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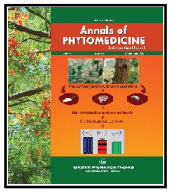

Original Article : Open Access

Screening, isolation and production of cellulases by *Aspergillus niger* JUC-2 under submerged and solid-state fermentation

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Abstract

A novel fungal isolate showing high cellulase activity was isolated from the garden soil sample of Jawaharlal Nehru Technological University, Hyderabad. The fungal isolate, on further analysis for strain identification and 18S rRNA sequencing was confirmed as a new strain *Aspergillus niger* JUC-2. Qualitative screening showed maximum enzyme activity of 2.1 ± 0.05 cm on a 1% carboxymethyl cellulose (CMC) plate. Further, cellulase production was carried out using agricultural wastes such as rice straw, groundnut shell, and wheat bran as carbon sources. Higher cellulase titres were obtained with rice straw in both submerged fermentation (SmF) and solid-state fermentation (SSF) on 4th day at 5.0 pH, 30°C with 10% inoculum. In SmF, the maximum activities of FPase, CMCase, and β-glucosidase were 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, and 2.6 ± 0.05 IU/ml, respectively. Whereas, in SSF, the maximum activity of CMCase, FPase, and β-glucosidase were 223.4 ± 2 IU/g, 24.24 ± 0.5 IU/g, and 6.8 ± 0.2 IU/g at relative humidity of 70% moisture content. Hence, the present microbial strain can be considered as a potential candidate for production of CMCase using untreated rice straw in an eco-friendly and economical basis.

1. Introduction

Cellulose is the most abundant organic matter and an inexhaustible renewable resource on earth. However, cellulose has not been effectively utilized because of its complex chemical structure. On the other side, alternate low-carbon, renewable fuels have attracted giving attention to fossil fuels as future transportation fuels. The agricultural crop residues such as wheat bran, rice straw, sugarcane bagasse, and sorghum bagasse are considered as potential feedstock in cellulosic ethanol production, since these are devoid of main issues like excess land use and food-fuel conflict (Ramiya *et al.*, 2020; Kamlesh *et al.*, 2020; Nidhi *et al.*, 2021).The better alternative to petroleum-derived fuels is cellulosic ethanol nonetheless, its commercial production is not cost-effective because of various economic challenges (Nitin *et al.*, 2020). Cellulase enzymes have industrial significance and are widely used for the production of bioethanol from lignocellulosic biomass. They are a cocktail of complex enzymes of 3 types, namely; cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCase (EC3.2.1.4), and β-glucosidases (EC 3.2.1.21) to convert cellulose into glucose by synergetic activity (Oscar *et al.*, 2019; Lodha *et al.*, 2020; Anuradha *et al.*, 2021). In recent years, the cellulases have been extensively studied in the production of second-generation ethanol (Hou *et al.*, 2020; Navnit *et al.*, 2019).

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Fungal genera such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Penicillium*, *Chaetomium*, and *Trichoderma* are in focus due to their cellulolytic activities and are receiving great attention in ethanol production (Johannes *et al.*, 2021; Tanwar *et al.*, 2020; Hussein *et al.*, 2013). The main reasons for studies on fungal cellulases are, firstly, as opposed to bacteria, downstream digestion is much simpler for fungi, and further steps in the purification of bacterial enzymes make their recovery cumbersome, resulting in higher costs for the substance. Secondly, the activity of fungal cellulases is much greater than that of bacteria-produced cellulases. Finally, as opposed to bacteria, fungi can grow on a relatively inexpensive substrate such as whey and other cellulosic waste and most fungal cellulases can digest cellulose because of their elongated hyphen, which causes mechanical strain on the structure of the cellulose.

To produce cellulases, fermentation is carried out by using two processes, submerged fermentation (SmF), and solid state fermentation (SSF). SmF process is traditionally equipped in the industries for the production of enzymes because of its easy handling system and control of several environmental conditions such as pH, temperature, agitation and aeration (Praveen *et al.*, 2015). In recent years, solid-state fermentation (SSF) has gained a substantial reputation in enzyme processing, where a fermented substance can be used directly as an enzyme source (Jimenez *et al.*, 2017). The purpose of this study was to investigate the ability of the newly isolated strain for the production of cellulases using various lignocellulosic biomass and comparing the process parameters of both SmF and SSF. To achieve maximum activity of crude enzymes and optimization of pH, moisture and temperature were conducted.

2. Materials and Methods

2.1 Sample collection

Various soil samples were collected from organic rich soils of JNTU Hyderabad (India) in sterile bags. The samples were suspended in sterile water and appropriate dilutions were plated on potato dextrose agar (PDA) by serial dilution, followed by spread plate method, which was incubated at 30ºC for 3-7 days. After incubation, the colonies were selected for further screening.

2.2 Carbon sources used in the present study

The agricultural wastes such as groundnut shells, wheat bran and rice straw were collected locally from the mills and fields in siddipet district, Telangana and all the lignocellulosic substrates were further processed, milled and used as carbon sources for the production of cellulase.

2.3 Screening and identification of cellulase producing fungal strains

Based on cellulase production efficiency, both qualitative and quantitative tests were carried out for the screening of cellulolytic fungi.

2.3.1 Qualitative screening of cellulase producing strains

The fungal isolates were tested for their ability to produce cellulase enzyme using a 1% carboxymethyl cellulose (CMC) agar plate. The plates were incubated at 28ºC ± 0.5ºC for 3 days and stained with 1% congo red dye for 1 h, followed by destaining with 1M NaCl solution for 15-20 min. Cellulose activity on CMC agar was determined by the relative cellulolytic activity index (RCAI). The enzyme activity was calculated as enzyme index (EI) which is equal to the diameter of the hydrolysis zone by the diameter of the colony. Zone of clearance can be observed around active fungal strain.

2.3.2 Fungal identification and phylogenetic analysis

Isolation of genomic DNA

Genomic DNA was isolated using a fungal genomic DNA isolation kit from Chromus Biotech, India. 100 mg of fungal culture was taken in mortar and pestle along with 750 µl of 1X suspension buffer. The fungal tissue was crushed till it formed a fine paste which was transferred to a 2 ml vial, followed by the addition of 5 µl of RNaseA solution and incubated at 65ºC for 10 min with intermittent mixing. The sample was incubated with 1ml of lysis buffer for 15 min at 65ºC, the mixture was centrifuged at 13,000 g for 1 min at room temperature and the clear supernatant was collected. This step was repeated twice. 500 µl of 1X wash buffer was then added to the sample and centrifuged at 13,000 g for 1 min at room temperature. The sample was incubated with 50 µl of elution buffer at 65ºC for 1 min and once again centrifuged at 13,000 g for 1 min. The pellet was dissolved in TE buffer and DNA content was extracted.

