Microbial Bio-production of Proteins and Valuable Metabolites

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15.1 Introduction

Microbes like bacteria, fungi, yeast, and microalgae are the prolific source of large number of valuable natural compounds of commercial and therapeutic interest. Microbes are capable of synthesizing structurally divergent compounds. According to a recent report by Business Communication Company (BCC), the global market of microbes and microbial products would reach to $302.4 billion by 2023 from $186.3 billion in 2018 (McWilliams 2012). The microbial products are comprised of either the whole microbial cells or the metabolites derived from the microbes. Various products including pharmaceuticals, bulk and fine chemicals, metabolites, proteins, nutraceuticals, biofuels, antibiotics, bioplastics, food supplements, and biofertilizers are produced using biocatalytic processes, microbial cell factories, or cell-free processes (Schmidt-Dannert 2017). The producer microbes are identified through various approaches followed by establishment of microbial growth as well as production parameters for target molecule under laboratory conditions and further optimizations for large-scale production of target molecule in fermenters. The microbial production platforms are successfully becoming an effective alternative to traditional chemical synthesis due to various advantages offered by them such that microbial biosynthesis does not require heavy metals, solvents, strong acids, or bases unlike chemical synthesis, enzymes exhibit broader substrate specificity resulting in lesser by-products, natural synthetic pathways are already available for some compound with complex structures, engineering of biosynthetic pathways can

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further improve yield, and productivity of the compound of interest or novel pathways can be constructed in host microbe (Du et al. 2011).

The advances in recombinant DNA technology have prompted the development of microbial systems for bio-manufacturing of various valuable chemicals and natural products (Chemier et al. 2009). Microbes with well-studied genetics, physiology, and biochemistry like *Escherichia coli* and *Saccharomyces cerevisiae* are commonly used as bio-production platform. *Pseudomonas putida*, *Bacillus, Cyanobacteria*, and *Streptomyces* species have also been used for biosynthesis of target compounds (Chemier et al. 2009). Metabolic engineering and synthetic biology approaches have significantly contributed in development of engineered microbes for production of various useful compounds from simple and cheap substrates not only at laboratory scale but also at the industrial scale (Jullesson et al. 2015). Computational softwares are tremendously used in metabolic engineering to extract the information from big datasets as well as to assist in designing and optimizing the novel pathways in microbes (Reed et al. 2011).

15.2 Microbial Enzymes

The enzymes are biological molecules, usually proteinaceous in nature with the exception of ribozymes (catalytic RNA molecules), and they play crucial role in different stages of metabolism or biochemical reactions as bio-catalysts (Cech and Bass 1986; Gurung et al. 2013). Enzymes possess several features that make them attractive candidates for various applications such that they enhance the rate of reaction under mild physico-chemical conditions without being consumed, they are non-toxic, and they exhibit remarkable chemoselectivity, enantioselectivity, regioselectivity, and substrate specificity.

15.2.1 Potential Sources of Enzymes

Nature contributes an extensive amount of enzyme resources. In the beginning of enzyme biotechnology era, the plant tissues and animals were the most important sources of enzymes. However, currently microbes represent the largest and useful sources of many enzymes (Demain and Adrio 2008; Volesky et al. 1984). Most of enzymes which are used commercially are obtained from aerobic strains. Majority of microbial enzymes are derived from *Aspergillus*, *Bacillus*, *Streptomyces*, and *Saccharomyces* species (Headon and Walsh 1994). Microbes are usually preferred over plants and animals as a source of enzymes because they represent amicable and economical way for enzyme production in short time, microbes have shorter generation time and genetic manipulation can be easily performed, microbial enzyme expression is controllable, microbial enzymes are more stable as well as active, and production in larger quantities can be achieved (Anbu et al. 2015; Gurung et al. 2013).

Microbial enzymes are obtained from different microorganisms. For example, proteases of commercial applicability are produced mainly by bacteria species such as *Pseudomonas*, *Clostridium*, and *Bacillus* and also by some fungal species (Nigam 2013). Studies on enzyme isolation, their characterization, and production on bench and pilot scale are continuously increasing. Owing to their commercial applications, the market for industrial enzymes is widespread (Sanchez and Demain 2017; Adrio and Demain 2014). The market for industrial enzymes will reach to nearly $6.2 billion by 2020 with annual growth rate (CAGR) of 7% (Singh et al. 2016b). In general, numerous microbial enzymes are already being exploited in many different industrial processes.

15.2.2 Microbial Enzyme Production

Microbes produce vast variety of enzymes but the absolute amount of produced enzyme differs markedly even between the strains of same microbial species. Thus, for the production of the desired enzyme for commercial applications, the strain that exhibit highest yield is ideally selected (Underkofler et al. 1958). The enzyme-based product, which is newly introduced in market, can become a commercial success if it has a large existing market share and if it is economically viable. For the successful development of a commercial enzyme process, various requirements should be fulfilled including the ability of producer microbe to grow at a rapid rate on an inexpensive medium, production of the enzymes in high yields as well as at high concentration, minimal generation of enzyme contaminants and other metabolites in the fermentation of broth, the possibility to grow the microbe on a concentrated medium in a dense culture which improves the enzyme productivity in fermenters, and easy as well as inexpensive recovery of the enzyme from the culture media (Headon and Walsh 1994; Volesky et al. 1984).

Generally, the production of the desired enzymes begins with the screening of the microbes present in the collected environmental samples to identify the producer strain using suitable selection procedures. It is followed by optimization of the culture conditions, physico-chemical properties, and process parameters to maximize the production of target enzyme. The screening processes on laboratory scale focuses on the search for a high titre enzyme-producing microorganism, and they are usually labour intensive, monotonous, and time consuming (Yoo et al. 2017). The advent of genetic engineering approaches facilitated the cloning of the gene encoding for the target enzyme in microbes with defined growth conditions, with controllable gene expression, and with GRAS status (generally recognized as safe), leading to impressive enzyme yields. The construction of metagenomic library by cloning of total isolated DNA from environmental samples in suitable vector system, and subsequent function-based screening is another powerful approach that allows to explore the potential of biological diversity in different ecosystems for the identification of target enzyme (Thies et al. 2016). This approach circumvents the need of culturing and isolation of individual microbe in laboratory (Guazzaroni et al. 2015). Another approach to obtain superior enzyme

producer strain is mutagenesis where the microbial cultures are exposed to muta-genic agents like chemicals, heat, and radiations. The screening for survival of cells is then performed to select the strain that can overproduce the target enzyme (Ghazi et al. 2014).

For production of target enzyme, the producer microbes are cultivated by inocu-lation of the pure culture into the suitable sterile medium. Submerged fermentation and solid-state fermentation (SSF) are the methods used for the enzyme cultivation (Renge et al. 2012). In submerged fermentation, the microorganisms are cultivated in a closed vessel (fermenter) containing liquid nutrient media and a high concentra-tion of oxygen. The growing microbes release the target enzyme in extracellular environment i.e. in fermentation broth. The biomass is then removed from fermen-tation broth by centrifugation and the enzymes in the broth are then concentrated by evaporation of media, membrane filtration, or crystallization. This approach was used traditionally to prepare the target enzymes due to easy handling and ability to control physico-chemical factors (Mrudula and Murugammal 2011). In solid-state fermentation, microbes are cultivated on a solid substrate like wheat bran, wheat straw, and rice straw. This method is used for the cultivation of fungi such as *Aspergillus and Penicillium* to obtain enzymes such as amylase, proteases, and pec-tinases (Volesky et al. 1984).

15.2.3 Applications of Microbial Enzymes

The demand of microbial enzymes in various industries is expanding rapidly. Their application in few sectors is summarized.

