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Endogenous Hydrolyzing Enzymes: Isolation, Characterization, and Applications in Biological Processes

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Abstract

Enzymes are the key substances responsible for a variety of biotechnological processes involved in the production of useful bioproducts. Malt and microbial species (bacteria, fungi, etc.) are the main sources of endogenous hydrolyzing enzymes (EHEs). EHEs are primarily involved in the digestion of complex substrates into simpler units and the resulting formation of biological products. Based on origin and substrate specificity, EHEs are categorized into cell wall-, starch-, protein-, lipid-, nucleic acid-, polyphenol-, and thiol-hydrolyzing enzymes. The substrate specificities and reaction mechanisms of individual EHEs and groups of EHEs have been verified through isolated and purified enzymes. A number of methods have been reported for high-yield, economically feasible isolation of enzymes. The endogenous enzymes contained in microbial cells are separated from cells, cellular fragments, and organelles through several cell lysis and separation methods. Analysis of the mechanism of action has revealed that most enzymes systematically undergo biological processes through a cascade of enzyme-specific reactions. The applications of these EHEs are involved in almost every aspect of human and animal life and are important in food, animal feed, textile, paper and pulp, fuel (energy), pharmaceutical, and chemical industries. In this chapter, we describe the origins, classes, isolation techniques, mechanisms, and applications of various EHEs with examples from updated literature.

Keywords

Endogenous hydrolyzing enzyme; Malt; Microbial species; Cell lysis; Mechanism of action

1 Introduction

Most of biochemical reactions occur spontaneously, while others depend on specific catalysts in order to reduce the magnitude of the energy difference essential for successful conversion of substrate into product (Illanes 2008). Enzymes are highly specialized catalysts that lead to increases in reaction rates and the specificity of metabolic reactions, from simple to highly complex biological reactions. Additionally, each biological reaction may be catalyzed by a specific enzyme, enabling reactions to function at rates required to sustain life. In theory, enzymes should not be consumed or altered during conversion of the substrate into product. However, in practice, the sustainability and functionality of any enzyme during reaction catalysis is strongly dependent upon the conditions adopted for substrate conversion because enzymes are primarily proteins (Shuler and Kargi 2001).

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The enzymes catalyzing a biological reaction can be broadly divided into two types (exogenous hydrolyzing enzymes and endogenous hydrolyzing enzymes [EHEs]), based on their source. Generally, exogenous enzymes are expensive and ultimately increase the final cost of a bioproduct. Moreover, optimization of operational conditions, such as selection of appropriate temperature, pH, and substrate and enzyme concentrations, is a tedious job. In contrast, EHEs are attractive candidates for industrial-scale production of various bioproducts. All biological processes and metabolic pathways are driven and regulated by a group of enzymes catalyzing a cascade of reactions. These enzymes can act on the substrate both in vivo and in vitro and mediate reactions that ultimately result in improved product yields, up to severalfold. However, in the absence of a biological catalyst, the reaction rate is retarded, and biological reactions are ultimately blocked. EHEs are also advantageous in that they are encoded by the genome of a particular cell, which controls the expression level and activity of the EHE, resulting in enhanced biological activity within the same cell. Generally, EHEs are expressed within the cell when required; however, their expression can be regulated (enhanced or suppressed) depending on the cell's needs and on environmental conditions. Khattak et al. (2012) reported the presence of several EHEs from waste fermentation broth; these EHEs included starch-hydrolyzing enzymes, cellulose-hydrolyzing enzymes, cell wall-hydrolyzing enzymes, protein-hydrolyzing enzymes, lipid-hydrolyzing enzymes, and several other important enzymes of industrial importance (Khattak et al. 2012).

Cell extracts containing EHEs can be isolated through cell disruption and further processed and utilized for various useful applications, such as development of synthetic pathways, cell-free enzyme systems, and commercialization. Cell disruption can be carried out by various methods, including chemical, enzymatic, alkaline lysis, and mechanical insults, such as ultrasonication, bead beating, and high-pressure homogenization. The obtained cellular extracts are further evaluated for extracellular and intracellular enzymes from cells and cellular fragments, respectively, through various approaches, such as filtration, centrifugation, flocculation, and flotation. Because the obtained extract possesses a lower concentration of enzymes, extracts can be further concentrated through various approaches, such as thermal methods, precipitation using different reagents, and ultrafiltration. For enhanced activity and storage to prior utilization for various purposes, enzymes can be further purified through crystallization, electrophoresis, and various chromatographic techniques.

The ease of use, cost-effectiveness, distinctive properties, and efficacy of EHEs make them attractive candidates for utilization in various industries, including breweries, food processing, and several medical and pharmaceutical industries. This chapter briefly describes the history of EHEs and then introduces various types of EHEs based on the type of substrate; discusses different techniques employed for the isolation, separation, and purification of EHEs from various sources; and describes the mechanisms of action of various EHEs.

2 History of EHEs

Hundreds of years have passed since humankind started to use enzymes for the production of beverages and baking products, as first described by ancient Egyptians. Indeed, ancient Egyptians used biological catalysts to produce beer, wine, bread (leavened bread), and cheeses, and later civilization used biological catalysts to make yogurt and other fermented milk products, pickles, vinegar, butter, and sauerkraut (Steinkraus 2002). In late eighteenth century, de Réaumur (1752) and Spallanzani reported that gastric juices secreted by the stomach are responsible for the digestion of meats (de Réaumur 1752; Modlin and Sachs 2004). In later years, a number of similar phenomena

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were observed, including endogenous secretions in plant extracts and saliva, which participate in the breakdown of starch into simple sugar. In 1814, glutinous (proteinaceous) secretions were reported to be responsible for the hydrolysis of starch into sugar (Segel 1993). Although the substances involved in digestion of meat, starch, and amygdalin have been identified, these early studies did not define these endogenous secretions and their mechanisms of action (Williams 1904).

After the first clear identification of endogenous enzymes made by Payen and Persoz in 1833 (Payen and Persoz 1833), it was unclear until the nineteenth century whether the molecules responsible for the catalysis of biological processes belonged to living species or were chemical substances. The substance they obtained (referred as "diastase") was employed for the hydrolysis of starch. A second well-known enzyme obtained from an animal source in 1836 was pepsin, described by Schwann as the water-soluble factor found in gastric juice that was responsible for albumin digestion (Modlin and Sachs 2004). The next year (1837), Berzelius elucidated the details of the catalyst, which, by its mere presence, could "exert its influence and arouse affinities and relativities in the complex body thereby causing a rearrangement of the constituent of the complex body." By studying the mechanism of action of both pepsin and diastase, along with phenomenon observed during yeast fermentation, Berzelius concluded that in all three cases, only a single chemical agent (called the catalyst) is responsible for conversion of one substance into other (Segel 1993). In 1838, Charles Cagniard de Latour investigated the nature of yeast and its involvement in alcohol fermentation. His findings supported the hypothesis that fermentation was caused by a living organism (Berche et al. 2009). After additional careful studies, Louis Pasteur reported the presence of some vital force, referred to as a "fermenter," which was responsible for fermentation, thus providing a strong foundation for the vitalistic protoplasm theory (Dubos 1951).

Like most notable chemists of the time, Stahl and Liebig initially accepted the existence of a "vital force" but subsequently introduced the concept of the inorganic world, in contrast to the "vital force," in the 1850s (Goujon 2001). In their era, a clear distinction was made between organized and unorganized ferment claim to be involved in the biological process. In 1858, Moritz Traube, a student of Liebig, clearly defined this entity as a specific protein-like compound, which catalyzes all cellular activity, including fermentation, respiration, and putrefaction; later, he proposed the theory of enzyme/substrate interaction that occurs inside the cell (Rollin et al. 2013; Renneberg 2008). In 1878, Kühne introduced, for the first time, the term "enzyme," meaning "in yeast," for both the organized and unorganized ferment (Whitehurst and Oort 2009; Stead 1987). The suffix "*ase*," most commonly used as the suffix in enzyme names, was proposed by Duclaux and became the standard for enzyme nomenclature in 1883; this suffix generally indicates the substrate on which the enzyme acts (Traut 2008). The concept of the lock-and-key model was proposed by Emil Fischer in 1894 to account for the high degree of specificity of an enzyme toward a particular substrate (Segel 1993).

Moritz Traube isolated an endogenous enzyme in its active form from potatoes and proved that an enzyme from a plant extract was able to catalyze a reaction in vitro. In 1897, the concept of a cell-free enzyme system, proposed by two brothers, Hans and Edouard Buchner, provided a solid and logical background to refute Pasture's assertion of "vital force" and Liebig's "organic world" (Segel 1993). The same year, Gabriel Betrand proposed the role of heat-stable, dialyzable material, which he called "*coenzymes*," in cell-free glucose fermentation (Semenza and Turner 2005). In 1902, Henri derived a mathematical equation for the effects of substrate concentration on the enzymatic reaction rate, and Sorenson described the effects of pH on enzyme activity in 1909 (Kuby 1990). The industrial use of endogenous enzymes began in 1913, when German chemist Otto Röhm evaluated the specific activity of pancreatic trypsin for the removal of proteinaceous stains from cloth (Aehle 2007). The same year, Michaelis and Menten reestablished the equation derived by Henri and further evaluated this equation after confirming careful control of pH. The Henri-Michaelis-Menten

equation is based on the chemical equilibrium of enzyme kinetics (Kuby 1990). In 1925, Briggs and Haldane explained the steady-state concept of enzyme kinetics and modified the Michaelis-Menten equation with the more general valid assumptions of the steady state (Palmer and Bonner 2007). Today, both approaches are still applied to enzyme kinetics. In 1926, Summer obtained the enzyme urease in crystalline form and announced it to be a simple protein (Sumner 1948). However, the crystalline forms of several other enzymes were not established until the 1930s, when Northrop and his fellows crystallized pepsin, trypsin, and chymotrypsin as pure crystals (Segel 1993). In the 1940s and 1950s, hundreds of new enzymes were discovered, purified, and crystallized (Segel 1993). In 1955, the complete sequence of amino acids of insulin was reported by Sanger (Sanger 1960). In 1960, ribonuclease was sequenced, and the first chemical synthesis of an enzyme (ribonuclease) was achieved in 1969 (Segel 1993). In 1957, Kendrew discovered the three-dimensional geometry of myoglobin through X-ray diffraction analysis (Kendrew 1963). In 1963, the primary structures of two enzymes, pancreatic ribonuclease and egg white lysozyme, were reported by Smyth et al. and Canfield as containing 120 amino acids, further supporting the protein-like nature of these enzymes (Smyth et al. 1963; Canfield 1963). In 1965, the isolation of *Taq* polymerase from *Thermus aquaticus* by Thomas D. Brock set the foundation of a new era in molecular biology and biotechnology (Rollin et al. 2013). Heat-shock proteins (HSPs), identified by Tissieres et al. in 1974, are group of polypeptides expressed under stress conditions that play an important role in the stability of endogenous enzymes under stress conditions (Tissieres et al. 1974).

Currently, the most common definition of an enzyme is "a protein with catalytic activity based on the specific activation of its substrate." However, with the discovery of other catalytic biomolecules, such as catalytic RNA, the identification of ribozymes in 1982 began a new debate regarding the definition of the enzyme (Yonaha and Soda 2007). The efficiency of an enzymatic reaction was increased to a certain extent by using different advanced techniques, including immobilization, in the early 1980s (Rollin et al. 2013). In contrast to cellular extracts, a reconstituted cell-free enzyme system was employed by Welch and Scopes in 1985 for the production of bioethanol from a glucose solution. Compared to the Buchner system, the Welch and Scopes reconstituted cell-free system was evaluated as an effective system for bioethanol production, characterized by near-100 % efficiency, which was quite an achievement (Welch and Scopes 1985).

The concept of the reconstituted cell-free system, as proposed by Welch and Scopes, was the beginning of studying the activities of enzymes in a sequential manner, similar to the methods for studying particular metabolic pathways. Later, this concept was further extended to include the production of certain others biocommodities, including biofuels, biochemicals, and food (You and Zhang 2013). Endogenous enzyme-based biotransformation systems have become an interesting tool for the production of certain other high-value biocommodities. Using a combination of selective endogenous enzymes as a single biosystem for the biotransformation of substrate into product has advantages over whole-cell systems; for example, this method is highly targeted and generally much more efficient. The best example is the cell-free enzyme system developed by Khattak et al. (2014); this system consists of both glycolytic and fermentation enzymes and can be used for bioethanol production at elevated temperatures at which conventional fermentation is impossible (Khattak et al. 2014).

