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STUDIES ON THE OPTIMIZATION OF AMYLASE PRODUCTION BY *Aspergillus* sp.

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Abstract

Amylases are a group of important enzymes which are mainly employed in the starch processing industries for the hydrolysis of polysaccharides like starch into simple sugars. Amylases accounts for about 30 % of the world's enzyme production. Due to wide range of application of amylases in various sectors like confectionary, baking, paper, textile, detergent and pharmaceutical many are gaining the attention of researchers. In the present study, the production of extracellular amylases was investigated employing our three laboratory isolates *Aspergillus niger* - I, *Aspergillus niger* - II and *Aspergillus niger* - III. The agricultural residual substrate, wheat bran was used for the enzyme production. Effects of various process conditions, namely: temperature, pH, supplementary carbon and nitrogen source on production of amylase have been studied and accordingly, optimum conditions have been determined. It was found that the amylase production was highest at 35 °C, pH 5.5, supplementation of carbon (starch at 2.0 %) and nitrogen source (yeast extract at 1.0 %) showed an increase in amylase production and the highest amount of amylase production obtained under all optimized conditions.

Key words: *Aspergillus niger*, Optimization and Wheat bran.

1. Introduction

Enzymes are biological catalysts, they speed up reactions without being used up in the reaction themselves, therefore, a small amount of enzymes can be added to a substance and providing that the substrate is complementary to the active site of the enzyme (Lock and Key Theory), a reaction will occur. Each enzyme has its own specific optimum pH, temperature and substrate concentration; these factors will affect the performance of the enzyme. When these factors are taken to their extremes however, the enzyme can often become denatured (permanently damaged) and will no longer be able to perform its function, it cannot be reverted because the bonds in the molecule is broken and the active site is not in correct shape.

Fungal glucoamylase is the most important industrial enzyme which has wide application in the starch processing industry. Fungal glucoamylases are known for their activity usually at acidic pH, and has low thermostability. Glucoamylases from different species differ in their properties such as resistance to proteolysis and urea denaturation. All the glucoamylase so far studied are glycoproteins the carbohydrate groups being necessary for maintaining the structural stability of the enzyme conformation (Shenoy *et al*, 1985). The use of enzymes in food preservation

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and processing pre-dates modern civilization. Fermentation of common substrates such as fruits, vegetables, meat and milk provides a diverse array of food items in human diet. Beer, wine, pickles, sausage, salami, yoghurt, cheese and buttermilk are all fermented products are all the result of the enzymatic modification of constituents in the substrate (Table - 1).

Table – 1: Commercial applications of Amylase

Industry	Application	Sources
Baking & Milking	Reduction of dough viscosity, acceleration of fermentation process, increase in loaf volume, improvement of crumb score and softness maintenance of freshness and softness	Fungal
Beer	Mashing	Fungal / Bacteria
Cereals	Precooked baby foods, breakfast foods	Fungal
Chocolate, coco	Manufacture of syrups	Fungal / Bacteria
Confectioner, Candy	Sugar recovery from scrap candy	Fungal / Bacteria
Corn syrup	Manufacture of high maltose syrup	Fungal / Bacteria
Distilled beverages	Mashing	Fungal / Bacteria
Feeds, Animal	Pig starter rations	Fungal
Flavour	Clarification (starch removal)	Fungal
Pharmaceutical and Clinical	Digestive acids	Fungal
Textiles	Digesting fabrics	Fungal
Vegetables	Liquefying soups	Fungal

The use of enzymes in food industry covers a wide range of effects including the production of food quality attributes such as flavors and control of color, texture and addition to their nutritive value (Carriere *et al*., 1991). Due to the ever increasing demand of this enzyme, people are still trying to increase the productivity of amylases by a variety of approaches like selection of a high enzyme producing strain, process optimization, usage of cheaper substrates, effective downstream processing, etc (Lalit *et al*., 2010). The present study revealed that the various process conditions, namely temperature, pH, supplementary carbon and nitrogen source on production of amylase have been studied and accordingly, optimum conditions have been determined.

2. Materials and Methods

Fungal strain

Isolation of the fungal strains was carried out from various soil samples collected from local market area near Chidambaram, Tamil Nadu, India. *Aspergillus* species were identified on the basis of morphological characteristics and certain standard confirmatory tests.

