Enzymes in Bioconversion and Food Processing

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Abstract

Enzymes are biological catalysts that can be found in every living system. It takes part in various reactions and is mainly found in plants, animals, and microorganisms. The introduction of enzymes in the food industries began with the application of chymosin, derived from the calf stomach, for the production of cheese. Since then, advances in biotechnology have paved way to the application of enzymes synthesized by recombinant microorganisms. Living organisms achieve bioconversion of a substance with the help of enzymes. Due to high substrate specificity, enzymes find applicability in baking, dairy, detergent, leather, and beverage industries. Additionally, enzymes also play a vital role in wastewater treatment, animal nutrition, paper manufacturing, and pharmaceutical and biofuel applications. Furthermore, the discovery of cellulose- and lipid-hydrolyzing enzymes along with the extensive applications of genetic engineering has provided momentum to producing alternative fuel sources from plant-based waste on a huge scale. This chapter deals with various enzymes typically applied in bioconversion and food processing.

Keywords

Enzymes · Hydrolysis · Fermentation · Food processing · Bioconversion

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2.1 Introduction

Enzymes are biological catalysts which regulate metabolic pathways in every living system. Owing to their inherent nature of substrate specificity and controlled reaction conditions, enzymes find commercial applications in various industries. Since their introduction into the manufacturing process in the 1970s, enzymes have paved a niche for themselves, and the "Global Enzyme Industry" is projected to be worth 6.3 billion dollars by 2020 (marketsandmarkets.com 2016). Industrial enzymes are widely accepted in food and beverage, pharmaceutical, textile, animal feed, biofuel, cosmetic, detergent, and paper and pulp industry.

Industrial enzymes are produced by growing bacteria and fungi in submerged fermentation or by solid-state fermentation. Submerged fermentation is preferred over the latter owing to better process control measures. Depending on the nature of enzymes being intracellular or extracellular, downstream processing techniques after fermentation may include cell disruption and filtration. The crude enzyme is then further purified by precipitation, centrifugation, and other effective protein purification techniques (Ravindran and Jaiswal 2016).

As mentioned earlier enzymes are highly specific in the reactions they catalyze. Furthermore, enzymatic reactions take place in mild conditions compared to chemical syntheses. Being readily biodegradable, an enzymatic process requires much less investment to make it environment-friendly while leading to very low or no toxicity. This gives manufacturers the flexibility to produce products with the same or higher quality without leaving a carbon footprint and releasing harmful chemicals into the environment. Although competent from an environmental point of view, enzymes must be commercially viable to displace chemical manufacturing processes that are traditionally extremely inexpensive. The beginning of commercial enzyme production was masked with the secretion of a mixture of enzymes with the yield of the target enzyme being less than 10 g/l. With the application of recombinant DNA technology, researchers have been able to devise truncated molecules that serve a unique purpose while using bacterial and fungal hosts to mass-produce superior quality of an enzyme with yields reaching as high as 40 g/l. Almost 90% of the enzymes in the industry today is manufactured by following these steps. Also, heterologous genes that code for enzymes are modified to optimize the performance under conditions they are intended to work in. The development of tailor-made enzymes is an iterative process which involves mutation and screening and is highly dependent on structural information of the enzymes and design concepts aiming to improve substrate specificity or thermostability (Cherry and Fidantsef 2003).

2.2 Classification of Enzymes

The International Commission of Enzymes, founded in 1955, established the most exclusive enzyme classification system based on the type of reactions each enzyme catalyzed. Under this system, enzymes and coenzymes, their units of activity and standard methods of assay along with symbols used to demonstrate reaction kinetics

were all encompassed into a single system. All the enzymes were designated with a number with four digits based on the class, subclass, and sub-subclass they were categorized into. There are six main categories each for enzymes and they are:

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases

Oxidoreductases catalyze oxidation reactions by facilitating the transfer of electrons from the substrate and in some cases oxygen to the substrate. These enzymes can vaguely be categorized into three based on their mode of reaction: hydrogenases that directly act upon the primary substrate, oxidases that require intermediate electron acceptor such as H_2O_2 or H_2O, and oxygenases that catalyze biological reactions that involve the addition of molecular oxygen. These categories of enzymes are further classified based on the electron acceptors or donors that come into play during the reactions begin catalyzed.

Common substrates for oxidoreductases include amines and imines, CH–CH groups, alcohol, aldehydes and ketones, sulfur-containing groups, and diphenols. A few examples of these enzymes include alcohol dehydrogenase, sarcosine oxidase, glutathione reductase, etc. (Aszalos 1978; Goldberg et al. 1993).

Transferases are enzymes that catalyze the transfer of chemical groups such as methyl, carbonyl, sulfur, phosphorus, and nitrogen radicals. The subgroup of this class of enzymes is based on the type of groups that are transferred during the catalysis. For example, the subgroup 2.1 is capable of transferring groups such as methyl, hydroxymethyl, carboxyl, and amino groups, subgroup 2.2 transfers aldehyde and ketone groups, meanwhile subgroup 2.3 catalyzes the transfer of acyl groups. Other groups that are transferred include glycosyl group (2.5); nitrogenous groups (2.6); phosphor-containing groups such as phosphate ester, phosphoryl, phosphoanhydride, etc. (2.7); and sulfur-containing groups (2.8). A few examples of transferase enzymes include pyruvate kinase, aspartate aminotransaminase, glycine amidinotransferase, etc. (Webb et al. 1992).

