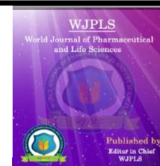
wjpls, 2018, Vol. 4, Issue 1, 80-84 *Research Article* ISSN 2454-2229

World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 4.223



PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM *ASPERGILLUS NIGER*

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Article Received on 23/10/2017 **Article Revised on 13/11/2017** **Article Accepted on 04/12/2017**

ABSTRACT

The fungal isolates from soil were screened for amylase production and the isolates identified as *Aspergillus niger*. The isolated organism was cultivated in PDA medium & spread plate techniques were used for isolation. The morphological character was identified using lacto phenol cotton blue stain. Strains were maintained in PDA slants and it is subcultured for every weeks. Screening procedure is carried out to identify the amylase production by *Aspergillus niger*. The mycelia matt from the production medium is obtained after incubation period and subjected for centrifugation. The crude enzyme obtained from the supernatant is used for further analysis and enzyme activity. Based on the results obtained the enzyme activity of amylase by *Aspergillus niger* were found to be 400 units/ml. Based on optimum pH, temperature, incubation period, carbon source and nitrogen source. The amylase production were maximum at pH 5(415 U/ml), temperature 30°C (400 U/ml), incubation period 120 hours (5th days) 400 U/ml, Starch 350U/ml, peptone 330 U/ml respectively. On the light of the obtained results, it could be conclude that both nutritional and cultural conditions were required for optimum growth and production of amylase from *Aspergillus niger*. *Aspergillus niger* isolated from soil can be industrially exploited for the synthesis of amylase and strain improvement studies can be carried out to enhance enzyme production.

KEYWORDS: *Aspergillus niger*, Amylase, synthetic growth medium, Substrate, Solid state fermentation.

INTRODUCTION

Amylase are enzymes that breakdown starch or glycogen. Amylases are produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carryout extra cellular digestion.

Amylases are important enzymes employed in processing industries for the hydrolysis of starch into simple sugars.[1] Evidences of amylase production by moulds, yeasts and bacteria have been reported and their properties documented.[2,3] Fungi among many microbes are good sources of amylolytic enzymes.[4,5] Amylases hydrolyze the alpha 1-4-glucosidc bonds of amylopectin, glycogen are related compounds.[6]

Among various extra cellular enzymes amylase ranks first in terms of commercial exploitation spectrum of applications amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industries.

These enzymes are found in animals (saliva, pancreas) plants (malt) bacteria and moulds have been reported. The amylase of fungal origin was found to be most stable than the bacterial enzyme.[7]

The cost of enzyme production in submerged production is high, which necessitates reduction in production cost by alternative methods. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by products for the reduction of cost of the medium. Starch degrading enzymes like amylases are received great deal of attention because of their perceived technological significance and economic benefits. This enzyme is also used for the commercial production of glucose.

The *Aspergillus niger* group is wide spread with many strains capable of producing amylases. The amylase producing strains of *Aspergillus niger* have spore bearing heads which are large, tightly packed, globular and may be black or brownish black. They are considered to be mesophillic with optimal temperature for growth between 25^{0}C and 35^{0}C. They are aerobic in nature and

can grow over a wide range of hydrogen ion concentration. These organisms can utilize different kinds of foods from simple to complex ones, which make them easy to cultivate and maintain in the laboratory.

The use of agricultural wastes makes solid state fermentation an attractive alternative method have reported d amylase production in solid state fermentation with wheat bran and rice husk as substrates. Starch degrading amolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries. The amylases can be derived from several sources such as plants, animals and microbes. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits.

In most developing countries of the tropics carbohydrate based agricultural products like starchy tubers and cereal occurs abundantly starch tubers such as cassava, yam, sweet potato and cocoyam are important stable foods I the diet of people in most developing countries of the tropics. The most effective amylases are those that are thermo stable. They are generally preferred as their application minimizes contamination risk and reduces reaction time thus enabling considerable energy saving.

Industrially important enzymes have traditionally been obtained from submerged fermentation because of the ease of handling and greater control of environment factors such as temperature and $_{P}$H. Enzymatic hydrolysis of starch has now replaced acid hydrolysis in over 75% of starch hydrolyzing processes due to many advantages. Alpha amylases have been derived from several fungi, yeasts bacteria and *Actinomycetes*. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. The most abundantly used bacterial alpha amylases were derived from *Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus stearothermophilus.* The hydrolyzed products are widely applied in the food, paper and textile industries.

They are used for starch hydrolysis and starch liquefaction process that convert starch into fructose and glucose syrups. They are also used as partial replacement for the expensive malt in the brewing industry to improve flour in the backing industry and to produce modified starches for the paper industry. In addition to this, they are used to remove starch in the manufacture of textile and as additives to detergents for both washing machines and automated dishwashers. Microbial amylases have successfully replaced chemical industries. Besides that use in starch saccharification, they also find potential application in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries, with the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields such as clinical, medical and analytical chemistry.

