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Degradation analysis of different agro-substrates by *Aspergillus flavus* SB04 using FT-IR.

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ABSTRACT

Solid state fermentation (SSF) is an eco-friendly process for the production of value added products. Different substrates such as rice husk, paddy straw, sugarcane bagasse, wheat husk, and leaves of mango, sapota and guava were used for the production of cellulase by *Aspergillus flavus* SB04. The process of SSF was characterized by carbon, hydrogen, and nitrogen (CHN) analysis. The microbial utilization and product formation resulted in lowering of CHN values at the end of the treatment process. Microbial fermentation resulted in conversion of complex polymeric lignocelluloses substrates into simpler monomers. Fourier transform infrared spectroscopy (FT-IR) analyses of metabolites confirmed the breakdown and complete degradation of lignin, cellulose and polysaccharides at different fermentation time intervals of 0, 15 and 30 days.

Keywords: SSF, substrates, *Aspergillus flavus*, CHN, FT-IR

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INTRODUCTION

Several strains of filamentous fungi from different environmental niche have been exploited for the production of industrially important chemicals and enzymes. *Aspergillus* species has been used traditionally for the safe production of value added products. They are the massive producers of many types of enzyme in immense concentration. Cellulolytic enzymes can be produced commercially by submerged or by solid state fermentation (SSF). However, the growth of fungi is most appropriate in SSF as it simulates the natural habitat of the fungi and enhances the production of enzymes. The agricultural residues are generated globally and a major portion is left underutilized leading to environmental pollution problems. Wide agricultural activities produce tons of by-products, such as sugarcane bagasse, sweet sorghum, citrus and agave, seeds and peels, rice, barley, wheat and oat straw, corn straw and corncobs.

Bioconversion of inexpensive agricultural wastes as substrates for enzyme production would hold a promising application in future fermentation technologies, mainly because of its cost effectiveness, eco-friendliness and feasibility in both developed and developing countries. Moreover, the elevated market price hinders the extensive production and application of cellulase enzyme [1]. Therefore it is essential to develop the valuable enzyme from inexpensive substrate to make the process economically viable.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected using aseptic techniques from different areas where humification process was observed to be more. The samples were serial diluted and plated on sterile potato dextrose agar (PDA; Himedia, Mumbai, India) incorporated with antibiotics (chloramphenicol 25 µg/mL). The inoculated plates were incubated at 28°C for 120 hours. The mixed cultures were subcultured by quadrant streaking on sterile PDA plates. The grown axenic cultures were stocked in slants and preserved at 4°C [2].

Identification of Fungi

Macroscopic identification

The surface and reverse mat colony morphology were used to study the size, texture and pigmentation.

Microscopic identification

Lactophenol cotton blue staining method was used to study the microscopic structural morphology of mycelium and conidial appearance in fungi.

Screening of Fungi for Cellulase Production

Congo red method

Pure cultures of fungal isolates were grown in carboxymethyl cellulose (CMC) broth medium under agitation for 120 hours. The medium was filtered and centrifuged at 10,000 rpm for 15 minutes. In sterile CMC agar the cell-free filtrate was dispensed in wells and incubated at 25–30°C for 72 hours. Petri plates were spread with 1% of Congo red and left for 15 minutes. The plates were then destained with 1 M NaCl solution for 15 minutes. The zone of clearance was observed for the cellulose hydrolysis around the colonies [3].

Inoculum Preparation

From the different cultures, *Aspergillus flavus* SB04 was identified as potential strain for the production of cellulase. The scale-up was done for the inoculum production. The SSF media [4] was inoculated with 1% (v/v) of spore suspension (6×10^7 spores/mL) prepared by suspending the spores obtained from end of logarithmic growth phase slants of *Aspergillus* SB04 on PDA in10 mL of sterile distilled water containing 0.01% (v/v) Tween 80 [5].

Solid State Fermentation

Different substrates such as rice husk [6], paddy straw [7], sugarcane bagasse [8], wheat husk [9], and dried leaves of mango, sapota and guava [10] were used in SSF. The experiment was started in 250 mL Erlenmeyer flasks that contained 30 g substrate and 15 mL of distilled water. The flasks were sterilized at 121°C for 15 minutes and cooled to room temperature. Approximately 1 mL of spore inoculum was added, mixed well and incubated at 28°C in a humidified incubator for 96 hours. The substrate aggregation was minimized by manual mixing periodically [11, 12].

