

# A novel strain of *Trichoderma viride* shows complete lignocellulolytic activities

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Received 5 September 2012; revised 11 October 2012; accepted 29 November 2012

## ABSTRACT

In this study we describe a novel dark-green strain of *Trichoderma viride* exhibiting complete ensemble of cellulase, hemicellulase and ligninase activities on specific plate assays. To assess the cellulase production in detail, basal salt medium (BSM) was fortified with synthetic (carboxymethyl cellulose (CMC), glucose, sucrose, dextrose, lactose or maltose) and natural (flours of banana, banana peel, jack seed, potato or tapioca) carbon as well as nitrogen (yeast extract, beef extract, peptone, NaNO<sub>3</sub> or NH<sub>4</sub>NO<sub>3</sub>) sources. Temperature and pH optima were 28°C and 4, respectively for the growth of the fungus in CMC-BSM with 137 U/mL cellulase activity, which was enhanced to 173 U/mL at 1.25% CMC concentration. Flours of potato and banana peel supported comparable yields of cellulase to that of CMC, while sodium nitrate was the preferred nitrogen source. The water soluble bluish-green pigment (a probable siderophore) extracted from the spores showed an absorption maximum at 292 nm. To sum up, the complete lignocellulolytic potential of this fungus offers great industrial significance, coupled with the production of a new pigment.

**Keywords:** *Trichoderma viride*; Morphology; Liquid Fermentation; Bluish-Green Pigment

## 1. INTRODUCTION

Lignocellulosic biomass comprises cellulose, hemicellulose and lignin, of which lignin is the most recalcitrant to biodegradation and only higher fungi are capable of degrading this polymer via an oxidative process involving an array of oxidases, peroxidases and hydrolytic enzymes [1,2]. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall [2]. The complex lignin polymer forms a ce-

menting envelop around cellulose in lignocellulosic wood; thus the success of the bioconversion of lignocelluloses into fermentable sugars lies in the removal of lignin wrap around the cellulose fibers packed inside. Cellulose is a fibrous, insoluble and crystalline polysaccharide consisting of D-glucose residues linked by  $\beta$ -1, 4-glucosidic bonds; owing to its highly ordered crystalline structure, cellulose is more resistant to hydrolysis than hemicellulose. Hemicellulose macromolecules are often polymers of pentoses (xylose and arabinose), hexoses (mostly mannose) and a number of sugar acids, while cellulose is a homogenous polymer of glucose [3]. Cellulose is the most abundant biopolymer in nature, being degraded to glucose through the synergistical hydrolysis of three classes of cellulases, viz., including endo- $\beta$ -1,4-glucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91) and  $\beta$ -glucosidase (EC 3.2.1.21). Glucose from the hydrolysis of cellulose can easily be fermented to useful industrial products such as ethanol, lactic acid, single cell protein and other value-added products [4]. There are different methods for the production of low-cost enzymes by the activity of microorganisms.

Some members of all classes of microorganisms, viz., fungi, actinomycetes and bacteria are known to produce cellulase isoforms [5,6]. Among them, mycelial fungi constitute a group unique of microorganisms showing prominent lignocellulolytic activity. Lignocellulolytic fungi are often divided into three groups, viz., white rot, brown rot and soft rot fungi. *Trichoderma* spp., the ascomycetous green-spored having cosmopolitan distribution are classified under white rot fungi. The surface colony colour is white and scattered greenish patches become visible as the conidia are formed and may form concentric rings. The highly branched conidiophores bear phialides singly or in groups. *Trichoderma* spp. are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants, where they can also induce systemic resistance against pathogens [6]. At pre-

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sent, the various strains of *Trichoderma* spp. are recognized by the researchers as the steadiest and safest fungi for the production of cellulase and hemicellulases. Mostly *T. reesei* and its variants are widely employed for the commercial production of hemicellulases and cellulases. *T. reesei* might be a good producer of hemicellulolytic and cellulolytic enzymes but is unable to degrade lignin. The ability of fungi to degrade lignocellulosic materials is due to their highly efficient enzymatic system [7]. *T. viride* have mostly been studied for cellulase production. There exist many studies describing the optimization of process parameters for the production of cellulase using submerged fermentation from *T. viride* [8]. Species of *Trichoderma* are shown to have lignolytic, hemicellulolytic and cellulolytic activities, but no organism is reported yet having the embodiment of all these enzyme complexes together. Based upon this background, the specific objectives of this study are to: 1) Confirm the novel isolate as *T. viride* by morphological characteristics; 2) Check the lignocellulolytic activities of *T. viride*; 3) Analyze cellulase production potential of *T. viride* in detail; and 4) Check the nature of pigment produced by *T. viride*.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism

