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1. Introduction

The use of enzymes dates from much longer than their ability to catalyze reactions was recognized and their chemical nature was known. The first completely enzymatic industrial process was developed in the years 1960 [1]. Starch processing, which is undertaken in two steps, involves liquefaction of the polysaccharide using bacterial α-amylase, followed by saccharification catalyzed by fungal glucoamylase.

After the Second World War, enzyme applications rose due to advances in industrial microbiology and biochemical engineering [1]. Nowadays, enzymes are employed in many different areas such as food, feed, detergent, textiles, laundry, tanning, as well as pharmaceuticals, cosmetics, and fine-chemicals industries. Industrial applications account for over 80% of the global market of enzymes [2]. At least 50% of the enzymes marketed today are obtained from genetically modified organisms, employing genetic and protein engineering. Food enzymes are the most widely used and still represent the major share in enzyme market.

Developments in process technology allied to the use of recombinant techniques during the last decades allowed for considerably improved yields by fermentation, increased stability, and altered specificity and selectivity of enzymes [3-5]. Those techniques thrust forward and are continuing to broaden the applications of enzymes in food technology and many different areas.
There are two scenarios regarding the use of enzymes, either the enzymes are used to convert the raw material into the main product, or the enzymes are used as additives to alter a functional characteristic of the product. In the first case, the enzymatic process is undertaken in optimized and controlled conditions to enhance the catalytic potential of the enzyme, whereas in the second situation it is more difficult to assure optimal conditions and to control the enzymatic reaction [1]. An example of the first case is the use of immobilized glucose isomerase for the production of high-fructose syrups (HFS), and an example of the second scenario is the use of fungal proteases in dough making [1,6,7].

Enzymes are an important ingredient used in most bakery products. More recently enzymes have assumed an even greater importance in baking, due to the restrictions on the use of chemical additives, especially in the manufacture of bread and other fermented products [8].

The aim of this review is to discuss current applications of enzymes in the bakery industry and to explore future trends in this sector of food industry.

2. Bakery enzymes market

The development of bread process was an important event in mankind. After the 19th century, with the agricultural mechanization, bread’s quality was increased while its price was reduced; thereby white bread became a commodity within almost everyone’s reach [9]. An important aspect that contributed to evolution of the baking market was the introduction of industrial enzymes in the baking process, where bakery enzymes represent a relevant segment of the industry.

Among the main industrial enzyme producers, according to Novozymes S/A report 2011 [10], Novozymes S/A occupies 47% of the market, DuPont 21%, DSM 6% and the rest is occupied by other players. Furthermore, in that year, food and beverage enzymes represented 29% of enzyme business and biobusiness sales by the industry [10].

The world enzyme market is in evolution and a growth of 6.8% per year is expected [11]. The world food and beverage enzymes demand requires attention, because it represented $1,220 million dollars in 2010, around 36.5% of the total world industrial enzyme demand, estimated in $3,345 million dollars. Moreover, the world food and beverages enzymes demand is expected to be responsible for 40.1% of the world industrial enzyme demand in 2020, accounting for $2,520 of $6,280 million dollars of the world industrial enzyme market (Figure 1) [11].

Table 1 summarizes the world bakery and enzyme demand between 2000 and 2020, segmented according to products. It is possible to observe that the enzymes market for baked goods is expected to increase from 420 million dollars in 2010 to 900 million dollars in 2020, although maintaining its representativeness in this segment, varying from 34.4 in 2010 to 35.7% in 2020 [11].
Source: Adapted from The Freedonia Group Inc., World Enzymes to 2015.

**Figure 1.** Estimated world food and beverage enzyme demand participation on the world industrial enzymes in million dollars from 2000 to 2020.

| Item                              | Years |
|----------------------------------|-------|
|                                  | 2000  | 2005  | 2010  | 2015  | 2020  |
| World food and beverage enzyme demand | 520   | 760   | 1220  | 1770  | 2520  |
| Baked goods                      | 140   | 250   | 420   | 625   | 900   |
| Dairy                            | 180   | 260   | 360   | 465   | 610   |
| Other foods and beverage         | 200   | 250   | 440   | 680   | 1010  |

Source: Adapted from The Freedonia Group Inc., World Enzymes to 2015.

**Table 1.** Estimated demand of baked goods, dairy and other food & beverage enzymes in million dollars from 2000 to 2020.

### 3. Main constituents of baked products

Baking is a common name for the production of baked goods, such as bread, cake, pastries, biscuits, crackers, cookies, pies and tortillas, where wheat flour is both the most essential ingredient and key source of enzyme substrates for the product [12]. Even though based on cereals other than wheat, baked goods such as gluten-free products or rye bread are also considered to be baked products [8]. Baked goods formulations vary significantly depending on the desired
final product, and typical ingredients, apart from starch, can include wheat flour (8-16% protein, 71-79% carbohydrate), fats, sugars, eggs, emulsifiers, milk and/or water [13].

Bread is usually made from wheat flour as raw material, which is a mixture of starch, gluten, lipids, non-starch polysaccharides and enzymes. After flour, yeast and water are mixed, complex biochemical and biophysical processes begin, catalyzed by the wheat enzymes and by the yeast, characterizing the dough phase. These processes go on in the baking phase, giving rise to bread. Extra enzymes added to the dough improve control of the baking process, allowing the use of different baking processes, reducing process time, slowing-down staling, compensating for flour variability and substituting chemical additives [14]. Starch is the main component of products such as bread and other bakery goods and is added to different foods, acting as a thickener, water binder, emulsion stabilizer, gelling agent and fat substitute [15]. It is the most abundant constituent and most important reserve polysaccharide of many plants, including cereals, occurring as intracellular, semi-crystalline granules. On a molecular level, its major components are the glucose polymers amylose and amylpectin [16]. Amylose is an essentially linear molecule, consisting of up to 6000 glucose units with α-(1,4)-glycosidic bonds (Figure 2). On the other hand, amylpectin is a highly branched polysaccharide constituted of short α-1,4 linked linear chains of 10–60 glucose units and α-1,6 linked side chains with 15–45 glucose units (Figure 3), containing on average 2 million glucose units [17].