Isolation for Amylolytic Organisms

The soil suspension was diluted 10^{-4} to 10^{-5} times and 0.5 ml of each diluted suspension was then transferred by Spread plate method with a sterile glass spreader on petriplates containing Czapex Dox starch agar medium. The petriplates were incubated at 37 °C for 4 - 5 days. To screen amylolytic fungal strains, isolates (most of which were grouped as *Aspergillus* black) were grown at 37 °C on solid agar medium supplemented with starch. After 4 - 5 days incubation fungal colonies were flooded with 1 % Iodine solution, to select isolates which show wide clear zone around their colonies. Based on the zone of clearance on the Czapex Dox starch agar plates, colonies were picked and individual amylase activity of selected colonies was carried out. The young colonies of fungal cultures were aseptically picked and transferred to Czapex Dox starch agar slants with 1 % starch. These slants were then incubated at 37 °C for 4 days and after sufficient growth, they were stored at 4 °C in the refrigerator.

Inoculums preparation

Fungal isolates were stored in Czapex Dox starch agar medium at 4 °C and were periodically transferred to a fresh media every month and

whenever spores for inoculation were required. Each isolates was inoculated on to agar medium by first suspending a loop full of spore on to 50 ml of saline solution (0.85 g/L), and a drop of the spore.

Screening for Fungal Amylases

To select the best amylase producing strains all fungal isolates with amylolytic activity were grown in liquid medium (50 ml broth in 250 ml capacity Erlenmeyer flasks) on a shaker, 120 rpm. The fungal isolates were grown at room temperature for 4 days. The crude enzyme from the fungal cultures was harvested by filtration through Whattman's No. 1 filter paper. The culture filtrate served as enzyme source.

Effect of carbon source on enzyme production

The effect of carbon source on enzyme production by *Aspergillus* sp. was determined by growing the test organism in liquid medium (pH adjusted to 6.0) in which 1.0 % of either of the following carbon sources: glucose, maltose, lactose, starch and sucrose was used as a sole carbon source. After 4 days incubation at 37 °C on a shaker, 120 rpm the crude enzyme was harvested and assayed to determine enzyme activity.

Effect of Nitrogen source on enzyme production

To determine the appropriate nitrogen source for amylase production by the fungal isolates, this test organism was grown in liquid medium (the pH adjusted to 6.0) in which 0.5 % (w/v) of either of the following nitrogen sources: Yeast extract, Peptone, Ammonium sulphate, Urea and Sodium nitrate was used as sole nitrogen source. At the end of 4 days incubation, the crude enzyme harvested was assayed to determine the enzyme activity.

Effect of pH on the enzyme production

To determine the pH profile of the amylase production by the fungal isolates, this test organism was grown in liquid medium with substrate prepared in a pH range of 4.5 – 7.0. At the end of 4 days incubation the crude enzyme harvested was assayed to determine the enzyme activity.

Effect of Temperature on the enzyme production

The effect of temperature on enzyme production by *Aspergillus* sp. was determined by growing the test organism in liquid medium (the pH adjusted to 5.5) with substrate and incubated in BOD incubator at various temperatures from 25 to 45 °C. At the end of the incubation periods, the crude enzyme harvested was assayed to determine the enzyme activity.

Solid state fermentation (SSF) medium for fungal amylase production

The SSF medium was used for enzyme production from selected isolates using wheat bran as a sole carbon source supplemented with the following nutrients g/100 ml (%): Starch, 1.0; Yeast extract, 0.5; NH_4SO_4 0.02; $MgSO_4$, 0.008; KH_2PO_4. 0.19; pH 5.5. Five gram of wheat bran moistened with above nutrient solution in 250 ml size. Erlenmeyer flasks were used for cultivation of the fungal inoculums and incubated at 35 °C for 96 hrs. The crude enzyme from SSF medium was harvested by centrifugation (5000 rpm, 10 mints). First the moldy bran was soaked in 10 mM acetate buffer pH 4.5 in (w/v) 1:10 ratio (Omemu *et al.*, 2005), and shaked for 1 hr at 120 rpm on the shaker, then it was centrifuged repeatedly at 5,000 rpm (Soni *et al.*, 2003). The supernatants were assayed for amylase.

