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Production, Partial Purification and Characterization of α Amylase by *Aspergillus niger* Using Wheat Bran as Substrate

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ABSTRACT

Aspergillus niger available at MRD LifeSciences, Lucknow was screened for production of α amylase by starch hydrolysis test on starch agar plate inoculated with *Aspergillus niger*. *Aspergillus niger* was studied for its growth parameters including growth curve, effect of pH and temperature. Culture reached the stationary phase on 4-5 days, it showed maximum growth at pH 6.2 and at 28 °C. Production of α amylase was done by solid state fermentation using wheat bran as substrate. Crude extract obtained was partially purified by ammonium sulphate precipitation and dialysis. Crude and partially purified enzyme showed an activity of 0.08 U/ml/min and 0.036 U/ml/min respectively. Purified enzyme was characterized for effect of pH and temperature and was found to be stable upto pH 10 and 50 °C giving an indication of its extremophilic nature.

Key words: *Aspergillus niger*, α Amylase, Ammonium Sulphate Precipitation, Dialysis, Solid State Fermentation.

INTRODUCTION

Amylase is an enzyme that catalyses the hydrolysis of starch into simple sugars. It has been classified into **alpha-Amylase** (EC 3.2.1.1) also named as 4-α-D-glucan glucanohydrolase; glycogenase) α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium. They act at random locations along the starch chain and break down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. **β Amylase** (EC 3.2.1.2) also called as 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase), works from the non-reducing end, β-amylase catalyzes the hydrolysis of the

second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. **γ-Amylase** (EC 3.2.1.3) also called as Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase, cleaves α (1-6) glycosidic linkages in addition to cleaving the last α (1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose.

Amylases have a wide range of applications in a large number of industries including food, textile, paper and pharmaceutical etc. Although amylases are obtained from plant, animal and microbial sources the microbial amylase production has been found to be practical and economical. In the microbial system fungal amylases have been found to be more economical because of the low rather no cost of substrates used for Solid state fermentation and also fungal α amylase is used for its use in formulations for human or animal consumption involving applications under acidic conditions and around 37°C [1]. Because of the importance of fungal α amylases a large number of fungal species have been explored for the production of α amylases by various researchers including [2-7].

Taking into consideration the previous research work and the need of lowering the cost of α amylase present investigation was also designed for production and partial purification of α amylase by *Aspergillus niger* using wheat bran as substrate.

MATERIALS AND METHODS

Aspergillus niger

Aspergillus niger available at MRD LifeSciences, Lucknow was sub cultured on PDA plates by point inoculation and used throughout the study.

Screening of *Aspergillus niger* for α amylase production

Aspergillus niger was screened for α amylase production efficiency in starch agar media comprising the following in gm L^{-1}yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15, pH 5.6 [8]. *Aspergillus niger* was inoculated at the centre on sterile solidified starch agar plates, a blank without inoculation was also maintained for comparison. Plates were incubated at 28 °C for 48 hours after that the test plates along with blank was flooded with iodine and observed for zone of hydrolysis.

Study of growth parameters of *Aspergillus niger*

Growth parameters of the *Aspergillus niger* which showed good starch hydrolysis was studied in terms of:

a) Growth Kinetics

Growth curve of the *Aspergillus niger* was studied in order to have an idea about the phases of growth. For studying the growth curve 100 ml PDB was prepared, divided in two flasks containing 80 ml and 20 ml and autoclaved. After cooling the flask containing 80 ml PDB was inoculated with a loop full of *Aspergillus niger* showing maximum starch hydrolysis. The inoculated flask was incubated at 28 °C at

100rmp and the uninoculated flask was stored as blank, the growth of isolate was tracked for 7 days by reading the absorbance at 600 nm against blank. After that a curve was plotted between days on X axis and OD at 600nm on Y axis.

b) Optimization of pH for maximum growth of *Aspergillus niger*

pH optimization was done so that the production could be carried out at that pH. Four flaks containing 20 ml PDB maintained at pH 5, 5.6, 5.9, and 6.2 respectively were prepared and autoclaved. All the four were inoculated with *Aspergillus niger* and incubated at 28 °C at 100 rmp for 48 hours. After that growth of *Aspergillus niger* in all the flasks was studied by reading the absorbance of inoculated flasks at 600 nm against uninoculated PDB.

c) Optimization of temperature for maximum growth of *Aspergillus niger*

Temperature optimization was done so that the production could be carried out at that temperature. Four sterile PDA plates were prepared and inoculated with *Aspergillus niger* by point inoculation, plates were incubated at 22 °C, 28 °C, 37 °C and 50 °C respectively for 48 hours. Growth was quantified based on visual identification.

