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A comparative study on extraction, purification and characterization of amylase enzyme from *Aspergillus niger* and *Aspergillus awamori*

Swaroop G[1*], Vinod Kumar G[2], Ashwini Karakaraddi[3]

[1]*Research Scientist I, research and development, Apotex Pharmachem India Pvt Ltd, Bangalore*
[2]*BSc student, Bangalore University, Bangalore*
[3]*Head of the department, biotechnology, k.l.e's s. nijalingappa college, Bangalore*
**Corresponding author E-mail: simhavahini23@gmail.com*

Abstract

Amylase is an enzyme, which is produced by the microorganisms, which has many applications in today's market. Amylase is required in digestion of carbohydrates into smaller units and eventually converting them into even smaller units such as glucose. It is also involved in inflammatory reaction, such as those caused by the release of histamine and similar substances. A number of digestive enzymes including amylase are required to produce fructose in large quantities [1]. Objective of our research is to study the comparative production of amylase enzyme from both the fungal strains of *Aspergillus Niger* and *Aspergillus awamori* which are used as the digestive enzymes in baby foods. In this study we have investigated the level of amylase produced by the each *Aspergillus* strain, isolation and characterization will be discussed in detail.

Keywords: *Amylase enzyme, aspergillus Niger, aspergillus awamori, batch fermentation, dialysis, ion exchange chromatography, immobilization, starch hydrolysis, streptomycin, SDS-page.*

1 Introduction

Much of the history of biochemistry is the history of enzyme research. Biological catalysis was first recognized and described in the late 1700s, in studies on the digestion of meat by secretions of the stomach and research continued in the 1800s with examinations of the conversion of starch into sugar by saliva and various plant extracts.

Since Amylase is having so many industrial advantages, the study of these enzymes will be helpful in better development of human race. Hence we concentrated on extraction and purification of enzyme amylase produced by *Aspergillus Niger* and *Aspergillus awamori* by solid state and submerged state fermentation method, its characterization and application to develop a method for high production of amylase. [1]

Amylase belongs to group, which is called amylolytic enzymes. Amylolytic enzymes represent a group of catalytic proteins of great importance to food industry. They were also the one of the first enzymes to be produced commercially by microorganism. Amylase refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) sugar and starch. Amylase digests carbohydrates (polysaccharides) into smaller disaccharide units, eventually converting them into monosaccharide such as glucose. People who are fat intolerant (can't digest fats) often eat sugar and carbohydrates to make up for the lack of fat in their diet. If their diet is excessive in carbohydrates, they may develop an amylase deficiency [18].

The amylases constitute a large group of enzymes. They are characterized by their ability to hydrolyse 1, 4-glucosidic linkages in polysaccharides.A-amylases are exo-enzymes. They attack all linkages between glucose units in the starch molecules. The bond hydrolyzed is between carbon-1 and oxygen atom linked to adjacent glucose group. A-amylases vary in their effectiveness, depending on their source. The substrate for α -amylase is starch. Two types of microbial α-amylase have been recognized on termed "liquefying" and "saccharifying" α-amylases. The main difference between them is that the saccharifying enzyme produces a higher yield of reducing sugar than the liquefying enzymes. [3].

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units.

The maximum growth yields of Aspergillus Niger are high when compared with other organisms. The fungal mycelium synthesized and excretes high quantities of hydrolytic exoenzymes [19].

Starch molecules are glucose polymers linked together by the alpha, 1-4 and alpha 1-6 glycosidic bonds. These polymers exist in two basic components amylase (16-30%) and amylopectins (65-85%) Amylase is a polymer of

glucose inked by alpha, 1-4 bonds, mainly in linear chains. Amylopectin is a large highly branched polymer of glucose including alpha, 1-6 bonds at the branch points.

The effect of yeast extract for the synthesis of glucoamylase by *Aspergillus Niger* in solid state fermentation revealed a 20% increasing in enzyme secretion at 0.5% yeast extracts. Filamentous fungi isolated from cereals were screened for their ability to produce alpha-amylase. A selected strain identified as *Aspergillus flavus* showed high enzymatic activity. A single extra cellular alpha amylase was purified to homogeneity by a starch adsorption method. *Aspergillus flavus* enzyme was mainly glucose as well as unidentified oligosaccharides.

Yam peel was used as a carbon to produce extracellular amylase in shake flask cultures of a thermophilic strain if Aspergillus Niger.Peak amylase activity was obtained on the 4th day and 6th day fermentation, period which corresponded with the early stationary phase of the organism.

A new starch degrading enzyme activity is induced by storage of potato tubers at low temperatures. The cold induced activity was separated from other amylolytic activities in Zymograms based on iodine staining of PAGE gets containing Amylopectin. The cold induced enzyme was separated by 10th exchange chromatography from other amylolytic activities.