Pure *T. viride* culture procured from the Kerala Agricultural University, Mannuthi, Kerala (Receipt No.17 dated 15-12-2011) was used for the present study. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4°C. Analytical grade chemicals from Merck India Ltd. and Himedia were used.

### 2.2. Morphological Characterization

Morphological characteristics of *T. viride* were determined by lactophenol cotton blue staining under binocular microscope attached with Image Analyzer (Nikon Eclipse 400). Phase-contrast images of *T. viride* spores were taken using Leica M80 (Germany).

### 2.3. Screening for Lignocellulosic Activity

6 day old *T. viride* on PDA slants was cultured on basal mineral salt medium (BSM) containing (g/L) NaNO<sub>3</sub>—2, K<sub>2</sub>HPO<sub>4</sub>—1, KCl—0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O—0.5, proteose peptone—2, agar—20 containing 0.5% CMC (for cellulase activity), xylan (for hemicellulase activity) or lignin (for ligninase activity) was used for preliminary screening of cellulase, hemicellulase or ligninase activities, respectively. The agar plates were incubated at 28°C. Growth after incubation indicates lignocellulolytic activity of *T. viride*.

### 2.4. Screening for Cellulolytic Activity on CMC Medium

Cellulolytic activity of *T. viride* on CMC medium was determined qualitatively by iodine plate assay. 6 day old *T. viride* from PDA slant were cultured on BSM-agar medium containing CMC for enrichment and incubated for 4 days, then the plates were flooded with iodine solution (containing 1% iodine crystals and 2% potassium iodide), incubated at 30°C for 15 min, and excess stain was washed off for visual observation. Cellulolytic activity was indicated by the clear zone around the colonies.

### 2.5. Screening for Hemicellulase Activity on Xylan Medium

Hemicellulolytic activity of *T. viride* on xylan medium was determined qualitatively by congo-red plate assay. 6 day old *T. viride* on PDA slant was cultured on BSM agar containing xylan and incubated for 4 days, then the plate were flooded with 10 ml Congo-red (0.1%) solution, incubated for 15 min, then excess stain was drained off, and 10 ml of 1 M NaCl was added for destaining; opaque clear zone formation around the colonies indicates positive result.

### 2.6. Screening for Ligninase Activity on Lignin Medium

6 day old *T. viride* from PDA slant was cultured on BSM containing lignin for enrichment. *T. viride* culture was grown for 5 - 6 days on BSM-lignin medium; growth on lignin medium indicates positive result.

### 2.7. Pigment Extraction

Pigmented biomass of five days old culture on PDA agar plate was collected in various solvents (distilled water, acetone, hexane, chloroform or methanol) and stirred well on a magnetic stirrer (10 min), followed by centrifugation (9000 × g for 15 min, 4°C). The supernatant was pooled and its absorption spectrum was determined using UV-visible spectrophotometer (Elico BL 200 double beam biospectrophotometer).

### 2.8. Cellulase Production Characteristics of *T. viride*

For making the inoculum uniform; spore suspension in sterile ddH<sub>2</sub>O was stirred well on a magnetic stirrer aseptically for 5 min, and number of spores in 1 mL of suspension was calculated by counting the spores using Neubauer haemocytometer. Different parameters like effects of incubation period, pH, temperature, CMC concentration, synthetic carbon sources, natural carbon sources and nitrogen sources were optimized.