15.2.3.1 Industrial Application

Microbial enzymes are used in various industrial applications including production of pharmaceuticals or pharmaceutically important intermediates, leather process-ing, textile industry, and paper and pulp, detergents, and biofuel production. In laun-dry detergents, proteases are extensively used to remove the proteinaceous dirt from the fabric. Proteolytic enzymes in many commercially available detergents are derived from the *Bacillus* species (Kumar et al. 2008). Other enzymes are also used in combination with proteases to improve the cleaning performance of the deter-gent, which includes lipases, amylases, and cellulases to remove fats or oils, remove starch residues, and brighten colour, respectively (Hasan et al. 2010). Several active pharmaceutical ingredients are being generated using the enzymes because of their remarkable specificity and selectivity. Carbonyl reductases have been used to obtain an intermediate for synthesis of blockbuster drugs and statins, by reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to ethyl (S)-4-chloro-3-hydroxybutanoate ((S)-CHBE) (Xu et al. 2016). Atorvastatin which is an important ingredient of Lipitor, a cholesterol-lowering drug, has also been shown to produce through enzy-matic synthesis (Bornscheuer et al. 2012). The commercial manufacturing of tela-previr, boceprevir, and esomeprazole drugs against hepatitis C virus involved in the oxidase-catalysed desymmetrization (Li et al. 2012).

Tyrosine phenol lyase expressed in *Erwinia herbicola* cells has been used to produce L-3,4-dihydroxyphenylalanine (L-DOPA), a drug for treatment of Parkinson's disease (Patel 2008). Most of the enzymes used in textile industry are hydrolases like cellulases, pectinases, laccases, amylases, and catalases. These enzymes are being used as a substitute of stone wash, in bio-finishing, in bio-scouring, and in improving the look of material (Doshi and Shelke 2001). The involvement of lipases, cellulases, and xylanases has been reported for bioethanol production by decomposition of lignocellulosic material and also synthesis of fatty acid methyl esters (Liew et al. 2014). In leather industry, proteases and lipases are involved at different stages of leather processing. They are used in curing, soaking, dehairing, degreasing, tanning, and waste processing of leather (Choudhary et al. 2004).

15.2.3.2   Food

Microbial enzymes are significantly used in processing of food products such as cheese, beer, bread, and soft drinks, and the use of enzymes in manufacturing is increasing (Fernandes and Carvalho 2017). Amylases from the malted cereal, bacterial, or fungal sources are added to flour at the bakery and mill (Taylor and Richardson 1979). Another example of a microbial enzyme used in food industry is microbial transglutaminase which catalyses isopeptide bond formation between proteins. This property is widely used in manufacturing cheese and other dairy products, meat processing, manufacturing bakery products, and producing edible films (Kieliszek and Misiewicz 2014). Proteases are used in meat tenderization, ripening of cheese, and milk coagulation (Aruna et al. 2014). Lipases are also used in cheese flavour development and improving its texture. They are also in used in flavour development in butter and improving the shelf life of baking products (Aravindan et al. 2007). Galactosidases are used in lactose hydrolysis of milk-based products for lactose-intolerant people, in preparation of prebiotic food ingredient like galacto-oligosaccharides, and in lactose hydrolysis in whey (Rosenberg 2006).

15.2.3.3   Medicines

Therapeutic enzymes derived from microbial sources are used to treat various diseases. Nattokinase from *Bacillus subtilis* decreases the blood coagulation and removes existing thrombus. It is also used to decrease the lipids that can increase the chances of cardiovascular disease (Banerjee et al. 2004; Milner 2008). Streptokinase and urokinase are used for dissolving the blood clots in blocked blood vessels (Banerjee et al. 2004; Olson et al. 2011). Collagenases have been used to assist in healing skin burns and tumours in combination with antibiotics (Ostlie et al. 2012). In dental hygiene, enzymes like dextranase and cariogenanase from *Penicillium funiculosum* and *Bacillus* sp. are, respectively, used to reduce plaques and dental carries. Toothpastes containing a mixture of enzymes from *Aspergillus niger* and *Aspergillus oryzae* reduce calculus and soft accretions (Singh et al. 2016a). Tyrosine hydroxylase is responsible for catalysing the conversion of L-tyrosine to L-dopa, which is a useful agent in the treatment of Parkinson's disease (Taylor and Richardson 1979).

15.2.4 Strategies for Enhancing Applicability of Existing Microbial Enzymes

In spite of the significant advances in screening and selection approaches for identifying the novel enzymes to combat the ever-increasing industrial demands, there still remains the need of efficient ways to obtain enzymes with better catalytic performance for relevant industrial processes. In this connection, protein engineering strategies have been devised to improve the efficiency of existing enzymes (Kaushik et al. 2018). Protein engineering focuses on tailoring enzymes to overcome inherent shortcomings in existing enzymes like low activity, lack of specificity, and low stability or to introduce new functionalities. One of the protein engineering approaches is directed evolution or in vitro evolution, which mimics natural evolution process and does not require detailed knowledge on structure, function, and mechanistic aspects of target enzyme (Chen et al. 2012). It involves exposure of the gene encoding for the enzyme of interest to iterative rounds of random mutagenesis resulting in construction of library of gene variants; the resulting library is then screened for the variant that exhibits desired level of improvement (Chen and Arnold 1993). The process of directed evolution basically relies on effective mutagenesis method that generates significant genetic diversity and a robust screening method that leads to identification of the enzyme variant with desired catalytic characteristics as compared to wild-type enzyme.

The genetic diversity can be introduced by random mutagenesis methods like use of mutator strains, UV irradiation, chemical mutagenesis, error-prone PCR, and sequence saturation mutagenesis (SeSAM), or it can be introduced by gene recombination methods like DNA shuffling and oligonucleotide primer-based methods (Labrou 2010). Another protein engineering approach is rational redesign, which involves use of sequence and structure-based information with computational modelling to predict the hotspot residues which on mutagenesis are likely to result in improved enzyme functionalities. This approach dramatically reduces the library size and subsequently eliminates need of high-throughput screening methods. A semi-rational approach involving both the components of random and rational mutagenesis to design smart libraries with small size and high quality has shown to be practically more effective in generating tailor-made enzymes for specific needs (Lutz 2010). In recent years, engineering of access tunnel residues in enzymes with buried catalytic site has become an attractive approach to alter the enzyme properties. Access tunnels are the transport pathways that connect the buried active site of the enzyme to the exterior environment and allow the access or egress of substrates, reactive intermediates, solvents, ions, and products to the catalytic site (Damborsky et al. 2010; Timmis et al. 2010). Modification of the access tunnel lining residues doesn't affect the architecture of catalytic site and thus increases the chances of getting functional clones with tailored properties. This strategy has been applied on several enzymes with buried active site to improve their catalytic properties (Kaushik et al. 2018; Prokop et al. 2012; Sandström et al. 2012). De novo protein design is another strategy that can allow to introduce new catalytic functions in protein scaffold such that de novo enzymes have been successfully designed that can catalyse

the Diels-Alder and Kemp elimination reactions (Blomberg et al. 2013; Siegel et al. 2010). Recently, possibility to introduce de novo functional tunnels in existing protein has been demonstrated to facilitate creation of better and efficient enzymes (Brezovsky et al. 2016).

15.3   Proteins

The word protein is derived from the Greek word 'protos' that means first or 'protieos' which means primary (Aronson 2012). Proteins are the primary constituent of living things and are part of the molecular machinery in living organisms. They form the fundamental basis of the structure and function of life. Peptides and proteins are polymers of amino acids. They are the products of translation of mRNA within the living cell (Berg et al. 2002; Nelson et al. 2008). Variety of proteins/peptides derived from microbial systems have direct implication in production of vaccines, as therapeutic agents and as food supplements (Akash et al. 2015).