![Figure 2. Structure of amylose chain, assumed as a left-handed spiral due to α(1→4) glycosidic bonds (n = 500 - 6000 α-D-glucopyranosyl units).](image)

Even though many flour components such as starch, arabinoxylans and lipids affect dough rheological properties [16,18-20], gluten provides dough with extensibility, viscosity, elasticity, cohesiveness and contributes to its water absorption capacity [21]. The unique ability of wheat flour to form visco-elastic dough with gas-holding properties is mostly due to the gluten proteins, the major storage proteins of wheat, which have an essential role in breadmaking [22].
Figure 3. Partial structure of amylopectin with amylolytic enzymes action sites represented by arrows: (a) α-amylases; (b) amyloglucosidases; (c) β-amylases; (d) isoamylases and pullulanases. Both α(1→4) glycosidic linkages between the glucose units in the linear chain and one α(1→6) glycosidic linkage to a side chain of the polysaccharide are represented.

Figure 4. Schematic drawing of gluten proteins structure, where gliadins are represented by spheres and glutenins by filaments. The bulkier structure in the upper part shows the gas retained in the gluten network and consequent dough volume expansion observed in the baking process. The slimmer structure in the lower part represents plasticity, extensibility and viscous properties of the gluten matrix.

Gluten proteins can be divided into monomeric gliadins and polymeric glutenins, based on solubility in 70% aqueous ethanol solutions [23]. Gliadins are globular proteins with molec-
ular weights ranging from 30,000 to 80,000, and are further classified into three groups: α-, γ- and ω-gliadins [24,25]. Except for the ω-gliadins which lack cysteine residues, gliadins contain intramolecular disulfide bonds [21]. Glutenins consist of a heterogeneous mixture of linear polymers with a broad molecular weight range from ca. 80,000 up into the millions [22], made up of disulfide cross-linked glutenin subunits which are biochemically related to the gliadins. The intermolecular disulfide bonds stabilize the glutenin polymers [21]. Gliadins mainly impart the plasticity, extensibility and viscous properties to wheat flour dough whereas glutenins are mostly responsible for the elasticity and cohesive strength of dough (Figure 4) [21,22]. Aspects such as the glutenin polymer structure, size distribution and subunit composition, as well as the gliadin/glutenin ratio are important to determine gluten quality and, consequently, the breadmaking potential of wheat flour [25-29].

Cereal non-starch polysaccharides are dietary fibre constituents, mostly composed of arabinoxylans, β-glucan and arabinogalactan-peptides. Arabinoxylans make up the largest non-starch polysaccharide fraction of cell walls of many cereals, such as wheat and rye [22,30]. They are polydisperse polysaccharides with similar structural properties, which are present in water-extractable (WE-AX) and water-unextractable (WN-AX) forms [16]. Arabinoxylans consist of a β-1,4 linked D-xylopyranosyl backbone substituted with α-L-arabinofuranose residues at the C(O)3 and/or C(O)2 positions [31-33]. Arabinose residues can be further coupled at the C(O)5 to ferulic acid through an ester linkage [34] (Figure 5). Even though minor flour constituents, arabinoxylans have the capacity to significantly affect the properties of dough and the final baked product [18]. Arabinoxylans and arabinogalactans possess important functional properties for the cereal industry. They can improve dough development and dough stability, by enhancing the water absorption capacity of the dough. These polysaccharides also confer viscosity and may increase gas permeability by contributing to the elasticity of the protein film around them. Additionally, during breadmaking they improve loaf volume, crumb firmness, reduce retrogradation and therefore, enhance the shelf life and storage stability of bread [35].

Figure 5. Partial structure of an arabinoxylan: a linear main chain formed by xylan (a pentosan consisting of D-xylose units connected by β(1→4) linkages), randomly attached to L-arabinofuranose residues by α(1→3) or α(1→2) linkages.
In addition to starch, gluten proteins and wheat flour non-starch polysaccharides such as arabinoxylans, lipids and enzymes can considerably improve the breadmaking performance [16,18,22,36,37]. Lipids are important components in breadmaking because they provide a variety of beneficial properties during processing and storage. In bread, lipids come from multiple ingredients, largely wheat flour, shortening and surfactants in a typical bread formula [38]. Wheat flour contains about 2% lipids [23], which occur free and bound to other wheat constituents. They are classified as starch lipids and free and bound non-starch lipids, based on their solubility in solvents of different polarities [39]. The bound non-starch lipids are mainly associated with flour protein and consist predominantly of non-polar lipids, while free non-starch lipids comprise mostly polar glyco- and phospholipids [40].

4. Baking process

Bread processing can be divided into three basic operations mixing, fermentation (resting and proofing) and baking. Through baking the mainly fluid dough or batter is transformed into a predominantly solid baked product. Indirectly, baking alters the sensory properties, improving palatability, and extending the range of tastes, aromas and textures of foods produced from raw materials [41].

Although baking has been practiced for a very long time, the whole process is not completely understood, possibly due to the occurrence of several coupled complex physical [42] and molecular processes [43]. The baking process therefore results in a series of physical, chemical and biochemical changes in the product. These changes include volume expansion, evaporation of water, formation of a porous structure, denaturation of protein, gelatinization of starch, crust formation and browning reactions [44].

Bread consists of an unstable, elastic, solid foam structure, containing a continuous phase made up of an elastic network of cross-linked gluten protein molecules and of leached starch polymer molecules, mainly amylose, uncomplexed and complexed with polar lipid molecules, and also a discontinuous phase of entrapped, gelatinised, swollen, deformed starch granules [45]. The nature and properties of the final product are influenced by physical and mechanical mixing, chemical reactions (including enzyme-catalyzed reactions), and thermal effects (baking time and temperature).

The simplest breadmaking procedure is a straight-dough system where all bread formula ingredients are mixed into developed dough [46]. A second process is the sponge and dough method where mixing of ingredients is performed in two steps. Leavening agent is prepared in the first step, by mixing together the yeast and certain quantity of water and flour. The mixture is left to develop for a few hours and then it is mixed with the other ingredients [42]. A third procedure is the Chorleywood method in which all the ingredients are mixed for a few minutes in an ultrahigh mixer [47].

