Enzymatic Processing in the Food Industry

Pedro Fernandes[a,b], [a] IBB – Institute for Bioengineering and Biosciences, Instituto Superior Tecnico, Universidade de Lisboa, Lisboa, Portugal; and [b] Universidade Lusófona, Lisboa, Portugal

A Brief Overview on Enzymes

Basics Considerations on Enzymes

Enzymes have been used for millennia in food processing, such as bread baking, brewing, cheese and wine making, although only in the later decades of the 20th century, processes were developed that allowed the production in well-characterized formulations, even at large scale (Kirk et al., 2002; Mishra et al., 2017). Enzymes are globular proteins that act as catalysts, thus they speed up the rate of a reaction by lowering the energy of activation. Some enzymes require cofactors, small organic molecules or metal ions, for catalytic activity. Unlike chemical catalysts, enzymes are natural in origin, operate under mild temperature and pressure, display high specificity and are biodegradable (van Oort, 2010; Subin and Bhat, 2015). Biologically active enzymes can be obtained from animals, microorganisms and plants, but microbial sources are favored. Microbial enzymes can be produced in high yield, in relatively low-cost and short time processes, and are typically more stable than enzymes from the remaining sources. Particularly preferred are microbial produced enzymes that are secreted to the fermentation medium, as this eases separation and purification. Genetically modified microorganisms expressing exogenous enzymes (from plant or animal sources and from pathogenic or difficult to grow microbial strains) are also used in commercial enzyme production (Chandrasekaran et al., 2015; Subin and Bhat, 2015). Enzyme activity and stability are influenced by operational conditions, e.g., pH, temperature, substrate concentration, presence of metal ions and enzyme concentration. Enzymes have optimal pH and temperature conditions for activity and stability that may not fully match in an industrial process. An increase in substrate concentration increases activity up to a given point, henceforth the rate of reaction stabilizes or may even decrease, in case of substrate inhibition. Also depending on the enzyme, given metal ions may be required for activity (e.g., Ca^{2+} for most α-amylases), or may inhibit enzyme activity (Subin and Bhat, 2015). Thus, careful selection of operating conditions is critical for high enzyme performance. This is relatively easy to implement in laboratory condition with model systems, but may prove difficult to reproduce with real systems, due to the complexity of the matrix to be processed, e.g., hydrolysis of lactose in buffer system or industrial scale hydrolysis of lactose in milk. Enzymes can be used in free form or immobilized, e.g., attached to/entrapped in an inert support, to allow the repeated/continuous use of the enzyme, and to also increase its stability. Still, the implementation of an immobilized enzyme based system at industrial scale requires a careful evaluation as in addition to technical issues, e.g., loss of activity during immobilization, mass transfer limitations, the economics of immobilized enzymes, e.g., cost of immobilization, cost of immobilization carrier and chemicals for immobilization, must be considered (DiCosimo et al., 2013; Sheldon and van Pelt, 2013).

Enzyme Classification

Enzymes are differentiated from one another by the type of reaction catalyzed, therefore this specific property is the basis for the classification and naming of enzymes. A systematic approach was implemented in the 1960s to avoid confusion caused by the increasing number of enzymes known, a few hundred then and over 5500 currently, which rendered the use of traditional and trivial names unpractical (Cornish-Bowden, 2014; Subin and Bhat, 2015). The nomenclature established then, that still stands largely unchanged, classifies each enzyme with a four-digit code preceded by EC, which stands for Enzyme Commission (**Table 1**).

The first digit is the most important since it ascribes the enzymes to one of six classes, which depend on the nature of reaction catalyzed (**Table 1**). The second and third digits correspond to further divisions of each class in subclasses and of each of these in

Table 1 Top-level classification of enzymes

Class	*Type of reaction catalyzed/comments*
EC 1 (oxidoreductases)	Oxidation-reduction, where electrons are transferred between molecules, e.g., oxidases, dehydrogenases. Co-factor required, two-substrate reaction
EC 2 (transferases)	Chemical group transfer between two molecules
EC 3 (hydrolases)	Hydrolysis, e.g., hydrolytic cleavage of peptide and ester bonds among others. Strictly, it involves group transfer to water, that is typically in excess. Under selected conditions many hydrolases can catalyze the reversal counterpart of the hydrolytic reaction
EC 4 (lyases)	Elimination reactions, where a group of atoms is removed from the substrate in a manner not involving hydrolysis nor redox reaction
EC 5 (isomerases)	Transfer of functional groups within a molecule to produce an isomer
EC 6 (ligases)	Covalent bond formation between two molecules trough condensation reactions. ATP, adenosine triphosphate, is required

sub-subclasses. Such divisions depend on noticeable differences of the enzymes in the class and subclass, respectively, and are related to the type of reaction catalyzed, such as substrate acted on and groups transferred. The fourth digit identifies the individual enzyme within a sub-subclass (Tipton and Boyce, 2000; Ako and Nip, 2012; Cornish-Bowden, 2014). Specific details can be found on-line at Explodens (http://www.enzyme-database.org/) and Brenda (https://www.brenda-enzymes.org/), among other sources (McDonald et al., 2009; McDonald and Tipton, 2014). Still, trivial names, which are either the original name, e.g., papain, pepsin, rennet, or those that result of adding the suffix "-ase" to either the substrate or to the reaction type, e.g., inulinases, glucose isomerase, are commonly used and are the recommended names once ambiguity is ruled out (Subin and Bhat, 2015).

Enzymes in Food Processing

Overall Perspective

The use of enzymes in the food industry presents several advantages, namely: a) allows for high product yields; b) minimizes formation of by-products and unwanted side reactions, concomitantly easing downstream processing; c) leads to environmentally friendly processes with low energy requirements and reduced carbon footprint; d) provides safe and high-quality products that comply with the requirements of increasingly demanding public and regulatory agencies (Chandrasekaran et al., 2015; Mishra et al., 2017). It is thus not surprising that the food and beverage area leads the application market for enzymes (Pellis et al., 2018). Major goals aimed at involving the use of enzymes are given in **Table 2**.

