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3. Fungal Enzyme Technology and Application for Mankind

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3.1 Information:

Biotechnology offers an increasing potential for the production of goods to meet various human needs. Enzyme technology, a subfield of biotechnology have been developed to manufacture both bulk enzymes as biocatalysts and high value-added products using enzymes in order to meet global needs like foods (e.g. bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins, etc.) and pharmaceuticals. Enzymes are the biocatalysts synthesized by living cells. They are complex protein molecules that bring about chemical reactions concerned with life. It is fortunate that enzymes continue to function (bring out catalysis) when they are separated from the cells i.e. *in vitro*. Enzymes are necessary in all living systems to catalyze all chemical reactions required for their survival and reproduction rapidly, selectively, and efficiently. Enzymes can catalyze these reactions outside living systems or inside the living cells. These excellent properties of enzymes are utilized in enzyme technology. For example, they can be used as biocatalysts either as isolated enzymes or as enzyme systems in living cells to catalyze chemical reactions on an industrial scale in a sustainable manner. Basically, enzymes are nontoxic and biodegradable. They can be produced in large amounts by microorganisms for industrial applications. The use of industrially produced enzymes increases every day with the development of native and recombinant proteins in modern biotechnology.

Enzymes provide services in washing and environmental processes or for analytical and diagnostic purposes. The driving force in the development of enzyme technology is to developes new and better products, processes and services to meet human needs and/or the improvement of processes to produce existing products from new raw materials. The goal of these approaches is to design innovative products and processes that not only are competitive but also meet criteria of sustainability. The concept of sustainability was introduced by the World Commission on Environment and Development (WCED, 1987) with the aim to promote a necessary "*development that meets the needs of the present without compromising the ability of future generations to meet their own needs.*" This definition is now part of the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, which aims to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on biological diversity.

Cartagena Protocol on Biosafety to the Convention on Biological Diversity is defined as an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on January 29, 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on September 11, 2003.

Enzyme technology broadly involves production, isolation, purification and use of enzymes (in soluble or immobilized form) for the ultimate benefit of mankind. In addition, recombinant DNA technology and protein engineering involved in the production of more efficient and useful enzymes are also a part of enzyme technology.

3.2 Enzyme Application:

Microbial enzymes have been used for many centuries without knowing them fully. The first enzyme produced industrially was taka-diastase (a fungal amylase) in 1896 in United States. It was used as a pharmaceutical agent to cure digestive disorders. Enzymes have wide range of applications (Table-3.1). Their application covers the production of desired products for human needs like food production, food processing, preservation, animal feed, pharmaceuticals, bulk and fine chemicals, detergents, fibers for clothing, hygiene, textile manufacture, leather industry, paper industry, medical applications and environmental technology, in scientific research as well as for a wide range of analytical purposes, especially in diagnostics.

As per recent estimates, a great majority of industrially produced enzymes are useful in processes related to foods (45%), detergents (35%), textiles (10%) and leather contribute 3% (Raveendran *et al*., 2018). The utilization of enzymes (chiefly proteases) for laundry purposes started in 1915. However, it was not continued due to allergic reactions of impurities in enzymes. Now special techniques are available for manufacture and use of enzymes in washing powders (without allergic reactions). Commercial enzymes can be produced from a wide range of biological sources. At present, a great majority (80%) of them are from microbial sources. The different organisms and their relative contribution are for fungi (60%), bacteria (24%), yeast (4%), Streptomyces (2%) and higher animals (6%). Some well-known enzyme and enzyme sources are stated bellow.

a. **Unique organism for production of bulk enzymes:** *Aspergillus Niger* is a unique organism for production of bulk enzymes. Among the microorganisms, *Aspergillus Niger* occupies a special position for the manufacture of a large number of enzymes in good quantities. There are well over 40 commercial enzymes that are conveniently produced by *A. Niger*. These include a-amylase, cellulase, protease, lipase, pectinase, phase, catalase and insulinase (Singh, 2016).
b. **Recombinant enzymes:** Recombinant fungi are one of the main sources of enzymes for industrial applications. The industrial enzyme market reached $1.6 billion in 1998 for the following application areas (excluding diagnostic and therapeutic enzymes). A recombinant strain of *Aspergillus oryzae* producing an aspartic proteinase from Rhizomucor miehei has been approved by Food and Drug Administration (FDA) for cheese production.
c. **Lipases:** Lipases are extremely important in the detergent industry. They are extensively used in household detergents, industrial cleaners and leather processing, where they can