15.3.1   Microbial Proteins and Their Utility in Vaccine Production

A vaccine is a biological substance that stimulates the active acquired immune system of the body to act against a particular germ, thereby preventing the disease caused by it. Microbial surface proteins are associated with pathogenesis and thus represents major target for vaccine development. The commercially available vaccines contain attenuated pathogenic microbes or the microbial antigenic protein (Table 15.1). Various in silico tools have been developed by researchers so as to rapidly identify the surface proteins which can possibly display antigenic properties (Giombini et al. 2010). Approaches like whole-genome sequencing, labeling of surface proteins by selective biotinylation of whole bacteria, identification of immunogenic proteins from pathogens on protein microarrays, and enzymatic shaving of surface of bacteria with proteases have made identification of the surface antigens easier for vaccine development (Grandi 2010).

Strategies used in the production of vaccines are attenuation of the live pathogenic microbe, structural vaccinology, reverse vaccinology, epitope mapping, recombinant protein synthesis, and microbial cell-surface display. Structural vaccinology or structure-based antigen design involves the use of high-resolution structural analysis in distinguishing structural components of the antigen that elicit protective and disease-enhancing immunity (Dormitzer et al. 2008). This strategy has effectively guided design of engineered RSV F subunit antigen against respiratory syncytial virus, GBS (group B *Streptococcus*) pilus-based fusion protein, and an improved MenB (serogroup B meningococcus) single-domain fHbp (factor H-binding protein) antigen against meningitis (Dormitzer et al. 2012). Reverse vaccinology uses whole genome sequencing and immunological information of the pathogen to identify the suitable candidate vaccine antigens (Sette and Rappuoli 2010). Bexsero™ is a meningococcal group B vaccine that was developed through

Table 15.1 Representative vaccines based on attenuated microbes and microbial proteins

Vaccine	Disease	Causative microbe	Components	Manufacturer	References
Tetravalent influenza vaccine (split virion) I.P. (TetIV)	Influenza	Influenza A and B viruses	Two A strains (H1N1 and H3N2) and two B strains (Yamagata and Victoria)	Cadila Healthcare Limited	Sharma et al. (2018)
Vaxigrip trivalent split-virion, inactivated influenza vaccine	Influenza	Influenza A and B viruses	Two A strains (H1N1 and H3N2) and either one of two B strains (Yamagata and Victoria)	Sanofi Pasteur	Haugh et al. (2017)
Zostavax	Herpes zoster (shingles)	Varicella-zoster virus	Live attenuated vaccine	Merck & Co.	Keating (2016) and Levin et al. (2018)
Rotavac oral	Rotaviral diarrhoea in children	Rotavirus	Live attenuated vaccine of human live rotavirus strain G9P11	Bharat Biotech International Limited	Chandola et al. (2017)
Dukora (monovalent oral cholera vaccine)	Cholera	*Vibrio cholerae*	Killed whole cell vaccine consisting of Inaba and Ogawa serotypes of *V. cholerae* O1 in conjunction with recombinant cholera toxin B subunit (WC-rBS)	Crucell Sweden AB, Stockholm, Sweden	Khan et al. (2017)
Shanchol (bivalent oral cholera vaccine)	Cholera	*Vibrio cholerae*	Killed whole cell cholera vaccine consisting of *V. cholerae* lacking cholera toxin B subunit	Shantha Biotechnics, Hyderabad, India	Ivers et al. (2015)
rVSVΔG-ZEBOV-GP vaccine	Ebola virus disease	Zaire Ebola virus	Recombinant vesicular stomatitis virus vaccine	Merck Phase 1 and phase 2 trial	Agnandji et al. (2017) and Kennedy et al. (2017)

(continued)

Table 15.1 (continued)

Vaccine	Disease	Causative microbe	Components	Manufacturer	References
ChinZIKV (recombinant chimeric ZIKV vaccine)	Zika virus disease	Zika virus	Replacement of the prM-E genes of Japanese encephalitis live attenuated vaccine JEV SA14-14-2 with the corresponding region of an Asian ZIKV strain FSS13025	Awaiting clinical development	Li et al. (2018)
Gardasil (recombinant human papillomavirus vaccine)	Cervical cancer	Human papillomavirus	Major capsid protein L1 of HPV types 6, 11, 16, and 18	Merck & Co.	Stanley (2007)

reverse vaccinology (Del Tordello et al. 2017). Recombinant protein subunit vaccines have been formulated with the help of protein antigens synthesized with heterologous host cells including *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris* and mammalian cells. To design new recombinant protein production strategies, the gene sequence should be optimized to be stably expressed in the recombinant host cell. Optimizing culture conditions and induction protocols increases recombinant protein yields and it has been demonstrated in cultures of both *P. pastoris* and *E. coli* (Bill 2015). For posttranslational modifications such as glycosylation of the expressed protein, baculoviral system in insect cells is ideal (Demain and Vaishnav 2009). Microbial cell surface display is another strategy, which deals with expressing the protein of interest as a fusion to various anchoring motifs like surface proteins or their fragments. The host strain selected for display must be compatible with the protein of interest being displayed with minimal activity of proteases and should be able to cultivate without lysis (Lee et al. 2003). This approach has been used for the development of live vaccine where the heterologous epitopes were exposed on human commensal or attenuated pathogenic bacterial cells to evoke antibody responses specific to the antigen (Lee et al. 2000; Liljeqvist et al. 1997). More strategies for vaccine production are being developed for optimum yield in research laboratories globally in an effort to reduce the manufacturing costs, and microbes as hosts for the production of vaccine would be very advantageous in achieving this goal.

15.3.2 Toxins and Antimicrobial Peptides

15.3.2.1 Toxins

Microbial toxins are poisons produced biologically by either bacteria or fungi. They function as autonomous molecules, attacking specific cells in an organism by punching holes into the cell membranes or modifying intracellular components. Some bacteria secrete toxins into their surroundings to overcome host defence and are responsible for the symptoms of bacterial infections (de Wit 2013). Microbial toxins are typically soluble, stable, non-volatile, and highly bioactive compounds that may have cytotoxic, inflammatory, immunosuppressive, and carcinogenic effects (Korkalainen et al. 2017). Bacterial toxins are classified into two endotoxins and exotoxins while fungal toxins are classified into peptidic toxins and non-peptidic toxins. Despite of their detrimental effects, the toxins have been used as therapeutics, cosmetic agents, and adjuvants or drug delivery agents (Fabbri et al. 2008) (Table 15.2).

15.3.2.2 Antimicrobial Peptides and Proteins

Antimicrobial peptides and proteins (AMPs) or host defence peptides (HDP) are a diversified group of very small, normally positively charged molecules composed of varying number of amino acids. Multicellular organisms produce them as a first line of defence. They are used by unicellular organisms to compete for nutrients with other organisms. AMPs can be classified based on various parameters such as biological activity, 3D structure, and peptide family (Wang 2015). In 1939, Rene Dubos discovered and isolated the first microbial peptidic antibiotic Gramicidin from *Bacillus brevis* (renamed as *Brevibacillus brevis*) (Dubos and Cattaneo 1939). Since then, new AMPs are being discovered and their biochemical aspects were studied to shed light on their mechanism of action as well as their potential in clinical therapeutics. Some of these AMPs are listed in Table 15.3. Existing AMPs are being genetically engineered to create recombinant peptides with greater potency against infectious microorganisms. Thus, they represent attractive alternative to antibiotics in controlling pathogenic microbes and maintenance of human lifespan.

