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Biocatalyst: Cellulase Production in Solid State Fermentation (SSF) Using Rice Bran as Substrate

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Abstract: The study was aimed to analyze the biological transformation of cellulose in rice bran by *Aspergillus flavus* SB04 in SSF for 28 days. The culture conditions such as pH, temperature, moisture content were optimized for the effective production of the enzyme in SSF. Effect of carbon and nitrogen sources on cellulase production was further estimated in SMF and were quantified for 24hrs intervals for 7 days Maximum cellulase production for rice bran was observed to be high in glucose (carbon source) and yeast extract (nitrogen source) at initial moisture 75ml, pH 6, temperature 33°C and fermentation period was 14th day that was optimized using response surface methodology. The enzyme production was analyzed individually by dinitrosalicylic acid (DNS) method, Lowry protein estimation, and filter paper assay. The lignocellulosic degradation was observed and confirmed by FTIR and SEM. The degradation of cellulose periodically increases after 7 days, which influences the yield of cellulase enzyme.

Keywords: Cellulase; JMP10; RSM; SEM; FTIR.

1. Introduction

Cellulose is a polymer of glucose units connected by β-1,4 bonds [1–3]. It is the most profused organic material and a major mechanical support constituent of flora and a renewable resource of energy in the earth. Cellulose comprising agrowastes serves as an inexpensive carbon source and other bioactive compounds. So, agrowaste can be effectively used as an alternative energy source to produce different products [4–6]. Cellulose is chiefly reduced by cellulase that is generally produced by microorganisms. Cellulases can efficiently breakdown cellulose into glucose units via the synergistic actions of the enzymes, known as an endo-β-1,4 glucanase, cellobiohydrolase, and β-d-glucosidase [7].

Isolation and screening of highly cellulolytic filamentous fungi were isolated from different sources such as soil, industrial effluent, seeds fruit, vegetable, bread, and wood. Although a large number of microorganisms were identified as the potential decomposers of cellulose, the research studies revealed that *Trichoderma viridae*, *Aspergillus niger*, and *Aspergillus flavus* are found to be relatively high in occurrence [8]. Fungi isolates are reported to be potential cellulase secretors than bacteria because of their accumulated mycelium that reduces the separation cost [9]. Due to the increase in demand for more thermostable, highly active, and specific cellulose, this study was designed to screening the native fungi isolate as

hyper-producers of cellulases by investigating the cellulose system of local fungi keeping in view the importance and application of the cellulases [10]. Hence the commercial demand in the near future of cellulase in industries has become an attentive study.

2. Materials and Methods

2.1. Microorganism.

A pure culture of *A. flavus* was isolated from soil and preserved in the Department of Biotechnology, Sathyabama Institute of Science and Technology. The fungus was maintained as direct stock culture from which inoculums were prepared. It was grown on PDA slants at 28º C for 5 days and stored at 4º C with regular sub-culturing [11].

2.2. Preparation of substrates.

Rice bran (RB) is one of the most popular agro-industrial waste residues preferred by many researchers to produce value-added metabolites by SSF from various microorganisms [4]. The SSF substrates RB is obtained from the local market, Chennai. The RB substrates were cleaned, sun-dried, and ground to a fine powder. Rice by-products are used to generate cellulase under SSF and have many operational benefits, including low cost, product stability, require low space, etc. [12].

2.3. Pre-treatment of substrates.

Powdered RB substrates were pre-treated by soaking in 1% NaOH solution in the ratio of 1:10 (substrate: NaOH solution) overnight at room temperature. The treated substrates were filtered and washed with distilled water until the pH reaches 7.0 in the substrate. Finally, the pre-treated substrates were autoclaved at 121º C for 15 minutes [13].

2.4. Response surface methodology (RSM).

The parameters pH, temperature, moisture content, and incubation time were optimized further by response surface methodology – central composite design [14]. The software used for the Design of Experiment (DOE) was JMP 10.

2.5. Optimization of cellulase production in SSF.

The influence of temperature (35 to 55º C), pH (4 to 8), the incubation period (24 to 168 h), particle size, inoculum size, moisture content, carbon (CMC, glucose, sucrose, and maltose) and nitrogen sources (yeast extract, ammonium nitrate, peptone, and sodium nitrate) was tested for 28 days at 28–30°C to optimize the production of cellulase by fungal isolate grown in RB. The cellulase activity is measured using reducing sugar and protein estimation method [15–18].