3 Types of EHEs

Each cell contains genetic information encoding thousands of different endogenous enzymes, and their expression and activity in specific cell compartments usually determine which of the many

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Table 1 Major classes of hydrolyzing enzymes involved in the hydrolysis of longer chain into their respective constitutive subunits

Substrate	Class	Enzyme	Reference
Cell wall	1	Endoglucanase	Khattak et al. 2012
		Xylanase	Khattak et al. 2012
		Arabinofuranosidase	Khattak et al. 2012
		Feruloyl esterase	Khattak et al. 2012
		Acetyl xylan esterase	Khattak et al. 2012
		Carboxypeptidase	Khattak et al. 2012
		Exo-β-glucanase	Khattak et al. 2012
		β-Glucosidases	Khattak et al. 2012
		Glucanase	Khattak et al. 2012
Starch	2	α-Amylase	Khattak et al. 2012
		β-Amylase	Khattak et al. 2012
		Limit dextrinase	Khattak et al. 2012
		α-Glucosidase	Khattak et al. 2012
Cellulose	3	Cellobiohydrolases	Aro et al. 2005
		Endoglucanase	Aro et al. 2005
		β-Glucosidases	Aro et al. 2005
Protein	4	Endopeptidase	Khattak et al. 2012
		Carboxypeptidase	Khattak et al. 2012
		Proteases	Khattak et al. 2012
		Lipid transfer proteins	Khattak et al. 2012
Lipids	5	Lipase	Khattak et al. 2012
		Lipoxygenase	Khattak et al. 2012
		Hydroperoxide lyase	Khattak et al. 2012
		Hydroperoxide isomerase	Khattak et al. 2012
		Hydrase	Khattak et al. 2012
Nucleic acid	6	Exoribonucleases	Mishra 1995
		Exodeoxyribonucleases	Mishra 1995
		Endoribonucleases	Mishra 1995
		Endodeoxyribinucleases	Mishra 1995
		Restriction endonucleases	Mishra 1995
		Damage-specific deoxyribonucleases	Mishra 1995
		Topoisomerases	Mishra 1995
		Recombinases	Mishra 1995
Phytin	7	Phytase	Khattak et al. 2012
Polyphenol	8	Peroxidase	Khattak et al. 2012
Thiols	9	Thiol oxidase	Khattak et al. 2012

possible biological reactions take place within the cell. Enzyme expression is either induced within the cell or supplied exogenously under definite circumstances. It is critical to understand the characteristics of the enzyme and its specificity to the available substrate before application in a specific biological process. Known EHEs are classified based on substrate specificity into the different categories shown in Table 1.

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3.1 Starch-Hydrolyzing Enzymes

Starch, the most common storage carbohydrate in plant, is made up of D-glucopyranose linked together via α-(1→4) and α-(1→6) glycosidic bonds. Structurally, starch consists of 20–25 % amylase, a linear and helical molecule, and 75–80 % amylopectin, a branched molecule. Amylopectin provides the starch with a greater tendency to resist enzymatic hydrolysis because the residues involved in α-(1→6) glycosidic branch points constitute 4–6 % of the glucose present. Although most of the hydrolytic enzymes are specific for α-(1→4) glycosidic linkages, α-(1→6) glycosidic bonds must also be cleaved for complete hydrolysis of amylopectin to glucose. The most recently developed processes carry out the hydrolysis of starch in three steps, involving gelatinization, liquefaction, and saccharification. In gelatinization, nanogram-sized starch granules are dissolved in water or some other liquid to form a suspension. This is followed by liquefaction, which involves partial hydrolysis of starch through enzymes. Finally, glucose and maltose are formed through saccharification by enzymes.

Malt contains several starch-hydrolyzing enzymes, such as α-amylase, β-amylase, limit dextrinase, and β-glucosidase (MacGregor 1987; Bamforth 2009). Alcohol precipitates of malt extracts (known as diastase), which liquefy starch into simple sugars (Payen and Persoz 1833), have two catalytic units (Maercker 1878) classified as α-amylase (or dextrinogen) and β-amylase (or saccharogen) (Ohlsson 1926). α-Amylases from different sources, such as *Bacillus amyloliquefaciens*, *B. licheniformis*, *A. oryzae*, *A. niger*, and *B. subtilis*, act as endo-acting enzymes that randomly cleave α-(1→4) glycosidic linkages to yield α-dextrins and maltose. These molecules play an important role in the metabolism of starches and are usually produced in the beginning of the malting process, after which they are moved to and stored in the mature endosperm of seeds (Svensson 1994). β-Amylase from malted barley is a typical exo-acting enzyme that cleaves the α-(1→4) glycosidic linkage from nonreducing ends, yielding limit dextrins, β-glucose, and β-maltose (Hoseney 1994; Svensson 1994). Glucosidases also function as exo-acting enzymes and cleave α-(1→4) glycosidic linkages in starch. Compared to β-amylase, glucosidase has the ability to bypass the side chain at α-(1→6) bonds. Glucoamylase from *A. niger* cleaves α-(1→4) and α-(1→6) glycosidic linkages from nonreducing ends to yield β-glucose. Pullulanase from *B. acidopullulyticus* cleaves α-(1→6) glycosidic linkages to yield straight-chain maltodextrins. Limit dextrinase cleaves amylopectin α-(1→6) linkages of branched dextrins to complete the hydrolysis of starch into its monomeric sugars at the end of the starch-hydrolyzing reaction (Bowles 1996).

3.2 Cellulose-Hydrolyzing Enzymes

Cellulose is an organic compound that constitutes the major portion of plant cell walls and vegetable fibers, such as cotton, ramie, and wood. It is a highly stable, water-insoluble polysaccharide consisting of chains of glucose monomers. The molecular formula of cellulose is $(C_6H_{10}O_5)_n$, producing an unbranched homopolysaccharide composed of α-D-glucopyranose units linked by (1→4) glycosidic bonds (Purves 1954). It is the most abundant biopolymer on earth and is produced at a rate of about 1×10^{11} tons per year. The enzymes responsible for cellulose hydrolysis are referred as cellulases.

Cellulases are *O*-glucoside hydrolases (GHs) that hydrolyze the β-1,4 linkages of cellulose. These enzymes are predominantly found among prokaryotes and fungi (Hildén and Johansson 2004). Several fungal species are known to produce cellulases with known catalytic abilities (Cunningham and López 1994). Cellulase genes have also been identified in the marine yeast *Aureobasidium pullulans* and *Bursaphelenchus xylophilus* (Chi et al. 2009). GHs are classified into cellulase families based on amino acid sequence similarity. Out of the 122 families of proteins identified to

date, 14 correspond to cellulases. Most cellulases form complexes with other GHs; these complexes contain a catalytic module, a highly *O*-glycosylated linker, and a cellulose-binding module (CBM). The latter domain facilitates cellulose hydrolysis by holding the catalytic module in close proximity to its substrate. Cellulases are classified, depending on their enzymatic activity, in three major groups: exoglucanases, endoglucanases, and β-glucosidases. Exoglucanases catalyze the successive hydrolysis of residues from the reducing and nonreducing ends of the cellulose polysaccharide, releasing cellobiose molecules as the main product of the reaction (Aro et al. 2005). These enzymes account for 40–70 % of the total components of the cellulase system and are able to hydrolyze crystalline cellulose. Exoglucanases are monomeric proteins with molecular weights ranging from 50 to 65 kDa, although smaller variants (41.5 kDa) have been identified in some fungi. Exoglucanases have low levels of glycosylation (from 0 % to 12 %), their optimum pH is 4.0–5.0, and their optimum temperature varies from 37 °C to 60 °C, depending on the specific enzyme-substrate combination. Endoglucanases randomly cleave internal linkages in amorphous cellulose filaments, generating randomly sized oligosaccharides and creating new chain ends that can in turn be attacked by exoglucanases (Aro et al. 2005). These are the enzymes that initiate the cellulolytic process, randomly cleaving internal linkages at amorphous regions of the cellulose fiber and creating new reducing and nonreducing ends that are susceptible to the action of cellobiohydrolases. Endoglucanases are monomeric enzymes with molecular weights ranging from 22 to 45 kDa. Endoglucanases are not glycosylated; however, they sometimes may be conjugated with relatively low amounts of carbohydrates (from 1 % to 12 %). These enzymes function best at an optimal pH of 4.0–5.0 and temperature of 50–70 °C. β-Glucosidases hydrolyze cellobiose, releasing two molecules of glucose, and thereby provide a carbon source that is easy to metabolize. These enzymes have molecular weights ranging from 35 to 640 kDa; they can be monomeric, reaching molecular weights of approximately 100 kDa, or exist as homo-oligomers, such as in the yeast *Rhodotorula minuta*. β-Glucosidases are generally glycosylated and have an optimal pH of 3.5–5.5 and temperature of 45–75 °C. The activity of a cellulase enzyme system is much higher than the sum of the activities of its individual subunits, a phenomenon known as synergism. Cellulase systems are not just simply a conglomerate of enzymes with components from all three cellulase types, but act coordinately to efficiently hydrolyze cellulose fibers (Lynd et al. 2002).

3.3 Cell Wall-Hydrolyzing Enzymes

The plant cell wall is a highly organized network of lignocellulosic material containing cellulose and cross-linked glycans. The cell wall constitutes the outermost boundary in most cell types. It is localized outside the cell membrane and provides the inner cell material with structural support and protection. The cell wall also acts as filtering mechanism and prevents the cell from expanding. Chemically, the cell wall of starch endosperm is composed of (1→3, 1→4)-β-D-glucans (75 %), arabinoxylane (20 %), cellulose (2 %), glucomannan (2 %), and traces of acetic and ferulic acids. Aleurone cells are composed of arabinoxylane (71 %), (1→3, 1→4)-β-D-glucans (26 %), and 3 % cellulose and glucomannan (Fincher 1992; Lazaridou et al. 2008). A number of cell wall-hydrolyzing enzymes are present in malt, including endoglucanase, exoglucanase, arabinofuranosidase, esterase (Bamforth et al. 2009), carboxypeptidase (Bamforth et al. 1979; Sørensen et al. 1989), xylanase (Chithra and Muralikrishna 2008), and β-glucosidase (MacGregor 1987). The cell wall-hydrolyzing enzymes are glycosyl hydrolases, which comprise one of the two main classes of carbohydrate-active enzymes (Jamar et al. 2011).

The (1→3, 1→4)-β-D-glucans are also referred as β-glucan, a linear polysaccharide composed of an unbranched chain of D-glucose residues linked together by β-(1→4) and β-(1→3) bonding with a ratio of 3.2:1–6.6:1 (Jamar et al. 2011). Based on their solubility in water, these compounds are

classified as either soluble or insoluble (Swanston and Ellis 2002). The molecular weight of β-glucan varies from 800 to 1,220 kDa (Jin et al. 2004). β-Glucans are hydrolyzed directly by β-glucan exohydrolases into glucose or indirectly by a number of enzymes involved in removal of the outer layer of the cell wall followed by hydrolysis into glucose. Enzymes participating in the breakdown of β-glucans include (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidase (Jin et al. 2004).

Xylan is a major structural component of plant cell walls and is the second most abundant renewable polysaccharide in nature (after cellulose) (Collins et al. 2005). Xylan interacts with lignin and cellulose via covalent and noncovalent linkages; these interactions are important for both protecting the cellulose microfibrils against biodegradation and maintaining the structural integrity of cell walls. Due to its heterogeneity and complex nature, the complete breakdown of xylan requires the action of a variety of hydrolytic enzymes (Biely 1985; Coughlan and Hazlewood 1993). Degradation of the xylose backbone depends on xylanases, which cleave bonds within the polymer, and β-xylosidases, which release xylose units from xylobiose and xylooligomers. Four enzymes, endo-β-(1→4)-xylanase, exoxylanase, β-xylosidase, and α-arabinofuranosidase, are involved in the hydrolysis of arabinoxylan (Hrmova et al. 1997). Although exoxylanase cleaves the outer β-(1→4) xylosidic linkages, endo-β-(1→4)-xylanase attacks inner β-(1→4) xylosidic linkages in arabinoxylan polymers, separating the arabinofuranosyl residues (Egi et al. 2004). β-Xylosidase catalyzes the hydrolysis of β-(1→4) xylosidic bonding within xylo-oligosaccharides, while arabinofuranosidase cleaves the α-(1→2) and α-(1→3) linkage formed between arabinofuranose units.