15.3.3 Microbial Proteins as a Food and Feed Source

Proteins are a dietary requirement for both humans and domesticated animals. Nutritious food is required in bulk quantities for livestock and pisciculture industry, both of which are among the major sources of proteins for humans. The concerns about future food security are raising due to rapidly increasing human population which is expected to reach ten billion in 2050 as per the United Nations report. Sustainable manufacturing of proteins in bulk will reduce the strain on the environment to provide sufficient nutritious food for the maintenance of these industries. Microbial proteins also known as single-cell proteins (SCP) can be a solution to this perplexing problem as bacteria already have high protein content and multiply exponentially using low-cost substrates under optimal conditions. SCP is a protein

Table 15.2 Microbial toxins, their mode of action, and applications

Toxin	Source microbe	Biochemistry	Deleterious effect	Potential applications	References
Viral toxin					
NSP4 viral *Rotavirus* enterotoxin		Viral replication and Ca^{2+} immobilization which may further induce signalling through other Ca^{2+}-sensitive cellular processes to potentiate fluid secretion while curtailing fluid absorption	Causes diarrhoea similar to rotaviral diarrhoea	Study of causes and possible vaccine for rotaviral diarrhoea	Morris and Estes (2001)
Bacterial exotoxins					
Botulinum neurotoxin	*Clostridium botulinum*	Single, inactive polypeptide (150 kDa). Acts by blocking the release of acetylcholine at neuromuscular junction	Causes botulism in humans	Treatment of dystonia, spasticity, hyperhidrosis, prophylactic migraine, strabismus, reduction of glabellar lines, and cosmetic surgery	Sharma (2016), Burroughs and Anderson (2015), and Foster et al. (2018)
Tetanus neurotoxin	*Clostridium tetani*	A heavy and a light chain linked via a disulphide bridge	Spastic paralysis in tetanus disease	Development of tetanus toxoid vaccine against tetanus	Masuyer et al. (2017) and Link et al. (1992)
		Inhibits neurotransmission of inhibitory interneurons			
Anthrax toxin	*Bacillus anthracis*	Consists of three proteins: protective antigen (83 kDa), lethal factor (90 kDa), and edema factor (89 kDa)	Anthrax disease	Tumour targeting and drug delivery	Bachran and Leppla (2016)
		Affects signalling pathways and modulates immunologic responses			
Cholera toxin B subunit	*Vibrio cholera*	Homopentameric protein, non-toxic in nature (55kD)	Subunit of virulence factor of *V. cholera* responsible for cholera	Part of a licensed oral cholera vaccine	Verier et al. (2006) and Baldauf et al. (2015)
		High affinity binding to the GM1 ganglioside present on mammalian cells		Adjuvant for mucosal vaccine	
				Immunomodulatory and anti-inflammatory agent	

Table 15.2 (continued)

Toxin	Source microbe	Biochemistry	Deleterious effect	Potential applications	References
Fungal toxins					
α-Sarcin ribotoxin	*Aspergillus giganteus*	Composed of a rigid hydrophobic core and some exposed segments, mostly loops	Protein biosynthesis inhibition	Immunotoxin in antitumor therapy	Lacadena et al. (2007) and Olombrada et al. (2014)
		Cleaves one phosphodiester bond of the sarcin/ricin loop, impairing its essential function		Bioinsecticides	
Candidalysin	*Candida albicans*	Amphipathic protein containing an amino-terminal α-helical hydrophobic region	Mucosal infection	Novel therapeutic agents	Pasricha and Pearson (2016) and Moyes et al. (2016)
		Causes damage to epithelial membranes, activates a danger response signalling pathway and epithelial immunity			
Bassiacridin	*Beauveria bassiana*	Single polypeptide chain (60 kDa)	Cytotoxic effect on insect cells	Microbial control of locusts and grasshoppers	Quesada-Moraga and Alain (2004)
		Exhibits β-galactosidase, β-glucosidase, and N-acetylglucosaminidase enzyme activities			
Ribotoxin hirsutellin A	*Hirsutella thompsonii*	Single polypeptide chain (15 kDa) with at least two cysteine residues involved in the formation of an intrachain disulphide bridge	Cytotoxic effect on insect larvae	Model for bioinsecticide development	Mazet and Vey (1995)

Table 15.3 Representative antimicrobial peptides with their properties and applications

AMP	Source microbe	Type	Biochemistry	Mechanism of action	Target microbe	Activity	Applications	References
Nisin	Lactococcus lactis subsp. Lactis	Cationic lantibiotic	Exists in multimeric forms (3500 Da)	Interact with cytoplasmic membranes of bacteria and form transient pores, causing efflux of ions and small molecules	Clostridium butyricum, C. tyrobutyricum, and C. aporogenes	Delayed toxigenesis of C. botulinum type A and type B	Increasing shelf life of processed cheese, dairy desserts, milk, fermented beverages, bacon, frankfurters, and fish	Vandenbergh (1993)
Linear gramicidin	Bacillus brevis (strain BG)	–	15-residue peptides with β-helix like structure	Forms transmembrane channels causing diffusion of intracellular component out of the cell leading to cell dysfunction	Gram-positive and Gram-negative bacteria	Antibacterial, antiviral, spermicidal	Ophthalmic use as solutions/drops (approved in 1940s)	Mishra et al. (2017), Yang and Yousef (2018), and Dubos (1939)
						Anti-HIV	Topical application as antibiotic cream	
Alamethicin	Trichoderma viride	Peptaibol	20-amino acid peptide with a backbone conformation resembling the flexible helix-bend-helix arrangement	Self-associates into hexameric barrel–stave transmembrane helices and permeabilizes membranes by forming channels through them	Gram-positive bacteria, such as Enterococcus faecalis, Staphylococcus haemolyticus, Streptococcus viridans, and Staphylococcus aureus	Inhibits methanogenesis and promotes acetogenesis in bioelectrochemical systems	Production of industrially relevant organic compounds by inhibition of methanogenesis	Bechinger (1997), Zhu et al. (2015), Ray et al. (2017), and Ageitos et al. (2017)
							Enhanced power generation in microbial fuel cells	

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Table 15.3 (continued)

AMP	Source microbe	Type	Biochemistry	Mechanism of action	Target microbe	Activity	Applications	References
Copsin	*Coprinopsis cinerea*	Defensin	CSαβ core fold interconnected by six disulphide bonds and N-terminal pyroglutamate	Binding to the peptidoglycan precursor lipid II and prevention of cell wall biosynthesis	Gram-positive bacteria, such as *Listeria*, *Enterococci*, and *Bacilli* including vancomycin-resistant *E. faecium*	Most potent against *L. monocytogenes*, a foodborne pathogen causing severe forms of listeriosis	Novel highly stabilized scaffold for antibiotics	Franzoi et al. (2017) and Essig et al. (2014)

source from microbial cultures such as bacteria, yeast, filamentous fungi, and algae with the potential to be animal feed as well as human protein supplements. They are either dehydrated microbial cell culture or purified proteins derived from microbial cell culture (Ugbogu and Ugbogu 2016).

Some SCPs that are available commercially or under study are indicated in Table 15.4. SCPs offer various advantages: they contain high protein content (60–82% of dry cell weight) along with other nutrients, they are good source of essential amino acids such as lysine and methionine which are limited in most plant- and animal-based foods (Suman et al. 2015), the microbes have rapid generation time, they are genetically modifiable (e.g. for composition of amino acids), and they require less space as compared to conventional agriculture. However, SCPs have some disadvantages like high nucleic acid content, accumulation of uric acid crystals caused by bacterial SCPs leading to gout, possibility of allergic reactions with fungal SCPs as mycotoxins are allergens, and slow digestibility due to rigid cell wall. Currently SCPs are produced using solid-state fermentation (Jaganmohan et al. 2013). Recent advances in fermentation, extraction, downstream processing techniques, and optimization of substrates/conditions resulted in large-scale production of protein biomass. Production and marketing of a wider range of SCPs could be a promising step to alleviate food shortage and malnutrition.

15.3.4 Microbial Factories for Production of Recombinant Proteins

Microbes represent convenient system for production of proteins which are difficult to obtain from their native sources (Ferrer-Miralles et al. 2009). The use of microbes for protein production has increased in recent times due to the low cost, high productivity, and rapid use (Terpe 2006). A range of microbes including bacteria such as *Escherichia coli and Bacillus megaterium*, filamentous fungi such as *Aspergillus niger* and *Trichoderma reesei*, and yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris* are exploited as recombinant cell factories. The first licensed protein drug successfully produced by recombinant DNA technology was human insulin in *E. coli* by Genentech and was commercialized by Eli Lilly in 1982. At present, nearly 400 drugs out of approved 650 protein drugs are produced by recombinant technologies (Sanchez-Garcia et al. 2016).

