The Industrial Applicability of Purified Cellulase Complex Indigenously Produced by *Trichoderma viride* Through Solid-State Bio-processing of Agro-Industrial and Municipal Paper Wastes

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An indigenous strain of *Trichoderma viride* produced high titers of cellulase complex in solid-state bio-processing of agro-industrial orange peel waste, which was used as the growth-supporting substrate. When the conditions of the SSF medium containing 15 g orange peel (50% w/w moisture) inoculated with 5 mL of inoculum were optimal, the maximum productions of endoglucanase (655 ± 5.5 U/mL), exoglucanase (412 ± 4.3 U/mL), and β-glucosidase (515 ± 3.7 U/mL) were recorded after 4 days of incubation at pH 5 and 35 °C. The enzyme with maximum activity (endoglucanase) was purified by ammonium sulfate fractionation and Sephadex G-100 column gel filtration chromatographic technique. Endoglucanase was 5.5-fold purified with specific activity of 498 U/mg in comparison to the crude enzyme. The enzyme was shown to have a molecular weight of 58 kDa by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). The shelf life profile revealed that the enzyme could be stored at room temperature (30 °C) for up to 45 days without losing much of its activity.

Keywords: Trichoderma viride; Cellulase; Agro-industrial residue; Municipal paper waste; Purification; SDS-PAGE; Shelf life

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INTRODUCTION

In nature, cellulose, hemicellulose, and lignin are the major components of plant cell walls. Cellulose, the most common and most abundant component of plant matter, composes about 35 to 50% of the plant cell wall. An important feature of cellulose that is relatively unusual in the polysaccharide world is its crystalline structure (Brown and Saxena 2000). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (proto-fibrils), which are packed into even larger units called microfibrils. These are in turn assembled into the familiar cellulose fibers (Koyama *et al.* 1997).

A wide spectrum of micro-organisms including *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* have the ability to produce enzymes such as cellulases, hemicellulases, and pectinases, under suitable growth conditions that together hydrolyze insoluble polysaccharides into soluble oligomers and then into monomers (sugars)

(Phitsuwan *et al.* 2010). *Trichoderma* is one of the most efficient cellulase producers (Iqbal *et al.* 2011a), and it is being studied for its potential in producing cellulose-degrading enzymes from various agro-industrial waste materials and their by-products, such as citrus peel, rice straw, wheat straw, banana waste, bagasse, and many others.

The cellulase enzyme system is a complex of three major enzymes that exhibit higher collective than individual activity and degrade cellulose into fermentable glucose. The cellulase complex can be divided into three main types: (i) endoglucanase, also called carboxymethyl cellulase, (ii) exoglucanase, and (iii) β-glucosidase (Iqbal *et al.* 2011a). Currently, cellulase is being used in many industrial applications, especially in the fields of cotton processing, paper recycling, and agriculture, as animal feed additives, and in research and development (Iqbal *et al.* 2010; Sun *et al.* 2010; Iqbal *et al.* 2012; Yano *et al.* 2012). The most promising technology for the conversion of lignocellulosic biomass into fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes (Yousuf 2012).

Keeping in view the extensive industrial applications of cellulase, this study on the purification of the endoglucanase from *T. viride* was performed in order to introduce its potential application in an industrial setting. In this regard, the applicability of purified endoglucanase in the saccharification of agro-industrial and paper wastes with the aim of generating fermentable glucose was also investigated.

EXPERIMENTAL

Chemicals and Solid Substrates

All the chemicals used were of analytical laboratory grade. The agro-industrial wastes, *i.e.* wheat straw, sugar cane bagasse, apple pomace, and orange peel, were obtained from a local fruit market in Gujrat, Pakistan, while a large quantity of municipal paper waste was collected from the routinely distributed local newspapers. All of the substrates were crushed into pieces, dried, and ground to fine particle size, and stored in plastic jars.

Fungal Culture and Inoculum Development

The pure culture of *T. viride* was obtained from the Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan. To develop homogeneous inoculums suspension, spores of *T. viride* were cultivated at 30±1 °C for 7 days in an Erlenmeyer flask (250 mL capacity) containing 30 mL of Potato Dextrose broth that had been sterilized at 15 lbs/in^2 pressure and at 121 °C in a laboratory scale autoclave for 15 min. This was then incubated under stationary conditions for the development of the fungal spore suspension.

