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Exploiting Local Fungal Isolates for Sequential Saccharification of Water Hyacinth (*Eichhornia crassipes*) Biomass to Wards Cellulase Production and Dye Degradation

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ABSTRACT

Cellulase enzyme production using water hyacinth (WH) biomass as a carbon source by local fungal isolates was studied. Dye degradation potential of fungal spent biomass after cellulase production was screened. *Trichoderma viride* and *Aspergillus niger*, isolated from decomposing WH produced maximum cellulase at optimum culture condition [substrate concentration- 10:5 (v/v); pH - 5.0; Temperature - 35° C; and incubation period - 6 d]. Maximum CMCase enzyme production of 83.4 IU by *T. viride* followed by *A. niger* (72.6 IU). Findings on dye de-colorization (66%) and laccase activity confirmed the ability of the spent fungal biomass after cellulase enzyme production for dye degradation that can be considered as a potential means of pollution control in dye effluent. **Rationale of the Work**: Water hyacinth (WH) - an aquatic weed, produces concentrated lingo-cellulosic biomass and has immense potential to colonise the pond/ lake where it grows. WH biomass has rich cellulose content that be exploited as substrate in SSF by cellulase producing fungal system. At the end of the fermentation process, large amount of unspent biomass goes waste. Present work is an attempt to exploit this unspent biomass for textile dye biodegradation in polluted water samples from textile industrial area in the state. Hence, WHB can be considered as a potential means of pollution control in dye effluent.

INTRODUCTION:

Unprecedented use of fossil fuel has led to increasing demand for alternative source for fuel[1]. Bioethanol produced from biomass is considered as an alternative to fossil fuel [2]. The first generation ethanol is produced from sugar and starch. However, the demand for these sources will not be sufficient to meet the current need of fuel industry, due to their use as a food source [3]. Terrestrial and aquatic residue is gaining much importance these days because of its low cost, easy and decentralized availability for the biological production of industrial chemicals such as lactic acid, acetic acid, propionic acid, and ethanol [4]. Enzymatic hydrolysis of cellulosic material is the most promising approach to get a high yield of monomeric sugars which is vital for the economic success of any bioprocessing method in ethanol production [5]. The successful enzymatic hydrolysis of the biomass can be performed with the help of a suitable pre-treatment method which will enable the enzyme to hydrolyze the cellulose and hemicellulose by removing the lignin and decreasing the degree of crystallinity of the cellulose. Many kinds of physical, chemical, and biological pre-treatments have been reported in literature [6]. Still, there is a need to optimize the pre-treatment method by choosing a suitable chemical/ biological method that will provide maximum enzymatic hydrolysis [7].

Ponds, lakes, backwaters, and canals, both natural and man-made play an important role in the cultural heritage and economic status of the nations. But now most of these aquatic systems are infested with many invasive aquatic weeds among which *Eichhornia crassipes* (water hyacinth) has a greater part as it is known as ‘blue devil’, which grows in wetlands, marshes, sluggish flowing water, large lakes, rivers, shallow ponds and reservoirs [8]. As it is non-endemic, there are no natural control mechanisms such as insects and fishes that feed on it. Also, other control mechanisms including physical, chemical, and biological methods have failed miserably or are too expensive to carry out regularly. Hence the concept of eradication through utilization is being adopted and researchers are focusing on new methods of utilizing these wastes [9].

Water hyacinth has a considerable amount of cellulose and was thought to be used as a cheap source for the production of cellulases by fungal isolates had a wide range of industrial applications such as starch processing, animal feed production, grain alcohol fermentation, paper, and textile industry. Several scientists reported that different agricultural wastes especially water hyacinth as one of the substrates for cellulase production by using fungi [10-11]. The

present study focuses on the enriched production of cellulases by natural fungal isolates using water hyacinth biomass as a carbon source and also to find out the reuse ability of fungal spent biomass after cellulase production for dye degradation.

MATERIALS AND METHODS:

Isolation and Identification of fungal strains

The fungal cultures were isolated from the naturally decomposed water hyacinth using serial dilution method. The isolated fungi were identified by their morphology and colony characteristics. Stock cultures of isolates were maintained on potato dextrose agar at 4ºC and sub-cultured twice a month [12].

Preliminary Screening of fungal isolates

The cellulose degradation potential of the fungal isolates was assessed by their growth and zone formation in carboxy-methylcellulose (CMC) agar medium [13] containing (g/l): $NaNO_3$ 2.0, $KH_2 PO_4$ 1.0, $MgSO_4 7H_2O$ 0.5, KCl 0.5, Carboxymethyl cellulose sodium salt 2.0, peptone 0.2 and agar 20. Plates were inoculated with 3 days old mycelium (8 mm agar block) of fungal strains and incubated at 30ºC for 6 days. Plates were flooded with 0.1% Congo red solution for 15 minutes then de-stained with 1M NaCl solution for 15 minutes. The diameter of zone of de-colorization around each colony was measured. Fungal colony the exhibited largest zone of de-colorization was selected for cellulase production. The reducing sugar productivity of the fungal isolates was also assessed using commercial cellulose (HiMedia RM 198).