Assay of Cellulase

At different growth period, the matrix was washed with phosphate-buffered saline and the cell-free extract was used for analysis. The fungal crude extract was prepared by centrifuging 10 mL of cell-free extract at 5000 rpm for 15 minutes. The activity of Cellulase was studied, using DNS assay method. The assay was carried out as follows. Culture filtrate 0.2 mL 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 1 mL of salt solution. The OD was taken at 575 nm against blank [13].

Enzyme Units

One unit enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar from the appropriate substrate per minute under the assay conditions. The results were shown in UmL^{-1} [14].

Instrumental Analysis

Fourier transform infrared spectroscopy (FT-IR)

Shimadzu IR Affinity-1 spectrophotometer was used for investigating the changes in surface functional groups of the biomass after SSF. The spectra were then subjected to perfect baseline correction and the bands were crosschecked to identify the changes in the structure of lignocellulose matrix [11].

RESULT AND DISCUSSION

Isolation and Identification of Fungal Strains

Different fungal strains were isolated from the soil samples. All the pure cultures were grown on PDA and CMC agar. The cultural morphology and microscopic observation were observed (Figure 1 and Table 1). The fungal isolates were purified and cultivated in CMC broth, the cell-free filtrate was screened using CMC cellulase method and *Aspergillus flavus* SB04 was found to have maximum ability to produce cellulase among other strains isolated. Thus this isolate was used in SSF of different substrates.

Table 1: Representation of individual colony morphology

S. no.	Colony morphology
1	Green spongy (SB1)
2	White hairy (SB2)
3	Light green (SB3)
4	Green mold (SB4)
5	Pink (SB5)
6	White rhizoid (SB6)

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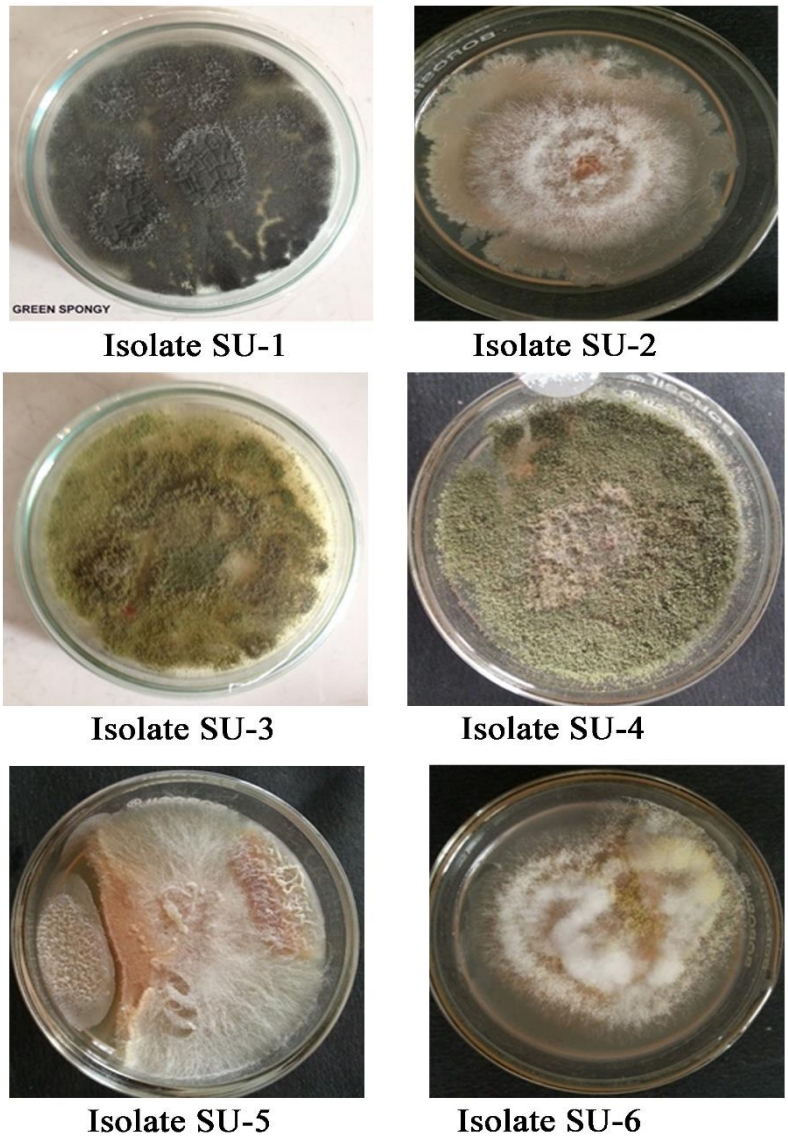

