representing the fermentation-vat where microorganisms, *Bacillus* species, work in a symbiotic relationship with the host to digest complex ingested feed. The ruminant microbiome is a relatively less exploited yet potentially rich source of biocatalysts of industrial, environmental, and therapeutic importance. However, no or minimal comprehensive review has been reported on biocatalysts of ruminant origin. Therefore, this review presents an in-depth analysis of biocatalysts of rumen microbiome with particular emphasis on *Bacillus* spp. of ruminant origin as an untapped source of diverse lipase-isoforms with potential industrial and therapeutic applications.

Keywords *Bacillus* species · Biocatalyst · Lipase · Microbiome · Rumen · Ruminant · Therapeutics

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12.1 Introduction

The exponential growth of the global population in the last centuries saw an increase in demand and consumption of daily life products: food, potable water, chemicals, cosmetics, medicine, transportation, and pharmaceutical (Sarmah et al. 2018). The industrial world has been revolutionized to meet the demand for daily life products, and it played a significant role in the improvement of the economy. The production of such consumables was deemed to be highly dependent on raw materials and require too much energy, thus, generating a considerable amount of waste which substantially affects the environment (Sarmah et al. 2018). To save the environment from industrial pollution and effects of climate change, death of aquatic and vegetable life, ozone layer destruction, and fossil depletion, industries globally have drifted their focus towards the development of technologies of less environmental effect and that would continue to meet the increasing demand for products while using fewer resources (Sarmah et al. 2018).

Enzyme catalysis or enzymatic processing among biotechnologies are promising alternatives for greener, cleaner, safer, and sustainable in comparison to the traditional or conventional processes (Sarmah et al. 2018). Enzymes or biocatalysts are proteins that are produced by living organisms for the synthesis of critical sustainable biomolecules and the digestion of foods. They accelerate the rate of various biochemical reactions that can also be mimicked in various industries (Gurung et al. 2013).

Enzymes possess significant advantages over harsh chemicals used in conventional processing due to their specificity, reusability, and biodegradability (Andualema and Gessesse 2012). Enzymes are highly specific and target some particular bonds to produce the necessary products. Thus, the enzymatic processes eliminate the generation of unwanted products and are reputed to withstand competitive conditions. Additionally, enzymes do not leave any ecological footprints because they are readily degraded by microscopic life. The resulting decomposed materials are always recycled back to nature (Leow et al. 2007).

The diverse functions that enzymes perform on different substrates during chemical reactions greatly influence their classification. They have been classified into six groups; oxidoreductase (EC 1), transferase (EC 2), hydrolase (EC 3) currently being the most used in biotechnology, lyase (EC 4), isomerase (EC 5), and ligase (EC 6), per the Enzyme Commission (Fig. 12.1). Hydrolase is comprised of carbohydrase, proteases, and Lipolytic enzymes which represent more than 70% of the enzyme market sales (Haki and Rakshit 2003).

12.2 Rumen Microbiota as a *Bacillus* Habitat

The genus *Bacillus* is composed of many species that are gram-positive, rod-shaped, motile, aerobic and facultative anaerobic, and gram-variable endospore-forming bacteria (Guncheva and Zhiryakova 2011). *Bacillus* genera and other

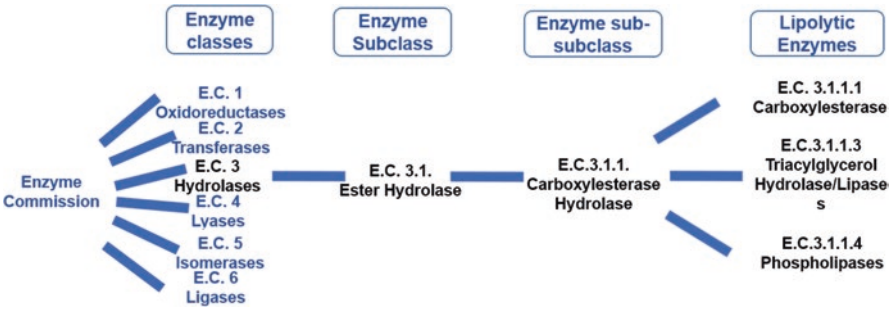

Fig. 12.1 A representation of the numerical classification of lipolytic enzymes by the Enzyme Commission (EC) (Casas-Godoy et al. 2012; Robinson 2015). The classification is based on the chemical reactions that enzymes catalyze. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme (Robinson 2015)

bacteria-producing lipases inhabit their nature from the temperature ecological environment to the extreme environment such as the soil, hot springs, marine sediments, volcanic water, industrial effluents, human gut, and the rumen (Guncheva and Zhiryakova 2011).

The rumen is a four-chambered stomach of the ruminants [the rumen, reticulum, omasum, and abomasum), herbivorous animals digesting their feeds through rumination. The rumination process starts by consuming or engulfing the raw material, regurgitating it into a semi-digested form known as cud, and by chewing the resulting cud (Oyeleke and Okusanmi 2008).

The rumen, which also qualifies as the unique digestive vessel, is inhabited by a group of microbes, namely protozoa, archaea, fungi, and bacteria, which work in a symbiotic relationship (Choudhury et al. 2015). Bacteria consist of 50-75% of the rumen microbiome (Choudhury et al. 2015). Ishaq et al. (2015) isolated 31 bacteria from the rumen of North American Moose (*Alces alces*) whereby, 26 were identified as *Bacillus* spp. thus, confirmed the genus *Bacillus* as one of the predominant bacterial genera inside the rumen. Similarly, Oyeleke and Okusanmi (Oyeleke and Okusanmi 2008) isolated microorganisms from the rumen of the cow, sheep, and goat and discovered that *Bacillus* spp. represented 37.8% of the bacterial population of the rumen microbiome.

The rumen is a harsh environment that is prevailed by a strict anaerobic condition, a physiological 5.5-6.0 pH range, and a temperature of about 40 °C (Hungate 1966; Miyagi et al. 1995). *Bacillus* genus survives in such a complex environment, and their biocatalysts, especially lipases, are believed to demonstrate exceptional qualities. The rumen microbiome plays a pivotal role in the digestion processes by synthesizing enzymes that are applied in the breaking down of complex ingested feeds into simpler compounds (Hungate 1966; Miyagi et al. 1995). Thus, the rumen, a very complex microbiota, can be explored for its microbiome sources of enzymes like lipases with potential in biotechnology, research, and industrial applications (Hess et al. 2011).

Lipolytic enzymes notably lipase have attracted the attention of the biotechnology world because of their excellent features (Javed et al. 2018). The subsequent section describes in detail different Lipolytic enzymes, their common and divergent features such as the lipase isoforms, the three-dimensional structure, their mechanism of action, different sources, mining methods of novel lipases, stabilization, and their potential in industrial and therapeutic applications.

12.3 Lipolytic Enzymes

Lipolytic enzymes are the third large subclass of the hydrolases after carbohydrases and proteases being the first and second respectively (Gurung et al. 2013). They both make up to 70% of the enzyme sales (Gurung et al. 2013; Ouyang et al. 2013). Lipolytic enzymes are further characterized by their chemo-, stereo-, enantio- and regio-specificity characteristics that render them extensively useful in different industries, like food, detergent, paper and pulp, leather, and textile industries (Haki and Rakshit 2003), biodiesel production, and therapeutic applications (Sarmah et al. 2018).

Lipolytic enzymes are comprised of esterases also called carboxylesterase (EC.3.1.1.), phospholipases [E.C. 3.1.1.4), and "true" lipases also called triacylglycerol hydrolases (EC.3.1.1.3) (Fig. 12.1) (Bornscheuer 2002; Casas-Godoy et al. 2012; Fan et al. 2012; Hudlicky and Reed 2009). Although the three listed lipolytic enzymes share the same functionality, mechanism of action, and α/β hydrolase canonical fold patterns, they differ in the three-dimensional structure or amino acid sequences of the active site or the substrate preference (Bornscheuer 2002; Casas-Godoy et al. 2012; Fan et al. 2012; Hudlicky and Reed 2009).

12.3.1 Esterases

Esterases, as phospholipases and lipases, catalyze the hydrolysis reactions (Bornscheuer 2002; López-López et al. 2014). They mainly fasten the hydrolysis of water-soluble esters which are usually made up of a short acyl chain, precisely less than or equivalent to twelve carbons. Esterases do not only catalyze the hydrolysis of esters, but they also catalyze a plethora of reactions under micro-aqueous conditions to form ester bonds. Such reactions are mainly called esterification, alcoholysis, and transesterification (Bornscheuer 2002; López-López et al. 2014).

Being a superfamily of the α/β hydrolase enzyme, esterases share a similar 3D structure with the enzymes of the α/β hydrolase family (Bornscheuer 2002). The structure is mainly determined by the presence of eight stranded β-sheets that are parallel and antiparallel at the center of the enzyme molecules. The stranded β-sheets are connected to the surrounded helices and connecting loops (Bornscheuer 2002). The β-sheets and α-helices are the support of the active site which is made up of

signatures of three amino acids residues namely serine, histidine, and aspartic acid or glutamic residue (Bornscheuer 2002). Serine residue is mainly located in the center of the pentapeptide motif (G-X-S-X-G-) with X representing any residue, and the nucleophile elbow is located on the pentapeptide motif between α-helix and β-sheet (Bornscheuer 2002). Unlike other Lipolytic enzymes, the active site of the esterases is always opened, and the binding pocket for the acid moiety of the substrate is remarkably large. Therefore, esterases have broad substrate profiles because they are specific for a broad range of substrates (Bornscheuer 2002).

Esterases act similarly to the enzymes of α/β hydrolase during hydrolysis. In order to release fatty acid and alcohol from an ester in the presence of water, the hydroxyl (-OH) group of the serine residue from the catalytic triad attacks the carbonyl carbon of the ester bond to be broken down. Upon attacks, an alcohol metabolite and an acylated enzyme (tetrahedral intermediate) are released (Bornscheuer 2002). The acylated enzyme is formed by the formation of a covalent bond between the enzyme and the fatty acid moiety of the substrate. The stabilization step then occurs by first adding hydrogen to the nitrogen atom of histidine residue thereafter, adding a carboxylic group of the aspartic or glutamic acid residue of the oxyanion hole to the histidine. Lastly, the stabilized histidine residue then gets attracted to the water molecules and form a bond with water to release an acid moiety and enzyme at its active state (Bornscheuer 2002). Therefore, esterases obey the Michaelis-Menten classical kinetics because they only catalyze water-soluble substrates. Their broad substrate specificity and versatility features make them useful for industrial applications. However, their limitation only acts on short acyl-chain substrate that reduces their application compared to phospholipases and lipases (Borrelli and Trono 2015; Javed et al. 2018).

12.3.2 Phospholipases

Just like esterases, phospholipases catalyze the hydrolysis of a classical substrate called phospholipids (Borrelli and Trono 2015). Phospholipids are water-insoluble triglycerides having a phosphate group at the third carbon of the glycerol group. Apart from acting on phospholipids, phospholipase can also cleave the ester bonds of the triacylglycerols at the first and second carbon of the glycerol backbone. Contrary to esterases, phospholipases catalyze the hydrolysis of the water-insoluble substrate that is simply esterases having a long acyl chain. Therefore, the reactions catalyzed by phospholipases take place at the water/lipids interphase (Borrelli and Trono 2015).

Phospholipases are not only involved in the hydrolysis of phospholipids or triglycerides (exceptional conditions), but they also catalyze diverse reactions (Borrelli and Trono 2015). Such diverse reactions include acyl transesterification during which free fatty acids are added to lysophospholipids, transphospahtidylation during which the phosphatidyl group from the phospholipid is transferred to the alcohol to give phosphatidyl alcohol under low water activity in the presence of organic

substrates or solvents. Phospholipases are a group of enzymes that are classified into six classes that are Phospholipases A, Phospholipases A1, Phospholipases A2, Phospholipases B, Phospholipases C, and Phospholipases D (Borrelli and Trono 2015). The classification of Phospholipases A and B is entirely based on the carbon position of the glycerol backbone at which the hydrolysis of the ester bond occurred, while the classification of phospholipase C and D entirely depends on the cleavage of glycerophosphate and phosphodiester bonds, respectively (Borrelli and Trono 2015).

Phospholipases have the 3D structure of the α/β hydrolase family i.e., they have the same amino acid signature on their active site. The active site is comprised of the catalytic triad Ser-, Serine the central or nucleophilic residue of the pentapeptide motif (GXSXG), Asp- Aspartic acid the catalytic acid residue, and His- Histidine the catalytic base residue (Borrelli and Trono 2015). Some phospholipases (PLA) have their catalytic acid Aspartic acid replaced with Asn- Asparagine while other phospholipases (PLA2) possess an additional Ca^{+2} binding loop (Borrelli and Trono 2015). Compared to esterases, phospholipases have shorter β5 and β9 sheets and a lid domain to cover the active site when it is in a closed conformation (Fig. 12.2); thus, the two mentioned differences render phospholipases substrate-specific (Borrelli and Trono 2015).

It is made up of two domains (C2 A in orange, indicated with a black arrow, and C2 B in blue, indicated with a red arrow). The two domains are linked to the α/β hydrolase core (in blue sky color, wrapped in the red box). The hydrolase core consists of 10 β sheets represented as blue tiny ribbons. The β sheets are connected to

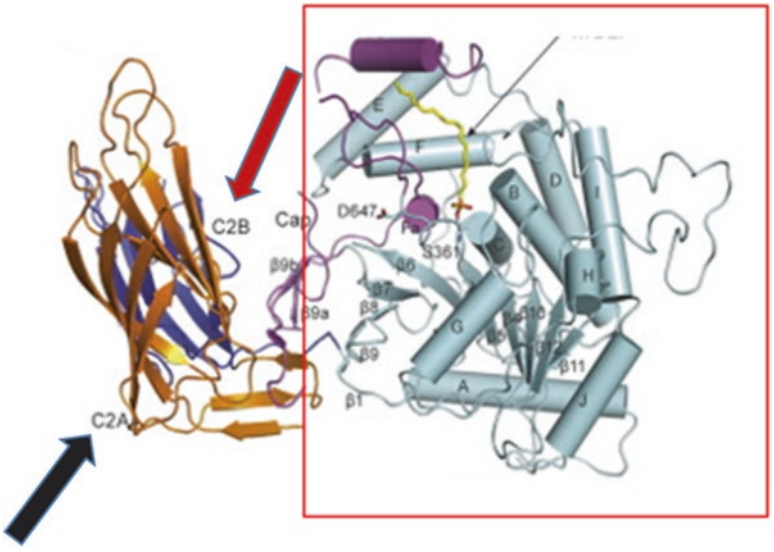

Fig. 12.2 The three-dimensional structure of phospholipase A2 from a human GIVD (cytosolic Group IV D) cytosol (Wang et al. 2016)

the 10 α helices (represented in long cylinders A-J in blue color) in the form of a can (Wang et al. 2016).

The mode of action of phospholipases is like the classical mechanism of action of esterases. However, some phospholipase A2 starts their nucleophilic attack with the Histidine residue playing the role of the nucleophilic residue (Borrelli and Trono 2015). The substrate specificity and versatility make phospholipases to be a great potential for application in various industries and therapeutic domains. Nevertheless, their limitation of reactions to the glycerol carbon position or sites leads to a search for more versatile and broad substrate-specific enzymes such as lipases (Borrelli and Trono 2015).

12.3.3 Lipases

Lipases are serine hydrolases that catalyze the cleavage of ester bonds between the glycerol group and fatty acids moiety to yield free fatty acids or acyl-glycerides and alcohol (Ramnath et al. 2017). Unlike esterases, lipases have an affinity for water-insoluble long-chain acyl triglycerides notably more than twelve carbons. Lipases are always activated by the occurrence of water and lipids interphase for lipids hydrolysis (Ramnath et al. 2017).

12.3.3.1 Lipase-Catalyzed Reaction

Hydrolysis of triglycerides usually takes place under natural conditions in the presence of excess water. In limited water conditions, lipases can catalyze the reverse reaction designated as esterification (Ramnath et al. 2017). Moreover, under deficient water activity, lipases are still capable of catalyzing diverse transesterification reactions such as alcoholysis, acidolysis, aminolysis, and interesterification. Therefore, based on the diverse reactions that lipases catalyzed (Table 12.1), they are always regarded as one of the versatile enzymes found in nature (Borrelli and Trono 2015). As a member of the serine hydrolase family, lipases rely on their serine residue to cleave the ester bonds of the triglyceride substrate as described in the subsequent section.

12.3.3.2 Lipase Structure and Mechanism of Action

12.3.3.2.1 Lipase Structure

As mentioned previously, lipases are serine hydrolases and are generally characterized based on the α/β folding signature. Lipases have a backbone that is made up of eight different strands constituting the central β sheet (Gupta et al. 2015). The eight strands are designated as β_1, β_2, β_3, β_4, β_5, β_6, β_7, and β_8. Studies performed on the three-dimensional structure of lipases from various sources except for the lipases

Table 12.1 List of reactions catalyzed by microbial lipases

Type of reaction	Industrial application	Source of enzyme	References
Hydrolysis	Bioorganic synthesis, Food industry Detergent industry	*Bacillus pumilus*	Casas-Godoy et al. (2012), Chaplin et al. (2004), Laachari et al. (2015), and Litantra et al. (2013)
	Oil and fat industry	*B. thermoleovorans*	Patil et al. (2011)
	Solid waste treatment	*Pseudomonas aeruginosa*	Bose and Keharia (2013)
	Medical and healthcare	*Staphylococcus aureus*	Vaquero et al. (2016)
Esterification	Chemical industry	*Acinetobacter* sp.	Thakur (2012)
	Food processing industry	*B. coagulans*	Thakur (2012)
	Medical and healthcare	*B. licheniformis*	Sharma et al. (2015)
	Flavor industry	*Staphylococcus epidermidis*	Patil et al. (2011)
Transesterification	Biodiesel	*B. subtilis*	Bajaj et al. (2010) and Treichel et al. (2010)
	Jet fuel	*Burkhodelia cepacia*	Bajaj et al. (2010), Salum et al. (2010)
	Light hydrocarbon oil	*Enterobacter aerogenes*	Bajaj et al. (2010)
	Fuel oil	*Pseudomonas cepacia*	Bajaj et al. (2010)
	Incorporation of different acids	*B. stearothermophilus*	Thakur (2012)
	Therapeutic agents	*Candida antarctica*	Hernández-Fernández et al. (2010)
Alcoholysis	Quick-drying oil	*Pseudomonas* sp.	Salis et al. (2009)
	Cosmetics	*Pseudomonas fluorescens*	Patil et al. (2011)
	Biodiesel production	*Pseudomonas* sp.	Salis et al. (2009)
Enantioselective hydrolysis	Medical and healthcare products	*Serratia marcescens*	Su et al. (2014)

from the pancreas revealed the presence of the eight β sheets that are sequentially connected to about six α helices. The helices are named as α1, α2, α3, α4, α5, and α6 (Fig. 12.3b) (Gupta et al. 2015).

The backbone holds the active site of the lipases, and the site is made up of some amino acids that are involved in the hydrolysis mechanism (Gupta et al. 2015). The

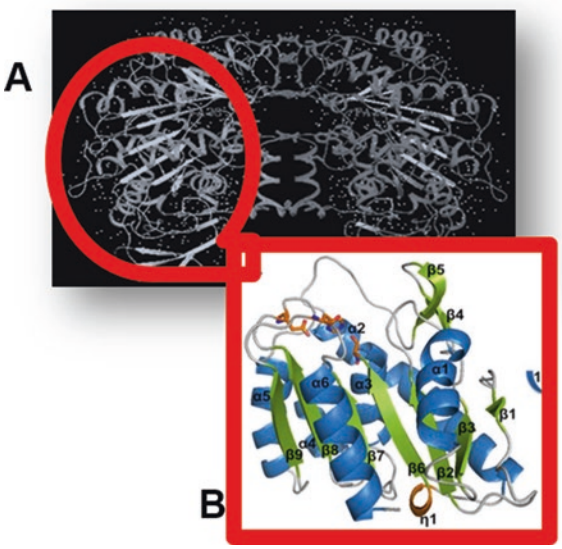

Fig. 12.3 (**a**) The Quaternary structure of lipase; (**b**) The α/β folding signature of the lipase formed of eight different strands (β_1, β_2, β_3, β_4, β_5, β_6, β_7, and β_8) in green that are connected to the six α helices (α1, α2, α3, α4, α5, and α6) in blue

three most essential amino acids called the catalytic triad, of the active site, are like those of esterases and phospholipases (i.e., the serine and nucleophilic residues respectively). The serine residue is the central residue of the pentapeptide motif and the other two amino acids of the active site are the Aspartic or Glutamic acid, catalytic acid, and Histidine residues (Gupta et al. 2015).

12.3.3.2.2 Mechanism of Action

Lipases possess the nucleophilic elbow containing the Serine residue that forms the pentapeptide motif and a lid covering the active site (Jaeger et al. 1999). Lipases are generally known to have a closed conformation in their inactive state i.e., the lid covers the active site when the lipases do not perform their catalytic activity (Ito et al. 1998; Jaeger et al. 1999). During activation, the lid is removed from the active site for ease of serine accessibility to the carbonyl carbon atom of the substrate [either fats or lipids). Triglycerides of long acyl-chain carbon are insoluble in water as mentioned previously, (Jaeger et al. 1999) but during their hydrolysis in the presence of lipases, they tend to dissolve partially in the water. Thus, the observation and formation of a water-oil emulsion activate the lipases by having their lid moved away from the active site leaving it open for catalysis. Therefore, one of the characteristics of the lipases is the activation by the water-lipids interphase (Jaeger et al. 1999; Sharma et al. 2001).

The hydrolysis of triglycerides by lipases has three steps of mechanism (Fig. 12.4).

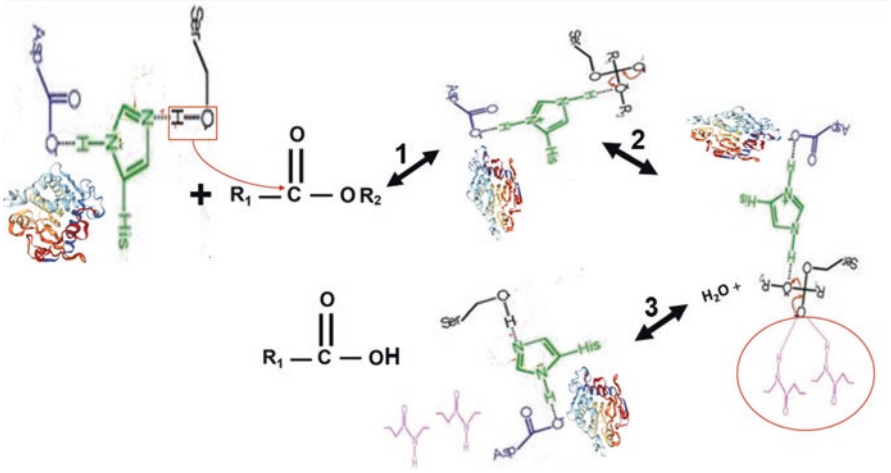

Fig. 12.4 The steps involved in lipid or triglycerides hydrolysis. (1), shows the covalent bonding of hydroxyl group from the serine residue and the carbonyl carbon of the triglyceride to form a tetrahedral intermediate. (2), tetrahedral intermediate stabilization step. (3) the water molecule hydrolysis of the covalent bonds between the enzyme and acyl product and stabilization of the enzyme in a closed conformation and the acyl (diacylglycerol) (Ribeiro et al. 2011)

When the active site of the lipases is opened, the –OH group of the serine residue binds to the carbonyl carbon of the substrate (Fig. 12.4, reaction 1) leading to the formation of tetrahedral intermediate (enzyme-acyl complex). The resultant intermediate is then stabilized by two hydrogen bonding between the negatively charged carbonyl oxygen atoms of the intermediate to nitrogen atoms of the amino acid residues of the oxyanion hole (Fig. 12.4, reaction 2) leading to the release of alcohol as a product (Ribeiro et al. 2011). The water molecule hydrolyzes the covalent bond between the enzyme and acyl product to release the stabilized enzyme in a closed conformation and the acyl can be the diacylglycerol (Fig. 12.4, reaction 3) (Ribeiro et al. 2011).

After hydrolysis, the lipases reclaim the closed conformation by having their helices and strands rearranging themselves in such a way to form a helical segment, the lid, to protect the active site of the lipases93. The active site of the lipases, with its catalytic triad, is also found in the serine proteases, thus, lipases are classified as serine hydrolase.

12.3.3.3 Properties of Lipases

Lipases are known to catalyze a wide range of reactions (Borrelli and Trono 2015; Javed et al. 2018; Thakur 2012). They are said to be versatile, a feature having great potential for industrial applications. The versatility of lipases originates from

various properties that lipases possess (Javed et al. 2018). Lipases are said to be solvent saline, high and low temperature tolerant, protease-resistant, and substrate specificity (Javed et al. 2018).

12.3.3.3.1 Stability in Organic Solvents

Organic solvents mainly used in industries improve the solubility of substrates, reduce microbial contamination, and side reactions. Therefore, the stability of lipases in organic solvents makes lipases a potential for industrial processes. An example of an organism producing such lipases is the *Pseudomonas* genera (Borrelli and Trono 2015; Bose and Keharia 2013; Thakur 2012).

12.3.3.3.2 Tolerance to High and Low Temperatures

Many industrial processes operate at high temperatures (Borrelli and Trono 2015). Such processing requires thermophilic enzymes to avoid loss of activity during the process. Some household chores such as laundry require high temperatures for effective removal of stains and thus, thermophilic enzymes are of great importance (Borrelli and Trono 2015).

12.3.3.3.3 Tolerance to Alkaline and Acidic pHs

Alkalinity condition is one of the primary requirements by the detergent industry (Chauhan et al. 2013); thus, alkaline enzymes attract such sectors. Since washing conditions are harsh, lipases, as one of the main components of the detergents for the removal of stubborn greasy stains, are required to be active and stable in the alkaline condition (Chauhan et al. 2013).

Some other industrial applications take place under acidic conditions (Andualema and Gessesse 2012). Therefore, lipases of great potential for industrial applications should not only be alkaline, but those required for acidic operations should be active at acidic pH (Andualema and Gessesse 2012).

12.3.3.4 Classification of Lipases Based on Substrate and Region-Specificity

Lipases having the same mechanism of action are said to differ among themselves in the order of amino acids or residue sequences making them up (Sarmah et al. 2018). There has been no formal classification of lipases as far as literature is concerned (Javed et al. 2018). However, many classifications (Fig. 12.5) have been made based on different properties such as their sources, substrate specificity (Fig. 12.6), and region-specificity (Sarmah et al. 2018).

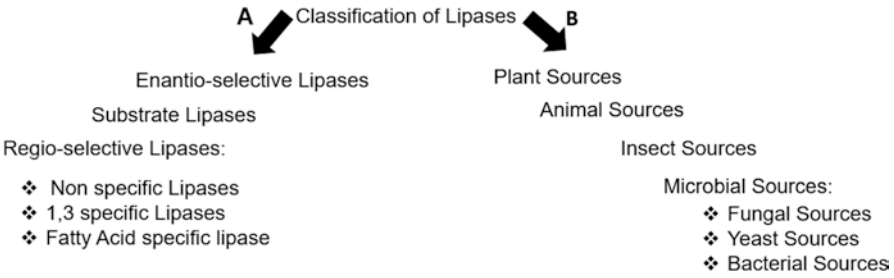

Fig. 12.5 Classification of lipases based on (**a**) properties or specificity and (**b**) their sources

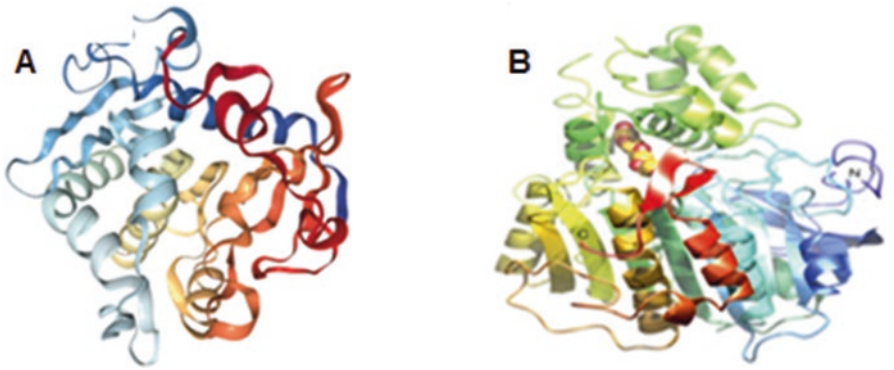

Fig. 12.6 The illustration of different lipase isoforms based on their folding pattern obtained from the X-ray crystallography of lipase from *Rhizomucor miehei* (**a**) from *Candida antartica* (**b**). They are all substrate specific because they target triglycerides with specific fatty acids (Gupta et al. 2015; Gurung et al. 2013)

The first class of lipases includes the nonspecific lipases which catalyze triglycerides into free fatty acids and glycerol. During the reaction, the intermediates that are produced comprise the mono- and diglycerides. Such lipases attack the carbonyl atom irrespective of their position in the molecule (Kapoor and Gupta 2012; Ribeiro et al. 2011). The second class of lipases includes the 1, 3- specific lipases which catalyze triglycerides into monoglycerides and fatty acids (Kapoor and Gupta 2012; Ribeiro et al. 2011). The third class of lipases comprises the fatty-acid specific lipases which catalyze triglycerides based on selective fatty acid. This isoform has an affinity for long-chain acyl group having a double bond between carbon 9 and 10 in cis position.

The ability of lipases to perform multiple types of reactions in heterogeneous media, large-scale substrate specificity stability under extreme conditions, readily available, are the primary factors influencing the choice of lipases isoforms for industrial and therapeutic applications over other chemical catalysts which are not eco-friendly and highly toxic (Ribeiro et al. 2011).

12.4 Sources of Lipase

Lipases are major enzymes employed in the turnover of the lipid's biomass in nature. They are mainly sourced from plants, animals, and microorganisms; thus, they are ubiquitous (Sarmah et al. 2018).

12.4.1 Plant Lipase

Plant lipases are in various parts of the plants such as the germinating seeds and cereals, and they appear to attract substantial attention in biotechnology due to their low cost, uniqueness, and specificity in their ability to cleave the three ester bonds of triglycerides and to synthesize designed esters (Sarmah et al. 2018). Plant lipases find their application in the food and beverage industries, pharmaceutical, and therapeutic applications (Sarmah et al. 2018). Plant lipases are not extensively studied and explored because their purification and bulk production are confronted with some difficulties (Sarmah et al. 2018).

12.4.2 Animal Lipase

On the other hand, animal and insect lipases, which are mainly composed of pancreatic lipase, hepatic lipase, and hormone-sensitive lipase, are primarily used for digestion inside the hosts (Wong and Schotz 2002). Animal lipases are used in medicine and diagnosis (Sarmah et al. 2018). Animal lipases are less studied because they are bulky and difficult to purify. The use of animal lipases has some disadvantages such as health implications and defective products (Sarmah et al. 2018).

12.4.3 Microbial Lipase

Lipases from microorganisms are the most industrially used owing to their high abundance, ease of handling, amenability to genetic manipulation, ease of purification, bulk production, stability, and substrate specificity (Ray 2012). The above-listed features influence the attractiveness of lipases for industrial applications and make lipases to be a potential tool for biotechnological applications. The majority of microbial lipases are extracellularly produced by fungi, yeast, and bacteria (Thakur 2012).

12.4.3.1 Fungal Lipase

Fungal lipases are produced by fungi and yeast either outside or inside the fungal cells depending on the composition of the crude enzyme production medium (Thakur 2012). Fungal lipases are obtained through Solid Substrate Fermentation (SSF) or Submerged Fermentation (SmF), and they can catalyze a range of reactions such as hydrolysis and transesterification, etc... Fungal lipases are the widely used lipases thanks to their attributes including thermal stability, low-cost extraction and production, alcohol and other organic solvents tolerance, and pH stability (Patil et al. 2011). Despite such qualities, an isoform of lipase named Lipase B from *Candida antarctica* (CALB) was noticed to be cold-active although widely applied in various industries (Sarmah et al. 2018). Some of the major genera involved in the production of commercially important fungal lipases include *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., *Rhizomucor* spp., *Rhizopus* spp., *Candida* spp., *Yarrowia* spp., *Pichia* spp., and *Saccharomyces* spp. (Miyagi et al. 1995). Such genera are ubiquitous and have so long been isolated from diverse microbial niches such as soil contaminated with oil, hot springs, compost, industrial wastes and effluents, dairy plants, and vegetable oil processing factories (Sarmah et al. 2018).

12.4.3.2 Bacterial Lipase

Bacterial lipases are mostly produced extracellularly depending on the composition of the production medium and are deemed to be glycoproteins while some are lipoproteins (Javed et al. 2018). They can also be intracellular or attached to the membrane (Javed et al. 2018). Additionally, bacterial lipases are considered more suitable for industrial applications because of their ability to withstand the harsh conditions imposed by the industrial environment, alkaline pH and organic solvent tolerance, substrate nonspecific, and abundance (Sarmah et al. 2018). Some of the important genera reputed to produce lipases of commercial importance include *Staphylococcus* spp. *Achromobacter* spp., *Micrococcus* spp. *Burkholderia* spp., *Pseudomonas* spp., *Chromobacterium* spp., and *Bacillus* spp., the most useful lipase producer (Jaeger et al. 1994).

Among the bacterial genera listed, *Bacillus* is identified as a much safer bacterium to use for industrial production, and it is generally regarded as safe (GRAS) (Rashid et al. 2013). Bacillus lipases with their fascinating feature (Table 12.2)**,** which is thermos-stability, are considered to be potential lipases for biotechnological applications (Rashid et al. 2013). *Bacillus* lipases differ from other lipases groups despite their diversified features or properties as demonstrated by the analysis of many *Bacillus* lipases (Table 12.2) that have been isolated to date.

The pentapeptide conserved motif of *Bacillus* lipases have their amino sequences as indicated: Ala-His-Ser-[Met or Gln)-Gly. Alanine residue substituted the Glycine residue present in the canonical pentapeptide conserved motif of other microbial lipases. Such substitution conveys the increased thermos-stability to the *Bacillus* lipases (Jeong et al. 2002). *Bacillus* lipases are divided into two groups: I.4

Table 12.2 Biochemical properties of bacterial lipases

Enzymes	Microorganisms	Sources	pH	Temp. (°C)	Tolerance	References
Lipases	*Bacillus alcalophillus*	Mangrove detritus	10-11	60 °C	7.5% NaCl tolerance	Ghanem et al. (2000)
	Bacillus sp. *A30-1*	Hot spring	9.5	60	H_2O_2, alkaline protease tolerant	Wang et al. (1995)
Monoacylglycerol lipase	*Bacillus* sp. *H-257*	Soil	6.0-8.0	75	–	Kitaura et al. (2001)
Lipase	*Geobacillus* sp. *T1*	–	9.0	70	Ions tolerant	Leow et al. (2007)
	Geobacillus thermocatenulatus BTL2	–	8.0-9.0	65–75	–	Eggert et al. (2003)
	Acinetobacter sp.	–	10.0	50	–	Ahmed et al. (2010)
	Ralstonia sp.	–	8.0-9.5	50–55	Methanol tolerant	Chouhan and Sarma (2011)
	Talaromyces thermophiles	–	9.5	50	–	–

subfamily and I.5 subfamily *Bacillus* lipases (Guncheva and Zhiryakova 2011). I.4 subfamily *Bacillus* lipases are produced by *B. subtilis, B. pumilus,* and *B. licheniformis* and have a low molecular weight of 19-20 kDa. Additionally, I.4 subfamily *Bacillus* lipases are extremely alkaline tolerant, not thermostable, and do not possess a lid to protect the active site. Therefore, their active Serine residue is opened to the organic solvent and the polar (Guncheva and Zhiryakova 2011; Van Pouderoyen et al. 2001).

I.5 subfamily *Bacillus* lipases are synthesized by *Geobacillus thermocatenulatus* and have a molecular weight doubled the size of the I.4 subfamily *Bacillus* lipases (Ahmed et al. 2010; Eggert et al. 2003; Ghanem et al. 2000; Kitaura et al. 2001; Leow et al. 2007; Wang et al. 1995) kDa. They have neutral to moderate alkaline pH and are thermostable. They possess a lid to cover the active site. *Bacillus* lipases are mostly produced by submerged culture fermentation (Guncheva and Zhiryakova 2011).

12.5 Mining of Bacterial Lipase

Despite the diversity, specificity, uniqueness versatility, and the ease of bulk production of microbial lipases, the quest for lipases with improved and novel catalytic and stability under competitive conditions are still intense. To facilitate the hunt for novel lipases, different approaches (Table 12.3) have been used to answer the above-stated issues.

Table 12.3 List of approaches used to mine Lipases

Enzymes	Sources	Methods of mining	References
Moderately thermostable (and thermally activated) lipase	Soil	Metagenomics	Faoro et al. (2012)
Protease insensitive feruloyl esterase	Cow rumen	Metagenomics	Cheng et al. (2012)
Two lipases	Cattle rumen	Metagenomics	Liu et al. (2009)
Two esterases	Sheep rumen	Metagenomics	Bayer et al. (2010)
Two esterases	Soil and water	Metagenomics	Ouyang et al. (2013)
Three carboxylic ester hydrolases	Soil and drinking water	Metagenomics	Elend et al. (2006)
Thermostable esterase	Mud-sediment rich water	Metagenomics	Rhee et al. (2005)
Lipase	Palm oil-contaminated waste	Culture-based methods	Hasan et al. (2018)
Lipase	Degrading oil cakes	Culture-based methods	Sarkar and Chatterji (2018)
Lipase	–	Culture-based methods	Navvabi et al. (2018)

12.5.1 Conventional Method

The conventional approach (Fig. 12.7), which consists of cultivation and isolating the potential lipases producing strains, has been used for a century to get access to the lipases of interest. The traditional methods or culture-dependent methods consist of growing the organism of interest on an appropriate substrate, usually the emulsified synthetic oil (Tributyrin) under laboratory conditions like the natural habitat of the organisms to be studied.

The detection of positive lipases producing strains is based on the formation of a transparent or clear zone of halos around the colony. Nevertheless, it is stated that the culture-dependent methods managed to tap 1% of the microbial diversity of an ecosystem leaving about 99%, the vast natural variety untapped (Liu et al. 2009). The 99% which are made of the viable but non-culturable microbes are believed to produce a novel family of lipase isoforms (Miyagi et al. 1995). Such findings implied that the exploration of sources of novel lipases could be limited and restricted. To explore the vast intrinsic diversity, the culture-independent method called metagenomics is applied (Fig. 12.7).

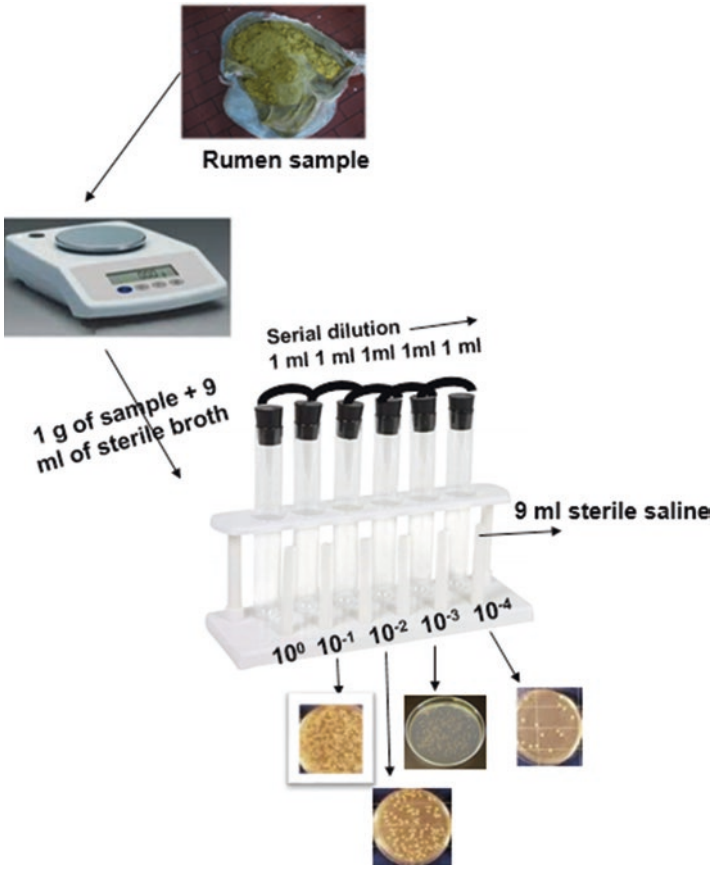

Fig. 12.7 The conventional culture-based technique used for the isolation of culturable organisms

12.5.2 Metagenomic Approach

The Metagenomic technique consists of three steps. The first step entirely relies on the extraction of the pooled genomes from an environmental microbiome, followed by the genomic libraries construction and the sequencing steps (Miyagi et al. 1995). The genomic library construction step helps in storing the genomes of microbes that are viable but non-culturable. Such genomes could be sources of genes coding for unknown or novel enzymes notably lipases possessing novel and unique properties. The progress that was made recently in the field of metagenomics favored the accessing of enzymes from unaccommodating habitats such as hot springs, compost, Antarctic soil, flat intertidal sediments, seawater, coastal environment, deep-sea marine sediments, marine sponges, mangrove sediments, and fat-contaminated soils (Miyagi et al. 1995). Treichel et al. (Treichel et al. 2010) reported the isolation of novel lipolytic enzymes that had variation in a protein sequence as compared to those

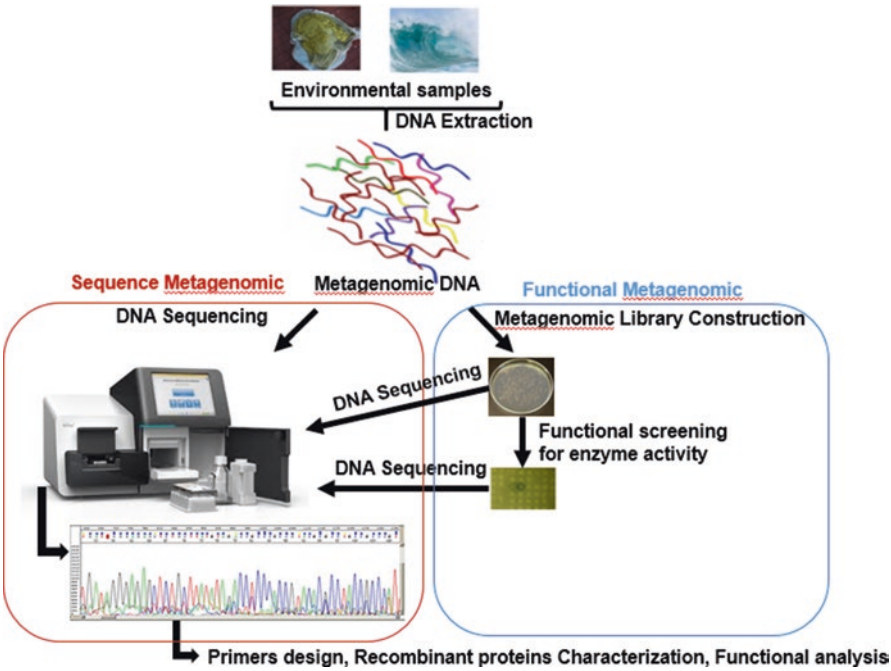

Fig. 12.8 The illustration of the metagenomic approach (Sequence and Function-based screening) used to exploit the unculturable microbial ecology

from the commonly reported classes of enzymes. Thus, the most significant accomplishment of the metagenomic approach in the discovery of novel families of biocatalysts, notably lipases, is the isolation of biocatalysts possessing unique protein sequences from deeply divergent lineages without exhibiting any close relativeness and representing brand new families. Metagenomic studies are revealed to continuously adding enzymes to the growing number of already existing industrial biocatalysts. It is hypothesized that biocatalysts discovered through metagenomics are on the verge to surpass biocatalysts determined through the conventional approach (López-López et al. 2014). There are two types of metagenomic approach: the sequence-based strategy and the function or phenotype-based strategy (Fig. 12.8).

12.5.2.1 Sequence-Based Metagenomic Approach

New enzymes are discovered through the analysis of the sequences obtained via the sequencing using platforms such as MiSeq and HiSeq Illumina from the Next-Generation Sequencing (Fig. 12.9). The study of sequences relies on the alignment and annotation of the metagenomic data against known and available sequences data

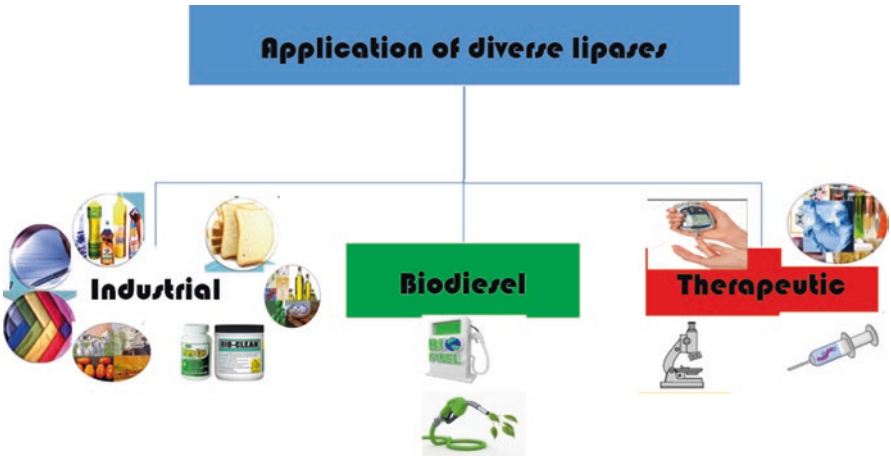

Fig. 12.9 Different industrial Applications of diverse lipases in terms of white, green, and red biotechnology

for enzymes homologous to known biocatalysts such as lipases/esterases (López-López et al. 2014). The sequence-based approach metagenomic is not readily used because this approach only detects genes coding for enzymes that are related to previously reported families. Consequently, the new sequences are overlooked because of a lack of similarity with known sequences (López-López et al. 2014).

12.5.2.2 Function-Driven Metagenomic Approach

Alternatively, with the function-based metagenomic approach, metagenomic libraries are screened based on their activity such as lipolytic activity reflecting on their phenotypes and many factors are influencing the success of this approach (López-López et al. 2014). The cloned genes should be compatible with the transcription and translation machinery of the heterologous host which is usually *Escherichia coli (*López-López et al. 2014*)*. The expression of lipases dramatically depends on the requirement for specific chaperones for the correct folding of the enzyme or can be hindered by its toxicity to the host cells (López-López et al. 2014). Using the function-driven metagenomic approach is advantageous because it allows the discovery of entirely new genes coding for novel biocatalysts. Currently, new families of lipases are still discovered through both traditional and metagenomic approaches (Nagarajan 2012).

Metagenomics like the conventional approach also experiences some challenges and bottlenecks such as the underrepresentation of genes of interest that are quickly picked through inefficient screening of metagenomic libraries. In this case, genes coding for enzymes of interest is represented in the minority in the metagenome

(Nagarajan 2012). The above bottlenecks made the metagenomic approach only recovered 40% of desirable enzymes from a metagenomic library through functional screening (Gabor et al. 2004). By 2009–2010, the functional metagenomic approach had so far only identified two lipases from the cattle rumen (Liu et al. 2009) and two esterases from the sheep rumen (Bayer et al. 2010). The isolated genes were less like genes coding for lipases from other environments. The resulting lipases also had their function unidentified.

12.6 Application of Lipases

Nature is known as one of the manufacturers of biocatalysts that are so searched for and such biocatalysts have been produced and used in vivo for centuries (Nagarajan 2012). Lipases, one of the biocatalysts, are said to contribute to the industrial world through the catalysis of a wide range of chemical reactions taking place in the transformation of chemicals (Biotransformation) (Sarmah et al. 2018). In virtue of their versatility, stability, regiospecificity, enantioselectivity, ease of production, catalytic properties, and microbial lipases constitute one of the critical groups of enzymes valuable to biotechnology and therapeutic applications (Nagarajan 2012) (Fig. 12.9).

12.6.1 Industrial Applications

12.6.1.1 Food Industry

In the food industry, lipases are used for many applications as discussed in the following sections:

12.6.1.1.1 Fat and Oil Industry

The value of triglycerides dramatically depends on their structure. Therefore, triglycerides of low cost can be transformed into valuable triglycerides using lipase. In the fat or oil industry, lipase is used for the modification of lipids by either changing the position of one fatty acid moiety from one carbon of the glycerol backbone to another or replacing one or more fatty acids moieties with new ones (Pabai et al. 1995; Undurraga et al. 2001). The newly produced lipids usually have the form of vegetable oils with nutritional importance, low calories, and oleic acid-enriched. An example of the use of lipase in the oil industry is the use of lipase for the interesterification of the palm (cheap) oil to produce cocoa butter equivalents. The palmitic acid of the palm oil at the second carbon position is replaced with oleic acid, and the cocoa butter is then used in chocolate manufacturing (Nakajima et al. 2000).

12.6.1.1.2 Dairy Industry

In the dairy industry, lipase is used for flavor modification by synthesizing esters of short-chain fatty acids moiety plus alcohols. The resulting esters are compounds of developed flavor for dairy products and fragrance (Alves Macedo et al. 2003), whereas the fatty acids moiety acts as flavor compounds or flavor precursors in cheese making of milk production (Andualema and Gessesse 2012). In the production of mayonnaise, phospholipases are used to treat the egg yolk (Sarmah et al. 2018).

12.6.1.1.3 Meat Processing Industry

In the meat processing industry, lipase is used to remove the fat from the meat products to obtain lean meat. Additionally, lipase is used to remove fat from fatty fish products through the bio-lipolysis process (Kazlauskas and Bornscheuer 1998).

12.6.1.1.4 Bakery Industry

Lipase aids in the extension of bread shelf-life in the baking industry, and as an enhancer that controls the nonenzymatic browning while increasing the loaf volume and improving the crumb structure. An example of such is the lipase from *Bacillus subtilis* (Ray 2012; Sangeetha et al. 2011).

12.6.1.2 Detergent Industry

Detergent is one of the cleaning agents used worldwide for household chores. It is used for either automatic or hand-wash laundry, dishwashing, and household cleaning. The addition of lipase in the detergent industry constitutes one of the most commercially essential applications of lipase. Lipase has been used in combination with other enzymes such as amylase and protease to increase the performance of the detergent (Ito et al. 1998). However, during the improvement of the detergent, some factors are said to be taken into consideration (Chauhan et al. 2013). Lipase is incorporated in the detergent to help in the removal of fatty stains like fried oil, sauce oil, and lipstick (Jaeger and Reetz 1998). Some of the lipase used in the detergent industry is sourced from *Staphylococcus arlettae*, *Burkholderia cepacia*, *P. fluorescens*, and *Candida* spp. (Ahmed et al. 2010; Phuah et al. 2015; Su et al. 2014; Su et al. 2015). The chelating agents in commercial detergents inactivate the incorporated enzyme, but the lipase from *B. licheniformis* had its activity restored through the addition of calcium chloride to the enzyme-detergent complex (Romdhane et al. 2010). Similarly, recent studies on anionic surfactants reported the retention of lipase stability and activity in the presence of sodium lauryl ether sulfate with two ethylene oxide units (Magalhaes et al. 2016). Novel lipases from *Talaromyces*

thermophilus, *P. aeruginosa*, and many more have been studied, and the findings gave a promising insight into their potential use in the detergent industry (Grbavčić et al. 2011; Kanjanavas et al. 2010).

12.6.1.3 Pulp and Paper Industry

Lipase is mainly used in the paper industry (Califano et al. 2014) as one of the solutions provided to the problem of pitch formation. Pitch is mostly made of hydrophobic triglycerides or waxes coming from the wood or lignocellulosic biomass components (Jaeger and Reetz 1998). Pitch makes its way through the paper manufacture process and is deposited in the paper mill machines ending up making holes and spots in the final paper. The reason why pitch control in the pulp and paper industry should be implemented is to enhance the quality of the paper to be produced, prolong the equipment shelf life, save energy, and reduce the level of pollution caused by the chemical process. Lipase does solve the problem by being applied to the wood to hydrolyze 90% of the pitch into monoglycerides and fatty acids. The hydrolysis renders the pitch less sticky, thus easily washable (Jaeger and Reetz 1998).

12.6.1.4 Leather Industry

Leather is a sturdy material produced from hides and skins of animals such as bovine. Hides and skins that are transformed into leather through tanning usually possess proteins and fats that pose a major problem in the tanning industry (Andualema and Gessesse 2012). To remediate the problem, Lipase is applied in the leather industry to decrease and remove fat from the skin prior to the initiation of the tanning process. The leather processing operation undergoes three steps: pre-tanning, tanning, and post-tanning (Thanikaivelan et al. 2004). During the stages of soaking, bating, and degreasing to remove subcutaneous fat, hair, and stuffing; lipases are applied to break down lipids found in the skins. The use of lipases has been deemed eco-friendly compared to the conventional use of solvents (Andualema and Gessesse 2012).

12.6.1.5 Textile Industry

Lipase has been utilized in the textile industry as a tool for sizing and improving features of clothing materials (Rowe 1999). Lipase is mainly used in the industry to render the polyester fabric capable of uptaking chemical compounds such as cationic compounds, fabric finishing compositions, dyes, antistatics, anti-staining, antimicrobial, antiperspirant, and deodorant compounds (Rowe 1999).

12.6.1.6 Cosmetics

In the cosmetic industry, lipase is used to yield esters such as isopropyl myristate, isopropyl palmitate, and 2-Ethylhexyl palmitate which is applied as a moisturizer or soothing ingredients in personal skin care products (Sarmah et al. 2018). It was discovered that the replacement of conventional acid catalysts with lipase generates higher quality products than those obtained from the traditional procedures (Andualema and Gessesse 2012). The downstream refining process was also minimized (Andualema and Gessesse 2012). Lipases from *P. fluorescens* and *P. cepacia* were used in the beauty industry to yield the menthol esters which provided a peppermint flavor in mouthwashes and shaving creams (Chaplin et al. 2004).

12.6.1.7 Agrochemical Industry

Lipase has been incorporated in the agrochemical industry to synthesize organic compounds used as herbicides or pesticides in crops (Barbosa et al. 2011). An example is the production of insecticides and fungicides by transesterification using *Pseudomonas* lipase as a catalyst. Notably, one of the herbicides (S)-indanofan, which was produced using lipases as a catalyst, is proven successful and useful against wild grass and weeds (Sangeetha et al. 2011). Lipases from *B. subtilis* was employed for the synthesis of optically active forms of pesticides from a racemic mixture of alcohol and carboxylic esters.

12.6.1.8 Waste Management

Lipase, a fat-splitting molecule, is incorporated in chemicals that are widely used to continuously remove thin layers of fats formed on the surface of aerated tanks used for aerobic waste processes of many industries (Sarmah et al. 2018). Lipase from organisms like *Bacillus, Pseudomonas, Candida,* etc. is also used to treat oily effluent from abattoirs, poultry waste processing, food processing, restaurants, and domestic sewage (Sarmah et al. 2018) Lipases from *Bacillus subtilis* COM-B6 has been used as one of the effective decontaminants of wastes from lipid-processing factories. Lipase was also found to be useful in the degradation of diesel oil in freshly contaminated, unfertilized, and fertilized soils.

12.6.2 Therapeutic Applications

Lipases constitute the primary enzyme involved in the metabolism of lipids in the body (Thanikaivelan et al. 2004). In virtue of its hydrophobicity, lipids are not assimilated by the body because they are insoluble in the blood which is majorly made of water. Therefore, lipase needs to break down lipids into small fatty acids to

be absorbed by the body system. Additionally, lipases are used as digestive aids to stimulate the digestion process of lipids ingested. Bacterial lipases are currently used to treat cystic fibrosis and pancreatitis (Ye et al. 2011). They replaced pancreatic lipases for such treatment (Ye et al. 2011). Bacterial lipases are also used in the treatment of malignant tumors by activating the tumor necrosis factor. Moreover, monoglyceride lipase is a promising drug target for cancer, neurodegenerative, and inflammatory diseases (Grabner et al. 2017).

Lipases that are generally isolated from the wax moth (*Galleria mellonella*) do possess a bactericidal action on *Mycobacterium tuberculosis* (MBT) H37Rv (Javed et al. 2018; Nagarajan 2012)). Infection initiated by *Mycobacterium tuberculosis* is diagnosed through the detection of lipases produced by *M. tuberculosis.* Therefore, lipases were deemed suitable for health care (Nagarajan 2012).

Lipases can also be used in the treatment of skin scalp disease and hair loss, while *Bacillus* lipase is used for the synthesis of enantio-pure compounds to be used as pharmaceutical products (Javed et al. 2018).

As biochemical biosensors, lipases are used in the immobilized form in combination with other enzymes to determine the concentration of lipids/ triglycerides or cholesterol through the quantification of glycerol (Javed et al. 2018). Lipases are used as biosensors in diagnosis to determine the concentration of lipids in the blood, in food, and in pharmaceutical products. Lipases can also be used as a diagnostic tool because it is one of the enzyme markers in medicine. Since some microbes produce lipase as a means of defense or as a poison for the adverse organisms, the presence of lipase at a concentration can indicate the initiation of an infection or disease. For further illustration, the presence and quantity of lipase in the blood serum prognosticate (Aronne et al. 2015; Califano et al. 2014; Solanki et al. 2016; Zehani et al. 2015).

12.6.3 Biodiesel Production

Biodiesel has been one of the alternatives to fuel since the raising of the environmental conservation issue (Sarmah et al. 2018). Biodiesel is a liquid produced from the transesterification of lipids with methanol or ethanol, short-chain alcohol. Industrial production is said to provide less amount of greenhouse gases [sulfur oxide) as compared to the burning of fossil fuels to produce diesel or petrol (Sarmah et al. 2018). Biodiesel production is additionally seen as a replacement for fossil fuel because of the depletion of the resources of fossil fuels. The transesterification process requires lipase as a catalyst because the use of lipase renders the production of biodiesel less harmful to the environment (Lukovic et al. 2011). Another advantage that the enzymatic transesterification has over the chemical one is the easy recovery of the purified glycerol which is a byproduct of the transesterification reaction (Lukovic et al. 2011).

The enzymatic production of biofuel consumes less energy and produces waste in a reduced quantity as compared to the chemical process. Examples of raw

materials used in the production of biodiesel are nonedible oils. The use of nonedible oils does not affect food production (Ahmia et al. 2014). Furthermore, edible oils can also be used for the production of biodiesel. The properties and features of lipases determine their relevancy in the industry; Aguieiras et al. (2015) reported on the current status and perspectives of the use of fungal lipases for biodiesel production. Aguieiras et al. (2015) tested the lipase activity and stability against variant raw materials, alcohols, the concentration of solvents, etc.. The type of lipase to be used for the transesterification of lipids/fats with alcohol is highly dependent on the variety of raw materials. For example, lipase from *M. miehi* (Lipozyme IM-20) was deemed more effective on animal fat, while lipase from *Geotrichum* spp. worked best for the transesterification of waste cooking oil. Whereas, lipase from *P. fluorescens* was suitable for the transesterification of waste sunflower oil. Further studies are also conducted to develop strategies to reduce the cost of biodiesel production (Ghaly et al. 2010).

12.7 Conclusion and Future Prospects

Lipolytic enzymes notably lipases, being a versatile biocatalysts in virtue of their catalytic activities towards a wide range of reactions are one of the biocatalysts that attract the attention of many industries. The lipase-catalysis reactions make them a potential candidate for industrial, biotechnological, and research applications. Therefore, lipases with improved features such as selectivity, region-specificity, enantio-selectivity, thermos-stability, pH stability, and organic solvent tolerance are necessary for such applications. The rumen, as one of the ecosystems possessing thermophilic microbes (data unpublished), should be explored for sources of potential biocatalysts with enhanced features discussed above, for biorefinery applications and other industrial applications. The recently developed "metagenomics" technique would be of great help for mining novel lipases. The mined lipases are to be characterized to serve industrial purposes.

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Chapter 13
Bacillus spp. Facilitated Abiotic Stress Mitigation in Rice

Meenu Thampi, Edna Mary Varghese, and M. S. Jisha

Abstract Rice (*Oryza sativa* L.) is the main source of staple food for human population. One of the significant challenges currently is to obtain higher crop yield. Environmental conditions, cultivar quality, and plant diseases enormously affect plants productivity. Understanding rice responses to stress may help breeding for more tolerant varieties. On the other hand, several endophytic *Bacillus* species have emerged as a complementary, efficient, and safe alternative to current crop management practices. This study focuses on the critical role of endophytic *Bacillus* spp. in plant health and their stimulatory different mechanisms to tolerance against abiotic stress in rice. Bacterial endophytes have the ability to act as plant growth–promoting agents through producing phytohormones and also enable plants to grow in contaminated soils through breakdown of hazardous compounds. These endophytes manage plant growth under adverse conditions such as salinity, drought, temperature, heavy metal stress, and nutrient stress through different mechanisms. This chapter presents new approaches for the utilization of endophytic *Bacillus* spp. to battle abiotic stresses in agricultural fields, which increments global crop production.

Keywords *Bacillus* spp. · Endophyte · Abiotic stress · Climate change

13.1 Introduction

Rice (*Oryza sativa*) is the most widely consumed cereal grain in the world's human population. Of all the continents, Asia is the chief producer of rice (FAO 2007). Studies show that rice is a prehistoric food source among other crops. Therefore, any stress on this crop will have a devastating effect on its production which can eventually lead to huge economic losses. Abiotic stresses, including drought,

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salinity, low temperatures, high temperatures, and toxic metals, can make rice production extremely problematic and limit agricultural productivity. The response to abiotic stress depends on the duration and progression of the stress and the different plant growth and development stages. (Feller and Vaseva 2014). Drought is the most important abiotic stress on crop productivity around the world. Salinity is one of the second most severe factors in crop survival, biomass, and yield (Mariani and Ferrante 2017). Major abiotic stresses adversely affect crop survival, biomass, and yield of up to 70%, mainly reducing plant growth and profitability (Thakur et al. 2010). Stressors affect plant morphology, physiology, and biochemistry, as well as the gene regulations by developmental stage, resulting in the loss of microbial diversity of soil, fertility of soil, and competition for nutrients (Chodak et al. 2015).

The internal metabolic abilities help plants to cope with rapid fluctuations and adverse environmental conditions (Simontacchi et al. 2015). Plants have acquired a range of conservation mechanisms to facilitate their regular biophysical-chemical processes regardless of the external conditions (Mickelbart et al. 2015). Therefore, plants can resist abiotic stresses with the aid of their associated microbial communities. Mycorrhizal fungus, a microbial association with plants, promotes plant growth and development under various abiotic stresses. The plant-microbial associations can be classified into beneficial, deleterious, and neutral microbes. *Pseudomonas* (Grichko and Glick 2001; Ali et al. 2009), *Bacillus* (Sorty et al. 2016), *Azospirillum* (Creus et al. 2004; Omar et al. 2009), *Azotobacter* (Sahoo et al. 2014a, b), *Bradyrhizobium* (Swaine et al. 2007; Tittabutr et al. 2013), *Enterobacter* (Grichko and Glick 2001; Nadeem et al. 2007; Sorty et al. 2016), *Methylbacterium* (Madhaiyan et al. 2007; Meena et al. 2012), *Rhizobium* (Alami et al. 2000; Remans et al. 2008; Sorty et al. 2016), *Trichoderma* (Ahmad et al. 2015) and cyanobacteria (Singh et al. 2011) help to promote plant growth and alleviate multiple types of abiotic stresses.

Plant growth-promoting bacteria (PGPB) can be free-living or colonize the phyllosphere, rhizosphere, or interior of plant tissues (endophytes) to produce phytohormones, solubilize mineral phosphate, antagonize plant pathogen (Beneduzi et al. 2012), and alleviate abiotic stresses. The colonization areas of PGPB in the plant are generally classified as rhizosphere and endosphere, and the inhabitants are called rhizobacteria and endophytes, respectively (Timm et al. 2015). PGPB stimulates plant growth through a wide range of mechanisms. These include the availability of nutrients to plants derived from processes such as biological nitrogen fixation, phosphate solubilization, stress mitigation through ACC deaminase expression modulation, and phytohormone and siderophore production. These create a favourable environment for growth and functioning. Non-symbiotic endophytic microbes colonize the intercellular spaces of plant cells with high amino acids, carbohydrates, and volatile nutrient levels (Bacon and Hinton 2007).

Among the various PGPB, the dominant species are *Bacillus* and *Pseudomonas* spp. (Kang et al. 2015a). Owing to their high endurance in varied abiotic and biotic environments, a few of the PGPB have been commercialized. Thus, organic fertilizers could be employed as a substitute for harmful chemical fertilizers and pesticides, as well as offer new insights into plant growth and development, even in the presence of pathogenic organisms (Choudhary 2011). Alinit, developed from *Bacillus spp.,* was the first commercial bacterial fertilizer that displayed a 40% rise

in crop production (Kilian et al. 2000). Kodiak (*B. subtilis* GB03), Quantum-400 (*B. subtilis* GB03), Rhizovital (*B. amyloliquefaciens* FZB42), Serenade (*B. subtilis* QST713), and YIB *(Bacillus spp*.) have been commercialized to improve crop production (Brannen and Kenney 1997; Ngugi and Nyariki 2005; Cawoy et al. 2011). The fertilizer properties of *Bacillus* spp. are high compared to *Pseudomonas*-based fertilizers owing to their effective spore-forming ability and metabolite production, which enhance cell viability in commercial formulations (Haas and Défago 2005). *Bacillus sp.* are ubiquitous, gram-positive organisms that can be found in all environment niches. They have been used in various agricultural, medicinal, and industrial products (Lyngwi and Joshi 2014). *Bacillus* spp. interact with roots and produce biofilms to enhance plant growth (Beauregard et al. 2013). Therefore, *Bacillus*-based fertilizers can be applied to the soil. Gingivitis can increase the available nutrients of plants in the rhizosphere, regulate the growth of pathogenic microorganisms, and stimulate pests (García-Fraile et al. 2015; Kang et al. 2015b). Therefore, this chapter focuses on the growth-enhancing potential of *Bacillus* spp. in paddy plants to cope with abiotic stresses in agricultural sectors that increase global crop production.

13.2 Abiotic Stresses

Abiotic stress is any factor that disrupts a plant's growth, development, or metabolism rate. It is any substance that decreases plant growth and productivity compared to that of its actual genotype (Krishnan et al. 2011). Therefore, in this chapter, we deliver an elucidation of different types of stresses—drought, salinity, heavy metal, cold, and heat stresses—and their effect on paddy cultivation, as well as their bias. In mitigating these stresses, the typical abiotic stresses affecting paddy cultivation are illustrated in Fig. 13.1.

13.2.1 Drought

Drought is a chief factor limiting the production of drought-affected crops. This has been described as a situation that brings plant water potential and turgor to the point where the plant encounters difficulties in performing normal physio-biochemical functions (Noel et al. 2018). Compared to other crops such as maize and wheat, crops such as paddy have a much higher drought challenge because it has a relatively high water requirement (Todaka et al. 2015). In rice, drought stress increases or prolongs the growth period and causes a series of physical and chemical changes in the plant and leaves, *viz.* decrease in size and root growth reduction, leaf drying, and leaf rolling (Lafitte et al. 2004; Lima et al. 2015; Prakash et al. 2016), increased ethylene production, changes in chlorophyll content, impairment of photosynthetic devices leading to inhibition of photosynthesis (Lata and Prasad 2011),

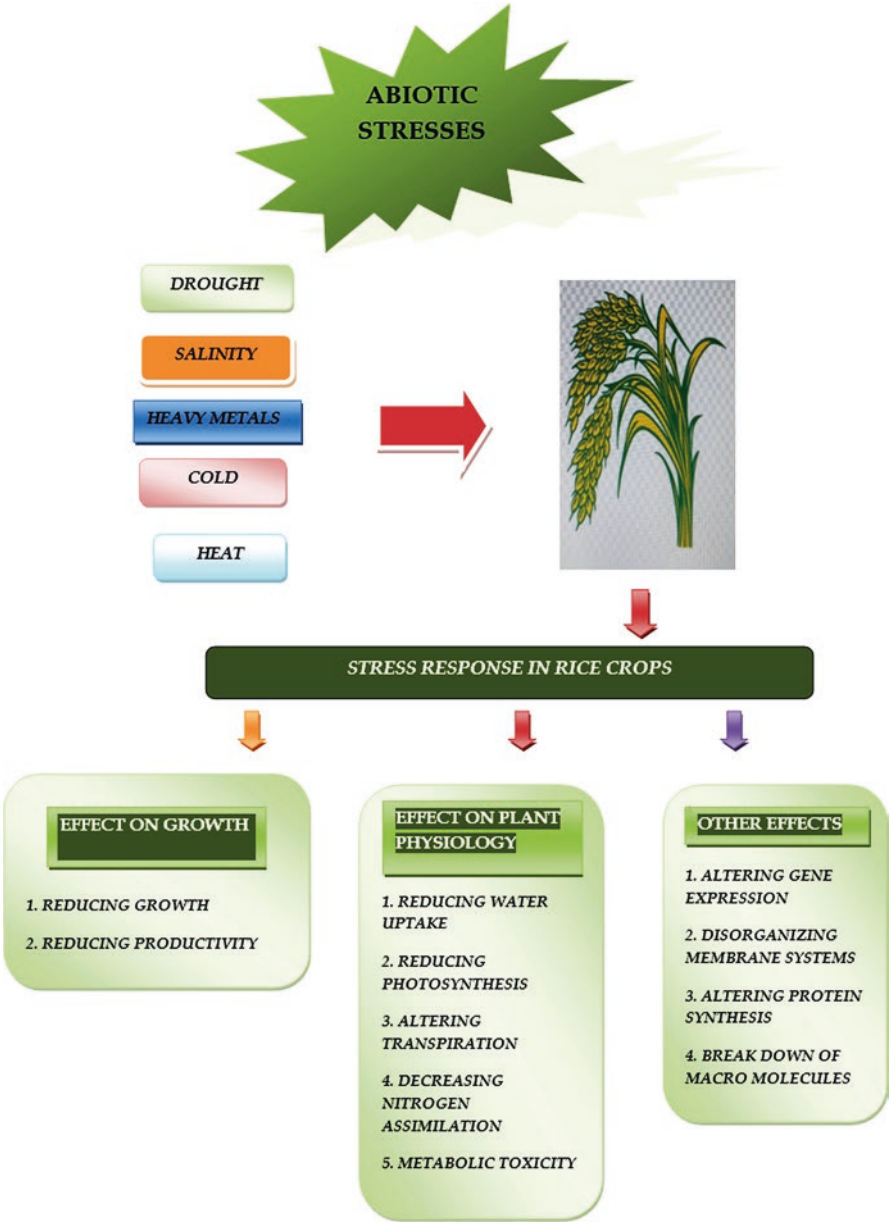

Fig. 13.1 Common abiotic stresses affecting rice

accumulation of free radicals altering membrane function, protein formation, lipid peroxidation, and eventually cell death (Tiwari et al. 2016).

13.2.2 Effect of Drought Stress in Rice Crop

There have been several investigations into the behaviour of various plants in drought conditions, including paddy cultivation (Chen et al. 2011; Gao et al. 2008). Effects of drought on different growth and development stages, vegetation stage, grain filling stage, and yield were studied. Drought conditions (Ahmad et al. 2009) affect the early developmental stages like seed germination and seedling development, which adversely affects germination rate, especially in polyethylene glycol concentrations (Kaya et al. 2006). Zeid and Shedeed (2006) reported that due to water scarcity initiated by polyethylene glycol, seed germination capacity, hypocotyl length, and root/shoot biomass decreased at the same time as root measurement was developed. Drought stress causes disabled mitosis and cell proliferation, leading to stunted growth and growth attributes of rice (Hussain et al. 2008). Swain et al. 2010 reported a decrease in panicle number (72%) and grain yield (12%) during the evaluation of eighteen rice genetic variants. Liu et al. (2006) and Rang et al. (2011) reported that drought negatively affects the production of viable pollen, panicle initiation exertion, pollen shed and germination, and embryo development, which are parts of fertilization and initiation of grain filling. This causes the spikelet fertility to decrease and the dry weight of the fertile spikelets to cause a loss of grain yield. Dehydration is considered multidimensional stress as it affects a wide variety of cells, tissues, and plants as a whole (Rahdari and Hoseini 2012). Nair et al. (2008) reported that drought stress produces free radicals that affect the antioxidant immune system and reactive oxygen species (ROS) and therefore cause oxidative stress which adversely affect the various levels associated with the paddy plant. Lack of water reduces photosynthetic pigmentation, resulting in photo-oxidation (Anjum et al. 2011, 2016a), and also causes decreased NO_3^- uptake, which restricts plant growth (Ali et al. 2014; Awais et al. 2017a, b).

During the grain filling stage, sucrose, ADP-glucose pyrophosphorylase, starch, and starch branched compounds are formed (Taiz and Zeiger 2002). In rice, the lack of water adversely affected the rearrangement of carbohydrates stored during the grain filling phase into rice grains (Yang et al. 2012; Naseem et al. 2016). This is due to low sucrose synthesis activity in drought conditions and ADP-glucose-pyrophosphate inactivation (Ahmadi and Baker 2001; Nasim et al. 2016). Lack of water disrupts the nature of the leaves by controlling the amount of source and sink tissues. In addition, foam stacking and drying tissues were disabled (Akram et al. 2019). Dry conditions cause stomatal closure, which regulates the entry of CO2, resulting in photosynthesis (Flexas et al. 2004), stunted vegetative growth and development, flower formation, and grains leading to reduced grain yield (Akram et al. 2019). Rice responses to drought stress through various physiological, morphological, and molecular modifications are illustrated in Fig. 13.2.

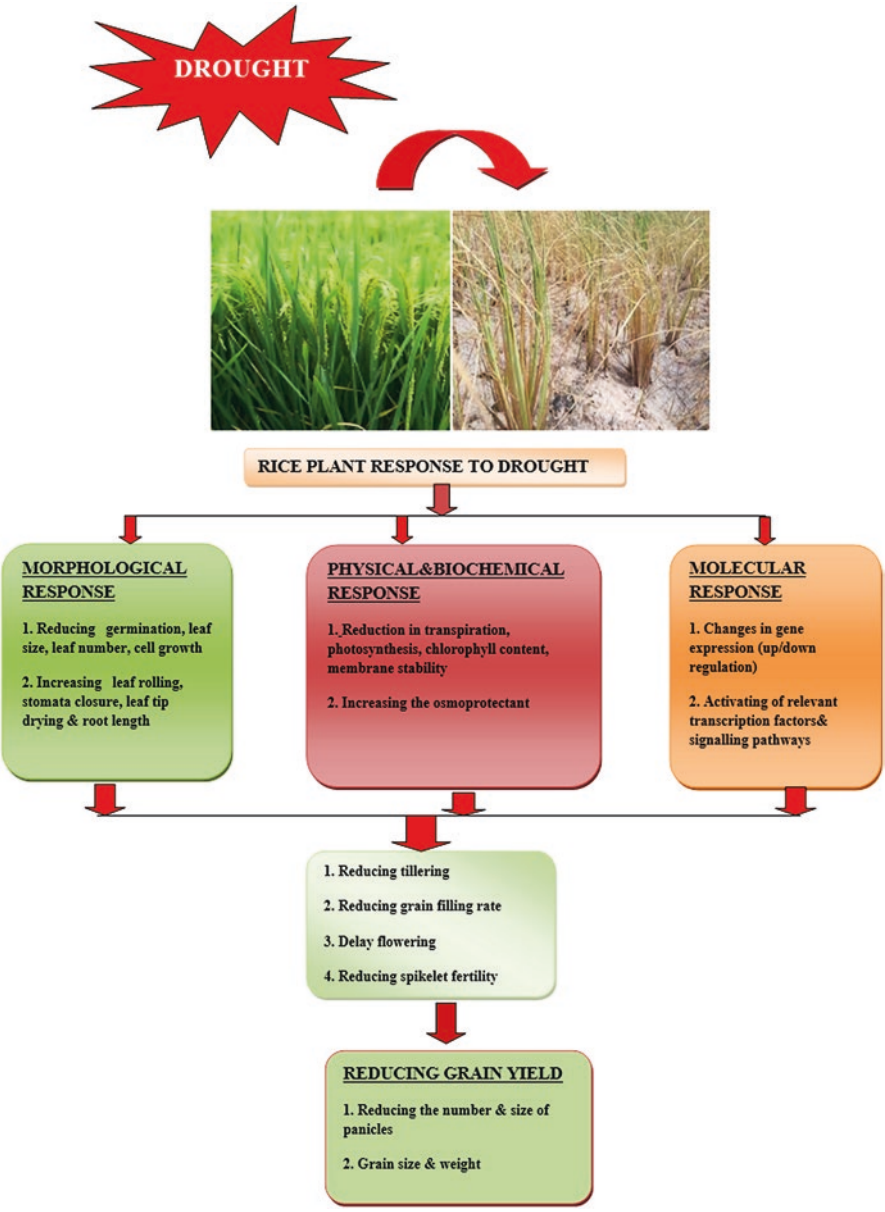

Fig. 13.2 Rice response to drought stress

Normal gene expression pattern of plants changes during drought conditions. The expression of OsDREB1A (dehydration-responsive element-binding protein 1A), OsWRKY11 (WRKY transcription factor 11), OsGAPDH (glyceraldehyde-3-phosphate dehydrogenase), OsDIL (drought-induced lipid transfer protein), P4H

(prolyl-4-hydroxylase), sHSP (small heat shock protein), Cadhn (dehydrin-like protein), VA (vacuolar H+-ATPase), cAPX (cytosolic ascorbate peroxidase), CaPR-10 (pathogenesis-related protein 10), rbcL (ribulose-1,5-bisphosphatecarboxy/oxygenase large subunit), and rbcS (ribulose-1,5-bisphosphate carboxy/oxygenase small subunit) genes of crop plants are affected in drought stress conditions (Radhakrishnan et al. 2017).

13.3 Salt Stress

Excessive accumulation of salt in soil is salt stress, which inhibits crop growth and eventually leads to crop death. In more than 100 countries, 1–10 billion hectares of salt-affected land are reported to exist, accounting for more than 7–10% of the total land area (Panta et al. 2014; Jesus et al. 2015; Zhang et al. 2017). Munns (2002) and Ruan et al. (2010) estimated that 20% of irrigated land is inclined to high salinity and 50% to moderate or secondary salinization. Rahman et al. (2019) suspected that salinity is related to irrigation, its quality, and saltwater use. Salt pressure affects the plant in many ways. Nutrient deficiencies, oxidative stress, ion toxicity, alteration of metabolic processes, genetics, cell division reduction, development, lack of essential nutrients, *viz.* K^+, and the toxicity of Na^+ within the plants hamper plant processes like lipid metabolism, photosynthesis, biomass accumulation and protein synthesis (Munns and Tester 2008; Asgari et al. 2012).

13.3.1 Effect of Salt Stress in Rice Crop

Paddy is considered a salt-sensitive crop where production is affected by the spread of salt-affected land in the croplands. Salt pressures applied to plants are of two types: osmotic pressure due to increased rhizosphere osmotic potential and ionic pressure due to enhanced ionic concentration (Ghosh et al. 2016). In rice, early vegetation and later reproductive stages are more susceptible to saltwater. Due to additive gene effects, rice genetic variants differ in salinity tolerance (Shannon et al. 1998; Mass and Hoffman 1977; Sahi et al. 2006). Salt causes mutations and anatomical changes in roots, stems, and leaf cells that inhibit cell division and development, reducing the size of the agricultural meristem, cortex, and vascular cylinder (Islam et al. 2019). The effects of salinity have been reported. They include the growth and establishment of seedling and grain yield components such as spikelet and tiller number. Increased salinity caused a decrease in the number of spikelets per panicle, 1000 grain weight, and infertility, irrespective of season and stage of development (Ghosh et al. 2016; Zeng et al. 2003). Rice response to salt stress is shown in Fig. 13.3.

Salinity affects ion status, leading to excessive accumulation of Na^+/Cl^- inside and outside the plant, which in turn affects ion homeostasis of essential elements causing nutrient imbalances or deficiencies (Naeem et al. 2010). And also saline

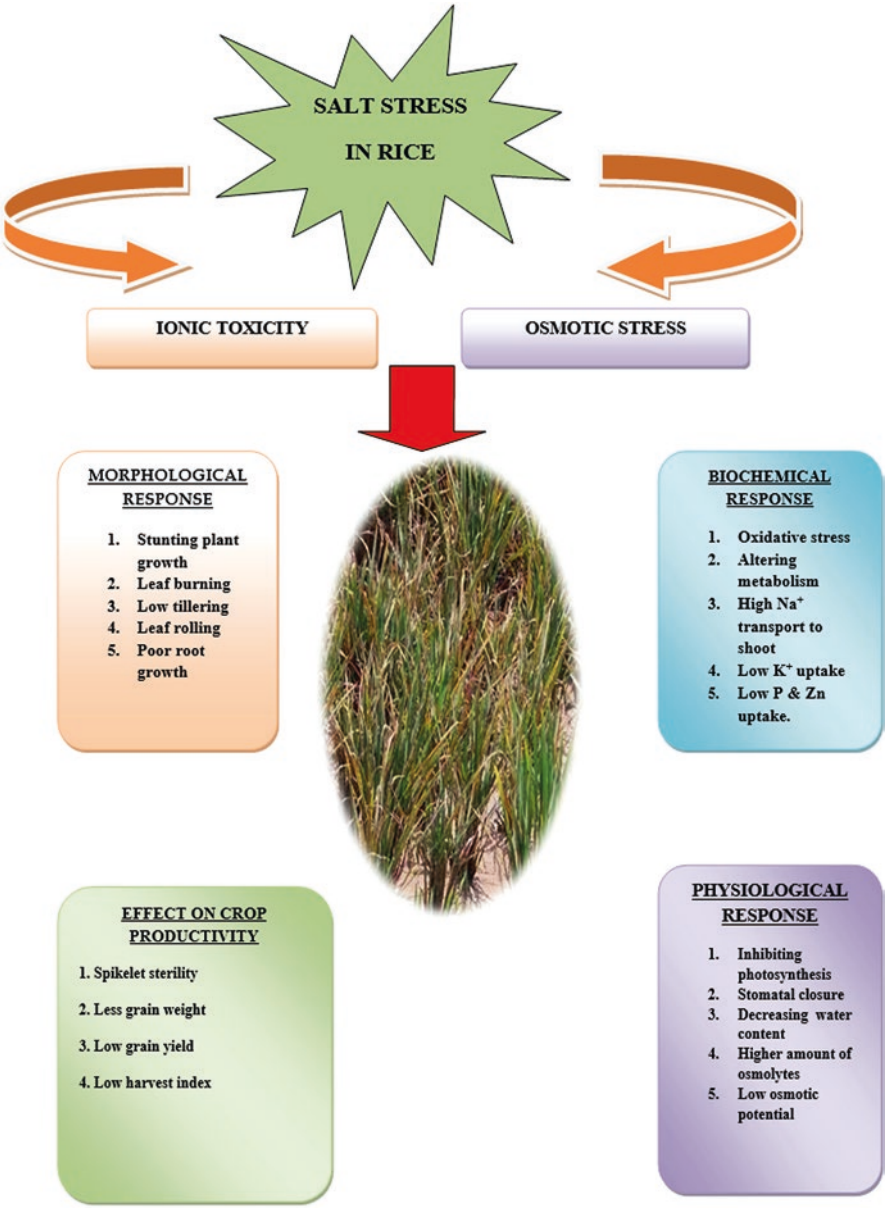

Fig. 13.3 Rice response to salt stress

stressed soil is greatly susceptible to Fe deficit affecting the morphology of the root and root/shoot biomass ratio due to the increased pH, insoluble Fe complex formation, and Fe chelator uptake in rice plants (Islam et al. 2019). Salt pressure creates oxidative stress through the production of ROS in rice cells. Lipid peroxide-derived short-chain carbon mediators are thought to lead to salt-producing cell death in

plants (Biswas and Mano 2015). Golldack et al. (2014) reviewed that signalling mediated by lipids in rice plant is activated in response to abiotic stresses, which leads to the maintenance and regeneration of membrane properties and affects membrane-related protein function.