3.4 Protein-Hydrolyzing Enzymes

Proteins are large biological molecules consisting of one or more chains of amino acid residues. Proteins perform several principle functions, such as catalyzing metabolic reactions, facilitating the replication of DNA, mediating stimulus responses, participating in transport, and maintaining the structures of different organelles in the cell, including the nucleus, nuclear membrane, and cell membrane. Chemically, the building blocks of proteins (i.e., amino acids) are linked together through peptide bonds. These bonds are hydrolyzed by a group of enzymes designated as peptidases, which account for about 2 % of genes within the genomes of various organisms. Peptidases are important for many biological processes, including digestion of food proteins, recycling of intracellular proteins, the blood coagulation cascade, antigen presentation, and activation of a variety of proteins, such as enzymes, peptide hormones, and neurotransmitters.

Certain proteolytic enzymes synthesized during germination are involved in the production of free amino nitrogen (FAN) and di- and tri-amino acids through their synergistic action. These enzymes can provide a greater exposed surface area for starch-hydrolyzing enzymes (Briggs 1998). All enzymes share the same substrate (polypeptides), but have different catalytic sites. The product is a modified protein that ultimately results in seed softening and increased friability being required for the effective growth of seedlings (Wentz et al. 2004). Some of the enzymes involved in protein modification already exist in mature barley grain, while some are synthesized de novo in aleurone cells during germination (Briggs 1992). Endopeptidases and exopeptidases are two principal groups of malt proteolytic enzymes. Endopeptidases cleave the bonds between two amino acids in a protein molecule at random, producing relatively smaller peptide chains, while exopeptidases attack these smaller peptide chains and cleave the links between terminal amino acids. Therefore, exopeptidase activity ultimately leads to protein molecules being hydrolyzed into FAN or di-/tri-amino acids. Endopeptidases function at a lower optimum temperature; thus, most of these enzymes are degraded during the malting process. However, exopeptidases can withstand high

temperatures and complete the protein hydrolysis process. Exopeptidases consist of two major enzymes: carboxypeptidase and aminopeptidase, both of which have different active sites. Specifically, carboxypeptidase has the ability to hydrolyze protein molecules from the carboxyl end, while aminopeptidase functions from the amino end (Lalor and Goode 2010).

3.5 Lipid-Hydrolyzing Enzymes

Lipids are a group of naturally occurring, small, hydrophobic, or amphiphilic molecules, including fats, waxes, sterols, fat-soluble vitamins, monoglycerides, triglycerides, phospholipids, and others. The amphiphilic nature of some lipids allows them to form structures, such as vesicles, liposomes, or membranes, in an aqueous environment. The principle biological functions of lipids include acting as a natural food reservoir (natural glycosides), storing energy, providing a source of hydrocarbons (waxes), functioning as a cofactor in intermediate metabolism (quinines) and structural component of cells membranes (polar glycerides), and participating in signaling (Subramaniam et al. 2011). Grain lipids consist of 65–78 % natural lipids, 7–13 % glycolipids, and 15–26 % phospholipids (Briggs 1978). Lipids in cereal grains are associated with starch granules on their surface or within the starch structure (Morrison 1988). The lipids inside starch granules alter the gelatinization properties of starch granules (Briggs et al. 1981). The hydrolysis of numerous biopolymers is governed by respective hydrolyzing enzymes in the initial stage of germination during malting. Commonly used lipid-hydrolyzing enzymes include lipases (Baxter 1982), lipoxygenases (Antrobus et al. 1997), hydroperoxide lyases, and hydroperoxide isomerases (Bamforth 2009; Bamforth et al. 2009).

Lipases are hydrolyzing enzymes that catalyze esters of long-chain aliphatic fatty acids to produce free fatty acids and glycerol. Their natural substrates are insoluble lipid compounds prone to aggregation in aqueous solution. In eukaryotes, lipases may be confined within an organelle (i.e., the lysosome) or can be found in the spaces outside cells, playing roles in the metabolism, absorption, and transport of lipids. In lower eukaryotes and bacteria, lipases can be localized either intracellularly or can be secreted, functioning to degrade lipid substrates present in the environment. Moreover, in some pathogenic organisms (*Candida albicans*, *Staphylococcus* and *Pseudomonas* species, and *Helicobacter pylori*), lipases can even act as virulence factors. Their ability to hydrolyze insoluble fatty acyl esters makes lipases different from all other esterases. Cereals contain storage fats or oils called lipid bodies, which are hydrolyzed into free fatty acids by lipase. The hydrolysis of lipid bodies is directly related to the concentration of lipase in the aleurones and scutellums of grains, including both preexisting and newly synthesized lipases, in response to physical and chemical changes (Huang 1992). Free fatty acids produced by triglyceride hydrolysis are readily utilized as metabolic energy in the synthesis of glucose when grains containing soluble sugars are depleted or as precursors for phospholipid synthesis, which is involved in the growth of the cell membrane. In malt, the enzyme lipoxygenase can hydrolyze the dioxygenation of polyunsaturated fatty acids (linoleic and linolenic acids) into hydroperoxy acids. This hydrolyzed product is used as a precursor for the production of the stale testing aldehydes used in the wine brewing industry. Barley genetic material encodes two types of lipoxygenases secreted in embryonic tissues, LOX-1 and LOX-2. LOX-1 is already present in raw barley, and its expression increases with germination. In contrast, LOX-2 is only expressed during germination of barley grain (de-Almeida et al. 2005). Lipoxygenase is heat sensitive and loses most of its activity (96–98 %) during the kilning process. Even with the dramatic reduction in hydrolyzing activity after the kilning process, almost 30 % of malt lipids are hydrolyzed by lipoxygenase during mashing. This enzyme also plays a crucial role in senescence, wound healing, infection, and resistance against pests. Hydroperoxide lyase (HPL), which is very common in plants, is known for catalysis of the hydrolysis reaction of fatty acid hydroperoxides into

oxo-acids and aldehydes. HPL is a member of the cytochrome P450 family, which plays a major role in phyto-oxylipin synthesis resulting from interactions among plant herbivores. HPLs can be divided into two groups based on substrate activity: 13-HPL (CYP74B) and 9-/13-HPL (CYP74C). Because it can change the constituents of volatile aldehydes, HPL plays an important role in determination of the characteristics of food products. The hydroxyperoxide isomerase catalyzes the conversion of hydroperoxylinoleic acid into α- or β-ketols. The localization of HPL expression changes with germination, and the enzyme is only expressed in the embryo in its dormant form during early development.

3.6 Nucleic Acid-Hydrolyzing Enzymes

Nucleic acids are the polymeric biological macromolecules essential for all known forms of life. Nucleic acids, such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are made up of basic repeating units called nucleotides. Each nucleotide contains three essential components: a nitrogenous base, a phosphate group, and a five-carbon sugar molecule. Each cell of a living organism contains nucleic acids in the nucleus and in some other organelles, such as mitochondria and chloroplasts (in plants only); these nucleic acids function to encode, transmit, and express genetic information. The building blocks of nucleic acids (i.e., nucleotides) are synthesized enzymatically (5′–3′ direction) and chemically (3′–5′ direction). During synthesis, the nucleotides are sequentially added to the growing oligonucleotide chain. Different types of oligonucleotides, such as antisense oligonucleotides, small-interfering RNA, primers (for DNA sequencing and amplification), and probes (for detecting the complementary DNA or RNA via molecular hybridization technique), have several applications in a variety of biological fields and act as tools for the targeted introduction of mutations and restriction sites and for the synthesis of artificial genes or gene fragments.

Several nucleic acid-hydrolyzing enzymes are used in genetic engineering approaches; these include restriction endonucleases, nucleases, alkaline phosphatases, and RNase P. Among these, restriction enzymes or, more specifically, restriction endonucleases (REases) are the principle nucleic acid-hydrolyzing enzymes. These produce discrete DNA fragments upon cleavage, which serves as a useful feature for analyzing and recombining the DNA molecules from different origins. These characteristics have led to the development of various genetic engineering techniques and the search for additional enzymes with similar functions. REases recognize a unique short sequence (four to eight base pairs) in the DNA fragment and produce a nick at the target site (type II), vicinity (type I), or away from the site of attachment (type III), depending upon the type of enzyme. This process produces fragments with sticky ends or blunt ends. Several applications of restriction enzymes have been reported, including manipulation of DNA, insertion of genes into plasmids, distinguishing between gene alleles, digestion of genomic DNA for gene analysis, etc. Nucleases are enzymes that cleave DNA or RNA (called DNases and RNases, respectively). DNases are further classified into exonucleases, which act on the ends or terminal regions of DNA, and endonucleases, which act at nonspecific regions in the center of DNA. Exonucleases require at least two 5′ and 3′ ends and thus cannot act on circular DNA, such as plasmids. However, endonucleases can act on both linear and circular DNA molecules. Exonucleases release single nucleotides, while endonucleases produce short DNA segments. Phosphatases remove the phosphate from DNA, RNA, and proteins. Most of these enzymes act in basic buffers and are known as alkaline phosphatases. Three common types of alkaline phosphatases used in molecular cloning experiments are bacterial alkaline phosphatases (BAPs), calf intestine alkaline phosphatases (CIAPs), and shrimp alkaline phosphatases. Alkaline phosphatases are metalloenzymes containing Zn^{2+}. RNase P, which specifically

cleaves at the 5′ end of RNA, is a complex ribozyme containing a 20-kDa protein and a 377-nucleotide RNA molecule possessing at least part of the enzymatic activity of the complex.

3.7 Other Hydrolyzing Enzymes

Besides the above-described enzymes, several other enzymes also catalyze hydrolytic reactions; these include glucose oxidase, galactose oxidase, cholesterol oxidases, catalases, phytase, peroxidase, and thiol oxidase. Glucose oxidase is a flavoprotein that catalyzes the oxidation of β-D-glucose by O_2 to δ-gluconolactone and H_2O_2. Galactose oxidase is a type II (non-blue) copper protein with a molecular mass of 69 kDa that catalyzes the oxidation of a wide range of primary alcohols and polysaccharides (e.g., D-galactose) to the corresponding aldehydes. Cholesterol oxidases are bifunctional flavoenzymes that catalyze two reactions at a single active site. The first is the oxidation of cholesterol to cholest-5-ene-3-one and the isomerization of the labile cholest-5-ene-3-one intermediate to a cholest-4-ene-3-one product. Catalases or hydroperoxidases catalyze the degradation of H_2O_2 to H_2O and O_2. Phytase is involved in the hydrolysis of phytate into phytic acid, inorganic phosphate, and myoinositol during the malting process and thus decreases its total contents in the final product. Two types of phytases, 3- and 6-phytase, are known for their dephosphorylation of phytate. 3-Phytase is found in microbial species, while 6-phytase is primarily found in seeds of higher plants, including barley. Thiol oxidase belongs to the family of oxidoreductases that specifically act on the sulfur group of donors, with oxygen as the acceptor.

4 Isolation of EHEs

As mentioned earlier, enzyme-catalyzed reactions require different types of enzymes either supplied externally or induced within the biological system. Different types of EHEs have been isolated from different sources at various states of purity before being fully characterized. However, partially purified EHEs isolated from different sources tend to be analyzed more completely for elucidation of their inhibition characteristics, substrate specificity, and interaction with activators molecules during the initial characterization. The conclusions from all these preliminary studies will guide us in understanding the mechanisms through which these proteins are controlled under different conditions. Therefore, it is important to understand the basic features of techniques most commonly used for the isolation of EHEs from different sources.

4.1 Occurrence/Sources of EHEs

Malt, comprised of germinated cereal grains that have been dried in a process known as “malting,” is considered an enriched source of EHEs (Khattak et al. 2012). During the malting process, cereal grains (mostly often wheat or barley) induce signals for the expression of different types of EHEs, including cell wall-hydrolyzing enzymes, starch-hydrolyzing enzymes, proteases, lipases, and phytases (Fig. 1) that mediate the internal physiological changes of seeds. Soaking of seed results in scutellum-induced gibberellin, which functions as a signal for the expression of starch-hydrolyzing enzymes; this is thought to be the first change in seeds that leads to the production of monosaccharides, including glucose and fructose, as well as disaccharides essential for the initial growth and development of the seed (Khattak et al. 2012). During malting, the expression of proteases involved in the hydrolysis of long-chain peptides into short-chain peptides and amino acids is also essential for seed growth (Celus et al. 2006). The expression of endogenous enzymes inside the seed is primarily involved in the hydrolysis of three major components of the grain: starch, protein, and cell wall polysaccharides (Hayes et al. 2003).

