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PRODUCTION OF CELLULASE USING CHEAP SUBSTRATES BY SOLID STATE FERMENTATION

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ABSTRACT: Four fungal isolates were isolated from soil and screened for cellulase production, out of the four isolates MJS1101 later identified as *Aspergillus niger* showed maximum zone of hydrolysis. Growth of the isolate was found to be maximum at 28°C and at pH 6.2. Four substrates (saw dust, corncob, wheat straw and newspaper) were used for fermentation and cellulase was produced by all of them but corncob gave maximum CMCase activity of 0.027U/ml/min followed by wheat straw (0.024U/ml/min), newspaper (0.020 U/ml/min) and saw dust (0.018U/ml/min).

Keywords: *Aspergillus niger,* CMCase, Solid state fermentation, Cellulases.

INTRODUCION

Cellulases are enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze hydrolysis of cellulose. The EC number of for this group of enzymes is EC 3.2.1.4. Cellulases have been divided into five types based on their mode of hydrolysis a) Endocellulase which breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains [1] b) Exocellulase which cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharides such as cellobiose. c) Cellobiase or beta glucosidase hydrolyses the exocellulase product into individual monosaccharides [2] d) Oxidative cellulases depolymerize cellulose by radical reactions, as for instance. e) Cellulose phosphorylases swhich depolymerize cellulose using phosphates instead of water. Cellulases have found a wide range of applications in coffee processing, textile industry and in laundry detergents, pulp and paper industry, pharmaceutical industry, biofuel industry etc [3]. Large demand of cellulases has increased their prices to a large extent and the major reason is the cost of substrate, and fermentation procedure it is the need of the time to search for cheaper substrates and reduced fermentation cost so that the production cost can be reduced to a large extent. The present investigation also focuses on the same and that's why we are trying to search for substrates with zero cost. We are also trying to use solid state fermentation which is a bit cheaper in comparison to submerged fermentation [4-5].

MATERIALS AND METHODS

Isolation of fungi

For isolation of fungi soil sample was collected from the area containing decaying leaves on surface in sterile polybag and transferred to the laboratory. 0.5 g soil was subjected to serial dilution upto 10^{-5} dilution and 50 µl diluted sample was spreaded on sterile PDA (Potato Dextrose Agar) plates. Inoculated pates were incubated at 28°C for 48 hours. After incubation four different fungal colonies were picked based on morphological differences and named as MJSU1101, MJSU1102, MJSU1103 and MJSU1104. All the four colonies were further sub cultured by point inoculation on sterile PDA plates.

Screening of isolates for cellulase production

All the four fungal isolates were screened for their cellulase producing abilities on CMC agar plates as reported [6] in which all the four isolates were streaked centrally on CMC agar media containing 1% CMC and incubated at 28°C for 48hours. After the completion of incubation period the plates were flooded with 0.1% Congo red solution and washed with 1 M NaCl for 15–20 min. Plates were observed for zone of cellulose hydrolysis.

Identification of the isolate showing maximum hydrolysis

The fungal isolate showing maximum hydrolysis was identified by the routine physical and microscopic identification procedure [6].

Study of growth parameters

a) **Growth Kinetics**

The fungal isolate showing maximum hydrolysis was studied for its growth pattern wherein 100ml PBD was prepared and divided into two flasks containing 80ml and 20ml media respectively. Both the flasks were autoclaved, cooled to room temperature and the flask containing 80ml media was inoculated with the fungal isolate showing maximum cellulose hydrolysis. Incubated at 28°C & 120rpm, growth of the isolate was studied by reading the absorbance at 600nm against uninoculated media for 8 days.

b) **Effect of Temperature on Growth of Isolate**

In order to know the optimum temperature for the growth of fungal isolate, it was streaked on four sterile PDA plates, and incubated at various temperatures as 28°C, 37°C, 18°C and 50°C for 48 hours. After incubation the growth of isolate was quantified based on visual identification.

c) **Effect of pH on Growth of Isolate**

In order to know the optimum pH for the growth of fungal isolate, it was inoculated in four flasks containing 20ml of PDB each maintained at different pH i.e pH 5.2, 5.6, 5.9 and 6.2 respectively. All the four flasks were incubated in shaker incubator at 120 rpm at 28 C for 48 hours. After that growth of fungal isolate was studied by reading the absorbance of the flasks at 600nm against uninoculated PDA.

