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Application of Microbial Enzymes in Dairy Products: A Review

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Abstract: Enzymes produced by microbial sources are biological molecules that known to catalysts biochemical reactions which roles involve in lead to stimulate the necessary chemical reactions, as well as to the formation of fermented products. Microbial protease, lipase and β-galactosidase are important examples of such interest in industrial food and dairy product. This is due to their thermoresistant, thermostability and thermoacidophilic properties. In brief, hydrolyses are the substrate which includes some enzymatic reaction that allows to avoid the health and environmental problems, and also to catalyses chemical reaction during the formation of flavor compounds or prebiotic and other products additives in the production and development of healthy dairy food products. Thus, enzymes are one of the relatively important factor that expected to be utilized in large-scale in the process of products development. This review focused on the importance and application of three major enzymes that microbial produce which are of great interest in dairy industries and have positive impact on consumer's health.

Keywords: Microbial enzymes, Protease, Lipase, β-galactosidase, Application.

Introduction

Enzymes are the catalysts of biological processes that are produced by living cells, which work to accelerate chemical reactions. Now a days, enzymes have a large share in the market of fermented products. In point of view of the importance and their role, the global production of enzymes is reached 53,000 tons per year, (industrial enzymes 75% are hydrolytic) and Denmark is lead known as lead producer. There are two companies viz. Novozymes and Danisco in Denmark are producing 70% of the world's production of enzymes sales of industrial enzymes were 625-700 million Dollar estimated since 1989-1990. Recently, it was estimated 5-5.5 billion Dollar in 2016 3.3 billion Dollar in 2010 and expected to 7.6 billion Dollar exceed by 2022 (Jaramillo *et al.,* 2015; Guerrand, 2017). Although, enzymes have been traditionally extracted from plants and animals, but today their production from microorganism is growing rapidly with 90% of the industrial enzymes. There are more than 3000 enzymes,but only 5% are used in 500 commercial products. The food industry is ranked first and accounted for 45 % of total enzyme application (Aehle, 2007; Li *et al*., 2012; Brahmachari *et al*., 2017). In past few decades, about 200 enzymes of microbial origin was traditionally used (Li *et al.,* 2012). Protease, lipase and β-galactosidase were produced by a wide range

of microbial sources such as mold, yeast and bacteria which have subjected to the attention of many researchers because of their easy extraction from their sources release into the growth media (Hsu *et al*.,2007; Naimah *et al.,* 2018). These enzymes may also be concentrated for their use in other application. So thus reducing the cost of production compared to other source (Jooyandeh *et al*., 2009; Feijoo-Siota *et al*., 2014; Jaramillo *et al.*, 2015; Kazemi *et al.*, 2016). Therefore, the aim of this review to study the microbial source of protease, lipase and β-galactosidase and it's important application in dairy products.

Microbial sources of enzyme

The microbial enzymes are preferred to produce from animal or plant sources due to their economic production and consistency. The ease of employing the microorganisms and the rapid adaptation for consumption of cheap agro-industrial residues available in nature, so it highly recommended use for large-scale production (Singh *et al.*, 2016).

Table (1): List of some important microbial produced enzymes

Microorganism	Fermentation	Enzyme	Reference
Aspergillus niger	SSF	Amylase,	Roses and Guerra, 2009
	SSF	Cellulase,	Salihu *et al.*, 2015
	LSF	Glucose oxidas,	Kiesenhofer *et al.*, 2017
	SSF	β-galactosidases	Kazemi *et al.*, 2016
	SSF, SmF	Invertase	Viniegra-González *et al.*, 2003
	SSF, SmF	Pectinase	Viniegra-González *et al.*, 2003
	SSF, SmF	Tannase	Viniegra-González *et al.*, 2003
Aspergillus giganteus	SSF	Pectinase	Ortiz *et al.*, 2017
Aspergillus oryzae	SSF	β-galactosidases	Nizamuddin *et al.*, 2008
	SSF	Protease,	Chutmanop *et al.*, 2008
	SSF	Amylase	Chutmanop *et al.*, 2008
Rhizopus oryzae	SSF	Lipase	Vaseghi *et al.*, 2012
Saccharomyces cerevisia	LSF	Invertase	El-Enshasy and Elsayed, 2017
Kluyveromyces marxianus	LSF	β-galactosidases	Al-Jazairi *et al.*,2015
Bacillus	LSF	Protease,	Laili and Antonius, 2015;
	LSF	Amylase	Simair *et al.*, 2017;
	LSF	Lipase	Ugras, 2017;
	LSF	Cellulase	Nandimath *et al.*, 2016
Pseudomonas	LSF	Cellulase	Nandimath *et al.*, 2016
Streptococcus thermophilus	LSF	β-galactosidase	Sangwan *et al.*, 2015
SSF: Solid-state fermentation, **LSF**: Liquid state fermentation, **SmF** :Submerged Fermentation			

