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Recent developments of industrial enzyme production in food biotechnology

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Abstract

The use of enzymes in industrial applications is of great commercial importance. The enzyme industry, which has a high market value, comprises many areas. One of them is the food industry. Enzymes have an important role in terms of properties such as taste, aroma fragrance components in foods and their ability to produce the desired flavor in food. In addition to these, it has features such as facilitating the digestion of the food, improving the quality, improving the nutritional value, facilitating production, increasing the efficiency and reducing the cost. Enzymes are found naturally in foods, as well as animal, vegetable and microbial sources and enzymes are produced. In this review, it is aimed to give information about researches and current developments related to the biotechnological production of enzymes that are widely used in the food industry.

Key words: food biotechnology, enzyme, industrial enzyme production, DNA

Introduction

Food, which is one of the basic elements of life, takes its place in tables as raw or processed material. One of the branches of the food industry covering these processes is biotechnology applications. These applications are the use of plant, animal and microbial systems in the production and processing of foods (Angold et al., 1989). Biotechnology applications, while using techniques that do not harm the environment, having low energy requirements, do not require high pressure, can be carried out at room temperature or at lower temperatures, can also enable the evaluation of pollutants and disintegrate them with the help of microorganisms (Telefoncu, 1996; Gundesli et al., 2019).

With the development of enzyme technology, the increase in the variety of usage areas and the high economic value made the researches about industrial enzymes in biotechnology more important. The growth rate of some of the organisms used in enzyme production is slow, production amounts are low and fluctuates due to various factors. At this point, the production of enzymes has reached great dimensions by using recombinant DNA technology, which is considered as a strategic field in recent years, and its usage has become widespread (Köksel et al., 1989; Kapucu, 2003; Eskimez et al., 2019). This technology has enabled new enzymes to be produced according to processing conditions in specific food product production. Protein engineering is involved in this field to detect and modify microorganisms that are the source of enzymes with new techniques (Yeşilçubuk, 2012). The mentioned method is also known to be useful for the development of strains. For example, mutagenesis deletion of the extracellular protease gene in bacterial and fungal species, many of which damage the target enzyme, yields non-protease-producing mutants (Çerçi et al., 2011).

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Enzymes

Enzymes are defined as organic and catalytic chemical compounds synthesized by living cells, consisting of highly organized protein molecules that catalyze all chemical reactions in the cell (Özata and Kutlu, 2000; Saldamlı, 2007; Dikmen, 2018). Enzymes are divided into two according to their intracellular and extracellular activity. Enzymes that accumulate in the cell after they are synthesized and catalyze reactions within the cell are called intracellular enzymes and enzymes that are sent out of the cell after they are synthesized are called extracellular enzymes. Extracellular enzymes are synthesized when nutrients are too large to pass through the cell membrane and break down these macromolecules into micro molecules that are large enough to pass through the cell membrane. Due to their extracellular activity, enzymes that can be produced in large quantities by microorganisms are isolated and used in various industrial fields (Fox and Cameron, 1977). The effect of enzymes is generally very specific (Koolman and Roehm, 2005). Enzymes are able to show their biological activities at optimum pH and temperature values. (Simon et al., 1959). Temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors and activators may affect the enzymatic presence at the initiation of enzymatic reactions (Kumar et al., 2012). In industrial applications, you prefer more than traditional processes and are used in industry. The reason for this can be shown that the catalytic power of enzymes is very high, they are specific, they are economical (Anonymous, 2011).

Enzyme in Food Biotechnology

Biotechnology can be defined as the use of biological systems in production technology. Biotechnology has two main characteristics. These are their relationship with practical applications and inter-scientific cooperation. Since food is of biological origin, any technological application in the food industry is referred to as biotechnology application by definition of biotechnology. Biotechnological processes in food sector are production, modification, product development and other applications (Jarvis and Holmes, 1982: Higgins, 1985; Anonymous; 2011).