Enzymes in the food industry have been typically assigned to the roles of processing aids or food additives (Chandrasekaran et al., 2015), although the latter denomination is under debate and the alternative designation of ingredients is being considered (https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes-guidance-categorisation.pdf). In any case, food enzymes are mostly used as processing aids, meaning that they are used during the production process and are not present in the final product, or if so, have no technological effect in the final product. Some enzymes, such as invertase or lactase can be used in both roles. Enzyme formulations used in the food industry are carefully scrutinized by regulatory agencies such as the

Table 2 Intended goals encompassing the use of enzymes in food industry

Purpose of use	*References*
Production of a given molecule, such as glucose from starch hydrolysis or fructose out of isomerization of glucose	Hobbs (2009) and White (2014)
Adjusting the rheology of processed suspensions to established parameters, e.g., reduction of the viscosity and clarification of fruit juices through hydrolysis	Moelants et al. (2014) and Sharma et al. (2017)
Extraction of compounds, such as amino-acids, flavors and colorants, assisted peeling of fruits and descaling of fish	Sowbhagya and Chitra (2010), Nielsen and Nielsen (2012), Feng et al. (2013), and Poojary et al. (2017)
Modification of organoleptic properties, e.g., debittering of juices, tenderizing meat, altering/adding flavor	Puri et al. (2008), Cheetham (2010), Bekhit et al. (2014a,b), and Ni et al. (2014)
Altering functionalities towards intended food formulations	Rajendran et al. (2009) and Ferreira-Dias et al. (2013)
Improving shelf-life	Miguel et al. (2013)
Digestive aids, e.g., α-galactosidase (EC 3.2.1.22) degrades non-digestible carbohydrates such as raffinose and stachyose, preventing flatulence	Chandrasekaran et al. (2015)
Incorporation in biosensors to monitor food quality	Economou et al. (2017)

Joint FAO/WHO Expert Committee on Food Additives (JECFA, http://www.who.int/foodsafety/areas_work/chemical-risks/jecfa/en/), or the European Food Safety Authority (http://www.efsa.europa.eu/en/topics/topic/food-enzymes), through the issue of adequate specifications (http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm). A key goal of these specifications is to make sure that enzymes and remaining components of the formulations, e.g., stabilizers, are safe and according to good manufacturing practices (Chandrasekaran et al., 2015; Subin and Bhat, 2015). Updated information on enzyme formulations commercially available can be obtained from the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), at http://www.amfep.org/.

Practical applications of enzymes may require the action of a single enzyme, e.g., lactase that promotes the hydrolysis of lactose in the production of lactose-free milk; or the concerted action of several enzymes, e.g., α- and γ-amylases, pullullanase and glucose isomerase in the production of high fructose syrup or a combination of amylases, lipases, oxidoreductases and xylanases in the production of bread (Miguel et al., 2013; Chandrasekaran et al., 2015; Hua and Yang, 2015).

Alongside with its application in well-established food production and processing systems, enzymes are also playing a role within the scope of functional foods, those that have a potential health benefit beyond basic nutrition, namely through the production of prebiotics. These are compounds that are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract so that they reach the colon, where they can have a positive impact in the health of the host related to the modulation of the microbiota (Corbo et al., 2014; Dominguez et al., 2014; Anadón et al., 2016). These are mostly non-digestible oligosaccharides, resistant to digestion in the stomach and small intestine. Currently inulin, fructo-oligosaccharides (FOS), lactulose, and galacto-oligosaccharides (GOS) are considered as established prebiotics, yet several other oligosaccharides are under evaluation (Dominguez et al., 2014; Osman, 2016).

Hydrolases such as carbohydrases, proteases or lipases, are the most commonly used enzymes in food processing (Brown, 2011; Ackaah-Gyasi et al., 2015; Ventura-Sobrevilla et al., 2015), yet enzymes from the remaining classes also find applications within the scope of food processing, such as: glucose oxidase, EC 1.1.3.4, in bakery (Miguel et al., 2013); transglutaminase, EC 2.3.2.13, in meat processing (Santhi et al., 2017); acetolactate decarboxylase, EC 4.1.1.5, in brewery (Choi et al., 2015); glucose (xylose) isomerase, EC 5.3.1.5, in the production of corn-based sweeteners (Tomasik and Horton, 2012); and L-amino acid ligases for the synthesis of functional peptides, some of which can replace sodium chloride (Arai et al., 2013; Nandakumar and Wakayama, 2015). The latter class is currently, and by far, the least common in food processing. More detailed information on the features of relevant enzymes in food processing and the rationale for their application is given in the following sections.

Relevant Enzymes in Food Processing

Hydrolases

Amylases

Amylases are widely known as starch degrading enzymes. Starch is composed of two polysaccharides, amylose and amylopectin. Amylose is mostly a linear polysaccharide formed by α-1,4 linked D-glucose residues with very few α-1,6-branching points. Amylopectin is highly branched, thus combining α-1,4 linked D-glucose with α-1,6 linked D-glucose residues. Amylases can be obtained from microbial, animal and plant sources, but the former is preferred (Gopinath et al., 2017). They are divided in α, β and γ, depending of the type of bond they cleave. α-Amylase (EC 3.2.1.1. α-1,4-D-glucan glucanohydrolase), typically a metalloenzyme that requires calcium ions for activity and stability, catalyzes the endohydrolysis of α-1,4-glycosidic bonds in starch polysaccharides containing three or more 1,4-α-linked D-glucose residues, releasing dextrins (Hua and Yang, 2015; Liu and Kokare, 2017). β-Amylase (EC. 3.2.1.2, α-1,4-D-glucan maltohydrolase) catalyzes the hydrolysis of the α-1,4-glycosidic bonds in starch from the non-reducing ends as to release successive maltose units. γ-Amylase (EC 3.2.1.3., α-1,4-D-glucan glucohydrolase, commonly known as glucoamylase or amyloglucosidase) instead hydrolyzes the α-1,4-linked D-glucose residues successively from the non-reducing ends of starch and malto-oligosaccarides with the release of β-D-glucose. Moreover, γ-amylase also hydrolyzes α-1,6- and α-1,3-D-glycosidic in bonds in other polysaccharides, but at a much lower catalytic efficiency as compared to the main activity (Divakar, 2013; Hua and Yang, 2015). Amylases are used in the starch industry in the production of corn syrups (maltose and glucose syrups) and crystalline glucose, and in the baking industry, to generate fermentable compounds for yeast and reduce the time of fermentation, to improve rheological properties of dough and ease its handling, to increase bread volume and softness, and to intensify the color and flavor of bread as result of Maillard reaction over the reducing sugars formed. Furthermore, they are used in brewing processes and fruit juice production, for the clarification of beer and fruit juices, and in the production of fermentable sugars from residual dextrins in the production of light beer. A further use is in confectionary, where high-maltose and high-glucose syrups produced by amylases are used for the manufacture of hard candies and soft cream candy (Simpson et al., 2012; Miguel et al., 2013; Chandrasekaran et al., 2015; Hua and Yang, 2015; Sharma et al., 2017).