For initial studies, BSM supplemented with CMC (0.5%) was used. In all studies, 100  $\mu\text{L}$  of spore suspension ( $\sim 7.2 \times 10^6$  spores/mL) was used for 10 mL medium. All flasks were incubated in an environmental shaker (Scigenics Biotech) at 150 rpm, 28°C for 10 days. Various culture parameters studied for cellulase production were: pH (1 to 8 using different buffers), temperature (25°C, 28°C, 31°C, 33°C, 35°C and 37°C), CMC concentrations (0.75%, 1.0%, 1.25% and 1.5%), various synthetic carbon sources (0.5% glucose, lactose, maltose, dextrose and sucrose), natural carbon sources (0.5% banana flour, banana peel flour, tapioca flour, potato flour and jack seed flour) and nitrogen sources (0.2% peptone, beef extract, yeast extract, sodium nitrate and ammonium nitrate). Growth and yield were monitored up to 7 days. The culture broth was centrifuged ( $9000 \times g$  for 15 min at 4°C) and the supernatant was used for cellulase assay.

## 2.9. Cellulase Assay

Enzyme production was quantitatively measured by DNS (3,5-dinitrosalicylic acid) spectrophotometric assay by the method of Miller [9]. Fermented culture broth was centrifuged at  $9000 \times g$  for 10 min at 4°C and supernatant was used as crude enzyme solution for assays. Reaction mixture contains 0.5 mL of 1% CMC in 0.1 M sodium citrate buffer (pH, 4.8) and 0.5 mL of crude enzyme solution added, then incubated at 50°C for 30 min. The reaction was stopped by adding 3 mL DNS and incubated in a boiling water bath for 5 min and cooled. The absorbance was measured at 540 nm (Elico double beam bl 200 bio spectrophotometer). One unit (U/mL) of cellulase activity is defined as the amount of protein (cellulase) required to liberate 1 micromole of reducing sugar (D-glucose) from CMC per min under the assay conditions. Cellulase activity was calculated using the formula,  $\Delta E \times V_f / \Delta t \times \sum \times V_s \times d$ . Where,  $\Delta E$  = absorbance at 540 nm,  $V_f$  = final volume of reaction mixture including DNS,  $V_s$  = crude supernatant (ml) containing cellulase used,  $\Delta t$  = incubation time for hydrolysis,  $\sum$  = extinction coefficient of glucose (0.0026),  $d$  = diameter of cuvette.

## 2.10. Statistics

All studies were repeated at least three times to check the reproducibility of the results, and average values were given with standard deviation. Microsoft Excel was used to draw the figures.

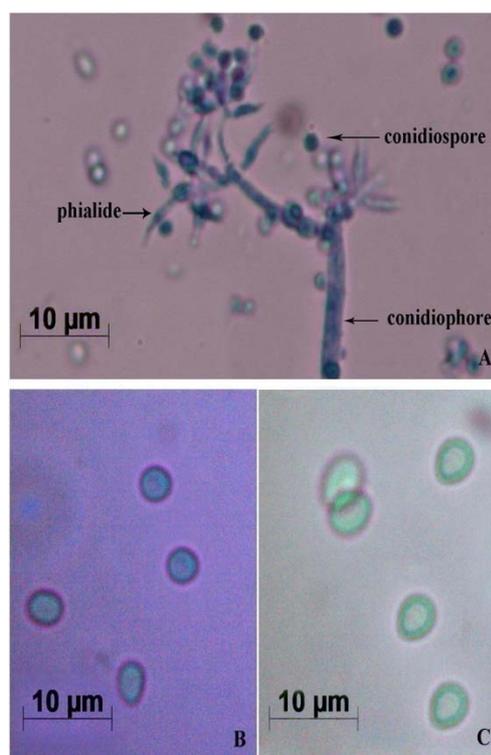
## 3. RESULTS AND DISCUSSION

Results shown here are the average values of at least 3 independent experiments. The fungal culture started to grow as white mycelia on PDA plate, then spread all over the surface of the medium as a dark-green mat. It

was stained using lactophenol cotton blue and observed under Image Analyzer. The mycelia, conidiophores bearing warted phialides singly or in clusters and numerous conidiospores were observed (**Figure 1A**). Usually the phialides were ellipsoidal or flask shaped inflated at the base dark-green coloured spores with smooth outer wall were found to be spherical and biconcave, resembling human erythrocytes (**Figures 1B and C**). This preparation was observed under phase-contrast microscope. Bissette [10] described that the species in *Trichoderma* (*Ascomycetes*, *Hypocreales*) have narrow and flexuous conidiophores and branches, with branches and phialides uncrowded, frequently paired, and seldom with more than three elements in a whorl. Its type species *viride* typically produced warted conidia [11], as we described (**Figure 1A**). Thus, the novel fungal culture used in this study can be confirmed as *T. viride*.

