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Research Article

Production, assay and optimisation of amylase by submerged fermentation using *Aspergillus niger*

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Abstract: Amylases are a class of enzymes that have wide industrial application due to their ability to catalyze the hydrolysis of starch into maltose. Amylase enzymes are produced extracellularly by many microorganisms like *Aspergillus niger*. Submerged fermentation is a widely used method of obtaining amylase enzymes for industrial application. Enhanced amylase production can be beneficial for industrial application. In this study an attempt has been made to optimise some of the parameters that affect the production of amylase enzyme by *Aspergillus niger* through submerged fermentation in potato dextrose medium. Maximum enzyme activity per millilitre of the extract was found after 96 hours of incubation following inoculation of the spores in sterile potato dextrose medium. The culture maintained at temperature of 40°C showed maximum enzyme activity per millilitre of the enzyme extract. Maximum enzyme activity per millilitre of the enzyme extract was obtained from the culture maintained at p^H 6.

Key Words: Amylase Production, *Aspergillus niger,* Submerged fermentation, p^H Optimisation, Temperature Optimisation.

Introduction

Amylases are a class of enzymes that catalyze hydrolysis of starch into smaller carbohydrate molecules such as maltose. Two categories of amylases, denoted alpha and beta, differ in the way they attack the bonds of the starch molecules. α-Amylase (EC 3.2.1.1) brings about endohydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units. α-Amylase acts on starch, glycogen and related polysaccharides in a random manner, and reducing groups are liberated in the α-configuration. The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed. β-Amylase (EC 3.2.1.2) brings about hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains. β-amylase acts on starch, glycogen and related polysaccharides producing β-maltose by an inversion. The term 'β' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed (UBMB Enzyme Nomenclature). α-Amylase is required for various industrial processes most often related to food industry. The α-amylase enzymes are produced by plants, animals and microbes. But microbial source of amylase is mostly preferred for industrial purposes because they can be produced in large scale at lower cost and in less time. It is produced mainly from microbial sources and is used in many industries. (Gopinath *et al.,* 2017) Both bacteria and fungi can produce α-amylase. Amylases are used for a variety of applications in different enterprises including food, paper, detergent and textile industry. Additionally, amylases may also have potential application in pharmaceutical and fine chemical industries (Singh *et al.,* 2016). The optimum temperature and P^H at which these enzymes function varies among the strains and the choice of microorganism to be used as enzyme source depends on the temperature and p^H required for the industrial process. *Aspergillus niger* BAN3E was identified to be the best producer of amylase. When *A. niger* BAN3E was incubated for 6 days at 37°C it showed high yield of amylase in groundnut oil cake substratein solid state fermentation. Sucrose and nitrogen improved the yield in the same medium (Ramaswamy *et al.,* 2011). Toledo *et al.,* (2007) purified and did biochemistry characterization of alpha-amylases from *Aspergillus niger*. Amylase can be produced for industrial purpose from various substrates including agricultural wastes. Research is focused on developing thermotolerant and pH tolerant a-amylase from microbes, modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzyme (Sivaramakrishnan *et al.,* 2006)

Materials and Methods

Isolation of the microorganism

In this study *Aspergillus niger,* which was used of amylase production, was isolated from the soil sample by serial dilution and pour plate method using PDA medium. The colony characteristics

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were observed. Fungal samples from selected colonies were stained with cotton blue lactophenoland observed under microscope. *Aspergillus niger* was identified using handbook for identification of fungi (Nagmani, 2006). The selected colony was streaked on solidified PDA medium to obtain pure culture of *A niger*.

Screening

Screening of fungi for amylase production was done using starch agar salt medium. Starch agar medium was prepared using starch, 20 g; KNO_3, 1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; water to 1 L and sterilised. Shirling E. B. and Gottleib D., 1966) The solidified starch agar plates were inoculated with loop full of *Aspergillus niger* pure culture and incubated at 30°C for 48 hours. The plates were then flooded with iodine solution. Presence of a colourless zone surrounding the fungal colony indicated conversion of starch to maltose by the fungal amylase.

