**Media for enzyme production**

Detailed description of the development and use of fermenters for the large-scale cultivation of microorganisms for enzyme production is outside the scope of this volume but mention of media use is appropriate because this has a bearing on the cost of the enzyme and because media components often find their way into commercial enzyme preparations. Details of components used in industrial scale fermentation broths for enzyme production are not readily obtained. This is not unexpected as manufacturers have no wish to reveal information that may be of technical or commercial value to their competitors. Also some components of media may be changed from batch to batch as availability and cost of, for instance, carbohydrate feedstocks change. Such changes reveal themselves in often quite profound differences in appearance from batch to batch of a single enzyme from a single producer. The effects of changing feedstocks must be considered in relation to downstream processing. If such variability is likely to significantly reduce the efficiency of the standard methodology, it may be economical to use a more expensive defined medium of easily reproducible composition.

Clearly defined media are usually out of the question for large scale use on cost grounds but may be perfectly acceptable when enzymes are to be produced for high value uses, such as analysis or medical therapy where very pure preparations are essential. Less-defined complex media are composed of ingredients selected on the basis of cost and availability as well as composition. Waste materials and by-products from the food and agricultural industries are often major ingredients. Thus molasses, corn steep liquor, distillers solubles and wheat bran are important components of fermentation media providing carbohydrate, minerals, nitrogen and some vitamins. Extra carbohydrate is usually supplied as starch, sometimes refined but often simply as ground cereal grains. Soybean meal and ammonium salts are frequently used sources of additional nitrogen. Most of these materials will vary in quality and composition from batch to batch causing changes in enzyme productivity.

**METHODS OF ENZYME PRODUCTION**

Submerged fermentations (SmF) and solid-state fermentations (SSF) are the two methods widely employed for the production of microbial Enzymes.

**Submerged fermentation:**

SmF is a traditional method for enzyme production from microorganisms which has been used for a longer period of time. In SmF, free-flowing liquid substrates like molasses and broths are used. The end products of the fermentation are liberated into the fermentation broth. Substrate utilization is very rapid in SmF; henceforth, substrate must be provided continuously for this fermentation process. This technique is well suited for the extraction of secondary metabolites from bacteria because it requires high moisture content for their growth. SmF has several advantages in which genetically modified organisms are grown well compared to SSF and media sterilization, purification, and recovery of the end products. Further, the control of process parameters such as pH, temperature, moisture, oxygen transfer, and aeration can be done easily.

 **Solid state fermentation:**

Solid-state fermentation (SSF) is suitable for the less moisture content required microorganisms. In SSF, nutrient rich waste materials such as bran, bagasse, and paper pulp can be used as substrate for the microorganisms and they are consumed very slowly and constantly. Hence, there is no need to supply the substrate for longer time. Major advantages of SSF are easy to handle, recovery of higher concentration of products, and generation of lesser effluent. Therefore, SSF is considered as a promising method for commercial enzyme production. α -Amylase production by SmF and solid-state fermentation techniques has been examined for fungal species. The results showed that SSF was well suited for developing countries due to cost-effective production process.



**COMMERCIAL PRODUCTION OF ENZYMES**

Use of an aerobic submerged culture in a stirred-tank reactor is the typical industrial process for enzymeproduction involving a microorganism that produces mostly an extracellular enzyme.

**Organism and enzyme synthesis:**

A variety of different microorganisms are used for the industrial production of enzymes. They cover thetaxonomic gamut from eukaryotic systems such as yeasts and fungi to prokaryotic systems from boththe gram-negative and gram-positive families. When biopharmaceutical enzymes are considered as well,mammalian and insect cell lines also come into play. For most of the history of enzyme applications,production occurred in the strain known to make the enzyme of interest. This explains why so manydifferent types of microorganisms have been employed to make enzymes.

**Examples:**

* Bacillus licheniform produces naturally Alkaline protease and a-amylase.
* Aspergillus produces glucoamylase
* Species of Steptomyces produce glucose/xylose isomerase.