Recombinant protein production involves manipulation of the gene expression system of microbes with the aim of producing large amounts of recombinant protein tailored for a specific function. For a microbe to express foreign protein, the gene encoding the protein of interest is cloned into an expression vector with a suitable promoter gene and then introduced into the microbe. If the gene contains introns, it is cloned from a cDNA library as bacteria cannot excise introns. The plasmid is then transformed into a suitable host that is able to produce the desired protein. The transformed strain is transferred to liquid media and cultured. At a specific stage of growth, a chemical inducer triggers the promoter of the expression vector and induces expression of recombinant gene. The polypeptide produced folds into the

Table 15.4 Representative antimicrobial peptides with their properties and applications

Type of microbe	Microbe	Substrate	Cultivation	Commercial name	Crude protein content (%)	Remarks/uses	References
Algae	*Euglena gracilis*	Cramer–Myers medium	Photo-bioreactor	Whole cell form marketed by Algaeon Inc.	47	Potential animal feed source	Chae et al. (2006) and
						Biotechnological production of metabolites like α-tocopherol and paramylon	Rodríguez-Zavala et al. (2010)
Bacteria	*Rhodococcus opacus*	Corn stover	Bioreactor	NA	47.0–56.9	Fortification to livestock and poultry feed	Mahan et al. (2018)
		Orange waste					
		Lemon waste				Food source in fish farming	
	Arthrospira platensis, Arthrospira maxima	Grows photosynthetically, molasses can be used to stimulate growth	Photo-bioreactor	Spirulina marketed by Cyanotech Corporation, DDW Inc., C.B.N. Spirulina Canada Co., Ltd	50–70	Supplement for both humans and animals	Andrade and Costa (2007)
	Methylococcus capsulatus	Methane	U-loop fermentation	*UniProtein®* marketed by UniBio	70–72.9	Animal feed supplement	Prado-Rubio et al. (2010)
Yeast	*Kluyveromyces marxianus*	Whey	Laboratory scale fermenter	NA	83.33	Food source	Nayeem et al. (2017)

Filamentous fungi	Fusarium venenatum	Wheat starch as carbon source	Batch culture,	Quorn® (mycoprotein) marketed by Marlow foods	56	Protein-rich food containing essential amino acids	Garodia et al. (2017)
		Ammonia as nitrogen source	Continuous culture			Fat or cereal replacer	
			Air-lift fermenters			Helps control blood lipids, blood glucose, and appetite due to dietary fibre content	
	Penicillium janthinellum	Sugarcane bagasse	Laboratory scale fermentation	NA	40–50	SCP as animal feed and xylanase by-product as prebleach agent in paper and pulp industry	Rao et al. (2010)

NA not available

recombinant protein of interest, which can be further purified by suitable purification approaches. The production of the target protein can also be scaled up from initial batch cultures to stirred tank bioreactors on fed-batch regimens to manufacture large protein biomass, which is then released and purified (Overton 2014).

E. coli has been one of the most commonly employed microbial cell factories for heterologous expression, and it has been used for the production of 30% of recombinant proteins approved by the FDA (Rosano and Ceccarelli 2014). It has been used for producing a range of biopharmaceuticals ranging from growth hormones (Goeddel et al. 1980; Olson et al. 1981), growth factors (Kwong et al. 2016), peptides (Zorko and Jerala 2010), and therapeutic proteins (Mane and Tale 2015). However, the major hurdles in exploiting *E. coli* as an expression host include inclusion body formation due to aggregation of overexpressed protein. Proteins derived from eukaryotes often undergo posttranslational modifications to achieve proper folding, but *E. coli* lacks such system and thus recombinant proteins expressed in *E. coli* microenvironment does not fold properly or misfolding occurs (Sharma and Chaudhuri 2017). The membrane proteins and the proteins with molecular weight more than 60 kDa are also difficult to express in *E. coli*. Toxic nature of heterologous protein and instability of the plasmid are other obstacles affecting successful expression in *E. coli*. *Saccharomyces cerevisiae* is another conventionally used host for recombinant protein production. Other non-conventional yeasts are *Hansenula polymorpha*, *Pichia pastoris*, and *Yarrowia lipolytica* (Kim et al. 2015).

The dominant role of yeast is seen in production of human blood proteins (Martinez et al. 2012), insulin analogues, and hepatitis vaccine (Wang et al. 2017). Efforts are being done to improve the titre, rate, and yield of the yeast cell factory through rational metabolic engineering in *Saccharomyces cerevisiae*. Multiple-genome integration was observed to be an ideal approach for generating stable strains with high copy numbers of heterologous genes. Strong glycolytic promoters (PGK1p, TPI1p, ADH1p) and inducible promoters have been developed to induce heterologous protein expression at various levels as the glycosylation capability of yeast is inappropriate for human proteins (Hou et al. 2012; Wang et al. 2017). One of the bottlenecks of protein production in yeasts is the protein secretory machinery, which may not be able to handle a high flux of proteins requiring specific posttranslational modification. This can result in missorting where the heterologous protein is targeted to the vacuole for degradation instead of being secreted. The use of systems biology integrates large-scale datasets (-omics) with mathematical modelling to direct metabolic engineering and site-directed mutagenesis towards overcoming the limitations of the protein secretion machinery (Martínez et al. 2012; Wang et al. 2017).

Information obtained using systems biology involving the study of the transcriptomics, proteomics, metabolomics, and metabolic flux analysis of *P. pastoris*, a methylotrophic yeast, is being utilized to enhance protein folding and secretion as well as engineer the recombinant protein process towards maximizing the yield and improving the yeast strain (Zahrl et al. 2017). High-throughput screening of improved strains with high protein yield in *S. cerevisiae* and *P. pastoris* specific to the target protein is the final step in the development of

recombinant strains (Ahmad et al. 2014; Wang et al. 2017). Filamentous fungi are other candidates for recombinant protein production. They have mostly been used as robust cell factories for producing pharmaceutically relevant enzymes. Examples of recombinant enzymes are catalase, glucose oxidase, and phytase from *Aspergillus niger* and cellulose and xylanase from *Trichoderma reesei* (Archer 2000). Filamentous fungi have enormous potential in efficient large-scale production of recombinant proteins as they are cheap to cultivate and downstream processing is easier as the proteins are secreted through hyphae (Nevalainen and Peterson 2014). Numerous efforts have been made to develop filamentous fungi as a host for recombinant proteins, but further improvement is required for the expression of wider range of heterologous proteins. To achieve this, proteome profile of filamentous fungi like recombinant strains of *Aspergillus nidulans* is being performed to identify the bottlenecks in heterologous protein expression (Zubieta et al. 2018). These findings help us to understand the mechanisms underlying protein production and to rationally manipulate target genes for the improvement of fungal strains.

15.4   Secondary Metabolites

Secondary metabolites derived from microbes represent the important group of compounds with a wide range of applications. The term secondary metabolite has been introduced by Bu'LocK in 1961. Secondary metabolites are the low-molecular-weight products with no direct involvement in physiology and development of microbe but may render several benefits to the organism (Bu'Lock 1961). For instance, antibiotics are one of the well-known secondary metabolites, which confer selective growth advantage and better survival ability to the host microbe. Other examples of secondary metabolite from microbial origin with varied biological functions include antibiotics, alkaloids, pigments, antitumour agents, toxins, growth promoters, carotenoids, and enzyme inhibitors.