2.6. Inoculum preparation.

To the fungal slants, 5mL of sterile distilled water was dispensed, and the spores were dislodged using an inoculation loop. The prepared spore suspensions were transferred to the conical flask under aseptic conditions [19].

2.7. Enzyme production.

The culture was grown in a 150 mL Erlenmeyer flask, which contains 30g of RB substrate mixed with sterile minimal salt medium (MSM). The MSM of composition given below was used in SSF experiments. The MSM composition includes (g/L): Ammonium sulphate-10g; Potassium phosphate-3g; Magnesium sulphate- 0.5g; Calcium chloride -0.5g, Yeast extract-7gm and Dextrose-15g. The initial moisture content of the RB mixed with MSM was determined before the onset of the experiment [20].

2.8. Scanning electron microscopy.

The RB from SSF of 0th and 28th day as well as untreated RBs (control) were initially dried in a hot air oven at 60º C for 8h, and the samples were selected using a light microscope. The selected samples were coated with the gold ions, and the coated stubs were placed in FESEM module, and samples were analyzed at different magnifications.

2.9. Fourier transform infrared spectroscopy (FTIR).

The dried RB samples obtained at different time intervals were mixed with KBr of spectroscopic grade 1MPa. The spectra were then subjected to baseline correction, and the bands were studied in Perkin Elmer infrared spectrophotometer to quantify the changes in the chemical structure of the lignocellulose matrix [21, 22].

2.10. FPase assay for cellulases.

To test cellulase activity, 1 mL buffer was added with a 0.5 mL enzyme. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly less than 2 mg of glucose in the reaction conditions. Whatman filter (50 mm) paper strip was inserted into the test tube and incubated at 50º C for 1 h. The mixture was boiled for 20 min, followed by an additional 20 mL water, and the mixture was filtered with a glass filter paper. The filtrate was measured against reference at 540 nm. A linear glucose standard was constructed using the absolute amounts of glucose (0.5 ml/mg) plotted against 540 nm. Using this standard, the absorbance values of the sample tubes (after subtraction of enzyme blank) were converted into glucose units [23–25].

3. Results and Discussion

3.1. Optimization in SSF.

3.1.1. Effect of incubation period on enzyme production.

The incubation period is directly related to the production of enzymes and other metabolic activity up to a certain extent. The incubation period to achieve peak cellulase activity by the isolate *Aspergillus flavus* was at 3rd day, which is suitable for the commercial point of view [13]. Sirohi *et al*. [26] reported that the precise condition of SSF influences greater production of value-added products than the submerged fermentation.

3.1.2. Effect of pH on enzyme production.

Cellulase yield by *Aspergillus flavus* appears to depend on pH value. It was then observed to decrease with more increase in pH, indicating that there was a reduction in the

cellulase activity. All three methods of enzyme estimation: DNS, Lowry's *et al*., and Filter Paper Assay were showed to be high at pH 6. The significant saccharification is influenced by the optimal temperature and the substrate concentration along with pH [27].

3.1.3. Effect of moisture content on enzyme activity.

Ricebran was used in solid-state fermentation for the production of cellulase. In this study, we investigated a moisture range for rice bran (75 ml) was used in order to accelerate the growth of *Aspergillus* species to generate cellulase production. The biomass coverage and spore formation on the substrate surface was positively associated with the increase in moisture content, indicating that the higher the moisture, the higher growth rates were within the moisture range.

3.2. RSM.

Like the design of the experiment, the estimation of cellulase activity was performed, and the results were tabulated, and maximum productivity was identified.

