Role of Hydrolytic Enzymes of Rhizoflora in Biocontrol of Fungal Phytopathogens: An Overview

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Abstract

Microbial community in the rhizosphere produces a variety of hydrolytic enzymes that are responsible for the degradation of various components of fungal pathogens. The extracellular hydrolytic enzymes excreted by soil rhizobia degrade cell wall components of plant pathogenic microbes. The enzymes of these types are able to breakdown glycosidic linkages present in the polysaccharide of the cell wall of phytopathogens. In this regard, plant growth-promoting rhizobacteria (PGPR) are known to colonize rhizosphere and enhance plant growth through different mechanisms that include (i) plant growth promotion and (ii) biological control of plant disease. Plant growth promotion mechanisms include mineralization of insoluble substances, production of plant growth hormones, biological nitrogen fixation, and promotion of root growth. Biocontrol mechanism involves competition, antibiosis, parasitism, induction of systemic acquired resistance (SAR), induction of systemic resistance (ISR), soil suppressiveness, and production of various antifungal metabolites; hydrolytic enzymes such as chitinase, glucanase, protease, and cellulase; and antibiotics such as 2,4-diacetyl phloroglucinol (DAPG), amphisin, oomycin A, hydrogen cyanide, phenazine, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, oligomycin A, zwittermicin A, kanosamine, and xanthobaccin. Production of hydrolytic enzymes by PGPR is an important mechanism directed against phytopathogens for sustainable plant disease management. These enzymes break down the cell wall of fungal pathogens causing cell death. This review focuses on the different aspects of various hydrolytic enzymes produced by rhizoflora and their role in sustainable biocontrol of phytopathogens.

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9.1 Introduction

Soilborne phytopathogens are responsible for causing infection of roots, stems, leaves, and fruits. These phytopathogens occur in a broad spectrum of susceptible hosts under favorable environmental conditions. Such diseases are known to cause severe economic losses to variety of food crops and are commonly controlled by using synthetic fungicides or nonspecific chemical fungicides. These plant diseases are known to cause a loss of 30% crop yield posing economic hardship to producers (Sayyed et al. 2012; Shaikh and Sayyed 2015). All over the world, population is increasing tremendously every day and hence the agricultural practices to increase the yield. This need has compelled to use synthetic agrochemicals, but the chemical pesticides and fertilizers have caused even more destructive effects to the agricultural field because these chemicals are not eco-friendly. The present need of sustainable agricultural practices is focused on the safer alternatives to conventional agrochemicals (Pane et al. 2013). The intensive use of fungicides, to control plant pathogens and excessive use of chemical fertilizers to increase crop productivity, has severally imbalanced the agroecosystem (Logemann and Schell 1993). In this regard, PGPR have been seen as a greener approach to control plant pathogens and to promote plant growth (Sayyed and Chincholkar 2009; Sayyed et al. 2010, 2013, 2015; Sayyed and Patel 2011; Shaikh et al. 2014, 2016).

The mechanisms of plant growth promotion by PGPR include production of plant growth regulators, asymbiotic N_2 fixation, and solubilization of mineral phosphates and other nutrients (Sarvanakumar et al. 2007; Sayyed et al. 2007; Sharma et al. 2013), while biocontrol involves antagonistic action toward plant pathogens by production of siderophores, antibiotics, cyanide, and hydrolytic enzymes (Shaikh et al. 2014; Shaikh and Sayyed 2015). Antagonistic or biocontrol activity of PGPR is attributed to the production of different types of cell wall-lysing enzymes such as chitinase, protease/elastase, cellulase, and β-1,3 glucanase.

9.2 Plant Growth-Promoting Rhizobacteria (PGPR)

Rhizospheric bacteria, having plant growth-promoting ability by colonizing the plant roots, are known as PGPR (Kloepper and Schroth 1978). PGPR are potentially useful in stimulating plant growth and increasing crop yields (Sayyed et al. 2010). Thus the rhizosphere of crop plants is a promising source of PGPR (Lucas et al. 2001 and Barriuso et al. 2005). PGPR can be differentiated into two categories on the basis of their relationship with the plants: symbiotic rhizobacteria and free-living rhizobacteria (Khan 2005; Freitas et al. 2007). Worldwide literature clearly states that the use of PGPR in agriculture is increased tremendously, and significant increase in growth and yield of agronomically important crops has been obtained (Asghar et al. 2002; Vessey 2003; Gray and Smith 2005; Silva et al. 2006; Figueiredo et al. 2008; Araujo 2008). The plant growth-promoting ability of some bacteria is highly specific to certain plant species, cultivar, and genotype (Bashan 1998; Gupta et al. 2000; Lucy et al. 2004). PGPR not only provide essential nutrients for plant

growth promotion, but they are also important in biocontrol of pathogen; they improve the health of soil in the long term and, hence, are potentially important in reducing the use of chemical fertilizers and chemical pesticides (Lugtenberg and Kamilova 2009). However, the better understanding of mechanisms of plant growth promotion and the biocontrol is vital aspect for the better utilization of PGPR in agriculture. The knowledge of structure and diversity of rhizosphere microbial consortium with respect to their complexity; natural selection; interpopulational relations like symbiosis, parasitism, mutualism, or competence; and succession is equally important in this aspect (Barriuso et al. 2008).