Enzyme is used in many areas of life and its use in food preparations is an age-old process. An example is given in Table 1 below. All of these processes are called ‘enzyme technology (Anonymous., 2019). Due to the wide range of enzyme technology, the market is expected to reach US $ 17.50 billion by 2024 (Pellis et al., 2018).

Enzyme technology includes microbial processes involving selection and development of producer strains, enzyme production by fermentation (optimization for medium production, medium conditions, etc.), modification of three-dimensional structures of enzymes (protein engineering), isolation, purification and immobilization studies. (Demiralp et al., 2015).

Enzymes are produced by animals, plants and microorganisms. Although the importance of plant and animal enzymes in enzyme production for some specific applications and uses is indisputable, the use of microbial sources is dominant for technical applications. Microorganisms complete their development with fermentation techniques due to very short generation times. In addition, it is possible to easily control the factors and conditions in microorganism development environments, that is, easy process modification and optimization, cost effective and stable properties increase the prevalence of microorganisms in production. When plant and animal resources are used for production, more plant and animal materials are needed. As a result, there may be difficulties in meeting these materials (Gurung et al., 2013; Anonymous, 2019).

Also, microorganisms are selected not only by their ability to produce enzymes, but also by their toxic and non-pathogenic properties (Kıran et al., 2006).

It was difficult to isolate and characterize intracellular enzymes in the 1940s, which led to the need for more efficient methods of protein purification techniques. First, the pure intracellular enzyme was obtained from skeletal muscle and yeast. The development of purification methods increased the number of enzymes obtained in pure form. The rapid development of separation and purification techniques and the use of these techniques in enzyme purification have enabled the production and introduction of around 3000 enzymes as pure preparations (Gerhatz 1990; Saldamli, 2007). The enzyme production, which consists of two basic steps as isolation and purification, is summarized below (Figure1).

The isolation step consists of extraction, separation of enzymes from solution and concentration. The appropriate enzyme source should be selected first in the enzyme to be isolated. The source may be plant, animal or microorganism (Saldamli, 2007).

Different fermentation techniques are used in microorganism production. Solid phase fermentation techniques, liquid phase fermentation techniques and in-depth fermentation techniques are examples. They have their own advantages and disadvantages. The solid phase fermentation technique is preferred since it does not require complicated basic processes such as pasteurization when compared to the liquid phase fermentation technique. The solid fermentation technique stands out from the rest as it has superior properties such as reduced fermenter volume, reduced bacterial contamination and its use in non-sterile substrates as compared to in-depth fermentation (Boyacioglu et al., 2012).

After obtaining animal, plant extract and microorganism cultures, the common step is the separation of enzymes from this medium. In this step, one of the techniques such as filtration and centrifugation can be applied. The next step is followed by concentration of the enzyme solution. The crude solution enzyme may contain other components such as protein, sugar, fat and nucleic acid, as well as solution enzymes. For separation of enzymes from this solution, the most suitable of precipitation, membrane filtration or thermal methods should be chosen. Different enzymes and proteins may be present in the concentrated enzyme solution obtained as a result of mentioned processes. The desired enzyme should be obtained as a pure enzyme preparation or a high-purity enzyme preparation by applying purification to the solution. Crystallization and electrophoretic methods are used for purification of enzymes. Gel, ion exchange, hydrophobic, affinity, immunoaffinity and covalent chromatography can be cited as chromatography types (Saldamli, 2007).

Table 1. Sources and Usage Areas of Enzymes in Food Industry (Saldamli 2007; Raveendran et all., 2018).