Amylase assay

One unit of α- amylase activity was defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under assay conditions and expressed as units per gram dry substrate. Amylase activity was determined (Pandey *et al.*, 1999). The reaction mixture containing 1.25 ml of 1 % soluble starch, 0.25 ml of 0.1 mM acetate buffer (pH 5.0) and 0.25 ml of crude enzyme extract was incubated for

10 min at 50 °C. After incubation, the reducing sugar was estimated by Dinitro salicylic acid (DNS) method (Miller *et al*., 1959). For the estimation of enzyme, read colour developed reaction mixture was taken at 420 nm using a Spectrophotometer with glucose standard. To a 0.9 ml of the substrate in a pair of test tube, 0.1 ml of appropriately diluted enzyme source was added and incubated for 10 min at 50 °C (fungal enzyme) in water bath. At the end of the incubation period the reaction was stopped by adding 2 ml DNS solution. The control used was prepared in such a way that enzyme source was added after the reaction was stopped by the DNS solution.

Qualitative estimation of sugar (Benedict's method)

The reaction mixture was then boiled for exactly 5 min in boiling water. Finally, the test tubes were cooled in running water for about 5 min and the resulting coloured solution was compare with standard Benedict's colored.

Quantitative estimation of sugar (Folin Wu method)

A protein free filtrate of whole sample is heated an alkaline tartarate solution. Glucose from the sample reduces the cupric ions in the soluble cupric tartrate to cuprous oxide produced was measured by the reduction of phosphor molybdate to molybdenum blue. The intensity of the blue colour produced was proportional to the glucose in the sample and compared with the colour given by a standard glucose.

Calculation

The amount of sugar was calculated as mg/dl in the test sample and standard with the formula.

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}$$

3. Results and Discussion

Isolation and screening

Screening for Glucoamylase producing fungal strains

Akpan *et al.* (1999) described a simple and rapid method for the detection of amylase producing microorganisms on solid media using Remazol Brilliant Blue Starch as substrate. The blue starch is incorporated into nutrient agar and amylase production is detected by the disappearance of the color around the colonies. The method, which is non-destructive, allows for direct visualization and isolation of amylolytic microorganisms from the environment without prior replication of colonies. Based on amylolytic activity which was reflected by wide clear zone formation around the colony on the solid starch containing agar media, out of a total 5 isolates, 3 isolates (3 *Aspergillu*s black groups) were selected for further screening. To select the best glucoamylase producing strain all the 3 amylolytic isolates were grown in liquid medium. Based on this step three isolates *Aspergillus* black groups, *Aspergillus niger* - I (ASP I), *Aspergillus niger* - II (ASP II) and *Aspergillus niger* - III (ASP III) were selected. Glucoamylase production was optimized with respect to various nutritional and environmental parameters such as carbon inducer (1 % w/w) (glucose, lactose, maltose, sucrose and starch), nitrogen sources (inorganic (0.25 % w/w) Ammonium sulphate, urea and Sodium nitrate; organic (1 %) – yeast extract, peptone); pH (4.5 - 7.0) and temperature (25 – 45 °C).

Effect of the carbon source on growth and amylase production by *Aspergillus* sp.

Gupta *et al.* (2008) reported on the carbon sources, starch at 0.5 % was recorded to be the best carbon sources for enzyme production. α-Amylases production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes (Bhella and Altosaar, 2004). Glucose and sucrose supplementation resulted in repression of enzyme production. Similar results of catabolite repression

