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Isolation, screening and selection of cellulolytic fungi for the production of cellulase using agricultural wastes as substrates

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Abstract : *Cellulolytic fungi synthesize cellulase enzyme for biodegradation of cellulose. Cellulase production from fungi is advantageous as the enzyme production rate is higher as compared to other microorganisms. In the present investigation, our aim was to isolate novel cellulase producing fungi from local fruit dump wastes so as to reduce the production cost of cellulase by optimizing the production medium and using an alternative carbon source. Four fungal strains were isolated from fruit dump's soil and screened for cellulase efficiency of which Aspergillus niger was found to be the most potent cellulase producer showing maximum hydrolysis zone. This strain has been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulases. Growth of the isolate was found to be maximum at 26°C and at pH 6.2. Different agricultural wastes (wheat straw, sugarcane bagasse, corncob, paddy straw and rice husk) were used for fermentation. Cellulase enzyme was produced by all out of which paddy straw showed maximum activity of 0.025 U/ml/min followed by corncob (0.024 U/ml/min), bagasse(0.011U/ml/min), wheat bran(0.009U/ml/min) and rice husk(0.01U/ml/min).*

Key words: *Aspergillus niger, CMCase, Solid state fermentation, Cellulases.*

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Introduction :

India is an agriculture based country where a lot of agricultural waste is available. The agriculture wastes contain a high proportion of cellulosic matter which may be decomposed by a combination of physical, chemical and biological processes. Some common examples of agricultural waste in India include rice husk, wheat straw, corn cob, sugarcane bagasse, paddy straw, etc. These are considered to be the cheapest source for the production of different utilizable products throughout the world (Saleem et al., 2012).

Cellulose is the most abundant carbohydrate found in plants that provides agricultural integrity to their cell walls (Bhat and Bhat, 1997). It is the world's most abundant natural biopolymer that contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by a-1, 4 linkages. The long-chain cellulose polymers are linked together by hydrogen and van der Waal bonds, which cause the cellulose to be packed into microfibrils. Cellulases are a group of hydrolytic enzymes which are capable of degrading cellulose (Rana and Kaur, 2012). Cellulases (E.C.3.2.1.4) are a class of enzymes produced chiefly by fungi, bacteria and protozoa that catalyze the hydrolysis of cellulose. Cellulase breaks down cellulose to beta-glucose.

Cellulase have attracted much interest because of the diversity of their applications such as their usage in coffee processing, textile industry, laundry detergents, pulp and paper industry, pharmaceutical industry and biofuel industry (Khan and Singh, 2011). 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 (Khan and Singh, 2011).

Materials and Methods :

Collection of soil sample : Waste samples from fruit dumps were collected from the localities of Gandhi Maidan in sterile polythene bags and brought to the laboratory for further study.

Isolation of fungi : For isolation of fungi, soil sample was collected from sites rich in decomposing plant cells in sterile polybags and transferred to the laboratory. 1 gram of soil sample was transferred to aliquots of 9.9 ml of sterile normal saline in vials. It was shaken vigorously at constant speed for 15 minutes. The soil suspension was then subjected to serial dilution upto 10^{-5} &100µL diluted sample was spreaded on sterile PDA (Potato Dextrose Agar) plates (Bhat and Bhat, 1997). Inoculated plates were incubated at 28° C for four to five days. After incubation different fungal colonies were picked on morphological differences and named as S1, S2, S3 and S4.All the four colonies were further sub-cultured on Potato Dextrose Agar (PDA) plates.

Screening of Isolates for Cellulase Production : The isolated fungal strains were tested for their cellulase producing abilities on CMC agar plates in which all the four isolates were point inoculated on CMCase Basal Salt media and incubated at 28 °C for 5-7 days (Shahriarinour et al., 2011). After the completion of incubation period the plates were flooded with 0.1% Congo red solution followed by counterstaining with 1 M NaCl solution for 15-20 minutes. Plates were observed for zone of cellulose hydrolysis.

Identification of the Isolates : Isolated cellulolytic fungi were identified based on their colonial and cultural characteristics and on the morphology of their sporulating structures. The fungal isolate that showed maximum hydrolysis during plate screening method was selected for further study.

