Fungal Enzymes: Sources and Biotechnological Applications

21

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Abstract

Fungi, being obligate heterotrophs, are natural decomposers and elaborate a number of enzymes. Currently, more than half of the industrial enzymes are of fungal origin and are being used successfully in diverse industrial processes and products. Some of the well-known areas are pulp and paper, textiles, detergents, food, feeds, nutraceuticals, and therapeutics. Production of industrial enzymes utilizes different fungal genera, *Aspergillus* being the most exploited one. Apart from protease, phytase, L-asparaginase, and few others, most commercial fungal enzymes are glycosyl hydrolases (cellulases, xylanase, mannanase, amylase, pectinase, β-fructofuranosidase, and others).

Cellulase and amylase (including glucoamylase) from *Trichoderma* sp. and *Aspergillus* spp., respectively, are exploited for bio-ethanol, textiles, and detergent industries. Fungal proteases, including keratinases, find application in detergent, food, leather, pharmaceutical, and waste management sectors. The role of fungal acidic pectinases in bringing down the cloudiness and bitterness of fruit juices is well recognized, while fungal phytases are being explored in enriching the nutritive value of poultry diets. L-Asparaginases sourced from molds are being examined for cancer therapy and mitigation of acrylamide formation in food. With the advent of biotechnological interventions, heterologous overexpression in suitable hosts, immobilization on novel matrices, and tailoring of fungal enzymes are being pursued. In this chapter, some of the important fungal enzymes are explored from recent perspective of their biotechnological applications.

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21.1 Introduction

Although the term enzyme (Greek "en" meaning "in," and "zyme" meaning "east" or "leaven") derives its origin from the yeast-mediated fermentation of sugary syrups, the first reference to the successful application of fungal enzymes dates back to 1894 on account of a patent on Taka-diastase, α-amylase from *Aspergillus oryzae* cultivated on rice by Jokichi Takamine. Fungi are natural decomposers and therefore are bestowed with a number of enzymes required for bioconversion of a variety of complex substrates (Berbee et al. 2017). Owing to ease of culturing, amenability to genetic manipulation and amazing enzymatic spectra, fungi predominate the scenario of microbial enzyme producers. Moreover, fungal cultivation in a variety of traditional preparations (brewing, baking) dates back to time immemorial, thus providing a firm and safe background for their modern-day exploitation. The advent of industrial enzymes geared up with a better understanding of their nature and function. Among the six classes of enzymes, hydrolases belonging to Class 3 make most of the industrial enzymes with predominating alkaline protease and glycoside hydrolases (Murphy et al. 2011).

Fungi are the natural factories that produce versatile enzymes which are proficient catalysts for various chemical reactions. Enzymes offer a number of favorable and economic factors over chemical catalysts. As a conventional estimate, more than half of the enzymes known are of fungal origin. Advent in fungal genomics is unraveling more number of enzymes that may play important role (Peciulyte et al. 2017). Many of these have been screened for their ability to produce industrially sound products. Fungi have been important in both ancient and modern biotechnological processes. Processes and products that make use of fungi include production of antibiotics, enzymes, organic acids, baking, brewing, alcohols, and numerous pharmaceuticals. The industrial production of numerous enzymes utilizes different fungal species. The use of fungal cells for most of the industrial enzyme production is based on their characteristics such as pH tolerance, thermostability, high yield, low operational cost, easy and cheap downstream processing etc.

Fungi being obligate heterotrophs secrete a battery of extracellular enzymes to hydrolyze complex polymeric substrates around (Kües 2015). Many a times these enzymes are robust enough to survive harsh conditions including low water activity level and high temperature. Solid state fermentation of complex substrates, particularly agro-industrial wastes such as sugarcane bagasse, palm kernel cake, copra meal, wheat bran, rice hulls, orange peel etc., naturally suits them as molds thrive well in xerophilic conditions (Hölker et al. 2004; Diaz et al. 2016).

Over the past few decades, the worldwide market of enzyme has rapidly grown. It was valued at $7.082 billion in 2017 and is projected to reach $10.519 billion in 2024, amounting to a compound annual growth rate of 5.7% from 2018 to 2024. As

per the Global Enzymes Market Report 2018, protease segment alone made one-fourth share of the global enzymes market in 2017, and it projects that lyase segment will grow at the fastest rate in the coming years (https://www.businesswire.com/news/home/20180628006408/en/Global-Enzymes-Market-report-2018). Surge in the demand of first- and second-generation biofuels has increased the demand of amylolytic and cellulolytic enzymes.

As per the Association of Manufacturers and Formulators of Enzyme Products (AMFEP 2009), out of about 260 commercial enzymes, 60% are sourced from about 25 fungal genera. The most dominating among microorganisms is the versatile genus *Aspergillus,* accounting for about 25% of total industrial enzymes. Enzymes sourced from *Trichoderma*, *Penicillium*, *Rhizopus,* and *Humicola* add up to another 20% of the industrial enzymes.

The industrial enzyme market is dominated by Class 3 (hydrolases), making 85% of the total, followed by Class 2 oxidoreductases (8%), Class 4 lyases (4%), Class 2 transferases (2%), and Class 1 isomerases (1%). Many enzymes find applications in more than one industry, especially hydrolases like cellulases, amylases, and proteases. Often fungi are known to produce a spectrum of enzymes desirable for efficient depolymerization of complex substrates like lignocelluloses. Some of the prominent industrial enzymes in the light of recent developments are discussed in the present chapter (Fig. 21.1).