Cellulase

Cellulase refer to a multi-enzyme system required for the full hydrolysis of cellulose, a polysaccharide composed of β-1,4-glycosil residues. Cellulase includes β-1,4-endoglucanase (EC 3.2.1.4), often simply referred to as cellulase, which catalyzes the endohydrolysis of β-1,4 D-glycosidic bonds in cellulose and in β--D-glucans; cellobiohydrolase or exoglucanase (EC. 3.2.1.91, β-1,4-D-glucan cellobiohydrolase), which also catalyzes the hydrolysis of β-1,4 D-glycosidic bonds but from the non-reducing end, releasing cellobiose units; and β-glucosidase or cellobiase (EC. 3.2.1.21, β-D-glucoside glucohydrolase), which hydrolyzes cellobiose and releases glucose from non-reducing ends of cello-oligosaccharides. Cellobiase has a key role in the cellulose hydrolysis, as both endo- and

exo-glucanases are inhibited by cellobiose. In synergy with other enzymes such as pectinases and hemicellulases, cellulases disrupt the structural integrity of the plant cell wall and enhance the extraction of the targeted molecules. Hence, they are used in any process involving processing of plant based materials. Cellulases are used in wine and fruit juice production to ease the maceration and accordingly, the extraction of pigments, flavor compounds and fermentable sugars and the extraction of juices. In bakery, cellulase is used to break down gums in dough structure, making the rise of dough and flavor distribution more even, improve the strength, elasticity and machinability of the dough and improve the crumb structure of the baked goods (Simpson et al., 2012; Miguel et al., 2013; Niemann, 2017). Cellulases are also used for the extraction of nutraceuticals from plants. Nutraceuticals are compounds from natural sources with additional health benefits, besides the nutrition role (Puri et al., 2012).

Glucanase

Alongside with cellulase (EC 3.2.1.4), laminarinase (EC 3.2.1.6., β-1,3(4)-*endo*-glucanase) which catalyzes the endo-hydrolysis of β-1,3- or β-1,4-glycosidic bonds in β-D-glucans, are the most widely used exogenous glucanases in brewing processes. More recently, the use of liceheninase (EC 3.2.1.73, β-1,3–1,4- glucan-4-glucanohydrolase), which cleaves β-1,4-glycosidic bonds adjacent to β-1,3-bonds, has also gained relevance. These enzymes are typically added during mashing and ease the filtration of beer by breaking down glucans which results in decreased viscosity and improves wort separation. Together with papain and α-amylase, glucanases are used to control haze in different stages of the manufacture of fruit juices. Glucanases are also used in wine making to enhance the release of aromatic compounds (Slaughter, 1985; Schauer and Borriss, 2004; Singhania et al., 2010; Simpson et al., 2012; Ackaah-Gyasi et al., 2015).

Inulinases

Inulinases are enzymes that mostly hydrolyze inulin, a polyfructan, but also levan and sucrose. Inulinases can have either endo- or exo-action. The former, endo-inulinase (EC 3.2.1.7. 2,1–β-D-fructan fructanohydrolase) catalyzes endohydrolysis of 2,1-β-D-fructosidic linkages in inulin, resulting in the formation of fructo-oligosaccharides (FOS), used as prebiotics; the latter, exo-inulinase (EC 3.2.1.80) hydrolyzes terminal, non-reducing 2,1- and 2,6-linked β-D-fructofuranose residues in fructans, and are used in the production of ultra-high fructose syrups. Fructose is sweeter than sucrose and does not crystalize easily, hence it is used as a sweetener in beverages and in confectionery (Singh et al., 2017).

Invertase

Invertase (EC 3.2.1.26, β-fructofuranosidase also known as sucrase) hydrolyzes terminal non-reducing β -D-fructofuranoside residues in β-D-fructofuranosides. It is essentially used for the hydrolysis of sucrose to glucose and fructose (invert sugar syrup). Invert sugar syrup is used as sweetener in baking, beverage, canning and dairy processes. The good humectant properties of the syrup also contribute to improved shelf-life in confectionery products. Within the scope of the latter, invertase is directly used to promote the formation of soft fondant centers (Serna-Saldivar and Rito-Palomares, 2008; Simpson et al., 2012; Subin and Bhat, 2015). Under adequate operational conditions (high initial sucrose concentration, pH, temperature, enzyme source) invertase displays *trans*-fructosylation activity, which enables the synthesis of short-chain fructo-oligosaccharides (scFOS) from sucrose. These scFOS are a mixture of 1-kestose, nystose and β-1-fructofuranosyl nystose and are formed upon the cleavage of the β-1,2- glycosidic bond of sucrose and the transfer of the fructosyl moiety onto an acceptor such as sucrose or a FOS. FOS are used several in food formulations such as jam, ice cream and in beverages and confectionery applications (Khandekar et al., 2014; Khanvilkar and Arya, 2015).

Lactase

Lactase (EC 3.2.1.23, β-D-galactoside galactohydrolase) also known as β-galactosidase hydrolyzes the β-glycosidic bond of lactose with the release of its monomers, glucose and galactose. Lactase is used for the hydrolysis of lactose in milk and whey. Lactose is present in milk at roughly 4.7% w/v, which prevents the consumption of this food by a significant fraction of adult population that suffers from lactose intolerance, unless it is processed with lactase. Moreover, lactose hydrolysis enhances the sweetness of the processed milk and decreases the risk of crystallization during spray-drying of milk, whey and cream. Hydrolyzed whey syrup is used in ice cream, milk desserts and sauces, and as a sweetener in cereal bars (Law, 2010; Fraatz et al., 2014). Lactase is also available as digestive aid (Ianiro et al., 2016). Under adequate operational conditions (temperature, initial lactose concentration, enzyme source, pH) lactase has also been shown to be able to carry out transgalactosylation reactions, where the galactosyl moiety released during lactose hydrolysis is accepted by lactose. The trisaccharide formed can act as acceptor of further galactosyl moieties released and so forth, so that a mixture of oligosaccharides, or galactooligosaccharides (GOS) can be synthesized. GOS are similar to human milk oligosaccharides which are lightly sweet, heat resistant and they influence the large intestinal microflora. They are currently used in infant formula and increasingly in milk and dairy products such as fermented milk, ice cream or lactic acid bacteria beverages. Moreover, GOS can be used in fruit juices and soft drinks, desserts, bakery and jams (Sangwan et al., 2011; Tzortzis et al., 2012; Osman, 2016).