These starches can be converted to reducing sugars by acid or enzymatic saccharification. In terms of energy utilization and process simplicity amylase convention of raw starches is believed to be superior to the conventional method that makes use of pregelatinized starch as substrate.[8] Amolytic enzymes of microbial origin rae divided into exoacting, endoacting, debranching and cyclodexrin producing enzymes. Glucoamylases hydrolyze-1,4 and α-1,6 linkages and produce glucose as the sole end product from starch and related polymers and amylases hydrolyze α-1,4 bonds but cannot bypass, 6 linkages in amylopectin and glycogen are exo-acting enzymes whereas amylases are endoacting. Alpha-amylase are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants. Amylases are among the most important enzymes in present day biotechnology.

Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand. A variety of microorganisms including bacteria, yeasts and filamentous fungi, have been reported to produce amylolytic enzymes. Amylases are used in a variety of industrial processes which require efficient saccharificattion of raw starch.[9]

Solid state cultivation systems and submerged liquid cultivation systems have been use for amylase production although most research has used culture conditions, which allow greater control of culture conditions. Such as extreme temperature and $_{P}$H.[10] Starch degarading enzymes are of great significances in biotechnological applications ranging from good fermenatation textiles and paper industries.

The amylase can be derived from several sources such as plants, animals and microbrs. The microbial amylases meet industrial demands; a large number of them are available commercially; and thay have almost completely replaced chemical hydrolysis of starch in starch processing industry. Alpha amylase has been derived from several fungi, yeast, bacteria and *Actinomycetes*, however enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and to only one species of *Penicillium*, *Penicillium brunneum*.

Strains of *Penicillium* species were isolated from the coastal soil of a mangrove habitat. One isolate produced extra cellular α-amylase which was confirmed by percentage blue value and by identification of the products obtained by starch hydrolysis. Since this natural isolate produced low concentration of amylase, attempts were made to increase the productivity by optimizing the cultural conditions.[11]

Amylases are starch degarading enzymes of industrial importance. Animal amylase is mainly α-amylase, while β-amylase occurs in plants was established few decades ago that β-amylase occurs in plants was established few decades ago that α-amylase occur and can be produced as an extra celluar enzyme by microorganisms.

First of such discovery was made in 1946 when β-amylase was found to be produced by *Bacillus polymyxa* and later by another Bacillus species identified as *Bacillus cereus var mycoides*, other amylolytic enzymes can also be obtainable in *Bacillus* strains.

AIMS AND OBJECTIVES

The aim of the present study is to isolate and produce amylase from *Aspergillus niger*.

OBJECTIVES

- Isolation and identification of *Aspergillus niger.*
- Production of amylase from the isolated fungi.
- Isolation of crude enzyme.
- To detect enzyme activity.

MATERIALS AND METHODS

Collection of sample

The soil was freshly collected from the ground by using sterile conical flask and spatula.

Isolation of fungi

The fungal cultures are isolated from the soil sample by the spread plate technique. 1ml of each of the dilution was transferred to a sterile PDA plate and spreaded with the help of L-Rod. The plates were incubated at 37^0C. The fungi grown were observed and the colony morphology was noted down.

Identification of fungal species

Clean glass slide was taken. One drop of lactophenol cotton blue stain was placed. Gently tease by using dissecting needle. Place the cover slip. Then observe under low and high power objective.

Production of Enzyme

All ingredients are prepared in 100 ml of distilled water {starch [1%], KH2PO4 [0.2%], NH[4]2SO4[0.14%], cacl2[0.03%],MgSo47H20[O.03%], Urea [0.03%], Peptone [0.1%], Trace element solution [0.01%],Triton X-100[0.021%]}. The pH of the medium was adjusted to 4.8 with concentrated HCL. Medium was sterilized by autoclaving at 121^0C and 15-17 for 30 min. a certain inoculation size of conidia [each ml of cells suspension contained 2.0x 10^6 cells] was transferred from a stock culture in 250 ml flask containing 50ml of growth medium. The flasks were incubated for 72h at 28 ± 20C on rotator shaker at 150 rpm.

Extraction of enzyme

On the last day of incubation period [72h]. The fungal separated by centrifugation at 4500 rpm for 10 minutes. The clear supernatant [crude enzyme] was used for enzyme activity. The enzyme activity was expressed in number of units. One unit of enzyme was defined as the amount of enzyme (protein) in milligram required for hydrolysis of starch to produce a milli molar of reducing sugar (glucose) in one hour under assay conditions. The specific activity was defined as number of units per gram protein. The protein was separated by centrifugation at 2000 Xg for 30 min at 4C dissolved in minimum volume of phosphate buffer [50mm and $_P$H 7.20] and used immediately for activity determination.

Enzyme Assay

The reaction mixture contained the following in a total volume of 2ml; 10mg starch, 1to 1.8ml of crude enzyme and 1ml of phosphate buffer. The reaction mixture was incubated at 37^0C for 30 minutes and the reaction was terminated by addition of 3ml of DNS solution(3,5 – Dinitro salicyclic acid). After stopping the reaction the tubes were placed in boiling water bath for 5 minutes and then cooled and absorbance was determined at 540nm. The amount of glucose producd was calculated by referring to the standard plot using glucose as the reducing sugar.