Optimization of culture conditions for cellulase production

The various process parameters that influence the enzyme production during submerged fermentation were optimized over a wide range [14].Process parameters thus standardized included substrate biomass load (carbon source), pH (3-8) adjusted with 0.05M sodium citrate (pH 3-5) or 0.05M sodium phosphate buffer (pH 6-8), incubation temperature (30-70ºC) and incubation period (1 – 7 days).

Enzyme production and characterization

The cellulase production was carried out by submerged fermentation process at optimized condition in 250ml conical flasks with 100ml of pre-sterilized (121°C for 15 min.) basal salt medium containing water hyacinth biomass (WHB) as a sole carbon source [15]. Culture suspensions of *T. viride* and *A. niger* (2% v/v) were introduced separately and fermentation proceeded at 37 °C with an agitation rate of 200 rpm for 7 days. The supernatant obtained from the fermentation flask was treated with different saturation levels of solid ammonium sulfate (20, 40, 60, and 80%), with continuous overnight stirring. Enzyme was collected by centrifugation (10000 rpm, 15 min) and dissolved in 0.1M citrate buffer (pH-5.0). The enzyme solution was dialyzed against the same buffer for 48h with several intermitted buffer changes. The partially purified enzyme obtained was lyophilized and used for further study [16].

Filter paper activity (FPase) (total cellulase activity) was determined according to the standard method [17]. 50 mg of Whatman No. 1 filter paper strip [1 × 6 cm] were immersed in 1 mL of 0.05M Sodium citrate buffer containing 0.1% of partially purified enzyme (pH 5.0). After incubation at 50 ± 2^0 C for 1 h, the reducing sugar released was estimated by the dinitrosalicylic acid [DNS] method [18]. Endoglucanase activity (CMCase) was measured in the same method using a reaction mixture containing 1mL of 1% carboxy-methylcellulose (CMC) in 0.5M citrate acetate buffer (pH 5.0). The enzyme activities were expressed as an international unit (IU) where 1 IU is equal to one micromole of reducing sugar released per milliliter per minute. the *β*-glucosidase activity was assayed by the method of [19] using a reaction mixture containing 0.2 mL of 5 mM ρ – nitro phenyl β-D-glucopyranoside (PNPG), 1.6 mL of 0.05 M sodium citrate buffer [pH 4.8], and 0.2 mL of enzyme solution. After incubation for 30 minutes at 50^0 C, the reaction was stopped by the addition of 4 mL of 0.05 M NaOH glycine buffer (pH 10.6), and the yellow-colored ρ - nitro phenyl was measured at 420 nm. One unit of glucosidase activity is defined as that released mole of PNP from PNPG per minute per ml. Cellulose [20] and proteins [21] were estimated by standard methods.

FTIR Analysis

Fourier Transform Infrared spectra were studied on treated and untreated WHB using a Shimadzu spectrometer (Japan). For this, 3.0 mg of the sample was dispersed in 300 mg of

spectroscopic grade KBr and subsequently pressed into disks at 10 MPa for 3 min. The spectra were obtained with an average of 25 scans and a resolution of 4 cm-1 in the range of 4000–400 cm^{-1}.

XRD analysis

The crystallinity of cellulose in the pre-treated and treated water hyacinth with fungal strains at optimum condition was analyzed by X-ray diffraction method in a PANalytical (Netherlands), X – pert pro diffractometer set at 40 KV, 30mA; radiation was Cu Kα(λ=1.54Å) and grade range between 10 to 30° with a step size of 0.03°. The crystallinity index (CrI) was determined based on the equation shown below [22].

$$CrI = \frac{I_{002} - I_{am}}{I_{am}} x\ 100$$

Where I_{002} is the intensity of the diffraction from the 002 planes at 2θ=22.6° and I_{am} is the intensity of the background scatter measured at 2θ=18.7°. It is known that the I_{002} peak corresponds to the crystalline fraction and the I_{am} peak corresponds to the amorphous fraction [23].

Dye degradation

Dye degradation was carried out in broth culture by the standard method [24]. The spent fungal biomass was recovered from the cellulase production media at an optimized period by centrifugation and inoculated in 250ml Erlenmeyer flask containing 100ml of basal medium with 3% sucrose as carbon source and 40 mg of filter-sterilized methylene blue. The above reaction mixture was incubated at 30°C with an agitation speed of 200 rpm for 6 days. The de-colorization of methylene blue corresponds to the dye degradation potential of the fungal biomass. The dye degradation (%) was measured using a spectrophotometer at a wavelength of 395 nm [25].