13.4 Heavy Metal Stress

Intensified industrialization processes lead to heavy metal pollution causing problems in environmental and agricultural sectors apart from human health deterioration problems (Fahad et al. 2015a, b, c, d, e). The major heavy metal pollutants are cobalt (Co), cadmium (Cd), mercury (Hg), lead (Pb), copper (Cu), chromium (Cr), and manganese (Mn) (Goyer 1997; Adrees et al. 2015; Anjum et al. 2015, 2016a, b; Bakhat et al. 2017; Shahzad et al. 2018). Some of these are essential metals such as Zn, Ni, and Mn, which are critical for plant physiological processes in small quantities (Goyer 1997; Kabata-Pendias 2010). Most metals are available in the rhizosphere (Wenzel et al. 2003), which pass through the root cell plasma membrane and form a wide substrate specificity through the transport of other cations (Manara 2012).

13.4.1 Effect of Heavy Metal Stress in Rice Crop

Mining, metal processing, wastewater, sludge, and fertilizer application are the major metalloid soil contaminants caused by rapid industrialization and urbanization (Zheng et al. 2010; Niu et al. 2013; Anjum et al. 2016a, b). Metal poisoning mainly affects plant growth by disrupting proteins, lipids, thylakoids, and cellular structures (Fahad et al. 2019).

Heavy metal toxicity is increasing in arable lands, particularly in paddy (Yu et al. 2016). Du et al. (2013) found that two-thirds of the soil in Hunan province was contaminated with Cd (above 0.3 ppm), in rice with 0.2 ppm density. In a study of paddy fields in the Yangtze River region of China by Liu et al. (2016), heavy metal transfer in paddy is mainly through agricultural involvements, *viz.* fertilizers, pesticides, irrigation, etc. Heavy metal poisoning first appears on young leaves with dark green rib formation, where the leaves develop chlorosis and eventually turn white. Cu, Pb, and Zn toxins in rice reduce germination rate and seedling growth characteristics (Fahad et al. 2019). Ashraf et al. (2015) reported that lead poisoning in rice can cause variations in physiological and biochemical characteristics. The first mechanical reaction to heavy metal poisoning is the production of ROS. Cd poisoning increased oxidative stress due to ROS accumulation, MDA content, and electrolyte leakage in rice seedlings (Srivastava et al. 2014; Yu et al. 2013). High Cd toxicity is regulated by the ups and downs of many genes (Lee et al. 2013) and the overexpression of the glutamine synthesis (GS) gene in rice causes plant death (Lee

et al. 2013). Cd pressure slows seed germination and seedling growth. Pb mainly affects seed germination, root/shoot ratio, and the dry and fresh weight of rice (Sethy and Ghosh 2013). Cd and Cu pressure accumulate high thiobarbituric acid reactive substance (TBRS) in plant biomass. Stress of excessive Cu is minimized by the control of antioxidant and stress-related protein genes such aldos reductase, peroxyroxine, glyoxalase I, allopi proteins, DNAc-type molecular chaperone, and receptor-like kinase, as well as other metabolic processes. Chatterjee et al. (2004) reported that PB toxins reduced levels of chlorophyll, carotene, nitrogen, and protein. Singh et al. (2010) estimated that lead poisoning adversely affected photosynthesis, chloroplast structure, its pigments, reduction of CO_2 fixation, and electron transport network. The reaction of rice to heavy metal toxins is shown in Fig. 13.4.

Nickel reduces rice seedling roots and is pH-dependent, although it also contributes to the activity of ferulic acid (FPOX) and syringaldazine peroxidase, and the lignin content is observed in $NiSO_4$ application (Lin and Kao 2005). Lead toxicity unfavourably affects photosynthesis by decomposition of chloroplast structure, biosynthesis of photosynthetic pigments, reduction of CO_2 fixation, and activity of enzymes responsible for carbon metabolism. Cd /Cu heavy metal accumulation causes lipid peroxidation, modulation of salicylic acid, abscisic acid, and jasmonic acid leading to decreased plant biomass (Kim et al. 2014a, b). Cultivation of paddy in Cd contaminated soil poses a serious challenge to food production quality (Jallad 2015). Heavy metal poisoning not only affects rice yield but also leads to human health problems due to the intake of heavy metal contaminated rice.

13.5 Temperature Stress

The main effect of the plasma membrane and water level (transpiration) is that during the growing season, plants can be exposed to one or more environmental stress factors; one is the intensity and frequency of temperature stress. It is a major detrimental factor in leaf emergence in plants (Ritchie 1993). Thermal pressure (HS) and cold pressure have become significant abiotic stress conditions for crop productivity and food security worldwide as they impair photosynthetic activity, enzyme activity, cell division, and plant growth (Kumar et al. 2018).

If not handled properly, heat stress can cause a lot of physical and biochemical effects on plants. According to Qu et al. (2013) and Kotak et al. (2007), plants undergo several mechanisms to surpass thermal stress, including the production and accumulation of osmolytes and enzymes. The main proteins are heat shock proteins (HSP20, HSP60, HSP70, HSP90, HSP100) and ROS-scavenging enzymes (catalase and ascorbate peroxidase).

Under cold pressure, ROS accumulate (Song et al. 2011) causing serious harm to the various cellular components, changing membrane lipid composite, and increasing the accumulation of malondialdehyde (MDA) (Sato et al. 2011).

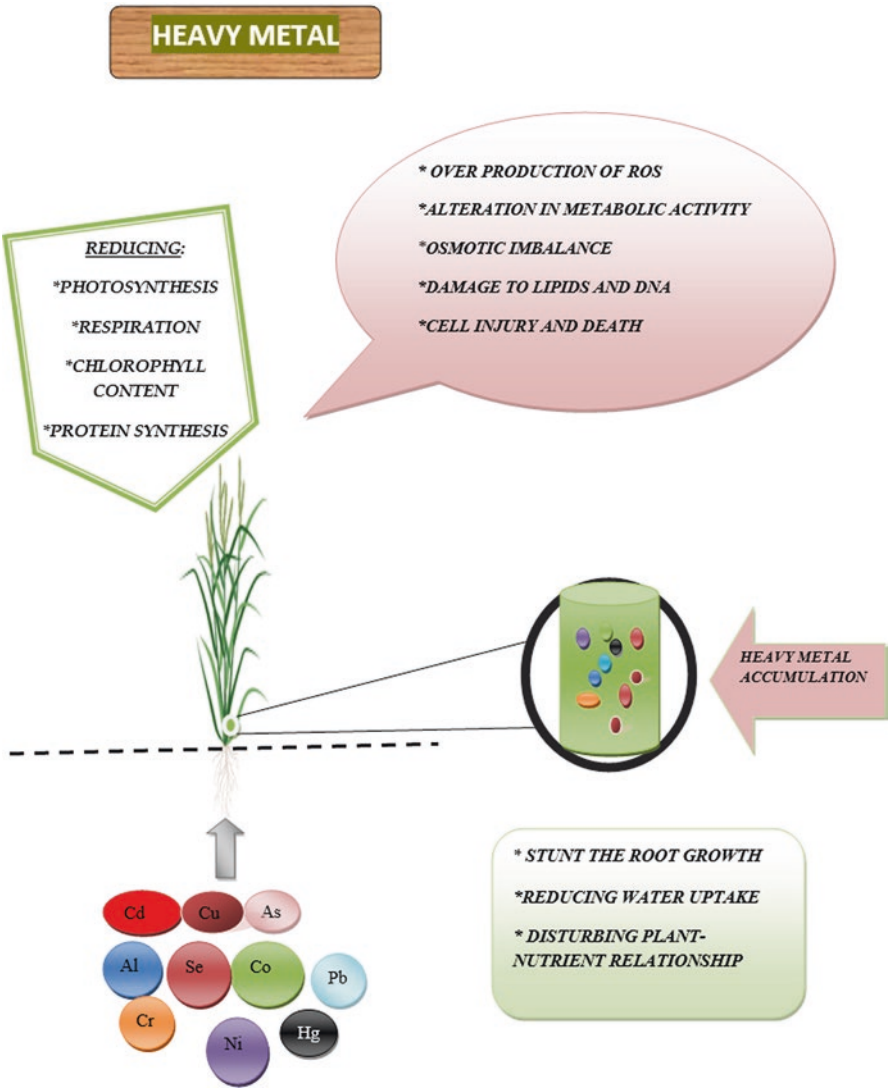

Fig. 13.4 Rice response to heavy metal toxicity

13.5.1 Effect of Temperature (Heat and Cold) Stress in Rice Crop

Rice (*Oryza sativa* L.) is an important grain and heat-sensitive plant (Jagadish et al. 2010; Fahad et al. 2015a). According to Bahuguna et al. (2015), the quality of the reproductive or grain filling stages is adversely affected by high temperature/

pressure. Heat stability of the cell membrane is a key factor determining the yield performance of the crop.

Paddy is now grown in areas with higher than growth (28/22 °C) temperatures. High temperature reduces photosynthesis (Oh-e et al. 2007; Fahad et al. 2016b, c, a) and is also reported to transfer the dry matter to roots and shoots. Fahad et al. (2018) reported that heat stress and high radiation may cause considerable pre- and post-harvest damages, thermal stress and high radiation, burns of leaves and twigs, sunburn on leaves, stems, and branches, elimination of leaf ageing, inhibition of shoot and root growth, damage to fruit, and decreased yield. Heat stress increases the growth rate, which in turn reduces the quality of assimilation in the grain, which leads to poor-quality millet rice grains (Kobata and Uemuki 2004). Rice responses to heat and cold stress are shown in Figs. 13.5 and 13.6, respectively.

Cold-stressed rice shows yellowing of leaves, low height, and crop failure (Zhang et al. 2014), causing the depletion of energy sources and photosynthetic machinery impairment, specifically to the chloroplasts modifying light-harvesting chlorophyll antenna complexes (Huner et al. 2012), altering thylakoid structures (Santarius 1992) leading to a decline in plant energy resources. Hughes and Dunn (1996) showed that the plasma layer alters sowing to the rearrangement of unsaturated and unsaturated fatty acids at low temperatures. Enerstvedt et al. (2017) have shown that the lack of hydration instigated by cooling can damage the plasma layer. These may include lysine, lamellar-to-hexagon-phase advances, additional lesions, and ROS presentation.

13.6 Plant-Microbe Interaction Assisting Stress Tolerance

When the plant senses the stress stimuli microbes, use both direct and indirect mechanisms to stimulate plant growth and development under stressful conditions. One strategy is to perceive signals in stressful situations and understand the best molecular level machinery. It includes descriptions of metabolic pathways and their regulatory genes in plants (Miller et al. 2010; Dahro et al. 2016), identifying the characteristic features involved in stress responses, and the linker marker of such genes. The other strategy we focused on was the micro-mediation determination of abiotic stresses. Based on stress stimuli, plants show a complex stress-specific signal cascade (Chinnusamy et al. 2004; Andreasson and Ellis 2010), accumulation of acid and ethylene, flavonoids, and phenolic acids (Singh et al. 2011; Tiwari et al. 2011), and activation of transcription factors (TFs), initiating the expression of defence-specific genes (Koussevitzky et al. 2008; Atkinson et al. 2013; Prasch and Sonnewald 2013). Plants could be made capable to withstand these adverse conditions by the utilization of stress-tolerant plant growth-promoting microorganisms (PGPM) and arbuscular mycorrhizal (AM) fungi (Nadeem et al. 2014). Others include strains belonging to the genera *Acinetobacter*,

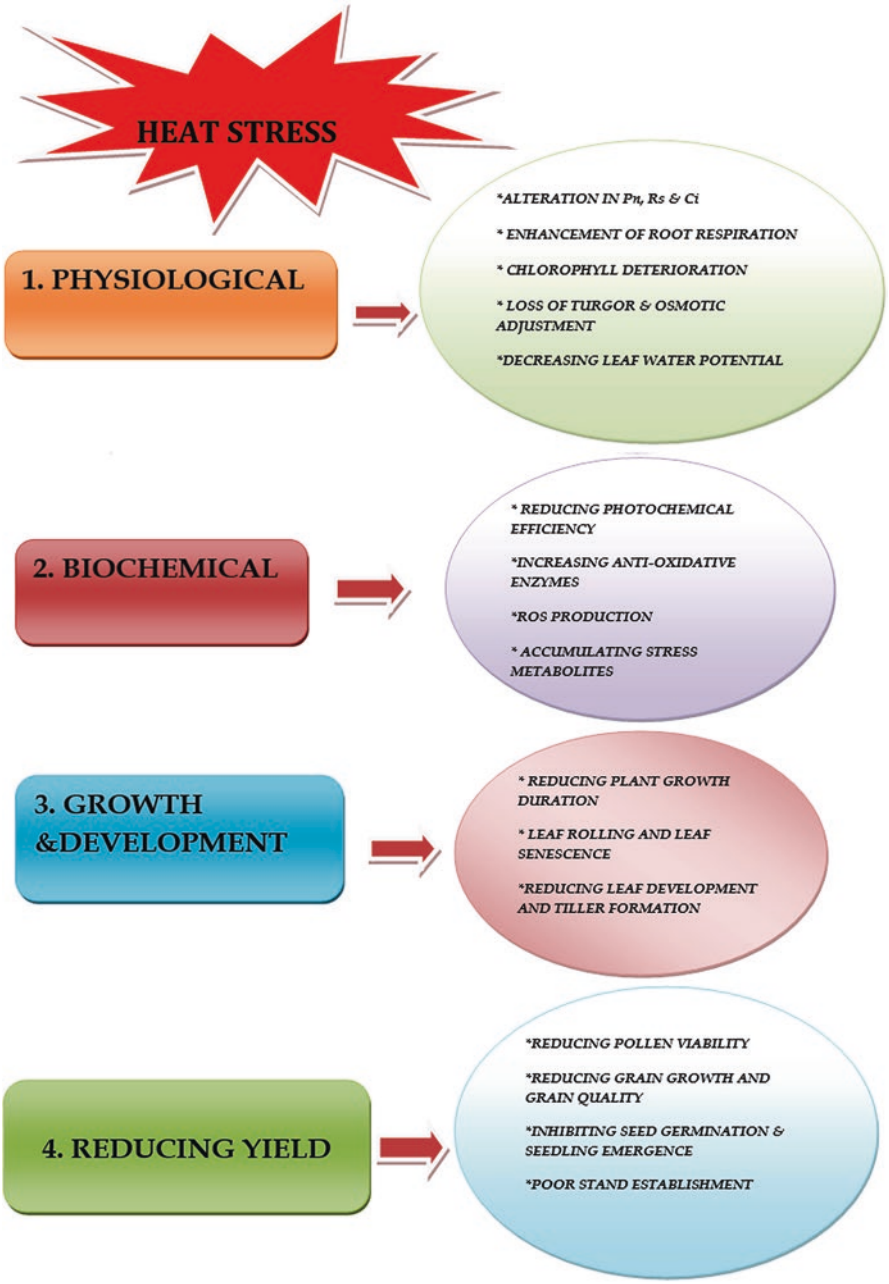

Fig. 13.5 Rice response to heat stress

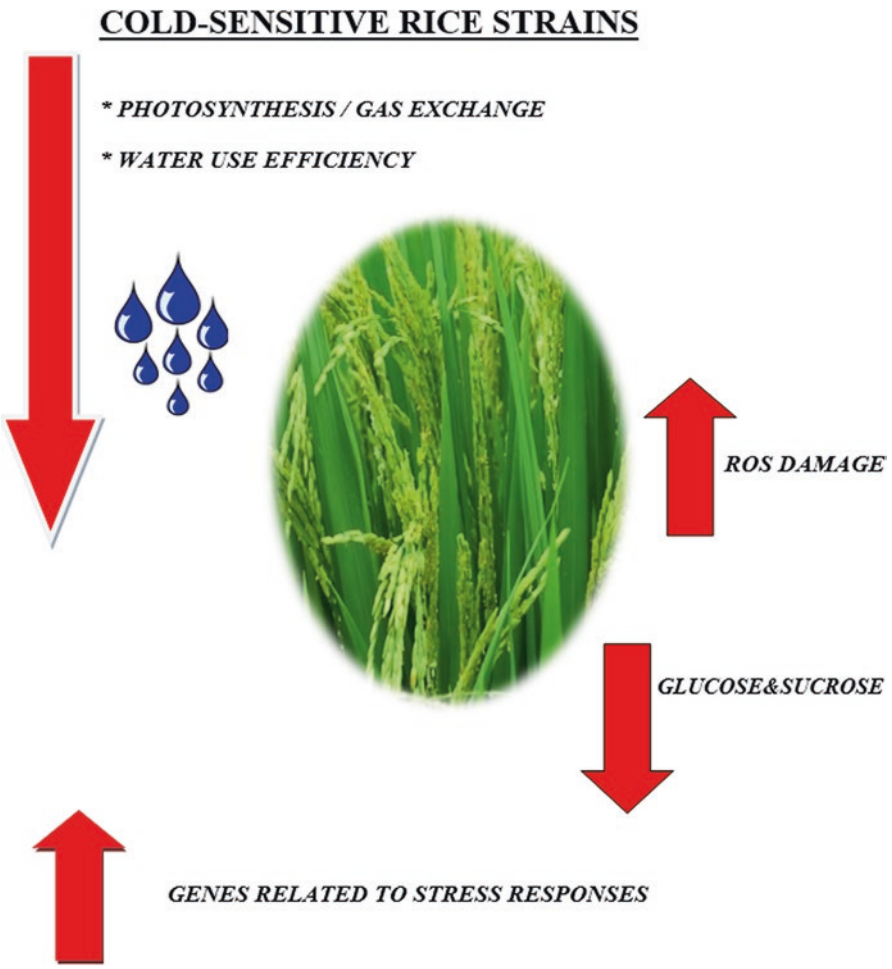

Fig. 13.6 Rice response to cold stress

Alcaligenes, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, Enterobacter, *Erwinia*, *Rhizobium*, *Flavobacterium,* and *Serratia* (Bashan and de-Bashan 2005; Maksimov et al. 2015). These microorganisms help plant growth by correcting atmospheric nitrogen by synthesizing plant hormones (Ahmad et al. 2011). Chauhan et al. (2015) stated that production of cytokinins through the process of induced systemic tolerance (IST), antioxidants, and bacterial ethylene precursor 1 aminocyclopropane-1-carboxylate (ACC) by plant-associated bacteria can help to tolerate abiotic stresses more proficiently. Microbes have systems for nutrient accumulation, exopolysaccharide production, and rhizobitoxine, which help the plant cope with adverse conditions (Vardharajula et al. 2011). These microorganisms develop important enzymes such as glucanase and chitinase under stress circumstances (Farooq et al. 2009). In sustainable

agriculture, the application of PGPM increases the yield and nutritional value of food grains. Fatnassi et al. (2015) reported that more than 1 mm of copper (Cu) inhibits the vegetative growth of *Vicia faba*, but with *Rhizobia* and PGPR, its effects subsided. Harmful effects of cadmium stress on the reduction of malonaldehyde and hydrogen peroxide in crops are investigated (Hashem et al. 2016). Ghosh et al. (2015) reported that arsenic-resistant bacteria (ARB) from *Pteris vittata* is an effective siderophore manufacturer, enhancing plant nutrition through phosphate solubilization.

Andrews (1992) and Pandey et al. (2012) stated that endophytic bacteria were found to be more potent in providing strong immune responses against stress than rhizospheric or bulk soil microorganisms. Endophytes, *viz.* bacteria and fungi, reside within healthy plant cells and can stimulate plant growth under stress without causing any harmful effects. Depending on the pathogen, they may or may not be pathogenic microorganisms in the endophytic relationship, and may be pathogenic to another host, or non-pathogenic, but can still be colonized by selection methods or genetics (Backman and Sikora 2008). An individual plant is considered to be a host for more than one endophyte (Strobel et al. 2004; Ryan et al. 2008). Environmental changes alter the metabolism of plants from homeostasis, forcing the plant to adapt to systems within the cellular system (Gill and Tuteja 2010). The significance of endophytes is greatly enhanced by the fact that they help plants grow by increasing nutrients, water efficiency (osmotic adjustment, stomata regulation), and reducing abiotic stresses. Instead, endophytes gain access to the nutrients of the host plants and their distribution to the next generation. However, *Bacillus spp.* has a special interest in, has been identified as a dominant community, and has been commercialized as it exists within the boundaries of biotic and abiotic environments (Kilian et al. 2000). *Bacillus*-based biofertilizers, such as Kodiak (*B. subtilis* GB03), Quantum-400 (*B. subtilis* GB03), Rhizovital (*B. amyloliquefaciens*FZB42), Serenade (*B. subtilis* QST713), and YIB (*Bacillus spp*.) have been commercialized for improved crop yield due to high metabolic production (Radhakrishnan et al. 2017). Endophytic bacteria are resistant to metals, thereby promoting plant growth under metal stress by providing a direct supply of mineral nutrients, plant growth regulators, and enzymes. Some PGPMs produce low-molecular-weight organic acids, which play a major role in phytoremediation. Ullah et al. (2015) and Janoušková et al. (2006) reported that citric, oxalic, and gluconic acids are the most effective in accumulating and supplying heavy metals to plants.

Numerous studies and statistics have attempted to identify bacteria belonging to *Bacillus* (Yu et al. 2011; Gaind and Gaur 1991; Zaidi et al. 2006) genera that can help to alleviate the abiotic stresses of many crops, especially rice (Bhambure et al. 2018). Not all PGPBs can withstand different environmental pressures. Therefore, research that incorporates endophytic bacteria as stress alleviators is a good job of combating the adverse effects of abiotic factors.

13.7 *Bacillus* spp.: A Multifunctional Toolbox

Many microorganisms are proved to be naturally beneficial to plants and helpful for maintaining plant growth and yield under abiotic and biotic pressures. PGPB and fungus, which inhabit the endophytic part, phyllosphere part, and rhizospheric part, cause plant growth and protect the plants from diseases and abiotic factors (Tonelli et al. 2010; Radhakrishnan et al. 2014). It includes many PGPB such as *Bacillus* spp., *Agrobacterium, Pseudomonas*, and Azo*spirillum.* Among them, genus *Bacillus* spp. is the most naturally occurring PGPB which produces phytohormones which are able to regulate the endogenous level of phytohormones in plants, thereby balancing the overall hormonal balance of the plant and its reaction to stress (Glick et al. 2007; Kundan et al. 2015).

Most of the PGP microorganisms have multifunctional features (e.g. indole acetic acid production, siderophore production, potassium, phosphate, zinc solubilization), abiotic stress tolerance, and resistance to plant pathogens and withstand extreme environmental conditions (Hassan 2017; Gond et al. 2015). In agricultural field, *B. pumilus*, *B. thuringiensis*, *B. amyloliquefaciens*, *B. subtilis*, *B. velezensis*, *B. cereus*, and *B. licheniformis* are the most studied *Bacillus* spp. (Lopes et al. 2018). Endophytic *Bacillus* spp. has been mainly documented to produce a vast variety of antimicrobials, including compounds that block a wide variety of pathogens that exhibit broad-spectrum activity (Lopes et al. 2015; Jasim et al. 2016a, b; Hu et al. 2017). They are also reported to control quorum sensing-regulated virulence in phytopathogenic bacteria (Lopes et al. 2018). They act as an elicitor of plant immunity and have the ability to induce plants to an improved immune system condition called induced systemic resistance (ISR). Kang et al. (2015a) and Kuan et al. (2016) investigated that many *Bacillus* spp. can convert the complex/complicated form of essential nutrients P and N into the simplest available form. *Bacillus* spp. releases ammonia from nitrogen organic matter (Hayat et al. 2010). Ding et al. (2005) stated that few *Bacillus* spp. can undergo nitrogen fixation and produce a gene that contains the enzyme nitrogenase, which delays senescence by increasing plant growth (Kuan et al. 2016). Shabanamol et al. (2018) reported the production of phytohormones (IAA, GA, cytokinin), synthesis of siderophores, ACC deaminase activity, biological N_2 fixation, phosphate solubilization, ammonia production, and biological resistance by rice endophytic diazotrophic bacteria *Lysinibacillus sphaericus*. The use of endophytic nitrogen-fixing bacteria with gibberellic acid (GA) production leads to effective and direct plant growth (Shabanamol et al. 2018).

Under controlled field conditions, *Bacillus* spp. adopt various mechanisms to promote plant growth such as synthesis of plant growth hormones (e.g. indole acetic acid, gibberellins, cytokinins, and spermidines) (Arkhipova et al. 2005; Xie et al. 2014; Radhakrishnan and Lee 2016; Hassan 2017, Shabanamol et al. 2018), secretion of 1-aminocyclopropane-1-carboxylate (ACC) deaminase for inhibition of ethylene synthesis (Yaish et al. 2015), solubilization of minerals through biofortification, nitrogen fixation or ammonia release from nitrogenous organic matter (Zhao et al. 2011; Hassan 2017; Yousaf et al. 2017; Shabanamol et al. 2020), and production of

volatile organic compounds (VOCs) which regulate the metabolism of phytohormones (Tahir et al. 2017; Rath et al. 2018). For, e.g. *B. amyloliquefaciens*, which produces gibberellins and regulates endogenous phytohormones (Shahzad et al. 2016), conformational nitrogen fixation ability by nif gene analysis by *L. sphaericus* (Shabanamol et al. 2018), *B. atrophaeus*, which produces IAA and siderophores (Zhao et al. 2015; Yaish et al. 2015), and *B. aryabhattai*, which produces IAA, ammonia, and ACC deaminase (Yaish 2017), Zhao et al. (2015) investigated that *B. megaterium* produces IAA, ACC deaminase, and siderophores, as well as have the ability to solubilizes phosphate, and also reported *B. mojavensis* produces plant growth-stimulating VOCs (2,3-butanediolandacetoin) (Rath et al. 2018).

13.8 *Bacillus* spp. Mediated Alleviation of Abiotic Stress in Rice

Plant growth and development are restricted due to environmental pressures and stresses created by living communities. Two mechanisms by which plants can survive the abiotic stress are (i) activation after stress exposure (Meena et al. 2017) and (ii) endophytic synthesis of biochemical agents, working as anti-stress agents (Schulz et al. 2002) to regulate abiotic stressors. As a result, plants undergo similar transformations, such as physiochemical, biochemical, and molecular changes (Potters et al. 2007; Huang et al. 2008). Numerous studies by researchers proved that various strains of endophytic *B. subtilis* protect host plants from a variety of abiotic stresses and contribute to increased plant growth and yield (Turan et al. 2012; Ahmad et al. 2017; Martin et al. 2017; Egamberdieva et al. 2017; Martins et al. 2018; Wani et al. 2018, Karthika et al. 2020).

13.8.1 Drought Tolerance by Bacillus *spp.*

Under drought conditions, *Bacillus* spp. colonized plants take more water (Marulanda et al. 2009) and are reported to take nutrients such as N, P, K, Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+} (Zawadzka et al. 2009; Barnawal et al. 2013; Armada et al. 2014). Sandhya et al. (2011) investigated the application of *Bacillus* spp. in soil, which increases the population of bacteria in roots resulting in the stimulation of root exudation promotes both bacterial and plant growth and also migitates the disturbances in normal cellular physiological and biochemical processes and prevents the formation of ROS (Zgallaï et al. 2005; Miller et al. 2010), which results in the degradation of proteins, lipids, and nucleic acids. Barnawal et al. (2013). In drought-affected plants, PGPB such as *Bacillus* spp. accumulate endogenous amino acids and synthesis of chlorophylls pigments such as chlorophyll a and b and carotenoids, thereby triggering the production of metabolites,

which causes oxidative stress reduction and increased photosynthesis. Under adverse conditions, EPS suppresses plant drought resistance by combining suitable solvents and fertilizers (Chitrasree et al. 2011). Bacteria retain membrane permeability by the accumulation of glycine, proline, betaine, and trehalose in drought conditions. Ortiz et al. (2015) stated that mycorrhizal interactions of *Pseudomonas aeruginosa* with specific bacteria, such as *B. thuringiensis*, reduce stomatal conductivity and electrolyte leakage as proline accumulates in the shoot and root.

Gusain et al. (2015) investigated that *Bacillus* spp. cultured rice was found to be mitigating oxidative damage by improving plant growth and activating antioxidant immune systems, which increases the membrane stability in plant cells. Inoculation of crops with *B. altitudinis* FD48 increased the accumulation of relative water content (RWC) and proline which suggested to contribute to osmoregulation during stress tolerance in plants (Kumar et al. 2017) and also showed increased catalase activity than the control plants. *Bacillus altitudinis* FD48 treated rice plant reported less ethylene emission, which usually increases when rice plant was subjected to drought conditions. *Bacillus altitudinis* FD48 was also reported to show inhibitory effect induced by drought stress through the ACC deaminase activity (Zahir et al. 2008). Gusain et al. (2014) stated that inoculation with *B. cereus* shows better parameters and prevention of forming ROS such as superoxide dismutase, catalase, and peroxidase as compared to uninoculated plants under drought stress.

During environmental stressed conditions, production of ROS such as H_2O_2 and catalase is regulated by bacteria, which makes them act as scavenging enzymes, thereby protecting oxidative damage of cells (Shukla et al. 2012, Sandhya et al. 2011 and Gusain et al. 2015), such as catalase and exopolysaccharides; producing ability of rhizobacteria has been already reported (Hussain et al. 2014). It has been recently reported in rice crop plants inoculated with *B. amyloliquefaciens* SN13 (Nautiyal et al. 2013; Kim et al. 2014a; Tiwari et al. 2016) improved the presence of stress-induced symptoms in rice such as membrane integrity, accumulation of osmoprotectants, and expression of marker genes. RoyChoudhury et al. (2007) and Kumar et al. (2014) reported that overexpression of two stress tolerance marker genes LEA and DHN provides abiotic stress tolerance in several crops as well as its expression is increased during the elevated expression of dehydration responsive element binding (DREB) in rice (Lata et al. 2011).

13.8.2 Salinity Tolerance by Bacillus *spp.*

Salinity is the commonest of all the abiotic stresses in modern agriculture. It affects the growth of plants and microorganisms through its osmotic effect and ion poisoning. Salinity has detrimental effects on seed germination and disrupts the physicochemical and ecological balance (Shrivastava and Kumar 2015). The role of PGPM and endophytic microorganisms in the mitigation of salt stress in various plants has been reported in detail. The formation of PGPB biofilms under salt pressure is the most effective strategy for mitigating adverse effects (Kasim et al. 2016). Under salt

stress, in order to maintain cell turgor and plant growth, crop inoculated with PGPB accumulates osmolytes in their cytoplasm, which counteract osmotic stress. Damodaran et al. (2013) demonstrated that *B. pumilus* and *B. subtilis* isolated from rhizospheric part of saline soil displayed PGP properties, *viz.* IAA production, ammonia production, phosphate, zinc, and potassium solubilization, and hydrogen cyanide (HCN) and also salinity tolerance.

Inoculation of paddy with *B. pumilus* and *B. subtilis* showed PGPR characteristics (Damodaran et al. 2013). Many PGPB such as *Mycobacterium phlei, P. alcaligenes* PsA15, *B. polymyxa* BcP26, and MbP18 have also shown their potential to survive in saline soils like calcisol soil. Rice plants inoculated with *P. pseudoalcaligenes* and *B. pumilus* will increase salinity as they accumulate high concentrations of glycine betaine (Jha et al. 2011). Jha and Subramanian (2014) investigated that the PGPB inoculated crops reduce lipid peroxidation and superoxide dismutase activity in GJ-17 in salt-sensitive rice during salt stress. Inoculation of salt-tolerant *B. amyloliquefaciens* NBRISN13 (SN13) into rice plants increases growth and salt tolerance through upregulation and repression of 14 genes in rice plants (Tank and Saraf 2010; Bal et al. 2013; Nautiyal et al. 2013). Whereas the inoculation of rice with *B. amyloliquefaciens* (SN13) downregulates the expression of LEA and DHN the stress marker genes under salt and heat stress, this demonstrates the crucial role of (SN13) in rice seedling stress alleviation as well as its active participation in osmolyte biosynthesis and subsequent osmotic adjustment (Tiwari et al. 2017). Shahzad et al. (2017) reported that inoculation of rice with *B. amyloliquefaciens* RWL-1 induced salinity stress by the production of ABA and auxin. Hashem et al. (2015) proved that *B. subtilis* enhanced lipid synthesis, *viz.* oleic, linolenic, and linoleic acids as well as phospholipids, in plants grown in saline conditions.

Jha and Subramanian (2014) investigated that *B. pumilus* associated with the roots plants reduce the activity of caspase, which is a protease that belongs to the cysteine endopeptidase family and engages in programmed cell death in plants. Inoculation with *B. subtilis* RH-4 enhances seed germination and plant growth and improves the synthesis of carbohydrates, photosynthetic pigments, proteins, proline, betaine, glycine, and choline in the injured chickpea plants. Plant-associated bacteria increases secondary metabolites such as gallic acid, caffeic acid, syringic acid, vanillic acid, ferulic acid, cinnamic acid, and quercetin, which allow plants to tolerate salinity and stimulate the expression of NADP-Me2 (NADP-malic enzyme 2), EREBP (ethylene-responsive element-binding protein), SOSI (salt overly sensitive 1), BADH (betaine aldehyde dihydride), and SERK1 (somatic embryogenesis receptor-like kinase 1) (Nautiyal et al. 2013; Tiwari et al. 2011). They found that reducing caspase activity reduced ROS formation and programmed cell death and restored the activity of antioxidants to increase plant tolerance. During saline conditions, SAPK4 (serine-threonine protein kinase) and GIG (gigantea) genes in plants are downregulated.

13.8.3 Heavy Metal Tolerance by Bacillus *spp.*

The use of microbial diversity to aid the solution of heavy metals is a recent strategy to protect and preserve the environment from their toxic effects. Microbes are good indicators for mitigating stress exhibited by heavy metals (Chen et al. 2014, Broos et al. 2004;). Mycorrhizae, heavy metal tolerant organisms, and plant-related microorganisms, have the potential for plant growth promotion and development under metal stress. Glick (2010) reported that these microorganisms are involved in several mechanisms, including EPS sequestration, water flow, metal insufficiency, destabilization, enzymatic detoxification, and metal complexation. This PGPM promotes the growth of plants by producing growth regulators such as IAA, ACC deaminase, and disease suppression. In addition, nitrogen fixation ability, nutrient accumulation, siderophore production, and phosphate, zinc, and potassium solubilization increase plant growth and removal of heavy metals (Verma et al. 2013; Ahmad et al. 2011). Vigliotta et al. (2016) reported that the combined use of heavy metal resistant bacteria from *Bacillus, Lysinibacillus (*Shabanamol et al. 2018*),* and *P. aeruginosa* might improve phytoremediation of heavy metals. *Bacillus* spp. associated plants showed to mitigate this stress effect by increasing amylase and protease, thereby reducing lipid peroxidation and SOD activity (Pandey et al. 2013). Some *Bacillus* spp. increase P and Ca content and reduce Ni accumulation in plants growing in contaminated soil (Jamil et al. 2014). Also, the inoculation *B. licheniformis* enhances Cu, Zn, Cd, Cr, and Pb accumulation and distribution in plants grown in heavy metal-contaminated soil, which causes a decrease in soil toxicity (Brunetti et al. 2012).

Ma et al. (2016) stated that heavy metal remediation by PGP beneficial microorganisms goes through different mechanisms such as the removal of metals, biosorption, precipitation, extrusion, active removal, biotransformation, or bioaccumulation of metals in the external and intracellular spaces of plants. When wheat is inoculated with *Bacillus* spp., AMP2 in chromium salts ($CrCl_3$, K_2CrO_4, $K_2Cr_2O_7$) reduced the Cr acquisition by 10–20 μg mL^{-1}.

Bacillus subtilis inoculated rice (*Oryza sativa* L.) can help to reduce Cd accumulation in all parts of rice roots, shoots (45 days), and grains (120 days) (Treesubsuntorn et al. 2018). Here, the heavy metals were effectively absorbed by *B. subtilis* than *B. cereus*, which might be the chief mechanism to decrease the heavy metal transport Cd, Ca, and Mg+ in rice plants. Plants that were inoculated with *B. subtilis* had the highest levels of Ca and Mg compared to plants inoculated with *B. cereus.* In addition to other stress factors, *B. subtilis* could increase the dry weight of the rice plant and protect them from Cd stress due to the ability to produce IAA, ACC deaminase activity, and phosphate solublization. They proved that sub-stylus can increase the dry weight of paddy and protect it from CD stress. Survival and plant growth intoxicated soil is acquired by the synthesis of mineral nutrients and pigments and by the *Bacillus* spp. association.

13.8.4 Temperature Tolerance by Bacillus *spp.*

Temperature plays an important role in the physiochemical and metabolic regulation of microorganisms living in extreme temperature environments. Plant-associated microbial enzymes help to adapt to low and high temperatures. Microorganisms contain special mechanisms that protect their proteins, membranes, and nucleic acids. Yadav et al. (2015) have shown that *Brundimonas terrei*, *P. cedrina*, *and Arthrobacter nicotianae* are suitable for thriving at lower temperatures and showing the ability to multifunctional plant growth promotion. Psychrophiles and psychotrophs are two groups of microorganisms. Psychotrophic microorganisms thrive at temperatures above 15°C, while the maximum growth of psychrophilic organisms is below 15°C. In comparing with other stress conditions, plant-associated *Bacillus* spp. makes a sustainable growth in a wide range of temperatures ranging from 31°C to 76°C (Desriac et al. 2013).

Chang et al. (2007) reported that during heat stress (HS) condition causes the production of heat shock proteins and production of normal cellular proteins. This allows the paddy plants to develop HS tolerance. Heat stress is an induced expression of microbial survival. The DnaK gene expression in *Alicyclobacillus acidoterrestris* increases the HSP code during heat stress, which protects microorganisms from thermal stress.

Bacillus subtilis strain 330-2 contains many genes associated with heat tolerance (elongation factor Tu; aspartokinase II; and dihydroorotase, *pyrC*) and cold stress (sporulation cortex protein, and *coxA*), and they are differentially expressed in the *B. subtilis* strain 330-2. Trehalose stimulates synthesis during heat stress and protects plant-associated microorganisms from temperature shock injuries and oxidative stress. The accumulation of trehalose in bacteria and fungi increases the heat stress by several times. Li et al. (2009) showed that trehalose deposited in the microbial cell during hot and cold pressures protects them from thermal deformation. Trehalose is the most active against freezing and desiccation. Trehalose can protect proteins from heat-induced protein denaturation. The amount of metabolites varies according to the plant and the microorganism. Psychrophilic bacteria from Antarctica show antimicrobial activity. In agricultural sectors, inoculation of thermotolerant phosphate solubilizing microbes or stress-tolerant microorganisms acts as a multifunctional biofertilizer (Javani et al. 2015).

13.9 Conclusion

Different types of abiotic stresses such as drought, salinity, heavy metals, cold, and heat stress adversely affect the different growth and transformation stages of crop plants. Morpho-physiological changes commonly occurring in rice due to abiotic stress include impaired seed germination due to cessation of meristematic cell activity in young seedlings, decreased photosynthetic rate, decreased leaf/plant size,

reduced leaf size, and reduced plant height. Crops and plants can change their physical and biological properties by exposure to cold, heat, drought, and saltwater, as well as alkali-tolerant proteins when subjected to stress. These barriers are major barriers to crop production, food quality, and global food security. Continuous effects include hormonal imbalances, nutrient instability, ion toxicity, and the risk of disease. The only long-term and environmentally friendly solution to this problem is to develop some microbial tools and technologies that will benefit from plant-microbial-soil interactions. Application of stress-tolerant microbial consortium of *Bacillus* genera PGPM strains and mycorrhizal fungus can be used to enhance plant growth. These microorganisms can lead to plant growth promotion by enhancing the antioxidant system by regulating plant hormones, nutrient enhancement, and siderophore production. Other systems include induced systemic resistance and systemic acquired resistance (SAR) in multiple mechanisms. Arbuscular mycorrhizae increase the supply of nutrients and water under stress conditions and increase the tolerance of plants to various stressful conditions. The use of microorganisms can solve future food security problems and maintain soil health. Therefore, this chapter aims to shed light on the important role that microorganisms play as environmental engineers in solving environmental stress problems. Based on these reviewed data, we recommend scientific societies and policy planners to use the versatile, *Bacillus* spp. in biofertilization formulations, which can be used to solve problems of biotic and biotic stress and to have a better plan for their impact globally. Therefore, considering the current situation, future research is needed to identify highly effective stress-tolerant PGPM. Of course, the diversity of microbial strains should be tested to form an effective microbial consortium to overcome the negative impact of the changing environment.

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Chapter 14
Growth Enhancement and Bioremediation of Heavy Metal in Crop Plants Through *Bacillus* Species Application

Saidu Abdullahi, M. A. Baset Mia, and Amir Hamzah Ghazali

Abstract Mitigating the effects of heavy metals and their subsequent remediation on plants is one of the hot topics of environmental research studies. Application of *Bacillus* species in this area of research has received considerable attention probably due to the high rate of adaptability and survivability of the species under extreme environments. *Bacillus* spp. have shown great potential in plant growth enhancement and bioremediation of heavy metal–contaminated soils. More *Bacillus*–plant physiological studies are required for better understanding of the mitigation mechanisms of *Bacillus* spp. against heavy metal stress conditions and plant growth promotion. Our findings have successfully shown that Bacilli have multiple beneficial traits which assisted the crop plants either directly or indirectly through plant growth–promoting activities and heavy metal tolerance enhancements.

Keywords Bioremediation · Heavy metal toxicity · Growth enhancement · Crop plants · Environmental pollution

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14.1 Introduction

Bacillus species represents one of the most important and dominant groups of microbes that exist in various environments with a high rate of survival in extreme and adverse environmental conditions (Islam et al. 2019). The gram-positive or facultative gram-negative spore-forming bacteria can survive for a long time under unfavourable environmental conditions (Radhakrishnan et al. 2017), and spore formation is one of the most important characteristics of *Bacillus* spp. The aerobic endospore-forming bacteria are ubiquitous in agricultural systems due to possession of certain physiological traits associated with their survival which include production of a multilayered cell wall, production of endospores that are stress-resistant, secretion of antibiotics, peptide signal molecules, and extracellular enzymes (Gardener 2004). The cell-wall-degrading substances (protease, cellulase, chitinase, glucanase, hydrogen cyanide, and lipopeptides) from *Bacillus* spp. damage the pathogenic organisms including bacteria, fungi, viruses, nematodes, and pests to control their populations (Radhakrishnan et al. 2017). *Bacillus* spp. also have great potential for applications in agriculture, industry, and medicine and are a good source of important metabolites and enzymes of various biotechnological interests. As an example, it was shown that *B. thuringiensis* H-14 is able to kill mosquito larvae within 24 h of treatment with the lowest concentration of spore-crystal suspension (0.05 mg/L) (Fun et al. 2016).

There is an increasing interest in *Bacillus* spp. on the aspects of agricultural and environmental biotechnology due to increasing demand for food production, recovery of degraded soils as well as the development of biofertilizers and biopesticides (Nascimento et al. 2020). The unfavourable biotic and abiotic stimuli affect normal plant metabolism, suppressing the growth and yield of plants, however, the stress factors on crops are mitigated by *Bacillus*-induced physiological changes (Radhakrishnan et al. 2017). Under unfavourable environmental conditions including heavy metal stress, *Bacillus* produces important substances such as siderophores and exopolysaccharides, which prevent the movement of toxic ions and adjust the ionic balance and water transport in plant tissues (Radhakrishnan et al. 2017). *Bacillus* can live both outside and within plant tissues, facilitating plant growth and development as well as protection from harsh environmental conditions through several mechanisms including N_2 fixation, production of indole acetic acid (IAA), siderophore, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Additionally, phosphate and potassium solubilization and production of exopolysaccharides show antagonistic actions against pathogens and other pests. There are many bacterial species that are being applied as plant growth-promoting bacteria (PGPB) to crop plants; however, members of the *Bacillus* group are more favoured for commercialization as PGPB due to some features including their ability to produce the heat and desiccation-tolerant endospores, which help to maintain high cell viability and prolong the shelf life of the bacteria in the carrier formulations (Akinrinlola et al. 2018). Bacterial colonization of roots provides a nutrient source, and the plants on the other hand, receive bacterial metabolites and other stimuli that

enhance growth and stress resistance (Hashem et al. 2019). In this relationship, the microsymbiont (e.g. *B. subtilis*) will form a thin biofilm layer on the root surfaces for long-term bacterial colonization of the host plants (Hashem et al. 2019). Additionally, the rhizosphere of the host plants is also heavily colonized by beneficial bacterial populations. Nowadays, plant growth-promoting *Bacillus* spp. are gaining prominence as biofertilizer, biopesticides, and bioremediator of certain mineral elements that are toxic to crop plants (Amir et al. 2005; Fun et al. 2016; Mia et al. 2010; Salwani et al. 2012; Tang et al. 2020). Salwani et al. (2012) revealed that the beneficial diazotrophic microsymbionts of leguminous cover crop *Mucuna bracteata* were not only from the conventional Alphaproteobacteria class (e.g. *Brevundimonas* sp.) but were also from the Betaproteobacteria class (e.g. *Achromobacter* sp. and *Burkholderia* sp.) and the Gammaproteobacteria class (e.g. *Stenotrophomonas* sp.). The authors also successfully obtained and identified *Bacillus* sp. from the root nodules of the host plants.

Heavy metal may be found naturally in the soils or added to the soils through various anthropogenic activities such as exploitation of mines and smelters, fossil fuel combustion, metallurgical and electronics industries, indiscriminate waste disposal, and military training. Additionally, the agricultural practices involving excessive application of inorganic fertilizers and chemicals for optimum crop improvement are also contributing towards the escalated accumulation of heavy metal in soils (Kapahi and Sachdeva 2019; Oves et al. 2016). Examples of heavy metals which are contaminating soils and water bodies include As, Cd, Pb, Hg, Cr, Co, Cu, Hg, Ni, Zn, Se, U, Mn, and Ni (Weissmannová et al. 2019). The continuous release of heavy metals into the environment severely affects soil and water quality. Despite the fact that waste water may serve as an important source of essential nutrients for plants, many risks can be attributed to the use of such waste water for crop irrigation due to presence of toxic contaminants including heavy metal (Khalid et al. 2018). Many heavy metal are toxic even at very low concentrations, and some (e.g. As, Cd, Cr, Hg, Ni, Cu, Pb, and Zn) are not only cytotoxic but also mutagenic and carcinogenic in their nature (Dixit et al. 2015).

Heavy metal contaminated soils are generally deficient in essential nutrients, and if the contaminated soils are used for crop production, there is a huge risk of metal being transferred into food chain at higher concentration. It can cause severe human health problems (Oves et al. 2016). Certain heavy metal is essential for plant growth and health at certain concentrations. For example, Co is required in nitrogenase enzyme as metal activator for N_2 fixation both in legume and non-legume crops, and Mo is needed as catalytic centre of many enzymes namely nitrate reductase. However, when the elements exceed the required quantities, they may cause serious injury or even lead to death of plants (Oves et al. 2016). Biological methods for remediation of heavy metal known as bioremediation are considered most eco-friendly, cost-effective, reliable, and have no adverse effects to the environment (Dixit et al. 2015). There is an increasing interest towards studying the role of microorganisms in biotransformation and detoxification of heavy metals and the production of plant growth promoting substances by them under stress conditions (El-Meihy et al. 2019; Nayak et al. 2018). Understanding various mechanisms of

metal accumulation and plant growth promotion has numerous biotechnological implications for bioremediation of heavy metal and crop productions in heavy metal contaminated soils. Mitigating the effects of heavy metal and their subsequent remediation on plants is one of the hot topics of environmental research nowadays. Application of *Bacillus* species in this area of research has received considerable attention (El-Meihy et al. 2019; Li et al. 2019; Tang et al. 2020) probably due to the high rate of adaptability and survivability of the species under extreme environments (e.g. heavy metal stress tolerance and their multiple beneficial mechanisms of actions) (Radhakrishnan et al. 2017). However, very few study reports are available on the application of *Bacillus* in bioremediation of heavy metal in plants. Most of the relevant studies were carried out on the physiological mechanisms of plant growth promotion or heavy metal tolerance of the individual *Bacillus* spp.. Rhizobacteria viz. *Bacillus* spp. (*B. megaterium, B. cereus,* and *B. pumilus*), isolated from *Ludwigia octovalvis,* were capable of absorbing As and can be good candidates for bioremediation of toxic effects of that element on plants (Titah et al. 2018). More studies on heavy metal tolerant and plant growth promoting *Bacillus* and other bacteria to harness their potentials as bioremediation or phytoremediation agents are therefore still needed. In this chapter, potentials of *Bacillus* spp. on promoting growth of associated selected host plants and their ability to remediate heavy metal are discussed.

14.2 Production of Plant Growth Promoting (PGP) Substances by *Bacillus* Species

14.2.1 Production of PGP Substances by Individual Bacillus *Species Under Culture Conditions Supplemented with Heavy Metal*

Some of *Bacillus* spp. namely *B. sphaericus* strain UPMB10 are able to produce phytohormone like auxin and gibberellin which resulted in increased root growth in banana and oil palm under hydroponics and pot culture conditions (Mia et al. 2010; Amir et al. 2005). A large body of studies reflected that *Bacillus* species can produce plant growth promoting substances even under heavy metal stress conditions (Wu et al. 2019). Rizvi et al. (2019) also stated that heavy metal tolerant *B. subtilis* strain BM2 was able to synthesise variable concentrations of indole acetic acid (IAA) when cultured under different concentrations of Pb and Ni. However, the highest concentration of IAA was produced by the bacterial strain (BM2) when cultured in metal free medium (51.6 μg/mL), which kept declining with increasing concentrations of the added metal. At 400 μg/mL Pb and Ni, IAA secretion was reduced by 58 and 47%, respectively. Despite the toxicity and inhibitory effects of the metal, the strain maintained reasonable amount of IAA production potentiality. The strain was also able to produce substantial amount of ACC deaminase and

siderophore when cultured in media supplemented with high concentrations of metal. However, the siderophore production by the strain was not detected on CAS agar with metal supplementation but detected under liquid medium with the metal supplementation. The cause of siderophore not detected in CAS agar but in liquid medium with metal supplementation remains unknown, but likely due to the presence of HDTMA (hexadecyltrimethyl-ammonium bromide) in the solid agar (Rizvi et al. 2019).

Bacterial IAA induce root elongation and development to overcome heavy metal toxicity and improve plant growth, while ACC deaminase, on the other hand, metabolizes the ethylene precursor ACC, thereby alleviating the effects of ethylene stress due to the metal and helps in plant survival under stress conditions (Mishra et al. 2017). Siderophore produced by *Bacillus* and other plant growth promoting bacteria is one the most important substances that can directly alleviate heavy metal toxicity by binding/chelating heavy metal ions and increasing the supply of iron to plants (Nayak et al. 2018). Therefore, maintenance in the production of plant growth promoting substances by *Bacillus* spp. under heavy metal stress could play an important role in improving growth of the host plants in addition to reduce the toxic effects of the metal on the plant.

14.2.2 Production of PGP Substances by Bacillus *Species in Association with Plants*

Nitrogen is an important essential nutrient which is required for growth and development of plants, taken up as NO_3^-, NH_4^+, and N_2 (*via* biological nitrogen fixation process) (Bellogín et al. 2014). Atmospheric nitrogen fixing bacteria form symbiotic association on the roots of plants where they convert nitrogen into form that can be used by plants, a process mainly restricted to leguminous plants. However, several non-symbiotic bacteria have been recognized including many *Bacillus* spp. as free living nitrogen fixers in many plants of agronomic importance. Various species of *Bacillus* were reported to fix atmospheric nitrogen which include *B. cereus, B. marisflavi, B. megaterium, Paenibacillus polymyxa,* and *P. massiliensis* (Ding et al. 2005; Halim et al. 2016; Nayak et al. 2018; Tang et al. 2020). Others were able to solubilize phosphates like *B. cereus, B. thuringiensis, B. megaterium, B. safensis, P. cineris,* and *B. subtilis* (Akinrinlola et al. 2018; Babu et al. 2013; Huang et al. 2020; Zaidi et al. 2006). Phosphorus, which mostly occurs in the soil in the form of non-soluble compounds and thus not always available for plant consumption (Bellogín et al. 2014), is converted to soluble form that can be absorbed and utilized by plants in the process of phosphate solubilization. Besides that, PGPB inoculation can also stimulate and increase uptake of other nutrients such as K, Ca, Fe, Cu, and Zn which may be achieved through acidification of the rhizosphere (e.g. by production of organic acids), and in general, decrease in soil pH increases solubilization of nutrients (Bellogín et al. 2014). Some *Bacilli* have iron-chelating properties through

the production of siderophores that are known to solubilize iron in the rhizosphere from minerals and organic compounds (Radhakrishnan et al. 2017). The siderophores produced bind insoluble Fe^{3+} and reduce it to soluble Fe^{2+}, which can then be utilized by plants.

14.2.3 Role of Phytohormone (IAA) and Enzyme (ACC Deaminase) in Mitigating the Heavy Metal Stress in Plants

The IAA production by plant-associated bacteria plays an important role in plant-bacterial interactions. IAA synthesized by beneficial bacteria can increase the number of root hairs and lateral roots as well as the total root surface area, leading to an enhancement of root exudation and mineral uptake from the soil (Kong and Glick 2017). Number of studies have also suggested that PGPB producing IAA may play essential role in improving metal phytoremediation, however, most did not provide definitive proof of the direct involvement of the IAA (Kong and Glick 2017). In a previous study, two PGPB strains *B. paralicheniformis* YSP151 and *Brevibacterium frigoritolerans* YSP40 isolated from *Brassica juncea* in a Pb-contaminated mine soils were used to inoculate similar host plants grown in a metal-contaminated soil (Yahaghi et al. 2018). Results from the trial showed enhanced growth and Pb uptake by the inoculated host plants and both bacterial strains were reported to have high IAA producing ability. Multiple plant growth promoting traits may together be responsible for survival and enhanced metal tolerance of the host plants grown in heavy metal contaminated soils. Some bacteria can stimulate plant growth either by synthesizing more IAA or by degrading excess synthesized IAA when it is detrimentally higher than normal levels (Kong and Glick 2017).

Increase in ethylene synthesis is mostly associated with various environmental stresses such as heavy metal stress, extreme temperatures, drought, high salinity, organic pollution, insect damage, radiation, wounding, and various pathogens including viruses, bacteria, and fungi. Much of the plant growth inhibition that occurs due to environmental stresses is the result of increased levels of stress induced ethylene, which affects the plant's response to the stressor (Kong and Glick 2017). Some PGPB have great potential to decrease ethylene production that in turn enhance plant growth. This potential is due to the production of enzyme ACC (1-Aminocyclopropane-1-Carboxylate) deaminase. ACC is the precursor to ethylene synthesis and ACC deaminase enzyme hydrolyses ACC into a-ketobutyrate and ammonia (Ashraf et al. 2017). The ammonia generated add to the nitrogen source of the plants. ACC deaminase producing PGPB can facilitate plant growth and development by converting the ACC into α-ketobutyrate and ammonia, thereby reducing the levels of plant ethylene and providing some protection against growth inhibition caused by heavy metal and other stress factors (Kong and Glick 2017).

14.3 *Bacillus* Species and Heavy Metal Transformation, Detoxification, and Mobilization

Bacteria are known to enhance plant growth and survival under heavy metal stress conditions due to their ability of consuming and converting complex waste into simple non-toxic products/compounds (Tiwari and Lata 2018). Some heavy metal tolerant bacteria are also involved in enzymatic oxidation or reduction of toxic metal to a non-toxic or less toxic forms. Application of *Bacillus* spp. into heavy metal-contaminated soils can reduce the deleterious and toxic effects of heavy metal on growth of crop plants. For example, the Cr and As resistant *B. firmus* strain TE7 isolated from tannery effluent was able to reduce Cr(VI) to Cr(III) and oxidize As(III) to As(V) (Bachate et al. 2013). It reduced 100 mg/L Cr(VI) and oxidized 150 mg/L As(III) within 60 hours in nutrient broth and 10 hours in minimal medium. The hexavalent chromium [Cr(VI)] is the most toxic form of chromium, and arsenite [As(III)] the most toxic form of arsenic, and thus, their respective reduction and oxidation have great environmental importance as they affect their toxicity and mobility (Bachate et al. 2013). Furthermore, two *B. cereus* strains (*B. cereus* D and 332) were recently shown to have high Cr tolerance and reduction ability, and *B. cereus* D achieved 87.8% Cr(VI) removal in 24 h (Li et al. 2020). According to the authors, Cu (II) significantly increased the removal rate of the Cr (VI). Inoculation of *B. cereus* WSE01 was reported to increase the level of leaf enzymatic activity in *Myriophyllum verticillatum*, which, on the other hand, mitigate the effects of oxidative damage caused by reactive oxygen species resulting from heavy metal stress. Li et al. (2019) found that metal-resistant *B. thuringiensis* HC-2 was able to reduce the concentrations of Cd and Pb by extracellular adsorption and increasing the pH of the solution. Treatments of metal contaminated substrates with *B. thuringiensis* HC-2, biochar, and biochar combined with *B. thuringiensis* HC-2 significantly reduced water-soluble Cd and Pb concentrations by 34–56% and 31–54%, respectively and also increased the pH and NH_4^+ concentration in solution, in comparison to the values in a control.

14.4 Physiological and Biochemical Mechanisms of Heavy Metal Tolerance of *Bacillus*

The PGPB may contribute in reducing metal phytotoxicity through biosorption and bioaccumulation (Ma et al. 2016). Metal biosorption by bacteria comprised of two steps, which are passive and active biosorption (Ma et al. 2016). Passive biosorption of metal usually by living and dead or inactive cells take place in the cell wall due to number of metabolism-independent processes. Here, metal ions are adsorbed rapidly to the cell surface by reactions between metal and functional groups on the cell surface. Metal binding mechanisms like ion exchange, complexation, coordination, sorption, chelation, and precipitation may be involved. Active biosorption

(bioaccumulation), on the other hand, is referred to the uptake of metal by living cells through a much slower active metabolism-dependent transport of metal into bacterial cells. In heavy metal detoxification, studies carried out using different species, *B. licheniformis* NSPA5, *B. cereus* NSPA8, and *B. subtilis* NSPA13, indicated a significant level of Pb biosorption, with *B. cereus* having the maximum of 87–90% (Syed and Chinthala 2015). The negatively charged functional groups such as hydroxyl groups, phosphate groups, carbonyl groups, etc. that are present in biomolecules of microbial cell wall surfaces bind readily to heavy metal ions (Ojuederie and Babalola 2017). One of the most essential constituent in bacterial cells having ion sequestration capability is exopolysaccharide (EPS) (Ojuederie and Babalola 2017) mainly composed of complex high molecular weight organic macromolecules. Exopolysaccharides are known to protect bacteria against environmental stresses such as heavy metal toxicity.

Other abiotic and biotic stress factors with adverse effects on growth and survival of crop plants are mitigated by *Bacillus*-induced physiological changes, which include the activation of antioxidant and defence systems (Huang et al. 2020), regulation of water transport, nutrient uptake, and enhancement of photosynthetic pigments (Babu et al. 2013), leading to increased crop tolerance and productivity.

14.5 Molecular and Genetic Basis for Plant Growth Promotion and Heavy Metal Tolerance Ability of *Bacillus* Species

Bacillus association triggers plant resistance and immunity against different stresses (including heavy metal stress) by altering stress-responsive genes, proteins, phytohormones, and related metabolites (Radhakrishnan et al. 2017). Studies on genomic analysis of species of *Bacillus* have revealed rich genetic elements involved in important plant growth promoting and heavy metal tolerance activities. For example, the genome of *B. megaterium* STB1 possess genes related to rhizosphere colonization, xenobiotic degradation, pathogen antagonistic activities and several other genes for multiple stress resistance (Nascimento et al. 2020). Genes associated with different plant growth promoting activities are found in many species of *Bacillus* under heavy metal stress. Ding et al. (2005) selected 29 isolates in a study aimed at identifying the possible nitrogen-fixing Bacilli from plant rhizospheres based on their ability to grow on nitrogen-free medium, out of which seven had *nifH* gene belonging to *Bacillus* and *Paenibacillus* genera. It was the first report of nitrogen fixation in *B. marisflavi* and *P. massiliensis* and the first of the *nifH* gene from *B. megaterium* and *B. cereus*. Also, the whole genome of *B. aryabhattai* AB211 was sequenced, with main focus on genomic elements related to plant microbe interaction (Bhattacharyya et al. 2017). Genome comparisons between the strain AB211 and other related strains of *B. aryabhattai* revealed about 3,558 genes which were conserved among all the genomes, with most genes involved in plant growth

promotion activities found within core genes of all the genomes used for comparison. The findings showed possible common plant growth promoting traits shared among the strains of *B. aryabhattai*. Functional annotation of genes in the *B. aryabhattai* strain AB211 revealed the presence of many PGP genes which include those responsible for phosphate solubilization, siderophore production, exopolysaccharides production, and IAA production, most of which were experimentally verified in the study. Earlier findings by Halim et al. (2016) also proven that *Paenibacillus durus* ATCC 35681 is a free-living nitrogen-fixing bacterium, and the complete genome of the strain was successfully sequenced too. Interestingly, the isolate can also be found in a symbiotic relationship with plant roots.

Similarly, genes for the resistance, mobilization, detoxification, or transformation were detected in many *Bacillus* species. For example, genomic analysis of *B. megaterium* STB1 revealed abundance of genes for heavy metal resistance and transport, which include those encoding arsenate (*arsC*) and chromate (*chrR*) reductases and several others for Zn, Cd, Cu, Co, Mn, Ni, Cr, and As (Nascimento et al. 2020). One of the arsenate reductase genes in the genome occur in a cluster that contain other arsenate transport and resistance related genes. BLASTn analysis showed that the cluster is rare, and that close homologs were only found in *B. weihaiensis* Alg07 chromosome and *B. oceanisediminis* 2691 pB01. These bring in new insights into the capabilities and roles of *Bacillus* spp. as potential and important plant growth promoting bacteria with ability of not only improving plant growth but also enhancing the tolerance and survivability of the host plants under heavy metal stress conditions.

14.6 Enhancement of Plant Growth and Heavy Metal Tolerance of Plants in Association with Different Species of *Bacillus*

Representatives of plant-beneficial bacteria are widely spread among gram-negative and gram-positive bacteria, with *Pseudomonas* and *Bacillus* attracting main attention (Borriss 2014; Radhakrishnan et al. 2017). Despite the great progress in *Pseudomonas* research, its commercial use in agriculture is unfortunately limited due to difficulties in preparing stable and long-living bioformulations compared to *Bacillus* (Borriss 2014). Bacilli are increasingly and interestingly used commercially in agriculture to enhance yield of crops and reduce use of harmful agrochemicals (Ahmad and Saghir 2010; El-Meihy et al. 2019; Minaxi et al., 2012). When the plant growth promoting attributes of *Bacillus* sp. RM-2 were tested in both laboratory and field conditions, the isolate significantly increased growth ($P<0.05$) and yield parameters and nutrients uptake of cowpea plants (Minaxi et al., 2012). Metal tolerant *B. subtilis* BM2 was able to mediate the phytotoxic impact and significantly reduced Ni and Pb uptake in winter wheat and also improved plant growth (Rizvi et al. 2019). The BM2 strain was able to increase the grain yield significantly by

49% and 50% under 870 mg/kg Ni and 585 mg/kg Pb, respectively. The exceptional ability of the strain to produce plant growth promoting substances like siderophore, IAA, ammonia, and ACC deaminase under the metal stress condition might have played important role in achieving the overall performance and enabling continuous growth and survival of wheat even under the metal stress condition (Rizvi et al. 2019).

The *Bacillus* spp. are as well employed in the field of phytoremediation aimed at improving the uptake of heavy metal by hyperaccumulator plants (Babu et al. 2013; Ndeddy Aka and Babalola 2016; Tang et al. 2020). Recently, *B. cereus* strain WSE01, that can tolerate up to 1500 mg/L Mn, was shown to increase the growth and leaf enzymatic activities in *Myriophyllum verticillatum* under 400 mg/L Mn stress condition. The bacterium was also able to increase the Mn content in the stems and leaves of the inoculated plants by 36.4% and 54.7% , respectively, compared to non-inoculated plants (Tang et al. 2020). In a study to assess bacteria with potential to enhance growth and metal accumulation in hyperaccumulator *Alnus firma* (Babu et al. 2013), the bacterium identified as *Bacillus thuringiensis* GDB-1 had better capacity and increased significantly the biomass, chlorophyll content, and accumulation of As, Pb, Cu, Ni, and Zn in the *A. firma* seedlings. A summary of different species of *Bacillus* with plant growth promoting and heavy metal tolerant activity applied for growth enhancement and bioremediation of heavy metal in plant is presented in Tables 14.1 and 14.2.

In our recent study, one of the most promising and potential heavy metal tolerant plant growth-promoting isolate, identified as CCB-MBL5001 *Bacillus cereus* 2M1, was tested for its ability to alleviate metal stress and improve the growth of rice seedlings under Pb stress. Sterilized rice seeds (treated with or without 2M1 inoculation) were sown in sterile petri plates before uniformly germinated seeds were transferred aseptically to test tubes containing Yoshida medium supplemented with 0, 100 and 150 mg/L Pb. For both inoculated and non-inoculated seedlings, all growth parameters were noted to decline as affected by increasing concentrations of metal tested. However, better improvement in plant growth parameters were observed for seedlings inoculated with the bacteria compared to the control (non-inoculated, supplied with similar Pb concentrations) (Table 14.3). On the effects on photosynthetic pigments, the bacterial inoculated treatments significantly increased chlorophyll a, chlorophyll b, and total chlorophyll contents in the rice seedlings despite the occurrence of high metal stress (Fig. 14.1). Increase in the chlorophyll contents due to bacterial inoculation was not only found under Pb stress but also under non-stressed (control) conditions. Toxicity of heavy metal can affect photosynthesis by causing distortion in the ultrastructure of chloroplast, preventing the synthesis of photosynthetic pigment in chlorophyll content and enzymes of Calvin cycle. To assess the extent of oxidative stress and degree of damage caused by the heavy metal (Pb), the electrolyte leakage was estimated as presented in Fig. 14.2a. Stress resulting from the tested metal was shown to cause oxidative stress in the leaves of the host plants, which increased in response to increasing concentrations of the metal. However, there was significant decrease in the electrolyte leakage in 2M1 inoculated plants, which is a clear indication in the stability of the membrane. The % EL was decreased by 58% under the highest concentration of Pb, and the

Table 14.1 Plant growth enhancement and bioremediation of heavy metal in crop plants as influenced by plant growth promoting *Bacillus* species

Bacillus species	Plant species	Heavy metal	Function/activity	Condition	References
B. thuringiensis HC-2	Radish plant	Cd and Pb	Decreased Cd (28–94%) and Pb (22–63%) content in radish; increased dry weight of roots (18.4–22.8%) and leaves (37.8–39.9%)	Field	Li et al. (2019)
B. cereus MG257494.1	Sorghum	Cu, Cd, Zn and Pb	Decreased metal uptake; increased growth and biomass, antioxidant enzymes activity, and photosynthetic pigments	Pot	El-Meihy et al. (2019)
B. subtilis BM2	Wheat	Ni and Pb	Decreased metal uptake; improved growth parameters; relieved metal stress; decreased in proline, malondialdehyde content, and antioxidant enzymes activities	Pot	Rizvi et al. (2019)
P. mucilaginosus	Alfalfa	Cu	Increased nutrient content in plants; improved plant growth; increased antioxidant enzymes activities; improved growth and Cu uptake	Pot	Ju et al. (2019)
Bacillus sp. PSB10	Chickpea	Cr	Improved growth and biomass; increased nodulation and chlorophyll content; reduced Cr uptake by chickpea plants	Pot	Ahmad and Saghir (2010)

lower percentages of EL in the inoculated rice plants proved metal stress alleviation by the microbe to the plant. Plants are always being exposed to several stress factors in the field which can include heavy metal, that can affect their growth, development, and productivity. The adverse effects generally induce the accumulation of reactive oxygen species (ROS), which can cause severe oxidative damage including electrolyte leakage to plants. The balance between the production and detoxification of ROS is usually sustained by enzymatic and nonenzymatic antioxidants. The catalase (CAT) and superoxide dismutase (SOD) enzymes activities determined in the leaves of rice seedlings were higher in plants inoculated with 2M1 compared to their uninoculated counterparts (Fig. 14.2b and c). It was also observed that the inoculated controls without any heavy metal had the least activities. Increase in CAT and SOD activities by bacterial inoculation indicated strong response towards coping with oxidative stress generated by exposure to the heavy metal.

Table 14.2 Influence of *Bacillus s*pecies on growth enhancement of hyperaccumulator plants and phytoremediation potentials of soils contaminated with heavy metal

Bacillus species	Plant species	Heavy metal	Function/activity	Condition	References
B. cereus HM5 *B. thuringiensis* HM7	*Broussonetia papyrifera*	Mn	Higher growth and biomass; increased accumulation of Mn by plant; reduced malondialdehyde and antioxidant activities in leaves	Pot	Huang et al. (2020)
B. thuringiensis GDB-1	*Alnus firma*	As, Ni, Cu, Pb and Zn	Increased growth and biomass, chlorophyll content, and heavy metal accumulation	Pot	Babu et al. (2013)
B. cereus WSE01	*Myriophyllum verticillatum*	Mn	Improved growth; increased leaf enzymatic activity; increased Mn accumulation in stems (36.4%) and leaves (54.7%)	Hydroponic culture	Tang et al. (2020)
B. subtilis SJ-101	*Brassica juncea*	Ni	Decreased Ni toxicity; enhanced Ni accumulation in B. juncea	Pot	Zaidi et al. (2006)
B. subtilis KP717559	*B. juncea*	Cd, Cr, and Ni	Increased growth and biomass; increased metal accumulation	Pot	Ndeddy Aka and Babalola (2016)
B. cereus T1B3	*Vetiveria zizanoides*	Cr and Fe	Enhanced growth and biomass; enhanced Cr and Fe accumulation	Pot	Nayak et al. (2018)

Table 14.3 Growth parameters of rice seedlings inoculated with locally isolated heavy metal tolerance *B. cereus* 2M1 under Pb stress condition

	Treatments		Plant growth parameter					
	Pb (mg/L)	Inoculation (2M1)	Root length (cm)	Shoot length (cm)	Root fresh weight (mg)	Shoot fresh weight (mg)	Root dry weight (mg)	Shoot dry weight (mg)
1	0	−	7.50 ± 0.29^{a}	25.67 ± 2.90^{a}	50.00 ± 0.00^{a}	103.33 ± 3.33^{a}	3.63 ± 0.58^{ns}	19.10 ± 0.55^{a}
2	0	+	7.83 ± 0.44^{a}	26.50 ± 3.28^{a}	50.00 ± 0.00^{a}	106.67 ± 3.33^{a}	3.80 ± 0.55^{ns}	17.87 ± 1.49^{a}
3	100	−	5.83 ± 0.44^{bc}	15.73 ± 0.37^{bc}	40.00 ± 5.77^{ab}	73.33 ± 3.33^{bc}	3.30 ± 0.44^{ns}	8.33 ± 0.20^{b}
4	100	+	5.33 ± 0.33^{c}	17.03 ± 0.26^{b}	43.33 ± 6.67^{a}	80.00 ± 5.77^{b}	3.50 ± 0.29^{ns}	10.26 ± 0.68^{b}
5	150	−	5.33 ± 0.60^{c}	10.83 ± 0.60^{c}	26.67 ± 3.33^{c}	56.67 ± 6.67^{c}	2.83 ± 0.44^{ns}	7.97 ± 0.55^{b}
6	150	+	7.07 ± 0.58^{ab}	13.83 ± 0.44^{bc}	40.00 ± 5.77^{ab}	73.33 ± 3.33^{bc}	3.57 ± 0.30^{ns}	9.83 ± 0.95^{b}

Values are presented as means ± standard error. Values with different superscripts along the columns are significantly different ($p < 0.05$)

Notes: − = uninoculated; + = inoculated; ns = not significant

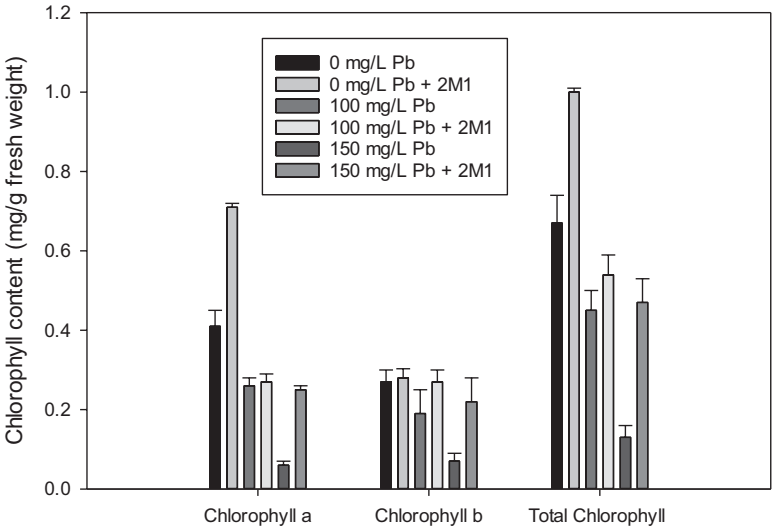

Fig. 14.1 Chlorophyll content in rice seedlings inoculated with locally isolated heavy metal tolerant *B. cereus* 2M1 after 15 days of growth under Pb stress and aseptic conditions

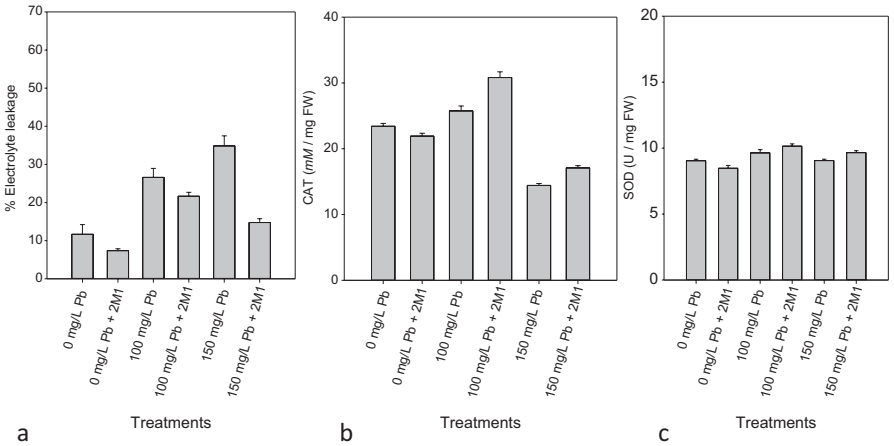

Fig. 14.2 Electrolyte leakage (**a**) CAT activity (**b**) and SOD activity (**c**) in rice seedlings inoculated with locally isolated heavy metal tolerant *B. cereus* 2M1 after 15 days of growth under Pb stress and aseptic conditions

14.7 Conclusions and Future Perspective

Bacilli have multiple beneficial traits, which mediate better growth and development of crop either directly or indirectly through their different plant growth-promoting activities and heavy metal tolerance enhancement. This has great application towards growth enhancement and bioremediation of heavy metal in crop plants. Information made available in this chapter are testament of great potential of

Bacillus spp. in growth enhancement and bioremediation of heavy metal in crop plants. However, most of the experiments were carried out under control conditions (lab or pot experiments), with paucity of information from field trials. Though the *Bacillus* group is one of the most commercially exploited bacteria in the agrobiotechnology industry, its potential has still not been realized sufficiently, and thus, the emphasis should be towards translating the relevant technologies from laboratory to the real world situation in the field (Saxena et al. 2019). More *Bacillus*-plant physiological studies are required to have better understanding of the *Bacillus* spp. mediated mitigation mechanisms against heavy metal stresses in plants.

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Chapter 15
Bacillus Probiotics and Bioremediation: An Aquaculture Perspective

Dibyendu Kamilya and Wangkheimayum Malemnganbi Devi

Abstract Probiotics confer health benefits to the host, and their application to control infectious diseases in aquaculture is well recognized. Probiotic bacteria exert beneficial effects in fish/shellfish when applied through the feed. Besides the application of probiotics as a feed additive, an additional concept of "water probiotic" is also pertinent in aquaculture, owing to the intricate relationship of aquatic organisms with their surrounding environment. In fact, an extended definition of probiotics has been suggested in aquaculture, incorporating the idea of improving the quality of the ambient aquatic environment by probiotics. Among different bacteria tested as probiotics in aquaculture, *Bacillus* constitutes the dominant genera. Besides the use of *Bacillus* as a feed additive to obtain the probiotic effect, administration of this bacterial group in the rearing water has also been investigated to improve water quality. The majority of the studies indicated an effective bioremediation capability of *Bacillus* spp. in terms of improving water quality. However, in a few studies, *Bacillus* spp. did not show any effect on all or some of the water quality parameters. This chapter provides an overview of the concept, application, and beneficial effects of *Bacillus* as a bioremediating probiotic in aquaculture.

Keywords Probiotic bacteria · Feed additive · Water quality · Bioremediation · Aquaculture

15.1 Introduction

Owing to the increasing demand for fish and fishery products by the ever-increasing global population, there has been a remarkable development in the aquaculture industry across the globe. To satisfy the increasing demand, a shift in aquaculture practices, from traditional to intensive systems, has taken place in several parts of

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the world. The intensification and diversification of aquaculture have led to the frequent occurrence of infectious diseases and deterioration of environmental conditions (Bondad-Reantaso et al. 2005). To counter the disease problems, antibiotics and other chemotherapeutants have been widely used in aquaculture. However, the emergence of antibiotic and drug-resistant bacteria has become a serious problem, leading to the restriction on their rampant usage (Akinbowale et al. 2006; Balcázar et al. 2006). The use of probiotic bacteria to control infectious diseases in aquaculture is increasingly being considered as an eco-friendly alternative to drug and antibiotic treatments (Balcázar et al. 2006; Dawood et al. 2019; Doan et al. 2020).

Even though various definitions have been proposed for probiotics, the two most widely quoted definitions are – "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller 1989) and "live microorganisms that when administered in adequate amounts confer health benefits on the host" (FAO/WHO 2001). In all the definitions, the concept of probiotic effects revolves around the realization that active modulation of the gastrointestinal tract could confer protection against pathogenic microorganisms, help stimulate the immune system, provide nutritional benefits, and assist the mucosal barrier of the intestine (Vaughan et al. 2002). Essentially, probiotics are considered as health-promoting "functional foods" for humans, as well as therapeutic, prophylactic, and growth supplements in animal production and human health (Kesarcodi-Watson et al. 2008). Like the human and animal production sectors, the concept and application of probiotics as health-promoting "functional foods" in aquaculture has also been extensive and well researched.

Besides the health-promoting attributes, probiotics have an extended application in aquaculture. The intricate relationship between an aquatic organism and its surrounding environment, as compared to that of terrestrial animals, legitimate the extension of the probiotic concept beyond the health-promoting feed supplements. Thus, bacterial treatments to improve water quality by directly applying the probiotics into the rearing water have been included in the broad concept of probiotics in aquaculture. In fact, this extended concept has been proposed to describe probiotics in aquaculture as microbial "water additives" (Moriarty 1998). It is expected that the bacteria which improve water quality may be beneficial to the health of the aquatic animals, and interestingly, several commercial products, referred to as "probiotics," have sought to exploit this idea (Gatesoupe 1999).

A large number of gram-positive and gram-negative bacteria have been investigated as probiotics in aquaculture. Among different probiotic bacteria, *Bacillus* constitutes the dominant genera, and the beneficial effects of their applications in the aquaculture field are well established (Gatesoupe 1999, 2008; Kuebutornye et al. 2019; Soltani et al. 2019). Apart from the health-promoting effects (as dietary additives), several species in the genus *Bacillus* have also been investigated for their bioremediation capability when applied directly into the rearing water. The gram-positive, endospore-forming, and rod-shaped *Bacillus* spp. are almost ubiquitous in nature, and the important physiological traits, such as their sporulation capacity and ability to produce a diverse range of antimicrobial compounds and extracellular enzymes, are responsible for contributing to the beneficial attributes (Soltani et al. 2019).

In view of the above, this chapter aims to present an overview of the published information on the concept, application, and beneficial effects of *Bacillus* as a bioremediator in aquaculture. The research gaps existing in the current state of knowledge about the bioremediation potential of *Bacillus* in aquaculture and suggestions for future studies are also delineated.

15.2 The Concept of "Water Probiotic" and Microbial Community Manipulation

Given the close relationship of aquatic organisms with their external environment, the classical probiotic concept was modified with respect to their aquatic usage. Accordingly, an extended definition was proposed by Verschuere et al. (2000) to define probiotic as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment." Unlike the terrestrial animals, probiotic application to the aquatic animals can be accomplished either through feed ("feed probiotic") or by directly applying them into the aquaculture pond or tank ('water probiotic"). Probiotics applied through feed, directly reach the intestine and modify the host-associated microbial community, resulting in health benefits. On the other hand, water probiotics can modify the ambient microbial community by competitive exclusion principle, thereby, creating a better environment from the health perspective. An additional advantage of water probiotics is their ability to degrade organic wastes present in the pond water or sediment. The addition of beneficial microbes in the rearing water improves the water quality, leading to stress minimization and, hence, render the animals less susceptible to infectious diseases.

