

Microbial Utilization of Agronomic Wastes for Cellulase Production by *Aspergillus niger* and *Trichoderma viride* Using Solid-State Fermentation

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ABSTRACT

Microbial utilization of agronomic wastes has attracted worldwide attention for conversion of these renewable resources into bio-based products and bioenergy. Microbial cellulases find enormous applications in various industries. The objectives of the present study were to optimize media and growth conditions for effective utilization of agronomic wastes such as wheat bran, rice straw, sugarcane bagasse, banana peel, Bengal gram husk and corn husk through cellulase production by *Aspergillus niger* and *Trichoderma viride* using solid-state fermentation. Optimization studies revealed maximum production of cellulase from rice straw by *A. niger* at 25°C, pH 4.5, when incubated for 4 days, with initial moisture content of 49.44% and inoculum size of 4%, supplemented with 1% (w/v) tryptone as the nitrogen source. *T. viride* produced maximum cellulase from banana peels at 30°C, pH 5.5, when incubated for 3 days, with a moisture content of 24.01%, inoculum size of 3%, supplemented with 1% (w/v) yeast extract as the nitrogen source. The enzymatic assay of cellulase was performed by the dinitrosalicylic acid method with absorbance at 540 nm. The *A. niger* and *T. viride* enzyme extracts, when subjected to ammonium sulphate precipitation, ion-exchange chromatography and dialysis, revealed a 2- and 8-fold increase in enzyme activity, respectively. The activities of the partially purified cellulases from *A. niger* and *T. viride* were 402.13 and 104.8 U/g dry substrate, respectively. SDS-PAGE revealed three protein bands with apparent molecular weights of 25, 30 and 75 kDa.

Keywords: banana peel, enzyme activity, molecular weights, optimization studies, rice straw

Abbreviations: CMC, carboxy methyl cellulose; DEAE, diethyl amino ethyl; DNSA, dinitro salicylic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; U/gds, units/gram dry substrate

INTRODUCTION

The current predominant energy resource, fossil fuel, is limited, causing enormous energy and environment problems. There has been an increasing worldwide interest in alternative sources of energy, such as agricultural biomass, to substitute fossil-fuel-based energy resources (Aristidou and Penttila 2000; Jeffries and Jin 2000; Zaldivar *et al.* 2001; Li *et al.* 2009). Microbial utilization of agronomic wastes has attracted worldwide attention for conversion of these renewable resources into bio-based products and bioenergy. Agronomic wastes such as corn husks, sugarcane waste, wheat or rice straw, forestry and paper mill discards, the paper portion of municipal wastes have plentiful cellulose, which can be converted into fuel ethanol (Leschine 1995; Singhanian *et al.* 2010).

Cellulose is a fibrous, insoluble, crystalline polysaccharide found in plant cell walls, composed of repeating D-glucose units linked by -1,4-glucosidic bonds, and the most abundant carbohydrate polymer on earth (Jagtap and Rao 2005; Guo *et al.* 2008). Each year, photosynthetic fixation of CO₂ yields more than 10¹¹ tons of dry plant material worldwide, and almost half of this material consists of cellulose. Cellulose is used as a food source by a wide variety of organisms including fungi, bacteria, plants and protists, as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, molluscs and nematodes (Watanabe and Tokuda 2001; Davison and Blaxter 2005).

Cellulase is an important enzyme for carrying out the depolymerization of cellulose into fermentable sugars. Microbial cellulases find applications in various industries and constitute a major group of the industrial enzymes

(Sukumaran *et al.* 2005). One of the most immediate and important applications of biomass energy systems using cellulases could be in the fermentation of fuel ethanol from biomass (Lin and Tanaka 2006). Application of solid-state fermentation technology could be an attractive possibility for economic utilization of agro-industrial residues (Pandey *et al.* 2000). Moreover, solid-state fermentation requires less energy input than liquid fermentation, making its potential application of interest (Kerem *et al.* 1992).

The objectives of this present study were isolation of cellulolytic fungi and optimization of parameters for effective utilization of agronomic wastes through cellulase production using solid-state fermentation.