Pretreatment of Agro-Industrial and Municipal Paper Wastes

The moisture-free, fine-powdered substrates (wheat straw, sugar cane bagasse, apple pomace, orange peel, and municipal paper waste), of which there were 10 g each, were pretreated with 2% HCl in an Erlenmeyer flask (250 mL) at room temperature (30 ^{o}C) for 2 hours of initial reaction time and then autoclaved at 121 ºC and 15 lb/in^2

pressure for 15 min. After this slurry of each substrate was filtered through three layers of muslin cloth, both the filtrates and the residues were saved. The filtrate was used for the analysis of total sugars and reducing sugars, while the residue was used for the production of cellulase enzymes and further analysis.

Solid-State Fermentation Protocol

A basal fermentation media in an Erlenmeyer flask (250 mL) was used to moisten the pretreated substrates for cellulase production. The initial pH value of the medium was adjusted to 4.5 before sterilization, inoculated with 5 mL of freshly prepared fungal spore suspension, and incubated at 30±1 ºC in an incubator for five days.

Optimization of Fermentation Parameters

The fermentation parameters, such as fermentation time period, pH, temperature, substrate level, and inoculum size, were optimized using the pretreated hydrolyzate (orange peel waste), which was selected from the initial substrate screening trial as the best yielded growth substrate.

Optimization of fermentation time

To optimize the fermentation time, each flask containing 5 g/100 mL of orange peel hydrolyzate was sterilized, inoculated, and fermented at 30 ^{o}C for a specified time period, *viz.* 24, 48, 72, 96, or 120 hours, in a still culture temperature controlled incubator. Flasks were harvested in triplicate after every 24 hours of fermentation time and analyzed for cellulase complex activity.

Optimization of pH

Fermentation media containing 5 g/100 mL hydrolyzate was adjusted to varying pH levels (pH 3 through 7), then was inoculated with 5 mL of fresh spore suspension and allowed to ferment for a stipulated fermentation time period.

Optimization of temperature

To determine the optimum temperature for the growth of *T. viride* and the desired enzyme complex production, triplicate flasks containing 5 g/100 mL hydrolyzate were adjusted to pH 5, inoculated, and subjected to fermentation at varying temperatures ranging from 25 to 45 ^{o}C.

Optimization of substrate level

To investigate the effects of substrate level on enzyme production, varying levels of pretreated hydrolyzate (5, 10, 15, and 20 g/100 mL) were used in a static culture environment. Flasks were inoculated in triplicate with *T. viride* and subjected to fermentation for the optimum time period at pH 5 and 35 ^{o}C.

Optimization of inoculum size

To determine the inoculum level that would give the best enzyme production by way of *T. viride*, the triplicate flasks were inoculated with varying volumes (1 to 7 mL) of freshly prepared inoculums and then processed for 72 hours at 35 ^{o}C.

Extraction of Cellulase Complex

After the stipulated fermentation time period, the cellulase complex was extracted from the fermented biomass via the addition of a citrate buffer, 0.05 M of pH 4.8 in a 1:10 (w/v) ratio, and then the flasks were shaken at 120 rpm for 30 min (Iqbal *et al.* 2010). The contents were filtered through muslin cloth and washed twice with the citrate buffer. The filtrates were centrifuged at 10,000×g (4 ºC) for 10 min, and the collected supernatants were used both as a crude enzyme extract to determine the activity and for purification purposes.

Cellulase Assays

The quantity of endo 1,4-β glucanases was assayed according to the methodology described earlier by Iqbal *et al.* (2011a). Exo 1,4-β glucanase was assayed according to the method of Deshpande *et al.* (1984). The β-glucosidase activity was determined by the method of Gielkens *et al.* (1999).

Determination of Protein Contents

The protein contents of the crude and purified enzyme extracts were determined with the Bovine serum albumin as standard as described earlier by Iqbal *et al.* 2011a.