An organism can be viewed as a metabolic system converting substrates into cell mass and byproducts.Enzymes function in this system as catalysts for the different reactions. Each cell is equipped withmechanisms that regulate the synthesis and activity of the enzymes to enable the cell to respondadequately to environmental changes. Therefore, in its elementary form, an organism can be describedas a set of metabolic components with a mechanism for enzyme synthesis and a regulatory apparatus.The kinetics of the process is determined by structural components of the organism and by variousphysical and chemical factors. The key role that enzymes play in biological processes has led to anextensive study of both the mechanism and the regulation of their synthesis and some of the results arerelated to the problem of process development.

**Strain Improvement:**

Most of the strains used for enzyme production have been improved through classical selection. Mutagenesis by chemical agents or UV radiation has been used to more quickly find useful variants. Many cells must be subjected to a mutation or recombination procedure and then tested for the desired combination of characteristics by selection. The success of strain improvement programs often depends on development of an effective selection method for finding one mutant among 10 000**–**100 000 cells. Methods range from plate selection to the continuous culture technique. Mutation changes the protein structure and most probably results in a deterioration of function. Changes in structural components by mutation are therefore rarely improvements unless the specific loss of function is required for production purposes, e.g., when a loss of regulatory function results in enhanced enzyme production.

Mutation and selection are directed primarily toward higher overall productivity rather than mutation of a specific function, but a loss of regulatory function is highly probable. However, some studies describe screening for a mutation in a specific function. Strain improvement has been revolutionized by the advent of genetic engineering. The ability to specifically improve strains by changing the host genome or the addition of extra-chromosomal DNA elements has greatly increased the speed of strain improvement. Genetic engineering also uses microorganisms to produce enzymes of higher organisms by placement of the corresponding gene into the microorganism. Novel methods to improve strain performance are under development. These include gene shuffling and directed evolution.

**Fermentation:**

Once the organism is selected and improved. Next step is growth of that organism to produce desired enzyme which is done by fermentation. There are two methods of fermentation used to produce enzymes. These are submerged fermentation and solid-state fermentation.

1. **Submerged Fermentation:**

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution.

Due to the development of large-scale fermentation technologies, the production of microbial enzymes accounts for a significant proportion of the biotechnology industry’s total output. Fermentation takes place in large vessels (fermentor) with volumes of up to 1,000 cubic meters.

The fermentation media sterilizes nutrients based on renewable raw materials like maize, sugars and soya. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources.

Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilized nutrients are added to the fermentor during the growth of the biomass. In the continuous process, sterilized liquid nutrients are fed into the fermentor at the same flow rate as the fermentation broth leaving the system. This will achieve a steady-state production. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimize the fermentation process.

Firstly, in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation. As most industrial enzymes are extracellular (secreted by cells into the external environment), they remain in the fermented broth after the biomass has been removed. The biomass can be recycled as a fertilizer, but first it must be treated with lime to inactivate the microorganisms and stabilize it during storage.

The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization depending on their intended application. If pure enzyme preparations are required, they are usually isolated by gel or ion exchange chromatography. Certain applications require solid enzyme products, so the crude powder enzymes are made into granules to make them more convenient to use. Sometimes liquid formulations are preferred because they are easier to handle and dose along with other liquid ingredients. Enzymes used in starch conversion to convert glucose into fructose are immobilized, typically on the surfaces of inert granules held in reaction columns or towers. This is carried out to prolong their working life as these enzymes normally go on working for over a year.

**Advantages:**

* Measure of process parameters is easier than with solid-state fermentation.
* Bacterial and yeast cells are evenly distributed throughout the medium.
* There is a high water content which is ideal for bacteria.

**Disadvantages:**

* High costs due to the expensive media
* Large reactors are needed and the behavior of the organism cannot be predicted at times.
* There is also a risk of contamination.

