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Optimization of Cellulase Producing *Aspergillus flavus* SB4 by Solid State Fermentation using Rice Bran

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This study was aimed to isolate and screen the cellulolytic ability of fungi from litter soil samples collected in and around Chennai, India. The culture conditions were optimized for the growth of fungi by SSF using Rice Bran. Fungi are the main cellulase producing microorganisms when compared with other microorganisms. Among eleven fungal isolates, a fungal isolates were identified as maximum cellulase producer and labelled as SB4. The cultures were maintained on PDA slants at 4°C. The morphology was studied by LPCB method and fungal identification was done based on 18s rRNA ITS sequencing. The zone of clearance was observed on CMC agar plates after 72 hours of incubation by staining with 1% Congo red. The zone of hydrolysis was found to be high for SB4 having the highest diameter of 17 mm. Maximum cellulase production for rice bran was observed to be high at initial moisture 85%, pH-6, temperature-31°C and fermentation period was 14th day. The enzyme production was analyzed individually by Dinitrosalicylic acid (DNS) method. The cellulosic degradation was confirmed by FTIR and SEM on 0th day and 28th day.

Key word: Cellulase, *Aspergillus flavus*, Solid state Fermentation, FTIR, SEM.

Cellulose constitutes the most abundant, renewable polymer resource available today worldwide. Cellulose is a complex carbohydrate, or polysaccharide consisting of 3,000 or more glucose units. Cellulose is an organic compound with the formula $(C_6H_{10}O_5)$ n, a polysaccharide consisting of a linear chain of several hundred to over ten thousand $\beta(1\rightarrow4)$ linked D-glucose units (Crawford 1981). There are cellulases produced by a few other types of organisms, such as some termites and the microbial intestinal symbionts of other termites (Watanabe *et al*., 1998). Several different kinds of cellulases are known, which differ structurally and mechanistically.

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Solid State Fermentation (SSF) is the growth and/or cultivation of microorganisms under controlled conditions in the absence of free water for the production of desired products of interest.

Cultivating of filamentous fungi is difficult when they are grown in liquid media and they form abundant viscosity which results in reduced oxygen intake and the mortality of the cell increases due to agitation. Hence solid state fermentation (SSF) enhances the culturing of filamentous fungi as they facilitate the aeration into the media as they spread over it (Biesebeke*et al*., 2002).

MATERIALS AND METHODS

Isolation of Fungal Strains

For isolation of fungi, sample was collected from the soil that is from the different areas containing decaying leaves on surface. The samples were dispensed into bags and were

brought to the Sathyabama University Lab and soil enrichment was done. 1g soil was subjected to the serial dilution up to 10^{-5}dilution and then 1ml of diluted sample was spread on sterile PDA plates incorporated with antibiotics (Chloramphenicol 25µg/ml). The inoculated plates were incubated at 28°C for 48 – 72 hours. The isolates were sub cultured on sterile PDA plates and incubated at 4°C.

Screening of fungi for cellulase production

Congo red method

The different fungi species were inoculated in CMC broth medium and were kept in the shaker for 5 to 7 days. The medium was then filtered and centrifuged at 10,000rpm for 15minutes.The CMC agar was prepared with the following composition and autoclaved at 121°C for 1 hour.

The CMC Agar medium was poured in the sterile petri plates and left for 15 minutes. 20µl of the supernatant of centrifuged fungi medium was added to the wells made in the CMC Agar plates using micro pipette. These were incubated at 25-30°C for 72 hours. The petri plates were flooded with 1% of Congo red and left for 15minutes. The plates were then de-stained with 1M NaCl solution for 15 minutes. The zone of clearance was observed for the cellulose hydrolysis surrounding the colonies. (Arijit Das *et al*., 2011)

Identification of fungi

Macroscopic identification

The colony was observed from the top and bottom to study the size, texture, pigmentation.

Microscopic identification

Lactophenol cotton blue staining method was performed to study the microscopic structure of the fungi.

Molecular identification

Using the primer G18F and 22R the PCR was performed. Re-amplified PCR fragments were cloned using the pGEM-T Easy vector system (Promega). Plasmid DNA containing the inserts were cycle-sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and a model 3100 automatic sequencer (Applied Biosystems, USA) according to the manufactures instruction. The closest known relatives of the new isolates were determined by performing a sequence database search. The sequences of closely related strains were retrieved from GENBANK and the Ribosomal Database Project (RDP) libraries.