Sources	Enzymes	Scope of application
Cattle and pig pancreas Barley malt *Aspergillus oryzae* *Aspergillus niger* *Bacillus subtilis*	α –Amylase	Baking, beer, starch liquefaction Improving bread quality Rice cakes Fruit juice treatment
Aspergillus niger *Endothia parasitica*	Glucoamylase	Beer production Improving bread quality High glucose and high fructose syrups
Aspergillus oryzae *Bacillus subtilis*	Protease	Beer Meat softening Coagulation of milk Improving bread quality
Aspergillus oryzae *Aspergillus niger* *Sacchromyces fragilis*	Lactase (β-galactosidase)	Prebiotic foodstuffs
Cattle and pig pancreas castor bean *Endothia parasitica*	Lipase	Cheese flavor development Cheddar cheese production
Clover, tomato, citrus	Esterase	Increasing flavor and aroma in fruit juice De-esterification of dietary fiber Production of short chain flavor esters
Aspergillus niger *Aspergillus niger*	Cellulase Pectinase	Animal feed Fruit juice treatment Fruit juice treatment
Aspergillus niger	Glucoseoxidase	Food shelf life improvement Improving food flavor
Bovine liver, erythrocytes *Aspergillus niger* *Micrococcusly sodeikticus*	Catalase	Removal of hydrogen peroxide from milk before cheese production

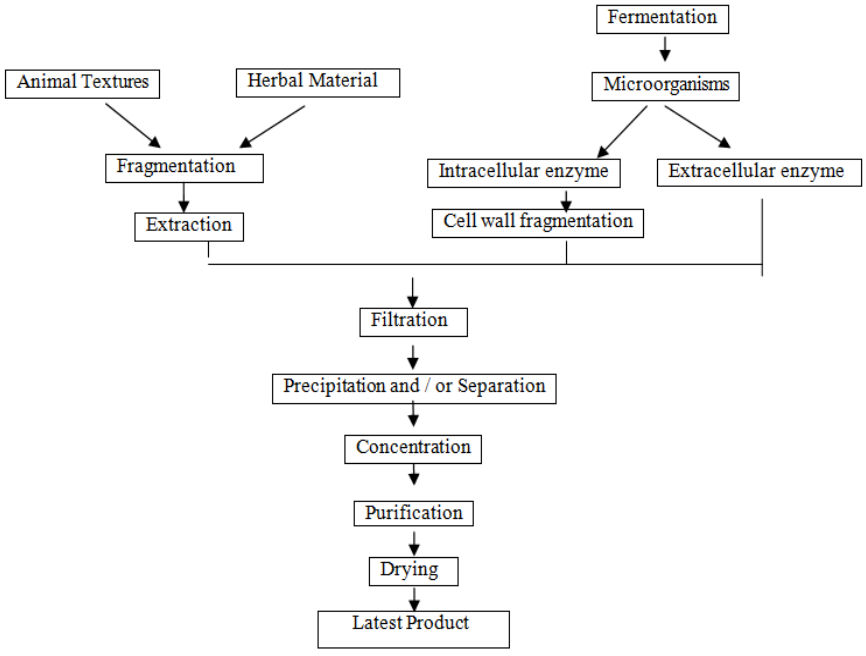

Figure 1. Enzyme Production Scheme (Saldamli, 2007).

We can list the types of enzyme separation and purification methods as below.

1.Size and mass-dependent methods (centrifugation, gel filtration, dialysis and ultrafiltration)

2.Load dependent methods (Ion exchange chromatography, isoelectric focusing)

3. Methods based on change in resolution (change in pH, change in ionic strength, triple phase separation technique).

The fact that the triple phase separation process, which has been popular in recent years, is one of the methods based on the change in resolution that is related to its superior usage advantages (Babagil, 2018).

A biotechnological product may need to be formulated, dried and stored under optimal conditions to maintain its activity and stability during transportation and storage. Therefore, the product formulation of the purified enzyme is made as a final step (Hatti-Kaul and Mattiasson, 2001).