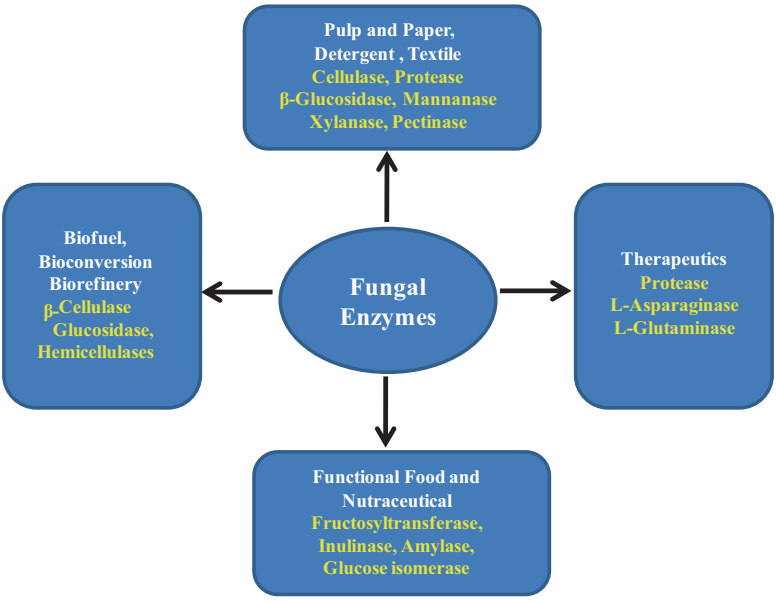

Fig. 21.1 Application of fungal enzymes

21.2 Cellulases

Cellulose is the most abundant renewable carbohydrate on the earth and the major constituent of plant cell wall. Cellulose is naturally embedded with lignin-hemicellulose matrix within plant cell wall. It is a homopolymer composed of glucose units linked by β-1,4-glycosidic bonds. Hydrogen bonding between individual cellulose fibrils gives rise to compact crystalline structure which is difficult to digest by a single hydrolase (Payne et al. 2015; Ghosh et al. 2019a).

Cellulases represent a complex group of synergistically acting enzymes. They principally contain endo-1,4-glucanase (EC 3.2.1.4) which cleave randomly at internal amorphous cellulose sites causing rapid reduction in the cellulose while liberating cello-oligomers in the process, cellobiohydrolases (EC 3.2.1.91) or exo-1,4-glucanases which act progressively on crystalline cellulose and primarily attack the reducing ends of polymer to produce cellobiose, and short-chain oligosaccharides and β-glucosidases (E.C. 3.2.1.21) which hydrolyze cellobiose to glucose monomers (Adlakha et al. 2011; Gastelum-Arellanez et al. 2014; Prajapati et al. 2018).

Biofuel generation from cellulosic biomass utilizes three steps, *viz*., pretreatment, enzymatic saccharification, and ethanolic fermentation. After pretreatment, generation of monosugars is catalyzed by cellulase, hemicellulases, and glucosidase. Alcoholic fermentation of the released sugars for bio-ethanol production is carried out by widely used yeasts like *Saccharomyces cerevisiae* (Huang et al. 2018).

Based on higher yields, the most prominent fungal cellulase producers belong to *Trichoderma* spp. and their cellulolytic enzymes are applied in food, feed, biofuel and biorefinery, and textile industry. A number of commercial cellulases sourced from different molds suiting to different applications are available (Table 21.1). *T. reesei* research has since pioneered the concept of enzymatic saccharification of cellulose by a synergistic amalgamation of different cellulase activities and laid the foundation for our recent understanding of the enzyme regulation. Cellobiohydrolase CBH1 (*cel7a*) was the first eukaryotic cellulase to be cloned and the first cellulase resolved structurally (Shoemaker et al. 1983; Divne et al. 1994).

An important step toward applying *T. reesei* cellulases industrially, was the development of strain mutagenesis and screening procedures in the 1970s. While the standard for cellulase production in industry was proclaimed to be higher than 100 g/L, strain RUT-C30 still is the prototype cellulase hyperproducer available with concentration of extracellular protein reaching 30 g/L (Bischof et al. 2016).

Over the past decades, the genome of *T. reesei* has been explored to help achieve overexpression and hyperproduction of cellulase heterologously. Better understanding of genomics and transcriptomics has helped in identifying constitutive and tunable promoters in the strain. All these lead to developing novel synthetic expression systems. Understanding of the gene expression mechanism and control will help in understanding gene function and enhance yields for biotechnological purposes (Fitz et al. 2018). *Trichoderma reesei* has been established

Table 21.1 Commercial cellulases and their fungal sources

Trade name	Manufacturer	Source	Composition	Application
Novozyme 188	Novozymes, Denmark	*A. niger*	Cellobiase	Biomass depolymerization
Multifect CL	Genencor, USA	*T. reesei*	Cellobiase	Bioblasting in textile mills
Biocellulase A	Quest Intl., USA	*A. niger*	Cellulase and xylanase	Improves the nutritional value substantially
GC 880	Genencor	*T. reesei* *T. longibrachiatum*	β-Glucanase and xylanase complex	Biomass hydrolysis
Accellerase® 1500	Genencor	*T. reesei*	Exo- and endoglucanases, hemicellulase, and β-glucosidase	Hydrolyzes lignocellulosic biomass into fermentable monosaccharides
Bio-feed beta L	Novozymes	*T. longibrachiatum* *T. reesei*	β-Glucanase and xylanase complex	Beta-Glucanase hydrolyzes the barley beta-glucans under formation of mono- or oligomers
Celluclast 1.5 LFG®	Novozymes	*T. reesei*	Cellobiase	To degrade oligosaccharides into glucose in order to investigate the biodegradability of bioabsorbable bacterial cellulose (BBC)
Rovabio	Excel Adisseo, France	*P. funiculosum*	Feedase	Improves the digestibility of feedstuffs from vegetal origin for animals, poultry, and swine; also contains arabinanase activities allowing to break down arabinose links
Cellic CTec2	Novozymes	*T. reesei*	Cellulase	Degrades cellulose to fermentable sugars