Lipase

Lipase (EC 3.1.1.3. triacylglycerol acyl hydrolase) hydrolyzes triacylglycerols into free fatty acids and glycerol. Most of the lipases operate at lipid–water interfaces enabled by a mobile lid domain located over the active site. Besides the standard reaction type catalyzed, lipases catalyze other types of reactions, namely (trans)esterification, lactonization and polymerization, displaying a high affinity to long chain substrates, as opposed to esterases (Divakar, 2013; Khan et al., 2017). Lipases are highly promiscuous

enzymes hence they have wide application within the food sector. In bakery processes, lipases are used to replace or supplement emulsifiers, to improve the flavor of baked products by releasing short-chain fatty acids through esterification, to improve shelf-life, softness and texture of baked goods in synergy with amylases and xylanases, to increase dough strength, stability and machinability, and to improve and even crumb structure. Phospholipases have also been used in complement to lipases; however, their application in baking is much more recent than that of amylases or proteases (Miguel et al., 2013; Guerrand, 2017). In dairy, lipases are used for flavor enhancement in cheese products, to obtain specific flavors milk/vegetable oil/fat and to accelerate cheese ripening (Jooyandeh et al., 2009; Guerrand, 2017). Lipases are also used for the production of cocoa butter alternatives through interesterification reactions involving edible oils (palm oil, soybean oil) (Abigor et al., 2003). The production of lipids with similarities to human milk fat for incorporation in infant formula has also been done using lipases. Human milk fat substitutes (HMFS) have been obtained through: acydolysis of cheap natural oils and fats with high amounts of palmitic acid at *sn*-2 position such as butterfat, lard, palm oil or palm stearin and a source of free fatty acids, e.g., coconut oil, olive oil, soybean oil, rapeseed oil, or fish oils, rich in ω-3-polyunsaturated fatty acids (PUFAs); interesterification between oils containing palmitic acid at *sn*-2 position and vegetable oils rich in oleic acid and/or PUFA. Overall, the former approach is preferred, and it is used in the production of the commercial brand Betapol®, out of lard and soybean fatty acids (Ferreira-Dias and Tecelão, 2014; Guerrand, 2017). In egg processing, lipases are used for the conversion of egg yolk phospholipids into lyso-phospholipids to increase the emulsion stability and enhance its performance in dressings and mayonnaise related products (Guerrand, 2017). Degumming of edible vegetable oils, a process that consists of the removal of phosphatides from the crude oil, to improve storage stability and ease downstream processing of the oil, is carried out enzymatically with phospholipases (Casado et al., 2012; Guerrand, 2017).

Pectinase

Similar to cellulase, pectinase (or pectic enzymes) is a collective term that covers a set of enzymes that allow the degradation of pectic substances, polysaccharides composed of chains of D-galacturonic acid linked by α-1,4- bonds, present in plant cell walls. Pectic substances include pectic acids, a polymer of galacturonic acid, and pectin, where roughly 80% of the carboxyl groups of the polymer of galacturonic acid are methyl-esterified (Khan et al., 2013).

Pectic enzymes are classified according to their activity. Pectinesterase (EC 3.1.1.11, pectin pectylhydrolase, also known as pectin methylesterase), which hydrolyze the methyl ester bonds in pectin, resulting in pectate and methanol. Due to the release of methanol, there are restriction to the use of pectinesterase in food processing. The action of pectinesterase eases subsequent depolymerization activity of polygalacturonases towards the degradation of pectin and ease further processing of the materials, namely facilitating extraction, maceration, filtration and clarification. The depolymerization implemented through the use of polygalacturonases involves the hydrolysis of the α-1,4-glycosidic bonds between D-galacturonic acid units in the unesterified polygalacturonic backbone. Commonly used polygalacturonases are endopolygalacturonases (EC 3.2.1.15, α-1,4-D-galacturonan glycanohydrolase, commonly known simply as polygalacturonase) which randomly hydrolyze α-1,4-D-galactosiduronic linkages in pectate and other galacturonans; and exopolygalacturonase (EC 3.2.1.67, poly[-α-1,4-D-galacturonide] galacturonohydrolase) which release monomers or dimers form the non-reducing end of the chain. Depolymerization can also occur through the action of pectin lyases, which cleave the α-1,4-glycosidic bonds by β-elimination to produce 4,5-unsaturated oligogalacturonates. Pectin lyases can depolymerize highly esterified pectin into small molecules without prior action of pectinesterase, hence the use of pectin lyases prevents tampering with the polymer chain ester content, which is accountable for the aroma of fruits. As with polygalacturonases, pectin lyases can cleave the polysaccharide chain either randomly with pectate lyase (EC 4.2.2.2., α-1,4-D-galacturonan lyase) and pectin lyase (EC 4.2.2.10, (1,4)-6-*O*-methyl-α-D-galacturonan lyase), the latter acting preferably on highly methylated pectins, or by releasing dimers from the reducing end with exopectate lyase (EC 4.2.2.9., α-1,4-D-galacturonan reducing-end-disaccharide-lyase) (Yadav et al., 2009; Mojsov, 2016; Sharma et al., 2017). Commercially available enzyme preparations are a mixture of these pectic enzymes (Pedrolli et al., 2009). Given their catalytic activities, pectic enzymes are naturally used when processing of cereals, fruits and vegetables is involved. Thus, in combination with (hemi)cellulases, pectinases enhance the extraction of fruit juices, vegetable oils, and aromatic compounds, therefore, they find applications in the production of edible oils, juices, wine and nutraceuticals. Pectinases are also used to decrease viscosity in fruit juices and to prevent jellification in the preparation of concentrated fruit juices, to prevent haze formation and ease filtration in wine and fruit juices, to improve maceration of vegetables in the production of pastes and purées, to speed up the fermentation of tea, and to remove the mucilage layer of the grain in the processing of coffee beans (Pedrolli et al., 2009; Khan et al., 2013; Molina et al., 2015; Sharma et al., 2017).