The beneficial effects of water probiotics in terms of exclusion of pathogenic bacteria and improvement of water quality parameters in the aquaculture system can be attributed to the interaction of the added bacteria with the resident microbial community and the factors facilitating those interactions. The species composition and development of a microbial community are determined by deterministic and stochastic factors. Deterministic factors include various environmental parameters that create a habitat in the aquaculture system in which a well-defined range of microbes can grow and proliferate more rapidly than others (Verschuere et al. 1997; Moriarty 1999). On the other hand, the species composition of a microbial community is also influenced by stochastic phenomena where chance favors those organisms which happen to be in the right place at the right time to respond and proliferate if the conditions are suitable (Moriarty and Body 1995; Verschuere et al. 1997). Thus, the species composition in an aquaculture environment can be manipulated by the addition of probiotic bacteria. If the probiotic cultures are well adapted to the prevailing environmental conditions, a single addition of a probiotic culture may

suffice to achieve colonization and persistence in its ambient environment (Verschuere et al. 2000). However, regular addition of probiotic cultures is recommended to maintain a persistent numerical dominance when there is a stable and well-established microbial community in the environment (Verschuere et al. 2000).

Typically, a change in species composition in an aquaculture environment can occur via competitive exclusion principles (Smith 1993). The probiotics can compete with the resident microflora by secreting antimicrobials substances. For example, *B. subtilis* is known to secrete several antimicrobial substances, such as coagulin, amicoumacin, and subtilisin, to suppress the growth of competing microbes (Cutting 2011).

Change in species composition can also occur via the addition of water probiotics that can favor the growth of other groups of beneficial bacteria. The dominance of probiotics and other beneficial bacteria facilitates efficient utilization of the available substrates, leading to improvement in water quality. For example, the addition of *B. subtilis* SC02 at a dose of 1×10^9 CFU m^{-3} per seven days in fish rearing water significantly altered the microbial diversity and improved the water quality, compared to the control group (Zhang et al. 2013). The 454-pyrosequencing analysis revealed that the *B. subtilis* SC02 treated group was dominated by Proteobacteria, Bacteroidetes, and Actinobacteria, all of which are in one or other way beneficial and might have facilitated the improvement of chemical quality of the culture water. A commercial probiotic significantly improved the water quality and increased the population density of *Bacillus* sp., ammonifying bacteria, and protein mineralizing bacteria in shrimp ponds (Wang et al. 2005). In another study, the addition of *B. subtilis* FY99–01 at a dose of 5×10^4 CFU ml^{-1} once every week had a pronounced effect on bacterial community structure and composition of shrimp culture water (Wu et al. 2016). The addition also improved the water quality, promoted beneficial microalgal growth, and inhibited the growth of pathogenic bacteria belong to Vibrionaceae. Thus, the water probiotics can inhibit pathogenic microorganisms and simultaneously improve the water quality, ensuring better health of the resident fish population.

A lot of commercial products and technologies are now available to establish the dominance of a particular or a group of microbial species to treat an aquaculture environment. This practice of bioremediation is being applied in many areas with varying degrees of success. The success of a bacterium or bacterial consortium as bioremediator depends on the selection of bacteria capable of performing specific functions compliant to bioremediation, and the addition of bacteria into the environment at a high concentration, and under favorable environmental conditions (Moriarty 1999).

15.3 Application of *Bacillus* as Bioremediator in Aquaculture

The addition of feed, generation of fecal matters, and inclusion of other organic matters into the aquaculture system lead to the accumulation of organic pollutants in the pond. This, in turn, deteriorates the pond water quality, making the environment unsuitable for the reared animals. Bioremediation of such contaminated rearing environment can be accomplished by the water probiotics. It has been suggested that gram-positive bacteria like *Bacillus* spp. are more efficient in converting organic matter back to CO_2 than gram-negative bacteria (Verschuere et al. 2000). Thus, the buildup of dissolved and particulate organic carbon during a culture cycle can be minimized by maintaining a high level of these bacteria in the culture pond (Balcázar et al. 2006). Additionally, *Bacillus* spp. can participate in nitrification, denitrification, nitrogen fixation, iron precipitation as well as oxidation, and reduction of metals (Priest 1993; Slepecky and Hemphill 2006). These attributes make *Bacillus* spp. a potent bioremediator of the contaminants in the aquaculture environment. Several bacterial species in the genus *Bacillus* have been found to improve the water quality parameters of the aquaculture pond or tank. Modulation of ammonia, nitrite, nitrate, pH, dissolved oxygen, biochemical oxygen demand, alkalinity, chemical oxygen demand, total dissolved solids, etc. has been recorded in rearing water after treatment with the *Bacillus* spp. A summary of the impacts of *Bacillus* spp. on water quality parameters in aquaculture systems is presented in Table 15.1.

15.4 Mechanism of Bioremediation by *Bacillus* spp.

The ability of *Bacillus* spp. to act as heterotrophic nitrifiers, denitrifiers, nitrogen fixers, metal oxidizers and reducers, iron precipitators, and others reflects a considerable diversity in their physiological properties (Abriouel et al. 2011). All these properties exhibited by a great variety of *Bacillus* strains indicate that these bacteria can colonize a wide variety of substrates and can perform a range of functions pertinent to bioremediation. Their widespread ecological diversification is potentiated by the production of endospores, which are resistant to environmental stress and can remain dormant for a considerable period (Abriouel et al. 2011).

It has been suggested that *Bacillus* spp. compete with the resident bacterial community for available organic matter in the aquaculture environment. The outcome of the competitive microbial interactions is greatly impacted not only by the relevant enzyme-producing capacity of the strains but also by the environmental conditions (Soltani et al. 2019). In fact, temperature as well as pH are considered as critical factors in influencing the growth of bacteria and their ability to absorb and utilize substrates (as enzyme activities are highly temperature-dependent) (Verstraete and Focht 1977; Wilks et al. 2009). For example, among nine strains of bacteria of wastewater origin, *B. amyloliquefaciens* showed the highest ammonia nitrogen

Table 15.1 *Bacillus* probiotics used as bioremediator in aquaculture

Species	Reared animal/ media	Origin	Dose	Impacts on water quality	References
Bacillus subtilis, B. megaterium, B. polymyxa, B. licheniformis	*Ictalurus punctarus*	Commercial	2×10^9 CFU mL^{-1}	↓COD, ↑BOD, ↑TAN, ↑ NO_2^--N	Queiroz and Boyd (1998)
B. subtilis, B. megaterium, B. polymyxa, B. licheniformis	*Litopenaeus vannamei*	Commercial	Unknown	↓ammonia, ↓ NO_3^--N, ↓ NO_2^--N, ↓phosphorus, ↓COD, ↓BOD, ↓TSS	McIntosh et al. (2000)
B. subtilis, B. megaterium	*C. citrinellum* × *C. synspilum*	Commercial	10^{10} CFU per trail, twice a week	↓TAN, ↓COD, ↑Transparency	Chen and Chen (2001)
Bacillus sp.	*Penaeus monodon*	Commercial	7×10^{11} cells in 0.8 ha pond	↑transparency, ↓total organic carbon	Dalmin et al. (2001)
Bacillus spp.+ non-*Bacillus* spp.	*P. monodon*	Commercial	10^8 CFU mL^{-1}	↑TAN, ↑ $NO_{2\_}^{\ -}$-N, ↑ammonia, ↑transparency, ↓hardness, ↔COD, ↔pH, ↔hydrogen sulfide, ↔ DO, ↔ BOD	Matias et al. (2002)
B. licheniformis, B. pumilus, B. subtilis	Shrimp hatchery	Brackish water	Unknown	↓TAN	Foon (2004)
Bacillus spp.+ non-*Bacillus* spp.	*L. vannamei*	Commercial	10^4–10^9 CFU mL^{-1}	↔pH, ↓ phosphate, ↑transparency, ↑DO, ↓total inorganic nitrogen, ↓COD	Wang et al. (2005)
B. subtilis, B. cereus, B. licheniformis	*Cyprinus carpio*	*C. carpio*	Each at 10^5 CFU mL^{-1}	↓ NO_2^--N, ↓NO_3^--N, ↓ammonia	Lalloo et al. (2007)
B. subtilis	*Xiphophorus helleri, X. maculates, Poecilia reticulata and P. sphenops*	*Cirrhinus mrigala*	5×10^5–5×10^8 cells mL^{-1}	↓dissolved organic matter, ↓TAN	Ghosh et al. (2008)
B. pumilus	*P. monodon*	Native	10^6 CFU mL^{-1}, every 3 days	↓TAN, ↓ NO_3^--N, ↔DO, ↔salinity,↔pH	Banerjee et al. (2010)

Bacillus sp.	*M. rosenbergii*	Native prawn, water, sediment	10^6 cells mL^{-1}	↓ammonia, ↓ NO_2^--N, ↓pH, ↔DO	Mujeeb-Rahiman et al. (2010)
B. subtilis, B. licheniformis, B. megaterium, B. laterosporous	*Thunnus albacares*	Commercial	1.5×10^6 CFU mL^{-1}	↓TAN, ↓un-ionized ammonia, ↑DO	Zink et al. (2011)
B. megaterium, B. licheniformis, B. thuringiensis, B. polymyxa, B. subtilis, B. circulans, B. pumilus	*L. vannamei*	Commercial	Each probiotic at 10^9 CFU mL^{-1}	↓pH, ↓ammonia, ↓ NO_2^--N, ↔ NO_3^--N, ↔salinity, ↔DO, ↔phosphate	Nimrat et al. (2012)
B. licheniformis, *B. natto*, *B. subtilis* + non-*Bacillus* spp.	Crucian carp	Unknown	Unknown	↑DO, ↓COD, ↓ NO_3^--N, ↓ NO_2^--N, ↓sulfide, ↔pH, ↓ammonia	Zhu et al. (2012)
B. subtilis, B. pumilus, B. licheniformis	*Paralichthys olivaceus*	Shrimp intestine	10^{10} CFU g^{-1}	↓ammonia	Cha et al. (2013)
B. amyloliquefaciens	Simulated aquaculture water	Activated sludge of a polluted river	Unknown	↓ NO_2^--N, ↓ammonia	Xie et al. (2013)
B. subtilis	*Ctenopharyngodon idellus*	Grass carp pond	10^9 CFU m^{-3}	↓NH_3, ↓ NO_3^--N, ↓total nitrogen	Zhang et al. (2013)
Bacillus sp. + non- *Bacillus* sp.	*L. vannamei*	Commercial	1.65×10^{10} CFU L^{-1}	↑pyrrophyta density, ↑water sediment quality, ↑water quality	de Paiva-Maia et al. (2013)
B. pumilus, B. licheniformis, B. subtilis	*P. monodon*	Native marine water and soil	10^6 CFU mL^{-1}	↓ NO_3^--N, ↔DO, ↔salinity, ↔pH	Devaraja et al. (2013)
B. subtilis	*L. vannamei*	Fermented pickle	10^5 and 10^8 CFU mL^{-1}	↓ammonia, ↓ NO_2^--N, ↓ NO_3^--N	Zokaeifar et al. (2014)
B. vietnamensis	Shrimp wastewater effluent	Shrimp wastewater	Unknown	↓ TAN, ↓ NO_2^--N	Muthukrishnan et al. (2015)
B. subtilis + non-*Bacillus* spp.	*C. carpio* var. koi	Commercial	Bacterial mixture @ 0.25, 0.50 and 1.0 g m^{-3}	↓phosphate-P, ↓BOD, ↓COD, ↓DO, ↓TAN	Sonia et al. (2015)

(continued)

Table 15.1 (continued)

Species	Reared animal/ media	Origin	Dose	Impacts on water quality	References
Bacillus sp. + non-*Bacillus* sp.	*Clarias gariepinus*	Sampled fish farm water	1 ml inoculum L^{-1} wastewater	↓phosphate, ↓sulphate, ↓NO_3^-, ↓ammonia$_3$, ↓BOD, ↓COD, ↓total suspended solids, ↑DO, ↑pH	Omitoyin et al. (2016)
Paenibacillus polymyxa	*C. carpio*	Lab culture	10^3–10^5 CFU mL^{-1}	↔pH, ↔ NO_2^--N, ↔ammonia	Gupta et al. (2016)
B. licheniformis, *B. laterosporus*, *B. polymyxa*, *B. subtilis*, and *B. circulans*	Common carp wastewater effluent	Commercial product	10^8 CFU L^{-1}	↓TAN, ↑NO_3^- –N, ↓Turbidity	Naderi-Samani et al. (2016)
B. subtilis	*Litopenaeus vannamei*	Shrimp pond soil	5×10^4 CFU mL^{-1}	↓pH, ↓ NO_2^--N, ↑transparency, ↑COD, ↓soluble reactive phosphorus, ↑Chlorophyll a density	Wu et al. (2016)
B. pumilus + non-*Bacillus* sp.	*C. carpio*	Lab culture	62.5×10^8 cells mL^{-1}	↓TAN, ↔pH, ↔DO, ↑ total suspended solids, ↑ total dissolved solids	Dash et al. (2018)
B. subtilis, *B. pumilus*, *B. licheniformis*	*Oreochromis niloticus*	Commercial probiotic	10^{10} CFU g^{-1}	↓ammonia, ↑electrical conductivity, ↑ total dissolved solids, ↑pH	Elsabagh et al. (2018)
B. cereus	*P. monodon*	Native pond water and soil	Unknown	↑DO, ↓pH, ↓NO_2^--N	Barman et al. (2018)
B. velezensis	*I. punctatus*	Soil and channel catfish	10^9 CFU mL^{-1}	↓eutrophication, ↓TAN, ↓NO_3^- –N, ↓ total nitrogen, ↓total phosphorus	Thurlow et al. (2019)
B. cereus, *B. amyloliquefaciens*, + non-*Bacillus* sp.	*O. mossambicus*	Water, sediment, soil samples from discharge points	10^8 CFU mL^{-1}	↓ammonia, ↓ NO_3^--N ↓ NO_2^--N	John et al. (2020)

'↑' increase; '↓' Decrease; '↔' No change; COD – chemical oxygen demand; TAN – total ammonia nitrogen; BOD- biochemical oxygen demand; TAN- total ammonia nitrogen; NO_3^- –N – nitrate nitrogen; NO_2^--N - nitrate nitrogen; DO – dissolved oxygen

degrading activity at 35 °C and pH 7.0, and a gradual reduction in this activity was observed at higher temperatures and pH (Yu et al. 2012). Additionally, bacterial cell surface hydrophobicity is considered to play a crucial role in the adhesion of microorganisms to bioremediation surfaces (Krasowska and Sigler 2014). With the increasing cell surface hydrophobicity, bacteria tend to aggregate and form cell-to-cell adhesion, and degradation of phosphorus, nitrogenous and other wastes occurs via various metabolic pathways (Adav et al. 2008; Liu et al. 2009). In a study, the role of hydrophobicity of two *Bacillus* probiotics was investigated in bioremediation of aquaculture, and the results showed a significantly higher bioremediation ability of the strain with greater cell hydrophobicity (Wang and Han 2007).

The feed and excreta of the reared animals in aquaculture are the major source of nitrogenous wastes. *Bacillus* spp. can effectively utilize these nitrogenous substances, and under aerobic, facultative aerobic, and anaerobic conditions, they can greatly impact nitrogen metabolism. Among different nitrogen species, nitrate or nitrite is used by *Bacillus* spp. as sole sources of nitrogen and also as electron acceptors for anaerobic respiration (Lalloo et al. 2007). *Bacillus* spp. have also been reported to carry out heterotrophic nitrification in which nitrate is produced by the oxidation of inorganic and organic reduced forms of nitrogen (Mevel and Prieur 2000). Heterotrophic nitrification is particularly more pronounced in acid soils or where heterotrophic bacterial biomass and carbon:nitrogen ratios are high (Prosser 2005). Simultaneous heterotrophic nitrification and aerobic denitrification is a recently discovered alternative nitrogen removal process where NH_4-N can be aerobically converted to nitrogenous gas (Khanichaidecha et al. 2019). *Bacillus* spp. isolated from soils and wastewater treatment systems have been found to have the capability of performing heterotrophic nitrification and aerobic denitrification simultaneously (Takenaka et al. 2007). Another interesting mechanism of direct uptake and assimilation of ammonia occurs in *Bacillus* spp. Different species in this bacterial group can employ either glutamate dehydrogenase or glutamine synthetase-glutamate synthase pathway to assimilate ammonia into glutamic acid (Kanamori et al. 1987). Thus, *Bacillus* spp. can effectively employ a wide range of mechanisms to act as a potent bioremediator.

15.5 Conclusions

Except for a few, the majority of the published literature indicates the potential of *Bacillus* spp. as a water probiotic in reducing the environmental impacts in the aquaculture system. Several commercial probiotic products are also available where different *Bacillus* spp. have been used to function as bioremediator to improve the pond water quality. These imply the ability and potential of *Bacillus* spp. in controlling the toxic contaminants of pond rearing water, particularly the nitrogenous wastes. Improvement of water quality parameters is translated into the reduction of stress, which ensures a better environment for growing a healthy population of fish and shellfish. Moreover, the addition of *Bacillus* spp. into the rearing water promote

the growth of other groups of beneficial bacteria and reduce the load of pathogenic bacteria. Therefore, the use of *Bacillus* as a water additive not only facilitates bioremediation of the aquaculture environment but improves the health status of the animals. However, it is important to have a thorough knowledge of the mechanisms by which *Bacillus* spp. control water quality and improve the health of the reared animals. A comprehensive understanding of the interaction of *Bacillus* spp. with the natural microflora, the degree and frequency of their addition into the rearing water to achieve dominance and persistence in the ambient environment, the influence of the prevailing environmental conditions on their growth dynamics, and identification of suitable strains capable for functioning optimally are some of the important considerations which warrant further research.

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Chapter 16
Enhanced Nutrient Accumulation in Non-leguminous Crop Plants by the Application of Endophytic Bacteria *Bacillus* Species

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Abstract Endophytic bacteria exert beneficial effects on various crop plants especially the non-legumes, and the effects are carried out in multidimensional mechanisms unlike the biological N_2 fixation (BNF) process. They create a conducive environment in the apoplastic area of root tissues as well as in shoot for providing benefits to the host plant. Among the endophytic bacteria, recently, *Bacillus* spp. are gaining prominence as a biofertilizer and bioenhancer for crop production. A large number of species and strains of this genera have been isolated and identified from the diversified crop plants such as rice, wheat, maize, alfalfa, banana, black pepper, canola, cucumber, clover, oil palm, and apple. Inoculation of plants with these endophytic Bacilli resulted in various beneficial effects on the colonization including better nutrition, improvement of growth, yield, and quality of crop plants. A significant amount of atmospheric N_2 is fixed and incorporated into a good number of non-legumes like rice, wheat, maize, banana, oil palm etc. that are confirmed by the ^{15}N isotopic dilution technique. The *Bacillus* spp. are found in the apoplastic area, produce phytohormone especially auxin, and excrete their fixed N_2 as ammonium to

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the host plant cells. Additionally, these endophytes are also able to enhance the accumulation of P, K, and Ca through the stimulation of cell membrane-ATP-ase activity. The *Bacillus* spp. are also capable to solubilize complex rock phosphate to a simpler form of phosphate, i.e. dihydrogen orthophosphate and monohydrogen orthophosphate, and help plant phosphorus nutrition. Besides, they produce siderophores, which are very much effective in iron uptake in diversified crop plants. Therefore, the endophytic Bacilli are considered as microbial agents for enhancing uptake and better utilisation of nutrients in different crop plants under normal and harsh environmental conditions. This chapter updates our understanding of nutrient accumulation in non-legumes crop plants by endophytic *Bacillu*s spp.

Keywords ^{15}N isotopic dilution · Phytohormones · Non-legumes · Endophytes · ATP-ase activity; · Cell membrane

16.1 Introduction

Endophytic bacteria thrive and colonize in the internal tissue of the plant showing no external sign of infection or adverse effect on the host plant (Afzal et al. 2019; Holliday 1989; Schulz and Boyle 2006). Generally, they complete their life cycle partially or wholly inside the plant and inhabited in the apoplastic area, i.e. intercellular space or middle lamella of epiblema, cortical tissue of roots, or in the xylem vessel of stem and even in the leaf as well (Xia et al. 2015). They can be regarded either as obligate or facultative endophytes where obligates are not culturable and require a more specific environment for their growth. On the contrary, the facultative endophytes are able to survive in soil, rhizosphere, artificial medium, and inside the plants. The facultative endophytes are widely distributed throughout the plant biota for their advantage of growth potentiality, higher adaptability under adverse conditions. The most common endophytes were isolated from wild or cultivated crops of monocotyledonous and dicotyledonous plants. They may be classified as actinomyces, bacteria, and fungi depending on the microorganism, with bacterial and fungal endophytes being the most studied organisms. The interactions between endophytes and the host plants are complex which involve mutualism and antagonism, and the association might be obligate or facultative. Nearly 300,000 plant species are present on earth, and each individual plant is host to one or more endophytes (Strobel et al. 2004). Only a few of these plants have ever been completely studied in relation to their endophytic biology. Hence, the potentiality to explore this arena is crucial, in order to boost up the biofertilizer development in a sustainable approach. Plant growth-promoting bacterial endophytes (PGPBE) have also been identified which promote plant growth and development in versatile approaches (Mia et al. 2016). Their identification as PGPEBE is attributed to their role in enhancing plant growth using various traits namely production of ammonia, indole-3-acetic acid (IAA), siderophores, N_2 fixation, and accumulation of essential nutrient elements viz. P, K, Ca, Mg, and Fe (Mia et al. 2009). Several rhizospheric or endophytic bacteria belonging

to the genera *Azospirillum, Azotobacter, Burkholderia, Bacillus, Enterobacter, Pseudomonas, Rhizobium*, and *Agrobacterium* have been noted as plant growth-promoting (PGP) microorganisms (Vessey 2003). Among them, *Bacillus* is frequently reported as a potential biofertilizer due to its multifunctional PGP traits namely, phosphate solubilisation, IAA production accumulation of nutrients, siderophore (iron chelator) production, and biopesticidal activity (Bahadir et al. 2018; Bjelić et al. 2018; Mohite 2013; Wahyudi et al. 2011, 2020). In addition, biofertilizers containing *Bacillus* strains are considered important because of their spore-forming capacity, allowing their adaptation to extreme abiotic conditions, like extreme temperatures, pH, or pesticide exposure. These species have been shown to have positive effects on soybean seed germination by enhancing the root and shoot length or the number of lateral roots of the seedling, which is related to the production of phytohormone and siderophore, and the capability of these bacteria to solubilize P complex. It has also been reported that *Bacillus* promoted seed germination and growth of tomato, pepper, eggplant, and rice (Mia and Shamsuddin 2013; Beneduzi et al. 2008). Other studies also revealed that *Bacillus* have improved plant growth under drought stress and produced a variety of compounds that can be used for the management of a wide array of plant pathogens. Bacteria viz. *B. megaterium, B. cereus, B. pumilus, B. circulans, B. licheniformis, B. subtilis, B. brevis,* and *B. firmus* have been recognised as N_2 fixers based on their nitrogenase activity (Radhakrishnan et al. 2017). It is the most abundant genus in the rhizosphere, and the PGP activity of some of these strains has been known for many years. There are several metabolites that are released by these strains, which strongly affected the environment by increasing nutrient availability in the plants (Barriuso and Solano 2008).

Currently, one of the major challenges of agriculture is to boost up crop productivity under adverse environmental conditions, especially poor fertile soils having less availability of nutrients. It is interesting to note that microbes that are beneficial to plants are used to enhance crop yield and are alternatives to chemical fertilizers. Additionally, several endophytic *Bacillus* species have emerged as a complementary, efficient, and safe alternative to current crop management practices. It is reported that endophytic *Bacillus* is not host-specific, can colonize a wide array of crop plants, and this gives it a great potential tool for increasing crop productivity in a sustainable manner. This chapter reviews current knowledge on the roles of endophytic *Bacilli* in nutrient accumulation in non-legume crop plants.

16.2 Beneficial Effects of Endophytes on Accumulation of Nutrients in Non-legumes

The major contributions of beneficial and biofertilizer endophytes are to supply nutrients like fixed N_2, enhanced uptake of other essential nutrients like P, K, Zn, and Fe. However, limited information are available on the direct contribution of endophytes to host plant like legume-*Rhizobium* symbiosis process (Hardoim et al. 2008). Nevertheless, endophytes mediate plant growth promotion through direct

and indirect mechanisms. Since they start their journey as rhizosphere bacteria, it is assumable that they may retain their attributes inside the plant. Their mechanisms of beneficial effects seem related to rhizosphere bacteria because most endophytes can be cultured and can survive outside of the host in the rhizosphere. A list of endophytic *Bacilli* performs beneficial effects has been presented in Table 16.1.

Table 16.1 Performance of endophytic *Bacillus* spp. inoculation on growth and development of different crop plants

Host plant	Name of endophytic *Bacillus*	Mode of beneficial effect	References
Rice (*Oryza sativa* L.)	*Bacillus aryabhattai* strain E7, *B. aryabhattai* MN1, *B. fortis* strain T9. *B. aryabhattai* strain HS-S05; *B. megaterium* strain KW7-R08; *B. subtilis* strain CB-R05	Increase rice plant growth Synthesis of indoleacetic acid (IAA)	Shen et al. (2019) Ji et al. (2014)
Sweet sorghum (*Sorghum bicolor*)	*B.* spp.	Increase plant growth	Mareque et al. (2015)
Banana (*Musa* spp.)	*B. sphaericus* strain UPMB10	Increase growth and nutrient uptake	Mia et al. (2007)
Beet (*Beta vulgaris* L.)	*B. pumilus*	Increased concentration of carbohydrates	Shi et al. (2010)
Sugar cane (*Saccharum officinarum* L.)	*Bacillus* sp. strain H15	Increase plant growth	Chauhan et al. (2012); Wang et al. (2020)
Cocco *(Theobroma cacao* L.)	*B. subtilis*	Promote plant growth	Leite et al. (2013)
Sunflower *(Helianthus annus* L.)	*B. pumilus*	Improve plant growth	Forchetti et al. (2007)
Maize *(Zea mays* L.)	*B. subtilis, B. lentimorbus*	Nitrogen fixation; IAA synthesis; growth promotion	Wang et al. (2010); Szilagyi-Zecchin et al. (2014)
Soybean *(Glycin max* L.)	*B. amyloliquefaciens, B. japonicum*	Production of siderophores; IAA synthesis; ACC-deaminase; antifungal activity; phytases; N_2 fixation	Sharma et al. (2013) and Hungria et al. (2013)
Wheat (*Triticum aestivum* L.)	*B. subtilis, Bacillus* spp.	IAA synthesis; phosphate solubilisation; growth promotion; increase in grain yield	Wang et al. (2010); Hungria (2011) and Upadhyay et al. (2012) Zhao et al. (2015)

16.2.1 *Mechanism of Beneficial Effects of* **Bacillus** *Spp. on Plants*

Unlike nutrient accumulation, they can directly benefit plants by providing nitrogen supply via N_2 fixing abilities iron chelators and siderophore production (Long et al. 2008), P complex solubilizing compounds and (Knoth 2014). In addition, they influence on plant growth through the production of phytohormones especially auxin which increased root growth through hair formation, increased lateral roots, volume, and surface area. Also, several S oxidizing endophytes are known which can oxidize elemental S into SO_4^{2+} to be used by plants (Banerjee and Yesmin 2009). Moreover, endophytes are prolific sources of phytochemicals (Nisa 2015) which impede plant hygiene (Chen 2011; Benhamou et al. 1998). They are also excellent sources of biologically active secondary metabolites and contributing to the production of metabolites (Brader 2014; Schulz and Boyle 2002).

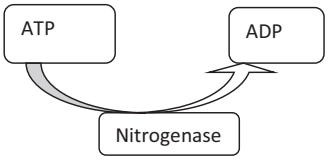
16.2.2 *Phytohormone Production in Relation to More Root Growth for Higher Nutrient Uptake*

Plant growth promotion by endophytic *Bacillus* spp., through phytohormone production, is perhaps the well-agreed method which causes morphological and anatomical changes in roots of the plant. The mechanism of phytohormones production by endophytic *Bacillus* in host plants is similar to plant growth-promoting rhizobacteria. But here, the roots get extra benefits for proper utilisation of synthesized auxin, since the endophytes remain in the apoplastic areas. There are several pathways to produce auxin where endophytes mainly follow the tryptophan precursor–mediated pathways. Whatever the pathways followed for auxin synthesis, the products are released in the apoplastic area of root cortical zone transported to endodermis through apoplastic and symplastic pathways via downhill process. The endodermis releases auxin to the pericycle, located just beneath it; the auxin stimulates the pericycle to convert into meristem consequently form the lateral roots primordia and finally, produced the lateral roots as well as the root hair (Péret et al. 2009; Waidmann et al. 2020). The increased root growth facilitated the plants to absorb more nutrients through root interception, which is one of the mechanisms of nutrient uptake (Marschner 1995).

16.2.3 *Atmospheric Nitrogen (N_2) Fixation*

Nitrogen is one of the most important nutrient elements which limits crop yield under deficient conditions. The main source of N is the atmospheric N_2, which is unavailable to crop plants due to strong triple bond ($N \equiv N$), which must be reduced

by the fixation process. Biological N_2 fixation is the most significant where a large amount of N_2 is fixed by various types of symbiotic associative free-living and endophytic bacteria in association with plants, both of dicotyledon and monocotyledon. The process is mediated by the activity of enzyme nitrogenase using ATP and the overall reaction is shown as follows:



$$N_2 + 8e^- + 8H^+ 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16Pi$$

Diazotrophic endophytes can fix a handsome amount of N_2, which have been recorded in rice, wheat, maize, sugarcane, oil palm, and bananas, and have been documented by various researchers (Mia et al. 2007, 2010a; Amir et al. 2001; Bashan and Holguin 1997). The mechanism of fixing N_2 by nitrogenase enzyme present in the endophytes are very much similar to *Rhizobium* species symbiosis with legumes, and their genomes possess an *nifHDK* operon, encoding both nitrogenase components: the nitrogenase protein (MoFe protein, *NifDK*), which contains a molybdenum–iron cofactor in the prosthetic group (Carvalho et al. 2014). The nitrogenase enzyme is encoded by ~20 genes which are termed as *nif* genes (N-fixation genes) that are organised in seven operons (*nif* cluster) spanning over 24 kb. The *nifH* gene encodes the Fe protein and *nif D* and *nifK* genes encode the Mo-Fe protein of the nitrogenase enzyme (Rubio and Ludden 2008; Seefedt et al. 2009). The fixed N_2 could be confirmed and measured by several methods like acetylene reduction assay (ARA), ^{15}N isotopic both enrichment and dilution techniques. The ARA technique is a qualitative measure for the activity of enzyme nitrogenase and can be used under certain cases and conditions (Mia and Shamsuddin 2010; Danso 1985). The ^{15}N isotopic technique is used to provide a direct method for detecting BNF (Danso 1995), which comprises enrichment and dilution techniques. The ^{15}N isotope dilution technique was used to quantify the contribution of BNF to clover legume by McAuliffe et al. (1958) which has the capacity to separate out any plant-associated contribution of BNF to plants and is recognised as a more accurate method (Boddey et al. 1996; Roger and Ladha 1992).

The microbial bioassay where isolated endophytes could be cultured specific N-free cultural media and specific conditions and can be quantified by the degree of growth of endophytic bacteria (Das and De 2018). Cell sap analysis is another technique of estimating N_2-fixation where analysis of N solute in xylem exudates and plant parts is based on the determination of the composition of N compounds in plant tissues or N flowing through the xylem sap to the shoot, although the method has some limitation as only a small proportion of known N_2 fixing plants are ureide exporters (Mia and Shamsuddin 2010). A list of N_2-fixing *Bacilli* spp. has been presented in Table 16.2.

Table 16.2 List of N_2-fixing endophytic *Bacillus* spp. beneficial to different non-legumes

Endophytic *Bacillus* sp.	Isolated from crop plant	Colonized into crop	Method used to confirm N_2-fixing ability	References
B. aryabhattai strain HS-S05 *B. megaterium* *B. subtilis* strain CB-R05	Rice (*Oryza sativa* sub sp. *japonica*)	Rice (*Oryza sativa* sub sp. *japonica*)	Amplification of *nifH* genes	Ji et al. (2014)
B. subtilis strain EB-04; *B. pumilus* strain EB-64, EB-169; *Paenibacillus* sp. strain EB-144	Banana cultivar 'Prata Anã' (*Musa acuminata* × *balbisiana*)	–	Amplification of *nifH* genes; acetylene reduction assay (ARA)	Andrade et al. (2013)
B. sp. strain CNPSo 2476, CNPSo 2477, CNPSo 2478;	Corn (*Zea mays* L.)	Corn (*Zea mays* L.)	Amplification of *nifH* genes; ARA	Szilagyi-Zecchin et al. (2014)
B. cereus strain KU097330	Sugarcane (*Saccharum officinarum* L.)	Sugarcane	ARA	Hossain et al. (2020)
B. sphaericus strain UPMB10	Oilpalm roots *(Elaeis guineensis)*	Oil palm, banana, soybean, sweet potato	ARA, ^{15}N isotopic dilution technique. ARA	Amir et al. (2001) Mia et al. (2007, 2010a, 2010b), Saad et al. (1999)

A good number of endophytic *Bacillus* spp. have been identified based on the nitrogenase activity like *B. megaterium, B. cereus, B. pumilus, B. circulans, B. licheniformis, B. subtilis, B. brevis,* and *B. firmus* (Xie et al. 1998). One of the plant-associated members, viz. *B. sphaericus* strain UPMB10, fixes a substantial amount of atmospheric N_2 in association with various non-legumes, including, banana, oil palms, and sweet potato (Mia et al. 2007, 2010a; Mia and Shamsuddin 2010; Mia et al. 2013). The strain UPMB10 has been evaluated for their N_2-fixing capacities in association with oil palm seedlings, banana, rice, and vegetable soybean through ARA and ^{15}N isotopic dilution technique (Mia et al. 2007). Inoculation with *B. spharecus* strain UPMB10 could fix 37–39%N_2 in banana tissue cultured plantlets enumerated by ^{15}N isotopic dilution technique. The highest % Ndfa (nitrogen derived from atmosphere) was recorded at the lowest fertilizer-N applied plant with strain UPMB10. Generally, % Ndfa declined with an increase in fertilizer N application, making a lower contribution of N_2 fixed to the total plant N. The confirmation of N_2 fixation was supported by higher ARA values (129 ηmole plant^{-1} hour^{-1}) in roots of inoculated plants (Mia et al. 2007). In field conditions, rice plants could obtain 20% of their total N requirement and 25–50% of N for oil palm in nursery condition by rhizobacterial inoculation (Shrestha and Ladha 1996; Amir et al. 2001). In a greenhouse study on sugarbeet, three different *Bacillus* isolates fixed N_2 and increased growth (Çakmakçi et al. 2006). Enhanced accumulation of N in sweet

sorghum by inoculation of *Bacillus* spp. were recorded by Ribeiro et al. (2018). In another study, Hafeez et al. (2006) noted that selected *Bacillus* sp. used as bioinoculants on wheat resulted in increased root length, plant biomass, and higher accumulation of N and P. Generally, they are widely used in crop production system and are important P-solubilizing inocula, resulting in improved growth and yield of crops (Prakash and Arora 2019).

The production of plant growth regulators such as auxin, cytokinin, and gibberellin by these bacteria may also give additional support to the growth and development of host plant species (Joo et al. 2009; Kang et al. 2009). Recent literature indicates that *Bacillus* sp. have a positive role in plant growth enhancement and biologically active metabolites production (Indiragandhi et al. 2008; Kang et al. 2009). Shen et al. (2019) isolated *B. aryabhattai* E7, *B. aryabhattai* MN1, and *B. fortis* T9 from rice seedling roots and found that these strains have both N_2 fixation potential and IAA production abilities. Sequence analyses of endophytic *Bacilli* in banana cv. 'Prata Anã' roots revealed that PCR amplification of the *nifH* gene was detected in 24 of the 102 bacterial isolates. Seven species of *Bacillus* were *nifH*-positive, including: *B. amyloliquefaciens*, *B. cereus*, *B. flexus*, *B. licheniformis*, *B. pumilus*, *B. subtilis,* and *B. tequilenses* (Suzane et al. 2013). Çakmakçi et al. (2006) observed that three different *Bacilli* isolates fixed N_2 and increased growth in sugar beet in a greenhouse study. A good number of endophytic and associative *Bacillus* spp. have been commercialized as bioinoculants for the growth of crop plants (Islam and Hossain 2013).

16.2.4 Transfer of Fixed N_2 to Host Cell

Whatever the amount of N_2 fixed by endophytes, the release and transportation of fixed N_2 to the host tissues is of the greatest importance. The release or excretion of NH_4^+ and subsequent transportation to the host cell is not clearly identified in the case of associative bacteria since very little amount of N_2 is fixed, which is most probably being utilized by the bacteria itself. Nevertheless, several strains of endophytic bacteria have a unique N regulation system where the NH_4^+ is excreted out of the cell through simple diffusion despite accumulation in its own cell (Brewin et al. 1999; Castorph and Kleiner 1984; Day et al. 2001; Kleiner 1982). The excreted NH_4^+ are transported to cytoplasm via ion channel or plasmodesmata either by downhill or uphill process thereafter being utilized via GS-GOGATT pathway for the synthesis of amino acid and subsequently, other amino acids through transamination process (Mia 2015).

16.2.5 Solubilisation of Soil Insoluble Phosphates

Apart from N_2 fixation, several endophytes release organic acids into the soil which solubilize the phosphate complexes and convert them into ortho-phosphate for plant uptake and utilisation. Although phosphorous exist abundantly in soils, most of it remains unavailable as an insoluble form (Miller 2010). Endophytic *Bacillus* is capable to solubilize the complex unavailable rock phosphate to an available form of P where inoculation of *Bacillus* spp. could solubilize the P-Fe minerals and enhanced uptake of P and Fe in pearl millet (Ribeiro et al. 2018). A good number of *Bacillus* spp. have been isolated from bananas and aerobic rice by Matos et al. (2017) and Panhwar et al. (2009), which are very much effective in the solubilisation of rock phosphate. Rhizobacteria *B. methylotrophicus* CKAM isolated from apple roots showed higher P- solubilisation (Mehta et al. 2014). Similarly, the application of *B. megatorium* in combination with fish bone produced the highest amount of available P for crop plants (Saeid et al. 2018). One of the most important mechanisms is the production of organic acid and release to the soil to solubilize monocalcium, bicalcium, and tricalcium complex phosphate to simpler phosphate as well as increase the activity of phosphatase. The concentration of organic acid is released through root exudates is decreased with increasing the presence of complex phosphate in the rhizosphere.

16.2.6 Enhancement of K Uptake by Plants

Potassium is an essential macronutrient element that contributes to the growth and development of crop plants by influencing the activities of various enzymes system. This element has non-specific functions not directly involved in any reaction system in the cell and do physiological functions in the various arena through osmoregulatory, enzymetic regulation, charge balance in the cytosole (Mia 2015). The greatest amount of K is found in the plant body which is highly mobile in the plant system. Although higher quantities of K present in the soil plant cannot take this element as those are fixed and unavailable for the plants. Recently, the use of potassium solubilizing bacteria (KSB) viz. *Acidothiobacillus ferrooxidans, Paenibacillus* spp., *B. mucilaginosus, B. edaphicus,* and *B. circulans* have shown to solubilize K- bearing minerals, like biotite, feldspar, illite, muscovite, orthoclase, and mica. This type of bacteria can dissolve silicate minerals and release K through the production of organic and inorganic acids, acidolysis, polysaccharides, and complexolysis, chelation, and exchange reactions. The application of *B. sphaericus* strain UPMB10 could not increase the K concentration but its total accumulation was greatly increased due to higher (36%) dry matter production in tissue-cultured banana plantlets growth under a hydroponic condition where dry matter production is directly related to K accumulation. The higher accumulation of K might be due to enhanced root proliferation from the inoculation process (Mia et al. 2010a). Anyway,

the KSB have a greater impact on the release of K by various mechanisms. Inoculation of KSB *Bacillus* strain B2084 and B2088 increased the higher accumulation K in a shoot of pearl millet (Ribeiro et al. 2018). The endophytes may also have functions in providing K for a plant by increasing root exudates through supplying different chemicals which can lower the pH by proton efflux, enhancing chelation of the cation bonds to K, and acidolysis of the fixed K area of the soil (Etesami et al. 2017; Meena et al. 2014).

16.2.7 Improvement of Ca Uptake and Absorption by Plants

The endophytic PGPB inoculation greatly increased Ca concentration (nearly 14%) in root especially in plants inoculated along with supplemental 33% fertilizer-N in banana. However, inoculation without fertilizer-N, which could not show any increment, indicated the requirement of starter inorganic N. As Ca is a non-mobile element in the plant system, a higher concentration is found in the root and no influence has been observed in the pesudostem and leaf. Similarly, inoculated plants provided with 33% fertilizer-N also showed lower concentrations when compared to inoculated plants without fertilizer-N. However, in leaf, application of 100% fertilizer-N showed lower Ca concentration (Mia et al. 2009).

The higher Ca uptake by the inoculated plants might not only be due to higher plant growth but also higher uptake capacity, which is induced by bacterial interaction through the acceleration of proton efflux, which results in the acidification of the rhizosphere. It is one of the important mechanisms in cation uptake by the roots, which is the consequence of stimulation of root membrane ATP-ase activity. The endophyte could increase this enzyme activity through bacteria-root interaction as both the strain successfully colonized banana roots. Higher activity of this enzyme resulted in higher Ca concentration in roots (Marschner 1995). Similarly, Bashan et al. (1989) concluded that *Azospirillum* could increase the proton efflux by stimulating ATP-ase activity in the root of wheat seedlings. Increased accumulation of Ca in the cytosole may be the stimulation of the Ca^{2+} ion channel consequently greater influx of Ca^{2+} from the apoplastic area.

16.2.8 Influence of Endophytic Bacilli on Uptake and Absorption of Mg by Plants

Magnesium is an essential macronutrient element which is mobile in the plant system. It plays an important role in the synthesis of chlorophyll molecule as it is the central atom of chlorophyll. Application of endophytic bacteria *B. sphericus* strain UPMB10 could not increase the concentration of Mg rather enhanced the total

accumulation which is due to increased root growth not to general uptake rate as in Ca.

16.2.9 Influence of Bacillus *Spp. on Siderophores Production and Iron Uptake*

Iron is an important micronutrient element that performs many metabolic activities in the plant through the activation of many enzymes taking part as a prosthetic group. However, it is a problematic element in case of uptake and absorption by the plants because the ferric form (Fe^{3+}) readily becomes insoluble in the soil. Therefore, crop plants suffer deficiency despite having sufficiency in the soil. Recently, the microbial siderophore technique in the uptake of iron is gaining prominence for boosting up crop productivity. It is a smaller molecule organic compound synthesize by microorganisms and is capable of absorbing ferric even under Fe-limited soil conditions thereby solving iron nutrition problems in crop plants. The Fe^{3+} siderophore complex can easily enter into cytosole and release the Fe^{3+} where it reduces as $Fe^{2+}$for further utilisation (Saha et al. 2016). Similarly, endophytes also synthesize siderophore which can absorb and utilize Fe^{3+}as like as rhizosphere bacteria. Endophytes produce small molecular compounds called siderophores, which are iron-chelating compounds that can make available iron to plants and deprive pathogens of iron (Compant et al. 2005). Out of the range of siderophore produced by endophytes, one with biocontrol properties are catecholate, hydroxymate, and/or phenolate types (Rajkumar 2010). Also, siderophores specifically help iron-deficient plants in fixing N_2, since diazotrophs require Fe^{2+} and Mo factors for nitrogenase synthesis and functioning (Kraepiel 2009). The interaction between plants and beneficial bacteria can have a profound effect on plant health, growth, development, production, and soil quality.

Ribeiro et al. (2018) reported the capacity of four endophytic *Bacillus* strains to solubilize iron phosphate (Fe-P), produce siderophores and indole-acetic acid (IAA) in vitro, and evaluate their plant growth promotion ability in greenhouse conditions by inoculation into pearl millet cultivated in a P-deficient soil. All strains solubilized Fe-P, and three of them produced carboxylate-type siderophores and high levels of IAA in the presence of tryptophan.

16.3 Conclusion and Future Perspectives

Endophytic *Bacillus* spp. promote better nutrition and increase the yield of crop plants. These biologicals are highly potential tools for boosting crop productivity in a sustainable agricultural system. They exert beneficial effects on plants through multifaceted mechanisms. Recently, *Bacillus* spp. gaining prominence as potential

candidates for endophytic biofertilisation. This chapter reviews the effect of these bacteria on the nutrient accumulation of non-legumes and discusses their mode of action. Our knowledge of molecular crosstalks between endophytic *Bacillus* spp. and host plants are limited (Alvin et al. 2014). Recent progress in genomics and post-genomics analytical methods enhances our understanding about the underlying molecular mechanisms of the beneficial effects of endophytic Bacilli on plants. Exploration of elite strains of endophytic Bacilli and their genes involved in exerting beneficial effects on plants would help us to apply them in sustainable and climate-smart agricultural systems. Furthermore, the application of the recently developed genome editing by the CRISPR-Cas toolkit would be helpful to design industrially powerful biofertilizers and growth promoters from the genus of plant endophytic *Bacillus*.

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Chapter 17
Role of *Bacillus* Species in Alleviating Biotic Stress in Crops

Neha Chausali and Jyoti Saxena

Abstract Feeding the growing world population has become a crucial issue with each passing year. At present, the prime focus of farmers and scientists is on maximizing yield and minimizing the damages to food crops by diseases and harsh environmental conditions. Synthetic pesticides and fertilizers are being used abundantly in agricultural fields to increase productivity but the indiscriminate use of synthetic chemicals has resulted in severe pollution of soil and water. Consequently, practices as the use of biopesticides and biofertilizers have become an eco-friendly alternative for harmful agrochemicals, thus encouraging sustainable agriculture. A group of bacteria characterized as plant growth-promoting rhizobacteria (PGPR) has been known to reinforce plant growth and development and also mitigating abiotic and biotic stresses. Many weeds and phytopathogens such as bacteria, fungi, viruses, and nematodes may induce biotic stress in their plant hosts resulting in reduced biomass, crop quality, and yield. Various species of *Bacillus* are well-known PGPR and are also considered as potential biocontrol agents for many plant diseases. These are used to combat biotic stresses by inducing physiological changes in plants and secreting several metabolites in response. The present chapter focuses on the biotic stress management by *Bacillus* spp. and the various mechanisms involved in it.

Keywords Biopesticides · Biofertilizer · Plant growth-promoting bacteria · Phytopathogens · Biocontrol · Biotic stress

17.1 Introduction

Human beings depend on agriculture to a large extent for their food necessities. India is an agriculture-based economy with 18% of its GDP contributed by the agriculture sector. Also, 70% of its rural households and 58% of the total population

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depend predominantly on agriculture for their livelihood (FAO 2020; Tripathi et al. 2020). The world population at present is about 7.7 billion (https://www.worldometers.info/worldpopulation) and is projected to increase by 10 billion in the next 50 years (Etesami and Maheshwari 2018; Glick 2014), hence, requiring 70% more food production (FAO 2009). To achieve this, the expansion of agricultural land and a significant increase in production will be the major target. Inadequate food supply may create an alarming situation worldwide in the future. So the agriculture sector requires much more attention from the research community and the government.

For many years, various synthetic chemicals have been used to enhance food production which has caused serious threats to the environment and human health. Moreover, the environmental stresses including abiotic and biotic stresses have also been a big hurdle and limiting factor for agricultural production (Etesami et al. 2020). Therefore, the use of biological environment-friendly alternatives to agrochemicals came into a trend to overcome problems associated with chemical-based products. Microorganisms play a significant role in enhancing plant growth and mitigating biotic and abiotic stresses posed by harsh environmental conditions and phytopathogens. PGPR is a well-known group of bacteria used extensively in plant growth and health promotion of various crops. Several microorganisms belonging to genera *Acetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, and *Serratia* are the most studied plant growth promoting bacteria and also reported for abiotic and biotic stress mitigation (Jha et al. 2013; Mishra et al. 2017; Verma et al. 2019). *Bacillus* and *Pseudomonas* are dominantly used PGPR for agricultural applications due to their beneficial role in plant growth and development, however *Bacillus* based biofertilizers are more effective than *Pseudomonas* due to their spore-forming nature and more efficient metabolite production capability which increases their commercial applicability (Haas and Défago 2005; Ongena and Jacques 2008). *Bacillus* species possess some unique characteristics that make it a potential candidate for biological control as it replicates rapidly and has large genetic biodiversity. Also, due to spore-forming ability, it can survive in extreme environmental conditions like high or low temperatures, unsuitable pH, and insufficiency of nutrients or water (Albayrak 2019). *Bacillus* is a ubiquitously found genera in nature, some species are free-living, while others are endophytic and can colonize the rhizospheric zone of plant root and internal tissues. It is a gram-positive spore-forming bacterium having immense applications in industry, agriculture, and medicinal fields (Lyngwi and Joshi 2014). Further, *Bacillus* spp. have been identified for their presence in stressed environments and also reported for alleviating biotic and abiotic stress (Yadav et al. 2016; Mishra et al. 2017; Ahmad et al. 2018). It has shown a good response in tolerating abiotic stresses like salinity, water deficit, heavy metal toxicity, flooding, extreme temperatures, and nutrient deficiency (Etesami et al. 2020). On the other hand, biotic stresses like weeds, nematodes, and phytopathogens (bacteria, fungi, and viruses) affect crop quality, biomass, and yield negatively and the species of *Bacillus* have been found very effective against them. Therefore, various species of *Bacillus* have been reported to act as a biocontrol agent for various phytopathogens and pests (García-Fraile et al. 2015; Kang et al. 2015). The phytopathogens can be controlled by the action of several cell wall degrading enzymes produced by

Bacillus such as cellulase, chitosanase, glucanase, protease, and other compounds viz. hydrogen cyanide and lipopeptides (Radhakrishnan et al. 2017). Also, a range of metabolites produced by *Bacillus* spp. including antibiotics, lipopolysaccharides (LPS), salicylic acid (SA), siderophores, and hydrolytic enzymes (Hassan et al. 2010, 2015; Qin et al. 2011) were found to be responsible for suppressing the growth of pathogens and boosting up the plant defense mechanisms (Rais et al. 2017).

As shown in Fig. 17.1, *Bacillus* can control plant diseases through various mechanisms such as competition for nutrients and ecological niche in the rhizosphere, production of inhibitory chemicals and metabolites, and induced systemic resistance (ISR) in plants (Cawoy et al. 2011; Rais et al. 2017). *Bacillus* spp. are also responsible for enhancing plant immunity by altering stress-responsive genes, phytohormones, proteins, and allied metabolites and also induce physiological changes including nutrient uptake, regulation of water transport, etc. (Radhakrishnan et al. 2017). In addition, *Bacillus* species significantly stimulate the production of antioxidant defense enzymes like superoxide dismutase, peroxidase, and other enzymes, which are known to suppress diseases in plants by diminishing the reactive oxygen species (ROS) causing oxidative stress (Liu et al. 2011; Shi et al. 2006; Yasmin et al. 2016). The association of *Bacillus* spp. with plant roots promoted plant growth by the formation of biofilm (Beauregard et al. 2013) and enhanced the availability of nutrients such as phosphate (by P solubilization) for plant uptake (Jha et al. 2012; Minaxi et al. 2012). Various species of *Bacillus* genera can produce an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which in turn can alleviate environmental stresses by reducing the ethylene level in the host plant (Minaxi et al. 2012; Misra and Chauhan 2020). This enzyme cleaves ACC (a precondition of ethylene production) to α-ketobutyrate and ammonia and thus reduces ethylene levels in plants (Etesami et al. 2020). In addition to ACC deaminase, the genera also produced indole-3-acetic acid and gibberellic acid that regulated intracellular

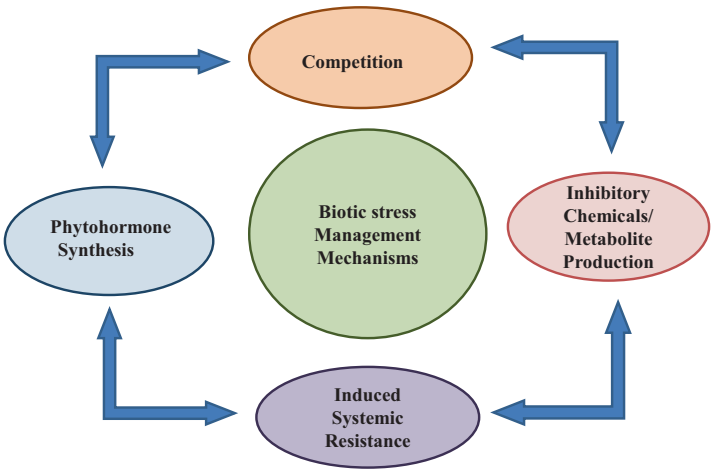

Fig. 17.1 Various mechanisms involved in the mitigation of biotic stresses

phytohormone metabolism, which consequently increased plant stress tolerance considerably (Minaxi et al. 2012; Radhakrishnan et al. 2017).

The global biopesticide market was estimated to grow about 3.0 billion USD in 2018 and is expected to grow about 6.4 billion USD by 2023, at a CAGR of 15.99%. Furthermore, the major driving force for the growth of the biopesticide market is the rise in the organic industry, increase in the cost of synthetic pesticides, growing insect resistance to these chemicals, and awareness towards hazards caused by chemical pesticides to the environment (https://www.marketsandmarkets.com). Among all bacterial biocontrol agents, approximately 70% of the total sale is contributed by *Bacillus thuringiensis* (Cawoy et al. 2011). This bacterium is the source of the Bt gene used in "Bt GMO crops" and about half of the commercial bacterial biocontrol agents belong to this species (Cawoy et al. 2011). There are numerous advantages of using biopesticides over chemical products. Microbial pesticides do not cause pollution as they decompose quickly and are not toxic for nontarget species (Cawoy et al. 2011). Also, they do not have any bad impact on health and the environment.

A limited number of studies are available on physiological changes induced by *Bacillus* species that occur in plants in stressed conditions. The present chapter deals with different biological stresses in crops and the beneficial effects of *Bacillus* species in alleviating biotic stresses through different mechanisms.

17.2 Alleviation of Biotic Stress in Plants by the *Bacillus* Species

Plants may encounter biotic stress due to the presence of weeds, phytopathogens, nematodes, etc. in agricultural fields which affect crop productivity inversely. *Bacillus* species and other PGPR have the capacity to promote the growth of plants as well as mitigate biotic and abiotic stresses. The effect of these PGPR on plant growth and their role in plant disease control has been well demonstrated (Etesami and Maheshwari 2018; Compant et al. 2005).

As illustrated in Fig. 17.2, plants under biotic stress generally employ two defense mechanisms. First, constitutive defense includes performed barriers like walls, waxy epidermal cuticles, bark, and metabolites, whereas the second is inducible defense, that is triggered by signal compounds, invaders, or herbivore attack and responds with the production of toxic chemicals, pathogen-degrading enzymes, and deliberate cell suicide (Freeman and Beattie 2008). Again, inducible mechanism has two categories, one is systemic acquired resistance (SAR), which relies on salicylic acid (SA) pathway, and another is induced systemic resistance (ISR), induced by some microorganisms such as mycorrhizal fungi and PGPR relying on ethylene (ET) and jasmonic acid (JA) signaling pathway (Boubakri 2020).

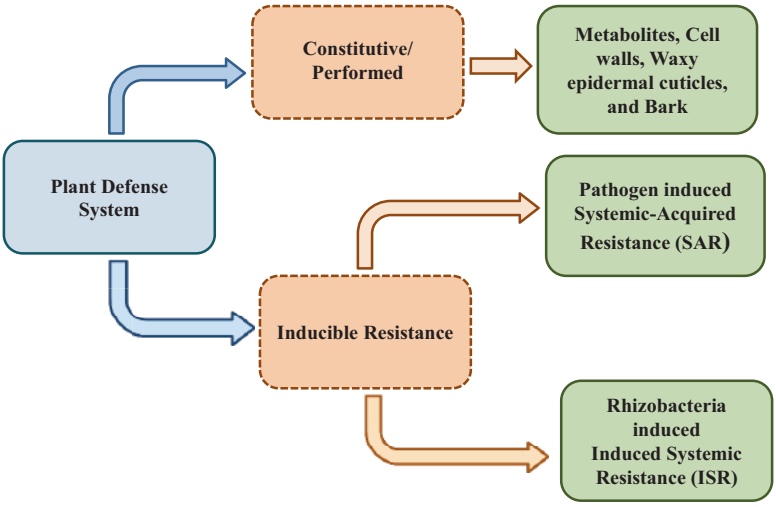

Fig. 17.2 Plant defense mechanism under biotic stress

Furthermore, Fig. 17.3 describes the direct and indirect mechanisms employed by *Bacillus* species to ameliorate biotic stress or plant diseases. Several PGPR including *Bacillus* species adopts one of these two basic mechanisms to combat biotic stress. The compound released in response to stress that stimulated plant growth and ameliorates stress comes under a direct mechanism (Goswami et al. 2016). This encompassed a number of compounds like secondary metabolites including antioxidants (superoxide dismutase and peroxidase) and antibiotics. Various hydrolytic enzymes (cellulose, chitosanase, glucanase, hydrogen cyanide, lipopeptides, and protease), siderophores, hormones (IAA, gibberellic acid, etc.), and other metabolites such as LPS and SA were found to be produced in response to biotic stresses directly by species of *Bacillus* (Hashem et al. 2019). Also, nitrogen fixation, mineralization of organic phosphates, and solubilization of insoluble inorganic phosphates are also part of this mechanism, through which plants get nutrition for their growth and are able to survive in stressed conditions (Etesami and Beattie 2017; Etesami and Maheshwari 2018; Glick 2012; Hayat et al. 2012). Further, induction of systemic resistance and competitive omission support plant growth through an indirect mechanism in stressed conditions (Tripathi et al. 2012).

17.2.1 Molecular Mechanisms Behind Inducible Resistance (SAR and ISR)

The ISR is a systemic resistance developed by some non-pathogenic rhizobacteria that are able to suppress disease in plants (Van Loon et al. 1998). In contrast, SAR is a type of induced resistance that is developed in plants by prior exposure to a

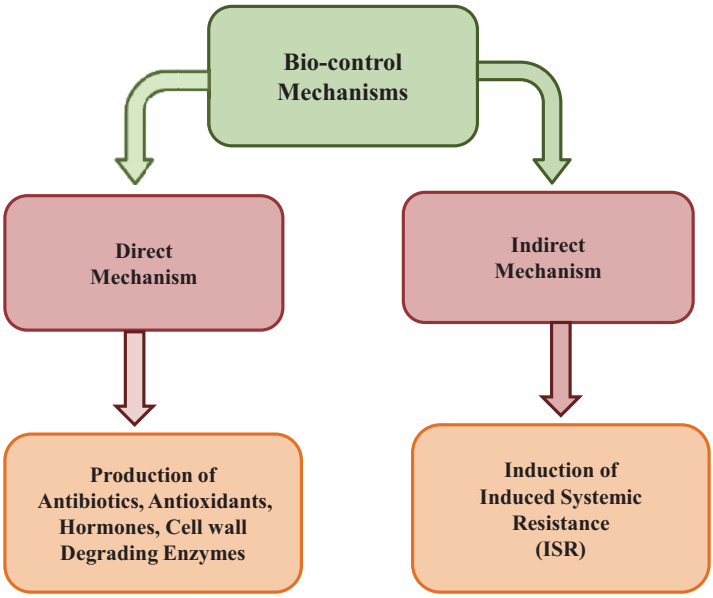

Fig. 17.3 Biocontrol mechanisms of *Bacillus* species

pathogen (Nie et al. 2017). A redox-sensitive transcription factor NIM1/NPR1 (nonexpressor of PR1) that regulates the expression of pathogenesis-related (PR) genes is a key player in both SAR and ISR mechanisms, as illustrated in Fig. 17.4 (Pieterse et al. 1998, 2014; Conrath et al. 2015). NPR1 induces the expression of pathogenesis-related (PR) genes in response to SAR signal molecule, salicylic acid (Hermann et al. 2013). The NPR1 translocates to the nucleus after getting activated by SA and functioning as a coactivator of PR genes providing SAR, whereas during the development of ISR, NPR1 was found to act in the cytosol, though its exact role is unidentified (Asari et al. 2017). Elicitation of ISR by plant-associated bacteria was first demonstrated in *Pseudomonas* spp. and other gram-negative bacteria. Besides *Pseudomonas*, various *Bacillus* spp. specifically *B. amyloliquifaciens*, *B. cereus*, *B. mycoides*, *B. pasteurii*, *B. pumilus*, *B. sphaericus*, and *B. subtilis* are also reported as elicitors of ISR (Kloepper et al. 2004). In most cases, these ISR eliciting species of *Bacillus* genera have also been found to elicit plant growth promotion (Kloepper et al. 2004). Several species of *Bacillus* were found independent of the salicylic acid pathway but dependent on jasmonic acid, ethylene, and the regulatory gene NPR1 in elicitation of ISR. However, some ISR eliciting species of *Bacillus* are independent of jasmonic acid and NPR1 and dependent on salicylic acid (Choudhary et al. 2007; Kloepper et al. 2004). Moreover, in some cases, ISR mediated by the rhizobacterium *Bacillus* species such as *B. cereus* strain AR156 employed both the JA/ET and SA signaling pathways, and NPR1 (Niu et al. 2011). Numerous *Arabidopsis* mutants and reporter lines revealed that the activation of JA-dependent genes VSP2 and PDF1.2 signifying the participation of MYC/ABA

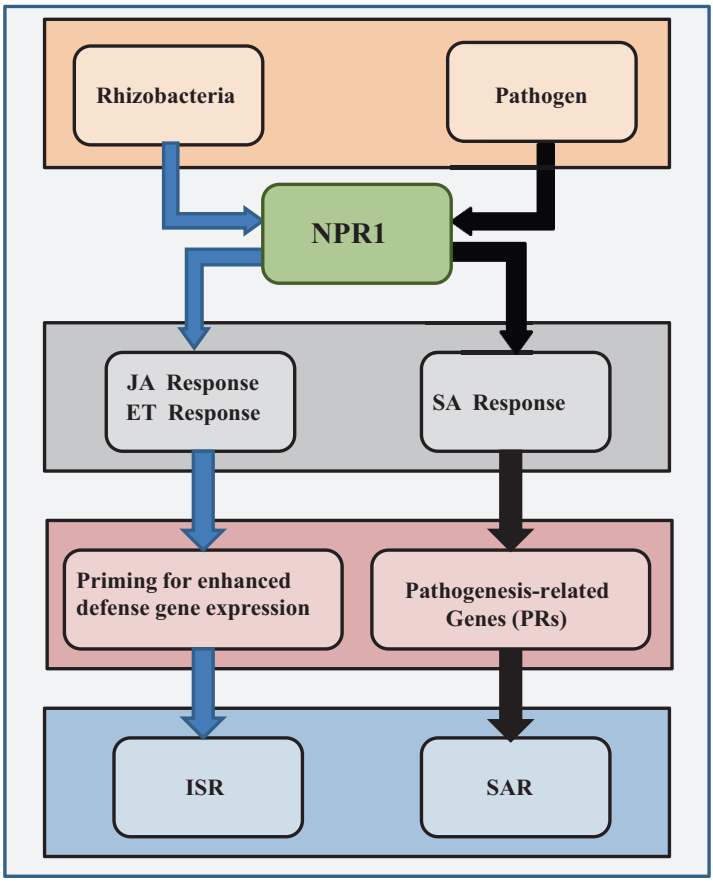

Fig. 17.4 Molecular mechanism of inducible resistance in plants

and ERF/ethylene, respectively (Pieterse et al. 2012). Further, SAR and ISR are well-characterized on the basis of key regulators such as NPR3 and NPR4 or COI1 (SAR) and MYB72 and MYC2 (ISR) with activation of defense genes, such as pathogenesis-related (PR) genes (SAR) or gene encoding plant defensin 1.2 (PDF1.2) and VSP2 (ISR) (Pieterse et al. 2014). However, the molecular mechanism for priming of ISR is not well acknowledged (Asari et al. 2017).

ISR is a sequential process involving three steps: (i) plant cells encounter elicitors produced by the inducing agents, (ii) initiation of signal transduction that propagates the induced state, and (iii) expression of defense mechanisms to inhibit the entry of the pathogen into the host tissues (Van Loon 2007). Salicylic acid and jasmonic acid pathways produce characteristic molecules like pathogenesis-related (PR) proteins (chitinases, β-1, 3-glucanases, proteinase inhibitors, etc.), phytoalexins (antimicrobial compounds), oxidative enzymes (peroxidases, polyphenol oxidases, and lipoxygenases) to diminish ROS and lignin for reinforcement of cell

walls (Boubakri 2018; Van Loon 2007). ISR-based biocontrol strategies have been investigated and some trials were successfully performed under field conditions. *Bacillus* spp. have been found to produce volatile compounds (VOCs) such as 2, 3-butanediol (Ryu et al. 2005), and lipopeptides that were recognized as elicitors of ISR (Cawoy et al. 2011).

17.2.2 Crop Protection from Pathogenic Fungi by the Application of Bacillus *spp.*

Crops are susceptible to various fungal diseases. They can adversely affect crop productivity and their growth leading to major losses in food production and storage worldwide (Savary et al. 2012). Various trends of *Bacillus* have been reported for controlling a wide range of plant diseases. Different *Bacillus*-based biocontrol agents and their target fungal diseases/fungi are listed in Table 17.1.

Members of the *Bacillus* genus are distinguished as the good source of biologically active molecules, which have antagonistic activities towards a wide variety of phytopathogens (Meena and Kanwar 2015). Direct and indirect mechanisms as discussed before are used to biologically control the growth of pathogenic fungi in the host plant. Under direct mechanism, *Bacillus* spp. produce a number of metabolites and enzymes which directly inhibit the growth of pathogenic microorganisms and are effective against a broad spectrum of fungal species (Stein 2005). Lipopeptides such as surfactin (bacillomycin D), iturin, fengycin, and kurstakin, which are commonly found in *Bacillus* genera, have been well-known for their antimicrobial properties. These lipopeptides are composed of a lipophilic fatty acid chain and a hydrophilic peptide ring (Toure et al. 2004). Surfactins and iturins are amphiphilic cyclic peptides composed of 7 α-amino acids and fengycins by 10 α-amino acids. Moreover, iturins are linked to a single β-amino fatty acid, while surfactins and fengycins linked to a β-hydroxy fatty acid (Dimkić et al. 2017).

On the other hand, lytic enzymes like β-1, 3-glucanase, protease, and chitinase play a key role in controlling the growth of fungi through their cell wall degrading activity. Other than that, the volatile organic compounds (VOC) recognized by their antifungal activity are 2, 3-butanediol, benzene acetic acid, benzaldehyde, 1-decene, phenylethyl alcohol, and tetradecane and have also been studied for their role in biocontrol activity against a variety of fungal pathogens by Ryu et al. (2005) and Dhouib et al. (2019). Studies on the indirect mechanism of biocontrol found in several *Bacilli* reveal that it has a significant role in enhancing and boosting up the plant defense system through inducible resistance, namely SAR and ISR. Characteristic molecules of inducible resistant such as pathogenesis-related (PR) proteins (chitinases, β-1, 3-glucanases, proteinase inhibitors, etc.), phytoalexins (antimicrobial compound), oxidative enzymes (peroxidases, polyphenol oxidases, and lipoxygenases), and VOCs have been studied and well-demonstrated in the findings of García-Gutiérrez et al. (2013), Jangir et al. (2018), Pingping et al. (2017), Myo et al. (2019), Rais et al. (2017), and Waewthongrak et al. (2014).

Table 17.1 *Bacillus* based biocontrol of fungal diseases

Biocontrol agent	Crops	Mechanism of control	Fungal diseases/fungi	References
B. subtilis	Tomato	Direct inhibition (lytic enzymes)	*Fusarium oxysporum*	Chebotar et al. (2009)
		Indirect inhibition (ISR)	*Fusarium semitectum*	Nihorimbere et al. (2010)
		Direct inhibition (antibiosis)	*F. oxysporum f.* sp. *lycopersici*	Abd-Allah et al. (2007) and Baysal et al. (2008)
	Banana	Direct inhibition (antibiosis)	*Pseudocercospora musae*, *Colletotrichum musae*	Fu et al. (2010)
	Cotton	Direct inhibition (hydrolytic enzymes)	*F. oxysporum*	Gajbhiye et al. (2010)
	Lettuce	Competition/direct inhibition (antibiosis)	*Pythium aphanidermatum*	Correa et al. (2010)
	Peach, nectarine	Direct inhibition (antibiosis/enzymes)	*Monilinialaxa*(Brown rot)	Casals et al. (2010)
			Moniliniafructicola	Fan et al. (2000)
	Pea	Direct inhibition (antibiosis/enzymes) and indirect inhibition (ISR)	*Fusarium* spp. (Fusarium wilt)	Khan et al. (2011)
	Chilli	Direct inhibition (mycolyticenzymes)	*Colletotrichum gloeosporioides*OGC1	Ashwini and Srividya (2014)
	Cucumber	Competition, direct inhibition (inhibitory metabolites), and indirect inhibition (ISR)	*Podosphaer axanthii* (powdery mildew), *Didymella bryoniae* (gummy stem blight) *F. oxysporum* f. sp. *radicis-cucumerinum* (Fusarium root), *Pythium* spp. (Pythium crown and root rot)	Ni and Punja (2019)
	Tomato	Direct inhibition (antibiosis and inhibitorymetabolites)	*P. aphanidermatum*	Kipngeno et al. (2015) and Shankar (2016)
		Direct inhibition (antifungal compounds) and indirect inhibition (ISR)	*F. oxysporum* f. sp. *lycopersici* (*Fusarium wilt*)	Abd-Allah et al. (2007), Akram and Anjum (2011) and Shafi et al. (2017)
		Direct inhibition (antibiosis)	*Penicillium* spp. (Blue mold rot)	Punja et al. (2016) and Soleyman et al. (2014)
*B. subtilis*BCB3-19	Tomato	Indirect inhibition (ISR)	*Botrytis cinerea* (Grey mold)	Siripornvisal (2010)

(continued)

Table 17.1 (continued)

Biocontrol agent	Crops	Mechanism of control	Fungal diseases/fungi	References
*B. subtilis*WXCDD105	Tomato	Direct inhibition (antibiosis)	*B. cinerea* (Grey mold)	Wang et al. (2018)
B. subtilis UMAF6639	Melon	Indirect inhibition (inducible resistance)	*P. xanthii* (Powdery mildew)	García-Gutiérrez et al. (2013)
B. subtilis CCTCC M207209	Table grape	Direct inhibition (inhibitory metabolites/ antibiosis)	*Aspergillus carbonarius* CCTCC AF2011004	Jiang et al. (2014)
B. subtilis 30VD-1	Pea	Direct inhibition (lytic enzymes/VOCs/ inhibitory metabolites)	*Fusarium* spp.	Khan et al. (2018)
B. subtilis CICC 10034	Apples	Direct inhibition (cell wall degrading enzymes/antibiosis)	*Penicillium expansum*	Wang et al. (2016b)
B. subtilis ABS-S14	Citrus fruit	Indirect inhibition (ISR)	*Penicillium digitatum*	Waewthongrak et al. (2014)
B. subtilis HZ-72	Flax	Direct inhibition (cell wall degrading enzymes/antibiosis)	*Rhizoctonia solani* (Flax seedling blight)	Tan et al. (2019)
B. subtilis, *B. megaterium*	Peanut	Direct inhibition (enzymatic lysis)	*Aspergillus niger* (Root rot disease)	Yuttavanichakul et al. (2012)
B. amyloliquefaciens 9001	Apple	Direct inhibition (lytic enzymes) and indirect inhibition (ISR)	*Botryosphaeria dothidea* (Apple ring rot)	Li et al. (2013)
B. amyloliquefaciens	Wheat	Direct inhibition (metabolites/ antifungal compounds)	*Fusarium graminearum* (Fusarium head blight)	Crane and Bergstrom (2014)
B. amyloliquefaciens Q-426	Spinach	Direct inhibition (antibiosis)	*F. oxysporum* f. sp. *Spinaciae*	Zhao et al. (2014)
B. Amyloliquefaciens subsp. *Plantarum*	Ginseng	Direct inhibition (antifungal activity/lytic enzymes) or antagonistic	*F.* cf. *incarnatum* (Ginseng root rot)	Song et al. (2014)
B. amyloliquefaciens W19	Banana	Direct inhibition (antibiosis, iturin and bacillomycin D)	*F. oxysporum* f. sp. *Cubense* (FOC)	Wang et al. (2016a)

B. amyloliquefaciens strain BLB369, *B. subtilis*BLB277, *Paenibacilluspolymyxa* BLB267	Durum wheat	Direct inhibition (antibiosis)	*F. graminearum*	Zalila-Kolsi et al. (2016)
B. amyloliquefaciens L-1	Pear	Direct inhibition (antioxidant enzymes)	*Botryosphaeria berengeriana* (Pear ring rot)	Pingping et al. (2017)
B. amyloliquefaciens, *B. megaterium* (B5)	Wheat plants	Direct inhibition (antagonistic)	*Cochliobolus sativus*, *Alternaria alternata*, and *F. graminearum*	El-Gremi et al. (2017)
B. amyloliquefaciens, *B. subtilis*	Pistachio	Direct inhibition (antibiosis)	*Aspergillus parasiticus*	Siahmoshteh et al. (2017)
B. pumilus	Tomato, melon	Direct inhibition (antibiosis/enzymatic lysis)	*Xanthomonas campestris* and *F. oxysporum* f. sp. *melonis*	Suárez-Estrella et al. (2013)
	Tomato	Direct inhibition (antibiosis/enzymatic digestion) and indirect inhibition (ISR)	*F. oxysporum* f. sp. *lycopersici* (Fusarium wilt)	Heidarzadeh and Baghaee-Ravari (2015)
B. cereus AR156	Sweet cherry fruit blue rot	Direct inhibition (cell wall degrading enzymes)	*P. expansum*	Wang et al. (2015)
B. toyonensis, *B. cereus*, *B. aryabhattai*, *B. megaterium*, *B. aerius*, *B. stratosphericus*, *Paenibacillus*, *Barcinonensis*	Tomato	Direct inhibition (antagonistic/enzymatic digestion)	*F. oxysporum*f. sp. *lycopersici* (Fusarium wilt)	Rocha et al. (2017)
B. stratosphericus (FW3)	Ginseng	Direct inhibition (antagonistic metabolites)	*Ilyonectria* sp., *Neurospora* sp., *Cladosporium* sp., *Eutypella* sp., *Aschersonia* sp. and *Fusarium* sp. (Ginseng root rot disease)	Durairaj et al. (2018)

(continued)

Table 17.1 (continued)

Biocontrol agent	Crops	Mechanism of control	Fungal diseases/fungi	References
B. atrophaeus B5	Anthracnose soursop and avocado	Direct inhibition (antibiosis)	*Colletotrichum*, *Gloeosporioides*	Guardado-Valdivia et al. (2018)
B. velezensis C2	Tomato	Direct inhibition (antibiosis, enzymatic lysis) and ISR (VOCs)	*Verticillium dahlia* (Verticillium wilt disease)	Dhouib et al. (2019)
B. velezensis NKG-2	Tomato	Direct inhibition (enzymatic lysis) and ISR (VOCs)	*F. oxysporum* (Wilt disease)	Myo et al. (2019)
Bacillus spp.	Rice	Indirect inhibition(ISR)	*Pyriculariaoryzae*	Rais et al. (2017)
	Tomato	Direct inhibition (enzymatic lysis, metabolite production) and indirect inhibition by ISR (VOCs)	*F. oxysporum* f. sp. *Lycopersici*	Jangir et al. (2018)
Bacillus sp. P12	Bean	Direct inhibition (metabolite production/lipopeptides)	*Macrophomina phaseolina*	Sabaté et al. (2019)

Pathogenic fungi cause diseases in plants and some of them also produce mycotoxins, which contaminate the food and feed. Mycotoxins are toxic secondary metabolites produced by toxigenic fungi (Albayrak 2019). In the literature survey, *Bacillus* spp. were also found active against a number of mycotoxin producing fungi and destroyed them by antibiosis. *B. subtilis* SQR9 synthesized fengycin and bacillomycin antibiotics which inhibit mycelial growth and conidial germination of *F. oxysporum* f. sp. *couperin* (Cao et al. 2012). Also, *B. subtilis* fmbJ produced bacillomycin D which was active against *Aspergillus flavus* and was liable for injury to cell wall and membrane (Gong et al. 2014). Ayed et al. (2014) reported that antibiotic fengycin, surfactin, and pumilacidin produced by *B. mojavensis* acted against gram (+ve), gram (−ve), and many fungal pathogens. Further, antibiotic bacillomycin D from *B. subtilis* fmbJ caused the distortion of mycelia and disruption of spores, induction of more ROS, and apoptosis of *Aspergillus ochraceus* through cell and DNA damage (Qian et al. 2016). Ochratoxin A (OTA), a mycotoxin mainly produced by species of *Aspergillus* and *Penicillium* was very efficiently removed by *Bacillus megaterium* through adsorption as reported by Shang et al. (2019).

17.2.3 Bacillus *spp. in Prevention of Bacterial Diseases*

A number of bacterial diseases that are biologically controlled by the various species of *Bacillus* are listed below in Table 17.2.

As it can be clearly seen from Table 17.2, the *B. subtilis* and *B. amyloliquefaciens* have emerged as the most potential biocontrol agent for bacterial diseases. Different strains of *B. subtilis* produced a good range of hydrolytic enzymes, including i.e., cellulases, beta-glucanases, and proteases. This bacterial species also produced several metabolites and antibiotics that could limit the growth of invading pathogens and microorganisms. It has been reported in the literature that the indirect mechanism like ISR played a significant role in suppressing bacterial diseases in plants. Remarkably, *B. subtilis* strains are well-recognized for synthesizing antibiotic lipopeptides, including fengycin, surfactin, and iturin (Hashem et al. 2019). Surfactants are antimicrobial compounds and can also have an important role behind inhibiting phytopathogens.

17.2.4 Bacillus *in Pest/Insect/Nematode Control and* Bacillus*-Based Commercial Products*

Some important species of *Bacillus* efficient in controlling pest/insects and nematodes are listed in Table 17.3.

*Bacillus thuringiensis*is, a well-known species of *Bacillus*, used as biopesticide worldwide since biopesticides came into existence. Approximately 95% of

Table 17.2 *Bacillus* species as a biocontrol agent for bacterial diseases

Biocontrol agent	Plant/crop	Mode of action	Target disease/bacteria	References
B. subtilis	Arabidopsis	Antibiosis (lipopeptide surfactin) and biofilm formation	*Pseudomonas syringae* pv. *tomato* DC3000 (root infection)	Bais et al. (2004)
	Mulberry	Biofilm formation and indirect inhibition (ISR)	*Ralstonia solanacearum* (bacterial wilt)	Ji et al. (2008)
	Tomato	Indirect inhibition (ISR and SAR)	*Xanthomonas euvesicatoria* *Xanthomonas perforans* (bacterial spot)	Roberts et al. (2008)
B. subtilis, *B. amyloliquefaciens*	Potato	Competition, fertilization, induction of antagonist (microbial) population	*R. solanacearum* (bacterial wilt)	Chen et al. (2013)
B. subtilis AP-01 (Larminar™), *Trichoderma harzianum* AP-001 (Trisan™)	Tobacco	Direct inhibition (antibiosis, metabolite secretion)	*R. solanacearum* (bacterial wilt)	Maketon et al. (2008)
B. subtilis 9407	Melon	Direct inhibition (antibiosis by surfactin)	*Acidovoraxcitrulli* (bacterial fruit blotch)	Fan et al. (2017)
Endophytic *B. subtilis* SR63	Grapes	Direct inhibition (antibiosis, metabolite secretion)	*Agrobacterium tumefaciens* (crown gall)	Ferrigo et al. (2017)
Endophytic *B. amyloliquefaciens*	Rice	Direct inhibition (metabolite secretion-siderophores, IAA etc. and root colonization)	*Xanthomonas oryzaepv. oryzae* (bacterial leaf blight)	El-shakh et al. (2017)
B. amyloliquefaciens BL10	Tomato	Direct inhibition (antibiosis)	*R. solanacearum* (bacterial wilt)	Nawangsih et al. (2011)
B. subtilis	Chili	Direct inhibition (growth promotion, metabolite secretion)	*R. solanacearum* (bacterial wilt)	Istifadah et al. (2017)
B. pseudomycoides NBRC 101232 *B. thuringiensis* ATCC 10792 *B. mycoides strain* 273		Direct inhibition (antibiosis etc.) and indirect inhibition (ISR)		Yanti et al. (2018)

Bacillus sp.	Eggplant	Direct inhibition (inhibitory compound production) and indirect inhibition (ISR)	*R. solanacearum* (bacterial wilt)	Achari and Ramesh (2014)
B. amyloliquefaciens strain S1	Tomato	Direct inhibition (antibacterial metabolite, siderophores and lytic enzymes production)	*Clavibactermichiganensis* ssp. *michiganensis* (bacterial canker)	Gautam et al. (2019)
B. subtilis, *B. amyloliquefaciens* (FZB 24), EPB 9, EPB10, EPCO 29 and EPCO 78	Rice	Indirect inhibition (ISR)	Bacterial leaf blight, sheath blight	Krishnan et al. (2013)
Endophyte B. velezensis	Citrus species	Direct inhibition (bioactive secondary metabolites production)	*Xanthomonas citri* subsp. *citri* (citrus bacterial canker)	Rabbee et al. (2019)
B. amyloliquefaciens and *Trichoderma asperellum*	Tomato	ISR and growth promotion by enhanced nutrients (P, K, Mg) availability	*Xanthomonas perforans* (bacterial spot)	Chien and Huang (2020)
B. velezensis	Potato	Bacteriostatic activity and antibacterial mechanisms	*Streptomyces* (potatp scab)	Cui et al. (2020)

Table 17.3 *Bacillus* species in controlling pests/nematodes/insects/weeds

Biocontrol agent	Crops	Pest	Mode of action	References
B. subtilis	Soybean	*Heterodera glycines*	Direct inhibition (antibiosis/metabolite production)	Araújo et al. (2002)
	Tomato	*Meloidogyne incognita* (root-knot nematode)	Direct inhibition (inhibitory metabolites)	Araújo and Marchesi (2009) and Siddiqui and Futai (2009)
	Pulses	*M. incognita* (root-krot nematode)	Direct inhibition (antibiosis) and indirect inhibition (ISR)	Khan et al. (2011)
Bacillus strains (EPCO 102 and EPCO 16)	Cotton	Cotton bollworm	Indirect inhibition (ISR)	Rajendran et al. (2007)
B. Thuringiensis	Potato	Coleopteran insects, boll weevil, Colorado potato beetle	Bt toxin	Herrnstadt et al. (1986)
	Soybean	Caterpillars, stink bugs	Membrane pore formation and cell lysis	Schünemann et al. (2014)
B. thuringiensis var. tenebrionis Xd3 (Btt-Xd3)	Alder	*Agelasticaalni* (Alder leaf beetle)	Bt toxin	Eski et al. (2017)
B. flexus JIM24	–	*Lathyrus aphaca* weed	Aminolevulinic acid production	Phour and Sindhu (2019)

biological control products for agricultural pests belong to this species (Lambert et al. 1992). It produces the toxic proteins Cry, Cyt, and vegetative (secretable) insecticidal proteins (Vip) known as Bt toxins, which are highly lethal against a wide range of insects but nontoxic for mammals (Schnep et al. 1998). Bt toxins present in spore get activated on cleaving with proteases in the alkaline environment of the insect gut. That is why they act in a very specific manner and do not have any toxic effect on nontarget species (Bravo et al. 2007). Coleoptera, Lepidoptera, and Diptera are the major insect families against which Bt toxins (Cry/Cyt) are very effective.