 **Solid State or Surface Fermentation**

Solid-state fermentation (SSF) is another method used for the production of enzymes. Solid-state fermentation involves the cultivation of microorganisms on a solid substrate, such as grains, rice and wheat bran. This method is an alternative to the production of enzymes in liquid by submerged fermentation. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment.

There are many substrates that can be utilized for the production of enzymes by SSF. These include wheat bran, rice bran, sugar beet pulp and wheat and corn flour. The selection of substrate depends on many factors, which is mainly related to the cost and the availability of the substrate. Other factors include particle size and the level of moisture. Smaller substrate particles have a larger surface area for the proliferation of the microorganisms, but if too small the efficiency of respiration will be impeded and poor growth and hence poor production of enzymes will result. Larger particles provide more efficient aeration and respiration, but there is a reduction in the surface area. A compromise must be reached, regarding the particle size of the substrate for a particular process. SSF requires moisture to be present on the substrate, for the microorganisms to produce enzymes. As a consequence the water content of the substrate must also be optimized, as a higher or lower presence of water may adversely affect the microbial activity. Water also has implications for the physicochemical properties of the solid substrate. Enzymes of industrial importance have been produced by SSF. Some examples are proteases, pectinases, glucoamylases and cellulases.

**IMPORTANT MICROBIAL ENZYMES**

The extensive application of microbes in different bioprocess is used to deliver a variety of products in applied industries. Table [2](https://link.springer.com/article/10.1007/s13205-016-0485-8#Tab2) summarizes several applications of microorganisms to deliver a variety of products. The Schematic representation of industrial production of microbial enzymes has been shown in

Fig as below:

| **Industry** | **Enzyme** | **Function** | **Microorganisms** |
| --- | --- | --- | --- |
| Dairy | Acid proteinase | Milk coagulation | *Aspergillus* sp. |
| Neutral proteinase | Faster cheese ripening, debittering | *Bacillus subtilis, A. oryzae* |
| Lipase | Faster cheese ripening, flavor customized cheese, | *Aspergillus niger, A. oryzae* |
| Lactase (β-galactosidase) | Lactose reduced milk and whey products | *Escherichia coli, Kluyveromyces*sp. |
| Aminopeptidase | Faster cheese ripening | *Lactobacillus* sp. |
| catalase | Cheese processing | *Aspergillus niger* |
| Transglutaminase | Protein cross linking | *Streptomyces* sp. |
| Baking | Amylase | Flour adjustment, bread softness | *Aspergillus* sp*., Bacillus* sp. |
| Maltogenicα-Amylase | Enhance shelf life of breads | *Bacillus stearothermophilus* |
| Xylanase | Dough conditioning | *Aspergillus niger* |
| Lipase | Dough stability and conditioning | *Aspergillus niger* |
| Glucose oxidase | Dough strengthening | *Aspergillus niger, Penicillium chrysogenum* |
| Transglutaminase | Laminated dough strength | *Streptoverticillium* sp., *streptomyces* sp. |
| Beverage | Pectinase | Depectinization | *Aspergillus oryzae, Penicillium funiculosum* |
| Glucose oxidase | Oxygen removal from beer | *Aspergillus niger* |
| Cellulase | Fruit liquefaction | *Aspergillus niger, Trichoderma atroviride* |
| α-Amylase | Starch hydrolysis | *Bacillus, Aspergillus* |
| β-Amylase | Starch hydrolysis | *Bacillus, Streptomyces, Rhizopus* |
| β-Glucanase | Restrict haze formation | *Bacillus subtilis, Aspergillus*spp. |
| protease | Restrict haze formation | *Aspergillus niger* |
| Pullulanase | Starch saccharification | *Bacillus* sp*., Klebsiella* sp. |
| Naringinase | Debittering | *Aspergillus niger* |
| limoninase | Debittering | *Aspergillus niger, A. oryzae* |
| Aminopeptidases | Protein breakdown during mashing | *Lactobacillus brevis, L. plantarum* |
| Animal feed | Phytase | Hydrolyze phytic acid to release phosphorous | *Aspergillus niger* |
| Xylanase | Enhanced digestibility of starch | *Aspergillus* sp*., Bacillus* sp. |
| β-glucanase | Digestive aid | *Aspergillus niger* |
| Pulp and paper | Lipase | Pitch control | *Candida Antarctica* |
| Protease | Biofilm removal | *Bacillus subtilis* |
| Amylase | Deinking, drainage improvement | *Bacillus licheniformis* |
| Xylanase | Bleach boosting | *Trichoderma reesei, Thermomyces lanuginosus, Aureobasidium pullulans* |
| Laccase | Non-chlorine bleaching, delignification | *Bacillus subtilis* |
| Cellulase | Deinking, drainage improvement | *Bacillus* sp*., Aspergillus niger* |
| Polymer | Lipase | Polycondensation, ring-opening polymerization of lactones, carbonates | *Candida Antarctica* |
| Laccase | Polymerization of bisphenol A | *Trametes hirsuta* |
| Glucose oxidase | Polymerization of anilines | *Aspergillus niger, Penicillium chrysogenum* |
| Transglutaminase | Crosslinking of protein | *Streptomyces mobaraensis* |
| Tyrosinase | Polymerization of lignin and chitosan | *Trichoderma reesei* |
| Detergent | Amylase | Carbohydrate stain removal | *Aspergillus* sp*., Bacillus subtilis* |
| Lipase | Fat stain elimination | *Aspergillus oryzae, A. flavus,* |
| Protease | Protein stain removal | *Aspergillus oryzae, Bacillus subtilis* |
| Cellulase | Color clarification | *Aspergillus niger, Bacillus* sp. |
| Cutinase | Triglyceride removal | *Fusarium solani f. pisi* |
| Mannanase | Mannan spot removal | *Bacillus* sp. |
| Leather | Alkaline protease | Dehairing, bating | *Alcaligenes faecalis* |
| Neutral Protease | Dehairing, soaking | *Aspergillus niger, A. flavus, Bacillus subtilis* |
| Lipase | Degreasing | *Aspergillus oryzae, A. flavus,* |
| Amylase | Fiber splitting | *Aspergillus* sp*., Bacillus subtilis* |
| Cosmetics | Superoxide dismutase | Free radical scavenging, skin care | *Corynebacterium**Glutamicum, Lactobacillus plantarum* |
| Protease | Removal of dead skin | *Aspergillus niger, A. flavus, Bacillus subtilis* |
| Endoglycosidase | Teeth and gum tissue care | *Mucor hiemalis* |
| laccase | Hair dye | *Bacillus subtilis, Trametes versicolor* |
| lipase | Skin care | *Aspergillus oryzae, A. flavus* |
| Organic synthesis | Lipase | Synthesis of pharmaceuticals, polymers, biodiesels, biosurfactants | *Aspergillus oryzae, A. flavus* |
| Glycosyl tranferase | Synthesis of oligosaccharides | *Bacillus* sp. |
| Nitrile hydratase | Synthesis of acrylamide, butyramide, nicotinamide | *Rhodococcus rhodochrous* PA-34*, Bacillus* sp*. APB*-*6* |
| Glucose isomerase | Production of High fructose corn syrup | *Corynebacterium* sp*., streptomyces murinus* |
| Acyltransferase | Synthesis of hydroxamic acids | *Bacillus* sp. APB-6 |
| Laccase | Production of textile dyes, cosmetic pigments, flavor agents, and pesticides | *Trametes versicolor, Bacillus subtilis* |
| Waste management | Amidase | Degradation of nitriles containing wastes | *Rhodococcus erythropolis* |
| Amylase | Bioremediation of vegetables wastes | *B. licheniformis, Aspergillus* sp. |
| Amyloglucosidase | Starch hydrolysis for bioremediation | *Aspergillus niger* |
| Lipase | Degradation of crude oil hydrocarbons | *Aspergillus oryzae, Candida tropicalis* |
| Nitrile hydratase | Degradation of nitriles containing wastes | *Rhodococcus* sp. |
| Protease | Bioremediation of keratinic wastes | *Chrysosporium keratinophilum* |
| Laccase | Degradation of waste containing olefin unit, polyurethane and phenolic compounds | *Trametes versicolor* |
| Cutinase | Degradation of plastics, Polycaprolactone | *Fusarium solani f. pisi* |
| Manganese peroxidase | Degradation of phenolic compounds | *Phanerochaete chrysosporium, Coprinus cinereus* |
| Lignin peroxidase | Degradation of phenolic compounds | *Phanerochaete chrysosporium, Coprinus cinereus* |
| Oxygenase | Degradation of halogenated contaminants | *Pseudomonas* sp*., Rhodococcus*sp. |