Laccase activity was determined using 2, 20-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the substrate [26]. The laccase reaction mixture contained 0.5 ml of 0.45 mM ABTS, 1.2 ml of 0.1 M phosphate buffer (pH 6.0), and 0.5 ml of fungal biomass to give a final reaction volume of 2.2 ml. The oxidation of the substrate (ABTS) was monitored by the increase in the

absorbance at 420 nm using Shimadzu UV-1800 spectrophotometer (ELICO, India) over 90 s at 30°C, using e = 3.6 9 104 cm-1 M-1. Enzymatic activity was expressed as 1U = 1 µmol of ABTS oxidized per min at 25°C.

RESULTS AND DISCUSSION:

Several industrial processes have been developed to utilize WHB as raw materials for the production of value-added products such as single-cell protein, extracellular enzymes, and secondary metabolites [10, 27]. The present investigation was concerned to explore the possibility of using WHB as a substrate for the production of cellulases using *T. viridie* and *A. niger.*

Screening of cellulolytic fungi

The cellulose degradation potential of the selected strains was assessed by their substrate utilization potential and release of maximum reducing the sugar by *T. viride* and *A. niger* using WHB. A significantly higher amount of reducing sugar was produced by *T. viride* (343.3mg g^{-1}) followed by *A. niger* (308.2mg g^{-1}). The maximum diameter of the zone of clearance was expressed by *T. viride* (41.2 mm) followed by *A. niger* (39.3 mm). The overproducing cellulase strains of *T. viride* and *A. niger* were selected for further experiments (Table 1).

Optimization of cellulase production

Effect of substrate concentration (carbon source)

The basal medium supplemented with different volumes of water hyacinth blend as a carbon source in different ratios (10:1, 10:2, 10:3, 10:4, 10:5, 10:6 and 10:7) (v/v) was prepared, inoculated with 2 ml of fungal spore suspension containing 10^4 spores per ml and incubated at 30ºC with agitation speed of 150 rpm in rotary shaker incubator for 6 days. The culture media was centrifuged at 4000 rpm for 20 min and the supernatant was used as a crude enzyme source. Both the selected strains had produced maximum cellulase enzyme activity at the carbon source of 10:5 ratio where the highest CMCase was exhibited by *T. viride* (88.2 IU) (Table 2). A similar trend in enzyme production was also obtained when the isolates were grown in submerged culture containing water hyacinth as the carbon source for *A.niger* and *A.nidulans*, where the best ratio obtained for cellulase production was 10:4 [28].

Effect of pH on cellulase enzyme production

Cellulase production was greatly influenced by pH during submerged fermentation. *T.viride* and *A.niger* had produced maximum CMCase activity of 72.5 IU and 68.1 IU respectively on the same pH 5.0. Cellulase activity was declined with the increase or decrease in pH beyond the optimum (Table 3). CMCase, FPase, and β-glucosidase activities reached their maximum values on the 6thday of fermentation. The pH had a significant effect on cellulase enzyme production and the best enzyme titers were achieved at pH 4.0 - 6.0 but the pH 3.0 and 9.0 was not suitable for cellulase enzyme production. Similar results were obtained with *Trichoderma hamatum* USD B 0008 in rotten wood shavings after 144 h cultivation [29].

Effect of Temperature on cellulase enzyme production

The incubation temperature is yet another factor regulating the enzyme synthesis. In the present study, maximal enzyme production was recorded at 35°C in the submerged fermentation of WHB (Table 4). *T. viride* produced maximum CMCase activity (70.3 IU/ml) at 35° C which was comparatively higher than other enzymatic activities. The results were consistent with previous findings of [10] in *Triboliumcastaneum*. Beyond 40°C, the specific activity was decreased which may be due to thermal denaturation of the enzyme. Maximum cellulase activity of both strains was achieved within the range of 30-40°C coinciding with the characteristic of mesophiles[30].

Effect of incubation period on cellulase enzyme production

T.viride and *A.niger* had produced maximum CMCase activity (98.4 and 72.3 IU/ml) respectively on the 6th day of fermentation, thereafter the activities were declined (Table 5). Results showed the activity of β-glucosidase activity was relatively close to CMCase activity. FPase activity was comparatively lower than other cellulase activities for both organisms. Among the selected organisms *T.viride* was the efficient organism in concern with cellulase production in a shorter period.