Purification of Endoglucanase

A crude extract of endoglucanase obtained from *T. viride* was centrifuged (10,000×g) for 15 min at 4 ^{o}C in order to maximize clarity. After obtaining maximal clarity, the ammonium sulfate fractionation methodology as described by Iqbal *et al.* (2011a) was adopted for the purification of the endoglucanase. Further purification was attained by gel filtration chromatography using a Sephadex-G-100 column. Both the enzyme activities as well as the protein contents were determined for each separate fraction, as mentioned in the previous section.

SDS-PAGE for Determination of Molecular Weight

To determine the molecular weight of the endoglucanase, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 5% stacking and a 12% resolving gel according to the method of Laemmli (1970).

Industrial Application

Shelf life/storage

For the commercial utilization of enzymes at the industrial level, their potential for shelf life/storage is an important consideration. To investigate their storage stabilities, both forms, the free crude and the purified endoglucanase, were incubated at room temperature (30 ^{o}C) for up to 60 consecutive days. After every 15 days, the residual activities of each case were measured using a spectrophotometric protocol under standard conditions as described earlier (Iqbal *et al.* 2011a).

Saccharification and glucose determination

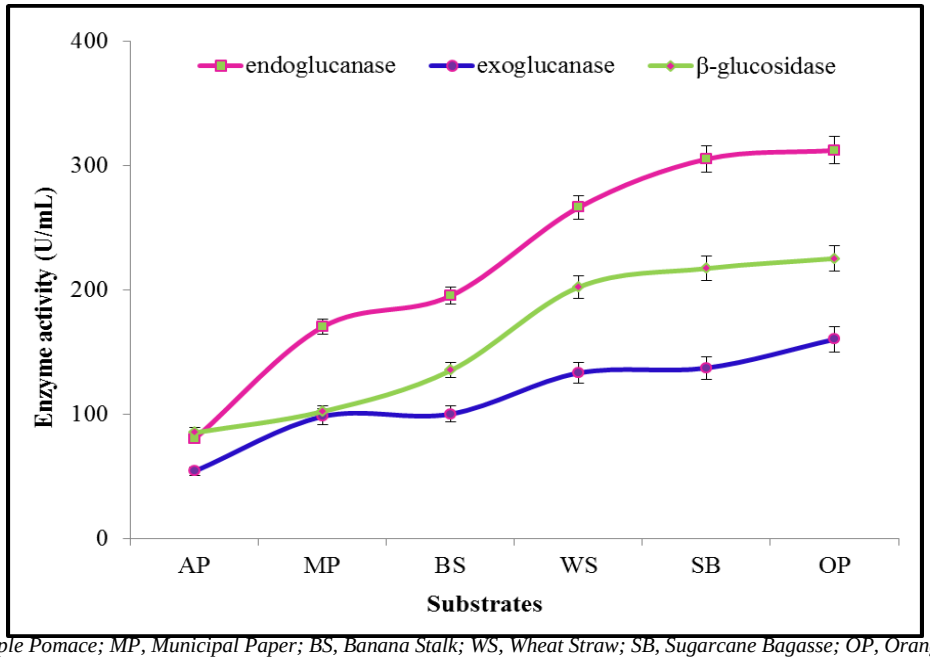
Purified endoglucanase was further applied in the saccharification of the remaining pre-treated substrates in order to investigate the applicability of the enzyme in

terms of the glucose release that could be further fermentable into bio-ethanol. The glucose estimation was performed using the method of Gadgil *et al.* (1995).

RESULTS AND DISCUSSION

Screening of Substrates

T. viride was cultured for five days in a fermentation medium containing pretreated substrates (wheat straw, sugar cane bagasse, apple pomace, orange peel, and municipal paper waste) for the production of a cellulase complex comprising endoglucanase, exoglucanase, and β-glucosidase. After the stipulated time, the fermented samples were harvested and analyzed individually for enzyme activity. The maximum production of endoglucanase (312 U/mL), exoglucanase (160 U/mL), and β-glucosidase (225 U/mL) was noted in the medium containing orange peel as a substrate, followed by the sugar cane bagasse, wheat straw, banana stalk, municipal paper waste, and finally apple pomace, respectively. Based on the results presented in Fig. 1, the orange peel waste, as the best yielded growth substrate, was selected for further product optimization. It has been reported in literature that low-cost substrates like wheat flour, wheat bran, rice straws, and molasses are suitably effective for growth and enzyme production (Shampa *et al.* 2009; Iqbal *et al.* 2011a). Ojumu *et al.* (2004) reported high cellulase activity using 3% pretreated saw dust, bagasse, and corncob as substrates.