2.4 Seed culture preparation

Seed culture was prepared by inoculating the fungal spores from fresh PDA slants into potato dextrose broth for 24 h at 30^{0}C in a shaking incubator before it was used for the fermentation process.

2.5 Production of cellulase enzyme by submerged fermentation process

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks with a working volume of 100 ml, using following medium components, urea 0.3 g/l; $(NH_4)_2SO_4$ 1.4 g/l; K_2HPO_4 2.0 g/l; $CaCl_2$ 0.3 g/l; $MgSO_4$ 0.3 g/l; yeast extract 3 g/l; peptone 0.75 g/l; $FeSO_4$ 5.0 g/l; $MnSO_4$ 1.6 g/l; $ZnSO_4$ 1.4 g/l; $CoCl_2$ 0.2 g/l; tween 80, 0.2 % (v/v); carbon source, 4%. The medium was sterilized by autoclaving at 121ºC for 15 min. Each flask was inoculated with seed culture (1×10^7 spores/ml). The cultures were incubated at 30°C for 7 days on a rotary shaker (120 rpm).

2.6 Production of cellulase enzyme by solid-state fermentation process

Solid-state fermentation was carried out in 250 ml Erlenmeyer flasks using 10 g of carbon source and 20 ml of above-mentioned production medium. The flasks were sterilized at 121ºC for 15 min and cooled to room temperature. About 1×10^7 spores/ml inoculum was added, mixed well, and incubated at 30ºC in a humidified incubator for 96 h.

2.7 Enzyme extraction process

In solid-state fermentation (SSF), the enzyme was extracted by mixing homogeneously with (1:7w/v) citrate buffer (0.05M), pH 5, and agitated at 30°C on a rotary shaker (100 rpm) with a contact time of 1h. The extract was filtered using dampened cheesecloth to obtain the enzyme. In both (SmF and SSF), samples were centrifuged separately at 8000 rpm for 15 min and the supernatant were collected for further analysis.

2.8 Cellulase enzyme activity

Carboxymethyl cellulose (CMCase), filter paper assay (FPase), and β-glucosidase activities were assayed according to the standard method described by Ghose *et al* (1987). Crude enzyme extract was serially diluted by 2-fold dilution before assay. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmole of reducing sugars per min with glucose as standard for FPase and CMCase, whereas one International Unit (IU) of cellobiase is defined as 2 µmole of glucose released in the assay and the values of enzymatic activity were expressed as IU/ml for SmF and IU/g of dry mycelial bran (DMB) for SSF.

2.9 Optimization of physicochemical parameters of fermentation for enzyme production

To obtain high yields of enzyme various physical and chemical parameters of fermentation were carried out. All the experiments were conducted in triplicates and its means results were represented with standard deviation.

2.9.1 Nitrogen source

The basal medium described by Mandels *et al.* (1969) was used for all fermentations. To investigate the effect of nitrogen sources on cellulase production, different types of nitrogen source [$(NH_4)_2SO_4$, peptone, urea, and yeast extract] at different concentrations (3, 6, 3, and 6 gl^{-1}) were used separately in the medium formulation.

2.9.2 Carbon source

The effect of carbon source on growth and enzyme production by the selected isolate was determined in SmF and SSF process using the following as a carbon source: rice straw, groundnut shell, and wheat bran with 4% in SmF and 10% in SSF.

2.9.3 Effect of moisture level on cellulase production for SSF

The effect of moisture level on cellulase production was tested by varying the rice straw to-moisture ratio (w/v) in the range of 1:1 to 1:3 where the medium was added as a moistening agent, to compare the enzyme activity for solid-state fermentation only.

2.9.4 Effect of pH on cellulase production

The effect of initial pH on growth and cellulase production was performed by adjusting the medium pH ranging from 3 to 7 using either in HCl or in NaOH. The flasks were kept at 30ºC for 7 days of cultivation.

2.9.5 Effect of temperature and incubation time on cellulase production

The optimum temperature for cellulase production was carried out at various temperatures (20, 25, 30, 35, 40, 45, and 50ºC). The effect of the incubation period on cellulase production was also studied in the flasks from 1, 2, 3, 4, 5, 6, and 7 days incubation both in submerged and solid-state fermentations.

2.10 Scanning electron microscopy

The surface properties of rice straw (inoculated and uninoculated with *Aspergillus niger* JUC-2) were investigated using scanning electron microscope (SEM). The fungal cells were collected by centrifugation (10,000 × g, 10 min) and rinsed 3 times with ultrapure water. Dried samples were coated with gold-plated coater (OPC80T; Filgen, Nagoya, Japan) and then examined by SEM (JSM 6320F; JEOL Ltd., Tokyo, Japan). (Lee *et al*., 2020).

2.11 Functional group identification and characterization of biomass by FTIR

A Perkin Elmer spectrum FTIR was used to obtain spectra from inoculated and uninoculated biomass. FTIR spectroscopy can be used to identify the functional groups. The instrument was equipped with a mercury cadmium telluride (MCT) detector and the spectra were recorded in the frequency range of 600 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. For FTIR measurement, the dried biomass samples of approximately 1.5 mg were ground with 100 mg of spectroscopic grade potassium bromide (KBr) powder in an agate mortar (Kiran *et al*., 2018).

3. Results

3.1 Isolation and qualitative screening of cellulase producing strains

All microbial isolates were incubated at 30°C for 48 h and were screened for the presence or absence of cellulolytic activity on CMC plates. The isolates which can utilize cellulose were selected for further study for the production of cellulase (Lynd *et al*., 2020; Islam *et al*., 2018). A clear zone around the colonies was observed after staining with Congo red, indicating the hydrolysis of CMC as a result of cellulases production (Fatima *et al*., 2014; Ramiya *et al*., 2020). Figure 1 depicts that all the fungal isolates were efficient in cellulase production with diameter of clear zone ranging between 0.43 to 2.1 cm. Out of the all the selected isolates, JUC-2 isolate showed the highest cellulolytic activity of 2.1 ± 0.05 cm (Table1). Hence further evaluationof phylogenetic sequence was carried out using JUC-2 isolate.The phylogenetic analysis based on 18s rRNA (Figure 2) showed 94% similarity with *Aspergillus niger* ISSFR-019 strain and can be considered as a new isolate and was named as *Aspergillus niger* JUC-2.