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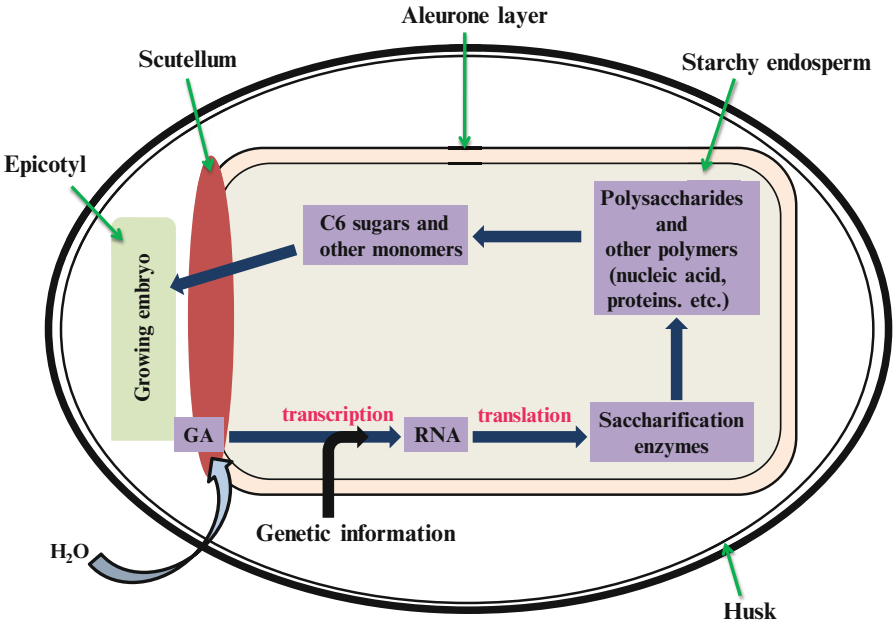

Fig. 1 The generalized scheme representation of the EHEs production in barley grain in response to external stimuli induced during malting process

A number of cell wall-hydrolyzing enzymes, including endoglucanase, exoglucanase, arabinofuranosidase, esterase (Bamforth 2009), carboxypeptidase (Bamforth et al. 1979; Sørensen et al. 1989), xylanase (Bamforth 2009; Chithra and Muralikrishna 2008), and β-glucosidase (MacGregor 1987), have been reported to be expressed and obtained from malt. In microorganisms, fungi in particular are often studied with respect to the expression and isolation of endogenous cell wall-hydrolyzing enzymes (Aro et al. 2005; Hasegawa and Nordin 1969). Antagonistic fungi use cell wall-hydrolyzing enzymes as weapons to attack their target fungi and thus maintain stability (Aro et al. 2005). *Trichoderma* and *Aspergilli*, two industrial fungal strains, are capable of producing abundant amounts of cell wall-hydrolyzing enzymes (Durand et al. 1988; Berka et al. 1991). In the case of bacteria, *Streptomyces* strains produce cell wall-hydrolyzing enzymes capable of digesting yeast cell walls (Bacon et al. 1968). Considering the above discussion, we can conclude that endogenous cell wall-hydrolyzing enzymes can be isolated from both plants and microorganisms depending on the nature of the substrate/host.

Malt extract is also a major source of starch-hydrolyzing enzymes. Payen and Persoz (1833) found that precipitates obtained after treatment of malt extract with alcohol have the potential to liquefy starch polysaccharides into glucose (Payen and Persoz 1833). At the time of their study, malt extract was called *diastase*; the functional enzymes within the extract were later classified as α-amylase and β-amylase based on their different activities (Maercker 1878). Similarly, the existence and isolation of other starch-hydrolyzing enzymes, including limit dextrinase and β-glucosidase from malt extract, has also been reported. In recent studies, the activities of starch-hydrolyzing enzymes have been reported in agro-industrial wastes (e.g., wastes from beer fermentation broth [WBFB]), which is thought to be an enriched source of saccharification enzymes (Khattak et al. 2013a, b). Germinated barley seeds possessing abundant quantities of starch-hydrolyzing enzymes have also been processed for enzyme isolation (Sun and Henson 1990). Certain microorganisms, including thermophilic *Archaea*, bacteria (Bertoldo and Antranikian 2002), and yeast (Kelly et al. 1985), have been used for isolation of starch-hydrolyzing enzymes.

Cellulose-hydrolyzing enzymes play a pivotal role in a variety of biotechnologies, particularly biofuel production. Similar to cell wall- and starch-hydrolyzing enzymes, malt has been shown to be an enriched source of cellulose-hydrolyzing enzymes (Khattak et al. 2012). In microorganisms, fungi (Nevalainen and Palva 1978), algae (Mohapatra et al. 2003), and bacteria (Han and Srinivasan 1968) are common sources of endogenous cellulase enzymes, which can be extracted following incubation of the microorganism in its respective media.

Peptidase enzymes are encoded by about 20 % of genes in all types living organisms and play an important role in the viability of living creatures. Germinated seeds are an economical and feasible source of such EHEs. A number of proteolytic enzymes such as endopeptidases and carboxypeptidases are synthesized during seed germination. Drzymała and Bielawski (2009) also reported the isolation of endogenous carboxypeptidase from germinated triticale grains (Drzymała and Bielawski 2009). Similarly, the isolation of endogenous proteolytic enzymes from others plant sources, including barley (Sørensen et al. 1989), wheat (Baulcombe et al. 1987), and triticale (Drzymała et al. 2008), has also been reported. Microbial sources of endogenous proteolytic hydrolyzing enzymes include bacteria (Fujimura and Nakamura 1981; Pacaud et al. 1976), fungi (Kamath et al. 2010), and algae (Matsubara et al. 2000). Certain agro-industrial waste materials, including WBFB (Khattak et al. 2012), cassava wastewater (Barros et al. 2013), slaughterhouse waste, dairy industry effluent, and sewage waste (Boominadhan et al. 2009), have been utilized for the production of proteolytic enzymes through microbial species.

Lipid-hydrolyzing enzymes have been obtained from different sources, including plants, animals, and microorganisms. Based on statistical data, different organisms, including bacteria (45 %), fungi (21 %), animals (18 %), plants (11 %), and algae (3 %), contribute to total lipase production with varying degrees (Patil et al. 2011). Among microbes, fungal strains have always been considered preferable because they usually excrete endogenous enzymes, allowing for simple and inexpensive isolation of enzymes from fermentation broth (Maia et al. 1999). Agro-industrial wastes (e.g., WBFB, waste from the fish industry, etc.) are composed of complex polysaccharides that can be exploited as economically feasible substrates for microbial growth and are responsible for endogenous enzyme production (Khattak et al. 2012; Rebah and Miled 2013). The availability and cost of these alternative media (agro-industrial waste) are the main determinants of the economics of the enzyme production process. Cereals crops (e.g., malt, wheat, and barley) can be processed for the production and isolation of endogenous lipase enzymes (Khattak et al. 2012). The lipase activity in cereal crops increases significantly, particularly during the initial stage of germination. The requirements for structural carbons and energy to initiate seed growth are met through the hydrolysis of stored food (insoluble tri-acylglycerols) by lipase (Pahoja and Sethar 2002).

Nucleic acid-hydrolyzing enzymes play an important role in certain biological processes. Complete hydrolysis of nucleic acids yields inorganic phosphate, deoxyribose sugar, and four different heterocyclic bases. As mentioned earlier, different types of nucleic acid-hydrolyzing enzymes have been isolated from different sources, including microorganisms, plants, fungi, and highly developed animals. In microorganisms, different bacterial strains (e.g., *Bacillus*, *Brevibacillus*, *Microbacterium*, *Pseudomonas*, and *Stenotrophomonas*) and fungi (e.g., *Alternaria*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Fusarium*, *Gliomastix*, *Humicola*, *Penicillium*, *Scopulariopsis*, *Wardomyces*, and *Periconia*) are commonly used (Balestrazzi et al. 2007; Balabanova et al. 2012). Roberts (2005) reported that more than 3,000 restriction enzymes with over 250 different specificities have been isolated from various organisms (Roberts 2005). These enzymes are thought to have evolved from a common ancestor and likely became widespread via horizontal gene transfer (Jeltsch et al. 1995; Jeltsch and Pinground 1996). Evidence also exists for their evolution as self-genetic elements (Naito et al. 1995). Isolation of restriction nucleases has been reported from

Escherichia coli (Linn and Arber 1968). Alkaline phosphatases are commonly found in the periplasmic space, external to the cell membrane in gram-negative bacteria, and are usually produced during phosphate starvation (Horiuchi et al. 1959). Additionally, RNase P has been described in several bacterial strains (Evans et al. 2006), Archaea (Hall and Brown 2002), and eukaryotes, such as yeast (Marquez et al. 2006).

Besides the abovementioned enzymes, several other enzymes have also been reported from different sources. Industrial-scale production of glucose oxidase is mainly carried out using *Aspergillus niger* or *Penicillium amagasakiense* (Crueger and Crueger 1990). Moreover, other *Penicillium* and *Aspergillus* sp., including *Penicillium notatum*, *Penicillium funiculosum*, *Penicillium piceum*, *Penicillium purpurogenum*, *Penicillium variabile*, *Penicillium chrysogenum*, and *A. fumaricus*, are also attractive candidates for industrial production of this enzyme (Leiter et al. 2004). Different fungal species, e.g., *Fusarium dendroides* (*Dactylium dendroides*), *Gibberella fujikuroi*, and *G. zeae*, as well as the basidiomycete *Polyporus circinatus*, are widely used for industrial-scale galactose oxidase production. Genes encoding galactose oxidase can also be expressed efficiently in either *Pichia pastoris* (Whittaker and Whittaker 2000) or *A. oryzae* (Xu et al. 2000). Cholesterol oxidases are produced by bacteria, including species from the *Rhodococcus*, *Streptomyces*, and *Nocardia* genera. However, several *Brevibacterium*, *Proactinomyces*, *Pseudomonas*, and *Cellulomonas* species also possess enzymes with cholesterol oxidase activity and are suitable candidates for industrial production of this enzyme (Watanabe et al. 1986). Recombinant *E. coli* strains expressing bacterial cholesterol oxidase genes have been reported (Sakka et al. 1994). Different microorganisms, including bacteria (e.g., *Micrococcus*, *Bacillus*, *Microscilla*, and *Alcaligenes* spp.), fungi (e.g., *Aspergillus*, *Penicillium*, *Thermomyces*, *Thermoascus*, and *Acremonium* spp.), and yeasts (e.g., *Saccharomyces*, *Candida*, and *Mycotorula* spp.), are known for their abundant production of catalases. However, catalases from animal sources (e.g., bovine liver) are generally inexpensive, and thus, their production from microbial sources is only economical when better producer strains (preferably recombinant strains) and inexpensive technology can be used or when enzymes with special properties (e.g., functional at high or low temperatures or at alkaline or acidic pH) are produced (Takeuchi and Isobe 1999).

4.2 Methods of Cell Lysis

Obtaining endogenous enzymes from respective organisms without rupturing their cell envelopes is a difficult task. However, certain organisms excrete endogenous enzymes into the surrounding microenvironment for a variety of purposes, most commonly for cell defense. The first attempt to obtain cellular contents, specifically the proteinaceous content of the cell, was made in microbial cells, with attempts to rupture the outer cell envelope by prolonged exposure to water, followed by heating in glycerin solutions. The method was successful only in that it allowed the researchers to obtain fractions of cellular contents; however, most of the contents were altered (Buchner 1966). Over time, a number of specialized, highly efficient techniques have been reported for the rupture of enveloped cells. These techniques are briefly described below.

4.2.1 High-Pressure Homogenizer

High-pressure homogenization was the first successful cell lysis method for obtaining endogenous enzymes from yeast cells. A hydraulic press supplied by Brink and Hubner was used for applying higher pressure to cells to squeeze out the cell contents. Using this mechanical lysis method, almost 1,000 g of yeast was squeezed for a few hours, resulting in about 500 mL of liquid solution that was yellowish brown in color and contained endogenous enzymes (Buchner 1897). Dilution of the yellowish brown solution with water and hydrogen peroxide led to formation of oxygen, inducing

violent foaming. Additionally, researchers concluded that the presence of catalase in the solution catalyzed the oxygen reaction. Later studies demonstrated that mixing the yellowish solution with concentrated glucose solution produced bubbles in the form of a thick layer of foam over the surface, clarifying the mechanism of carbon dioxide formation as result of a cell-free fermentation process (Buchner 1966).

