CHAPTER

5

Application of Microbial Enzymes in the Dairy Industry

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5.1 INTRODUCTION

Microorganisms from barley yeast have been used in the commercial production of alcoholic beverages by the Sumerians and Babylonians since 6000 BCE. Globally, enzymes of the microbes have been known for their prevalent applications in many industries; for example, agriculture, chemicals, energy, food, and medicine. Microbial enzymes are rapidly attracting attention and mediated processes due to the fact they consume little energy, have a decreased process time, are eco-friendly, nontoxic, and cost effective (Choi et al., 2015; Li et al., 2012). With the emergence of protein engineering and DNA technology, microorganisms could be cultured and manipulated in huge quantities to fulfill needs due to the increased demand of industrial implementations such cost reduction, consumer goods, and depletion of natural resources, (Choi et al., 2015; Liu et al., 2013). The world market for microbial enzymes was constituted in 2014 at nearly $4.2 billion USD and it will be expected to increase by 7% by 2020. Enzymes are biological molecules, are protein in nature, and are considered catalysts that activate the bio-chemical reactions inside the biological system (Cech and Bass, 1986). Enzymes are specific, lowering the activation energy without consuming it (Aldridge, 2013; Fersht, 1985; Piccolino, 2000). Typically, enzymes require mild conditions such as pressure and temperature to catalyze reactions, instead of needing hazardous materials and pollutants (Choi et al., 2015; Illanes et al., 2012). The optimal pH and temperature of mammalian enzymes are 7.4 and 37°C, respectively. In addition, a pH higher than 7.4 and temperatures over 40°C lead the enzymes to denature which limits their use in non-physiologically conditions. Moreover, mammalian enzymes are affected by product and substrate inhibitions and can cause allergies; there is also the higher fee of purification, isolation, and difficulty in reusing them. Enzymes are large macromolecules consisting of a building block made of amino acids; their molecular mass is measured by Dalton (Da). Often the active site of the enzyme is deep within a pocket that causes their specificity of a certain substrate. Large

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enzyme numbers are characterized and purified and, as a result, the enzyme nomenclature was established. In consultation between the IUBMB and IUPAC, EC was established to guide the systematic classification and naming of enzymes. Microorganisms are considered preferable sources for industrial enzymes because of their fast growth rate and easy availability. Altering genes of microbial cells using recombinant DNA technology could be easily done to elevate the production of enzymes and development of science (Illanes et al., 2012). In Industrial applications, the manufacture of microbial enzymes is an important issue. This importance came from the excellent and high-efficiency achievements of various microbial enzymes, enzymes that work in numerous chemical and physical cases. It has been stated that the enzymes of microorganisms can be used in the treatment of the shortage of human enzymes resulting from genetic disorder (Anbu et al., 2017; Vellard, 2003). As an example, patients who are unable to digest sucrose (sucrase-isomaltase deficiency) were treated orally with sacrosidase (EC 3.2.1.26) to aid sucrose digestion (Treem et al., 1999). It has been reported that the treatment for phenyl ketonuria disorders were treated with ammonia phenylalanine lyase to break down the substrate phenylalanine (Sarkissian et al., 1999).

Enzymes of microorganisms have numerous industrial applications such as in the dairy, pharmaceutical, food, paper, textiles, and leather industries. Moreover, their use is rapidly increasing more than other conventional methods because of their greater efficiency, higher quality, and low impact on the environment (Gurung et al., 2013; Jordon, 1929; Kamini et al., 1999). This chapter will focus on the application of microbial enzymes in the dairy industry.

5.2 THE DAIRY INDUSTRY

In the dairy industry, the microbial enzymes utilized have a significant role, where they are used to improve and enhance organoleptic features like aroma, color, and flavor, as well as giant yield of milk products. There are many types of microbial enzymes used in the dairy industry, such as catalase, aminopeptidase, proteases, lactoperoxidase, lipases, transglutaminase, etc. They are well known in this field and are different from coagulants as they help improve shelf life. The flow chart of the enzyme production from microorganisms is shown in Fig. 5.1. It has been reported that enzymes of microorganisms are used in yogurt and cheese production (Pai, 2003; Qureshi et al., 2015). The mixture of pepsin and chymosin (rennet) is applied to coagulate milk for cheese and whey production. The world requirements of cheese produced by rennet microorganisms constitutes about 33% per day (Van Kampen et al., 2013).