Production of Cellulases By Solid State Fermentation Using Cheap Substrates

Substrates Used: Saw dust, Corncob, Wheat straw and Newspaper. Corncob and wheat straw were pretreated with 0.1% H2SO4, Saw dust with 2N NaOH and Newspaper with distilled water.

Production

20 gm each of pretreated substrates mentioned above were taken in 250 ml flasks, moistened with 50 ml of Mineral salt media i.e MSM comprising the following in gm/l (0.8 g NaCl , 0.8 g KCl , 0.1 g CaCl2 , 2.0 g Na2HPo4 , 0.2g MgSO4 , 0.1 g FeSO4, 8.0 g Glucose, 2.0 g NH4Cl pH 6.2), all the four flasks were autoclaved and cooled to room temperature. After cooling all were inoculated with 1ml of 48 hour old broth culture of the culture showing maximum cellulose hydrolysis during screening. Flasks were incubated in shaker incubator (120rpm) at 28°C for 120 hours.

Extraction of crude enzyme

100ml of 100mm Tris buffer was added to each flask after completion of incubation period, shaken at 150 rpm for 1hour and filtered by the help of masculine cloth, later by whatman's filter paper No.1 and filtrate was centrifuged at 8000rpm for 5 minutes at 4°C. After centrifugation supernatant was collected and used as crude enzyme.

Protein Estimation in Crude Enzyme

Amount of protein in crude enzyme was determined by Lowry's method [7] of protein estimation, in which 0.5ml of crude enzyme, 0.5ml of distilled water was reacted with 5ml of Lowry's reagent C and 0.5ml of Reagent D and the absorbance was read at 660nm. Absorbance was compared with the standard graph prepared by reacting known concentration of protein ranging from 0.02 mg/ml to 0.20mg/ml with the Lowry's reagents and plotting a graph between concentration of protein BSA (X axis) and OD at 660 nm (Y axis).

Enzyme Assay in Crude Enzyme

CMCase activity in crude enzyme was determined by DNS method of [8] in which 0.1ml of enzyme was reacted with the substrate (1% CMC in 100 mm Tris buffer) for 15 minutes at 28°C and the reaction was stopped by adding DNS reagent and the test tubes were boiled for 15 minutes and absorbance was read at 540nm. Absorbance was compared with the standard graph plotted by reacting known concentration of glucose (0.05 to 0.5mg/ml) with DNS reagent and plotting a graph between concentration of glucose (X axis) and OD at 540nm (Y axis). One unit CMCase activity was defined as amount of enzyme that releases 1 micromoles of glucose per minute under standard reaction conditions.

RESULTS

Isolation of Fungi

Four different fungal isolates namely MJSU1101, MJSU1102, MJSU1103 and MJSU1104 were obtained in the mixed culture plate and were sub cultured by point inoculation.

Screening of Isolates for Cellulase Production

All the four cultures were screened for their cellulase producing potential, hydrolysis zones were observed, Table 1 and Figure 1 below show the results of screening.

Table 1: Screening for cellulase

S. No.	ISOLATE	RESULT
1.	MJSU1101	++++
2.	MJSU1102	-
3.	MJSU1103	+++
4.	MJSU1104	++

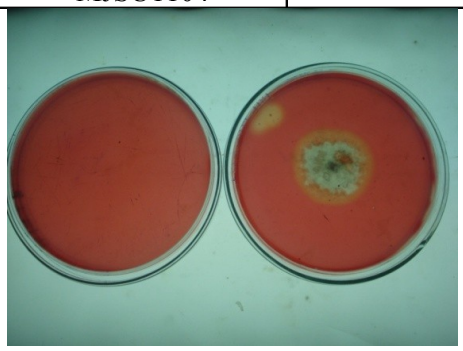

Figure 1: Screening Plates

Identification of the Isolate Showing Maximum Hydrolysis

Based on morphological studies, and Lactophenol cotton blue staining characteristics (the isolate MJSU1101 was identified as *Aspergillus niger.* Figure 2 below shows the *Aspergillus niger* plate.