Due to the promising results in controlling a range of pathogenic diseases, the commercial applicability of *Bacillus*-based biocontrol agents has increased. Hence, a good range of *Bacillus*-based biocontrol agents is available in the market. Some commercial *Bacillus*-based biocontrol agents are given in Table 17.4.

Table 17.4 Commercial *Bacillus* based biocontrol agents

Biocontrol agent	Product	Manufacturer	Target disease/organism	Crops
B. subtilis	Avogreen®	Ocean Agriculture South Africa	*Colletotrichum gloeosporioides* and *Cercospora* spot	Avocado
	Biosubtilin	Biotech International Ltd. India	*Fusarium*, *Verticillium*, *Pythium*, *Cercospora*, *Colletotrichum*, *Alternaria*, *Ascochyta*, *Macrophomina*, *Myrothecium*, *Ramularia*, *Xanthomonas*, and *Erysiphe polygoni*	Cotton, cereals, ornamental plants, and vegetable crops
	Stanes Sting®	Stanes Company, India	*M. incognita*	Tomato
B. subtilis strain GB34	GB 34	Gustafon, USA	*Rhizoctonia*, *Fusarium*	Soyabean
B. subtilis strain GB03	Kodiac companion	Growth Products, USA	*Rhizoctonia*, *Aspergillus*	Wheat, barley, pea
B. megaterium	Bioarc®	Sphere Bio-Arc Pvt Ltd.	*Tylenchulus semipenetrans* (nematode)	Cooton, beans, orange
B. firmus	BioNem®	Agro-green Minrav group of Israel	*M. incognita*	Tomato
B. amyloliquefaciens	RhizoVital® 42 liand RhizoVital 42 TB	ABiTEP GmbH, Germany	Soilborne pathogens	Potato, corn, strawberry, tomato, cucumber, ornamental plants
B. thuringiensis aizawai	Agree-WP	Certis USA L.L.C., USA	Armyworms, diamondback moth	Fruits, nuts, vegetables
	Florbac	Valent Biosciences Libertyville, USA		
	XenTari WG	Nufarm, Canada		
	Xantari®	Valent Biosciences Libertyville, USA		

(continued)

Table 17.4 (continued)

Biocontrol agent	Product	Manufacturer	Target disease/organism	Crops
B. thuringiensis kurstaki	Biobit®	GroChem, New Zealand	Lepidoptera	Apple, avocado, citrus, flowers, grapes etc.
	Cordalen®	Agrichem Bio, Madrid, Spain		
	Costar-WG	SKL Biosynthesis, Italy		
B. thuringiensis israelensis	Teknar® SC, VectoBac®	Valent Biosciences, Libertyville, USA	Mosquitoes and black flies	–
	Vectobar™	AgriLife, AP, India		
B. thuringiensis tenebrionis	Novodor®	Valent Biosciences, Libertyville, Illinois, USA	Colorado potato beetle	Potato
	Trident®	Certis USA L.L.C., USA		
B. thuringiensis sphaericus	VectoLex®, VectoMax®	Valent Biosciences, Libertyville, USA	Mosquitoes	–
B. pumilus	Yield Shield®	Bayer Crop Science, USA	*R. solani* and *Fusarium* (root rot)	Soybean
B. thuringiensis var. kurstaki	DiPel 2x®	Nufarm, Canada	*M. incognita* , Lepidoptera pests	Several vegetables

17.3 A Comparison of Biopesticides and Synthetic Pesticides

There are several advantages of using biopesticides. *Bacillus* species are recognized as safe bacteria that produce substances that are beneficial for crops and the production of industrial compounds (Stein 2005). As a biocontrol agent, *Bacillus* has the advantage of long-term storage and reduced complexity of formulation process due to its ability to form spores that help it to survive in adverse environmental conditions (Collins and Jacobsen 2003). In addition, biopesticides are nontoxic and easily degradable, which makes them more beneficial than any chemical pesticide. Although biopesticides offer a lot of advantages but have not replaced conventional pesticides completely as they are not so popular and common in use, and have specific requirements. Since they are highly specific, farmers will need different biopesticides for different pathogens or insects. Furthermore, maintaining the viability of these biocontrol agents is extremely important (inside.battelle.org).

17.4 Future Perspective

Developing new biopesticides itself is a very tedious process due to several challenges like cost, efficacy, and commercialization process. Delay in the authorization process is common due to the lack of enough expertise and regulatory model for biopesticides in India (Tripathi et al. 2020). Besides the investigation of new biomolecules, recombinant DNA technology is also being used for improving the efficiency of biopesticides. Novel fusion proteins, made up of toxins combined with a carrier protein, have been developed as next-generation biopesticides, and this technology makes this fusion protein toxic to target insects or pests after it is consumed orally (Fitches et al. 2004). More research is required in order to have an effective pest management in production systems. Funding agencies and government policies are influencing factors in biopesticide research and promotion. Government can control the use of hazardous pesticides by enforcing laws and encouraging the biopesticide industry for organic agriculture (Moosavi and Zare 2016).

Also, a strict regulatory mechanism is equally important for the desired quality and reasonable cost of biopesticides (Kumar and Singh 2015). Other than that, biological control agents (BCAs) may behave differently in different environmental and climatic conditions, hence, every country needs to develop indigenous BCAs (Keswani 2020). Moreover, limitations like slow in killing pests, cost, production, and formulation problems are the major drawbacks associated with biological pesticides. Therefore, working on these limitations to improve the performance may help in the global acceptance of biopesticides. Nanoformulations may play a significant role in improving the residual action and stability of biopesticides (Damalas and Koutroubas 2018; Tripathi et al. 2020). Recombinant DNA technology, molecular biology, and biotechnology can help to enhance the performance of biopesticides in their field use.

17.5 Conclusion

Biological stress is considered as one of the major restrictions to crop production in agricultural fields, which also exacerbates with climate change (Etesami et al. 2020). The use of biological agents to control plant diseases has become a very good alternative to conventional pesticides as they are nonhazardous for living beings and the environment. Several species of *Bacillus* are able to suppress plant diseases through various mechanisms, categorized into direct and indirect mechanisms. These mechanisms are responsible for the production of a broad range of antibiotic compounds (lipopeptides), lytic enzymes, antioxidants, siderophores, formation of biofilms, and various other metabolites which inhibit the growth of pathogens by their action. Moreover, through indirect mechanisms such as ISR, *Bacillus*-based biocontrol agents induce the plant immune/defense system and help them to grow in harsh conditions of stress. Also, *Bacillus* has a prominent role in alleviating induced ethylene levels under biological and nonbiological stresses, which suppress plant growth. Biopesticides and *Bacillus*-based products are gaining much attention that is why huge numbers of commercial products are available in the market belonging to *Bacillus* species. It has been well demonstrated that *Bacillus* species have immense potential to mitigate biotic stresses and encourage the growth and development of plants. However, these agents are not able to provide full protection against diseases but biopesticides combination with synthetic pesticides, fertilizers, and different types of tillage, incorporated into integrated pest management systems can fulfill the purpose to some extent. Apart from this, extensive research in new active ingredients, biopesticide formulation, and efficacy will give a new insight into biopesticide application in agriculture.

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Chapter 18
Bacilli and Polyhydroxyalkanoates: An Intracellular Granule Having Promising Feature as a Resource for Production of Bioplastics

Priya Patel and Nasreen S. Munshi

Abstract Polyhydroxyalkanoate (PHA) is biodegradable biopolymer produced by microorganisms as lipid inclusion body under the stressful environmental conditions. They possess the properties analogous to petrochemically derived synthetic plastics and can serve as novel resource for production of bioplastics. Varieties of prokaryotes in diverse niches have been reported to accumulate PHA when there is excess carbon and/or limited nitrogen or phosphorous. *Bacillus* spp. are prominent source for industrial production of PHA as they are predominant in nature. Different *Bacillus* spp. are reported to utilize a wide range of substrates such as sucrose, glucose, fructose, starch and others for production of PHA. On the other hand, few *Bacilli* accumulate PHA while using inexpensive biowastes such as pea-shell slurry, fish solid waste, activated sludge, sugar industry wastewater and others as substrates. This allows sustainable management of waste along with generating a valuable by-product. They are known to synthesize PHA homopolymer as well as copolymers. The Food and Drug Administration (FDA) has considered *Bacillus* as Generally Regarded As Safe organisms (GRAS), allowing its application for large-scale bioplastic production. Further, the absence of immunogenic lipopolysaccharide layer in *Bacillus* spp. allows biomedical applications of produced PHA. The main emphasis of this article is to summarize the generalized, metabolic and genetic features of *Bacillus* spp. associated with PHA production and providing substantial information for exploiting capabilities of *Bacillus* spp. for industrial PHA production.

Keywords Polyhydroxyalkanoate · *Bacillus* spp. · Biodegradable · Biopolymer · Industrial PHA production · Bioplastic · Biowastes · PHA granule

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18.1 Introduction

Plastics and their products are basic necessity of individual's life. Low cost, light weight, water resistance, rust free and robustness are promising features of plastic, permitting its wide applications for societal benefits (Thompson et al. 2009). Concurrent to its enormous applications, plastic pollution has become threat for biosphere. Approximately more than 300 Mt. plastic is produced annually. Plastic manufacturing and utilization rate in India, China and Brazil are increasing exponentially (Koller 2017). These non-biodegradable petrochemically derived synthetic plastics accumulate as such in environment causing harm to wildlife, marine animals, humans and environment (Sathya et al. 2018).

Biodegradable polymers having properties similar to synthetic plastics are considered to be potential substitute of petrochemically derived plastics (Koller 2017). When discarded in surroundings, the polymers which get entirely converted into CO_2 and H_2O within fixed duration are designated as "biodegradable polymers" (Sathya et al. 2018). Nature-based and chemical-based polymers are the two main categories of biodegradable polymers. Further, as depicted in Fig. 18.1, animal-based, agro-based and microbe-based polymers are subcategorized under naturally derived polymers (Mulchandani and Katiyar 2020). Nature-based polymers are also called as "biodegradable biopolymers" as they are obtained from biological materials (Pittmann and Steinmetz 2017). Chemical-based polymers are initially extracted from biological sources in the monomeric form and then polymerized by using various chemicals. For example, polylactic acid (PLA) and polybutylene succinate

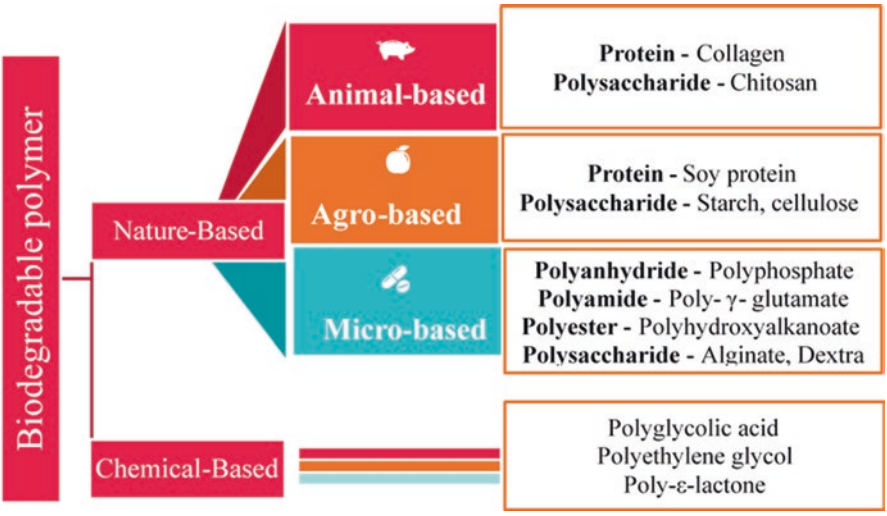

Fig. 18.1 Categories of biodegradable biopolymers: there are two main types of biodegradable polymers: nature-based and chemical-based. Further, nature-based polymer is divided into three subtypes, i.e. animal-based, agro-based and microbe-based. Examples are illustrated in different categories of polymers

(PBS) get polymerized in the presence of lactic acid and succinic acid, respectively. In contrast to this, nature-based biopolymers are advantageous as they do not require polymerization after extraction (Kourmentza et al. 2017). Amongst all the nature-based biopolymers, starch is widely used as packaging material due to its biodegradable nature other than being cost-effective and non-hazardous. But the major drawback is that it lacks thermoplastic properties (Sanyang et al. 2015). Chemical treatment of starch with plasticizer can impart thermoplastic properties to it (Coats et al. 2016). Microbe-based biopolymer – polyhydroxyalkanoate (PHA) – is an intracellular granule synthesized by bacteria in the presence of excess substrate and/or nutrient limiting condition. They possess features similar to polypropylene including thermoplasticity and is also biodegradable in nature (Pagliano et al. 2017; Hassan et al. 2016; Mokhtarani et al. 2012; Gamba et al. 2017). Also, biodegradability of PHA is higher than PLA and starch (Coats et al. 2016). Different bacterial strains have been reported to accumulate PHA by utilizing several carbon sources as well as agricultural and industrial waste as feedstock (Chua et al. 2003). The stated characteristics of PHA allow its wide acceptance as a source of bioplastics suitable for applications in packaging, medical, pharmaceutical, agriculture and food industries (Coats et al. 2016; Goudarztalejerdi et al. 2015).

Prokaryotes such as *Ralstonia eutropha*, *P. putida* CA-3, *P. putida* mt-2, *P. putida* F1, *Sphingobacterium* sp. ATM, *Bacillus odyssey* SUK3, *P. desmolyticum* NCIM 2112, etc. are capable of PHA production (Nikodinovic et al. 2008; Tamboli et al. 2010; Sato et al. 2008). Although many types of PHA producers are reported till date, *Bacillus* spp. are considered as valuable bioresource for industrial PHA production as they are predominant in nature (Mohapatra et al. 2017). They are capable of accumulating PHA homopolymer as well as copolymers (Singh et al. 2009). Moreover, they are also stated as "Generally Regarded As Safe organisms" (GRAS) by the Food and Drug Administration (FDA) (Singh et al. 2009; Mohapatra et al. 2017). Hence, *Bacillus* spp. are appropriate bio-resource for industrial PHA production. In this chapter, the generalized characteristics of PHA are described along with an account on how the abilities of *Bacillus* spp. can be exploited for industrial PHA production.

18.2 Biodegradable Biopolymer: Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are water insoluble polyester granules situated inside bacterial cytoplasm (Colombo et al. 2017; Goudarztalejerdi et al. 2015; Kumar et al. 2004). They are synthesized by bacteria in the presence of excess carbon and/or limited phosphorus, sulphur, oxygen or nitrogen (Goudarztalejerdi et al. 2015; Sathya et al. 2018).

18.2.1 Monomeric PHA and Its Derivatives

PHA biopolymer comprises of hydroxyalkanoic acid monomers (Goudarztalejerdi et al. 2015; Sathya et al. 2018). The general structural formula of hydroxyalkanoic acid or PHA monomer is shown in Fig. 18.2, where R represents an alkyl group and n ranges from 1 to 3. A total number of carbon atom in PHA monomer ranges from 3 to 14 on the basis of type of R group incorporated (Pradhan et al. 2020). The PHA biopolymer consists of long chain of monomeric PHA ranging from ~100 to 30,000 units (Pradhan et al. 2020; Jacquel et al. 2008; Jiang et al. 2016; Basnett and Roy 2010). Table 18.1 denotes the type of PHA monomer and a number of C atoms present in it according to incorporated alkyl group.

PHAs are classified into four categories on the basis of biopolymer chain length and the type of monomeric unit incorporated in biopolymer chain. On the basis of chain length, they are classified as short-chain length PHA (*scl*-PHA) and medium-chain length PHA (*mcl*-PHA). The *scl*-PHA comprises of 3–5 carbon atoms, whereas *mcl*-PHA contains 6–14 carbon atoms (Kourmentza et al. 2017; Ciesielska and Kiewisz 2016). On the basis of monomeric unit, two types of PHAs are there, one is homopolymer PHA and the other is copolymer PHA. In homopolymer PHA, biopolymer chain is consisted of identical type of PHA monomer, whereas biopolymer chain of copolymer PHA contains different types of PHA monomers (Pradhan et al. 2020). Classification of PHA is defined in Fig. 18.3.

The *mcl*-PHAs have properties similar to elastomers and are semi-crystalline in nature. They are used for preparation of drug delivery matrix, surgical sutures and implants. On the other hand *scl*-PHAs have tensile strength similar to polypropylene, they are used for formulating food packaging material and disposable items (Kourmentza et al. 2017; Pradhan et al. 2020). The *scl*-PHAs are predominantly accumulated in bacteria as compared to *mcl*-PHAs (Pradhan et al. 2020). More than 150 different kinds of PHAs have been reported so far (Pittmann and Steinmetz 2017; Sathya et al. 2018; Pradhan et al. 2020; Kourmentza et al. 2017). Table 18.2 illustrates the types of PHAs produced by different species of bacteria (Singh et al. 2015).

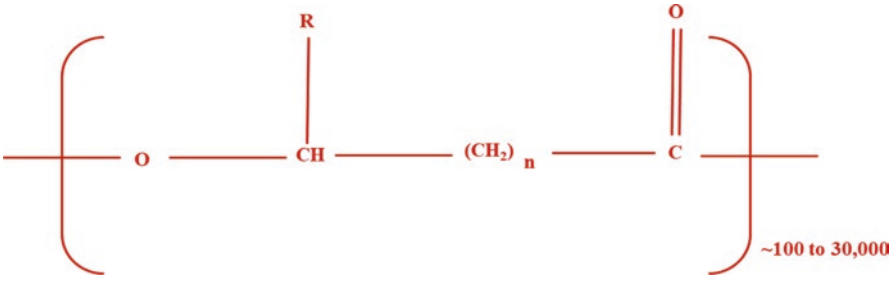

Fig. 18.2 General formula of monomeric PHA: the formula shown is of hydroxyalkanoic acid, the monomeric subunit of PHA biopolymer where R indicates an alkyl group and n ranges from 1 to 3. Long chain of hydroxyalkanoic acid in PHA biopolymer contains multiple monomers

Table 18.1 Type of alkyl group and number of C atoms present in monomeric PHA

Alkyl group (−R)		Total number of C atoms in PHA monomer	n	Type of PHA monomer
Type	Molecular formula			
Hydrogen	–H	C_3	1	Poly(3-hydroxypropionate)
		C_4	2	Poly(3-hydroxybutyrate)
		C_5	3	Poly(3-hydroxyvalerate)
Methyl	$–CH_2$	C_4	1	Poly(3-hydroxybutyrate)
		C_5	2	Poly(3-hydroxyvalerate)
		C_6	3	Poly(3-hydroxyhexanoate)
Ethyl	$–C_2H_6$	C_5	1	Poly(3-hydroxyvalerate)
		C_6	2	Poly(3-hydroxyhexanoate)
		C_7	3	Poly(3-hydroxyheptanoate)
Propyl	$–C_3H_8$	C_6	1	Poly(3-hydroxyhexanoate)
		C_7	2	Poly(3-hydroxyheptanoate)
		C_8	3	Poly(3-hydroxyoctanoate)
Butyl	$–C_4H_{10}$	C_7	1	Poly(3-hydroxyheptanoate)
		C_8	2	Poly(3-hydroxyoctanoate)
		C_9	3	Poly(3-hydroxynonanoate)
Pentyl	$–C_5H_{12}$	C_8	1	Poly(3-hydroxyoctanoate)
		C_9	2	Poly(3-hydroxynonanoate)
		C_{10}	3	Poly(3-hydroxydecanoate)
Hexyl	$–C_6H_{14}$	C_9	1	Poly(3-hydroxynonanoate)
		C_{10}	2	Poly(3-hydroxydecanoate)
		C_{11}	3	Poly(3-hydroxyundecanoate)
Heptyl	$–C_7H_{16}$	C_{10}	1	Poly(3-hydroxydecanoate)
		C_{11}	2	Poly(3-hydroxyundecanoate)
		C_{12}	3	Poly(3-hydroxydodecanoate)
Octyl	$–C_8H_{18}$	C_{11}	1	Poly(3-hydroxyundecanoate)
		C_{12}	2	Poly(3-hydroxydodecanoate)
		C_{13}	3	Poly(3-hydroxytridecanoate)
Nonyl	$–C_{9–}H_{20}$	C_{12}	1	Poly(3-hydroxydodecanoate)
		C_{13}	2	Poly(3-hydroxytridecanoate)
		C_{14}	3	Poly(3-hydroxytetradecanoate)
Decyl	$–C_{10–}H_{22}$	C_{13}	1	Poly(3-hydroxytridecanoate)
		C_{14}	2	Poly(3-hydroxytetradecanoate)
Undecyl	$–C_{11–}H_{24}$	C_{14}	1	Poly(3-hydroxytetradecanoate)

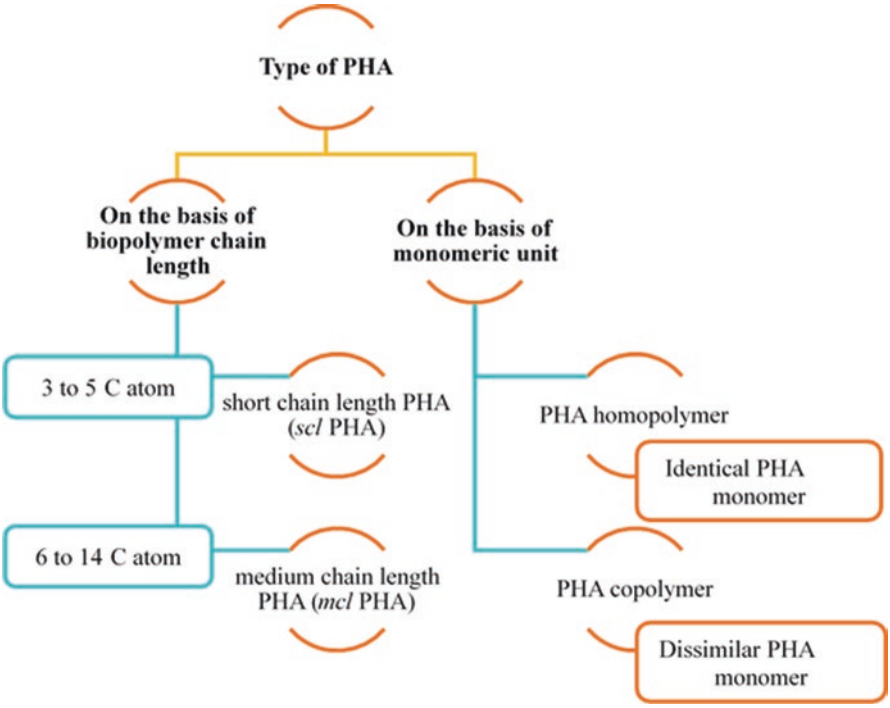

Fig. 18.3 Classification of PHA: there are four types of PHA classified on the basis of biopolymer chain length and on the basis of monomeric unit. Short-chain length PHA (*scl*-PHA) contains 3–5 carbon atoms, whereas medium chain length PHA (*mcl*-PHA) contains 6–14 carbon atoms. PHA biopolymer chain having identical monomeric unit is termed as PHA homopolymer while that with different monomeric units is termed as PHA copolymer

18.2.2 PHA as Carbon and Energy Reserves for Prokaryotes

PHAs are the inclusion bodies acting as intracellular carbon reservoir in bacterial strain (Sowinski et al. 2010). PHA granules constitute about 90% of the cell dry weight (CDW) (Bhuwal et al. 2013; Strong et al. 2016). *Ralstonia eutropha* was capable of accumulating 80% PHA of cell dry weight (Mokhtarani et al. 2012). When availability of carbon sinks in surrounding, PHA granules are used as carbon and energy source. Hence, they augment the survival of bacterial strain under stressful conditions existing in water and soil environments. UV irradiation, salinity, desiccation, osmotic shock, thermal stress and oxidative stress are the few stressful conditions occurring in water and soil environments where PHAs are used as carbon and energy source. Taxonomically diverse species of bacteria are inhabiting in soil niches. They have to deal with fluctuating conditions existing there. It has been reported that PHA-producing strain is protected during starvation as compared to mutant strain deficient in PHA production. But the co-relation

Table 18.2 Types of PHAs produced by bacteria

Name of PHA	Type of PHA		Name of PHA-producing bacteria	Substrate	Yield	References
	Chain length	Monomeric unit				
P(3HB)	NA	Homopolymer	*Enterococcus* sp. NAP11	Cardboard industry wastewater	79.27%	Bhuwal et al. (2013)
P(3HB)	NA	Homopolymer	*Brevundimonas* sp. NAC1	Cardboard industry wastewater	77.63%	Bhuwal et al. (2013)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain SDS3(HQ 230975)	Crude oil	23.13%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain XB7(KF 44738)	Crude oil	21.87%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. stutzeri* strain PS-SRU-ICU (JF 264901)	Crude oil	23.26%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(2HDDE), P(3HTD), P(3HHDE)	NA	Copolymer	*P. aeruginosa* strain H1 (JX 100389)	Crude oil	20%	Goudarztalejerdi et al. (2015)
Biopolymer having P(3HO), P(3HD), P(3HDDE), P(3HHDE).	*mcl*-PHA	Copolymer	*P. putida* F1, *P. putida* mt-2 and *P. putida* CA-3	BTEX mixture	0.25 ± 0.04 g/l	Nikodinovic et al. (2008)

NA not available, *P* poly, *(3HB)* 3-hydroxybutyrate, *(3HO)* 3-hydroxyoctanoate, *(3HD)* 3-hydroxydecanoate, *(2HDDE)* 2-hydroxydodecanoate, *(3HTD)* 3-hydroxytetradecanoate, *(3HHDE)* 3-hydroxydecahexanoate, *(3HHDE)* 3-hydroxydodecanoate

between PHA accumulation and survival strategy is strain specific depending on the suboptimal growth preceding to starvation (Sowinski et al. 2010). PHA-producing bacteria are reported in microbial mats pursuing an essential role in stress tolerance and biofilm formation (Campisano et al. 2008; Sowinski et al. 2010). Reducing equivalents produced during PHA degradation plays a vital role in energizing chemotaxis process in the surrounding environments having lower quantity of reducing power (Kadouri et al. 2003; Sowinski et al. 2010). Moreover, EPS production is observed in PHA accumulating strains (Aneja et al. 2004). NADH generated during PHA degradation gets accumulated into EPS which serves as energy reservoir, useful for bacteria under stressed conditions (Sowinski et al. 2010).

Apart from this, biodegradable nature of PHA permits its utilization as carbon and energy source by microbial communities existing in the environment. PHA gets depolymerized into oligomer by intracellular PHA depolymerase (i-PhaZ) and extracellular depolymerase (e-PhaZ) (Grage et al. 2009; Philip et al. 2007). Former acts on native PHA granules and later converts the partially degraded PHA granules into oligomers. These oligomers are transformed into monomers by hydrolases. The monomers generated can be utilized as source of carbon and energy by surrounding microbial community (Sowinski et al. 2010). Figure 18.4 displays the possible role of PHA in stressful environment.

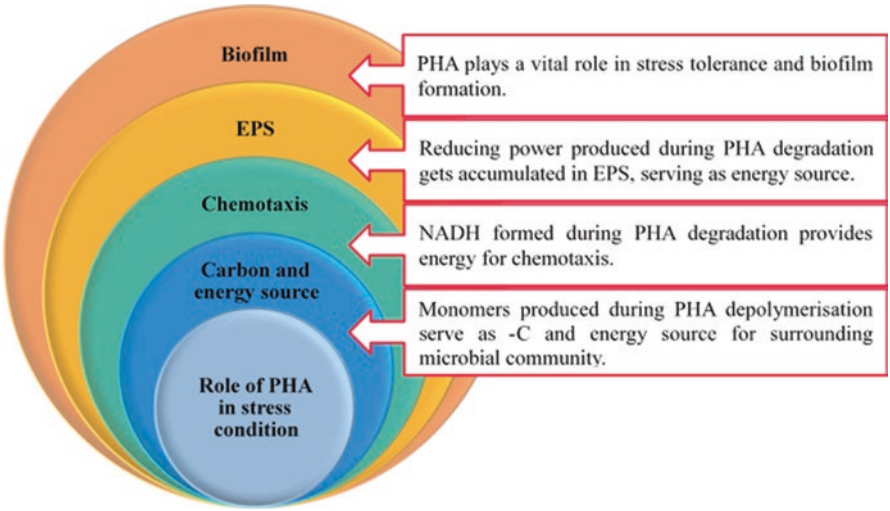

Fig. 18.4 Possible roles of PHA in stressful environment: monomeric PHA synthesized during biodegradation of polymeric PHA by depolymerase enzyme is used as carbon and energy source by diverse microbial communities existing in the surrounding environment. During its breakdown, reducing equivalent formed is used as energy source for chemotaxis. These reducing powers also get accumulated in EPS serving as energy source. Even PHA is essential in stress tolerance and for formation of microbial mats

18.2.3 Structure of PHA

Carbonosomes PHA granules are composed of proteins and phospholipid layer which is resistant to physical and chemical agents (Jendrossek 2009; Jendrossek and Handrick 2002). The innermost part of granule is made up of polyesters (Grage et al. 2009). PHA synthase (PhaC), PHA depolymerase (PhaZ), regulatory protein (PhaR) and phasins (PhaP) are four proteins present in PHA granule (Grage et al. 2009; Potter and Steinbuchel 2005). Structure of PHA granule is shown in Fig. 18.5.

PHA synthase plays a crucial role in converting hydroxyalkanoic acid into PHA polyester. They are classified into four classes – I, II, II and IV – as described in Fig. 18.5b. PHA synthase of class I and II is composed of 61–70 kDa PhaC subunit. Class III PHA synthase comprises of PhaC similar to that of class I PhaC and PhaE, both having 40 kDa molecular weight, whereas class IV PHA is composed of 40 kDa PhaC and 20 kDa PhaR. Class I, III and IV PhaC are reported to synthesize *scl*-PHA in contrast to class II PhaC which forms *mcl*-PHA (Grage et al. 2009; Potter and Steinbuchel 2005, 2006). As mentioned earlier, PhaZ are of two types, i-PhaZ present on the surface of PHA inclusion bodies and e-PhaZ secreted by

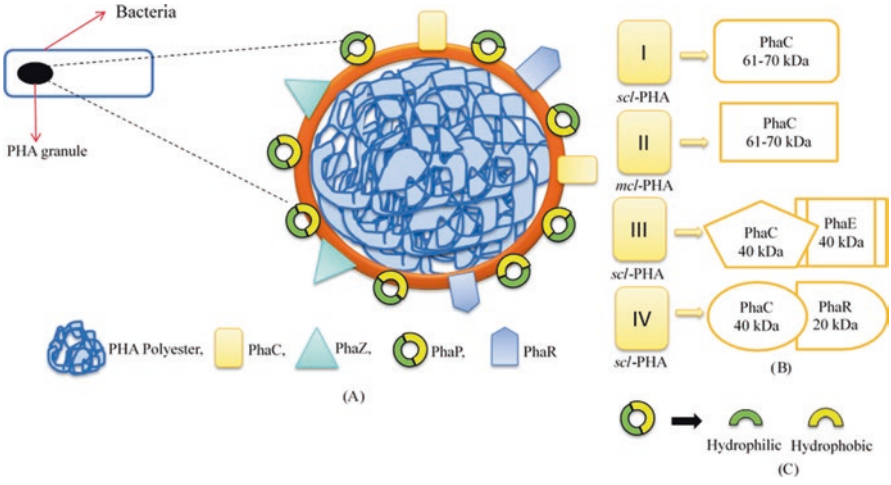

Fig. 18.5 Structure of PHA granule and its components: (**a**) intracellular PHA carbonosomes, (**b**) four classes of PhaC, (**c**) PhaP protein components. PHA carbonosomes are composed of hydroxyalkanoic acid polyester core surrounded by PhaC, PhaZ, PhaP and PhaR proteins. PhaZ are PHA depolymerase, important for degradation of PHA, while PhaR is a regulatory protein, essential for regulating the process of granule formation. PhaC is PHA synthase and PhaP is phasin proteins. There are four classes of PhaC which are essential for synthesizing biopolymer PHA. Class I and II of PhaC have single subunit of 61–70 kDa and are essential for synthesizing *scl*-PHA and *mcl*-PHA, respectively. Class III PhaC leads to formation of *scl*-PHA and is a combination of 40 kDa PhaC and 40 kDa PhaE. Class IV PhaC is made up of 40 kDa PhaC and 20 kDa PhaR, playing vital role in formation of *scl*-PHA. PhaP protein which forms a major part of the granule is amphibolic in nature having outer hydrophilic layer facing towards cytoplasm and inner hydrophobic layer facing granular inner side

many bacteria. Both are very essential for biodegradation of PHA granules (Grage et al. 2009; Sowinski et al. 2010). PhaR is the transcriptional regulatory protein required for regulation of PHA synthesis and PhaP production (Grage et al. 2009). Phasins are non-catalytic surface proteins having low molecular weight ranging between 11 and 25 kDa. They are produced in huge amount, comprising ~5% of cellular proteins (Grage et al. 2009). Their amphipathic layer as shown in Fig. 18.5c is composed of hydrophobic domain facing inside of granules and hydrophilic domain facing towards cytoplasm, thus, creating an interface between granule and cytoplasm (Grage et al. 2009; Mezzina and Pettinari 2016). They are essential for expression and activation of PhaC and PhaZ and, hence, are essential for PHA synthesis as well as degradation. They are also important for sorting of PHA granules and even affect size and number of granules. Few phasins act as chaperone proteins. Further, they belong to four families – PF09361, PF09602, PF09650 and PF05597 (Mezzina and Pettinari 2016).

Two models as presented in Fig. 18.6 have been reported till date for formation of PHA granules – micelle formation model and budding model (Potter and Steinbuchel 2006; Rehm 2006). According to the first model, initially PhaC proteins are randomly distributed in the cytoplasm, and as polymerization event initiates, it gets arranged in the form of micelle. Later on with increase in the biopolymer chain length, it gets distributed on the surface of PHA granules. After this, phasins and PhaR get accumulated on the granule surface. On the other hand, the budding model states that the PhaC are located between the phospholipid bilayer and carry out the polymerization of granule. The formed granule is then released into the cytoplasm. Then, the other proteins get attached to the outer surface of granule. Budding model is similar to the formation of eukaryotic neutral lipid. Micelle formation model is widely accepted as compared to the budding model (Potter and Steinbuchel 2006). *B. megaterium* seems to follow the budding model for PHA granule formation (Valappil et al. 2007a). It is believed that the PhaC possesses all the characteristics essential for formation of granule (Rehm 2006).

18.2.4 Comparative Aspects of Plastics and PHA

PHA have properties similar to petrochemically derived synthetic plastics like polyethylene and polypropylene (Numata et al. 2009; Bhuwal et al. 2013). Table 18.3 describes the general properties of PHA (Bugnicourt et al. 2014). Average molecular mass of PHA is 4.0×10^6 Da (Verlinden et al. 2007).

Further, the mentioned general property of PHA varies according to the type of biopolymer polymerized. Poly(3-hydroxybutyrate) denoted as P(3HB) is the most common type of PHA biopolymer accumulated by bacteria (Numata et al. 2009). It was the first biopolymer to be isolated from *B. megaterium* in the year 1920 by Maurice Lemoigne at the Pasteur Institute (Philip et al. 2007; Verlinden et al. 2007; Numata et al. 2009; Potter and Steinbuchel 2005). It has 162–181 °C melting temperature, −4 to 18 glass transition temperature, 19–44 MPa tensile strength, 1.2–4

Gpa Young's module, 0.8–4.5% elongation to break and 50–80% degree of crystallinity (Pradhan et al. 2020). Table 4 elucidates the properties of diverse types of PHA and polypropylene.

18.3 Bacilli and PHA

18.3.1 Diversity of PHA-Producing Bacillus *Species*

Members belonging to the genus *Bacillus* are able to accumulate varieties of PHA by utilizing ample of carbon sources. Table 18.5 displays the list of PHA-producing *Bacilli*. For PHA production, they were able to feed on simplest nutrient source such as glucose to complex hydrocarbons such as dyes and industrial effluents. Cost associated with PHA production decreases when any waste is used as substrates (Wen et al. 2010). *Bacillus* spp. were able to accumulate PHA while feeding on low-cost C source (Mohapatra et al. 2017).

18.3.2 Nutrients Essential for PHA Production by Bacilli

As described earlier, microorganisms accumulate PHA when there is excess substrate and/or growth limiting conditions. Feast and famine are the two terminologies describing the substrate availability. Former describes excess substrate and later indicates limited substrate availability. In famine conditions, the microorganisms will limit their cellular activity to minimum level essential for cell viability. They limit their activity by lowering the level of RNA transcription and/or enzyme activity. Further, when famine is followed by feast, initially the available substrate is utilized for PHA production instead of cellular growth. This happens because of the absence of essential enzymes required for cell growth. Thus, PHA accumulation is the physiological adaptation of microorganisms depending on substrate concentration (Albuquerque et al. 2010; Beun et al. 2002). In other words, such type of microorganisms does not require nutrient limiting conditions for PHA accumulation, and hence, they show growth-associated biopolymer production (Shi et al. 2007). Thus, type of microorganisms and microbial growth rate are the two internal factors affecting PHA accumulation. Moreover, the growth limiting conditions also arise by external factors such as limiting nutrients like N and P or in the presence of electron acceptors, viz. O_2, nitrate and phosphorous (Albuquerque et al. 2010; Shahid et al. 2013; Saharan et al. 2014). Growth-limiting nutrients will minimize the cellular growth as described earlier, and excess substrate is directed towards PHA production. Such microorganisms require limited nutrients and excess substrate for PHA production. In few organisms, it has been reported that C:N ratio also affects PHA

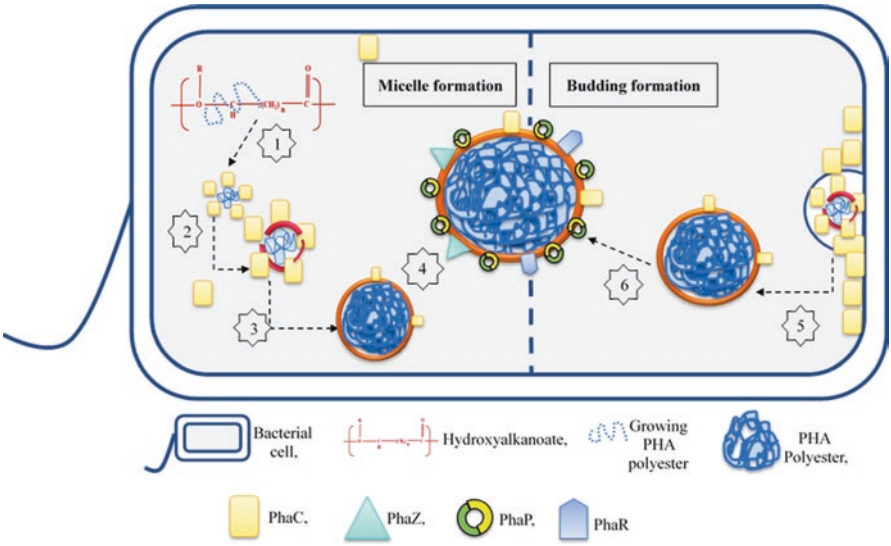

Fig. 18.6 Mechanism of PHA granule formation: two mechanisms for PHA granule formation are known – micelle formation (shown on left) and budding formation (shown on right). According to the most widely accepted micelle formation model, randomly distributed PhaC in cytoplasm initiates formation of granular body from hydroxyalkanoic acid (1) and (2). The granular body then enlarges into native granule acquiring PhaP, PhaZ and PhaR (3) and (4), while budding formation model depicts that the PhaC are located in the intracellular membrane and buds off into the granule (5) and (6)

accumulation (Shi et al. 2007; Wen et al. 2010; Saharan et al. 2014). Figure 18.7 indicates the pictorial outline of PHA production in varied nutrient conditions.

B. megaterium PNCM 1890 prefer urea as nitrogen source over sodium nitrate, ammonium chloride and ammonium sulphate, showing high PHB production. Researchers believe that uptake of low molecular weight, polar and uncharged urea for PHB formulation is higher as compared to remaining inorganic and ionic sources. The strain was capable to accumulate 3.91 g/L of PHB when C:N and C:P ratios were 14.3 g/g and 21.4 g/g, respectively, within 24 h (Danez et al. 2020). *B. megaterium* BA-019 produced 42% PHB of CDW in the presence of sugarcane molasses and urea as carbon and nitrogen source, respectively. The C:N ratio was 10 mol/mol, and incubation period was 24 h (Kulpreecha et al. 2009) (Table 18.4).

PHA production by *B. megaterium* DSM 509 was observed when grown in MM medium with different carbon sources such as glucose, glycerol, succinic acid, citric acid, acetic acid, pentanoic acid and octanoic acid. Then from this, medium cells were transferred into MM medium without N (MM-N), and PHA was extracted. Monomeric composition of PHA extracted from MM medium indicated *scl*-PHA, whereas that from MM-N indicated *mcl*-PHA. Authors suggested that the *scl*-PHA may get degraded when transferred from MM to MM-N in order to provide energy for synthesizing *mcl*-PHA (Shahid et al. 2013). *Bacillus flexus* is reported to

Table 18.3 General properties of PHA

Property	Unit	PHA
Glass transition temperature (T_g)	°C	2
Melting temperature (T_m)	°C	160–175
Degree of crystallinity (X_{cr})	%	40–60
Young's modulus (E)	GPa	1–2
Tensile strength (σ)	MPa	15–14
Elongation to break (ε)	%	1–15

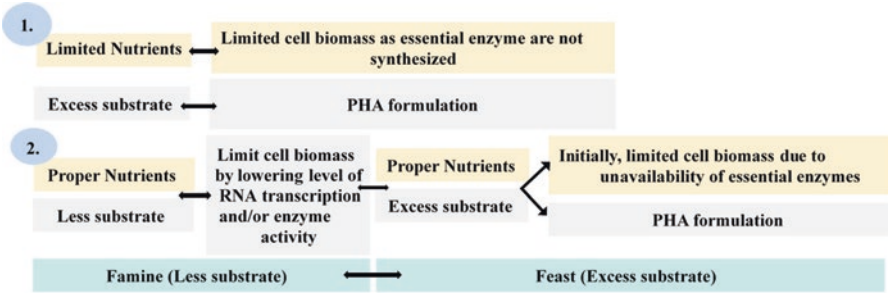

Fig. 18.7 Generalized mechanism for microbial PHA production under different nutrient conditions: as indicated in mechanism 1, limited nutrients hinder synthesizing of enzymes essential for cell biomass and hence limit growth. Excess substrate is directed towards PHA formulation, whereas as seen in mechanism 2, instead of nutrients, substrate is limited (famine) which again limits cell biomass development by lowering the level of RNA transcription and/or enzyme activity. When famine is followed by feast (excess substrate), initially cell biomass is limited as essential enzymes are unavailable, and excess substrate is directed for PHA formulation

produce high PHA in nitrogen-limiting conditions (Somashekara et al. 2009). In contrast to this, *Bacillus thuringiensis* EGU45 showed higher P(3HB-co-3 HV) (1.5–3.5 g/L) copolymer production in the presence of excess nitrogen or low C:N ratio and crude glycerol as carbon source (Kumar et al. 2015). *Bacillus mycoides* RLJ B-017 was unable to accumulate PHB at high oxygen transfer rate (OTR). It accumulated 69.4 ± 0.4% PHB of CDW in the presence of sucrose as carbon source and di-ammonium sulphate (Borah et al. 2002). The non-photosynthetic microorganisms, *Bacillus thuringiensis* EGU45 and *Bacillus cereus* EGU44 were capable of producing 11.3% PHB of CDW in the same medium where they were initially subjected for H_2 production (Patel et al. 2011) (Table 18.5).

Table 18.4 Properties of different types of PHA and polypropylene

Type of PHA and polypropylene	T_g (°C)	T_m (°C)	X_{cr} (%)	E (GPa)	σ (MPa)	ε (%)	References
Polypropylene	−14 to −6	160–169	50	1.1–2	28–40	20–75	Pradhan et al. (2020)
Polypropylene	−10	176	NA	1.7	38	400	Strong et al. (2016)
Polypropylene	−10	176	50–70	NA	38	400	Verlinden et al. (2007)
LDPE	−30	130	NA	0.2	10	620	Strong et al. (2016)
LDPE	−125 to −90	105–125	43	0.14–0.3	7–17	200–900	Pradhan et al. (2020)
HDPE	−125 to −90	130–137	79.8–81	0.7–1.4	20–40	100–1000	Pradhan et al. (2020)
P(3HB)	−4 to 18	162–181	50–80	1.2–4	19–44	50–80	Pradhan et al. (2020)
P(3HB)	−1	180	NA	3.5	40	5	Strong et al. (2016)
P(3HB)	2	177	60	NA	43	5	Verlinden et al. (2007)
P(3HO)	−35	60	30		10	300	Basnett and Roy (2010)
P(3HB-co-3 HV)	10 to −6	137–170	NA	0.7–2.9	Up to 690	30–38	Ciesielska and Kiewisz (2016)
P(3HB-co-3 HV)	−13 to 10	64–171	53–56	0.14 to 8.7	1.8 to 51	1–970	Pradhan et al. (2020)
P(3HB-co-3 HV)	−1	145	56	NA	20	50	Verlinden et al. (2007)
P(3HB-co-20%3HD)	−8	130	NA	NA	17	680	Ciesielska et al. (2016)
mcl-PHA	−48.46	43.95	NA	NA	NA	NA	Nikodinovic et al. (2008)
mcl-PHA	−40	80	NA	NA	20	300	Ciesielska and Kiewisz (2016)
mcl-PHA	~ − 40	~60	40	NA	Higher	Higher	Pradhan et al. (2020)
scl-PHA	179	4	NA	3.5	5	40	Ciesielska and Kiewisz (2016)
scl-PHA	~ − 180	~ − 0	70	NA	Lower	Lower	Pradhan et al. (2020)
P(3HB-co-20 mol% 3 HV)	−1	145	NA	0.8	20	50	Strong et al. (2016)
P(3HB-co-6 mol% 3 HV)	−8	133	NA	0.2	17	680	Strong et al. (2016)
P(3HB-co-mol16% 4B)	−7	150	45	NA	26	444	Verlinden et al. (2007)
P(3HB-co-mol10% Hx)	−10	176	34	NA	21	400	Verlinden et al. (2007)
P(3HB)	−11	161	NA	NA	NA	NA	Contreras et al. (2013)
P(3HB)	−16	136.8	NA	NA	NA	NA	Contreras et al. (2013)

NA not available, *LDPE* low-density polyethylene, *HDPE* high-density polyethylene, *P* poly, *(3HB)* 3-hydroxybutyrate, *(3HO)* 3-hydroxyoctanoate, *3(HV)* 3-hydroxyvalerate, *3(HD)* 3-hydroxydecanoate, *(4B)* butyrate, *(Hx)* hexanoate, T_g glass transition temperature, T_m, melting temperature, X_{cr} degree of crystallinity, E Young's module, ε elongation to break, σ tensile strength

Table 18.5 Diversity of PHA-producing *Bacilli*

Type of *Bacilli*	Type of PHA	PHA yield	Nutrient source	Incubation period	Study level	References
B. megaterium	P(3HB)	186.8 mg/g	Glycerol reagent grade (GRG)	48 h	Flask level	Cardozo et al. (2016)
B. megaterium uyuni S29	P(3HB) with 161Tm	30%	Glucose	NA	Bioreactor level	Contreras et al. (2013)
	P(3HB) with 136.8 T_m*	70%	Glucose	NA	Bioreactor level	
Isolate AWW belonging to genus *Bacillus*	P(3HB)	41.66%	Glucose	48 h	Flask level	Getachew and Woldesenbet (2016)
	P(3HB)	54.16%	Fructose	48 h	Flask level	
	P(3HB)	48.83%	Sucrose	48 h	Flask level	
	P(3HB)	51.61%	Corn cob	48 h	Flask level	
	P(3HB)	38.55%	Teff straw	48 h	Flask level	
	P(3HB)	26.92%	Banana peel	48 h	Flask level	
	P(3HB)	63.41%	Peptone	48 h	Flask level	
	P(3HB)	51.25%	Ammonium nitrate	48 h	Flask level	
Isolate ASS belonging to genus *Bacillus*	P(3HB)	35.45%	Glucose	48 h	Flask level	
Isolate LAW belonging to genus *Bacillus*	P(3HB)	28.88%	Glucose	48 h	Flask level	
Isolate FPS belonging to genus *Bacillus*	P(3HB)	23.59%	Glucose	48 h	Flask level	
Isolate KAS belonging to genus *Bacillus*	P(3HB)	16.66%	Glucose	48 h	Flask level	
Isolate KIS belonging to genus *Bacillus*	P(3HB)	28.57%	Glucose	48 h	Flask level	
Isolate WW belonging to genus *Bacillus*	PHA	46.28%	Glucose	48 h	Flask level	Getachew and Berhanu (2016)
Isolate RS belonging to genus *Bacillus*	PHA	35.45%	Glucose	48 h	Flask level	
Isolate SS belonging to genus *Bacillus*	PHA	34.04%	Glucose	48 h	Flask level	
Bacillus sp. N-2	P(3HB)	20%	Glucose	5 days	Flask level	Hassan et al. (2016)

(continued)

Table 18.5 (continued)

Type of *Bacilli*	Type of PHA	PHA yield	Nutrient source	Incubation period	Study level	References
6 BC1 *Bacillus* co-culture	P(3HB)	150 mg/L	Glucose	48 h	Flask level	Kumar et al. (2014)
	P(3HB)	855 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	20 mg/L	Pea-shell slurry	48 h	Flask level	
5 BC1 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	1620 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3 HV)	25 mg/L	Pea-shell slurry	48 h	Flask level	
5 BC2 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	1595 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	15 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC1 *Bacillus* co-culture	P(3HB)	250 mg/L	Glucose	48 h	Flask level	
	P(3HB)	430 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	30 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC2 *Bacillus* co-culture	P(3HB)	205 mg/L	Glucose	48 h	Flask level	
	P(3HB)	960 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	20 mg/L	Pea-shell slurry	48 h	Flask level	
4 BC3 *Bacillus* co-culture	P(3HB)	190 mg/L	Glucose	48 h	Flask level	
	P(3HB)	570 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	30 mg/L	Pea-shell slurry	48 h	Flask level	
3 BC1 *Bacillus* co-culture	P(3HB)	185 mg/L	Glucose	48 h	Flask level	
	P(3HB)	875 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	25 mg/L	Pea-shell slurry	48 h	Flask level	
2 BC1 *Bacillus* co-culture	P(3HB)	230 mg/L	Glucose	48 h	Flask level	
	P(3HB)	780 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	40 mg/L	Pea-shell slurry	48 h	Flask level	
2 BC2 *Bacillus* co-culture	P(3HB)	220 mg/L	Glucose	48 h	Flask level	
	P(3HB)	835 mg/L	Pea-shell slurry	48 h	Flask level	
	P(3HV)	35 mg/L	Pea-shell slurry	48 h	Flask level	

Bacillus sp.	PHA	NA	Marine sample	NA	NA	Wecker et al. (2015)
Bacillus odysseyi SUK3	PHA	58%	Mixture of red HE8B, red M5B, remazol red, orange 3R, rubine, golden yellow HER and direct blue GLL	48 h	Flask level	Tamboli et al. (2010)
Bacillus megaterium BA-019	P(3HB)	42%	Molasses and sucrose	24 h	Fed-batch	Pagliano et al. (2017)
Bacillus cereus SPV	P(3HB)	61.07%	Sugarcane molasses	50 h	Shaken flask	Akaraonye et al. (2012)
	P(3HB)	51.37%	Sugarcane molasses	50 h	Fermenter level	
B. megaterium DSM 509	PHA	48%	Octanoic acid	24 h	Flask level	Shahid et al. (2013)
Bacillus megaterium	PHB	5.61 g/L	Glucose and ammonium sulphate	64 h	Bioreactor	Mohanrasu et al. (2020)
Bacillus megaterium SRKP-3	PHB	11.32 g/L	Dairy waste, Rice bran and Sa water	36 h	Feed-batch	Pandian et al. (2010)
Bacillus drentensis BP17	PHB	5.55 g/L	Pineapple peel solution	36 h	Flask level	Penkhrue et al. (2020)
Bacillus sp. *CFR 67*	PHA	524 mg/L	Wheat bran hydrolysate	72 h	Flask level	Sreekanth et al. (2013)
Bacillus tequilensis	P(3HB-co-3 HV)	59%	Synthetic acids	48 h	Flask level	Reddy et al. (2014)
		36%	Acidogenic fermented food waste (AFW)			

NA not available, T_m melting temperature, *P* Poly, *3(HB)* 3-hydroxybutyrate, *3(HV)* 3-Hydroxyvalerate

18.3.3 Metabolic Overview for PHA Production in Bacilli

The monomeric PHA/hydroxyalkanoic acid is synthesized by different metabolic pathways. The type of –R group incorporated in monomeric PHA depends on the type of substrate and the capability of microorganisms to metabolize the available substrate (Sudesh et al. 2000). The common intermediate in the pathway is hydroxyacyl-Co-A, as shown in Fig. 18.8a (Chen 2010). The type of acyl group present in it reveals the formation of a particular type of PHA. For example, 3-hydroxybutyryl-Co-A contains butyryl group which gets polymerized into 3-hydroxybutyric acid/polyhydroxybutyrate [3(PHB)] (Chen 2010; Pradhan et al. 2020). Polymerization event is catalysed by PHA synthase (PhaC) (Grage et al. 2009; Chen 2010; Pradhan et al. 2020). It is the most common enzyme involved in all PHA production pathways. The metabolic pathway of PHB, the most common kind of PHA, is well established. As shown in Fig. 18.8b, it involves two enzymes, viz. β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB), along with PhaC. The microorganisms act upon available substrate and convert it into acetyl CoA. Further, PhaA transforms it into acetoacetyl-CoA which gets converted into 3-hydroxybutyryl-CoA by PhaB (Pradhan et al. 2020; Chen 2010; Beun et al. 2002). Like all other biochemical pathways, PHA synthesis also involves wide varieties of enzymes and a few of them are listed in Table 18.6.

Apart from this, *B. thuringiensis* strain YBT-1520 was reported to show PHB production from acetyl Co-A via two dissimilar pathways. One involved three traditional enzymes, viz. PhaA, PhaB and PhaC, while other involved enzymes designated as AtoB, FadB and Crt. The enzyme AtoB converts acetyl CoA into acetoacetyl-CoA. Then it gets transformed into Crotonoyl CoA via

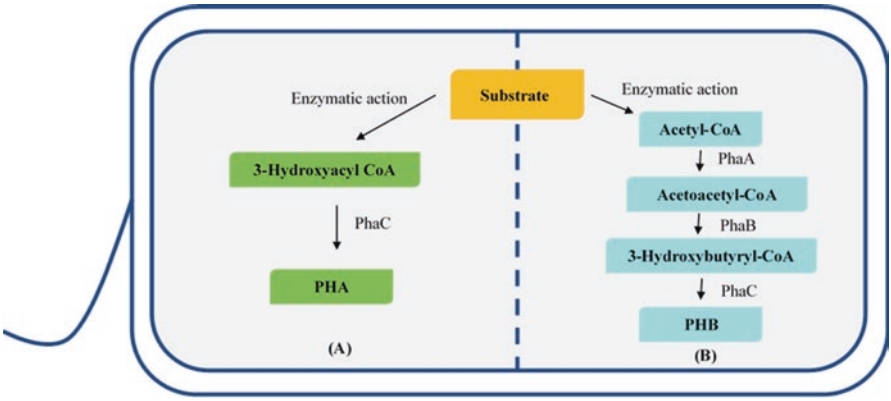

Fig. 18.8 Metabolic pathways for PHA production in bacterial cells. (**a**) PHA synthesis via 3-hydroxyacyl CoA intermediate and (**b**) PHB synthesis via acetyl-CoA: Formation of particular type of PHA depends on the type of acyl group. For example, butyryl is the acyl group leading to formation of PHB

Table 18.6 Enzymes involved in biosynthesis of PHA and gene encoding them

Enzyme	Gene	Type of PHA	Organism	References
β-ketothiolase	*pha*A	P(3HB-*co*-3HHx)-	*Aeromonas caviae*	Fukui and Doi (1997) and Fukui et al. (1998)
NADH-acetoacetyl-CoA dehydrogenase	*pha*B			
(*S*)-specific enoyl-CoA hydratase (crotonase)	$phaJ_{Ac}$			
PHA synthase	*pha*C			
β-ketothiolase	NA	P(3HB)	*Rhizobium* (*Cicer*) sp. strain CC 1192	Chohan and Copeland (1998)
NADPH-dependent acetoacetyl-CoA reductase	NA			
PHA synthase	NA			
3-ketoacyl-ACP synthase III (FabH)	*fab*H	PHA	*Aeromonas caviae*	Taguchi et al. (1999)
Malonyl-CoA-ACP transacylase (FabD)	*fab*D			
PHA synthase (PhaC*Ac*)	$phaC_{Ac}$			
3-ketothiolase	NA	Poly(3HB-co-4HB)	*Clostridium kluyveri*	Valentin and Dennis (1997)
Acetoacetyl-CoA reductase	NA			
PHA synthase	NA			
Succinic semialdehyde dehydrogenase	*suc*D			
4-hydroxybutyrate dehydrogenase	4*hb*D			
4-hydroxybutyrate-CoA: CoA transferase	*orf*Z			
β -ketothiolase	NA	Poly(3HB-co-3HV-co-4 HV	*Alcaligenes eutrophus*	Valentin and Steinbuchel (1995)
Acetoacetyl-CoA reductase	NA			
3-hydroxyacyl CoA dehydrogenase	NA			
Acyl CoA dehydrogenase	NA			
Acyl CoA synthase	NA			
Acyl CoA transferase	NA			
β-hydroxyacyl-CoA hydrolase	NA			
PHA synthase	NA			
PHA synthase	*pha*C	PHA	*Bacillus megaterium*	Mccool and cannon (2001)
Class IV PHA synthase	$phaRC_{Bm}$	P(3HB)	*Bacillus megaterium* NBRC15308T	Tomizawa et al. (2011)
Class IV PHA synthase	$phaRC_{By}$	P(3HB)	*B. cereus* YB-4	Tomizawa et al. (2011)
PHA synthase	*pha*C and *pha*R	PHA	*Bacillus* sp. INT005	Satoh et al. (2002)

NA not available

(S)-3-hydroxybutynyl Co-A by FadB. Lastly, Crt acts upon crotonoyl CoA and forms (R)-3-hydroxybutynyl CoA, the common intermediate of both pathways which is polymerized by PhaC (Gong et al. 2012). *B. cereus* has been reported to formulate PHA via acetyl CoA metabolism or via fatty acid oxidation. It contains enzymes such as PhaA, PhaB, PhaJ, PhaRC and FadB for PHA production. PhaJ converts crotonoyl CoA to R-3-hydroxybutyryl CoA. PhaRC functions like PhaC while the remaining enzymes function as described before (Tsugeet et al. 2015).

Generally, PHA biosynthesis competes with TCA cycle for assimilating acetyl-CoA. Under stable growth condition, acetyl-CoA gets oxidized via TCA cycle and NADH generated is utilized for ATP production. In nutrient-deprived state, cell growth is limited, and the NADH accumulates (Faccin et al. 2013). This NADH will inhibit citrate synthase indicating the availability of sufficient amount of ATP and precursor for biosynthesis. Thus, rate of TCA cycle declines (Kim and Gadd 2008). Further, acetyl CoA generated due to the presence of excess substrate is directed towards PHA biosynthesis (Faccin et al. 2013). Decline in TCA cycle is even related with the absence of oxygen. Due to unavailability of O_2, NADH does not get oxidized via electron transport chain (ETC) and gets accumulated there. This inhibits citrate synthase, and rate of TCA cycle reduces (Kim and Gadd 2008; Faccin et al. 2013). Though unavailability of O_2 causes failure of TCA cycle, PHA accumulation is also limited. The reason behind this is oxidation of enzymes essential for PHA production. The inhibitory effect of O_2 over PHA production is solely dependent on the individual organisms (Borah et al. 2002). For appropriate PHA production, optimum aeration is required. Availability of O_2 above and below optimum level reduces PHA accumulation (Faccin et al. 2013).

18.3.4 Molecular Evidences for PHA Production by Bacilli

Biosynthesis of PHA includes numerous classes of enzymes. Three genes termed as *pha*A, *pha*B and *pha*C encode three key enzymes β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase of PHB production pathway. Other genes involved in PHA production are listed in Table 18.6.

B. thuringiensis R1 possesses *pha*P, *pha*Q, *pha*R, *pha*B and *pha*C genes, as depicted in Fig. 18.9. Gene designated as *pha*P encodes for phasins protein and *pha*Q encodes for transcriptional regulatory protein essential which regulates *pha*P activity. Apart from this, *pha*R encodes for unknown product essential for activity of *pha*C. Gene length of these genes along with their promoter regions and

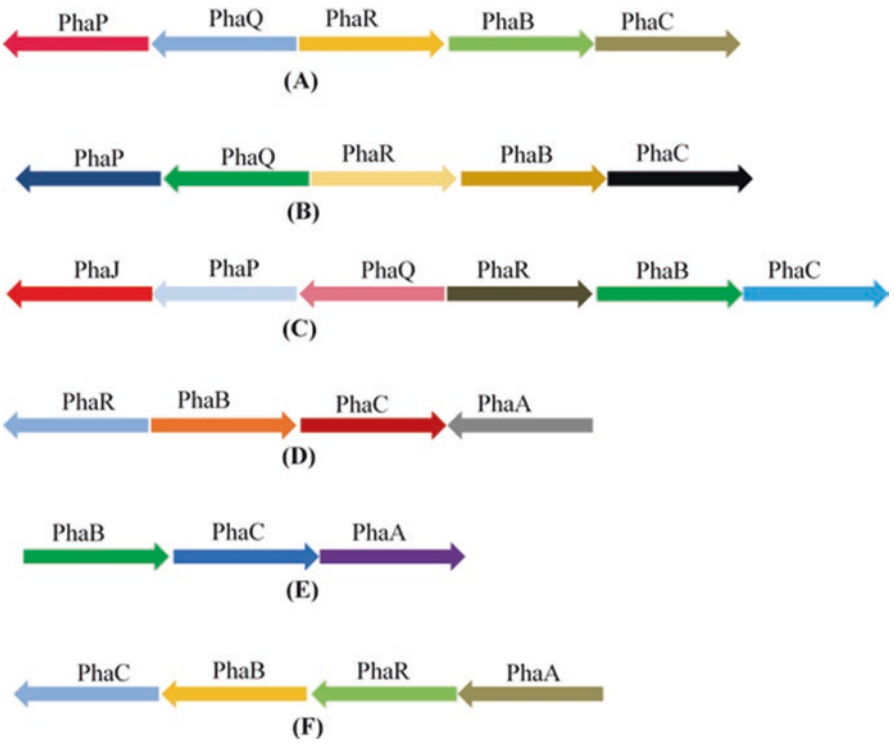

Fig. 18.9 Arrangement of PHA synthesis operon in different *Bacillus* spp. (**a**) *B. thuringiensis* R1, (**b**) *B. megaterium*, (**c**) *B. cereus* subgroups, (**d**) numerous *Bacillus* spp., (**e**) *B. thuringiensis* serovar chinensis CT-43 and *B. megaterium* QM B1551 and (**f**) *B. anthracis* CDC 684 and *B. megaterium* WSH-002

Table 18.7 Nucleotide sequence of R1 promoter and RBS of genes essential for PHA formulation in *Bacillus thuringiensis* (Desetty et al. 2008)

Gene	Size (bp)	Promoter sequence	RBS sequence
*pha*P	522	−10 (^{1099}CACATTTAA^{1091})	^{556}TTGGGG^{551}
*pha*Q	450	−35 (^{1119}AACTGA^{1114})	^{1053}GAGGTG^{1048}
*pha*R	528	−10 (^{1143}AAATAAAAT^{1130})	^{1171}CAGAAT^{1176}
*pha*B	741	−35 (^{1765}TTTCTA^{1770})	^{2682}AAGGAG^{2687}
*pha*C	1083	−10 (^{2606}ATATGTAAT^{2614})	^{2682}AAGGAG^{2687}

ribosomal binding sites (RBS) is listed in Table 18.7 (Desetty et al. 2008). *B. megaterium* have *phaP* (513 bp), *phaQ* (441 bp), *phaR* (609 bp), *phaB* (744 bp) and *phaC* (1089 bp) as shown in Fig. 18.9b (Valappil et al. 2007a). Few *B. cereus* subgroups as shown in Fig. 18.9c have *pha*R-*pha*B-*pha*C operon located in same direction and *pha*P-*pha*Q-*pha*J operon in its contradictory direction. Later operon is found

upstream of former one. The gene *pha*J was found to be involved in directing monomer supply via β-oxidation for PHA formation (Tsugeet et al. 2015).

Wide variations in orientation of genes crucial for PHA production were reported in numerous *Bacillus* spp. It was reported that operon *pha*RBCA as shown in Fig. 18.9d was found in *B. anthracis* A0248, *B. anthracis* Ames, *B. anthracis* "Ames Ancestor", *B. cereus* Q1, *B. cereus* 03BB102, *B. cereus* AH820, *B. cereus* B4264, *B. cereus* G9842, *B. cereus* AH187, *B. cereus* biovar anthracis str. CI, *B. cereus* ATCC 10987, *B. cereus* E33L, *B. megaterium* DSM 319, *B. thuringiensis* BMB171 and *B. thuringiensis* serovar konkukian, 97–27. Moreover, authors had reported operon *pha*BCA as illustrated in Fig. 18.9e, in *B. thuringiensis* serovar chinensis CT-43 and *B. megaterium* QM B1551. They had even shown that *B. anthracis* CDC 684 and *B. megaterium* WSH-002 have *pha*CBRA operon, as shown in Fig. 18.9f (Kumar et al. 2013).

18.3.5 Strategy for PHA Accumulation and Recovery

Microorganisms show higher PHA accumulation in favourable environmental conditions. As described earlier in nutrient-depleted environments, few microorganisms ensure higher PHA yield. Growth conditions favourable for microbes to accumulate PHA vary from organism to organism (Albuquerque et al. 2010; Shahid et al. 2013; Saharan et al. 2014; Beun et al. 2002). Also, yield of PHA obtained varies with the time at which PHA is extracted (Pandian et al. 2010; Kumar et al. 2015; Pagliano et al. 2017; Rathika et al. 2018). Moreover, inoculum size and pH also affect the yield of extracted PHA. Higher PHA yield extracted from *B. subtilis* RS1 was obtained with 10% inoculum size and at pH 7 (Rathika et al. 2018). *Bacillus* sp. BPPI-14 and *Bacillus* sp. BPPI-19 showed higher PHA yield at pH 7 and 37 °C using glucose as sole source of carbon (Mohammed et al. 2019). Hence, optimization of PHA accumulation media is required for enhancing PHA yield. Utilization of cheap nutrient source will reduce the cost of industrial PHA production (Verlinden et al. 2011; Mohapatra et al. 2017; Kourmentza et al. 2017).

Another factor which affects industrial level PHA production is the cost associated with PHA recovery. Effective PHA recovery scheme plays an important role in gaining higher PHA yield. As demonstrated in Fig. 18.10, approaches for PHA recovery can be divided into six steps, viz. cellular biomass harvesting, pretreatment, non-PHA cellular biomass disruption, PHA extraction, drying and purification (Kourmentza et al. 2017; Sathya et al. 2018; Jacquel et al. 2008). As PHA is intracellular product, concentration of cellular biomass is carried out. It is harvested using centrifugation or filtration (Kourmentza et al. 2017). The harvested biomass is subjected for pretreatment prior to cell lysis. The main aim of pretreatment is to weaken the microbial cell wall, which involves physical techniques such as lyophilization, ultrasonication and high temperature. The cell lysis of pretreated biomass is carried out using chemical, enzymatic or biological method. The agents used for cell lysis should not deteriorate PHA. Chemical method involves usage of sodium

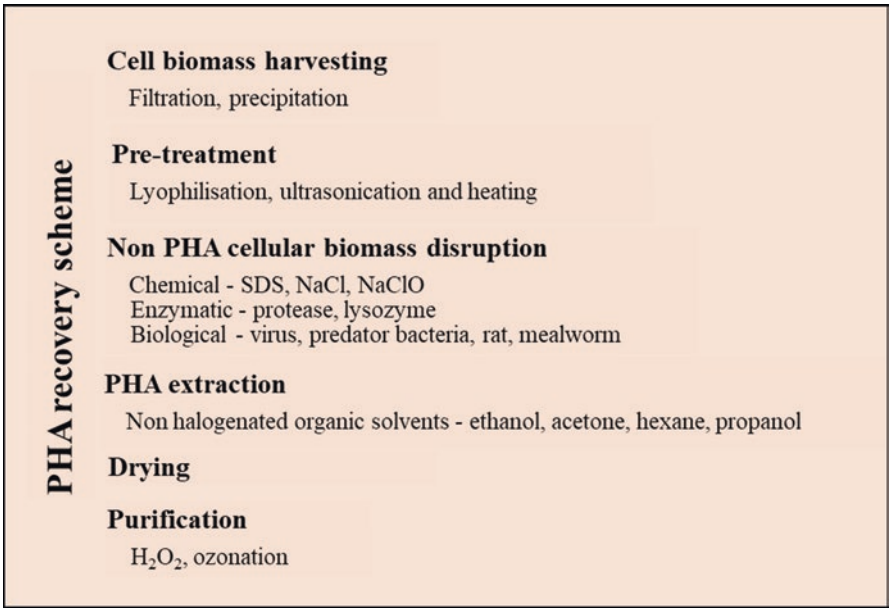

Fig. 18.10 PHA recovery scheme: it includes six steps, viz. cell biomass harvesting, pretreatment, non-PHA cellular biomass disruption, PHA extraction, drying and purification

chloride (NaCl), sodium hypochlorite (NaClO) or sodium dodecyl sulphate (SDS). Enzymatic method includes application of enzymes such as proteases and lysozyme (Kourmentza et al. 2017; Jacquel et al. 2008). For biological cell lysis, researchers have used virus and predatory bacteria. Some researchers have fed the rats and mealworms with biomass, and PHA was recovered from faeces (Kourmentza et al. 2017). Amongst all, chemical methods are widely accepted as they are eco-friendly and do not involve the use of halogenated solvents. Moreover, enzymatic methods are costly, and biological methods are time-consuming (Kourmentza et al. 2017; Jacquel et al. 2008). Traditionally, PHA was extracted using hazardous chlorinated halogenated solvents, such as chloroform, 1, 2-dichloroethane and methylene chloride (Jacquel et al. 2008). Nowadays, PHA extraction is carried out using non-halogenated organic solvents in which PHA is soluble. It includes solvents such as acetone, n-hexane, propanol and ethanol (Kourmentza et al. 2017; Jacquel et al. 2008). Extracted PHA is dried and obtained in the form of powder (Kourmentza et al. 2017; Jacquel et al. 2008). For purification of biopolymer, H_2O_2 or ozonation has been employed (Horowitz et al. 2001; Madkour et al. 2013). Application of cheap and environment-friendly PHA recovery technique is beneficial which needs to be optimized (Kourmentza et al. 2017).

18.3.6 Techniques Involved in Characterization of PHA

A wide range of sophisticated techniques are employed for characterization of extracted PHA powder. Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and gas chromatography-mass spectroscopy (GC-MS) are the most common techniques used for determining the functional group incorporated in PHA. Apart from this, X-ray diffraction (XRD), gel permeation chromatography (GPC), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were used for determining mechanical and thermal properties of PHA (Pradhan et al. 2020; Sathya et al. 2018; Mohapatra et al. 2017; Gumel et al. 2012; Godbole 2016; Johnston et al. 2018). Details of all these techniques are described in Table 18.8.

PHA extracted from *B. licheniformis* AS3-2 was characterized using FTIR (Shah 2012). PHA extracted from *Bacillus cereus* was characterized using scanning electron microscopy (SEM), FTIR, XRD, NMR, DSC and thermal gravimetric analysis (TGA) and confirmed as PHB (Babruwad et al. 2015). PHB-co-PHV extracted from *Bacillus megaterium* OUAT 016 was characterized using FTIR, NMR, XRD and TGA (Mohapatra et al. 2020).

18.3.7 Challenges for Bacilli to Produce PHA

Bacillus spp. are capable of producing PHA using cheap substrates including waste materials. Moreover, they are capable of tolerating high pH and high osmotic pressure. Despite of such facts, the major drawback associated with its application for industrial scale PHA production is its sporulating nature (Wu et al. 2001; Mohapatra et al. 2017). Spore formation may utilize energy generated via PHB degradation (Wu et al. 2001). Normally, it was supposed that PHB degradation diverts energy and carbon source for sporulation (Kominek and Halvorson 1965). *B. cereus* has been reported to accumulate PHB prior to sporulation which subsequently gets degraded during spore formation event (Navarro et al. 2006; Kominek and Halvorson 1965). PHB accumulated by *B. thuringiensis* using glucose is utilized for spore formation (Benoit et al. 1990). *Bacillus* sp. JMa5 showed spore formation in nutrient-limiting conditions with low PHB yield. Besides this, it showed growth-associated PHB production. Hence, authors conclude that low levels of nutrients induce sporulation which may limit PHB accumulation (Wu et al. 2001). Further, acidic pH and low level of potassium show decline in spore formation (Mohapatra et al. 2017). *Bacillus* spp. SPV was unable to sporulate in acidic pH and showed PHB accumulation (Valappil et al. 2007b). The antisporogenic agent α-picolinic acid prevents conversion of vegetative *Bacillus cereus* T cells to sporulating cells. TCA cycle enzymes essential for sporulation seem to be synthesized during shift from vegetative phase to sporulation phase. α-picolinic acid is inhibitory to aconitase synthesis and prevents TCA cycle essential for spore formation and not for

Table 18.8 Techniques used for characterization of PHA

Name	Role	Information obtained			References
FTIR	Used for identification of functional group of PHA	–R group	Wave frequency (cm^{-1})		Pradhan et al. (2020)
		–CH	2962–2853		
		–C=O	1742–1709		
		–C–O or –C–C	1300–1709		
		–OH	3460–3407		
NMR	Used to recognize functional group and polymeric content of PHA biopolymer	–R group	^{1}H NMR (ppm)	^{13}C NMR (ppm)	Pradhan et al. (2020)
		–CH	5.2–5.26	67.8–68.5	
		$–CH_2$	2.17–2.7	31.09–41.3	
		$–CH_3$	1.25–1.6	19.95–21.4	
		–C=O	NA	169.1–169.5	
GC-MS	Used for analysing monomeric composition of PHA	Methyl esters of PHA are subjected to GC-MS analysis and functional groups are identified on the basis of retention time			Lee and Choi (1997)
XRD	Useful for studying crystalline nature of PHA	% Xc = At − Aa/At × 100 At = area of crystalline peak Aa = area of amorphous peak			Pradhan et al. (2020)
GPC	Used for determining mw, Mn and polydispersity index	Polydispersity index indicates molecular mass distribution and was calculated by determining Mw/Mn ratio			Pradhan et al. (2020)
DSC	Used for understanding thermal properties such as T_m and X_c of PHA	% $X_c = \Delta H_m/\Delta H_f \times 100$ ΔH_m = measured enthalpy of polymer ΔH_f = enthalpy of 100% pure PHB (146 J/g)			Pradhan et al. (2020), Gunaratne and Shanks (2005), and Kulkarni et al. (2010)
TGA	Used for analysing thermal stability and degradation as well as resistance temperature of PHA	TGA graph indicates the reduction in weight of PHA with rise in temperature. PHA degradation takes place as the temperature increases. Degradation of PHA correlates with decrease in weight of PHA			Pradhan et al. (2020)

NA not available, *Mw* average molecular weight, *Mn* number average molecular weight, T_m, melting temperature, X_c crystallinity

biomass development. Thus, this antisporogenic agent prevents spore formation (Hanson et al. 1963). Such sort of manipulation of environmental conditions may be supportive in regulating the sporulating nature of *Bacillus* spp. and thus permitting its usage for industrial scale PHA synthesis. Moreover, study by Wang et al. (2016) shows that sporulation in *B. thuringiensis* is not associated with PHB degradation.

They even observed that many *Bacillus* spp. lack *pha*C and *pha*Z genes but still sporulate, indicating individuality of spore formation over PHB degradation. There arises a need of strategies for controlling sporulation for application of such strains in industrial PHA production.

Apart from this, the *Bacillus* spp. possess thick cell wall which makes PHA extraction difficult (Wu et al. 2001; Mohapatra et al. 2017). *B. flexus*, grown in inorganic rich medium contains less diaminopimelic acid and amino acids in cell wall. This allows easier cell lysis, and so higher PHA recovery was found (Divyashree and Shamala 2010). Similar techniques can be used for efficient recovery of PHA from *Bacillus* spp.

18.3.8 Approaches for Improving Properties of PHA for Industrial Application

Most of the biopolymers possess poor mechanical properties. Brittle and fragile nature of biopolymers limits their industrial scale application (Vieira et al. 2011). In order to improve their mechanical properties, they are either blended with plasticizer and/or cross-linking agent (Jantrawut et al. 2017) or blended with other polymers (Mohapatra et al. 2017; Narancic et al. 2018). Plasticizers have low molecular weight and are non-volatile compounds. They are widely used in polymer industry for enhancing the properties of polymers. They are known to reduce Tg of polymers and supplement their biodegradation (Vieira et al. 2011). Usage of eco-friendly nature-based biodegradable plasticizer is advantageous over conventional plasticizer such as phthalates (Vieira et al. 2011). Thermal and mechanical properties of PHBV films improve after blending with biodegradable plasticizers such as soybean oil (SO), epoxidized soybean oil (ESO), dibutyl phthalate (DBP) and triethyl citrate (TEC) (Vieira et al. 2011). Cross-linking agent forms intermolecular cross linkages with biopolymer, permitting suitable biopolymer film formation (Jantrawut et al. 2017). Further, application of some additives along with plasticizer enhances enzymatic degradation of PHB (Vieira et al. 2011).

Blending of P(3HB) extracted from *B. megaterium* Ti3 with polyethylene glycol enhances biocompatibility of P(3HB) film (Israni et al. 2020). The blends of PHB extracted from *B. cereus* strain VIT-SSR1 isolated from industrial effluents were prepared with chitosan. Biocompatibility of these blends was investigated on L929 mouse fibroblast cell line with MTT assay. They proved to be biocompatible and hence can be used for drug delivery (Evangeline and Sridharan 2019). PHBV extracted from *B. aryabhattai* PHB10 was blended with polyethylene glycol, and cytotoxicity was analysed on human keratinocytes (HaCat cells). Approximately 99% cells were viable, and hence this blend can be employed for skin graft application (Pillai et al. 2020).

18.3.9 Commercial Applications of PHA Obtained from Bacilli

Microbially originated biodegradable PHA have plenty of applications. They are used for manufacturing of packaging material and biomedical products (Mohapatra et al. 2017; Chen 2010; Sathya et al. 2018). Moreover, they are used as drug delivery carriers, as pharmaceutical products/drugs and as biofuels (Chen 2010). PHA are non-toxic in nature and hence are biocompatible, allowing its biomedical applications (Pradhan et al. 2020). They are even used for agricultural purposes (Pradhan et al. 2020; Sowinski et al. 2010).

Commercially, manufacturer entitled as PHB Industrial S.A., Brazil, have employed *Bacillus* spp. under Biocycle trademark. They are exploited for P(3HB) production from sugarcane (Ciesielska and Kiewisz 2016). PHB extracted from pigmented *Bacillus* sp. C1 (2013) (KF626477) was biocompatible in nature and hence can be used as drug delivery carrier (Pati et al. 2020). PHB of *B. thuringiensis* is non-toxic and is suitable for biomedical purpose (El-Abd et al. 2017). PHB-co-PHV extracted from *B. megaterium* OUAT 016 was found to be biocompatible and can be used as drug delivery carrier (Mohapatra et al. 2020). P(3HB-co-HV) extracted from *B. thermoamylovorans* was esterified using methanol and H_2SO_4. This, P(3HB-co-HV) methyl ester can be used as biofuel (Sangkharak et al. 2020). PHA levofloxacin nanoparticles were prepared using PHA extracted from *B. subtilis* NCDC0671, and its levofloxacin releasing efficacy was proved to be efficient. Hence, they can be used for delivering levofloxacin drug (Umesh et al. 2017).

18.3.10 PHA Depolymerase of Bacilli *and Biodegradation*

The most attractive feature of PHA is its biodegradability. It is composed of 100% natural biobased resources (Pradhan et al. 2020). In aerobic conditions, they get transformed into CO_2 and H_2O, whereas in anaerobic conditions, CH_4 is obtained additionally. It undergoes thermal degradation as well as enzymatic and non-enzymatic degradation (Pradhan et al. 2020; Nesic et al. 2020). It gets degraded when exposed to soil or compost and even in marine sediments (Nesic et al. 2020). It is prone to get degraded by microbial PHA depolymerase (i-PhaZ and e-PhaZ) or non-enzymatically inside animal tissues. It takes about few months to a year to get degraded in anaerobic conditions. Degradation rate boosts up in UV light. PHA polymers with high T_m take longer duration for degradation. Apart from this, PHA with low molecular weights gets degraded faster. Factors affecting rate of PHA degradation are temperature, pressure, moisture, surface area, pH and type of microorganism (Pradhan et al. 2020).