**IMMOBILIZATION OF ENZYMES**

As enzymes are biological catalysts that promote the rate of reactions but are not themselves consumed in the reactions; they may be used repeatedly for as long as they remain active. However, in most of the processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the reaction and are generally wasted. Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor for further catalysis. This is done by Enzyme immobilization which may be defined as-

                   “The process whereby the movement of enzymes, cells, organelles, etc. in space is completely or severely restricted usually resulting in a water-insoluble form of the enzyme.”

Immobilized enzymes are also sometimes referred to as sound, insolubilized, supported or matrix-linked enzymes.

**SALIENT FEATURES OF ENZYME IMMOBILIZATION:-**

The enzyme phase is called as carrier phase which is water insoluble but hydrophilic porous polymeric matrix, e.g. agarose, cellulose, etc.

* The enzyme phase may be in the form of fine particulate, membranous, or microcapsule.
* The enzyme in turn may be bound to another enzyme via cross linking.
* A special module is produced employing immobilization techniques through which fluid can pass easily, transforming substrate into product and at the same time facilitating the easy removal of catalyst from the product as it leaves the reactor.
* The support or carrier utilized in immobilization technique is not stable at particular pH, ionic strength, or solvent conditions. Hence, may be disrupted or dissolved releasing the enzyme component after the reaction.

**Advantages of enzyme immobilization:-**

* Multiple or repetitive use of a single batch of enzymes.
* Immobilized enzymes are usually more stable.
* Ability to stop the reaction rapidly by removing the enzyme from the reaction solution.
* Product is not contaminated with the enzyme.
* Easy separation of the enzyme from the product.
* Allows development of a multienzyme reaction system.
* Reduces effluent disposal problems.

**Disadvantages of enzyme immobilization:-**

* It gives rise to an additional bearing on cost.
* It invariably affects the stability and activity of enzymes.
* The technique may not prove to be of any advantage when one of the substrate is found to be insoluble.
* Certain immobilization protocols offer serious problems with respect to the diffusion of the substrate to have an access to the enzyme**.**

**TECHNIQUE OF ENZYME IMMOBILIZATION:-**

**1.     Carrier binding.**

  Physical adsorption.

  Covalent bonding.

  Ionic bonding.

**2.     Cross linking.**

**3.     Entrapment.**

  Occlusion within a cross linked gel.

  Microencapsulation.

**PHYSICAL ADSORPTION:-**

This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Examples of suitable adsorbents are ion-exchangematrices, porous carbon, clay, hydrous metal oxides, glasses and polymeric aromatic resins**.**

The bond between the enzyme and carrier molecule may be ionic, covalent, hydrogen, coordinated covalent or even combination of any of these.

Immobilization can be brought about by coupling an enzyme either to external or internal surface of the carrier.

The external surface binding method is advantageous as it does not involve conditions like pore diffusion. The disadvantages, however, include exposure of enzymes to microbial attack, physical abrasion of enzyme due to turbulence associated with the bulk solution.