Hydrolases cleave covalent bonds that are commonly found in biological compounds such as peptide bonds, glycosidic bonds, ester bonds, anhydride bonds, phosphoric acid bonds, and thioester bonds. Hydrolases that catalyze the hydrolysis of ester bonds are categorized in subgroup 3.1. A most common example of hydrolase, alkaline phosphatase, which is found in milk and the liver, catalyzes transphosphorylation. Phospholipases are another example of hydrolases that act upon on phospholipids and are found in the muscle, liver, kidney, heart, and pancreas. The enzymes categorized in subgroup 3.2 split glycosyl bond which is found in polysaccharides such as dextran and starch. A very important enzyme belonging to this category is α-amylase which digests starch into glucose. Most polysaccharide-degrading enzymes such as amylase, invertase, β-galactosidase,

hyaluronidase, etc. belong to this group. The enzymes in subgroup 3.3 cleave ether bonds. An example of enzyme belonging to this category includes adenosylhomocysteinase. Subgroup 3.4 has enzymes that act upon proteins and peptides to digest the peptide bonds in them (e.g., urokinase, chymotrypsin A and B, pepsin, etc.). Enzymes in the subgroup 3.5 catalyze C–N bonds that are not peptide bonds. An interesting enzyme of this subgroup, L-asparaginase, has been used to combat acute lymphoblastic leukemia. The enzyme acts by converting L-asparagine into L-aspartate and ammonia, thus depriving the malignant cell of essential nutrient (Cantarel et al. 2009).

Lyases catalyze the addition of a radical to a double bond or the removal of a functional group by the formation of a double bond in the substrate. Compounds such as carbon dioxide, water, aldehydes such as acetaldehyde and formaldehydes, amines, pyruvate, keto acid, etc. are some of the groups that may be added or removed from the substrate by lyases. Enzymes that catalyze the removal of carbon dioxide from the substrate are categorized in the subgroup 4.1. (e.g., malate synthase). The subgroup 4.2 encompasses enzymes that hydrolyze the substrate resulting in the removal of a water molecule. The most common enzymes belonging in this category are carbonic anhydrase, which is found in red blood cells and gastric mucosa. It cleaves carbonic acid resulting in the formation of carbon dioxide and water. Carbon-nitrogen lyases form the subgroup 4.3, and examples of this class of enzymes include aspartate ammonia lyase, histidine ammonia lyase, phenylalanine ammonia lyase, etc. Subgroup 4.4 and 4.5 categories are carbon-sulfur lyases and carbon-halide lyases, respectively (Aszalos 1978).

As the name suggests, isomerases catalyze isomerization reactions. The nature of the reactions catalyzed may vary from racemization, epimerization, *cis-trans* isomerization, intramolecular oxidoreduction, and intramolecular group transfer. Enzymes that facilitate racemization and epimerization fall in the subgroup 5.1. Racemization is the conversion of an optically active compound from one form to another (e.g., L-form to the D-form). Epimerization, on the other hand, is the conversion of the optical configuration of an optically active molecule. Racemases and epimerases fall in the subgroup of 5.1. Examples of racemases and epimerases are alanine racemase and hydroxyproline epimerase, respectively. Enzymes categorized in 5.2 catalyze *cis-trans* conversions (e.g., maleylacetoacetate isomerase). Intramolecular conversion reactions between aldoses and ketoses such as the synthesis of ribulose from arabinose and the formation of dihydroxyacetone phosphate from glyceraldehyde-3-phosphate are catalyzed by isomerases belonging to the subgroup 5.3 (e.g., arabinose isomerase and triose phosphate isomerase) (Aszalos 1978).

Ligases are important enzymes in DNA replication and protein synthesis. Ligases synthesize two molecules at the expense of energy bonds in ATP. Further classification of ligases is based on the nature of the bond formed by catalysis. Ligases that bind two molecules by the formation of C–O bond are grouped into subgroup 6.1. Tyrosyl t-RNA synthase which binds t-RNA to amino acids during peptide synthesis is an example for ligases that belong to subgroup 6.1. Enzymes that catalyze the formation of C–S bond between two molecules are categorized in subgroup 6.2. Acetyl-CoA synthase is an enzyme that catalyzes the reaction acetate and CoA to

form acetyl-CoA at the expense of a single ATP molecule. Subgroup 6.3 comprises of enzymes that catalyze the formation of C–N bond. This is maybe in the form of linkages between ammonia and carboxylic acids (sub-subgroup 6.3.1) or amino acids with acids (sub-subgroup 6.3.2). Subgroup 6.4 catalyzes the formation of C-C bonds between two molecules. Examples of enzymes belonging to this subgroup include pyruvate carboxylase and γ-glutamyl carboxylase. Subgroup 6.5 comprises of enzymes that form phosphodiester bonds between nucleotides chains that are broken. This subgroup only has one sub-subgroup (6.5.1). All the ligases commonly known to act upon DNA and RNA can be classified into this subgroup (e.g., DNA ligase and RNA ligase). Like 6.5, the subgroup 6.6 also consists of only one sub-subgroup 6.6.1. These groups of enzymes catalyze the formation of coordination complexes between nitrogen and metal atoms. An example of an enzyme which belongs to this subgroup is magnesium chelatase. This enzyme is a part of the chlorophyll biosynthesis (Fodje et al. 2001).

2.3 Biochemistry of Enzymes and Their Various Sources

Most industrial enzymes are proteinaceous in nature with a defined primary, secondary, tertiary, and in most cases a quaternary structure. The primary structure of enzymes involves covalent bonds known as peptide bonds, which bind amino acids in a sequence. The primary structure of the enzyme is a single molecule of protein devoid of any cofactors. Such a molecule is called apoenzyme. A protomer is a single unit of protein that in combination with other proteins forms heterologous protein molecules called oligomers. Enzymes are made of several numbers of protomers depending on its nature and activity. Minor changes in the primary structure of an enzyme will have far-reaching alteration in its enzyme activity. For example, protomers coded for the same activity but with different sequences of amino acids may associate to form different enzymes. These enzymes may differ in their physical and catalytic properties but will catalyze the same reaction. Such enzymes are called isozymes. Such enzymes enable an organism to catalyze the same reaction under different physiological conditions (Soltis 2012).