The role of i-PhaZ and e-PhaZ is described in earlier section, i.e. in functions of PHA and structure of PHA granule. *Bacillus megaterium* has i-PhaZ designated as PhaZ1 and degrades PHB into 3-hydroxybutyric acid monomers (Chen et al. 2009). *B. thuringiensis* possess P(3HB) depolymerase designated as PhaZ (Huang et al.

2012). *Bacillus* sp. strain NRRL B-14911, *B. megaterium*, *B. pseudofirmus* and *Bacillus* sp. strain SG-1 have e-PhaZ (Ma et al. 2011). *B. megaterium* N-18-25-9 contains e-PhaZ gene designated as $phaZ_{Bm}$ (Takaku et al. 2006). *B. thuringiensis* subsp. *israelensis* ATCC 35646 contains gene designated as *phaZ* have function similar to intracellular P(3HB) depolymerase (Tseng et al. 2006).

18.4 Future Prospects

Biodegradable biopolymers are recognized as potential substitute for conventional petrochemical-based plastics. *Bacillus* spp. are considered as promising agents for PHA production. A wide range of lab level investigations on *Bacillus* and PHA have been carried out till date. But their application in actual biopolymer industry needs attention. Moreover, varied spectrum of microbial PHA is known to exist, but most of the studies are constricted to P(3HB). Finding out additional forms of PHA having plastics like properties might be advantageous. Genetically engineered *Bacillus* strain ensuring higher PHA-producing capabilities can be the targeted aim of future research. Methyl esters of PHA have properties similar to biofuel so exploring the application of PHA is obtained using waste substrates, as biofuel will allow sustainable management of waste along with formation of valuable by-product. The PHA polymeric proteins such as PhaP, PhaZ and PhaC may be applicable as potential drug delivering tools.

18.5 Conclusions

Plastic pollution is one of the major concerns in the world. Biodegradable biopolymers being solutions to such issue have gained much attention amongst scientific community. Primarily, microbial PHA are significantly valuable as they are solely biobased and totally biodegradable. They are carbon-rich inclusion bodies synthesized by microorganisms in response to stress conditions. The polyester PHA granules are comprised of hydroxyalkanoic acid, PhaP, PhaC, PhaZ and PhaR. Micelle formation and budding are the two strategies known for PHA formulation in microorganisms. *Scl*-PHA have properties identical to polystyrene, whereas *mcl*-PHA are widely accepted in medical industry.

First and foremost concern for using PHA in bio-industry depends on the choice of microbial strain and cost-effective nutrient source. *Bacillus* spp. are known to serve as appropriate bacteria for industrial application. They are also recognized to produce PHA from varieties of inexpensive waste. They are known to produce PHA in the presence of excess substrate with either nutrient-deprived or non-nutrient-deprived conditions. Metabolic pathway of PHA production and operons encoding enzymes of pathway in *Bacilli* are well known. The media conditions known to enhance PHA accumulation in bacilli need to be optimized in order to increase the

yield. Accomplishing appropriate cost-effective eco-friendly PHA recovery strategy can enable us to meet the need of industries. Advances in modern science have led to development of many sophisticated techniques for characterization of PHA. Features such as sporulation and thick cell wall associated with *Bacillus* spp. hinder its applicability for industrial scale PHA manufacturing. Abundant research had been done to solve such issues. Moreover, blending of PHA extracted from *Bacilli* with plasticizer or cross-linking agent has increased its biocompatibility, making them suitable for biomedical applications. Blending also decreases the time required for PHA degradation. The enzymes i-PhaZ as well as e-PhaZ from *Bacilli* origin play a vital role in biodegradation of PHA. Thus, *Bacillus* spp. are promising resources for bioplastic industry.

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Chapter 19
Bacillus as a Versatile Tool for Crop Improvement and Agro-Industry

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Abstract Agriculture and the agro-industry form the backbone of any economy, whether traditional or modern sector. Though it contributes less in terms of GDP percentage but employs a significantly large amount of human power. Agriculture depends upon numerous biotic and abiotic factors which are essential for productivity. Alteration in the fine balance of these factors has the potential to result in the development of stress that may affect the plants. The chemical fertilizer and pesticide routinely tackle nutrient nonavailability and competition from weeds and pathogenic infection. However, the impact of these chemicals on the environment and health is a serious concern with addition to the increasing cost of production. Metabolites produced by various microbes have been considered as an effective biological agent to enhance productivity, prevent stress conditions, and provide chemical-free food products. *Bacillus* spp. have been a striking biological agent owing to its ability to produce a wide range of metabolites that enhance crop productivity by availing plant various micronutrients, volatile compounds, and antimicrobials targeting pathogens. Many species of *Bacillus* are known to produce antibiotics, chitinase, lipopeptides, glucanase, and hydrogen cyanide which kill bacteria, fungi, and pests. Some other *Bacillus* species promote plant growth indirectly by elicitating systematic resistance against pathogens or by producing growth hormones like cytokinins, IAA, gibberellin, and spermidines, which results in root and

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shoot growth. Introduction of *Bacillus* in soil has also shown to provide tolerance towards harsh environmental stimuli like heavy metal, droughts, and salinity to the crops. In this chapter, we try to elaborate on these essential roles played by the *Bacilli* species in soil and its future directions.

Keywords Agriculture · *Bacillus* · Crop protection · Metabolites · Biological agents

19.1 Introduction

Agriculture plays a vital role in the world economy. However, crop cultivation is affected by various biotic and abiotic factors. Agriculture is affected by biotic stresses that include plant infection with viruses, bacteria, and fungi, as well as infestation by the pest and arachnids (Singh et al. 2018). Several weed species are competing for the nutrients with the plant also a major cause of biotic stresses. At the same time, the abiotic stress includes an imbalance in soil salinity, temperature, water, and nutrients deprivation. This abiotic and biotic stress upon manifestation creates a negative outcome on plant growth, development, and yield (Bacon et al. 2012; Shafi et al. 2017a). To overcome such stresses and enhance productivity, many chemical-based management strategies are being used since industrialization. Long-term and excessive utilization of these chemical and synthetic products on crop production has resulted in severe environmental pollution, degradation in the quality of soil, and adverse effect on human health. Due to these adverse effects of chemicals on human health and environment, the scientific community is trying to develop a sustainable and green solution for the improvement of plant productivity (Shafi et al. 2017a; Hussain and Khan 2020a; Dunlap et al. 2013; Torres et al. 2019).

Biological methods provide a new ray of hope to enhance plant productivity and simultaneously curtailing the role of chemical fertilizer and pesticide (Dhankar et al. 2020). These biological agents in the form of live cells or their by-products are a positive, sustainable source for agricultural development by increasing the productivity and reducing the health risk caused by the use of chemicals (Berg et al. 2005; Bacon et al. 2015; Hussain and Dhanker 2021).

Microbes associate with the plant as a symbiotic relationship at multiple surfaces such as leaves and the rhizome. Microbial endophytes dwelling within the plant tissues consist of the beneficial symbiont. Such plant-microbe interaction is a mutually beneficial relationship, unlike the pathogen, which negatively regulates plant growth. Of all the beneficial microbes in the entophytic system, bacteria are quite varied and are known to provide the plant with several essential products (Ahamd et al. 2019; Haris et al. 2020; Hussain et al. 2020e, f). These endophytic bacteria have the potential for phytoremediation, biological control of pest/pathogens, and agronomic enhancements of plants' maturity and growth (Berg et al. 2005; Bacon et al. 2015; Hussain and Khan, 2020d; Hussain et al. 2021). The numerous bacteria *Bacillus* spp. are considerable interest for its role as plant

growth-promoting rhizobacteria (PGPR) and as the producer of beneficial secondary metabolites (Kang et al. 2015). *Bacillus* consists of a diverse group of rod-shaped, gram-positive motile bacteria owing to peritrichous flagella. Their presence across a variety of environment can be attributed to their ability to form hard resistance endospore which helps to survive during the stress conditions and ability to live both as aerobe and facultative anaerobe (Radhakrishnan et al. 2017). These *Bacilli* form the active part of the (PGPR) that could be utilized in agricultural practice to alleviate stresses and to produce environmental-friendly management tool (Grover et al. 2011; Vejan et al. 2016).

The gram-positive bacteria are ubiquitous and are acquired from various environmental niches. Many of the *Bacillus* spp. are used commercially, as a fertilizer, resulting in a 40% increase in crop production (FZB24® Bacillus subtilis – mode of action of a microbial agent enhancing plant vitality n.d.). Some other *Bacillus*-based products such as Kodiak (*Bacillus subtilis* GB03), rhizovital (*B. amyloliquefaciens* FZB42) quantum-400 (*B. subtilis* GB03), serenade (*B. subtilis* QST 13), and YIB (*Bacillus* spp.) have also been commercialized for agricultural industry (Brannen and Kenney 1997; Ngugi et al. 2005; Cawoy et al. 2011). *Bacillus*-based products are more active compared to other biofertilizers due to their more effective metabolite production and spore-forming character, which enhances the viability of the cells for a long time (Haas and Défago 2005). Here we describe the different metabolites produced by the *Bacillus* and their role in increasing the agricultural productivity (Fig. 19.1).

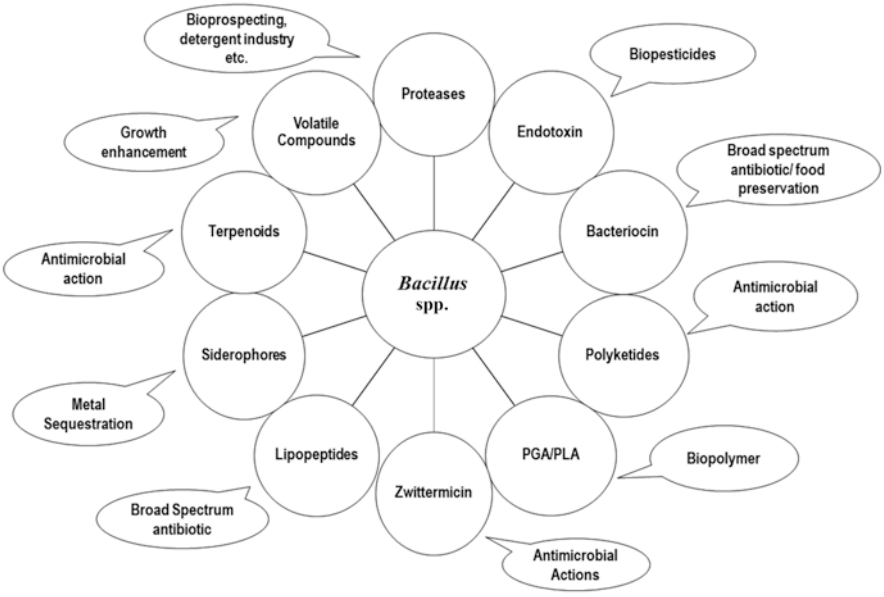

Fig. 19.1 Flow chart depicting the classification of various metabolites produced from different *Bacillus* spp. involving different synthesis pathways and their importance

Bacterial metabolites can be broadly classified into two categories, i.e. primary and secondary metabolites based upon the stage they are produced and their role. Primary metabolites are produced during the growth phase (log phase) of the bacterial life cycle and are essential for growth and survival. At the same time, the secondary metabolites are produced in the stationary phase and play subsidiary roles such as defence and pigmentation, etc. Owing to its presence across a broad diversity of environment, *Bacillus* produces several metabolites, especially the secondary metabolites.

19.2 Functions of *Bacillus* Various Metabolites

Bacillus as a PGPR has been described to stimulate plant growth through various direct and indirect mechanisms, or by a combination of both mechanisms. Direct interaction comprises of fixing of nitrogen, providing phytohormones to plants, solubilization of phosphates, iron uptake via bacterial siderophores, and production of ACC deaminases that can reduce the levels of stress-induced ethylene in plants (Glick 2004). Extracellular hydrolytic enzymes, antimicrobial agents, and siderophores produced by these microorganisms eliminate the pathogenic microbes present in the rhizosphere and the surrounding. This type of interaction between plant and microbes is termed as indirect interaction (Yuwono et al. 2005; Mandal et al. 2021).

19.2.1 Enzymes

Several *Bacillus* spp. are commercially important due to their ability to secrete industrially essential enzymes. These enzymes are essential in industries like food, detergent, textiles, feed, and beverages (Siddiqua et al. 2021). Alkaline serine proteases secreted from *Bacillus* are of considerable importance in several industries. Different species of *Bacillus* such as *B. halodurans*, *B. clausii*, *B. amyloliquefaciens*, and *B. subtilis (*Schallmey et al. 2004*;* Saeki et al. 2007*)* produce several proteases of commercial importance that includes amylases, cellulases, ligininlases, and lipases. α-galactosidase produced from *B. megaterium* and *B. stearothermophilus* has shown wide application in the pulp, beer sugar, paper, soyfood, and pharma industries.

Apart from the above-mentioned enzymes, the *Bacillus* species also produce peptidoglycan hydrolase enzymes which de-polymerizes and hydrolyse specific bonds in the cell wall. This type of hydrolase has great perspectives in different biotechnological fields such as in bioenergy production and as biocontrol against other microbes (Raddadi et al. 2004; Raddadi et al. 2005). Adding to the antimicrobial property of *Bacillus* are also the quorum quenching enzymes like AHL- lactonase (Dong et al. 2002).

19.2.2 Biopolymer

Biopolymers have gained extensive interest because of their eco-friendly production and vast areas of application. Various types of polymers have been reported to be produced by *Bacillus* spp. Poly (γ-glutamic acid) PGA, which is an anionic water-soluble polymer, has been used in pharmaceutical, cosmetics, food, and wastewater management as a heavy metal chelator or flocculant (Bajaj and Singhal 2011). *B. licheniformis*, *B. subtilis*, and *B. amyloliquefaciens* are known to be producing such biopolymers (Yamashiro et al. 2011; Cao et al. 2011).

19.2.3 Protein Crystal

Bacillus thuringiensis during the sporulation phase produce parasporal crystals. These Cry protoxins or δ endotoxin, when ingested by insects, convert into its active form which leads to pore formation (Lüthy and Wolfersberger 2000; Schwartz and Laprade 2000), and ultimately membrane permeability disrupts resulting in the death of the insect larvae (Porcar and Juárez-Pérez 2003). Cry proteins are active against dipteran, lepidopteran, coleopteran, and nematodes like *Nippostrongylus brasiliensis (*Schnepf et al. 1998*;* Wei et al. 2003*;* Kotze et al. 2005*)*. Besides the endotoxins, *B. thuringiensis* also produced δ-exotoxins, a non-proteinaceous toxin which is secreted into the medium. This is active against lepidopterans, dipteran, and coleopteran but has also shown an adverse effect against beneficial organisms like *Apis mellifera* (honeybee) (Espinasse et al. 2002, 2004).

19.2.4 Antimicrobial Agents

19.2.4.1 Bacteriocin

Bacteriocin is 20–60 amino acid long ribosomally synthesized, cationic, and hydrophobic antimicrobial peptides (AMP) (Riley and Wertz 2002a, b). These peptides inhibit both gram-positive and gram-negative bacteria (Walsh et al. 2015; Suganthi and Mohanasrinivasan 2015; Nes and Holo 2000). *Bacillus* produces immunity proteins to protect themselves from the toxic effect of their bacteriocin (Oppegård et al. 2007). Bacteriocin synthesis is regulated by structural genes involved in the synthesis of bacteriocin peptide with an N terminal leader sequence and several accessory genes involved in the processing and transporting the peptide molecule outside the cell. The bacteriocin production from the bacterial cell is often regulated by cell density or quorum sensing (Lee and Kim n.d.).

Bacteriocin targets the bacterial membrane using membrane surface charge (Rashid et al. 2016; Moll et al. n.d.). Bacterial surfaces are negatively charged due

to the presence of phosphatidylglycerol (PG), lipopolysaccharide (LPS), phatidylethanolamine (PE), cardiolipin (CL), and lipoteichoic acid (LTA). These serve as targets for the cationic bacteriocins (Rashid et al. 2016; López-Lara and Geiger 2017). The positively charged part of the bacteriocin interacts with the negative bacterial surface via electrostatic interactions. Simultaneously, the hydrophobic part of the bacteriocin penetrates the lipid membrane. Upon piercing the bilayer, the peptides self-polymerize and form channel complexes (Shahnawaz and Soto 2012), resulting in permeabilization of the cell membrane by ion-selective pores which result in proton motive force and exhaustion of intercellular ATP (Christensen and Hutkins 1992). This, in turn, results in intercellular substrate leakage and eventually death of the organism (Minahk et al. 2000).

Many *Bacillus* species are known to produce a range of bacteriocin, and these include lichenin, megacin, coagulin, and polyfermenticin from *B. licheniformis*, *B. megaterium*, *B. coagulans*, and *B. polyfermecticus*, respectively. Similarly, *B. thuringiensis* subsp. *kurstaki* produces a bacteriocin known as Bacthuricin F4. Bacthuricin F4 is a 3160.05 Da molecular weight bacteriocin and has demonstrated antibacterial and highest bacteriocin activity. Another bacteriocin produced by *B. amyloliquefaciens* from mangrove has shown to inhibit *Ralstonia solanacearum (*Hu et al. 2010*)*. Subtilin, a bacteriocin produced by *B. subtilis*, is prominently used in the dairy industry owing to its broad-spectrum antimicrobial activity. These bacteriocins not just protect the phytopathogen present in the soil they are living, but also their antimicrobial activity is being exploited for their use as food preservatives in packed food and beverages.

19.2.4.2 Lipopeptides

Lipopeptides are non-ribosomal peptides synthesized by large multienzyme peptide synthetase complex (Bacon et al. 2015). Lipopeptides are powerful biosurfactant having anti-mycoplasm, antibacterial, and antiviral activity. These lipopeptides are stable over an extensive range of pH 3–11 and temperature from 15 to 100 ℃ (Makkar and Cameotra 1998; Cameotra and Makkar 1998; Joshi et al. 2008). Many of these properties are commercially explored to provide sustainable solutions for the environment and industrial applications. Cyclic lipopeptides extracted from *Bacillus subtilis* ABS-S14 have shown to activate defence mechanism at translation and transcriptional levels by inducing defensive proteins and genes (Tunsagool et al. 2019). Surfactins are a class of cyclic lipopeptides synthesized by surfactin synthetase in *B. subtilis* synthetase (Das et al. 2008; Nicolas 2003). It has a broad-spectrum antimicrobial, anti-coagulation, anti-tumour activity. Apart from that, Bacillus also possesses bioremediation property (Franzetti et al. 2010) as well as exhibits antimicrobial property against phytopathogens (Mulligan 2005). Surfactins owing to their bioremediation activity are routinely used to reclaim the degraded land and making them fit for cultivation by degrading the organic pollutants (Franzetti et al. 2010).

Bacillus spp. producing a combination of these lipopeptides form prominent biocontrolling agents (Bais et al. 2004; Kearns et al. 2004; Ongena and Jacques 2008; Hussain et al. 2019a; Hussain and Khan 2018a). Globally about 33 *Bacillus* spp. strains have been isolated, which produce surfactins with varying carbon length ranging from 11 to 17. Most of these strains produce surfactins with 15 carbon lengths, which are amongst the most active kind of surfactins (Bacon et al. 2012; Snook et al. 2009); *B. mojavensis* produces varieties of surfactins that have shown to be plant-friendly and symptomlessly infect the tissues except for the xylem (Bacon and Hinton 2002; Bacon and Hinton n.d.). *B. mojavensis* is osomophile tolerant and anaerobic in its character (Folmsbee et al. 2004). It has been studied for its protective role in maize from *Fusarium (*Bacon and Hinton 2002*;* Bacon and Hinton 2011*)* and for their capability to decrease the building up of Fumonisin mycotoxins (Bacon et al. 2001). The surfactins are inhibitory in feature to bacteria, fungi, virus and insects; however, the in vitro fermentation capacity of *B. mojavensis* of these bio-surfactins is rather poor and limits its use to technological exploitation (Bacon and Hinton 2014). *B. subtilis* produces three different lipopeptides (mycosubtilin, fengycin, and surfactin) which show potent inhibitory activity (Bacon and Hinton 2014; Hussain and Khan 2018b, 2020b; Hussain et al. 2019b). Ornamental plant cultivation shares a vital place in agro-industry. One of the significant phytopathogenic threats to ornamental plants has been the soil-borne fungus *F. oxysporum*. Mycosubtilin-like fungicide will protect these ornamental plants from the fungus and help in the agro-industry development and growths (Mihalache et al. 2018).

19.2.4.3 Polyketides

Polyketides are a member of a large family of metabolites which consist of bioactive compounds with immunosuppressive, antibacterial, and other bioactivities. The biosynthesis of these compounds is accomplished in a stepwise decarboxylative condensation (claisen condensation) of malonyl-CoA units. The PKS (polyketide synthase) multienzyme system utilizes acyl carrier proteins to channel the extending chain in the course of elongation processes (Cane 1997). The condensation process takes place between the growing polyketide chain and the extender unit with a simultaneous generation of β-ketoacyl as intermediates.

Bacterial type I PKSs is modularly arranged synthase, with each module containing an acyltransferase (AT), a β-ketoacyl synthase (KS), the ACP, and some other domains. The order of these modules decides the sequence of the polyketide biosynthesis events (Cheng et al. 2003). Genome analysis revealed the presence of several gene clusters responsible for coding PKS in *B. amyloliquefaciens* FZB42 (Koumoutsi et al. 2004) and in *B. mojavensis* strain RRC101 (Gold et al. 2014).

B. amyloliquefaciens FZB42 has three operons for the synthesis of polyketides. psk1 and psk2 have been ascribed with bacillaene and difficidin/oxydifficidin synthesis, whereas the psk2 has been associated with the synthesis of four members from the macrolactin family, namely, macrolactin A, macrolactin D 7-O-malonyl,

and 7-O succinyl macrolactin (Schneider et al. 2007). This macrolactin has also been related to an antimicrobial activity like other polyketides. Macrolactin A, 7-O-succinyl macrolactin A, 7-O malonyl macrolactin A, and two homologous bacillomycin D isolated from *B. amyloliquefaciens* NJN-6 showed antagonistic effects against *Ralstonia solanacearum* and *Fusarium oxysporum*, respectively (Yuan et al. 2012).

19.2.4.4 Zwittermicin A

Zwittermicin A, a linear aminopolyol, possesses antibiotic properties (Silo-Suh et al. 1998). Chemically, zwittermicin consists of D amino acids, gylcosyl moieties, ethanolamine, and an unusual terminal amide modifications (Sansinenea and Ortiz 2011). High polarity, sensitivity to alkaline condition, and charged state at physiological pH make its large-scale production difficult (Rogers and Molinski 2007). Biosynthesis of zwittermicin A follows mixed pathway involving both the NRPS and PKS systems.

B. cereus UW85 produces a novel antibiotic that showed activity against oomycetes, algal protist, bacteria, and plant fungi. When combined with kanosamine another antibiotic by *B. cereus*, they act synergistically against *Phytophthora medicaginis*, which is an oomycetes (Silo-Suh et al. 1998).

19.2.4.5 Terpenoids

Terpenoids are amongst the most diverse class of natural compounds. They occur in various chemical structures and have been reported to have antifungal, antiviral, anti-parasitic, antimicrobial, anti-allergic, immunomodulatory, and chemotherapeutic properties (Abdallah and Quax 2017). Isoprene(2-methyl-1-,3-butadiene), also known as isopentenyl diphosphate, is a precursor for all terpenoids. In archaea and eukaryotes, the precursor is formed via the mevalonate pathway (Kuzuyama 2002), whereas in plant plastids and some eubacteria, this is formed through the methylerythritol phosphate pathway (MEP) (Rohmer et al. 1993; Rohmer 1999). In *B. subtilis*, only homologous forms of the genes involved in MEP pathway have been reported (Wagner et al. 2000). *B. subtilis* for MEP pathways consists of five genes, namely, *yluB*, *yacM*, *yabH*, *yacN*, and *yqfP* to be essential for the isoprene production (Julsing et al. 2007). When a synthetic operon with up to eight regulated gene expression was introduced in *B. subtilis*, it resulted in a stable cell factory with high carotenoid production (Abdallah et al. 2020). Comparing the lipid profile of wild-type *B. subtilis* and SqhC-deficient strain also led to the detection of three novel tetracyclic terpenoids, the sporulenes from *B. subtilis (*Kontnik et al. 2008*)*. These sporulenes are produced by the cyclization of regular polyprenes by

B. subtilis cyclase. The presence of these tetracyclic isoprenoids in the lipids helps the bacteria against oxygen species (Bosak et al. 2008).

19.2.5 Insecticides and Pesticides

An inappropriate use of agrochemicals can lead to decrease in the microbial metabolic activities (Dhankar et al. 2021). Insecticidal activity of the *Bacillus* proteins is utilized to produce transgenic plant lines resistant to various Lepidopteron, thus eliminating the need for insecticide. An elite rice line KMD1 formed by introducing cry1Ab gene into Japonica Chinese rice variety shows insect-resistant attributes, with no damage being caused by Lepidopteran pests like *Chilo suppressalis*, *Scirpophaga incertulas*, and *Cnaphalocrocis medinalis (*Shu et al. 2000*)*. A similar introduction of a novel synthetic cry2A gene in Indica rice via *Agrobacterium*-mediated transformation has shown to develop transgenic rice lines resistant to Lepidopteran rice pest (Chen et al. 2005). Transgenic castor oilseed crop with cry1Aa gene has also been genetically developed to resist pests like *Achaea janata* and *Spodoptera litura (*Muddanuru et al. 2019*)*. Apart from the Cry protein, another protein from *B. thuringiensis* has also proved to exhibit pest-controlling attributes. This protein Vip3A (vegetative insecticidal protein 3A) can be isolated from the culture medium and has a wide spectrum of targets. Vip3A protein has shown to have a toxic effect on larva growth, leading to operative mortality (Chakrabarty et al. 2020).

19.2.6 Nematocides

Plant-parasitic nematodes lead to massive loss in crop production sector every year worldwide. Proteins produced by various *Bacillus* sp. have gained a role as nematocide. A 28 kDa serine protease enzyme isolated from *Bacillus* sp. strain B16 has shown to hydrolyse substrates like collagen and nematode cuticle. The protease reportedly kills 80% of nematodes, *Panagrellus redivivus* within 24 hours (Qiuhong et al. 2006). Another instance of biocontrol of nematodes was reported by the dipeptide cyclo(D-Pro-I-Leu) from *B. amyloliquefaciens* Y1, *B. subtilis HussainT-AMU (*Hussain et al. 2020*)*, and *B. firmus HussainT:Lab.66 (*Hussain and Khan 2020c*)*. This has shown to reduce gall per root of the tomato plant as well as a decrease in egg counts and stage 2 juvenile population of nematode, *Meloidogyne incognita*. The increased root, shoot length and fresh and dry weight have also been observed after the introduction of strain Y1 (Jamal et al. 2017). Few more examples of the functions are given in Table 19.1.

Table 19.1 Functions of various metabolites of *Bacillus* spp.

Metabolites	Compounds	Organisms	Functions	References
1. Enzymes	AHL lactonase	*B. thuringiensis*	Antipathogenic against *E. carotovora*	Dong et al. (2002)
2. Biopolymer	Poly γ-glutamic acid	*B. amyloliquefaciens*LL3	Hydrogels, flocculants, thickeners, feed additives, cosmetics, drug deliveries	Cao et al. (2011)
3. Protein crystal	Cry protoxin or δ endotoxin	*B. thuringiensis*	This microbial protein has been exploited to produced bioinsecticides and transgenic plants	Roh et al. (2007)
	Bac14B (bacteriocin)	*B. subtilis*14B	Antimicrobial effect against *agrobacterium tumefaciens*	Hammami et al. (2009)
	Tochicin (bacteriocin)	*B. thuringiensis* subsp. *toichigiensis*HD868	A narrow spectrum of bactericidal activity against *B. Thuringiensis* and *B. subtilis*	Paik et al. (1997)
4. Antimicrobial agents	Mycosubtilin (lipopetides)	*B. subtilis*	Fungicidal	Mihalache et al. (2018)
	Difficidins and bacilysin (polyketides)	*B. amyloliquefaciens*	Biocontrol against fire blight of orchard trees by *Erwinia amylovora*	Chen et al. (2009)
	Zwittermicin A	*B. cereus*	Suppress fungal diseases	Silo-Suh et al. (1998)
	Terpenoids	*B. amyloliquefaciens*S5Ia	Antibacterial and antifungal	Shafi et al. (2017b)
5. Insecticides	Cry9Cb1 (MP1489)	*B. thuringiensis strain* SP663	Insecticidal activity towards *Plutella xylostella*, *Chilo suppressalis*, and *Ostrinia furnacalis*	Shan et al. (2019)
6. Nematocides	Endotoxins (AI, AII, AIII, AIV)	*B. thuringiensis*	Inhibits *M. incognita*	Abd El-Moneim (2014)

19.3 Stress Tolerance

19.3.1 Water Stress

Drought refers to the non-availability of soil moisture over a prolonged period. Drought is one of the common stresses that plants suffer across the globe and serves as the deciding factors in the production and growth of agro-industry. Most of the

PGPRs have been reported to initiate drought tolerance and help the crops in arid and semi-arid regions to cover this problem. *B. licheniformis* K11 upon inoculation in the pepper plant led to the up-regulation of four differentially up-regulated proteins. These proteins were, namely, CaPR protein 10, sHSP, VA, and Cadhn (Lim and Kim 2013). CaPR protein 10 is related to plant defence against abiotic and biotic stress (Lee et al. 2008). sHSP (heat shock protein) generally works as molecular chaperon and plays a vital role during stress by preventing irreversible aggregation of denatured proteins (Sarkar et al. 2009). VA (V ATPase) energizes sodium sequestration in the central vacuoles (Golldack and Dietz 2001), and Cadhn, which functions through stabilization of large-scale hydrophobic interactions (Borovskii et al. 2002). Thus, the over-expression of all these four proteins may perhaps result in the induction of drought stress tolerance in pepper (Lim and Kim 2013).

Another example of an endophytic relationship with *Bacillus* mitigating drought stress was seen with *Phleum pratense* L (Timothy). The plant with *B. subtilis* strain B26 inoculation under stressed condition showed an increase in photosynthesis and an increase in shoot and root biomass by 26.6 and 63.6%, respectively. It also leads to an increase in stomatal conductance by 214.9% compared to non-inoculated plants. A higher amount of sugars like fructans, sucrose, and amino acids like glutamic acids, glutamine, and asparagine were also recorded in the shoot and roots of the colonized plants (Gagné-Bourque et al. 2016). Inoculation of PGPR also results in an increase in the production of secondary metabolites and antioxidants in plants under drought stress. *Bacillus pumilus*, when inoculated with Glycyrrhiza, had shown to increase its biomass by 34.9% along with the increase in oxygen species, antioxidants, and few important enzymes expression like that of SQS, HMGR, and beta-AS which plays a vital role in the synthesis of glycyrrhizic acid. Its association has also marked a decrease in lipid peroxidation level in the plant (Xie et al. 2019).

19.3.2 Salt Stress

Another type of stress which plants face is salt stress. Microbial volatile compounds (mVOCs) released from *B. amyloliquefaciens* GBo3 upon administration to *Mentha piperita* without any physical contact with the bacteria showed a generation of tolerance towards salt stress. VOCs-exposed plants showed a better morphological and chlorophyll content compared to the control. It also showed an increase in salicylic acid content and a decrease in abscisic acid, which aid in stress tolerance (del Rosario Cappellari and Banchio 2019). In a similar example, halotolerant bacteria *Bacillus subtilis* HG-15 has also shown to effectively improve the growth and systematic tolerance level of heat when associated with it (Ji et al. 2020). PGPR strains *B. siamensis* (PM13), *B. methylotrophicus* (PM19), and *Bacillus* sp. (PM15) have also exhibited induced NaCl tolerance. Wheat seeds showed an increase in germination and seedling growth under salt stress when grown along with bacteria. The emission of exopolysaccharides and ACC deaminase by these strains played an important role in providing tolerance towards salt (Amna et al. 2019).

19.3.3 Heavy Metal Stress

Contamination of soil with heavy metals poses great adverse stress scenario towards the growth of the crops in soil. Phytoremediation of heavy metal contaminated soil is an important issue for better crop production (Kumar et al. 2021; Haris et al. 2021; Hussain et al. 2021). Arsenic remediation is seen in *Valliseriadense serrulata–Bacillus s*pp XZM union. There was an increased uptake of the arsenic when the plant was colonized with the bacteria. This arsenic is then detoxified and metabolized by the *V. denseserrulata* leaves (Irshad et al. 2020). Similarly, *Bacillus thuringiensis* and biochar both individually and when taken together showed their ability to immobilize Cd and Pb in culture solution. These treatments increase the dry weight of radish leaves and roots and also resulted in a decrease in the content of Pb and Cd in the roots. Simultaneously, this also leads to an increase in ammonium concentration, pH, organic content, and the $NH^{4+}/NO^{3-}$the ratio of the rhizosphere soil (Li et al. 2019). A mutant of *Bacillus megaterium* strain BM18, BM18–2, has shown to enhance the phytoremediation potential of the hybrid *Pennisetum* by improving its growth, and Cd absorption property, when grown on Cd-stressed soil (Wu et al. 2019). A wastewater bacterium *Bacillus s*pp. KUJM2 has been reported to be efficient in removing potential toxic elements like As, Cd, Cu, and Ni under both mono- and co-contaminated conditions (Mondal et al. 2019). All these can be very useful in turning the land around the mining area to bearable again.

19.3.4 Temperature Stress

Temperature is another significant abiotic factor in plant growth and development. As the global temperature is increasing and negatively affecting the crop yield and quality, some sustainable solution needs to be worked out. Soybean, when grown with *B. tequilensis* SSB07, has shown promising effects countering to heat stress. It results in plant growth and increases in jasmonic acid and salicylic acid, whereas a considerable decrease in ABA as a stress response mechanism was noted (Kang et al. 2019a). Under heat stress, soybean plants when inoculated with *B. aryabhattai* SRB02 showed tolerance by containing steady levels of ABA and exhibiting ABA-induced stomatal closure (Park et al. 2017).

19.3.5 Radiation Stress

Bacillus spp. have shown to provide tolerance to plants against ozone toxicity. Ozone being a phytotoxic compound is capable of reducing crop productivity. Addition of *Bacillus* sp. N7 with *Oryza sativa* L. var. *indica* cv. *Pathum Thani1* (PTT1) and *Oryza sativa* L. var. *indica* cv. RD41(RD41) resulted in increased O_3

tolerance along with high rice quality. An evident decrease in malondialdehyde (MDA) due to increased levels of ascorbic acid was noticed. This colonization also resulted in high photosynthesis, stomatal conductance, and chlorophyll content in plants (Anusaraporn et al. 2020).

Another way by which the PGPR enhances ozone tolerance in plants was seen through the production of different kinds of volatile organic components, which in return triggers the induced systemic tolerance of plants. Such an example is seen when the Tobacco plant is colonized with PGPR *Bacillus velezensis* Gj11, which produces volatile organic compound consisting of acetoin and 2,3-butanediol. These compounds trigger the IST (induced systemic tolerance) and lead to stomatal closure and prevent plant against O_3 injury (Liu et al. 2020).

19.4 Plant Growth Promotion

19.4.1 Plant Growth Promotion Hormone

Volatile organic compounds (VOCs), apart from playing a significant role in stress tolerance and defence, also promote plant growth. These volatile compounds work similarly to plant growth hormones and enhance plant growth; some other stimulates the secretion of the phytohormone itself. VOCs composed of acetoin from *B. methylotrophicus* M4–96, when applied to strawberry plant without physical contact with bacteria, resulting in improved growth and development, and also it increases callus deposition by fivefold in leaves and increased petiole length and root number. Increase in root length and petiole number in strawberry plants were seen after subjecting them to *B. methylotrophicus* M4–96 inoculum (Vicente-Hernández et al. 2019). VOCs emitted by *Bacillus* sp. JC03 included tetrahydrofuran-3ol, 2-heptanone, and 2–ethyl-1-hexanol promoted the growth of *Arabidopsis* seedlings via the action of strigolactone and auxin. This also increases biomass accumulation in *Arabidopsis (*Jiang et al. 2019*)*.

Many PGPRs stimulate the growth and development of plants by modulating the necessary phytohormones. Root architecture variability was also observed in rice plants upon association with *B. altitudinis* (strain FD48). This change of architecture in rice and FD48 partnership was attributed to increase in IAA accumulation in the root of the plant compared to non-inoculated control (Ambreetha et al. 2018). Similarly, *B. aryabhattai* SRB02, which produces IAA along with cytokinin, jasmonic acid, ABA, and different gibberellic acids (GA1, GA7, GA4) in culture, showed longer root and shoot for soybean plant (Park et al. 2017). Another bacterium, *B. tequilensis* (SSB07), which produced IAA with several gibberellins and abscisic acid also showed improvement in Chinese cabbage seedling and soybean cultivation (Kang et al. 2019a). PGPR like *Bacillus subtilis* JW1 was seen to produce high concentration of gibberellins, GA1, GA4, and GA7, along with other organic acids, fatty acids, and tricalcium phosphates. These chemicals showed to

result in a substantial rise in plant height, chlorophyll contents, biomass, and nutrient uptake when Chinese cabbage was inoculated with it (Kang et al. 2019b).

Another critical aspect of plant growth is nutrient uptake from its surrounding. PGPR promotes growth by enhancing the accumulation of essential nutrients in plants. Endophytic *Bacillus* strains B1923, B2084, and B2088 when inoculated with pearl millet resulted in better growth and enhanced nutrient uptake. B1923 strain produced IAA and solubilized Fe-P, which also resulted in the increase of root and shoot dry weight and N, P content of root. At the same time, the B2084 and B2088 strain showed enhanced biomass production and N, K, and P accumulations in pearl millet shoot (Ribeiro et al. 2018).

Similarly, *B. subtilis* QST713 has shown to improve the P uptake in wheat grain. It has also reported improving Zn concentration in edible parts of the plants like grains by 24% relative to control, thus stimulating Zn biofortification effect (Moreno-Lora et al. 2019). *Bacillus subtilis* strain QST713 also promotes root growth in cucumber by enhancing the overall uptake of P by 40% (García-López and Delgado 2016). *Bacillus subtilis* STU6 has also shown to ameliorate iron deficiency in tomato plant (Zhou et al. 2019).

19.4.2 Siderophore

Iron is an essential micronutrient required by the plant for its growth, and iron deficiency leads to a significant loss in crop production, especially in the region of calcareous soils (Kobayashi et al. n.d.). Siderophores are low molecular iron chelators produced by microbes that bind with iron with high affinity and shuttle them into the cells (Zawadzka et al. 2009). Several types of siderophores have been found in various *Bacillus* spp. like schizokinen from *B. licheniformis*, *B. megaterium*, *B. cereus*, and *B. subtilis*, petrobactin and bacillibactin from *B. cereus (*Park et al. 2005*)*, *B. thuringiensis (*Wilson et al. 2006*)*, and *B. anthracis (*Cendrowski et al. 2004*)*. These siderophores also help in biocontrol of phytopathogens like in the case of *B. thuringiensis* against fungi (Raddadi et al. 2007).

19.5 Conclusion

Bacillus, a group of spore-forming bacteria, is known to be found in the varied habitats owing to its ability to produce a diverse array of metabolites upon interaction with different environmental condition. These bacteria synthesize various primary and secondary metabolites that help in their survival in the given environmental condition. *Bacillus* spp. are of considerable importance owing to its ability to produce new and essential metabolites. Metabolites secreted by them are up of considerable importance in agro-industry, pharmaceuticals, beverage, and other industries. Many of the metabolites secreted by *Bacilli* are significant to plant as they act as

antimicrobial agents against the phytopathogens and many times as the nutrient quenchers. All these functions make these bacteria referred to as the "good bacteria" for the plant/soil, as they enrich the soil by various primary and secondary metabolites upon their presence there.

Metabolites such as bacteriocin and lipopeptides secreted by *Bacillus* are known for their antimicrobial activity against pathogenic bacteria, virus, and fungi and thereby acting as the phytoprotectant against these pathogens. Further, similar antimicrobial activity is also shown by numerous polyketides secreted by these bacteria to protect themselves and help the symbiont. Zwittermicin A, an aminopolyol known to be synthesized by the mixed pathway (NRPS/PKS), also shows antimicrobial activity against a broad range of microbes. Apart from providing protection against microbe, some other groups of secondary metabolites work by increasing the plant productivity. These include the molecules such as siderophores, which quench out micronutrient from the soil and provide the associated symbiont plant with a much-needed advantage over the others and enhances the plant productivity. Some *Bacillus* secret volatile compounds are similar to the IAA, gibberellin, and jasmonic acid that enhance the plant growth. Apart from having the antimicrobial and growth enhancement activity, they also help in combating various abiotic stresses. Heat shock protein V, ATPase secreted by these *Bacilli*, helps in water stress tolerance. Further, many other *Bacillus* provide stress tolerance to temperature and salinity by hormonally regulating the stomatal opening and closing. In addition to growth enhancement, microbial protection, and stress tolerance to various abiotic stresses, they are also essential agents of bioremediation. These microbes help in bioremediation by removing heavy metal pollutants from the soil and making soil suitable for other crops.

Several secondary metabolites produced by *Bacillus* have been commercialized, and many more are in the pipeline. These include various proteases that are used in the detergent industry, several antimicrobial used in the dairy industry. These products being natural are gaining popularity over synthetic fertilizer and pesticides for increasing the soil productivity and soil health, as they also eliminate the entry of toxic chemicals in the human food chain. Many of these bacteria are used as inoculum along with seeds which provides protection and increases productivity. Several protease-producing species of *Bacillus* are used in biofuel production from the lignocellulosic waste. In the current decade, when there is gaining popularity for organic food and greener and cleaner development, these microbes provide cost-effective, affordable, and easy benefits to fight with various biotic and abiotic stresses. Further, many of these secondary metabolites are being explored for their industrial-scale production through various fermentation processes. These processes suffer at the scale of per unit yield, downstream processing, and cost issues. However, they also provide a ray of hope to commercialize many of these bacteria and their metabolites to counter the agricultural distress developing due to varied reasons and add to the agricultural productivity.

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Chapter 20
Mechanisms of the Beneficial Effects of Probiotic *Bacillus* spp. in Aquaculture

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Abstract The usage of probiotic bacteria in aquaculture is profoundly ranging from growth promotion of cultured fishes to improvement of the rearing environment. Among the different groups of probiotic bacteria, the *Bacillus* spp. stand out due to their capability to withstand the harsh environment in nature and in fish gut. Although the probiotic *Bacillus* spp. in sustainable aquaculture is widely applicable, the mechanisms of their beneficial effects are not comprehensively reviewed. This chapter aimed to update our knowledge of the mode of actions of *Bacillus* spp. as probiotic in the promotion of aquaculture. The probiotic *Bacillus* spp. secrete diverse enzymes that help fishes in the digestion of complex feedstuffs thus decrease the feed conversion ratio and increase the specific growth rate of the host fishes. They can colonize the fish gut and produce diverse antimicrobial substances like bacteriocins (antibiotics) and lytic enzymes and promote immunity in fishes to protect from pathogens. They also compete for nutrient, energy, and binding sites and thus exclude pathogens from the fish guts. The probiotic *Bacillus* spp. also boost the host's genes expression associated with the growth and development of the fishes. Conversion of toxic nitrogenous wastes by *Bacillus* spp. detoxifies the rearing water

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and environment. This chapter is compendium of existing knowledge on the mechanism of beneficial actions of *Bacillus* spp. in aquaculture.

Keywords Green aquaculture · Biocontrol of fish diseases · Quorum quenching · Gene expression · ISR · Fish probiotics

20.1 Introduction

The attention of researchers to the field of beneficial bacteria or microorganisms has arisen many folds since the first hypothesis of beneficial microorganisms was given by Elie Metchnikoff in 1907. The term "probiotic" has gained popularity over the years, and its application has expanded in many areas of life today. "Probiotics are living microorganisms, which when deliver in adequate amount provides benefits to the health of the host" (FAO/WHO 2002). There are various formulations of probiotic microorganisms that are being established and standardized for both human and animal intake. To prevent gastrointestinal infections, probiotics are used extensively in the aquaculture and poultry feed industries (Hong et al. 2005). They are also applied as organic fertilizers and biopesticides in crop production (Yadav et al. 2017). Due to consumer awareness, the exploration of safe and cost-effective treatment/feed and the pathogens antibiotic resistance capability have forced researchers to find an alternative to current therapeutic practice, which mainly rely on antibiotics. Moreover, it is expensive to use antibiotics in large water volumes (Harikrishnan et al. 2011). Probiotic therapy appears to be the most effective one, with a long history of guaranteed safety, among loads of recommended options.

Bacillus is the globally recognized genus of bacteria that are being used for their potential benefits to plants (Rahman et al. 2018; Surovy et al. 2019, Dutta et al. 2018), animals (Xu et al. 2017; Sun et al. 2013), and even in humans (Piewngam et al. 2018), and the significance of *Bacillus* sp. for sustainable aquaculture is systematically reviewed (Rahman et al. 2019). Some *Bacillus* strains have been selected for their possible probiotic application, in several in vitro and in vivo representations. Beside maintaining probiotic standard, *Bacillus* spp. can tolerate higher acidity and stability in heat processing and low storage temperature (Bader et al. 2012). They can exclude pathogens and possess antioxidant, antimicrobial, and immunomodulatory activities (Lefevre et al. 2015; Shobharani et al. 2015; Ripert et al. 2016). Species in the genus *Bacillus* are capable of endospore production. They are Gram-positive bacteria with a rod-shaped structure and can be either aerobic or facultative anaerobes, sized between 2.5 and 10 mm. *Bacillus* spp. belong to the large phylum Firmicutes and classified as *Bacilli* in the order *Bacillales*. About 200 species are included in the genus, which are almost omnipresent in the environment. *Bacillus* spp. can be found in compost (*B. composti* and *B. thermophiles*) (Yang et al. 2013), in high temperature (*B. thermophiles*) (Yang et al. 2013), in high pH

conditions (*B. firmus* OF4) (Sturr et al. 1994), and high salt (*B. halodurans*) (Annamalai et al. 2013), in all types of the waterbody (Ichimatsu et al. 2000) and the gut of aquatic animals (Ray et al. 2012). Yi et al. (2018) reported that endospore formation capability enables *Bacillus* spp. to withstand extreme stresses. The capacity to produce tannase, chitinase, protease, phytase, lipase, cellulase, and xylanase are some of their varied biological characteristics (Ray et al. 2012, Ghosh et al. 2019). Bacteriocins and antimicrobial substances are among the other important features of *Bacillus* spp. (Abriouel et al. 2011). Therefore, they earn the ability to take possession of different habitats and patronize host nutrition, and they also serve as environmental and feed probiotics (Moriarty 1998; Hong et al. 2005) in fish farming. It is proven that the host health heavily relies on the optimal and effective functionality of the gastrointestinal (GI) tract and its gut microbiota (Clemente et al. 2012; Xiong et al. 2017), and numerous intricate mechanisms are involved. General immunity and function are weakened, reduced protection against infections, and decreasing gut health is reported in the absence of intestinal microbiota (Ringø et al. 2018; Rawls et al. 2004, 2006; Gómez and Balcázar 2008; Ray et al. 2012; Wang et al. 2018a, b; Li et al. 2019).

Bacteria, virus, and parasites are the attributes of aquaculture diseases (Carbone and Faggio 2016; Bastos Gomes et al. 2017) that linked with the genus *Streptococcus*, *Aeromonas*, *Vibrio*, *Yersinia*, *Acinetobacter*, *Lactococcus*, and *Pseudomonas* (Santos et al. 2018). *Bacillus* spp. as probiotics have already verified experimentally to fight against the diseases causing pathogens (Balcázar et al. 2006; Kavitha et al. 2018; Ramesh and Souissi 2018; Yi et al. 2018) and improve feed utilization, which increases growth (Aly et al. 2008; Goda et al. 2018), to boost the immunity of farmed fish species (Yu et al. 2019; Buruiană et al. 2014) and ameliorate the culture water quality (Camargo and Alonso 2006), as well as to reduce environmental stresses (Eissa et al. 2018).

Although the significance of *Bacillus* spp. and their commercial application in aquaculture as fish probiotics have been reported (Rahman et al. 2019; Acosta et al. 2016; Adorian et al. 2019), the underlying molecular mechanisms of their beneficial effects on fishes are poorly understood (Kuebutornye et al. 2020). The probiotic *Bacillus* spp. exert beneficial effects to the host fishes through various mechanisms that include the antibiotics and lytic enzymes production, promotion digestion of feeds, trigger immunity against disease pathogens, and gene expressions associated with growth and tolerance to the abiotic stresses (Lin et al. 2019; Liu et al. 2012). Some of the probiotic *Bacilli* also improve the water quality in the cultured environment (Zokaeifar et al. 2014). However, no comprehensive review has so far been published on the mechanisms of the beneficial effects of probiotic *Bacilli* in aquaculture. This chapter summarizes the mechanisms of probiotic *Bacillus* spp. to improve the productivity and sustainability in aquaculture.

20.2 *Bacillus* in Aquaculture

In previous decade, the evaluation of different types of probiotic bacteria has tremendously been increased particularly in aquaculture (Hoseinifar et al. 2018). The beneficial effects of probiotic *Bacillus* spp. on growth performance and disease resistance are well documented for many fish species like carps and salmonids (Zhang et al. 2014; Dawood and Koshio 2016; Hoseinifar et al. 2016; Dawood et al. 2018; Fečkaninová et al. 2017). Not only in fish but also in white shrimp, *Litopenaeus vannamei* farming, the probiotic *Bacillus* spp. have been proved promising (Zokaeifar et al. 2012a, b) with a different mode of actions that include the secretion of antimicrobial peptides and extracellular substances. These findings encourage Pacific white shrimp (*L. vannamei*), tiger shrimp (*Penaeus monodon*), and giant freshwater prawn (*Macrobrachium rosenbergii*) health and immunity to combat pathogens thus increases the chance of survival as well as promote growth by improved digestion (Shen et al. 2010; Chai et al. 2016; Laranja et al. 2017). The use of prebiotics as a growth promoter in aquaculture (Gatlin III et al. 2006; Li and Gatlin 2004; Ng and Koh 2016) stimulates the production of exogenous enzymes from probiotic *Bacillus* spp. that improves the intestinal physiology (Gatlin and Peredo 2012; Zhou et al. 2010a, b) with the dietary administration in different fish species such as sturgeon (*Acipenser transmontanus*) (Hoseinifar et al. 2017), tilapia (*Oreochromis niloticus*), and totoaba (*Totoaba macdonaldi*) (Van Hai 2015; González-Félix et al. 2018). Application of probiotic bacteria is vital to increase the feed efficiency as feed constitutes around half of the production cost of rainbow trout (*Oncorhynchus mykiss*) in aquaculture (Amiri et al. 2018). *B. subtilis* significantly improved growth parameter and feed efficiency at the rate of 3.0×10^9 CFU/kg in grass carp (Wu et al. 2012), and in Nile tilapia with *B. subtilis* at either 5×10^6 CFU/g (Telli et al. 2014), 1×10^7CFU/g for 2 months (Aly et al. 2008), or 1×10^8 CFU/g for 2 months (Liu et al. 2017), and in olive flounder (*B. subtilis* at 1×10^{10} CFU/g). A higher dosage of *B. subtilis* ($0.42–1.35 \times 10^7$ CFU/g) administered with feed juvenile large yellow croaker (*Larimichthys crocea*) (Ai et al. 2011). In contrast to this, *B. licheniformis* Dahb1 was more productive at a lower dosage (1×10^5 CFU/mL) in the growth of catfish (*Pangasius hypophthalmus*) than a higher dosage (1×10^7 CFU/mL) (Gobi et al. 2016). Besides oral administration, *Bacillus* is also effective in bath application as reported by Zhou et al. (2010a, b) with *B. coagulans* (1×10^7 CFU/mL) and *B. subtilis* (1×10^7 CFU/mL) in Nile tilapia which had significantly greater final weight and SGR compared to control.

The intestinal microbiota of fish can colonize the host's epithelial surface, allied with the microvilli, or in the lumen (Ringø 1999). Gastrointestinal (GI) tract is among the key infection routes for some pathogens in fish (Groff and LaPatra 2000; Birkbeck and Ringø 2005; Ringø et al. 2007, 2010; Bøgwald and Dalmo 2014); therefore, evaluation of the intestinal microbiota is of immense importance. Figure 20.1 represents the different fish species from where the *Bacillus* is reported with especial emphasis on the Indian major carps where the presence of *Bacillus* reported most.

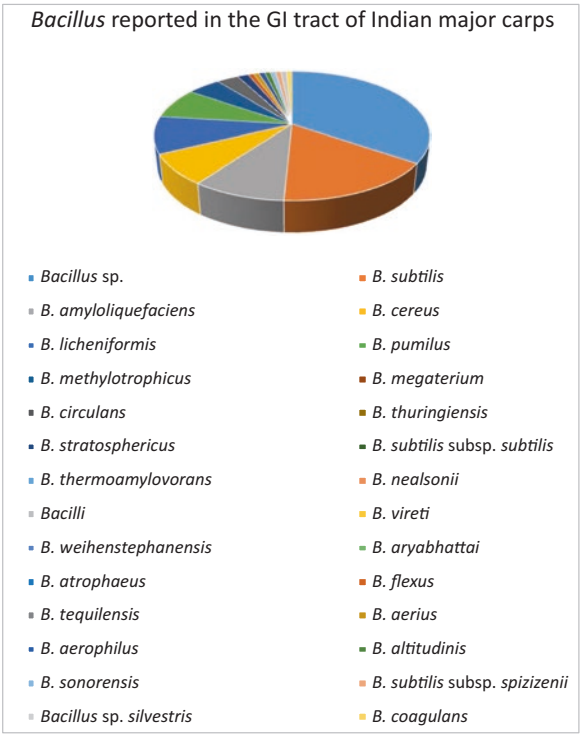

Fig. 20.1 *Bacillus* reported in different fish species and in the GI tract of Indian major carp
Sources: Trust (1975); Ramirez-Torrez et al. (2018); Kim et al. (2007); Trust et al. (1979); Sugita et al. (1985); Ramachandran et al. (2005); Sugita et al. (1988b); Yang et al. (2017); Nedoluha and Westhoff (1995); Nedoluha and Westhoff (1997); Sugita et al. (1987); Sugita et al. (1998); Al-Harbi and Uddin (2005); Ramirez and Romero (2017); Rasheeda et al. (2017); Bindiya et al. (2015); Sanchez et al. (2012); Sirtori et al. (2006); Kavitha et al. (2018); Ran et al. (2012); Giri et al. (2011); He et al. (2013); Nyman et al. (2017); Bairagi et al. (2002); Miao et al. (2018); An et al. (2015); Chen et al. (2016a); Chen et al. (2016b); Ghosh et al. (2002); Esakkiraj et al. (2009); Austin and Al-Zahrani (1988); Ray et al. (2007); Saha et al. (2006); Yilmaz et al. (2018); Ma et al. (2010); Kumar et al. (2018); Banerjee et al. (2017); Zhu et al. (2016); Jasmin et al. (2016); Gao et al. (2019); Tzeng et al. (2015); Vargas-Albores et al. (2017); Hindu et al. (2018a); Al-Hisnawi et al. (2015); Ray et al. (2010); Nandi et al. (2017b); Dey et al. (2016); Khan and Ghosh (2012); Mukherjee et al. (2016); Ghosh et al. (2010); Mondal et al. (2010); Ghosh et al. (2017); Banerjee et al. (2016); Mukherjee and Ghosh (2016); Khan and Ghosh (2013); Ringø et al. (2006); Zhou et al. (2013); Dutta et al. (2015); Dutta and Ghosh (2015); Kim et al. (2013); Ringø et al. (2016); Askarian et al. (2012); Banerjee et al. (2013); Mukherjee et al. (2017); Talukdar et al. (2016); Green et al. (2013); Mente et al. (2018); Sugita et al. (1988a); Sugita et al. (1989); Das et al. (2014); Sarkar and Ghosh (2014); Das and Ghosh (2013); Das and Ghosh (2015); Hovda et al. (2007); Hovda et al. (2012); Wong et al. (2013); Peixoto et al. (2011); Ramachandran and Ray (2007); Yang et al. (2018); Strøm and Olafsen (1990); Ricaud et al. (2018); Ringø et al. (2014); Li et al. (2015a,b); Guo et al. (2016); Salam et al. (2021).

20.3 Competition for Nutrients and Energy

Competition between probiotic bacteria and fish pathogenic microorganisms for nutrients and energy leads to the competitive exclusion of the loser (Fig. 20.2). As pathogens and probiotic bacteria hunt energy and nutrients from the same resources, this competition has a great influence on the domination of probiotic over pathogenic microbes (Verschuere et al. 2000a; Hassanein and Soliman 2010). Heterotrophs largely rely on carbon and other energy sources especially in the aquatic environment for their survival (Mohapatra et al. 2013). *Bacillus* species make starve the pathogens by higher organic carbon consumption and uptake of iron by synthesizing siderophores (Verschuere et al. 2000b; Winkelmann 2002; Kesarcodi-Watson et al. 2008; Lalloo et al. 2010) that result in reduced growth of the pathogens since most microbes depend heavily on iron and carbon for their growth (Braun and Killmann 1999). Lalloo et al. (2010) demonstrated that because of the siderophore production, *B. cereus* had significantly better growth with inadequate iron or glucose than pathogenic *A. hydrophila*. Similarly, winning over pathogens in the competition of carbon sources for energy makes *Bacillus* species as a strong probiotic as shown by many scientists in in vitro studies (Lee et al. 2017; Meidong et al. 2017; Kavitha et al. 2018).

20.4 Exo-enzymes Produced by *Bacillus*

Autochthonous gut-adherent bacteria reduce the chances of negative effects by the microorganisms or their metabolites as the microorganisms obtained from tropical and subtropical finfishes can safeguard their colonization and enzyme supplementation within the intestine which is ideal for aquaculture (Ghosh et al. 2019). *Bacillus*

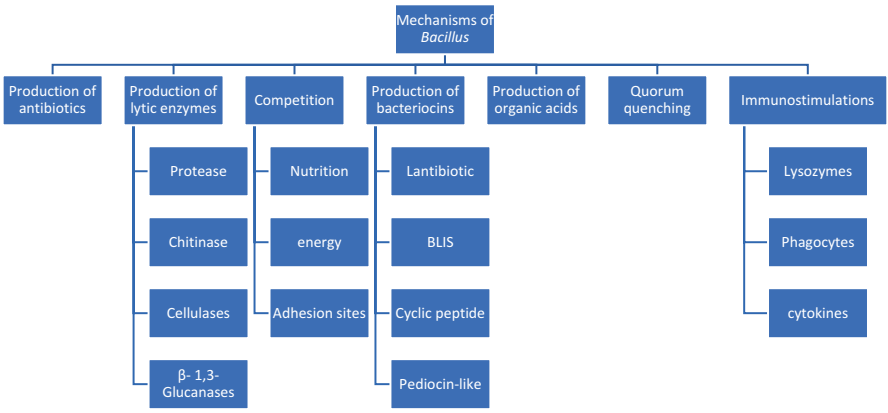

Fig. 20.2 Mechanisms of beneficial actions of probiotic Bacilli in aquaculture species
BLIS bacteriocin-like inhibitory substances

spp. produce a variety of beneficial enzymes, antimicrobial compounds (Prieto et al. 2012), and proteinaceous substances (Zokaeifar et al. 2012a) that include enzymes and bacteriocins or bacteriocin-like inhibitory substances (BLIS) with diverse biological functions. Numerous studies on teleosts like Atlantic salmon (*Salmo salar*), Arctic charr (*Salvelinus alpinus* L.), and Indian major carps revealed that autochthonous *Bacillus* spp. can produce exogenous enzymes such as carbohydrases, phosphatases, esterases, lipases, and peptidases which contribute to the better digestion process and nutrition in fish (Cahil 1990; Ringø et al. 1995, Ringø and Song 2016). Ringø et al. (1995) reported that either by enzymatic breakdown of triglyceride or by shifting pancreatic lipase action with bacterial proteases, gut bacteria might prompt lipolysis. Lipase-producing *Bacillus* spp. were identified in the guts of Atlantic salmon (Askarian et al. 2012), Indian major carps (Dutta and Ghosh 2015), wishers catfish (Das et al. 2014), catfishes (Dey et al. 2016), and Nile tilapia (Ghosh et al. 2017). Apart from these digestive enzymes, the non-starch polysaccharide (NSP) – degrading enzymes, phytase, tannase, and chitinase produced by gut bacteria also attributes significantly to fish nutrition and well-being where autochthonous gut *Bacilli* play a major part (Ray et al. 2012). Among the NSPs, cellulose and hemicelluloses (e.g., xylans) are abundant in natural food like algae, phytoplankton, detritus, aquatic macrophytes, and in different oil cakes which are mostly indigestible for fishes (monogastric animals) because beta-glucanases and beta-xylanases (essential to digest NSPs) are either rare or not present in fish (Kuzmina 1996). However, the degradation of cellulose and hemicelluloses may be accomplished by the fermentative symbiotic gut microorganisms for the host fish (Clements 1997). Recently, studies on the omnivorous fish such as rohu, catla, and mrigal (Ray et al. 2010); grass carp and tilapia (Saha et al. 2006); bata (Mondal et al. 2010); walking catfish (Dey et al. 2016); and striped dwarf catfish (Nandi et al. 2017a) revealed a huge number of cellulose-degrading *Bacilli*. Tannins, which are prevalent in plant feedstuffs, can be degraded by *Bacillus* spp. (Deschamps et al. 1980), and tannase in the alimentary canal of ruminants is well known (Goel et al. 2005). But reports on tannase-producing bacteria from the fish gut are rare. Talukdar et al. (2016) documented that *B. subtilis* KP765736 and *Brevibacillus agri* KP765734 isolated from Nile tilapia have tannase activity. Like tannins, the chitin is abundant in voluminous fish food organisms, viz., crustaceans, mollusks, protozoans, fungi, and green algae (Ray et al. 2012). *B. subtilis*, *B. thuringiensis*, *B. cereus*, and *Bacillus* sp. obtained from the gut of Atlantic salmon were reported to possess chitinase enzyme (Askarian et al. 2012), although the ability of *Bacilli* to degrade chitin was first recorded in *B. chitinovorus* in the early twentieth century from an aquatic source.

Plant feedstuffs for aquaculture feed can be processed by solid-state fermentation (SSF) using exo-enzymes produced by *Bacilli*. The *Bacillus* spp. minimize the level of anti-nutritional factors present in the plant ingredients (Bairagi et al. 2004; Ramachandran et al. 2005; Ramachandran and Ray 2007; Khan and Ghosh 2013) which is responsible for the bio-unavailability of different nutrients in feed. Banerjee and Ghosh (2016) predicted that during the bioprocessing of plant feedstuffs through SSF, essential biomolecules such as amino acids, fatty acids, and vitamins might increase in nutrient levels through microbial synthesis. This improves the

bioavailability and digestibility of nutrients which can be ensured from plant feedstuffs. To avoid the detrimental effects of harmful metabolites, microbial symbionts isolated from the fish gut were used for the application of SSF-processed substrate as fish feed ingredients (Khan and Ghosh 2013). *Bacilli* can supplement cellulase (Bairagi et al. 2002) and phytase (Roy et al. 2014) in the processing of plant feedstuffs or in a complete diet. They are not only found potential in reducing phytic acid, cellulose, hemicelluloses, crude fiber, tannins, and trypsin inhibitor but also in increasing the levels of free amino acids (especially arginine, cysteine, valine, histidine, isoleucine, tryptophan, methionine, phenylalanine and threonine), fatty acids, and different minerals (Das and Ghosh 2015). Ray et al. (2007) reported enhanced cellulase-producing ability under SSF of *B. circulans* TP3 and *B. subtilis* CY5 from the gut of Mozambique tilapia and common carp, respectively, while maximum residual cellulase activity of *B. subtilis* P6 and *Bacillus velesensis* P11 was found at pH 7.0–9.0 (Peixoto et al. 2011). Phytase supplementation hydrolyzes the phytate compounds of minerals bound to phytic acid which might increase the availability of phosphorus and other minerals of plant feedstuffs (Oatway et al. 2001). Phytase-producing *Bacilli* first found within fish gut was *B. licheniformis* from rohu (Roy et al. 2009) and, subsequently, autochthonous *B. subtilis*, *B. thuringiensis*, and *B. cereus* from the gut of Atlantic salmon (Askarian et al. 2012). Overall, the autochthonous exo-enzyme-producing *Bacilli* are effective in the fermentation of plant feedstuffs to improve the digestibility and quality of aquafeed. Therefore, the application of these bacteria has a great prospect ahead.

20.5 Production of Organic Acids

Lindgren and Dobrogosz (1990) revealed that lactic acid bacteria (LAB) such as *Lactobacillus* sp. can produce organic acids during lactic fermentation subject to strain and type of LAB. Antimicrobial effects through the reduction of pH with organic acids of LAB result in inhibition of pathogenic microbes which is well documented (González et al. 2007; Maeda et al. 2014). The acidic pH causes an interruption of substrate transport systems across cell cytoplasm, and the undissociated acid collapses the electrochemical proton gradient when diffused passively across the membrane or by modifying the permeability of the cell membrane (Ammor et al. 2006; Musikasang et al. 2009). Organic acids play a robust inhibitory role against deleterious bacteria using this mechanism of action (Musikasang et al. 2009). *B. mojavensis* inhibits the fish pathogen *Edwardsiella piscicida* by secreting low-molecular-weight organic acids (Etyemez and Balcazar 2016). The organic acids secreted by *Bacillus* spp. also help to solubilize insoluble nutrients in the sediments that promote the growth of phytoplankton and zooplanktons in the aquaculture system.

20.6 Production of Antioxidant Enzymes by the *Bacillus* spp.

Various kinds of reactive oxygen species and superoxides are produced in the eukaryotic cells through the metabolic process and due to environmental stresses. Overproduction of ROS damages cell membranes and many macromolecules in cells including DNA. Neutralization of these ROS by antioxidants is therefore needed for maintaining the sound health of fishes. When the antioxidant capacity of cells or tissues fails to surpass the superoxides produced through phagocytosis and/or cellular metabolism (Gobi et al. 2018), it leads to oxidative stresses (Mouthuy et al. 2016; Bermejo-Nogales et al. 2016) in the cells. Neutralization of the oxidative stresses is directly linked to the production of antioxidant enzymes in the cells (Messaoudi et al. 2009; Hindu et al. 2018b). According to the findings of Li et al. (2012), probiotic *Bacillus* species can produce some common antioxidant enzymes that are also found in fishes (Di Giulio et al. 1993). Castex et al. (2010) and Li et al. (2015a, b) found that SOD decomposes O^{2-} to H_2O_2, CAT catalyzes the disproportionation of H_2O_2 into H_2O and O_2 (Wang et al. 2017), and glutathione reduces the free radicals effectively (Hindu et al. 2018a). *B. licheniformis* Dahb1 is capable of modulating antioxidant responses either in serum or in mucus as mentioned by Gobi et al. (2016, 2018) in the study of Asian catfish (*Pangasius hypophthalmus*) and Mozambique tilapia (*O. mossambicus*). Similar findings were reported by Esteban et al. (2014), in gilthead seabream (*Sparus aurata*) where expressions of antioxidant enzyme GPx and SOD were enhanced in mucus after dietary supplementation of *Shewanella putrefaciens* and *Bacillus* species. *B. coagulans* and *B. subtilis* supplementation improved the antioxidant potentiality of gibel carp (*Carassius auratus gibelio*), while a similar effect was observed with *B. licheniformis* supplementation in Nile tilapia (Abarike et al. 2018). These antioxidant enzymes produced by the colonized probiotic *Bacillus* spp. may also improve the abiotic stress (high salinity, pH, temperature fluctuation, etc.) tolerance capacities in the treated fishes.

20.7 Mechanisms of Suppression of Fish Pathogens by the Probiotic *Bacillus* spp.

As the antimicrobial action of bacterial strains are reliant on their ability to modulate varied substances in a very definitive spectrum (Urdaci and Pinchuk 2004), understanding the mode of actions such as the production of bacteriocins, bacteriolytic enzymes, and antibiotics by *Bacillus* spp. is needed for efficient application of probiotic *Bacillus* spp. in aquaculture. It is reported by many scientists that *Bacillus* sp. can provide immunostimulation to host that enable them to survive against many deadly fish pathogens. For instance, *B. subtilis* through immunostimulation can prevent *A. hydrophila*, *A. salmonicida*, *V. anguillarum*, *Y. ruckeri*, *E. ictaluri*, and *F. columnare* in Nile tilapia (*O. niloticus*) (Addo et al. 2017; Mohamed and Refat 2011), grass carp (*C. idella*) (Tang et al. 2019), rainbow trout (*O. mykiss*)

(Newaj-Fyzul et al. 2007; Raida et al. 2003), giant freshwater prawn (*Macrobrachium rosenbergii*) (Keysami and Mohammadpour 2013), and Pacific white shrimp (*L. vannamei*) (Cheng et al. 2017).

20.7.1 Production of Bacteriocins

A way out from the antibiotics can be the bacteriocins (Bierbaum and Sahl 2009) and discussed elaborately regarding their differences with conventional antibiotics by several investigators (Zou et al. 2018). Bacteriocins (form in the ribosome) are antimicrobial peptides that are bioactive and released extracellularly to kill or inhibit the prolification of pathogenic prokaryotes including bacteria. These natural products have the potential antimicrobial action against various pathogenic bacteria including antibiotic-resistant strains (Riley and Wertz 2002). Bacteriocinogenic *Bacillus* spp. have been identified in different aquatic habitats like in Amazon basin (Cladera-Olivera et al. 2004), in seaweeds (Prieto et al. 2012), and in marine sediment (Rajesh et al. 2012, Smitha and Bhat 2013, Chopra et al. 2014).

Subtilin, ericin S, ericin A, sublancin 168, mersacidin, and paenibacillin are the bacteriocins produced by *B. subtilis* (Parisot et al. 2008; Dubois et al. 2009). Some other important bacteriocins such as coagulin, thurincin H, cerein 7A, subtilosin A, and megacin A-216 are produced by *B. coagulans*, *B. thuringiensis*, *B. cereus*, *B. amyloliquefaciens*, and *B. megaterium*, respectively (Le Marrec et al. 2000; Gray et al. 2006; Oscariz and Pisabarro 2000; Kiss et al. 2008; Sutyak et al. 2008).

Bacteriocins that produce antimicrobial compounds (such as by *Bacillus*) are confirmed by genome sequencing (Grubbs et al. 2017; Rahman et al. 2020). They are antibiotics and nonribosomally produced compounds, i.e., amino sugars, polyketides, and phospholipids (Stein 2005). Bacteriocins and similar substances produced by genus *Bacillus* (Abriouel et al. 2011; Al-Thubiani et al. 2018) are effective against various fish pathogens (Urdaci and Pinchuk 2004). Yi et al. (2018) identified four bacteriocins gene clusters, five non-ribosomal peptide synthetase gene clusters, and three polyketide synthases which are bacteriocins-associated gene clusters from *B. velezensis* isolated from carp. The *B. velezensis* inhibits various fish pathogenic bacteria including *Aeromonas hydrophila*, *Lactococcus garvieae*, *Vibrio parahaemolyticus*, and *Streptococcus agalactiae*. Rahman et al. (2020) identified several peptide genes coded for bacillaene, subtilin, bacillibactin, surfactin, fengycin, bacilycin, and subtilosin A in the genome of *B. subtilis* strain WS1A isolated from a marine sponge from the Saint Martin's island of the Bay of Bengal. The *B. subtilis* strain WS1A exhibited antimicrobial activity against fish pathogenic *A. veronii*. Dietary supplementation of the strain and its extracellular products significantly strengthened the resistance against motile *Aeromonas septicaemia* in rohu fish (*L. rohita*) (Rahman et al. 2020). Bacteriocins from *Bacillus* species such as coagulin isolated from *Bacillus coagulans* acts as food preservatives (Gálvez et al. 2007; Fu et al. 2018). *B. licheniformis* can increase the shelf-life of food and feed by inhibiting spoilage bacteria through bacteriocins (Teixeira et al. 2009).

20.7.2 *Quorum Quenching*

Quorum quenching (QQ) can be defined as the interruption of quorum sensing (Roy et al. 2011). Quorum sensing (QS) is the bacterial communication through signaling molecules that regulates antibiotic resistance, biofilm formation, bioluminescence, and bacterial virulence. QS is bacterial coordinated gene expression performed by releasing and distinguishing tiny signaling molecules to regulate their density and harmonize target gene expression (Zhang and Dong 2004). QS can control phenotypes by virulence factors, bioluminescence, and biofilm development (Miller and Bassler 2001; Menit et al. 2003; Mellbye and Schuster 2011).

Many microbes including *Bacillus* spp. can produce enzymes that interrupt N-acyl homoserine lactone (Tang et al. 2013) signal to disrupt QS (Wee et al. 2018). The mechanism of QQ can be used effectively in aquaculture against fish pathogens (Defoirdt et al. 2004) since QS regulates the pathogenicity of bacteria. Musthafa et al. (2011) discovered that pathogenicity and biofilm production capability of *Chromobacterium violaceum* and *Pseudomonas aeruginosa* were reduced by *Bacillus* sp. SS4 through interfering the AHL signaling of the pathogenic bacteria. Decreased mortality of common carp with *A. hydrophila* was observed in a challenge test due to QQ attributed to AHL lactonase produced by *Bacillus* species (Chen et al. 2010). Similarly, regarding *Bacillus*, it is reported by Zhang et al. (2011) that a gene (AiiO-AIO6) can inhibit the virulence factors by blocking the signaling molecules of *A. hydrophila*. An N-acyl homoserine lactone degradation gene from *Bacillus* has been reported by Reimmann et al. (2002) that was effective against pathogenic *P. aeruginosa*. In zebrafish, AiiAAI96 gene of *Bacillus* species reduces *A. hydrophila* infection through oral administration (Cao et al. 2012, 2014). These results suggest that *Bacillus* species degrade the QS AHL of pathogenic bacteria by producing degradation enzymes, and genes thus protect the fishes in the aquaculture system (Fig. 20.2).

20.7.3 *Suppression of Fish Pathogens by the Production of Lytic Enzymes by Probiotic* Bacillus *spp.*

Bacillus spp. are reported to secrete variety of lytic enzymes with antimicrobial (Urdaci and Pinchuk 2004) and antifungal properties (Kim et al. 1999). However, how these enzymes alone act upon pathogens of fishes is not clear and needs further investigations. The cell wall of pathogenic microbes is made up of proteins, cellulose, chitins, etc. that can be broken by proteases, cellulases, and chitinases enzymes, respectively (Jadhav et al. 2017). *Bacillus* is reported to produce those cell wall lytic enzymes which make them important for aquaculture industry and in the digestion of fishes (Thankappan et al. 2015; Zaineldin et al. 2018).

20.7.4 Production of Antibiotics

The antibiotic production capability of *Bacillus* spp. is one of their key strengths against fish pathogens. The chances of the development of antibiotic-resistant in pathogens are less as the mode of actions of antibiotics produced by *Bacillus* species is diverse and wide. Genus *Bacillus* was reported to produce 167 antibiotics, 40% of which are from *B. subtilis* (Béahdy 1974; Mondol et al. 2013). Roughly 4–5% of the genome of *B. subtilis* dedicates to antibiotic production (Stein 2005), and to combat *Helicobacter pylori*, amicoumacin antibiotic synthesized by *B. subtilis* 2335 was found effective (Pinchuk et al. 2001). Antibiotics from *Bacillus* species include bacitracin, laterosporin, gramicidin, tyrocidin, polymyxin, mycobacillin, and zwittermicin (Suva et al. 2016).

20.7.5 Enhancement of Immune Response in Fish

Probiotic bacteria enhance the host's innate immunity when supplemented with the diet (Nayak 2010) by humoral immune responses modulation and expression of host genes related to immunity (Verschuere et al. 2000a). This section highlights the significance of *Bacillus* probiotics in mediating humoral immunity and host gene expression against fish pathogens.

20.7.5.1 Nonspecific Immunity

The nonspecific and the acquired immune system perform decisive roles against pathogens in the host's defense mechanism (Munir et al. 2016). Superoxide dismutase, lysozyme, peroxidase, protease and antiprotease, catalase, and myeloperoxidase are some of the immune parameters of serum of fishes (Nayak 2010). Higher activities of these parameters indicate the higher immune responses (Alexander et al. 2010; Rauta et al. 2012) against infection (Fig. 20.3). Modulation of these parameters and immune system of fishes by the supplementation of probiotic *Bacillus* species in the fish diet is well documented (Wilson 2017). Increased disease resistance attributed to enhanced humoral and cellular immune responses was observed in fish fed with *Bacillus* supplementation (Yi et al. 2018). As the frontliner against pathogens, mucus has vital function in the fish immune system (McNeilly et al. 2008). The mucus contains antimicrobial peptides, immunoglobulin, lysozyme, and proteolytic enzymes, (Nigam et al. 2012; Jung et al. 2012). The research on mucosal immunity enhancement with probiotic bacteria is scarce. However, there are a few reports that suggest that *Bacillus* species can increase the mucosal immunity in fishes (Sheikhzadeh et al. 2012; Sangma and Kamilya 2015). Thy et al. (2017) reported that dietary administration of a mixture of *B. amyloliquefaciens* and *B. pumilus* increases the phagocytic activity in striped catfish (*P.*

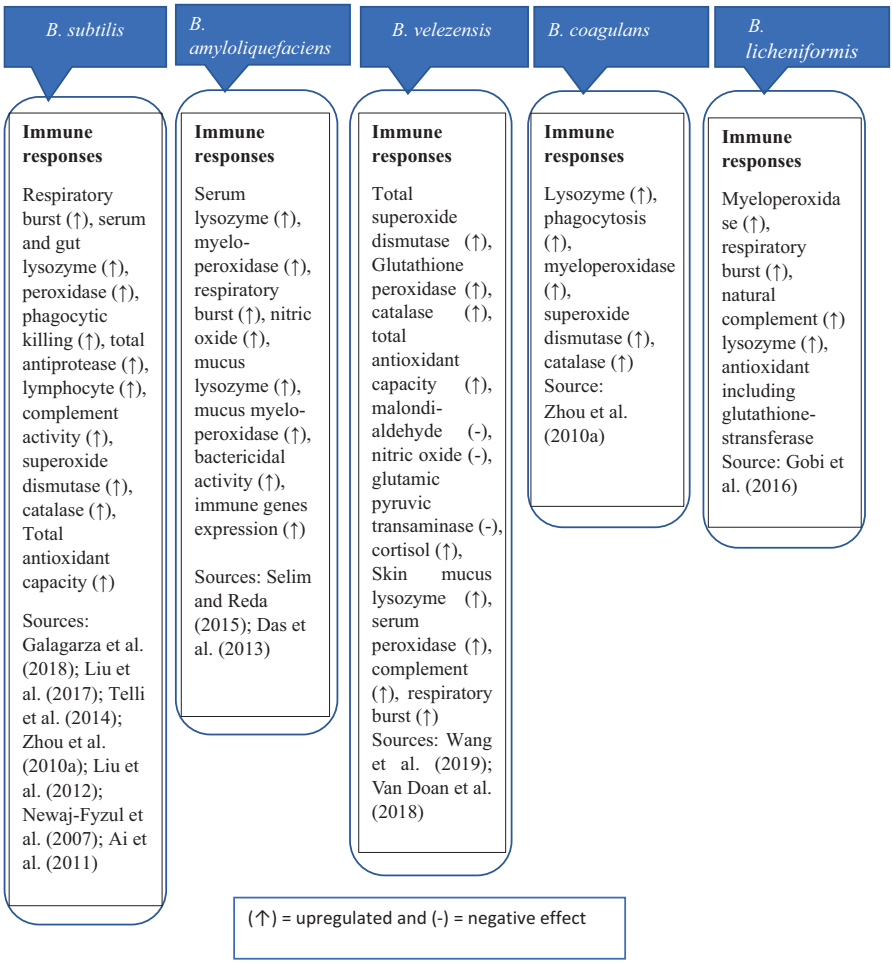

Fig. 20.3 Immune responses of different species of *Bacillus* in fish

hypophthalmus). Similarly, *B. subtilis* E20 and *B. licheniformis* proved phagocytic interaction with different fish species was observed after dietary supplementation (Gao et al. 2018). Nandi et al. (2017a) observed enhanced activities of lysozymes in *L. rohita* after supplementation of *Bacillus* probiotics. *Bacillus* is also reported to have a positive effect on pro-inflammatory cytokines (Yan et al. 2016), IgM (Nandi et al. 2017a), and respiratory burst (Thy et al. 2017) of fishes.

20.7.5.2 Immune Gene Expression in Host

The importance of *Bacillus* spp. in improving fish growth and metabolism is well established. Expression of genes linked with antioxidants, digestion processes, growth, metabolism, inflammation, cytoskeleton, and transport proteins is enhanced by the application of *Bacillus* spp. (Esteban et al. 2014; Rajanbabu and Chen 2011; Peng et al. 2012; Avella et al. 2010). Diet supplemented with suitable strains of *Bacillus* spp. augments the expression of genes associated with the immunity of fishes (Fig. 20.4) (Midhun et al. 2019). Avella et al. (2010) demonstrated that the adjustment to the rearing environment of the seabream larvae was improved due to lessening cellular stress by suppressing the functionality of HSP70 gene with a mix of *Bacillus* species in rearing water. Immune-related genes involved in biosynthesis of peroxinectin, prophenoloxidase, lipopolysaccharide, β-1,3-glucanbinding protein, and serine protein are highly expressed in fishes by the application of *B. subtilis* L10 and G1. These gene expressions help white shrimp, *L. vannamei*, to combat pathogen *V. harveyi* (Zokaeifar et al. 2012b). Supplementation of probiotic *Bacillus* species modulates the expression of the mucosal gene, immune-related genes in the head kidney and pro-inflammatory cytokines in head kidney and intestine of gilthead seabream (*S. aurata*) (Esteban et al. 2014), goldfish *(C. auratus)*, orange-spotted grouper (*E. coioides*) (Wang et al. 2018b), and Nile tilapia (*O. niloticus*) (Galagarza et al. 2018), respectively. Yi et al. (2018) reported that *B. velezensis* JW can boost the expression of the interferon- gamma gene (IFN- γ) and tumor necrosis factor-α (TNF-α) plus the gene expression of interleukin-1 (IL-1) and interleukin-4 (IL-4) all of which has significance in the defense mechanism of goldfish (*C. auratus*). Pro-inflammatory genes, interleukin-8 (IL-8), and the expression of caspase-1 (CASP-1) were increased by *B. subtilis* plus microalgae (*Tetraselmis* chuii) fed a diet in gilthead seabream (*S. aurata* L.) (Cerezuela et al. 2013). Similarly, as feed additives in tilapia (*O. niloticus*), it was evident that probiotic *B. licheniformis* (HGA8B) can enhance growth-related genes like growth hormone receptor genes (GHR-1 and GHR-2), insulin-like growth factor genes (IGF-1 and IGF-2) expression in the tissue of liver, and anti-inflammatory cytokine such as IL-10 (Midhun et al. 2019). Significantly higher heat-shock protein (HSP70), interleukin (IL-1 β), tumor necrosis factor (TNF-α), and interferon-gamma (INF-γ) were also obtained in Nile tilapia (*O. niloticus*) fed with *B. subtilis* (BS7 and BS8) (Won et al. 2020).