Usually, commercially used microbial enzymes are intracellular if produced from bacteria and yeasts while intra or extracellular from molds (Sangwan *et al.*, 2015; Kazemi *et al*., 2016).

In general, microbes produce enzymes either in an induced or constitutively (Aehle, 2007). indicated in Table 1 that the fungi are the most important microorganisms (including *Aspergillus niger, Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) whose produced enzymes have been extensively studied by many researchers. Thus, they are classified as generally recognized as safe (GRAS) by Food and Drug Administration, (Mahoney, 2003; Saad, 2004; Brahmachari *et al*., 2017).

Enzymes production of different fermentation methods

There are two methods of fermentation for production of enzymes, Submerged Fermentation (SmF) and Solid-State Fermentation (SSF).

Liquid or Submerged fermentation

The Submerged fermentation is an important method which includes batch and continuous culture, that has been used in the production of many enzymes. This process is involved in the development of microorganisms in a liquid broth, but good growth is not enough to obtain a higher enzyme yield. (Viniegra-González *et al.*, 2003). Submerged fermentation is best suited for bacteria that require high water activity (a_w) (Sangwan *et al.*, 2015 ; Laili and Antonius, 2015), Ugras *et al.*(2017) produced the lipase enzymes from *Bacillus licheniformis* isolated from water and soil of the Hayran thermal springs in Giresun. Furthermore, many studies were used lactic acid bacteria(LAB) for production of enzymes in liquid state fermentation (Feijoo-Siota *et al*., 2014; Sangwan *et al*., 2015). Whereas O'Connell and Walsh (2010) produced the β-galactosidase from *Aspergillus niger* and *Kluyveromyces marxianus* in submerged fermentation using wheat bran medium with 200 rpm/minute. In another study of Lanka *et al*. (2017) who produced protease enzyme from *Aspergillus niger* which was isolated from dairy form effluent.

Solids State fermentation

Solid-state fermentation is an advances in fermentation technologies that have become the most important alternative and advantages from the economic point of view over conventional submerged fermentation for enzyme production (Aguilar *et al.,* 2008). Most of the enzymes from microorganisms are produced by Solid-state fermentation method due to the fact that it is more concentrated, stable and high yielding products (up to 5.5 times more than in SmF), a low water demand which helps to minimize contamination with low costs for extraction of pure enzymes also the highest enzyme activity. The volume of medium per unit weight of substrate is low and easy aeration process. Additionally, the extracellular nature of their enzymes and the stability at wide ranges of pH and temperature (Chutmanop *et al*., 2008; Kazemi *et al.*, 2016). The production medium in this method is often simple which employs natural raw materials as the substrate such as agro industrial by-products like rice bran, wheat bran, Sugar cane waste, maize bran, cassava waste or wheat straw (Aguilar *et al.*, 2008), Several studies have reported interesting advantages of enzyme produce by solid state culture (SSC) over submerged culture (SmC) (Shaikh *et al*.,1997; Vaseghi *et al*., 2012). This was found by Al-Manhel (2011) who produced the extracellular β-galactosidases enzymes by 8.15 fold from *Aspergillus oryzae* and results showed that solid state fermentation method was better than the submerged fermentation.