as a model organism for cellulase development and regulation machinery. In this context, many researchers have recently started working on the role of mitogen-activated protein kinases (MAPKs) in cellulase formation (Wang et al. 2017; de Paula et al. 2018). MAPKs, extremely conserved family of serine/threonine protein kinases, regulate diversity of essential cellular processes that help fungus differentiate in carbon sources, stress response, transport, proliferation, etc. (de Paula et al. 2018). The integration of light and nutrient signals has been used for strain improvement and adaptation of enzyme production in *T. reesei* (Schmoll 2018). Transcriptome

and secretome analysis for cellulases is being explored in fungi such as *Aspergillus fumigatus* and *Aspergillus tamarii* grown on sugarcane bagasse (de Gouvêa et al. 2018; Midorikawa et al. 2018).

Heterologous expression of cellulases can also be triggered in rich growth media by utilization of inducible or auto-inducible promoters. Upward of 20 g and reportedly up to 100 g of crude cellulases per liter are reachable with engineered *Trichoderma reesei* strains (Cherry and Fidantsef 2003). Furthermore, other fungi, such as *Penicillium, Acremonium,* and *Chrysosporium,* are viewed as probable and promising alternatives to *Trichoderma* (Gusakov et al. 2005). For the alteration of biomass to biofuels on an industrial scale, several hurdles need to be overcome. For example, continued high production costs of cellulases, which comprise up to 20% of the total ethanol production costs as evaluated by the US National Renewable Energy Laboratory (NREL), reduced production efficiency on a commercial level. In addition, to achieve efficient biomass conversion, concerted action of a set of enzymes is required as per the composition of particular substrate. The use of traditional fungal host organisms for cellulose degradation is constrained by the need for special culturing and induction conditions. To triumph over these limitations, researchers are not only working on increasing the expression level of fungal cellulases to lower the fabrication costs but also on the optimization of recombinant expression systems in plants or microorganisms (Lambertz et al. 2014).

β-Glucosidases carry out hydrolysis of β-1,4-glycosidic bonds in aryl- and alkyl β-D-glucosides through non-reducing terminal and act in combination with endoglucanase for complete hydrolysis of cellulose to glucose (Maitan-Alfenas et al. 2015). The commercial β-glucosidase (Novozyme 188) is obtained mainly from *Aspergillus niger*, and other filamentous fungi such as *Penicillium decumbens* (Chen et al. 2010), *Phanerochaete chrysosporium* (Tsukada et al. 2006), *Paecilomyces thermophila* (Yang et al. 2009), *Aspergillus unguis* (Rajasree et al. 2013), and *Penicillium verruculosum* (Korotkova et al. 2009) are also reported to be potent β-glucosidase producers. Some of the fungal β-glucosidase producers are listed in Table 21.2. The hydrolysis carried out by glucosidase is a two-step process. The first step is nucleophilic addition reaction which results in an α-glycosyl enzyme intermediate which ultimately hydrolyzed to β-glucose in the presence of H_2O. Sawant et al. (2016) studied the two-way dynamics with the release of glucose from cellobiose and

Table 21.2 Some fungal sources of β-glucosidases

Source	References
Myceliophthora thermophila	Bonfa et al. (2018)
Clavispora sp.	Wang et al. (2016a)
Aspergillus unguis	Rajasree et al. (2013)
Penicillium piceum	Gao et al. (2013)
Trichoderma reesei	Nakazawa et al. (2011)
Neosartorya fischeri	Kalyani et al. (2011)
Humicola insolens	Souza et al. (2010)
Paecilomyces thermophila	Yang et al. (2009)
Daldinia eschscholzii	Karnchanatat et al. (2007)
Thermomyces lanuginosus	Lin et al. (1999)

cello-oligosaccharides by β-glucosidase. Soluble cellodextrin hydrolyzing β-glucosidase also helps to avoid cellulase inhibition by cellobiose (Karnaouri et al. 2013). Recently, two isoforms of β-glucosidase (50 and 200 kDa) were obtained when thermophilic *Myceliophthora thermophila* M.7.7 was grown on a mixture of sugarcane bagasse and wheat bran (1:1). The lower molecular weight β-glucosidase showed thermostability at higher temperature (60 °C) with half-life of 855.6 min (Bonfa et al. 2018).

21.3 Hemicellulases

Hemicelluloses, comprising a significant part of plant biomass, are a diverse group of structural polysaccharides. Xylan, mannan, arabinan, and other hemicelluloses make up to 30% of the lignocelluloses. Hemicelluloses have dissimilar compositions (heteropolymeric) as they contain both hexose and pentose sugars (Sjostrom 1993; Chaikumpollert et al. 2004; Bajpai 2014). Their enzymatic hydrolysis using fungal hemicellulases has found a number of applications, *viz*., biobleaching; waste paper deinking; fruit juice maceration; upgradation of feed, fodder, and fibers; and saccharification of biomass. Hemicellulases include backbone hydrolyzing enzymes xylanase, mannanase, and arabinase and accessory enzymes α-glucuronidase, α-arabinofuranosidase, β-mannosidase, acetyl xylan esterase, and feruloyl xylan esterase (Saha 2003; Juturu and Wu 2012; Obeng et al. 2017).