Production of amylases by Solid state fermentation using wheat bran as substrate

Production of amylase was carried out by SSF, 15gm of powdered wheat bran was taken in 250 ml flask and moistened with nearly 50 ml of MSM (minimal salt media) containing the following in gm/l (0.8 g NaCl , 0.8 g KCl , 0.1 g CaCl2 , 2.0 g Na_2HPO4 , 0.2g $MgSO_4$, 0.1 g $FeSO_4$, 8.0

g Glucose, 2.0 g NH_4Cl pH 6.2). Flask was autoclaved, cooled to room temperature, inoculated with 1 ml of 48 hour old grown broth culture of *Aspergillus niger* and incubated at 28 °C for 5 days.

Extraction of crude enzyme

Crude enzyme was extracted from fermented media by adding 50ml of 0.1 M Tris buffer pH 6.2, agitating the flask in shaker at 180 rpm for 1 hour, the mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4ºC for 15 min. The supernatant was collected and treated as crude enzyme.

Partial purification of crude enzyme

Crude enzyme was partially purified by ammonium sulphate precipitation upto 70 % saturation, wherein ammonium sulphate was added pinch by pinch when the beaker containing crude extract was kept on magnetic stirrer under ice cold conditions. Once all the salt was dissolved the beaker was kept at 4 °C, later the salt precipitated crude extract was transferred to centrifuge tubes and spun at 10000 rpm for 15 minutes at 4 °C, pellet obtained were dissolved in 10 ml of 0.1 M Tris buffer pH 6.2. Pellet dissolved in buffer was tied in activated dialysis bag of 1000 Daltons cut off, and left for dialysis in 0.1 M Tris buffer pH 6.2, whole procedure was performed at 4 °C, buffer was changed after every 2 hours. The

enzyme in the dialysis bag was collected and treated as partially purified enzyme.

Protein estimation in crude and partially purified enzyme

Concentration of protein in crude and partially purified enzyme was determined by Lowry's method [9] of protein estimation in which enzyme was reacted with the Lowry's reagents and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein with known concentrations (0.02 mg/ml to 0.2 mg/ml) with the Lowry's reagents and plotting a graph between concentration of standard protein (BSA) on X axis and absorbance at 660nm on Y axis.

Enzyme assay in crude and partially purified enzyme

Enzyme assay was carried out by DNS method [10] in which 0.5 ml enzyme was reacted with 0.5 ml of substrate (1% starch in 0.1M Tris buffer pH 6.2) under standard reaction conditions and the reaction was stopped by adding DNS reagent, amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540 nm with the standard graph plotted by reacting the known concentration of maltose (0.05mg/ml to 0.5mg/ml). One unit amylase activity was defined as amount of enzyme that releases 1 micromoles of maltose per minute under standard reaction conditions.

Characterization of partially purified enzyme

Partially purified enzyme was characterized for the effect of pH and temperature. For the effect of pH enzyme substrate reaction was carried out at different pH (5.9, 6.2, 6.5, 7.0, 8.0, 9.0 and 10) based on the method [10] described earlier. Similarly for the effect of temperature enzyme substrate reaction was carried out at different temperatures (22 °C, 28 °C, 37 °C, and 50 °C).

RESULTS

Screening of fungal *Aspergillus niger* for α amylase production

Aspergillus niger showed good starch hydrolysis and was marked as +++ on the basis of visual identification.

Study of growth parameters of *Aspergillus niger*

Growth parameters including growth curve, effect of pH and temperature on growth of *Aspergillus niger* were studied in order to have a proper idea of the stationary phase, optimum temperature and pH of the isolate so that the same environment could be provided during fermentation procedure.

a)Growth Kinetics

Table 1 and Figure 1 below show the growth kinetics statistics of the *Aspergillus niger* it can be seen that stationary phase reached between day 4-5.

b)Optimization of pH for maximum growth of *Aspergillus niger*

pH optimization experiment was carried out in order to have an idea of the best pH for the growth of *Aspergillus niger.* Table 2 and Figure 2 below shows the data of the pH optimization experiments.

c)Optimization of temperature for maximum growth of *Aspergillus niger*

Temperature optimization experiments were carried out in order to have an idea of the optimum temperature for growth of *Aspergillus niger.* Table 3 below show the result of the same.

d)Protein estimation in crude and partially purified enzyme

Concentration of protein in crude and partially purified enzyme was determined by Lowry's method described earlier, results of the same can be seen below in Table 4 below.

e) Enzyme assay in crude and partially purified enzyme

Enzyme assay was performed by standard method described earlier, and the results of the same can be seen in Table 5 below.

f)Characterization of partially purified enzyme

Purified enzyme was characterized for the effect of temperature and pH and it was found that the enzyme was stable upto 50 °C, and it was also stable upto pH 10. Results of the same can be seen in Table 6-7 and Figure 3-4 below.