MATERIALS AND METHODS

Chemicals and reagents

Potato dextrose agar, yeast extract powder, DEAE sepharose and dialysis membrane were purchased from Himedia, Mumbai, India. Tryptone and carboxy methyl cellulose (CMC) were procured from Loba Chemie, Mumbai, India. Beef extract powder, peptone and phenol were supplied by Qualigens Fine Chemicals, Mumbai, India. Tris, sodium sulphite and dinitro salicylic acid (DNSA) were obtained from SD Fine-Chem Ltd., Mumbai, India. All the other chemicals were purchased from Nice Chemicals, Kochi, India. All the chemicals and reagents used in this study were of analytical grade. Pure distilled water was obtained with a Milli-Q system (Millipore, Tokyo, Japan).

Microorganisms and taxonomic study

Various fungal isolates were obtained from the rhizosphere soil within the institute campus and isolated on potato dextrose agar containing 1% (w/v) CMC. The sporulating fungi were identified based on the colony morphology and microscopical features using standard protocols. Pure cultures of fungal isolates were maintained on CMC agar slants at 4°C in the dark.

Screening for cellulolytic activity

Pure cultures of the fungal isolates were grown on CMC agar plates and incubated at 25°C for 7 days under aerobic conditions. The plates were flooded with 0.1% Congo Red dye for 30 min, destained with 1M NaCl for 15 min and observed for the clear zone of cellulose hydrolysis surrounding the colonies (Vu *et al.* 2009).

Source of agronomic wastes

Cheap agronomic wastes like wheat bran, rice straw, bagasse, banana peel, Bengal gram husk and corn husk were procured from the local market in Bangalore city, India and used for the optimization of carbon sources for cellulase production.

Inoculum preparation and production of cellulase

Rice straw, bagasse, banana peel and corn husk were chopped into small pieces using sterile scissors and were weighed separately. 25 g of each substrate was packed in sterile glass Petri dishes, wetted with 15 ml of distilled water, wrapped in aluminium foil and autoclaved at 121°C and 15 lbs for 15 min. Moisture content was determined by considering the moist weights of the substrates. Spore suspensions of the fungal cultures were prepared in sterile distilled water and aseptically added to each plate at 1% (v/v). The substrate with added inoculum was mixed properly using a sterile spatula. The inoculated plates were incubated at 25°C for 6 days under aerobic conditions.

Following incubation, the substrates along with the fungal mycelia were crushed in a sterile mortar with a pestle after adding a known volume of distilled water. It was filtered through normal filter paper and then through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) and the filtrate was collected. The filtrate was centrifuged at 5000 rpm for 30 min at 4°C using a Hettich Universal 32R (Germany). The clear supernatant was subjected to cellulase assay and further purification.

Cellulase assay

Reducing sugar content was determined by the DNSA method using 1% CMC in 0.02 M acetate buffer, pH 5.2, as the substrate and glucose as the standards (Miller 1959; Begum *et al.* 2009). The absorbance was recorded at 540 nm using a SANYO Gallenkamp UV-VIS spectrophotometer (UK). One unit (U/gds) of cellulase activity was defined as the amount of enzyme that releases 1 µg of reducing sugar as glucose per g of dry substrate per minute, under standard assay conditions.

Optimization of different nitrogen sources

The effect of different nitrogen sources on cellulase production was investigated by incorporating tryptone, peptone, beef extract and yeast extract (1% w/v) into the production medium.

Effect of different environmental conditions on cellulase production

The influence of physical factors such as temperature and pH were also determined. Production of cellulase was noted at 25, 30, 37, 42 and 50°C. The effect of pH was studied by adjusting the substrate pH such as 4.5, 5.5, 6.5, 7.5 and 8.5. The effect of inoculum size was investigated using different volumes of fungal spore suspensions such as 0.5, 1, 2, 3, 4 and 5% (v/v).

Time course of cellulase production

The effect of incubation time on fungal cellulase production was determined every 24 hrs for 6 days.

Scale-up studies and purification of cellulase

Mass production of cellulase was carried out using 500 g of the substrates in 1000-ml Erlenmeyer flasks, fungal cultures and all the optimized conditions. The crude enzyme extract obtained after fermentation was subjected to 80% (w/v) ammonium sulphate precipitation. Ion-exchange chromatography was performed using DEAE-sepharose (Chen *et al.* 2004). Approximately 8.5 ml of clear crude extract was loaded onto a DEAE-sepharose column (17.5 × 1.6 cm; 1 ml/min) previously equilibrated in 50 mM Tris-HCl buffer (pH 7.4). The cellulase was eluted in 50 mM Tris-HCl buffer (pH 7.4) with a 0.1 to 0.5 M NaCl gradient. Fractions (1.5 ml) were collected and tested for cellulase activity. Active fractions were pooled, dialyzed with 10 mM Tris-HCl buffer (pH 7.4) supplemented with 0.15 M NaCl. The insoluble residue was removed after dialysis by centrifugation at 8000 rpm for 30 min, and equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Protein concentration was also estimated by Lowry's method (Lowry *et al.* 1951).