In conventional breadmaking, the most commonly used leavening agent is the yeast *Saccharomyces cerevisiae*, although other *Saccharomyces* species such as *S. cariocanus*, *S. mikatae*, *S. para-
doxus and S. kudriavzevii can be also employed [48]. Furthermore, lactic acid bacteria, mainly Lactobacillus species are used as leavening agents for sourdough bread production [49].

The breadmaking process begins with the formation of dough through mixing of flour, water, yeast, sugar, salt, shortening and other ingredients. Flour particles are hydrated and sheared during mixing, and dough develops when gluten proteins form a continuous cohesive network in which the starch granules are dispersed [40]. Depolymerisation and polymerisation reactions possibly give rise to the gluten network, mostly made up of glutenin [50]. Incorporation of air during dough mixing is extremely important, affecting the final crumb structure because the carbon dioxide produced by yeast during fermentation diffuses to pre-existing air bubbles [40,51]. An optimal gluten network confers dough machinability, good gas retention, high bread volume and fine crumb structure [29]. After resting, the dough is divided into loaf-sized pieces, rounded, moulded, placed on a baking tray, proofed and baked.

The combined effects of heat, moisture and time induce starch gelatinisation and pasting which together with heatsetting of gluten proteins occur during baking, giving rise to the typical solid foam structure of baked bread [22]. The partially crystalline starchy starch is converted into amorphous, transient, gelatinised starch networks. The swollen gelatinised starch granules are deformed, part of the starch polymers leach out of the granules and form a continuous network in the bread crumb [40,52]. Besides accumulation of amylose outside the granules, the presence of an amylose-rich region in the centre of gelatinised starch granules was found after baking [22,52].

During baking the transient gluten network formed in dough is transformed into a continuous, permanent network probably due to modifications in protein surface hydrophobicity, sulfhydryl/disulfide interchanges and formation of new disulfide cross-links [22,38,50,53]. Moreover, heat-induced sulfhydryl-disulfide exchange reactions can lead to incorporation of α- and γ-gliadins into the glutenin network [54]. Gas cell opening occurs, and besides becoming gluten continuous the bread is also gas-continuous [38,40].

Macroscopic changes during baking include further expansion of the dough and crust formation and browning [40]. The oven spring is due to continued production of carbon dioxide by yeast, its expansion by heating and vaporisation of ethanol and water. The bread bakes from the outside to the inside, resulting in a baked crumb [38].

The crust browning is directly related to the reducing sugars (glucose, fructose, maltose, etc.) formed by hydrolysis of starch and complex sugars of the flour, during dough making and leavening. Under heating, the sugars can undergo caramelisation, and/or the reducing sugars can react with the free amino acid groups of proteins in the Maillard reaction [54,55]. Besides, different flavour compounds are produced, giving bread its appealing smell and taste [55].

Additional interactions between biopolymers in the bread crumb occur during cooling. Amylose chains form helices, self-associate and crystallise [22,52,57]. Moreover, amylose may form more inclusion complexes with polar lipids. As a consequence, a permanent and in part crystalline amylose network is formed, providing a soft crumb in fresh bread. The gluten network organized during baking and the amylose network developed while cooling thus account for the plasticity of freshly baked bread [22].
Fresh bread consists mainly of a continuous gluten network, which forms a compressed matrix between the swollen, gelatinised starch granules, and the starch network, consisting of entangled, gelatinised starch polymers [22]. It usually presents an appealing brownish and crunchy crust, a pleasant aroma, fine slicing characteristics, a soft and elastic crumb texture, and a moist mouthfeel [47]. However, when a loaf of bread is removed from the oven after baking, a series of undesirable changes called staling starts, eventually leading to deterioration of quality [46].

Staling implies a relatively short shelf life for fresh bakery products. The loss of freshness is paralleled by an increase in crumb firmness and a decrease in flavour and aroma, leading to loss of consumer acceptance. Loss of moisture and starch retrogradation are accepted as two of the basic mechanisms in the firming of the crumb [58]. This subject has been extensively reviewed and discussed in [16,22,45]. In this context, mechanization, large scale production and increase in consumer demand for consistent product quality and longer shelf life of baked goods have led to the use of a wide range of additives (bread improvers) in the baking industry, which include emulsifiers, soy flour, chemical redox agents and enzymes [29,42,59].

5. Enzymes used in baked products

Baking comprises the use of enzymes from three sources: the endogenous enzymes in flour, enzymes associated with the metabolic activity of the dominant microorganisms and exogenous enzymes which are added in the dough [60].

The supplementation of flour and dough with enzyme improvers is a usual practice for flour standardization and also as baking aids. Enzymes are usually added to modify dough rheology, gas retention and crumb softness in bread manufacture, to modify dough rheology in the manufacture of pastry and biscuits, to change product softness in cake making and to reduce acrylamide formation in bakery products [8]. The enzymes can be added individually or in complex mixtures, which may act in a synergistic way in the production of baked goods [60-62], and their levels are usually very low.

5.1. Hydrolases

Enzymes as technological aids are usually added to flour, during the mixing step of the breadmaking process. The enzymes most frequently used in breadmaking are the α-amylases from different origins [63].

5.1.1. Amylases and other starch-converting enzymes

The industrial processing of starch is usually started by α-amylases (α-1,4-glucanohydrolase). Most of the starch-converting enzymes belong to the α-amylase family or family 13 glycosyl hydrolases (GH), based on amino acid sequence and structural similarities [64,65,66,67].
α-Amylases (EC 3.2.1.1) are endoenzymes that catalyze the cleavage of α-1,4-glycosidic bonds in the inner part of the amylose or amylopectin chain. The end products of α-amylase action are oligosaccharides, with an α-configuration and varying lengths, and α-limit dextrins, which are branched oligosaccharides [17]. These enzymes can be obtained from cereal, fungal, bacterial and biotechnologically altered bacterial sources. Differences in the number of binding sites and location of catalytic regions determine substrate specificity of α-amylases, the length of the oligosaccharide fragments released after hydrolysis and, consequently, the carbohydrate profile of the final product. The different forms of α-amylases also have diverse thermal stability profiles [15].