Secondary structure of proteins is due to the hydrogen bonds formed between nitrogen in an amino acid and the carboxyl group of the fourth amino acid in the peptide chain sequence. Since the peptide chain is planar, it has only two degrees of freedom. The peptide chains are assumed to form either an α-helix or a β-pleated sheet structure since they both are the most stable of configurations formed by a polypeptide chain. For example, α-helices contribute to 25% of the structure of the enzyme lysozyme (Dalal et al. 1997). The percentage of each configuration differs from molecule to molecule (Blake et al. 1965). The three-dimensional conformation of a protein molecule is known as the tertiary structure. The activity of an enzyme relies on its specific tertiary structure. The tertiary structure of an enzyme can be lost by denaturation due to variations in temperature. This will result in loss of enzyme activity. Interestingly, enzymes are much more stable when they are attached to their substrates or coenzymes (Iyer and Ananthanarayan 2008). The tertiary structure

brings amino acids closer to each other that otherwise would be located far away from each other in the polypeptide chain. Therefore, cleaving an enzyme between two adjacent amino acids may not affect the activity of the enzyme as a whole (Richards and Vithayathil 1959). Also, there is no "fixed" structure of enzymes, and they may change their conformation based on the presence of its substrate near. This is the mechanism behind "induced-fit" theory of enzyme activity (Koshland 1995). Quaternary structure is a combination of all the protomers held together in a tight fit to form the oligomeric enzyme. The forces that act upon the protomers may be a combination of many weak forces such as electrostatic interactions, hydrogen bonds, hydrophilic bonds, ionic binding, and dipole-dipole interaction (Robinson-Rechavi et al. 2006). The bonds that form the quaternary structure can be broken by using urea or guanidine hydrochloride (Klotz et al. 1975).

Almost all living organisms produce enzymes as a part of their metabolic systems. However, most of the industrial enzymes are derived from bacterial and fungal sources. The advent of recombinant DNA technology has enabled the production of animal and plant enzymes by microorganisms. A great variety of enzymes used in the industry come from some limited genera of microbes, viz., *Bacillus*, *Aspergillus*, *Kluyveromyces*, *Trichoderma*, *Rhizopus*, *Saccharomyces*, etc. Table 2.1 provides an insight on the various enzymes produced by different microbial species and their industrial applications.

2.4 Enzymes in Food Processing

2.4.1 Enzymes that Act on Polysaccharides

2.4.1.1 α-Amylase

α-Amylases (endo-1, 4-α-D-glucan glucanohydrolase EC 3.2.1.1) are a family of enzymes that randomly cleave α-1, 4 linkages between adjacent glucose subunits in polysaccharides resulting in the release of short-chain oligomers and α-limit dextrin. α-Amylases find a wide range of applications in bread and brewing industry; textile, paper and pulp industry; and pharmaceuticals (Sahnoun et al. 2015). α-Amylases are industrially produced via submerged fermentation using genetically improved *Bacillus* and *Aspergillus* species. A lot of other bacterial and fungal species have found to produce α-amylase enzyme with different characteristics such as thermostability, halo-tolerance, psycho-tolerance, and alkali-stability (Prakash et al. 2009; Sen et al. 2014; Roohi and Kuddus 2014). α-Amylase can also be synthesized following the solid-state fermentation method (Sundarram and Murthy 2014).

2.4.1.2 Amyloglucosidase

Amyloglucosidases (E.C. 3.1.2.3) are also known as glucoamylases and can cleave the α-1, 4 linkages found in starch to release glucose molecules. It is an exoamylase; it cleaves β-D glucose from the nonreducing ends of amylose, amylopectin, and glycogen (James and Lee 1997). Amyloglucosidase (AMG) also breaks α-1, 6 glycosidic bonds but at a slower rate (Espinosa-Ramírez et al. 2014).

Table 2.1 Approved enzymes designated as orphan drugs in the USA

Trade name	Generic name	Indication	Sponsor
Adagen®	Pegademase bovine	For enzyme replacement therapy for ADA in patients with SCID	Enzon Inc.
Ceredase®	Alglucerase injection	For replacement therapy in patients with Gaucher's disease type I	Genzyme Corporation
Pulmozyme®	Dornase α	To reduce mucous viscosity and enable the clearance of airway secretions in patients with CF	Genentech, Inc.
Cerezyme®	Imiglucerase	Replacement therapy in patients with types I, II, and III Gaucher's disease	Genzyme Corporation
Oncaspar®	Pegaspargase	Treatment of acute lymphocytic leukemia	Enzon, Inc.
Sucraid®	Sacrosidase	Treatment of congenital sucrase-isomaltase deficiency	Orphan Medical, Inc.
Elitek®	Rasburicase	Treatment of malignancy-associated or chemotherapy-induced hyperuricemia	Sanofi-Synthelabo Research
Fabrazyme®	Agalsidase beta	Treatment of Fabry's disease	Genzyme Corporation
Aldurazyme®	Laronidase	Treatment of patients with MPS I	BioMarin Pharmaceutical, Inc.
Replagal™	α-Galactosidase A	Long-term enzyme replacement therapy for the treatment of Fabry's disease	Transkaryotic Therapies, Inc.