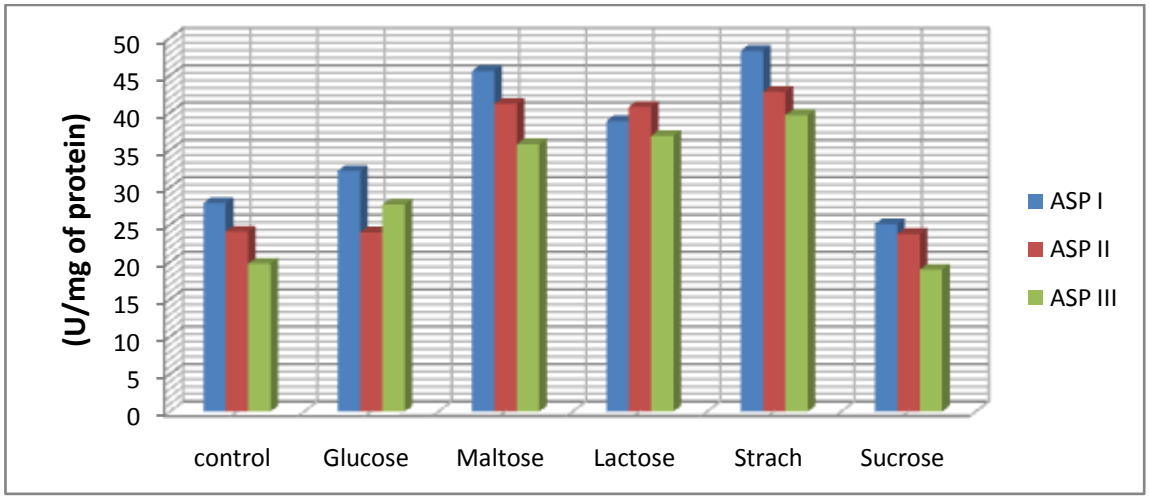

Fig. 1 Effect of carbon sources on amylases production by *Aspergillus* sp.

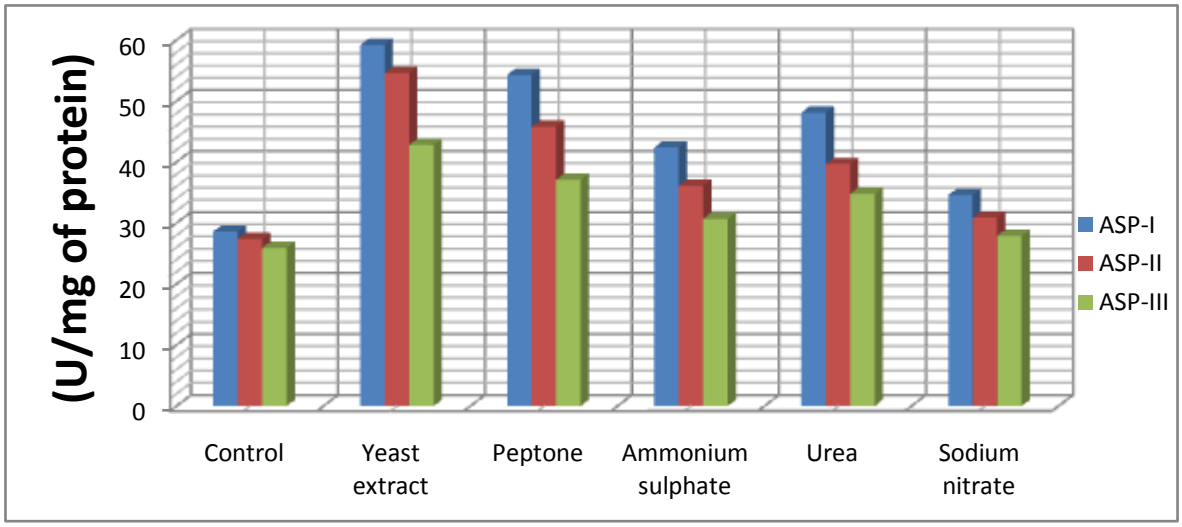

Fig. 2 Effect of nitrogen sources on amylases production by *Aspergillus* sp.