## 3.1. Lignocellulolytic Activity

**Figure 2** clearly shows that the fungus harbours an en-



**Figure 1.** Morphological characterization of *T. viride*. (A) Mycelia bearing conidiophores and conidiospores after staining with lactophenol cotton blue (Images by Image Analyzer, Scale bar = 10  $\mu\text{m}$  (Nikon Eclipse E400, Towa Optical, Japan, fitted with Nikon digital camera, DXM 1200F, Japan); (B) Conidiospores under Bright Field Microscope (Scale bar = 10  $\mu\text{m}$ , Leica M80, Germany); and (C) Conidiospores under Phase Contrast Microscope (Scale bar = 10  $\mu\text{m}$ , Leica M80, Germany).

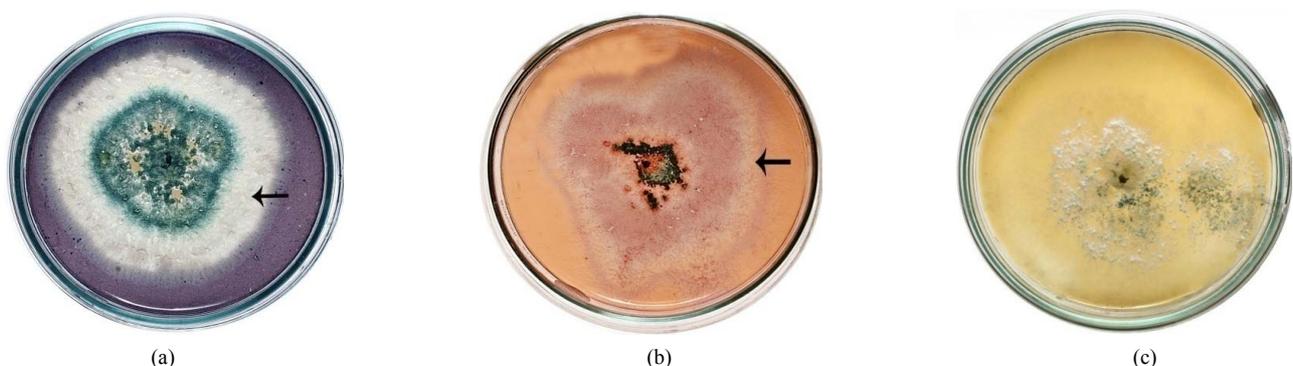
semble of enzymes for the complete degradation of lignocelluloses. Upon iodine plate assay of *T. viride* on BSM-CMC agar plate showed a clear zone around the mycelial colony, which indicates cellulolytic activity (**Figure 2(a)**). Xylan degradation around the colonies appeared as yellow and opaque zone against a reddish purple colour for undegraded xylan (**Figure 2(b)**). On lignin medium, mycelia were grown as bluish-green patches (**Figure 2(c)**), however, its growth was not as fast as in CMC or xylan medium. These tripartite plate assays clearly show that *T. viride* harbours an ensemble of lignocellulolytic enzymes for the complete degradation of lignocellulosic materials. The biodegradation of wood constituents is currently understood as a multi-enzymatic process with the mediation of small molecules [12]. Cellulolytic fungi can easily be screened within two days for the production of cellulolytic enzymes (especially, endoglucanase and exoglucanase) by staining technique or by measuring the amount of reducing sugar (glucose) produced with the dinitrosalicylic acid reagent method [13]. Thus, we employed DNS method for analyzing the cellulase activity of the culture in liquid media.