be combined with proteases, oxidases and peroxidases. To be suitable, lipases should be alkalophilic, able to work at temperatures above 45ºC and at pH values of about 10, and capable of functioning in the presence of the various components of wash-product formulations such as oxidants and surfactants. In 1994, Novo Nordisk introduced Lipolase, the first commercial recombinant lipase for use in a detergent by cloning the *Humicola lanuginose* lipase gene into the *A. oryzae* genome (Shintre *et al*., 2002).

d. Microbial Lipases: Microbial lipases have a huge potential in areas such as food technology, biomedical sciences and chemical industries since they are: (1) stable in organic solvents, (2) possess broad substrate specificity, (3) do not require cofactors and (4) exhibit high enantioselectivity. In the food industry, lipases are commonly used in the production of fruit juices, baked foods, desirable flavors in cheeses and interesterification of fats and oils to produce modified acylglycerols (Bharathi and Rajalakshmi, 2019). There are three fungal recombinant lipases currently used in the food industry, *Rhizomucor miehi, Thermomyces lanuginosus* and *Fusarium oxysporum*, all of which are produced in *A. oryzae*.

Table 3.1: Enzymes from Fungal Sources and Application

Enzyme	Source	Application
Lactase	*Saccharomyces fragilis*	Removal of lactose from whey
Lipase	*Aspergillus niger* *Candida lipolytica*	Flavour production Preparation of cheese
Pectinase	*Aspergillus sp*	Clarification of fruit juice and wines
Takadiastase	*Aspergillus oryzae*	Supplement of bread, digestive acid
Protease alkaline	*Aspergillus oryzae*	Meat tenderiser
Protease acid	*Aspergillus niger*	Digestive acid
α -Amylase	*Aspergillus oryzae* *Aspergillus niger*	Beer and alcohol production Glucose syrups
Amyloglucosidase	*Aspergillus niger* *Rhizopus niveus*	Starch hydrolyse
Cellulase	*Aspergillus niger* *Tricoderma koningi*	Alcohol and glucose production
Glucoamylase	*Aspergillus nige*	Beer and alcohol production
Glucose oxidase	*Aspergillus niger*	Antioxidant in prepared food
Invertase	*Saccharomyces cerevisiae*	Sucrose inversion, Preparation of artificial honey and confectioneries

3.3 Enzyme Production Technology:

In general, the techniques employed for microbial production of enzymes is comparable to the methods used for manufacture of other industrial products. The major steps are briefly discussed (Figure 3.1). There are 4 major steps- selection of organisms, formulation of medium, production process & recovery and purification of enzymes.

3.3.1 Selection of Organism:

The most important criteria for selecting the microorganism are that the organism should produce the maximum quantities of desired enzyme in a short time while the amounts of other metabolite produced are minimal. Once the organism is selected, strain improvement for optimising the enzyme production can be done by appropriate methods (mutagens, UV rays). From the derised microorganism, inoculum can be prepared in a liquid medium.

3.3.2 Formulation of Medium:

The culture medium chosen should contain all the nutrients to support adequate growth of microorganisms that will ultimately result in good quantities of enzyme production. The ingredients of the medium should be readily available at low cost and are nutritionally safe. Some of the commonly used substrates for the medium are starch hydrolysate, molasses, corn steep liquor, yeast extract, whey and soy bean meal. Some cereals (wheat) and pulses (peanut) have also been used. The pH of the medium should be kept optimal for good microbial growth and enzyme production. Formulation of medium with different ingredients is important for microbial growth and enzyme production.