Protease

Proteases (peptidase or proteinase) encompass a large array of enzymes that share the ability to hydrolyze covalent peptide bonds. The resulting smaller peptides typically display novel properties, including solubility, gelation, emulsification, foaming and taste (Liu and Kokare, 2017; Zeeb et al., 2017). An example of the advantageous use of these features is the production of protein hydrolysates, which can be included in human diet either for protein supplementation (e.g., energy drinks, sports nutrition, weight-control diets) or for clinical application, as ingredients of functional foods (e.g., tackling metabolic syndromes such as short bowel syndrome, hypertension by inhibiting angiotensin converting enzyme, ulcerative colitis; production of hypoallergenic infant formula) (Clemente, 2000; He et al., 2013; Lafarga and Hayes, 2017). Proteases are among the most commonly used enzymes in food processing and examples to illustrate this include the use of rennet (mostly chymosin, complemented by a minor amount of pepsin) as a coagulant in the manufacture of cheese and the use of papain for meat tenderization (Sumantha et al., 2006; Law, 2010; Liu and Kokare, 2017).

Proteases can be classified depending on pH, mode of action and nature of the active site, as illustrated in **Table 3**, where further examples on the application of proteases in the food industry are presented.

Other proteases representatives of the relevance of this type of enzymes within the scope of use in the food industry include (Subin and Bhat, 2015): a) aminopeptidases (EC 3.4.11) and carboxypeptidases (EC 3.4.16–3.4.18), used for the production of flavor-precursors and taste active compounds, for the production of enzyme modified cheeses and for debittering of protein hydrolysates (Raksakulthai and Haard, 2003; Fu et al., 2011; Tchorbanov et al., 2011; Katsimpouras et al., 2014); b) chymosin (EC. 3.4.23.4, also known as rennin), an acidic protease, that is the major component of rennet, and cleaves the bond -Phe105-Met106- in the κ -chain of casein, to promote milk clotting and separation of the curd, or cheese from the whey. Chymosin is also a key proteolytic agent in cheese ripening. The specificity of chymosin is of interest since non-specific proteolysis of α- and β-casein during curd formation can decrease the yield in cheese manufacture (O'Mahony et al., 2005; Law, 2010; Roy et al., 2015); c) pepsin (EC 3.4.23.1, pepsin A), which is a minor component of rennet, and displays a wider range of proteolytic activity, thus expanding its range of action. Besides its clotting role in cheese making, it is suggested, although without hard evidence, to improve cheese ripening. Pepsin, alongside with trypsin and papain, has been used to produce bioactive peptides from dairy products through proteolysis. Pepsin, as well as other proteases such as ficin or papain, has also been used to prevent haze formation in beer, by degrading leftover proteins which partially precipitate during chilling. Descaling of fish assisted by pepsin has been also reported (Law, 2010; Van Oort, 2010; Choi et al., 2012; Nielsen and Nielsen, 2012; Roy et al., 2015); d) papain (EC 3.4.22.2.), a plant enzyme, has a broad specificity for peptide bonds, although it privileges an amino acid containing a large hydrophobic side chain at the P2 position. Accordingly, papain is commonly used when proteolytic activity is required. The primary use of this enzyme involves meat tenderization, where it promotes noticeable degradation to both myofibrillar and collagen proteins (Ashie et al., 2002; Arshad et al., 2016). Ficin (EC. 3.4.22.3) and bromelain (EC 3.4.22.32, stem bromelain and EC 3.4.22.33 - fruit bromelain), both also plant enzymes are used as meat tenderizers, yet less common than papain due to economic reasons (Van Oort, 2010; Bekhit et al., 2014a,b); e) subtilisin (EC. 3.4.21.62), is an alkaline protease with broad specificity for peptide bonds, privileging large uncharged residues in P1 position, which hydrolyzes peptide amides. P1 is the first amino acid residue in a substrate undergoing cleavage in the N-terminal direction from the cleaved bond. Subtilisin, commercialized under the brand Alcalase®, is used in the production of protein hydrolysates that can be used in the manufacture of soups, dressings, protein bars and sports drinks (Nielsen, 2010; Subin and Bhat, 2015; Tavano, 2016); f) thermolysin (EC 3.4.24.27) preferentially cleaves sites with bulky and aromatic residues, e.g., Leu (leucine) and Phe (phenylalanine) respectively (Leu, in P1′ position. P1′ is the first amino acid residue in a substrate undergoing cleavage in the C-terminal direction from the cleaved bond It is used in the production of bioactive compounds from milk hydrolysates (e.g., angiotensin I-converting enzyme inhibitor) and of the artificial sweetener Aspartame® (Nielsen, 2010; Tavano, 2016); g) trypsin (EC. 3.4.21.4), an animal protease, has been used in the production of hydrolysates for food flavoring, yet is gradually being replaced by microbial proteinases for economic reasons. The latter are used in the production of protein hydrolysates of vegetable or animal origin that may be used for protein supplementation, emulsification or flavor enhancement in food and beverages (Van Oort, 2010; Tavano, 2016).

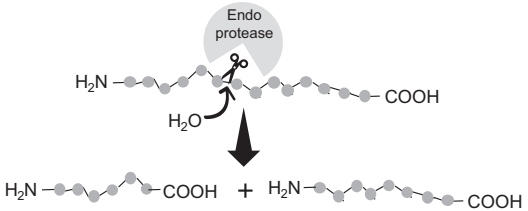
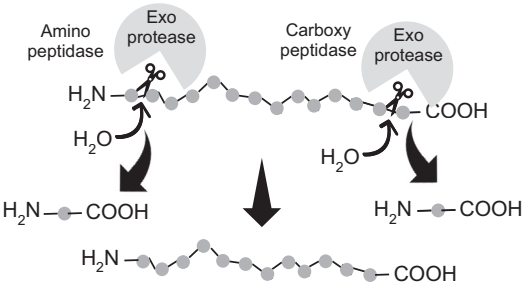
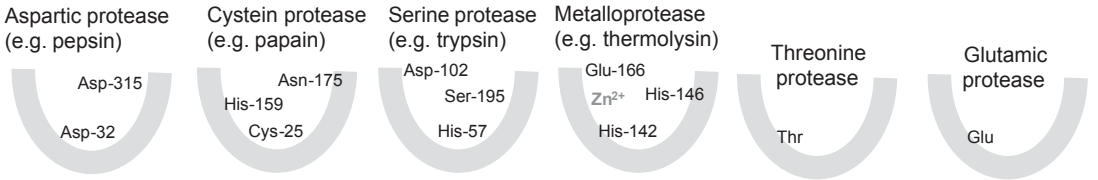
Pullulanase