(AP, Apple Pomace; MP, Municipal Paper; BS, Banana Stalk; WS, Wheat Straw; SB, Sugarcane Bagasse; OP, Orange Peel)

Fig. 1. Cellulase complex activities on different agro-industrial and municipal wastes materials

Optimization of Culture Conditions

Based on the results of the substrate screening trial, orange peel waste was selected for further optimization of the SSF production process. The optimization of

various growth factors caused an increase in enzymatic activities; therefore, in this study the aim was to investigate the effects of such growth parameters in order to achieve optimized enzyme production.

Optimization of fermentation time period

To investigate the incubation time effect, pretreated orange peel was incubated for 10 days at room temperature (30 ^{o}C). The maximum activity of each individual enzyme, endoglucanase (398 U/mL), exoglucanase (205 U/mL), and β-glucosidase (365 U/mL), occurred after the 4th day of inoculation with *T. viride* fresh spores (Fig. 2), while beyond this time period, feed-back inhibition of each enzyme was observed. In an earlier study, Quiroz-Castañeda *et al.* (2009) reported that the maximum activity of the cellulases (CMCase) occurred after 8 days of fermentation with wheat straw as a growth substrate. In 4 days, *T. viride* produced higher levels of cellulase activity without any additional nutrient supplements in comparison to different fungi previously studied, which produced significantly lower maximum activities after 6 and 8 days (Quiroz-Castañeda *et al.* 2009).

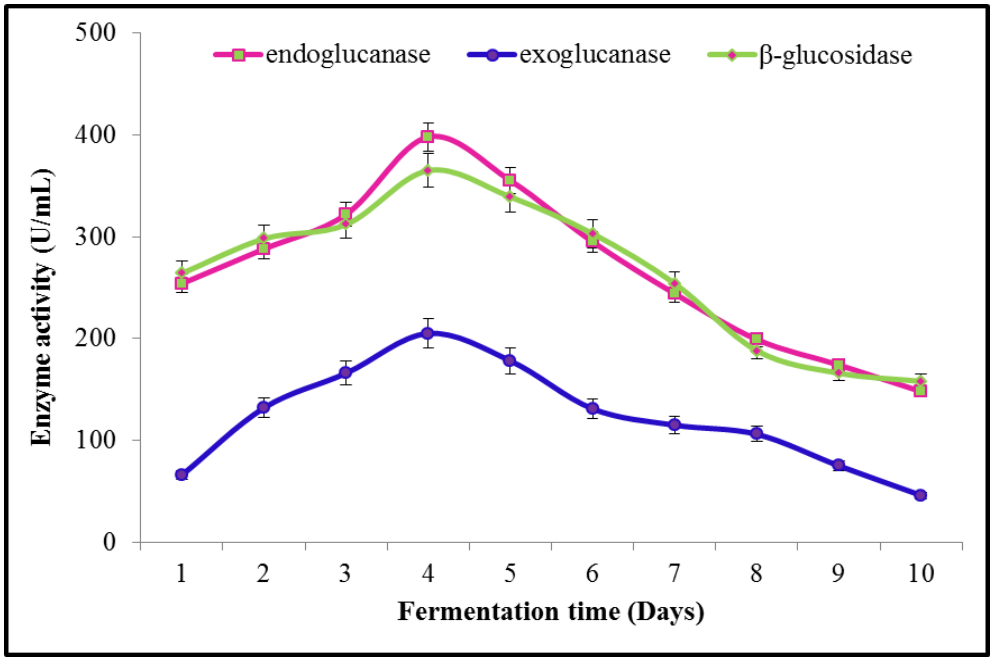


Fig. 2. Cellulase complex activities under different incubation time period

Optimization of Initial pH

The effect of the initial fermentation medium pH on enzyme production was determined, and the maximum endoglucanase (435 U/mL), exoglucanase (233 U/mL), and β-glucosidase (392 U/mL) activities were recovered at pH 5 (Fig. 3). Further increase or decrease in pH from that optimum level retarded the enzyme activity. It has been reported that the optimal pH for cellulase varies from species to species and has a broad range between 3 and 9 (Ishfaq *et al.* 2011). Similar results are also reported by Pushalkar *et al.* (1995), who found that β-glucosidase was more active on the substrate at pH 5.8 and between 4.0 and 5.5.