When the studies on purification method are examined, we encounter numerous examples. For example, in a study conducted by Grabski and Jeffries (1991), *Streptomyces roseiscleroticus* NRRL B-11019 produced from β-1-4-endoxylanase, ammonium sulfate precipitation and two different cation exchange chromatography reported that they are 45 times purified. Chen et al. (1997), oat xylan produced in culture medium with *Trichoderma longibrachiatum* CS-185 produced xylanase, ultrafiltration, ammonium sulfate precipitation, cation exchange chromatography and gel filtration chromatography using all 56-fold purification techniques performed. Carmona et al. (2005) continued purification from two xylanases obtained from *Aspergillus versicolor* and started by chromatography and one of the xylanases bound to the column and the other from the column binding. Xylanase bound to the column was also applied by gel filtration chromatography. Reported that they purify this xylanase 28 times. Güder (2014) used corn cobs as a carbon source for enzyme production in order to evaluate the wastes, and produced enzyme production with 21.8-fold purification by applying anion exchange column chromatography techniques with gel filtration in purification. Yadav et al. (2008) with their research, *Aspergillus flavus* MTCC 7589 purified a pectin lyase, ammonium sulfate fractionation, anion exchange chromatography and gel filtration chromatography applications to electrophoretic homogeneity. The obtained enzyme was effective in softening the plant fibers of *Crotalaria juncea*. In another study Sandri et al. (2013) purified pectinase from *Filamentous fungi.* A strain of *Aspergillus nigerian,* called LB23, obtained the extract enzyme by solid culture method (SSF), used it for processing juices, and compared some properties with two commercial enzyme preparations, obtained statistically similar or superior results. Marrufo-Hernandez et al. (2017) obtained polyphenoloxidase enzyme from apples. Applications were in the form of phenyl sepharose hydrophobic interaction chromatography, anion exchange chromatography. As a result, they purified 319 times enzymes in 1.6% yield.

In studies of increasing activity, Sookheo et al. (2000) reported that three extracellular protease enzymes produced by *Bacillus stearothermophillus* increased enzyme activity by 5 mM $CaCl_2$ and inhibited EDTA. Aijun et al. (2005) informed that applied air pressure vibration in protease production from *B. pumilu.* When the width of its vibration reached 0.10 MPa, the period of air pressure vibration was determined as 860 U / ml, 4300 U / g wheat bran, which was obtained in a period of 1 hour with a humidity level of 65%. They stated that this value was two times higher than the values obtained with static SSF. Pinheiro (2003) reported that in the production of beta-galactosidase obtained by applying the batch system from *Kluyveromyces marxianus* CBS 7894, pressure was applied to the reproductive medium at different rates. At the end of the study, it was observed that applying 6 bar pressure increased the biosynthesis of beta-galactosidase enzyme.

In a study conducted on the evaluation of wastes, enzymes were produced as an alternative to SmF (Submerged Fermentation) in SSF medium and SSF technique was reported to be more efficient. (Karatas, 2008).

Enzyme Immobilization

The Enzymes are disposable free and cannot be recovered, thus increasing the cost. To remedy this, the enzyme molecules are bound or trapped in an insoluble support (called a carrier or matrix). Or a system which is formed by cross-linking enzyme molecules without losing their catalytic activity is used. With this application, enzymes are used repeatedly to reduce the cost (Anonymous 2011).

Immobilized enzyme; enzyme, matrix (carrier / support), the interaction between the enzyme and the matrix (carrier / support) consists of three main components. Re-use of enzymes, filtration, easy isolation by solid-liquid separation techniques such as centrifugation, use in continuous type reactors, features such as high efficiency yields the use of immobilized enzymes advantageous provides widespread use area (Swaisgood, 2003; Boyacioglu et al., 2012). However, there are disadvantages of immobilized enzyme. These disadvantages have been shown to be due to the loss of enzyme activity due to the technique used in immobilization and the adverse effect of the carrier on the mass transfer by making it difficult to transport the substrate molecule to the enzyme molecules (Anonymous, 2011).

Types of enzyme immobilized methods are chemical bonding (ionic, cross and covalent bonding) and physical methods (immobilization by asorption and immobilization by confinement) (Babagil, 2018).

In one study, pectinolytic enzymes polygalacturonase (PG), pectin lyase (PL) and pectin methylesterase (PME) were isolated from *Aspergillus ornatus*. The purification process uses ammonium sulfate fractionation, dialysis and gel chromatography. Specific activities and purification coefficient were determined after PG, PL and PME passed through the column. Active PG, PL and PME were also superficially immobilized and Michaelis-Menten kinetic values of each free and immobilized enzyme were determined. It was emphasized that there was a significant improvement in pH and temperature values after immobilization. In some fruit juices, apple, mango, peach and apricot fruit juice clarification, color and viscosity tests have been emphasized that positive results were obtained and said that these enzymes are suitable for potential applications in the food industry (Irshad et al., 2017).