Table 1: Hydrolysis capacity of carboxymethyl cellulose (CMC) by various cellulase-producing fungi isolated from JNTUH garden soil sample

No of isolates	Zone of clearance (cm)
JUC-1	0.81 ± 0.02
JUC-2	2.1 ± 0.05
JUC-3	0.92 ± 0.01
JUC-4	1.14 ± 0.03
JUC-5	1.31 ± 0.02
JUC-6	0.43 ± 0.01
JUC-7	1.18 ± 0.05
JUC-8	1.13 ± 0.06
JUC-9	0.59 ± 0.01
JUC-10	0.64 ± 0.03

Table 2: FTIR band and corresponding groups present in lignocellulosic biomass

Name of the molecule	Corresponding peaks (cm^{-1})	Correspond to
Cellulose	900	Glycosidic bond
	1075	C-O-C
	2910	CH
	3300-3500	OH
Hemicellulose	875-930	Glycosidic bond
	1720	C=O
Lignin	1268	Guaiaryl
	1320	Syringyl
	1310-1390	Phenol
	1465	C-H deformation
	1515-1650	Aromatic
	1720	C=O

3.2 Optimization of physicochemical parameters of fermentation for enzyme production.

The microbial growth dependents on several macro and micronutrients and few physical conditions. Therefore, the effect of macronutrients like nitrogen and carbon; and physical conditions like temperature and pH on microbial growth were optimized as one factor at a time model.

3.2.1 Nitrogen source

Nitrogen is one of the major nutrients for stimulation of cellulase activity. The effect of various nitrogen sources on cellulase production by *Aspergillus niger* JUC-2 was investigated using urea, peptone, yeast extract, and ammonium sulphate at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 g/l). The maximum cellulase activity was obtained at 0.3 g/l urea, 0.6 g/l peptones, 0.6 g/l yeast extract, and 0.3 g/l ammonium sulfate. The activities of

FPase, CMCase, and β-glucosidase were 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, 2.6 ± 0.05 IU/ml in SmF, and 24.24 ± 0.5 IU/g, 223.4 ± 2 IU/g, 6.8 ± 0.2 IU/g in SSF, respectively. Similar studies were reported with 0.03% urea, peptone, $NaNO_3$ as nitrogen source, with the activities of cellulase of 0.824, 0.421 and 0.401 IU/ml, respectively (Narasimha *et al*., 2006). In another study (Hitesh *et al*., 2016) urea at 0.04 % showed maximum CMCase activity 0.25 IU/ml, exoglucanase 0.03 IU/ ml, β-glucosidase 0.20 IU/ml, FPase 0.23 IU/mlat 96 h. Figure 3 shows that among the various sources of nitrogen, ammonium sulphate resulted a 3-fold increase in enzymatic cellulase activity in both SmF and SSF. Ammonium sulphate might help in the stimulation of cellulase activity (Sajib *et al*., 2021). Jaspreet *et al*. (2020) reported maximum CMCase of 126.26 IU/g using *A.niger* P-19 strain whereas, in the present study, the isolated strain (*Aspergillus niger* JUC-2*)* resulted CMCase titres of 223.4 ± 2 IU/g with ammonium sulphate.

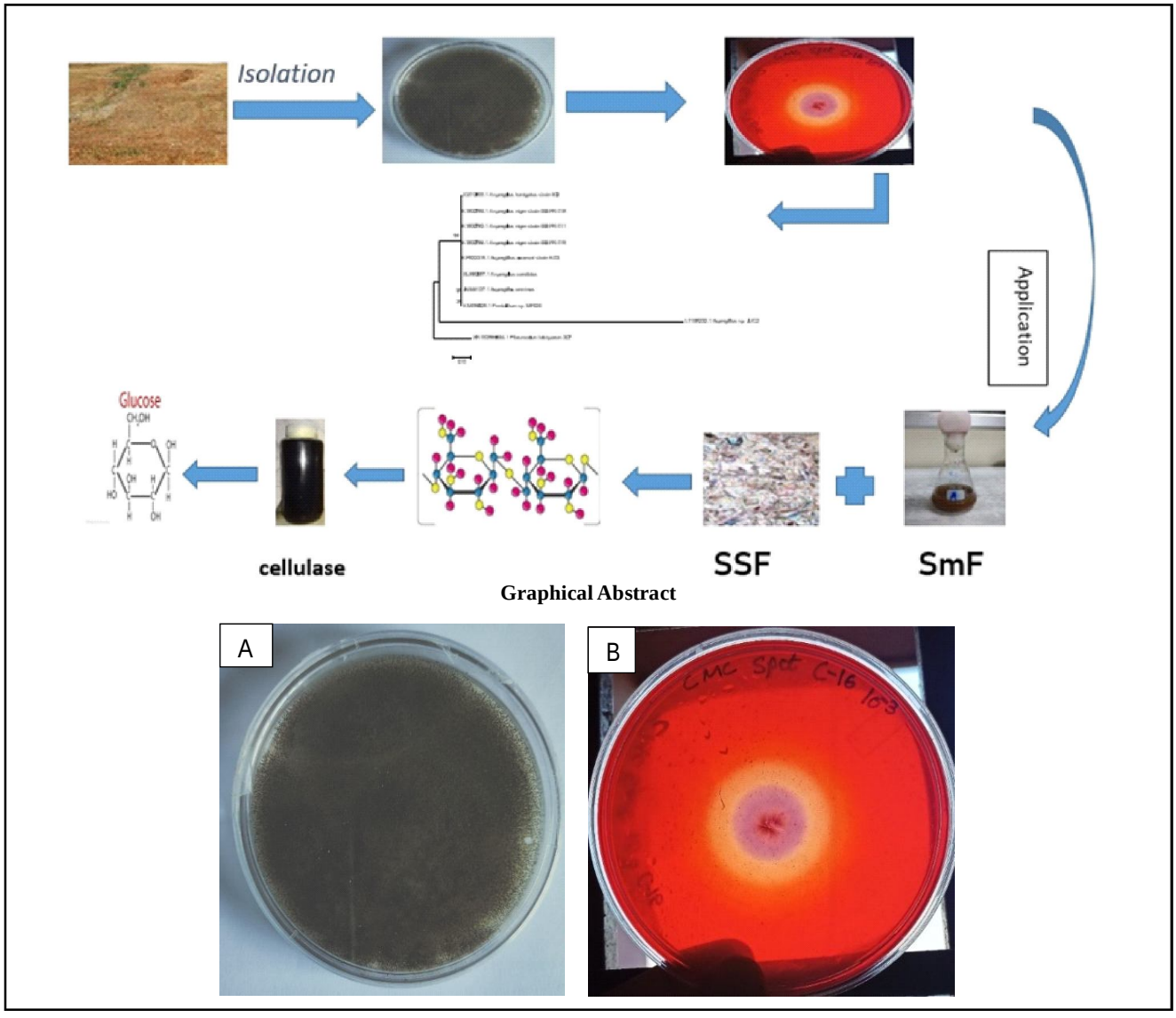

Figure 1: (A) *Aspergillus niger* JUC-2 with spore after growth on cellulose agar medium. (B) *Aspergillus niger* JUC-2 forming clear zone on a cellulose agar plate.