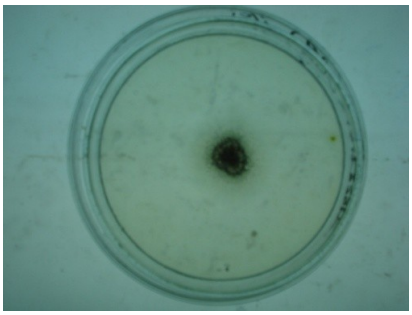

Figure 2: *Aspergillus niger*

Study of Growth Parameters

Growth parameters including growth curve, pH and temperature 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 MJSU1101 **(*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	0.05
5.	4	0.07
6.	5	0.07
7.	6	0.06
8.	7	0.05
9.	8	0.04

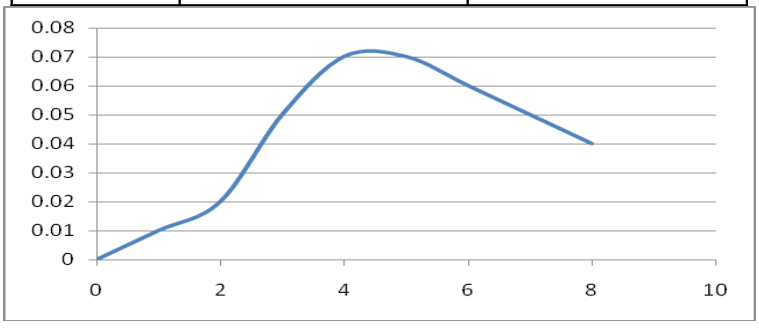

Figure 3: Growth Kinetics (X axis: Time in days; Y axis: OD at 600nm)

b) **Effect of Temperature on Growth of Isolate MJSU1101**

Effect of temperature was studied on growth of isolate MJSU1101 and it can be seen from the **Table 3** below that the isolate grows maximally at MJSU1101.

Table 3: Effect of Temperature on Growth

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

c) **Effect of pH on Growth of Isolate MJSU1101**

Effect of pH of media was studied on the growth of isolate MJSU1101, Table 4 and Figure 4 show that maximum growth was seen at pH 6.2

Table 4: Effect of pH on growth

S. No.	pH OF MEDIA	OD AT 600nm
1.	5.0	0.17
2.	5.6	0.13
3.	5.9	0.12
4.	6.2	0.21

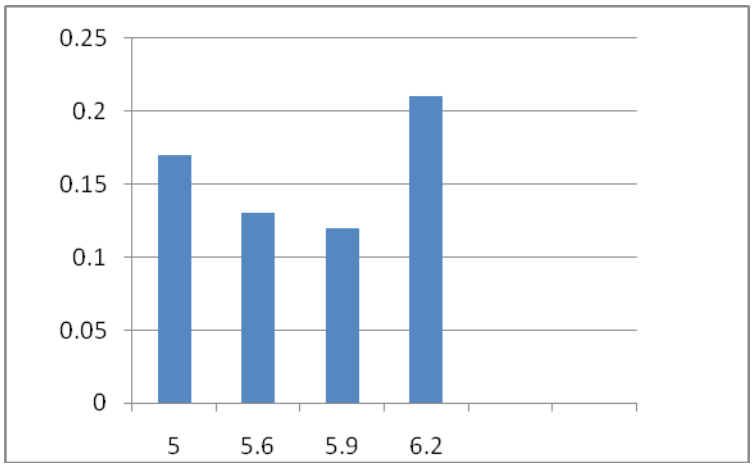

Figure 4: Effect of pH (X axis: pH; Y axis: OD at 600nm)

Production of Cellulases By Solid State Fermentation Using Cheap Substrates

Solid state fermentation was carried out in flaks using cheap substrates, Figure 5 below shows the solid state fermentation flasks.

