

Original Research Article

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Production of Cellulase in Submerged Fermentation using Water Hyacinth as Carbon Source and Reutilization of Spent Fungal Biomass for Dye Degradation

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ABSTRACT

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Cellulase enzyme production using water hyacinth biomass (WHB) as carbon source in submerged fermentation by *Trichoderma viride* and *Aspergillus niger* was studied. The FTIR spectral studies revealed that WHB had fermentable content of cellulose and hemicellulose. The fungal strains *T. viride* and *A. niger*, produced maximum cellulase at optimum culture condition with a substrate concentration of 5% (w/v) and incubation period of 6 days (72hrs). The partially purified crude enzyme obtained from the 72 hr culture of *T. viride* showed total cellulase activity (FPase) of 68.3 (IU) whereas, *A. niger* showed 46.3 (IU). The maximum CMCase enzyme production of 98.4 (IU) was exhibited by *T. viride* followed by *A. niger* 72.3 (IU). The spent fungal biomass from cellulase production media was reutilized for dye degradation potential. The present study showed that both the fungal spent biomass had moderate dye de-colourization (66%) potential and significant laccase activity (3.4 IU).

Introduction

Cellulases are enzymes largely focused on by researchers and industries for the production of liquid fuel and other chemicals which are potential substitutes for petroleum derivatives (Bozell and Petersen, 2010). Cellulase is a group of enzymes which have the capability of hydrolyzing cellulose into fermentable sugars such as glucose, which can be used for producing many useful products such as ethanol, biofuel and other useful chemicals from the

cellulosic feedstocks. Cellulase could be produced from many lignocellulosic feed stocks such as straws, bagasse, wheat bran, corn stover, corncob, etc (Camassola and Dillon, 2009). Terrestrial and aquatic weedy biomass are gaining much importance in 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. Several scientists reported that

different agricultural wastes especially water hyacinth as one of the substrate for cellulase production by using fungi (Fayyaz *et al.*, 2009; Okunowo *et al.*, 2010).

Water hyacinth (*Eichhornia crassipes*), was known as one of the fastest growing plants and a kind of unwanted species and the large-scale outbreak of it leads to many environmental problems in India. In order to resolve the problems, water hyacinth has been used for producing biogas (Singhal and Rai, 2003), ethanol (Mishima *et al.*, 2008) and high caloric fuel (Lu *et al.*, 2009). Water hyacinth have high content of hemicellulose (35 to 55% of dry mass), cellulose (18% of the dry mass) and protein (13% of the dry mass), which can provide enough nutrients for cellulase production by many microbial strains (Zhao *et al.*, 2011). Therefore, the present study focuses on the enriched production of cellulases by natural fungal isolates using water hyacinth biomass as carbon source under controlled environment. The extensive use of cellulase in many industries depends on the cost of the enzyme which in turn depends on the method of production. Hence, research all over the world focuses on isolating new, hyper producing microbial strains and also to develop new fermentation processes aimed at reducing the cost of the enzyme with a view to bring down the overall process cost (Tengerdy and Szakacs, 2003).

Physical and chemical methods have been widely used for the decolorization of dyes liberated by textile industries, but these methods are not feasible techniques since they are very expensive and forming hazardous by-products or intensive energy requirement (Patel *et al.*, 2006). Microbial decolorization methods are commonly applied to treat textile industry effluents because various microorganisms, such as bacteria, yeasts, algae and fungi, are able to remove different classes of dyes (Tan *et al.*,

2013; Kelewou *et al.*, 2014). Earlier researches confirmed that some ligninolytic fungi have the capacity to degrade synthetic dyes (Muthuchezian *et al.*, 2008) because of high laccase activity. Hence, the present study also aims to find out the reuse ability of fungal spent biomass after cellulase production for dye degradation.

Materials and Methods

Biomass and culture organisms

Fresh water hyacinth biomass (WHB) was collected from a local pond at Karaikudi, Tamilnadu, India (10.07°N, 78.78°E). The collected samples were washed to remove adhering dirt, cut into small pieces (2 or 3 mm) thickness and dried at sunlight. The fungal strains *Trichoderma viride* (NCIM 1195) and *Aspergillus niger* (NCIM 1255) were procured from the National Collection of Industrial Microorganism, Pune. Stock cultures of isolates were maintained on potato dextrose agar at 4°C and sub-cultured twice a month.