Adopted with permission from Vellard (2003)

Amyloglucosidases exhibit optimum activity at a pH range of 4.5–5 and a temperature range of 40–60 °C (Kumar and Satyanarayana 2009). AMG finds applications in the food, brewery, and pastry industry (Diler et al. 2015). *A. niger* and *A. oryzae* are the most common strains that are used by the industry to produce commercial AMG (Espinosa-Ramírez et al. 2014; Singh and Soni 2001). However, *Bacillus* sp., *Rhizopus* sp., and *Saccharomyces* sp. have been reported to synthesize AMG (Shin et al. 2000; Ali et al. 1989).

2.4.1.3 Xylanase

Xylanases (E. C. 3.2.1.8, 1, 4-β-xylanxylanohydrolase) are enzymes that break down xylan which is an integral part of plant polysaccharide. Xylan is a complex polysaccharide made of xylose-residue backbone with each subunit linked to each other by a β-1, 4-glycosidic bond (Ramalingam and Harris 2010). Xylanases are produced by several bacterial and fungal species. Some insects, crustaceans, and seeds of plants have also been reported to produce xylanase. Filamentous fungi that synthesize this enzyme are of particular interest because they secrete the enzyme into the media in large quantities in comparison to bacteria (Knob et al. 2013). Xylan, being a complex polysaccharide, requires a consortium of enzymes for total

hydrolysis. Thus, xylanases are an enzyme complex comprising of different enzymes that are specific to different substrates and cleave chemical bonds of different nature. This enzyme system consists of endoxylanases, β-xylosidases, ferulic acid esterase, *p*-coumaric acid esterase, acetylxylan esterase, and α-glucuronidase. Endoxylanases and β-xylosidases are most extensively studied components of this system (Polizeli et al. 2005). Xylanases have wide applications in the food industry, biomedical industry, animal feed industry, and bioethanol production (Goswami and Pathak 2013; Ramalingam and Harris 2010; Das et al. 2012).

2.4.1.4 Inulinase

The importance of inulinase arises from the emergence of fructose and fructooligosaccharides as a safe sweetener compared to sucrose in the food and pharmaceutical industry. Inulinase acts upon insulin, which is a poly-fructose chain terminated by a glucose molecule. The fructose units in inulin are bonded together by *β*-2, 1-linkage (Vandamme and Derycke 1983). Commercially, fructose syrup is produced by the combined activity of α-amylase and amyloglucosidase followed by glucose isomerase which converts glucose into fructose. However, the best yield that can be procured from such a process is 45% of fructose, 50% of glucose, and the rest being oligosaccharides. The activity of inulinase results in the complete conversion of the substrate to fructose (Zittan 1981). Inulinase also finds applications in the production of bioethanol, citric acid, butanediol, and lactic acid as well. Inulinases can be classified into exo-inulinases (*β*-D-fructanfructohydrolase, EC 3.2.1.80) and endo-inulinases (2, 1-*β*-D-fructanfructohydrolase, EC 3.2.1.7) depending upon their modes of activity (Vijayaraghavan et al. 2009). Several bacterial and fungal species such as *Streptococcus salivarius*, *Actinomyces viscosus*, *Kluyveromyces fragilis*, *Chrysosporium pannorum*, *Penicillium* sp., and *Aspergillus niger* have been known to synthesize different forms of inulinase (Chi et al. 2009).

2.4.1.5 Lactase

Lactase, otherwise known as *β*-D-galactohydrolase (EC 3.2.1.23), hydrolyzes lactose into glucose and galactose (Nakkharat and Haltrich 2006; Duan et al. 2014). Lactose is the sugar which is found in milk. Humans produce this enzyme only as infants, and this ability lessens as they grow older which leads to lactose intolerance (Kies, 2014). Hence, lactase is a very important enzyme in the dairy and food industry. Industrial lactase is produced by employing selected strains of *Kluyveromyces lactis* (Bonekamp and Oosterom 1994). However, several articles can be found that report other organisms that produce lactase. *Candida pseudotropicalis* has been reported to produce lactase when grown on deproteinized whey (de Bales and Castillo 1979). *Trichoderma viride* ATCC 32098 produces a high thermostable lactase enzyme which showed 90% activity in a pH range of 3.0–7.5 (Seyis and Aksoz 2004). Macris (1981) reported the production of extracellular lactase from *Fusarium moniliforme* using the wheat bran solid medium. The addition of agriculture by-products such as molasses and whey increased enzyme yield.

2.4.1.6 β-Glucanase

1,3-1,4-*β*-Glucans are polysaccharides made up of glucose units that are found in endosperm cell walls of cereals such as barley, rye, sorghum, oats, etc. (Celestino et al. 2006). Endo-1,3-1,4-*β*-glucanases are enzymes that can hydrolyze the *β*-1,4 glycosidic linkages at the nonreducing ends of glucan to release cellobiosyl-D-glucose and 3-*O*-*β*-D-cellotriosyl-D-glucose. This enzyme is particularly important in the brewing industry. β-glucanases along with a consortium of xylanase and cellulase facilitate the reduction of viscosity in fluids with higher solid content. This leads to less water consumption during the production process, saves energy, and eliminates the need for several raw materials (Tang et al. 2004). β-glucanases also find application in the wine industry along with pectinases by facilitating a smooth and fast filtration process along with increasing the quality of the product (Villettaz et al. 1984).

2.4.1.7 Invertase

Invertase, technically known as β-fructofuranosidase (EC.3.2.1.26), is a glycoprotein which catalyzes the hydrolysis of sucrose into glucose (dextrose) and fructose. Invertase exhibits optimum activity at a pH of 4.5 and a temperature of 55 °C. *Saccharomyces cerevisiae* is the chief strain used in the production of the invertase enzyme in the industry (Neumann and Lampen 1967). Invertase is used to produce invert sugar, which was earlier done by acid hydrolysis. Acid hydrolysis of sucrose results in the 50% conversion of sucrose into invert sugar. Moreover, the acid hydrolysis product also contains impurities whose formation cannot be controlled during inversion. The use of invertase results in 100% inversion of sucrose without the formation of impurities (Kulshrestha et al. 2013).