3.3.3 Production Process:

Industrial production of enzymes is mostly carried out by submerged liquid conditions and to a lesser extent by solid-substrate fermentation. However, solid substrate fermentation is historically important and still in use for the production of fungal enzymes e.g. amylases, cellulases, proteases and pectinases. The medium can be sterilized by employing batch or continuous sterilization techniques. The fermentation is started by inoculating the medium with desirable microorganism. The growth conditions (pH, temperature, O_2 supply, nutrient addition) are maintained at optimal levels. The bioreactor system must be maintained sterile throughout the fermentation process. The duration of fermentation is variable around 2-7 days, in most production processes. Besides the desired enzyme(s), several other metabolites are also produced. The enzyme(s) have to be recovered and purified.

3.3.4 Recovery and Purification of Enzymes:

The desired enzyme produced may be excreted into the culture medium (extracellular enzymes) or may be present within the cells (intracellular enzymes). Depending on the requirement, the commercial enzyme may be crude or highly purified. Further, it may be in the solid or liquid form. The steps involved in downstream processing i.e. recovery and purification steps employed will depend on the nature of the enzyme and the degree of purity desired. In general, recovery of an extracellular enzyme which is present in the broth is relatively simpler compared to an intracellular enzyme.

For the release of intracellular enzymes, special techniques are needed for cell disruption by physical means (sonication, high pressure, glass beads). The cell walls of bacteria can be lysed by the enzyme lysozyme. For yeasts, the enzyme β-glucanase is used. However, enzymatic methods are expensive. The recovery and purification steps will be the same for both intracellular and extracellular enzymes, once the cells are disrupted and intracellular enzymes are released. The most important consideration is to minimise the loss of desired enzyme activity. The major focus is to be given in the following steps.

- **Removal of cell debris:** Filtration or centrifugation can be used to remove cell debris.
- **Removal of nucleic acids:** Nucleic acids interfere with the recovery and purification of enzymes. They can be precipitated and removed by adding poly-cations such as polyamines, streptomycin and polyethylene mine.
- **Enzyme precipitation:** Enzymes can be precipitated by using salts (ammonium sulfate) organic solvents (isopropanol, ethanol, and acetone). Precipitation is advantageous since the precipitated enzyme can be dissolved in a minimal volume to concentrate the enzyme.
- **Liquid-liquid partition:** Further concentration of desired enzymes can be achieved by liquid-liquid extraction using polyethylene glycol or polyamines.
- **Separation by chromatography:** There are several chromatographic techniques for separation and purification of enzymes. These include ion-exchange, size exclusion, affinity, hydrophobic interaction and dye ligand chromatography .Among these, ion-exchange chromatography is the most commonly used for enzyme purification.
- **Drying and packing:** The concentrated form of the enzyme can be obtained by drying. This can be done by film evaporators or freeze dryers (lyophilizers). The dried enzyme can be packed and marketed. For certain enzymes, stability can be achieved by keeping them in ammonium sulfate suspensions.
- All the enzymes used in foods or medical treatments must be of high grade purity, and must meet the required specifications by the regulatory bodies. These enzymes should be totally free from toxic materials, harmful microorganisms and should not cause allergic reactions.