4.2.2 Chemical Lysis

Chemical lysis is a well-established method for obtaining endogenous enzymes, particularly from microbial cells (Arnold 1972). Freshly prepared microbial cultures are incubated in organic solvent at room temperature for few days in order to permeabilize the microbial membrane. Addition of organic solvent, primarily toluene, induces the expression of β-fructofuranosidase, classified as a cell wall enzyme; the secretion of this enzyme primarily depends on the action of cell wall-modifying enzymes (endogenous enzymes). The addition of organic solvents (e.g., toluene and ammonium hydroxide) creates hypertonic conditions, resulting in the alteration of the membrane composition, which subsequently activates the EHEs located within the cytoplasm (Arnold 1972). Although the method is well established, certain limitations are associated with chemical lysis, particular the stability of the endogenous enzymes in the presence of endogenous peptidases (Boonraeng et al. 2000).

4.2.3 Enzymatic Lysis

Enzymatic lysis is another approach for the isolation of EHEs that has been used with a great degree of success (Salazar and Asenjo 2007). In enzymatic lysis, specific cell wall-hydrolyzing enzymes are used to digest the cell envelope. The microbial cell wall (e.g., that of yeast) is a highly dynamic structure composed of mannoprotein and fibrous β(1→3) glucans with a few β(1→6) glucan branches. The β(1→6) glucans make connections between the inner and outer cellular components of the yeast cell (Lipke and Ovalle 1998). A number of enzymes, including β(1→3) glucanase, proteases, β(1→6) glucanase, mannanase, and chitinase, can be applied directly in groups and act synergistically to digest the outer cell wall (Salazar and Asenjo 2007). This approach has a major limitation: cellular proteins released into the medium after cell wall digestion are subjected to the activity of the protease.

4.2.4 Freeze-Thawing

Rapid freeze-thawing is an uncommon method used for lysis of the yeast cell wall. A number of traditional methods, mostly mechanical methods, have been used to disrupt the cell wall; however, there are few inherent disadvantages to using these methods. For example, heat generation within a sample is commonly encountered during application of traditional techniques for cell lysis and can cause protein denaturation or aggregation. This problem can be overcome by keeping the sample at a low temperature. Liquid nitrogen is used for rapid freezing of the targeted cells, followed by grinding using a sterilized mortar and pestle prechilled with liquid nitrogen. The freeze-thawing method causes the target cells to swell and ultimately rupture due to the formation of ice crystals during freezing and subsequent contraction during thawing. However, this process takes a long time because several cycles of freezing and thawing are required. However, freeze-thawing has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols. The fine powder obtained from this method can be stored at −80 °C, and for experimental purposes, cell lysates can be produced by the addition five volumes of buffer solution per volume of cells (Amberg et al. 2006).

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4.2.5 Ultrasonication

The process of sonication uses ultrasound pulses, i.e., high-frequency sound waves, for disruption of the cell wall. This approach to cell lysis is an alternative method that is now commonly used for rupturing the cells of bacteria, spores, and even diced tissues. The ultrasonic homogenizer is a common tool used for partial or complete disruption of the cell wall. This tool produces sound waves delivered through a vibrating probe that is immersed in the liquid cell suspension. The device consists of a piezoelectric transducer, temperature probe, horn tip, and digital system for controlling parameters, such as frequency, amplitude, and temperature. Due to the capacity for well-controlled sonication parameters and the comparatively high selectivity for cellular material release and purity, ultrasonication for disintegration of the cell wall has been described as a reliable and efficient approach (Balasundaram et al. 2009).

4.2.6 Bead Beating

Bead beating is one of the most common and frequently used approaches for obtaining cellular proteins through cell lysis. The method involves the mechanical disruption of the cell envelope of microorganisms, plants, and animals (Khattak et al. 2014). Glass beads of different sizes (0.425–0.60 mm) are used, depending on the nature of the cell, and beads are vortexed at high speeds using a vortexer. Because of the nature of this mechanical approach, localized heating within a sample can occur because of the continuous collision of beads with the wall of the container or with one another. Therefore, special measures should be taken to avoid the thermal denaturation of the cellular materials; every cycle of the agitation step must be interspersed with a cooling cycle. The beat beating procedure for cell lysis is depicted in the schematic diagram in Fig. 2. Table 2 summarizes the benefits and limitations of each approach used for the production of cell-free systems.

4.3 Separation of EHEs from Cell Lysates

Recent developments in the identification of new enzymes have also facilitated the improvement of recovery and separation methods. Following cell lysis, enzymes are separated from cells or cellular fragments and organelles. This process is rather sensitive due to the small size of microbial cells, which results in slight differences between the densities of cells and the components of the fermentation medium. Moreover, no effective techniques have been developed for recovery of all enzymes, and selection of an appropriate technique based on the type of enzyme and the specific microbial cells used will ensure better separation of enzymes from other constituents. A few of the techniques commonly used today are described below.

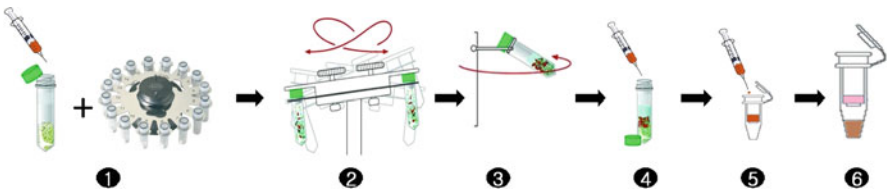

Fig. 2 Bead beating is the most common techniques employed for the cell lysis in order to obtained EHEs from cell lysate. The technique consists of (*1*) isolation of biomass from different sources in a specialized tube, (*2*) vertexing at different speed, (*3*) centrifugation and isolation of supernatant, (*4*) concentration of enzymes mixture through specialized column and obtains highly concentrated endogenous enzyme mixture

Table 2 Comparative study of different cell lysis methods employed for the rupturing of cell enveloped in order to release endogenous enzymes into the medium

Method of cell lysis	Limitations	Advantages	References
Hydraulic press	Need expensive equipment, difficulty in maintenance	Quick and ideal for large-scale lysis, effective for all cells lysis	(Buchner 1966; Goldberg 2008)
Organic solvent lysis	Possibilities of alteration in cellular materials, difficult in purification	Economical as no specialized equipment required and fast process	(Breddam and Beedfeldt 1991)
Enzymatic lysis	Can cause alteration in target protein structure, required expensive exogenous enzymes	Required nonspecialized equipment, highly specialized approach	(Lam and Wassink 1990)
Freeze grinding	Slow process, chances of contamination	Economical, obtained proteins with original activity	(Taskova et al. 2006; Yeng et al. 2013)
Centrifugation	Only effective for weal cell wall, slow process	Economically feasible	(Martz 1966; Lodish et al. 2000)
Cell bomb	Only applicable to specialized cells	Fast, ensured the protein stability and activity	(Goldberg 2008; Simpson 2011)
Ultrasonification	High ultrasound waves show negative effect on endogenous enzyme function	Really effective for large-scale cell disruption, controllable	(Liu et al. 2013; Stathopulos et al. 2004)
Beads beating	Difficulty in target proteins purification, heat produced can cause alteration in target proteins structure	Effective for all kind of cells, economical, sample, fast	(Khattak et al. 2014; Griffiths et al. 2006)

4.3.1 Filtration

The rate of filtration can be determined as a function of filter area, pressure, viscosity, and resistance offered by the filter cake medium. Generally, the flow rate becomes more uniform as the medium becomes clearer. Moreover, the cumulative filtrate volume increases linearly with time. The gradual increase in the thickness of the formed filtrate resists the flow rate across the filtration media. This limitation can be overcome to some extent by increasing the pressure applied. However, beyond certain limits, the applied pressure may cause collapsing of the filtrate cake, leading to complete blockage of the filter. The most recent developments in this area have resulted in the application of cross-flow membrane filtration and microfiltration; these techniques have unique advantages, such as enhanced efficacy, ease of operation and cleaning, improved downstream ultrafiltration purification, and, most importantly, cost-effectiveness. During the cross-flow filtration process, the input stream flows parallel to the filter area and thus prevents the accumulation of filter cake and increased resistance to filtration. Additionally, maintaining a sufficiently high filtration rate usually requires large amounts of energy in the form of high flux rates over the membranes.

4.3.2 Centrifugation

EHEs can be separated from components of lysed cells through gentle fractionation techniques, such as centrifugation; often, this method can help to preserve the functions of these enzymes. An effective and economical separation can be achieved only by sedimentation in a centrifugal field based on the molecular weight of the enzyme and the sizes of the media components. The effective operation of centrifugation technique requires a combination of high centrifugal force and short sedimentation distances. Commercially, this is achieved by using either a sieve centrifuge or a solid-wall centrifuge. Another type of centrifuge, called a decanter or scroll-type centrifuge, works with

low centrifugal forces and is specifically used for the separation of cells and protein precipitates. These types of centrifuges are feasible for continuously operating processes. Tubular-bowl centrifuges are used for separation of very small-sized particles. However, these centrifuges cannot be employed for continuous operation. Separators or disk-stack centrifuges are used for continuous removal of solids from suspensions.

4.3.3 Extraction

This technique is based on the principle of liquid-liquid extraction in an aqueous two-phase system (Hustedt et al. 1985). Cellular fragments are separated in the first step, followed by subsequent purification using other methods if greater purity is required. This may involve affinity ligand binding, modified chromatography gels, etc.

4.3.4 Flocculation and Flotation

During this process, destabilized particles are induced to come together, make contact, and subsequently form larger aggregates. Separation of cells by filtration or centrifugation is limited by the small sizes and physical properties of microbial cells and cell debris, as well as the molecular weights of the enzymes. Flocculation of cell suspensions is assisted by both filtration and centrifugation (Bajpai et al. 1991; Sitkey et al. 1992a, b). Flocculating agents are additives capable of increasing the degree of flocculation of a suspension. They can be organic or inorganic and natural or synthetic.

The flocculation of cells or enzymes by polyelectrolytes is a two-step process. In the first step, the surface charge on the suspended material is neutralized. The second step involves the linkage of these particles to form large aggregates. Flocculant selection for a specific cell separation process is a challenge because many factors can affect flocculation. These factors can have their origin in the broth (cell surface charge and size, ionic strength, pH, cell concentration, and the presence of other charged matter), the polymer (molecular weight, charge, charge density, structure, and type), and engineering parameters (mixing and mode and order of addition). The final criteria for flocculant selection should take into consideration all aspects of the flocculation process. These include the cost of the added flocculant, subsequent separation performance, process robustness, and yield. In some cases, flocculation can also provide purification by selectively removing unwanted proteins, nucleic acids, lipids, and endotoxins from the cell broth. However, in cases in which no stable agglomerates are formed, the substances can be separated by flotation. Here, the enzymes or cells are adsorbed onto gas bubbles, rise to the top of the mixture, and accumulate in froth. A typical example of flotation is the separation of single-cell protein (Mayer and Woernle 1985).

4.4 Concentration of EHEs

Protein samples separated from cell debris often contain very small concentrations of enzymes. The volume of the material to be processed is generally very large because large amounts of waste materials must be removed. Thus, there is a tremendous need to concentrate the starting material in order to achieve economic, efficient purification. However, the approaches selected must be such that they do not inactivate or denature the enzymes. Several commonly used approaches are described below.

4.4.1 Thermal Methods

Enzymes are often unstable at elevated temperatures. Thus, only a brief heat treatment can be used for concentration. Evaporators with rotating components that achieve a thin liquid film (thin-layer

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evaporators and centrifugal thin-layer evaporators) or circulation evaporators (long-tube evaporators) can be employed for the concentration of enzymes.

4.4.2 Precipitation

Enzymes are very complex protein molecules possessing both ionizable and hydrophobic groups, which can both interact with the solvent. Indeed, proteins can be made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes (Bell et al. 1982) and can be achieved with various reagents, such as salts, organic solvents, and polymers, or at the isoelectric point.