The data were subjected to analysis of variance, and the contour plot was generated by using JMP 10.0.0. The regression analysis showed that 93 %, and the best fit of the model was also justified.[28]. Figure 1 showing the interaction between the actual and predicted values. Figure 2 shows the prediction profile of various parameters. Table 2 shows the parameter estimates and gives a t-test for the hypothesis that it equals zero. Table 3 shows scaled estimates (Nominal factors expanded to all levels). Figure 3 shows the Bivariate fit of enzyme activity by pH, Temperature, Moisture content, Incubation time, which is a continuous relationship study between the variables in a specific time frame. The values were shown in a scatterplot. The R^2 value was considered to be significant. In the summary of the fittable (Table 1), the regression analysis values show a somewhat similar relationship between R^2 and Adjusted R^2 values. The experimental values and the predicted results were significantly correlated with the obtained R^2 value (0.93), and also the R^2-adj was close to the value of R^2. Based on the results, the yield of the enzyme on the various condition can be predicted [27]. RSM study has reported that it could assist in attaining the maximum enzyme production with the evaluated different parameters on tea residue for the mixed strains (*B. subtilis, A. niger, S. cerevisiae*) on SSF [(29]. Figure 4. Influence of variable represented in contour plot for Biomass production: a) pH vs. Temperature b) Incubation time vs. Temperature c) Incubation time vs. pH.

3.3. Response Enzyme activity.

Actual by Predicted Plot

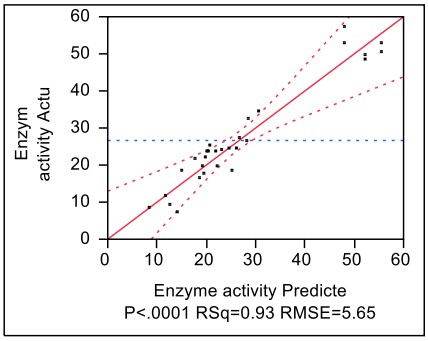

Figure 1. Least squares fit graph for actual and predicted.

Table 1. Summary of Fit.

R^2	0.931089
R^2Adj	0.846275
Root Mean Square Error	5.656044
Mean of Response	26.63067
Observations (or Sum Wgts)	30

Table 2. Parameter Estimates.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	51.878333	2.30907	22.47	<.0001*
pH (4,8)	0.26875	1.154535	0.23	0.8196
Temperature (25,45)	–1.020417	1.154535	-0.88	0.3928
Moisture content (30,70)	-0.197083	1.154535	-0.17	0.8671
Incubation time (3,5)	-1.337917	1.154535	-1.16	0.2674
pH*Temperature	-1.055625	1.414011	-0.75	0.4686
pH*Moisture content	-1.235625	1.414011	-0.87	0.3981
Temperature*Moisture content	1.185625	1.414011	0.84	0.4169
pH*Incubation time	1.083125	1.414011	0.77	0.4574
Temperature*Incubation time	0.369375	1.414011	0.26	0.7980
Moisture content*Incubation time	-0.133125	1.414011	-0.09	0.9264
pH*pH	-8.322396	1.079969	-7.71	<.0001*
Temperature*Temperature	-9.349896	1.079969	-8.66	<.0001*
Moisture content*Moisture content	-8.922396	1.079969	-8.26	<.0001*
Incubation time*Incubation time	-4.964896	1.079969	-4.60	0.0005*
Block [1]	0.2593333	1.460384	0.18	0.8618
Block [2]	3.7343333	1.460384	2.56	0.0239*

Table3. Scaled Estimates (Nominal factors expanded to all levels).

Term	Scaled Estimate	Plot Estimate	Std Error	t Ratio	Prob>|t|
Intercept	51.878333		2.30907	22.47	<.0001*
pH (4,8)	0.26875		1.154535	0.23	0.8196
Temperature (25,45)	-1.02041		1.154535	-0.88	0.3928
Moisture content (30,70)	-0.19708		1.154535	-0.17	0.8671
Incubation time (3,5)	-1.33791		1.154535	-1.16	0.2674
pH*Temperature	-1.05562		1.414011	-0.75	0.4686
pH*Moisture content	-1.23562		1.414011	-0.87	0.3981
Temperature*Moisture content	1.185625		1.414011	0.84	0.4169
pH*Incubation time	1.083125		1.414011	0.77	0.4574
Temperature*Incubation time	0.369375		1.414011	0.26	0.7980
Moisture content*Incubation time	-0.13312		1.414011	-0.09	0.9264
pH* pH	-8.32239		1.079969	-7.71	<.0001*
Temperature*Temperature	-9.34989		1.079969	-8.66	<.0001*
Moisture content*Moisture content	-8.92239		1.079969	-8.26	<.0001*
Incubation time*Incubation time	-4.96489		1.079969	-4.60	0.0005*
Block [1]	0.2593333		1.460384	0.18	0.8618
Block [2]	3.7343333		1.460384	2.56	0.0239*
Block [3]	-3.99366		1.460384	-2.73	0.0170*