Also part of the GH13 family are the exoenzymes maltogenic α-amylase (glucan 1,4-α-glucanohydrolase, EC 3.2.1.133) and other maltooligosaccharide forming amylases (EC 3.2.1.60, for instance). While maltogenic α-amylase mainly releases maltose from starch, maltooligosaccharide producing amylases give rise to maltotetraose or maltotriose, among others. On the other hand, debranching enzymes, such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), grouped as well in the GH13 family, hydrolyse α-(1,6)-bonds removing the side-chains from amylopectin [16,17].

β-Amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) are encompassed in the GH14 and GH15 families, respectively. Both are exoamylases that employ the inverting mechanism to cleave α-glycosidic bonds at the non-reducing ends of amylose and amylopectin, producing low molecular weight carbohydrates in the β-anomeric form [15,68]. β-Amylases are unable to cleave α-1,6-linkages and the final products consist of maltose and β-limit dextrin. Therefore hydrolysis of amylopectin is incomplete, resulting in only 50-60% conversion to maltose. In the case of amylose, the maximum degree of hydrolysis is 75-90% due to the slightly branched structure of this polysaccharide [15]. On the other hand, glucoamylase has a limited activity on α-1,6-linkages and would possibly be able to catalyse total conversion of starch into β-glucose [16].

Malt and microbial α-amylases have been widely used in the baking industry. The malt preparation led the way for the commercial use of many other enzymes in baking [69]. Fungal α-amylases or malt are usually added to optimize amylase activity of the flour, initially aiming to increase the levels of fermentable and reducing sugars. In view of their lower thermostability, fungal α-amylases are more appropriate than malt amyloses for flour standardization. The α- and β-amylases have different but complementary functions during the breadmaking process [70]. The supplemented α-amylases break down damaged starch particles into low molecular weight dextrans during the dough stage, while endogenous β-amylase converts these oligosaccharides into maltose which is used as fermentable sugar by the yeast or sourdough microorganisms [15,16]. The increased levels of reducing sugars lead to the formation of Maillard reaction products, intensifying bread flavour and crust colour. In addition, these enzymes can improve the gas-retention properties of fermented dough and reduce dough viscosity during starch gelatinization, with consequent improvements in product volume and softness [8,22,71].

Certain amylases are able to decrease the firming rate of bread crumb, acting as anti-staling agents. Amylase-containing anti-staling products typically consist of bacterial or fungal α-
amylases with intermediate thermostability [16,22]. In this context, one of the most effective anti-staling amylases is the Bacillus stearothermophilus maltogenic α-amylase [22]. The anti-staling action of amylases has been attributed to the modified retrogradation behaviour of the hydrolysed starch [72-74]. Yet, other researchers ascribe the effect to the interference of the low molecular weight dextrins with starch-starch and/or gluten-starch interactions [74-76].

5.1.2. Proteases

Proteases can be subdivided into two major groups according to their site of action: exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate [77]. Most of the proteolytic activity of wheat and rye flours corresponds to aspartic proteases and carboxypeptidases, which are both active in acid pH. Additionally, aspartic proteases of wheat are partly associated with gluten [78]. Nevertheless, the proteolytic activity of sound, ungerminated grain is normally low [79].

Proteases are used on a large commercial scale in the production of bread, baked goods, crackers and waffles [80]. These enzymes can be added to reduce mixing time, to decrease dough consistency, to assure dough uniformity, to regulate gluten strength in bread, to control bread texture and to improve flavour [16,60]. In addition, proteases have largely replaced bisulfite, which was previously used to control consistency through reduction of gluten protein disulfide bonds, while proteolysis breaks down peptide bonds. In both cases, the final effect is a similar weakening of the gluten network [79].

In bread production, a fungal acid protease is used to modify mixtures containing high gluten content. When proteases are mixed in the blend, it undergoes partial hydrolysis becoming soft and easy to pull and knead [7,60]. Proteases are also frequently added to dough preparations. These enzymes have great impact on dough rheology and the quality of bread possibly due to effects on the gluten network or on gliadin [7].

Proteases are also applied in the manufacture of pastries, biscuits and cookies. They act on the proteins of wheat flour, reducing gluten elasticity and therefore reducing shrinkage of dough or paste after moulding and sheeting [8,81]; for instance, hydrolysis of glutenin proteins, which are responsible for the elasticity of dough, has considerable improving effects on the spread ratio of cookies [81].

5.1.3. Hemicellulases

Hemicellulases are a diverse class of enzymes that hydrolyse hemicelluloses, a group of polysaccharides comprising xylan, xylobiose, arabinoxylan and arabinogalactan [82]. This group includes xylanase or endo-1,4-β-xylanase (4-β-D-xylan xylanohydrolase, EC 3.2.1.8), a glycosidase that catalyses the endohydrolysis of 1,4-β-D-xylidosic linkages in xylan and arabinoxylan.

Xylanase, also designated endoxylanase, was originally termed pentosanase [83]. A wide variety of xylanases have been reported from a plethora of microorganisms including bacteria,
archaea and fungi [84]. These enzymes are mainly classified in the glycosyl hydrolase (GH) families 10 and 11 [16,64,65], although putative xylanase activities have been reported in GH families 5, 7, 8 and 43 [84,85]. GH10 xylanases are regarded to have broader substrate specificity and release shorter fragments compared to GH11 xylanases, while the latter enzymes are more susceptible to steric hindrance by arabinose substituents [86,87]. In addition, different endogenous xylanase inhibitors occur in cereals: *Triticum aestivum* L. xylanase inhibitor (TAXI) [88,89], xylanase inhibitor proteins (XIP-type inhibitors) [90] and TLXI-type (thamatin-like endoxylanase inhibitors) [91].