Aspergillus flavus and *Aspergillus niger* produce extracellular amylase into the culture medium when grown on basal medium containing 2% (WlV) soluble starch or cassava peel as a sole carbon source On soluble starch hydrolysed min/per mg. Protein for *A.flavus* and *A niger*, respectively it is concluded that cassava peal might be a better substrate for the production of amylase by *Aspergillus flavus* than commercial soluble starch.

Starch substrates constitute the major part of the human diet for most the people in the world, as well as many other animals. They synthesized naturally in a variety of plants. Some examples with high starch content are corn, potato, rice, sorghum, wheat and cassava. Enzymatic hydrolysis of starch is catalysed by alpha amylase and disbranching enzymes such pullulanase.

The amylase from different sources shows a wide variety of reaction properties such as kinetic parameters, pH optimum and substrate specifically.

Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market.

It is desirable that alpha gelatination (100-110ºC and liquefaction (80-90ºC) to economical processes, therefore there has been a head for more thermophilic and thermostable alpha amylases.

The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistries, as well as their wide spread application in starch saccharification and in the textile, food, fermentation, paper, brewing and distilling industries.

The alpha amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through an alpha 1-1, alpha 1-4 linkage, alpha-1-6 linkage glycosidic bonds members of this family share a number of common characteristic properties. Amylases can be divided into two categories exoamylases and endoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths.Exoamylases act from the non-reducing end successively resulting in short end product. [20].

2 Materials and methods

2.1 Collection of aspergillus strains

The protein rich soil sample with sandy texture and grey to black in color were collected from the basement of our lab in J.P. Nagar 2nd block, Bangalore and was also indented from Broad Spectrum Laboratory.

2.2 Agar plating technique

Though various methods are available to isolate and enumerate microorganisms from soil, serial dilution agar plating method or liable plate count method is one of the commonly used for the isolation and enumeration of microorganisms [17].

2.3 Potato dextrose agar media

Potato (20g) is boiled in a heating mantle and filtered in a muslin cloth and to the filtrate, Dextrose (2g) is added and pH is adjusted to 5.6, Agar (1.8g) is added and the volume is made up to 100 ml and a pinch of antibiotic (Streptomycin) is added. Sterile Petri plates, test tubes, pipettes, inoculation loop, Distilled water.

9.0ml of sterile water blanks and sterile Petri plates were labeled as 10-1, 10 -2, 10-3, 10-4, 10-5 1gm of finely pulverized, air-dried soil was added into (10-1) water blank to make 1: 10 dilution.
Vigorously the dilution was vortexed for 10 minutes to obtain uniform suspension of microorganisms. 1ml of suspension was transferred from 10-1 to 10-2 with a sterile pipette under aseptic conditions.
Further dilutions were made by pipetting 1.0ml of suspension into additional water blanks 10-3, 10-4, 10-5 as prepared above 0.1 ml aliquots each test tubes dilution was transferred to the Petri plates. Approximately 15.0ml of the cooled agar medium (45ºC) was poured into each Petri plate and the inoculums is spread by 'L' shaped rod by gentle rotation of the Petri plates.
Upon solidification of the media, the plates were incubated in an inverted position at 37ºC for 7-8 days. This plating was done for each sample [4]

2.4 Isolation of colonies from mixed culture

Perform the spread plates or the streak-plate inoculation procedure for the separation of cells of a mixed culture so that discrete colonies can be isolated.

2.5 Inoculation of aspergillus Niger and aspergillus awamori colonies

The spread-plate technique requires that a previously diluted mixture of microorganisms is used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent rod while the Petri dish is spun on a "Lazy-Susan" turntable [4].

2.6 Lacto phenol cotton blue staining of fungi

Lactophenol Cotton Blue Stain is formulated with Lactophenol which serves as a mounting fluid and cotton blue. *Aspergillus* strains suspended in the stain are killed due to the presence of phenol. The high concentration of the phenol deactivates lytic cellular enzymes thus the cells do not lyse. Cotton blue is an acid dye that stains the chitin present in the cell walls of fungi [6].

2.7 Starch hydrolysis for amylase enzyme activity

Starch hydrolysis test is for the production of extracellular amylase by inoculating *Aspergillus Niger* and *Aspergillus awamori* on starch agar medium.
Melt the starch agar medium and cool to 45ºC and pour into the sterile Petri dishes. Label each of the starch agar plate with the name of the *Aspergillus* species to be inoculated. Using sterile technique, make a single streak inoculation of the each *Aspergillus* species into the centre of its appropriately labeled plate.
Incubate the fungal inoculated plates for 72 – 96 hrs at 25ºC in an inverted position. Flood the surface of the plates with Iodine solution with a dropper for 30 seconds [13].