20.8 Enhancement of Abiotic Stress Tolerance

Stress in commercial aquaculture is unavoidable. It is caused by handling, feeding, vaccination, water exchange, fish transportation, high stocking densities, and ammonia levels in water all of which distress the health and physiology of fishes (Abdollahi-Arpanahi et al. 2018; Fuchs et al. 2017; Hoseinifar et al. 2015; Segner et al. 2012; Thy et al. 2017; Liu et al. 2010). Higher levels of stress reduce the survivability and increase the incidence of diseases in aquaculture (Eissa and Wang

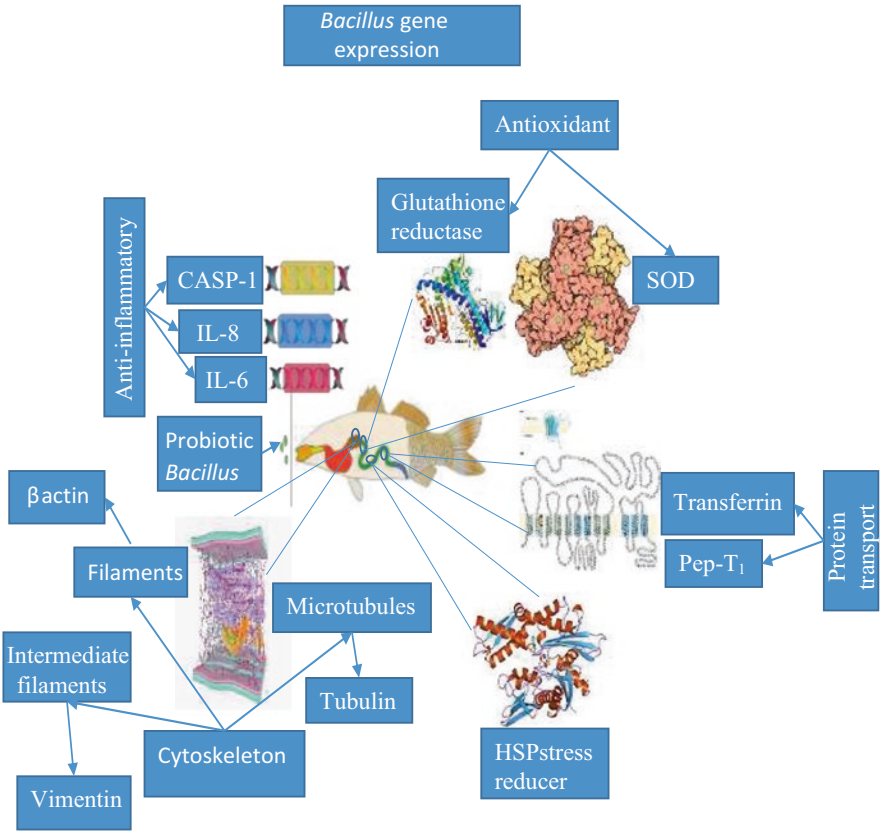

Fig. 20.4 Molecular mode of actions of probiotic Bacillus spp. on aquaculture species
HSP heat-shock proteins, *CASP-1* caspase-1, *Pep-T1* peptide transporter 1, *SOD* superoxide dismutase, *IL-6* interleukin 6, *IL-8* interleukin 8

2016). The global climate change also exerts various stresses such as high temperature to the aquaculture system. In fishes, the retaliation of stress occurs at the cellular level indicator of which includes glucose and cortisol (Abdollahi-Arpanahi et al. 2018), and heat-shock proteins that have a self-protective role in preserving the hemostasis (Eissa and Wang 2016) by reducing the stressors that produce healthy fishes (Yamashita et al. 2010). The probiotic bacteria make the fish resilient to stresses through various mechanisms including the expression of host-related genes in the host fishes (Forsatkar et al. 2017; Shaheen et al. 2014). Stress resistance to asphyxiation and air exposure of yellow perch observed to be improved with a mixture of *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens*, and *B. licheniformis* (Eissa et al. 2018) which is evident to the potentiality of *Bacillus* against different stressors. Similarly, Abdollahi-Arpanahi et al. (2018) reported that whiteleg shrimps, *Litopenaeus vannamei*, treated with *B. subtilis* and *B. licheniformis* were more resistant to environmental and chemicals stressors than the untreated control. Dietary administration of *B. subtilis* in Nile tilapia and *B. amyloliquefaciens* 54A

and *B. pumilus* 47B in striped catfish (*P. hypophthalmus*) reduces the stress of high stocking density (Telli et al. 2014) and ammonia toxicity (Thy et al. 2017), respectively. As climate change is posing a threat to aquaculture productivity worldwide, advanced research is needed to explicate the precise mechanisms of stress tolerance in fishes and shrimps by means of probiotic *Bacillus* spp.

20.9 Repair Tissue Damage in Farmed Fishes

Tissue damage of the farmed fishes is often associated with environmental or dietary toxicants which lead to various diseases like gall syndrome and liver enlargement (Kunjiappan et al. 2015). *Bacillus* spp. can play a significant role in reducing tissue damage of the cultured fish as suggested by many researchers (Abdollahi-Arpanahi et al. 2018; Hassaan et al. 2018). To understand the mechanism of action by which *Bacillus* species exert its beneficial effect, we have to know the causes of tissue damage in fishes. Some biochemical reactions of metabolism produce enzymes such as aspartate transaminase and alanine transaminase that in turn synthesize other metabolic intermediates by interconversion of amino acids with an upsurge in their amount leads to tissue damage (in heart, liver, muscle, and kidney) in farmed fishes (Abdollahi-Arpanahi et al. 2018; Babazadeh et al. 2011). As these enzymes are cytoplasmic and liberated into the blood after cellular damage, they can be used as an indicator to diagnose hepatic damage (Kunjiappan et al. 2015). Adorian et al. (2019) demonstrated that the dietary supplementation of *B. licheniformis* and *B. subtilis* in feed lessen the aspartate transaminase and alanine transaminase levels in fishes. Similar findings were reported in Nile tilapia fed with *B. subtilis*, *B. megaterium*, and *B. licheniformis* (Sutthi et al. 2018; Hassaan et al. 2018), in Rohu (*L. rohita*) treated with *B. amyloliquefaciens* CCF7 (Nandi et al. 2017a, b), and in shrimp with *B. subtilis* and *B. licheniformis* (Abdollahi-Arpanahi et al. 2018). These results demonstrate that *Bacillus* species play a role in reducing the toxic effects of enzymes and ensuring the better liver functions of the treated fishes.

20.10 Water Quality Enhancement

Better water quality for rearing fish with optimal physicochemical properties is crucial for successful aquaculture since diseases and infections are largely attributed to poor water quality (Zokaeifar et al. 2014). It is well recognized that probiotic *Bacillus* spp. enhance the water quality by detoxifying the harmful substances and provide fish a better environment to live in (Eissa et al. 2010; Hura et al. 2018; Tuan et al. 2013). Extracellular enzymes with antimicrobial peptides produced by the *Bacillus* species help to develop the culture water quality apart from killing harmful microbes (NavinChandran et al. 2014; Nimrat et al. 2012; Xu et al. 2013). Administration of *Bacillus* spp. directly in shrimp aquaculture can maintain the ammonia level in a

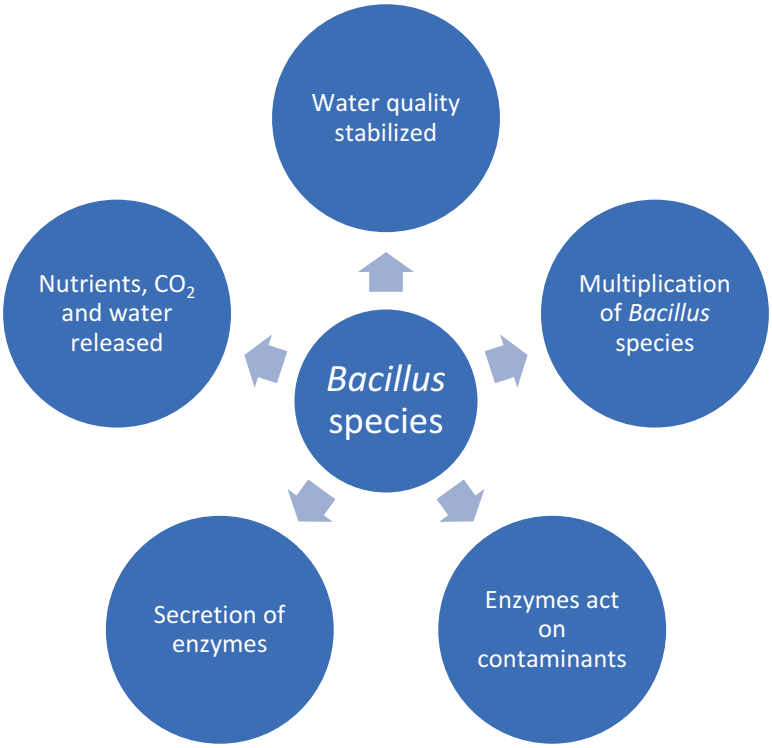

Fig. 20.5 Mode of actions of probiotic *Bacillus* spp. in improving water quality of aquaculture system

suitable range (Zokaeifar et al. 2014; Nimrat et al. 2012) through nitrification and/or denitrification of nitrogenous wastes and thus enhance water quality (Zink et al. 2011; Mujeeb Rahiman et al. 2010; Song et al. 2011; Lakshmanan and Soundarapandian 2008; Nimrat et al. 2011; Wang et al. 2005; Xie et al. 2013). Removing organic matter from rearing water is another important feature of *Bacillus* spp. that also improves water quality (Luis-Villaseñor et al. 2011). Figure 20.5 illustrated the mode of action of *Bacillus* species in improving water quality. The role of *Bacillus* spp. in modulating the microbiome in the aquaculture system and in the fishes is poorly understood. A better comprehension of the interactions of applied probiotics with fishes and environmental organisms would facilitate their effective use in the aquaculture industry.

20.11 Conclusions

Probiotic *Bacillus* spp. exert beneficial effects on treated fishes and aquaculture systems. Molecular systems involved in their beneficial effects are partially being understood. The mode of actions of *Bacillus* spp. to protect treated fishes includes

the production of antibiotics and lytic enzymes, the formation of biofilm, induction of host immune system, and battling for scope and nourishment with the fish pathogenic microorganisms. The applied *Bacillus* spp. also promotes the growth of fishes and shrimps through enhancing digestion of feeds, metabolism of nutrients, and expression of growth-related genes. However, additional experiments are needed to reveal the underlying molecular cross-talks between probiotic *Bacillus* spp. with treated fishes and shrimps using genomic and post-genomic analyses. Although the beneficial effects of *Bacillus* spp. on improving the water quality and environment of aquaculture systems are well documented, further research is needed on how the applied probiotics modulate the microbiome of the aquaculture system and the guts of the treated fishes and shrimps. We anticipate that the proper utilization of probiotic *Bacillus* spp. would enhance productivity and facilitates sustainability in the aquaculture industry.

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Chapter 21
Bacillus spp.-Mediated Drought Stress Tolerance in Plants: Current and Future Prospects

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Abstract Drought is a common environmental stress that threatens the sustainability of agricultural productivity in many parts of the world. Numerous morphological, physiological, and molecular changes may occur in plants as a response to this stress, thus affecting their growth and development. Some rhizosphere microorganisms, including *Bacillus* species, have proved efficiency in increasing drought tolerance to plants growing in regions with water scarcity. These endophytic bacteria can survive in the soil under a wide range of conditions and, when colonizing rhizosphere, may enhance drought stress tolerance of plants by promoting their growth.

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This can be ensured directly by improving the uptake of water and essential nutrients; the production of phytohormones, siderophores, and enzymes; and indirectly by suppressing phytopathogens and/or activating induced systemic resistance (ISR). To mitigate the impact of drought stress, *Bacillus* spp. employ various mechanisms including the inhibition of the formation of reactive oxygen species (ROS); the modification of phytohormonal activities; the accumulation of osmolytes, soluble sugars, and other molecules; and the induction of stress-response genes expression. This paper provides an overview of the most remarkable achievements in *Bacillus* spp. research done in agricultural crop plants. These researches could improve the understanding of the physiological and molecular mechanisms deployed by these bacteria to alleviate drought stress and lead to cope with the impacts of climate change and satisfy food demands, especially when there is scarcity of water or drought.

Keywords PGPR · *Bacillus* spp. · Drought stress · Drought stress tolerance · Proline · Antioxidant

21.1 Introduction

Crop production is constantly confronted with a range of biotic and abiotic stresses that negatively affect its yield and economic returns. General signaling pathways often are activated to help plants withstand dominant abiotic stresses (Lastochkina 2019), including pathways that target cell regulation, as well as morphological, physiological, biochemical, molecular, and genetic changes. Abiotic stresses, including drought, are among the main limiting factors for agricultural production all over the world (Abd El-Daim et al. 2019). Drought is considered as a severe environmental constraint to crop productivity (Vurukonda et al. 2016) and is one of the most known environmental stresses that has brought much attention from researchers in agriculture. Several aspects of crop growth can be affected by drought stress, including genes responses, transpiration, plant cell size, and physiology (premature senescence), which all contribute to decreased crop productivity (Disante et al. 2011; Kumar and Verma 2018; Mishra and Singh 2010).

The most known approach to deal with this challenge is the development of drought-tolerant varieties via genetic engineering and/or conventional breeding methods. However, this approach was shown to have limited effectiveness in improving plant tolerance to drought (Singh et al. 2015). Recent research indicates that microorganisms, in particular plant growth-promoting rhizobacteria (PGPR), can help crops mitigate abiotic stress. The role of these PGPR in promoting plant growth, improving crop nutrition, and suppressing diseases has been well documented (Grover et al. 2014). In other words, the application of PGPR may provide tolerance to drought, thus allowing plant survival even under a lower water potential. *Bacillus* species, gram-positive bacteria, are a major group of rhizobacteria that has been investigated broadly. This is directly linked with the population stability of

these bacteria which facilitates their storage, encapsulation, and subsequent applications in the field, and their ability to survive in the soil for a long period even under harsh conditions (Hashem et al. 2019).

Bacillus spp. can enhance plant tolerance to drought by regulating water transport and nutrient uptake, activating antioxidants, and other defense systems (e.g., the production of phytohormones and related metabolites), and regulating stress-responsive genes (e.g., the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase) (Radhakrishnan et al. 2017).

The impact of drought stress on crop productivity, the ability of *Bacillus* spp. to colonize root system, and its role in plant growth improvement as well as in mitigating drought stress are discussed in this review. Furthermore, the mechanisms deployed by *Bacillus* spp. to mediate drought stress tolerance, molecular basis involved in alleviating drought stress, and the role of *Bacillus* spp.-based commercial products in agricultural applications will be highlighted.

21.2 Impact of Drought on Crop Productivity

Climate change and the rapid worldwide population growth affect the global food security (Lesk et al. 2016). Indeed, the climatic conditions that prevail in different parts of the world result in anomalies in precipitation, thus generating severe periods of drought. This abiotic stress significantly affects agriculture due to the low availability of water resources and its management issues (Guoju et al. 2016).

The negative impact of drought stress on the yield has been investigated on many crops (Table 21.1). It has been shown that its impact depends largely on two factors: (*i*) the duration and the severity of the stress period and (*ii*) the crop stage. Several studies highlighted the occurrence of various physiological, biochemical, and

Table 21.1 Examples of yield losses caused by drought stress in field crops

Crop	Yield loss (%)	References
Maize (*Zea mays* L.)	21–40	NeSmith and Ritchie (1992)
	79–81	Monneveux et al. (2006)
	25–92	Atteya (2003)
	15	Edmeades (2008)
Pigeon pea (*Cajanus cajan* L.)	50	Nam et al. (2001)
Soybean (*Glycine max* L.)	46–71	Samarah et al. (2006)
Wheat (*Triticum aestivum* L.)	57	Balla et al. (2011)
Chickpea (*Cicer arietinum* L.)	45–69	Nayyar et al. (2006)
Rice (*Oryza saliva*)	24–84	Venuprasad et al. (2007)
	53–92	Lafitte et al. (2007)
Sunflower (*Helianthus annuus* L.)	60	Mazahery-Laghab et al. (2003)
Barley (*Hordeum vulgare*)	49–57	Samarah (2005)
Common bean (*Phaseolus vulgaris*)	58–87	Martínez et al. (2007)

molecular changes during plant growth, under drought stress (Kunert and Vorster 2020; Melandri et al. 2020; Ye et al. 2020). In cereals, for example, the negative effects of drought on pollen development, kernel number (Ji et al. 2010), size, and filling (Yang and Zhang 2006) have been widely investigated. Estrada-Campuzano et al. (2008) showed that drought occurring after anthesis shortened the period of grain filling while that occurring at pre-anthesis stages reduced the time to anthesis in cereals. The process of grain filling in cereals is attributed to four enzymes, viz., sucrose synthase, adenosine diphosphate-glucose-pyrophosphorylase, starch-branching enzyme, and starch synthase (Ahmadi and Baker 2001). The same author reported that the reduction in grain yield of cereals could be the result of the reduction in the activity of these enzymes under drought stress.

21.3 *Bacillus* spp. Root Colonization and its Role in Plant Growth Improvement

Plant root colonization by *Bacillus* spp. can be mutually beneficial to plants and bacteria. This is a remarkable strategy adopted by members of this genus to use carbon-rich root exudates, thus facilitating rhizobacterial colonization and mutualistic associations with the host plant (Allard-massicotte et al. 2016). In particular, up to 30% of the carbon fixed by plants are secreted via its root exudates, thereby feeding the bacteria (Allard-massicotte et al. 2016). In exchange, the bacteria provide plants with several growth-promoting elements (Allard-massicotte et al. 2016). The role of intact chemotaxis machinery in the localization and early root colonization by *Bacillus* spp. has been highlighted by several studies, especially with *Bacillus subtilis* for efficient colonization of plant rhizosphere in natural environments (Allard-massicotte et al. 2016). Furthermore, it has been shown that *B. subtilis* is attracted particularly by some components of root exudates, such as amino acids, as environmental cues to locate the source of nutrients and initiate symbiosis or even pathogenesis (Yang et al. 2015).

The long-term root colonization by the bacteria is determined by the formation of a biofilm which is a multicellular bacterial community covered by an extracellular matrix (Allard-massicotte et al. 2016). This biofilm may be formed hours after motile bacterial cells first settle, but for *B. subtilis*, the root colonization and biofilm formation often require 24 h (Beauregard et al. 2013). It is important to note that the root colonization is robustly stimulated by plants (Beauregard et al. 2013); the biofilm formation depends on the production of certain plant-derived molecules. Plant polysaccharides, the major component of plant cell wall, are among these essential compounds. The polysaccharides can act as an environmental cue for the biofilm formation and can also be used by bacteria as a carbon source to build the extracellular matrix (Beauregard et al. 2013). It has been demonstrated that another plant-derived molecule, malic acid (MA), can be involved in biofilm formation; elevated levels of MA promote the binding and biofilm formation of *B. subtilis* FB17 on

Arabidopsis thaliana roots (Rudrappa et al. 2008). It was later shown that MA would stimulate biofilm formation in a histidine kinase-dependent manner, but only at high concentrations (Chen et al. 2012). As mentioned above, root exudates are known to recruit beneficial bacteria and trigger biofilm formation. The *B. subtilis* RR4 strain can stimulate MA biosynthesis and exudation, and it is highly attracted to rice roots (Rekha et al. 2018). Specific molecules secreted by *Bacillus* spp., especially exopolysaccharides, appear important to the root colonization; an exopolysaccharide-deficient mutant, constructed from *B. amyloliquefaciens* FZB42, was less able to support biofilm formation and, subsequently, the colonization of *A. thaliana* roots (Lu et al. 2018).

In some cases, rhizobacteria penetrate the roots and enter the plant tissues as a harmless endophyte. This entry of commensal microbes into plants may involve the breaking of plant defense system (Deng et al. 2019). However, the mechanism deployed by endophytes in overcoming plant defense in order to establish a steady commensal relationship with their host plants has rarely been reported (Hacquard et al. 2017). Most of the compromised plant defense mechanisms are due to the adaptation by pathogens. In an attempt to decrypt how an endophyte evades plant defense and achieve harmonious commensalism, Deng et al. (2019) used *B. subtilis* BSn5 and showed that this strain would reduce plant defense responses via the production of antibiotic subtilomycin. Bond to self-produced flagellin, the antibiotic producer demonstrates advantages in colonizing plant root systems on *A. thaliana* and *Amorphophallus konjac* (Deng et al. 2019).

Endophytic bacteria have shown notable benefits to their plant hosts directly or indirectly (Afzal et al. 2019), especially endophytic *Bacillus* spp. These endophytes, some considered as PGPR, can significantly increase plant growth in early stages, forming a mutualistic plant-microbe relationship. *Bacillus* spp. are one of the predominant PGPRs (Radhakrishnan et al. 2017) with spores that can survive in the soil for long periods even under harsh environmental conditions (Hashem et al. 2019). Moreover, the ability to form spores is a characteristic trait of *Bacillus* spp., which differentiates them from other PGPRs, such as *Pseudomonas* spp. (Radhakrishnan et al. 2017). It has been shown that endophytic bacteria ameliorate plant growth in two different ways: (*i*) directly through the production of plant growth beneficial substances, including solubilization/transformation of mineral nutrients such as phosphate, nitrogen, and potassium; phytohormones; siderophores; and enzymes, and (*ii*) indirectly by controlling plant pathogens and/or activating induced systemic resistance (ISR) of plants (Ma et al. 2016). These mechanisms can help plant's growth and development under normal and stressful conditions (Ma et al. 2016), especially due to the fact that *Bacillus* spp. can survive for a long time under unfavorable environmental conditions (Radhakrishnan et al. 2017). For instance, *Bacillus* spp. can produce exopolysaccharides and siderophores under the conditions such as lack of water, salinity, and heavy metal accumulation in soil, thus preventing the movement of toxic ions and adjusting the ionic balance and water transport in plant tissues while suppressing soil-borne pathogens (Radhakrishnan et al. 2017). The synthesis of indole acetic acid (IAA), gibberellic acid, and ACC deaminase by *Bacillus* spp. may help regulate intracellular

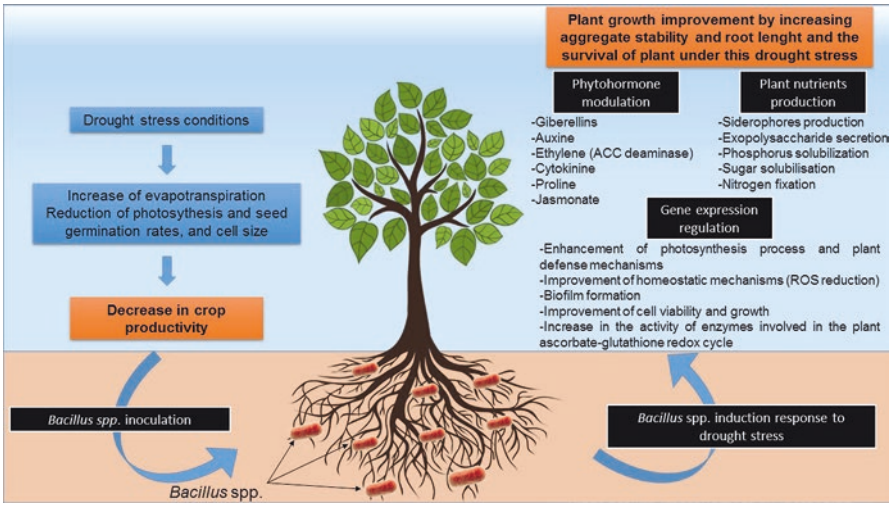

Fig. 21.1 Impact of drought stress on plant health and productivity and molecular and physiological mechanisms offered by *Bacillus* spp. to alleviate the adverse effect of drought stress

phytohormone metabolism and increase plant stress tolerance under certain conditions (Radhakrishnan et al. 2017). Cell-wall-degrading substances released by these bacteria, including chitosanase, protease, cellulose, glucanase, lipopeptides, and hydrogen cyanide, may be antagonistic to a range of pathogens and crop pests (Radhakrishnan et al. 2017). Taking together, it is noteworthy that biotic and abiotic stress factors to crops may be mitigated by *Bacillus*-induced changes, including the regulation of water transport, nutrient uptake, and the activation of the antioxidant and defense systems (Radhakrishnan et al. 2017). Below and in Fig. 21.1 are some of the examples on *Bacillus* spp. and their mechanisms involved in plant growth promotion.

21.3.1 Nutrient Acquisition and Phytohormones Production by Bacillus spp. in Plants

Both seed germination and plant growth are significantly influenced by nutrients available in the soil (Afzal et al. 2019; Fernández Bidondo et al. 2012)**.** Plants absorb phosphorous and nitrogen from the soil via root transporters, but soils usually lack a sufficient quantity of the bioavailable forms of these nutrients for optimal plant growth (Afzal et al. 2019; Fernández Bidondo et al. 2012). Some *Bacillus* spp. can convert the complex form of essential nutrients, including phosphorous and nitrogen, to simpler forms for easy uptake by plant roots (Kang et al. 2015). *Bacillus megaterium* CDK25 showed a remarkable solubilizing ability for zinc which is indispensable for better growth and development of plants (Bhatt and Maheshwari

2020)**.** This isolate can also help solubilize both inorganic and organic phosphate, thus leading to the amelioration of phosphate uptake by chili plants (*Capsicum annuum* L.), resulting in improved vegetative growth and other biological outputs. Furthermore, this *B. megaterium* isolate was able to produce siderophore, which positively influenced plant growth through the amelioration of soluble iron which is considered one of the requisite micronutrients for proper functions of several plant species. Thus, the availability of this element to plants led to the increase of their vegetative growth (Bhatt and Maheshwari 2020). The ability of four endophytic *Bacillus* spp., including *Bacillus cereus* EPP5, *B. cereus* EPP71, *B. amyloliquefaciens* EPP62, and *B. subtilis* subsp. *subtilis* EPP65, to transform insoluble nutrients to soluble forms has been reviewed by Kushwaha et al. (2020). *B. megaterium* HX-2 was also able to dissolve phosphate, providing more nutrients for improved plant growth (Li et al. 2019).

A broad range of genomic traits pertaining to plant growth promotion and potential agricultural applications has been raised following the study of *B. megaterium* RmBm31. This strain is proven to be an IAA-producing endophytic bacterium possessing a large set of genes that regulate plant growth-promoting traits. This bacterium enhanced the biomass and modified the root architecture of *A. thaliana* seedlings through a physical contact with roots and the production of volatile organic compounds (Dahmani et al. 2020). The *B. megaterium* CDK25 strain is endowed also with the IAA production (Bhatt and Maheshwari 2020). The *B. subtilis* RR4 strain has been shown to influence the biosynthesis of MA profoundly; the biosynthesis and accumulation of MA by this bacterium can improve primary metabolism, thus leading to plant growth promotion (Rekha et al. 2018). On pearl millet (*Pennisetum glaucum* (L.) R. Br.), rapid activation of several biochemical and molecular mechanisms was the key to the growth promotion occurred during plant-*Bacillus* interactions (Kushwaha et al. 2020), including the biosynthesis of IAA and siderophores; rapid solubilization and mobilization of nutrients such as zinc, potassium, and phosphate; and increased production of extracellular hydrolytic enzymes, etc. (Kushwaha et al. 2020). *Bacillus cereus*, for instance, has been shown to remarkably improve plant biomass increase by escalating nutrient uptake efficiency and releasing plant growth hormones (Kushwaha et al. 2020). *B. subtilis* can help solubilize phosphate in soil, improve nitrogen fixation, and produce siderophores that promote plant growth and suppress pathogens (Hashem et al. 2019). It has also been shown that *B. amyloliquefaciens* possesses immense potential to increase the growth of chili plants (*C. annuum*); a seed treatment with this bacterium resulted in substantial increases in germination (85%), seedling vigor, and vegetative growth (Gowtham et al. 2018). Another strain of *B. amyloliquefaciens*, Bc2, promoted the growth and development of strawberry seedlings, and its role in plant growth promotion has been demonstrated (Es-Soufi et al. 2020). The *B. megaterium* HX-2 strain, which also improved the growth of maize, was believed to confer the effect via IAA production and phosphate solubilization; seed soaked in a HX-2 suspension showed significantly improved plant growth, including the increase in plant height, root length, and plant dry weight (Li et al. 2019).

21.3.2 Indirect Plant Growth Promotion by Suppressing Phytopathogens

It has been well documented that the application of *Bacillus* spp. can activate ISR in several crop species as well as promote the growth of these plants (Hashem et al. 2019). The majority of endophytic strains of *Bacillus* spp. dwelling inside pearl millet showed antagonism against several important plant pathogens, including *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium solani*. This is achieved via several mechanisms, including the secretion of antimicrobial peptides (AMP). An isolate of *B. cereus*, for instance, was able to increase plant biomass by disrupting the mycelium growth of fungal pathogens through the biosynthesis of siderophores and extracellular fungal cell wall degrading enzymes (Kushwaha et al. 2020). In another study, an isolate of *B. subtilis* was reported as one of the promising microorganisms for sustainable agriculture as it promoted plant growth and showed efficacy against a wide range of pathogens both in vitro and in greenhouse/field trials. Multiple mechanisms have been identified for disease suppression by *B. subtilis*, including direct plant growth promotion, antibiosis, competition for space and nutrients, lysis of pathogen hyphae, and ISR (Wang et al. 2018). Many *B. subtilis* strains are able to activate ISR in plants, thus increasing disease resistance as reflected by lower levels of infections. Indeed, the number and diversity of microorganisms used for the production of commercial biocontrol products have increased (Hashem et al. 2019). The antimicrobial nature of *B. subtilis* BSn5 against a bacterial pathogen, *Erwinia carotovora* subsp. *carotovora*, is a good example of such application, although the exact mechanism underlying the antagonism remains unknown (Deng et al. 2011). Antifungal activity against fungal pathogens has been reported for *Bacillus stratosphericus* LW-03. This strain targets several fungal pathogens known to cause serious diseases on several crops, with considerable inhibition of *Fusarium oxysporum*, *Botryosphaeria dothidea*, *F. fujikuroi*, and *Botrytis cinerea* (Khan et al. 2020). Significant disease reduction (~71%) against the anthracnose disease has been observed on chili plants treated with *B. amyloliquefaciens* Bc2 (Gowtham et al. 2018).

21.4 *Bacillus* spp. for Mitigating the Impact of Drought Stress

Several adaptation and mitigation strategies of physiological, biochemical, and cellular/molecular nature can be activated in plants exposed to drought to maintain cell homeostasis (Krasensky and Jonak 2012; Lastochkina 2019). Those strategies include (*i*) accumulation of abscisic acid (ABA); (*ii*) reduction in photosynthetic rate; (*iii*) expression of aquaporins and vacuolar (H+)-pyrophosphatases for maintaining cell turgor through osmotic adjustments; (*iv*) accumulation of compatible osmolytes like proline, sugars, betaines, polyamines, quaternary ammonium

compounds, and amino acids; and (*v*) accumulation of drought stress-related proteins like dehydrins that maintain cell water status and help bacteria to maintain membrane permeability, proteins in their functional forms, and enzymes to maintain their integrity under stress conditions (Kumar and Verma 2018). In addition, drought stress induces reactive oxygen species (ROS) enzymes which are critical to cell integrity and functionality, as well as plant survival. ROS signaling is activated with the increase of ABA levels and calcium flows (Kaur and Asthir 2017). Therefore, a better understanding of the strategies used by plants, in response to drought stress, may facilitate the development of treatment that helps to alleviate the impact of drought and improve crop productivity.

21.4.1 Antioxidant Defenses and ROS

The lowered uptake of water and nutrients can affect plant physiological processes and generate ROS including superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Under drought stress, these ROS can damage plant organization at various levels including lipid peroxidation initiation, electrolyte leakage, membrane integrity alteration, and degradation of proteins, lipids, and nucleic acids (Vurukonda et al. 2016). The plant's antioxidant defense system involves several mechanisms to reduce the biosynthesis of ROS, including increased activities of specialized enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as other synthesis (ascorbic acid, cysteine, and glutathione) that alleviate the impact of oxidative stresses (Kaushal and Wani 2016). Several studies identified that *Bacillus* spp. played the role of an elicitor to mediate tolerance to drought in plants via a variety of mechanisms (Lastochkina 2019). Plant inoculation with these bacteria was reported to improve the efficiency of water uptake that is considered as an important mechanism in mitigating the impact of drought stress (Marulanda et al. 2009). *Bacillus* spp. were also found to enhance the levels of antioxidant enzyme activities in wheat (Chakraborty et al. 2013), maize (Vardharajula et al. 2011), and cucumber (Hou et al. 2018). For example, an increase in the activity of antioxidant enzymes like APX, CAT, and glutathione peroxidase (GPX) has been reported in drought-stressed maize plants inoculated with drought-tolerant *Bacillus* spp. (Vardharajula et al. 2011). Treatment of drought-stressed cucumber plants with a *Bacillus* spp. strain, *Bacillus methylotrophicus* CSY-F1, was often accompanied by the improvement of plant growth and the enhancement of antioxidant enzyme activities, including those of SOD, catalase, guaiacol peroxidase, APX, dehydroascorbate reductase, monodehydroascorbate reductase, and GR. Those enzymes are generally considered as biochemical indicators of drought tolerance (Hou et al. 2018). It is important to emphasize that the use of *Bacillus* spp. under drought stress has led to the improvement of plant growth and nutrition, as well as the enhancement of physiological and metabolic plant activities which may contribute to drought stress mitigation. This includes, for example, the deployment of *B. megaterium*,

Bacillus thuringiensis, and *Bacillus* spp. in drought-stressed lavandula and salvia plants which was accompanied by the increase of the concentration of some plant nutrients such as calcium, magnesium, zinc, manganese, and copper (Armada et al. 2014). Calcium is known for its role as a stabilizer of membrane systems. As to magnesium, it is involved in regulating ion homeostasis in some organelles such as chloroplasts, vacuoles, and stomata (Radhakrishnan et al. 2017).

21.4.2 Modification of Phytohormonal Activities to Impart Drought Tolerance in Plants

Phytohormones, in particular IAA, gibberellins, ethylene (ET), ABA, salicylic acid (SA), and cytokinins, play an essential role in plant growth and development by regulating some of the critical biological processes. The latter include the induction of germination, cell division and enlargement, and root extension. These processes are often involved in supporting plants to cope with environmental stresses (Forni et al. 2016). The role of *Bacillus* spp. in enhancing drought stress tolerance by altering endogenous phytohormone levels has already been highlighted. Such is the case, for instance, of *B. subtilis* for which a role in mediating tolerance to wheat against drought stress has been emphasized. Indeed, this bacterial strain increases the IAA content of wheat exposed to drought stress. Furthermore, it counteracts the increase of ABA and ACC content under the same conditions (Barnawal et al. 2017). IAA plays a crucial role in regulating plant growth, including the differentiation of vascular tissues and roots, cell division, and shoot growth (Goswami et al. 2016). ABA accumulation in plants improves drought tolerance by inducing stomatal closure, which helps to limit the water loss, activate antioxidant enzymes and water-deficit-induced genes, and improve root hydraulic conductivity (Forni et al. 2016). The role of cytokinins under the stress may be due to the antioxidant activity related to the protection of purine breakdown (Javid et al. 2011) since a low level of this hormone would favor carbon reallocation to root growth and prompt stomatal closure (Arkhipova et al. 2007). Jasmonic acid (JA) is also involved in drought tolerance by enhancing the activities of antioxidant enzymes and pathogenesis-related proteins (Forni et al. 2016). Both salicylic acid (SA) and ethylene (ET) were reported as stress-response hormones; SA can induce the expression of drought-responsive genes involved in maintaining the cell membrane stability and in preventing ROS accumulation (Jumali et al. 2011).

21.4.3 Control of ET Levels by ACC Deaminase

Under drought stresses, plants generally respond by synthesizing ACC synthase, which is the precursor for ET, a gaseous phytohormone produced when plants are exposed to abiotic (flooding, drought, temperature, and various contaminants) or biotic (phytopathogens) stressors. The increase in ET levels in plants leads to senescence, chlorosis, and abscission (Afzal et al. 2019). The modulation of ACC deaminase expression is the key to ACC biosynthesis and consequently to ET production (Kumar and Verma 2018). PGPR that stimulate the activity of ACC deaminase can prevent a jump in plant ET levels via ACC hydrolysis to convert the immediate ET precursor into products which can be easily assimilated by plants (α-ketobutyrate and ammonia). This helps reduce the negative effect of drought stresses on plants (Afzal et al. 2019).

21.4.4 ROS-Scavenging Antioxidant Enzymes

Bacillus spp. are known for their capacity as "biofertilizers" which can help plants grow under drought stress by optimizing the uptake of water and essential nutrients due to suppressed ROS levels as well as reduced cellular disruption to plants (Lopes et al. 2018). Treated plants may show increased physiological activities, especially photosynthesis, accompanied by a reduction in ROS levels (Armada et al. 2014).

21.4.5 Drought Stress Tolerance Induced by the Accumulation of Osmolytes, Soluble Sugars, Exopolysaccharides (EPS), and Bacterium-Derived Volatiles

The accumulation of osmoprotectants—a group of small molecules with low molecular weight, such as osmolytes, soluble sugars, sugar alcohols, and amino acids—is another mechanism deployed by plants to maintain homeostasis under drought stress (Singh et al. 2015; Vardharajula et al. 2011). Exopolysaccharides are carbohydrates with high molecular weight and are produced by some bacteria known to be involved in drought stress tolerance (Kumar and Verma 2018). These molecules, reported to be produced by *Bacillus* spp., may facilitate water uptake by roots and maintain plant ionic balance under drought stress (Hashem et al. 2019).

Volatile compounds (VOCs) produced by some bacteria may play an important role in inducing drought tolerance by serving as a signal to plants for developing proper responses (Vurukonda et al. 2016). Although ROS-targeting enzymes, including GR, monodehydroascorbate reductase, superoxide dismutase, and catalase (Timmusk et al. 2014), are activated commonly, VOCs produced by *Bacillus*

spp. may also induce plants to produce hydrogen peroxide and nitric oxide that are associated with plant drought tolerance (Liu and Zhang 2015).

21.5 Mechanisms of *Bacillus* spp.-Mediated Drought Tolerance

The rhizosphere nests a variety of microbe fauna, and their diversity has been characterized using metagenomics and high-throughput screening tools. A better understanding of this plant-microbe relationship helps lay the foundation for studying plant stress tolerance interacting with soil microorganisms (Kumar and Dubey 2020).

During the natural evolution, some microorganisms have developed mechanisms that could help them to survive harsh conditions, such as the lack of water supplies for an extended period of time. Some of these microbes have evolved with plants in the rhizosphere, becoming mutualistic to each other. *Bacillus* spp. are well known to promote plant growth through several direct and indirect mechanisms, including the production of phytohormones, bacterial exopolysaccharides, induction of systemic stress tolerance, and ACC deaminase (Kumar and Verma 2018). An intimate relationship between plants and their rhizosphere microbiota appears essential for improved plant tolerance to drought stresses (Goswami and Deka 2020). Application of biostimulants, which could induce physiological and molecular responses of plant for nutritional and functional improvements, has been reported to reduce the impact of drought stress (Basile et al. 2020). PGPR are among the microorganisms that produce a range of plant hormones to help reset some of the plant physiological process in response to drought stresses (Kumar and Verma 2018). Some of the hormones may act as a signal to trigger a cascade of further plant responses against drought stresses, including enzymatic activities and modification of root structure (Goswami and Deka 2020).

Drought tolerance involves several mechanisms in plants, including physiological, biochemical, and molecular responses to adapt to the drought stress (Goswami and Deka 2020). Under drought stress conditions, some rhizobacteria may accumulate solutes like, quaternary amines, sugar, and amino acids which can prevent degenerative processes and help cell growth under osmotic-deficient conditions (Vardharajula et al. 2011).

The knowledge of plant-microbe communication may be used to manipulate the abundance of microorganisms, altering plant development, and/or defense against pests or diseases (Arif et al. 2020). PGPR, especially *Bacillus* spp., have been shown repeatedly to promote plant growth and development and reduce the impact of stresses. Many *Bacillus* spp. are known to elicit tolerance against drought stress via the augmentation of antioxidants (AOX), followed by modification of root architecture, activation of early signaling, increase in osmoregulation, and finally the reduction of ET through ACC deaminase (Carlson et al. 2020). Some *Bacillus* spp. including *B. circulans*, *B. firmus*, *B. globisporus*, *B. licheniformis*, and

B. mojavensis have been reported to alleviate drought stress through ACC deaminase activities (Carlson et al. 2020).

B. sphaericus has been reported to absorb heavy metals and bind them extracellularly (Ramakrishna et al. 2020). Four *Bacillus* spp. isolates exhibited growth-promoting properties and drought stress tolerance in vitro under sterile and non-sterile soil conditions, via the production of ammonia and IAA, and solubilization of phosphorus (Grover et al. 2014). The treatment improved root growth and lateral root formation. *B. licheniformis* produces ACC deaminase under drought stress conditions, which limits the production of ET that impairs normal plants growth (Bleecker and Kende 2000; Lim and Kim 2013).

B. amyloliquefaciens, *B. licheniformis*, *B. thuringiensis*, and *B. subtilis* have been observed to stimulate root exudation and promote plant growth. These *Bacillus* spp. also were able to synthetize osmolytes under stress conditions, thus alleviating the negative impact on plants (Timmusk 2003) through the maintenance of the stability of plant cell membrane and proteins (Kogut and Russell 1987; Vardharajula et al. 2011).

Bacillus spp. strains also secrete exopolysaccharides under drought conditions that can form an organo-mineral sheath around the cells. The exopolysaccharide layer may lead to increased microaggregates as a secondary effect that increases the aggregate stability and root survival under drought stress (Alami et al. 2000). *Bacillus* spp. also increase the proline content in roots, which helps protect plant cell membranes, proteins, and water status (Yoshiba et al. 1997). Moreover, these bacteria are able to increase soluble sugars in root tissues, which support the biosynthesis against the effects of drought stress (Vardharajula et al. 2011). Armada et al. (2014) reported that *B. thuringiensis* mitigated the effect of drought stress depressing stomatal conductance to reduce water losses, increasing the production of potassium to support plant functionality under drought conditions, and controlling proline secretion and accumulation. Drought tolerance conferred by *B. thuringiensis* was linked to the decreased level of APX and GR which reduced the oxidative damage to plant cells (Armada et al. 2014). The rhizobacteria were also able to produce IAA under stress conditions, which is responsible for root growth enhancement. IAA also improves water use efficiency (Armada et al. 2014). *B. thuringiensis* produces a great amount of IAA and proline under drought stress conditions; this bacterial strain was able to survive and multiply under drought conditions. The use of *B. thuringiensis*, in combination with a consortium of arbuscular mycorrhizal (AM) in maize, decreased the oxidative damage to lipids, as well as the accumulation of proline in plant cells under drought stress conditions. The combined use enhanced the function of aquaporins for improved transport of water, carbon dioxide, glycerol, urea hydrogen peroxide, and boric acid (Armada et al. 2015).

Timmusk et al. (2014) reported that *B. thuringiensis AZP2* strain increased the growth of wheat seedlings, which was attributed to higher rates of photosynthesis and lower stress volatile emissions. *B. amyloliquefaciens* also increased the proline and total soluble sugar (TSS) levels in rice seedlings subjected to drought stress and positively modulates the expression of stress-responsive genes including glutathione S-transferases (GSTs) (Tiwari et al. 2017). GSTs are an enzyme with known

antioxidant properties that contribute to the reduction of glutathione (Tiwari et al. 2017).

The reduction of stomatal conductance is often the first response to drought stresses. The stomatal closure, however, has the disadvantage of reduced rates of photosynthesis due to limited gas exchange, but *B. subtilis* (B26 strain) treatment seemed also help to reduce the negative impact on photosynthesis under drought stress due to optimized stomatal conductance and increased root biomass (Gagné-Bourque et al. 2016). The same study also reported notably higher sucrose and fructan concentrations in shoots and roots of treated plants under drought conditions. The increase of sugar biosynthesis may allow an osmotic adjustment, contributing to mitigating the impact of drought stress (Gagné-Bourque et al. 2016). Furthermore, the treatment also leads to the production of amino acids in greater quantities in plant shoots and roots. These amino acids mainly belong to glutamate and aspartate families.

Inoculation of *Platycladus orientalis* (L.) Franco (conifers) with *B. subtilis* induced an increased level of ABA and stomatal conductance in shoots, in connection with drought-tolerance responses (Liu et al. 2013). Inoculation of *Lavandula dentata* L. (fringed lavender) with the IAA-producing strains of *B. thuringiensis* induced the increase in both proline and potassium levels but reduced APX and GR activities (Armada et al. 2014). Cytokinin-producing *B. subtilis* strains also proved their role in alleviating drought stress (Liu et al. 2013). Under drought stress, *B. megaterium* (BOFC15 strain) was able to excrete spermidine, a type of polyamine that plays a major role in plant growth and development. Zhou et al. (2016) showed that the bacterium enhanced the plant tolerance against drought stress, and this was related to the decrease in ABA levels and the improvement of adaptive responses. The treatment also increased plant biomass, photosynthetic capacity, proline, and soluble sugars under drought stress conditions (Zhou et al. 2016). Moreover, *B. megaterium* var. *phosphaticum* and *B. mucilaginosus* can solubilize phosphorous for plant by releasing organic acids (Arif et al. 2020), which may further add to plant drought stress tolerance.

Bacillus altitudinis is known for its ability of IAA and ACC deaminase production in rice under drought stress via an increase in relative water content, chlorophyll and membrane stability, proline and phenolics content, catalase activity, and a reduction in malondialdehyde (MDA). In addition, a reduction of ET emission under drought stress was reported with inoculated plants (Kumar et al. 2017). Forty-four *Bacillus* spp. isolates have been tested by Chari et al. (2018) in India, with 28 of them showing plant growth-promoting activities, 4 exhibiting water-stress tolerance ranging from −0.05 to −0.73 Mpa, and 6 displaying high-temperature tolerance up to 50 °C (Damodara Chari et al. 2018). Some isolates showed high phosphate and potassium solubilization, and many produced high levels of ACC. Rhizobacterial strains were able to utilize ACC as a source of nitrogen to variable degrees, and all the strains had ACC-deaminase activity (Damodara Chari et al. 2018).

B. subtilis (RJ46 strain) alleviated the impact of drought stress on two different plant species, *Pisum sativum* L. and *Vigna mungo* L. via the activation of ACC

deaminase (Saikia et al. 2018). The study also showed that several physiological changes occurred in treated plants during a period of drought including an increase in seed germination and shoot and root biomass. Furthermore, the treatment showed a higher-level osmolytes, which helps decrease the osmotic potential in plant cells. An upsurge in relative water potential and leaf chlorophyll contents was detected after the bacterial treatment compared to the non-inoculated plants under the same drought conditions (Saikia et al. 2018).

Hou et al. (2018) showed that *B. methylotrophicus* could alleviate drought stress even in the presence of a plant-growth inhibitor such as ferulic acid (FA). The study showed that the applied bacterial treatment decreased FA in the soil, with the capacity to increase soil water content, catalase, sucrose activities, and polysaccharide levels at each of the tested FA concentration. The bacterial treatment seemed to limit leaf wilting under drought stress. As in some of the other cases of *Bacillus* spp. treatment, proline, soluble sugars, ascorbate, and glutathione contents were all increased by the *B. methylotrophicus* (Hou et al. 2018). *Glycyrrhiza uralensis* Fisch. ex DC (Chinese liquorice) treated with *B. pumilus* under drought stress showed an increase in total biomass, flavonoids, polysaccharide, glycyrrhizic acid, and antioxidant enzymes, but a reduction in ROS and lipid peroxidation. These changes were believed to confer drought tolerance to the cultivated *G. uralensis* (Xie et al. 2019).

21.6 Molecular Mechanisms in Alleviating Drought Stress by *Bacillus* spp.

Despite the benefits of PGPR in alleviating drought stress to crop species (Saikia et al. 2018), key mechanisms that elicit the plant stress tolerance are yet to be well understood (Abd El-Daim et al. 2018, 2019; Lu et al. 2018; Wang et al. 2019). PGPR have been shown to play a role in plant resistance and adaptation to drought stress (Vurukonda et al. 2016), but changes at molecular levels in response to PGPR priming are not well characterized. Some of the possible molecular modes of action induced by *Bacillus* spp. in alleviating drought stresses are illustrated in Fig. 21.2. Transcriptomic analysis appears promising for revealing key pathways affected by plant-PGPR interactions (Malviya et al. 2020), and several studies have been carried out, with some candidate genes identified for potential drought stress tolerance (Abd El-Daim et al. 2018, 2019; Lu et al. 2018; Wang et al. 2019). Studies on the application of *Bacillus* spp. for drought tolerance are summarized in Table 21.2.

PGPR can trigger osmotic responses and activate genes involved in pathways that help plant survival under drought stresses (Vurukonda et al. 2016). Several *Bacillus* species that improve plant tolerance to drought stresses (Hou et al. 2018; Kasim et al. 2013; Li et al. 2019; Lim and Kim 2013; Vardharajula et al. 2011) have been studied for molecular modes of action (Table 21.3). Differential RNA and protein accumulation patterns were observed in pepper plants (*C. annuum*) inoculated with *B. licheniformis* K11 under drought stress conditions via two-dimensional

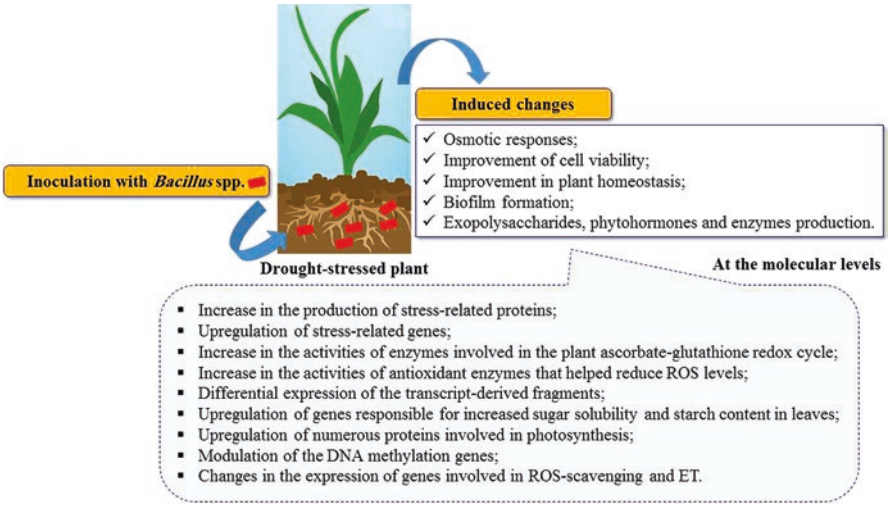

Fig. 21.2 Molecular mechanisms induced by *Bacillus* spp. to mediate drought stress tolerance in plants

Table 21.2 Examples of using *Bacillus* species for drought-stress tolerance in different plant species.

Bacillus species (strain)	Plant	Effects or putative mechanisms	References
Bacillus methylotrophicus (CSY-F1)	*Cucumber (Cucumis sativus* L.*)*	The increase of polysaccharide levels, plant relative water content and osmotic potential; Increased antioxidant enzyme activities (superoxide dismutase (SOD), catalase, guaiacol peroxidase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase); Increased proline and soluble sugar ascorbate and glutathione contents levels in soil; Decreased levels of superoxide radical, hydrogen peroxide, and malonaldehyde; Increased gene expression of drought-responsive genes CsPYL1 and CsPYL2	Hou et al. (2018)
B. amyloliquefaciens SN13	Rice (*Oryza sativa* L.)	Phytohormone induction; Increased proline and total soluble sugar content	Tiwari et al. (2017)

(continued)

Table 21.2 (continued)

Bacillus species (strain)	Plant	Effects or putative mechanisms	References
B. megaterium (BOFC15)	*Arabidopsis* (*A. thaliana* (L.) Heynh.)	The production of spermidine (Spd) and a type of polyamine (PA); Increased the level of abscisic acid (ABA) and polyethylene glycol (PEG) in plants	Zhou et al. (2016)
B. subtilis (B26)	Timothy grass (*Phleum pratense L.*)	Metabolic changes; Increased root and shoot biomass; Increased the activity of photosynthetic and stomatic conductance, synthesis of chlorophylls a and b and carotenoids which increases photosynthesis, and decreasing concentration of carbohydrates and amino acids	Gagné-Bourque et al. (2016)
B. subtilis (B26)	Purple false brome *Brachypodium distachyon* (L.) P. Beauv.	Increased root and shoot weights, accelerated growth rate and seed yield, and total soluble sugars and starch contents; Upregulation of the drought- responsive gene expression (DREB2B-like, DHN3-like, and LEA-14-A-like) and modulation of the process of DNA methylation genes (MET1B-like, DRM2-like, and CMT3-like)	Gagné-Bourque et al. (2015)
B. licheniformis (Rt4M10)	Grapes (*Vitis vinifera* L. *cv. Malbec*)	Increased ABA synthesis, indole-3-acetic acid (IAA) and the gibberellins.	Salomon et al. (2014)
B. thuringiensis	Fringed lavender (*Lavandula dentate* L.)	increasing K content and IAA, by depressing stomatal conductance, and the control of shoot and proline accumulation**,** decreased the activity of glutathione reductase and ascorbate peroxidase which improve nutritional, physiological, and metabolic activities	Armada et al. (2014)
B. amyloliquefaciens (5113)	Wheat (*Triticum aestivum* L)	The increase of enzyme activity involved in the plant ascorbate–glutathione redox cycle; the improvement of homeostasis	Kasim et al. (2013)
B. licheniformis (K11)	Chili/pepper (*Capsicum annuum L.*	Modification of proteins and gene expression patterns and the increase of production of auxin and ACC deaminase	Lim and Kim (2013)

(continued)

Table 21.2 (continued)

Bacillus species (strain)	Plant	Effects or putative mechanisms	References
Bacillus spp.	Maize (*Zea mays* L.)	The increase of plant biomass, longer root and shoot length, relative water content, leaf water potential, root adhering soil/root tissue ratio, aggregate stability, decreasing leaf water loss; The increase of production and accumulation of osmoregulation proline, sugars, free amino acids and exopolysaccharides and decreased electrolyte leakage reduced the activity of antioxidants enzyme (catalase, glutathione peroxidase)	Vardharajula et al. (2011)
B. altitudinis	Rice	Production of aminocyclopropane-1-carboxylate (ACC) and indole acetic acid; Increased relative water content, chlorophyll stability index, and membrane stability index Increased proline content, phenolics content, catalase activity and reduced malondialdehyde (MDA)	Kumar et al. (2017)
B. subtilis RJ46	Pea and mungo bean (*Pisum sativum L.* and *Vigna mungo L.*)	Increased seed germination rate; Increased shoot and root length and biomass; Decreased in ACC accumulation and expression of the ACC oxidase gene regulating plant ethylene levels; Increased ROS-absorbing cellular enzymes and osmolytes, leaf chlorophyll content, relative water content (RWC) and intensified root recovery	Saikia et al. (2018)
B. thuringiensis	Maize	Decreased oxidative damage due to the accumulation of lipids and proline Regulate several aquaporins that are able to transport water, CO_2 and other compounds such as glycerol, urea hydrogen peroxide (H_2O_2) or boric acid	Armada et al. (2015)
B. pumilus	Chinese liquorice *Glycyrrhiza uralensis* Ledeb.)	Increased total biomass, total flavonoids, total polysaccharide, glycyrrhizic acid and antioxidant enzymes; Reduced ROS and the level of lipid peroxidation	Xie et al. (2019)
B. thuringiensis AZP2	wheat	Increased plant biomass; Reduction of stress volatile emission; Higher photosynthesis activity	Timmusk et al. (2014)

Table 21.3 Induction of drought-responsive genes in plants by *Bacillus* spp.

Bacillus species and strain	Plant	Drought responsive genes	References
B. cereus AR156, *B. subtilis* SM21*	Cucumber (*Cucumis sativa*)	Reduced expression of drought-triggered genes *cAPX, rbcL, rbcS.*	Wang et al. (2012)
B. methylotrophicus CSY-F1	Cucumber (*Cucumis sativa*)	Enhancement of transcript levels of the drought-responsive genes *CsPYL1* and *CsPYL2*.	Hou et al. (2018)
B. licheniformis K11	Pepper (*Capsicum annuum*)	Increased of stress protein genes *Cadhn*, *VA, sHSP* and *CaPR-10.*	Lim and Kim (2013)
B. pumilus DH-11 and *B. firmus* 40	Potato (*Solanum tuberosum*)	Putative changes in the expression of genes encoding the ROS-scavenging enzymes and ethylene biosynthesis.	Gururani et al. (2013)
B. amyloliquefaciens 5113*	Wheat (*Triticum aestivum*)	Upregulation of stress related genes *APX1*, *SAMS1*, and *HSP17.8*.	Kasim et al. (2013)
B. amyloliquefaciens 5113	Wheat (*Triticum aestivum*)	Upregulation of stress related genes *IA20* and *IA24* which are homologous to abscisic acid (ABA) responsive proteins.	Abd El-Daim et al. (2018)
B. amyloliquefaciens 54	Tomato (*Solanum lycopersicum*)	Increase in the expression levels of stress-responsive genes such as lea, tdi65, and ltpg2.	Wang et al. (2019)
B. amyloliquefaciens FZB42	Arabidopsis (*Arabidopsis thaliana*)	Increase in the relative expression levels of drought defense-related marker genes such as *RD29A, RD17, ERD1,* and *LEA14*.	Lu et al. (2018)
B. velezensis 5113	Wheat (*Triticum aestivum*)	Upregulation of proteins ID 194, 192 and 218 involved in photosynthesis process.	Abd El-Daim et al. (2019)
B. subtilis LDR2	Wheat (*Triticum aestivum*)	Upregulation of *TaCTR1* gene-a regulatory component of ethylene signaling pathway- and the increase in the expression of *TaDREB2* gene encoding a transcription factor which is crucial for improving the tolerance of plants to abiotic stress conditions, including drought.	Barnawal et al. (2017)
B. subtilis B26	Stiff brome (*Brachypodium distachyon* Bd21)	Upregulation of the drought-response genes, *DREB2B*-like, *DHN3*-like and *LEA-14-A*-like and the modulation of the DNA methylation genes, *MET1B*-like, *CMT3*-like and *DRM2*-like.	Gagné-Bourque et al. (2015)

**B. cereus* AR156, *B. subtilis* SM21 and *B. amyloliquefaciens* 5113 have been tested in mixture with *Serratia* sp. XY21 and *Azospirillum brasilense* NO40, respectively.

electrophoresis (2D-PAGE) and differential-display PCR (DD-PCR), respectively. Six differentially accumulated stress-related proteins were identified in the inoculated plants, among which Cadhn, VA, sHSP, and CaPR-10 showed more than 1.5-fold increases over those in non-inoculated plants. These changes were attributed to the increases in auxins and ACC deaminase in inoculated plants (Lim and Kim 2013). The use of quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) discovered the upregulation of stress-related genes APX1, SAMS1, and HSP17.8 in the leaves of wheat and increased activities of enzymes involved in the plant ascorbate-glutathione redox cycle in plants treated with *B. amyloliquefaciens* 5113 and *Azospirillum brasilense* NO40. The treatment showed an attenuation in transcript levels that indicates an improvement in plant homeostasis. In other words, the treatment resulted in increased activities of antioxidant enzymes that helped reduce ROS levels in drought-stressed plants (Kasim et al. 2013). Also on wheat, these molecular changes caused by the treatment of *B. amyloliquefaciens* 5113 helped extend the water holding in plants for 5 additional days, transcript profiling based on cDNA-amplified fragment length polymorphism (cDNA-AFLP) showed an upregulation of numerous genes in the leaves under drought stress condition, while the majority of these genes were downregulated in control plants. Differential expression of the transcript-derived fragments (TDFs) IA20 and IA24 in the leaves of treated wheat seedlings was also observed; functional studies by virus-induced gene silencing (VIGS) suggested that IA20 and IA24 silencing can compromise the effect of *B. amyloliquefaciens* 5113 treatment in mediating the drought tolerance (Abd El-Daim et al. 2018). Gagné-Bourque et al. (2015) have demonstrated that *B. subtilis* (B26 strain) confers drought stress resistance through the upregulation of genes responsible for increased sugar solubility and starch content in leaves, and the production of phytohormones, especially the solubilization of phosphorous and production of IAA, cytokinin, and zeatin riboside.

The mechanisms of another *B. amyloliquefaciens* strain (54) in drought stress relief were further elucidated by analyzing the expression of several stress-related genes in tomato plants (*Lycopersicon esculentum* Mill.); the relative transcript levels of *lea*, *tdi65*, and *ltpg2* genes increased under drought stress conditions, based on RT-qPCR (Wang et al. 2019). Biofilm formation also stimulated helpful to the overexpression of these genes and the drought stress tolerance. The *B. amyloliquefaciens* FZB42 strain induced systemic drought tolerance in *Arabidopsis*, with the involvement of RD29A, RD17, ERD1, and LEA14, genes although the underlying molecular processes associated with the improvement of cell viability under the stress still need to be elucidated (Lu et al. 2018). It seems that the bacterial treatment affects phytohormone-mediated pathways, especially those influenced by ET and jasmonic acid (JA) in the induction of drought tolerance. Exopolysaccharides produced by the bacteria also appeared beneficial in protecting the plants from drought stress, based on the root length and plant branching, biomass, and survival rate. It was hypothesized that exopolysaccharides may comprise a microbe-associated molecular pattern that, once recognized by plants, induces drought tolerance (Lu et al. 2018). Inoculation of wheat with the *B. velezensis* strain 5113 induced metabolic modulation and the abundance of several proteins in leaves, which resulted in

increased drought stress tolerance (Abd El-Daim et al. 2019). Proteomic profiling of leaves from treated plants showed differential accumulation of numerous proteins involved in photosynthesis, including the ID 194, 192, and 218 proteins which were downregulated in control plants under drought. This suggests that *B. velezensis* 5113 provides the protection of photosynthesis in stressed plants through adjusting the expression of several proteins pertaining to stress defense, as well as energy supply (Abd El-Daim et al. 2019).

The molecular effect of *B. subtilis* B26 on the full life cycle of Purple false brome grass (*Brachypodium distachyon* L.), a well-established model species used for functional genomics in cereal crops and temperate grasses, has been studied, and both seedlings and mature plants inoculated with the bacterial inoculum showed reduced phenotypic effect under acute and chronic drought stress via the upregulation of drought response genes, including DHN3-like and LEA-14-A-like genes and the transcription factor that modulates the DREB2B-like gene, which binds dehydration-responsive elements (Gagné-Bourque et al. 2015). The modulation of the DNA methylation genes, i.e., MET1B-like, CMT3-like, and DRM2-like, involved in the process regulation is also involved in drought tolerance mediated by *B. subtilis* B26 (Gagné-Bourque et al. 2015). Another strain of *B. subtilis* (LDR2), when applied with other PGPR, enhanced the drought stress tolerance in wheat by modulating the regulatory component of ET signaling pathway, with increased expression of TaDREB2 gene. It is worth pointing out that this gene encodes a transcription factor crucial to improved plant tolerance to abiotic stresses (Barnawal et al. 2017). Treating cucumber plants (*Cucumis sativus* L) with a consortium of three PGPR strains, i.e., *B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21, produced a range of physiological indicators relevant to drought tolerance, without involving ACC deaminase (Wang et al. 2012). Drought tolerance was achieved via multiple mechanisms that help protect leaf cell membranes from being damaged by reduced water availability. The reduced expression of drought-triggered genes such as cAPX, rbcL, and rbcS that encode cytosolic ascorbate peroxidase and ribulose-1,5-bisphosphate carboxy/oxygenase (Rubisco) subunits was among those mechanisms (Wang et al. 2012). Changes in the expression of genes involved in ROS-scavenging and ET biosynthesis have been identified following the inoculation of potato plants with *B. pumilus* DH-11 and *B. firmus* 40 under drought stress conditions (Gururani et al. 2013).

21.7 Mass Production, Formulation, and Applications of *Bacillus* spp.

So far, not many PGPR have been commercialized, mainly due to their poor shelf lives and resilience under field conditions (Gotor-Vila et al. 2017; Radhakrishnan et al. 2017). *Bacillus* spp. can be a good candidate for commercial applications because this genus of bacterium forms endospores that resist adverse environmental conditions and are able to maintain the stability and efficacy throughout the

commercial process (Mari et al. 2014; Stojanović et al. 2019). Efficient mass production and effective formulation are crucial factors for the viable development of bacterial inoculants (Lobo et al. 2019; Schisler et al. 2004). Optimized medium composition and fermentation conditions are often the first step for stable and efficacious bacterial products (Schisler et al. 2004).

Bioformulation begins with microbial fermentation to obtain cell biomass with desired density and sporulation efficiency (Stojanović et al. 2019). Standard medium such as nutrient broth or Luria Bertani (LB) is commonly used to start inoculum cultures before they are transferred to an industrial growth medium consisting of sources of carbon, nitrogen, inorganic salts, and other ingredients needed for bacterial growth. The onset of sporulation is often initiated or triggered by a reduction in glucose concentration after the exponential phase of growth (Stojanović et al. 2019). The optimal temperature for fermentation is between 30 °C and 37 °C, depending on the strain, with pH ranging from six to seven for most bacteria. Increasing the concentration of available oxygen can sometimes enhance cell numbers, sporulation, and the yield of metabolites (Suresh et al. 2009), as members of the genus *Bacillus* are aerobic or facultative anaerobic bacteria. Agitation during fermentation helps increase the amount of oxygen dissolved in liquid media but may affect the sporulation negatively, as higher agitation rate may result in shear stress, which may reduce the sporulation. For example, an agitation rate of 200 rpm was recommended for efficient sporulation of *B. amyloliquefaciens* in liquid fermentation (Tzeng et al. 2008). The scaled-up fermentation and formulation may use dry and liquid processes (Schisler et al. 2004), and the products can be wettable powders, dusts granules, cell suspensions in water, oil, or emulsions (Schisler et al. 2004). Additives and carriers (fillers, extenders) often are added during the mass fermentation and formulation, and these materials should be cost efficient and easy to handle. Carriers are used also to enhance the chemical, physical, and/or nutritional properties of formulated products; additives can act as stimulant to enhance the product efficacy (Schisler et al. 2004). Commonly used carriers include talc, coal, peat, clays, vermiculite, perlite, and plant waste materials (Tripti et al. 2017). Additional substances in carriers may include polymers, sugars, milk, honey, polyols, amino acids, albumin, vegetable oils, pero-dexin (a by-product of the starch industry), and coconut water (an industrial waste) (Gotor-Vila et al. 2017). Waste proteins have been used as additives in the solid-state fermentation of *B. amyloliquefaciens* as a bio-organic fertilizer (Huang et al. 2015). With a low water content, dry formulations generally allow PGPR to survive longer and tolerate higher temperatures better than in a liquid. Several methods may be used for drying, including air drying, desiccation, freeze drying (lyophilization), spray drying, and shade drying, with freeze drying being used most widely in industrial scales. Freeze drying and spray drying are more expensive than other drying methods due to the need for specialized equipment (Gotor-Vila et al. 2017; Lobo et al. 2019). In contrast, liquid formulations can be used directly without rehydration (Lobo et al. 2019). To meet the quality standards, the inoculum concentration often is required in the range of 10^7 to 10^9 colony-forming units (CFU) per mL (Malusá and Vassilev 2014). Table 21.4 lists several examples of liquid and dry formulations from recent publications.

Table 21.4 Selected examples of *Bacillus*-based formulations from recent publications

Microorganism	Carrier/ additive	Drying method	Number of viable cells	Shelf life	Assayed crop	Application	References
Liquid formulation							
Bacillus siamensis SCFB3-1	carrageenan	-	10^9 CFU/ml	-	Sweet pepper (*Capsicum annuum*)	biofertilizer	Pastor-Bueis et al. (2017)
B. polymyxa, *B. macerans*, *B. circulans*		-	10^8 CFU/ml	-	Potato (*Solanum tuberosum*)	biofertilizer, biocontrol	Abbas et al. (2014)
dry formulation							
Bacillus sp. A30	biochar, flyash	-	1.35×10^8 CFU/g (biochar); 10^7 CFU/g (flyash)	240 days at 27°C	Tomato (*Lycopersicon esculentum*)	biofertilizer	Tripti et al. (2017)
B. endophyticus 77-NS2, *B. sphaericus* 77-CS-S1, *B. safensis* PSB5, *B. megaterium* PSB12	biogas sludge, soil	-	10^6-10^8 CFU/g	-	Wheat (*Triticum aestivium*)	biofertilizer	Mukhtar et al. (2017)
B. amyloliquefaciens FZB42	alginate, gum arabic, talc	freeze-drying	1.3×10^7 CFU/g	-	Maize (*Zea mays*)	biocontrol	Berninger et al. (2016)
B. cereus B25	talc	55°C for 36 hours	10^9 CFU/g	360 days at room temperature	Maize	biocontrol	Martínez-Álvarez et al. (2016)
B. subtilis SB-MYP-1	soybean flour	freeze-drying	1.3×10^8 CFU/g	-	-	-	Mahidsanan et al. (2017)
Bacillus sp. CaB85	talc	shade air drying	9×10^8 CFU/ml in culture	45 days	Cowpea (*Vigna unguiculata*), lady's finger (*Abelmoschus esculentus*)	biofertilizer	Basheer et al. (2019)

CFU: colony forming units

Bacillus spp.-based products are among the most promising biofertilizers, biopesticides, and bioremediators for agro-industrial applications. These products often are positioned as environmentally friendly alternatives to conventional agrochemicals. Biofertilizers are promoted to improve plant nutritional status by either increasing soil nutrients or making them more accessible to plants (Malusá and Vassilev 2014). *Bacillus* spp.-based biofertilizers can increase the intake of nitrogen and phosphorus by plants; the first bacterial fertilizer Alinit was developed from *Bacillus* spp. in 1897 by Bayer AG, which enhanced crop yield by 40% (Kilian et al. 2000). Another biofertilizer based on the *Bacillus* sp. strain A30 promoted the growth and yield of tomato (*L. esculentum*) (Tripti et al. 2017). Biopesticides (also called bioprotectants) based on *Bacillus* spp. may promote plant growth by controlling diseases and pests (Malusá and Vassilev 2014). Be present in rhizosphere in a large quantity, *Bacillus* spp. may stimulate plants to release a wide range of metabolites, including plant hormones, siderophores, cyanides, and antibiotics (Stojanović et al. 2019), as well as enzymes that degrade fungal cell walls (Hashem et al. 2019). *B. amyloliquefaciens* (SF14 and SP10 strains) controlled brown rot disease caused by *Monilinia* spp. on apple by reducing the mycelial growth and inhibiting spore germination of pathogen (Lahlali et al. 2020). Furthermore, *Bacillus* spp.-mediated bioremediation of environmental pollutants has been shown to break down contaminants in soils (Stojanović et al. 2019). A mixture of bacterial suspension consisting of *B. pumilus* and *B. cereus* degraded up to 84% of total petroleum hydrocarbons in contaminated soils (Patowary et al. 2016).

21.8 Conclusion and Future Prospects

Drought is a destructive abiotic stress that significantly influences crop yield and life span by inducing negative morphological, physiological, biochemical, molecular, and genetic changes. These changes often coincide with the production of free radicals, decrease in chlorophyll, and increase in the biosynthesis of ET membrane lipid peroxidation. Plant rhizosphere has intense microbial activities with a wealth of exuded metabolites. *Bacillus* spp. are frequently attracted to the rhizosphere, resulting in the biofilm formation. The root colonization by *Bacillus* spp. is mutually beneficial, and the bacteria have often been observed to assist plant survival and growth under stress conditions by inducing a range of stress tolerance mechanisms in plants, including stress signaling, improved regulation of water transport, efficient uptake of nutrients from soil, and activation of antioxidants and plant defense systems. Many of these pathways are triggered by the production of phytohormones and volatile compounds (VOCs)-related metabolites, activity of ACC deaminase, accumulation of osmoprotectants and exopolysaccharides, and the regulation of stress-responsive genes in response to drought stresses. The research on *Bacillus* spp. for stress mitigation has only scratched the surface, and more can be done to develop a variety of viable products for novel options in addressing drought and other environmental stresses.

New studies to further decipher the mechanisms employed by *Bacillus* spp. to mitigate the impact of drought stress will be valuable to develop sustainable strategies to cope with climate changes. Nowadays, research on the involved mechanisms is in progress but remains largely speculative. In this context, a combination of proteomics and metagenomics (high-throughput DNA sequencing), metabolomics, and advancing computational data-mining approaches might be useful to understand bacteria belonging to *Bacillus* genus and their interactions with their host plants and to reveal more information on mechanisms underlying tolerance to drought stress. Similarly, it is important to conduct a deep analysis of both plant and *Bacillus* genes expressed during drought stress to increase our knowledge on how the inoculation with *Bacillus* spp. confers tolerance to stress in several plant species.

Although the research mentioned here highlights the effectiveness of *Bacillus* spp. to alleviate the impacts of drought stress in agricultural crop plants, more systemic studies seem to be needed. One weakness of the research described here is the use of sterilized soils to study the role of *Bacillus* spp. in drought stress alleviating. In other words, it is difficult to take results achieved through the use of sterilized soils and to generalize them for "real" field conditions. The applicability of these results to real conditions is not straightforward. Furthermore, as it has already been raised by Ngumbi and Kloepper (2016) for PGPR, the diversity of physical and chemical characteristics of soils, which may be impacted by both drought stress and the PGPR and which may also further impact beneficial microorganisms, need to be taken into account when studying the role of *Bacillus* spp. in alleviating drought stress. Since under natural conditions, drought often occurs with other biotic and abiotic stresses; further studies are needed to address the role of *Bacillus* spp. to simultaneously mitigate numerous stresses. Biotic stressors such as pathogens and insects are among the most important stresses damaging crops. Since numerous *Bacillus* spp. showed effectiveness to suppress phytopathogens (Deng et al. 2011; Gowtham et al. 2018; Khan et al. 2020; Kushwaha et al. 2020; Lahlali et al. 2020), the identification of *Bacillus* spp. strains with potential to confer tolerance to both drought and biotic stress would be of great interest. Furthermore, the comprehension of the basic mechanisms behind ISR and those behind the observed *Bacillus*-mediated drought tolerance overlap would be of great benefit for future applications.

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Chapter 22
Unveiling the Potential of *Bacillus* sp. in Bioremediation and Biocontrol

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Abstract Bioremediation emerges as sustainable and environment-friendly approach in which role of microbes is most prominent after hyperaccumulator plants. Microbes including many *Bacillus* spp. and *Pseudomonas* spp. are highly efficient by producing various enzymes and secondary metabolites that enhance the bioremediation process. Many *Bacillus* spp. produce diverse secondary metabolites such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, and isocoumarins. These metabolites have the ability to decontaminate the heavy metal(liods) and organic pollutants. Bioremediation is an efficient and desirable management tool that can be used to regain the environmental sustainability. On the other hand, biocontrol of plant diseases by the application of some elite strains of *Bacillus* spp. is an ecofriendly approach alternative to the hazardous synthetic pesticides. This chapter is focused on the use of *Bacillus* sp. for bioremediation. The present chapter describes the bioremediation processes by the application of *Bacillus* species and briefly discusses the mode of action of *Bacillus* spp. on these two processes.

Keywords *Bacillus* sp. · Bioremediation · PGPR · Secondary metabolites · Types of bioremediations

22.1 Introduction

Bioremediation is the process of removing contaminants from the environment using microbial agents (such as fungi, yeast, or bacteria). Out of many definitions, the most appropriate and self-explanatory one is "the use of biological processes to eliminate, mitigate, or transform contaminated substances" (Abatenh et al. 2017). In

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many countries, aquatic ecosystems are mostly affected by point and nonpoint sources of pollution. There are many sources of pollution in water which depend upon directly discharged of pollutants in the aquatic water bodies. Among the known point sources, municipal and industrial wastewaters are the most common. However, landfill leachate, industrial site runoff and drainage, and ship runoff are the nonselective water sources in farmland and orchard rivers and urban runoff in open areas (Divya et al. 2015).

It is known that microorganisms can remediate pollutants from contaminated soil and water systems either by transforming, sequestering, or immobilizing these. To survive in an environment contaminated by high concentrations of pollutants, many microorganisms have developed resistance mechanism either by producing certain enzymes or secondary metabolites, and it is known that most microorganisms have specific genes for tolerating such high concentrations of pollutants (Adams et al. 2015). Bacteria are the most frequently used microorganisms and make up a large part of the total terrestrial biomass of 1018 g (Jayashree et al. 2012). Bacteria are used as remediating agent due to its ubiquitous capacity to grow and nature of adoptability in adverse conditions (Venosa et al. 2002). Some microorganisms used in the pollutants remediation and sequestration processes are *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Acinetobacter*, *Corynebacterium*, *Flavobacterium*, *Micrococcus, Mycobacterium*, *Nocardia bacteria*, *Pseudomonas*, and *Vibrio*. These microbes have also been reported to remediate the highly polluted sites even with multiple kinds of pollutants either organic or inorganic (Abatenh et al. 2017).

Bacillus sp. are rod-shaped, Gram-positive bacterium that normally needs aerobic or facultative aerobic environments. Under stress conditions, these can produce oval endospores that can retain their efficacy for both plant growth promotion and remediation (Arkhipova et al. 2005). *Bacillus* sp. produces a variety of enzymes that allow them to degrade/decontaminate various complex substrates under dynamic environmental conditions (Santoyo et al. 2012). The biological remediation of anthropogenic pollutants depends on the nature of the microbes, the chemical species of pollutants, geo-environmental conditions of the polluted site (Ojuederie and Babalola 2017). Microbial degradation depends on the nature of microorganism and the production of certain enzymes which are required for the degradation/decontamination of the pollutants (Goyal et al. 2019).

Bacillus sp. has been used due to its potential to remediate under biological remediation of water pollution in septic tanks and fish-farming ponds. The use of *Bacillus brevis*, *B. sphaericus*, and *B. megaterium* leads to the decomposition of 3-hydroxybenzoate (Zokaeifar et al. 2012). Various bacteria can vigorously metabolize contaminants by using them as an energy source, but others produce the enzymes which break down pollutants into a less toxic substance (Igiri et al. 2018). *Bacillus* bacterium can selectively take in gold from solutions that include Cu, Zn, Fe, and Au (Boopathy 2000). Biodegradability of petroleum hydrocarbon contaminated soil by micrococcus, *Bacillus*, and coryneform bacteria. Regarding the biological decolorization and biodegradation of dye molecules, methods for the removal of dyes from wastewater were investigated (Mondol et al. 2013).

Bioremediation of heavy metals in polluted soil can be done by using *Bacillus*, *Pseudomonas*, and fungi under various environments and under influence of geochemical behavior of the polluted sites (Idris et al. 2004).

This chapter includes brief introduction of *Bacillus* sp. and its efficacy in removing pollutants, while its metabolites and enzymes have also been elucidated. Many studies and books had been published on these aspects of the *Bacillus* spp., but this chapter has the brief and better understandable review of the topic.

22.2 Role of *Bacillus* sp. in Bioremediation Process

Remediation technology using microbes can inexpensively remove pollutants to protect human health and the environment (Abatenh et al. 2017). In many facilities, exogenous or genetically modified microorganisms are used for biodegradation/decontamination of pollutants. An efficacious, cost-effective microbial bioremediation process depends on hydrogeological circumstances, pollutants concentration, microbial ecosystems, and other considerations that change over time which can either be favorable or harsh for the process. In bioremediation procedure, microbes use pollutants as nutritional supplements or energy (C) resources. Enhancing nutrients source (N and P), electron acceptors (O_2), and a substrate (CH_4, C_6H_6O, and C_7H_8) or by introducing microorganisms with the necessary catalytic capabilities can improve the bioremediation activities in soil, and this process can either be biostimulation or bioaugmentation later discussed in this chapter (Jayashree et al. 2012).

Heterotrophic microbes use electron acceptor nitrite or NO_3 rather than O_2 and use organic matter as a source of energy, carbon to reduce oxidized nitrogenous substances (nitrite to nitrate) to nitrogen gas. Denitrification is widespread in Gram-negative bacteria like the *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Paracoccus*, and *Thiobacillus*. Denitrification is a slowdown process, especially for industrial wastewater with a high nitrate concentration. It was found that after adding *Bacillus thuringiensis* FQ1, the total amount of cadmium increased by 14.29% to 97.67%. When treated with bacterial and fungal consortium, 95.07% of the phenanthrene was removed, and the peak concentration was 500 mg/kg phenanthrene (Goyal et al. 2019; Pratush et al. 2018). Liu et al. (2015) reported that both lead and cadmium can affect the degradability of *Bacillus*. *Bacillus* sp. affects the level, composition, and activity of enzymes during the degradation of phenanthrene, while cadmium is more toxic to *Bacillus* sp. than lead, which also interferes with the *Bacillus* sp. efficiency to remediate the polluted sites.