Optimization of temperature

The temperature of the fermentation medium is one of the vital factors that have a deep influence on the end product. Figure 4 illustrates that the enzyme activity increased with the initial increase in temperature to 35 ºC. When cultivated at temperatures higher than 35 °C, the activities of the enzymes decreased substantially, possibly because of the inhibition factor. Similar to our findings, an incubation temperature of 30 ºC was optimal for the production of CMCase from *Trichoderma harzianum* (Iqbal *et al.* 2010), whereas the maximum production of cellulase by *Trichoderma* sp. was recorded on apple pomace under SSF at 32 ºC by Sun *et al.* (2010).

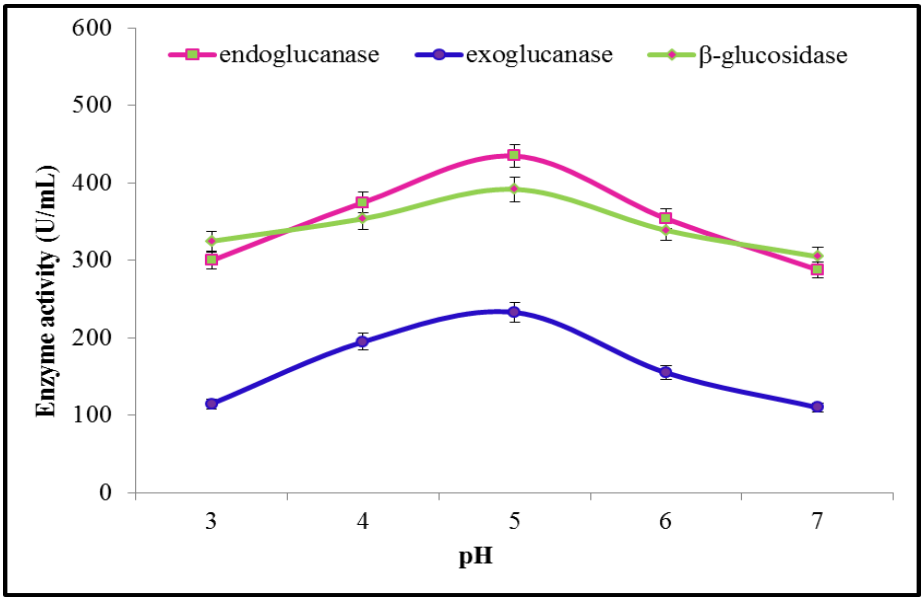


Fig. 3. Cellulase complex activities at different pH levels

Optimization of substrate concentration

As shown in Fig. 5, different concentrations of substrate ranging from 5 to 25 g/100mL were used, and the maximum cellulase (endoglucanase, 512 U/mL; exoglucanase, 288 U/mL; and β-glucosidase, 435 U/mL) activities were obtained at 15 g substrate concentration. Substrate concentration is a dynamic influencing feature that affects the product yield and the initial hydrolysis rate of cellulose (Iqbal *et al.* 2010). A low substrate concentration results in an increase in yield and reaction rate of the hydrolysis, while a high substrate concentration can cause substrate inhibition, which substantially lowers enzyme formation (Liu and Yang 2007).