Up to five different types of pullulanases have been identified, all sharing the ability to cleave α-1,6-D-glycosidic bonds, a behavior that is not observed in amylases, except marginally in γ-amylase (Martínez and Gómez, 2016). Pullulanases are thus called debranching enzymes, since α-1,6-D-glycosidic bonds occur where branches connect to the glucan backbone. Of all pullulanases, the most commonly used is pullulanase (EC 3.2.1.41, pullulan 6-α-glucanohydrolase) which hydrolyzes α-1,6-D-glycosidic linkages in pullulan and branched polysaccharides. The role of pullunase is complemented by neopullulanase (EC 3.2.1.135) and isopullulanase (EC 3.2.1.57) that additionally also cleave α-1,4-D-glycosidic bonds (Hua and Yang, 2015; Serna-Saldivar and Rubio-Flores, 2017). Pullulanases thus have a relevant role in starch processing, namely in the production of corn derived sweeteners. Pullulanase, combined with β-amylase in starch saccharification process, allows an increase in maltose yield. The combined use of γ-amylase and pullulanase in the starch saccharification process enhances the maximum amount of glucose that can be obtained, as compared to the saccharification without pullulanase, even with a decreased dosage of γ-amylase. The incorporation of pullulanase in starch processing thus enhances the yield in maltose and dextrose syrups and, considering the latter, better conditions are also provided in the production of high fructose syrups. The syrups are commonly used in confectionary and as sweeteners in beverages. Pullulanases are also used in brewing to increase the amount of substrate for β-amylase and maximize the fermentability of the wort, and to decrease the amount and wort viscosity in the production of light beers. In baking, pullulanases are used to prevent staling, and thus improve flavor, texture and volume of baked goods (Hi et al., 2012; Hua and Yang, 2015; Serna-Saldivar and Rubio-Flores, 2017).

Xylanase

Xylanase refers to an enzyme complex able to fully hydrolyze xylan. Xylan is a linear polymer of β-D-xylopyranosyl units linked by 1,4-glycosidic bonds. In nature, several residues, such as 4-*O*-methyl-α-D-glucuronopyranosyl units are added to the polysaccharide backbone. Xylan is the second most abundant natural polysaccharide and is present in the cell wall and in the middle lamella of plant cells (Polizeli et al., 2005). Xylanases are thus paramount in lysing the cell walls of grains and grapes, typically in synergy with other enzymes, such as cellulases and pectinases, and thus release aromas, fermentable sugars, pigments, etc., and thus find several applications in food processing (**Fig. 1**).

Table 3 Some common methodologies for the classification of proteases and examples of applications in food industry (Nielsen, 2010; Jain et al., 2010; Lalor and Goode, 2010; Fellows, 2017; Liu and Kokare, 2017)

Method	*Comments*
Based on pH	Acidic ($4.5 < pH_{optima} < 7.5$), e.g., pepsin. Used for milk coagulation, cheese processing, meat tenderization and production of food protein hydrolysates. Neutral ($7.0 < pH_{optima} < 8.0$), e.g., bacillolysin (Neutrase®), thermolysin. Used for the degradation of proteins in flour in bakery; to optimize the level of free α-amino nitrogen levels in high adjunct brewing; for meat and fish tenderization; to speed up cheese ripening; for debittering, and to produce aspartame, enzyme-modified cheese, hypoallergenic milk-based foods and food protein hydrolysates. Alkaline ($8.0 < pH_{optima} < 11.0$), e.g., subtilisin. Used for the production of food protein hydrolysates
Site of action	Endoprotease (e.g. pepsin, trypsin)  Exoprotease 
Catalytic mechanism	

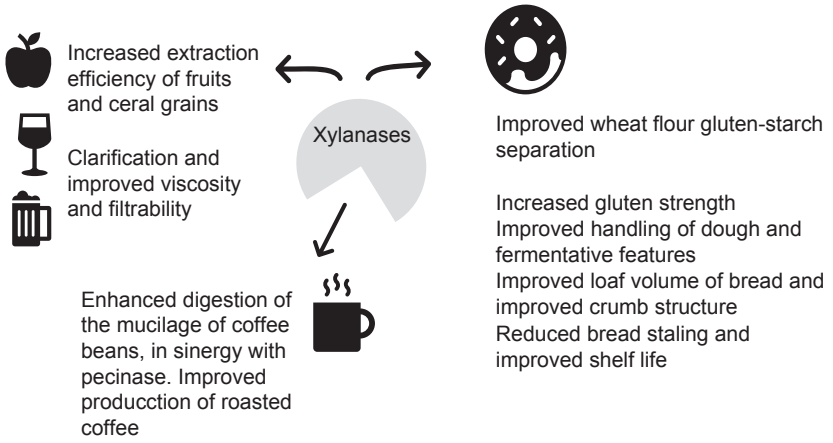


Figure 1 Relevant examples of roles of xylanase in food processing (Miguel et al., 2013; Chandrasekaran et al., 2015; Serna-Saldivar and Rubio-Flores, 2017).

The enzyme complex includes: a) xylanase (EC. 3.2.1.8, endo-1,4-β-xylanase), which hydrolyzes β-1,4-D-xylosidic linkages in xylan and arabinoxylan, forming arabinoxylo-oligosaccharides; b) β-xylosidase (EC. 3.2.1.37, xylan-β-1,4-xylosidase), which releases xylose monomers from the nonreducing end of arabinoxylo-oligosaccharides; c) arabinofuranosidase (EC. 3.2.1.55, α-L-arabinofuranosidase), which releases the arabinose residues; and d) ferulic acid esterase (EC. 3.1.1.73), which cleaves ester linkages between arabinose residues and ferulic acid (Rosell and Dura, 2015; Liu and Kokare, 2017).