CMC broth medium (gm/100ml)

Enrichment broth with cellulose as carbon source and peptone as nitrogen source was used for isolation of cellulolytic fungi. Carboxymethyl cellulose medium with pH 7 was employed to get desired fungi.

Inoculum Preparation

Culture of *Aspergillus flavus* was maintained by stock culture in PDA agar slants. They were grown at 27°C for 72 hours and stored at 4°C for regular subculturing. The solid state fermentation media was inoculated with 1%(v/v) of spore suspension (5×10^7 spores per ml) prepared by suspending the spores from 7days old sporulated slant of *Aspergillus* SB4 on PDA in 10ml of steriled distilled water containing 0.01% (v/v) Tween 80.

Solid State Fermentation

Solid state fermentation was caused out in 250ml Erlenmeyer flasks that contained 30g of rice barn and 15ml of distilled water (Moistening agent). The flask were Sterilized at 121°C for 15 min and cooled to room temperature. About 1ml of inoculums was added, mixed well and incubated at 27°C in a humidified incubator for 96h. The flask were periodically mixed by gentle shaking.

Assay of Cellulase

After the incubation time is over, the media was washed with PBS and the cell free extract was used for analysis. The fungal crude was prepared by following method. 10 ml of cell free extract was centrifuged at 5000 rpm for 15 minutes. The activity of Cellulase was assayed using DNS method.The DNS assay was carried out as follows. 0.2 ml of culture filtrate was mixed with 1% CMC in a test tube and incubated at 40°C for 30 minutes. The reaction was terminated by adding 3 ml of DNS reagent. The tube was then incubated at 100°C for 15 minutes followed by the addition of 1ml of salt solution. The OD was taken at 575 nm against blank.

Enzyme units

One unit (U) enzyme activity was defined as the amount of enzyme required to liberate 1 µmol reducing sugar from the appropriate substrate per min under the assay conditions. The results are shown in $U \cdot mL^{-1}$ for submerged and solid state cultivation.

Statistical tests

The results were statistically analyzed using IBM, SPSS software and analysis of variance with $p<0.05$.

Optimization

Substrates and Solid State Fermentation

Pre-treatment of substrate

Raw substrates were sun dried individually to reduce the moisture content to make them more susceptible for crushing. The crushed substrates were then sieved individually to get powdered form, then it is used as a substrate for SSF.30g of the substrate was weighed and added to the flasks. Initially the moisture content of the substrates is determined.(AnuradhaJabasingh and Valli Nachiyar, 2013)

Effect of incubation period on enzyme production

Fermentation period is important parameter for enzyme production by *Aspergillus* species. In this study experiments fermentation was carried out up to 7 days and production rate measured at 16h intervals (Gauthame*t al*2010).

Effect of pH on enzyme production

Optimization was carried out by using buffer of 4, 5, 6, 7, 8, 9 and10.The pH was adjusted by using 1N HCL or 1N NaOH. Then 35ml of minimal salt medium was added to 30g of Rice bran. Autoclaved and then fungi is inoculated.

Effect of temperature on enzyme production

Optimization was carried out by placing the substrates containing minimal salt medium and the fungi at different temperatures of 25°C, 27°C, 29°C, 31°C, 33°C, 35°C, 38°C and 40°C.

Scanning Electron Microscopy

Scanning Electron Microscope uses secondary electron emitted by electron beam to display the image (McMullan, 2006).

The treated lignocellulose sample from SSF of 0th and 28th day and the untreated lignocellulose samples were initially dried in hot air oven at 60°C for 48h and the samples were selected using light microscope. The selected samples were placed in SEM module, Hitachi 5415 A, and micrographs were taken at different levels of magnification (Ganesh Kumar*et al.*,2005).

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infraredspectroscopy (FTIR)collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time (Griffithsand deHasseth, 2007). Perkin Elmer infrared spectrophotometer was used for investigating the change in surface functional groups of the biomass after SSF. The dried biomass sample were mixed with KBr of spectroscopic grade 1MPa. The spectra were then subjected to baseline correction andthe bands were studied toquantify the changes in the chemical structure of lignocellulose matrix. (Ganesh Kumar*et al.*,2005)

RESULTS AND DISCUSSION

Screening and Isolation of Fungal isolates for cellulolytic activity

Eleven fungal species belonging to same genera i.e. *Aspergillus* were isolated from the forest region in and around Chennai, screened and compared for their ability to degrade cellulose. Screening of fungal isolates was performed by plate method. Among 11 fungal isolates, single fungal isolates were identified as maximum cellulose producer.