Figure 1: Colony morphology of the isolated organism on PDA plates

CHN Analysis

The percentage content of carbon, hydrogen and nitrogen (CHN) of different substrates were estimated by CHN analysis. The result showed that sapota leaves have highest carbon content whereas rice husk contains maximum amount of hydrogen and guava leaves have highest nitrogen content in comparison to other substrates (Table 2).

Table 2: CHN composition of different substrates

S. no.	Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)
1	Wheat husk	46.36	4.12	0.27
2	Sugarcane bagasse	46.57	5.15	1.25
3	Rice husk	39.99	6.46	2.20
4	Paddy straw	44.32	5.21	2. 51
5	Sapota leaves	46.99	6.16	1.45
6	Guava leaves	40.10	4.77	1.46
7	Mango leaves	45.71	4.32	2.02

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FT-IR Analysis

FT-IR spectroscopy analysis of sugarcane bagasse

Broad peak at around 3400 cm^{-1} represents OH groups either from cellulose or lignin. The peak at around 2922 cm^{-1} represents the C–H a symmetric stretching in aliphatic methyl. An intense peak appears at around 1734 cm^{-1} is due to ester carbonyl stretching (Figure 2). In a similar research carried out by Jurick et al. [14], the spectra of unmodified sugarcane bagasse were found within the range of 3500 to 700 cm^{-1}. The absorption bands were found near 1730, 1620, 1596 and 1513 cm^{-1}.

Figure 2: FTIR for sugarcane bagasse – untreated and treated sample

FT-IR spectroscopy analysis of rice husk

Band around 3400 cm^{-1} is due to stretching vibration of intermolecular hydrogen-bonded –OH groups in cellulose fibers. Absorption vibration at 1738 cm^{-1} is due to the vibration of carbonyl from carboxylic groups. A sharp peak around 1050 cm^{-1} is due to vibration of silica bonds (Figure 3).

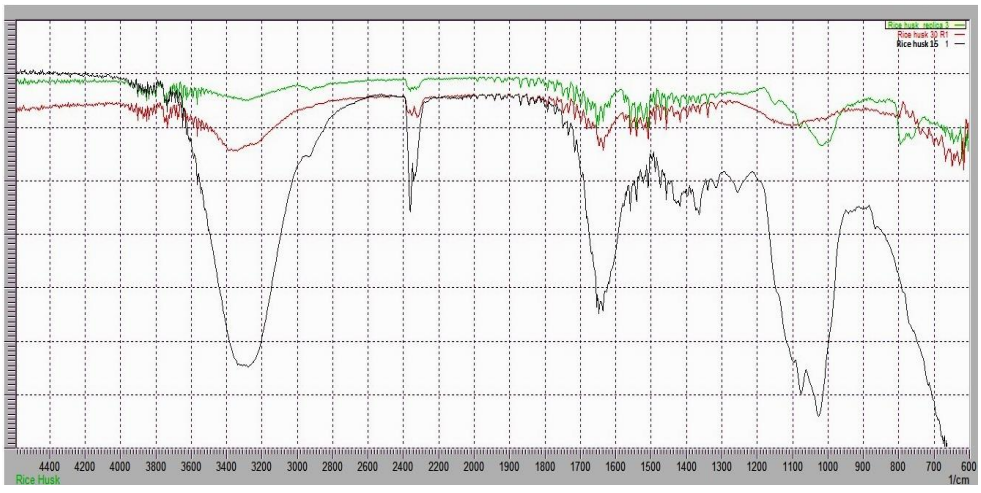

Figure 3: FTIR results for rice husk – untreated and treated sample

FT-IR spectroscopy analysis of wheat husk

The results show that there is large amount of SiO_2 (1070 cm^{-1}) and non-aromatic esters (1733 cm^{-1}) in wheat husk. The lignin peaks at 1731, 1650, 1595, 1505, 1455, 1425, 1039 and 835 cm^{-1} and cellulose peaks at 2900, 2850, 1650, 1455, 1430, 1367, 1317, 1160, 1034 and 891 cm^{-1} (Figure 4) [15].