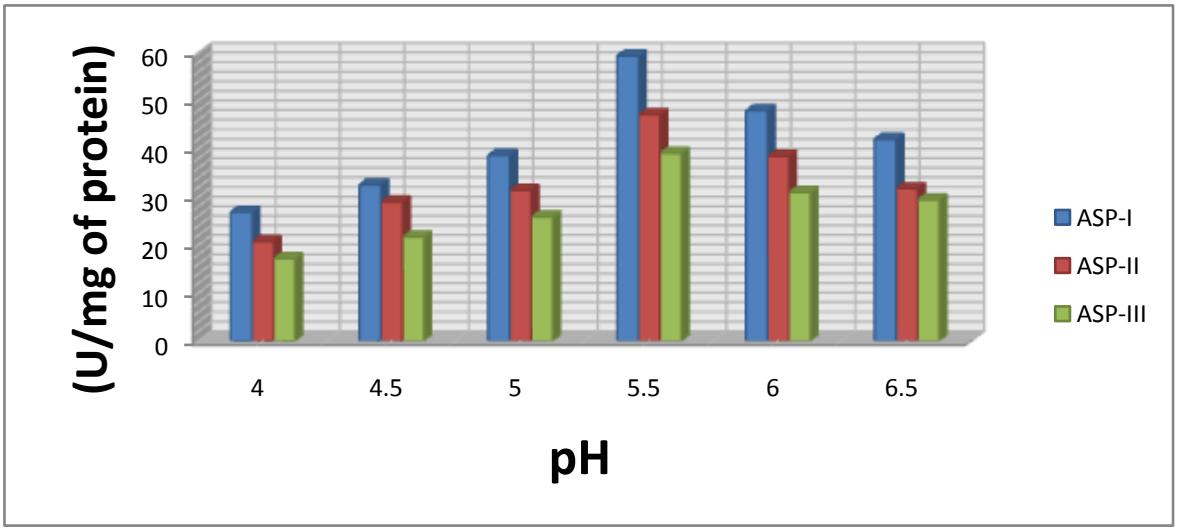

Fig. 3: Effect of pH on amylases production by *Aspergillus* sp.

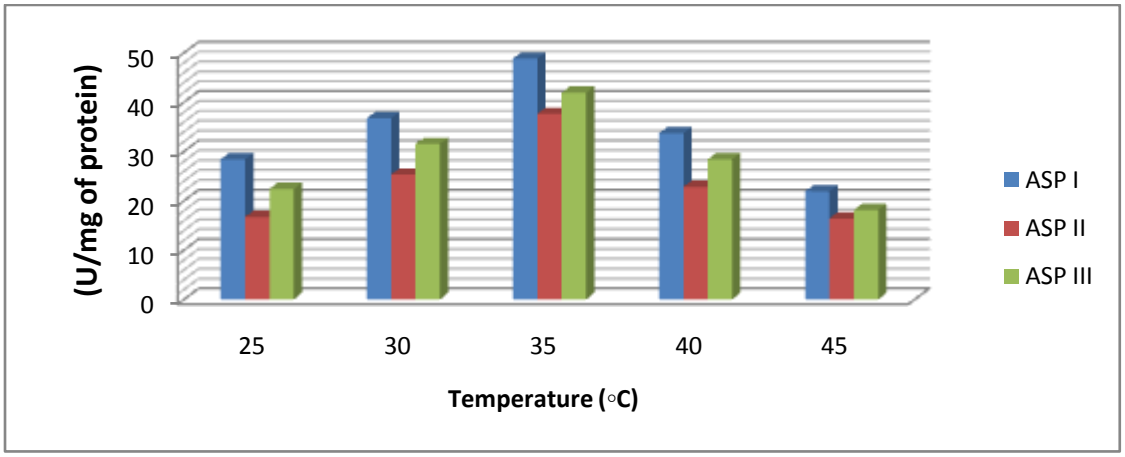

Fig. 4: Effect of temperature on amylases production by *Aspergillus* sp.

Table - 2: Standard Table for Benedict's test

S. No	Colour	Conclusion	Approximate Glucose mg/dl
1	Blue	Sugar: Absent	—
2	Green and slight yellow precipitate	Sugar: Present (+)	250 - 500
3	Green and thick Yellow precipitate	Sugar: Present (+ +)	500 -1000
4	Yellow and Orange	Sugar: Present (+ + +)	1000 -1500
5	Orange to Red Precipitate	Sugar: Present (+ + + +)	1500 - 2000
6	Brick red	Sugar: Present (+ + + + +)	more than 2000

Table – 3: Amylase activity in *Aspergillus* sp. (Benedict's Method)

S. No	Organism	Broth Conc.	Colour	Conclusion	Approximately Glucose (mg/ml)
1	ASP - I	1 %	Green and slight yellow precipitate	+	250-500
		2 %	Green and thick yellow precipitate	++	500-1000
		3 %	Green and thick yellow Precipitate	++	500-1000
2	ASP – II	1 %	Green and slight yellow precipitate	+	250-500
		2 %	Green and thick yellow precipitate	++	500-1000
		3 %	Green and thick yellow Precipitate	++	500-1000
3	ASP – III	1 %	Green and thick yellow Precipitate	++	500-1000
		2 %	Yellow and orange precipitate	+++	1000-1500
		3 %	Yellow and orange precipitate	+++	1000-1500