FTIR characterization of cellulose of Water Hyacinth

Fourier Transform Infrared spectra were studied on WHB using a Shimadzu spectrometer (Japan) in order to explore the characteristics of cellulose which was the sole carbon source for enzyme production. 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.

Enzyme production

The cellulase production was carried out by submerged fermentation process at optimized condition (pH 6.0; temp 32°C) in

250ml conical flasks with 100ml of pre-sterilized (121°C for 15 min.) basal salt medium containing 5% (w/v) powdered vegetative biomass of water hyacinth as a sole carbon source (Ariffin *et al.*, 2006). The culture suspensions of *T. viride* and *A. niger* (2% v/v) were introduced separately in two sets of media and fermentation proceeded at 32 °C with agitation rate of 200 rpm for 7 days. The supernatant obtained from the fermentation flask was treated with different saturation levels of ammonium sulphate (20, 40, 60 and 80%), with continuous overnight stirring. The precipitated enzyme was collected by centrifugation and dissolved in 0.1M citrate buffer (pH 5.0). The enzyme solution was dialyzed against the same buffer for 48hrs with several intermitted buffer changes. The partially purified enzyme obtained was lyophilized and used for further study (Arunachalam *et al.*, 2010).

Enzyme Assay

Filter paper activity (FPase) (total cellulase activity) was determined according to the standard method (Ghose, 1987). 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 $50 \pm 2^{\circ}$ C for 1 hr, the reducing sugar released was estimated by dinitrosalicylic acid [DNS] method (Miller, 1959). *Endoglucanase activity* (CMCase) was measured in the same method using a reaction mixture containing 1mL of 1% carboxy methyl cellulose (CMC) in 0.5M citrate acetate buffer (pH 5.0). The enzyme activities were expressed as IU (1 IU = μ mol of reducing sugar released per minute per ml) (Tribak *et al.*, 2002). β -glucosidase activity was assayed by the method of Ray *et al.*, (1993) using a reaction mixture containing 0.2 mL of 5 mM p - 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^o 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 p - 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 and the enzyme activity was expressed as micromole (μ mol) product (PNP) released per minute per ml.

Laccase Assay

The laccase activity was determined using 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the substrate (Rasera *et al.* 2009). 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^o C, using $e = 3.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$. Enzymatic activity was expressed as micromole (μ mol) product (ABTS) oxidized per min per ml.

Dye degradation

Dye degradation was carried out in broth culture by the standard method (Talekar *et al.*, 2011). The spent fungal biomass was recovered from the cellulase production media at the optimized period and inoculated into 250ml Erlenmeyer flask containing 100ml of sterilized basal medium containing azo dye. The above reaction mixture was incubated at 30°C with agitation speed of 200 rpm for 6 days. The de-colorization of dye corresponds to the dye degradation potential of the fungal biomass. The dye degradation (%) was measured using a spectrophotometer at a

wavelength of 395 nm (Selvakumar *et al.*, 2013).

Results and Discussion

Characterization of cellulose of water hyacinth

FTIR spectra of water hyacinth (Fig. 1) showed increased absorption bands at 1000 - 1200 cm^{-1} that were related to structural features of cellulose and hemicelluloses. The peak at 1653 cm^{-1} was observed due to either the acetyl and uronic ester linkage of carboxylic group of the ferulic and p-coumeric acids of lignin and/or hemicelluloses (Hu *et al.*, 2011). 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 (Marimuthu and Atmakuru, 2012). 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.