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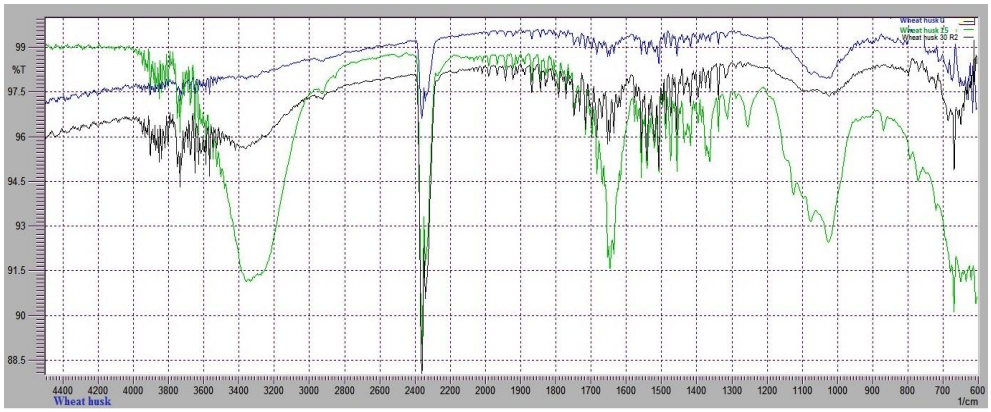

Figure 4: FTIR for wheat husk – untreated and treated sample

FT-IR spectroscopy analysis of paddy straw

The peak near 3400–3500 cm^{-1} is representative of the C–H and O–H groups. The peak at 1300 cm^{-1} symmetry of the C–H group. The peak around 1266–1200 cm^{-1} refers to the bending frequency of C–H, O–H, or CH_2, 1060–1050 cm^{-1} and 890 cm^{-1} refers to the C–H stretching vibration of C–O (Figure 5) [16].

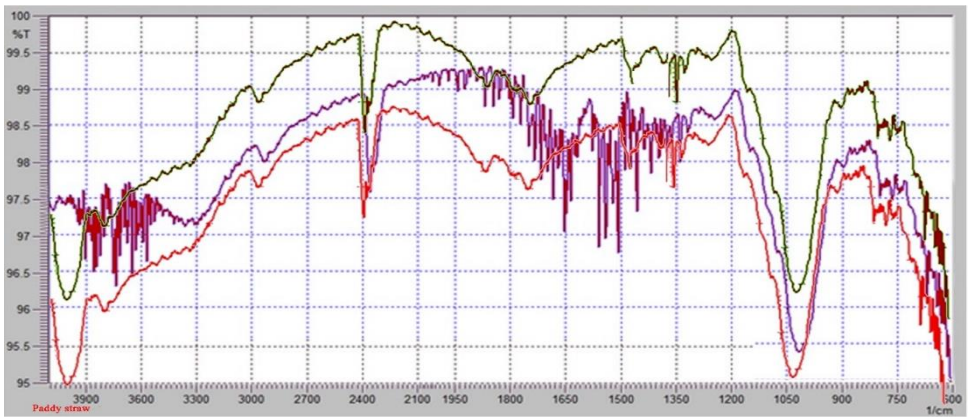

Figure 5: FTIR for paddy straw

FT-IR spectra of guava

FT-IR spectra of guava leaves reveal the functional groups of OH (3400–3600 cm^{-1}) stretching (CH_2) asymmetrical (2926 cm^{-1}), CH_2 stretching symmetrical (2856 cm^{-1}), C=O stretching of ester (1746 cm^{-1}), and stretching (C–O–C) of carbohydrate (1159 cm^{-1}) groups (Figure 6) [17].