Isomerases

Glucose Isomerase

Glucose (xylose) isomerase (EC 5.3.1.5, D-xylose aldose-ketose-isomerase) catalyzes the reversible isomerization of glucose to fructose, to produce a mixture of glucose and fructose, known as high fructose corn syrup (HFCS), a sweetener alternative to sucrose and invert sugar syrup. HFCS is widely used in foods and beverages; examples include: baked goods, such as biscuits, bread, cookies; soft drinks and juice drinks; cereals and cereal bars; jams and jellies; ice creams, flavored milks, eggnog, yogurts, and frozen desserts; and canned ready-to-eat foods, among other processed foods (Chandrasekaran and Beena, 2015). The compromise between thermal stability/activity of the enzyme and thermodynamic equilibrium limits operation to temperatures from 55 °C to 65 °C, depending on which, the reaction mixture can contain 42% to 47% fructose. Typically, isomerization is performed at 60 °C and under current immobilized glucose isomerase formulations, a reaction mixture containing 42% fructose, HFCS-42 is obtained, which is as sweet as sucrose. The titer in fructose can be increased to 55% through chromatography. This step leads to a syrup with 90% fructose (HFCS-90), which is blended to produce HFCS-55, about 1.3-fold sweeter than sucrose, required for most soft drinks (DiCosimo et al., 2013; Thum et al., 2014; Hua and Yang, 2015; Chandrasekaran and Beena, 2015; Desai et al., 2017). HFCS production has the largest scale within the scope of industrial processes utilizing immobilized biocatalysts, with the global production reaching 10^7 ton/year, and despite some controversy on the impact of HFCS on health, its consumption is foreseen to continue to increase (DiCosimo et al., 2013; Chandrasekaran and Beena, 2015; Desai et al., 2017).

Sucrose Isomerase

Another relevant isomerase in sugar modification is sucrose isomerase (EC 5.4.99.11), used in the production of isomaltulose (β-D-fructofuranosyl-1,6-α-D-glucopyranoside), by rearranging the α-1,2 bond between glucose and fructose into an α-1,6 bond. The resulting disaccharide, commercialized under the trade name Palatinose™, is half as sweet as sucrose but it is non-cariogenic, has a low glycemic index and has a prebiotic role. Hence, it is used as replacement of sucrose in the formulation of dietary foods for diabetics, in confectionery, chocolates, chewing gum and beverages, e.g., dairy drinks, sports drinks, alcohol free beer and helps preventing tooth decay. The enzymatic reaction also produces a secondary product, trehalulose, where glucose and fructose are linked through an α-1,1 bond. Trehalulose is about 60% as sweet as sucrose and shares most of the isomaltulose features and applications (Thum et al., 2014; Arruda et al., 2017).

Oxidoreductases

Glucose Oxidase

Glucose oxidase (EC 1.1.3.4., β-D-glucose:oxygen 1-oxidoreductase) oxidizes glucose to D-glucono-δ-lactone and hydrogen peroxide. Glucose oxidase acts mostly as oxygen scavenger and thus stabilizes foods and beverages, e.g., fruit juices, dried milk, mayonnaise, preventing changes in color and taste during storage. Glucose oxidase is also used in bakery processes to strengthen gluten, as a replacement of chemical oxidants, leading to more stable dough and improved bread quality (Miguel et al., 2013; Patel et al., 2017).

Laccase

Laccases (EC. 1.10.3.2, benzenediol:oxygen oxidoreductase) are multi-copper enzymes, which oxidize a wide array of compounds, such as phenolic compounds, coupled with the reduction of molecular oxygen to water (Mate and Alcalde, 2017). Laccases are versatile biocatalysts and accordingly find application in the processing of juices and alcoholic beverages, as well as in bakery processes, among other application (**Fig. 2**).

Lipoxygenase

Lipoxygenase (EC. 1.13.11.12, linoleate:oxygen 13-oxidoreductase) catalyzes the oxygenation of PUFAs to fatty acid hydroperoxides (Baysal and Demirdöven, 2007). Lipoxygenases are used in dough processing as an oxidative improving agent. Oxidizing agents increase the molecular weight of dough proteins, ultimately improving dough development, strength and workability, and enhance the reproducibility of the final product. The products resulting of lipoxygenase oxidation of flour fatty acids promote the cross-linking of flour proteins. Besides contributing to improving dough rheology, lipoxygenase has also a bleaching effect on the carotenoid pigments of flour (Hayward et al., 2017).

Transferases

Cyclodextrin Glycosyltransferase

Cyclodextrin glycosyltransferase (EC 2.4.1.19, 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing) catalyzes the cleavage of starch into cyclic, non-reducing, 1,4-α linked cyclomaltodextrins, commonly designated as cyclodextrins. Cyclodextrins act as molecular hosts and are used in the food industry for several purposes, namely: a) to provide a protective environment to lipophilic food components sensitive to oxygen, light or heat degradation; b) in the solubilization of food pigments and vitamins; c) for the controlled release of specific food ingredients; d) to prevent tampering with essential oils, flavors and vitamins; e) to suppress unwanted odors/tastes; f) to act as dietary fiber. Cyclodextrins are used as ingredients in baked goods, cereals, dairy products and transparent soft drinks among others (Liese et al., 2006; Astray et al., 2009; Li et al., 2014). Besides the cyclization reaction, cyclodextrin glycosyltransferase also catalyzes glycosylation, acceptor or transfer reactions between cyclodextrins and acts as a carbohydrate acceptor, e.g., glucose and sucrose. This ability is used to reduce the bitter taste of stevioside, a natural, high intensity sweetener with low glycemic index, used in soft drinks, fruit products and confectionery (Leemhuis et al., 2010; Martínez and Gómez, 2016). In bakery processes, cyclodextrin glycosyltransferases are used to prevent staling of baked goods by cleaving maltose units from amylopectin side-chains, and to improve bread volume (Martínez and Gómez, 2016).

Fructosyltransferases

Fructosyltransferases are enzymes with the ability to transfer fructosyl residues between or within molecules. Although some invertases display transfructosylation ability, three enzymes display the latter as primary role, namely sucrose:2,1-β-D-fructan 1- β -D-fructosyltransferase (EC 2.4.1.9, inulosucrase), sucrose:sucrose 1′-β-D-fructosyltransferase (EC 2.4.1.99) and 2,1-β-D-fructan:2,1-β-D-fructan 1-β-D-fructosyltransferase (EC 2.4.1.100). Of these three, the first is used in the production of scFOS, as an alternative to invertases (Michel et al., 2018; Herrera-González et al., 2017).

Transglutaminase

Transglutaminase (2.3.2.13, protein-glutamine:amine γ -glutamyltransferase) cross-links proteins by transferring the γ-carboxyamide group of the glutamine residue of one protein to the ε-amino group of the lysine residue of the same or another protein.