1)Effect of temperature on enzyme activity

Table 7 and Figure 3 below shows the results of effect of Temperature on the activity of partially purified enzyme.

2)Effect of pH on enzyme activity

Table 6 and Figure 4 below shows the results of effect of pH on activity of partially purified enzyme

Table 1: Growth Kinetics

S. No.	TIME (IN DAYS)	O.D AT 600nm
1.	0	0.0
2.	1	0.01
3.	2	0.02
4.	3	0.03
5.	4	0.05
6.	5	0.05
7.	6	0.02
8.	7	0.01

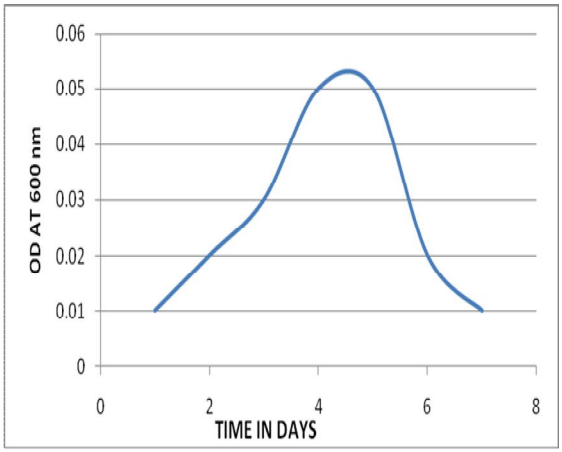

Figure 1: Growth curve

Table 2: Effect of pH on growth

S. No.	pH OF MEDIA	OD AT 600nm
1.	5.0	0.13
2.	5.6	0.10
3.	5.9	0.15
4.	6.2	0.16

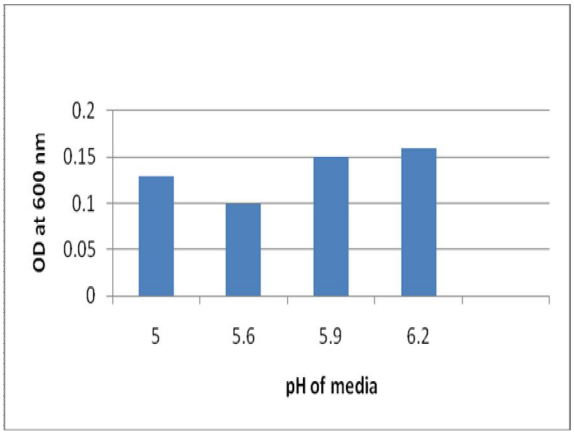

Figure 2: Effect of pH on growth

Table 3: Effect of temperature on growth

S. No.	INCUBATION TEMPERATURE (In °C)	REMARKS
1.	22	-
2.	28	+++
3.	37	++
4.	50	–

Table 4: Protein estimation in crude and purified enzyme

S. No.	ENZYME (IN ml)	DISTILLED WATER (IN ml)	REAGENT C (IN ml)	INCUBATED AT ROOM TEMP. FOR 15 MINUTES	REAGENT D (IN ml)	INCUBATED FOR 30 MINUTES IN DARK	O.D.	CONC. OF PROTEIN (mg/ml)
BLANK	0.0	1	5		0.5		0.0	0.0
CRUDE EXTRACT	0.5	0.5	5		0.5		0.51	0.187
PURE ENZYME	0.5	0.5	5		0.5		0.36	0.13

Table 5: Enzyme assay of crude and partially purified enzymes

S No.	ENZYME (in ml)	1% STARCH (in ml)	INCUBATED AT 37°C FOR 15 MINUTES	DNS (in ml)	BOIL FOR 15 MINUTES AT 100°C	O.D AT 540 nm	Enzyme activity (U/ml/min)
BLANK	0	0		1		0.0	0.0
CRUDE EXTRACT	0.5	0.5		1		1.62	0.08