Optimization of fermentation conditions

For improving the production of enzymes, firstly including the nutritional and culture conditions, and the second is the genetically treated with isolate, the parameters like the pH of the culture media plays a very important role on the growth of microorganisms, its affected on the process of metabolism, synthesis and solubility of nutrients in the media also its impact on the activity and stability of enzymes production (Aehle, 2007). There are several studies

which have shown "slight" variation in the pH values of the medium, used in the β-galactosidase production from the molds while this enzyme is certainly different in the case of its production from bacteria. Nizamuddin *et al.* (2008) reported that the best production of β-galactosidase at pH 5 from the *Aspergillus oryzae*, similar result also found by Chutmanop *et al*. (2008) in the protease production at pH 7.5 from *Aspergillus oryzae*, Whereas the optimum pH of β-galactosidase obtained ranged between 6.5-7.5 from *Lactobacillus acidophilus* ATCC 4356 (Carević *et al.*, 2015).

On the other hand, most of the studies that indicated the major impact of the incubation period on the production of protease, lipase and β-galactosidase by different microorganisms which was influenced by some factors, including types of microorganism, the components of the production medium and available growth factors as well as other culture factors such as temperature and pH (Table 2). Several studies confirm that the incubation period for the production of β-galactosidase enzyme from molds using solid state fermentation is not less than four days and not more than seven days. Nizamuddin *et al.* (2008) stabilized the incubation period for 6 days to study the effect of other production conditions on the β-galactosidase, while Ugras *et al.* (2017) showed that the production of the lipase from *Bacillus licheniformis* was taken 24 hours of incubation period and had the best enzymatic productivity. Sangwan *et al*. (2015) also produced enzyme from *Streptococcus thermophilus* after 18h. Moreover, temperature is another factor necessary for microbial activity which effects to the growth rate and production (Aehle, 2007). The used temperatures in the production of enzyme vary according to the different strains of microorganisms.

Ugras *et al*. (2017) indicates that the best temperature in fermentation for the production of the lipase from *Bacillus licheniformis* was 90°C, whereas Cruz *et al.* (1999) showed that the best temperature for the hydrolysis of the lactose ranged between 60-55°C while that 30°C is the best temperature for it's produced from *Penicillium simplissium*.

Table (2):Some optimization of fermentation conditions of microbial enzymes (Protease, lipase and β-galactosidase

Microbial source	pH	enzyme	Temp.	Incubat. Period (h)	References
Bacillus licheniformis	9	Lipase	90	24	Ugras *et al*., 2017
Rhizopus oryzae	8	Lipase	45	72	Vaseghi *et al*., 2012
Bacillus sp.	7	Protease	60	24	Laili and Antonius, 2015
Aspergillus oryzae	7.5	Protease	30	120	Chutmanop *et al*., 2008
Aspergillus niger	10	Protease	50	92	Lanka *et al*., 2017
Aspergillus oryzae	4.5	β-galactosidase	30	120	Al-Manhel, 2011
Kluyveromyces marxianus	3	β-galactosidase	20	64	Al- jazairi *et al*., 2015
Lactobacillus delbrueckii	6	β-galactosidase	45	48	Sharma and Singh, 2014

Protease

Proteases (mixture of peptidases and proteinases) also known as hydrolytic enzymes, peptidases and proteolytic enzymes. They are one of the largest group of enzymes in biotechnology and most important enzyme which produced on a large scale, nearby 60% of the world enzyme market (Sharma *et al*., 2017). This enzyme that catalyze the

hydrolytic protein into amino acid or smaller peptide fractions, which depends upon the optimum pH. They are defined as alkaline, neutral or acidic proteases. Now a days, microbial proteases are classified as exopeptidases and endopeptidases on basis of site of protein action which recognized into six families included : serine carboxy proteases (EC 3.4.16), metallo carboxy proteases (EC 3.4.17), serine proteases (EC 3.4.21) family includes tryosin, chymotrypsin, elastase, subtilisin, cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23) which have tow aspartic acid residues in the catalytic of the active site. Microbial rennin is also one of the most significant enzymes, produced by GRAS microorganisms like *Mucor pusilis*, *Mucor miehei* and *Bacillus subtilis*. It has been used instead of calf's rennin in cheese manufacture. Thus, this enzyme has a low proteolytic activity and high milk-clotting activity. Finally, metallo proteases I (EC 3.4.24) is another such most important enzyme known as exopeptidases (Whitaker, 2003; Brahmachari *et al*., 2017). Generality, most of the commercial protease produced from bacteria viz. *Bacillus* that is the major source (Laili and Antonius, 2015). It is one of the most important industrial enzymes in the world markets. The major application is recognized in dairy industry for cheese ripening, hydrolyzing whey protein and flavor development. Moreover, enzyme used to debittering of protein hydrolysates, synthesis of aspartame and accelerating cheese ripening times, Furthermore, proteases were also used for the production of milk proteins with low allergenic, which used as an ingredients in milk formulas of baby (Feijoo-Siota *et al*., 2014; Brahmachari *et al*., 2017).