4.4.3 Ultrafiltration

For ultrafiltration, a semipermeable membrane is used; this membrane permits the separation of solvent molecules from larger enzymes under high osmotic pressure. This is the principle on which all membrane separation processes, including ultrafiltration, are based. Ultrafiltration and cross-flow filtration are based solely on the sieve effect. In processing enzymes, cross-flow filtration is used to harvest cells, whereas ultrafiltration is employed for concentrating and desalting.

4.5 Purification of EHEs

Enzymes can be used in the partially purified form (e.g., industrial use) or the highly purified form (e.g., analytical and medical applications). Highly purified enzymes are obtained through various techniques, such as crystallization, electrophoresis, and chromatography. Some enzyme purification techniques are described below.

4.5.1 Crystallization

Crystallization offers a cost-effective, industrial-scale method for purification. Crystallization involves the formation of solid enzyme particles of defined shapes and sizes. An enzyme can be induced to crystallize or form protein-protein interactions by creating solvent conditions that result in enzyme supersaturation. Most studies of enzyme crystallization have been focused on obtaining crystals for X-ray diffraction analysis rather than as a purification process. However, crystallization is attracting increasing interest as a purification process. Some recent advancements have been made concerning the use of self-interaction chromatography for measuring the protein osmotic second virial coefficient to predict protein crystallization behavior (Tessier et al. 2002) and the development of high-throughput screening and analysis systems for establishing robust protein crystallization conditions (Brown et al. 2003). Various enzymes have been crystallized for commercial purposes, including cellulase, glucose isomerase, subtilisin, and alcohol oxidase.

The desired characteristics of industrial-scale enzyme crystallization are product purity, process yield, ease of crystal recovery, and short overall process time. To achieve these aims, the crystallization process must be carefully designed and developed to produce crystals with relatively large sizes and the desired morphology. Many factors, including salt type and concentration, pH, temperature, the presence of variable amounts and types of impurities, mixing, and crystal seeding, can affect enzyme crystallization. Controlling the level of supersaturation throughout the crystallization process is essential for optimization of crystal size, which can be controlled by the use of precipitants, such as salt, pH, and temperature (Scopes 1993; Judge et al. 1995).

4.5.2 Electrophoresis

This involves the separation and analysis of enzymes based on their size and charge. Enzymes are separated by charge in agarose because the pores of the gel are too large to sieve proteins. This

technique is mostly employed for the isolation of pure enzymes on the laboratory scale. A few limitations are associated with this approach, including generation of heat and interference caused by convection, as well as the necessity for scale-up due to these two factors.

4.5.3 Chromatography

In chromatographic techniques, enzymes are separated based on various physical properties (such as size, shape, charge, and hydrophobic interactions), chemical properties (such as covalent bonding), and biological properties (such as biospecificity). Several types of chromatographic techniques are described below.

Gel Chromatography In gel chromatography (also called gel filtration), hydrophilic, cross-linked gels with pores of finite sizes are used in columns to separate biomolecules. Concentrated solutions are necessary for separation because the sample volume that can be applied to a column is limited. Molecules are separated according to size and shape. Molecules larger than the largest pores in the gel beads, i.e., above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules, which enter the gel beads to varying extents depending on their sizes and shapes, require longer times to pass through the column. As a result, molecules are eluted in order of decreasing molecular mass.

Ion-Exchange Chromatography In this method, enzymes are separated based on charge. Enzymes possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged). The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration. Because of the concentrating effect of ion-exchange chromatography, samples can be applied in dilute form. The ability to process large volumes and the elution of dilute sample components in a concentrated form make ion-exchange methods very useful. The matrix used to produce ion-exchange resins should be sufficiently hydrophilic to prevent enzyme denaturation and must have a high capacity for large molecules with a rapid equilibration. In addition, industrial applications require ion exchangers that give high resolution, allow the use of rapid flow rates, suffer small changes in volume with salt gradients or pH changes, and exhibit high regenerative capacity.

Affinity Chromatography For affinity chromatography, the enzyme to be purified is specifically and reversibly adsorbed on an effector attached to an insoluble support matrix. Suitable effectors are substrate analogs, enzyme inhibitors, dyes, metal chelates, or antibodies. During separation, insoluble matrix is contained in a column. The biospecific effector, e.g., an enzyme inhibitor, is attached to the matrix. A mixture of different enzymes is applied to the column. The immobilized effectors specifically bind the complementary enzyme. Unbound substances are washed out, and the enzyme of interest is recovered by changing the experimental conditions, e.g., by altering pH or ionic strength.

5 Mechanism of Action of EHEs

5.1 Cell Wall-Hydrolyzing Enzymes

Cell wall-hydrolyzing enzymes include endoglucanase, exoglucanase, arabinofuranosidases, esterase, carboxypeptidase, xylanase, and β-glucosidase (Khattak et al. 2012). The solubilization of

β-glucans is the first step in cell wall hydrolysis. The enzymes involved in this process are termed "solubilases." There are two pathways that lead to the solubilization of β-glucans (Jamar et al. 2011). Specifically, β-glucans are either directly hydrolyzed into glucose by β-glucan exohydrolases or first hydrolyzed by a number of enzymes involved in removal of the outer cell wall layer and then further hydrolyzed into glucose. Enzymes involved in the hydrolysis of β-glucans include (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidases (Jin et al. 2004; Georg-Kraemer et al. 2004; Kuntz and Bamforth 2007). Hydrolysis of arabinoxylan is carried out by endo-β-(1→4)-xylanase, exoxylanase, β-xylosidase, and α-arabinofuranosidases (Hrmova et al. 1997). Exoxylanase is involved in the cleavage of the outer β-(1→4) xylosidic linkages, while endo-β-(1→4)-xylanase attacks the inner β-(1→4) xylosidic linkages in arabinoxylan polymers, separating the arabinofuranosyl residues (Egi et al. 2004). β-Xylosidase catalyzes the hydrolysis of β-(1→4) xylosidic bonding, while arabinofuranosidase hydrolyzes the α-(1→2) and α-(1→3) linkage between arabinofuranose units. Arabinofuranosidases consists of two subgroups α-L-arabinofuranosidases and α-D-glucuronidases. α-L-arabinofuranosidases catalyze the cleavage of terminal arabinose residues from the side chains of xylan and other arabinose-containing polysaccharides (Saha 2000). Xylanases are also classified into debranching and nondebranching enzymes, depending on whether or not they produce free arabinose in addition to cleaving the xylan backbone (Matte and Forsberg 1992). However, the reported release of arabinose by xylanases may have been due to the presence of arabinofuranosidases as contaminants. Indeed, the synergistic activity of xylanases and arabinofuranosidases allows small amounts of contaminants to yield detectable amounts of free arabinose (Coughlan et al. 1993). α-D-glucuronidases hydrolyze the linkages between 4-*O*-methylglucuronic/glucuronic acid and xylose residues in glucuronoxylan. Despite the biodegradation of xylan, α-D-glucuronidases also show activity only toward short xylooligomers, while others can release glucuronic acid from polymeric xylan (Puls 1992). Acetyl esterase and ferulic acid esterase also influence the hydrolysis of arabinoxylane (Humberstone and Briggs 2000a, b).

5.2 Starch-Hydrolyzing Enzymes

The two basic steps in the enzymatic conversion of starch are liquefaction and saccharification. Endoamylase (EC 3.2.1.1) protects against the rapid increase in starch solution viscosity caused by the release of amylose during liquefaction (Guzman-Maldonato and Paredes-Lopez 1995). α-Amylase randomly cleaves α-(1→4) glycosidic linkages in starch until the chain lengths of the reaction products are about 10–20 glucose units. At this point, the starch fragments fail to bind to the active site of the enzyme. Hydrolysis of amylopectin produces a mixture of linear malto-oligosaccharides and fragments that contain the α-1,6-bond, which cannot be cleaved by α-amylase. Xylanases and cellulases are glycosyl hydrolases that do not act on starch, but yield improvements in starch processing. Both are involved in the cleavage of the β-1,4-glycosidic bonds in cellulose and xylans, respectively. Xylanases reduce the viscosity of starch slurries by degrading xylans, whereas cellulases positively affect starch hydrolysis when it is contaminated by cellulose fibers. Moreover, β-amylase, an exo-acting enzyme involved in the cleavage of α-(1→4) glycosidic linkages at the nonreducing end of linear chains in starches and other polysaccharides, produces β-maltose and β-glucose successively (Hoseney 1994; Svensson et al. 1985). The catalytic activity of β-amylase continues until α-(1→6) linkages are reached in starch molecules. Glucosidases are also exo-acting enzymes and hydrolyze α-(1→4) glycosidic linkages in starch molecules. The main advantage of glucosidases over β-amylase is their ability to bypass the side chain at α-(1→6) bonds (Lalor and Goode 2010).

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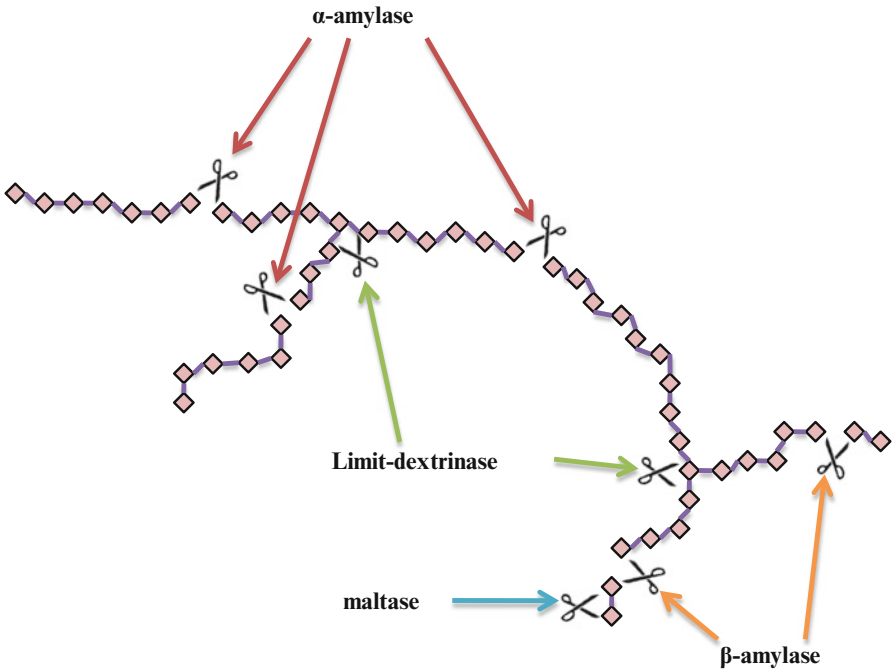

Fig. 3 Starch-hydrolyzing enzymes system consists of four major enzymes that cleave different bonding at different positions and produced glucose molecules as final product

Limit dextrinase (EC 3.2.1.41) has the ability to break the amylopectin α-(1→6) linkages of branched dextrins and complete the hydrolysis of starch, producing its monomeric sugar at the end of the hydrolyzing reaction (Bowles 1996). In short, the initial solubilization of starch is catalyzed by α-amylase, while the hydrolysis of the resulting dextrins to oligosaccharides and glucose is subsequently carried out by the synergistic action of α-amylase, β-amylase, limit dextrinase, and glucosidases (Fincher 1989; MacGregor 1996). Figure 3 represents the generalized schematic view of starch hydrolysis.

5.3 Cellulose-Hydrolyzing Enzymes

The hydrolysis of cellulose, the most abundant polysaccharide on Earth, requires the concerted activity of a number of enzymes with different substrate specificities. Cellobiohydrolases (e.g., EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolases) hydrolyze cellobiose units from the ends of long polysaccharide chains. Then, endoglucanases (e.g., EC 3.2.1.4; 1,4-β-D-glucan-4-glucanohydrolase) cleaves the middle, amorphous region of the cellulose chain, thus providing more active sites for the activity of cellobiohydrolases. Finally, the third most important hydrolyzing enzyme participating in the hydrolysis of cellulose is β-glucosidase (EC 3.2.1.21), which cleaves cellobiose to glucose. This enzyme has been available in feedstock as an easily metabolizable carbon source for a number of microorganisms and is known to facilitate the production of desired products.