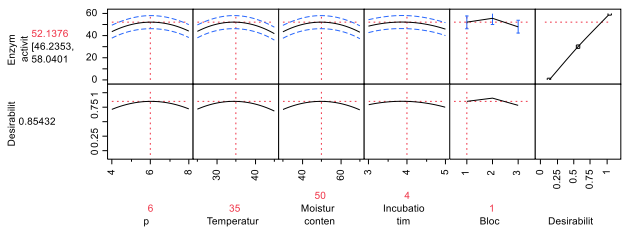

Figure 2. Prediction Profiler.

Fit Y by X Group

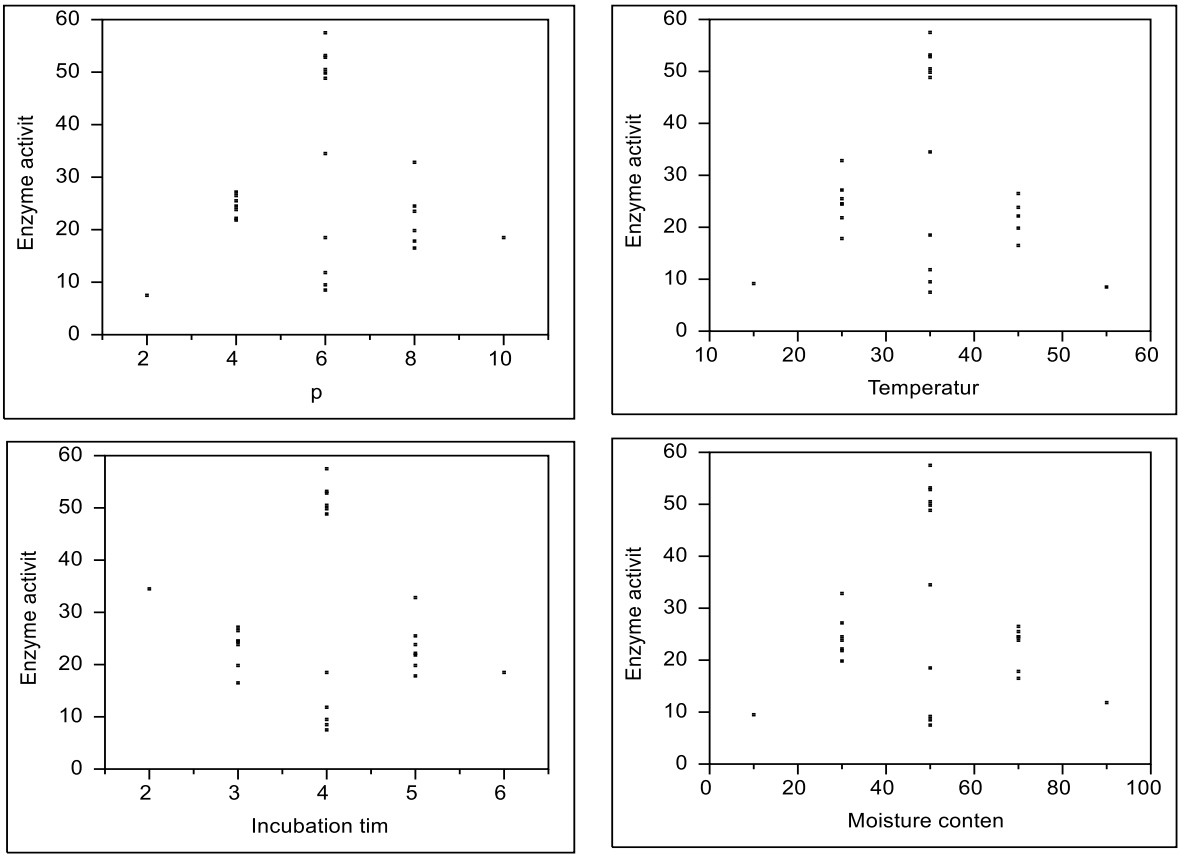

Figure 3. Bivariate fit of enzyme activity by pH, Temperature, Moisture content, Incubation time.