5.3 MICROBIAL ENZYMES IN DAIRY INDUSTRIES

In the dairy industry, microbial enzymes have been utilized to produce diverse products, such as yogurt, cheese, syrup, bread, etc. to enhance their quality. Traditional ancient arts such as brewing, cheese making, and tenderization of meat by papaya leaves were developed before we knew about enzymes. Moreover, early dairy processes included proteolysis, an insensible consequence of enzyme activity in food production. Here are the most important microbial enzymes used in the dairy industry (Table 5.1).

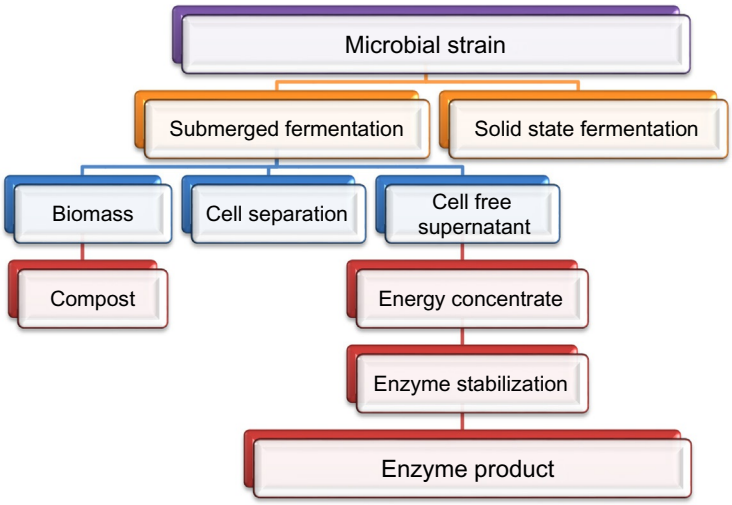

FIG. 5.1 Preparation of enzymes by microorganisms.

TABLE 5.1 Application of Microbial Enzymes in the Dairy Industry

Enzyme	Role(s)	Microorganism(s)	Reference
Aminopeptidase	Faster cheese ripening	*Lactobacillus* sp.	Masoud et al. (2017)
Acid proteinase	Milk coagulation	*Aspergillus* sp.	Qureshi et al. (2015)
Catalase	Cheese processing	*Aspergillus niger*	Perin et al. (2017)
Lipase	Faster cheese ripening	*Aspergillus niger*	Sharma and Sharma (2017)
Neutral proteinase	Faster cheese ripening	*Bacillus* sp., *Aspergillus oryzae*	Palomba et al. (2017)
Transglutaminase	Protein cross linking	*Streptomyces* sp.	Domagała et al. (2016)

5.4 RENNET

Rennet is considered a famous exogenous enzyme used in dairy processing, and has been used since 6000 BCE. The cheese production in the US increased from 8000 to 471,434 metric tons by April 2017 according to (USDA/NASS). This translated to a large demand of the exogenous enzyme rennet from different sources. The rennet enzyme usage by cheese manufacturers is one of the largest application of enzymes in dairy processing. Traditionally, animal rennet is utilized as a milk-coagulant in the dairy manufacturing industry to produce high-quality cheeses with unique features such as good texture and flavor (Fig. 5.2). The demand for cheese production has increased worldwide, while at the same time there has been a reduction in the supply of calf rennet. This led to the search for alternative sources of rennet such as rennet extraction from microorganisms. Worldwide, rennet from microorganism constitutes 30% of the total cheese produced. Rennin has enzymatic and nonenzymatic action that causes the milk to coagulate. The milk transforms to a gel-like structure during

FIG. 5.2 Different types of cheese produced by rennet.