9.3 Fungal Plant Diseases

The vast range of phytopathogens causes various types of diseases by infecting the whole or a specific part of the plants. Their effect ranges from mild symptoms to catastrophes in which huge plantations of food crops are destroyed and hence causes loss of yield. Catastrophic plant disease exerts the current deficit of food supply in which at least 800 million people are not properly fed. The strengths of phytopathogens like their populations are variable in time and space, and genotype increases the difficulties to control them (Strange and Scott 2005). The continuous use of fungicides has developed the resistance which causes the loss in productivity. The biological controls have been found more promising than chemical fertilizers, discussed in Sect. 9.5.

The worldwide reporting shows that not all but various fungal species are found to be pathogenic to the plants and their products. Some of the plants affected by phytopathogenic diseases are listed in Table 9.1.

9.4 Composition of Fungal Cell Wall

The cell wall of fungal or any pathogen is meant for protection of its internal constituent from various environmental factors. The structure of fungal cell wall is unique and is therefore an excellent target for the development of antifungal metabolites. The structure and biosynthesis of various antifungal metabolites have been reviewed. These studies have clearly demonstrated that fungal cell walls are mainly composed of chitin, glucans, mannans, and glycoproteins (Bowman and Free 2006).

The fungal cell walls contain fibrillar materials attached to sugars, proteins, lipids, and a variety of polysaccharides (Fig. 9.1). These fibrillar materials are inert. The functional components of cell wall are needed for nutrient transport, extracellular degradation of non-permeable substrates, communication, and modifications of cell wall structure.

About, 80% of the cell wall of fungi is made up of polysaccharides. The fibrillar structure is built on chitin, chitosan, ß-glucans, and a variety of heteropolysaccharides (Table 9.2). These fibers are encompassed in a complex gel-like matrix. Proteins in the form of glycoprotein are present in small amount, i.e., 20%. All

Table 9.1 List of phytopathogens infecting plants with various diseases

No.	Disease	Target plant or plant part	Phytopathogen	References
1	Brown patch	Patches of brown and yellow color appear on the lawn in irregular shapes	*Rhizoctonia solani*	Giesler and Yuen (1998)
2	Cankers	Woody plants	*Gibberella circinata* (*Fusarium circinatum*)	Wingfield et al. (2002)
3	Damping off	All types of plants	*Pythium* and *Fusarium*	Mao et al. (1997)
4	Powdery mildew	Grains, alfalfa, onions, cucumbers	*Uncinula necator*	Doster and Schnathorst (1985)
5	Ergot	Rye, barley, wheat, and other grasses	*Claviceps purpurea*	Giesbert et al. (2008)
6	Root rots	All types of plants	*Phytophthora* sp.	Thomas et al. (2003)
7	Rusts	Wheat, oats, barley, rye	*Puccinia*	Uchida et al. (2006)
8	Scab	Wheat, rye, barley, potatoes	*Fusarium graminearum*	O'Donnell et al. (2000)
9	Seed decay	All types of plants	*Phomopsis*	Li et al. (2015)
10	Smuts	Oats, barley, grasses, corn, wheat	*Ustilaginomycetes*	Müller (2015)
11	Soft rots, dry rots	Potatoes, onions, carrots, fleshy organs, etc.	*Syncephalastrum racemosum*, *Fusarium* sp.	Misra (2016) Heltoft (2016)
12	Wilts	Potatoes, alfalfa, trees	*Fusarium oxysporum*	Pietro et al. (2003)

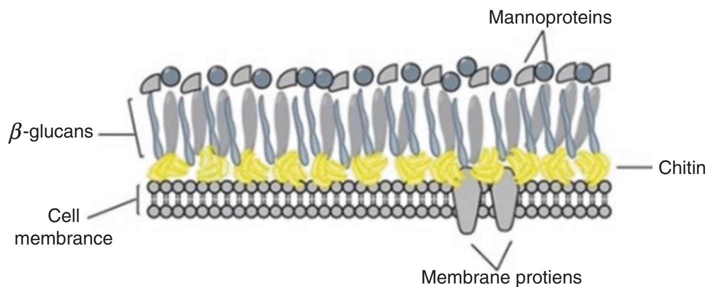

Fig. 9.1 Typical structure of fungal cell wall (Adapted from Vega and Kalkuma 2011)

proteins are not generally the structural components. Lipids are present only in small amount. Proteins and lipids regulate movement of water and protect the fungal cell from desiccation (Cox and Hooley 2009).