Macroscopic morphology

Colonies on potato dextrose agar at 25°C are golden green with a cream reverse. Very mature colonies turn greenish black. Rapid growth. Texture is woolly to cottony to somewhat granular over the surface.

Microscopic morphology

Lacto phenol Cotton Blue Staining (LPCB)

The Lacto Phenol Cotton Blue (LPCB) wet mount preparation is the most widely used method of staining for observing fungi.Hyphae are septate and hyaline. Conidial heads are radiate to loosely columnar with age. Conidiophores are coarsely roughened, uncolored, vesicles globose to subglobose and biseriated which has a similar features of *Aspergillus fumigatus*

Phylogenetic analysis

Input Parameters	
Program	FASTA
Sequence type	DNA
Matrix	none
Match/mismatch scores	+5/-4
Gap open	-14
Gap extend	-4
Display of multiple high-scoring alignments (HSPs)	false
Expectation upper limit	10.0

Expectation lower limit	0.0
Nucleotide strand	both
Histogram	false
Scores	50
Alignments	50
Score report format	default
Statistical estimates	1
Annotation Features	false
Sequence range	START-END
Database range	START-END
Filter	none
Database	em_rel
KTUP	6

Congo Red method

CMC Agar is a selective media and selectively supports the growth of the cellulolytic fungi because cellulase producing organisms can only utilize cellulose as the carbon source. The screening of the cellulolytic fungal isolates was performed based on the diameter of the clearing zone surrounding the colony on the CMC medium. All fungi examined produced zone of clearance in CMC agar plates within 2 diameter. Particularly, SB4 showed higher cellulase activity against CMC having a maximum diameter of 17 mm. Thus, SB4 was selected for the further studies into the enzyme production and the ability to degrade cellulose(Sazci et al., 1986).

Optimization by SSF

Effect of moisture content on Enzyme activity

Rice Bran was used in Solid State Fermentation for the production of cellulase. In this study, we investigated a moisture range for Rice Bran in order to accelerate the growth of *Aspergillus* species to generate cellulase production(Anshika Grover et al.,2013;Delabona*et al*, 2012).. The biomass coverage and spore formation on the substrate surface were positively associated with the increase in moisture content, indicating that the higher the moisture, the higher growth rates were within the moisture range (Reeta Rani Singhania et al., 2006).