2.4.1.8 Pectinase

Pectinases are a class of enzymes that catalyze the disintegration of pectin-containing compounds. Pectin compounds are an integral part of the plant cell wall. Pectinases can be classified into two groups according to their mode of action. Pectin esterases catalyze the de-esterification of methyl groups found in pectin to produce pectic acid. Depolymerase enzymes cleave the glycosidic bonds found in pectic acid to release various simpler compounds based on their mode of enzyme action. Protopectinases solubilize protopectin into a highly polymerized form of soluble pectin (Sakai et al. 1993). Pectinases are used in the fruit juice industry and winemaking for clarification and removal of turbidity in the finished product. It also intensifies the colors in the fruit extract while aiding in stabilization, clarification, and filtration (Servili et al. 1992).

2.4.2 Enzymes that Act on Proteins

2.4.2.1 Protease

Proteases (EC 3.4.21.62) are enzymes that perform proteolysis by hydrolyzing the peptide bonds that link amino acids together in polypeptide chains that forms

proteins. Proteases are among the most important hydrolytic enzymes and have been studied since the introduction of enzymology. They have always been in the limelight not only because of the active roles they play in cellular metabolism but also because of their application in the industry. Microorganisms are the source of many proteases which can be intracellular or extracellular in nature. Intracellular proteases are associated with various cellular and metabolic processes such as sporulation and differentiation, while extracellular proteases are important for the hydrolysis of proteins in the cell-free environment facilitating the absorption and utilization of the hydrolysates. Proteases were first used for detergent manufacturing in 1914, and since then they have been inducted into various other commercial processes such as food, pharmaceutical, animal feed, leather, diagnostics, waste management, and silver recovery. In fact, proteases dominate the total enzyme sales with a market share of almost 60% (Sawant and Nagendran, 2014). The dominance of proteases in the detergent industry is contributed by alkaline proteases due to their unique ability to remain stable and active in the alkaline pH range (Gupta et al. 2002). Serine proteases especially subtilisin A, neutrase, and trypsin are some of the commercially important proteases. Most of the proteases applied in the industry are produced by genetically modified strains of *Bacillus* and *Aspergillus* (Pillai et al. 2011; Radha et al. 2011).

2.4.2.2 Transglutaminase

Transglutaminases (E.C. 2.3.2.13, protein-glutamine-γ-glutamyl transferase) are a class of transferase enzymes, which catalyze the formation of an isopeptide bond between the γ-carboxyamide groups of glutamine residues and the γ-amino group of lysine residues (Kieliszek and Misiewicz 2014). Transglutaminases are produced in the mammalian muscle tissues as well as microbial agents. However, this enzyme is industrially produced by employing superior strains of *Streptoverticillium mobaraense* as it is calcium independent and lower in molecular weight compared to other isozymes (Motoki and Seguro 1998). Two fungal species, viz., *Pythium* sp. and *Phytophthora* sp., have sparked interest in researchers as potential organisms for the production of transglutaminases (Andersen et al. 2003). Several organisms have been tested as hosts for the overproduction of recombinant transglutaminase including *Escherichia coli*, *Corynebacterium glutamicum*, and *Streptomyces lividans* (Noda et al. 2013).

Transglutaminases find various applications in the industry; it is used in the flour, baked products, cheese, milk products, meat products, fish products, cosmetics, gelled food products, and leather finishing (Andersen et al. 2003). Treating wool with transglutaminase after undergoing protease treatment increases the strength and in turn the longevity of the wool fibers (Cortez et al. 2004). Food waste and agro-waste have been tried and tested by researchers as potential media components for the production of transglutaminase.

2.5 Enzymes in Bioconversion

2.5.1 Cellulase

The depolymerization of cellulose into component glucose molecules requires a combined hydrolysis of three key enzymes: endoglucanase (E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (E.C. 3.2.1.176) (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21). They are categorized in the glycoside hydrolase family, and they catalyze the cleavage of glycosidic bonds (Juturu and Wu 2014). Cellulases are enzymes of great commercial importance, especially because of the inevitable role they play in bioethanol production (Singhania et al. 2014). Besides biofuels, cellulases find application in bread and brewing industry, textile and detergent industry, and paper and pulp industry (Ferreira et al. 2014). A wide range of bacterial and fungal species produce cellulase enzyme. In bacteria, cellulases are found in the form of large, extracellular aggregates which are called cellulosomes (Doi and Kosugi 2004). Some of the bacterial species which produce cellulosomes include *Clostridium thermocellum*, *Bacillus circulans*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *E. coli*, and *Cellulomonas* sp. Fungal species have also been found to synthesize cellulases (Juturu and Wu 2014). Commercial cellulases are produced by improved strains of *Trichoderma reesei.* Examples of other fungal organisms that produce cellulase enzyme are *Schizophyllum commune*, *Melanocarpus* sp., *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. (Várnai et al. 2014).