Optimization of inoculum size

The maximal endoglucanase (655 ± 5.5 U/mL), exoglucanase (412 ± 4.3 U/mL), and β-glucosidase (515 ± 3.7 U/mL) activities were noted at the 5 mL inoculum size, while any further increase in inoculum size showed a decline in enzyme activity (Fig. 6). Optimum fungal spore density (the number of spores per unit weight of substrate) is an important consideration for SSF process. Lower inoculum sizes shortened the early lag

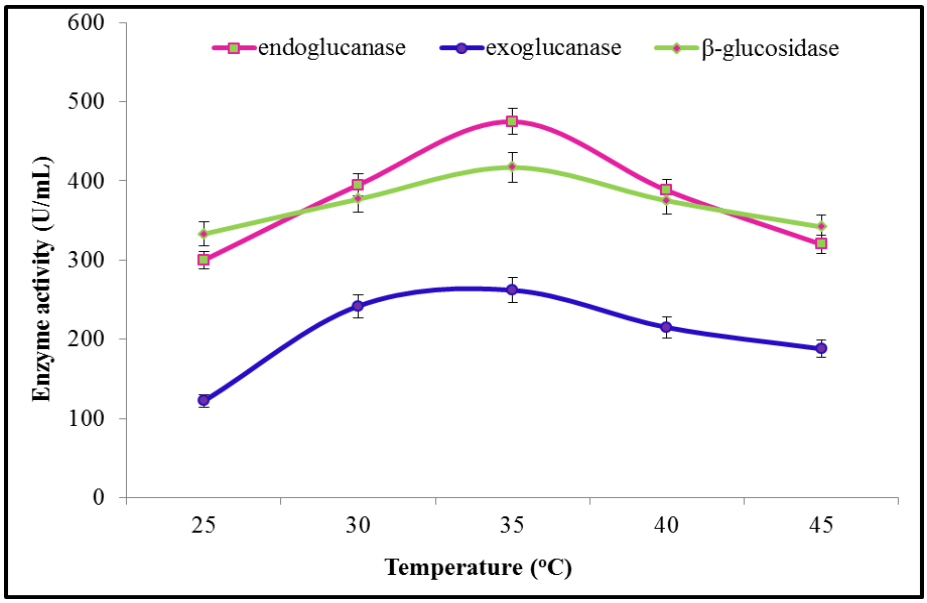


Fig. 4. Cellulase complex activities at varying temperatures

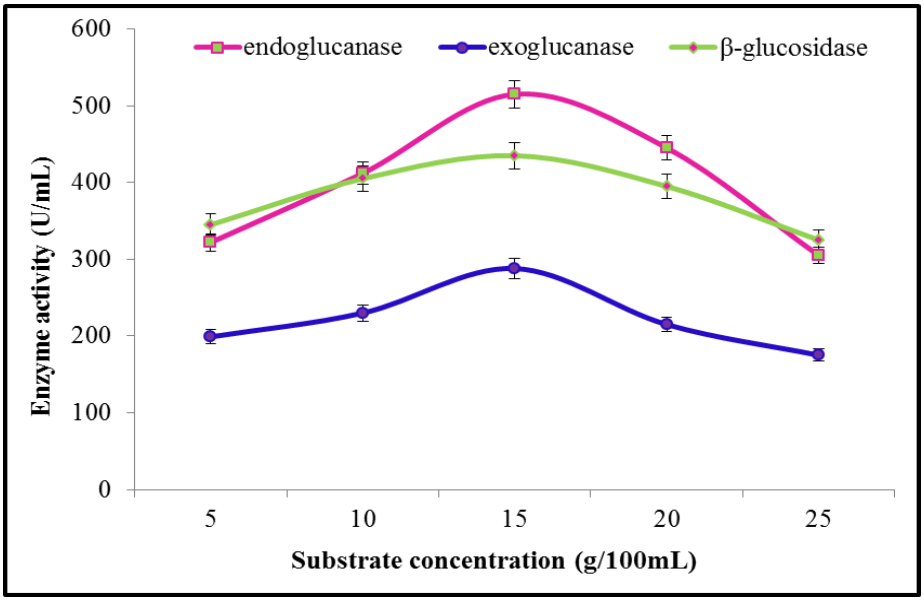


Fig. 5. Cellulase complex activities on different substrate levels

phase, whereas larger inoculum sizes increased the moisture content to a considerable extent and caused lower levels of enzyme formation. Fadel (2000) reported the maximum enzyme activity (216.2 IU/g) at an inoculum size of 10% with wheat straw as the substrate. Omojasola and Jilani (2009) reported the maximum cellulase activity with an inoculum size of 8%.