3.2.2 Carbon source

Cellulase production was found to be dependent upon the nature of the carbon source used in the culture media. The carbon source supplied to microorganisms also effect induction and repression of cellulases at genetic level. Different types of carbon substrates such as rice straw, wheat bran, and groundnut shell were studied for enzyme production. The carbon sources used in this study exhibited variable effects on the selected parameters, of which rice straw supported the maximal activities of FPase, CMCase, and β-glucosidase were 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, and 2.6 ± 0.02 IU/ml in SmF and 24.24 ± 0.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/g in SSF for rice straw; 0.24 ± 0.02 IU/ml, 12 ± 0.2 IU/ml and 1.01 ± 0.002 IU/ml in SmF and 12.4 ± 0.3 IU/g, 90.06 ± 0.5 IU/g and 2.65 ± 0.1 IU/g in SSF for wheat bran; 0.52 ± 0.03 IU/ml, 22 ± 1 IU/ml and 1.5 ± 0.02 IU/ ml in SmF and 16.74 ± 0.5 IU/g, 156.2 ± 2 IU/g and 4.25 ± 0.2 IU/g

in SSF for groundnut shell, respectively as shown in Figure 4. The results are comparable with study reported by Mrudula *et al.* (2011) on wheat bran with titres of FPase, CMCase, of 0.25, 0.31 IU/ml and 0.47, 0.49IU/g (SmF and SSF).

3.2.3 Effect of temperature

Temperature plays a crucial role in the metabolic activity of organisms and the stability of the enzyme. Hence, it is essential to optimize temperature to obtain maximum cellulase production. The effect of temperature on cellulase activity was determined at 20, 25, 30, 35, 40, 45, and 50ºC in SmF and SSF. The summary of the results showed maximum activities of FPase, CMCase, and β-glucosidase were 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, and2.6 ± 0.05 IU/ml in SmF and 24.24 ± 0.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/g in SSF, respectively at 30ºC on 4th day (Figure 5). Eriksson *et al.* (1976) suggested that the optimal temperature for cellulase production range between 20-30^{0}C for *Aspergillus* sp. both in SMF and SSF. According to previous studies, the maximum cellulase activity was observed at 28ºC, the activity of CMCase, FPase and β-glucosidase was 0.51 IU/ml, 0.29 IU/ml, and 0.23 IU/ml in SmF, respectively (Hitesh *et al.*, 2016). Other studies by Neeraj *et al.* (2017) reported maximum production of CMCase by *A. niger*, BK01 was achieved at 28ºC, resulting in 9.86 ± 0.05 IU/g. The optimum temperature might lead to mass transfer of nutrients and faster metabolic activity with an increase in protein content, and enhanced extracellular enzyme production. Similar studies were also carried out by Pooja *et al.* (2016) reporting that higher incubation temperature could lead to thermal denaturation and loss of protein and cell integrity. Whereas, transportation of nutrients is hindered below the optimum temperature (30ºC).

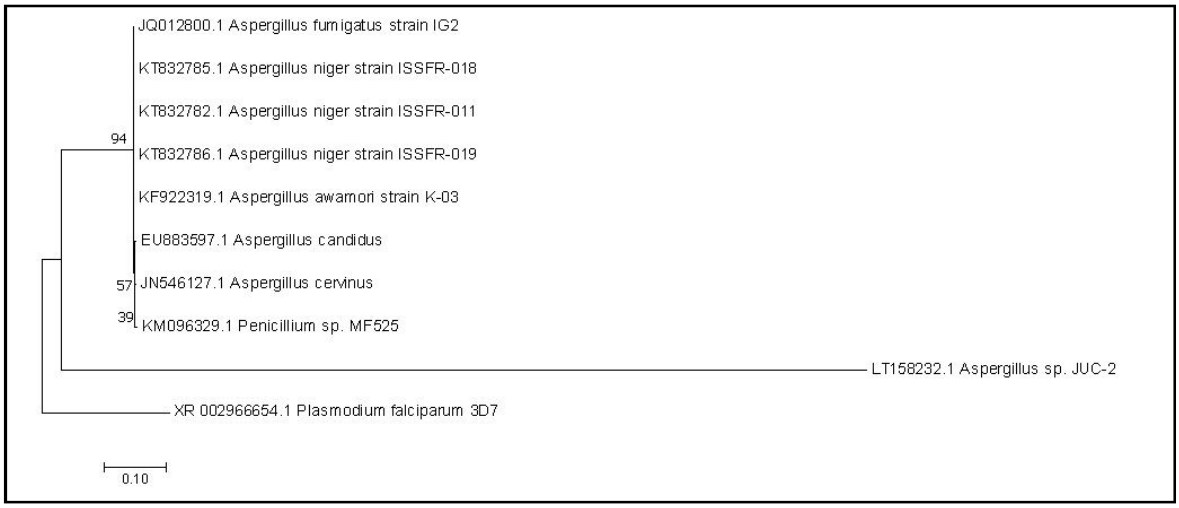

Figure 2: Phylogenetic trees based on the 18s r RNA sequence.