Inoculation of medium for enzyme production by submerged fermentation.

Liquid potato dextrose medium was prepared using an infusion of 200g of potato, 20g dextrose and 1 litre water. The media was divided equally in conical flasks, plugged with cotton and sterilised in the autoclave under 15 pound pressure and 121°C for 15 min.

Aspergillus niger pure culture producing α-amylase was streaked out and thoroughly mixed in 5.0 ml of sterile deionised water. Then, from the spore suspension, 1.0 ml was inoculated to fresh sterilized potato dextrose broth medium for development of working culture and incubated at 30 ± 1°C up to 7 days to attain about 5.0×10^8 spores ml^{-1}. For the entire study, 1×10^7 spores ml^{-1} was used as inoculum to carry out the fermentation process (Sethi *et al.,* 2013). The cooled sterile media was inoculated inside laminar air flow chamber and incubated according to experimental design.

Extraction

Extraction of the crude enzyme was done by centrifugation of the fermented media at 2000 rpm (revolution per minute) for 5 min, supernatant collected and filtered off using Whatman No.1 filter paper. The filtrate was used as crude enzyme extract (Oyeleke *et al.,* 2010)

Determination of optimum temperature for fermentative production of amylase:

To determine the optimum temperature, the liquid potato dextrose medium containing fungal spores were incubated at 25°C, 30°C, 35°C and 40°C temperatures at P^H 7 for 48 hours.

Determination of optimum pH for fermentative production of amylase

The effect of pH was observed by incubating the *A. niger* culture in liquid medium of potato dextrose at 30°C having different pH values. (pH: 4.5, 5, 5.5, 6, 6.5 and 7.)

Enzyme Assay

The α- amylase activity was assayed by the (3, 5-dinitrosalicylic acid) (DNS) procedure (Bernfeld 1995), using 1% soluble starch. To prepare maltose standard graph, about 1g of DNS is dissolved in 50ml of distilled water. To this solution about 30g of sodium potassium tartarate tetrahydrate is added in small lots, the solution turns milky yellow in colour. Then 20ml of 2N NaOH, is added which turns the solution to transparent orange yellow colour. The final volume is made to 100 ml with the distilled water. This solution is stored in an amber coloured bottle.

For the preparation of standard graph of maltose, a stock solution is prepared by taking 50 mg of maltose in 50 ml of water, and then from the stock solution, 10 ml of solution is dissolved with 40 ml of distilled water. This is now the working solution. From the working solution, different amounts i.e. 0.2, 0.4, 0.6, 0.8 and 1 ml is taken in 5 test tubes, and one test tube is kept as blank. The final volume in each test tube is made to 1 ml and then, 1 ml of DNS is added to each test tube. The contents in the test tubes are heated in a boiling water bath for 5 minutes. The test tubes are cooled and 10 ml of distilled water is added to each test tube. Optical density of each test tube is determined by a spectrophotometer at 540 nm. A graph is plotted with the amount of maltose on X axis Vs OD at 540nm on Y axis.

To test the enzyme activity, 1 % starch solution is prepared by using 1 gram of starch in 100ml of distilled water. To 1 ml of starch solution, 1 ml of enzyme extract produced by fermentation under different pH values and temperature, is added. In one of the test tube, to be treated as blank, 1ml if distilled water is added instead of enzyme with 1 ml starch solution the solution. Both the test tubes containing the sample and the blank are incubated for 10 minutes at 30°C. Then 1 ml of DNS was added and incubated in boiling water bath for 5 min. Both test tubes are then cooled and 10 ml of distilled water is added and mixed well. The optical density of the sample is then determined against blank at 540 nanometre. All assays were performed in duplicate and each reading was taken three times.