Table 9.2 Fungal cell wall-forming polymers

No.	Classification	Fibrous polymers	Gel-like polymers
1	Basidiomycota	Chitin β-(1-3), β-(1-6) glucan	Xylomannoproteins α (1-3) glucan
2	Ascomycota	Chitin β-(1-3), β-(1-6) glucan	Galactomannoproteins α (1-3) glucan
3	Zygomycota	Chitin chitosan	Polyglucuronic acid, glucuronomannoproteins, polyphosphate
4	Chytridiomycota	Chitin glucan	Glucan

Adapted from Gooday (1995)

9.5 Biocontrol Through Hydrolytic Enzymes

It has been studied that many rhizobacteria/biocontrol agents (BCAs) synthesize extracellular hydrolytic enzymes that are involved in hydrolysis of fungal cell wall components such as chitin, proteins, cellulose, hemicellulose, and DNA; these hydrolytic enzymes have the potential of inhibiting phytopathogens (Pal and Gardener 2006).

9.5.1 Hydrolytic Enzymes

The term biocontrol/biological control denotes the direct or indirect manipulation of microbes for reducing plant disease (Baker and Cook 1974; Maloy 1993). Among the wide genetic biodiversity of prokaryotes, PGPR play crucial role in the biocontrol of plant diseases and in improvement of crop productivity through various mechanisms (Fernando et al. 2005). Biotic agents like harmful insects, parasitic weeds, and phytopathogens are among the major causes of serious loss and damage to agricultural crop and products. This needs to be controlled to sustain the quality and quantity of agriculture products. Currently numerous strategies are employed to combat this problem (Bargabus et al. 2002; Benhamou 2004; Kloepper et al. 2004, Islam et al. 2005; Chisholm et al. 2006; Heydari 2007; Heydari et al. 2007). A natural, safe, and productive option for the control of these pathogens is the use of BCAs. BCAs include the number of microbial genera from rhizosphere including PGPR. Consequently, to improve biocontrol strategies by manipulating soil environment, the study of mechanism of biocontrol of plants diseases through the interaction between BCAs and pathogens is the key to create successful biocontrol conditions (Fravel 1998). The biocontrol of plant disease includes the secretion of microbial metabolite which controls the diseases by acting on or by inhibiting the growth of phytopathogens.

Hydrolytic enzymes (chitinase, glucanase, protease, and cellulase) produced by PGPR are responsible for the lysis of phytopathogens through hyperparasitism. The antagonistic properties of hydrolytic enzymes against various phytopathogens play a major role in biocontrol (Kim et al. 2003; Shaikh and Sayyed 2015). BCAs

producing hydrolytic enzymes are used in biocontrol of phytopathogens thereby improving plant growth. These attributes make PGPR an effective BCA (Garbeva et al. 2004; Ran et al. 2005). The cell wall of most of the phytopathogenic fungi (except oomycetes) is made up of chitin ($C_8 H_{13}O_5N)_n$, which is an unbranched, long-chain polymer of glucose derivatives, composed of β-1,4-linked units of the amino sugar N-acetyl-D-glucosamine (NAG).

The biocontrol activity of BCAs/PGPR can be achieved through the following mechanisms:

(a) **Niche competition –** this excludes the growth of phytopathogens from soil or host tissue.
(b) **Mycoparasitism –** leading to the lysis of fungal pathogen.
(c) **Production of antibiotics** – that interfere with the metabolism of phytopathogen.
(d) **Production of hydrolytic enzymes** – that degrade the cell wall of phytopathogens (Sayyed et al. 2013).

9.5.1.1 Cell Wall Lysis

Hydrolytic enzymes are capable of breaking down glycosidic bonds in chitin. Thus, they play a vital role in the biological control of many plant diseases by degrading the cell walls of phytopathogens.

It affects fungal growth by its lytic action on cell walls, hyphal tips, and germ tubes (Kim et al. 2003) and partial swelling in the hyphae and at the hyphal tip leading to hyphal curling or bursting of the hyphal tip (Fig. 9.2; Someya et al. 2000). Among the huge population of hydrolytic enzymes, chitinase, glucanase, protease, and cellulase are of major interest due to their ability to degrade and lyse fungal cell wall, and thus hydrolytic enzymes are employed in biocontrol of fungal phytopathogens (Mabood et al. 2014). Cell wall-degrading enzymes of rhizobacteria damage the structural integrity of the cell wall of phytopathogen (Budi et al. 2000). Felse and Panda (1999) reported the control of *Sclerotium rolfsii* and *F. oxysporum* through the cell wall degradation on beans.