15.4.1 Microbial Source of Secondary Metabolites

Secondary metabolites or small molecule natural products are synthesized by prokaryotes like bacteria to eukaryotes like fungi, plants, and animals, although the secondary metabolite producing ability is unevenly distributed. Secondary metabolites are formed by the biosynthetic pathways which branch off from the primary metabolic pathways. Secondary metabolism in fungi occurs during stationary phase in the liquid cultures and is often linked to the onset of morphological developments in surface-grown cultures. Similarly, in bacteria the secondary metabolites are formed during the late growth phase. Nearly 20,000 so-called microbial secondary metabolites are known (Marinelli 2009). Among prokaryotes, the filamentous actinomycetes species has been reported to produce over 10,000 bioactive compounds, streptomyces produces 7600 compounds, and rare actinomycetes produces nearly

2500 bioactive compounds, and they produce 45% of known bioactive microbial metabolites, representing the largest producer group (Bérdy 2005). Streptomyces is the largest antibiotic-producing genus and it alone provides more than 60% of the antibiotics (Esnault et al. 2017).

The genome sequencing of model actinomycete *Streptomyces coelicolor* A3(2) led to identification of more than 20 gene clusters capable for coding the secondary metabolites (Bentley et al. 2002). The gene clusters (polyketide synthases type I and II, nonribosomal peptide synthetases) were found in its genome. This strain also produces metabolites like methylenomycin, prodigiosin, actinorhodin, and a calcium-dependent antibiotic. The microbes with lesser ability to produce secondary metabolites include mycoplasma, mycoplasmatales, and spirotheces. Among the eukaryotic fungi, ascomycetes and endophytic fungal species are frequent producers, while yeasts, phycomycetes, and slime moulds are less frequent producers. The fungal bioactive compounds constitute 38% of known microbial products (Bérdy 2005). It has been shown that a large number of microbial species that cannot grow under standard laboratory conditions, known as 'unculturable' strains, can also be potential source of novel secondary metabolites. Development of methods to culture such microbes would further allow the exploitation of microbial diversity to produce interesting metabolites (Lewis et al. 2010; Newman 2016).

15.4.2 Approaches for Isolation and Identification of Bioactive Secondary Metabolites

In 1929, the serendipitous discovery of antibiotic penicillin G from *Penicillium notatum* (Fleming 1929) established the therapeutic potential of this fungal secondary metabolite and further expedited the exploration of novel bioactive metabolites. Since then various microbial metabolites have been isolated including β-lactams, aminoglycosides, glycopeptides, tetracyclines, and cephalosporins. The classical approach leading to the antibiotic discovery was based on the growth inhibition of target microbes. However, in recent times the screening methods based on growth inhibition has turned out to be unsuccessful in identifying new antibiotics. This propelled the development of modern methodologies and techniques to accelerate the discovery process (Davies 2011).

15.4.2.1 Isolation of Secondary Metabolite Producing Microbes and Strain Improvement

The screening of microbial fermentation extracts to identify biologically active compound was practiced previously. For successful screening, the selection of growth conditions that can initiate the synthesis of secondary metabolites in microbes and the bioassays or analytical methods that allow detection of the secondary metabolite are the general requirements. Once the desired strain that can overproduce a particular compound is isolated, the next step involves improving the concentration of the compound. It may be achieved by optimization of the culture conditions like medium composition, pH, temperature, agitation, and aeration.

Various additives can also be tested in culture media as limiting precursors of desired compound; e.g. lysine is added to the culture media as a precursor and cofactor to enhance the production of cephamycin by *Streptomyces clavuligerus* (Demain 1998; Gonzalez et al. 2003; Khetan et al. 1999).

The advent of recombinant DNA techniques led to manipulation and improvement of microbial strain for enhanced production of target secondary metabolite. In classical genetics, mutations are introduced randomly or on rational basis followed by screening/selection to identify the mutants with desired improvements (Sharma et al. 2014). The random screening method requires the limited knowledge of genetics, biochemistry, and physiology of biosynthetic pathway. On the other hand, rational screening requires basic knowledge of pathway regulation and product metabolism. For example, *Streptomyces hygroscopicus* mutant strain producing higher titre of rapamycin was obtained after mutagenesis and screening of parent culture (Cheng et al. 2001).

15.4.2.2 Mining Microbial Genomes for New Natural Products

The whole genome sequencing enabled rapid identification of the producer strains. Only specific regions of genome, namely, biosynthetic gene clusters, are involved in formation of valuable bioactive molecules. These gene clusters encode for proteins, which participate in synthesis of bioactive molecule using building blocks derived from primary metabolism. The ribosomal peptide synthetases and polyketide synthases have particularly much attention in recent years as they account for majority of structurally diverse, clinically and commercially important molecules (Naughton et al. 2017). Recently, microbial genome sequencing analysis has revealed the presence of numerous cryptic or orphan gene clusters which are responsible for production of a number of unknown secondary metabolites (Chiang et al. 2009). Various strategies have been devised to identify the metabolic products of the microbial cryptic gene clusters. It includes isotopic tracer technique, in vitro reconstitution, sequence analysis to predict physico-chemical properties of product, gene knockout or comparative metabolic profiling, and heterologous expression of cryptic gene cluster (Bentley 1999; Challis 2008; Davati and Habibi Najafi 2013). Web-based platforms like antiSMASH 2.0 (Blin et al. 2013), ClustScan (Starcevic et al. 2008), and CLUSEAN (Weber et al. 2009) have also been developed to automate the identification and characterization of bioactive secondary metabolites.

15.4.2.3 Metabolic Engineering

Metabolic engineering is the approach to modify the existing metabolic pathway or combining the pathways or enzymes from different host to single microbe with an objective of improved production of target compound or to produce new compounds in host cells from simple, inexpensive starting material (Keasling 2010). The important design parameters in production of secondary metabolite are yield and productivity. Thus, in optimizing the production of microbial metabolite, the primary aim is to enhance the metabolic flux towards the compound of interest and to minimize the flux towards the by-products. Increasing the flux towards the product increases both the overall productivity and yield (Nielsen 1998). Metabolic engineering has

been successfully applied for the efficient production of amino acids like L-threonine and L-valine, antimalarial drugs like artemisinin, anticancer drugs like taxol, antibiotics like β-lactams and cephalosporins, and benzylisoquinoline alkaloids (Davati and Habibi Najafi 2013; Minami et al. 2008).

15.4.3 Biosynthesis of Secondary Metabolites and Its Regulation

The secondary metabolite production is not only strain dependent but it is also influenced by diverse regulatory conditions like growth stage, optimum supply of nutrients, and the regulatory effects imparted by them (Liu et al. 2013). The production of particular secondary metabolite initiates due to the recognition of specific signal, transduction of this signal to generate the required regulators followed by regulator-mediated activation of biosynthetic gene cluster to produce the secondary metabolite, and then transport of the produced metabolite (Chang and Stewart 1998). The physiological regulation for production of secondary metabolites usually differs with the kind of microbe and metabolic pathway involved. It has been shown that when antibiotic-producing strain like streptomyces are cultivated under conditions that leads to nutritional stress, the stationary growth phase conforms to the onset of biosynthesis of secondary metabolite (Bibb 2005). Nutrients in culture media have been reported to be exerting their regulatory effects by activating or repressing the transcription factors and regulatory proteins.

Fine-tuning of optimal concentration of carbon source in medium is an important parameter to balance the qualitative production of the secondary metabolite and growth of the microbe. Presence of glucose as a carbon source usually improves the growth of the host but could interfere with the production of varied secondary metabolites like cephalosporin, alkaloids, and actinomycin. However, in some cases glucose acts as a good substrate for growth and differentiation as well as for the secondary metabolite production like aflatoxin (Luchese and Harrigan 1993). Glucose in high concentration of 100 g/L maximizes the production of the anticapsin by *Streptomyces griseoplanus.* The type of nitrogen sources employed in the medium affects the secondary metabolic pathways differently. Ammonium ions cause inhibition of novobiocin, cephamycin, and rifamycin production (Aharonowitz 1980). The biosynthesis of gibberellins by the fungus *Gibberella fujikuroi* was shown to be suppressed by the presence of ammonium ions and glucose as well (Brückner 1992). L-amino acids were found to positively influence the production of actinomycin D by *Streptomyces parvulus* (Bennett et al. 1977). The type of L-amino acids added to synthetic media strongly influenced the production of mycotoxins like emodin, catenarin, and islandicin by isolates of *Pyrenophora tritici-repentis* from wheat (Bouras et al. 2016). The concentration of inorganic phosphate that favours growth of the microbes generally exerts negative control on synthesis of secondary metabolites. However, in some cases high phosphate concentration is well tolerated for production of secondary metabolite as reported in the case of avermectin biosynthesis by *Streptomyces avermitilis* (Čurdová et al. 1989).