Another important source of cellulose readily available in complex form is hemicellulose. Hemicellulose is a heteropolymeric structure; however, the process through which hemicellulose hydrolyzes into simple sugar is well understood. Endoenzymes are the major class of hydrolyzing enzymes that cleave the main polymer chain internally, providing active sites for exoenzymes that liberate the simple sugars from the chain. Along with endo- and exoenzymes, a number of other enzymes also contribute to the hydrolysis of hemicellulose by cleaving the side chains of the polymers or oligosaccharides, producing various mono- and disaccharides depending on the type

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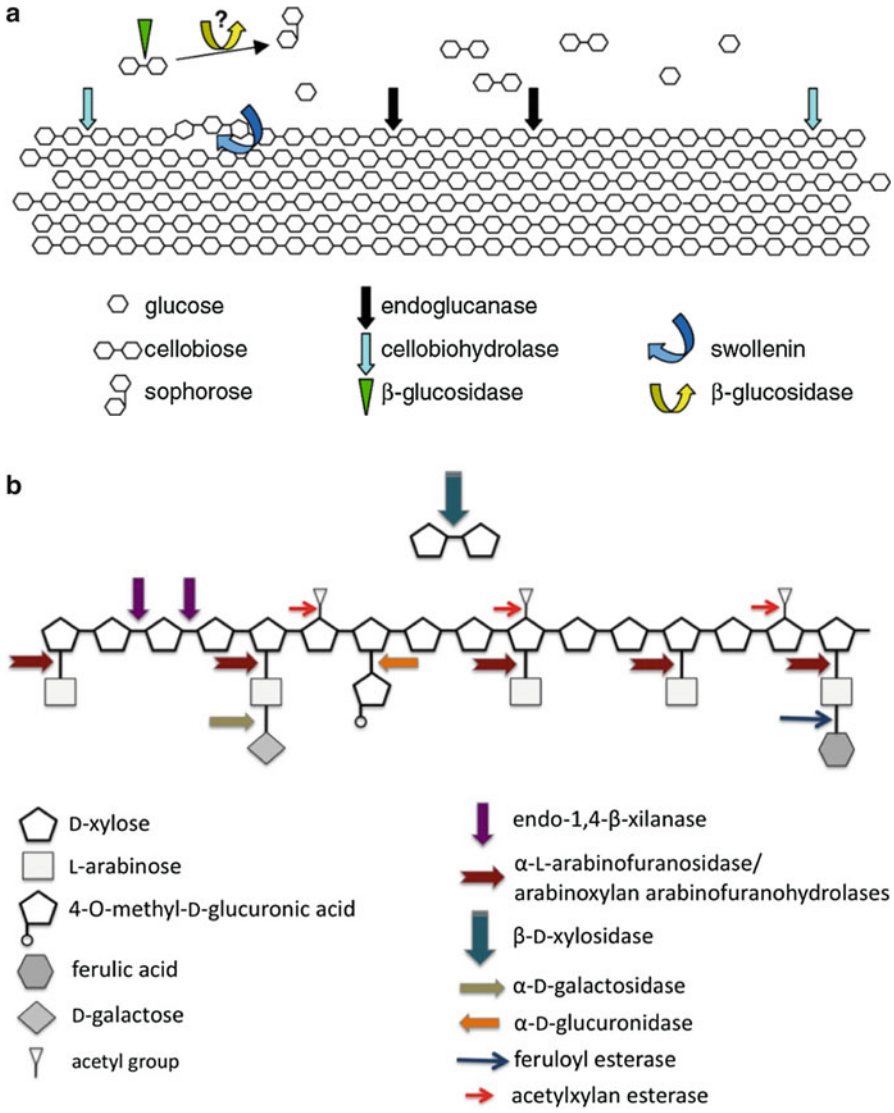

Fig. 4 Hydrolyzing enzyme system for the degradation of cell wall components. A number of hydrolyzing enzymes are participating in the degradation of (**a**) cellulose and (**b**) hemicellulose (Image taken from Aro et al. 2005)

of hemicellulose being hydrolyzed. For example, the breakdown of xylan involves at least endo-1,4-β-D-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37), which act on the main sugar chain, and, depending on the type of xylan, side-chain cleaving enzymes, such as α-glucuronidase (EC 3.2.1.131) and acetyl xylan esterase (EC 3.1.1.72). Similarly, different combinations of endoenzymes, exoenzymes, and ancillary enzymes are required for complete hydrolysis of other types of hemicelluloses into monomeric sugars. De Vries and Visser (2001) provided a complete list of enzymes involved in the degradation of hemicellulose and their mechanisms of action (de Vries and Visser 2001). Figure 4a, b provides a complete schematic view of the cellulotic and hemicellulolytic system.

5.4 Protein-Hydrolyzing Enzymes

Certain proteolytic enzymes are involved in the production of FAN and di-/tri-amino acids. The enzymes involved in this protein modification system share the same substrate

(polypeptides), but have different active sites. The final products, including, FAN, di-amino acids, and tri-amino acids, are produced as result of their synergistic action. Endopeptidases and exopeptidases are two principal groups of protein-hydrolyzing enzymes. Endopeptidases randomly cleave peptide bonds, producing relatively smaller peptide chains, while exopeptidases attack these smaller peptide chains and cleave the bonds between terminal amino acids producing FAN or di-/tri-amino acids. Exopeptidases comprise two major enzymes, carboxypeptidase and aminopeptidase, which have different active sites. Specifically, carboxypeptidase hydrolyzes proteins from the carboxyl end, while aminopeptidase hydrolyzes proteins from the amino end (Lalor and Goode 2010; Evan and Taylor 1990).

Endopeptidases hydrolyze internal, α-peptide bonds in a polypeptide chain. Endopeptidases include chymotrypsin (EC S01.001) (Graf et al. 2004), pepsin (EC A01.001) (Tang 2004), and papain (EC C01.001) (Menard and Storer 2004). Endopeptidases acting on substrates smaller than proteins are termed as oligopeptidases. Thimet oligopeptidase (EC M03.001) is an example of an oligopeptidase (Barrett and Chen 2004). Endopeptidases initiate the hydrolysis of proteins, producing new N- and C-termini, which subsequently act as substrates for exopeptidases that complete the hydrolytic process. Endopeptidases are also involved in the removal of signal peptides from secreted proteins (e.g., signal peptidase I, EC S26.001) (Dalbey 2004) and the maturation of precursor proteins, such as enteropeptidase (EC S01.156) (Sadler 2004) and furin (EC S08.071) (Creemers and Van de Ven 2004).

Exopeptidases require a free N-terminal amino group, C-terminal carboxyl group, or both and hydrolyze a bond up to three residues from the terminus. This group of enzymes is further divided into aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, tripeptidyl-peptidases, and dipeptidases.

Aminopeptidases release a single amino acid residue from the unblocked N-terminus of the substrate. This group includes aminopeptidase N (EC M01.001) (Turner 2004) and aminopeptidase C (EC C01.086) (Chapot-Chartier and Mistou 2004).

Dipeptidases hydrolyze dipeptides when both of the termini are free. They include dipeptidase A (EC C69.001) (Dudley and Steele 2004) and membrane dipeptidase (EC M19.001) (Hooper 2004).

Dipeptidyl-peptidases hydrolyze a dipeptidyl bond, i.e., release an N-terminal dipeptide from the substrate. They include dipeptidyl-peptidase I (EC C01.070) (Turk et al. 2004) and dipeptidyl-peptidase III (EC M49.001) (Chen and Barrett 2004).

Tripeptidyl-peptidases cleave a tripeptidyl bond, producing a tripeptide from the N-terminus of the substrate. Examples of tripeptidyl-peptidases are tripeptidyl-peptidase I (EC S53.003) (Sohar et al. 2004) and tripeptidyl-peptidase II (EC S08.090) (Tomkinson 2004).

Peptidyl-dipeptidases hydrolyze dipeptides from the C-terminus of the substrate. An example is peptidyl-dipeptidase A (EC XM02-001) (Corvol et al. 2004).

Carboxypeptidases hydrolyze a single residue from the unblocked C-terminus of the substrate. Examples include carboxypeptidase A1 (EC M14.001) (Auld 2004), cathepsin X (EC C01.013) (Menard and Sulea 2004), and carboxypeptidase Y (EC S10.001) (Mortensen et al. 2004).

5.5 Lipid-Hydrolyzing Enzymes

Lipid-hydrolyzing enzymes include lipase, lipoxygenase, hydroperoxidase lyase, and hydroperoxide isomerase (Khattak et al. 2012). Lipases hydrolyze esters of long-chain aliphatic fatty acids, producing free fatty acids and glycerol. Lipases are different from all other esterases due to their ability to hydrolyze insoluble fatty acyl esters (Ward 1985). The potential of lipases to act as biocatalysts is associated with their sophisticated selectivity for the modification of triglycerides.

Three features are relevant: (1) regioselectivity, i.e., the position of the fatty acid on the glycerol backbone; (2) fatty acid specificity, i.e., concerning the length or unsaturation of the chain; and (3) the class of acylglycerols, i.e., mono-, di-, or triglycerides. Most lipases are 1,3 regiospecific, hydrolyzing primary alcohol 1,3 positions, and only a few lipases are able to hydrolyze the sn-2 position to allow the complete conversion of triglycerides to free fatty acids. With regard to fatty acid selectivity, lipases are able to hydrolyze fats into medium- to long-chain molecules (C4–C18, rarely up to C22), but with different efficiencies. Lipolytic enzymes possessing different selectivity can therefore be used to obtain valuable products, such as structured triglycerides with improved nutritional value, as well as an impressive range of mono-acylglycerols, di-acylglycerols, tri-acylglycerols, fatty acids, esters, and intermediates (Bornscheuer 2000). The enzyme lipoxygenase hydrolyzes the dioxygenation of polyunsaturated fatty acids into hydroperoxy acids. Hydroperoxide lyase (HPL), common in plants, is mainly involved in the hydrolysis of fatty acid hydroperoxides into oxo-acids and aldehydes. Hydroxyperoxide isomerase catalyzes the conversion of hydroperoxylinoleic acid into α- or β-ketols.

5.6 Nucleic Acid-Hydrolyzing Enzymes and Other Hydrolyzing Enzymes

Nucleic acid-hydrolyzing enzymes, called nucleases, can be regarded as molecular scissors and hydrolyze the phosphodiester bonds between the nucleotide subunits of nucleic acids. These enzymes were previously called polynucleotidases or nucleodepolymerases. Nucleases can be classified into two main categories based on the site at which they cleave: endonucleases, which act within the strand, and exonucleases, which act at the terminal end of the strand. Some nucleases have the ability to cleave the phosphodiester bond at both terminal ends and in the middle of the nucleotide chain, therefore falling into both categories. The most well-known, highly applicable, and well-characterized nucleases are deoxyribonuclease and ribonuclease. Exonucleases have the ability to start cleavage of the nucleotides chain from either the 5′ end or the 3′ end of the nucleotide strand, depending on the encoded information. The generalized mechanism of action of nucleases and their vital roles in DNA repair are summarized in Fig. 5a, b.

Beside cell wall-, starch-, protein-, and lipid-hydrolyzing enzymes, phytase, peroxidase, and thiol oxidase (Bamforth 2009) also have hydrolytic activity. Phytase hydrolyzes phytate into phytic acid, inorganic phosphate, and myoinositol (Khattak et al. 2012). Two types of phytases, 3- and 6-phytase, function to dephosphorylate phytate. 3-Phytase is produced by microbes, while 6-phytase is found in seeds of higher plants, including barley. Inorganic phosphate, a constituent of nucleotides, is also utilized by metabolic machinery during in vivo nucleotide synthesis (purines and pyramidines).

6 Applications of EHEs

Endogenous enzymes of various classes have multiple applications in day-to-day life. The major applications are in food, animal feed, textile, paper and pulp, fuel (energy), pharmaceutical, and chemical industries (Carvalho et al. 2003; Hsu and Lakhani 2002). EHEs also have numerous potential applications in advanced biosciences and engineering fields. Some of the potential applications of endogenous enzymes are described below.

6.1 Food Industry

Hydrolyzing enzymes are a vital part of various processes within the food industry. Their hydrolyzing activities are responsible for the formation of different products from the raw food materials.