Determination of molecular weight

The molecular weights of the purified cellulases were determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protein Tetra Cell vertical electrophoresis unit. Electrophoresis was carried out on 10% polyacrylamide slab using the buffer system of Laemmli (1970). Samples were diluted twice with sample buffer consisting of 50 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue. Gel protein was stained with 0.2% (w/v) Coomassie brilliant blue solution. Apparent molecular weights of the purified cellulases were evaluated using broad range pre-stained protein marker (New England BioLabs, UK).

Statistical analysis

All the optimization studies were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Isolation of cellulolytic fungi

There has been much research aimed at obtaining new microorganisms producing cellulases with higher specific activities and greater efficiency (Subramanian and Prema 2000). In this study 15 isolates of *Aspergillus* and five isolates of *Trichoderma* were obtained from the rhizosphere soil rich in humus content. *A. niger* and *T. viride* were identified based upon macro- and microscopic characteristics. Both the fungal isolates yielded clear zones of cellulose hydrolysis on CMC agar plates. Prior studies have revealed many cellulolytic microorganisms including fungi (*Trichoderma reesei*, *Aspergillus* and *Rhizopus* sp.) and bacteria (*Cellulomonas* and *Bacillus* sp.) (Penttila *et al.* 1986; Lowe *et al.* 1987; Tomme *et al.* 1988; Murashima *et al.* 2002; Kim *et al.* 2003; Saito *et al.* 2003; Lynd *et al.* 2005).

Optimization of cellulase production from agronomic wastes

Media optimization is an important aspect to be considered in the development of fermentation technology. Formulation of a medium that is cost effective for the production of cellulase can greatly reduce the cost of the enzyme (Das *et*

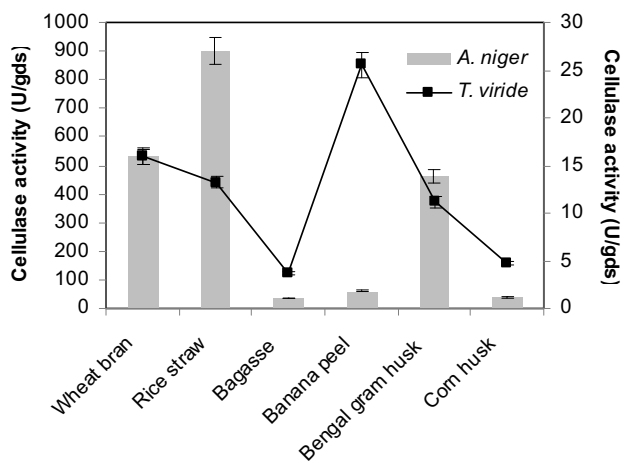


Fig. 1 Optimization of different agronomic wastes for cellulase production from *Aspergillus niger* and *Trichoderma viride*. Data represent mean \pm S.D. (n=3); $P < 0.05$.

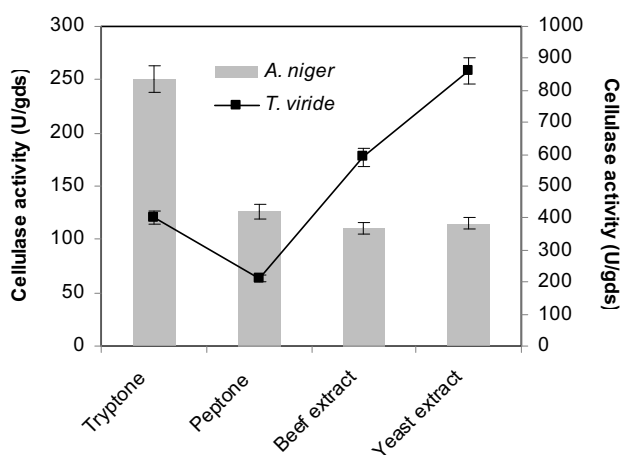


Fig. 2 Optimization of various nitrogen sources for cellulase production. Data represent mean \pm S.D. (n=3); $P < 0.05$.