Enzyme Production

Several industrial processes have been developed to utilize WH biomass as raw materials for the production of value added products such as single cell protein, extracellular enzymes and biologically active secondary metabolite etc (Fayyaz *et al.*, 2009; Okunowo *et al.* 2010). The present investigation was concerned to explore the possibility of using WH biomass as a substrate for the production of cellulases using *T. viride* and *A. niger*. The lignocellulosic biomass composition of WH includes cellulose (20.2 g 100g⁻¹ DM), hemi-cellulose (34.3 g 100g⁻¹ DM), lignin (4.4 g 100g⁻¹ DM) and crude protein (13.3 g 100g⁻¹ DM) (Pothiraj *et al.*, 2014).

The partial purification of crude enzyme by ammonium sulphate precipitation from the

72 hour culture (6d) gave total cellulase activity (FPase) of 68.3 (IU) for *T. viride* and 46.3 (IU) for *A. niger* which indicated significant increase in activity by *T. viride* over *A. niger* (Table 1). A similar result was reported by Milala *et al.*, (2014) where ammonium sulphate precipitation of the 72 hour *R. stolonifer* culture gave cellulase activity of 33.00U ml⁻¹ at 80% saturation for millet stalk and 28.00U ml⁻¹ at 50% saturation in sawdust feed substrate.

Both the selected strains had produced maximum cellulase enzyme activity where the highest CMCase was exhibited by *T. viride* (98.4 (IU)) (Table 2).

A similar trend in enzyme production was also obtained by Usama *et al.* (2008) when the isolates were grown in submerged culture containing water hyacinth as the carbon source for *A.niger* and *A.nidulans*. Similarly, *T. viride* showed significantly increased β -glucosidase activity (71.9 IU) when compared with *A. niger* (58.9 IU) (Table 3).

Similar results were obtained with *Trichoderma hamatum* in rotten wood shavings after 144 hours cultivation (Chang *et al.*, 2001) and the results were also basically consistent with previous findings of Fayyaz *et al.*, (2009) in *Tribolium castaneum*. The results clearly showed that among the selected organisms *T. viride* was the efficient organism in concern with cellulase production in a shorter period.

Compositional changes of WH biomass during fermentation

Cellulose Utilization

Microorganisms which utilized cellulose as the preferred substrate for growth and enzyme activity could be more efficient in bioconversion process (Clavert, 2002).

T.viride showed higher and faster utilization of cellulose than *A.niger*. More than 80% of cellulose (80.15%) from water hyacinth biomass was utilized by *T.viride* while *A.niger* had utilized 70.7% of cellulose at the end of fermentation period (7d) (Table 4). Potential of these organisms can be well correlated with the activities of cellulose digesting enzymes such as CMCCase, FPase and β -glucosidase activity. Similar results have been reported with earlier studies on cassava waste under solid state fermentation (Pothiraj *et al.*, 2006).

Reducing Sugar

The maximum release of reducing sugar in WH during the fermentation process was expressed by *T.viride* (321.4 mg g⁻¹) followed by *A.niger* (303.4 mg g⁻¹) on the

6th day of fermentation (Table 5). *T. viride* giving a higher yield of reducing sugar may be due to its higher utilizing potential of WH biomass as carbon source. Baig *et al.*, (2004) and Ojumu *et al.*, (2003) 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.

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. 2).

Table.1 Production of CMCCase (IU) in submerged fermentation using Water hyacinth

| Organisms | Period of fermentation (Days) | | | | | | |
|------------------|-------------------------------|------------|------------|------------|------------|------------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| <i>T. viride</i> | 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 |
| <i>A. niger</i> | 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 |

Results are mean ± SE of three replicates

Table.2 Production of total cellulase (FPase) (IU) in submerged fermentation using Water hyacinth

| Organisms | Period of fermentation (Days) | | | | | | |
|------------------|-------------------------------|------------|------------|------------|------------|------------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| <i>T. viride</i> | 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 |
| <i>A. niger</i> | 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 |

Results are mean ± SE of three replicates

Table.3 Production of β -glucosidase (IU) in submerged fermentation using Water hyacinth

| Organisms | Period of fermentation (Days) | | | | | | |
|------------------|-------------------------------|------------|------------|------------|------------|------------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| <i>T. viride</i> | 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 |
| <i>A. niger</i> | 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.4 Cellulose utilization (%) during the submerged fermentation of WHB

| Organisms | Period of fermentation (Days) | | | | | | |
|------------------|-------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| <i>T. viride</i> | 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 |
| <i>A. niger</i> | 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.5 Reducing sugar release (mg g⁻¹) during the submerged fermentation by fungal isolates

| Organisms | Period of fermentation (Days) | | | | | | |
|------------------|-------------------------------|-------------|-------------|--------------|--------------|-------------|--------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| <i>T. viride</i> | 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 |
| <i>A. niger</i> | 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.

