MICROBIAL ENZYMES - AMYLASES PRODUCTION

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α-Amylases (E.C.3.2.1.1) are enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose and maltotriose units.

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market .

Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry.

The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α-amylases.

The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics.

α-Amylase has been derived from several fungi, yeasts and bacteria. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors.

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF α-AMYLASE

The α-amylase (α-1,4-glucan-4-glucanohydrolase) can be found in microorganisms, plants and higher organisms. The α-amylase belongs to a family of endo-amylases that catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds.

Neither terminal glucose residues nor α-1,6-linkages can be cleaved by α-amylase . The end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins , which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6-8 glucose units that contain both α-1,4 and α-1,6 linkages .

Others amylolytic enzymes participate in the process of starch breakdown, but the contribution of α-amylase is the most important for the initiation of this process.

Starch is a polymer of glucose linked to another one through the glycosidic bond. Two types of glucose polymers are present in starch: amylose and amylopectin . Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units with α-1,4 glycosidic bonds.

Amylopectin consists of short α-1,4 linked to linear chains of 10-60 glucose units and α-1,6 linked to side chains with 15-45 glucose units. Granule bound starch synthase can elongate malto-oligosaccharides to form amylose and is considered to be responsible for the synthesis of this polymer. Soluble starch synthase is considered to be responsible for the synthesis of unit chains of amylopectin.

α-Amylase is able to cleave α-1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chain.

Starch is hydrolyzed into smaller oligosaccharides by α-amylase, which is one of the most important commercial enzyme processes.

Amylases find application in all the industrial processes such as in food, detergents, textiles and in paper industry, for the hydrolysis of starch .

Saccharide composition obtained after hydrolyze of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. Specificity, thermo-stability and pH response of the enzymes are critical properties for industrial use .

α-AMYLASE PRODUCTION

The production of α-amylase by <u>submerged fermentation</u> (SmF) and <u>solid state fermentation</u> (SSF) has been investigated and depend on a variety of physicochemical factors.

SmF has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture

solid state fermentation (SSF) systems appear <u>promising due to the natural potential</u> and advantages they offer. SSF resembles the natural habitat of microorganism and is, therefore, the preferred choice for microorganisms to grow and produce useful value added products.

SmF can be considered as a violation of their natural habitat, especially of fungi.

<u>Fungi and yeast</u> were termed as suitable microorganisms for SSF according to the theoretical concept of water activity, whereas bacteria have been considered unsuitable.

Bacterial cultures can be well managed and manipulated for SSF processes .There are others advantages of SSF over SmF, including <u>superior productivity, simpler technique, lower capital investment, lower energy requirement and less water output, better product recovery and lack of foam build up,</u> besides it is reported to be the most appropriate process for developing countries.

Recently, researches evaluated whether SSF is the best system for producing enzymes. They found that SSF is appropriate for the production of enzymes and other thermolabile products, especially when higher yields can be obtained when compared to SmF.