XRD - Cellulose crystallinity

Cellulose crystallinity, usually measured as crystallinity index (CrI), is considered an important parameter determining the structural changes in the biomass of cellulose. CrI of a cellulose sample is an indication of the degree of formed crystallinity in the sample when the cellulose

aggregates. XRD profile of WH indicated that the crystalline index (CrI) of untreated WHB is 25.6% and that of microbial treated WHB is 45.7% (Fig. 1). The increase in CrI may be due to the breakdown of amorphous cellulose [31]. The X-ray diffractogram showed that the microbial treatment could increase the crystallinity of water hyacinth. According to [32] the crystallinity increase can also be attributed to the preferential removal of lignin.

FTIR analysis

FTIR spectra of the untreated and treated samples indicated structural changes of functional group in the biomass upon microbial treatment (Fig. 2 - 4). The increased absorption bands at 1000 - 1200 cm^{-1} were related to structural features of cellulose and hemicelluloses [33]. The change in the peaks in the given region (Fig. 3), suggests that there was an increase in absorbance in these regions. The peak at 1653 cm^{-1} was observed due to either the acetyl and uronic ester linkage of the carboxylic group of the ferulic and p-coumeric acids of lignin and/or hemicelluloses [34]. A sharp band at 896 cm^{-1}, corresponding to the C1 group frequency or ring frequency, was attributed to the glycosidic linkages between xylose units in hemicelluloses [35]. These peaks in the microbial treated sample had the highest absorbance suggesting an increase in cellulose and hemicellulose content. In the FTIR spectrum, the peaks observed at 1098 and 779 cm^{-1} were attributed to C–O stretching and C–H rocking vibration of cellulose structure. At these peaks, the treated sample had the maximum absorbance, suggesting an increase in the cellulose content. This study can be applied for the bioconversion of lignocellulose material to fermentative products, the improvement of animal feedstock digestibility, and bio-bleaching in the pulp and paper industry to reduce or even eliminate the need for chlorine-based compounds [36].

The digestibility of lignocelluloses is hindered by many physicochemical, structural, and compositional factors which required a suitable microbial treatment to enhance the susceptibility of the biomass for hydrolysis. So the microbial pre-treatment is essential for the enhancement of cellulase enzyme production. The FTIR and XRD data in the present study suggested that the microbial treatment increase the availability of polysaccharide for degradation process. The microbial treatment is preferable because it is less expensive safer as compared to either acid or alkaline treatment and it can be easily recovered from the hydrolysate. Fungi have been used to

pre-treat many lignocellulosic biomasses such as wheat straw [37], poplar wood [29], and corn [38].

Compositional changes of water hyacinth biomass during the bioprocessing

Cellulose

The cellulose content of the water hyacinth was 227.0 mg/ml. Microorganisms that utilized cellulose as the preferred substrate for growth and enzyme activity could be more efficient in the bioconversion process [39-40]. *T.viride* showed higher and faster utilization of cellulose than *A.niger*.Table 6 showed 75% utilization of cellulose in the water hyacinth biomass by *T.viride* within 6 days while *A.niger* had utilized 69% of cellulose during the same period of fermentation. The potential of these organisms can be correlated with the activities of cellulose digesting enzymes such as CMCase, FPase, and β-glucosidase activity. Similar results have been reported with earlier studies on cassava waste under solid-state fermentation [12].

Reducing Sugar

The maximum release of reducing sugar in WHB during the fermentation process was expressed by *T.viride* (321.4 mg/ml) followed by *A.niger* (303.4 mg/ml) on the 6th day of fermentation. *T.viride* giving a higher yield of reducing sugar may be due to its higher utilizing potential of WHB as a carbon source. [41-42,12] had shown that the yield of reducing sugar was positively correlated with the production of cellulases in submerged fermentation of cellulosic substrates using fungi and actinomycetes.

Protein

The microbial protein content increased (1.8 and 1.6 fold) over the control value on the 6th day of fermentation by *T.viride* and *A.niger* respectively. *T.viride* giving a higher yield of microbial protein was also observed to have a higher amount of enzyme production and their growth [43].

Reuse of spent fungal biomass for dye degradation

The de-colorization of methylene blue in the reaction mixture confirmed the degradation potentials of spent fungal biomass. The maximum dye degradation (66% and 61%) was exhibited by the spent biomass of *T. viride* and *A. niger* respectively (Fig. 5). Similar dye decolorization

was observed by fungal isolates from dye industry waste [25]. The present results demonstrated the dye degradation potential of isolated fungus and confirmed the ability of spent fungal biomass for dye decolorization after cellulase enzyme production. The laccase activity was measured and the results were summarized in Fig. 4. It was inferred from the results that the laccase activity increased up to the 6th day with a maximum activity of 3.4 U/ml for *T. viride* and 2.9 IU/ml for *A. niger*. The de-colorization process depends on the increased or decreased enzyme production by the spent fungal biomass [44].