Fig.2 Dye decolourization potential (%) and laccase activity (IU) of the spent fungal biomass

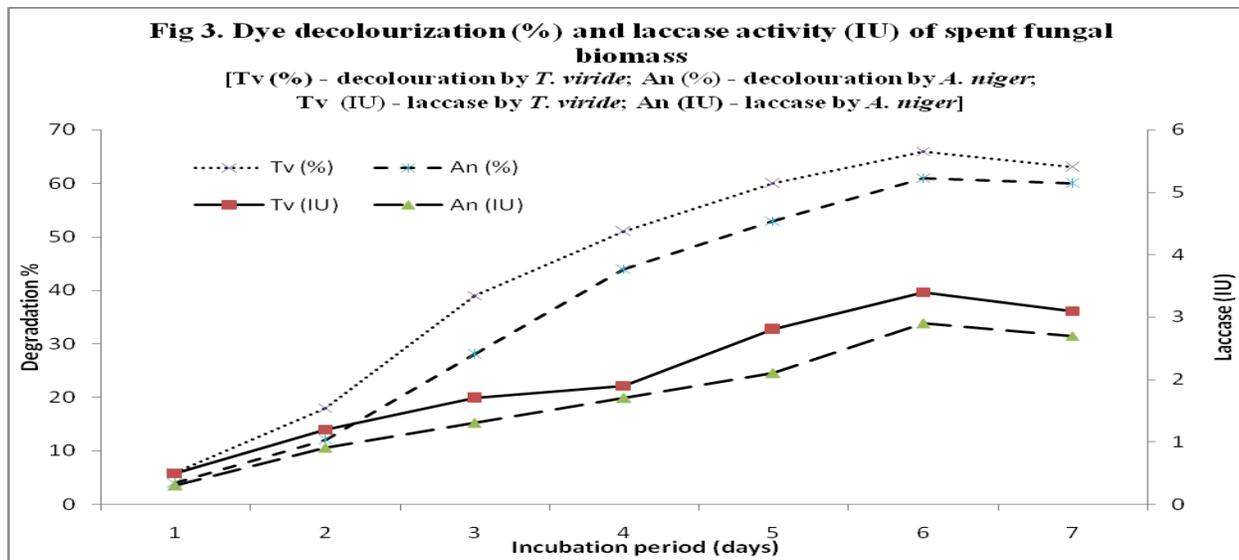
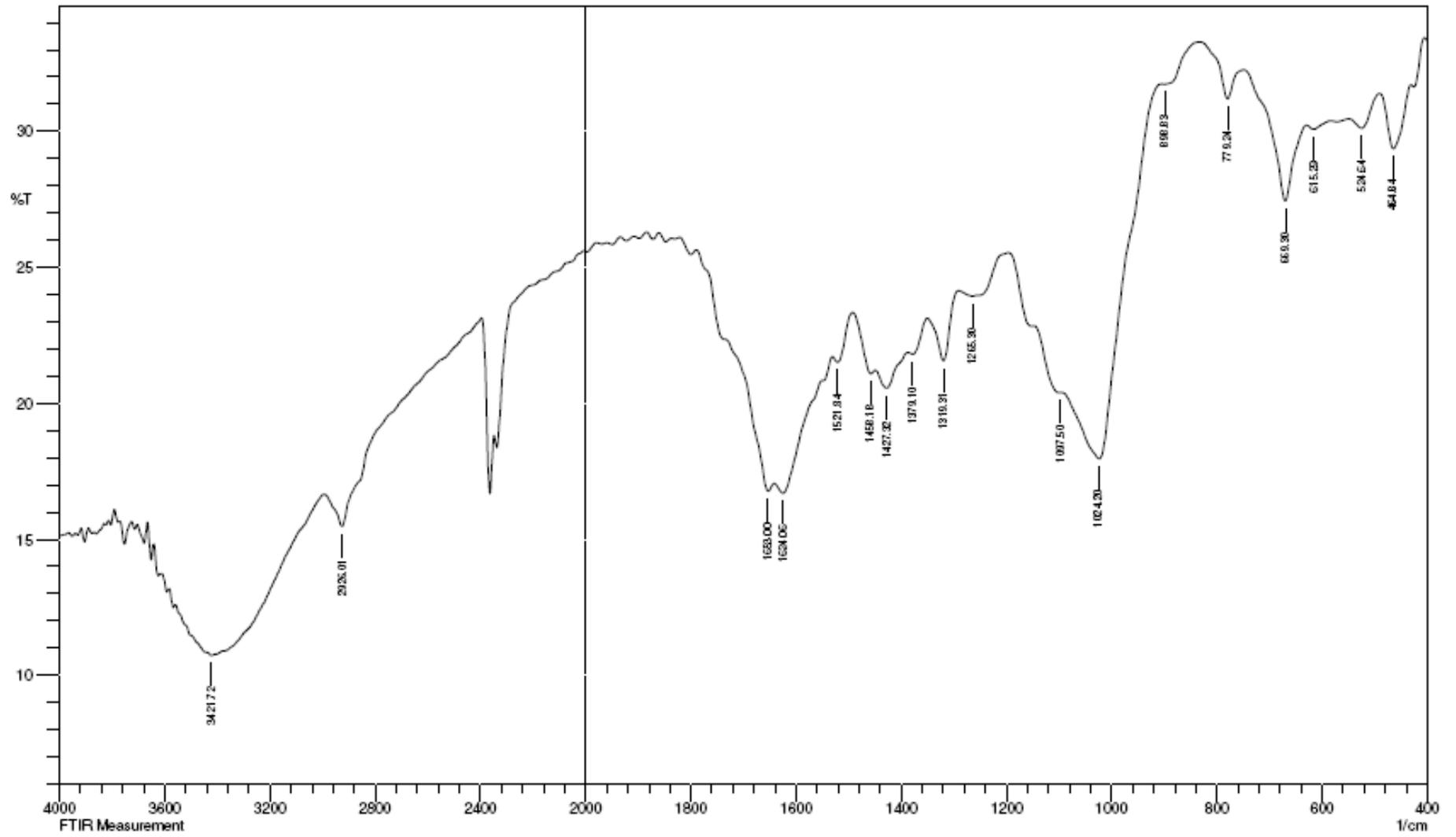


