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PRODUCTION OF ALPHA AMYLASES BY *ASPERGILLUS NIGER* USING CHEAPER SUBSTRATES EMPLOYING SOLID STATE FERMENTATION

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ABSTRACT : Four fungal isolates from soil were screened for alpha amylase production and the isolate MJSU1101 later identified as *Aspergillus niger* was found to have best activity among all the four isolates. Growth of *Aspergillus niger* was found to be optimum at 28°C and pH 6.2. SSF was carried out using four substrates namely wheat bran, rice husk, vegetable waste (potato, tomato, brinjal) and banana peel. Alpha amylase produced using all the four substrates was having good activity but wheat bran as a substrate was the best giving an activity of 0.08U/ml/min followed by vegetable waste (0.06U/ml/min), banana peels (0.05U/ml/min) and rice husk (0.045U/ml/min).

Key words: Alpha amylase, *Aspergillus niger*, Substrate, Solid state fermentation.

INTRODUCTION

Alpha-Amylase (EC 3.2.1.1) also named as 4-α-D-glucan glucanohydrolase, has found its application in a range of industries including food, brewing, distilling industry, textile, paper pharmaceutical and bioconversion of solid waste etc [1,2]. Large range of applications is the triggering factor for the industrialization of alpha amylase production. Amylases have been reported to be produced by plant, animal and microbial sources, although the microbial amylase production has been reported to be most effective. The synthetic media used for the production of amylases have been a bit costlier and that's why is a matter of concern for the researchers. Researchers are now busy in search of procedures to cut short the cost of production. Solid state fermentation which has been reported to be a bit cheaper because of the enzyme extraction procedures [3] is a ray of hope. In case of SSF the cost of the substrate also plays a key role in deciding the cost of production. Agro industrial wastes have been reported to be good substrate for the cost effective production of alpha amylases [4, 5] and are thus attracting researchers for using agro industrial waste as a substrate for alpha amylase production. Fungal species have been studied a lot for the production of alpha amylase [3, 5, 6, 7, 8] because of the low cost of substrates used for the production of alpha amylases. Thus the present study was designed in the search of cheaper carbon sources for the production of alpha amylase enzyme by fungal strains.

MATERIALS AND METHODS

Isolation of Fungi from Soil

Fungal colonies were isolated form soil samples enriched for amylase producing microorganisms by serial dilution method wherein PDA (potato dextrose agar) media was prepared, autoclaved and poured in sterile petriplates. 50µl of soil samples diluted upto 10^{-5} dilutions were spread on respective solidified PDA plates. The inoculated petriplates were incubated at 28°C for 48 hours. Four different fungal isolates differentiated on the basis of physical characteristics obtained after incubation were named as MJS1101, MJS1102, MJS1103 and MJS1104. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours in order to obtain pure fungal plates.

Screening of Fungal Isolates for Amylase Production

All the four fungal isolates were screened for amylase production efficiency in starch agar media comprising the following in gm L-1yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15, pH 5.6. All the four isolates were streaked centrally 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 all the plates along with blank were flooded with iodine and observed for zone of hydrolysis.

Identification of the Isolate (MJS1101) Showing Maximum Starch Hydrolysis During Screening

The isolate showing maximum zone of hydrolysis was identified based on its physical and staining/microscopic (Lactophenol cotton blue) characteristics [9].

Study of Growth Parameters of the Isolate showing Maximum Starch Hydrolysis During Screening

Growth parameters of the isolate MJS1101 showing maximum hydrolysis were studied in term of growth kinetics, effect of pH, effect of temperature on growth.

a) Growth Kinetics

Growth curve of the isolate MJS1101 was studied in order to get an idea about phases of growth. For studying the growth curve 100ml PDB was prepared, divided in two flasks containing 80ml and 20ml respectively and autoclaved. After cooling the flask containing 80 ml PDB was inoculated with a loop full of fungal isolate showing maximum 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 600nm against blank. After that a curve was plotted between days on X axis and OD at 600nm on Y axis.

b) **Optimization of Temperature for Maximum Growth of MJS1101**

In order to get optimum production of Amylases by showing maximum hydrolysis during screening the temperature optimization experiment was carried out so that the same can be used during fermentation procedure. For optimizing the temperature for the best growth of the isolate showing maximum hydrolysis during screening, 60 ml of autoclaved PDA media was poured in four sterile petriplates and after solidification all the four plates were inoculated with the fungal isolate showing maximum hydrolysis during screening by point inoculation. Plates were incubated at 28°C for 48 hours and observed for growth of the isolate.