The complete hydrolysis of arabinoxylans requires the concerted action of different enzymes. The xylan backbone will be cleaved randomly by endo-1,4-β-xylanases, the main arabinoxylan hydrolysing enzymes, yielding arabinoxylo-oligosaccharides. β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of arabinoxylo-oligosaccharides. The arabinose residues are removed by α-L-arabinofuranosidases (EC 3.2.1.55), while ferulic acid esterases (EC 3.1.1.73) cleave ester linkages between arabinose residues and ferulic acid [30,83].

Xylanases were introduced to the baking segment in the years 1970 and are most often used combined with amylases, lipases and many oxidoreductases to attain specific effects on the rheological properties of dough and organoleptic properties of bread [85]. These enzymes have also been used to improve the quality of biscuits, cakes and other baked products [71]. The most favourable xylanases for breadmaking are those that preferentially act on WU-AX and are poorly active on WE-AX, because they remove the insoluble arabinoxylans which interfere with the formation of the gluten network, giving rise to high molecular weight solubilised arabinoxylans, resulting in increased viscosity and thus enhancing dough stability [92-94]. As a consequence, a more stable, flexible and easy to handle dough is obtained, resulting in improved oven spring, larger loaf volume, as well as a softer crumb with improved structure [43]. Moreover, the addition of xylanases during dough processing is expected to increase the concentration of arabinoxylo-oligosaccharides in bread, which have beneficial effects on human health [95].

The potential of GH family 8 xylanases as technological aids in baking was shown for a psychrophilic enzyme from *Pseudoalteromonas haloplanktis* and a mesophilic enzyme from *Bacillus halodurans*. Although both enzymes had a positive effect on loaf volume, psychrophilic GH8 xylanase was apparently much more efficient than the mesophilic enzyme from the same family, because much lower concentrations of the former enzyme were required to produce a similar increase in bread volume. Additionally, a psychrophilic GH10 xylanase from *Cryptococcus adeliae* was found to be ineffective [85].

Recently, a purified GH11 xylanase from *Penicillium occitanis* was evaluated as an additive during mixing of wheat flours. Significant improvements of bread characteristics, including higher final moisture content, volume and specific volume, were observed. Enhancements in sensory and textural properties were also obtained [96].
5.1.4. Lipases

Lipases (EC 3.1.1.3) or triacylglycerol acylhydrolases hydrolyse triacylglycerols (TAG) producing monoacylglycerols (MAG), diacylglycerols (DAG), glycerol and free fatty acids. These enzymes are widely found in nature [97]. Besides TAG lipases there are phospholipases A1 (EC 3.1.1.32), A2 (EC 3.1.1.4), C (EC 3.1.4.3), D (EC 3.1.4.4) and galactolipases (EC 3.1.1.26). Even though they are present in all cereal grains; lipase activity of white flour is usually low enough to avoid rancidity due to hydrolysis of native lipids and of baking fat [71,79,98].

The use of lipases in the baking segment is much more recent in comparison to α-amylases and proteases. The first generation of commercial lipase preparations was introduced to the market in the years 1990 and recently a third generation became available [59]. The latter are protein engineered enzymes, claimed to give a better effect in high speed mixing and no-time dough processes. Moreover, third generation lipases have lower affinity for short chain fatty acids, which reduces the risk for off-flavour formation on account of prolonged storage of the baked goods and the use of butter or milk fat in baked products [12].

Lipases (TAG lipases) of the first generation are 1,3-specific, removing preferentially fatty acids from positions 1 and 3 in TAG. These enzymes can improve dough rheology, increase dough strength and stability, thus improving dough machinability [62,99,100]. In addition, lipases lead to an increase in volume which results in an improved, more uniform crumb structure; hence a softer crumb is obtained [99].

The second generation lipases act simultaneously on TAG, diacylgalactolipids and phospholipids, producing more polar lipids, providing a greater increase in volume, better stability to mechanical stress on the dough, and a fine, uniform bread crumb structure compared to the first generation lipases [43,59,101]. Moreover, a third generation lipase was found to increase expansion of the gluten network, increase the wall thickness and reduce cell density, enhancing volume and crumb structure of high fibre white bread [102].

The surface active properties of the hydrolysis reaction products (MAG, DAG, monoacylgalactolipids and lysophospholipids), along with modifications on the interactions between lipids and gluten proteins caused by the lipases, as well as the effect of these enzymes on the incorporation of air during mixing are possible mechanisms by which they affect bread volume [101]. In this context, the roles of lipids and surfactants in breadmaking have been extensively reviewed elsewhere [38,45].

The addition of lipases has been claimed to retard the rate of staling in baked products [8,103,104]. The effect of these enzymes has been attributed to in situ production of MAG following TAG hydrolysis, although this mechanism is not completely accepted because the amount of MAG would be insufficient to account for the antistaling effect [45,99]. Lipases may also be used for the development of particular flavors in bakery products [100].

The effect of a third generation lipase on the quality of high-fibre enriched brewer’s spent grain breads has been evaluated. The enzyme produced beneficial effects during bread making, positively affecting loaf volume, staling rate and crumb structure [102].
A recent study compared three generations of lipase enzymes with the emulsifier, diacetyl tartaric esters of monoglycerides (DATEM), on white wheat flour bread. Lipases and DATEM improved most aspects of bread quality. In shorter fermentation times, DATEM, a second generation (Lipopan F-BG) and a third generation (Lipopan Xtra-BG) lipase were more effective. In longer fermentations, unlike the third generation lipase (Lipopan Xtra-BG), moderate amounts of the second generation lipase (Lipopan F-BG) significantly increased the bread volume [59].

The application of lipase and MAG to produce fiber enriched pan bread using the straight dough method was assessed. The use of lipase dosages up to 50 ppm and MAG up to 2% indicated the possibility of replacement of MAG by lipases in fiber enriched pan bread [105].

Recently, the effects of two lipases and DATEM on the rheological and thermal properties of white and whole wheat flour doughs were compared. Lipases were able to cause modifications in the dough components (gluten proteins and starch). The enzymes improved dough handling properties to a similar or greater extent than DATEM, increasing dough stability, maximum resistance to extension and hardness, and decreasing softening degree and stickiness. The possible role of lipases in delaying starch retrogradation was indicated by the greater extent of formation of amylose-lipid complexes promoted by lipases in comparison to DATEM [106].