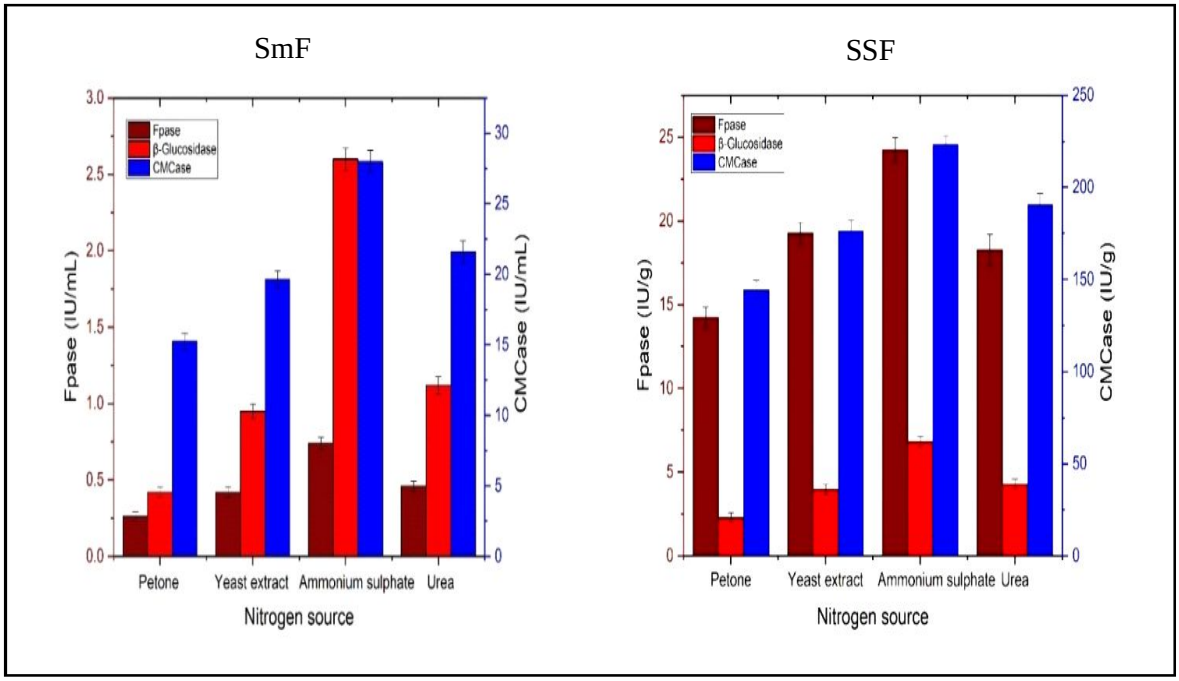

Figure 3: Effect of nitrogen source on cellulases production with *Aspergillus niger* JUC-2 in SmF and SSF.

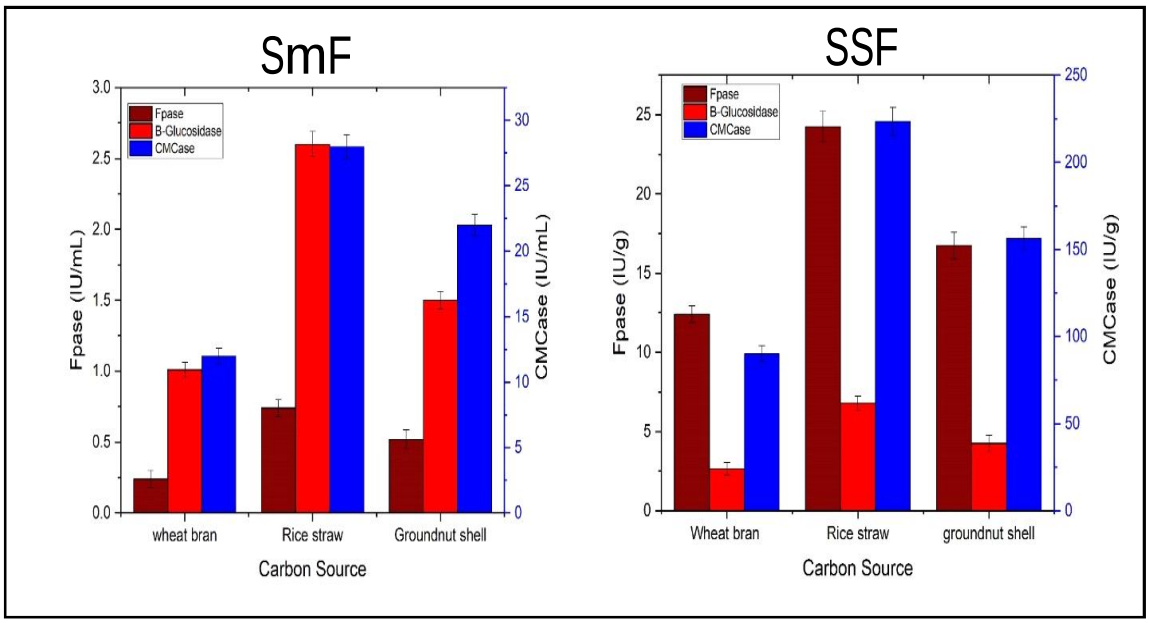

Figure 4: Effect of carbon source on cellulase enzyme production with *Aspergillus niger* JUC-2 in SmF and SSF.

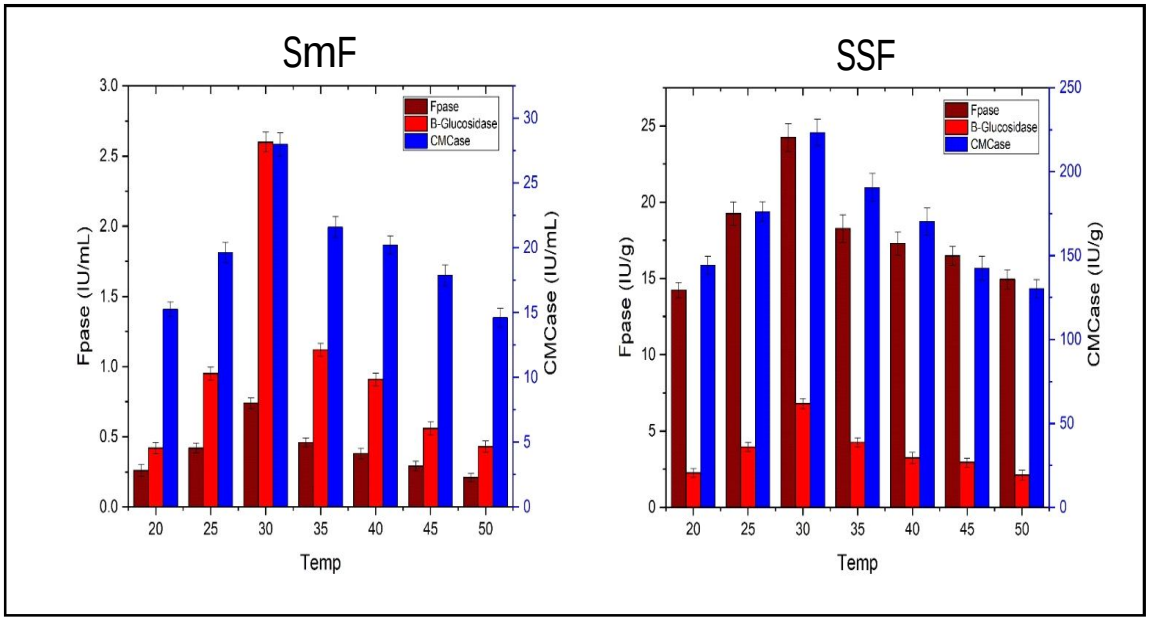

Figure 5: Effect of temperature on cellulase enzyme production with *Aspergillus niger* JUC-2 in SmF and SSF.