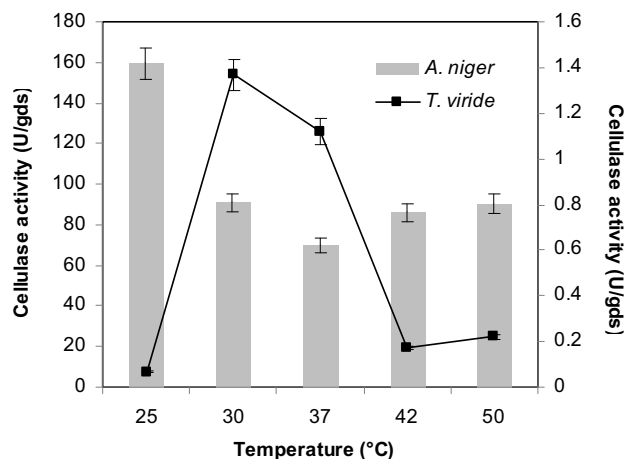


Fig. 3 Effect of temperature on cellulase production. Data represent mean \pm S.D. (n=3); $P < 0.05$.

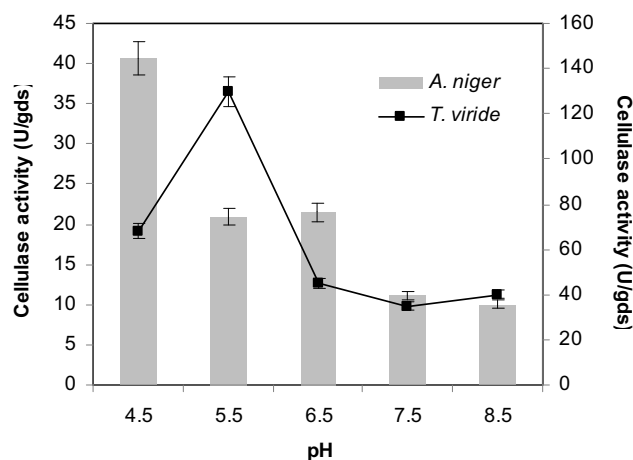


Fig. 4 Effect of pH on cellulase production. Data represent mean \pm S.D. (n=3); $P < 0.05$.

al. 2010). It has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as cheap carbon sources (Kim *et al.* 2003). The production cost of cellulases may be brought down by multifaceted approaches, which include the use of cheap lignocellulosic substrates for fermentative production of the enzyme and the use of cost-efficient fermentation strategies like solid-state fermentation (SSF) (Singhania *et al.* 2007). Therefore, in the present study an attempt was made to utilize different cheap renewable agricultural wastes such as wheat bran, rice straw, bagasse, banana peel, Bengal gram husk and corn husk as carbon sources for cellulase production through SSF (Fig. 1). Among the various substrates used, maximum activity of cellulase was recorded from rice straw (900.46 U/gds) by *A. niger* at 25°C, pH 4.5, when incubated for 4 days, with initial moisture content of 49.44% and inoculum size of 4% (v/v), supplemented with 1% (w/v) tryptone as the nitrogen source (Fig. 2). It has also been noted that the addition of nitrogen sources into the production medium improves enzyme production (Pandey *et al.* 1994). Previously, maize wastes fermented by *A. niger* for 72 h revealed high protein contents (12.58 g/100 gds) which could be attributed to the ability of the microorganism to secrete some extracellular enzymes (proteins), which degrade the cellulosic materials during fermentation (Oseni and Ekperigin 2007). In our study, *A. niger* produced 38.25 U/gds of cellulase from corn husks. Cellulase production from pineapple waste using *Trichoderma longibrachiatum*, *A. niger* and *Saccharomyces cerevisiae* was previously assessed, wherein, highest amount of glucose produced by