### 3.2. Cellulase Production by *T. viride*

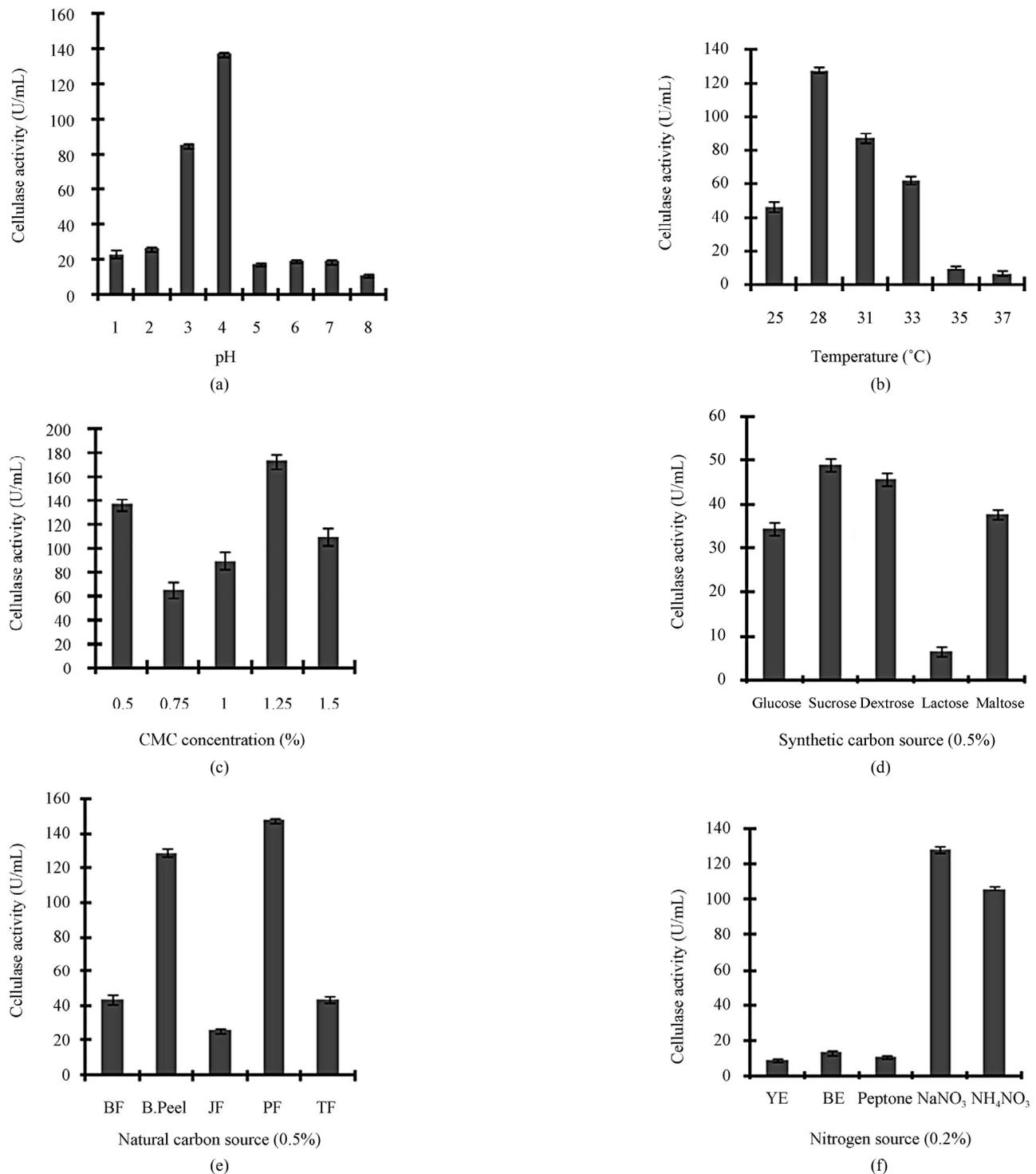
**Figure 3** shows a view of the cellulase production profile of the culture in BSM supplemented with various carbon and nitrogen sources. Optimum pH was 4, with maximum yield 137 U/mL (**Figure 3(a)**). Like pH, temperature also played a crucial role, whose optimum was 28°C with 128 U/mL at pH 4 (**Figure 3(b)**). Optimum concentration of CMC was 1.25% in the BSM, at this concentration the cellulase yield was 173 U/mL (**Figure 3(c)**). However, when CMC was replaced by simple sugars, the yield was reduce tremendously (**Figure 3(d)**); on the contrary, natural starch supplements (0.5%) showed best improvement in cellulase production (148 U/mL), which was comparable to that of CMC (**Figure 4(e)**). Simple

nitrogen source like sodium nitrate (0.2%) was the preferred nitrogen source (**Figure 4(f)**).