Study of Growth Parameters of the Selected Fungi

(a) Effect of Temperature on growth of isolate: In order to know the optimum temperature for the growth of the fungus showing maximum hydrolysis was streaked on four sterile PDA plates, and incubated at various temperature as 18°C, 28°C, 37°C and 50°C for 48 hours. After incubation the growth of isolate was quantified based on visual identification (Khan and Singh, 2011)

(**b) Effect of pH on growth of isolates:** In order to know the optimum pH for the growth of fungal isolates, it was inoculated in four flasks

containing 20ml of PDB (potato dextrose broth) each maintained at different pH i.e pH 5.35, 5.6, 6.2 and 6.5 respectively. All four flasks were incubated in shaker incubator at 120rpm 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 Potato Dextrose Broth (PDB) (Khan and Singh, 2011).

(**c) Growth kinetics:** The fungal isolate showing maximum hydrolysis was studied for its growth pattern where in 100ml Potato Dextrose Broth (PDB), was prepared and divided into two flask containing 80ml and 20ml media respectively. Both the flask were autoclaved and cooled to room temperature and the flask containing 80ml media was inoculated with the fungal isolate showing maximum hydrolysis and incubated at 28°C at 120 rpm. Growth of the isolate was studied by reading the absorbance at 600nm against uninoculated media for 8 days (Khan and Singh, 2011).

Production of Cellulases By Solid State Fermentation Using Agricultural Wastes as Cheap Substrate

Substrate used: Paddy straw, corncob, sugarcane bagasse, wheat bran and rice husk were pretreated individually in 1N sodium hydroxide solution (NaOH) in the ratio 1: 10 (substrate: alkali solution) for two hours at room temperature and autoclaved at 121°C for one hour. The treated substrates were then filtered and washed with distilled water until the wash water become neutral (Saleem et al., 2012) then dried overnight at 60°C in hot air oven. The dried substrates were packed in polypropylene bags until use.

Production: 20 gm of each of pretreated substrates mentioned above were taken in 250 ml flasks, moistened with 50 ml of mineral salt media (MSM) was autoclaved and cooled to room temperature. After cooling all were inoculated with 1ml of 48 hour old broth culture showing maximum cellulose hydrolysis during screening. Flasks were incubated in shaker incubator (120rpm) at 28 °C for 8 days.

Extraction of Crude Enzyme : 100ml of 0.05M Citrate Buffer (pH 4.8) was added to each flask after completion of incubation period, shaken at 150 rpm for 1 hour and filtered by the help of masculine cloth, later by Whatman's filter paper No.1 and filtrate was collected and used for the estimation of extracellular protien content and total activity of cellulases.

Protein Estimation in Crude Enzyme : Amount of protein in crude enzyme was determined by Lowry's method (Lone et al., 2012) of protein estimation in which, 0.5ml of crude enzyme, 0.5ml of distilled water was reacted with 5ml Alkaline solution C.Afterwards,0.5 ml of diluted Folin ciocalteau reagent was added and the absorbance was taken against blank at 750nm. 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 Bovine Serum Albumin (X axis) and OD at 750nm (Y axis).

Enzyme Assay In Crude Enzyme : CMCase activity in crude enzyme was determined by DNS method of Miller (Lone et al., 2012) in which 0.5ml of enzyme was mixed with 0.5ml of 1% CMC in citrate buffer for 30 minutes at 37°C and the reaction was terminated by adding DNS reagent and reaction mixture was boiled for 5 minutes, and absorbance was taken at 540 nm against the blank. Absorbance was compared with the standard graph plotted by reacting known concentration of glucose 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 and Discussion :

Isolation of Fungi : Four different fungal isolates namely S1, S2, S3 and S4 were obtained in the mixed culture plate (shown in Figure 1) and were sub cultured on Potato Dextrose Agar (PDA) slants.

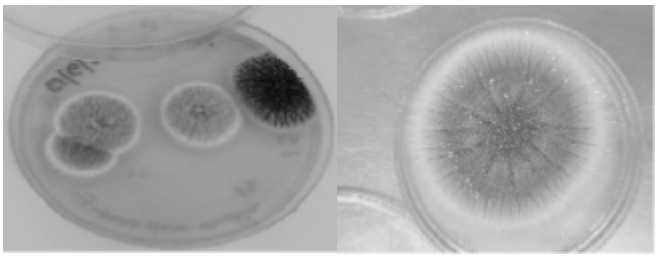

Figure1. Culture plate of the isolated colonies

All the four cultures were screened for their Cellulase producing potential on the basis of zone of hydrolysis as shown in Table 1 and Fig 2.