solid state fermentation

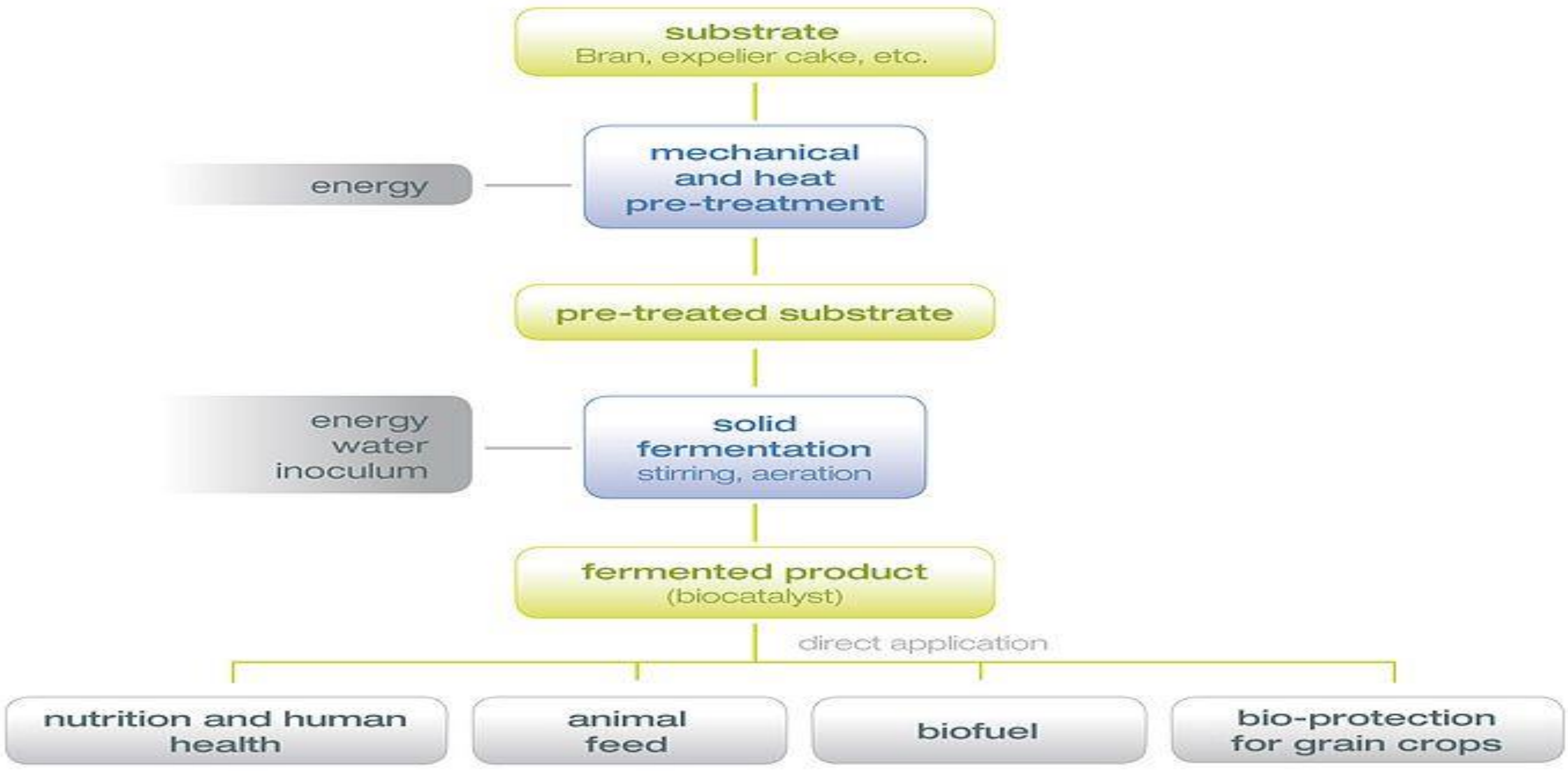

Fig. 1 Steps of Solid State Fermentation process to obtain enzyme from the fungus Aspergillus niger

The optimization of fermentation conditions, particularly physical and chemical parameters, are important in the development of fermentation processes due to their impact on the economy and practicability of the process.

The role of various factors, including pH, temperature, metal ions, carbon and nitrogen source, surface acting agents, phosphate and agitation have been studied for α-amylase production.

The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-independency must be matched to its application.

For example, α-amylases used in starch industry must be active and stable at low pH, but at high pH values in the detergent industry.

Most notable among these are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source .

The physical and chemical parameters of α-amylases from bacteria and fungi have been widely studied and described .

BACTERIAL AMYLASES

α-Amylase can be produced by different species of microorganisms, but for commercial applications α-amylase is mainly derived from the genus *Bacillus*.

α-Amylases produced from *Bacillus licheniformis* , *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries

Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability.

As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100-110ºC), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins .

***Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications**

Thermostable α-amylases have been reported from several bacterial strains and have been produced using SmF as well as SSF.

However, the use of SSF has been found to be more advantageous than SmF and allows a cheaper production of enzymes.

The production of α-amylase by SSF is limited to the genus *Bacillus* , and *B. subtilis* , *B. polymyxia* , *B. mesentericus* , *B. vulgarus*, *B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF.

Currently, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are being used in starch processing industries.

Enzymes produced by some halophilic microorganisms have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions.