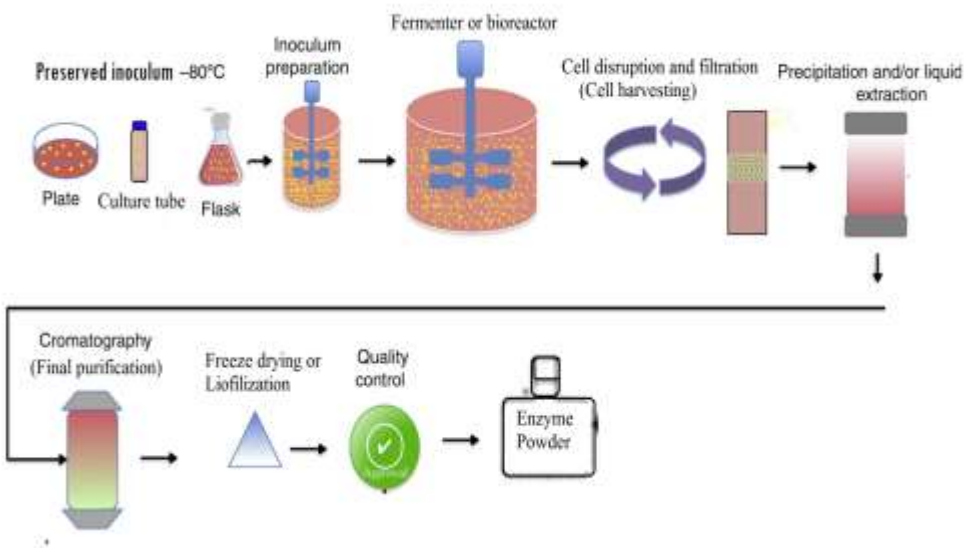

Figure 3.1: An Outline of the Flow Chart for Enzyme Production by Microorganisms

3.4 Immobilized Enzyme:

An immobilized enzyme is an enzyme attached to an inert, insoluble material such as calcium alginate (produced by reacting with a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature.

It also lets enzymes be held in place throughout the reaction, following which they are easily separated from the products and may be used again. An alternative to enzyme immobilization is whole cell immobilization.

Immobilized enzymes are very important for commercial uses as they possess many benefits.

3.4.1 Enzyme Immobilization Process:

There are various ways by which one can immobilize an enzyme. Various methods used for immobilization of enzymes are adsorption, covalent binding, and entrapment, affinity-tag binding and cross-linkage.

a. **Affinity-tag binding:** Enzymes may be immobilized to a surface e.g. in a porous material using non-covalent or covalent protein tags. This technology has been established for protein purification purposes.
b. **Adsorption on glass, alginate beads or matrix:** Enzyme is attached to the outside of an inert material. In general, this method is not suitable as the active site of the immobilized enzyme may be blocked by the matrix or bead greatly reducing the activity of the enzyme.
c. **Cross-linkage:** Enzyme molecules are covalently bonded to each other to create a matrix consisting of almost only enzyme. The reaction ensures that the binding site does not cover the enzyme's active site.
d. **Covalent bond:** The enzyme is bound covalentely to an insoluble support (such as silica gel or macroporous polymer beads with epoxide groups). This approach provides the strongest enzyme/support interaction and reduces lowest protein leakage during catalysis.
e. **Entrapment:** The enzyme is trapped in insoluble beads or microspheres. Various types of gels are used for entrapment of enzymes. The enzyme may be entrapped within polymeric mesh such as agar, polyacrylamide gel or calcium alginate by carrying out the polymerization reaction and /or cross-linking reaction in the presence of enzyme.
f. **Entrapment in polyacrylamide:** Polyacrylamide gel is the most commonly used material for entrapment. For the preparation of 15% gel, 7.5 g acrylamide, 0.5 g bisacrylamide, 50 mg ammonium per sulfate was added to 25 ml of phosphate buffer, pH 6.8 and mixed to dissolve these solids. Then 25 ml of amylase solution added. Mixed properly and added 50 µl of TEMED. Mixed gently and poured into glass Petri dishes or gel casting vertical electrophoresis unit in order to get the gel of uniform and desired thickness. Polymerization was done at room temperature for 1h. The gel was cut into small pieces and suspended in 0.1M phosphate buffer till further use.

Entrapment in calcium alginate gel -For calcium alginate beads, 25ml of 0.5, 1, 2, 3 and 4% solutions of sodium alginate were prepared and mixed with equal volume of amylase solution to get the final concentration of sodium alginate 0.25, 0.5, 1, 1.5 and 2%, respectively.

Entrapment of enzyme in calcium alginate gel was done by modifying the method of Kierstan and Bucke. Different $CaCl_2$ concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 M) were used to optimize the best concentration. For 50ml sodium alginate-enzyme mixture, 500 ml of $CaCl_2$ solution was used.

3.5 Immobilized Whole Cell:

Immobilized whole cell system is an alternative to enzyme immobilization. Unlike enzyme immobilization, where the enzyme is attached to a solid support (such as calcium alginate) in immobilized whole cell systems, the target cell is immobilized. Such methods may be implemented when the enzymes required are difficult or expensive to extract, an example being intracellular enzymes. Also, if a series of enzymes are required in the reaction; whole cell immobilization may be used for convenience. This is only done on a commercial basis when the need for the product is more justified.

3.6 References:

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