The major disadvantage of the internal immobilization method is the pore diffusion**.**

Advantages of adsorption:-

****Little or no confirmation change of the enzyme**.**

****Simple and cheap.

****No reagents are required**.**

****Wideapplicability and capable of high enzyme loading**.**

Disadvantages of adsorption:-

****Desorption of the enzyme protein resulting from changes in temperature, pH, and ionic strength**.**

****Slow method.

**Methods of immobilization by adsorption:-**

The absorptive immobilization of enzymes can be done by following methods:

1.     **Static Process:-** This is most efficient technique but requires maximum time. In this technique, enzyme is immobilized by allowing it to be in contact with the carrier without agitation.

2.     **Dynamic Process:-** This process typically involves the admixing of enzyme with the carrier under constant agitation using mechanical shaker.

3.     **Reactor loading:-** This process is employed for the commercial production of immobilized enzymes. The carrier is placed into the reactor and enzyme solution is transferred to the reactor with agitation of the whole content in the reactor.

**4.     Electro-Deposition:-** In this technique, carrier is placed in the vicinity of one of the electrode in an enzyme bath and electric current is applied leading to migration of enzyme towards the carrier. This results in deposition of enzyme on the surface of the carrier.

**Covalent bonding:-**

Covalent binding is the most widely used method for immobilizing enzymes. The covalent bond between enzyme and a support matrix forms a stable complex. Thefunctional group present on enzyme, through which a covalent bond with support could be established, should be non-essential for enzymatic activity.

The most common technique is to activate a cellulose-based support with cyanogen bromide, which is then mixed with the enzyme.

The protein functional groups which could be utilized in covalent coupling include:

* Amino group
* Carboxylic group
* Phenol ring
* Indole group
* Imidazole group

On the other hand examples of the polymeric supports include:

**·**Amino and related groups of polysaccharides and silica gel etc**.**

**·**Carboxylic acid and related groups of polyglutamic acid, carboxy methyl cellulose**.**

**·**Aldehyde and acetal groups of polymers**.**

**·**Amide gr. Of polypeptide.

The polymers may be engaged in direct coupling as well as could be modified by other coupling groups or activating groups. The most commonly used polymers are polysaccharides, polyvinyl alcohol, silica and porous glasses.

**Advantages of covalent coupling:-**

* The strength of binding is very strong, so,leakage of enzyme from the support is absent or very little.
* **T**his is a simple, mild and often successful method of wide applicability

**Disadvantages of covalent coupling:-**

* Enzymes arechemically modified and so many are denatured during immobilization.
* Only small amounts of enzymes may be immobilized (about 0.02 grams per gram of matrix).

**Cross linking:-**

This method is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents, leading to three dimensional cross linked aggregates.

The most common reagent used for cross-linking is glutaraldehyde.

**Advantages of cross linking:-**

* Very little desorption(enzyme strongly bound)
* Best used in conjunction with other methods.

**Disadvantages of cross linking:-**

* Cross linking may cause significant changes in the active site.

**Entrapment:-**

In entrapment, the enzymes or cells are not directly attached to the support surface, but simply trapped inside the polymer matrix. Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction.

Polymers like polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan etc are used as the matrices.

**Advantages of entrapment:-**

* Loss of enzyme activity upon immobilization is minimized.

**Disadvantages of entrapment:-**

* The enzyme can leak into the surrounding medium.
* Another problem is the mass transfer resistance to substrates and products.
* Substrate cannot diffuse deep into the gel matrix.

**1. Occlusion within a cross linked gel:-**

In this entrapment method, a highly cross-linked gel is formed as a result of the polymerization which has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cages.

Amounts in excess of 1 g of enzyme per gram of gel or fibre may be entrapped.

Some synthetic polymers such as *polyarylamide, polyvinylalcohol, etc...* and natural polymer (starch) have been used to immobilize enzymes using this technique.

**2. Microencapsulation:-**

This entrapment involves the formation of spherical particle called as “microcapsule” in which a liquid or suspension of biocatalyst is enclosed within a semi permeable polymeric membrane.