the enzymatic activity due to the temperature and calcium ion effect (Bhoopathy, 1994). There are very familiar microorganisms used to manufacture rennet, such as proteinases, which can replace calf rennet. In cheese production, some microorganisms such as *Aspergillus oryza*e, *R. miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, and *Irpex lactis* are widely used to make rennet. Many researchers have reviewed many studies done so far on rennet substitutes (Farkye, 1995; Fox, 1998; Green, 1993). Predominantly, many manufacturers use different strains of *Mucor* to produce rennet from microorganisms, while the best yields of milk-clotting are done by the protease of *Rhizomucor pusillus* derived from semi-solid media made up of 50% wheat bran. Furthermore, *Endothia parasitica and R. miehei* are shown to be suitable for submerged cultures. Excellent milk-clotting by protease yields could be achieved by utilizing a culture medium composed of 4% potato-starch, 10% barley and 3% soybean-meal. Lipases are excreted with the proteases through the microbial growth. Hence, the activity of lipases becomes wasted by minimizing the pH before the culture preparation. A study was conducted on the effect of incorporating whey protein concentrate (WPC) on the quality characteristics of Mozzarella cheese analogue (MCA) based on rennet casein (RC) (Dhanraj et al., 2017). It is recommended to use a blend of RC and WPC (90:10) as the protein source in the formulation of MCA to obtain a nutritionally superior cheese product that has the desired functional properties for its end use in baking applications. The effects of the size and stability of native fat globules on the kinetics of rennet-induced coagulation were revealed by determining the caseinomacropeptide (CMP) is a 64-amino-acid-residue peptide which is released from kappa-casein by gastric proteinases. Release rates and rheological properties of milk has been studied (Luo et al., 2017). It was concluded that a better understanding of the size of the globules' effect on milk gelation could lead to the development of cheese with specific properties.

5.5 CATALASE

The enzyme catalase could be utilized in a special application in order to produce cheese. In the case of producing some types of cheeses like Swiss, hydrogen peroxide, a strong oxidizer that is toxic to cells, is used in the state of pasteurization. It is used to retain natural milk enzymes that are useful for the finished product and flavor development of the cheese

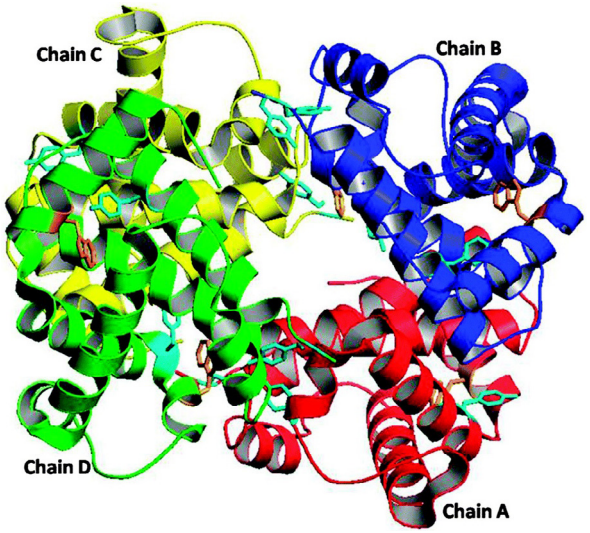

FIG. 5.3 Protein structure of catalase.

(Perin et al., 2017). Even though the high-level heat of pasteurization could break down these enzymes, residues of hydrogen peroxide in the milk prevent the bacterial cultures that are needed for the actual cheese production, so all traces of it must be removed. There are many resources used to get catalase enzymes like bovine livers or microbial sources. To change hydrogen peroxide into water, as well as molecular oxygen, catalase enzymes are added (Fig. 5.3).