Table 1. Screening for Cellulase by plate screening method

Serial No	Isolate	Result	Zone(cm)
1	S1	+	0.3-0.5
2	S2	+	0.5
3	S3	+	0.7
4	S4	++++	2.5-3

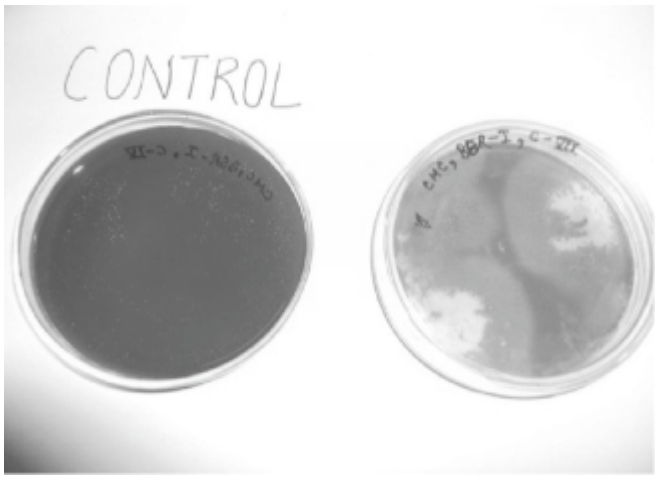

Figure 2. Plate screening of CMCase in the surrounding of colonies and Congo Red dye staining displaying clearing zone

Based on morphological studies and lactophenol cotton blue staining characteristics, the isolate S1 was identified as *A. flavus*, S2 as *A. fumigatus*, S3 *A. parasiticus* and S4 was *A. niger*. Fig 3 below shows the *Aspergillus niger* plate

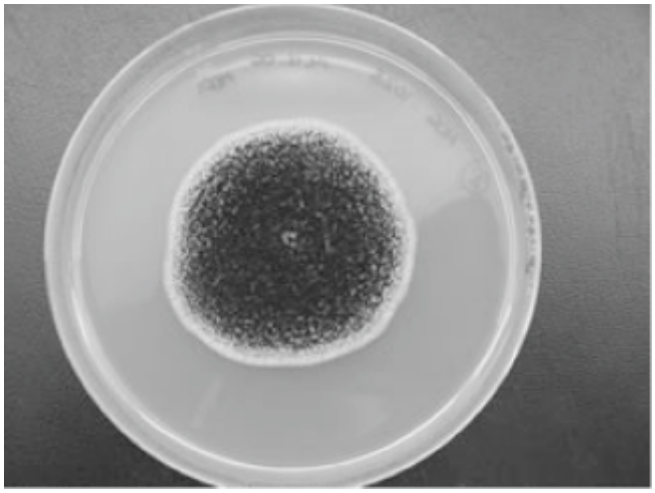

Figure 3. Plate showing growth of *A. niger*

Study of Growth Parameters : Growth parameters including optimal pH, optimal temperature and growth curve were studied in order to have a proper idea of the stationary phase, optimum temperature and growth of the isolate so that this environment could be provided during fermentation procedure according to the method of Khan and Singh (2011).

Effect of pH on growth of isolate S4 : Effect of pH was studied on the growth of isolate S4. Table 2 and Fig 4 show the maximum growth of the isolate at pH 6.2.

Table 2. Absorbance of the isolate at different pH

Serial No	pH of Broth(PDB)	OD at 600 nm
1	5.2	0.067
2	5.6	0.073
3	6.2	0.093
4	6.5	0.061

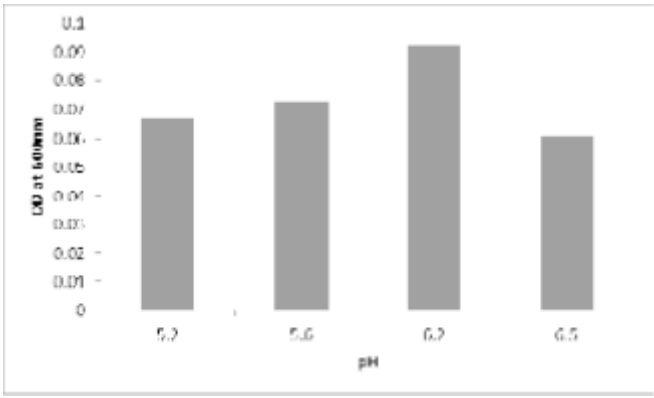

Figure 4. Effect of pH on the growth of *A. niger*

Effect of Temperature on growth of Isolate S4 : Effect of Temperature on the growth of isolate S4 are shown in the Table 3 and Figure 5 which shows that the isolate grows maximally at 26°C.