Table - 4: Glucose quantitative analysis (Folin Wu method) for amylase activity

Starch broth conc.	OD Value						Amount of Glucose (mg/dl)		
	ASP-I		ASP-II		ASP-III				
	Standard	Test sample	Standard	Test sample	Standard	Test sample	ASP-I	ASP-II	ASP-III
1 %	0.13	0.68	0.11	0.57	0.10	523.3	280.0	581.8	0.28
2 %	0.13	0.76	0.11	0.60	0.10	1169.2	760.3	1090.9	0.38
3 %	0.13	0.79	0.11	0.65	0.10	1823.0	1320.1	1772.7	0.44

of enzyme production by Glucose, has been reported by Nandakumar *et al*. (1999) for *A. niger* CFTRI 1105 and Alva *et al.* (2007) for *Aspergillus* sp. JGI 12. Our studies showed that soluble starch (48.3 U/mg), maltose (45.6 U/mg) and followed by lactose significantly increased enzyme production when compared to the control (Fig. 1).

Effect of the nitrogen source on growth and amylase production by *Aspergillus* sp.

The nitrogen source supplemented to the culture medium was also important to amylase production. The amylolytic activity with yeast extract (54.3 U/mg) was higher compared to peptone, Ammonium sulphate, urea and Sodium nitrate in this order. The initial pH of the culture medium decreased to 3.2. Fungal growth was considered good on all N sources (Fig. 2).

Effect of pH on the activity of the Glucoamylase

The selection of an appropriate pH is also essential for a-amylase production (McMahon *et al*., 1999). The pH of the growth medium induces morphological change in the microorganism and enzyme secretion (Asgher *et al*., 2007). To determine the pH profile of the α-amylase this enzyme was assayed using a substrate prepared in a pH range of 4.5 – 7.0. The highest activity was observed in the pH range of 4.5 to 7.0 and the maximum activity was observed at pH 6.0 (59.3 U/mg). The activity of this enzyme sharply decreased above pH 7.0 values (Fig. 3).

Effect of Temperature on the activity of the Glucoamylase

Gupta *et al.* (2008) reported on the temperature for enzyme production was 30 °C. The isolated *Aspergillus* strain was tested in a wide range of temperatures ranging from 25 to 40 °C. Maximum amylase production was obtained at a temperature of 35 °C (48.8 U/mg). The least α-amylase activity was observed at 25 ^{0}C (28.4 U/mg), but with increase in assay temperature α-amylase activity also increased (Fig. 4).

Solid-state fermentation (SSF) medium for fungal amylase production

In SSF, wheat bran was used for substrates of growth and α-amylase production by the ASP I, ASP II and ASP III. Culture media containing 5.0 g of substrate were subjected to fermentation for 4 - 5 days at pH - 5.5, temperature (35±1 °C). Wheat bran medium gave high titer of α-amylase activity (62.6 U/mg) after 96 hrs, Wheat bran had previously been reported to be the best substrate for α-amylase production (Soni *et al*., 1996; Mahmood *et al*., 1997; Ellaiah *et al*., 2002). The biosynthesis of α-amylase decreased after 96 hrs. Ramadas *et al.* (2008) reported that 96 hrs is the optimum period for amyloglucosidase synthesis by *Aspergillus niger* in SSF. Alava *et al.*, 2007, *Aspergillus* sp isolated from various seeds were screened for their ability to produce amylase. A select strain, *Aspergillus* sp. JGI 12, showed the highest amylase activity in solid state fermentation. Different substrates were screened for enzyme production. Coconut oil cake, groundnut oil cake and rice bran were found to be very good substrates for enzyme production by *Aspergillus* sp. After 96 hours, Qualitative analysis for glucose sugar present in starch broth sample was performed. In this test, *A. niger* - I type sample processed more glucose compared to the other types. The results for the qualitative test were tabulated (Table - 3) comparing with the standard chart (Table - 2). In the same way, quantitative analysis (Folin method) for glucose in the starch broth is exactly read by spectrophotometer at 420 nm (violet filter). The result obtained here also indicated that *A. niger* - I type sample contained more sugar than the other types. The result of quantitative method was shown in Table - 4.