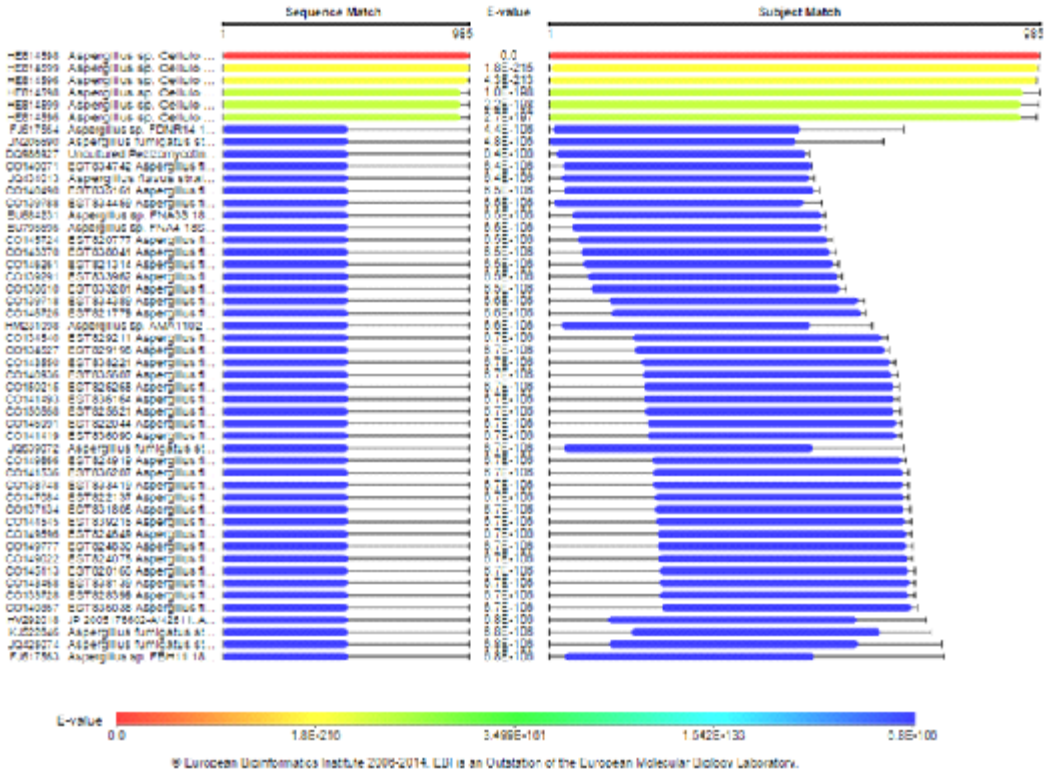

Fig. 1. FASTA analysis by EMBL

Effect of temperature on Enzyme activity

Temperature is also an important factor that influences the cellulase yield. The effect of temperature on cellulase production using SB4 was studied by varying the temperature range from 25°C to 35°C. The maximum production of cellulase was obtained at 33°C for rice bran. The rate of enzyme catalyzed reactions increased with temperature up to a certain limit. Enzyme activity decreases with increase in temperature because of enzyme

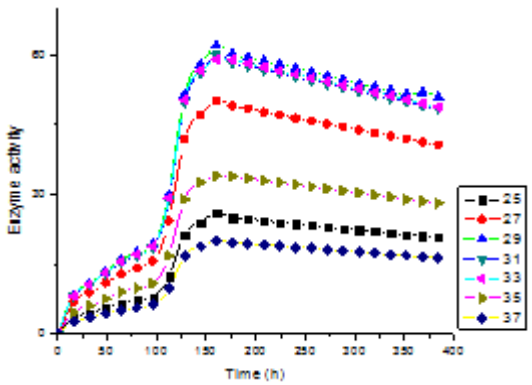

Fig. 2. Effect of temperature on Enzyme production in SSF

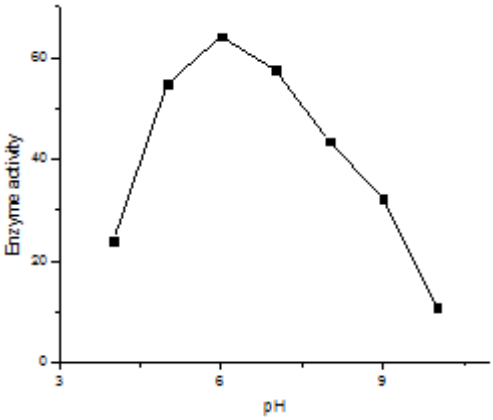

Fig. 3. Effect of pH on Enzyme activity

Scanning Electron Microscopy (SEM)