5.6 PROTEASES

The proteases of lactate bacteria are necessary for its growth in substrate (milk) and help dramatically in enhancing flavor of fermented milk products. The proteolytic enzyme system constitutes proteinases that first break down the protein of the milk into peptides. Peptidases then breaks down the peptides into amino acids and small peptides, then the transport system takes charge of the uptake of amino acids and small peptides (Fig. 5.4). It has been reported that milk bacteria have a complicated proteolytic activity which can transform milk casein into free peptides and amino acids required for its growth. The proteolytic proteinases involve amino-peptidases, tri-peptidases, *endo*-peptidases, extra cellular proteinases, and proline peptidases (serine proteases) (Qureshi et al., 2015). Other studies have been shown that lactic streptococcal proteinases have many proteinases of a non-lactostreptococcal source. Moreover, proteinases from *L. lactis*, *L. helveticus*, *L. delbrueckiisp*, *L. bulgaricus*, *L. plantarum* and *Lactobacillus acidophillus* are serines type of proteinases. Aminopeptidases play a significant role in enhancing the flavor of fermented milk products because they can secrete single residues of amino acid from large oligopeptides established by activity of extracellular proteinase. They were also used to minimize allergic properties of milk products and accelerate cheese processing. A development of a new laboratory technique to evaluate protease activity

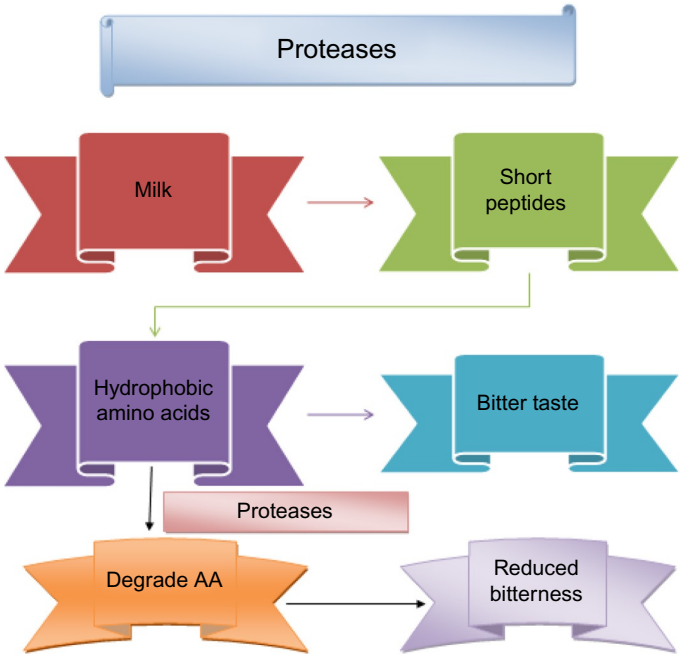

FIG. 5.4 Role of proteases in dairy industries.

in sheep and goat's milk has been done (Palomba et al., 2017). The study showed that the technique is useful for the proteolytic activity in different media and its effectiveness depends on chemical-nutritional characteristics of the sample.

5.7 LIPASES

Lipases are used to enhance flavor, speed the process of cheese, create customized milk products, and break down milk fat. Lipases are water-soluble enzymes, and help catalyze the hydrolysis of ester bonds in substrates (lipids) (Svendsen, 2000). Microbial lipases are produced by microorganisms together or individually with esterases. Examples of lipase-producing microorganisms are *Serratia marcescens*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococococcus aureus*. For the production of free fatty acids, glycerol, and various esters, lipase is used as biocatalyst. Furthermore, lipase is used for the production of fat and glycerides, which are esterified or amended from the cheaper substrates, such as oil of palm. Widely, many industries, such as pharmacological, chemical, and food use those products. Different lipases of animal or microbe origin created clear cheese, low bitterness, improved flavor, and potent malodors, whereas proteinases, in combination with lipases or/and peptidases, developed cheeses with excellent flavor and low bitterness levels. In order to accelerate the ripening of cheese content of peptidases and proteinases we can use attenuate cell-free extract or starter cells to do that (Sharma and Sharma, 2017).