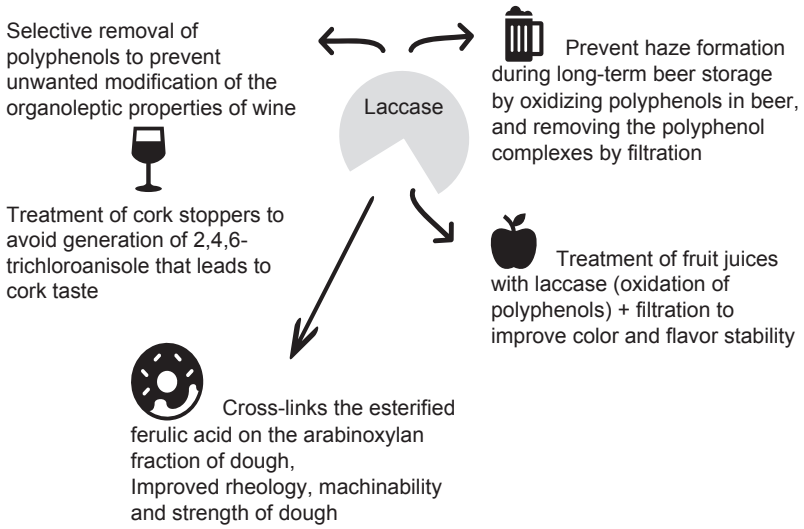


Figure 2 Relevant examples of roles of laccase in food processing (Miguel et al., 2013; Mate and Alcalde, 2017).

Concomitantly, the protein conformation is modified, leading to changes in the structure, gelation stability and water-binding ability, ultimately with modification of the rheological properties of protein products. Additionally, transglutaminase can also hydrolyze the γ-carboxyamide group of the glutamine residue of a protein (Nandakumar and Wakayama, 2015; Błażejak and Kieliszek, 2017). These features resulted in this enzyme being used for different application in the food industry, namely in bakery, dairy, meat, fish, fruit and vegetables processing as illustrated in **Fig. 3**.

Lyases

Acetolactate Decarboxylase

Acetolactate decarboxylase (EC. 4.1.1.5) is used to reduce the maturation time of beer. This enzyme is added in the fermentation step and is used to shunt the formation of diacetyl (**Fig. 4**). This compound is slowly formed out of acetolactate excreted by yeast during fermentation, and it imparts an unpleasant, buttery flavor to beer, even at 0.15 ppm. Diacetyl is then transformed into acetoin, a molecule that conveys fruity flavor to beer by yeast. The whole process can take a few weeks, and is considerably shortened by the addition of acetolactate decarboxylase, with no negative impact in the organoleptic and functional properties of beer (Dulieu et al., 2000; Serna-Saldivar and Rubio-Flores, 2017).

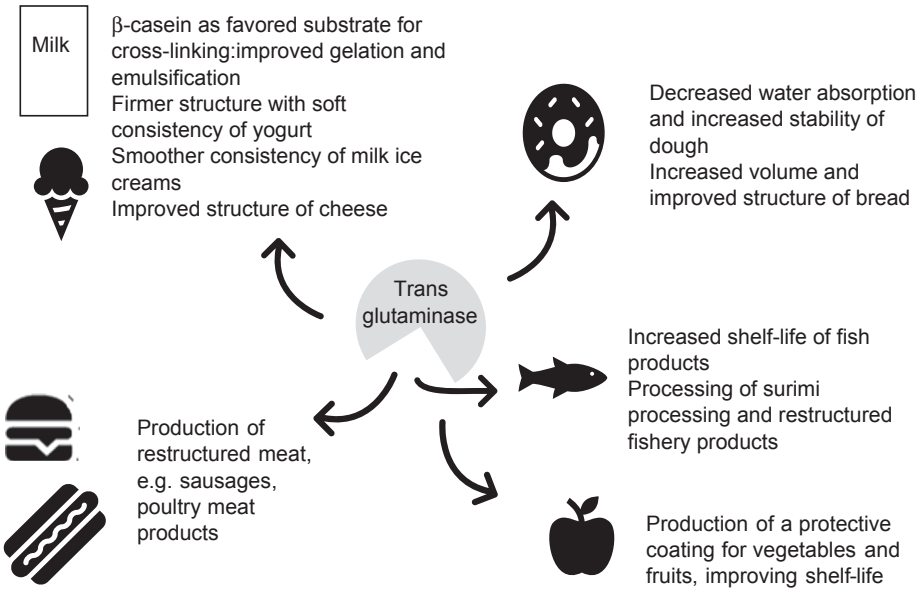


Figure 3 Representative examples of the application of transglutaminase in food processing (Nandakumar and Wakayama, 2015; Błażejak and Kieliszek, 2017).

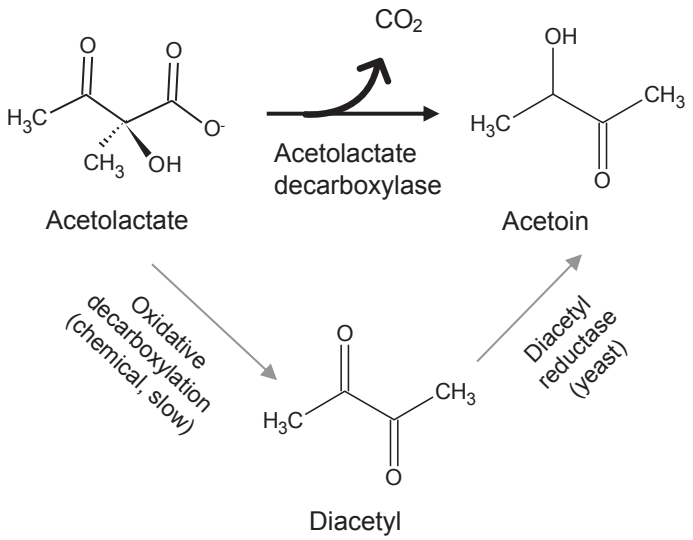


Figure 4 Action of acetolactate decarboxylase bypasses the formation of diacetyl in beer and speeds up the production process.

Concluding Remarks

The examples presented are illustrative of the widespread use of enzymes in the food industry, both in traditional sectors, such as bakery and dairy processing, as well as in the development of new sectors, such as functional foods. The increased insight of the catalytic mechanisms of the different enzymes and their structures, as well as of the materials they act upon, is providing an increasing rationale for their applications, gradually leaving behind some empiricism associated with their traditional use. As the number of identified enzymes increases, and methodologies for enzyme production are improved, novel and/or improved functionalities and further commercial food enzymes can be expected, with their practical application potentiated with suitable formulations, all under good manufacturing practices, to ensure that efficacity is coupled with health and safety for the consumer.

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