Since the immobilization technique caused loss of activity due to loss of binding and leak age through the pores, different methods were tried to obtain higher quality products. One of the methods used to increase the yield is to reduce the carrier size (Boyacioglu et al., 2012). With the development of nano-technology and biotechnology together, the increase in

knowledge accumulation has revealed nano-biotechnology and enzyme technology has started to be fed from here (Gürsel, 2006). Enzyme activity immobilized with nano-scale materials was as effective as free enzymes. It has been reported that with the synthesis of flower-shaped enzyme-inorganic hybrid nano-structures, which is a different immobilization method applied in this field, the enzymes increase the yield and stability by showing significant differences compared to free enzymes and other immobilization techniques. Hybrid nano-flower organic-inorganic production, development, growth and the formation of a flower-like structure consists of three steps.

These nano-hybrid structures are called nano-flowers due to their flower appearance (Lee et al., 2015; Koç, 2016; Babagil, 2018). There are varieties such as copper, calcium, manganese, protein and capsule hybrid structures. Enzymes have a strong binding strength against metal ions and are generally immobilized on the metal surface (Polaina et al., 2007).

Streptococcus sanguinis, Bacillus licheniformis and *Bacillus pumilus* microorganisms were isolated and identified from tomato and apple. From these bacteria, pectin lyase enzyme was produced in solid culture medium and purified by 126.7-181.2-191.8 times in 76.71-78.9-78.2% yield with triple phase separation method. For the first time the purified pectin lyase enzymes were immobilized in the nano-flower hybrid structure and the characterization of the nano-flowers was made. Then, free and nano-flower enzymes were used to clarify the fruit juices. It is stated that hybrid enzyme is more effective than free enzyme (Babagil, 2018).

One of the methods used to improve immobilized enzyme techniques is genetic engineering. Changes in the protein structure and properties of immobilized enzymes during the arrest are eliminated by this method (Kilara and Desai, 2002; Ueda et al., 2003; Boyacioglu et al., 2012).

Enzyme Production by Genetic Engineering Techniques

Recombinant DNA technology, called modern biotechnology, realizes the redesign of cells by interfering with enzymes. In this way, different properties are also given to the enzymes being developed. In this method, a host such as an animal, plant or microorganism is used to produce the enzyme. By cloning genes to encode enzymes, they can be produced in host microorganisms, which can easily conform to high-volume applications in industrial fermentations. It is also possible to produce enzymes that are compatible with the temperature and pH conditions at which the foodstuffs are processed. With the demand for efficient and reliable enzyme production in food technology, the increase in the information obtained about molecular biology, recombinant DNA technology and cloning makes it possible to produce enzymes at fast, high efficiency and industrial scale (Çerçi et al., 2011; Boyacioglu et al., 2012).

Production of recombinant enzymes; development of the host strain, structuring of the expression vector, transformation of the host strain, determination of the best recombinant strain, additional improvements and characterization of the production strain can be divided into stages (Yeşilçubuk, 2012). At this stage, it is necessary to pay attention to the formation of conditions suitable for the structure and properties of the host organism. In addition, the pathogenic and toxigenic potentials of host microorganisms are important.

The basic logic in rDNA technology is the use of microorganisms that are generally regarded as harmless (GRAS) as hosts, and the use of techniques to transfer genes to encode the desired enzymes and convert them into gene products. (Koksal et al., 1989).

Productivity differences are observed in the enzymes originating from microorganisms depending on the temperature conditions in which the microorganism can develop. In previous studies, it was stated that enzymes produced from microorganisms that can develop in cold (below 20 ° C) are more efficient in applications. In one study, the protease enzyme, which is active in the cold, was produced and this enzyme was used in the production of bioactive peptide. The optimization of fermentation conditions and the use of genetically modified yeast were carried out to increase enzyme production and yield higher. As a result, cold active enzyme production was carried out (Dikmen, 2018).