2.5.2 Laccase

Laccase (EC 1.10.3.2), categorized in the multicopper oxidase family, catalyzes the oxidation of phenolic compounds with the help of molecular oxygen (El-Batal et al. 2015). They were first isolated from the lacquer tree *Toxicodendron vernicifluum* and have since been found in over 20 bacterial species (Yoshida 1883). However, fungal laccases are more abundant in nature, especially wood rotting fungi. While bacterial laccases are periplasmic in nature, fungal laccases are extracellular. Three kinds of fungal organisms produce laccase enzyme, viz., white rot fungi, brown rot fungi, and soft rot fungi (Mate and Alcalde 2015). Out of the three, white rot fungi have been found to be the most efficient lignin-degrading microbe (Mishra and Kumar 2007). Laccases are commercially important enzymes due to their ability to degrade phenolic and non-phenolic lignin along with recalcitrant pollutants. They are used for the decolorization of dyes, bio-bleaching, synthesis of dyes, baking, biopulping, degradation of xenobiotics, and effluent treatment (Rodríguez Couto and Toca Herrera 2006). Glucose, fructose, mannose, lactose, and maltose are the commonly used carbon sources to produce laccase. Monosaccharides have a detrimental effect on laccase production, and a polymeric substrate like cellulose is a necessary media component (Shraddha et al. 2011).

2.5.3 Lipases

Lipases find a wide variety of applications owing to the type of reactions they can catalyze. In a living system, these enzymes facilitate the breakdown and mobilization of lipids within the cells. Within the spectrum of reactions, lipases catalyze some of the industrially interesting processes including hydrolysis, transesterification, alcoholysis, acidolysis, aminolysis, and esterification. They can hydrolyze fats into fatty acids and glycerol at the water-lipid interface and can catalyze the reverse reaction in nonaqueous media. All these features make lipases a widely sought out enzyme in the oil industry, dairy industry, pharmaceuticals, bakery industry, biopolymer synthesis, and the treatment of fat-containing waste effluents (Aravindan et al. 2007). Lipases from two species, viz., *Thermomyces lanuginosus* and *Rhizomucor miehei*, are extensively used for various industrial purposes in soluble as well as immobilized form, the latter, owing to low stability and difficulty in enzyme-product separation (Fernandez-Lafuente 2010; Mohammadi et al. 2014). However, other microbial species such as *Bacillus*, *Serratia*, *Pseudomonas*, and *Staphylococcus* have also been reported to produce lipases (Prasad and Manjunath, 2011).

Biodiesel production is one of the most novel applications of lipases. Lipases for biodiesel production can be derived from bacterial sources as well as fungal sources. *Burkholderia cepacia*, *Pseudomonas fluorescens*, and *Pseudomonas cepacia* are some of the bacterial lipases used as biodiesel production. Novozyme produces a widely popular yeast lipase enzyme called Novozym 435 which is derived from *C. Antarctica*, which is also used for biodiesel production (Talukder et al. 2009). Lipases are hydrolases that catalyze the breakage of carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. In transesterification reactions, lipases convert triglycerides into fatty acid methyl esters (FAME) and glycerol. Biodiesel can be derived from various sources such as vegetable oils or animal fats. Recent studies have focused on producing biodiesel from rubber seed oil, which is an agro-industry waste product and algae (Collet et al. 2014; Kynadi and Suchithra 2017). Transesterification of triacylglycerides using lipase enzymes is preferred over chemical catalysis due to substrate specificity, non-toxicity, low operational cost, and the ability to be reused in the case of immobilized lipases. In some cases, immobilized lipases can be used over ten cycles (Suali and Sarbatly 2012). Some of the drawbacks of employing lipases for biodiesel production are the formation of methanol and glycerol as by-products which may call for the need for downstream processing measures.

2.6 Enzymes in Pharmaceutical Applications

In the pharmaceutical industry, enzymes form an integral part of the biocatalysts along with whole-cell systems. Enzymes are invaluable to the pharmaceutical industry due to their unique ability to catalyze reactions based on regioselective and stereoselective properties. This enables the creation of synthetic routes to

procure complex molecules of interest by reducing the number of steps required in attaining the intermediate of interest. This favorably influences the process economics of chemical synthesis. Also, this property of enzymes has assisted in the discovery and formulation of pharmaceutical intermediates (Pollard and Woodley 2007). Chemoselectivity, regioselectivity, and stereoselectivity are the three main purposes of enzymes in the pharmaceutical industry.

A molecule with higher number of functional groups is hard to negotiate with when it comes to selectivity. Especially in the case of oligosaccharides, selectivity has been an issue due to the high density of functionalized groups. This calls for extensive use of protection techniques to ensure selective synthesis. However, the application of enzymes can circumvent the need for extensive process measures by facilitating the formation of the required compound directly. Glycosyltransferases are examples of enzymes that are extensively researched for their regiospecific glycosylation. Figure 2.1 illustrates the various applications of glycosyltransferase in catalysis of regioselective reactions. *Arabidopsis thaliana* is a source of glycosyltransferase (Cartwright et al. 2008; Chang et al. 2011). Enzymes have been used to reconstitute antibiotics as well. A study investigating the production of chloroeremomycin, a glycopeptide (Lu et al. 2004), was able to reconstitute the antibiotic by using sequential actions involving three enzymes, viz., glycosyltransferases A, B, and C. Heptapeptide aglycone was used as the substrate.

Lipases have extensively been used in studies to obtain regiospecific reactions. Irimescu et al. (2001) studied the effect of *C. antarctica* lipase (Novozym 435) for the synthesis of regio-isomerically pure 1, 3-dicapryloyl-2-docosa-hexaenoyl glycerol (CDC). According to their study, the composition of the reaction medium played a major role in obtaining the regiospecific product. Nonetheless, pure CDC was obtained in just two steps with a high yield of CDC (85.4%). The human milk fat, 1,3-dioleoyl-2-palmitoylglycerol (OPO), is commercially produced by the application of sn-1, 3-specific lipase enzyme. Cai et al. (2015) achieved 49.54-fold increase in yields of OPO by immobilizing sn-1, 3-specific lipase obtained from *Aspergillus oryzae* which was immobilized in polystyrene resin. In another study, lipases were used for the regiospecific interesterification of exotic oils. Two lipases, one from *Thermomyces lanuginosus* (which was commercially available) and the other from *Rhizopus* sp., were used individually and in combination to produce homogenous tri-glycerols from exotic oils such as buriti oil and murumuru fats. The active area of β-carotene was preserved in the triglycerols by all the enzyme systems. The newly formed inter-esterified lipids were found useful in the formulation of cosmetics and pharmaceuticals (Speranza et al. 2016).