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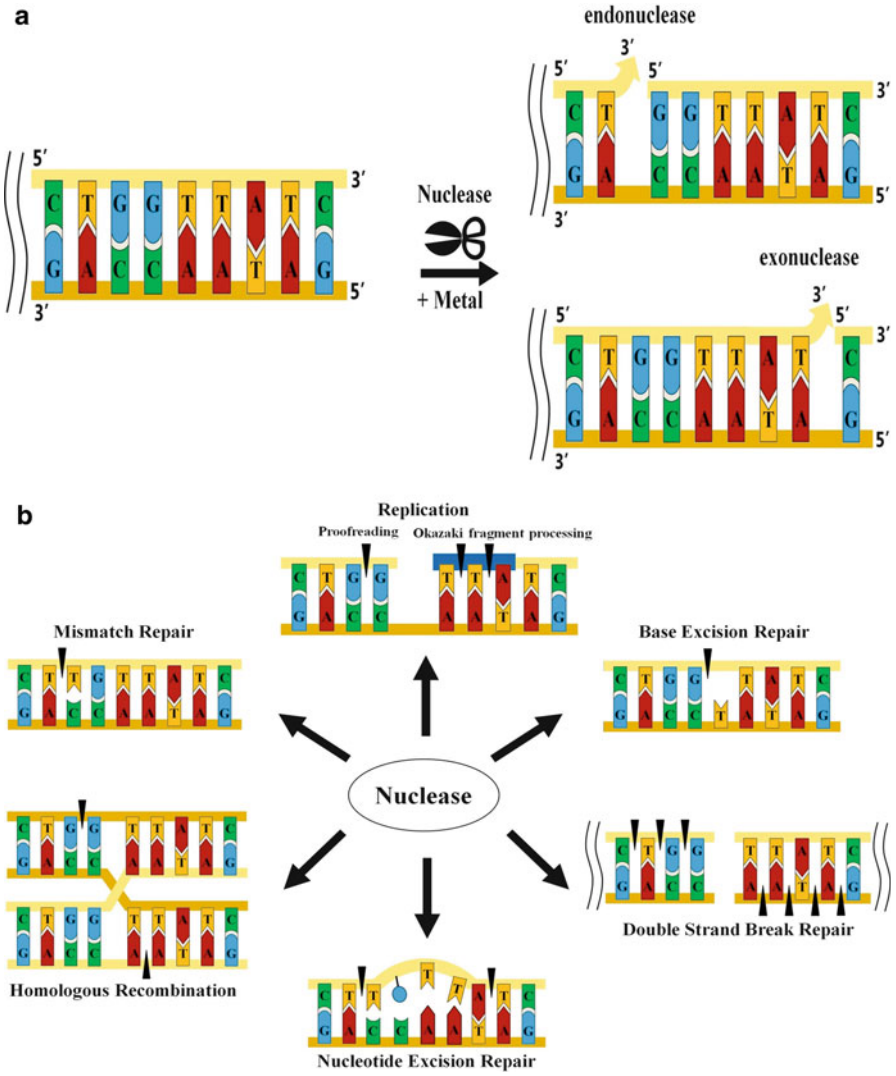

Fig. 5 Schematic diagram of the nuclease enzyme activity. The two strands of DNA are schematically drawn. (**a**) The cleavage made by the nuclease is represented by *arrows*. (**b**) The involvement of nuclease enzymes in the DNA repair system is summarized. Single-stranded region is shown in *blue* color

Starch-hydrolyzing enzymes play a significant role in the baking and brewing industries. Almost all hydrolyzing enzymes, such as α- and β-amylases, are found in raw food materials, e.g., flour and malt. Both of these amylases perform individual, but complementary, roles in food processing. Starch is initially broken down into low-molecular-weight dextrins by α-amylases; these dextrins are then further converted to maltose by β-amylase, and maltose is converted into whatever components are necessary for yeast growth (Khattak et al. 2012). Glucoamylases help to facilitate the breakdown of starches into simple fermentable sugars in the baking industry and have been shown to intensify the bread crust color through released glucose (Polaina and MacCabe 2007).

Xylanases hydrolyze hemicelluloses and are used, together with amylases, glucose oxidase, and proteases, in the bread-making process. Wheat flour contains hemicelluloses, which can be broken down to allow the redistribution of water, making the dough softer. Xylanases help to delay the formation of crumbs, thus increasing bread volume (Harbak and Thygesen 2002; Camacho and Aguilar 2003). Xylanase is recommended for making lighter cream crackers and improving the texture and regularity of the wafers. Currently, xylanases have been employed, together with

cellulases, amylases, and pectinases, in order to enhance the yield of juice through the liquefaction of fruit and vegetables; achieve better recovery of aromas, important oils, mineral salts, edible dyes, and pigments; reduce viscosity; and suppress breakdown of materials that obstruct the physiological features or chemical nature of the juice.

Cellulases have multiple applications in the food industry. For example, cellulases can be used for fruit juice and oil extraction. As mentioned earlier, along with other groups of enzymes, cellulases are used for the purification of fruits juices. An important feature of cellulases is their use in the isolation of proteins from soybeans and coconuts. The efficiency of starch isolation is increased with the activity of cellulases. Cellulases can digest ball-milled lignocelluloses, which are commonly used as food additives (Carvalho et al. 2003; Hsu and Lakhani 2002). Cellulases play a vital role in the hydrolysis of cellulosic waste, which results in the production of cello-oligosaccharides and fermentable sugars. These are essential for the digestion of cell wall components, which facilitates the release of flavor-related compounds, enzymes, and polysaccharides (Kuhad et al. 2011).

6.2 Brewing Industry

Enzymes have been used to make beverages since ancient times. Currently, the brewing industry relies on several groups of enzymes for preparing beverages using different feedstocks as substrate materials.

The traditional brewing process is mainly based on the germination and processing of barley grains. The germination period, which lasts about 7 days, involves such enzymes as α-amylase, β-amylase, and proteinases originating from barley grains. The germinated grains are then heated to develop color and flavor. These enzymes further hydrolyze the starches and proteins contained in crushed starchy cereals, resulting in the formation of simple sugars (e.g., mono- and oligosaccharides), which are fermented by yeast to produce ethanol. Amylolytic enzymes are also used for the development of low-molecular-weight carbohydrates (fermentable sugars) used by yeast cells for the production of alcoholic drinks.

Currently, a major focus in the brewing industry is immobilized enzyme technology, which can be used to effectively produce low-calorie beers and other beverages. Conventional fermentation systems produce high quantities of dextrins from starch hydrolysis, which are carried along to the final product. Immobilized glucoamylases are used to convert these dextrins to fermentable sugars for further fermentation. By passing the fermenting beer through a reactor containing immobilized glucosidase, these dextrins can be broken down into glucose, which is then almost completely transformed into alcohol. A great advantage of this technique is that the enzyme does not contaminate the product.

Glucanases obtained from microbes play a vital role in the fermentation processes of beers and wines (Singh et al. 2007; Bamforth 2009). The quality and yield of the fermented products is increased with the use of glucanases and related polysaccharides (Bamforth 2009; Bamforth et al. 2009). Enzymes including glucanase, pectinase, and hemicellulases improve the color extraction, clarification, quality, and stability of wine (Singh et al. 2007; Galante et al. 1998). Wine aroma is also improved by β-glucosidase, which modifies glycosylated precursors. Currently, many enzymes are commercially available for use in the preparation of wine, contributing to the improvement of wine yield and quality. A number of improved enzymes, such as cellulase and pectinase, can be exogenously added during wine making in order to improve the productivity of accessible brewing processes in the near future (Bamforth 2009; Bamforth et al. 2009).

6.3 Feed Industry

The use of enzymes as feed additives is also well established. A number of enzymes from various enzyme classes are used in various applications in the feed industry. Production of feed stock using enzymes is currently an important part of agricultural business. Most of these types of enzymes are involved in the digestion of cellulose-based feeds.

Xylanases, together with certain other essential enzymes, including glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases, and lipases, are used in the animal feed industry. These enzymes hydrolyze the arabinoxylans contained in feed components and reduce the viscosities of raw materials (Twomey et al. 2003). The addition of xylanase to feed containing maize and sorghum improves the initial digestion of nutrients in the early part of the digestive tract, improving energy utilization. Furthermore, the combined action of the remaining enzymes mentioned above produce a well-digestible food mixture. Endogenous enzymes are produced in much smaller quantities in young fowl and swine as compared to adults. Therefore, their performance as livestock is improved by the added food supplements.

Cellulases are widely used as supplements in the feeds of ruminants and monogastric animals. Additionally, cellulases are used in the pretreatment of lignocellulosic materials and in the dehulling of cereal crops. Their activities influence the digestibility of feeds in monogastric animals and ruminants (Kuhad et al. 2011). Supplementation with cellulase in feedstocks enables dairy animals to consume more feed, resulting in increased milk production. The nutritional values of agricultural wastes and grains can be improved through cellulases and xylanases (Godfrey and West 1996). These enzymes can degrade certain feed constituents and provide additional digestive enzymes, such as proteases, amylases, and glucanases.

Monogastric animals generally cannot digest and utilize plant-based feeds containing cellulose and hemicelluloses. Certain enzymes, such as xylanases and glucanases, are used in cereal-based feeds for these animals, enabling them to digest these feedstocks. β-Glucanases and xylanases are also used in the feeds of monogastric animals to promote the hydrolysis of nonstarch polysaccharides, such as β-glucans and arabinoxylans. Glucanases and xylanases decrease the viscosity of high-fiber rye- and barley-based feeds in poultry and pigs. These enzymes are effective in causing weight gain in chickens and piglets by improving the digestion and absorption of feed materials (Singh et al. 2007; Bhat 2000).

Phytase is currently the most abundant enzyme used in the feed industry. Phytase is used in cereal feeds (containing natural phosphorous bound to phytic acid) consumed by monogastric animals. The addition of phytase to these feeds results in a significant reduction in phosphorus excretion from monogastric animals (Lei and Stahl 2000; Kies et al. 2001).

6.4 Pharmaceutics and Medical Applications

The health-promoting activities of certain enzymes, their derivatives, and combinations of enzymes have facilitated the investigation of the therapeutic potential of these enzymes. For example, certain enzymes have been shown to have antimicrobial effects and have therefore become key elements in various therapeutic drugs and materials. Some of the potential therapeutic applications of EHEs are described below, and additional applications of EHEs in this industry are available in the literature.

Enzymes can be administered orally or by injection for treatment of certain diseases. Sacrosidase (β-fructofuranoside fructohydrolase from *Saccharomyces cerevisiae*) is used in the treatment of congenital sucrase-isomaltase deficiency (CSID). In this disease, patients are unable to use the disaccharide sucrose. Drugs containing sacrosidase hydrolyze sucrose, thus allowing patients with CSID to consume a more normal diet (Treem et al. 1999). Certain pancreatic enzymes, including lipases, proteases, and amylases (in the form of a mixture), have been shown to be effective in the

treatment of fat malabsorption in patients with human immunodeficiency virus (HIV) (Carroccio et al. 2001). Furthermore, the same enzymatic mixture has been successfully used to treat pancreatic insufficiency (Schibli et al. 2002).

Lysozyme is a naturally occurring bactericidal agent used in a variety of food and consumer materials. This enzyme has the ability to break down the carbohydrate chains in the bacterial cell wall, thereby prohibiting the growth of bacterial cells and preventing food poisoning and other maladies. Lysozyme has also been shown to possess activity against HIV.

Chitinases are another group of enzymes that possess natural bactericidal potential. Chitin is the basic structural unit of the cell wall in a variety of pathogenic microorganisms, such as protozoa, fungi, and helminths. Chitinases effectively target chitins and possess good antibacterial activities (Fusetti et al. 2002). Certain lytic enzymes derived from bacteriophages target the cell wall of *Streptococcus pneumonia*, *Bacillus anthracis*, and *Clostridium perfringens* (Zimmer et al. 2002). These enzymes have the potential to halt the growth and activities of drug-resistant bacterial strains.

Xylanase and xylan have a few potential applications in the pharmaceutical industry. Xylanases are sometimes added in combination with a complex of enzymes (hemicellulases, proteases, and others) to produce certain medicinal products. Hydrolytic products of xylan, such as β-D-xylopyranosyl residues, can be converted into combustible liquids (ethanol), solvents, and artificial low-calorie sweeteners. The resulting sweeteners are noncarcinogenic and are suitable for diabetic and obese individuals. A variety of commercial products containing xylitol, such as chewing gum, can be found on the market.

7 Conclusion

EHEs are involved in almost every known biological process. With the advent of novel techniques of enzyme isolation and purification, their applicability is broadening and increasing. Current approaches toward developing cell-free lysates from lysed microbial cells and their isolation, concentration, and purification have generated a great amount of interest in the potential use of EHEs in various industrial applications. Newly developing biotechnological techniques are allowing the identification of various novel applications of purified EHEs, specifically in genetic engineering. Current methods using grouped enzyme approaches for process simplification are providing insight into the broad future applications of EHEs.

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