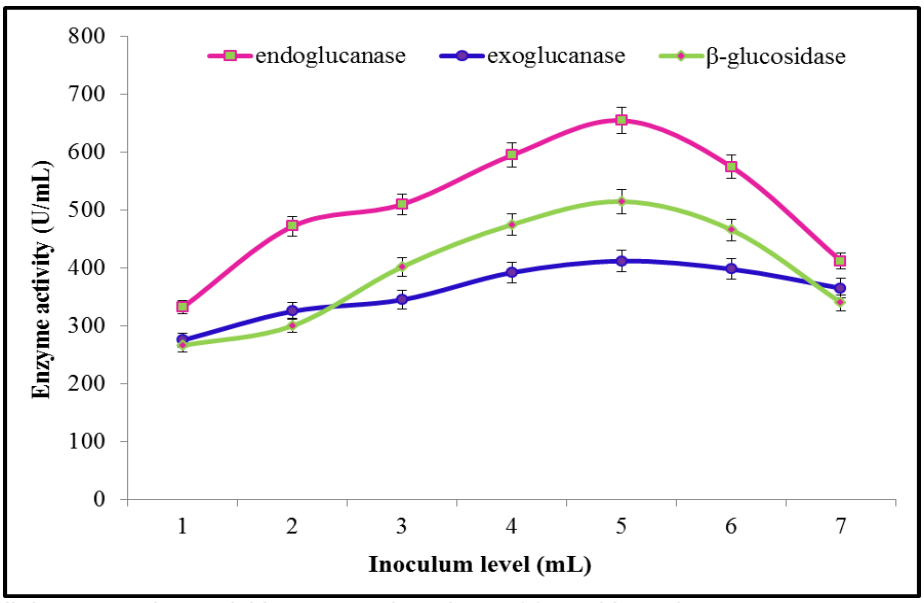


Fig. 6. Cellulase complex activities on varying sizes of fungal inoculum

Purification of Endoglucanase

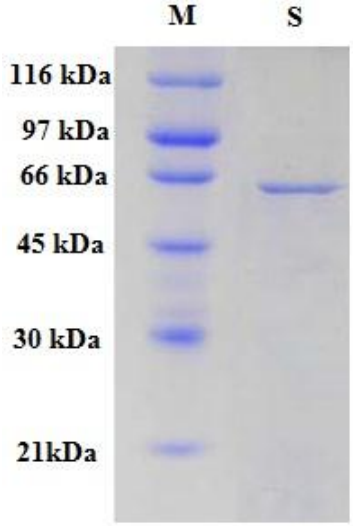
The separated crude supernatant solution containing endoglucanase unit activity and specific activity of 131000 U/200 mL and 91 U/mg, respectively, was subjected to purification. The crude enzyme was maximally precipitated at 80% ammonium sulfate saturation with specific activity and purification fold of 98 U/mg and 1.1, respectively. The optimally active fraction was loaded on a Sephadex G-100 column, and after gel filtration the enzyme was purified up to 5.5 fold with specific activity of 498 U/mg (Table 1). Previously, we have successfully developed and reported the Sephadex G-100 column gel filtration technique for the purification of various fungal enzymes, laccase, MnP, cellulases, and protease (Iqbal *et al.* 2011b; Ahmed *et al.* 2011; Asgher and Iqbal 2011; Asgher *et al.* 2012). Xue *et al.* (2008) also used the Sephadex-G-100 gel filtration chromatographic technique to purify β-glucosidase from *R. flaviceps*.

Table 1. Purification Summary of Endoglucanase Produced from *T. viride* Under Optimum Fermentation Conditions

Purification Steps	Total Volume (mL)	Enzyme Activity (IU)	Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
Crude Enzyme	200	131000	1440	91	1	100
$(NH_4)_2SO_4$ Precipitation	30	20550	210	98	1.1	15.7
Ultrafiltration	22	16170	105	154	1.7	12.3
Sephadex-G-100	11	8965	18	498	5.5	6.8

SDS-PAGE

The purified active fraction obtained from the gel filtration column was further purified to homogeneity and resolved on a 5% stacking and 12% running gel. *T. viride* endoglucanase was found to be a homogenous monomeric protein, as evidenced by the single band corresponding to 58 kDa on SDS-PAGE, which is within the range of the cellulase family (Fig. 7). In the literature, the endoglucanase from *Trichoderma* sp. is reported as a single band with different molecular weights based on the genetic information of the species (Quiroz-Castañeda *et al.* 2009).