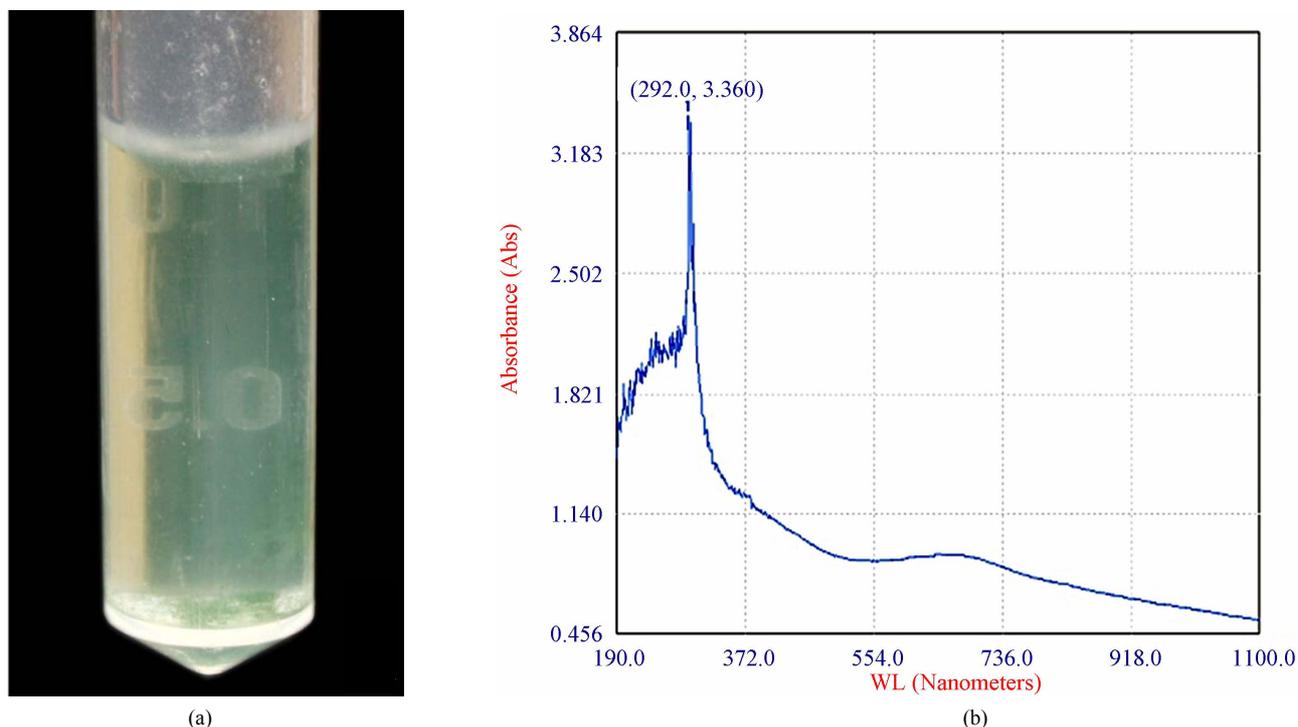
Optimization of the time course is of prime importance for the biosynthesis of cellulases by fungi [14]. CMC is the preferred carbon source used for the selective enrichment culture of cellulase producing microorganisms. Using CMC as the carbon source, Gashe [15] showed that the CMCCase (cellulose) activity of *Trichoderma* sp. as 167 U/mL. The incubation period was directly related to the production of enzymes and other metabolites up to a certain extent, and we showed that maximum cellulase activity was mostly obtained on the day 6. *Aspergillus niger* KK2 showed CMCCase (129 U/g),  $\beta$ -glucosidase (100 U/g) and  $\beta$ -xylosidase (193 U/g) activities concurrently after 5 to 6 days of solid-state fermentation [16], which is comparable to our results. Most of the filamentous fungi, especially *Trichoderma* spp. have been found to be able to grow and metabolize within a range of pH, 3 to 5 [17]. At the declining phase of cellulase yield, acidic proteins would be released that would adversely affect the yield and fermentation process [4]. Carbon sources in majority of commercial cellulase fermentations are cellulosic biomass including straw, spent hulls of cereals and pulses, rice or wheat bran, rice or wheat straw, sugarcane bagasse, water hyacinth, paper industry waste and other cellulosic biomass [18]. Other commonly used substrates are wheat bran, orange peel, corn cob residue, rice straw, sugarcane bagasse, etc. [19]. Lactose, cellobiose, sophorose, gentibiose, are the known inducer of cellulase production but only lactose can be used for economically feasible industrial production [19]. Though addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and cellulase production, they were not effective replacement for inorganic nitrogen sources because of their higher cost [20]. High concentration of nitrogen sources may lead to vitrification (the medium appears to be yellow and glassy) that is usually unsuitable for the microorganisms.