2.8 Mass production of amylase enzyme in starch broth media

Mass production of the enzyme can be done either by submerged fermentation or solid substrate fermentation. In submerged fermentation, the fungus is cultivated in liquid media in the flasks for the enzyme production where as in the solid substrate fermentation, the culture is inoculated across the surface of production medium and the culture remains on the surface throughout the fermentation.
The 96 hours old *Aspergillus Niger* and *Aspergillus awamori* is grown in Starch Broth medium for extracellular enzyme production. The fermentation using Starch broth medium is a liquid substrate in which the culture is inoculated over the surface of the medium. After inoculation, the flask is incubated at 28ºC in an orbital shaker at 220 rpm for 7-8 days [8].

2.9 Salt precipitation technique

The broth containing enzyme amylase is centrifuged for 5 minutes at 6000 rpm in 4ºC. The Supernatant is collected; then filtered through Whatman Filter paper No 1;
Volume is measured graduated cylinder. 80% salt cut is given to the supernatant, 80ml of supernatant is collected from the solid state fermentation system. To this supernatant 41.84 g of Ammonium Sulphate is added respectively. Ammonium Sulphate should be added very slowly with continuous stirring of the solution on a magnetic stirrer in cold condition. And the solutions are kept for overnight incubation.

The solutions are centrifuged at 8000 rpm for 10 minutes at 4ºC. The pellet is collected and dissolved in 10mM of Tris HCl. This solution contains the enzymes precipitate by Ammonium Sulphate. Precipitate is collected by centrifugation the extract at 8000 rpm for 10min at 4ºC. The precipitate is dissolved in 10ml of 10mM of Tris HCl and subjected to dialysis [14].

2.10 Dialysis of amylase enzyme

The Dialysis Bag is first processed to activate it. Dialysis bag of about 8 cm was boiled in 100ml of distilled water for 10 minutes. The bag is then boiled in 100ml of 2% Sodium Bicarbonate solution for 10 minutes. The bag was again boiled in 100ml distilled water for 10 minutes. Now the mouth of the bag was gently rubbed to open it. One end of the bag is tightly tied and the sample is loaded into it. After loading the sample the other end was also sealed. The dialysis bag was then suspended in a beaker containing distilled water and a magnetic bead and tied with the help of the glass rod and subjected on magnetic stirrer. The distilled water in a beaker is changed every half an hour for about three times. This setup was kept in refrigerator overnight [2, 13].

2.11 Purification of proteolytic enzymes by ion exchange chromatography

The chromatography column is packed with DEAE cellulose was washed using distilled water one or two times. The column was then washed with an Activation Buffer (10 ml of 25mM Tris HCl + 25mM NaCl). Then the column is filled with Elution buffers, firstly the chromatography column is washed with Elute 'A' (10 ml of 25mM Tris HCl + 25mM NaCl). The dialysed enzyme sample was poured into the column. The enzymes were then eluted using Elute 'B' (10ml of 25mM Tris HCl + 50mM NaCl).The Elutants were collected in the same test tubes. The process of elution is carried out using solutions C, D, E and F. This contains the different concentrations of NaCl [3].

2.12 Estimation of proteins by lowry's method

The blue colour developed by the reduction of the phosphomolybdate and phosphotungstic components in the Folin-Ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by biuret reaction of the amyloptic protein with the alkaline cupric tartrate are measured in the Lowry's method [6].

2.13 Assay of amylase enzyme by dns method

Add Starch 1.0 mL, Mix by swirling and equilibrate to 20ºC. Then α-Amylase Solution 1.0 mL was added. Mix by swirling and incubate exactly for 3 minutes at 20ºC.
Reagent E (Color Reagent Solution) Reagent G (Enzyme Solution) each of 1mL was adder and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and deionized water 9.0 mL was added.
Mix by inversion and record the absorbance 540 nm for both the test and blank using a suitable spectrophotometer [6].

2.14 Separation of proteins by SDS-page

The analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly the strongly anionic detergent Sodium Dodecyl Sulphate (SDS) is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is always is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS – polypeptide complexes migrate through polyacrylamide gels in accordance with the size of polypeptide. At saturation, approximately 1.4g of detergent is bound per gram of polypeptide. By using markers of known molecular weight of the polypeptide chains [17].

2.15 Immobilization of amylase enzyme

The reagent Calcium Chloride was poured in to the Petri dish. The amylase enzyme solution was mixed with the Sodium Alginate solution due to which the enzyme gets entrapped into the Alginate gel and cannot escape any permeation. Using a pipette, the enzyme entrapped in the gel was taken and released into the Calcium Chloride solution drop wise. Since the two phases or solutions are not miscible the enzyme in the gel formed bead-like round structures floating on top of the Calcium Chloride solution. To make the beads visible more clearly, dye was added [10].