Lipase

Lipase (EC 3.1.1.3) is also called as glycerol ester hydrolases, a lipolytic enzyme. Lipases are the key enzymes involved in fat digestion, catalyze the triacylglycerols reaction to fatty acids and glycerol, mono or di-glycerides and extracellular enzyme. They are mainly produced from fungi when induced by adding oils and fats. Microbial lipases constitute as an important group of biotechnologically valuable enzymes (Wong, 2003). Nowadays, bacterial lipases are receiving attention due to it's function in extreme conditions (Ugras *et al.,* 2017). Generally, commercial source of microbial lipase including fungi such as *Aspergillus, Mucor, Rizopus* and *Candida* while *Pseudomonas, Achromobacter, Staphylococcus* and *Bacillus* reported as bacterial lipases producers (Brahmachari *et al*., 2017). Most of the researches indicated to the lipase application in dairy industry. for acceleration cheese ripening, hydrolysis of milk fat, development of lipolytic flavours in some cheese ripening which production of a wide range of compounds by primary and secondary biochemical pathways. Moreover, the lipolysis of fat in butter, margarine and cream (Wong, 2003; Jooyandeh *et al*., 2009).

β-galactosidase

β-galactosidase (EC 3.2.1.23) is another name lactase, Lactosase and Lactosidase enzymes are classified in hydrolases class belongs to family 35 of the glycoside hydrolases (GH-35). This enzyme is located in the brush border membrane of the small intestine of humans and other mammals, which responsible to hydrolyze the glycosidic bond of ß, (1-4) in lactose and produce glucose and galactose, (Mahoney and Whitaker, 1978; Mahoney, 2003). This disaccharide is present in mammalian in concentrations up to 10% (w/w) and foremost sugar present in dairy