PURE ENZYME	0.5	0.5		1		0.73	0.036

Table 6: Effect of temperature on enzyme activity

S. No.	Incubation temperature (In °C)	OD AT 540 nm	Enzyme activity (U/ml/min)
1	22	0.69	0.029
2	28	0.73	0.036
3	37	0.77	0.038
4	50	0.80	0.039

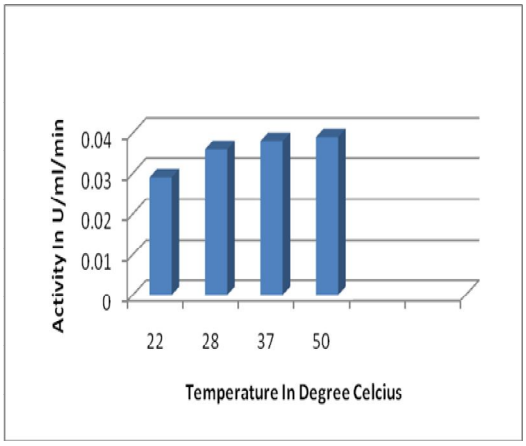

Figure 3: Effect of Temperature on enzyme activity

Table 7: Effect of pH on enzyme activity

S. No.	pH	OD AT 540nm	Enzyme activity (U/ml/min)
1	5.9	0.71	0.030
2	6.2	0.70	0.029
3	6.5	0.72	0.036
4	7.0	0.59	0.020
5	8	0.74	0.041
6	9	0.65	0.022
7	10	0.63	0.020

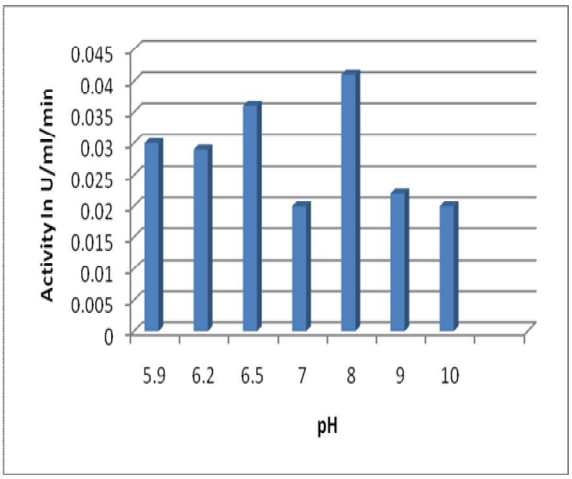

Figure 4: Effect of pH on enzyme activity

DISCUSSION

Aspergillus niger was screened for the production of α amylase enzyme by inoculating it on starch agar media and later performing the iodine test for starch hydrolysis as has been reported by [8].

After screening *Aspergillus niger* was studied for its growth parameters including growth curve, effect of pH on growth and effect of temperature on growth. It was found that the stationary phase was reached on 4-5 day of inoculation, growth was optimum at pH 6.2 and at 28 °C.

Production of enzyme was carried out by solid state fermentation using wheat bran as a substrate, solid state fermentation has been user earlier by [11] for production of α amylases.

Wheat bran as a substrate has been of interest because of the low cost and easy availability, earlier wheat bran has been used for the production of α amylase by.

Crude enzyme was extracted by adding a buffer to the fermented media, agitating the same filtering and centrifuging it, supernatant was collected and treated as crude α amylase as done earlier by [11].

Crude enzyme was partially purified by ammonium sulphate precipitation and dialysis, similar procedure has been used earlier by [7].

Partially purified enzyme was characterized for the effect of temperature and pH and it was found to be stable upto a temperature of 50 °C, giving an indication of its stability at higher temperature. Enzyme was also found to be stable upto pH 10, giving an indication of its alkaline nature which is an added advantage of it being used for detergent industry.

CONCLUSION

Based on the above investigation it can be stated that *Aspergillus niger* used in this study can be a good source for the production of α amylase using wheat bran as substrate. The enzyme purified here was found to be stable upto 50 °C and upto pH 10 also giving an indication of its extremophilic nature giving it added advantage for its industrial application.

Future aspects of the present investigation includes optimization of some pretreatment procedures for enhancing the activity of enzyme. It also includes further purification of the enzyme using sophisticated techniques like ion exchange chromatography, affinity chromatography etc. Further characterization of the purified enzyme in terms of effect of substrate concentration, activators and inhibitors.

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