22.2.1 Mechanism of Microbial Bioremediation

Microbes use different strategies to convert heavy metals. Microbes can alter the ionic nature of the different noxious elements which reduced their mobility, translocation, and bioavailability (Ayangbenro and Babalola 2017). The bioremediation of heavy metals can be achieved by the following methods, like biosorption, bioconcentration, immobilization, biomethylation, or organometallic complexation. The substance turns into a radionuclide converted. A brief description of the basic metalworking processes used by different microorganisms is as under:

22.2.1.1 Mobilization

Redox reaction technique enables to dissolve and transform hazardous metals and their radionuclides into inorganic-organic acids in this process. It can lower the pH of contaminants as well. Four subprocesses are further divided into complete mobilization processes, namely, enzymatic oxidation, enzymatic reduction, complexation, and siderophore. Soil Pb immobilization mechanisms were explored using *B. subtilis* DBM, a bacterial strain extracted from heavy-metal-contaminated soil. In the removal of Pb^{2+} from the liquid process, adsorption and desorption experiments with active bacterial cells and dead cells showed that both extracellular adsorption and intracellular accumulation were involved. Of the sequestered Pb(II), 8.5 percent was retained inside the cell wall by physical entrapment, 43.3 percent was held by ion exchange, 9.7 percent was complexed or precipitated on the cell surface with cell surface functional groups, and 38.5 percent was accumulated intracellularly. Fourier transform infrared spectroscopic study revealed the complexity of Pb^{2+} with carboxyl, hydroxyl, carbonyl, amino, and phosphate groups. X-ray diffraction study revealed that precipitates of $Pb_5(PO_4)_3OH$, $Pb_5(PO_4)_3Cl$, and $Pb_{10}(PO_4)_6(OH)_2$ formed on the cell surface during the biosorption process (Bai et al. 2014).

22.2.1.2 Enzymatic Oxidation

In general, in more than one oxidation state, certain inorganic compounds exist, and it is commonly found that the higher oxidation state is less soluble than the lower ionic phase. In this case, by oxidizing higher states to lower states, the enzymatic oxidation mechanism (catalyzed by enzymes released by microorganisms) plays a very important role and increases the solubility of compounds. The elimination of inorganic materials from the solution is one of the essential methods. Heavy metals lose electrons in this step and turn into a useful or less toxic state. The more frequent example of enzymatic oxidation is *Thiobacillus ferrooxidans*, and *T. thiooxidans* oxidizes uranium from ore (Cumberland et al. 2016).

22.2.1.3 Enzymatic Reduction

The mechanism is reversed in contrast to the enzymatic oxidation process. Inorganic compounds with several oxidation states in their reduced form remain insoluble. The enzymatic reduction mechanism was found to be useful in removing these elements from the solution. Enzymatic reduction reactions are carried out by facultative and obligatory anaerobic microorganisms, in an in situ bioremediation. Božič et al. (2010) carried a study in which A novel β-nicotinamide adenine dinucleotide (NADH) disodium salt-dependent reductase has been isolated from *B. subtilis*, which can convert the redox dye CI Acid Blue 74, CI Natural Orange 6, and CI Vat Blue 1 is reduced to a leuco form soluble in water. In reference to pH and temperature conditions, enzymatic reactions were enhanced. In the presence of NADH cofactor consumed stoichiometrically, the reductase can reduce acid blue 74 and natural orange 6. At the same time, Vat Blue 1 required the presence of the intermediate 1,8-dihydroxyanthraquinone (Božič et al. 2010).

22.2.1.4 Complexation

When complexing, inorganic metal complexes are produced by adding ligands. Due to the formation of metal complexes, hazardous inorganic substances are transferred and can be easily extracted from solid waste (Ayangbenro and Babalola 2017). There are two main types of microbial chelating agents: (i) organic acids with low molecular weight (citric acid, tricarboxylic acid, and alcohol) and (ii) high molecular weight ligands, siderophores, and toxic metal bond compounds. In addition, certain amino acids that are synthesized by certain bacteria can also be used as complexing agents. It has been observed that the complexation of heavy metals and radionuclides with microorganisms is strongly dependent on the pH value (Ayangbenro and Babalola 2017). Gorman-Lewis (2014) Gibbs energy, enthalpy, and entropy of Cd and Zn complexation between *B. licheniformis* and zinc on *B. subtilis* were measured by surface complexation model and isothermal titration calorimetry. Their results indicate that the Cd and Zn complexes of *B. licheniformis* are driven by entropy at low pH and enthalpy at ambient pH. Complexation of zinc on *B. subtilis* is driven by entropy, indicating that *B. licheniformis* has different donor ligands, which dominate the pH response of the surrounding medium.

22.2.1.5 Siderophores Production

When microorganisms grow in iron-deficient media, some microorganisms produce iron-specific chelating agents, also called siderophores. These siderophores have specific linking groups, such as catecholate, phenolate, or hydroxamate. Due to the presence of these specific groups, the complexing properties of these siderophores are improved, and they form various complexes with toxic metals, thus increasing their solubility (Khan et al. 2018). In recent years, from different biological

systems, several siderophores or siderophore-like compounds have been identified. Siderophores are unique to iron (III), according to literature records, but recent studies have shown that they can also be complex with other metals and radionuclides (Ahmed and Holmström 2014). *Bacillus* sp. PZ-1's siderophore-producing characteristics and conditions were studied, and the improvement of siderophores on Pb uptake and translocation in *Brassica juncea* was determined. Single-factor experiment results showed that glucose, $Pb(NO_3)^2$, and pH could stimulate PZ-1 growth and development of siderophores. Optimized by the response surface method, under the conditions of glucose concentration of 21.84 g/l, pH of 6.18, and Pb (NO3) 2 concentration of 245.04 μmol/l, the maximum yield of siderophores obtained is 90.52%. The type of siderophore is hydroxamate, and its concentration in the fermentation broth is 32.24 μg/ml. The pot experiment results showed that the siderophore made the mustard rape absorb more Pb from the soil, with an absorption rate of 1.04–2.74, and absorbed more Pb from the underground soil to the soil with a TF value of 1.21–1.48. The results showed that the genus *Bacillus* PZ-1 can produce abundant siderophores, which can be used to increase the extraction of lead in the soil by plants (Yu et al. 2017).

22.2.1.6 Immobilization

To restore metal polluted soils, ex situ and in situ restriction procedures are used. Ex situ technology is used in heavily polluted areas. The soil in such areas is excavated from the original location and then stored in such a location for fixing metallic ions in that soil by treating it with various microbial systems. In terms of local technology, the soil contaminated with metals must be treated in its original location. During the fixation process, organic nitrogen is resulted from nitrate nitrogen conversion, which is the opposite to the process of mineralization. The fixation process is controlled by several soil bacteria due to which it is considered a biological process. The process is influenced by moisture and temperature of the soil. The fixation takes place through energy-dependent (active or passive) methods. According to the observations, passive fixation is not specific for metallic species, while active fixation depends mainly on metabolism of microbial cells and is relatively slow. In most cases, specific proteins such as metallothionein are used in active immobilization, which forms complexes with heavy metals. Immobilization of heavy metals occurs mainly through bioaccumulation, precipitation, and biosorption. These methods are commonly used to treat wastewater polluted by heavy metals (Ayangbenro and Babalola 2017). Effects of interaction between soil minerals and *B. subtilis* DBM for the adsorption of Pb(II) and Cu(II) were studied (Bai et al. 2019). The adsorption capacities of kaolinite and goethite for Pb(II) and Cu(II) improved after combination with DBM as compared to the independent mineral application. After combination with DBM, the site concentrations increased by 80% and 30% of kaolinite and goethite, respectively, based on the modeling outcomes by potentiometric titration. However, lower concentrations of observable sites in comparison to theoretical values were observed due to the presence of functional groups in DBM/

mineral combinations, and the combinations contributed to an improvement in NH_4NO_3 and EDTA-Na_2 desorption rates (Bai et al. 2019).

22.2.1.7 Precipitation or Solidification

Metal ions in solution or soil can be precipitated or solidified by a variety of approaches. Sulfate reduction is the most common method of precipitation. Reducing sulfate bacteria (SRB) are used to treat metal contaminants in technical natural systems such as built-up wetlands. SRB forms metal sulfide precipitates by removing toxic metals from the solution. Most toxic metal sulfides have very low solubility, and their toxicity is almost negligible to the environment after precipitation. SRB also creates solution conditions that are conducive to the chemical reduction of metals. The conversion of organophosphates to orthophosphates by microorganisms can also proceed to the precipitation of metals (intracellular phosphates can also fix metals) by forming metal phosphates (especially at a pH above 7) (Nguyen et al. 2020). The process of precipitation can be performed ex situ or in situ. The interactive effect of each approach has been discussed later in this chapter.

22.3 Factors Affecting Bioremediation Process

Natural soil microorganisms play a significant role in soil bioremediation as biogeochemical reagents and can convert complicated organic substances into simple inorganic substances and their derivatives; this process is known as mineralization (Adams et al. 2015). As a whole, soil particles are negatively charged, so by multivalent cations, soil and bacteria can be linked together (Bai et al. 2014). In bioremediation, microbes reduce, remove, and convert pollutants from soil, sediment, air, and water. Bioremediation explains how to use microbes to obliterate or remediate waste (Shanahan 2004). Such a process is dependent upon the optimal use of nutrients and other factors that support the biological functions (Divya et al. 2015; Jayashree et al. 2012). These are the following:

Pollutant concentration: This directly affects the activity of microorganisms if the concentration is too high. Conversely, small amounts of contaminants can prevent the degradation/decontamination by resisting the bacterial enzymes production.

Bioavailability of contaminants: This depends on the extent to which they are adsorbed on the solid or sequestered by molecules in the contaminated medium, the extent to which they diffuse into the macropores of the soil or sediment and on other factors like its form. If the bioavailability of the pollutant is low, then microbial reaction is lower because the pollutants are more strongly adsorbed in the solid, are contained in the molecular matrix in the contaminated medium, and continue to diffuse into the macropores of the aquatic bodies, soil, and sediments.

Geo-environmental conditions: This has a major impact on the efficacy of bioremediation strategies. For bioremediation applications, pH (6–8 optimal range), nutrient utilization, water content, temperature, and redox potential are important environmental conditions at the site.

Redox potential and oxygen content: These represent oxidation or reduction circumstances. Due to electron acceptors (like sulfate, manganese oxide, iron oxide, and nitrate), redox potential is influenced.

Nutrients: Microbial cell development and division are required. There are usually micronutrients that are suitable for bacterial growth, but these nutrients can be applied in available forms or by organic substrate modifiers (which can also be used as electron donors) to improve bioremediation.

Moisture content: The growth of microorganisms requires the optimal available of water in the ecological matrix. Microorganisms need 12%–25% water for optimal growth and proliferation.

Temperature: This directly disturbs the metabolic rate of microorganisms, which in turn affects the activity of microorganisms in the environment. To a certain extent, the rate of biodegradation increases or decreases with the increasing or decreasing temperatures (Goyal et al. 2019; Igiri et al. 2018; Ojuederie and Babalola 2017).

22.4 Role of *Bacillus* sp. in Remediating Different Contaminants

A few years ago, it is assumed that plants can only receive cytokinin from soils that have been synthesized by rhizosphere microorganisms (Arkhipova et al. 2007). Many researchers have explained the fact how plants evacuate their hormones, like cytokinins (Arkhipova et al. 2007). But *Bacillus* bacteria have demonstrated the capability to generate and secrete cytokinins in the rhizosphere, which has a positive impact on plant growth. Arkhipova et al. (2005) analyzed various *B. subtilis* strains to synthesize zeatin riboside-type cytokinin (ZR), dihydro-cellulose nucleoside (DHZR), and pentene to synthesize adenosine capacity (IPA). In an experiment inoculated with *Bacillus* strains in lettuce plants, that it had been detected later 2 weeks, root and shoot tissues contained more cytokinin than non-inoculated plants. The frequency of cytokinin is related to a 30% rise in plant weight. It is worth noting that extreme concentrations of plant hormones like indolyl-3-acetic acid (IAA) and abscisic acid (ABA) have also been noticed.

It indicates that *Bacillus* strains get double response on the growing plant. Enrichment of cytokinin and other ways to increase hormone synthesis (like IAA and ABA) promote plant growth and destroy the synthesis of other hormones such as erythromycin GA (Arkhipova et al. 2005). *Bacillus* bacteria synthesize various hormones that affect plant development, such as auxin (Valencia-Cantero et al. 2007). In the laboratory experiments, these were used to inoculate legumes with

B. megaterium UMCV1. Compared to non-inoculated plants, the tissues of legumes have higher iron (Santoyo et al. 2012).

22.4.1 Volatile Organic Compounds

It suggested that *B. megaterium* UMCV1 promotes development in an unknown way in *Arabidopsis* model plants. Ryu et al. (2004) provide a clear suggestion of PGPR's new PGP mechanism in *Arabidopsis*, including *B. subtilis* GB-3 and *B. amyloliquefaciens* IN-937a. By applying distinct compartments (separate petri dishes), one on the side of the petri dish is inoculated with different PGPR, while the seeds of *Arabidopsis* are placed on the far side to prevent physical contact. Attractively, the existence of *Bacillus* strains appreciably stimulated plant biomass. Since no plants showed an induction, *E. coli* DH5a bacteria were used as negative controls. The author believes that volatile chemicals are responsible for the chemical signal transmission among *Bacillus* strains and *Arabidopsis*. It was demonstrated that volatile substances, such as 2,3-butanediol and acetosyringone, are synthesized with bacteria and therefore play a crucial role in this interface.

In a supplement, individual compounds have similar effects on plants. Compared to the wild strain, this mutant strain showed a significant plant growth-promoting effect in the production of 2,3-butanediol and acetylase by *B. subtilis* BSIP1173 and BSIP1174. Several compounds identified involving aldehydes, ketones, and alcohols by gas chromatography may be involved in root development (Santoyo et al. 2012). Other volatile substances like 1-octen-3-ol and butyrolactone can also be involved in this collaboration between plants and bacteria. The effects of volatile compounds, particularly on plants, whether specific overall or for tissue, impact the growth of plant. This is also essential to explore the requirements for PGPR growth that can induce production of compounds, whether they have the beneficial effects, because of a particular compound or one synergies activity of numerous compounds (the combination shows better stimulatory activity) (Blom et al. 2011). To determine whether the discoveries are common, more detailed analysis of plant is needed as the results of using agricultural plants have significant interaction with microbes.

22.4.2 Heavy Metal Remediation by Bacillus *sp.*

Since bioremediation is the phenomenon of surface, the cell wall is of the genus *Bacillus*. The morphology and physiology change after the adsorption of cadmium. Research was carried out using FTIR and SEM-EDX (Nithya et al. 2011). After the adsorption of cadmium, the zeta potential analysis of *Bacillus cereus* RC1 confirmed that electrostatic interaction and surface complexation are of key importance for biosorption (Huang et al. 2014). This biosorbent capacity of *Bacillus* is due to several functional groups that are present on cell wall. Extracellular polymers (EPS)

are also involved in biosorption in addition to these functional groups. The composition of EPS includes carbohydrates and proteins as well as their derivatives in the form of homopolymers or heteropolymers (Shameer 2016). Bacterial cells have many benefits from EPS production, but they are essentially benefitting from biofilm formation, which contributes to antibiotic and metal resistance (Shameer 2016). According to a study, *B. subtilis* NSPA13, *B. licheniformis* NSPA5, and *B. cereus* NSPA8 produce EPS, which makes them cadmium resistant and helps reduce cadmium levels (Shameer 2016). Another study confirmed these results (Chauhan et al. 2017). A TEM analysis of the EPS of the unidentified isolate showed the presence of cadmium entrainment in the same study. This may be due to the existence of active carboxyl groups as determined by FTIR of the *Bacillus* extracellular polymer substrate (Chauhan et al. 2017; Shameer 2016)).

Studies conducted by several scientists have observed that dead and living cells of *Bacillus* can remove nickel time to time which highlights the probability that biosorption is the main mechanism of nickel bioremediation. Aryal (2015) recommended that with the help of FTIR, it could be proven that the -NH_2 and -COOH groups of *B. sphaericus* are accountable for the binding of nickel. It was further proved by studying the removal of nickel under the influence of temperature. It was observed that with increasing temperature from 20 °C to 40 °C, the bioremediation capacity of organisms also increased (Aryal 2015). However, if the temperature continues to rise, the potential decreases because of the decrease in surface activity. The study was concluded with the report that the bioremediation of nickel by *B. sphaericus* occurred through exothermic processes and biosorption. One more published report showed related results (Sari et al. 2008) in which after getting exposed to nickel, the surface morphology of the dead *B. subtilis* MTCC 1628 examined by SEM-EDX, which further confirms that biosorption was responsible for the bioremediation of nickel. In *B. licheniformis*, similar changes were observed (Jain et al. 2017). The results show that it is a passive process, namely, the biosorption of surface phenomena.

Bacillus cell walls supply many functional groups with negative charge such as hydroxyl, amino, sulfate, and carboxylate groups (Vijayaraghavan and Yun 2008). After the initial formation of metal complexes and the neutralization of chemically active sites, these functional groups showed ionic interactions with Ni^{2+} ions (Shekharaiah et al. 2020; Abou-Aly et al. 2019). When the cells of *B. subtilis* 117S were pretreated with formaldehyde, mercury chloride, and sodium azide, it was observed that the Ni-removing ability of the cells was decreased. It occurs due to methylation of amino groups and esterification of carboxyl groups. It concludes that in nickel bioremediation, the functional groups present on the cell wall of *Bacillus* are important (Abdel-Monem et al. 2010).

De et al. (2008) used bacteria which were mercury-resistant, such as *Pseudomonas aeruginosa*, *Brevibacterium* iodide, and *B. pumilus*, to remove lead and cadmium. In this study, *Pseudomonas aeruginosa* and *Aspergillus faecium* removed 70% and 75% cadmium, and *Pseudomonas aeruginosa* reduced cadmium (by 1000 mg/L and A by 19.2 mg/L. The removal is approximately 72 hours. *Brevibacterium iodinum* and *B. pumilus* can remove more than 96% lead by more than 87% and 88% from

1000 mg/L to 1.8 mg/L. In another study by Singh et al. (2013), native facultative anaerobic *Bacillus cereus* was used to detoxify hexavalent chromium. *Bacillus cereus* has an ability to remove chromate of 72% at a chromate concentration of 1000 μg/ml. The bacteria can remediate chromate in a wide temperature range (25 to 40 °C) and pH value (6 to 10). The best temperature range is 37 °C, and the initial pH is 8.0 (Singh et al. 2013).

Various heavy metals were tested using the bacterial species (such as *Pseudomonas*, *Flavobacterium*, *Micrococcus*, *Enterobacter*, and *Bacillus*). Their high bioremediation capacity is based on their potential active chemisorption sites on the cell wall and high surface-to-volume ratio (Mosa et al. 2016). Abioye et al. (2018) used *B. subtilis*, *B. megaterium*, *Aspergillus niger*, and *Penicillium* to investigate the bioremediation of lead, chromium, and cadmium in tannery wastewater. *B. megaterium* recorded the highest reduction in lead, i.e., 2.13 to 0.03 mg/L, followed by *B. subtilis*, i.e., 2.13 to 0.04 mg/L. *Aspergillus niger* showed the highest ability to reduce chromium concentration (1.38–0.08 mg/L), followed by *Penicillium* (1.38–0.13 mg/L), while *B. subtilis* showed the highest ability to reduce the cadmium concentration (0.4–0.03 mg/L) after 20 days, followed by *B. megaterium* (0.04–0.06 mg/L).

22.5 Production of Bioactive Compounds as Tools for Biocontrol of Phytopathogens

22.5.1 Polypeptides

Non-ribosomal peptides (NRP) are synthesized by expanding the activated monomers of amino acid building blocks through non-ribosomal peptide synthesized (NRPS) modules. The NRPS is organized in three consecutive stages per module. These modules are liable for the integration of particular amino acids incorporated in the polypeptide chain: as well as for adenylation, thiolation, and condensation (Fickers 2012). *Bacillus* strains generally produce two types of polypeptides, linear peptides, and de-peptide peptides (Table 22.1). The polypeptide antibiotics are produced by *Bacillus* (Zokaeifar et al. 2012), and these antibiotics are efficient and effective against Gram-positive bacteria, though there is relatively little production of broad-spectrum antibiotics and antifungals effective against Gram-negative bacteria (Deng et al. 2011). *Bacillus* has a broad range of antibacterial activities because they are used as antifungal agents, antiviral agents, and cellular anti-mutant agents as well as anti-mycoplasma drugs (Aziz 2018).

Table 22.1 List of some important bioactive compounds isolated from *Bacillus* strains

Compounds	Producing strains	Inhibitory concentrations	Nature of bioactivities	References
Halobacillin	*Bacillus* sp. CND-914	0.98 μg/mL (IC50)	Anticancer	(Kim et al. 2010)
Mixirin	*Bacillus* sp.	0.68 μg/mL (IC50)	Anticancer	(Zhang et al. 2004)
Bogorol A	*Bacillus* sp.	2 μg/mL (MIC)	Antibacterial	(Blunt et al. 2017)
Loloatin B	*Bacillus* sp.	1–2 μg/mL (MIC)	Antibacterial	(Yang and Yousef 2018)
Bacillistatins 1	*B. silvestris*	10–4–10 − 5 μg/mL (GI50)	Anticancer	(Shukla 2016)
Bacillamide	*Bacillus* sp.	LC50 after 6 h: 3.2 μg/mL	Antialgal	(Jeong 2019)
Bacilosarcin A	*B. subtilis*	82% inhibition at 50 μM	Plant growth regulator	(Komaki et al. 2016)
Macrolactin S	*B. Amyloliquefaciens*	0.3 and 0.1 μg/mL (MIC)	Antibacterial	(Raimundo et al. 2018)
Macrolactin V	*B. Amyloliquefaciens*	0.1 μg/mL (MIC)	Antibacterial	(Raimundo et al. 2018)
Basiliskamides A	*B. Laterosporus*	1.0 and 3.1 μg/mL 2.5 and 5.0 μg/mL	Antifungal	(Baindara et al. 2017)
α-Amylase	*Bacillus subtilis S8–18*	*N/A*	*N/A*	(Karakus et al. 2018)
Lipase	*Bacillus smithii* BTMS11	*N/A*	*N/A*	(Zin et al. 2017)

22.5.2 Lipopeptides

Lipopeptide (LP) biosurfactants consist of lipid tails associated with the short linear as well as the circular oligopeptides. They are generated by fungi (involving *Aspergillus*) and different types of bacteria like *Streptomyces*, *Pseudomonas*, and *Bacillus*. Lipopeptide has got great attention due to its antimicrobial, cytotoxic, antitumor, immunosuppressive, and surfactant-containing attributes (Pirri et al. 2009). The main mechanism of LP is the formation of pores in the membrane, which leads to a discrepancy in transmembrane plasma flow and cell demise (Baltz 2009). Cyclic lipopeptide (CLP) is a universal metabolite delivered by a wide range of bacterial species. They consist of short loop oligopeptides that are associated with the fatty acid tail and have effective surfactant characteristics (Ongena and Jacques 2008). CLP is produced in the non-ribosome of the large NRPS hybridase synthase (non-ribosomal peptide synthase) -PKS (polyketide synthase). Formation of endospores *B. subtilis* produces various antibacterial peptides, which are synthesized by ribosomes and modified after translation (such as lantibiotic and lantibiotic-type peptides) or non-ribosomes (Caulier et al. 2019).

Wheat blast is a devastating disease caused a filamentous fungus *Magnaporthe oryzae Trticum* pathotype which poses a serious threat to food and nutritional security in South America, South Asia, and Africa. Several strains of *Bacillus* spp. significantly biocontrol this destructive fungus both in vitro and in vivo. Recently, some linear lipopeptides produced by a strain of marine *B. subtilis* have been suppressed devastating wheat blast fungus *M. oryzae Triticum*.

Due to its antibiotic action, CLP has received considerable attention from humans and phytopathogenic microbes, including mycoplasmas, viruses, trypanosomes, fungi, bacteria, and sporangia (Raaijmakers et al. 2006). Several strains of *Bacillus* can synthesize lipopeptide chemical compound, and the antagonistic activity of lipopeptide compounds against phytopathogenic agents has been studied (Leclere et al. 2005). *Bacillus* LPs are divided into three types of cyclic complexes: surfactants, iturin, and fengycin. Each family includes variants that have an identical peptide length then separate residues at certain positions. Besides, every variant may have several homologs with different lengths of fatty acid chains and different isomers, which leads to significant structural differences (Ongena et al. 2007), the heptapeptide variants of the esperin, lichenin, pumilacidin, and surfactin groups in surfactin family. The peptide part is combined with β-hydroxy fatty acids (C12-C16) with linear, hybrid, or pro-branched acids. In the iturin family, iturin A and C; bacitracin D, F, and L; and the subtilisin of mold are all six key variants. In all circumstances, the heptapeptide binds to chains of β-amino acid fatty acids of varying lengths (C14-C17) (Branquinho et al. 2014). The third family contains toyomycins A and B. If Tyr 9 is in configuration D, it is also called pravastatin. These decapeptides are combined with β-hydroxy fatty acid chains (C14-C18). The β-hydroxy fatty acid chains are also in linear, hetero- or trans-isomeric forms and can be saturated or unsaturated. In addition, the three *Bacillus* LP families showed a specific cyclization model (Ongena and Jacques 2008).

Surface actin and toymycin are large lactone rings, but for surfactants, the closed-loop between β-hydroxy fatty acids and the C-terminal peptide is catalyzed, while toymycin cyclase is in the peptide sequence. Like many *Pseudomonas* LP, the Tyr side chain in position 3 forms the ester bond together with the terminal C deposit, establishing an internal loop. Similar to surfactants, fatty acid chains are also implicated in the cyclization of iturins, but because of their β-amino nature, amide bonds are developed along with C-terminal groups that form large lactic acid structures (Ongena and Jacques 2008). Recently, two-dimensional NMR and the neutron reflectometer were used to carry out a detailed structural characterization of the representative members of these three families (Tsan and Gao 2007).

22.5.3 Fatty Acids

Bacillus sp. is well-known to use the conventional way of FAS (fatty acid synthase) to synthesize fatty acids with chain sizes between C12 and C19 (Fang and Kato 2007). The fatty acids in precursors of acetyl-CoA and malonyl-CoA are produced by an action of an enzyme called fatty acid synthase (FAS). In many organisms, there are fatty acids (methyl branched) together with methyl groups on linear carbon atoms (methyl substituents on carbons 2, 4, and 6), the source of which is selectively incorporated by the fatty acid synthase methylmalonyl-CoA (Mondol et al. 2013). The equivalent isocyanate branch in new methyl branched fatty acids is likely originating from leucine and isoleucine and then undergoes a sequence of extensions via malonyl-CoA. In the penultimate extension step, methylmalonyl-CoA appears to be a selective way to be incorporated by fatty acid synthase from bacteria that produce methyl branched-chain fatty acids (Mondol et al. 2013). So far, the question of whether it is a methyl branched fatty acid synthase known or unknown in bacteria remains a speculative question. The natural activities carried out of these new fatty acids may not have been described.

22.5.4 Macrolactins

Macrolactins are cyclic polyene macrolactones composed of 24-membered cyclic lactones, and their modified forms (such as adding of the glucose-β-glucopyranoside) might also appear as linear analogs (Mondol et al. 2013). The carbon skeleton of the macrolactin comprises three distinct diene structural elements in the 24-membered lactone cycle. Macrolactone compounds are mostly produced by terrestrial and marine strains (Hamdache et al. 2011). Through the genome of *B. amyloliquefaciens* FZB42, plants have environmental strains colonized by the root system, which can stimulate plant growth in the rhizosphere and inhibit soil-bound plant pathogens (Idris et al. 2004). There are 3 positions with a total of approximately 196,340 lead operon PKS. The origin of genome replication is equivalent to about 1.4 Mbit/s (PKS2), 1.7 Mbit/s (PKS1), and 2.3 Mbit/s (PKS3) clockwise, and the size is of 3916 kb (Chen et al. 2006). These three groups of genes show the typical modular structure of the PKS type I system, which indicates that the FZB42 strain has a biosynthetic mechanism and can produce at minimum three distinct types of polyketides. PKS1 and PKS3 are ascribed to the production of bacteriocins of which pks2 is engaged in the biosynthesis of large endocrine substances. The macrolide ring of the macrolide is formed by the cyclization of a polyketide chain composed of the PKS type I enzyme. These enzymes carry out a condensation of repeated decarboxylation of carboxylic acids by activated carboxylic acid initiating units (Schneider et al. 2007).

22.5.5 *Induced Systemic Resistance as a Mode of Biocontrol by* Bacillus

So far, the genus *Bacillus* contains species with different genetic traits, making it an ideal candidate for biological control agents. Its capability to induce systemic resistance (ISR) is another significant feature of *Bacillus* species. Induced systemic resistance constitutes a system through which bacteria (mainly PGPR) can cause plant defense systems to resist infection by pathogens (Kloepper et al. 2004). Several authors have compared ISR to acquired system resistance (ASR), and that is bacteria-free. Even if the induction mechanism is different from the plant response, the phenotypic response may be similar (Compant et al. 2005). For *Bacillus*, it's interesting to determine the biodiversity that can cause resistance in plant systems. Some examples are *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. cereus*, *B. sphaericus*, and *B. pumilus* (Santoyo et al. 2012). *Bacillus* can induce this reaction in plants, its beneficial effect being to protect plants against different pathogens like viruses, bacteria, nematodes, and fungi (Kloepper et al. 2004).

Certain lipopeptides produced by *B. subtilis*, such as surfactants and antibiotics, may also be able to induce ISR in plants (Ongena et al. 2007). During the past few years, some experiments have tried to analyze the mechanism of induction of resistance in plants and discovered that the volatile chemicals contribute a substantial role in that induction. The GBO3 and FB17 strains of *B. subtilis*, *B. cereus* AR156, and *B. amyloliquefaciens* IN937a are all specimens of strains that have ISR system by volatile emissions (Ryu et al. 2004). Lately, it has been proposed that the FB17 strain of *B. subtilis* produces acetal (3-hydroxy-2-butanone) and causes ISR in *Arabidopsis* (Rudrappa et al. 2010).

To interrupt the signaling pathway by volatile substances, *B. subtilis* GB03, ISR is induced by an ethylene-dependent pathway independent of jasmonic acid and salicylic acid (Ryu et al. 2004). However, some writers believe that PGPR has a generic mechanism used for triggering ISR in plants by pathways dependent on jasmonate and ethylene (Santoyo et al. 2012). ISR is essential to prevent or reduce plant pathogens. When a pathogen is detected, the plant synthesizes several hormones involved in ASR (like the ISR response). Most recent hormones, like brassinosteroids (BR), are important for plant development and have just recently been endowed with systemic resistance to various biological and abiotic loads. They are tolerating the various environmental impacts of PGP bacteria that produce 1-amino cyclopropane-1-carboxylic acid deaminase (ACC) on the environment. PGP bacteria can stimulate plant growth by reducing their ethylene content (Santoyo et al. 2012). It will be fascinating to assess whether the PGP bacteria cannot only induce protection from the pathogens but similarly resist environmental stress beyond ISR or any other processes.

22.6 Conclusions and Prospects

Bacillus genera are the rich resources of different secondary metabolites, comprising polypeptides, lipopeptides, macrolides, polyketides, fatty acids, lipoamides, isocoumarin, and carotenoids. The structure of these marine isolates has multiple uses and can be extracted from complicated biosynthetic ways. Several biologically active ingredients have great potential for advancement of potent drugs and agrochemicals. Owing to the genetic ability to adjust at extreme environments and the *Bacillus* strains isolated from unique conditions (such as hydrothermal vents, salt lakes, and deep sea, pH > 9.0) can create beneficial biologically active compounds. Still in marine isolates, recessive biosynthetic gene clusters can be triggered to determine new organic products through cultivation under changing stress environment (such as nutrients, temperature, pH, or salinity stress). Additional primary characteristic of *Bacillus* is that it will cause reduction of heavy metals, which can be viewed as a candidate for the remediation of toxic heavy metals. Bioremediation is an ecofriendly and inexpensive approach to eliminate xenobiotics from contaminated environments. The next generation of sequencing technology provides important insights into the molecular and biological mechanisms that play a role in the biological remediation of ecological pollutants (such as heavy metal pollutants). These findings will enhance the strategy of bacterial bioremediation, monitor its progress, and determine its success. Biological pesticides based on *Bacillus* can progress plant health by a unique mode of action and therefore have great commercial potential. As the occurrence of *B. subtilis* is frequent in ecosystem, its production capacity of large quantities of antibiotics should be accounted when combating pests. Biological pesticides based on *Bacillus* have great potential for future sustainable agricultural practices.

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Chapter 23
Impacts of *Bt* Brinjal on Economic Benefit of Farmers and Environmental Sustainability in Bangladesh

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Abstract Brinjal or eggplant (*Solanum melongena* L.) is severely attacked by the brinjal shoot and fruit borer (BSFB) insect, which reduces the yield and causes massive loss in brinjal production. This crop plant belongs to the family of Solanaceae, which is one of the common cultivated vegetables throughout the Asia including Bangladesh. This chapter aimed to comprehensively review the knowledge and information of genetically engineered (GE) brinjal called *Bt* brinjal on economic benefit of growers and environmental sustainability in Bangladesh. As a potential solution to protect eggplant from BSFB, Bangladesh developed and released genetically modified (GM) four *Bt* brinjal varieties. This transgenic brinjal carries an additional *Cry1Ac* gene from *Bacillus thuringiensis* (*Bt*) that provides built-in protection against the devastating BSFB insect. The generated *Bt* protein is activated in the insect's alkaline gut and ruptures the gut membrane which is detrimental for the insects, and thus no insecticide spray is required. But, in case of non-*Bt* brinjal, farmers have to spray insecticides frequently to control that insect but without much success. The residue of applied insecticides is detrimental for the environment and human health. The recent data showed that approximately 17% of total brinjal growers of the country were adopted and directly benefited from this GM technology. By cultivating the *Bt* brinjal, farmers save approximately USD 115 per hectare as an input cost mainly due to no or less insecticide spray is required for *Bt* brinjal compared to the non-*Bt*. By the use of *Bt* technology, the cost for insecticide use could be reduced up to 61% compared to conventional varieties. Additionally, *Bt* brinjal farmers receive 30% price premium compared to non-*Bt*. As a result, *Bt* brinjal farmers get five times higher returns per hectare than the farmers cultivating non-*Bt* varieties. It is worth noting that reduced application of insecticide is remarkably beneficial to the environment. No significant negative impacts of *Bt* brinjal have so far been reported on soil arthropods and other nontarget beneficial organ-

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isms. However, the durability of insect resistance by a single *Cry1Ac* gene and its consequence to the environment are still unclear. Overall, *Bt* brinjal is a successful GM crop in Bangladesh, which is found profitable for the farmers and good for the environment and human health.

Keywords Genetically modified (GM) crops · *Bacillus thuringiensis* · *Bt* brinjal · *Cry1Ac* gene · Sustainable environment · Economic benefit

23.1 Introduction

Brinjal or eggplant (*Solanum melongena* L.) is one of the most popular vegetables cultivated all over Asia, including Bangladesh (Prodhan et al. 2018). It is an important year-round vegetable which is popularly known as begun in this country. The quantity of brinjal is increasing in terms of consumption, which ranks third after potato among the vegetables cultivated in the country (Rashid et al. 2018). Brinjal is grown year-round in this country, and approximately, 50,956 hectares of land are under cultivation by 150,000 farmers (BBS 2018). But, in the field level, the vegetable is seriously infested with brinjal shoot and fruit borer (BSFB) (*Leucinodes orbonalis* Guenee), one of the top destructive pests among others. In Bangladesh, up to 86% yield losses of brinjal have been described due to BSFB. To reduce brinjal shoot and fruit injury by BSFB, farmers depend on repeated application of insecticides (Prodhan et al. 2018). Practically, the larva of BSFB burrows into the petioles as well as tender shoots. As a result, leaves drooping and shedding of flower buds are common phenomena. In the developing fruits, severe damage is mainly occurring because the caterpillars make tunnel inside the fruits through feeding the fleshy part. However, Raza et al. (2018) reported that under different trade names, 16 insecticides were usually sprayed to tackle this devastating enemy of brinjal. Additionally, for controlling this insect, farmers usually apply broad-spectrum insecticides two to three sprays per week and in some cases twice a day (Del Prado-Lu 2015). Consequently, it is not surprising to spray over 100 times per growing season, resulting in high residual effects on consumable fruits. Such a hazardous insecticide-dependent strategy poses threats to the atmosphere as well as human health. In addition to health and environmental concerns, populations of BSFB have also developed resistance to various foliar insecticides (Shirale et al. 2017). Most significantly, about 68.75% of brinjal growers have awareness, while 31.25% brinjal growers did not have proper knowledge about the deleterious effects of using high amounts of insecticides. The brinjal growers are frequently using same active ingredients under different commercial names (Raza et al. 2018). However, inefficient use of insecticides poses a series of ecological problems, such as development of pesticide resistance, nonpoint pollution, bioaccumulation in food chain, and a huge loss of biodiversity (Zhao et al. 2017). Moreover, the loss and decomposition rate of insecticides on crop foliar is typically more than 99%, which are caused by runoff, spray drift, and rolling down at the time of field application (Nuruzzaman

et al. 2016; Song et al. 2017; Zhao et al. 2017). As a result, the insect management based on insecticides is detrimental to humans, livestock, and environment (Javed et al. 2017).

A potential solution to control BSFB is the genetically modified *Bt* eggplant which carries an additional gene *Cry1Ac* from a soil bacterium, *Bacillus thuringiensis* (*Bt*). This gene provides brinjal built-in protection against the *Lepidopteran* insect pest (Rashid et al. 2018). As the first South Asian country, Bangladesh has developed and released four GM brinjal varieties in 2014 by introducing *Cry1Ac* gene (*Bt* brinjal) for commercial cultivation of brinjal as food crops (Ahmed et al. 2019; Prodhan et al. 2018). In the last two decades, utilization of *Bt* technology has revolutionized the agricultural practices in different countries by developing and commercializing the transgenic plants that have a built-in insecticidal toxin-expressing genes (Castagnola and Jurat-Fuentes 2012). The remarkable increase in acceptance of biotech crop technique can be recognized to its contribution such as higher food production which improves food availability for the native community and global levels. On the other hand, improvement of food quality through biofortification of important nutrients as well as social positive impact of the growers through economic development are also considered as important role of this technology (James 2014a, b). However, the approval issue of GM crops for commercial cultivation remains controversial in Bangladesh and globally. The common criticisms include GM crops, which are destructive to the ecology and environment and harmful to human health. On the other hand, small farmers will lose their accessibility due to higher cost and intellectual property rights of the seed producing company. Additionally, it is also claimed that GM crops, including *Bt* brinjal, deliver no yield paybacks (Ahmed et al. 2019). This book chapter aimed to provide an independent rigorous scientific information based on review of reputed research articles that could address some of these key criticisms. The specific objectives of this book chapter were as follows: (i) to describe the concept of *Bt* brinjal development; (ii) to illustrate the economic benefit of *Bt* brinjal cultivation over non-*Bt* varieties; and (iii) to demonstrate the impact of *Bt* brinjal cultivation on maintenance of environmental sustainability.

23.2 The Developmental Concept of *Bt* Brinjal in Bangladesh

23.2.1 History of Bt Brinjal

The development of transgenic plants and commercialization of their cultivation have revolutionized agricultural practices. Improvement of this ground-breaking insect pest control technology was backed by the identification and characterization of insecticidal *Bt* proteins. Moreover, the development was assisted by the advanced plant transformation technique and genetic engineering. In most of the cases, the developed transgenic crops are occupying the space for conventional crop varieties

due to their insect resistance characters, lesser insecticide requirements, as well as greater yield potentials (Castagnola and Jurat-Fuentes 2012). In the year 2005, two BARI (Bangladesh Agricultural Research Institute) scientists visited the Mahyco Agricultural Research Station, Jalna, Maharashtra, India, with nine popular brinjal cultivars from Bangladesh. These cultivars were hybridized with the candidate brinjal plants (Fig. 23.1) containing the insecticidal toxin-expressing *Bt* gene from the bacterium *Bacillus thuringiensis* (*Bt*) as well as collected F1 seeds.

After that, backcrosses (BC1) were done there, and the resultant seeds were brought back to Bangladesh. Later on, successive backcrosses were produced at BARI from 2006 to 2011. While targeted plants were selected, multilocation confined field trials were conducted at seven different locations of BARI (APAARI 2018).

Over a 10-year period, public sector Bangladeshi agricultural researchers, with support from Maharashtra Hybrid Seeds Co. Pvt. Ltd. (Mahyco) and researchers based in the United States, have developed a series of GM varieties of *Bt* brinjal that are resistant to BSFB (APAARI 2018). Therefore, in 2013 after compiling all the results, BARI applied to National Committee of Biosafety (NCB), MoEF (Ministry of Environment and Forest) following Biosafety Guidelines/Rules of the country through MoA (Ministry of Agriculture) for the approval of four *Bt* brinjal genotypes. The applied *Bt* brinjal genotypes were BARI *Bt* begun-1, BARI *Bt* begun-2, BARI *Bt* begun-3, and BARI *Bt* begun-4.

Finally, on October 28, 2013, Bangladesh's National Committee on Biosafety (NCB) approved for commercial cultivation of four indigenous varieties of *Bt* brinjal, which were resistant to attacks by the BSFB, a common pest in South and Southeast Asia (Shelton et al. 2018).

Fig. 23.1 Cultivar Uttara (brinjal variety) showing injury by the brinjal shoot and fruit borer to non-*Bt* brinjal (**a**) and lack of injury in *Bt* brinjal (**b**) (Shelton et al. 2019)

23.3 Structure and Mode of Action of *Cry1Ac* Protein

Different insecticidal proteins such as Vip, Cyt, or Cry toxins were produced against different insect specificities by a gram-positive bacterium, *Bacillus thuringiensis* (*Bt*). Among these toxins, the three-domain *Cry* toxins (3d-*Cry*), the biggest *Cry* toxin family, have been shown to be highly effective for agricultural pest control (Pardo-López et al. 2013). Some of these 3d-Cry toxins like *Cry1Ab*, *Cry1Fa*, and *Cry1Ac* have already been introduced and expressed successfully to make transgenic crops such as maize, cotton, soybean, or brinjal reducing the use of chemical insecticides and in some cases increasing yields (Sanahuja et al. 2011; APAARI 2018). Herein, some of the *Bt* crops which are commercially available for cultivation are presented in Table 23.1. Generally, the *Cry1Ac* structure comprises seven distinct domains. The domain (D) number starts from D-I to D-VII. Among them, three canonical toxin core domains are D-I, D-II, and D-III, while four protoxin domains are D-IV, D-V, D-VI, and D-VII. *Cry1Ac*-FL is a sickle-shaped structure (Fig. 23.2) where the toxic core looks like handle and the protoxin domains as the blade (Evdokimov et al. 2014).

However, around thousands of individual toxin proteins have already been discovered. Among them, the majority belong to the "three-domain" toxin family. The insecticidal *Bt* proteins contain three structural domains within the toxic core. This is the most populous fraternity with thousands of known members, of which more than 50% are produced by their parent *Bt* strains as ~1200-residue protoxins. These protoxins are consisting of a proteolytically labile C-terminal segment that is sometimes mentioned as the protoxin domain. On the other hand, another part is N-terminal ~600-residue segment that encodes the three-domain toxic core (Schnepf et al. 1998; Crickmore et al. 1998; de Maagd et al. 2001). In fact, the isolation of C*ry1Ac* gene is done from the soil-living bacterium *Bacillus thuringiensis*. After the ingestion of C*ry1Ac* gene introduced plant parts by the BSFB larvae, the *Bt* protein is activated in the insect's alkaline gut and binds to the gut wall. After that, the gut wall breaks down and allowing the *Bt* spores to invade the insect's body cavity. However, current data suggested that the mode of action of *Cry1Ac* protoxins may not similar with that of activated toxins. Because the pore formation resulted from the binding of protoxins to insect receptor is different from that resulting from activated toxin. Based on these data, Gómez et al. (2014) proposed a dual mode of action of *Cry* proteins. They suggested that the two pre-pore structures may have differential roles in toxicity against selected targets by increasing their range of action. Moreover, insect may harbor different receptors, but different midgut proteases would influence the rate of protoxin/toxin activation. In support to the dual mode of action of *Cry* proteins, it was reported that several insect populations have developed resistance to *Cry* proteins and showed significantly lower resistance ratios to *Cry1Ac* protoxins than the activated toxins (Tabashnik et al. 2015). Therefore, by activating with the insect gut proteases, *Bt* 3d-*Cry* toxins are produced as protoxins which yield an active three-domain toxin. Herein, either *Cry1Ac* activated toxin or protoxin will create binding with the cadherin receptor forming

Table 23.1 *Bt* crops which are commercially available for cultivation (Adapted from ISAAA's GM Approval Database, 2020 http://www.isaaa.org/gmapprovaldatabase/; Castagnola and Jurat-Fuentes 2012)

Crops	Trade/commercial name	Targeted insects[a]	Involved genes	Method of transgene insertion[b]	GM developers
Maize (*Zea mays* L.)	Agrisure™ CB/LL	ECB	*Cry1Ab*	Direct DNA transfer	Syngenta
	Agrisure™ GT/CB/LL				
	Agrisure™ 3000GT	ECB, WCR	*Cry1Ab*, *Cry3Aa*	Direct DNA transfer, Agrobacterium-mediated transformation	
	Agrisure™ CB/LL/RW				
	Agrisure® Viptera™ 3110	ECB, FAW, CEW, BCW, WBC	*Cry1Ab*,*Vip3Aa20*	Direct DNA transfer, Agrobacterium-mediated transformation	
	Agrisure® Viptera™ 3111	ECB, WCR, FAW, CEW, BCW, WBC	*Cry1Ab*, *Vip3Aa20*		
	Agrisure® Viptera™ 4				
	YieldGard™ CB + RR	ECB	*Cry1Ab*	Biolistics	Monsanto
	YieldGard™ VT Triple	CRW	*Cry1Ab*		
	YieldGard™ Plus	ECB	*Cry3Bb1*		
	Genuity® VT Double Pro™	CEW, ECB, FAW	*Cry1A.105/Cry2Ab2*	Agrobacterium-mediated transformation	

Maize (*Zea mays* L.)	Genuity® VT Triple Pro™	CEW, CRW, ECB, FAW	*Cry1A.105/Cry2ab2*, *Cry3Bb1*	Agrobacterium-mediated transformation Agrobacterium-mediated transformation	Monsanto
	Genuity® SmartStax™	BCW, CEW, CRW, ECB, FAW, WBC, SCB, SWCB, SCSB, CEW, SCB, WBC, WCR	*Cry1A.105/Cry2Ab2,Cry1Fa2 Cry3Bb1*, *Cry34/35Ab1*	Agrobacterium-mediated transformation Biolistics Biolistics Agrobacterium-mediated transformation	
Cotton (*Gossypium hirsutum L.*)	Bollgard™ Cotton, Ingard™	PBW, TBW	*Cry1Ac*	Agrobacterium-mediated transformation	Monsanto
	Bollgard II™ Cotton	CBW, PBW, TBW	*Cry1Ac/Cry2Ab2*	Agrobacterium-mediated transformation/biolistics	
	Optimum™ Intrasect	ECB	*Cry1Ab*	Biolistics	DuPont
		ECB, WBC, BCW, FAW	*Cry1F, Cry1Ab*	Biolistics, biolistics	
Cotton (*Gossypium hirsutum L.*)	WideStrike™ Cotton	*Lepidopteran* insect resistance	*Cry1AcCry1F*	NA	Dow AgroSciences LLC
Eggplant (*Solanum melongena*)	BARI Bt Begun-1, 2, 3 and 4	BSFB	*Cry1Ac*	Agrobacterium-mediated transformation	MAHYCO
Soybean (*Glycine max L.*)	Conkesta Enlist E3™ Soybean	*Lepidopteran* insect resistance	*Cry1AcCry1F*	NA	Dow AgroSciences LLC
	Intacta™ Roundup Ready™ 2 Pro		*Cry1Ac*	NA	Monsanto

(continued)

Table 23.1 (continued)

Crops	Trade/commercial name	Targeted insects[a]	Involved genes	Method of transgene insertion[b]	GM developers
Rice (*Oryza sativa L.*)	BT Shanyou 63	*Lepidopteran* insect resistance	*Cry1AbCry1Ac*	NA	Huazhong Agricultural University (China)
	Huahui-1				
Potato (*Solanum tuberosum L.*)	Atlantic NewLeaf™ potato	*Coleopteran* insect resistance	*Cry3A*	Agrobacterium-mediated transformation	Monsanto
	New Leaf™ Russet Burbank potato		*Cry3A*		
	New Leaf™ Plus Russet Burbank potato		*Cry3A*		
	Superior NewLeaf™ potato		*Cry3A*		

[a]Common insect name abbreviations are *ECB* European corn borer, *WCR* western corn rootworm, *FAW* fall armyworm, *CEW* corn earworm, *BCW* black cutworm, *WBC* western bean cutworm, *CBW* cotton bollworm, *TBW* tobacco budworm, *PBW* pink bollworm, *SCB* sugar cane borer, *SWCB* southwestern corn borer, *SCSB* southern cornstalk borer, *SPB* spotted bollworm, *BSFB* brinjal shoot and fruit borer

[b]NA, not available

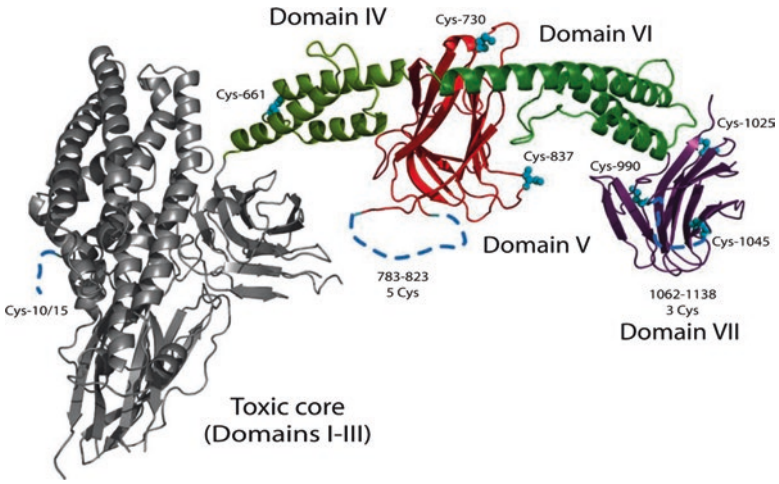

Fig. 23.2 Overall structure of *Cry1Ac* monomer that is domain-wise colored. The toxic core (domains I to III) are light gray, domain IV is dark green, domain V is red, domain VI is green, and domain VII is violet in color (Evdokimov et al. 2014)

distinct oligomers that insert into the membrane forming lytic pores. This consequences the reduction of potential evolution of resistance and possibly to broaden the range of insect targets. However, for the sustainable use of *Bt* crops, it is important to achieve a stable expression of *Cry* full-length proteins which will pose the same consequences. These phenomena are delaying resistance and giving protection from a wider number of insect pests (Soberón et al. 2016).

23.4 The Economic Benefit Incurred by the *Bt* Brinjal Growers

BSFB is a destructive pest of brinjal, which adversely affects quality and yield of brinjal fruit throughout the world (Javed et al. 2017). In modern world, widespread adoption of productivity-enhancing technologies has shifted production, with economic and environmental effects. Agricultural technologies like *Bt* brinjal offer new opportunities that must be evaluated in an increasingly complex world. A number of factors influence the effect of new or improved agricultural technologies on production and consumption. These include the characteristics of the existing agricultural and market systems, the agroecological conditions, socioeconomic status, and sources of information about these technologies, as well as beliefs, norms, and cultural practices. Adoption of agricultural technologies has proven to be effective in improving food availability and food quality, increasing farmers benefit from agricultural production system, as well as responsive to environmental risks and uncertainties (Ahmed et al. 2019).

23.4.1 Rapid Adoption

In Bangladesh, the first *Bt* brinjal seedling distribution program was held on January 22, 2014, where 20 farmers received seedlings to grow on a trial basis (APAARI 2018), and in the following years, *Bt* brinjal adoption increased tremendously (Shelton et al. 2018). In 2014–2015, BARI conducted demonstration trials in 108 farmer's fields of different climatic conditions (19 districts) of Bangladesh through its On-farm Research Division (OFRD). In 2015–2016 and 2016–2017, they conducted demonstration trials in 250 farmer's fields of 25 districts and 512 farmer's fields of 36 districts, respectively. In 2017–2018, BARI distributed seeds among 569 growers in 40 districts of Bangladesh. Besides, the Department of Agricultural Extension (DAE) also distributed the *Bt* brinjal seeds to the farmers. In this connection, for the production of *Bt* brinjal on commercial basis at the field level, 6000 and 7001 farmers got seeds in 2016–2017 and 2017–2018, respectively. In 2018, Bangladesh Agricultural Development Corporation (BADC) took the initiative to sell the seeds to an additional 17,950 farmers (Fig. 23.3).

In the year 2018, an estimate proved that approximately 150,000 farmers grown brinjal crops in Bangladesh. Among them, an estimated ~17% of brinjal farmers enjoyed the benefits of the *Bt* technology directly. A research team led by Professor Tony Shelton of Cornell University published a paper on *Bt* brinjal in the peer-reviewed journal *Frontiers in Bioengineering and Biotechnology* showed that during the year 2018, 27,012 Bangladeshi farmers harvested benefit due to rapid adaptation of the pest-reducing technology (Shelton et al. 2018). This trend of rapid adaptation proved that farmers are getting benefit from *Bt* technology, and the target pest reduction through built-in toxin mechanism is working successfully in the field level.

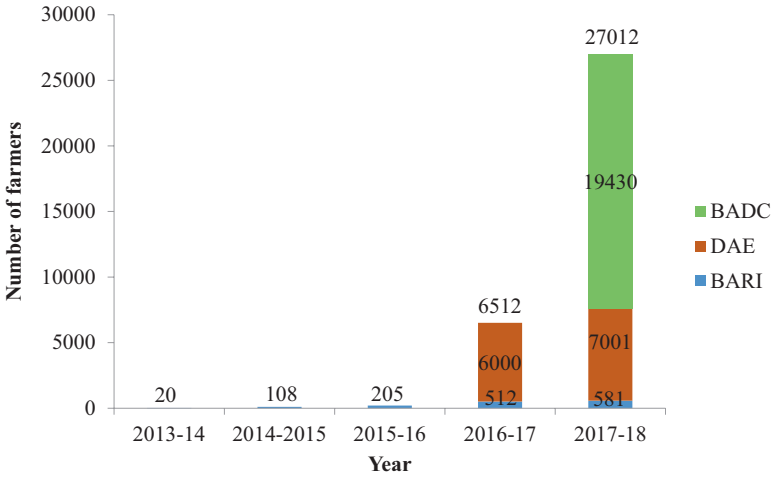

Fig. 23.3 Year-wise adoption rate of *Bt* brinjal in Bangladesh. (Redrawn from Shelton et al. 2018)

23.4.2 Cost of Production

In brinjal cultivation, the main cost is pesticides, but in case of *Bt* brinjal varieties, there was less pesticide required than the regular varieties. Because *Bt* brinjal deters BSFB, so no spraying is required for that pest. Spraying once in a week was enough for *Bt* brinjal to control other paste, whereas non-*Bt* varieties required as often as three times a week. This is financial savings for farmers (Ahmed et al. 2019). The researchers stated features which provided a breakdown of input costs per ha for treatment farmers cultivating *Bt* brinjal (*Bt* begun-4) and control farmers growing non-*Bt* brinjal (ISD-006) (Table 23.2).

The total costs of production for *Bt* brinjal per ha (BDT 72,109) were lower than non-*Bt* brinjal (BDT 81,902). The total gains in terms of input cost (BDT 9793) were mainly due to considerably less spent by *Bt* brinjal farmers on pesticides compared to the non-*Bt* farmers (Ahmed et al. 2019). Similarly, this trend of data was supported by another experimental result which described that pesticides were applied 11 times to *Bt* eggplant, whereas it was 41 times to non-*Bt* eggplant for controlling sucking pests during the whole season. Herein, the *Bt* eggplant farmers selected for this study saved 61% pesticide cost compared to non-*Bt* eggplant farmers. The *Bt* brinjal growers experienced no losses due to BSFB attack and got higher net returns. At the end of this study, Rashid and his co-scientists declared that the *Bt* eggplant technology was good for the farmer. In the future, of course, this technology will potentially improve the socioeconomic status of the *Bt* respondents (Rashid et al. 2018).

23.4.3 Net Return from Brinjal Production

During focus group discussions (FGD) with treatment farmers, a scientist team led by Akhter U. Ahmed of the International Food Policy Research Institute (IFPRI) revealed that although brinjal prices fluctuated with market demand, farmers more often sold *Bt* brinjal to market traders at higher prices than conventional varieties. It

Table 23.2 Input costs per hectare for *Bt* brinjal (*Bt* begun-4) and non-*Bt* brinjal (ISD-006) cultivation (Adapted from Ahmed et al. 2019)

	Cost of production (BDT per ha)	
Inputs	*Bt* brinjal	Non-*Bt* brinjal
Seed/seedlings	5,461	5,539
Fertilizer	30,326	32,026
Irrigation	11,241	11,867
Pesticide	14,852	22,145
Machinery	7,600	8,097
Total hired labor	2,505	2,227
Total cash cost	72,109	81,902

was found that market traders tend to buy *Bt* brinjal at higher prices but also sell at higher prices in the market. In the study period, during IFPRI's monitoring field visits, *Bt* brinjal adopted farmers and monitoring officials from DAE, which frequently reported that farmers received higher prices for *Bt* brinjal compared to traditional brinjal varieties because the physical appearance of *Bt* brinjal was better and had no marks of infestation or holes. Therefore, buyers preferred *Bt* brinjal and paid higher prices (Ahmed et al. 2019). These trends of qualitative results were strongly supported by another study which was conducted by Rashid et al. (2018), and the results were presented in Table 23.3. They revealed that gross returns from *Bt* eggplant cultivation were (Bangladeshi Taka) BDT 394,570/ha as compared to BDT 312,945/ha for non-*Bt* eggplant. In case of net returns, the amount was BDT 179,602/ha for *Bt* eggplant which was five times larger as compared to BDT 29,841/ha for non-*Bt*. The yield difference between the two groups was only 3.02 tons, but the non-*Bt* farmers applied almost three times more pesticides as well as more fertilizer to maintain the yields. The sprayed pesticide costs were BDT 14,215/ha for *Bt* eggplant and BDT 36,057 for non-*Bt* eggplant. As a result, the net returns were higher in case of *Bt* eggplant than non-*Bt* eggplant due to lower production costs and higher yields. Based on total cost, production cost of *Bt* eggplant was BDT 9.26/kg, and for non-*Bt* eggplant, it was BDT 14.20/kg (Rashid et al. 2018).

Previously, another study was conducted in the year 2016–2017 cropping season and compared 505 *Bt* brinjal growing farmers with 350 non-*Bt* brinjal growing farmers. The results indicated a 61% saving in pesticide cost, which translated to a 650% (six-fold) increase in returns, from $2151/ha for *Bt* brinjal as compared to just $357/ha for non-*Bt* brinjal. These cost savings and significant increase in returns are only due to reductions of pesticide costs (Shelton et al. 2018). Other studies suggest that these *Bt* brinjal varieties convey higher yields with lower applications of pesticides. Therefore, *Bt* brinjal farmers received higher revenues than conventional brinjal (ISD-006) (Ahmed et al. 2019). Moreover, an economic analysis proved that all adopted Bt brinjal lines provided higher gross returns compared to their non-*Bt* isolines (Prodhan et al. 2018). Another experimental result showed that all *Bt* and 86% non-*Bt* farmers desired to grow *Bt* eggplant in the next year by receiving higher yield and significant financial returns (Rashid et al. 2018).

Table 23.3 Per hectare return from eggplant production in the study areas (Adopted from Rashid et al. 2018)

	Return (BDT/ha)	
Items	*Bt* eggplant	Non-*Bt* eggplant
Fresh eggplant yield (ton/ha)	23.21	20.19
Gross return	394,570	312,945
Gross margin	248,651	101,590
Net return	179,602	29,841
Benefit cost ratio	1.84	1.11
Production cost (total cost basis) (BDT/kg)	9.26	14.02

23.4.4 *Higher Market Price of* Bt *Brinjal*

The major reasons mentioned by the respondents for higher prices of *Bt* brinjal varieties were that they look fresher and most importantly, they have no holes like non-*Bt* eggplants due to pest invasions. This finding contrasts with most of the previous literature which assumed that consumers are willing to pay less for genetically engineered (GE) varieties than non-GE varieties. Here, it was found that when GE products had lucrative observable characteristics, they obtained a positive premium in price for the growers. Surveying the market price *Bt* and non-*Bt* eggplant at both retail and wholesale levels, researchers found that *Bt* prices are higher than non-*Bt* prices at both the retail and wholesale levels (Table 23.4) (Ahsanuzzaman and Zilberman 2018).

A higher wholesale price suggested that farmers were receiving higher prices, while higher retail prices indicated that consumers were willing to pay more for *Bt* brinjal varieties. The retailers also received a higher premium for *Bt* varieties. The results estimated around a 30% price premium at all levels for the *Bt* eggplant varieties (Ahsanuzzaman and Zilberman 2018). Similarly, another result showed that *Bt* brinjal had positive impacts on sales revenue for farmers. Ahmed et al. (2019) revealed that *Bt* varieties gained a 27.3% increase in sales revenue per ha and a 10.9% more sales revenue per kg.

23.5 The Economic Impact of *Bt* Technology Worldwide

Bt crops are plants which are genetically modified (GM) and resistant to specific insect pests. The commercialization of GM crops has occurred at a rapid rate since 1995 when the EPA (Environmental Protection Agency) in the United States gave approval for commercial production and dissemination of the *Bt* products like corn, potato, tobacco, and cotton. These GM crops contain *Cry* toxins which are lethal to certain species of insects belonging to orders such as Coleoptera, Nematoda, Lepidoptera, Hymenoptera, and Diptera. In the year 2016, the total global cultivated area of *Bt* crops reached approximately 185 million ha (Abbas 2018). Additionally, there are several studies estimated the value of using GM crop technology in the agricultural farm level. These studies mainly evaluated impacts on yields, main variable costs of production, gross income, and vital impacts on the production

Table 23.4 Market price information of *Bt* and non-*Bt* eggplant according to Ahsanuzzaman and Zilberman (2018)

Items	Price (BDT/Kg)		*Bt* premium (%)
	Bt brinjal	Non-*Bt* brinjal	
Wholesale	15.45	11.7	32
Retail	28.6	22.35	28
Mark up	13.5	10.65	27

system. However, the annual updated analysis regarding *Bt* crops adaptation shows very noteworthy net financial benefits at the farm level since the year 1996 to 2016. Based on these gains, 48% farmers have been benefited in developed countries and 52% of the farmers in developing countries. Among the gains, about 65% have been achieved from yield and production system as well as the rest 35% came from savings of the production cost (Brookes and Barfoot 2018). Furthermore, in Brazil, another study estimated the efficacy of *Bt* maize expressing proteins like *Cry1F* to control *Spodoptera frugiperda* in the field conditions. The result showed that this *Bt* maize technique efficiently controlled seedling cutting injury as well as successfully reduced foliar feeding, and the estimated protection was near 100% from kernel feeding (Moscardini et al. 2020).

23.6 The Maintenance of Environmental Sustainability by the Cultivation of *Bt* Brinjal

Before introduction of new GE plants, environmental risk assessments are conducted on case-by-case basis. In this case, some of the points are taking into account such as plant biology, transgene nature, protein produced by the respective transgene, the phenotypic character of inserted transgene, the ultimate use of the plant, as well as the released environment where it will be cultivated for use (CERA 2011).

23.6.1 Reduction of Pesticide Use Ensures Toxic-Free Environment

Four *Bt* brinjal varieties were developed to resist the BSFB pest. The transgenic *Bt* brinjal successfully repels the BSFB pest, and as a result, farmers can reduce the use of pesticide. Reduction of pesticides application and less exposure of farmers to toxicity reflect positive environmental outcomes (Hautea et al. 2016). Herein, some of the experimental data are graphically presented in Fig. 23.4 based on percentage of farmers using pesticides of varying toxicity levels for BSFB control (Ahmed et al. 2019).

In the Fig. 23.4, data were summarized by constructing a toxicity score, the Pesticide Use Toxicity Score (PUTS), which assigns a score based on the GHS (Globally Harmonized System) oral hazard category of the selected pesticides and the frequency of the use of the respective pesticides. In the GHS Hazard Classification scale, lower numbers (1, 2) correspond to more severe levels of toxicity. For PUTS to be easily interpretable, the GHS scale is inverted so that higher values correspond to higher toxicity levels (Ahmed et al. 2019). Similar trend of results demonstrated that *Bt* eggplant lines containing *Cry1Ac* provide outstanding control against BSFB and can dramatically reduce the need for application of conventional insecticides

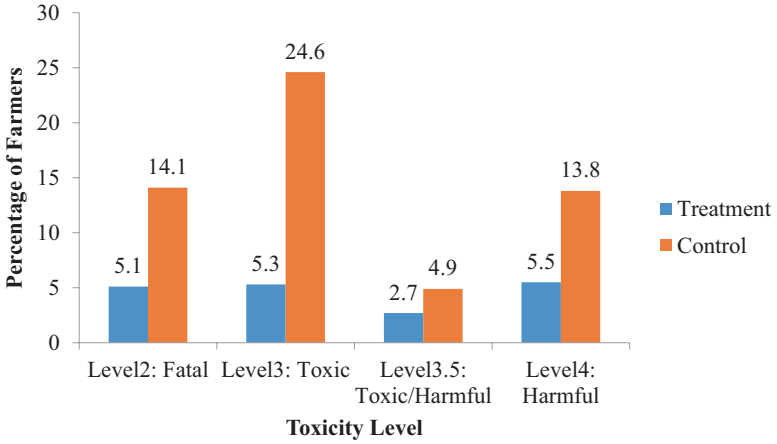

Fig. 23.4 Percentage of farmers using pesticides for brinjal shoot and fruit borer by toxicity level (Redrawn from Ahmed et al. 2019)

(Hautea et al. 2016). Furthermore, adoption of *Bt* eggplant and the reduction of pesticide applications associated with better environmental status (Ahsanuzzaman and Zilberman 2018).

Farmer's knowledge about the harmful effect of conventional pesticides plays an important role on injudicious use of chemicals application to control insect paste in brinjal field. Typically, those who spray the crop are not aware of the hazards of pesticides and do not use any personal protective equipment. These problems have been well documented in Bangladesh and other countries which are associated with our environmental degradation (bteggplant.cornell. Edu/content/facts; bic.searca.org; Del Prado-Lu 2015). These findings were supported by another result which showed about 68.75% brinjal growers have knowledge while 31.25% brinjal growers did not have knowledge about the negative impacts of using higher amounts of insecticides. The brinjal growers are randomly using same insecticides under different brand name (Raza et al. 2018). However, increasing farmer's knowledge about proper use of insecticide depends on many factors; rather it is better to introduce *Bt* brinjal which has built-in mechanism for BSFB control. Finally, according to scientists of the Bangladesh Agricultural Research Institute (BARI) who developed the four varieties, the protein in *Bt* brinjal disrupts the digestive systems of certain pests, causing them to die within 3 days of ingestion and significantly reduce the need to use pesticides (Shelton et al. 2018).

23.6.2 The Effect of Bt *Crops on Nontarget Organism (NTO)*

Development of GM crops was started about 25 years before, and various new cultivars of diverse range have already been released into the environment. Transgene cultivars were developed by introducing new desired genes from different sources using advanced molecular toolkit.

23.6.2.1 Effect on Soil Arthropods

Though the GM crops pose higher capacities for increasing crop production, their impact on the nontarget organisms like soil biota is still partly understood. Recently, some laboratory experimental results proved that horizontal gene transfer is occurring between native soil biota and GM crops (Fig. 23.5) (Mandal et al. 2020). A further metagenomics study is needed to precisely understand whether GM technology modulates microbiome in plants and the rhizosphere.

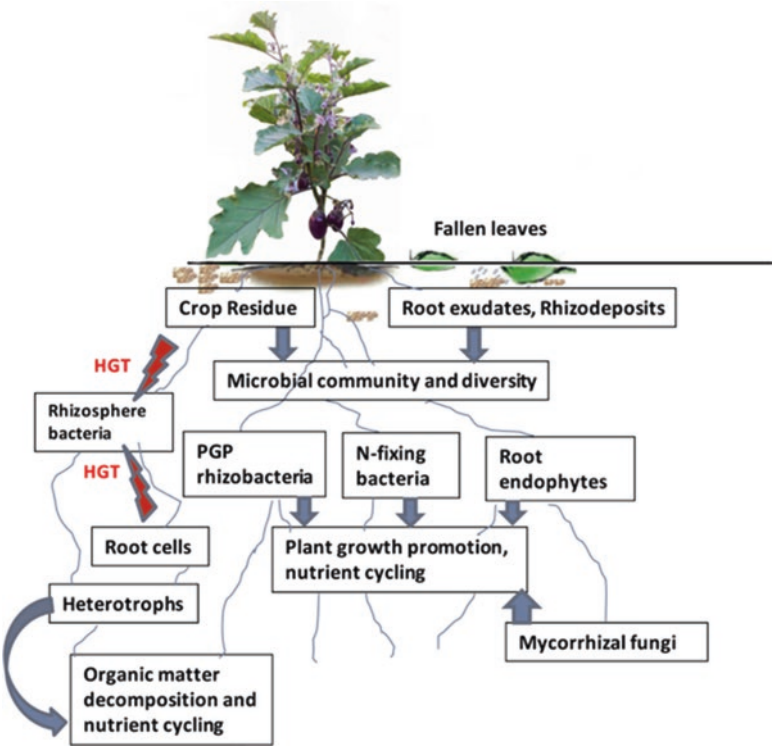

Fig. 23.5 Schematic diagram representing impact of genetically modified crops on soil microbial communities and microbe-mediated processes (PGP: Plant Growth Promoters; HGT: Horizontal Gene Transfer) (Adopted and modified from Mandal et al. 2020)

Transgenic *Bt*-crops have been generated by introducing a *Bacillus thuringiensis* gene, and the respective plant produces a *Cry* toxin to protect crops from insect pests. Mainly, in the rhizospheric zone of the soli, microorganisms are responsible for plant nutrient availability and organic matter decomposition. So, it is relevant to protect these invertebrate taxa from the negative impact of *Cry* toxin. In this connection, a good number of studies have showed comparison between the abundance of populations and biomass of soil microbes in cultivated lands planted with *Bt* crops as well as their traditional counterparts. Herein, some selected studies on Protista, mites, earthworms, nematodes, enchytraeids, and Collembola were reviewed and analyzed critically to strengthen the evidence that whether population richness and biomass of soil invertebrates are altered due to *Bt* crops cultivation in comparison with original cultivars. However, no significant effect of *Cry* protein on soil invertebrates was investigated (Krogh et al. 2020). Previously, the result showed that long-term cultivation of *Bt* cottons had no major effect on the population of soil invertebrates. Impacts on soil invertebrates are considered as a vital aspect of ecological risk assessment, as well as post-release observation of transgene introduced insect-resistant plants (Li et al. 2012). Another study proved that when *Cry1Ac* protein was released into the soil by *Bt* gene, it declined very quickly. Soil particles are absorbed most of the protein and decomposed even within a couple of days. The study also demonstrated that the population sizes, evenness, and diversity of soil microbiota such as fungi, archaea, as well as bacteria were not significantly or consistently affected by *Cry1Ac* protein. These findings suggest that *Bt* protein which resulted through cultivations of *Bt* crops is unable to cause transient or even major persisting changes in soil microbes in the field (Zhaolei et al. 2018). Similarly, transgenic soybean that is producing *Cry1Ac* and *Cry1F* toxins does not adversely affect the nontarget association with soybean (Marques et al. 2018). Therefore, studies on transgenic brinjal in Bangladesh (Prodhan et al. 2018) and the Philippines (Navasero et al. 2016) that are producing *Cry1Ac* protein have also confirmed that it does not destroy nontarget arthropods (Romeis et al. 2019). Despite minor variations that were found in the population size and the distribution pattern across the non-*Bt* and *Bt* brinjal cultivated soil, plant-growth-dependent variability was more prominent in comparison with genetic alteration. However, this study determines that genetic change of brinjal crop has insignificant effect on the fungal population living in the soil (Singh et al. 2013). However, a comprehensive metagenomics study is needed for making a final conclusion on this regard.

23.6.2.2 Effect on Pollinators

As a nontarget organism (NTO), honey bee (*Apis mellifera* L.) plays a very vital role in the pollination of various plants. During their foraging period as pollinator, they are likely to encounter GE crops, especially insect-resistant crops because they produce toxins for particular insect. Thus, it is important to measure probable impacts of these crops on honey bee. Finally, it is concluded that the studied *Cry* proteins do not have adverse effect for the successful survival of honey bees (Ricroch et al. 2018).

23.6.2.3 Effect on Biological Control Agents

Biological control, popularly known as biocontrol, is a method of controlling destructive pest population like weeds, plant disease, insects, and mites using other beneficial organisms. The natural enemies of insect pests can be considered as biological control agents including pathogens, predators, any kind of competitors, and parasitoids. In case of BSFB, natural enemies include predators such as praying mantis, ladybird beetles, earwig, green lacewing, and spiders. In this connection, an experimental result proved that *Bt* brinjal shows excellent pest suppression. But no undesirable nontarget effects on other arthropods were observed in the system, especially those beneficial living organisms that exert important ecosystem services like biocontrol agents (Prodhan et al. 2018). Another study confirmed that the *Cry* proteins of *Bt* crops to control Lepidopteran insect had no detrimental effects to the important natural biocontrol agents of these and other pest species (Shelton et al. 2016).

23.6.3 Establishment and Persistence of Cry1Ac*-Expressing Plants in the Environment*

Environmental risk assessments of GE plants start from its receiving environment where biologically similar non-transformed or host species are existing (OECD 2006). Significant movement of transgenes from GM crops into its wild relatives is evaluated for a new pest-resistant gene in a new crop (Koch et al. 2015). On the other hand, lower fitness was observed due to unintended interruption of endogenous genes. Herein, Indian variety, Swarna, was crossed with Golden Rice and observed a growth disturbance. In this case, precursors for vitamin A-producing gene constructs interfered with own gene of the plants for growth hormone production. The introgression of the transgene interrupted the native plant growth-related *OsAux1* gene function (Bollinedi et al. 2017). However, this effect was not detected in other rice varieties. Actually, such metabolic disruptions do not occur in conventional breeding process. Conventional gene transfer brings gradual changes, whereas genetic rearrangements and abrupt/disruptive additions of additional DNA coding for novel proteins cause drastic metabolic changes, termed as "metabolic meltdown" (Wilson 2018). Moreover, study results concluded that the ecological risk of *Bt* rice regarding the persistence of *Cry* toxins may be much smaller than which were assessed on the basis of ELISA quantification. It was assumed that partially degraded *Cry* proteins retain their immune reactivity but have actually lost their bioactivity (Deng et al. 2019). Another study showed that several plant species were used for genetic modifications, such as alfalfa, maize, creeping bentgrass, oilseed rape, cotton, poplar, eggplant, and rice, which were well known for their persistent nature and gene flow in the regions where they were cultivated (Bauer-Panskus et al. 2013; Devos et al. 2018). Therefore, recent understanding reveals that GM crops

impart destructive effects on the environment, such as weediness, modification in crop pervasiveness or invasiveness, the emergence of herbicide and insecticide tolerance, transgene stacking, as well as disturbed biodiversity. To prove these impacts and unveil further facts, a more in-depth view and critical research is required (Tsatsakis et al. 2017). Till today, in case of *Bt* brinjal, there is an insufficient amount of available data regarding establishment and persistence of *Cry1Ac* expressing plants in the environment. But the findings would convey remarkable in-depth information to the scientists, industry people, and policy-makers to realize various facets of struggle and invention of possible assistance for adopting sustainable commercialization of GM crops like brinjal in Bangladesh.

23.7 Conclusions

The BSFB is a destructive insect pest of brinjal which significantly reduces yield and profit of brinjal cultivation. To tackle this notorious pest, farmers intensively use various expensive chemical insecticides that are detrimental to human health, environment, and cost of production. To increase the productivity, reduce the application of insecticides, and increase the farmers' income, Bangladesh released first GM crop *Bt* brinjal by introducing a *Cry1Ac* gene to the genome of four local cultivars of brinjal. In fact, the *Bt* protein is activated within the insect's gut and severely affect the gut membranes of the BSFB larvae. Adoption of this genetically engineered *Bt* eggplant has significantly been increased yield and profitability of brinjal growers. The input costs per hectare of *Bt* brinjal production were lower due to reduced pesticide cost for crop protection. It gives higher yield because no BSFB infestation was found during field level study. Moreover, due to good physical appearance of the products, unit sale price was higher than the non-*Bt*, which gains per unit higher margin and ultimately farmers got more profit. Rapid adoption of *Bt* eggplant by the farmers and consumers also considerably reduces spray volume of pesticides compared to non-*Bt* varieties and thus improves environmental safety. On an average, the non-*Bt* brinjal growers sprayed insecticides 41 times during the whole period of crop cultivation, and the *Bt* adopted farmers applied only 11 times to protect brinjal from other sucking pests. Furthermore, when all the soil invertebrate taxa including collembolans, mites, earthworms, nematodes, and protists were considered together, there was no significant effect of *Bt* technology on these soil invertebrates. Similar results were found in case of other nontargeted insects like pollinator, honey bee. But there were no available data to draw a strong conclusion regarding the durability of this monogenic new technology and any negative effect of *Bt* gene on surrounding environment and biota. It can be concluded that the *Bt* brinjal technology significantly increased yield and farmers' profitability and ensured environmental and human health safety by drastically reducing the requirement for insecticides use for brinjal cultivation.

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Chapter 24
Bacillus subtilis: A Multifarious Plant Growth Promoter, Biocontrol Agent, and Bioalleviator of Abiotic Stress

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Abstract Increased agricultural production is the global requirement to nourish the rising people with limited cultivable agricultural land. To achieve this goal, fertilizers and pesticides have been gratuitously used, which has caused environmental impairment. Replacing chemicals with biocontrol agents is an environment-friendly alternative. Microorganisms are useful to plants and carry out the same role as chemical fertilizers and pesticides, performing as a biofertilizer and biopesticide. This chapter discusses the multifarious potential of plant growth-promoting *Bacillus subtilis* in plant growth promotion in different cereals, vegetables, and other plants. Inoculation with *B. subtilis* resulted in healthier seed germination, seedling vigor, and improved growth via both direct and indirect mechanisms. *B. subtilis* forms a thin biofilm on the roots for long-term colonization of the rhizosphere. Since various strains of *B. subtilis* possess several traits that can positively influence plants, they can be utilized in the advancement of innovative, protected, and resourceful seed treatments for the sustainable and environment-friendly strategy to cope with

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the adverse effects of abiotic and biotic stresses on plants. This is one of the best bacterial champions in implied enzyme manufacturer, and it can be used on a commercialized scale by biotechnology companies.

Keywords *Bacillus subtilis* · Plant growth-promoting (PGP) traits · Biofertilizer · Biopesticide · Stress bioalleviator

24.1 Introduction

Plant growth-promoting bacteria (PGPB) existing in soil (free-living bacteria) and plant roots (rhizosphere bacteria) promote plant development by enhancing pathogen resistance, increasing water uptake, and utilizing nutrients (Yuan et al. 2015), and have been commercialized as PGPB (Goswami et al. 2016). The utilization of PGPR as microbial inoculants is more emphasized nowadays as an environmentally affable method of increasing crop production compared to the use of chemical fertilizers. Their use as bioinoculants for sustainable agricultural manufacturers in drought-prone areas is on the rise (Ojuederie et al. 2019).

B. subtilis is present in the soil and gut of ruminants and humans. It is Gram-positive, rod-shaped, obligate aerobe, and catalase-positive (+) (Hashem et al. 2019). It can form endospores that promote its survival in adverse environments and also secrete metabolites that enhance plant growth and health. Some volatile organic compounds (VOCs) produced by *B. subtilis* strain (GB03) assist plants to recover from stress (Hashem et al. 2019). It stimulates seed germination and supports the general health and vigor of the plant. *B. subtilis* has been regarded as biofertilizers, phytostimulators, and biopesticides (Bhardwaj et al. 2014; Pérez-Montaño et al. 2014). The increasing climatic change and the need to reduce chemical fertilizers lead to the production of abiotic stress-tolerant plants as an international priority, which will require the wide use of PGPB.

24.2 *Bacillus subtilis* as Plant Growth-Promoting Bacterium (PGPB)

The promotion of plant growth and development is achieved by *B. subtilis* through direct and indirect mechanisms. The direct mechanism involves nitrogen fixation, solubilization of complex organic or inorganic nutrients, mobilization of iron via siderophore production, and production of plant growth regulators such as indole acetic acid (IAA), gibberellins, and cytokinin. Based on a different mode of action, direct plant growth-promoting rhizobacteria can be grouped into three categories including biofertilizers, phytostimulators, and rhizoremediators (Wang et al. 2018; Hashem et al. 2019). The indirect mechanism involves the production of various

compounds such as hydrogen cyanide (HCN), antibiotics, and volatile compounds that encourage plant development (Radhakrishan et al. 2017) and the induction of acquired systemic resistance. *B. subtilis* improves nutrient uptake, root growth, and the proliferation of plants (Table 24.1 and Fig. 24.1).

Two PGPB initially labeled as PR30 and PR31 isolated from an organic farm, SHUATS, Allahabad, analyzed phylogenetically by 16S rRNA sequences as *B. subtilis* PR30 and *B. subtilis* PR31. These organisms displayed multiple plant growth-promoting (MPGP) traits such as the production of ammonia (NH3), hydrogen cyanide (HCN), siderophore (SD), indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase (ACCD), and phosphate solubilization (PS) activity. Also, the organisms were tolerant of high levels of trace elements, a wide range of pH 5-9, and resistant to multiple antibiotics (Sagar 2017).

B. subtilis improves tolerance to biotic stresses. This mechanism of disease resistance begins with the expression of explicit genes and hormones, such as ACCD. ACCD prohibits the ethylene synthesis from its precursor ACC into α-ketobutyrate and ammonia. Ethylene limits root and shoot development and sustains plant homeostasis. The degradation of the ethylene precursor (ACC) by bacterial ACCD helps to relieve plant stress and maintain normal growth under stressful conditions (Glick 2014; Tiwari et al. 2018).

B. subtilis changes the complex form of essential nutrients, such as P and N, to an easily accessible form, such as NH_4^+ and PO_4^{3+}, that is uptaken by plant roots. Some of the *Bacillus* spp. release ammonia from nitrogenous organic matter (Hayat et al. 2010). Ding et al. (2005) reported that some of the *Bacillus* spp. synthesis of the enzyme nitrogenase (EC 1.18.6.1) is controlled by the nifH gene, which can fix atmospheric N2 and supply it to plants to enlarge plant growth and yield by delaying senescence. This property is exploited by scientists to use *B. subtilis* as a potent biofertilizer (Qiao et al. 2014; Garcia-Fraile et al. 2015; Kuan et al. 2016; Chandrasekaran et al. 2019).