RESULTS AND DISCUSSION

The Table 1 represents the quantitative assessment of the amylase productivity by *Aspergillus niger* using solid state fermentation process. The enzyme activity was found to be 400 units/ml. (Table 2).

Table 1: Determination of Enzyme activity by DNS method.

Particulars	Glucose (ml)	Distilled water (ml)	Phosphate buffer (ml)	DNS solution (ml)	O.D at 540 nm
Blank	-	1	1	3	0.00
S1	0.2	0.8	1	3	0.6
S2	0.4	0.6	1	3	0.12
S3	0.6	0.4	1	3	0.18
S4	0.8	0.2	1	3	0.24
S5	1	-	1	3	0.30

Table 2: Activity of Amylase.

S.No	Name of fungi	O.D at 540nm T1	Enzyme activity U/ml
1	*Aspergillus niger*	0.12	400

The Table 3 represents the enzyme production at various temperature ranges from 25^{0}C -45^{0}C for 72 hours. It was found that *Aspergillus niger* produced considerable amount of enzyme activity at 30°C (410U/ml). When compared to 25°C (200U/ml). Based on the results obtained the enzyme production was found to be optimum at 30°C.

Table 3: Effect of temperature on Enzyme production.

Temperature	Enzyme activity units/ml
25	200
30	410
35	400
40	300
45	210

The Table 4 represents the effects of pH on enzyme activity ranging from pH 3-8. The production of amylase was found to be best at pH 5.0 (415 units/ml). Below and above this pH the production was significantly lower.

Table 4: Effect of pH on Extracelluase enzyme production.

S. No	pH	Enzyme activity U/ml
1	3	150
2	4	175
3	5	415
4	6	200
5	7	100
6	8	50

The below Table 5 represents the amylase activity after every 24 hours of incubation in order to determine the optimum incubation period for maximum production of extra cellular amylase. The enzyme production however, started after 24 hours of inoculation and showed maximum production on 5th day of incubation period at 30°C.

Table 5: Effect of incubation period on extracellular amylase production.

S.No	Incubation period	Enzyme activity U/ml
1	24 hrs (1 Day)	20
2	48hrs (2 Day)	25
3	72hrs (3 Day)	100
4	96hrs (4Day)	200
5	120 hrs (5 Day)	400
6	144 hrs (6 Day)	250

The table 6 represents the role of different carbon sources for amylase production. Based on the results obtained sucrose, galactose and starch were good carbon sources for amylase production, but glucose produce low amount of enzyme.

Table 6: Effect of different carbon sources on amylase production.

S.No	Carbon sources	Concentration of carbon sources	Enzyme activity
1	Sucrose	0.5	200
2	Starch	0.5	350
3	Glucose	0.5	150
4	Galactose	0.5	250

The Table 7 represents the role of different nitrogen sources for amylase production. Based on the results obtained peptone, urea, gelatin were good nitrogen sources for amylase production but casein produced low amount of enzyme.

Table 7: Effect of different nitrogen sources on amylase production.

S.No	Carbon sources	Concentration of nitrogen sources	Enzyme activity
1	Casein	0.5	100
2	Peptone	0.5	250
3	Urea	0.5	200
4	Gelatin	0.5	150

The occurrence of amylolytic organism from the soil agrees with earlier reports that soil is known to be repository of amylase producer. This study showed that the susceptibilty of the raw starch to the crude enzyme of *Aspergillus niger* was significantly dependent upon the starch source and time of incubation.

In the present study the solid state fermentation technique used for production of industrial enzymes like amylase has great potential in developing countries due to its simplicity of operations low capital cost and high volume productivity which correlates with the reports given in table 4. A number of reports exhibit regarding the influence of various environmental conditions like effect of pH, temperature, incubation period, carbon source, nitrogen source. The enzyme is very sensitive to $_p$H therefore the selection of optimum $_p$H is very essential for the production of amylase.

Acidic medium was required for the optimum production of amylase. In the present study the enzyme production decreases (144 hours 6th day) as the incubation period is increased. This may be due to the depletion of nutrients

and by products by *Aspergillus niger*. The influence of temperature on amylase production is related to the growth of the organism. The results from the table – 3 shows that the enzyme yield was maximum (410U/ml) at 30^0C.

Based on the influence of carbon source, nitrogen source and starch, peptone has great influence in the production of amylase.

CONCLUSION

On the light of the obtained results, it could be conclude that both nutritional and cultural conditions were required for optimum growth and production of amylase from *Aspergillus niger*. *Aspergillus niger* isolated from soil can be industrially exploited for the synthesis of amylase and strain improvement studies can be carried out to enhance enzyme production.

ACKNOWLEDGEMENTS

The authors are grateful to the Director SF & Head of the Department of Microbiology, The Madura College (Autonomous), Madurai, for providing the necessary facilities and constant encouragements.

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