3.2.4 Effect of pH

pH is a key parameter that influences fungal growth, enzyme stability and production. pH of the broth during microbial growth was maintained from 3-7 in both SmF and SSF (Figure 6). It was observed that with the increase in pH from 3.0 to 5.0, the enzyme activity of cellulase had also been increased in both SmF and SSF. The maximum activity of FPase, CMCase, and β-glucosidase was 0.7 ± 0.02 IU/ml, 28 ± 0.15 IU/ml and 2.6 ± 0.05 IU/ml in SmF and 24.4 ± 0.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/gin SSF, respectively at pH 5. Results showed that cellulase activities decreased as the pH increased further towards alkalinity. This could be attributed that a slight change in pH can affect the enzyme catalytic activity on cellulase production (Eriksson *et al*., 1976; Pooja *et al*., 2016). These results were also supported with the literature where the maximum cellulase activity was observed at pH 4.2-5.2 with the enzyme activities of CMCase, FPase and β-glucosidase as 0.51 IU/ml, 0.29 IU/ml, and 0.23 IU/ml, respectively (Hitesh *et al.*, 2016). Sahar *et al.* (2017) also reported that pH 5.0 is a favourable condition for *Aspergillus* sp. Hence, it was concluded that pH 5.0 was found to be favourable conditions for cellulase enzyme production in SmF and SSF.

3.2.5 Effect of incubation period

Incubation period is directly related to the production of enzymes and other metabolites. Enzymes being the primary metabolites are

synthesized during the logarithmic phase of growth cycle. The type of cellulase production varies based on the type of carbon source utilization. The rice straw used in the study contains cellulose, hemicellulose and lignin as major components. Figure 7 depicts that *Aspergillus niger* JUC-2 cellulolytic activity varies during different incubation periods (days). The optimum incubation period for enzyme production by the isolate *Aspergillus niger* JUC-2 was determined on the 4th day with the maximum activity of FPase, CMCase, and β-glucosidase was 0.7 ± 0.02 IU/ml, 28 ± 0.15 IU/ml and 2.6 ± 0.05 IU/ml in SmF and 24.4 ± 0.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/g in SSF, respectively. According to Hitesh *et al.* (2016) the CMCase, FPase, and β-glucosidase were 0.24 IU/ml, 0.21IU/ml, and 0.21IU/ml, respectively at 96 h in SSF. In another study, the CMCase production by *Aspergillus* BK01 observed maximum levels after 96 h of incubation 9.06 ± 0.06 IU/g (Neeraj *et al.*, 2017). A drastic decline in cellulose acidity was observed, this might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the repression of the genes involved in the production of enzymes. Melo *et al.* (2007) reported that the enzyme level decreased with prolonged incubation, due to loss of moisture or denaturation of the enzyme resulting in variation in pH during fermentation. Singh *et al.* (2009) reported that the decrease of enzyme activities may be due to the accumulative effect of cellulobiose.

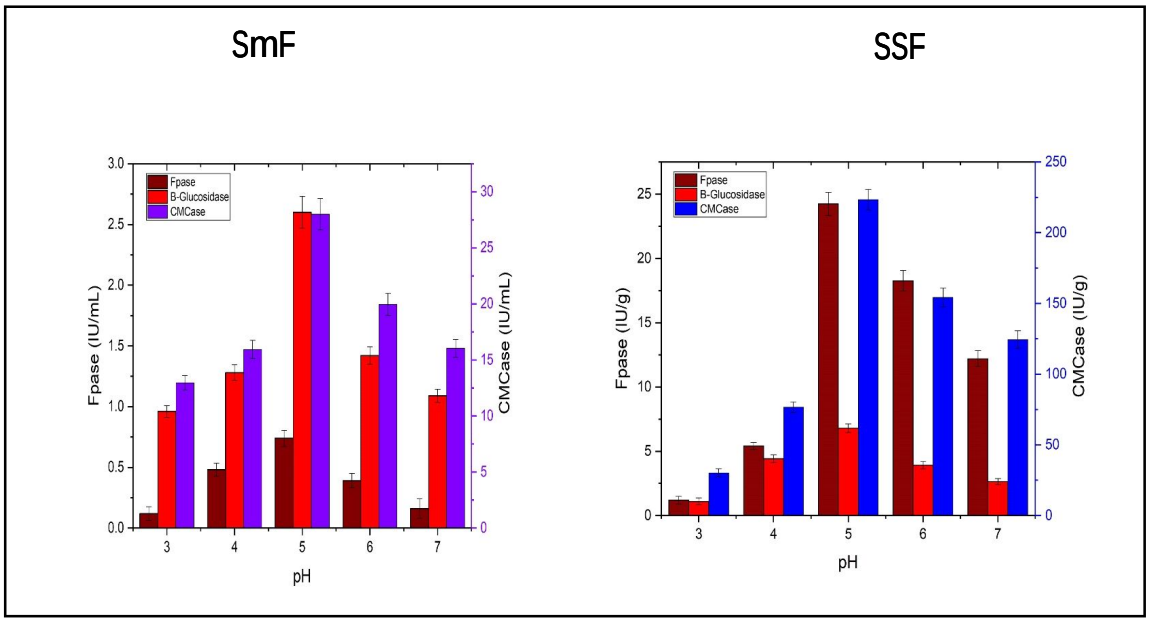

Figure 6: Effect of pH on cellulase enzyme production with *Aspergillus niger* JUC-2 in SmF and SSF.

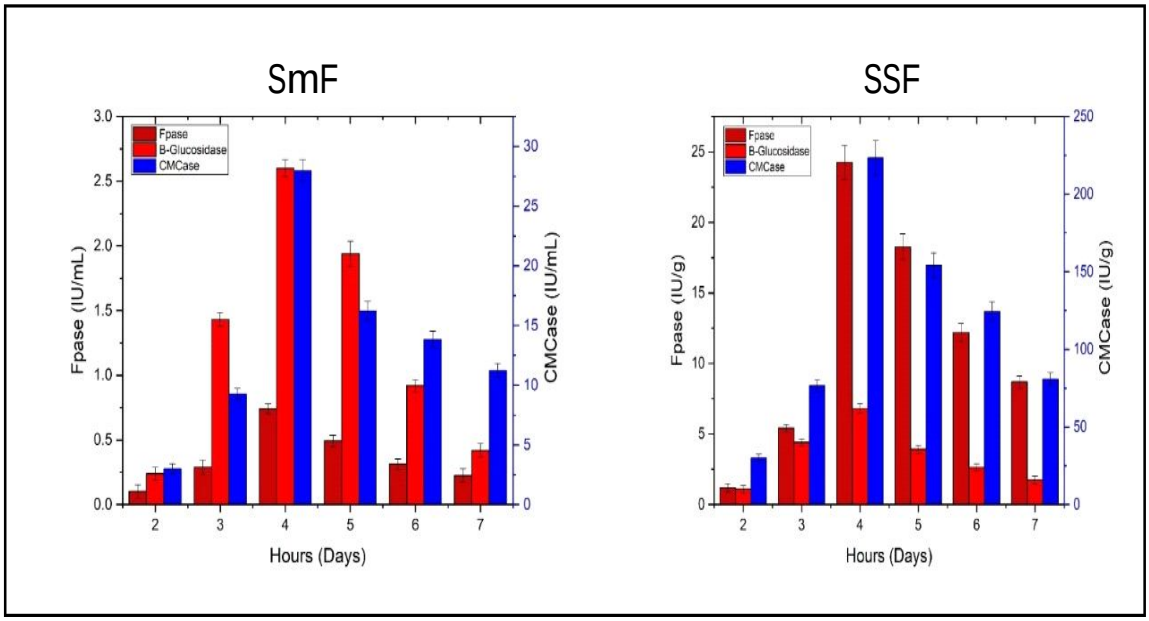

Figure 7: Cellulase enzyme activity with *Aspergillus niger* JUC-2 in SmF and SSF.