c) **Optimization of pH for Maximum Growth**

In order to get optimum production of α amylases by isolate showing maximum hydrolysis during screening the pH optimization experiment was carried out so that the same can be used during fermentation procedure wherein four flaks containing 20 ml PDB maintained at pH 5, 5.6, 5.9, and 6.2 respectively were prepared and autoclaved. Before inoculation 3ml of PDB from each flask was transferred to sterile and labeled eppendrof tubes and stored as blank. After that each flask was inoculated with 10µl of 48 hour grown broth of showing maximum hydrolysis during screening. The inoculated flasks were incubated at 28°C at 100rmp for 48 hours. Growth in the flasks was studied by reading the absorbance at 600nm against blank.

Production of Alpha Amylases by Solid State Fermentation Using Cheap Substrates Cheap Substrates Used

Wheat Bran, Rice Husk, Vegetable Waste (Potato, Tomato, Brinjal) and Banana Peel.

Production

Production of amylase was carried out by SSF using the substrates of zero cost namely Wheat Bran, Rice Husk, Vegetable Waste (Potato, Tomato, Brinjal) and Banana Peel. For SSF 20gm of powdered wheat bran and rice husk were taken in 250ml flasks and moistened with nearly 50ml of MSM containing the following in gm/l (0.8 g NaCl , 0.8 g KCl , 0.1 g $CaCl_2$, 2.0 g Na_2HPO_4 , 0.2g MgSO4 , 0.1 g FeSO4, 8.0 g Glucose, 2.0 g NH_4Cl pH 6.2). Flasks were autoclaved, cooled to room temperature, inoculated with 1ml of 48 hour old grown broth culture of showing maximum hydrolysis during screening and incubated at 28°C for 5 days. Vegetable waste and peels of banana to be used as substrate were washed several times with distilled water, dried under shed, rinsed with 0.1% H_2SO_4, cut into small pieces and ground by the help of sterile mortar and pestle. 20 gm of the resultant pastes of vegetable waste and banana peels were transferred into 250ml flask, and moistened with MSM. Both the flasks were autoclaved, cooled to room temperature, inoculated with 1ml of 48 hour old grown broth culture of showing maximum hydrolysis during screening and incubated at 28°C for 5 days.

Extraction of Crude Enzyme

Crude enzyme was extracted from fermented media by adding 100ml of 100mM Tris buffer pH 6.2, agitating the flask in shaker at 180rpm for 1hour, the mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4ºC for 5 min. The supernatant was collected and treated as crude enzyme.

Protein Estimation in Crude Enzyme

Concentration of protein in crude enzyme (extracted from four flasks containing different substrates) was determined by Lowry's method [10] 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 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 Enzyme

Enzyme assay was carried out by DNS method of [11] in which 0.5ml enzyme was reacted with 0.5ml of substrate (1% starch in 100mM Tris buffer) 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 ranging from 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.

RESULTS

Isolation of Fungi

Four different fungal isolates differentiated on the basis of colony morphology were obtained after spreading, and were named tentatively as MJS1101, MJS1102, MJS1103 and MJS1104. All the four isolates were subcultures by point inoculation and used for further studies.

Screening of Fungal Isolates for Alpha Amylase Production

All the four fungal isolates were subjected to screening procedure and after completion of incubation period plates were flooded with iodine solution and observed for zone of hydrolysis. The results of the same can be seen below in Table 1 and Figure 1.

Table 1: Screening for Alpha Amylase Production

S. No.	ISOLATE	RESULT
1.	MJS1101	+++
2.	MJS1102	+
3.	MJS1103	++
4.	MJS1104	+

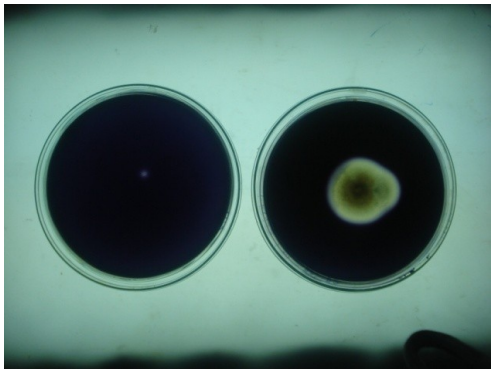

Figure 1: Screening Plates

Identification of the Isolate Showing Maximum Hydrolysis

Based on morphological studies, and Lactophenol cotton blue staining characteristics the isolate MJS1101 was identified as ***Aspergillus niger.*** **Figure 2** below shows the pure culture of ***Aspergillus niger.***