Xylanases are hemicellulases which act upon β-1, 4-xylosidic bonds in xylan, a polymer of xylose, and include endo-1, 4-β-xylanase, and β-xylosidase (Walia et al. 2017). Complete hydrolysis of xylan requires combined action of endo-1, 4-β-xylanase, β-1,4-D-xylan-xylanohydrolase, β-xylosidase, and some accessory enzymes (Kango et al. 2003). Many microbes such as bacteria, yeast, fungi, and actinobacteria are known for their xylanase production; however, filamentous fungi are the most proficient and most explored xylanase producers among these (Kumar et al. 2018). *Thermomyces, Trichoderma,* and *Aspergillus* are the most exploited genera for xylanase production (Kango and Jain 2005). *Thermomyces lanuginosus* (previously known as *Humicola lanuginosa*) has gained considerate interest due to its ability to produce high titers of thermostable endoxylanase (Mchunu et al. 2013). Apart from being used in conjunction with cellulases for biofuel production, xylanases have numerous applications in various industries such as food and animal feed, paper and pulp processing, textiles, etc. (Cesar and Mrša 1996; Kang et al. 2004; Kango et al. 2017). Enzymatic hydrolysis of xylan and mannan is much relevant to biobleaching and efficient saccharification of lignocellulosic biomass (Viikari et al. 1994, Maijala et al. 2012). Cellulase-free xylanases are desirable for biobleaching where they replace chlorine-based bleaching agents, and thus, release of toxic organo-chloro compounds is avoided.

Commercial production of xylanases at industrial level is being done in several countries (Table 21.3). The main microorganisms used to obtain these enzymes are *Aspergillus niger*, *Trichoderma* sp. and *Humicola* (Bajpai 2014). The desirability of cellulase-free xylanase for biobleaching is to ascertain the selective removal of

Table 21.3 Some commercial hemicellulases and their manufacturers

Trade name	Company
Commercial xylanases	
Biobrite 100 series	Iogen, Canada
Sumizyme X	Shin Nihon, Japan
Ecopulp, Econase	AB Enzymes, Germany
Multifect XL Optimase CX 72 L	Genencor, USA
FibreZyme PBL 100, FibreZyme LBL	Dyadic International, USA
SEBrite BL 1	Advanced Enzyme, India
Bleachzyme P	Aumgene Biosciences, India
Ecozyme	Thomas Swan Co., UK
Sternzym HC 46	SternEnzym, Germany
Pulpzyme HC, NS 51024, NS 51025	Novozymes, Denmark
Commercial mannanases	
Gamanase	Novo Nordisk, Denmark
Mannaway	Novo Nordisk, Denmark
Hemicell	Elanco Animal Health, USA
Purabrite	Genencor, USA
CTCzyme	CTC Bio Inc., South Korea
DigeGrain	Advanced Enzyme, India
Mannazyme XP	Aumgene Biosciences, India

hemicellulose fraction from the pulp (Archana and Satyanarayana 2003). Fungi produce xylanase extracellularly into the medium and their titers are much higher than yeasts and bacteria (Polizeli et al. 2005).

Mannans, chiefly composed of mannose, are plant polysaccharides commonly known as gums and occur in a variety of forms. These being heteropolymeric require a number of enzymes for complete degradation (Suryawanshi et al. 2019). β-Mannanase (EC 3.2.1.78) and β-mannosidase (EC 3.2.1.25) act upon the β-1-4 mannopyranosyl linkages of the mannan backbone, while β-glucosidase (EC 3.2.1.21), α-galactosidase (EC 3.2.1.22) and acetyl esterase (EC 3.1.1.6) cleave the respective moieties from the side chains (Soni and Kango 2013). β-Mannanase, the main enzyme, and other accessory enzymes are synthesized by a variety of microorganisms. Fungal mannanases have been investigated by various workers (Moreira and Filho 2008; van Zyl et al. 2010). Some of the prominent fungal mannanase producers are reported from the genus *Aspergillus* (Soni et al. 2016; Jana et al. 2018) followed by *Penicillium* sp. (Blibech et al. 2011) and *Trichoderma* sp. (Chai et al. 2016). About 50% of commercial mannanase preparations are sourced from genetically engineered microorganisms (Dhawan and Kaur 2007). A 1345 bp gene encoding mannanase (ManN) from *Aspergillus sulphureus* was expressed in *Pichia pastoris* (Chen et al. 2007). Malherbe et al. (2014) have expressed *Aspergillus aculeatus* endo-β-mannanase (Man1) and *Talaromyces emersonii* α-galactosidase (Agal) genes in *S. cerevisiae* Y294. Mannans occurring in animal feeds made from soybean and legumes are anti-nutritive and elicit a Feed-Induced Immune Response (FIIR) in animals (Hsiao et al. 2006; Zhang and Tizard 1996). Commercial mannanase

preparations specifically designed to mitigate the problem of immunogenicity, Hemicell digest the immunogenic mannan in feed and improve the poultry health (Korver 2006).