Table 3. Effect of growth of the isolate at variable Temperature

Serial No	Incubation Temperature (in°C)	Remark
1	18°C	+
2	26°C	+++
3	37°C	++
4	50°C	–

(+ shows fair growth, ++ shows good growth, +++ shows maximum growth – shows no growth)

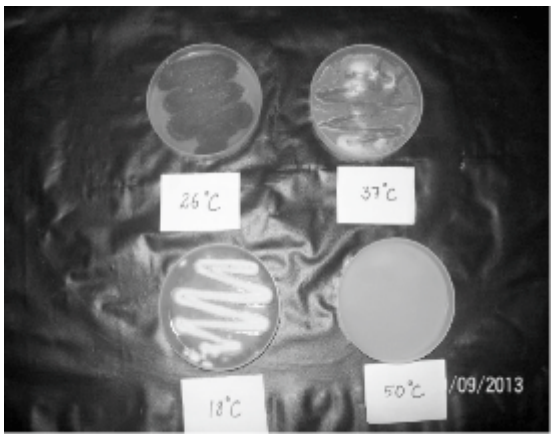

Figure 5. Plates of *A. niger* at different temperature

Growth kinetics : Table 4 and Fig 6 show the growth kinetics statistics of the isolate S4 (*Aspergillus niger*) and indicates that stationary phase reached between 8-9 days.

Table 4. Growth kinetics of the isolate

Serial No	Time (in days)	OD at 600 nm
1	0	0
2	1	0.058
3	2	0.089
4	3	0.110
5	4	0.136
6	5	0.162
7	6	0.198
8	7	0.212
9	8	0.212
10	9	0.198
11	10	0.176

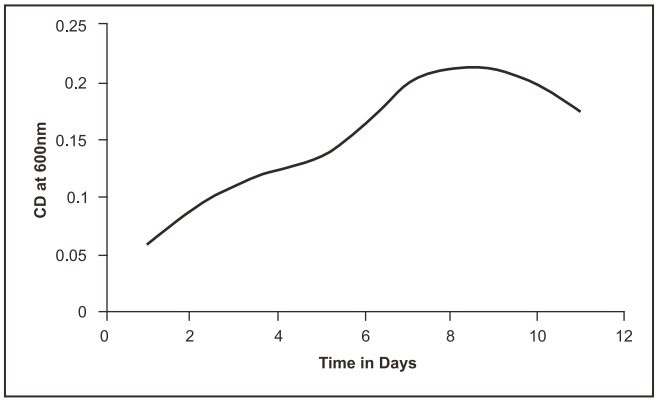

Figure 6. Growth curve of the isolate *A. niger*

Production of Cellulases by using agricultural wastes as cheap substrates : Solid state fermentation was carried out in flaks using cheap substrates.

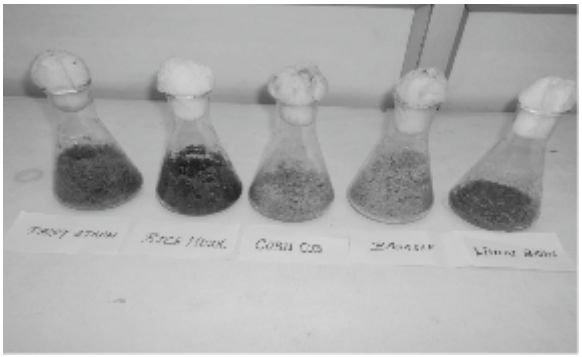

Figure 7. Production of enzyme using pretreated substrates

Protein Estimation In Crude Enzyme : Concentration of protein in crude extract was determined by Lowry's method and the result showed that maximum protein concentration was obtained in the flask containing Corn cob (0.47mg/ml) followed by Paddy straw (0.44mg/ml), Rice husk (0.25mg/ml), Wheat bran (0.20mg/ml), Bagasse (0.07mg/ml) . The results are shown in Figure 8.