5.2. Oxidoreductases

5.2.1. Lipoxygenases

Lipoxygenase (linoleate oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing dioxygenase, found in a wide variety of plant and animal tissues, which with molecular oxygen catalyses the oxidation of polyunsaturated fatty acids (PUFA) containing a cis,cis-1,4-pentadiene system, such as linoleic or linolenic acid, to form fatty acid hydroperoxides [107,108]. These enzymes are abundant in grain legume seeds (beans and peas) and potato tubers, being minor constituents of wheat flour [107]. Multiple isoforms of lipoxygenases are found in plants; for example a multigene family encodes soybean lipoxygenases, three members of which encode the three major seed isoforms L1, L2 and L3 [109].

The main commercial sources of lipoxygenases are enzyme-active soybean flour and, to lower extent, flour from other beans, such as fava beans [12]. Wheat lipoxygenase catalyses the oxidation of PUFA in the free or MAG forms [110] while soybean or horse bean lipoxygenases also catalyse the oxidation of PUFA present in TAG [111]. The transient alkyl, peroxyl and hydroxyl radicals formed during lipoxygenase catalysed reactions are able to oxidise carotenoid pigments and sulfhydryl groups in peptides and proteins present in the dough, mainly giving rise to hydroxyacids [112].

In fact, the initial application of lipoxygenases in doughs was based on their ability to bleach fat-soluble carotenoid flour pigments, through co-oxidation of carotenoids with PUFA [113,114]. However, since the endogenous lipoxygenase content of wheat flour is insufficient to give enough bleaching effect, enzyme-active soybean or fava bean flour is added [114].
Lipoxygenases are also employed to improve mixing tolerance and dough handling properties [115]. In this case, the effect of these enzymes may be explained by oxidation of thiol groups of gluten proteins which can lead to rearrangement of intra- or inter-chain disulfide bonds [21] and also to formation of tyrosine cross-links [116], with consequent strengthening of the gluten network. As a result, improvement in dough rheology occurs, with increase in dough strength through proofing and baking, finally leading to improved loaf volume.

On the other hand, the action of lipoxygenase can lead to undesirable flavors in bread [79,114]. These flavors are possibly due to some of the breakdown products (ketodienes) formed during the anaerobic reaction [117,112].

5.2.2. Glucose oxidase

Glucose oxidase (β-D-glucose: oxygen: 1-oxidoreductase; EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide [118,119]. This enzyme has been obtained from different fungal sources, mainly from genus *Aspergillus* and *Penicillium*, being *Aspergillus niger* the most commonly used [120-123].

Glucose oxidase has been used successfully to remove residual glucose and oxygen in foods and beverages aiming to increase their shelf life. The hydrogen peroxide generated by this enzyme presents antimicrobial properties, and is easily removed by catalase utilization, which is an enzyme that catalyzes the conversion of hydrogen peroxide to oxygen and water [12,124-127]. Glucose oxidase can be used as alternative oxidizing agent instead of potassium bromate in breadmaking. Potassium bromate is an oxidizing agent that was traditionally used in baking, and its use was prohibited in many countries after it was recognized as carcinogenic [128,129].

Although the mechanism of action of glucose oxidase is still not completely understood, a possible explanation is that the hydrogen peroxide formed during catalysis promotes, indirectly, the formation of either disulfide bonds or dityrosine crosslinks, or both, in the gluten network [116,130,131]. Therefore, the increase in disulfide crosslinking and/or promotion of gelative oxidation on the gluten matrix confers dough machinability, good gas retention, high bread volume and fine crumb structure [54,132-134]. Addition of increasing glucose oxidase concentrations to wheat flour dough produced significant changes on dough rheology and bread quality; and the extent of the effect was highly dependent on the amount of enzyme and the original wheat flour quality [130]. Furthermore, glucose oxidase was able to recover the breadmaking ability of damaged gluten [135]. Another possibility to explain the improvements on crumb properties, in both bread and croissants, as a result of glucose oxidase catalysed reactions would be the crosslinking of the albumin/globulin fraction with both disulfide and non-disulfide bonds, and the slight occurrence of non-disulfide crosslinking in the gluten proteins [131].

5.3. Other enzymes

Among the enzymes which have attracted attention for use in bakery is asparaginase. Differently from other enzymes, its use is not associated with improved bread volume, crumb softening
or reduced staling. Instead, asparaginase is claimed to have a high potential of reducing formation of acrylamide during baking [136-138]. Asparaginase (L-asparagine amidohydrolases, EC 3.5.1.1) catalyses the hydrolysis of asparagine to aspartic acid and ammonium, removing the precursor of acrylamide formation [139]. Acrylamide, classified as a probable human carcinogen, is formed in heated foods via Maillard reaction between asparagine and a carbonyl source [137,138,140,141]. Although asparaginase can be found among living organisms, including animals, plants and microorganisms, filamentous fungi as Aspergillus oryzae and A. niger have been explored for enzyme preparation aiming commercial purposes [142-144].

| Enzyme (classification) | Substrate in foods | Reaction | Applications in baked products | References |
|-------------------------|--------------------|----------|-------------------------------|------------|
| Amylolytic enzymes      | Starch             | Hydrolysis of linkages          | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22,175] |
| α-Amylases (EC 3.2.1.1) or α-(1,4)-glucanohydrolases | Amylose and amylopectin | α(1→4)-D-glycosidic [endo], liberating α-dextrins | Improvement in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| β-Amylases (EC 3.2.1.2) | Amylose and amylopectin | α(1→4)-D-glycosidic [exo], liberating β-dextrins and β-maltose | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Glucoamylase (EC 3.2.1.3) or amyloglucosidase | Amylose and amylopectin | α(1→4)-D-glycosidic, liberating β-glucose | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Pullulanase (EC 3.2.1.41) | Amylopectin | α(1→6)-D-glycosidic | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Isoamylase (EC 3.2.1.68) | Amylopectin | α(1→6)-D-glycosidic | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Maltogenic α-amylase (EC 3.2.1.133) | Amylose and amylopectin | α(1→4)-D-glycosidic, liberating maltose | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Maltoligosaccharides forming amylases (glucan 1,4-α-maltotetraohydrolase) (ex., EC 3.2.1.60) | Amylose and amylopectin | Liberation of maltotetrose or maltotriose | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [16,17,22] |
| Transfases | Amylose, amylopectin and dextrins | Hydrolysis of α(1→4) glycosidic bonds and transference of a reducing group to a non-reducing acceptor (monosaccharide unit) | Generation of fermentable compounds; Increase in bread volume; Reduction in fermentation time; Improvement in dough viscosity, rheology and bread softness; Improvement in bread texture; Formation of reducing sugars and subsequent Maillard reaction products; Intensifying bread flavor and color; Decrease of bread crumb firming rate; Anti-staling effects. | [17] |