Rhizomucor miehei mannanase showed classical (β/α) 8-TIM barrel-fold structure which provides high specific activity and hydrolyzing property. Using directed evolution strategies such as error-prone polymerase chain reaction (error-prone PCR), DNA shuffling, site-directed mutagenesis (SDM), and site-saturation mutagenesis (SSM), the catalytic activity of mannanase in acidic and thermophilic conditions was further improved (Li et al. 2017). β-Mannanases have extensive applications in industries such as food and feed processing. For the enhancement in the activity of mannanase, rational design strategy was applied which included N-glycosylation in the loop area intern. Improved thermal stability, pH stability, and protease resistance of the *Armillaria tabescens* β-mannanase were noticed (Hu et al. 2017). Structure of *Rhizopus microsporus* endo-β-mannanase was elucidated and it showed different binding behaviors with different oligosaccharides (You et al. 2018). Recently, fungal β-mannnanases from *Malbranchea cinnamomea*, *Aspergillus oryzae,* and *A. terreus* that generate mannooligosaccharide (MOS) from locust bean gum, guar gum, and konjac gum have been reported (Ahirwar et al. 2016; Li et al. 2017; Jana et al. 2018).

21.4 Amylases

Starch is the most abundant storage polysaccharide on the earth and major component of potato, wheat, corn, and rice. Apart from being a staple food such as bread or rice, it also finds use as a thickener and a gelling agent in food industry. Starch consists of linear insoluble amylose and branched soluble amylopectin. In amylose, glucose is linked by β-1,4-glycosidic bonds in a linear fashion, while in amylopectin some of the chains are linked by α-1,6 linkages giving it a branched structure (Buléon et al. 1998). A number of enzymes are known to act upon starch, among which the α-amylases and glucoamylases are the prominent ones (Parashar and Satyanarayana 2017). Starch being the most common source of energy, amylases occur in a wide array of organisms including bacteria and fungi. As mentioned earlier, α-amylase bears historical relevance from the point of view of industrial application of enzymes. After application of Taka-diastase in 1894 from *A. oryzae* as a digestive enzyme, α-amylase was also used as a textile desizing agent in Japan in 1905. Later in 1959, *Rhizopus* sp. was used for production of glucoamylase. Amylolytic enzymes account for about 30% of total industrial enzymes (Vaidya et al. 2015). Due to enormous advantages of enzymatic processing of starch over chemical hydrolysis**,** amylases have replaced the harsh chemicals in industries**.**

α-Amylases (EC 3.2.1.1) are extracellular endo-acting enzymes that randomly hydrolyze α-1,4-glycosidic bonds in starch and produce maltose and dextrins. β-Amylases (E.C.3.2.1.2) are starch hydrolyzing enzymes that cleave α-1,4 linkages from non-reducing end of starch and cause inversion of maltose to its β-form (Zhang et al. 2017a). However, most industrial applications employ *α*-amylases

for saccharification or liquefaction purposes. Fungal sources of industrial α-amylases are mostly confined to *Aspergillus, Penicillium,* and *Rhizopus* spp. (Li et al. 2011). *Aspergillus* is one of the prominent and notably the most explored genera for α-amylases. *Aspergillus oryzae* (Taka-diastase) and *Aspergillus niger* have been used extensively in starch industry (Kammoun et al. 2008; Porfirif et al. 2016; Avwioroko et al. 2018). Fungal amylases sourced from these two molds are preferred over other sources as they enjoy GRAS (generally regarded as safe) status. These molds are prolific producers of hydrolases and due to secretion of organic acids help avoid contamination. However, being mesophilic, the enzymes are not thermostable, and thus bacterial α-amylases replace them in the very first step of gelatinization (or cooking) at high temperature. Some workers have explored some thermophilic molds including *Thermomyces lanuginosus*, *Humicola griseus, Malbranchea pulchella, Rhizomucor pusillus,* and *R. miehei* for production of extracellular thermostable α-amylases (Arnesen et al. 1998; Jensen et al. 2002; Kumar and Satyanarayana 2003). Recently, Abdulaal (2018) has described occurrence of five α-amylases (A1-A5) from *Trichoderma pseudokoningii* and purified one A4 (Mr 30 kDa) stable at 80 °C.

The amylolytic enzyme to be discovered after α- and β-amylase is another glucose liberating enzyme referred as γ- or glucoamylase (Azzopardi et al. 2016). It is a very important enzyme for successive and complete degradation of starch into glucose. It is an exo-acting enzyme that cleaves α-1,4 linkages from the nonreducing ends, but can also cleave α-1, 6 linkages, thus leading to complete saccharification. Most commercial glucoamylases are sourced from *Aspergillus* or *Rhizopus* spp. (Carrasco et al. 2017). *Thermomucor indicae-seudaticae* produced thermostable glucoamylase optimally at 60 °C and pH 7.0 (Kumar and Satyanarayana 2003; Kumar and Satyanarayana 2007).

To achieve saccharification of starch in a single step, a chimeric biocatalyst (Amy-Glu) was prepared using α-amylase of *Bacillus acidicola* and glucoamylase of *A. niger* linked by a peptide. The chimeric enzyme (145 kDa) was expressed in *E. coli* (Parashar and Satyanaryana 2017). In an effort to co-immobilize alpha- and gluco- amylase, Salgaonkar et al. (2018) have used metal organic framework (MOF) by mixing zinc acetate and 2-methylimidazole with enzyme mixture in one pot. The product showed remarkable thermal stability (temperature ranges of 55–75 °C) and catalytic efficiency (V_{max}).