**Figure 2.** Lignocellulolytic activities of *T. viride*. (a) Iodine plate assay showing cellulase activity on 4<sup>th</sup> day of incubation at 28°C; (b) Congo-red plate assay showing xylanase activity on 4<sup>th</sup> day of incubation at 28°C; and (c) Growth of *T. viride* on lignin medium on 6<sup>th</sup> day of incubation at 28°C.



**Figure 3.** Optimization of parameters for cellulase production: (a) Effect of pH on cellulase production from *T. viride* on BSM supplemented with 0.5 % CMC on 6<sup>th</sup> day of incubation at 150 rpm and 28°C; (b) Effect of temperature on cellulase production from *T. viride* on BSM supplemented with 0.5% CMC on 6<sup>th</sup> day of incubation at 150 rpm, 28°C and pH 4; (c) Effect of CMC concentration on cellulase production from *T. viride* on BSM on 6<sup>th</sup> day of incubation at 150 rpm, 28°C and pH 4; (d) Effect of synthetic carbon sources on cellulase production from *T. viride* on BSM on 6<sup>th</sup> day of incubation at 150 rpm, 28°C and pH 4; (e) Effect of natural carbon sources (0.5%) on cellulase production from *T. viride* on BSM on 6<sup>th</sup> day of incubation at 150 rpm, 28°C and pH 4, where BF (banana flour), BP (banana peel), JF (jack seed flour), PF (potato flour), TF (tapioca flour); and (f) Effect of nitrogen sources on cellulase production from *T. viride* on BSM supplemented with 0.5% CMC on 6<sup>th</sup> day of incubation at 150 rpm, 28°C and pH 4, where YE (yeast extract) and BE (beef extract).



**Figure 4.** Characterization of the bluish-green pigment: (a) Pigment extracted from spores of *T. viride* in distilled water and centrifuged ( $9000 \times g$ , 15 min); and (b) Absorption spectrum of pigment of *T. viride* extracted as above (by ELICO BL 200 Double Beam Bio Spectrophotometer).

### 3.3. Pigment Production

The fungus used in this study produced a water-soluble greenish pigment (**Figure 4(a)**). In fact the mycelia were colourless and only the spores were colored. Upon stirring, the pigment bound to the wall of the spores partially diffused in to water, and yet more pigment diffused into it after centrifugation. The absorption maximum of the pigment was at 292 nm (**Figure 4(b)**). *T. viride* is shown to produce variously coloured pigments, which range from green [21], yellow [22] to brown [23]. Very recently, Xiaoyi *et al.*, [22] showed optimization of fermentation conditions in liquid state for a *T. viride* strain to produce yellow pigment, the viridin. From this, the pigment produced by the strain reported herein seems to be a new one, which needs to be elucidated further.

### 4. CONCLUSION

Characteristics of the fruiting bodies show that the novel strain described in this study is *T. viride*. From the screening tests, it is evident that apart from cellulases, it is an efficient producer of ligninases and hemicellulases. From the optimization studies in the liquid medium, *T. viride* shows good cellulase yield. Moreover, this strains produces an extractable and water soluble bluish-green pigment, probably a siderophore, which needs to be elucidated. Briefly, this study shows that *T. viride* we used in

this study is a potent strain for the complete degradation of lignocelluloses and subsequent fermentation for valuable products like ethanol, preferably by solid-state fermentation.

### 5. ACKNOWLEDGEMENTS

The authors are thankful to the Kerala State Council for Science, Technology and Environment for a research grant, No. (T) 422/SRS/2009/-CSTE.

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