Stereoselectivity is another property of enzymes that is widely exploited in the pharmaceutical industry. Due to this nature, enzymes are able to produce a higher yield of a compound in a single isomeric state instead of a racemic mixture which makes them an invaluable tool in organic synthesis. N-acylases are indispensable tools for obtaining the perfect enantioselective versions of a pharmaceutically viable compound. *Aspergillus melleus* has been conventional sources of commercial obtaining N-acylase (Dong et al. 2010). Penicillin acylase has been emerging as an enzyme of great commercial importance due to its application to produce essential

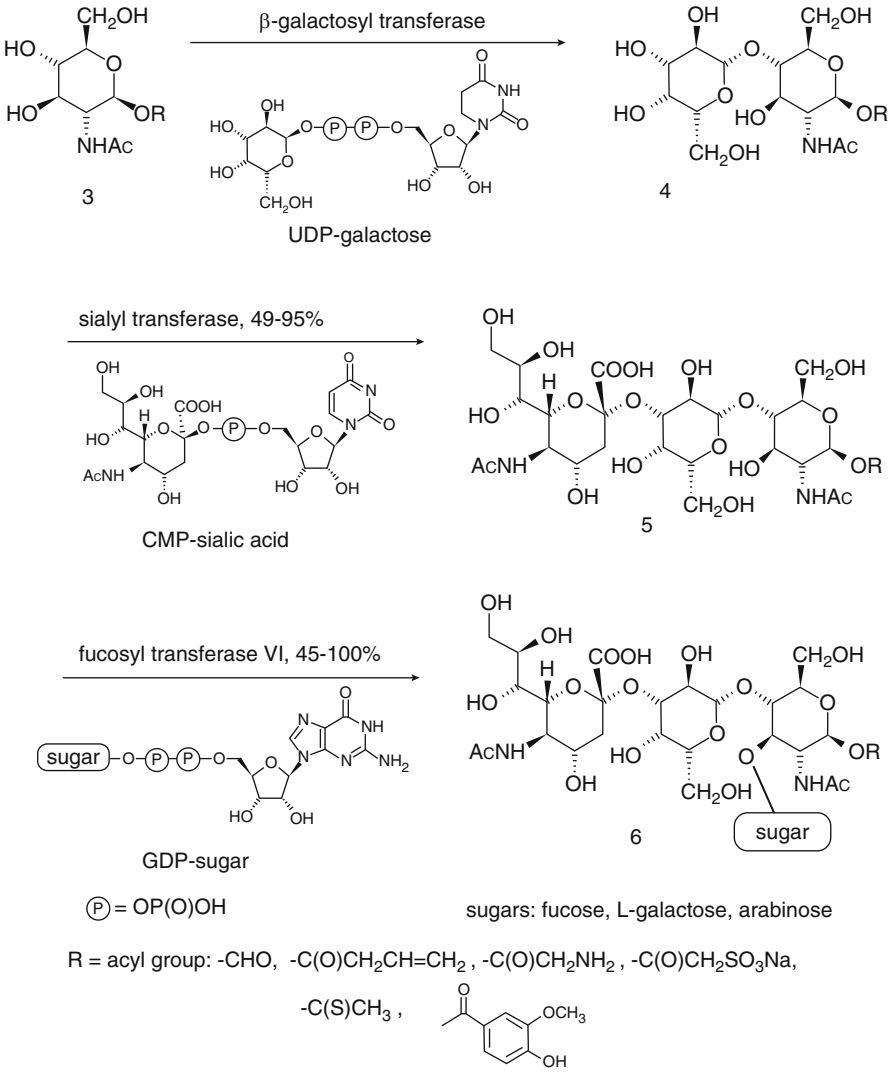

Fig. 2.1 Glycosyltransferase-catalyzed reactions for chemo-regiospecificity. (Adopted with permission from Rasor and Voss 2001)

beta-lactam nucleus which is the precursor molecule for several semisynthetic penicillins. *Kluyvera citrophila* and *E. coli* are the two major sources of penicillin acylase in the industry due to their resistance to harsh environmental conditions and ease of immobilization (Mukherji et al. 2014).

Lipases and esterases have also been known to possess enantioselective activity encompassing a wide range of substrates such as esters, alcohols, carboxylic esters,

and amines. As mentioned before lipases catalyze the hydrolysis of triglycerols in an aqueous environment. These reactions are in general 1,3-regioselective. In this case, primary hydroxyl functions as a favorable mediator in the acylation of 1,2-diols in an organic medium. Furthermore, the corresponding diesters undergo alcoholyses to liberate a primary alcohol in a regioselective manner. These reactions occur far from the stereocenter and often result in poor enantioselectivity. This often requires protective groups which further complicate the overall process. A combination of *C. antarctica* lipases A and B coupled with acylase I was used to engineer the preparation of enantiomers of 1-phenylethan-1,2-diol (Virsu et al. 2001).