9.5.1.2 Mycoparasitism

The other concept regarding the inhibition of phytopathogens is mycoparasitism that directly attacks which is defined as a direct attack on a fungal thallus leading to its lysis (Chet et al. 1997). According to Barnett and Binder (1973), mycoparasites play an important role in biocontrol. Mycoparasitism can be divided into two types: necrotrophic and biotrophic. Necrotrophic mycoparasites are those that kill the host cells before or just after invasion and use the released nutrients. These mycoparasites are more aggressive and destructive than biotrophs. They have a broad host range and are relatively unspecialized in their mode of parasitism. The antagonistic activity of necrotrophs is due to the production of antibiotics, toxins, or hydrolytic enzymes (Manocha and Sahai 1993). In biotrophic parasitism, the development of the parasite is favored by a living rather than a dead host structure (Chet et al. 1997). Biotrophic mycoparasites have a more restricted host range and in many cases

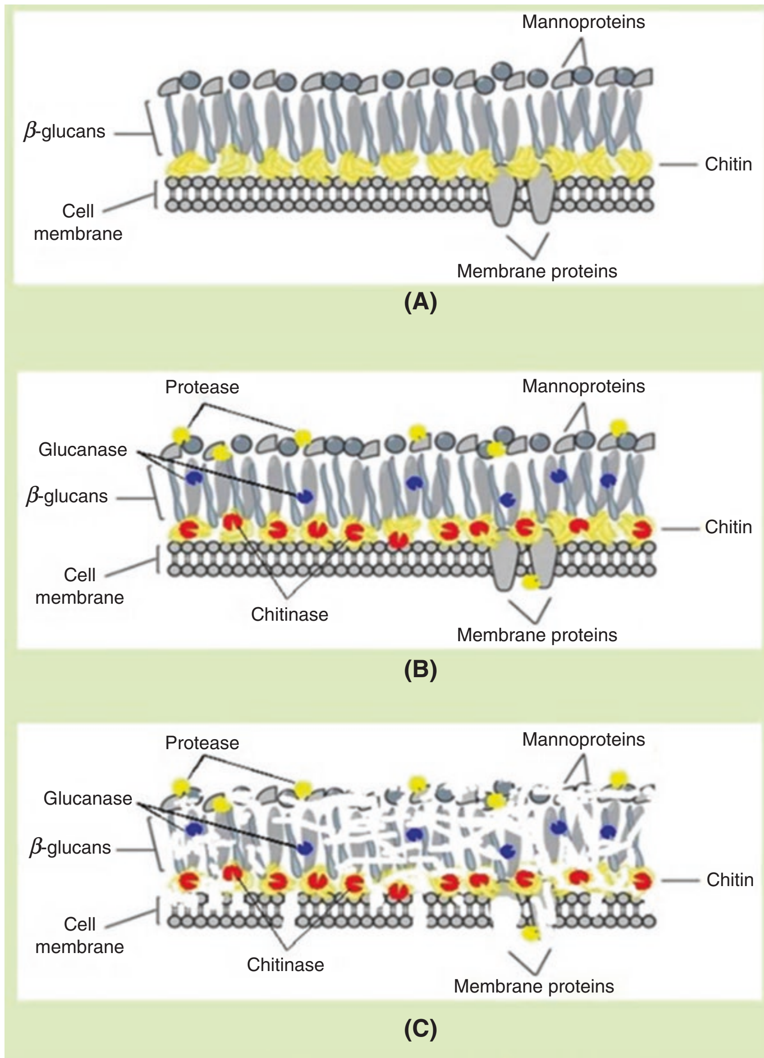

Fig. 9.2 Mechanism of fungal cell wall hydrolysis. (**a**) Typical structure of fungal cell wall. (**b**) Hydrolytic enzymes (chitinase, glucanase, and protease) acting on chitin, β-glucan, and proteins. (**c**) Fungal cell wall losing integrity after hydrolysis

Table 9.3 List of microorganisms showing hydrolytic activity

No.	Microbes showing hydrolytic activity	Hyd. enz. produced	Target phytopathogen	References
1	*S. marcescens*	Chitinase	*R. solani* and *F. oxysporum*	Someya et al. (2000)
2	*B. subtilis* NPU 001	Chitinase	*F. oxysporum*	Chang et al. (2010)
3	*S. plymuthica* C48	Chitinase	*Botrytis cinerea*	Frankowski et al. (2001)
4	*Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385	β -1,3-glucanase	*F. oxysporum*	Singh et al. (1999)
5	*Bacillus subtilis* YJ1	Cellulase	–	Li-Jung et al. (2010)
6	*Cellulomonas* sp. ASN2	Cellulase	–	Muhammad et al. (2012)
7	*Bacillus coagulans*	Carboxymethyl cellulase and polygalacturonase	–	Odeniyi et al. (2009)
8	*Bacillus cereus*, *Bacillus subtilis*, *Bacillus thuringiensis*	Cellulase	–	Basavaraj et al. (2014)
9	*P. aeruginosa* PGPR2	Protease	*Macrophomina* sp., *Rhizoctonia* sp., and *Fusarium* sp.	Illakkiam et al. (2013)
10	*Bacillus subtilis* PE-11	Alkaline protease	–	Adinarayana et al. (2003)
11	Paenibacillus and Streptomyces	–	*F. oxysporum*	Compant et al. (2005)
12	*B. cepacia*	–	*R. solani*, *P. ultimum*, and *S. rolfsii*	
13	*P. fluorescens LRB3W1* and *S. marcescens* B2	–	*F. oxysporum*	Someya et al. (2007)

produce specialized structures (haustoria) to absorb nutrients from their host (Manocha and Sahai 1993). Rhizobacteria capable of producing hydrolytic enzymes and inhibiting phytopathogens are listed in Table 9.3.