5.8 TRANSGLUTAMINASE

Transglutaminase (Tgase) is a catalysts in the polymerization of milk proteins and enhances the properties of milk products (Fig. 5.5). Human lactose intolerance is due to the inability to digest lactose because of a deficiency in the secretion of the lactase enzyme. Rennet cheese with modified textural and nutritional properties and improved yield could be obtained upon transglutaminase modification but simultaneous addition of rennet and transglutaminase is recommended (Domagała et al., 2016). Moreover, transglutaminase crosslinking and calcium reduction were investigated as ways to improve the texture and storage stability of high-protein nutrition (HPN) bars formulated with milk protein concentrate (MPC) and micellar casein concentrate (MCC). Hardness, crumbliness, moisture content, pH, color, and water activity of the HPN bars were measured during accelerated storage. The HPN bars prepared with MPC were harder and more cohesive than those prepared with MCC. Higher levels of Tgase crosslinking improved HPN bar cohesiveness and decreased hardening during storage (Banach et al., 2016). Also, a study evaluated panela cheeses—made from dairy-plant protein blends, using soybean or peanut protein isolates, supplemented with transglutaminase—has been done. The results showed that panela cheeses can be elaborated following a traditional procedure, but with the addition of soybean or peanut protein to the dairy ingredients. Cheeses containing these protein isolates showed higher protein content than the milk control cheese and had similar textural characteristics (Salinas-Valdes et al., 2015).

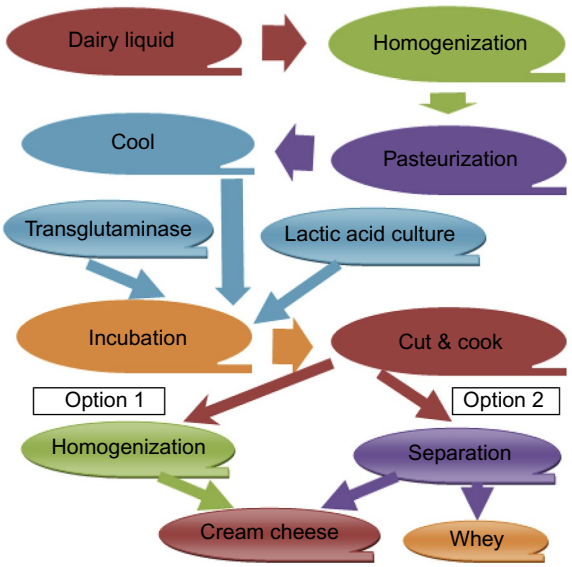

FIG. 5.5 The steps of the cream cheese production by transglutaminas.

5.9 LACTASE

Lactase (EC 3.2.1.23) accelerates the breakdown of lactose into galactose and glucose. It is used to improve the sweetness, solubility, as well as digestive agent, for milk products (Qureshi et al., 2015). Lactases are important in reducing and removing lactose in milk products for lactose-intolerant patients in order to protect them from severe diarrhea, fatal consequences, and tissue dehydration. Among various features of milk treated with lactase is the increased sweetness, hence it can avert the need for adding sugars during the production of flavored milk drinks. Lactase is used by the producers of ice cream, yogurt and frozen desserts to enhance spade and creaminess, sweetness, tastiness, and digestibility, and to decrease sandiness because of crystallization that occurs in lactose converged preparations (Fig. 5.6). Cheese produced from hydrolyzed milk ripens more quickly compared to cheese produced from normal milk. In the last decade, much research has been conducted on lactose existing in whey and milk, and the fatal consequences of β-galactosidase, lactase or hydrolyase (Mehaia and Cheryan, 1987). These are due to the immobilization techniques of enzymes that have provided great and new potential uses for lactose. Due to the incapability of intestinal enzymes for some individuals, there is evidence of sensitivity to lactose—drinking of milk and eating dairy products is difficult. Subsequently, product aids containing small amounts of lactose or food that does not contain lactose is suitable for people who are unable to tolerate lactose. This protects them from tissue dryness, diarrhea, and sometimes death. Scientifically, lactose crystallizes readily and quickly and puts outlines to some operations in the manufacturing of dairy. As a result of high costs, this method of usage of lactase is not viable. Furthermore, environmental damage is considered the major obstacle related to separating high amounts of cheese whey. Moreover, through the fermentation process, the isolated whey can be used to produce lactic acid because it is an inexpensive source of lactose. Due to the fact that it is a derivative product in the production of concentrated whey protein by filtration through a medium, the whey permeate can be fermented effectively by *Lactobacillus bulgaricus* (Mehaia and Cheryan, 1987). Manufacturers could obtain lactose from different sources—plants, animal organs, bacteria, as well as yeasts (intracellular enzyme) and molds, which are utilized to prepare enzyme for commercial purposes. Lactase production from *Aspergillus niger*, *Aspergillus oryzae*, and *Kluyveromyce slactis* are deemed safe due to the safe usage history of those resources. It has been subject to multiple safety examinations, whereas the most developed lactase, rerived from *E. coli* lactase, is not reliable in food treatment due to its high cost and poisoning issues. The enzyme's immobilization, immobilization method, and carrier type can also affect these optima rates. Generally, lactase derived from fungus has a pH in the ideal acidic range of 2.5–4.5, and yeast and lactases derived from bacteria in the neutral area 6–7 and 6.5–7.5,