[(Lane M, Molecular weights in kDa of standard marker (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa); lane S, Purified endoglucanase (58kDa)]

Fig. 7. Molecular mass determination of purified endoglucanase by SDS-PAGE

Industrial Application

Storage stability/shelf life

The effects of storage on the activity of crude (free) and purified endoglucanase were determined via incubation at room temperature (30 °C) for 60 days, and the results obtained are presented in Fig. 8. The residual activity profile revealed that the purified enzyme was stable in its activity at room temperature for up to 45 days, whereas the free enzyme started losing its activity rapidly within 15 to 30 days. This suggests that the enzyme in its purified form may be stored for up to 45 days at 30 °C without much loss in its activity.

Saccharification and glucose fermentation

Pretreatment refers to the complete or partial degradation of agricultural biomass in order to expose cellulose polymers for convenient cellulose hydrolysis into sugars by cellulase enzyme action. The 2% HCl-treated residues were hydrolyzed using cellulase extract from *T. viride*, and the quantity of sugars released was determined. After hydrolysis of the pretreated residue with cellulase for 24 h at its optimum temperature (35 ^{o}C), the cellulose was converted into a concomitant release of 45.8 g/dL glucose. The maximum glucose release was observed from sugarcane bagasse (45.8 g/dL), followed by

wheat straw (35.9 g/dL), banana stalk (32.3 g/dL), municipal paper waste (30.9 g/dL), and apple pomace (28.8 g/dL), respectively. Cellulose can be converted into bio-fuel by a multistep process that includes pre-treatment, enzymatic hydrolysis, and fermentation (Yeh *et al.* 2010; Xiao *et al.* 2011).

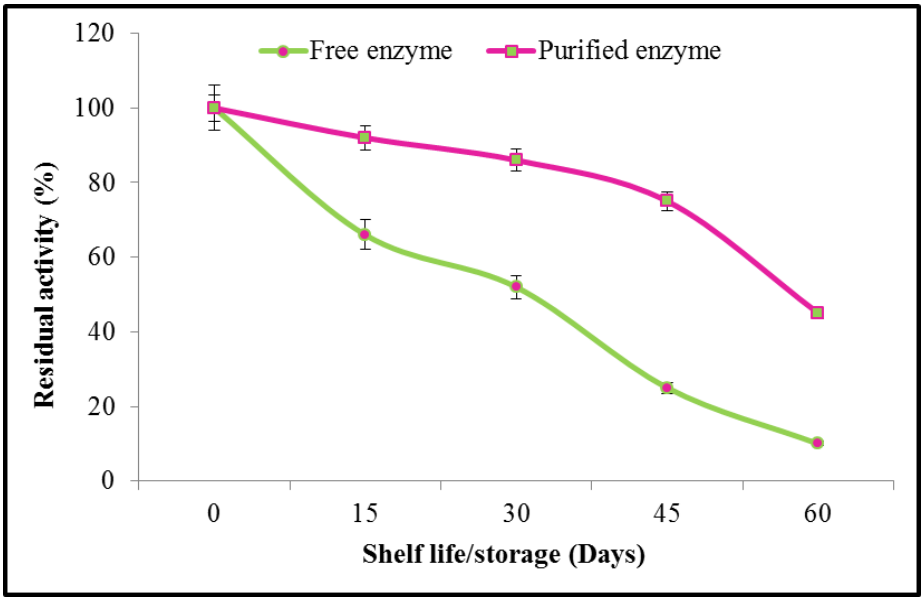


Fig. 8. Shelf life/storage of purified endoglucanase

CONCLUSIONS

1. In summary, the present proposed approach should be a superior strategy for proper management of agro-based materials.
2. The results of this study indicate the remarkable enzyme production potential of *T. viride* when using agro-industrial residue orange peel waste as a substrate.
3. The enzymatic treatment of waste biomass could be of particular interest, since it seems to be an eco-friendly approach to carrying out waste biomass treatment.

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