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Isolation and Screening of Enzymatic Hydrolysis of Starch by Enzyme Amylase from Soil Isolate Aspergillus Niger

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ABSTRACT:

Amylase has become the most promising enzyme in the industrial field due to its economical values and high catalytic rate. This class of enzyme constitutes approximately 25–30% of the enzyme stock around the world. It is commercially used for various purposes such as: hydrolysis of starch, resizing of textile fabrics, in pharmaceutical industries, in food industries, in paper industry, in detergent industry etc. α-Amylase can be produced by multitudunial sources i.e.plants, animals and microorganism but fungal amylase has become a new field of interest for the researchers.20 different soil samples were collected for the isolation of Aspergillus.niger. Selected strains from soil samples are subcultured on SDA agar and screened their ability for producing α- amylase by starch hydrolysis on a starch agar, clear sharp zones are observed from all the collected samples showing a clear hydrolysis of starch around the colonies. These fungal species are further processed for solid state fermentation in which wheat bran is used as a substrate from agro industrial wastes, resulted in high amount of production of enzyme amylase. Amylases hydrolyze complex molecules of starch into small oligosaccharides in step wise pattern. The determination of reducing sugar is done by Dinitrosalicylic acid method and observed by spectrophotometry at 575nm. A confirmatory starch-iodine test is also done, a drop of iodine is added in the tubes containing starch which turns the color blue but by adding raw enzyme that have been collected from the supernatant disappears the blue color which evident the production of enzyme α-amylase.

Keywords: *α- Amylase, Aspergillus .niger, starch hydrolysis, solid state fermentation (SSF), Dinitrosalicylic acid (DNS)*

INTRODUCTION

Enzymes are biological catalyst produced in biochemical reaction by living organisms during metabolisms. They are substrate specific (Abd-Elhalem *et al.*, 2015) The enzyme use in industries have high catalytic rate and need moderate temperature and pressure for its maximum activity (Kafilzadeh, and Dehdari, 2015). Microorganisms have been known with the variety of meaningful enzymes but type of enzyme produced depends on type of genera (Saranraj. 2014). Amylase has become a most promising enzyme in industrial field and in the field of biotechnology. This class of enzyme constitutes approximately 25–30% of the enzyme store around the world. (Saleem. *et al.*, 2014, Vijayaraghavan, *et al.*, 2015). In 1811 first time the starch degrading enzymes was discovered by Kichoff (Saranraj, Stella, 2013). Amylase constitutes a family of hydrolyses, It is a calcium metaloenzyme unable to show its activity without calcium (Saranraj. 2014). The two most dominant classes of amylase that has been spotted in microorganisms are amylase and glucoamylase. Amylases There are of three types of amylase α -amylase, β –amylase and amylase. Salivary and pancreatic amylases are α- amylase while β-amylase is of plant origin.

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Molecular weight of amylase is 50,000 Da and gives its maximum activity at optimum ph 6.9 and requires chloride for its activity(Salt, William B. et all, 1976). α-Amylases are starch-detoriating enzymes that do catalysis, by hydrolyzing the internal α-1,4 glycosidic bonds of polysaccharides into small oligosaccharides. (Gangadharan, *et al.*, 2006) Fungal amylase production can be found in both solid state fermentation (SSF) and also in submerged fermentation (SMF) (Saranraj.P, 2014). α – amylase attack α -1, 4 glycosidic linkages and do hydrolysis of starch which help in removing tough stains (Sundarram, A. *et al.*, 2014). The structure of α-amylase (α-1,4-glucan-4-glucanohydrolase) is an Endo-acting enzyme that attack internally on the interior side of the substrate (Sundarram, A. *et al.*, 2014, Abd-Elhalem et all, 2015) Amylase is having a 3-dimensional structure which is capable to bind to the substrate, hence they have highly specific catalytic groups, it promotes the breakdown of the glycosidic linkages. (Abd-Elhalem *et al.*, 2015) amylase is a calcium metaloenzyme as calcium act as co-factor. Amylase enzyme is having multiple domains of protein. C-terminal is considered as the active site of the (α/β) Barrel structure. (MacGregor, *et al.*, 2001) The protein structure of amylase have 3 major regions: A, B, and C .The A is considered as the larger domain, The B domain is present in between the A and C domains and it is linked with domain A by disulphide linkage.(MacGregor E, *et al.*, 2001, Monteiro,*et al.*, 2010) Calcium (Ca2+) a metallic co-factor is inserted between the A and B domains may do stabilization of the three-dimensional structure and regulate the allosteric activating site.(Saleem, *et al.*, 2014,)