Secondary metabolite production also requires trace elements like manganese, iron, and zinc, although their required optimal concentration may vary depending on the metabolite to be produced.

15.4.4 Applications of Secondary Metabolites

Secondary metabolites are valuable compounds with a wide range of applications (Williams et al. 1989). The microbial secondary metabolites are now progressively used as drugs for the treatment of various diseases in place of synthetic drugs. They are widely used as uterocontractants, anti-inflammatory agent, anticancer drug, cholesterol-lowering agent, hypotensive agent, immunosuppressant, antibacterial/antifungal agent, and antiparasitic agent (Gonzalez et al. 2003). They are also being used for non-medical applications like weed management and plant growth regulation (Cutler 1995; Sadia et al. 2015).

Secondary metabolites in addition to their known activities have also shown alternative activities, and thus they have been unexpectedly used as possible solution to other diseases for which the effective treatment is not available. β-Lactams are known for their antibiotic action, and their derivatives have also displayed antitumour prodrug activity (Xing et al. 2008). Prodigines, pigmented antibiotics, display antifungal, antiprotozoal, antimalarial, anticancer, and immunosuppressive activities in addition to their antibiotic activity (Williamson et al. 2006). Squalestatin, a fungal metabolite known for lowering the cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase enzyme of cholesterol biosynthesis pathway, has been identified as a potential drug against prion disease (Bate et al. 2004). Thus, exploring the new functions of existing secondary metabolites along with speeding up the process of identification of the novel secondary metabolites can allow the better targeting of the diseases for which currently no effective solutions are present (Vaishnav and Demain 2011).

15.5   Valuable Chemicals

Numerous chemicals are used in everyday life to serve various purposes such that they act as drugs for treating diseases, as fertilizers, as disinfectants, as industrial solvents, as pest control agents, and as health or hygiene products. These chemicals are produced using defined chemical synthesis reactions where simple chemicals are reacted to generate target products. The chemicals can be categorized into bulk chemicals, fine chemicals, and speciality chemicals. Bulk or commodity chemicals are produced on large scales and used as intermediates for production of other chemicals. Fine chemicals are produced as pure chemical substance in small quantities unlike bulk chemicals and are often used for production of speciality chemicals such as agrochemicals and pharmaceuticals.

15.5.1 Microbial Platform for Production of Bio-based Chemicals

The need of improved biotechnological processes for production of target chemicals is increasing with each passing year owing to the limited fossil resources and serious climate changes (Wu et al. 2018). The popularity of microbial systems as a tool for biological synthesis of chemicals is gaining momentum as they can produce a variety of complex molecules, and they require relatively less energy resources as compared to chemical synthetic techniques, thus making it a feasible option to produce fine chemicals. Many fine chemicals have been found to be ideal for microbial biosynthesis as they are intermediates or products of the natural metabolic pathways of various microbes. Industries that benefit from microbial biosynthesis include food, agriculture, chemical, pharmaceutical, and cosmetics (Gurung et al. 2013). The natural biosynthetic pathway in a microbial cell can also be modified by combining various approaches to produce target chemicals.

1. Enzymatic synthesis of fine chemicals where enzymes with or without coenzymes convert the substrate to the chemical of interest. The genes responsible for expressing the enzymes capable of catalysing the bio-based reaction are identified and isolated. The computational tools are used to mine genome and transcriptome data to identify novel biosynthetic pathways and enzymes (Lautru et al. 2005; Zhao et al. 2013). The identified enzymatic synthesis system is introduced into microbial cell to create a microbial cell factory.
2. Metabolic engineering is used to increase yield as well as productivity by redesigning the existing biosynthetic pathway to optimize the production of target compound. Various tools used in designing metabolic pathways are biochemical network integrated computational explorer (BNICE), RetroPath, GEM-Path, OptStrain, and DESHARKY (Chae et al. 2017). Flux balance analysis is a method that indicates how gene deletion and expression can be manipulated to distribute carbon towards chemicals of interest without blocking or reducing cell proliferation. This is a standard method to optimize metabolic pathways (Orth et al. 2010).
3. Genetic manipulation according to the redesigned pathway map obtained by computer simulation can be performed to give a relatively efficient recombinant strain of the selective microorganism. This involves heterologous expression, overexpression, downregulation, deletion, or mutation of the gene of interest.

Despite of various advantages offered by microbial systems for bio-based production of chemicals and other valuable materials, their potential could not be fully exploited as new alternative energy sources are coming to existence. Moreover, higher production cost of bio-products, lower yields, relatively decreased efficiency of bioprocesses as compared to chemical processes, and longer production periods due to slow microbial growth are other factors hindering the development of bio-based products at commercial scale (Chen 2012).

15.5.2 Bio-manufacturing of Bulk and Speciality Chemicals

The production of bulk chemicals is primarily driven by petrochemical feedstocks. However, as demand for bio-based chemicals is increasing, the chemical processes are being replaced with microbial catalysts and improved fermentation methods. Thus, the possibilities to utilize renewable resources for sustainable production of commodity chemicals are rapidly progressing in the current scenario (Hermann et al. 2007). Bio-based production of several commodity chemicals including alcohols, organic acids, amino acids, aromatic amines, diols, polyhydroxyalkanoates, and polysaccharides through fermentation has been successfully reported (Table 15.3). In parallel to fermentation approaches, system metabolic engineering has also been successfully used in production of commodity chemicals like amino acids (Ma et al. 2017). Such engineering strategies have been applied mainly in *Corynebacterium glutamicum* and *Escherichia coli* for amino acid production. Dedicated attempts are being made by researchers worldwide to construct novel pathways in microbes for bio-manufacturing of target bulk chemicals (Shin et al. 2013) (Table 15.5).

Like bulk chemicals, the fine chemicals were also conventionally produced by energy-intensive multistep chemical processes that resulted in high levels of wastes and by-products. However, the efforts are being made to exploit biological routes for chemical production on par with chemical synthetic techniques. The fine chemicals are synthesized by microbes, either as products of their natural metabolic pathways or by genetically engineering their metabolic pathways to produce the desired product (Hara et al. 2014). A range of speciality chemicals like isoprenoids, flavonoids, alkaloids, aromatic compounds, polyphenols, peptides, drugs, organic acids, and oligosaccharides has been reported to be produced by microbes using synthetic biology principles. The production strategy of few chemicals in microbial systems has been summarized.