Acetylated sugars are commercially important compounds due to their altered properties which make them viable to be used in biosurfactants in biomedicine, foods, detergents, and cosmetics (Kanelli and Topakas 2017). Esterases find wide applications in the production of regioselectively substituted polysaccharides. Acetyl esterases are the most common esterases that have been widely researched upon for the deacetylation of cellulose esters and cellulose acetate in particular. Currently, 13 classified carbohydrate esterase (CE) families exist, and acetyl xylan esterases are found in CE families 1–7 (Altaner et al. 2003). Esterase-catalyzed reactions have known to catalyze regioselective 6-deacylation of hexopyranose per-acetates such as per-acetylated glucose 4, mannose 6, *N*-acetylgalactosamine 8, galactose 10, methyl α-D-glucoside 12, methyl α-D-mannoside 14, and methyl α-D-galactoside (Horrobin et al. 1998).

Orally active peptides encounter various impediments during clinical development due to the lack of stability in an unfavorable physical environment and susceptibility to enzymatic degradation. This limits their permeation and proper assimilation in the body. In order to tackle this issue, studies have been conducted in formulating prodrugs to transiently alter the properties of the peptides to overcome pharmacokinetic problems. The best strategy would be to enable the prodrug to exhibit enhanced membrane penetration while resisting enzymatic degradation. Once at the site of action, the prodrug needs to release the peptide spontaneously or by enzyme-mediated transformation. Cyclic prodrugs consist of a linker between the N terminal and C terminal of the peptide that imparts structural stability and flexibility and protects the peptide from metabolic degradation. Pauletti et al. (1997) prepared a cyclic peptide using phenylpropionic acid and reduced the liability of the peptide to peptidase digestion, substantially increasing the permeation through biological membranes. Esterase was used as the enzyme-mediated mechanism to release the parent peptide from the prodrug.

Besides the application of enzymes for the production of enantiomers, stereoisomers, and pure racemic molecules, enzymes have also been used as therapeutic agents. Activase® (also known as alteplase) was the first enzyme that was approved by the US Food and Drug Administration to be used as a drug (Hill and Buchan 2002). Activase was a recombinant protein engineered in lieu of treating heart patients and individuals who had a higher chance of acute ischemic stroke due to clot formation in blood vessels.

2.7 Enzymes Used in Other Industrial Applications

2.7.1 Phytase

Phytases are important enzymes in the animal feed and nutrition industry. They were discovered by Suzuki et al. (1907) while performing the hydrolysis of rice bran. Phytases essentially degrade phytic acid which is a phosphate storage compound found in grains and seeds. They can be classified into three according to position of the phosphate they hydrolyze (3-phytase, 4-phytase, and 5-phytase) (E. C. 3.1.3.8, E. C. 3.1.3.26, and E. C. 3.1.3.72) (Joshi 2014). Monogastric animals that consume grains are unable to digest phytic acid due to less or no production of phytase. Substances that are used for animal feed such as oatmeal and wheat bran are rich in phytic acid, which is excreted undigested, and thus minerals such as calcium and phosphorus need to be supplemented. Treating animal feed with phytases eliminates the need for addition of minerals and increases nutritive value and also reduces the phosphate concentration in effluents from pig and poultry farms (Selle and Ravindran 2007).

2.7.2 Mannanase

Mannanase are a cluster of enzymes that degrade mannan, which is an integral part of the plant cell wall. Mannan is a representative of hemicellulose which is the second most abundant polysaccharide found in plants. Three major enzymes are involved in the degradation of linear mannans, viz., 1, 4-β-D mannohydrolases or β-mannanases (EC 3.2.1.78), 1,4-β-D mannopyranoside hydrolases or β-mannosidases (EC 3.2.1.25), and 1,4-β-D glucoside glucohydrolases or β-glucosidases (EC 3.2.1.21) (Chauhan et al. 2012). β-mannanases show endo-hydrolysis activity by cleaving the internal glycosidic bonds resulting in the release of short-chain β-1,4-manno-oligosaccharides (McCleary and Matheson 1983). On the other hand, β-mannosidases possess exo-hydrolase activity attacking the polymer at the nonreducing terminal and degrading mannobiose into individual mannose units (Gomes et al. 2007). β-glucosidase activity results in the excision of 1,4-glucopyranose units from the nonreducing terminal ends of oligomers released from glucomannan and galactoglucomannan hydrolysis (Mamma et al. 2004). Several *Bacillus* spp. including different *B. subtilis* strains have been reported to produce several mannan-degrading enzymes. Among fungal organisms, many *Aspergillus* spp. have been found to produce mannanase (Dhawan and Kaur 2007). Other bacterial and fungal species such as *Clostridium* sp., *Penicillium* sp., and *Streptomyces* sp. have also been known to synthesize mannanase (Chauhan et al. 2012). Due to its ability to effectively remove hemicellulose, mannanase has sparked an increasing interest in the paper and pulp industry (Clarke et al. 2000). Mannanase also find applications in the food, oil, feed, and textile industries (Cuperus et al. 2003; Naganagouda et al. 2009; Christgau et al. 1994).

2.8 Future Trends and Concluding Remarks

Enzymes have traditionally been applied to assist mankind in several processes. Today, over 4000 enzymes have been identified, and almost 500 enzymes are being used in industrial processes that range from the food and beverage industry to the pharmaceutical industry, fine chemical industry, and textile industry. Technological innovation has enabled the rapid growth of the enzyme industry. Advancements in this industry are heavily dependent on the identification and characterization of new enzymes from natural resources as well as the development in techniques to modify these enzymes for high-level expression and optimize them for various industrial processes. The existence of databases that allows researchers to catalogue existing enzymes and evaluate variations in naturally occurring proteins enables the development of engineering schemes for enzymes. Apart from enzymes, whole-cell catalysts are emerging as a promising technique in biocatalyst technology. Whole-cell catalysts are advantageous for the fact that they can be metabolically engineered for specific biochemical reactions in order to obtain a certain product. Techniques such as gene shuffling and directed evolution will enable the development of enzymes that better suit industrial requirements and for completely novel applications.

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