CONCLUSION:

The convergent spreading of aquatic weeds is a major global problem. Intensive efforts with different control measures and huge expenditure brought no success in controlling the growth of the aquatic weed - water hyacinth. Greater attention needs to be focused on finding ways and means for the utilization of these weeds. The present study reveals that WH biomass-associated fungi can be used to produce industrially valuable enzymes by submerged fermentation. The spent fungal biomass recovered after the fermentation process had significant dye decolorization potential. Hence, it can be used for dye bio-degradation in polluted water bodies.

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Table No. 1: Preliminary screening of fungal isolates for the production of cellulase enzyme using WH biomass (on 6th day)

Organisms	Zone of Clearance (mm)	Reducing sugar productivity (mg g^{-1} WHB)
Trichoderma viride	41.2 ± 2.4	343.3 ± 12.4
Aspergillus niger	39.3 ± 1.7	308.2 ± 21.2

Results are mean ± SE of three replicates

Table No. 2. Substrate concentration on cellulase activity [µmol mg-1 (protein)] in submerged fermentation using WHB

Organisms	Cellulase activity	Concentration ratio (WHB:Basal medium) (mg/ml)						
		1:10	**2:10**	**3:10**	**4:10**	**5:10**	**6:10**	**7:10**
T. viride	*CMC ase*	20.2 ± 1.1	31.3 ± 2.1	43.4 ± 1.3	65.9 ± 1.9	88.8 ± 2.1	63.5 ± 2.7	60.1 ± 1.3
	FPase	14.1 ± 1.1	23.2 ± 0.8	31.3 ± 0.9	48.4 ± 1.8	51.5 ± 2.1	44.3 ± 2.7	40.1 ± 3.1
	β-glucosidase	18.7 ± 0.5	26.5 ± 1.3	40.4 ± 2.1	56.1 ± 2.7	64.5 ± 3.1	58.4 ± 3.7	51.5 ± 3.3
A. niger	CMC ase	15.3 ± 0.9	21.8 ± 1.1	33.1 ± 1.9	48.4 ± 2.7	70.5 ± 3.5	60.5 ± 2.3	54.5 ± 1.9
	FPase	9.8 ± 0.7	15.7 ± 1.1	26.3 ± 1.8	33.8 ± 1.9	44.9 ± 2.1	39.7 ± 3.3	30.6 ± 2.7
	β-glucosidase	15.3 ± 0.9	20.2 ± 1.7	31.5 ± 1.9	47.5 ± 2.7	56.1 ± 3.3	48.7 ± 2.1	40.6 ± 1.9

Results are mean ± SE of three replicates

Table No. 3. pH on cellulase activity [µmol mg-1 (protein)] in submerged fermentation usingWHB

Organisms	Enzymes	pH					
		3	**4**	**5**	**6**	**7**	**8**
T. viride	CMCase	13.5 ± 0.9	41.8 ± 1.1	72.5 ± 2.4	63.8 ± 2.7	35.9 ± 1.9	11.5 ± 0.9
	FPase	8.4 ± 0.4	21.4 ± 1.3	44.5 ± 2.9	40.7 ±2.1	14.2 ± 0.8	10.3 ± 0.9
	β-glucosidase	10.6 ± 0.7	33.8 ± 2.1	61.4 ± 1.8	54.3 ± 3.1	28.7 ± 2.7	11.1 ± 0.9
A. niger	CMC ase	8.3 ± 0.4	26.3 ± 1.9	68.1 ± 4.3	56.5 ± 2.7	30.3 ± 1.3	10.3 ± 0.9
	FPase	9.3 ± 0.1	18.6 ± 1.1	38.7 ± 3.1	28.3 ± 1.3	11.7 ± 0.5	6.91 ± 0.1
	β-glucosidase	11.3 ± 0.1	29.3 ± 1.3	45.8 ± 3.6	38.1 ± 1.8	23.8 ± 1.3	7.31 ± 0.3

Results are mean ± SE of three replicates

Table 4 Temperature on cellulase activity [µmol mg-1 (protein)] in submerged fermentation using WHB

Organisms	Enzyme	Temperature °C					
		25	**35**	**45**	**55**	**65**	**75**
T. viride	CMC ase	20.4 ± 1.1	70.3 ± 2.7	65.4 ± 3.1	53.1 ± 2.1	43.0 ± 1.9	17.3 ± 1.1
	FPase	15.1 ± 0.2	41.2 ± 1.8	38.4 ± 2.7	33.1 ± 2.4	26.7 ± 1.7	11.5 ± 0.8
	β-glucosidase	19.4 ± 1.1	58.5 ± 2.7	51.6 ± 2.8	44.3 ± 2.1	33.3 ± 1.9	12.7 ± 0.4
A. niger	CMC ase	14.3 ± 1.1	54.1 ± 2.7	50.8 ± 3.4	39.3 ± 2.7	31.4 ± 1.3	12.7 ± 0.9
	FPase	9.5 ± 0.1	30.3 ± 1.3	28.3 ± 1.9	21.8 ± 1.3	15.3 ± 1.3	8.3 ± 0.3
	β-Glucosidase	11.3 ± 0.5	39.3 ± 0.4	40.4 ± 1.4	28.3 ± 1.3	17.5 ± 0.7	10.6 ± 0.8