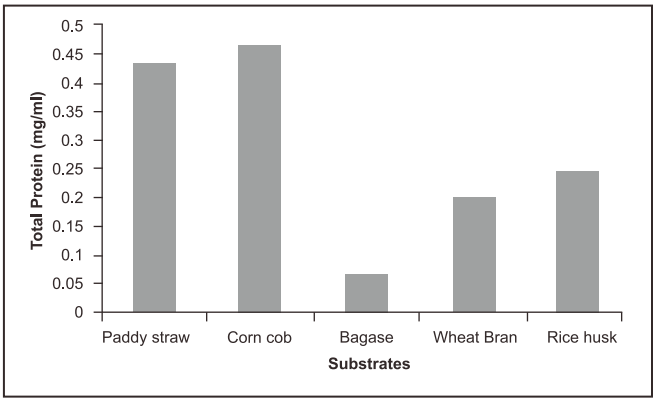

Figure 8. Total protein estimation using Agricultural wastes

Enzyme assay in Crude enzyme : Enzyme assay was performed by DNS method and it can be seen from the results in figure 9 that maximum activity was obtained in the flask containing paddy straw (0.025U/ml/min) followed by Corncob (0.024U/mg/ml), Bagasse (0.011U/mg/ml), Wheat bran (0.009U/mg/ml), Rice husk (0.001U/mg/ml).

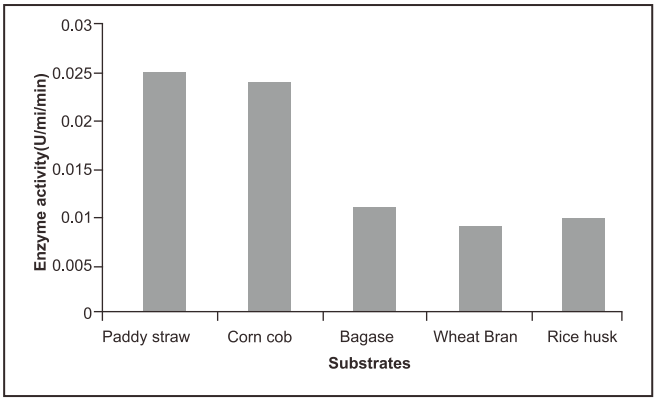

Figure 9. Cellulase enzyme activity using different agricultural wastes

In the present study four fungal strains were isolated from by Serial Dilution Method. Fungal strains were examined for zones of hydrolysis in CMC agar plates, isolate S4, *A niger* showed high activity against CMC (2.5-3cm).

The potent isolate S4 identified as *Aspergillus niger*, was studied for its growth curve, optimum temperature and pH. 26°C was found to be the optimum temperature, pH 6.2 as optimum pH and 8 days as the time for maximum production of enzyme. The results were in accordance to the work of Khan and Singh (2011).

Cellulase production was done by solid state fermentation using substrates such as paddy straw, corncob, sugarcane bagasse, wheat bran and rice husk as has been done earlier by Bamigboye (2013), Khan and Singh (2011), Saleem et al. (2012), Kiranmayi et al. (2011) and Omojasola and Jilani (2008). All the five substrates used were found to show good enzyme production but out of all the substrate used, paddy straw showed maximum activity of 0.025U/ml/min and protein content 0.44mg/ml. Similar work has been reported by Sakthi et al. (2011) that paddy straw is one of the cheap agricultural waste that produces high yield of cellulase enzyme by using *A niger* .The other substrate showing good and almost similar enzyme activity was corncob of 0.024 U/ml/min , total protein content of 0.47mg/ml which coincides with the work of Khan and Singh (2011) who reported that corncob is most efficient substrate that can be used for cellulose production showing 0.027U/ml/min activity.

Conclusion :

Fungal strain *A. niger* was successfully screened and found to be one of the potential and easy to isolate fungal strain from local fruit dump sources for cellulase enzyme. Based on the above study it can also be said that all the agricultural

substrates studied can be a good source for cellulose production, and could be used for economical production of cellulase enzyme at lower cost. Although good activity was seen in all the substrates but paddy straw and corncob being the best can be studied in order to increase the activity by trying different pretreatment procedures and optimizing various paramaters that could enhance the yield of cellulase enzyme on a large scale.

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