21.5 Protease

Proteases make a large class of enzymes that are involved in peptide bond (CO-NH) hydrolysis within a protein molecule. A wide variety of proteases are produced by different microbial sources. Generally, bacteria produce alkaline proteases and fungi are known to be good producers of acid proteases. Proteases have been utilized for a number of industrial applications and thus attracted attention of researchers to explore microbial diversity. Although bacterial proteases dominate the commercial scenario, fungal proteases have gained considerable interest due to

their broad pH activity range and stability over diverse industrial conditions (Banerjee and Ray 2017). Proteases are the most important industrial enzymes that make approximately 60% of the total enzyme market (Budak et al. 2014). A number of endo- and exopeptidases belonging to different families are produced by aspergilli in protein-rich medium (Machida et al. 2005). Filamentous fungi produce peptidases with varying specificities which must be taken into account in choosing a peptidase to catalyze the protein hydrolysis for the desired application (Hamin Neto et al. 2017a).

Proteases are subcategorized into two major classes, exo- and endopeptidases, based on their site of action. Exopeptidases (also known as peptidases) are known to cleave off N- or C-terminal amino acid from the peptide chain (Jain et al. 2010). Endopeptidases (also called proteinases) hydrolyze internal peptide bond within the protein molecule. Endo- and exopeptidases are further subdivided into four major groups, *viz*., cysteine, aspartic, serine, and metalloproteases, based on the functional groups present on active site. Most of the metalloproteases act as virulent factors of pathogenic fungi to the plants (Barrett and Rawlings 1991). Aspartic proteases, having aspartic acid residue in their active site, are generally produced by a number of filamentous fungi such as *Aspergillus*, *Rhizopus*, *Mucor,* and *Rhizomucor*. They are industrially important because they are unaffected by serine protease inhibitors, reagents having thiol group, and various chelating agents. Most of the aspartic proteases share similarities with pepsin and rennin and therefore can be used in bakery and animal feed industries (Mandujano-González et al. 2016). Some of the proteases sourced from molds are listed in Table 21.4.

Keratinases (EC 3.4.99.11) are proteases with the unique ability to attack highly cross-linked, recalcitrant structural proteins such as keratin (Pawar et al. 2018). Unlike most proteins which are easily degraded by common proteolytic enzymes like papain, pepsin, or trypsin, feather keratin protein is not degraded by these enzymes. Feather keratin is stabilized by disulfide bonds, hydrogen bonds and hydrophobic interactions (Ghosh et al. 2019b). Although keratinolytic proteases are produced by many microorganisms, keratinophilic fungi deserve special mention for colonizing keratin and production of keratinolytic enzymes (Lange et al. 2016). Feathers are almost pure keratin protein and hence can be used as a cheap alternative for production of protein-rich animal feed. Among various agriculture segments in India, poultry is considered to be one of the fastest growing segments, increasing at a rate of 8–10% per year. Thus, in the approaching years, there will be substantial increase in the generation of poultry waste which, if not handled properly, can lead to environmental pollution and health hazard (Farag and Hassan 2004). Current methods to convert feathers into animal feed include physical and chemical processing requiring significant amounts of energy and chemicals. Further, these processes also cause destruction of certain essential amino acids, reducing nutritional value of the feed. Chemicals used in feather processing are responsible for environmental pollution as the bulk effluents are released into water bodies. In this context, biodegradation of feathers by keratinolytic fungi is seen as a potential eco-friendly alternative to chemical treatment. They have gained importance in various biotechnological and pharmaceutical applications, yet the commercial availability of keratinases is still

Table 21.4 Types of some fungal proteases and their applications

Protease	Trade name/fungus	Application	Source/references
Exopeptidases			
Aminopeptidases (EC 3.4.11)	Flavourzyme	Food industry	Novo Nordisk (Denmark)
	Neutrase	Dairy industry	
	Aspergillus oryzae	Fish industry	
	Corolase	–	Rohm enzyme (Finland)
	Aspergillus sojae		
Carboxypeptidase (EC 3.4.16–3.4.18)	*Aspergillus oryzae*	–	Salamin et al. (2017)
Serine-type protease (EC 3.4.16)	*Neurospora sitophila*	Fibrinolysis and plasminogen activation	Deng et al. (2018)
Metalloprotease (EC 3.4.17)	*Candida kefyr* 41PSB	–	Yavuz et al. (2017)
	Eupenicillium javanicum	–	Hamin Neto et al. (2017b)
Cysteine-type protease (EC 3.4.18)	*Aspergillus nidulans*	–	Futai et al. (2001)
Peptidyl-dipeptidases (EC 3.4.15)	*Aspergillus oryzae* (Flavourzyme)	Food processing	Merz et al. (2015)
Dipeptidases (EC 3.4.13)	*Aspergillus oryzae* (Flavourzyme)	Food processing	Merz et al. (2015)
Endopeptidases			
Serine protease (EC 3.4.21)	*T. harzianum* CECT 2413	Biocontrol	Suárez et al. (2007)
Aspartic protease (EC 3.4.23)	*Piptoporus soloniensis*	Dairy industry	El-Baky et al. (2011)

Table 21.5 Keratinases from some fungal sources

Fungus	Optimum pH	Reference
Chrysosporium articulatum	7.58	Bohacz (2016)
Onygena corvina	8.0	Huang et al. (2015)
Purpureocillium lilacinum	–	Cavello and Cavalitto (2014)
Aspergillus parasiticus	7.0	Anitha and Palanivelu (2013)
Myrothecium verrucaria	8.3	Moreira-Gasparin et al. (2009)
Trichoderma atroviride	8.0–9.0	Cao et al. (2008)
Paecilomyces marquandii	8.0	Gradisar et al. (2005)
Aspergillus oryzae	8.0	Farag and Hassan (2004)
Aspergillus fumigatus	8.0	Noronha et al. (2002)
Doratomyces microsporus	7.5	Gradisar et al. (2000)
Microsporum canis	9.0	Mignon et al. (1998)
Chrysosporium keratinophilum	9.0	Dozie et al. (1994)
Trichophyton schoenleinii	5.5	Qin et al. (1992)

limited (Noronha et al. 2002). Keratinophilic fungi include hyphomycetes and several other taxa (Table 21.5). Hyphomycetes include dermatophytic (e.g. *Microsporum* sp.) and non-dermatophytic (e.g. *Chrysosporium* sp.) keratinophilic molds (Gopinath et al. 2015).