Results are mean ± SE of three replicates

Table No. 5. Incubation period on cellulase activity [µmol mg-1 (protein)] in submerged fermentation using WHB

Organisms	Enzyme activity (IU)	Period of fermentation (Days)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
T. viride	CMC ase	10.3 ± 0.5	28.3 ± 1.9	33.9 ± 3.1	55.8 ± 3.3	79.3 ± 6.5	98.4 ± 6.1	78.3 ± 5.1
	FPase	6.7 ± 0.4	13.5 ± 0.9	22.3 ± 1.7	31.4 ± 2.8	43.9 ± 3.2	68.3 ± 4.3	57.5 ± 3.8
	β-glucosidase	8.3 ± 0.3	17.5 ± 0.9	28.7 ± 1.9	39.3 ± 1.9	58.7 ± 3.5	71.9 ± 5.4	63.4 ± 2.9
A. niger	CMC ase	7.3 ± 0.3	21.8 ± 1.3	28.1±1.3	48.3 ± 3.9	63.3 ± 5.5	72.3 ± 6.5	61.7 ± 4.1
	FPase	4.1 ± 0.1	9.8 ± 0.3	18.3 ± 1.1	21.8 ± 1.1	31.8 ± 2.9	46.3 ± 2.7	35.3 ± 2.1
	β-Glucosidase	5.3 ± 0.3	13.5 ± 1.1	21.3 ± 1.8	33.4 ± 1.9	44.8 ± 2.8	58.9 ± 4.1	48.7 ± 2.8

Results are mean ± SE of three replicates

Table No. 6. Cellulose utilization (%) during the submerged fermentation of WHB by the fungal isolates

Organisms	Period of fermentation (Days)						
	1	2	3	4	5	6	7
T. viride	6.73 ± 0.62	25.15 ± 1.9	31.93 ± 2.2	48.81 ± 3.7	61.73 ± 4.7	80.05 ± 4.2	80.15 ± 5.3
A. niger	5.15 ± 0.41	17.48 ± 1.3	21.98 ± 1.7	35.25 ± 2.4	51.39 ± 3.9	70.40 ± 4.7	70.69 ± 4.1

Results are mean ± SE of three replicates.

Table No. 7. Reducing sugar release (mg g^{-1}) during submerged fermentation by fungal isolates

Organisms	Period of fermentation (Days)						
	1	2	3	4	5	6	7
T. viride	63.1 ± 3.8	121.3 ± 9.4	198.8 ± 9.3	227.6 ± 18.3	287.5 ± 16.5	321.4 ± 9.6	313.5 ± 18.3
A. niger	54.5 ± 3.3	114.8 ± 9.8	168.7 ± 8.4	208.3 ± 16.3	273.5 ± 14.1	303.4 ± 8.5	290.5 ± 13.4

Results are mean ± SE of three replicates.

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Table No. 8. Extracellular protein (mg ml^{-1}) produced by fungal isolates during fermentation

Organisms	Period of fermentation (Days)						
	1	2	3	4	5	6	7
T. viride	195.2 ± 10.6	263.7 ± 11.3	298.4 ± 10.6	311.3 ± 18.6	347.3 ± 11.3	398.1 ± 8.8	384.6 ± 19.4
A. niger	178.4 ± 11.3	228.4 ± 15.1	263.5 ± 14.3	294.6 ± 17.1	323.4 ± 18.1	360.4 ± 8.3	352.6 ± 10.3

Results are mean ± SE of three replicates.