Table 2. Applications of starch modifying enzymes in baking.
Transglutaminases (EC 2.3.2.13) from microbial sources also have potential for application in bakery products. Food proteins can be modified through cross-linking by transglutaminases, resulting in textured products, protecting lysine in food proteins from undesired chemical reactions, encapsulating lipids and lipid-soluble materials, forming heat and water resistant films, improving elasticity and water-holding capacity, modifying solubility and functional properties, and producing food proteins of higher nutritive value [29,145-153].

Laccase (EC 1.10.3.2) is a copper containing enzyme that catalyses the oxidation of a wide variety of phenolic compounds via one-electron removal, generating reactive phenolic radicals [29,154]. This enzyme is very interesting for baking due its ability to cross-link the esterified ferulic acid on the arabinoxylan fraction of dough, resulting in a strong arabinoxylan network [155]. It was also reported that laccase may improve crumb structure and softness of baked products. Furthermore, increases in strength and stability, as well as reduced stickiness of dough, which confers improvement of machinability, have been described [149,155-157].

A summary of the main applications of different classes of enzymes in the baking industry is presented in tables 2, 3 and 4.

| Enzyme (classification) | Substrate in foods | Reaction | Applications in baked products | References |
|-------------------------|--------------------|----------|--------------------------------|------------|
| Cellulases and Hemicellulases | Non-starch components of cereals | Hydrolysis of linkages | | |
| Cellulase (EC 3.2.1.4) | Cellulose and β-glucan | β(1→4)-D-glycosidic (endo) | Removal of insoluble arabinoxylans, contributing to gluten network formation; Increase in dough viscosity, stability, with better moldable form; Improvements on rheological properties of dough; Reduction in fermentation time; Increase of bread volume; Synergistic action of glucanases on xylanolytic attack of cereals structure, providing more soluble dietary fiber in bread products; Production of prebiotic oligosaccharides in bread. | [30,71,83,95,165,176,177] |
| Laminarinase (EC 3.2.1.6) | β-glucans | β(1→3)- and β(1→4)-D-glycosidic | Improvement of dough machinability | [71] |
| Lichenase (EC 3.2.1.73) | β-glucans | β(1→3)- and β(1→4)-D-glycosidic | Improvement of dough machinability | [30,71,83] |
| Endo β(1,4)-D-xylanase (EC 3.2.1.8) or endoxylanase | Arabinoxylan | β(1→4)-D-xylidosidic bonds | Improvement of dough machinability | [71,83,85,95,165,176,177] |
| α-L-Arabinosidase (EC 3.2.1.55) | Arabinoxylan | Terminal α-L-Arabino-furanoside residues | Improvement of dough machinability | [71,83,95,102] |
| β-D-Xylosidase (EC 3.2.1.37) | Arabinoxylan | β(1→4)-D-xylosidic bonds (non-reducing end) | Improvement of dough machinability | [83,95] |

Table 3. Applications of cellulases and hemicellulases in baking.
| Enzyme (classification) | Substrate in foods | Reaction | Applications in baked products | References |
|-------------------------|--------------------|----------|--------------------------------|------------|
| **Proteases** (EC 3.4.) | Gluten proteins    | Hydrolysis of peptide bonds | Reduction of dough mixing time; Control of dough rheology or viscoelastic properties of gluten strength in bread; Enhance dough extensibility; Increase loaf or bread volumes; Formation of aminoacids and flavors; Crispsness feature on bread crust; Production of gluten-free products. | [78,79,101,178,179,180] |
| **Transglutaminases** Protein-glutamine γ-glutamyl-transferase (EC 2.3.2.13) | Gluten Proteins | Acyl-transfer reaction between γ-carboxyamide and primary amines | Cross-link between gluten and other peptides, forming a new protein network; Increase volume and improve structure of breads, better retention of gas; Improve bread crumb strength, height increase in puff pastry and croissants volume; Improve dough stability; Improve properties of gluten-free breads; Protect frozen doughs from damage. | [29,145-150] |
| **Lipases and esterases** | Lipids | Hydrolysis of ester bonds | Improvement in bread volume and dough stability; Formation of emulsifiers; Retard staling; Development of flavors. | [59,106,158] |
| Lipase (EC 3.1.1.3) | Triacylglycerols | Liberation of free fatty acids | Improvement in bread volume and dough stability; Formation of emulsifiers; Retard staling; Development of flavors. | [59,106,158] |
| **Oxidoreductases** | Various | Oxi-reductions | Control on browning for Maillard reaction; Improvements in crumb properties. | [130,131,181] |
| Glicose oxidase (EC 1.1.3.4) | β-D-glucose:oxygen 1-oxidoreductase | Oxidation of β-D-glucose to gluconic acid | Bleaching of fat-soluble flour pigments; Hydroperoxides formed can oxidize sulfhydryl groups in proteins. | [79,182] |
| Lipoxygenase (EC 1.13.11.12) | Polyunsaturated fatty acids | Oxidation of fatty acids | Bleaching of fat-soluble flour pigments; Hydroperoxides formed can oxidize sulfhydryl groups in proteins. | [79,182] |
| Laccase (EC 1.10.3.2) | Feruloyl esters of arabinoxylans; | Oxidation of phenol groups | Dough strength, stability and reduced stickiness; Increase in volume; Improved crumb structure and softness. | [155,163] |
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| Enzyme (classification) | Substrate in foods | Reaction | Applications in baked products | References |
|-------------------------|--------------------|----------|--------------------------------|------------|
| or benzene-diol:oxygen oxidoreductase | sulfhydryl groups in gluten proteins | | | |
| Sulfhydryl oxidase | sulfhydryl groups in proteins | Oxidation of sulfhydryl groups Help gluten network formation and increase dough stability. | [79,162] |

Table 4. Applications of proteases, transglutaminases, lipases and esterases, and oxidoreductases in baking.