21.6 L-Asparaginase

L-Asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) hydrolyzes L-asparagine (essential amino acid) to aspartic acid and ammonia. Since several types of tumor cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase, thus resulting in starvation and death of leukemic cells. Low levels of the non-essential amino acid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirement for asparagine. This is because normal cells produce enzyme asparagine synthetase, which is able to synthesize asparagine, whereas, in cancer and tumor cells, enzyme is present in low levels. L-Asparaginase enzyme is being used effectively in the treatment of acute lymphoblastic and myelocytic leukemia, Hodgkin's lymphoma, lymphocytic leukemia, and lymphosarcoma treatment (Saxena et al. 2015; Agrawal and Kango 2019).

Commercially available L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* elicits a relatively high rate of immune response including silent hypersensitivity, thrombosis, pancreatitis, and hyperglycemia (Li et al. 2018). Hence, efforts are underway to find newer sources of L-asparaginase, and fungal L-asparaginases may be a promising alternative due to their eukaryotic origin. Vala et al. (2018) have reported a marine-derived *Aspergillus niger* AKV-MKBU L-asparaginase with anticancer properties. *A. terreus* L-asparaginase gene encoding a protein of 376 amino acids (42.0 kDa) was expressed in *E. coli* (Saeed et al. 2018). The gold nano-biocomposite was also prepared by immobilizing fungal L-asparaginase (*Aspergillus terreus* MTCC 1782) onto gold nanoparticles which showed anticancer activity against lung cancer cell line A549 (Baskar et al. 2018). Microbial production of L-asparaginase depends on a variety of environmental factors such as temperature, pH, oxygen availability, nutrient type and availability, etc. Commercial production of enzyme requires complete analysis of various optimum conditions and genetic makeup for highest yield. Native microbial strains produce asparaginase either constitutively or after induction by asparagine. Sarquis et al. (2004) reported L-asparaginase production by filamentous fungal species *Aspergillus tamarii* and *Aspergillus terreus*. Some fungal L-asparaginases are listed in Table 21.6.

Besides being an anticancer agent, L-asparaginase has application in food industry as well. Acrylamide, a potent carcinogen, is formed by Maillard reaction between reducing sugars and asparagine present in starchy foodstuff (Agrawal et al. 2018). In a report, L-asparaginase from *Aspergillus terreus* was used for the pretreatment of banana slices before frying to mitigate acrylamide formation during frying. The soaking and frying conditions were optimized using free and chitosan-immobilized fungal L-asparaginase (Aiswarya and Baskar 2017). L-asparaginases sourced from fungi with GRAS status are more suitable for application in food industry.

Table 21.6 Production of some fungal L-asparaginases

Fungus	Method	Yield	References
Aspergillus niger	SmF, cloned	15.78 U/ml	Vala et al. (2018)
Aspergillus terreus	SmF, cloned	42.46 U/mg	Saeed et al. (2018)
Saccharomyces cerevisiae	SmF, cloned	–	Costa et al. (2016)
Aspergillus terreus	SSF	273 U/gds	Varalakshmi and Raju (2013)
Penicillium digitatum	SmF	363.80 U/ml	Shrivastava et al. (2012)
Aspergillus terreus	SmF	24.10 U/ml	Gurunathan and Sahadevan (2011)
Fusarium equiseti	SSF	8.51 U/ml	Hosamani and Kaliwal (2011)
Emericella nidulans		1.1 U	Jayaramu et al. (2010)
Aspergillus niger	SSF	12.52 U/ml	Mishra (2006)

21.7 Inulinase and Fructosyltransferase (FTase)

Prebiotics have attracted eager interest of people as well as nutraceutical industries to process food due to their high therapeutic and nutritional properties (Rawat et al. 2017; Choukade and Kango 2019). Prebiotics contain short-chain non-digestible carbohydrates (NDC) which selectively nourish healthy gut microbiota and ultimately facilitate better health. Fructooligosacharides consist of 1-kestose (GF2), nystose (GF3), and β-fructofuranosyl nystose (GF4) produced from sucrose upon action of fructosyltransferase (FTase) from plants, bacteria, yeasts, and fungi (Flores-Maltos et al. 2014). FOS, a leading prebiotic, has various health-promoting properties as it is bifidogenic, non-cariogenic, and hypolipidemic and helps in ion absorption through gut. Inulinases hydrolyze plant fructan, inulin into inulooliogsaccharides (endoinulinase) and fructose (exoinulinase) by breaking on glycosidic linkages (Kango 2008; Kango and Jain 2011; Rawat et al. 2016).

Fructosyltransferase (FTase; EC 2.4.1.9) is known to hydrolyze sucrose and transfer fructosyl group to an acceptor molecule to generate fructooligosaccharides (FOS) along with glucose and fructose (Ganaie et al. 2013, 2014). FTase cleaves the β-1,2 linkage of sucrose and transfers fructosyl group to an acceptor molecule leading to the formation of fructooligosaccharides and release of glucose. β-Fructofuranosidase (FFase, EC 3.2.1.26) catalyzes both hydrolytic and transfructosylating reactions; however, the latter is evidenced only with higher sucrose concentrations (Rawat et al. 2015a, b).