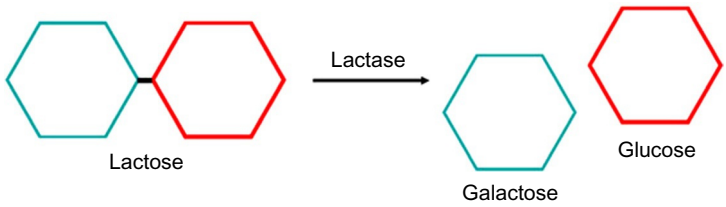

FIG. 5.6 Hydrolysis of lactose milk into glucose and galactose.

consecutively. The differences in ideal pH levels of lactases are the reason for their convenience for various uses. For instance, lactases derived from fungus can be utilized for acid whey decomposition, whereas yeast and lactases derived from bacteria are appropriate for milk with a pH level of 6.6, as well as sweet whey with a pH level of 6.1 decomposition. Galactose inhibition, as a type of product inhibition, is one characteristic that relies on the lactase resource. Galactose can inhibit the enzyme from *Aspergillus niger* more highly than the enzyme from *Aspergillus oryzae*. At minimum, hydrolyzed lactose can solve the product inhibitions through utilizing systems of immobilized enzymes or through improving the enzyme by the process of filtration using a medium after batch hydrolysis. Bacillus species lactases are the top lactases associated with thermo stabilization, the average of pH process, inhibition of product, and the sensitivity toward concentration of high substrate. Enzymes of thermo stabilization can keep their active condition under 60°C or more at protracted times. In this case, they have two distinguishing features: they give higher shorter residence time or higher conversion rates for certain transformation rates. Moreover, the microbial contamination is less because of higher growing temperature.

Bacillus sp. have low inhibition activity by galactose and high activity toward skim milk. For these reasons, *Bacillus* sp. are essential for the production of lactase (Gekas and Lopez-Levia, 1985). Lactose hydrolysis is perhaps accomplished by using the following enzymes: (1) free enzymes, commonly used in the impulse fermentation process, (2) immobilized enzymes, and (3) immobilized total cells manufacturing intracellular enzymes. Many hydrolyzed systems have been examined; few numbers have been successesful and fewer numbers have been used at the semi-industrial or industrial level. Different hydrolysis systems for acids have been improved to serve many industries. By using *K. lactis* lactase, numerous systems that utilize the free-enzyme process have been improved to treat and prepare whey and UHT-milk (Maxilact, Lactozyme).

Many commercially immobilized systems have been improved for large-scale production. In Italy, industrial milk treatment technology operations represent one as a cooperative method. Fiber-entrapped yeast lactase can be beneficial in a batch operation. Additionally UHT is a system used to sterilize the milk used. For leader factories, two other operations have been improved and designed for treating milk by Gist-Brocades (Germany), and Sumitomo (Japan). These are uninterrupted operations having average length of time. The UF-permeate operation of the whey is carried out through this system that was established by Connecticut, Corning Glass, Lehigh, Valio, and Ameracecorp. The process developed by Corning Glass is used in a commercial level in the production of baker's yeast by the use of hydrolyzed-whey (Gekas and Lopez-Levia, 1985). It has been shown that the in-pack addition of lactase after milk sterilization can have a negative sensorial and nutritional consequences, mainly related to the enzyme side of proteolytic activity, especially for prolonged storage times (Troise et al., 2016).