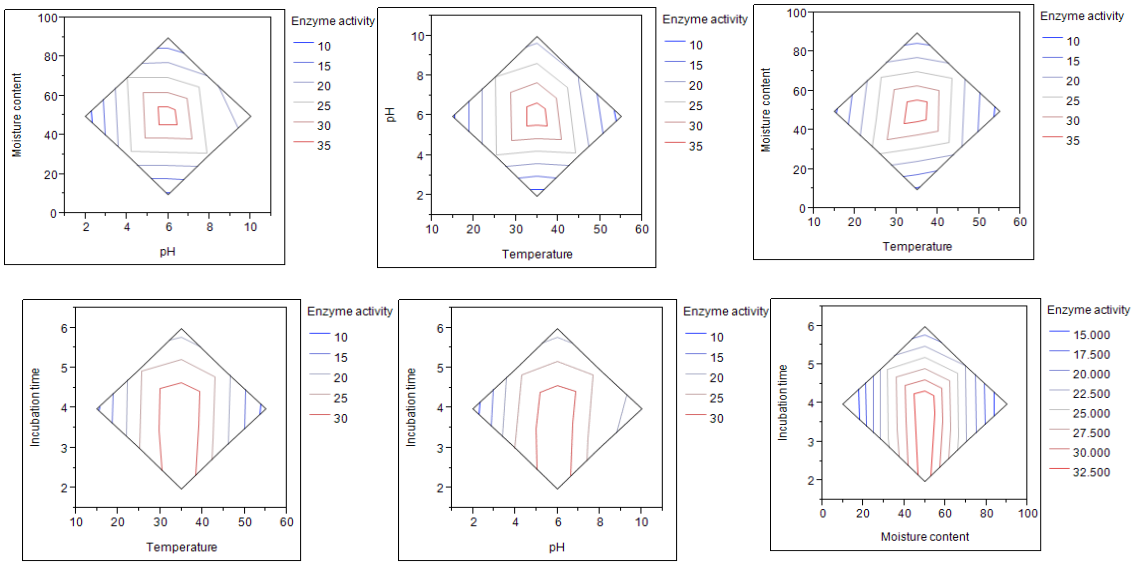

Figure 4. Influence of variable represented in contour plot for Biomass production: a) pH vs. Temperature b) Incubation time vs. Temperature c) Incubation time vs. pH.

3.4. Effect of carbon source.

Cellulase production by *Aspergillus flavus* was significantly influenced by the type of carbon source in MSM. Among the carbon source used, glucose influenced cellulase production (Table 4). Glucose was the most effective as a sole carbon source for cellulase production in both SSF [30-32]. Carbon sources (methylcellulose, hydroxyethylcellulose,

glucose) acts as an effective inducer for the secretion of cellulase in many fungal organisms [33]. A report revealed that the olive pomace also renders as a good carbon source for the production of cellulase in both liquid and solid-state fermentation [34].

Table 4. Results for carbon source estimation in SMF.

Content	1st day (mg/ml)	2nd day (mg/ml)	3rdday (mg/ml)	4th day (mg/ml)	5th day (mg/ml)
CMC	19.1 ± 0.2	21.2 ± 0.5	22.2 ± 0.4	19.2 ± 0.2	16.2 ± 0.9
Sucrose	21.2 ± 0.3	19.2 ± 0.1	18.2 ± 0.8	11.2 ± 0.1	31.2 ± 0.2
Glucose	18.2 ± 0.4	21.2 ± 0.6	38.2 ± 0.1	40.2 ± 0.4	45.2 ± 0.4

3.5. Effect of nitrogen source.

Cellulase production by *Aspergillus flavus* was significantly influenced by the type of nitrogen source in MSM (Figure 5) (Table 5). Yeast was the most effective as a sole nitrogen source for cellulase production in SSF. The optimal conditions of carbon and nitrogen sources with other physical parameters highly influenced the production of multienzyme in *A.clavatus* and *P.citrinum* [35].