Bali et al. (2015) have reviewed microbial production of FOS and mentioned fungi such as *A. niger*, *Aspergillus japonicus*, *A. sydowii*, *A. foetidus*, *A. oryzae*, *Aureobasidium pullulans*, *Penicillium citrinum*, *P. frequentans,* and *Fusarium oxysporum* as the prominent producers. Rawat et al. (2015a, b) have also provided a comparative account of fructosyltransferase, inulinase, and sucrase activities in some aspergilli and penicillia. Jiang et al. (2016) isolated a novel yeast *Aureobasidium* sp. P6 from a mangrove ecosystem and cloned inulinase gene. It produced inulin hydrolyzing enzyme (30.98 ± 0.8 U/ml) that showed transfructosylating activity at 30.0% sucrose concentration and generated fructooligosaccharides (FOS).

Zhang et al. (2017b) have used an industrial strain, *Aspergillus niger* ATCC 20611, to enhance the production of FOS wherein they have used polyethylene glycol (PEG)-mediated protoplast transformation system for strain improvement. The transformed *A. niger* ATCC 20611 displayed a 58% increase in β-fructofuranosidase production (507 U/g), compared to the parental strain *A. niger* ATCC 20611 (320 U/g). Production of an extracellular, thermostable inulinase was carried out by *Aspergillus tubingensis* CR16 using wheat bran and corn steep liquor (CSL) under solid state fermentation (SSF). The fungus produced 1358.6 U/g inulinase after parametric optimization which was fivefolds higher (Trivedi et al. 2012).

Tanriseven and Aslan (2005) have immobilized commercially available *Aspergillus aculeatus* FTase (Pectinex Ultra SP-L) in Eupergit C with 96% efficiency and maintained the recycling up to 20 days effectively to obtain GF4, GF3, GF2, glucose, and fructose. Immobilized enzyme also showed a higher temperature optimum at 65 °C. Heteroexpression of endoinulinase encoding gene from *Aspergillus ficuum* in *E. coli* with high inulooligosaccharide (IOS) yield of 94.41% has been reported by Wang et al. (2016b). Some heterologously expressed FTases and inulinases are listed in Table 21.7.

Table 21.7 Cloning and heterologous expression of fungal FTase, FFase, and inulinase

Organism	Enzyme	GenBank accession no.	Cloning host	Expression host	References
A. niger ATCC 20611	Inulinase	–	*Escherichia coli* DH5α	*A. niger* ATCC 20611 protoplast	Zhang et al. (2017b)
A. oryzae FS4	Inulinase	bfrAFS4 (CGMCC no. 9087)	*Escherichia coli* DH5α	BL21, *Pichia pastoris*	Xu et al. (2014)
Aspergillus oryzae	FTase	EU130944	*Escherichia coli* DH5α	BL21	Wang et al. (2016c)
A. terreus NIH2624	FFase ATEG 04996	XP 001214174.1	*Escherichia coli* strain GB05	*K. lactis* GG799	Spohner and Czermak (2016)
Aspergillus sp.	Endoinulinase	–	*Escherichia coli*	*Escherichia coli*	Raba'atun Adawiyah et al. (2011)
Aspergillus awamori	Exoinulinase	AJ315793	*Escherichia coli*	*Escherichia coli*	Arand et al. (2002)
Aspergillus fumigatus Cl1	Endoinulinase	AFUA 5G00530	*Escherichia coli* DH5α	*P. pastoris* GS115	Chen et al. (2014)
Aspergillus kawachii	Exoinulinase	CAC44220	*Escherichia coli* TOP10F	*P. pastoris* GS115 and X33	Chesini et al. (2018)
A. oryzae	FTase	–	*Escherichia coli* DH5α	*Y. lipolytica* CGMCC7326	Zhang et al. (2016)

High-yielding strain of *Aspergillus oryzae* was developed using strains with high fructosyltransferase (FTase) activity for intraspecific protoplast fusion via genome shuffling. The resulting strain produced 353 U/g FTase activity (Wang et al. 2016c). More recently, Wang et al. (2016d) have cloned endoinulinase in *Saccharomyces cerevisiae* and deleted its sucrase gene, resulting in high-content FOS production (90%) from inulin in a single step.

21.8 Future Perspectives and Conclusions

Fungi produce a number of industrial enzymes which find multifarious applications in a variety of industrial processes. Owing to their ability to utilize low-value substrates, amenability to manipulation, and ability to produce high enzyme titers, fungi are being explored extensively for industrial enzymes. Often, fungal species are noticed to elaborate spectra of hydrolases including main and accessory enzymes that can be used as consortia for efficient and complete depolymerization of complex substrates. Out of about 260 commercial enzymes, 60% are sourced from about 25 fungal genera. The enzyme market is projected to grow up to $10.5 billion by 2024. The rapid growth in enzyme market is indicative of the ever-increasing demand of enzymes in various sectors like biofuel, food, detergents, pharmaceuticals, etc. To realize the aim of replacing harmful toxic chemicals in industries, enzymes should be able to work under harsh or extreme conditions. This is one bottleneck where fungal enzymes lag behind bacterial extremozymes. Development of strains expressing robust and multifunctional (chimeric) enzymes using recombinant DNA technology, high-throughput screening of novel isolates, metagenomic screening, *in silico* enzyme engineering, site-directed mutagenesis, and directed evolution will pave a way to cater future demands.

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