3 Results

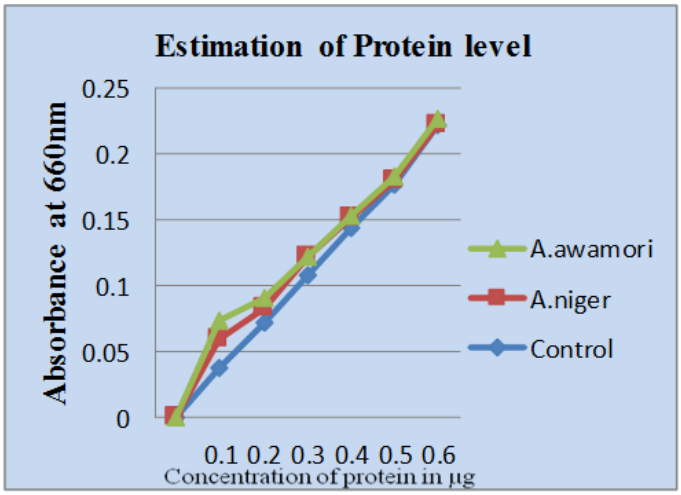

Fig. 1: Estimation of protein by lowry's method

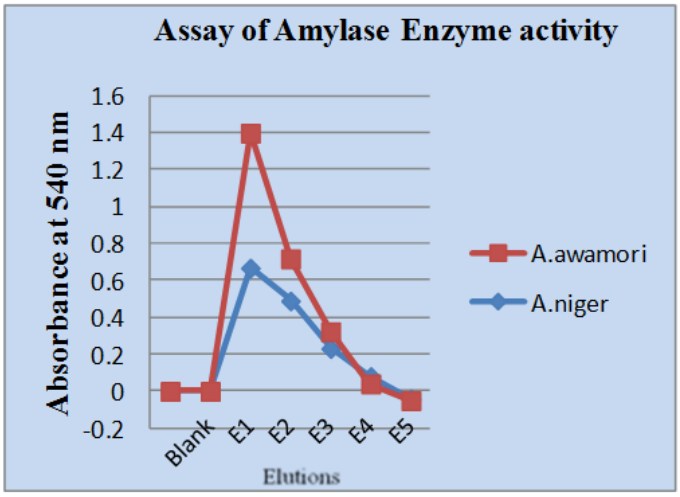

Fig. 2: Estimation of enzyme by DNS method

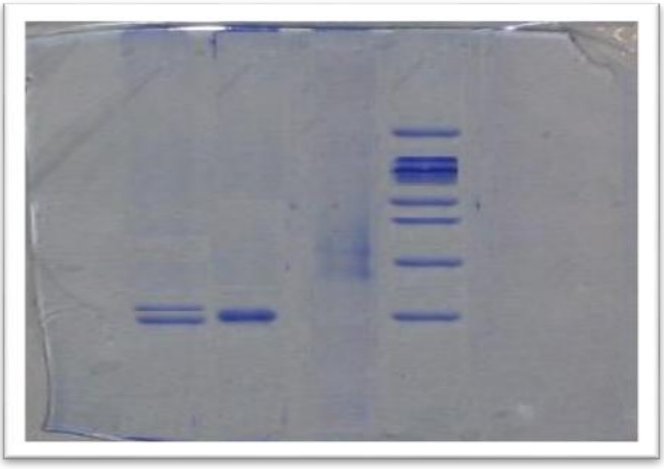

Fig. 3: Separation of proteins by SDS-PAGE

4 Discussion

Aspergillus strains were selected for our research as it is used for batch production of amylase enzyme. We have made an attempt to compare between the two strains *Aspergillus awamori* and *Aspergillus Niger*. These broths containing enzyme was used for various steps protein purification as mention using the chromatographic techniques. As per the standard methods of protein estimation and determination of Amylase enzyme activity and characterization of molecular weight by SDS-PAGE, our research studies revealed that *A.awamori* shows the maximum activity of amylase enzyme than the other stain *A.awamori*. The graph 1 shows the higher amylase production level in *A.awamori*. Graph 2 also proves the maximum amylase activity at the absorbance of 540 nm. Therefore *A.awamori* is a rich source of amylase which can be implemented for amylase production in food and enzyme industries.

5 Summary

Amylase is the unique class of enzymes, since they are the immense physiological as well as commercial importance. Studies were carried out to purify and characterize the amylases from *Aspergillus Niger* and *Aspergillus awamori* to improve the strain of amylase production. The fungal isolates were inoculated in various media for the mass production of amylases. The A. awamori broths that showed maximum amylase activity were chosen and purified to homogeneity by salt and solvent precipitation, Ion exchange chromatography and SDS-PAGE. Molecular weights of amylases were determined by SDS-PAGE. Finally the isolated enzymes were immobilized for the future usage. Immobilization of enzymes enables their efficient and continuous use. The rationale behind immobilization is the easy separation of product from the biocatalyst.

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