4. Reference

1) Akpan, I., Bankole, M. O and Adesemowo, A. M. 1999. A rapid plate culture method for screening of amylase producing microorganisms. *Biotechnology Techniques*, 13(6): 411- 413.

2) Alva, S., Anupama, J., Savla, Chiu, Y.Y., Vyshail, P., Shruthi, M., Yogeetha, B. S., Bhaya, D., Purvi, J., Ruchi, K., Kumudini, B. S and Varalakshmi, K. N. 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *Biotechnology,* 31: 578 - 584.
3) Asgher, M., Asad, M. J., Rahman, S. U and Legge, R. L. 2007. A thermostable a- amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *Journal of Food Engineering*, 79: 950 – 955.
4) Bhella, R. S and Altosaar, I. 2004. Role of CAMP in the mediation of glucose catabolite repression of glucoamylase synthesis in *Aspergillus awamori*. *Current Genetics,* 14 (3): 247 - 252.
5) Carrier, F., Raphel ,V., Moreau, H., Bernadac, A., Devadac, M. A., Grimaud, R., Brawman, J., Benicourt, C., Junien, J. L., Laugier, R and Verger, R. 1991. *Gasteroenterology*, 10 (2): 1535 - 1545.
6) Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P and Srinivasulu, B. 2002. Optimization of process parameters for glucoamylase production under solid - state fermentation by a newly isolated *Aspergillus* sp., *Process Biochemistry,* 38 (4): 615 - 620.
7) Gupta, A., Gupta, V. K., Modi, D. R and Yadava, L. P. 2008. Production and Characterization of α-Amylase from *Aspergillus niger*. *Biotechnology*, 7 (3): 551 - 556.
8) Lalit, S., Bharti, K and Iyer, L. A. 2010. Purification of a bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana)* by chromatography and its use as an affinity ligand, *Journal of Chromatography,* 878 (19): 1549 - 1554.
9) Mahmood, A., M. Aurengzeb, M. A., Baig, R. Ahmad. 1997. Production of glucoamylase from agrowastes by *Rhizopus nigricans*, *Pakistan Journal of Biochemistry and Molecular Biology,* 30: 49 – 54.
10) Marian, H., Seyed, H. R., Seyed, A. S., Seyyed, M. M., Khosro, K and Mohammad, S. 2010. Development of a solid state fermentation process for production of an alpha amylase with potentially interesting properties. *Journal of Bioscience and Bioengineering* (In Press).
11) McMahon, H. E. M., Kelly, C. T and Forgarty, W. M. 1999. High maltose - producing amylolytic system of a *Streptomyces* sp. *Biotechnology Letters*, 21: 23 – 26.
12) Miller, G. L. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Annals of Chemistry,* 31: 426 - 429.
13) Omemu, A. M., Akpan, I. Bankote, M. O and Tenioda, O. D. 2005. Hydrolysis of raw tuberstarches by alpha amylase of *Aspergillus niger* isolated from the soil. *African Journal of Biotechnology*, l4 (1): 19 - 25.
14) Pandey, A. 1990. Improvement in solid state fermentation for glucoamylase production. *Biological Wastes,* 34: 11 - 19.
15) Popovic, M. K., Jamrath, T and Linder, C. 2009. Production of thermostable alpha amylase using food industry wastes. *New Biotechnology*, 25 (1): 206.
16) Sangeeta, N and Rintu, B. 2009. Characterization of amylase and protease produced by *Aspergillus awamori* in a single bioreactor. *Food Research International,* 42 (4): 443 - 448.
17) Shenoy, B. C., Katwa, L. C., Appurao, A. G. and Raghavendrarao, M. R. 1985. Fungal Glucoamylase. *Bioscience,* 7 (3 & 4): 399 – 419.
18) Soni, S. K. Kaur, A. and Gupta, J. K. 2003. A solid state fermentation Based bacterial alpha amylase and fungal glucoamylase synthesis and its suitability for hydrolysis of wheat starch. *Process Biochemistry*, 39: 185 – 192.
19) Soni, S. K., I. K. Sundhu, K. S. Bath, U. C. Banerjee, P. R. Patnaik. 1996. Extracellular amylase production by *Saccharomycopsis capsularis* and its evaluation for starch. *Folia Microbiology,* 41: 243 – 248.