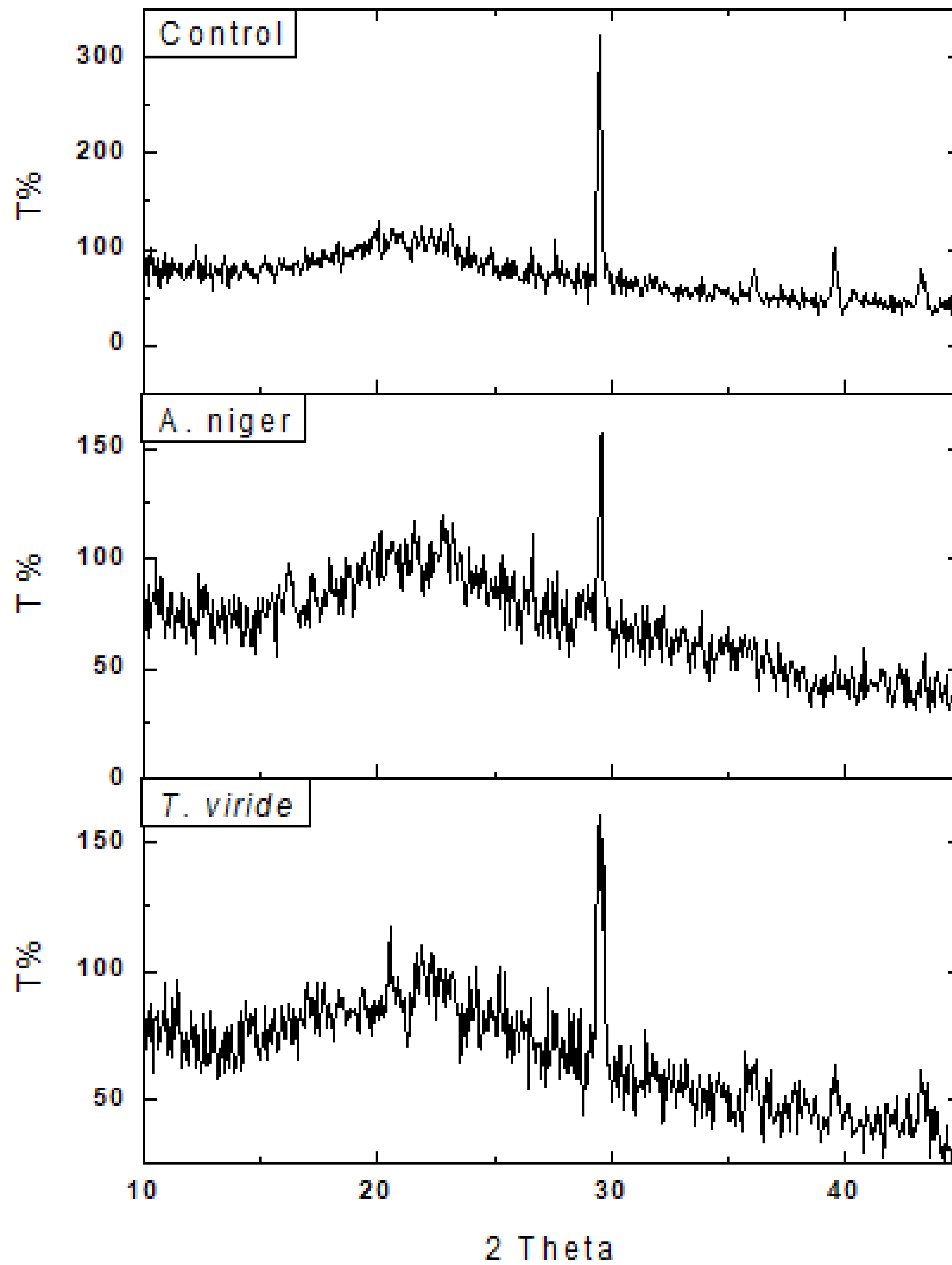


Figure no. 1. XRD analysis of WHB untreated and treated with *A. niger*&*T. viride*