A. niger was from pineapple pulp (0.63 mg/0.5 ml) (Omojasola *et al.* 2008).

On the other hand, *T. viride* produced maximum cellulase from banana peel (25.53 U/gds) at 30°C (Fig. 3), pH 5.5 (Fig. 4), when incubated for 3 days (Fig. 5), with initial moisture content of 24.01%, inoculum size of 3% (v/v) (Fig. 6), supplemented with 1% (w/v) yeast extract as the nitrogen source. Our results are in accordance with the findings of Singhania *et al.* (2006, 2007), wherein cellulase production from lignocellulosic substrates by *Trichoderma reesei* occurred maximally at 30°C (3.8 U/gds) and after 72 hrs (154.58 U/gds). Earlier reports suggest that the cellulolytic fungus, *T. reesei* has the strongest cellulose-degrading activity (Penttila *et al.* 1986; Tomme *et al.* 1988). In a previous study conducted by Omojasola *et al.* (2008), it had also been found that *T. longibrachiatum* produced the highest amount of glucose from pineapple wastes (0.92 mg/0.5 ml) among the cultures tested. In general, species of *Trichoderma* are ubiquitous colonizers of cellulosic materials and efficiently utilize cellulosic wastes (Schuster and Schmöll 2010).

The cultivation temperature has a marked influence on the growth rate as well as on the level of cellulase production. *A. niger* exhibited maximum bioconversion (159.5 U/gds) at 25°C, whereas, *T. viride* at 30°C (1.37 U/gds) (Fig. 3). *A. niger* favoured a lower incubation temperature for the enzyme production than *T. viride*. Similar findings were reported in a study conducted by Omojasola *et al.* (2008), wherein, it was observed that *A. niger* produced highest cellulase activity (0.63 mg/0.5 ml) at 40°C, whereas,

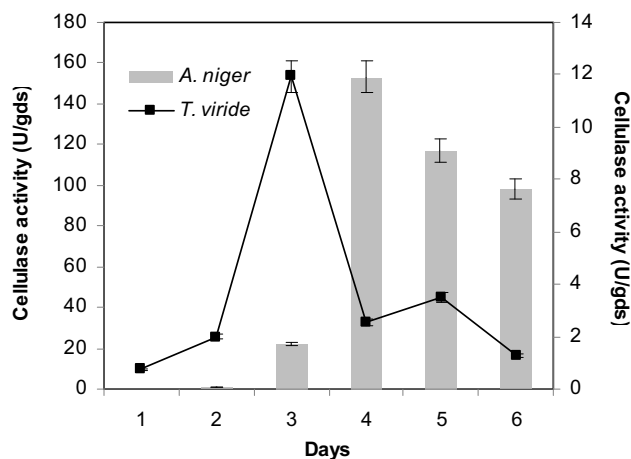


Fig. 5 Effect of incubation time on cellulase production. Data represent mean \pm S.D. (n=3); $P < 0.05$.

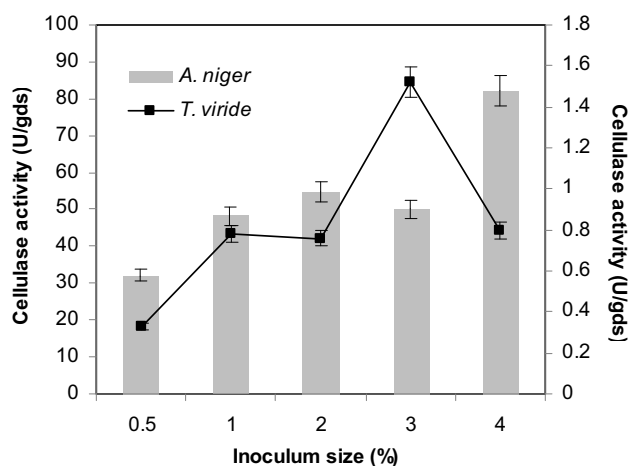


Fig. 6 Effect of inoculum size on cellulase production. Data represent mean \pm S.D. (n=3); $P < 0.05$.

T. longibrachiatum produced the highest amount of glucose (0.92 mg/0.5 ml) at 45°C.

The pH of the production medium greatly affects the growth rate of the fungus as well as the enzyme production. As evidenced from the optimization studies, *A. niger* demonstrated optimum cellulase synthesis (40.6 U/gds) at pH 4.5, but *T. viride* exhibited highest cellulase production (130 U/gds) at pH 5.5 (Fig. 4). Comparatively, a lower pH facilitated maximum cellulase production from *A. niger* than for *T. viride*. These results are in accordance with the data reported by Omojolasola *et al.* (2008), wherein, *A. niger* and *Trichoderma* sp. favoured a pH of 3.5 and 4.5, respectively.