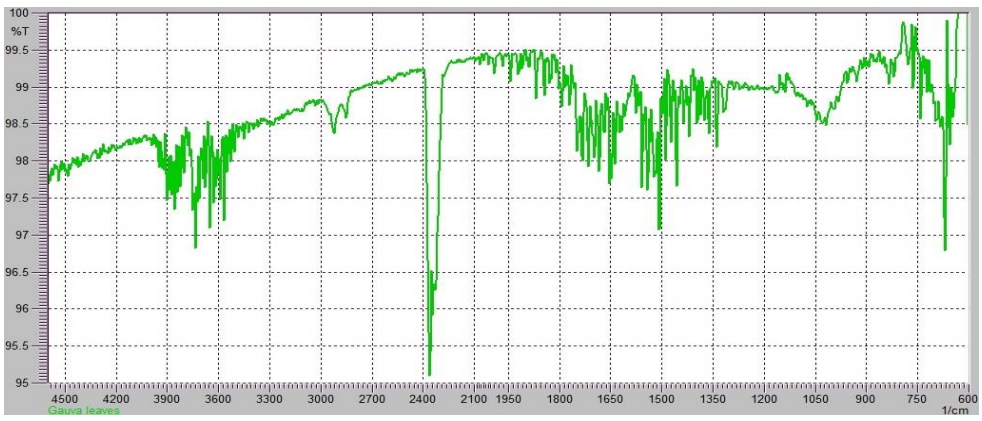

Figure 6: FTIR spectroscopy analysis of guava leaves-treated sample

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FT-IR spectroscopy analysis of mango leaves

FT-IR spectra of mango leaves reveal the functional groups of OH (3400 cm^{-1}) stretching (CH_2) asymmetrical (2924 cm^{-1}), CH_2 stretching symmetrical (2854 cm^{-1}), 1733 cm^{-1} is stretching vibration of C=O and 1373 cm^{-1} is symmetric stretching of –COO– (Figure 7).

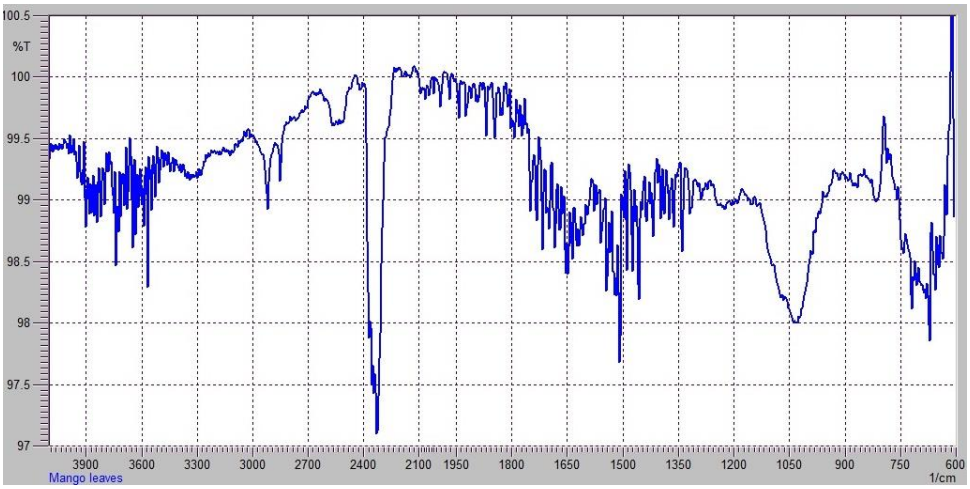

Figure 7: FTIR spectroscopy analysis of mango leaves-treated sample

FT-IR spectroscopy analysis of sapota leaves

A strong hydrogen-bonded (O–H) stretching absorption is seen at 3400 cm^{-1} for, 1440 cm^{-1} for C–H in lignin, 1380 cm^{-1} for C–H in cellulose and hemicellulose, 1320 cm^{-1} for C–H vibration in cellulose and C–O vibration in syringyl derivatives, 1100 cm^{-1} for aromatic skeletal and C–O stretch in cellulose and hemicellulose (Figure 8) [17].

Figure 8: FTIR spectroscopy analysis of sapota leaves-treated sample

CONCLUSION

FT-IR analysis elucidated the changes in the functional groups after treatment. The results confirmed the effective bioconversion of different substrates in SSF that can be utilized as a carbon source for the production of cellulase using *Aspergillus flavus*.

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