15.5.2.1 Artemisinin

The antimalarial drug artemisinin is a sesquiterpene lactone with an endoperoxide bridge. It is naturally produced by *Artemisia annua* (sweet wormwood) (Liu et al. 2006; Rathod et al. 1997). However, the methods for extraction of artemisinin were not economical and resulted in insufficient production levels. This led to development of recombinant strains as microbial factories to produce artemisinic acid, which is a precursor of artemisinin. This precursor was then converted to artemisinin by following synthetic organic chemistry steps (Paddon and Keasling 2014). In one of the studies, *E. coli* strain was engineered to synthesize the precursor amorphadiene by introduction of heterologous, high-flux isoprenoid pathway from *S. cerevisiae* to *E. coli* (Martin et al. 2003). The pathway genes were coexpressed with a codon modified amorphadiene synthase (Martin et al. 2003) resulting in a recombinant strain that could produce amorphadiene up to 24 mg/L. In a follow-up study, a higher yield of amorphadiene was achieved by utilizing a two-phase partitioning bioreactor (TPPB) strategy that resulted in efficient separation of amorphadiene from the fermentation broth (Newman et al. 2006). Much later, production of

Table 15.5 Representative examples showing bio-production of commodity chemicals by fermentation methods

Commodity chemical	Involved microbe	Applications	References
Ethanol	*Saccharomyces cerevisiae* *Zymomonas mobilis* Ethanologenic *Escherichia coli* strains	Industrial chemical, motor fuel, gasoline additive, solvent, preparation of tonics and cough syrups, beverages	Stewart et al. (1983) and Dien et al. (2000)
Lactic acid	Lactic acid bacteria like *Lactobacillus fermentum, Lactobacillus bulgaricus*	Pharmaceutical, food, and cosmetic industry	Wee et al. (2006)
Polyhydroxyalkanoates	*Ralstonia eutropha, Alcaligenes latus, Chromobacterium violaceum*	Substitute of synthetic plastic, biomaterial for tissue engineering	Singh Saharan et al. (2014)
1,3 propanediol	*Clostridium pasteurianum, Klebsiella pneumoniae, Citrobacter freundii*	Production of polyesters, polyurethanes, polyethers	Biebl et al. (1999)
Butanol	*Clostridium acetobutylicum*	Fuel, paint thinner, solvent	Abou-Zeid et al. (1978)
Citric acid	*Aspergillus niger, Bacillus licheniformis, Saccharomycopsis lipolytica*	Food and pharmaceutical industry	Vandenberghe et al. (1999)
L-lysine	*Brevibacteria, Corynebacterium glutamicum*	Animal feed supplement, medicament, chemical agent, food industry	Hodgson (1994)
Acetic acid	Acetic acid bacteria like *Acetobacter aceti*	Food and beverage industry, production of industrial chemicals	Raspor and Goranovič (2008)
L-glutamic acid	*Corynebacterium glutamicum*	Food additive, flavour enhancer, therapeutic agent, infusion compound	Nakamura et al. (2007)
Acrylic acid	*Rhodococcus rhodochrous*	Chemical industry, manufacture of plastics, coatings, polymers	Nagasawa et al. (1990)
Glycolic acid	*Candida tropicalis, Gluconobacter, Pichia naganishii*	Leather industry, texture washing, cosmetics	Kataoka et al. (2001)
Succinic acid	*Basfia succiniciproducens, Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens*	Food, surfactants, pharmaceutical products, detergents, plastics, precursor molecule	Tan et al. (2014) and Zheng et al. (2009)

artemisinic acid at gram scale (25 g/L) was achieved by optimizing the expression of CYP71AV1:CPR1 along with co-expression of cytochrome b5 and two dehydrogenases (Paddon et al. 2013).

15.5.2.2   γ-Aminobutyric Acid (GABA)

GABA, a non-protein amino acid, is synthesized by microbes, plants, and animals. It acts as an inhibitory neurotransmitter in the central nervous system of mammals and as a stimulant for immune cells (Dhakal et al. 2012). In microbes, it is involved in spore germination in the case of *B. megaterium* and *N. crassa* (Foerster and Foerster 1973; Schmit et al. 1975), while it provides resistance to acidic pH in *L. lactis, E. coli*, *and other microbes* (Castanie-Cornet et al. 1999; Sanders et al. 1998). It has a wide application in food, cosmetic, and pharmaceutical industry. The biosynthetic route of GABA involves a single-step reaction involving decarboxylation of glutamate to GABA, catalysed by glutamate decarboxylase (GAD) (Ueno 2000). The main GABA-producing microbes are lactic acid bacteria (LAB) (Dhakal et al. 2012). *Corynebacterium glutamicum* expressing Escherichia coli glutamate decarboxylase (GAD) has been engineered for production of GABA, and in order to further enhance its production, protein kinase G has been disrupted resulting in increased intracellular concentration of glutamate precursor and eventually improved yield of GABA (Okai et al. 2014).

15.5.2.3   Resveratrol

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a plant-derived polyphenol that is present in red wine. It is used as an antioxidant, in cosmetic and food industry and as therapeutic agent due to its anticarcinogenic, anti-inflammatory, anti-diabetic, and anti-ageing properties (Beekwilder et al. 2006; Mei et al. 2015). As such, production of polyphenols in microbes is a challenging task due to antibacterial and antifungal activity of these compounds (Daglia 2012). However, still metabolic engineering principles have been utilized to produce such compounds via microbial systems. Engineered *E. coli* and *S. cerevisiae* strains expressing 4-coumarate:coenzyme A ligase from tobacco and stilbene synthase from grapes has been developed to achieve resveratrol accumulations in the culture medium by supplying *p*-coumaric acid as a precursor molecule. These engineered strains showed relatively low production titres (Beekwilder et al. 2006). Another research group investigated various constructs for resveratrol synthesis, different *E. coli* strains, promoters and gene expression combinations, sequence, and structure analysis to achieve high titres (g/l) of resveratrol from biotransformation of *p*-coumaric acid (Lim et al. 2011).

15.5.2.4   Cinnamic Acid

Cinnamic acid is a phenylpropanoid acid, which is used as a cinnamon flavouring agent, in high performance thermoplastics, as precursor for chemical compounds, and as nutraceutical and pharmaceutical products (Vargas-Tah and Gosset 2015). It can be obtained by either chemical synthesis or by extraction from source plant. It can also be produced by engineered microbes like *Escherichia coli*, *Streptomyces lividans, Saccharomyces cerevisiae*, and *Pseudomonas putida.* Genes encoding

phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) have been expressed in *E. coli* and *S. cerevisiae* to allow the conversion of L-phenylalanine and L-tyrosine to cinnamic acid and *p*-hydroxycinnamic acids (*p*-coumaric acid) (Vannelli et al. 2007). *Pseudomonas putida* S12 strain was engineered for conversion of p-hydroxycinnamic acid from glucose (Nijkamp et al. 2007). The heterologous expression of PAL encoding gene from *Streptomyces maritimus* in *Streptomyces lividans* resulted in production of cinnamic acid from glucose with maximum titre of 450 mg/L (Noda et al. 2011).

15.6   Conclusion

Microbes play a significant role in maintaining the ecological sustainability. They synthesize a wide range of products like antibiotics, toxins, antimicrobial peptides or proteins, and enzymes that help them to thrive in the varied environmental conditions and provide them an ability to compete with other species in their ecological niche. These products are valuable due to their application in industrial bioprocesses, as they are used in nutraceuticals, in agriculture for production of drugs or vaccine, and for generation of clean fuel and bioremediation. For instance, enzymes derived from microbial source have potential applicability in different fields as they are used in pharmaceutical industry, in processing of food products, as therapeutic agents, and in production of biofuels and bioplastics. In order to further enhance their usefulness, protein engineering methods are being employed to generate custom-made biocatalysts for the desired processes.

Microbial surface proteins with antigenic properties represent major target for generation of vaccines, and various strategies have been devised to utilize microbial systems for production of recombinant vaccines to decrease the production costs. Antimicrobial peptides derived from microbes are another group of interesting biomolecules with therapeutic applications owing to their utility as alternative to antibiotics. Similarly, microbial toxins produced by bacteria or fungi are utilized in cosmetic industry, as therapeutic agent, and for drug delivery. Microbial proteins or whole microbial cells are used as food source and feed supplement. A variety of bioactive secondary metabolites have been derived from microbes. The discovery of new bioactive compounds has been achieved by advent of modern techniques like genome sequencing, metabolic engineering, proteomics, and advance computational tools. Metabolic engineering and synthetic biology principles have been successfully employed to develop the engineered microbial strains as cell factories for heterologous expression of recombinant proteins and bio-based production of bulk and speciality chemicals. The natural biosynthetic pathways can be either fine-tuned or novel pathways can be assembled in host microbe to optimize the production of the target compounds. In conclusion, microbes share a major role in bio-production of valuable chemicals, toxins, metabolites, proteins, and peptides with broad scope of applications.

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