There are many types of enzymes developed and produced by the rDNA technique in the food sector and their number is increasing day by day.

Advances in Protein Engineering

Most of the enzymes isolated from the microorganism cannot be used directly as biocatalysts and the enzymes need to be adapted to the demands of the industry. Natural enzymes catalyze reactions under mild conditions (ie. low temperature, atmospheric pressure and neutral pH), but recent advances in protein engineering enable the production of economically important biocatalysts adapted to a range of non-physiological process conditions such as high temperatures, alkali or acidic environments (Uday et al., 2016).

There are three basic approaches in protein engineering. These are rational design, semi-rational design and guided evolution (random mutagenesis). With these three basic strategies, different properties of enzymes are changed or improved. The directed evolution method has a large database. With this feature, it can be referred to as library-based methods in the literature. Evolution, measured by millions of years, reduces the time scale to years, months, weeks and in this way mimics natural evolution. It allows screening of enzyme variations of the desired property. In this method, having a large information network can be a waste of time. By applying this method to *E. coli* b-glucuronidase gene, a mutant enzyme was obtained which catalyzed the hydrolysis of beta-galactoside 500 times better than wild type enzyme. The semi-rational approach is the method that is carried out partially or full three-dimensional structure and amino acid sequence information from smart library. Compared to the other method, it is advantageous because the library size is narrowed. The thermostability of lipase enzyme was increased from 48 ºC to 98 ºC with semi-rational design approach (Anonymous, 2013a, b). The rational approach in different protein engineering strategies is known to be the most effective method for producing enzymes with the desired reaction kinetics using bioinformatics tools. In the rational approach, amino acid sequence information, three-dimensional structure and function information of the protein is required. In this method, a single amino acid or sequence length change can be made (Bornscheuer et al., 2012; Anonymous, 2013a, b; Rehm et al., 2016; Mahmood et al., 2019). In a study, the 1,4-β-xylanase (Xyl-11A) endogen of *Bacillus halodurans* C-125 was cloned into the pET-22b (+) vector and expressed in the expression strain *Escherichia coli* BL21 (DE3). Xyl-11A showed good tolerance for high pH and temperature as well as good

properties for industrial applications. To enhance the thermal stability of Xyl-11A, the amino acids responsible for the thermostability of the enzyme that underwent bioinformatics were identified (Mahmood et al., 2018).

Optimization

Optimization is a necessary step in order to ensure high process and product quality and effective results in processes (Koç and Ertekin, 2009). Various methods are used to measure both the interaction of the independent variables of enzyme production processes and the effects of processing alone and studies are ongoing to develop these methods. The aim is to achieve the highest yield and lowest cost of enzyme production (Boyacioglu et al., 2012).

Conclusion

While there have been discussions about enzyme production on more techniques in the past, today this situation continues. But new searches have to be emerged for enzyme production for more reliable, more effective and more efficient results. These searches foresee the study of different branches of science together. The rapid advancement of technology enables the integration of the disciplines of the sciences with higher rates and higher quality products. The development of biotechnology in various fields has also diversified the production of enzymes by biotechnological means. Examples include genetics, molecular biology, nano-technology, and protein engineering. In enzyme production; different strategies are used based on the resources to be used for obtaining the highest yield, activity and amount of enzymes, development of the production environment and process. Recombinant DNA technology enables the production of enzymes with new properties, and these enzymes produced by nano-technology are easier to apply to the products. Protein engineering is one of the branches that can develop in this field by using bioinformatics tools to obtain enzymes having the desired reaction kinetics that can develop under extreme conditions. In light of all this information, it will be possible to produce artificial enzymes with high catalytic activity in the following years. In addition, the conversion of wastes into high-value-added food products or their use in these products has an important place for economic and ecological balance. The fact that microorganisms can easily develop on wastes supports the use of microorganisms in enzyme production.

Conflict and Interest

Authors declare no conflict and interest.

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