products. Also, has an ability to catalyze the reverse reaction of the hydrolysis called as transglycosylation (Nivetha and Mohanasrinivasan, 2017). The β-galactosidase contains a proportion of carbohydrates associated with the covalent protein part, so it is a Glycoproteins, (Manzanares *et al.,*1998) Moreover, Many researchers studied the molecular structure of the β-galactaseidase enzyme produced from microorganisms and found that it differs in the number of subunits between the microorganism (Seyis and Aksoz, 2004; El-Gindy *et al*., 2009; Maksimainen *et al*., 2011). Although, structure of the enzyme in terms of the length of the amino acid chain and the effective site varies according to the microbial source (Mlichova and Rosenberg, 2006). There is a significant similarity between the enzymes of the β-galactosidase produced by yeasts, molds and bacteria in terms of their high content of acid amino acids and their low content of sulfur containing amino acid (Mahoney, 2003; Chen *et al.*, 2008). The main function of the β-galactosidase is to hydrolysis the lactose into glucose and galactose. Table 3 summarized the work of various researchers which described about the rate of hydrolysis of β-galactosidase. In fact, this depends on some of factors, i.e. types of microorganism, time of hydrolysis, lactose concentration, temperature and pH. Thus, improve solubility, sweetness (about 50%) and digestion of dairy products (Mlichova and Rosenberg, 2006). The use of β-galactosidase to hydrolysis of lactose in milk and its products is one of the most promising applications of β-galactosidase enzymes in food processing. The β-galactosidase could reduce the crystallization problem during storage due to low dissolvability of lactose (Nivetha and Mohanasrinivasan, 2017). Furthermore, Marrakchi *et al*. (2008) developed to a biosensor to apply for the quantitative detection of lactose in commercial milk samples from two enzymatic activities; one of them is glucose oxidase and second is β-galactosidase. Whey lactose hydrolyze has a number of beneficial uses, which is characterized by high sweeter. So, it is a source of "sugars which can be used in the manufacture of sweets and syrup as an alternative to sucrose used for bread and pastry, and also in ice cream. Moreover, *Saccharomyces cerevisae* can ferment to the whey (contain lactose hydrolysis) as a carbon source for production of alcohol and other a wide range of the bio products (Rech and Ayub, 2006). Furthermore, β-galactosidase is one of the most popular enzyme in the dairy industry, which works on the Lactose hydrolysis either from milk to glucose and galactose. As a result, Many characteristics and qualities of the dairy products were improved such as solubility, flavor, sweetness, digestibility, problem of crystallization, resulting a sandy or gritty texture and mealy (in ice cream, condensed milk and frozen milks) was decreased while increasing of nutritional value by adding galactooligosaccharides (Geiger *et al.,* 2016). Moreover, β-galactosidase could employed to converts the whey lactose into the useful products such as sweet syrups that are used in soft drink, confectionery and bakery industry (Saqib *et al*., 2017). On the other hand, milk lactose hydrolyze with β-galactosidase was also used in the various dairy products such as yoghurt, processed cheese and some other products. As it accelerates ripening because it's more easily to fermented of mono sugars. These products improved to the flavour as well as the reduction of the acidity period, which is due to the availability of a Monosaccharide's that are easy to consume and ready to be metabolized by the starter,

that increased formation of acid by the bacteria. (Mahoney, 2003). Therefore, recent studies have directed the use of free and immobilized β-galactosidase in reducing the level of lactose in milk and whey. The hydrolysis of lactose in milk and whey. This depend upon the β-galactosidase activity which in turn depends on the temperature, processing time, concentration of enzyme and pH, (Haider and Husain, 2009). Indeed, the β-galactosidase is an important in commercial scale for treatment of lactose intolerance or hypolactasia, which affects more than 70% of people unable to digest food containing lactose properly due to the inactivity or the lack of the intestinal enzyme. By this cause, they suffer from intestinal dysfunctions gas, diarrhea and abdominal pain if their diet contains lactose. However, with low β-galactosidase activity, lactose remains unabsorbed which results in intestinal discomfort due to bacterial activity (Nivetha and Mohanasrinivasan, 2017), Furthermore, some people are born with the ability to make enough amounts of β-galactosidase, the production of β-galactosidase normally decreases with age, this is called lactase deficiency. (Simpson, 2012). The β-galactosidase also catalyze transgalactosylation reactions in which lactose serves as a galactosyl donor and an acceptor to form di-, tri- or higher galactooligosaccharides, lactosucrose and lactulose (Sangwan *et al.,* 2015; Silverio *et al.,* 2016). These are indigestible compounds which act as a dietary fibre (prebiotics). They are enzymatically obtained which is considered as a substrate that is selectively utilized by probiotic bacteria conferring to the health benefit with the reduction of a significant number of potential pathogenic bacteria (Gibson *et al*., 2017).

Table (3): Lactose hydrolysis rates using microbial β-galactosidase.

Microorganism	Milk lactose%	Whey lactose%	Hydrolysis Time (h)	Reference
Aspergillus japonicus	35	55	4	Saad (2004)
Aspergillus oryzae	70	61	3-4	Haider and Husain (2009)
Aspergillus oryzae	40.72	49.35	4	Al-Manhel, 2011
Aspergillus niger	23	61	1	Woychik and Wondolowski, 1973
Penicillium simplicissimum	67	84	10	Cruz *et al.,*1999

Conclusions

Nowadays, microbial protease, lipase and β-galactosidases are being important to use in various fields such as health and dairy product. The probability of industrial utilization of such microbial enzymes in many industries has continuously increased because of their potential and beneficial roles which fulfil to meet the demand of the rapidly growing population and to be proved as a substitute for exhaustion natural resources in a more intensified manner in the future.

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