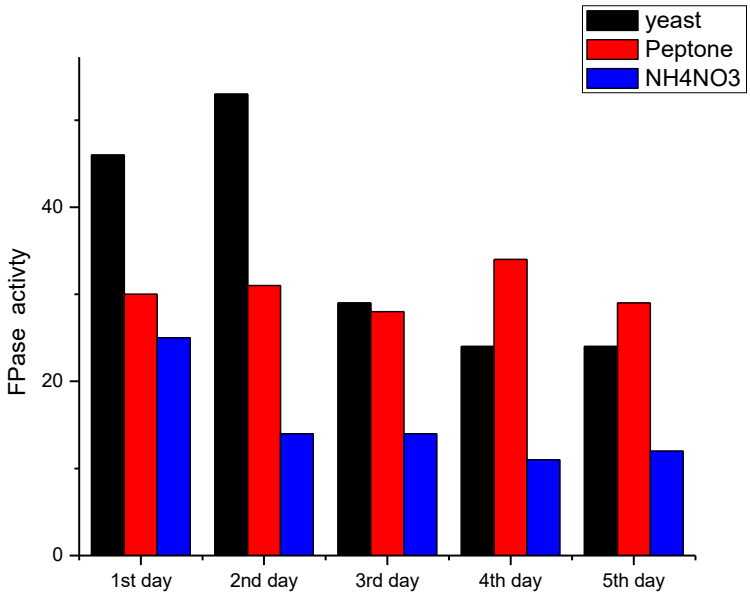

Figure 5. Effect of nitrogen source on FPase.

Table 5. Effect of various nitrogen source on FPase.

Content 2%	1st day (mg/ml)	2nd day (mg/ml)	3rdday (mg/ml)	4th day (mg/ml)	5th day (mg/ml)
Yeast	46.2 ± 0.5	53.2 ± 0.9	29.4 ± 0.4	24.2 ± 0.1	24.4 ± 0.2
Peptone	30.1 ± 0.5	31.3 ± 0.5	28.5 ± 0.6	34.9 ± 0.2	29.2 ± 0.6
$NH_4(NO_3)_2$	25.4 ± 0.9	14.1 ± 0.1	14.9 ± 0.1	11.7 ± 0.7	12.6 ± 0.1

3.6. Scanning electron microscope (SEM).

The SEM was used to study the morphological changes of cellulosic degradation by *Aspergillus flavus*. The longer incubation period of 28 days was required for the breakdown of cellular fibers(Fig 6). The enzyme degradation was observed on 0^{th} day and 28^{th} 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 28^{th} day compared to 0^{th} day (Figure 6).

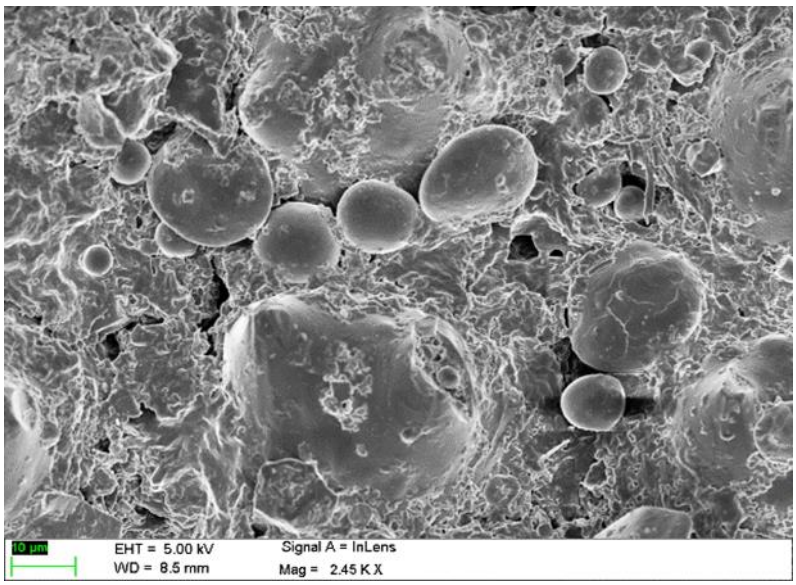

Figure 6. SEM.

3.7. Fourier transform infrared spectroscopy (FTIR).

The FTIR spectra for fungal treated samples and the compounds present on different days are the compounds observed on 0th day in rice bran [36-38] (Figure 7).