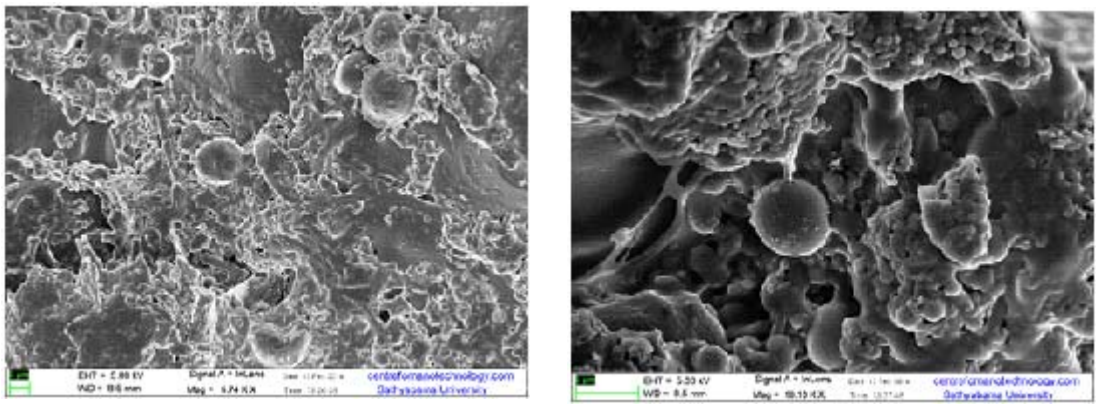

Fig. 4. SEM for Rice bran on 0th day and 28th day

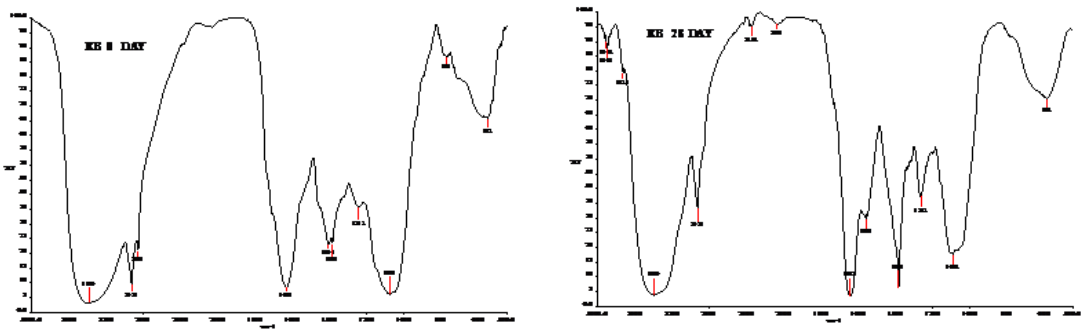

Fig. 5. FT-IR for Rice bran on 0th day and 28th day

denaturation (Moses Jeyakumar Rajesh*et al*.,2012).

Effect of pH on Enzyme activity

Among physical parameters, pH of the growth medium plays an important role by including morphological changes in microbes and in enzyme secretion. Cellulase yield by SB4 appeared to depend on pH value. Results illustrated by Figure - 3 clearly show that the optimum pH for maximum production of cellulase was 6 on 14th day when grown in SSF for both rice bran (Soma Mrudula and RangasamyMurugammal, 2011). It was then observed to decrease with more increase in pH indicating that there was a reduction in the cellulase activity. All the three methods of enzyme estimation: DNS, Lowry's *et al* and Filter paper Assay were showed to be high at pH 6.

Biomass

The fungal biomass concentration was increased by SB4. The level of increase showed the growth of the organism. However, the biomass value was observed to decrease after the 21st day. This decrease is due to the reduction in moisture content of the substrate after 21 days. The biomass for Rice Bran was optimum on 14th day and weighed to be 0.056 mg/ml.

The SEM was used to study the morphological changes of cellulosic degradation by SB4. The longer incubation period of 28 days was required for the breakdown of cellular fibres. The enzyme degradation was observed on 0th day and 28th day. The cellulose reacted with the enzymes secreted by the organism and resulted in the changes in surface morphology indicating the enzyme degradation. The substrate surface was degraded on 28th day compared to 0th day (Figure 3.4.5 (a) and 3.4.5 (b)).

Fourier Transform Infrared Spectroscopy (FT-IR)

The FTIR spectra of fungal treated samples of 0th day and 28th day of the substrates are shown in figure. The peaks for rice bran on 0th day were observed to be 3379 cm^{-1}, 2925 cm^{-1}, 1631 cm^{-1}, 1403 cm^{-1}, 1242 cm^{-1}, 1075 cm^{-1}, 773 cm^{-1} and 552 cm^{-1}. On the 28th day the rice bran showed the peaks to be 3390 cm^{-1}, 2925 cm^{-1}, 1642 cm^{-1}, 1262 cm^{-1}, 1092 cm^{-1} and 588 cm^{-1}.

CONCLUSION

The cellulolytic ability of *Aspergillusflavus* SB04 from soil samples collected in and around Chennai, India. The identification of the fungi was confirmed by morphological and molecular (18s rRNA ITS sequencing) identification. The homology search and phylogenetic analysis was done by FASTA. The confirmation of the cellulose activity was confirmed by Congo red method. Maximum cellulase production for rice bran was observed to be high at initial moisture 85%, pH-6, temperature-31°C and fermentation period was 14th day. The enzyme production was analyzed individually by Dinitrosalicylic acid (DNS) method. The cellulosic degradation was confirmed by FTIR and SEM on 0th day and 28th day.

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