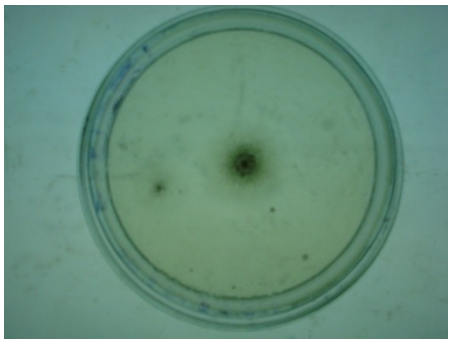

Figure 2: ***Aspergillus niger***

Study of Growth Parameters of the Isolate (MJS1101)

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

a) Growth Kinetics

Table 2 and Figure 3 below show the growth kinetics statistics of the isolate MJS1101 **(*Aspergillus niger*),** it can be seen that stationary phase reached between day 4-5.

Table 2: 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

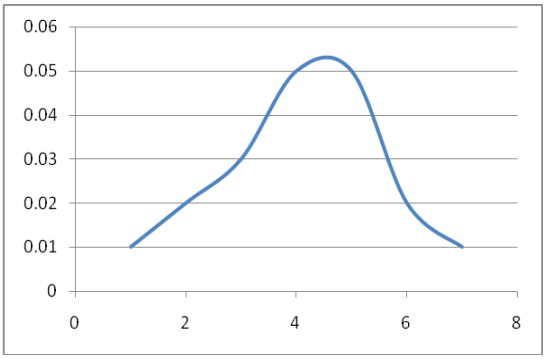

Figure 3: Growth curve (X axis: Time in Days; Y axis: OD at 600nm)

b) **Optimization of Temperature for Maximum Growth of MJS1101**

The fungal isolate was grown at various temperatures and it can be seen from the **Table 3** below that 28 °C is the temperature at which maximum growth was seen.

Table 3: Effect of Temperature on Growth

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

c) Optimization of pH for Maximum Growth of MJS1101

The fungal isolate showing maximum starch hydrolysis was grown at various pH and pH 6.2 was found to be the optimum pH for growth of the same.

Table 4: 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

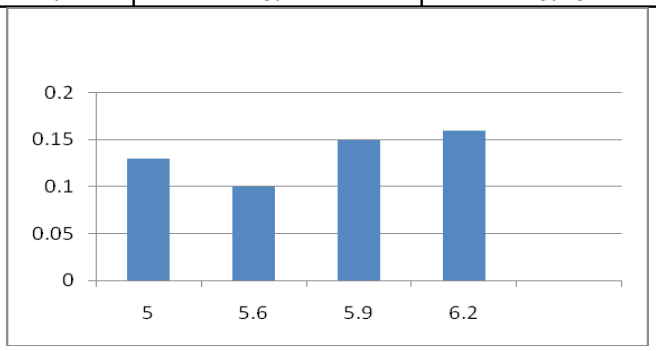

Figure 4: Effect of pH (X axis: pH; Y axis: OD at 600nm)

Production of Alpha Amylases by Solid State Fermentation Using Cheap Substrates

The culture showing maximum zone during screening later identified as *Aspergillus niger* was inoculated in SSF flasks containing different substrates, Figure 5 below shows the SSF flasks.