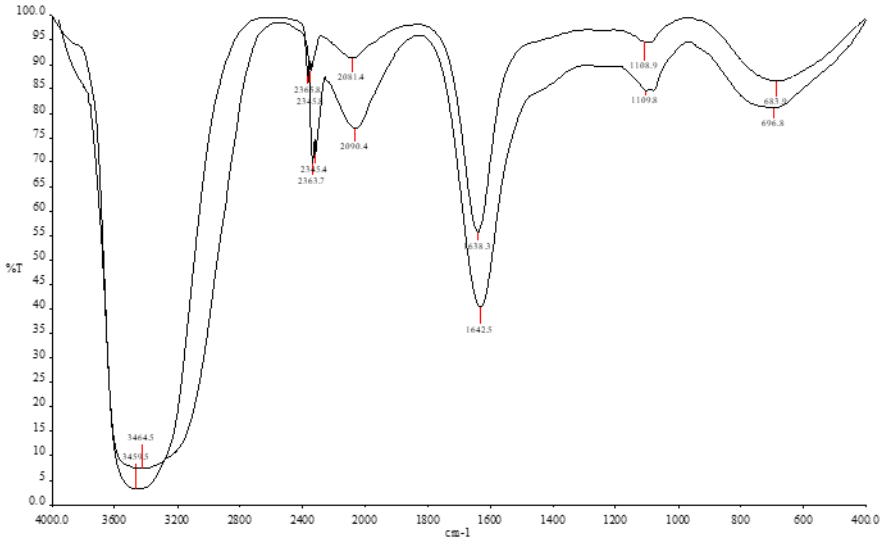

Figure 7. FTIR results.

Strong hydrogen-bonded (O–H) stretching absorption is seen at 3901 cm-1 and (C–H) stretch at 2928 cm-1. In addition, well defined peaks were shown at 1640 cm^{-1}, 1261 cm^{-1}, 1121 cm^{-1}, 899 cm^{-1} , 619 cm^{-1} and 592 cm^{-1}.

The compounds observed on 7th day in rice bran (Fig 7) 3459.5 cm^{-1} indicate O–H group (alcohol), 2081.4cm^{-1} indicates –C≡C– represent alkynes.1638.3cm^{-1} indicates C═C that represents alkene, aromatic ring.1108.9cm^{-1} indicate C–O that represent secondary alcohols,683 cm^{-1} indicate C–Br that represent alkyl halides. The FT-IR spectra of rice straw reports showed similar peaks around 3300cm-1 and 1640-1 with the hydroxyl bond(O-H) stretching and bend of free cellulase[39]

The compounds observed on 14th day in rice bran 3821.7 cm^{-1} indicate O–H group (alcohol), 3802 cm^{-1} indicates O–H represent alcohol group.3462.8 cm^{-1} indicate N–H that

represents primary amine or amide. 1636 cm^{-1} indicate C═C that represents alkene, aromatic ring,1083.8 cm^{-1} indicate C–O that represent secondary alcohol, 702 cm^{-1} represent phenyl group.

The compounds observed on 21st day in rice bran 3464.5 cm^{-1} indicate O–H group (alcohol), 2090 cm^{-1} indicates –C-represent alkyne.1648.5 cm^{-1} indicate C═C that represents alkene, aromatic ring.1109.8 cm^{-1} indicate C–O that represent secondary alcohol,696 cm^{-1} represent phenyl group The compounds observed on 28th day in rice bran. The peaks were observed to be 3421 cm^{-1}, 2923 cm^{-1}, 1734 cm^{-1}, 1642 cm^{-1}, 1501 cm^{-1}, 1259 cm^{-1}, 1055 cm^{-1} and 618 cm^{-1} showing all the stretches of O–H bond, aromatics, -C=C bond, –C–N bond and C–Cl bond.

The immobilization of the enzyme helps in the reuse and storage of enzymes [40].

4. Conclusions

This study could establish that rice bran, which is usually disposed of could serve as ideal substrates for the production of cellulases. Hence, the technology using these cheap and readily available substrates for the production of cellulases in optimum quantities holds promise for the future. The results are significant for the study on cellulase production and provide a potential approach for the industries. The culture conditions were optimized for the higher yield of the cellulase enzyme. Rice bran was selected as the best substrate for cellulase production using *Aspergillus flavus*. Cellulase production with *Aspergillus* was highest at temperature 30˚C, pH- 6.0, and incubation time-14th day. The best carbon source and nitrogen source for the growth of *Aspergillus flavus* is glucose and yeast extract. The morphological changes wereobserved by SEM and FTIR analysis. The high activity of cellulase enzymes will be of use in various industrial and biotechnological applications.

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Conflicts of Interest

The authors declare no conflict of interest.

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