9.5.2 Chitinases in Biocontrol of Phytopathogenic Fungi

Chitinase [EC 3.2.1.14] plays a vital role in the biocontrol of many plant diseases by lysing fungal cell wall through degradation of chitin polymer present in the cell walls of fungal phytopathogens. The enzyme can either be used directly in the

biocontrol on microorganisms or indirectly by using purified proteins or through manipulation of genes coding for chitinase (Kim et al. 2003). Chitinases have been reported from various microorganisms, such as insects, crustaceans, yeasts, and fungi, and also organisms that do not contain chitin, such as bacteria, higher plants, and vertebrates (Kramer et al. 1997). Chitinase was isolated, purified, and characterized in 1992 (Cruz et al. 1992). Chitinase produced by rhizobacteria exhibits antagonism in vitro against fungi (Gay et al. 1992; Fridlender et al. 1993). Schlumbaum et al. (1986) and Skujins et al. (1965) demonstrated the inhibition of fungal growth by chitinases of *Streptomycetes*. The importance of chitinase activity was further demonstrated by the loss of biocontrol efficacy in *Serratia marcescens* chitinase mutants in which the *chiA* gene had been inactivated (Jones et al. 1986). The potential BCAs can be produced by cloning *chiA* gene into rhizosphere competent model organisms. Oppenheim and Chet (1992) cloned the *chiA* gene of *S. marcescens* into *E.coli* for the control of *S. rolfsii* and *R. solani* and found *E.coli* to be better in reducing disease incidence. Likewise the chitinase genes from *S. marcescens* were expressed in *Pseudomonas* and the plant symbiont *Rhizobium meliloti* to control the pathogens *F. oxysporum* var. *redolens* and *Gaeumannomyces graminis* var. *tritici* (Sundheim 1992). The antifungal activity of the transgenic *Rhizobium* during symbiosis on alfalfa roots was verified by lysis of *R. solani* hyphal tips treated with cell-free nodule extracts (Sitrit et al. 1993).

The fungal spp. *Trichoderma* and *Gliocladium virens* have been studied more extensively (Cook 1993; Chet et al. 1997). Weindling (1932) reported the potential of *Trichoderma* species as BCAs. The chitinase of *T. harzianum* was used as a means of biocontrol of phytopathogens such as *Rhizoctonia solani* (Chet and Hornby 1990). Several species of *Trichoderma* have been tested as BCAs; among them *T. harzianum* was found to be more effective and can be used to control the number of economically important soilborne phytopathogens (Chet 1987). Using genetic modification technology, Lorito (1998) cloned the tobacco and potato with gene encoding endochitinase from *T. harzianum* (P1) and reported the high level and broad spectrum of resistance against a number of phytopathogens.

9.5.2.1 Mode of Action of Chitinase

Chitinases are chitin-degrading enzymes which play an important role in biological control and plant defense mechanisms against phytopathogens. Chitin is the second most abundant polymer in nature, an unbranched homopolymer of 1,4-β-linked *N*-acetyl-D-glucosamine (GlcNAc) after cellulose. It is abundant as a structural polymer in most fungi and insects, including those that are agricultural pests (Havukkala 1991).

On the basis of mode of action, chitinase is divided into three types:

(A) **β-1,4-*N*-acetyl-glucosaminidases** (EC 3.2.1.30) split the chitin polymer into GlcNAc monomers in an exo-type pattern.
(B) **Endochitinases** (EC 3.2.1.14) cleave randomly at internal sites over the entire length of the chitin microfibril.

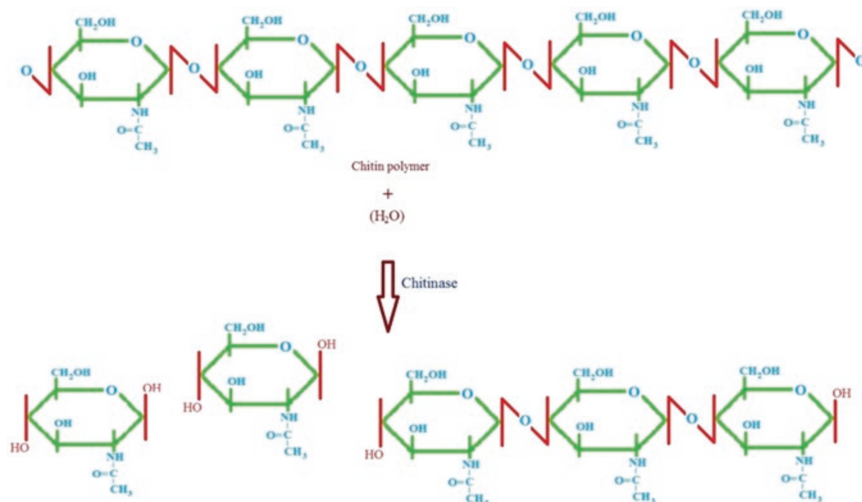

Fig. 9.3 Chitinolysis of 1,4-β-linked *N*-acetyl-D-glucosamine (GlcNAc)