The effect of incubation period on cellulase production was estimated for 6 days. Enzyme activity increased steadily with an increase in incubation time. Maximum production was observed after 4 days for *A. niger* (153.4 U/gds) and 3 days for *T. viride* (11.94 U/gds), while the minimum was noted at 24 hrs in both cases (Fig. 5). On further incubation of the fungal plates, cellulase production substantially decreased, probably due to the depletion of essential nutrients in the media and/or accumulation of toxic secondary metabolites produced by the fungi.

The amount of inoculum used also affected cellulase production. The effect of inoculum size on cellulase production was examined: 4% (v/v) inoculum resulted in highest enzyme activity (82.2 U/gds) in *A. niger* compared to 3% in *T. viride* (1.52 U/gds) (Fig. 6). Improved distribution of oxygen and more effective uptake of nutrients contribute to the high production of the enzyme. When the inoculum sizes were too small (0.5, 1 and 2%), the amount of cellulase production was less.

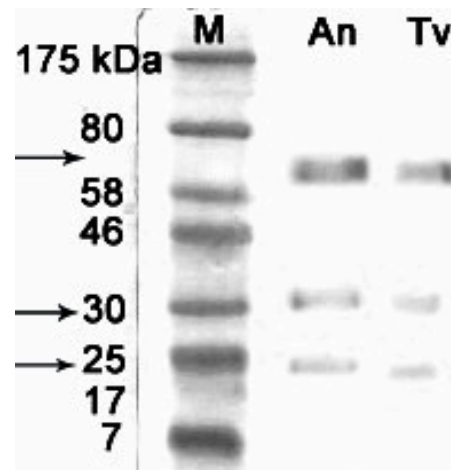


Fig. 7 Protein profile of the purified fungal cellulases by SDS-PAGE. Lane M, standard protein marker; Lane An, cellulase from *A. niger*; Lane Tv, cellulase from *T. viride*. The arrows indicate the three bands corresponding to the three subunits of fungal cellulase. The molecular sizes of the marker proteins are shown on the left.

Acid or alkali treatment of lignocellulosic wastes also affects the cellulase production. Cellulase is an inducible enzyme. Raw untreated cellulosic substrates, often acting as crude inducers, have been found to be very effective in inducing cellulase production (Nigam *et al.* 1987; Mekala *et al.* 2008). In the present study various cheap, agronomic cellulosic wastes were utilized as crude inducers for the enzyme production. It has also been found that higher initial moisture content of the medium had a negative effect on cellulase production (Singhania *et al.* 2007). In our study untreated substrates like sugar cane bagasse and other agronomic wastes with moderate levels of initial moisture content (49.44 and 24.01%) supported cellulase production from *A. niger* and *T. viride*, respectively, in solid-state fermentation. The requirement for lower moisture level for *T. viride* might be attributed to the inherent moisture content of banana peels than that present in rice straw for *A. niger*.

Scale up studies and characteristics of cellulases

Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al.* 2005). The activities of the purified cellulases from *A. niger* and *T. viride* were recorded as 402.13 and 104.8 U/gds, respectively. The purified cellulases from *A. niger* and *T. viride* revealed a 2- and 8-fold increase in the enzyme activity, respectively, indicating that both of these fungal isolates are potential producers of cellulase and hence can be effectively employed for the large scale bioconversion of agronomic wastes. *A. niger* demonstrated higher cellulase yield from rice straw whereas *T. viride* produced cellulase at a faster rate from banana peel, with comparatively low inoculum size than *A. niger*. This property of *T. viride* may be exploited for quicker utilization of banana peels.

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes (Das *et al.* 2010). Four classes of cellulolytic enzymes have been identified in aspergilli, although the number of isozymes produced by different species or even strains of the same species can differ (De Vries and Visser 2001). In the present study, SDS-PAGE analysis revealed three protein bands with apparent molecular weights of 25, 30 and 75 kDa, respectively (Fig. 7). This may be attributed to the presence of three enzymes such as exo-1,4-glucanase, endo-1,4-glucanase and 1,4-glucosidase, comprising the complete cellulase system found in some cellulolytic microorganisms (Kim and Kim 1994; Wilson and Irwin 1999).

CONCLUSIONS

Enhancing the activity of cellulase enzyme and reducing its production cost are two key issues that need to be addressed. In the present study, both *Aspergillus niger* and *Trichoderma viride* have been found to possess the potential for utilization of low-cost cellulosic materials such as agricultural wastes for cellulase production through solid-state fermentation. This could provide a cheap and eco-friendly approach for cellulase production in future.

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