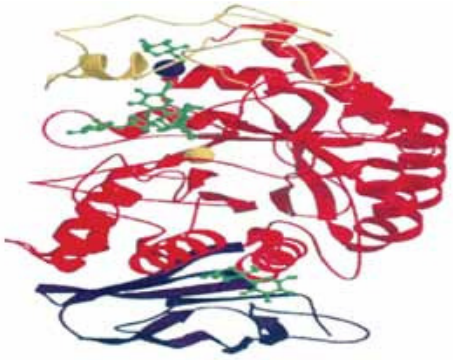

Figure 1: Structure of α –amylase. A domain is shown in red which is the largest domain, B domain is shown in yellow and c-domain is shown by purple color, calcium in a center of catalytic site is seen in sphere of blue color. The green color structure by surface binding sites is bound with the active site of enzyme. (Paula Monteiro De Souza, 2010)

α-Amylases are endoacting enzyme that internally that do catalysis of α-1,4 glycosidic linkages of starch polymers amylose or amylopectin into glucose units. Amylase has two major mechanism of action; random attack (single attack) action and multiple (multi-chain) attack action, the molecule is completely hydrolyzed before disengagement of the enzyme-substrate complex. (El-Fallal, and Dobara *et al*, 2012)

The enzyme Amylase is ubiquitous enzymes originated from many sources like animals, plants and also by microorganisms. Number of amylase-producing microorganisms; bacteria, fungi and other have been isolated, purified and characterized over many centuries. (Nigam. and Singh 1995). Bacteria and fungi release amylases outside their cell walls to carry out the mechanism of extra-cellular digestion. (El-Fallal, Dobara *et al.*, 2012). Among bacteria's, *Bacillus spp*. are generally used for heat stable

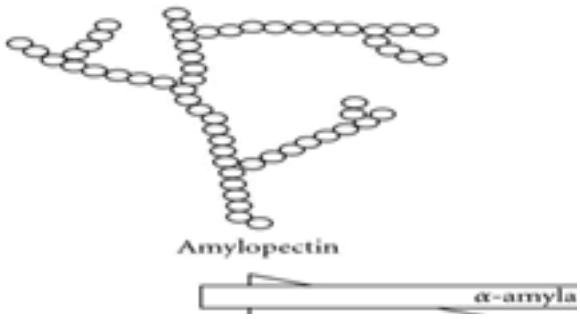

Figure 2: Hydrolysis of starch by α -amylase (Jensen and Norman, 1984)

α-amylase production, to overcome industrial based requirements most common strains of bacillus are *B.subtilis*, *B.stearothermophilus, B.licheniformis* and *B.amyloliquefaciens*. Fungal amylases had also been extensively used in preparation of Asian foods. (Nigam.P, and Singh 1995) Fungal species *Aspergillus. niger*, *Aspergillus.oryzae* is considered as the best fungal strains used in amylase production. (El-Fallal, and Dobara *et al.*, 2012) Many of *Streptomyces spp.*, *S. albus,S. griseus*, *S. thermocyaneoviolaceus* production of involve in the α-amylase.(Swetha Sivaramakrishnan *et al.*, 2006)

In late years solid state fermentation has shown a remarkable important evolution in industrial area, because of its potential applications in producing industrially important enzymes and also shows an attractive way to use it for pharmaceutical and chemical industries, (Ali, Zulkali, 2011) in Bioremediation, bioleaching, biopulping, biobeneficiation and in biological delignification etc. (Nigam, Pandey, 2009). The best considered substrate for solid state fermentation are agro-industrial residues some common are wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk etc. (Pandey, A. *et al.*, 1999). These substrates have been employed for the production of enzymes by using microorganisms. (chakraverty R & Chandra, 1999)There are various factors which effect the production of amylases in SSF. These physicochemical factors includes the type of substrate, nutrient composition of the substrate, incubation period, given temperature, pH, amount of oxygen present, concentration of substrate , amount and type of carbon source, phosphate and nitrogen concentration, humidity, (Balkan and Ertan,2007) The maximum activity of α -amylase produced shown in SSF was at peak at 70˚C and of pH 4.0, solid state fermentation method has commonly used in different countries due to its economic value obtaining grate biomass from cheap raw materials. (Suanthi, Rg. Et all, 2011)

MATERIAL AND METHODS:

Collection of Soil Samples: 20 different soil samples have been collected from various places and cultured on SDA agar after serial dilution of the soil sample till 1×10-6 dilution factorincubate the plate at 25˚C for seven days.
Lactophenol Blue Staning: A drop of Lactophenol Cotton Blue Stain is placed in the slide then a thumb impression is taken with the scotch tape method from the fungal plate. Observe under low power 40 x lens.