Table 24.1 PGP traits of *B. subtilis* and their influence on the physiological functions of crop plants

Characteristics	Physiological functions	References
Phytohormones/ IAA	Enhance plant growth, elongation of root and shoot, and cell division	Chowdappa et al. (2013), Xie et al. (2014), Kang et al. (2015b), Shao et al. (2015), Radhakrishnan and Lee (2016), Barnawal et al. (2017)
ACCD	Enhances plant amplification	Xu et al. (2014), Pourbabaee et al. (2016), Glick (2017); Tiwari et al. (2018)
Siderophores	Reduce the movement of toxic ions, assist to solubilize iron as of minerals and organic compounds in the rhizosphere	Patil et al. (2014), Lastochkina et al. (2017), Radhakrishna et al. (2017)
P solubilization	Conversion of inorganic phosphate into soluble phosphates	Sivasakthi et al. (2013), Kang et al. (2014a, 2015a); Kuan et al. (2016)

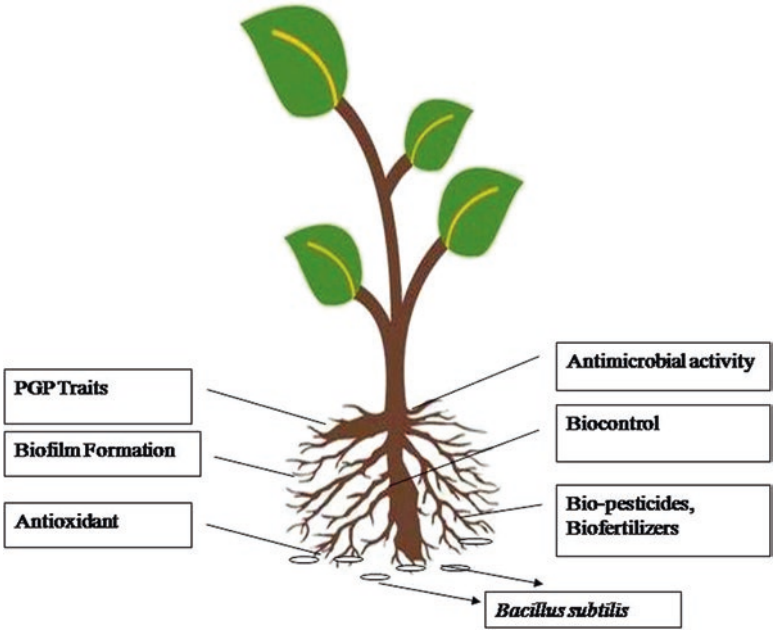

Fig. 24.1 The multifarious potential of *Bacillus subtilis*

24.2.1 Influence of B. subtilis *on Physiological Parameters of Vegetables, Cereals, and Other Plants*

Vegetables and cereals comprise an integral part of human healthy foods. These are important sources of dietary nutrients, such as calcium, magnesium, potassium, iron, beta-carotene, vitamin B complex, vitamin C, vitamin A, vitamin K, and antioxidants, along with providing soluble and insoluble dietary fibers, collectively recognized as non-starch polysaccharides (NSP), for example, cellulose, mucilage, hemicellulose, gums, pectin, etc. Hence, it is recommended to use PGPR in vegetable cultivation to enhance vegetable production without the use of chemicals. Here, an effort is made to enlighten the PGPR role in vegetable production under both regular and decrepit soil (Rai and Nabti 2017). Significant increases in seedling growth of tomato, mung bean, broccoli, and chickpea in response to inoculation with *B. subtilis* have been reported (data not published). Inoculation with PGP *B. subtilis* exhibited a higher percentage of seed germination, seedling vigor, and enhanced growth parameters in vegetables crop seeds such as chickpea (Pusa-256), mung bean (B1Zaid 2012), broccoli (Palam Samridhi), and tomato (NTLI86) compared to non-treated control plants (Qiao et al. 2017; Bahramisharif and Rose 2019), chickpea (Egamberdieva et al. 2017a, b; Abd_Allahet al. 2017; Khan et al. 2019) and mung bean (Hashem et al. 2017; Shen et al. 2017; Akinrinlola et al. 2018; Arif et al. 2019).

A large number of mechanisms are involved in growth promotion and increasing crop yield. The most common is the production of phytohormones, which are IAA

and auxin, which enhances plant growth parameters by various mechanisms (Qiao et al. 2017, Bahramisharif and Rose, 2019); phosphate-solubilizing property has an impact on growth parameters in tomato and pepper (Radhakrishnan and Lee 2016; Barnawal et al. 2017). N, P, and K, which are the highest requirement of a plant for its development, are supplied to the plants by these bacteria (Table 24.2).

24.3 Biocontrol Potential of *B. subtilis*

The disease process is an interplay between susceptibility of host or resistance to pathogen environmental conditions and disease-causing potential of a pathogen (Wang et al. 2018). *B. subtilis* profoundly manipulates these interactions and shows its antimicrobial activity through (Ashraf et al. 2019; Lima et al. 2019; Khan et al. 2019) biofilm formation (Gingichashvili et al. 2017; Dragos et al. 2018; Dinh et al. 2019; García et al. 2020; Gingichashvili et al. 2020), which thus act as biocontrol (Bahramisharif and Rose, 2019; Hashem et al. 2019; Zhang et al. 2019) (Table 24.3 and Fig. 24.1).

24.3.1 Mechanisms Involved in Biocontrol

Biocontrol of fungal and bacterial phytopathogens involves the competition for nutrients or colonization sites, cytolytic effects, and antibiotic production (Han et al. 2015; Wang et al. 2015; Mnif et al. 2016). *B. subtilis* produces antimicrobial metabolites such as antibiotics the lipopeptides representing various antifungal and antibacterial antibiotics, which include fengycins, iturins, and surfactins (Mnif and Ghribi 2015); secretes hydrolytic enzymes, which have cell lysis effect; produces endospores; and alters the microenvironment conducive for plant growth, which favors its use as a biocontrol agent.

The success of biocontrol agents depends upon their capability to protect crops against target pathogens and pests; these must be eco-friendly. *B. subtilis* colonizes the root of plants in the rhizosphere; this potential is increased by genetically engineered strains that overexpress one or more characteristics so the strains with several different anti-pathogen traits can act synergistically when used together (Bahramisharif and Rose 2019; Hashem et al. 2019; Wang et al. 2018; Zhang et al. 2019, Dotaniya et al. 2016). *B. subtilis* isolates B4 used as a biofungicide to reduce peanut soil-borne diseases under both greenhouse and field conditions keeping Rizolex-T (fungicide) as standard (Ahmad et al. 2019).

Recovery from oxidative stress-induced damage is possible via antioxidant synthesis, which inhibits ROS formation. *Bacillus*-based bacterial association in plants can reduce the synthesis of ROS in cells via various scavenging enzymes (Ashraf et al. 2019; Lima et al. 2019; Khan et al. 2019) (Table 24.3 and Fig. 24.1). *B. subtilis* checks the growth of pathogens by the following mechanisms: to gain an insight

Table 24.2 Influence of *B. subtilis* inoculation on vegetables and other crops for seed germination and growth parameters

Crop name	Function	References
Tomato	Enhances fruit and grain yield	Xu et al. (2014), Abbasi and Weselowski (2015), Akram et al. (2016), Qiao et al. (2017), and Bahramisharif and Rose (2019)
Pepper plants	Improves photosystem II effectiveness and boost photosynthesis	Samaniego-Gámez et al. (2016)
Chickpea	Enhances chlorophyll, protein, and sugar contents	Patil et al. (2014), Egamberdieva et al. (2017a, b), Abd_Allah et al. (2017) and Khan et al. (2019)
Bean	Augments leaf water content and the parameter of stomata	Li Y et al. (2016a, b), Lima et al. (2019) and de Lima et al. (2019)
Mung bean	Improves growth	Hashem et al. (2017), Shen et al. (2017) and Arif et al. (2019)
Cucumber	Seed germination, plant growth, and yield	Radhakrishnan and Lee (2013, 2014)
Soybean	Increases growth significantly	Kang et al. (2014b) and Akinrinlola et al. (2018)
Pigeon pea	Promotes cardiovascular health and can be build up as a new dietary complement or useful food that avert hypertension	Lee et al. (2015)
Barley	Enhances grain yield, straw, total yield (TY), and plant nutrient element (PNE) content	Baris et al. (2014).
Rice	Enhancing plant growth	Elshakh et al. (2016) and Ahmad et al. (2017)
Maize	Significant increases in root dry weight and increased total biomass	Ahmad et al. (2017), Akinrinlola et al. (2018), Lima et al. (2019), Lima et al. (2019) and Misra and Chauhan (2020)
Wheat	Increases shoot length, increase the crop yield	Pourbabaee et al. (2016), Barnawal et al. (2017), Lastochkina et al. (2017), Reiss (2017), Akinrinlola et al. (2018) and Ashraf et al. (2019)
Arabidopsis thaliana roots	Utilizes carbon-rich root exudates	Allard et al. (2016)
Sesame	Increases the length and biomass of shoot, roots, and leaves	Radhakrishnan and Lee (2016)
Cacao	Stimulates both foliar and root growth	Falcäo et al. (2014)
Timothy plants	Improves the yield	Gagné-Bourque et al. (2016)
Cannabis	Improves marijuana and hemp yield and quality	Lyu et al. (2019)

Table 24.3 Disease control by various strains of *B. subtilis*

Strain no.	Crop	Disease	Causal agent	Mode of action	References
B. subtilis	Tomato	Root knot	Nematodes	Activates ISR	Adam et al. (2014)
B. subtilis	Tomato	*Botrytis* rot	*Botrytis cinerea*	Uses as one of the biocontrol agents for disease suppression	Lee et al. (2014)
4812	Tomato	Bacterial wilt	*R. solanacearum*	Considers as a more effective biocontrol agent	Almoneafy et al. (2013)
IAGS174	Tomato	*Fusarium* wilt	*Fusarium* sp.	ISR	Akram et al. (2014)
OTPB1	Tomato	Early and late blight	*P. infestans*	PGP and ISR	Chowdappa et al. (2013)
QST 713	Tomato	Bacterial spot	*Xanthomonas*	Biofilms formation; surfactin and iturin	Abbasi and Weselowski (2015)
QST 713	Tomato	Bacterial speck	*Pseudomonas syringae*	Reduces disease significantly	Fousia et al. (2016)
B. subtilis	Potato	Black scurf of	*R. solani* (PRS1)	Produces antifungal substances against plant pathogens	Singh et al. (2017)
B. subtilis	Rice	Sheath blight	*F. oxysporum f.* sp. *melonis*, *R. solani*	Effective formulation as an alternative to chemical fungicides	Singh et al. (2017)
NUU4	Chickpea	Root rot	*F. solani*	Reduces the infection	Egamberdieva et al. (2017b)
BS21-1	Chinese cabbage and lettuce	Soft rot	*Pectobacterium carotovorum* SCC1	Uses as biocontrol agents for disease suppression	Lee et al. (2014)
BS21-1	Cucumber	Anthracnose lesions	*Colletotrichum orbiculare*	Uses as biocontrol agents for disease suppression	Lee et al. (2014)
BERA 71	Mung bean	Goid disease	*Macrophomina phaseolina* (Tassi)	Modulate the metabolism of pigments, hormones, antioxidants	Hashem et al. (2017)

(continued)

Table 24.3 (continued)

Strain no.	Crop	Disease	Causal agent	Mode of action	References
LCA1 and M24	Mung bean	Seed sprouts pose	*Salmonella enterica* and *Enterohemorrhagic Escherichia coli* (EHEC)	Production of antimicrobial substance and/or nutrition/space competition	Shen et al. (2017)
PHYS77, PHYS78	Onion	Cape and umbel blights of onion	*Botrytis* sp.	Biocontrol agent	Elyousr et al. (2020)
B. subtilis	*Cannabis* crop	Powdery mildew	*Fusarium*, sooty molds	Growth promotion and disease biocontrol	Lyu et al. (2019)
IIHR BS-2	Carrot	Root-knot nematode and soft rot	*E. carotovorum*	Increases in carrot yield	Rao et al. (2017)
GLB191	Grape	Grape downy mildew	*Plasmopara viticola*	Uses as a new biocontrol product	Yan et al. (2019)
UMAF6639	Melons	Cucurbit powdery mildew	*Podosphaera fusca*	Biological control agent	Garcia-Gutierrez et al. (2013)
Y-1	Apple	Root rot	*Fusarium* sp.	PGP and ISR	Ju et al. (2014)
EA-CB0015	Banana	Black sigatoka	*Mycosphaerella fijiensis*	Reduces the severity of the disease	Gutierrez-Monsalve et al. (2015a, b)
HJ5	Cotton	*Verticillium* wilt	*Verticillium dahlia*	Biofilm formation	Li et al. (2013)
B15	Rice seed	Leaf blight (BLB)	*Xanthomonas oryzae pv. Oryzae*	Enhances activities of defense-related enzymes	Elshakh et al. (2016)
SYX04	Rice	rice blast	*M. oryzae*	Compete for colonization sites; ISR	Mnif et al. (2015)
OH 131.1	Wheat	Head blight	*F. graminearum*	Bioactive metabolites	Dunlap et al. (2015)
QST713	Wheat	Yellow rust	*Puccinia striiformis*	Enhance growth	Reiss and Jørgensen (2017)
Tpb55	Tobacco	Tobacco black shank	*P. nicotianae*	Biofilm formation of antibiotic substances	You et al. (2016) and Han et al. (2015)

into the type of molecules that activate the AHL biosensors, it was decided to test whether these compounds were substrates for the AiiA AHL-lactonase, a quorum quenching enzyme from *Bacillus* sp. The *Bacillus* aiiA gene encoding the AiiA protein was cloned, expressed, and purified to homogeneity as a His6-tagged protein (Degrassi et al. 2007).

Copper hydroxide along with *B. subtilis* QST 713 was tested against *Pseudomonas syringae* under greenhouse conditions. The plant showed protective activity of QST 713, as Pin2 expression was significantly higher in the QST 713-treated plants challenged with Pst compared to the control Pst-inoculated plants (Fousia et al. 2016). Induced systematic tolerance and improve the health of plants (Kang et al. 2014b). *Bacillus* strain 6051produces surfactin, indicating very stable biofilm formation and that it is a good biocontrol agent against pathogenic bacteria (Bais et al. 2004). As previously mentioned, the lipopeptides produced by *B. subtilis* characterize different antifungal and antibacterial antibiotics including fengycins, iturins, and surfactins (Mnif and Ghribi, 2015). *B. subtilis* forms a thin biofilm on the roots for long-term colonization of the rhizosphere. *B. subtilis* locates and colonizes young roots during chemotaxis (Allard et al. 2016). Biofilms consist of a multicellular bacterial community enclosed in a self-secreted matrix. The timing of the formation of a *B. subtilis* biofilm on host roots is also dependent on the promoter of the genes responsible for the production of the matrix when the bacterium initially contacts a root (Beauregard et al. 2013) in the soil; *B. subtilis* forms biofilms on plant roots which help to produce lipopeptides and augment their antimicrobial activity (Davey et al. 2003). *Bacillus* species are used for rhizosphere applications and also function as plant endophytes (McSpadden Gardener and Driks 2004) that also protect plants from pathogens (Romero et al. 2004).

24.3.1.1 Induction of Host Enzymes Such as Peroxidase, Polyphenol Oxidase, Superoxide Dismutase

B. subtilis can secrete antibiotics and hydrolytic enzymes; it can be used as environment modifier in a self-beneficial manner and also produces resistant endospores to sustain itself under adverse conditions (Dotaniya et al. 2016). Antibiotic secretion and release of transcription factors (Malfanova et al. 2012; Beauregard et al. 2013; Allard et al. 2016; Yang et al. 2016a, b). *B. subtilis* serves as a potent insecticide due to the production of lipopeptides (surfactin C14, C15, iturin 15) (S499 strain). The strain BS-Y9 is active against green peach aphid *Myzus persicae*, and the active components are the surfactins isomers of C14leu 7, C14 val7, and C15 leu 7 with leucine moieties showing higher activity. The strain SPB1 lipopeptide showed activity against ectomyelois *Ceratoniae zeller* (Assié et al. 2002; Ghribi et al. 2012; De Faria et al. 2011; Basaid et al. 2020).

24.3.2 B. subtilis *as a Biocontrol Representative in Different Crops*

B. subtilis strains are rhizospheric endophytes and are widely studied as biocontrol agents both in vitro and in greenhouses and fields for sustainable agriculture (Rao et al. 2017; Hashem et al. 2017; Singh et al. 2017; Yan Li et al. 2019; Lyu et al. 2019).

Table 24.3 lists some *B. subtilis* strains as biocontrol agent allied with plant disease control, e.g., *Fusarium* head blight of wheat (Dunlap et al. 2015), wheat take-all (Yang et al. 2015) head blight (Dunlap et al. 2015), yellow rust (Reiss and Jørgensen, 2017), rice blast (Mnif et al. 2015; Yang et al. 2015), leaf blight (BLB) (Elshakh et al. 2016), and sheath blight (Singh et al. 2017). *B. subtilis* is also utilized to manage tomato *Fusarium* wilt (Akram et al. 2016), bacterial spot (Abbasi and Weselowski 2015), and cucumber root rot (Senol et al. 2014). Moreover, *B. subtilis* also controls some post-harvest diseases, e.g., soft rot of fruits (Tang et al. 2014). *B. subtilis* suppresses tobacco black shank (Han et al. 2015; You et al. 2016).

Notably, *Bacillus* species can act as plant endophytes that protect plants against pathogens (Lastochkina et al. 2019) (Elshakh et al. 2016). *B. subtilis* is beneficial for plant productivity (Venkateshwaran et al. 2013) as they promote plant growth by improving plant nutrition, synthesis, and regulation of phytohormones, and suppression of disease-causing organisms.

24.4 Alleviation of Abiotic Stress by *B. subtilis*

Drought and salinity due to industrial pollution engulfing the agricultural land are the most atrocious abiotic factors restricting the yield of crop plants because most crops cannot tolerate these stresses. Microorganisms come in front to save the agricultural land and increase the agriculture produce such as tolerance to saline conditions, synthesis of compatible solutes, production of plant growth-promoting hormones, and biocontrol potential, which interact with crop plants and impart tolerance to stress conditions (Shrivastava and Kumar 2015). Inoculations of plants with these bacteria stimulate root exudation by plants, which in turn promote both bacterial and plant growth (Kakar et al. 2016).

The plants colonized by bacillus absorb more water so it assists plant combat drought and salt stress (Allah et al. 2017; Egamberdieva et al. 2017a, b; Lastochkina et al. 2017; Misra and Chauhan 2020; Woo et al. 2020; Kakar et al. 2016; Kang et al. 2014b) and water stress (Lima et al. 2019; Li et al. 2016a, b). The extenuating property of *Bacillus*-induced physiological alterations in plants is exposed in Table 24.4.

The chemical fungicides and insecticides hamper the number and growth of valuable soil microbes that help to augment plant growth. Thus, the replacement of chemical pesticides with beneficial bacteria, such as *B. subtilis*, can reduce the use of synthetic pesticides and insecticides in modern agriculture. *B. subtilis* is known as protected bacteria. *B. subtilis* constructs endospores, allowing them to germinate

under different environmental cues that permit long-term storage, and reduces the intricacy of the formulation procedure (Hashem et al. 2019).

24.4.1 Antioxidant Enzymes in Stress Management

Abiotic stresses impede seed germination, hinder plant growth, and lower crop productivity. When plants are subjected to abiotic stresses such as salinity, drought, temperature extremes, herbicide treatment, and mineral deficiency, excessive salt ions absorbed by the plant break the ion homeostasis of plant cells, which in turn affects the activity of relevant enzymes, resulting in the accumulation of a large number of toxic substances such as reactive oxygen species (ROS)s. A balance between the ROS production and the appeasing action of antioxidants does exist. Any disturbance of the balance results in peroxidation, which causes oxidative damage to macromolecules of cells, viz., lipids, protein, and nucleic acids. Superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) are some of the enzymes which act as scavengers of ROS, ensuing enhanced plant resistance to stresses (Hu et al. 2019).

The APX, SOD, and GR degrade H_2O_2 in cell organelles such as chloroplast and mitochondria. Similarly, CAT and POD eliminate H_2O_2 and can counterbalance for ageoxy intermediates and free radicals. SOD curbs relative amounts of O2− and thus diminishes the hazard of −OH radical formation. CAT is the chief scavenger of H_2O_2 in peroxisomes, and it converts this to water and molecular oxygen. CAT and POD also have the same role against oxidative stress by detoxifying H_2O_2 in chloroplast, cytosol, mitochondria, and peroxisome of plant cells. SOD and CAT help GSH in its role as a free radical scavenger physiologically by decreasing levels of hydrogen peroxide, CAT, which is present frequently in glyoxysomes of lipid-storing tissues in plants; this enzyme contains a tetrameric heme which catalyzes the switch of hydrogen peroxide to water and molecular oxygen formed from the β-oxidation of fatty acids. The APX is present in different active isomeric forms in chloroplasts, cytosol, and microsomes of plants, which is one of the vital antioxidant enzymes that scavenge H_2O_2 by using ascorbate for reduction (Abd_Allah et al. 2017; Ashraf et al. 2019; de Lima et al. 2019; Khan et al. 2019).

24.5 Conclusion

The ability of *B. subtilis* to enhance plant growth has been commercialized. *B. subtilis* enhances plant growth through the direct and indirect mechanism. The endospore-forming ability of *Bacillus* allows its application in the harsh environmental conditions such as abiotic and biotic stress. Plant growth-promoting *Bacillus* spp. showed growth of roots, shoots, and leaves as well as enhanced yields. *Bacillus* sp. can be used as biofertilizers, and soil fertility control agents inhibit

Table 24.4 Management of abiotic stress in different crops by *B. subtilis*

Crops	Abiotic stress	Mode of action	References
Wheat	Drought	Increase macronutrients	Barnawal et al. (2017)
Rice	Drought	Induced efficient tolerance and develop the health of plants	Kakar et al. (2016)
Soybean	Salt and drought stress	Enhance plant growth, increase the water, nutrients, antioxidants pigments, and hormones	Kang et al. (2014b)
Chickpea	Salinity	Ameliorate seed germination and plant development, carbohydrates, proteins, and osmolytes	Qurashi and Sabri (2013)
Rice	Salinity	Prompt the expression of the NADP-Me2, EREBP, SOSI, BADH, and SERK1 genes	Nautiyal et al. (2013)
Cucumber	Salinity	Seed germination, plant enlargement, and yield	Radhakrishnan and Lee (2013, 2014)
Soybean	Salinity	Enhance plant growth, increase the water, nutrients, antioxidants, pigments, and hormones	Kang et al. (2014b)
White clover	Salinity	Augmentation of the K^+/Na^+ ratio in plants grown	Han et al. (2014)
Bassia indica	Salt stress	Prompt the synthesis of chlorophylls a and b and carotenoid, increase photosynthesis	Hashem et al. (2015a, b)
Solanum lycopersicum	Salinity	Amend lipid synthesis, specifically that of oleic, linoleic, and linolenic acids as well as phospholipids	Hashem et al. (2015a, b)
Acacia gerrardii Benth	Salt	Induction of osmoregulation	Hashem et al. (2016a, b)
Acacia gerrardii	Salinity	Enhance plant growth	Hashem et al. (2016a, b)
Rice	Salinity	Sledding in lipid synthesis might mitigate lipid peroxidation and oxidative stress, CAT and POD activities are enhanced	Jha and Subramanian (2015)
Chenopodium quinoa	Salinity	Growth and productivity of quinoa could be improved by inoculating	Yang et al. (2016a, b)
Chickpea	Salinity	Renovate plant biomass and the synthesis of photosynthetic pigments	Abd_Allahet al. (2017)
Chickpea	Salinity	Improve plant growth and symbiotic performance and to control root rot disease	Egamberdieva et al. (2017a, b)
Wheat	Salinity	Increase growth	Lastochkina et al. (2017)
Maize	Salinity	Prompt plant response for defense enzymes, chlorophyll, proline, and soluble sugar	Misra and Chauhan (2020)

(continued)

Table 24.4 (continued)

Crops	Abiotic stress	Mode of action	References
Faba bean	Water stress	Efficacy of the closure of stomata and promoted water use efficiency	Li et al. (2016a, b)
Common bean and maize	Water stress	Magnification of leaf water content and the regulation of stomata	Lima et al. (2019)
Mung bean	Heavy metal, lead (Pb)	Improve mung bean growth potential	Arif et al. (2019)

disease-causing pathogenic microbial growth and provoke pest defense systems in plants. Since these PGP *B. subtilis* inoculants exhibited multiple characterizations valuable to the plants, it can be useful for the development of newer, safer, and effective seed treatments as an alternative to chemical fertilizer. *B. subtilis* carves a space in the agriculture market as a potent biofertilizer, biocontrol agent, and mico-remediator of increased drought, salinity, and a whole host of industrial pollutions.

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Chapter 25
Bacillus thuringiensis Proteins: Structure, Mechanism and Biological Control of Insect Pests

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Abstract *Bacillus thuringiensis* (Bt) produces a wide variety of insecticidal proteins. It synthesizes δ-endotoxins as parasporal crystalline inclusion bodies during its sporulation and the stationary growth phase. It also produces vegetative insecticidal proteins that are initially given off during the bacterial vegetative growth stage. The insecticidal proteins are widely used as biopesticides either by spraying or by incorporating into transgenic crops. The application of these insecticidal proteins significantly reduced the use of synthetic chemical insecticides. It also controls the major agricultural pests with increased target specificity and environmental safety.

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This chapter updates the structure and specificity, insecticidal activity, mechanism of action, and application of Bt proteins for biological control of target insect pests in various crops. Furthermore, we discuss the benefits of using Bt biopesticides over chemical insecticides to promote sustainable and eco-friendly agriculture.

Keywords *Bacillus thuringiensis* · insecticidal protein · Bt crops · biopesticide · biological control

25.1 Introduction

Biological control of destructive insect pests is not a new approach. However its importance is increasing day by day due to the emergence and re-emergence of insect resistance to chemical insecticides as well as increasing risk to public health and environmental safety (Usta 2013; Islam et al. 2016, 2019). Insects have a strong capacity of developing genetic resistance which can restrict the prolonged use of insecticidal chemicals or toxins (Jin et al. 2019a; Zhang et al. 2020; Datta et al. 2021). The remarkable progress in functional genomics and genetic engineering makes it possible to know the gene function and to transfer the desired gene from one organism to another organism of a totally different genetic background. An improved understanding of the mechanism of action, target specificity and genetics of the biocontrol agents will provide a basis for their future improvement. Instead of chemical insecticides, different entomopathogenic viruses, bacteria, and fungi can be efficiently applied to manage insect pests. Among the biocontrol agents, Bt is broadly used for controlling insect pests as well as other organisms such as mites, nematodes, and protozoans (Schnepf et al. 1998).

Bt is a spore-forming gram-positive bacterium that can be isolated from soil, water, dead insects, different conifers, and leaves of deciduous trees (Höfte and Whiteley 1989; Palma et al. 2014). It was first isolated and identified in 1901 by a Japanese sericulturist Ishiwata Shigetane in *Bombyx mori* that had flacherie or flaccid disease. Later, it was isolated from infected *Ephestia kuehniella* and rediscovered by German microbiologist Ernst Berliner in 1911 (Berliner 1915; Knowles 1994). Bt is a member of the bacterial group *Bacillus cereus* complex, and they have a strong phylogenetic relationship (Fayad et al. 2019). The entomopathogenic properties of Bt distinguish itself from other members of *B. cereus* group (Helgason et al. 2000). In 1927, Bt was again isolated by Mattes, and it was used for the biological control of *Ostrinia nubilalis* in the next years (Beegle and Yamamoto 1992). Bt produces entomotoxic Crystal (Cry) and Cytolytic (Cyt) proteins or δ-endotoxins as parasporal crystals during its sporulation and the stationary growth phase (Höfte and Whiteley 1989; Schnepf et al. 1998; OECD 2007; Palma et al. 2014). Biopesticides that are produced from insecticidal crystal proteins are widely used to control the major agricultural pests worldwide (Tabashnik et al. 2013; Wu et al. 2019). Since 1996, transgenic crops expressing Cry proteins are comprehensively cultivated, which significantly reduced the use of synthetic insecticides. Bt also synthesizes vegetative insecticidal proteins (Vip) and secreted insecticidal protein

(Sip) during its vegetative growth stage. Novel Vip, first discovered in 1996, can be isolated from the culture media forthrightly (Estruch et al. 1996). Vips differ from Cry toxins in terms of structure and genetic makeup. Their binding sites and mode of action are also different from Cry toxins (Estruch et al. 1996; Yu et al. 1997). Vips have a broad insecticidal range and are compatible with other Cry proteins that facilitate the pyramiding of multiple *Bt* genes encoding different insecticidal proteins with multiplex modes of action. Pyramiding of *Bt* genes greatly increase the susceptibility of major damaging insects and help prevent the rapid emergence of insect resistance to Bt crops (Lee et al. 2006; Sena et al. 2009; Gouffon et al. 2011; Liu et al. 2011). Vip3 proteins exhibit a wide variety of insecticidal activities against pest Lepidoptera (Chakrabarty et al. 2020), and Vip1, Vip2 and Sip are toxic against Coleopteran insects (Donovan et al. 2006; Milne et al. 2008; Hernández-Martínez et al. 2013; de Escudero et al. 2014; Chakroun et al. 2016). However, these variations in function and target specificity among Bt proteins help to overcome the risk of the emergence of insect resistance to Bt toxins. It also provides a worthy alternative to chemical insecticides to control damaging insect pests in an environmentally safe way. This chapter aims to discuss the structure, insecticidal function, and biological control of insect pests by Bt. The benefits of using Bt technology in green farming are also discussed.

25.2 Bt Toxin Diversity

Bt species have been categorized into different subspecies based on phylogenetic and serotyping, phase susceptibility, and plasmid features. Approximately, 100 subspecies of Bt have already been identified to date. Most of these subspecies synthesize more than one Bt toxin that has a specific insect host range. For instance, *Bt* var. *kurstaki*, *Bt* var. *israelensis* and *Bt* var. *morrisoni* show specific insecticidal activity against Lepidopteran, Dipteran, and Coleopteran insects (Schnepf et al. 1998; Sanahuja et al. 2011). Among the δ-endotoxins produced during sporulation, 800 different *Cry* genes under 75 families and 38 different *Cyt* genes under three families have been identified based on their primary amino acid sequence (Jouzani et al. 2017; Sajid et al. 2018) (Fig. 25.1). Cry proteins are active against different insects belonging to the orders Lepidoptera, Coleoptera, Diptera, Homoptera, Hymenoptera, Orthoptera, Mallophaga and some other non-insect invertebrate species such as nematodes, mites, and protozoa (Crickmore et al. 1998; Ye et al. 2012). Bt strains that synthesize Cry5 and Cry6 proteins are found to be active against nematodes (Wei et al. 2003). Cyt toxins are mainly active against Dipteran insects. However, a few Cyt toxins are found to be active against Coleopteran insects (Federici and Bauer 1998; Guerchicoff et al. 2001). The Cry proteins that are not related phylogenetically have been grouped into 3D (three-domain) Cry toxins, Mtx-like (mosquitocidal) Cry toxins and binary (Bin)-like Cry toxins (Crickmore et al. 1998, 2018). Among the Cry toxins, 3D-Cry toxins formed the biggest group that consists of more than 53 different Cry toxin subgroups (Crickmore et al. 2018) (Fig. 25.1). The molecular structure of the members of the 3D-Cry toxin group is globular and

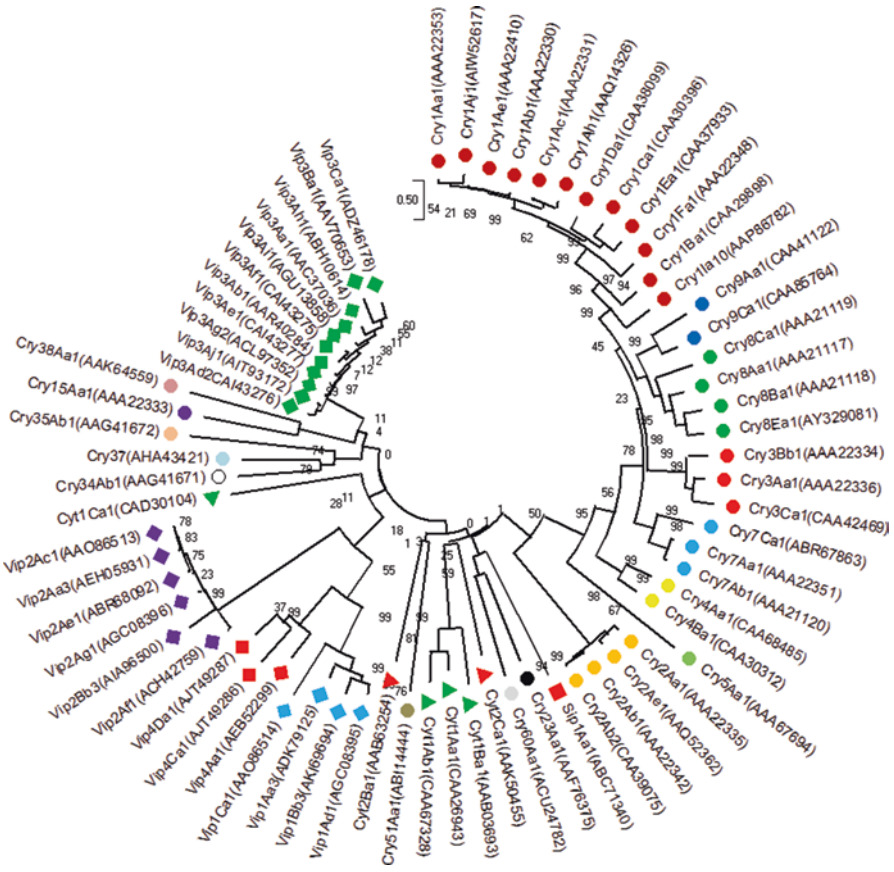

Fig. 25.1 Phylogenetic relationship among Bt toxins. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al. 1992). Evolutionary analyses were conducted in MEGA X program (Kumar et al. 2018). The NCBI GenBank accession number for specific protein is presented in the parenthesis

consists of three different domains which are connected by single linkers. The Cry toxins such as Cry34, Cry35 and Cry36 show their insecticidal activity against some Coleopteran larvae. They are named Bin-like Cry toxin as they have some similarities with the binary (Bin) toxins produced by *Lysinibacillus sphaericus*, which are specific to Dipteran pests. These toxins have a pore-forming capacity, and in an acidic environment, they produce ionic channels in the lipid bilayer (Masson et al. 2004). The members of the Mtx-like Cry toxin group have similarities to the Mtx toxin produced by *L. sphaericus* that shows mosquitocidal activity. However, Mtx-like Cry toxins such as Cry15, Cry23, Cry33, Cry37 and Cry38 are active against Coleopteran insects. The toxin Cry60 shows toxic activity against the larvae of Dipteran insects, while Cry51 shows toxicity against the insects belonging to the family Coleoptera and Hemiptera (Crickmore et al. 2018). In contrast to Cry toxins,

genetically and structurally different Vip proteins are produced during the vegetative growth stage. Their binding sites and mode of action are also different from Cry toxins (Estruch et al. 1996; Yu et al. 1997). There have been 15 Vip1, 20 Vip2, 110 Vip3, and 1 Vip4 proteins identified so far. The binary toxins that are produced from Vip1 and Vip2 proteins exhibit synergistic entomotoxicity against *Aphis gossypii* and some Coleopteran insects (Sattar and Maiti 2011). The Vip3 proteins are active against a wide range of Lepidopteran insects (Milne et al. 2008; Hernández-Martínez et al. 2013; de Escudero et al. 2014; Chakroun et al. 2016). To date, the insecticidal action of Vip4 protein is unknown (Crickmore et al. 2018; Chakrabarty et al. 2020).

25.3 Structure and Specificity of Bt Proteins

25.3.1 Cry Toxins

The structure of a protein reveals the placements of functional domains that are required for a toxin's specificity and proteolytic stability. Among the Cry proteins, 3D-Cry toxins are mostly used for controlling insect pests and their structures are well studied. The 3D-Cry toxins are produced as ~130 and ~70 kDa protoxins. The 130 kDa protoxin is not responsible for toxicity, but shares a highly conserved C-terminus that is essential for forming intermolecular disulfide bonds at the time of crystal formation (Bietlot et al. 1990; de Maagd et al. 2001). The structure of ~70 kDa protoxin is the same as the N-terminal portion of the large toxin group. In some cases, these small protoxins require the availability of accessory proteins for crystallization (Agaisse and Lereclus 1995; Berry et al. 2002). The C-terminal region is implicated in crystal formation, but in mature toxins, it is cleaved out in the insect midgut. Though there is very little similarity among the amino acid sequences of the Cry proteins, they share an overall similar structural topology. The X-ray crystallography revealed that active 3D-Cry proteins consist of three structurally and functionally distinct domains. Among the Cry proteins, the first 3D structure of Cry3A was elucidated in 1991 (Li et al. 1991), then three-dimensional structures of other members of Cry family such as Cry1Aa, Cry1Ac, Cry2Aa, Cry3Bb1, Cry4Aa, Cry4Ba, Cry5B, Cry7Ca1 and Cry8Ea1 have been revealed (Grochulski et al. 1995; Derbyshire et al. 2001; Galitsky et al. 2001; Morse et al. 2001; Boonserm et al. 2005, 2006; Guo et al. 2009; Hui et al. 2012; Jing et al. 2019). Later, three-dimensional structures of 120 kDa Cry1Ac1 protoxin have been determined, which consist seven different structural domains (DI-DVII) (Evdokimov et al. 2014). Domain I spans at the N-terminal end of the 3D-Cry toxins. It is a bundle of seven α-helix where a central helix α-5 is surrounded by six outer helices. These six helices are amphipathic in nature and are long enough to span the 30 Å thick hydrophobic region of a membrane bilayer. Domain I remains highly conserved, and shares strong structural similarity with the domain of α + PFTs (Pore-Forming Toxins) Colicin A. It is involved in membrane insertion, oligomerization of toxin and pore

formation (Berry et al. 2002; Pardo-Lopez et al. 2013; Domínguez-Arrizabalaga et al. 2020; Vílchez 2020). Domain II, a β-prism comprising three anti-parallel β-sheets packed around a hydrophobic core with exposed loop sections, is responsible for receptor recognition in the insect midgut. Domain III is a β-sandwich of two anti-parallel β-sheets that plays a role in receptor binding and formation of pores in the cell membrane (de Maagd et al. 2001; Pardo-Lopez et al. 2013). Domains IV and VI are α-bundles, just as the spectrin domain or the bacterial fibrinogen-binding complement inhibitor. Domains V and VII, on the other hand, are β-rolls like carbohydrate-binding proteins (Evdokimov et al. 2014). Though the functions of domains IV and VII are unknown, they may have important roles in the formation of crystal and stability of toxins in the insect gut. Moreover, recent studies suggest that the domains V and VII could interact with the proteins present in gut membranes, and therefore, are involved in receptor recognition (Zghal et al. 2017).

Though the structure and function of ETX_MTX2 proteins are still not clear, they show similar features with the epsilon toxin of *Clostridium perfringens*. It implies that they may possess an extended β-sheet and the capacity of a forming pore. A single β-stranded domain is found in the crystal structure of Cry23Aa (Fig. 25.2) that is structurally similar to β-pore-forming proaerolysin. The proaerolysin is produced by *Aeromonas hydrophila* and other related bacteria species (Palma et al. 2014; Domínguez-Arrizabalaga et al. 2020). Cry37Aa has a C2 β-sandwich fold, similar to the calcium phospholipid-binding domain identified in human cytosolic phospholipase A2 (Fig. 25.2) (Rydel et al. 2001). Bt toxins belong to the Toxin_10 family include Cry35 and Cry36 toxins. The crystal structure of Cry35Ab1 toxin shows an aerolysin-like fold which has a β-trefoil N-terminal domain containing QxW motifs similar to a carbohydrate-binding domain found in proteins such as ricin and Mtx1 from *L. sphaericus* (Fig. 25.2). The Cry34Ab1 is

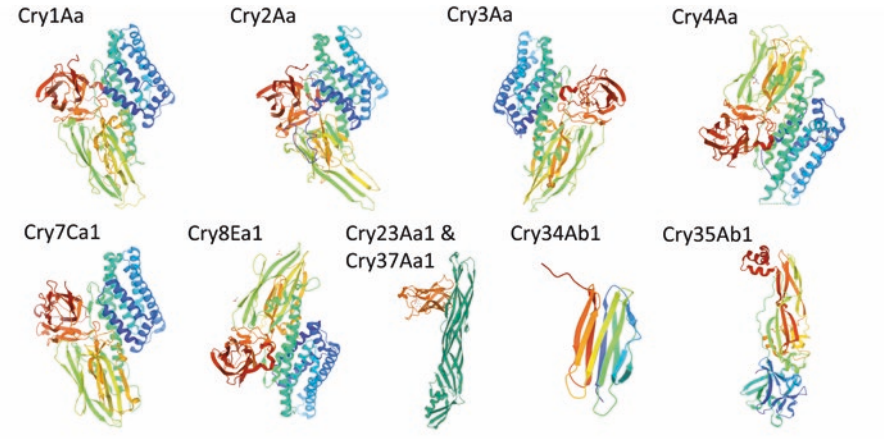

Fig. 25.2 Three-dimensional structures of some Cry toxins produced by Bt. The PDB (Protein Data Bank) accession numbers for Cry1Aa, Cry2Aa, Cry3Aa, Cry4Aa, Cry7Ca1, Cry8Ea1, Cry23Aa1 and Cry37Aa1, Cry34Ab1 and Cry35Ab1 are 1CIY, 1I5P, 1DLC, 2C9K, 5ZI1, 3EB7, 4RHZ, 4JOX and 4JPO, respectively

also a member of aerolysin family that contains a β-sandwich fold and a hydrophobic core (Fig. 25.2) (de Maagd et al. 2003). Cry22 is a non-3D protein, showing structural similarity with the domain III of 3D toxin by containing four cadherin-like proteins and a C-terminal region (Kelker et al. 2014).

25.3.2 Cyt Toxins

The Cyt toxins constitute another group of insecticidal toxins produced by Bt. The size of proteolytically active Cyt protoxins is around 25 kDa (Soberón et al. 2013). Like some Cry proteins, the 3D structure of Cyt proteins has also been known. The 3D structures of Cyt1Aa, Cyt2Aa, and Cyt2Ba revealed that they have a similar structure comprising a single α-β domain with two layers of α-helix hairpins wrapped around a β-sheet (Fig. 25.3) (Li et al. 1996; Cohen et al. 2008, 2011). Analysis of membrane insertion ability with peptides of Cyt1Aa exhibited that the α-helix peptides are major structural components involve in the membrane interaction and also in the toxin oligomerization (Gazit et al. 1997; Promdonkoy et al. 2008), while the β-sheet is involved in membrane insertion by forming an oligomeric pore with a β-barrel structure into the membrane (Li et al. 1996). However, Cyt1Ca is different from other Cyt toxins, as it has a further domain at the C-terminal end with homology to the carbohydrate-binding domain of the ricin (Manasherob et al. 2006).

25.3.3 Vip Toxins

Bt produces novel insecticidal proteins known as Vips during its vegetative growth stage. Their structure, genetics as well as the manner of action are different from Cry proteins (Estruch et al. 1996; Yu et al. 1997). The Vip1 and Vip2 proteins are produced from *B. cereus* strain AB78, and they are transcribed together from a ~4 kb single operon. Vip1 encodes ~100 kDa protein as a protoxin and produce as mature

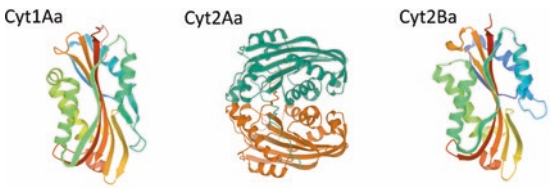

Fig. 25.3 Three-dimensional structures of Cyt toxins produced by Bt. The PDB accession numbers for Cyt1Aa, Cyt2Aa and Cyt2Ba are 3RON, 1CBY, and 2RCI, respectively

toxin of ~80 kDa after secretion. Furthermore, Vip2 generates a ~ 50 kDa trypsin-resistant fragment. The Vip1 and Vip2 together form a binary toxin, and they both are required for toxicity (Warren 1997; Bi et al. 2015). The crystal structure of Vip2 revealed that it is maixed α/β protein and contains two domains (Fig. 25.4). The homology of Vip1 and Vip2 to other binary toxins indicates that they form a classical A + B type binary toxin, where Vip2 is the A domain responsible for cytotoxicity, and Vip1 is the B domain responsible for receptor-binding and translocation of the cytotoxic Vip2 into the susceptible insect cell (de Maagd et al. 2003; Barth et al. 2020). The Vip2 toxin shares structural and sequence similarities with the *Clostridium difficile* toxin CdtA and the *C. perfringens* toxin iota. These toxins possess ADP-ribosyltransferase activity that targets actin protein, and thus the activated form of these toxins could cause cytoskeleton disruption and cell death (Han et al. 1999; Jucovic et al. 2008). The Vip1 binds to the membrane receptor in monomeric form, and their interaction cause a subsequent structural changes followed by the formation of homoheptamers that translocate Vip2 toxin into the cytoplasm through acid endosomes (Barth et al. 2020). After translocation of Vip2 toxin inside the cytoplasm, it destroys filamentous actin and causes cell death by cytoskeleton disruption (Shi et al. 2004, 2007).

The Vip3 proteins are secreted from Bt strain AB88 and AB424 without processing at the N-terminus (Estruch et al. 1996). The number of amino acids in the Vip3A proteins ranges from 657 to 795 with an average of around 787-amino acids, and the average molecular weight is ~89 kDa (Chakroun et al. 2016). The N-terminal region could function as a secretion signal peptide and plays a role in protein activation. It is contingent that the exceptionally conservative N-terminal region of Vip3A protein conserves the protein's structure and control its insecticidal function, whereas the varied C-terminal domain play a role in target precision (Zack et al. 2017). A number of recent studies have reported some important conformational information of Vip3A proteins. The cleavage of Vip3Aa protoxin happened at the primary cleavage site despite the amount of treated trypsin used. However, the resulting peptides of 66 and 19 kDa remained together, indicating the presence of secondary structure with a cluster of β-sheets at the C-terminus (Bel et al. 2017). Alanine scanning revealed five structural domains in the Vip3Af1 protein's 3D structure (Banyuls et al. 2018). In solution, both the protoxin and the functional Vip3 proteins form

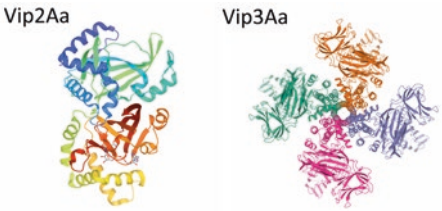

Fig. 25.4 Three-dimensional structures of Vip proteins produced by Bt. The PDB accession numbers for Vip2Aa and Vip3Aa are 1QS2 and 6TFK, respectively

homo-tetramers (Zack et al. 2017). It has also been observed that an interaction between the amino-terminal and the carboxy-terminal is essential for lethal toxicity, as this interaction increases oligomerization and provides proteolytic stability (Zack et al. 2017).

The Vip4 protein is the latterly identified from Bt strain Sbt016. The complete structure and function of Vip4 protein is still unknown (Crickmore et al. 2018). The length of the Vip4Aa1 gene is 2895 bp, and its protein sequence contains 965 amino acid residues with a molecular weight of ~108 kDa. The Vip4Aa protein is phylogenetically close to Vip1 proteins than other Vip proteins. It exhibits 34% sequence similarity with Vip1Aa1 protein. As Vip4 protein resembles the B component of the binary toxin, it is likely that it might interact with an unidentified A component to show its toxicity. Conversely, it might also be possible that the Vip4 is a B component that has lost its A component and forfeit its toxicity (Crickmore et al. 2018; Chakroun et al. 2016). However, deep analysis of the genome of Bt strain Sbt016 may shed some light on the structure and mechanism of this protein.

25.4 Mechanism of Action of Bt Proteins

25.4.1 Mechanism of Action of Cry Proteins

The mechanism of action of Cry toxins has been widely studied, but still there are some controversies. The pore-forming model of Cry toxin activity against various insects is the most commonly accepted mode of action (Schnepf and Whiteley 1981; Zhuang et al. 2002; Bravo et al. 2004; Rodríguez-Almazán et al. 2009) (Fig. 25.5a). The toxin crystals were dissolved at high pH (acid or alkaline, depending on the Cry toxin) and proteolyzed by proteases on the midgut under suitable physicochemical conditions. By traversing through the peritrophic matrix, activated toxins can enter the apical brush border membrane of the insect's midgut. To form pores, Cry toxins need to bind to specific receptors in brush-border membranes (Bravo et al. 2004, 2007, 2005; Jin et al. 2019b). In this case, Domain I of Cry toxins fixes into the cell membrane through its hydrophobic helical hairpin, where highly variable loops from Domain II plays a significant role in the identification of Cry toxin receptors such as aminopeptidase N (APN), alkaline phosphatase (ALP) and ATP-binding cassette (ABC) transporter (Bravo et al. 2004, 2005; Pardo-López et al. 2006; Pigott and Ellar 2007; Adang et al. 2014; Palma et al. 2014; Wu et al. 2019; Jin et al. 2021). Domain III is a crucial structure, involved in receptor binding and pore formation. It interacts to the APN receptor's N-acetylgalactosamine (GalNAc). In *Manduca sexta* and *Bombyx mori*, APN has been identified as a binding receptor for Cry1A toxins (Grochulski et al. 1995; Bravo et al. 2004; Pacheco et al. 2009). Some studies suggested that, for high cytotoxicity, binding of Cry protein to ABC transporters is critical, while the interaction of Cry proteins with the other receptors may increase toxicity (Tanaka et al. 2013; Bretschneider et al. 2016). The detailed mechanism of

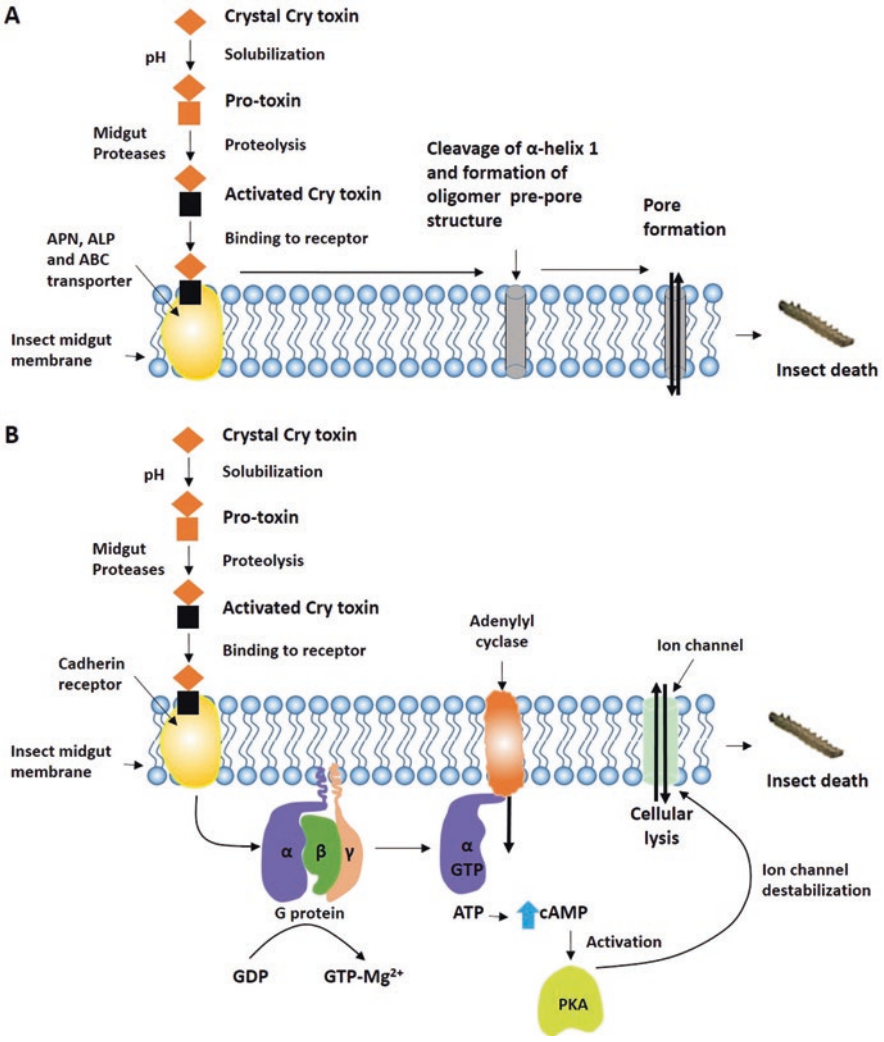

Fig. 25.5 Simplified models of the mechanisms of action of Cry proteins. (**a**) Pore-forming model, (**b**) signaling pathway model

the post-binding events that results in cell death is controversial (Vachon et al. 2012). However, most studies support that insertion of the Cry protein oligomer into the enterocyte membrane is essential to form a cation-selective pore that results in osmotic imbalance and cell death forwarded by reparative water influx through aquaporins (Endo et al. 2017). The midgut epithelial barrier is destroyed by massive enterocyte death that allows invasion of the hemocoel by Bt and other bacteria present in the gut, thus causing the death of the host by septicemia (Raymond et al. 2010; Jurat-Fuentes et al. 2021).

The second proposed model for Cry toxin mode of action is the signaling pathway model. A cell culture toxicity experiment is performed by expressing a cadherin receptor from *M. sexta* in the Sf9 cell line to explore the mechanism of action of Cry toxins in a signaling pathway model (Wickham et al. 1992; Kwa et al. 1998; Castella et al. 2019). The signaling pathway model hypothesizes that cytotoxicity of Cry proteins is reconciled by recognizing and binding to a cadherin receptor, which stimulates an Mg^{2+}-dependent cellular signal cascade pathway that directs cell death (Castella et al. 2019). The binding of Cry toxins to the cadherin receptor stimulates adenylyl cyclase activation that triggers an increase in cAMP and activation of protein kinase A (PKA). These circumstances stimulate a cascade that causes cell death by the formation of an ion channel on the membrane and cytoskeleton destabilization (Fig. 25.5b) (Zhang et al. 2006).

25.4.2 Mechanism of Action of Cyt Proteins

The insecticidal mechanism of Cyt proteins is still unknown, and it is also not clear whether Cyt proteins have a specific receptor for target specificity. Nonetheless, the cytotoxicity mechanism of Cyt toxins follows two main models. In the pore-formation model, similar to the Cry toxins, the monomer of Cyt toxins bind to the specific receptors of the membrane surface. According to this model, Cyt toxins interact straight with saturated membrane lipids such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (Thomas and Ellar 1983; Butko 2003; Mendoza-Almanza et al. 2020). Then, a conformation change occurs in Cyt toxin that supports in recruiting six monomers of Cyt toxin and conjoins the monomers into an open umbrella-like structure. In this structure, the strands span the lipid bilayer crosswise, while the alpha-helices remain on the surface of the membrane. These events result in succeeding membrane permeabilization, pore-formation, and finally insect death (Butko 2003; Mendoza-Almanza et al. 2020).

Another model for the mechanism of action of Cyt proteins is the detergent-effect model. According to this model, the Cyt toxins exhibit their cytotoxicity against target cells through a solubilization effect, where Cyt toxins concentrate on the surface of the cell membrane and dissolve the lipid bilayer in a detergent-like manner (Butko 2003; Mendoza-Almanza et al. 2020). These models are not mutually exclusive, thus, one or both may act against susceptible cells based on the concentration of the toxins (Butko 2003). The detergent effect could be induced only at higher concentration of Cyt toxin, while the oligomerization and pore formation has occurred at low concentrations of Cyt toxin (Butko et al. 1996; Mendoza-Almanza et al. 2020). Thus, at high toxin concentration, the target cell membranes are not able to assemble oligomers; alternately, it forms a toxin-lipid complex in which the integrity of the membrane is entirely lost (Butko 2003; Mendoza-Almanza et al. 2020).

25.4.3 Mechanism of Action of Vip Proteins

The studies on the mechanism of action of Vip proteins against insects are very limited. However, some studies on the mechanism of action of Vip3A proteins have been conducted with the members of the Vip3Aa subfamily. When the larvae fed Vip3A proteins, the midgut cells showed damaging symptoms including cell swelling, lysis of epithelial cells and leakage of cell content (Chakroun and Ferré 2014; Boukedi et al. 2015). Vip3Aa protoxin is hydrolyzed by insect midgut juice into 62–66, 45, 33, and 22 kDa polypeptide fragments in vitro. In susceptible insects, the activated Vip3A can function when it is inserted into the brush border membrane vesicles (BBMV). However, for specificity and insecticidal activity of Vip3A, the hydrolysis and activation by midgut protease is not an essential step. Non-target insects can also use midgut juice to hydrolyze Vip3A protein, resulting in a 65 kDa fragment that has toxic activity against target insects. This variation in the hydrolysis of Vip3A protein by different insects causes a difference in the insecticidal activity of the protein (Chakroun et al. 2012; Hernández-Martínez et al. 2013). According to competitive binding experiments, Vip3A proteins do not have a shared binding site with most of the Cry proteins. Vip3A and Cry toxins bind to the different receptors in the different binding sites on the surface of insect midgut cells (Lee et al. 2003).

The binding proteins of Vip3A have been identified on the surface of Sf21 cells by the yeast two-hybrid system, which revealed that Vip3A could interact with ribosomal S2 protein. In vitro pulldown assay and RNAi were used to validate the interaction of Vip3A and ribosomal S2 protein. Reduced toxicity of Vip3A proteins in Sf21 cells was observed by downregulating the ribosomal S2 protein. Vip3A protein interacts with ribosomal S2 protein that causes Sf21 cells to lyse. It implies that ribosomal S2 protein is Vip3A protein's interacting partner (Singh et al. 2010). Recently, it has been reported that Vip3A could bind to tenascins-like (a family of extracellular glycoproteins) BCW Vip3Aa-Receptor in the midgut tissue of *A. ipsilon* larvae. Vip3A toxin is thought to cause insecticidal toxicity by forming channels or transporting nutrients to the insect intestines (Osman et al. 2019). In Sf9 cells, the fibroblast growth factor receptor-like protein (FGFR) binds Vip3Aa protein. Vip3Aa and Sf-FGFR could co-localize on the surface of Sf9 cells and be internalized into Sf9 cells simultaneously (Jiang et al. 2018a). Vip3Aa binding to Sf-FGFR may also alter an apoptotic signaling pathway and cause apoptosis in Sf9 cell lines and *S. exigua* larvae (Hernández-Martínez et al. 2017; Jiang et al. 2018a; Osman et al. 2019). The Vip3Aa protoxin has been found to bind to the scavenger receptor class C-like protein (Sf-SR-C). The RNAi-mediated knockdown of the expression of SR-C in Sf9 cells and *S. exigua* larvae can reduce the toxicity of Vip3Aa. Furthermore, the Sf-SR-C-mediated endocytosis of the Vip3Aa is linked to its insecticidal action (Jiang et al. 2018b). The existence of pore formation structure of Vip3A has been confirmed in voltage-clamp assays, and the perforation ability of activated Vip3Aa on the BBMV of *Helicoverpa armigera* midgut was confirmed by fluorescence quenching test (Liu et al. 2007). Ion-channel formation is the most widely accepted mechanism of action for activated Vip3Aa toxin.

25.5 Bt Products for Biological Control of Insect Pests

25.5.1 Bt Biopesticides

Bt is one of the most important microbes in agricultural pest control, which has a significant contribution to crop protection as a biopesticide (Lord 2005; Brar et al. 2006; Bravo et al. 2011). It is widely accepted and being used because of its efficacy and safety compared to synthetic chemical pesticides. As Bt pesticides are target-specific, this is not harmful for non-target or other beneficial organisms.

Bt spray has been used to destroy the mosquito larvae (WHO 1999; Bravo et al. 2007). It is extensively used for controlling the pests of organic vegetables such as cauliflower, carrot, lettuce, broccoli, cucumber and tomato. Most of the people eat those vegetables as raw with minimal wash. But no human health hazard has yet been reported (Federici and Siegel 2008). There are a number of Bt commercial pesticides used for controlling insect pests. Bt technologies provide a large and competitive market (Table 25.1). These are effective in controlling destructive Lepidopteran and Coleopteran insect pests (Table 25.1). Most of these products are from wild subspecies *Bt* var. *kurstaki* (Btk). Btk is effective mostly against leaf leaf-feeding Lepidopteran insects. *Bt* var. *aizawai*-derived insecticides are also used for controlling Lepidopterans insects that feed on stored grains. Products that are made from *Bt* var. *san diego* and *Bt* var. *tenebrionis* subspecies are used for the management of Coleopteran insects (Soberon et al. 2009). As soon as Bt subspecies *tenebrionis* was developed, it was formulated as an insecticide to control the Colorado potato beetle. The commercial biopesticides Di-Terra®, M-One®, M-One Plus®, Novodor® and Trident® are manufactured from *Bt* var. *tenebrionis*. It has been reported that Novodor® is effective for the management of *Leptinotarsa decemlineata*. The utility of Novodor® was also examined in laboratory condition on some species of beetles such as *Chrysophtharta bimaculata*, *C. agricola* and *Chrysomela scripta* (Coyle et al. 2000; Beveridge and Elek 2001). *C. scripta* was found to be susceptible to this product in the field condition. The leaf-feeding insects are mostly controlled by Bt-based insecticides, which use natural Bt strains. Bt biopesticides have a limited range of host specificity; thus, advanced studies should be carried out to improve the efficacy of the pesticides through developing new natural strains with more insecticidal properties. Bt subspecies *kurstaki* have been modified, which expresses several *Cry3* genes. These manipulated biopesticides have more host range, including Lepidopteran and Coleopteran pests (Gawron-Burke and Baum 1991).

25.5.2 Bt Crops

Bt biopesticides have many advantages over commercial pesticides but they have some limitations as well. Poor stability and early inactivation under sunlight is major problem for using Bt pesticides. It is also known to be susceptible to the soil

Table 25.1 Bt commercial products used for biological control of insect pests

Commercial Bt biopesticides	Producing company	Bt subspecies	Target insects
Agree®	BioControle	*Bt* var. *kurstaki* and *Bt* var. *aizawai*	Lepidoptera
Bac-control®	Agricontrol	*Bt* var. *kurstaki*	Lepidoptera
BactoSpeine®	Solvay	*Bt* var. *kurstaki*	Lepidoptera
Bactur®	Milenia Agrociências	*Bt* var. *kurstaki*	Lepidoptera
Biobit® and Foray®	Novo-Nordisk	*Bt* var. *kurstaki*	Lepidoptera
Certan®	Novartis	*Bt* var. *kurstaki*	Lepidoptera
Cutlass® and Condor®	Ecogen	*Bt* var. *kurstaki*	Lepidoptera
Delfin®, Javelin® and Thuricide®	Sandoz	*Bt* var. *kurstaki*	Lepidoptera
Dimy Pel®	Dimy	*Bt* var. *kurstaki*	Lepidoptera
DiPel®	Abbott	*Bt* var. *kurstaki*	Lepidoptera
Di-Terra®	Abbott	*Bt* var. *san diego*, *Bt* var. *tenebrionis*	Coleoptera
Ecotech Pro®	Bayer	*Bt* var. *kurstaki*	Lepidoptera
Florbac®	Abbott	*Bt* var. *aizawai*	Lepidoptera
Foil®	Ecogen	*Bt* var. *kurstaki*	Lepidoptera/ Coleoptera
LarvoBt®	Fermone	*Bt* var. *kurstaki*	Lepidoptera
Lepinox®	DuPont	*Bt* var. *kurstaki*	Lepidoptera
M-One® and M-One Plus®	Mycogen	*Bt* var. *san diego*, *Bt* var. *tenebrionis*	Coleoptera
M-Track®	Mycogen	*Bt* var. *tenebrionis*	Coleoptera
MVP®	Mycogen	*Bt* var. *kurstaki*	Lepidoptera
Novodor®	Novo-Nordisk	*Bt* var. *san diego*, *Bt* var. *tenebrionis*	Coleoptera
Nubilacid®	Radonja	*Bt* var. *kurstaki*	Lepidoptera
Spicturin®	Phyllom BioProducts	*Bt* var. *galleriae*	Coleoptera
Steward®	Thermo Trilogy Co	*Bt* var. *kurstaki*	Lepidoptera
Trident®	Sandoz	*Bt* var. *san diego*, *Bt* var. *tenebrionis*	Coleoptera
XenTari®	Abbott	*Bt* var. *aizawai*	Lepidoptera

Source: Adapted from Schünemann et al. (2014), Fernández-Chapa et al. (2019), and Sanahuja et al. (2011)
Bt, *Bacillus thuringiensis*

and environment. As a result, the commercial production of Bt insecticide has become limited. Nowadays, Bt crops are more expedient to use as several *Bt* genes have been identified as well as transgenic technologies have been improved. In Table 25.2, we summarize the crops expressing Bt toxin that has been popularized and commercially produced. Bt transgenic plants are mostly produced in major grain and economic crops such as cotton, maize, potato, rice, soybean, and eggplant. These possess different kinds of *Bt* genes which are mostly used to control Lepidopteran and Coleopteran pests (Table 25.2).

Table 25.2 Commercially available Bt crops expressing Bt genes

Crop	Trade name	Developer	Introduced Bt gene(s)	Bt gene source	Target insects
Cotton	Bollgard™ cotton, Ingard™ and Roundup Ready™ Bollgard™ cotton	Monsanto Company	*Cry1Ac*	*Bt* var. *kurstaki* strain HD73	Lepidoptera
	BXN™ Plus Bollgard™ cotton	Monsanto Company	*Cry1Ac*	*Bt* var. *kurstaki* strain HD73	Lepidoptera
	VIPCOT™	Syngenta	*Vip3A(a)*	*Bt* strain AB88	Lepidoptera
	Bollgard II™ cotton and Roundup Ready™ Flex™ Bollgard II™ cotton	Monsanto Company	*Cry1Ac* and *Cry2Ab2*	*Bt* var. *kurstaki* strain HD73 and *Bt var. kumamotoensis*	Lepidoptera
	Fibermax™ Liberty Link™ Bollgard II™	Bayer CropScience	*Cry1Ac* and *Cry2Ab2*	*Bt* var. *kurstaki* strain HD73 and *Bt* var. *kumamotoensis*	Lepidoptera
	TwinLink™ cotton and Glytol™ × Twinlink™	Bayer CropScience	*Cry1Ab* and *Cry2Ae*	*Bt var. kurstaki* and *Bt* var. *Dakota*	Lepidoptera
	Widestrik™ cotton	Dow AgroSciences LLC	*Cry1Ac* and *Cry1F*	*Bt* var. *kurstaki* strain HD73 and *Bt* var. *aizawai*	Lepidoptera
	Widestrik™ Roundup Ready cotton and Widestrik™ Roundup Ready Flex™ cotton	Monsanto Company and Dow AgroSciences LLC	*Cry1Ac* and *Cry1F*	*Bt* var. *kurstaki* strain HD73 and *Bt* var. *aizawai*	Lepidoptera
	VIPCOT™	Syngenta	*Cry1Ab* and *Vip3A(a)*	*Bt* var. *kurstaki* and *Bt* strain AB88	Lepidoptera
	VIPCOT™ Roundup Ready Flex™ cotton	Syngenta and Monsanto Company	*Cry1Ab* and *Vip3A(a)*	*Bt* var. *kurstaki* and *Bt* strain AB88	Lepidoptera
	Bollgard® III and Bollgard® III × Roundup Ready™ Flex™	Monsanto Company	*Cry1Ac*, *Cry2Ab2* and *Vip3A(a)*	*Bt* var. *kurstaki* strain HD73, *Bt* var. *kumamotoensis* and *Bt* strain AB88	Lepidoptera
	Glytol™ × Twinlink™ × VIPCOT™ cotton	Bayer CropScience	*Cry1Ab*, *Cry2Ae* and *Vip3A(a)*	*Bt* var. *kurstaki, Bt* var. *dakota* and *Bt* strain AB88	Lepidoptera
	Widestrik™ × Roundup Ready Flex™ × VIPCOT™ cotton	Dow AgroSciences LLC	*Cry1Ac*, *Cry1F* and *Vip3A(a)*	*Bt* var. *kurstaki* strain HD73, *Bt* var. *aizawai* and *Bt* strain AB88	Lepidoptera

(continued)

Table 25.2 (continued)

Crop	Trade name	Developer	Introduced Bt gene(s)	Bt gene source	Target insects
Maize	Agrisure™ CB/LL, Agrisure™ GT/CB/LL, NaturGard KnockOut™ and Maximizer™	Syngenta	*Cry1Ab*	*Bt* var. *kurstaki*	Lepidoptera
	YieldGard™, MaizeGard™ and Roundup Ready™ YieldGard™ maize	Monsanto Company	*Cry1Ab*	*Bt* var. *kurstaki*	Lepidoptera
	Mavera™ YieldGard™ Maize	Renessen LLC and Monsanto Company	*Cry1Ab*	*Bt* var. *kurstaki*	Lepidoptera
	Liberty Link™ Yieldgard™ Maize	Monsanto Company and Bayer CropScience	*Cry1Ab*	*Bt* var. *kurstaki*	Lepidoptera
	Bt Xtra™ Maize	Monsanto Company	*Cry1Ac*	*Bt* var. *kurstaki* strain HD73	Lepidoptera
	Herculex™ I, Herculex™ CB and Herculex™ I RR	Dow AgroSciences LLC and DuPont	*Cry1Fa2*	*Bt* var. *aizawai*	Lepidoptera
	YieldGard™ Rootworm RW, MaxGard™ and YieldGard™ VT™ Rootworm™ RR2	Monsanto Company	*Cry3Bb1*	*Bt* var. *kumamotoensis*	Coleoptera
	Starlink™ Maize	Bayer CropScience	*Cry9c*	*Bt* var. *tolworthi* strain BTS02618A	Lepidoptera
	Agrisure™ Viptera	Syngenta	*Vip3Aa20*	*Bt* strain AB88	Lepidoptera
	Agrisure™ RW and Agrisure™ GT/RW	Syngenta	*mCry3A (*modified *Cry3A)*	*Bt* var. *tenebrionis*	Coleoptera
	Agrisure® Duracade™	Syngenta	*ecry3.1Ab* (chimera of *Cry3A* and *Cry1Ab*)	*Bt*	Insects from multiple order
	Optimum™ Intrasect	DuPont	*Cry1Ab and Cry1Fa2*	*Bt* var. *kurstaki* and *Bt* var. *aizawai*	Lepidoptera
	Agrisure™ CB/LL/RW and Agrisure™ 3000GT	Syngenta	*Cry1Ab* and *mCry3A*	*Bt* var. *kurstaki* and *Bt* var. *tenebrionis*	Coleoptera and Lepidoptera
	YieldGard™ VT triple and YieldGard™ plus	Monsanto Company	*Cry1Ab* and *Cry3Bb1*	*Bt* var. *kurstaki* and *Bt* var. *kumamotoensis*	Coleoptera and Lepidoptera

	YieldGard™ VT Pro™ and Genuity® VT Double Pro™	Monsanto Company	*Cry2Ab2* and *Cry1A.105*	*Bt* var. *kumamotoensis*	Lepidoptera
	Optimum™ TRIsect	DuPont	*Cry1Fa2* and *mCry3A*	*Bt* var. *aizawai* and *Bt* var. *tenebrionis*	Coleoptera and Lepidoptera
	Herculex™ RW and Herculex™ RW Roundup Ready™ 2	Dow AgroSciences LLC and DuPont	*Cry34Ab1* and *Cry35Ab1*	*Bt* strain PS149B1	Coleoptera
	Agrisure® Viptera™ 3110	Syngenta	*Cry1Ab* and *Vip3Aa20*	*Bt var. kurstaki* and *Bt* strain *AB88*	Lepidoptera
	Agrisure® Viptera™ 2100	Syngenta	*Cry1Ab* (truncated) and *Vip3Aa20*	*Bt* var. *kumamotoensis* and *Bt* strain *AB88*	Lepidoptera
	Herculex XTRA™	Dow AgroSciences LLC and DuPont	*Cry1F*, *Cry34Ab1* and *Cry35Ab1*	*Bt* var. *aizawai* and *Bt* strain PS149B1	Coleoptera and Lepidoptera
	Genuity® VT Triple Pro™	Monsanto Company	*Cry2Ab2*, *Cry1A.105* and *Cry3Bb1*	*Bt* var. *kumamotoensis*	Coleoptera and Lepidoptera
	Power Core™	Monsanto Company and Dow AgroSciences LLC	*Cry2Ab2*, *Cry1A.105* and *Cry1Fa2*	*Bt* var. *kumamotoensis*, *Bt* var. *aizawai*	Lepidoptera
	Agrisure™ Viptera 3220	Syngenta	*Cry1Ab*, *Cry1Fa2* and *Vip3Aa20*	*Bt* var. *kurstaki*, *Bt* var. *aizawai* and *Bt* strain *AB88*	Lepidoptera
	Power Core™ × MIR162 × Enlist™	Dow AgroSciences LLC	*Cry1Ab*, *Cry1Fa2* and *Vip3Aa20*	*Bt* var. *kurstaki*, *Bt* var. *aizawai* and *Bt* strain *AB88*	Lepidoptera
	Agrisure® Viptera™ 3100, Agrisure® Viptera™ 3111 and Agrisure® Viptera™ 4	Syngenta	*Cry1Ab*, *mCry3A* and *Vip3Aa20*	*Bt* var. *kurstaki, Bt* var. *tenebrionis* and *Bt* strain *AB88*	Coleoptera and Lepidoptera
	Agrisure® Duracade™ 5122	Syngenta	*ecry3.1Ab, mCry3A*, *Cry1Ab* and *Cry1Fa2*	*Bt*, *Bt* var. *tenebrionis*, *Bt* var. *kurstaki* and *Bt* var. *aizawai*	Insects from multiple order
	Agrisure® Duracade™ 5222	Syngenta	*ecry3.1Ab*, *Cry1Ab*, *Cry1Fa2* and *Vip3Aa20*	*Bt*, *Bt* var. *tenebrionis*, *Bt* var. *kurstaki, Bt* var. *aizawai* and *Bt* strain AB88	Insects from multiple order

(continued)

Table 25.2 (continued)

Crop	Trade name	Developer	Introduced Bt gene(s)	Bt gene source	Target insects
	Agrisure® 3122	Syngenta	*Cry1Ab*, *Cry1Fa2*, *mCry3A*, *Cry34Ab1* and *Cry35Ab1*	*Bt* var. *kurstaki, Bt* var. *aizawai*, *Bt* var. *tenebrionis* and *Bt* strain PS149B1	Coleoptera and Lepidoptera
	SmartStax™ Pro × Enlist™	Monsanto Company	*Cry2Ab2*, *Cry1A.105*, *Cry3Bb1*, *Cry1F*, *Cry34Ab1* and *Cry35Ab1*	*Bt* var. *kumamotoensis*, *Bt* var. *aizawai* and *Bt* strain PS149B1	Coleoptera and Lepidoptera
	Genuity® SmartStax™	Monsanto Company and Dow AgroSciences LLC	*Cry2Ab2*, *Cry1A.105*, *Cry3Bb1*, *Cry1Fa2*, *Cry34Ab1* and *Cry35Ab1*	*Bt* var. *kumamotoensis*, *Bt* var. *aizawai* and *Bt* strain PS149B1	Coleoptera and Lepidoptera
Potato	Atlantic NewLeaf™ potato, New Leaf™ Russet Burbank potato, Shepody NewLeaf™ Y potato and Superior NewLeaf™ potato	Monsanto Company	*Cry3A*	*Bt* var. *tenebrionis*	Coleoptera
Rice	Huahui-1	Huazhong Agricultural University	*Cry1Ab* and *Cry1Ac*	*Bt* var. *kurstaki* and *Bt* var. *kurstaki* strain HD73	Lepidoptera
Soybean	Intacta™ Roundup Ready™ 2 Pro	Monsanto Company	*Cry1Ac*	*Bt* var. *kurstaki* strain HD73	Lepidoptera
	Conkesta Enlist E3™ Soybean	Dow AgroSciences LLC	*Cry1Ac* and *Cry1F*	*Bt* var. *kurstaki* strain HD73 and *Bt* var. *aizawai*	Lepidoptera
Eggplant	BARI Bt Begun-1, -2, -3 and -4	Maharashtra Hybrid Seed Company	*Cry1Ac*	*Bt* var. *kurstaki* strain HD73	Lepidoptera

Source: Adapted from International Service for the Acquisition of Agri-biotech Applications (ISAAA) based on *Bt* gene introduced

25.5.2.1 Bt Maize

The *Cry1Ab, Cry1Ac,* and *Cry2A* genes were primarily inserted into the genome of maize to produce transgenic plants (Huang and McGaughey 1999). In the last few years, more advanced research accelerated the development of Bt maize with multiple genes. As an example, the transgenic maize expressing *Cry1Ab + Vip3Aa20* or chimeric protein *ecry3.1Ab + Cry1Ab + Cry1Fa2 + Vip3Aa20* is resistant against Coleopteran pests (Carriere et al. 2015). In 2017, six genes namely, *Cry2Ab2, Cry1A.105, Cry1F, Cry34Ab1, Cry35Ab1,* and *Cry3Bb1* stacked together and developed another transgenic maize. Nowadays, more transgenic maize expressing multiple *Bt* genes is being produced worldwide (http://www.isaaa.org/gmapprovaldatabase/default.asp). *Chilo partellus* larvae causes more damage to non-Bt maize than the hybrid Bt maize expressing the *Cry1Ab* gene. In laboratory conditions, a 79.4–100% mortality rate was observed in *C. partellus* larvae when feeding with Bt maize (*Cry1Ab*) (Hari et al. 2008). Most Bt maize is effective against Lepidopteran and Coleopteran pests.

25.5.2.2 Bt Cotton

Bt cotton plays an important role in controlling Lepidopteran and Coleopteran pests. First Bt cotton expressing *Cry1Ac* was cultivated in Australia and USA, followed by China. It had great control over a wide range of insects including cotton bollworm (*H. armigera*), pink bollworm (*Pectinophora gossypiella*), and other population of the target pests (Wu and Guo 2005). Cultivation of this single gene transgenic Bt cotton for a long time increases the resistance in targeted insect pests. As a result, at the end of twentieth century, studies were performed with two or more combinations of Bt toxins such as *Cry1Ac + Cry2Ae*, *Cry1Ab + Vip3A(a)*, *Cry1Ab + Cry2Ab2*, *Cry1Ab + Cry2Ae*, *Cry1Ac + Cry2Ab2*, *Cry1Ac + Cry1F + Vip3A(a)*, *Cry1Ac + Cry2Ab + Vip3A(a)*, and *Cry1Ab + Cry2Ae + Vip3A*. Transgenic cotton expressing *Cry1Ab + Cry2Ab2* genes performed better than previously cultivated single toxin expressing cotton (Qiman et al. 1998). Bollgard™ cotton that expresses a combination of Cry1Ac + Cry2Ab2 + Vip3A proteins, shows higher insecticidal activity against *H. virescens, H. zea* and *P. gossypiella* instead of any of the single protein.

25.5.2.3 Bt Soybean

Transgenic Bt expressing *Cry1Ac, Cry1F, Cry1A105,* and *Cry2Ab2* have been developed, which have a great impact in controlling target pests. Monsanto has developed Bt soybean varieties, MON87701 and MON87701RR2Y by introgression of *Cry1Ac* and *EPSPS* genes. These Bt soybeans provide significant protection against *H. armigera* in the whole growing season (Yu et al. 2013).

25.5.2.4 Bt Rice

Bt Huahui-1 is the first Bt rice, which expresses a fusion of Cry1Ab/Cry1Ac protein. It has high insecticidal activity against *Chilo suppressalis*, *Scirpophaga incertulas* and other Lepidopteran pests (Cheng et al. 1998; Shu et al. 2000). A transgenic rice line, Bt-Shanyou 63 was developed expresses a Bt fusion gene derived from *Cry1Ab*/*Cry1Ac.* Bt-Shanyou 63 exhibited high toxicity against *S. incertulas* and *Cnaphalocrocis medinalis* (Tu et al. 2000). In a recent study, it is suggested that *Cry64Ba* and *Cry64Ca* can be used to produce transgenic rice, which in turn may provide a novel strategy to control Hemipteran pests of rice, *Laodelphax striatellus*, and *Sogatella furcifera* (Liu et al. 2018).

25.5.2.5 Bt Eggplant

Insect-resistant transgenic eggplant can be produced by inserting specific *Bt* gene through genetic engineering. Coleopteran-specific *Cry3B* gene was first successfully incorporated into eggplant, which exhibited resistance to Colorado potato beetle (*L. decemlineata*) (Chen et al. 1995; Arpaia et al. 1997). The main damaging insect of eggplant is eggplant (brinjal) shoot and fruit borer, *Leucinodes orbonalis*. A synthetic *Cry1Ab* gene was transferred to brinjal, which was resistant to *L.orbonalis* larvae (Kumar et al. 1998). Bangladesh is the pioneering country in commercially cultivating Bt eggplant. The commercially available Bt eggplant varieties in Bangladesh are BARI Bt Begun-1, BARI Bt Begun-2, BARI Bt Begun-3 and BARI Bt Begun-4 that express the *Cry1Ac* gene and effective against target Lepidopteran insect, *L. orbonalis* (Shelton et al. 2018). These varieties are getting popular in Bangladesh day by day. Cultivation of Bt brinjal increased an average of 19.6% yield compared to non-Bt brinjal (Shelton et al. 2020). In Chap. 23 of this volume, Paul et al. (2022) reviews the impacts of Bt-brinjal on the economic benefit of the farmers and environmental sustainability in Bangladesh.

Besides, Bt potato expressing *Cry3A* showed entomotoxicity against Coleopteran pests (Table 25.2). As Bt crops are environment-friendly and not harmful to human health, more emphasize should be given to the commercialization of Bt transgenic crop. In addition, the discovery of Bt genes is also needed to prompt the development and commercialization of Bt crops.

25.6 Conclusion

In conclusion, this chapter reviews and updates the structural and functional diversities of Bt toxins and discusses the mechanism of insect control by these natural toxins. Bt-based biopesticides are environment-friendly and effective in controlling various insect pests and nematodes. The application of Bt transgenic technologies in

increasing crop production by reducing hazardous synthetic pesticides are also discussed. Stacking of multiple *Bt* genes in various crop plants using recently developed CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) technology would enhance sustainable agriculture for ensuring food and nutritional security of the ever-increasing population of the world.

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