(C) **Exochitinases** (EC 3.2.1.14) catalyze the progressive release of diacetylchitobiose in a stepwise manner such that no monosaccharides or oligosaccharides are formed (Fig. 9.3) (Harman et al. 1993; Manocha and Sahai 1993).

An extracellular chitinase of *Myrothecium verrucaria* inhibits germination and germ tube elongation of the groundnut rust fungus *Puccinia arachidis*. Similarly, *Acremonium obclavatum* produces and secretes a chitinase in vitro which inhibits germination of uredospores of the peanut rust (Manocha and Balasubramanian 1994).

9.5.2.2 Molecular Characterization of Chitinase

Cruz et al. (1992) reported the purification and characterization of three chitinases from *T. harzianum*; the isozymes' mol. wt. were 37, 33, and 42 kDa, respectively. Only the purified 42 kDa chitinase hydrolyzed *B. cinerea* purified cell walls in vitro, but this effect was heightened in the presence of either of the other two isoenzymes. According to Haran et al. (1995), the chitinolytic system of *T. harzianum* was more complex, consisting of six distinct enzymes. The system is apparently composed of two ß-(1,4)-N-acetylglucosaminidases of 102 and 73 kDa, respectively, and four endochitinases of 52, 42, 33, and 31 kDa, respectively. Among these, the 42 kDa endochitinase was found more effective because of its ability to hydrolyze *B. cinerea* cell walls in vitro. The 1,4-β-*N*-acetyl-glucosaminidases of 72 kDa have been purified from *T. harzianum* strain (Lorito et al. 1994). Haran et al. (1995) reported the chitinase isolated from respective *T. harzianum* had different molecular weights: 73 kDa heat-stable glucosaminidase (CHIT 73), isolated from *T. harzianum* strain TM, an endochitinase of 52 kDa (CHIT 52), an endochitinase of 42 kDa (CHIT 42), the endochitinases produced by the other strains of *T. harzianum* which

are of 33 kDa (CHIT 33) and 31 kDa (CHIT 31), and two endochitinases, having molecular weights of 37 kDa and 33 kDa, which were expressed by *T. harzianum* strain CECT 2413.

9.5.3 Proteases in Biocontrol of Phytopathogenic Fungi

Proteases [E.C. 3.4.24] play a significant role in cell wall lysis of phytopathogenic fungi, since chitin and/or fibrils of β-glucan are embedded into the protein matrix. Thus proteolytic activity is prerequisite to lyse whole fungal cells (Elad and Kapat 1999). Proteases are wide spread in nature; microbes are the preferred source of these enzymes due to their fast growth and easy cultivation and the ease in genetic manipulation to get the enzyme with desired properties for specific applications (Anwar and Saleemuddin 1998; Beg and Gupta 2003). *Bacillus* sp. produces extracellular proteases; several *Bacillus* species like *Bacillus cereus*, *Bacillus stearothermophilus*, *Bacillus mojavensis*, *Bacillus megaterium*, and *Bacillus subtilis* are known to produce protease (Sookkheo et al. 2000; Beg and Gupta 2003; Banik and Prakash 2004; Gerze et al. 2005). Bacterial proteases are generally extracellular, easily produced in greater amounts, and active under various environmental conditions.

Proteases purified from *Bacillus* have significant activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification, and low-cost production process (Maurer 2004; Haddar et al. 2009). Extracellular proteases of *Trichoderma* sp. also play a significant role in the lysis of cell walls of phytopathogenic fungi. Some of the proteases produced by *Trichoderma* sp. are involved in inactivating extracellular enzymes of phytopathogenic fungi (Elad and Kapat 1999). The protease enzymes break down major proteins into peptide chains and/or their constituent amino acids of phytopathogens and thereby destroy their capacity to act on plant cells.

9.5.3.1 Mode of Action of Protease

Proteins are degraded by a hydrolysis that involves cutting of one or more peptide bonds by addition of water to liberate peptide or amino acids. Enzymes that hydrolyze the proteins are called proteases. Each protease recognizes the chemical structures of certain specific amino acids and then catalyzes the breaking of the peptide bond (Fig. 9.4).