Subculturing: Selected strains of Aspergillus. niger are subcultured on SDA agar for further screening.

Screening For Starch Hydrolysis: *Aspergillus. niger spps*, have inoculated on starch agar for seven days at 25˚C starch hydrolysis is screened by flooding iodine on plate after 5 minutes remove the iodine and observe clear hydrolysis around the colonies with blue, purple background

Solid State Fermentation for Enzyme Production: The fungi were grown at 25˚C in 250 ml of Erlenmeyer flask. Add 5 ml of coarsely ground substrate of wheat barn and also add distilled water.

Enzyme Processing: 22ml of 0.1M phosphate buffer of pH 6.5 is added to the culture. Shake the mixture for 30 minutes at 19˚C and 140 rpm on shaking water bath. The mixture is filtered by cheesecloth and centrifuged at 8000 rpm for 15 minutes. The supernatant collected is again filtered through whattman number 1 filter paper. The collected filtrate is used for enzyme activity.

Assay of Enzyme Activity: Assay of enzyme activity is observed by using Dinitro Salicylic Acid (DNS) method .Add 3ml (DNS) reagent to 3ml of reducing sugar in a tightly covered test tube. The mixture is then heated at 90˚C for

5-15 minutes to develop reddish brown color. Add 1ml of potassium sodium tartrate solution 40% to stabilize the color. After cooling the tubes, record O.D at 575nm

Confirmatory Test for the Presence of Amylase: 3ml of starch is taken in a test tube. Add one or two drops of lugol's iodine in the test tube which turns the color of tube blue by forming a starch-iodine complex. Add 3ml of collected filtered supernatant containing enzyme amylase. The disappearance of blue color after adding the supernatant indicates the presence of enzyme amylase. (Figure 6)

OBSERVATION AND RESULTS:

20 different soil samples were screened for production of enzyme amylase by soil isolate Aspergillus.niger. All the selected strains produce high amount of enzyme amylase. The Amylase activity was estimated by determining the amount of reducing sugar by Dinitro Salicylic Acid (DNS) method. The optical density of the filtrate through solid state fermentation is observed by spectrophotometry at 575nm. The following results are obtained (Table 1)

The highest O.D is observed in a sample no. 12; 0.294 (Graph 1)

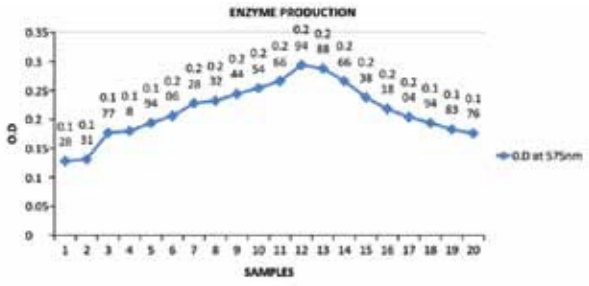

Figure 5: OD of different samples.

Table 1: O.D of samples at 575nm

Samples	O.D 575 nm	Samples	O.D 575nm
1	0.128	11	0.266
2	0.131	12	0.294
3	0.177	13	0.288
4	0.180	14	0.266
5	0.194	15	0.238
6	0.206	16	0.218
7	0.228	17	0.204
8	0.232	18	0.194
9	0.244	19	0.183
10	0.254	20	0.176

DISCUSSION

Amylase producing fungal isolates has been reported in earlier studies. Various agro industrial wastes such as: Wheat bran (WB), gram bran (BGB) and rice bran (RB) are used for amylase production by using solid-state fermentation (SSF) by filamentous Fungi. Wheat barn is considered as the best substrate for commercial scale production of α- amylase. In the following study the colonies of *Aspergillus. niger* is subcultured from the collected samples of different soil that are obtained by serial dilution method, then screened for starch hydrolysis on a starch agar, remarkable results are obtained as expected, clear hydrolysis is seen by Aspergillus spp. The Amylase activity was estimated by determining the amount of reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1M phosphate buffer in pH 6.5, at 25°C for 20 min(Miller GL, 1959,). The optical density of the filtrate of solid state fermentation is determined at a wavelength of 757 nm. The increase in O.D shows amylase production by the filamentous fungi isolated from the different soil samples. Among all the samples the highest amount of enzyme produced by the sample no.12 having highest O.D of 0.294 due to highest turbidity. The presence of enzyme α- amylase is confirmed by confirmatory starch-iodine test.

CONCLUSION

Enzymes are the biological catalyst which speeds up the biological metabolism. Amylase is endohydrolase enzyme; it was discovered first time in 1811 the starch by kichoff, also referred as the starch degrading or starch degrading enzymes. There are three types of amylases: α-amylase, β- amylase and ϒ- amylase

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