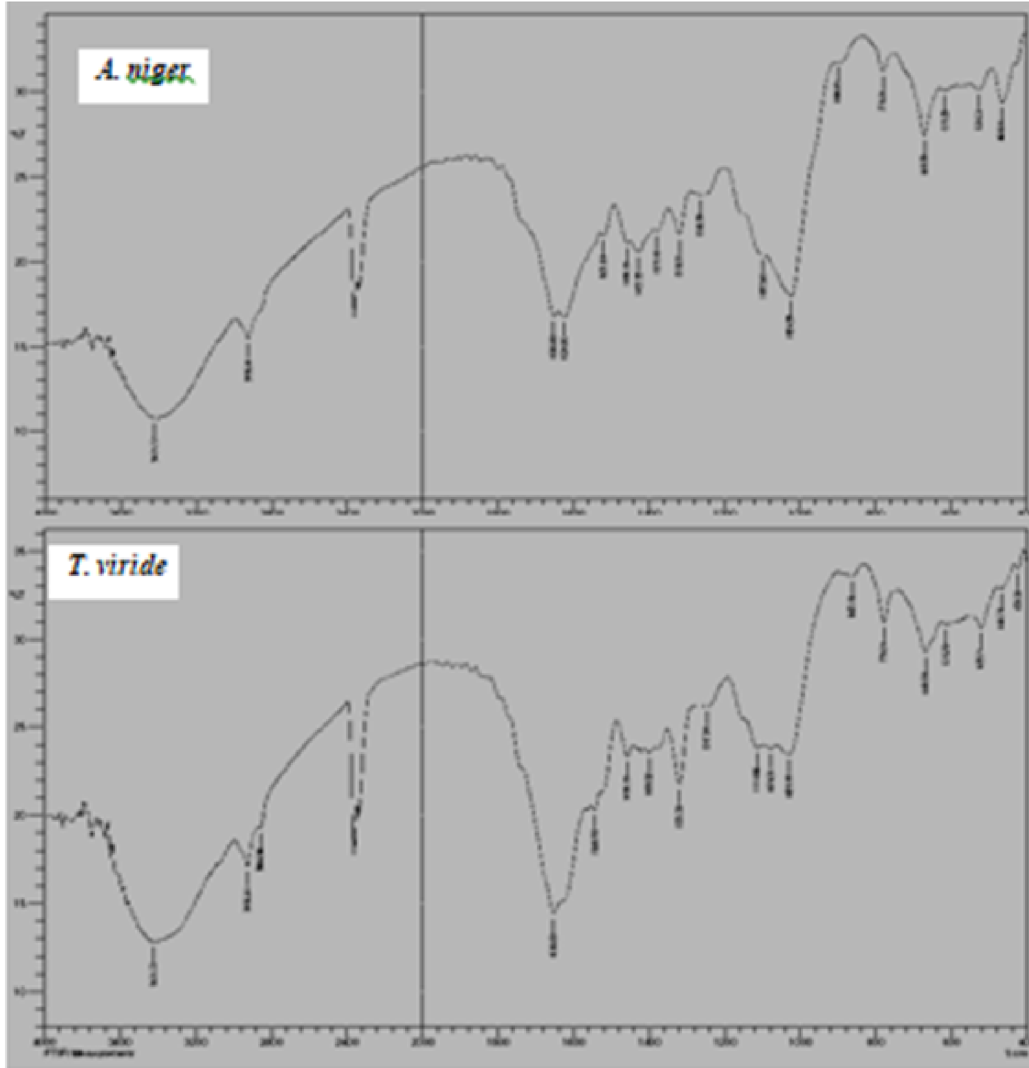
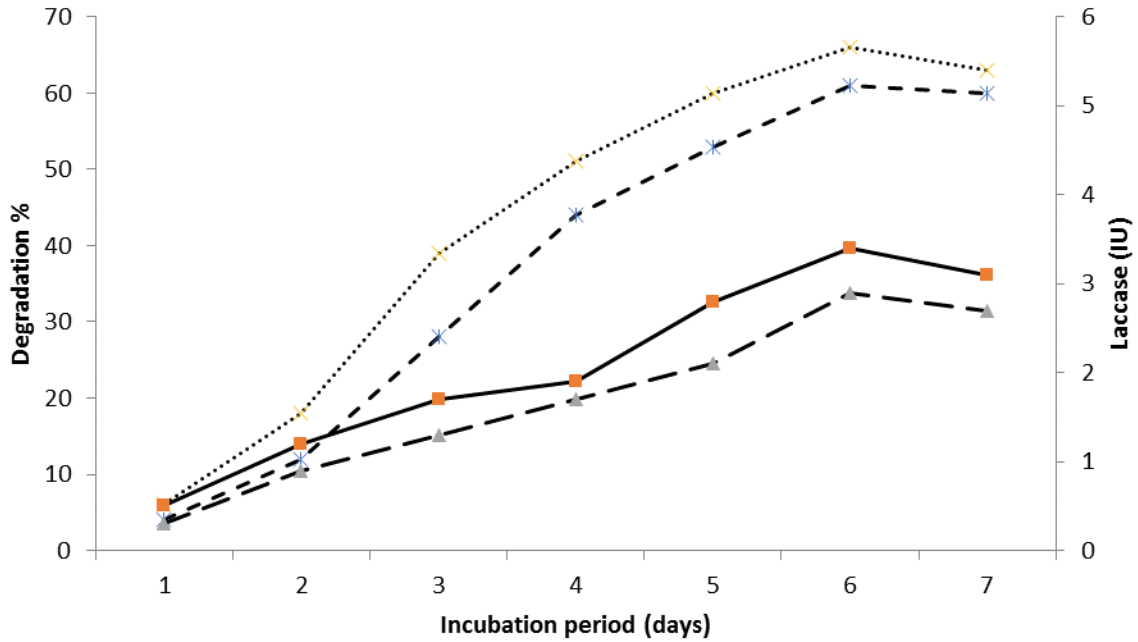


Figure no.2 FTIR spectra of WHB treated with *A. niger* & *T. viride*



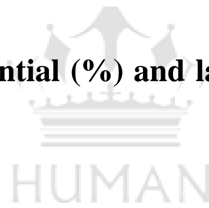

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Figure no.3 Dye decolourization potential (%) and laccase activity [μmol mg^{-1}(protein)] of the spent fungal biomass