9.5.3.2 Molecular Characteristics of Protease

The recent studies by Asker et al. (2013) reported the molecular weight of the purified proteases P1 and P2 as 28 and 25 kDa, respectively. The purified P1 and P2 were rich in aspartic acid and serine and relatively have higher amounts of alanine, leucine, glycine, valine, threonine valine, and glutamic acid. Gessesse et al. (2003) purified an alkaline protease of 24 kDa from *Bacillus pseudofirmus* AL-89. Adinarayana et al. (2003) purified an alkaline protease of 15 kDa from *B. subtilis* PE-11. A halotolerant alkaline protease of 28 kDa was purified from *Bacillus*

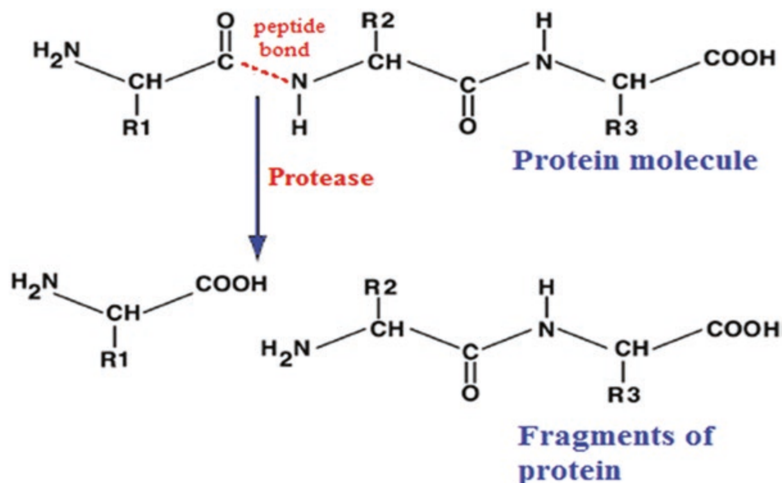

Fig. 9.4 Schematic representation of proteolysis (Modified from Donohue and Osna 2003)

clausii I-52 using a combination of Diaion HPA75, phenyl-Sepharose, and DEAE-Sepharose column chromatography (Joo and Chang 2005). Gupta et al. (2005) purified an alkaline protease from *B. pseudofirmus* to tenfold purity with an 85% yield using a single-step method with a phenyl-Sepharose 6 fast-flow column. The apparent molecular weight of this protease was 29 kDa. Sareen and Mishra (2008) purified a 55 kDa alkaline protease from *Bacillus licheniformis* RSP-09-37.

9.5.4 Cellulase in Biocontrol of Phytopathogenic Fungi

Cellulases [EC 3.2.1.4] catalyze the hydrolysis of 1,4-β-D-glycosidic linkages in cellulose and play a significant role in nature by recycling this polysaccharide. Cellulose is a linear polymer of β-D-glucose units linked through 1,4-β-linkages with a degree of polymerization ranging from 2,000 to 25,000 (Kuhad et al. 1997). Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble, crystalline microfibrils. Cellulose is structurally heterogeneous having both amorphous and crystalline regions. Resistance to microbial degradation depends on the degree of crystallinity, and highly crystalline regions are more resistant to enzymatic hydrolysis. Cellulases belong to a class of enzymes that catalyze the hydrolysis of cellulose and are produced chiefly by fungi, bacteria, and protozoa as well as other organisms like plants and animals. The cellulolytic enzymes are inducible since they can be synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo 2001).

9.5.4.1 Mode of Action of Cellulase

Complete degradation of cellulose involves a complex interaction between different cellulolytic enzymes. It has been widely accepted that three types of enzymes

including cellulose/endoglucanases [EC 3.2.1.4], exo-cellobiohydrolase/exo-glucanases [EC 3.2.1.91], and β-glucosidases [EC 3.2.1.21] act synergistically to convert cellulose into β-glucose (Lynd et al. 2002). Cellulases are a mixture of endo-1,4-β-glucanase enzymes and exo-1,4-β-glucanase enzymes. Endo-1,4-β-glucanase cleaves the internal bonds, while exo-1,4-β-glucanase cleaves two to four units from the ends of cellulose strands and cellobiase, which cleaves the disaccharide cellobiose into two glucose moieties (Fig. 9.5).

9.5.4.2 Molecular Characterization of Cellulase

Hurst et al. (1977) reported the cellulase of molecular weight of 26,000 on the basis of amino acid composition and PAGE analysis. Carboxymethyl cellulase produced by *B. pumilus* EB3 was having the range of a molecular weight from 30 to 65 kDa (Ariffin et al. 2006). Li-Jung et al. (2010) reported the strain *Bacillus subtilis* YJ1 producing cellulase; they purified and characterized cellulase, having a molecular mass of 32.5 kDa.