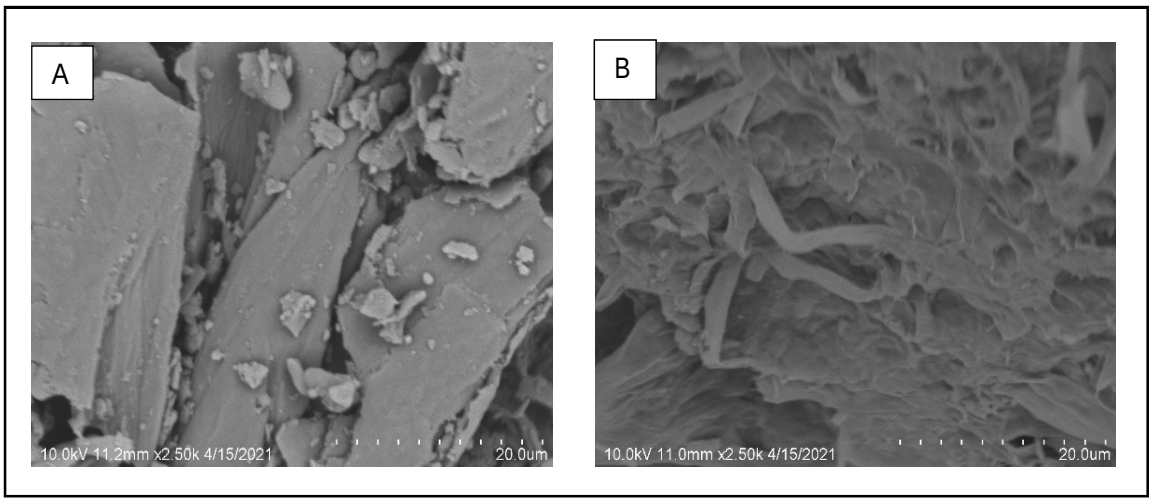

Figure 8: Images of scanning electron microscope at x100 magnification. [A]: Uninoculated rice straw. [B]: Rice straw inoculated with *Aspergillus niger* JUC-2.

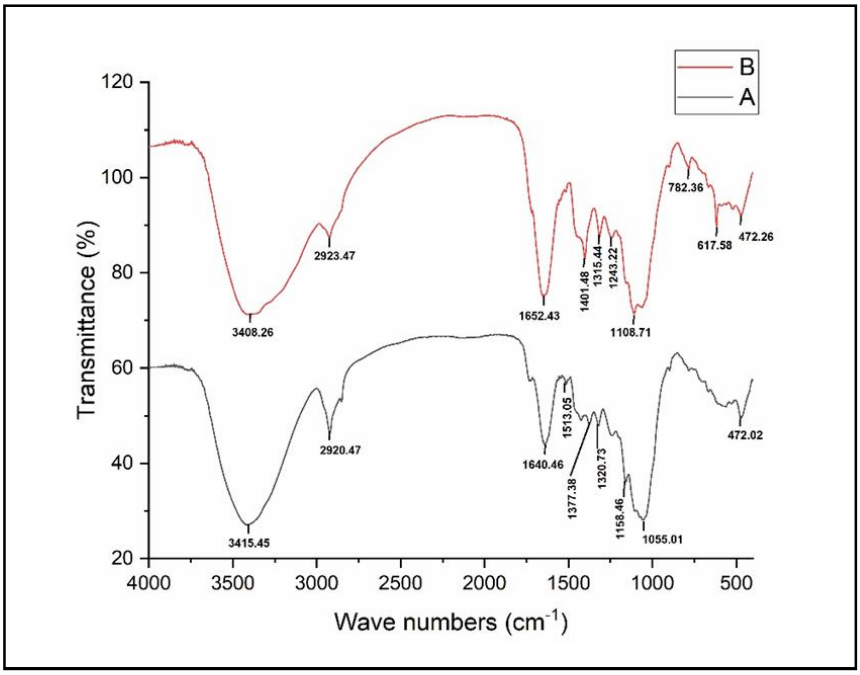

Figure 9: FT-IR of rice straw (A): Uninoculated and (B): Inoculated.

3.2.6 Effect of moisture level

Moisture content is a crucial factor in the SSF process because this variable can influence the growth and biosynthesis of the microbe as well as the secretion of different metabolites such as enzymes. The result demonstrated that FPase, CMCase, and β-glucosidase were 24.4 ± 0.5 IU/g, 223.4 ± 2 IU/g, and 6.8 ± 0.2 IU/g in SSF, respectively on the 4th day of the fermentation process with 70% of moisture content. Jaspreet *et al*. (2020) reported maximum cellulase production at 83.3% moisture content with a 1:5 solid-liquid ratio, exhibiting the yields corresponding to 70 IU/g, 27 IU/g, and 37 IU/g for CMCase, FPase, and β-glucosidase, respectively. These results are also supported from the earlier reports indicating 70% (w/v) as the most suitable moisture content range for cellulase production (Lee *et al*., 2011). Low moisture contents limit nutrient solubility and effective uptake by the fungi. However, higher level of moisture content (80%) in the medium also affects the microbial growth. The adversative effect of high-level of moisture content is recognized for the reduction of substrate porosity, mass transfer, low heat through the culture and decrease of air conversation, which in turn result in a decrease of fungal growth and formation of product (Sanjay *et al*., 2011).

3.3 Quantitative evaluation of cellulases production

Cellulases production were carried out in both SmF and SSF with the above-optimized conditions resulted in the following enzyme activities.