In addition, most halobacterial enzymes are considerably thermotolerant and remain stable at room temperature over long periods.

Halophilic amylases have been characterized from halophilic bacteria such as *Chromohalobacter sp.* *Halobacillus sp.* , *Haloarcula hispanica* , *Halomonas meridiana* ,and *Bacillus dipsosauri* .

FUNGAL AMYLASES

Most reports about fungi that produce α-amylase have been limited to a few species of mesophilic fungi, and attempts have been made to specify the cultural conditions and to select superior strains of the fungus to produce on a commercial scale .

Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium*

The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry.

A. oryzae has received increased attention as a favourable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α-amylase .

***Aspergillus oryzae* has been largely used in the production of food such as soy sauce, organic acid such as citric and acetic acids and commercial enzymes including α-amylase.**

***Aspergillus niger* has important hydrolytic capacities in the α-amylase production and, due to its tolerance of acidity (pH < 3), it allows the avoidance of bacterial contamination.**

Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate.

The fungal α-amylases are preferred over other microbial sources due to their more accepted GRAS (Generally Recognized As Safe) status.

The **thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase**. Jensen and Kunamneni purified the α-amylase, proving its thermostability.

PURIFICATION OF α-AMYLASE

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations.

The commercial use of α-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases.

The enzyme in the purified form is also a prerequisite in studies of structure-function relationships and biochemical properties .

Different strategies for purification of enzymes have been investigated, exploiting specific characteristics of the target biomolecule.

Laboratory scale purification for α-amylase includes various combinations of ion exchange, gel filtration, hydrophobicity interactions and reverse phase chromatography.

Alternatively, α-amylase extraction protocols using organic solvents such as ethanol, acetone and ammonium sulfate precipitation and ultrafiltration have been proposed .

These conventional multi-step methods requires expensive equipments at each step, making them laborious, time consuming, barely reproducible and may result in increasing loss of the desired product.

However, liquid-liquid extractions consist of an interesting purification alternative since several features of the early processing steps can be combined into a single operation.

Liquid-liquid extraction is the transfer of certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other.

This process is widely employed in the chemical industry due to its simplicity, low costs, and ease of scale up. Purification of biomolecules using liquid-liquid extraction has been successfully carried out on a large scale for more than a decade.

Advantages of using this system are lower viscosity, lower cost of chemicals and shorter phase separation time. The dynamic behavior of these systems has to be investigated and understood to enhance plant-wide control of continuous liquid-liquid extraction and to assess safety and environmental risks at the earliest possible design stage.

INDUSTRIAL APPLICATION OF α-AMYLASE

Starch conversion

The most widespread applications of α-amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups.

The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis.

Initially, the α-amylase of *Bacillus amyloliquefaciens* was used but it has been replaced by the α-amylase of *Bacillus stearothermophilus* or *Bacillus licheniformis.*

The enzymes from the Bacillus species are of special interest for large-scale biotechnological processes due to their remarkable thermostability and because efficient expression systems are available for these enzymes.

Detergent industry

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe.

Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes .These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, dextrins and other smaller oligosaccharides .

Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing.

Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. Examples of amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus.*

Fuel alcohol production

Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world . In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars.

The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* .

The production of ethanol by yeast fermentation plays an important role in the economy of Brazil . In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* .

Among bacteria, α-amylase obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions .

Food industry

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups.

The α-amylases have been widely used in the baking industry. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins , which are subsequently fermented by the yeast.

The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product.

Moreover, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products.

Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry .

Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber.

Textile industry

Amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process.

Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry.

Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process.

The α-amylases remove selectively the size and do not attack the fibres .

Amylase from *Bacillus* stain was employed in textile industries for quite a long time.

Paper industry

The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch.

The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases in a batch or continuous processes.

Starch is a good sizing agent for the finishing of paper, improving the quality and erasebility, besides being a good coating for the paper. The size enhances the stiffness and strength in paper.

Examples of amylases obtained from microorganisms used in paper industry includes Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and α-amylase G9995® (Enzyme Biosystems, USA).

REFERENCE

Paula Monteiro de Souza; Pérola de Oliveira e Magalhães, Application of microbial α-amylase in industry - A review