9.5.5 Glucanases in Biocontrol of Phytopathogenic Fungi

β-1,3-Glucanases [EC 3.1.1.6] are widely spread in bacteria, fungi, and higher plants (Simmons 1994). This enzyme has interesting and important physiological roles and practical applications in the degradation of cell wall in fungi, yeasts, and higher plants (Pang et al. 2004). These enzymes are classified as either exo- or endo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase). Fridlender et al. (1993) reported the hydrolytic inhibition of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* by β-1,3-glucanases of *Bacillus cepacia*. Singh et al. (1999) reported two strains of *Paenibacillus* and *Streptomyces* sp. which produce β-1,3-glucanases that inhibited the growth of *F. oxysporum*. Vazquez et al. (1998) reported the seven β-1,3-glucanases produced by *T. harzianum* strain under diverse growth conditions.

9.5.5.1 Mode of Action of Glucanase

β-1,3(1,6)-Glucans are major components in cell wall of yeasts and fungi. The cell wall polysaccharide glucan is consisted of predominantly β-1,3-linked backbone having some branches via β-1,6-linkages, 4,6,8,9. Glucanase causes degradation of cell wall and further penetration into the host mycelium (Fridlender et al. 1993). These enzymes can hydrolyze the substrate by two possible mechanisms: (a) exo-1,3-glucanases (EC 3.2.1.58) hydrolyze the substrate by sequentially cleaving glucose residues from the nonreducing end and (b) endo-1,3-glucanases (EC3.2.1.39) cleave linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides (Noronha and Ulhoa 1996).

9.5.5.2 Molecular Characteristics of Glucanase

Cruz et al. (1992) and Noronha and Ulhoa (1996) have reported two 1,3-glucanases having molecular weights of 78 and 36 kDa, respectively, purified from the supernatants of *T. harzianum* grown in minimal medium, supplemented with chitin as

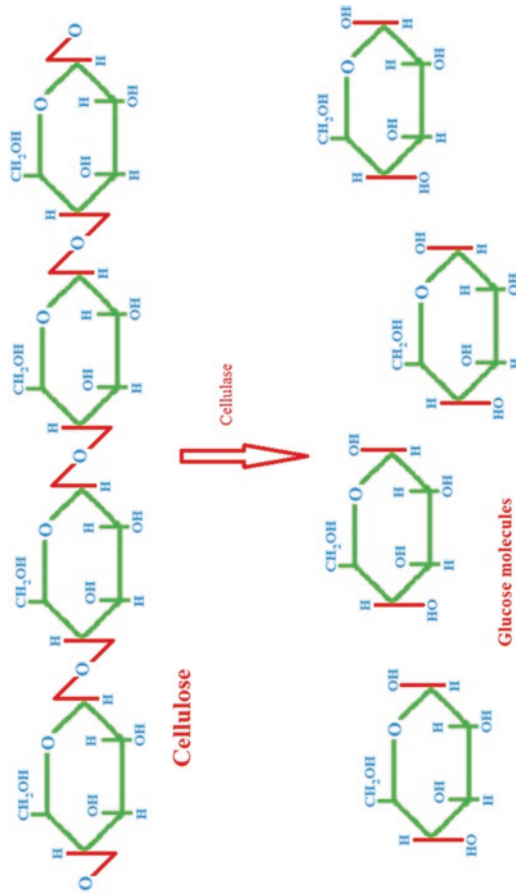

Fig. 9.5 Structure of cellulose and its digestion to glucose

carbon source. The characterization of these enzymes revealed that they are endo-1,3-glucanases, as confirmed by the production of oligosaccharides rather than glucose from the laminarin. Noronha and Ulhoa (2000) purified and characterized the 29 kDa extracellular-1,3-glucanase produced by *T. harzianum*, grown on chitin-containing medium. Maria et al. (2003) report the two purified 83.1 kDa extracellular exo-β-1, 3-glucanases produced by *T. asperellum*.

9.6 Conclusion

In concern with the current scenario toward chemical pesticides and fertilizers, and their huge consumption, there is a prominence/focus on utilization of microbial inoculants and organic inputs for its application in agricultural field. Hence, the potential of rhizobacteria in crop protection by producing different defensive antifungal metabolites like antibiotics, hydrolytic enzymes, and other metabolites is hoped to provide sustainable and eco-friendly plant disease control. Application of these rhizobacteria in agricultural field in the form of formulated product will give the greener and eco-friendly approach for the sustainable agriculture to combat the fungal diseases. Application of efficient rhizobacterial strain secreting various hydrolytic enzymes will help to reduce the liberal use and doses of agrochemicals which is the most important prospect in rhizobacterial/PGPR research. Commercial production of these organisms will have sustained release of antifungal metabolites in the environment, and these metabolites do not develop the resistance to target organism as in chemical pesticides.

Application of single or consortium of these organisms has shown the promising prospect in the field of biocontrol and plant growth promotion. These microbes can successfully utilize their potential for agricultural integrated plant disease management (IPDM) strategies. Study of hydrolytic enzymes of rhizobacteria will help in manipulating the bacterial community with biological control and plant growth promotion ability in rhizospheric zone of different sites. So these rhizobacteria will be the key determinant in plant health and productivity with sustainability.

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