Submerged fermentation and solid-state fermentation

Out of the three substrates studied, rice straw showed higher cellulase production in both SmF and SSF. The production of FPase, CMCase, and β-Glucosidase activities using rice straw as a substrate was carried out for 7 days. Figure 7 depicts that at the initial stage of fermentation, the yields of FPase, CMCase, and β-Glucosidase were lower or in the undetectable range on day-1 incubation. The production of cellulase started from day 2 and reached its maximum activity on the 4th day. The maximum cellulase activities of FPase, CMCase, and β-Glucosidase were found to be 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, and 2.6 ± 0.05 IU/ml for SmF and 24 ± 0.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/g for SSF, respectively. In solid-state fermentation (SSF), natural fermentation of solid waste is cultivated under favourable conditions which are closer to their natural habitat, results in the production of extracellular enzymes and other enzymes than that of submerged fermentation (Shruthi *et al*., 2019). After the incubation period of the 4th day, a decline phase of cellulolytic enzyme activity was observed with rice straw. The decline trend of the cellulase enzyme production might be due to the end products (protease and xylanase) accumulation in the media that are produced by certain metabolic pathway which hinder the enzymatic activity (Vinod *et al*., 2014). Shaymaa *et al*. (2020) reported cellulase enzyme activity of 37.481 IU/g after 7 days of the fermentation period, which was further optimized to reach 124.94 IU/g. In another study, *A. terreus* showed maximum CMCase activity in SSF with different crude substrates, such as banana peels, with maximum activity of 1.11 and 1.5 U/ml in mono and co-culture with *Aspergillus niger* MS23, respectively (Rehman *et al*., 2014). Whereas, sugarcane-bagasse and sweet sorghum bagasse reported maximum activity of 145.32 IU/g and 105.2 IU/g, respectively (Kiran *et al*., 2015; Sharma *et al*., 2014). Kiran *et al*., (2015) also studied on rice straw as a carbon source for the production of cellulase and observed maximum titres of 96.6 IU/g CMCase at optimized cultural conditions in SSF using *A. terreus*. When compared between SmF and SSF, production of total cellulase in SSF was 32.7, 8, and 2.6 folds higher than SmF. From the present investigation, the highest cellulase productivity was obtained with solid-state fermentation. The advantage of SSF process in the enzyme production is by obtaining concentrated form and adjusting the extraction conditions (1gsolid: 7 ml of buffer), so that it is more economical at commercial scale.

3.4 Scanning electron microscope (SEM) analysis

Figures 8. A and B show the morphological changes observed in rice straw uninoculated and inoculated with microorganisms *via* scanning electron microscope. The structure of uninoculated rice straw (Figure A) looks intact without any disruption on its surface, whereas rice straw inoculated (Figure B) with microorganism's shows surface disturbance along with hyphal growth. The selected enzymatic of microorganisms covered disintegration of biomass.

3.5 Identification of functional group through fourier transform Infrared (FTIR) spectroscopy

To study the functional groups in biological samples, fourier transform infrared (FTIR) spectroscopy has proven and recognized to be a powerful tool. FTIR analysis was performed to identify the specific chemical functional groups of cellulose, hemicelluloses and lignin in the sample (Table 2). The FTIR transmittance peaks obtained from the inoculated and uninoculated rice straw is represented in Figure 9. In IR spectra, the broad band at 3000-3500 cm^{-1} is associated with OH stretching of cellulose in inoculated and uninoculated of rice straw, in addition band at 2920-2923 cm^{-1} was attributing to C–H stretching. The band near 1075-1108 cm^{-1} is confirming a significant characteristic of the C-O-C of the organic compounds (aldehydes and ketones) linkage of cellulose presence in both samples of rice straw. On the other hand, absorption peak at 1315-1320 cm^{-1} was observed as prominent and usually it is defined as functional group, syringyl moiety of lignin presence in both samples of rice straw. The results correlated with Bhattacharya *et al*. (2019) were also observed the functional group, syringyl moiety of lignin (1315-1320 cm^{-1}) which was more prominent in his studies. The bonds between 1513 to 1652 cm^{-1} have been assigned to aromatic skeleton stretching as shown in the (Table 2). Major distinct bands were observed in the lignin molecules corresponds to aromatic skeleton region of 1513, 1640 and 1652 cm^{-1} in the inoculated and in uninoculated samples of rice straw, which reveals the presence of aromatic skeleton in the present studied samples. Based on the above criteria compared to lignin and cellulose, the hemicelluloses content was less in inoculated and uninoculated samples of rice straw. Similar findings were in accordance with those reported by Bhattacharyya *et al.* (2020); Purakayastha *et al*. (2016); Sudha *et al.* (2020).

4. Discussion

In the present study, a newly isolated strain *Aspergillus niger* JUC-2 was found to be effective for the production of cellulosic enzyme. The optimized conditions for cellulase enzyme production with *Aspergillus niger* JUC-2 were studied. Nitrogen is one of the major nutrients for stimulation of cellulase activity. The effect of various nitrogen sources on cellulase production by *Aspergillus niger* JUC-2 was investigated using urea, peptone, yeast extract, and ammonium sulfate at different concentrations. The maximum cellulase activity was found using ammonium sulfate as nitrogen source with a 3-fold increase in enzymatic cellulase activity in both SmF and SSF. This can be attributed that ammonium sulphate might help in the stimulation of cellulase activity. Different types of carbon substrates such as rice straw, wheat bran, and groundnut shell were studied for enzyme production.The study exhibited variable effects on the selected parameters of the carbon sources applied, of which rice straw supported the maximal activities. The results are comparable with literature reported by Soma *et al*. (2011).The effect of various temperatures on cellulase activity was determined by incubating at different temperature. The summary of the results showed maximum activities at 30^{o}C. The optimum temperature might lead to mass transfer of nutrients and faster metabolic activity with an increase in protein content, and enhanced extracellular enzyme production.

The pH is a key parameter that influences fungal growth and plays a major role in enzyme stability and production. The maximum activity was observed at pH5.0 and the results showed that cellulase activities decreased as the pH increased further towards alkalinity. This could be attributed that a slight change in pH can affect the enzyme catalytic activity on cellulase production. *Aspergillus niger* JUC-2 showed the most active cellulolytic species during different

incubation periods (days) and the optimum incubation period for enzyme production was determined on the 4th day with the maximum activity. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology, resulting in the repression of genes involved in the production of enzymes and moisture content 70%.When compared between SmF and SSF, production of total cellulase in SSF was 32.7, 8, and 2.6 folds higher than SmF. From the present investigation, the highest cellulase productivity was obtained with solid-state fermentation.

5. Conclusion

The newly isolated strain *Aspergillus niger* JUC-2 was found to be effective for the production of the cellulosic enzyme. The optimized conditions for cellulase enzyme production with *Aspergillus niger* JUC-2 were nitrogen source (ammonium sulfate 3 g/l), carbon source (4% rice straw), temperature 30ºC, pH 5.0, incubation period of 4 days, and moisture content 70%. The maximum cellulase activities of FPase, CMCase, and β-Glucosidase in SmF were found to be 0.74 ± 0.02 IU/ml, 28 ± 0.15 IU/ml, and 2.6 ± 0.05 IU/ml and 24.240.5 IU/g, 223.4 ± 2 IU/g and 6.8 ± 0.2 IU/g in SSF, respectively. Therefore, *Aspergillus niger* JUC-2 can be considered as a potential candidate for the production of cellulases.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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