Fig.1 FTIR spectra of Water Hyacinth Biomass



Similar dye de-colorization was observed by fungal isolates from dye industry waste (Selvakumar *et al.*, 2013). The present results clearly demonstrated the dye degradation potential of isolated fungus and confirmed the ability of spent fungal biomass for dye de-colourization after cellulase enzyme production. The laccase activity was measured and the results were showed in Fig. 2. It was inferred from the results that the laccase activity increased up to 6th day with a maximum activity of 3.4 (IU) for *T. viride* and 2.9 (IU) for *A. niger*. The de-colourization process depends on the increased or decreased enzyme production by the spent fungal biomass (Vinothkumar *et al.*, 2011).

In conclusion, using water hyacinth as carbon sources, cellulase was produced in submerged fermentation by *T.viride* and *A. niger* and the production parameters were optimized. The partially purified crude enzyme of *T. viride* exhibited significantly higher total cellulase activity (FPase) of 68.3 (IU), CMCase (98.4 IU) and glucosidase (71.9 IU) compared to *A. niger*. The dye degradation potential of both fungal strains (66% & 61%) confirmed the ability of spent fungal biomass for dye de-colourization after cellulase enzyme production. Hence, it can be used for dye degradation in water bodies polluted with textile effluents. The present study reveals that the WHB associated fungi can be used to produce industrially valuable enzymes by submerged fermentation.

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