5.10 AMYLASES

Amylases are enzymes that originated from fungi and bacteria. They are playing an important role in food and beverage, baking, brewing, starch, and sugar industries. In order to hydrolyze starch into a water-soluble product, amylase is used for this purpose (Fig. 5.7).

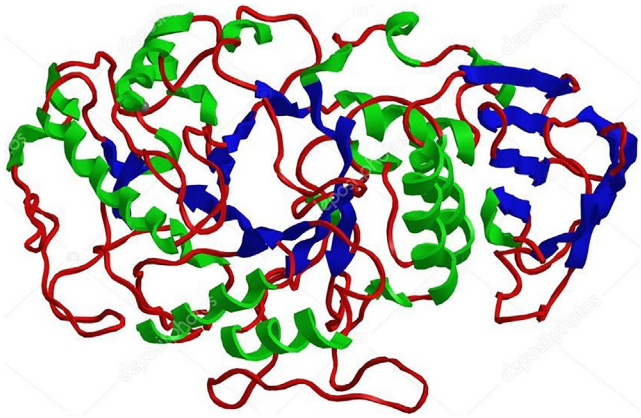

FIG. 5.7 Bacterial α-amylase used in food industry.

It is important to note that amylase is characterized by a glucose of low molecular weight. Widely, amylase enzymes can be applied in both drink and textile industries. In drinking, for instance, it is utilized for the production of High Fructose Syrup (HFS) (Ziegler, 1999). There are many microbial sources that can produce amylases such as *Bacillus*, *Pseudomonas* and *Clostridium* family. Nowadays, and at industrial level *Bacillus licheniformis* and *B stearothermophillus* are the probable bacteria used to produce amylases (Ploss et al., 2016). For the sake of reducing costs, *B stearothermophillus* is widely used because this strain is able to produce thermo-stable enzymes (Sundarram et al., 2014). α-Amylases are characterized by their impact on baked goods. It is important to add α-amylase and sugar to compensate for the grain deficiency. The addition of enzymes provides various advantages on the sugar. At a flour mill, adding enzymes to the flour may be standard so that a same product could be offered. Moreover, enzymes gradually form sugar that meets with the yeast growth requirements (Kulp et al., 1981). While the paste is placed in the bakery, steadily the temperature increases which causes an increase in the reaction rate of the enzyme and more sugars are produced. Malt extract and malt flour may be utilized as an enzyme supplement because malt is rich in α-amylase. However, it is preferable to use fungal α-amylase. The α-amylase hydrolyzes the wheat starch flour to small units of dextrins, hence the yeast is allowed to work constantly during dough fermentation and on the earlier stages of baking (Chi et al., 2009). This causes enhancements in bread volume and crumb texture. Furthermore, when three enzymes produce sugars such as maltose and glucose and small oligosaccharides, it helps in improve the baked flavor and reactions of the crust browning. Supposedly, ß-amylases in cereals are extensively studied as they play a very significant role in the release of facilely fermentable sugars of cereal grain starch and convert it to alcohol (bio-fuel) by yeast. Cereal beta-amylases can be used in many applications including analysis of starch, as well as in the food industry, and they make useful markers in breeding studies and cereal estimation. Finally, amylase splay an important role in degrading milk into monomeric molecules such as fatty acids oligosaccharides, and amino acids; molecules responsible for flavors in cheese (Konkit and Kim, 2016).

5.11 CONCLUSION

Only a relatively small number of microbial enzymes are used commercially in the dairy industry. But the number is increasing day by day, and their field of application will be expanded more and more in near future. The purpose of this chapter is to describe the practical applications of microbial enzymes in the field of the dairy industry.

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