Substrate: Saw dust

Substrate: Corncob

Substrate: Newspaper

Substrate: Wheat Straw

Figure 5: Solid State Fermentation Flasks

Protein Estimation in Crude Enzyme

Concentration of protein in crude extract was determined by Lowry's method and the result 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. AT 660 nm	CONC. OF PROTEIN (mg/ml)
BLANK	0.0	1	5		0.5		0.0	0.0
Crude Extract (Saw dust)	0.5	0.5	5		0.5		0.66	0.226
Crude Extract (Corncob)	0.5	0.5	5		0.5		0.37	0.125
Crude Extract (Wheat Straw)	0.5	0.5	5		0.5		0.35	0.120
Crude Extract (Newspaper)	0.5	0.5	5		0.5		0.13	0.044

Enzyme assay in crude enzyme

Enzyme assay was performed by DNS method and it can be seen from the results in **Table 6** below that maximum activity was obtained in the flask containing corncob as substrate.

Table 6: Enzyme assay of crude enzymes

S No.	ENZYME (in ml)	1% CMC (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	0.5ml+0.5 ml D/W		1		0.0	0.0
Crude Extract (Saw dust)	0.5	0.5		1		0.52	0.018
Crude Extract (Corncob)	0.5	0.5		1		0.76	0.027
Crude Extract (Wheat Straw)	0.5	0.5		1		0.66	0.024
Crude Extract (Newspaper)	0.5	0.5				0.57	0.020

Comparative Study of All the Substrates

Histogram below in **Figure 6** shows the comparative analysis of cellulase activity in the used substrates and it can be seen that maximum activity was seen when corncob was used as a substrate.

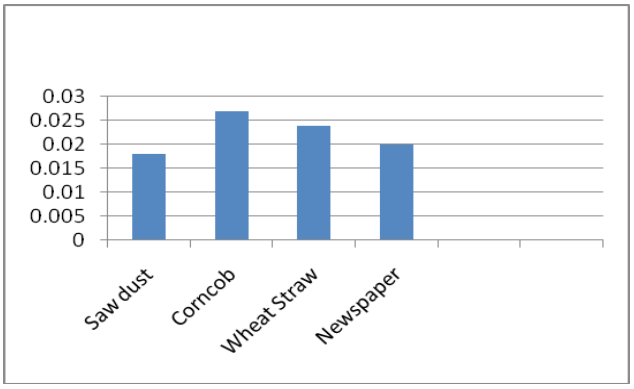

Figure 6: Comparative Study of All the Substrates

(X axis: Substrate; Y axis: Activity in U/ml/min)

DISCUSSION

Fungal strains were isolated from soil by serial dilution method on PDA plates. Fungal strain was screened for cellulase production on CMC agar plates, further stained with 0.1% congo red and washed with 1M NaCl as reported in **[6].**

The isolate MJSU1101 identified as *Aspergillus niger* was studied for its growth curve, optimum temperature and pH. 28°C was found to be the optimum temperature, pH 6.2 as optimum pH and 120 hours as the time for maximum production of enzyme.

Cellulase production was done by solid state fermentation using substrates such as corncob, saw dust, wheat straw and newspaper as has been done earlier by **[9-10]**

All the four substrates used were found to show good enzyme production but out of the four corncob showed maximum activity of 0.027 U/ml/min followed by wheat straw which gave an activity of 0.024 U/ml/min, newspaper (0.020U/ml/min) and saw dust (0.018U/ml/min).

CONCLUSION

Based on the above study it can be said that all the four substrates studied can be a good source for cellulase production, and could be used for economical production of cellulase enzyme. Although good activity was seen in all the substrates corncob being the best they can be studied in order to increase the activity by trying different pretreatment procedures and optimizing the incubation time.

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