5.4. Use of enzyme combinations

It is common practice to use mixtures of enzymes, some of which are commercially available. The enzymes may act individually or present a synergistic effect. The trend is to choose and control the use of complex mixtures of enzymes which may act in a synergistic way and can exert a better effect (than the individually used) on the different flour components [60]. Recent advances in understanding of the dough forming and overall baking processes at the molecular level have focused attention on improvements that can be achieved by application of more specially tailored enzymes alone or in combinations. Usually, integrated experimental design and optimization followed by chemical analyses, rheological experiments and baking trials are necessary in order to provide answers to the more complicated questions [158].

The use of a combination of enzymatic preparations of amylases, xylanases and lipases has been reported by different authors [60,102,159]. This specific mixture is claimed to increase bread volume and shelf-life. The use of α-amylase and glucose oxidase to replace bromate led to a significant improvement in dough extensibility and bread volume [160]. Addition of commercial enzyme mixtures, containing α-amylase and lipase activities to produce bread samples, using the straight dough method, had a beneficial effect on bread keeping properties and resulted in the formation of a more thermostable amylose-lipid complex compared to the control bread [161]. Amylopectin retrogradation was inhibited by the use of the enzyme combinations and this effect was strongly related to a decrease in crumb-firming rates.

The combined use of different enzymes, classified as gluten degrading (like proteases) or adjuvants, such as amylases and xylanases, with a group of crosslink promoting enzymes, such as transglutaminases and glucose oxidase, was also studied [149]. Better shaped bread could be obtained after the use of gluten degrading or adjuvant enzymes, and association with transglutaminase resulted in improvements on texture and rheological properties. The crumb firmness which can further lead to staling, can result from transglutaminase action, but it may be reversed with opposite amylase, xylanase and protease effects.

In a similar way, combinations of enzymes classified as carbohydrate degrading, including amylases and xylanases (pentosanases), and crosslink promoting enzymes, like transglutaminases and oxidases, including glucose oxidase, laccase [149], lipoxygenase and sulfhydryl oxidase [79,162] were evaluated. The most frequent associations contained xylanases and glucose oxidase, but addition of laccase and transglutaminase was also employed. The hy-
drogen peroxide formed by glucose oxidase catalysis may interfere in gluten network, via oxidized glutathione reaction, leading to gluten disulfide bonds formation [43], and it also interferes in the formation of a soluble pentosan gel (from xylans) that increased dough consistency [146]. Because both oxidases and xylanases influence the xylan properties, xylanases and oxidases could be used advantageously in combination, resulting in a mesh of gluten and gelified xylans matrix, which increases gas retention, dough stability and bread volume.

Laccase is reported to catalyze dimerization of feruloylated esters in feruloylated arabinoxylans in doughs [163,164], forming a xylan network, contributing to increase strength of dough and volume.

Lipoxygenases oxidize polyunsaturated fatty acids during dough mixing. The hydroperoxides formed can oxidize the sulfhydryl groups of gluten proteins and thus be advantageous in the formation of the gluten network of dough. Sulphydryl oxidase combined to glucose oxidase and xylanases has been used to strengthen weak doughs [79,162].

The use of a combination of commercial preparations of glucolipase, hemicellulase and hexose oxidase in formulations of frozen pre-baked French bread, substituted with whole wheat flour, improved parameters such as proofing time, oven spring and cut opening and cut height [158]. An interaction among the three enzymes was observed for most of the parameters, because the responses of each enzyme to variations in dosing were influenced by the doses of the other two.

**6. Future trends**

Besides the demand for replacement of chemical additives by others from natural sources, there is an increasing concern among the consumers and consequently an increased demand for preservation and/or enrichment of foods with products that have beneficial effects on human health. Regarding baked goods, the use of enzymes to obtain dietary fiber enriched bread [102,165], for the development of gluten free products [145], to obtain products with increased contents of arabinoxylan oligosaccharides with prebiotic potential [165], has been reported.

Several aspects can be pointed out for the development of enzyme preparations able to provide the desired effects or with adequate characteristics for use under process conditions. Some of the possible strategies include selection of novel enzymes from different sources [166], especially from microorganisms obtained from the vast biodiversity of the planet, production of recombinant proteins from genetically modified organisms [167], as well as protein engineering.

Psychrophilic enzymes usually have higher optimal activity and stability at lower temperatures than their mesophilic counterparts [168]. Due to the fact that the temperatures most frequently used in dough mixing and proofing are around or below 35 °C, it has been suggested that psychrophilic enzymes would be advantageous candidates for use as additives in the baking industry [83,85]. In this context, researchers have shown that much lower dos-
ages of psychrophyllic xylanases than of the mesophilic enzymes could be used to attain maximal bread volumes [85,169,170].

Directed evolution is a powerful tool of protein engineering to design and modify the properties of enzymes [171]. This technology can be employed for a wide range of proteins, most of which are of interest for biocatalytic processes. Within a decade, directed evolution has become a standard methodology in protein engineering and can be used in combination with rational protein design and other standard techniques to meet the demands for industrially applicable biocatalysts capable of withstanding process conditions such as high substrate concentrations, high temperatures and long-term stability, as well as presenting desired specificity and/or selectivity [172]. For instance, a recent study reported the combined use of directed evolution and high-throughput screening to improve the performance of a maltogenic α-amylase from Bacillus sp. for low pH bread applications. One of the resulting variants showed an important increase in thermal stability at pH 4.5 and a considerable antistaling effect in low pH breads [173].

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