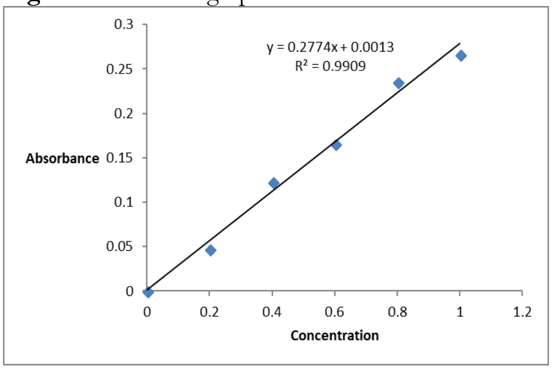
Results and Discussion

Standard graph preparation

Table 1. Standard Graph Values

Concentration Of Maltose	(Average) Optical Density At 540nm
0	0
0.2	0.048
0.4	0.123
0.6	0.166
0.8	0.236
1	0.267

Figure 1. Standard graph for maltose



In the standard graph,

$$y = 0.2774x + 0.0013$$

y = Absorbance, X = concentration of maltose from the working solution, slope (m) = 0.2774 and b = 0.0013

$$y = mx + b$$

So,

$$X = (y - b)/m$$

= (Absorbance – 0.0013)/0.2774

Maltose units per ml of enzymes = (micro grams of maltose released) (dilution factor)/ml of enzyme.

Table 2. Effect of P^H on Amylase production

P^H	Absorbance	µg of maltose units per ml of enzymes
4.5	0.18	132.66
5	0.52	374.91
5.5	0.76	550.11
6	1.25	904.11
6.5	0.59	421.77
7	0.72	521.27

Table 3. Effect of temperature on Amylase production

Temperature	Absorbance	µg of maltose units per ml of enzymes
25	0.473	341.02
30	0.686	494.59
35	1.964	1416.01
40	2.166	1561.64
45	0.77	555.16

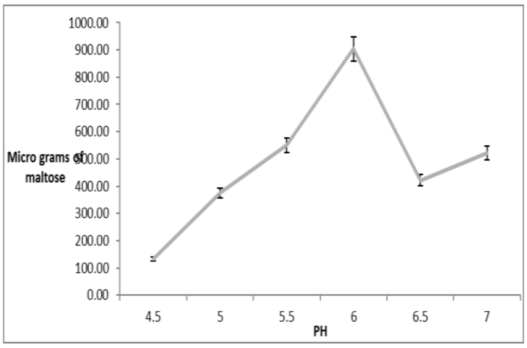
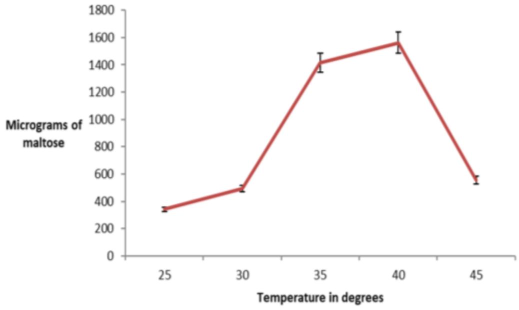


Figure 2. Effect of P^H on Amylase production graph

Figure 3. Effect of temperature on Amylase production graph



Conclusion

In the study it was found that p^H 6 (904.11microgram of maltose/ml of enzyme) and temperature of 40°C (1561.64 microgram of maltose/ml of enzyme) centigrade is most suitable for amylase production by submerged fermentation after 96 hours of incubation. Mathew *et al.,* (2016) observed maximal α-amylase activity after 7 days of submerged fermentation on white Yam water at pH 7.0 and room temperature 28°C. Pasin *et al.,* (2014) found that influence of the initial pH of the medium for the production of amylases by *A. japonicas* cultivated for 4 days in static condition was 30°C and it was also observed that pH 5.5 was the best initial pH for amylase production. Wang (2016) found enzyme production reached maximum at temperature of 30°C, pH 7, with 40 g/L starch in the medium inoculated with 1.4% v/v spore.

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