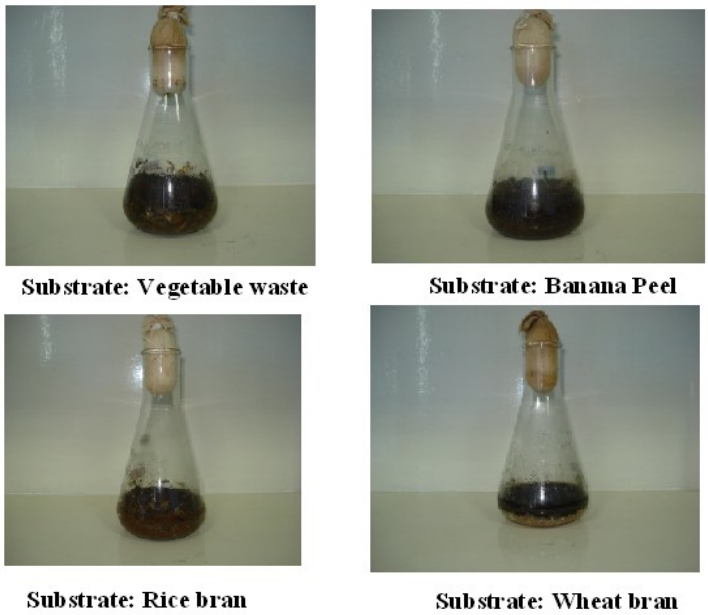

Figure 5: Solid State Fermentation Flasks

Protein Estimation in Crude Enzyme

Amount of protein in the crude enzymes extracted from fermented flasks was determined by Lowry's method and the results of the same can be seen in **Table 5** below.

Table 5: Protein Estimation in Crude 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 Enxyme (Rice husk)	0.5	0.5	5		0.5		0.16	0.059
Crude Enzyme (Banana Peel)	0.5	0.5	5		0.5		0.19	0.07
Crude Enzyme (Vegetable Waste)	0.5	0.5	5		0.5		0.34	0.125
Crude Enzyme (Wheat Bran)	0.5	0.5	5		0.5		0.51	0.187

Enzyme Assay of Crude Enzyme

Enzyme activity in the extracted enzymes was determined by DNS assay and the results of the same can be seen in **Table 6.**

Table 6: Enzyme Assay of Crude Enzymes

S No.	ENZYME (in ml)	1% STARCH (in ml)	INCUBATED AT 28°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 Enzyme (Rice Husk)	0.5	0.5		1		0.93	0.047
Crude Enzyme (Banana Peel)	0.5	0.5		1		1.03	0.05
Crude Enzyme (Vegetable Waste)	0.5	0.5		1		1.37	0.06
Crude Enzyme (Wheat Bran)	0.5	0.5		1		1.62	0.08

Comparative Study of Alpha Amylase Activity of all the Substrates

The histogram below in **Figure 6** shows a comparison between the alpha amylase activity in the flasks containing different substrates used in the present study. It can be seen that maximum amylase activity was seen when wheat bran was used as substrate followed by vegetable waste, banana peel and rice husk.

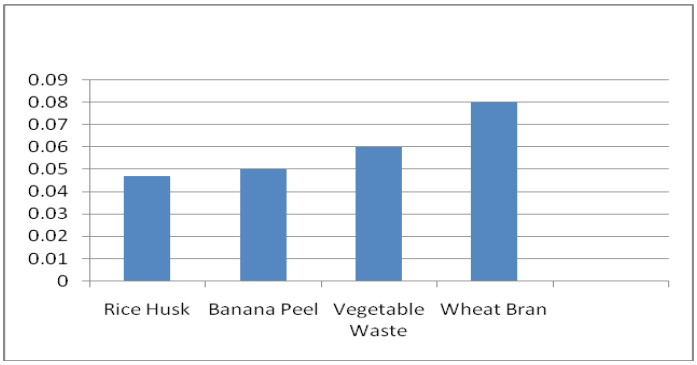

Figure 6: Comparative Study of Alpha Amylase Activity of all the Substrates (X axis: Substrate; Y axis: Activity in U/ml/min)

DISCUSSION

Isolation of fungal strains was done from soil enriched for amylase producing microorganisms using serial dilution agar plate method. Screening of the fungal isolates for amylase production was carried out in starch agar plates followed by iodine test as done earlier by **[12]** For SSF various agro industrial wastes including wheat bran, rice husk, vegetable waste (potato, tomato, brinjal) and banana peel were used as substrate. All the four were found to be good substrates as the alpha amylase activity was seen in all the four flasks. Enzyme activity was maximum in the flask containing wheat bran as substrate and it was found to be 0.08U/ml/min followed by vegetable waste (0.06U/ml/min), banana peel (0.05U/ml/min) and rice husk (0.047U/ml/min).

CONCLUSION

Based on the above study it can be concluded that wheat bran can be a very good substrate for the production of alpha amylase and can be help full in reducing the production cost. Other substrates (vegetable waste, rice husk and banana peels) used in the study can also be used industrially for alpha amylase production but after proper optimization.

Future prospects of the present study includes further optimization of the pre treatment procedures of the substrate, optimization of incubation time in order to increase the production of enzyme.

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