

# Detergent-Compatible Purified Endoglucanase from the Agro-Industrial Residue by *Trichoderma harzianum* under Solid State Fermentation

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A robust process of purification, characterization, and application of endoglucanase from the agro-industrial waste was performed using solid state fermentation (SSF). *Trichoderma harzianum* as a micro-organism and wheat straw as a growth supportive substrate were used in SSF under pre-optimized conditions. The maximum activity of  $480 \pm 4.22$  U/mL of endoglucanase was attained when a fermentation medium was inoculated using 10% inoculum size and 3% substrate concentration with pH = 5.5 at 35 °C for an optimized fermentation period. In comparison with crude extract, enzyme was 1.83-fold purified with a specific activity of 101.05 U/mg using Sephadex-G-100 column chromatography. Sodium dodecyl sulfate (SDS) poly-acrylamide gel electrophoresis revealed that the enzyme exhibited a low molecular weight of 43 kDa. The purified enzyme displayed maximum activity at pH = 6 and a temperature of 50 °C, respectively. The maximum activity ( $V_{max}$ ) of 156 U/mL and  $KM$  value of 63  $\mu$ M were observed. Ethylenediaminetetraacetic acid (EDTA), SDS, and  $Hg^{2+}$  inhibited enzyme activity, while  $Co^{2+}$  and  $Mn^{2+}$  enhanced enzyme activity at 1 mM concentration. The maximum substrate affinity and specific activity of biosynthesized endoglucanase revealed that it can be potentially useful for industrial applications.

**Keywords:** *Trichoderma harzianum*; Endoglucanase; Wheat straw; Solid state fermentation

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## INTRODUCTION

The major constituents in the cell wall of plants are lignin, cellulose, and hemicellulose. The most predominant polysaccharide found in plants is cellulose, which comprises 35% to 50% of all plant materials (Lynd *et al.* 1999). Cellulose is produced by terrestrial plants and marine algae (Teeri 1997). Among polysaccharides, the annual production of cellulose is approximately  $4 \times 10^9$  tons, and is extremely consistent, including a linear biopolymer of anhydroglucose units comprised of  $\beta$ -1, 4-linked glycosyl, a unique residue (Coughlan 1990; Yin *et al.* 2010). The crystalline structure of cellulose is an essential and unique feature and is moderately rare in polysaccharides (Brown and Saxena 2000). The formation of cellulose fibers constitutes approximately 30 entities of cellulose molecules in each subunit assembled to produce large units known as micro-fibrils. Ultimately, these micro-fibrils assemble to make long cellulose fibers (Koyama *et al.* 1997; Kroon-Batenburg and Kroon 1997).

The synthesis of enzymes such as protease, oxidoreductase, esterase, pectinase, cellulase, and hemicellulase has been carried out using various micro-organisms with high

capabilities, including *Penicillium*, *Trichoderma*, *Fusarium*, and *Aspergillus* (Iqbal *et al.* 2010; Ahmed *et al.* 2011; Irshad *et al.* 2012; Ahmed *et al.* 2015). The synthesis of these enzymes requires favorable growth conditions (Farinas *et al.* 2010) to transform insoluble polysaccharides into solvable oligomers, and ultimately into monomers (Beukes and Pletschke 2006; Phitsuwan *et al.* 2010). The main aim has been to produce cellulases from roughage such as corncobs, rice and wheat straw, wood, wheat bran, bagasse, corn stover, rice husk, and other agro-processing waste products (Brijwani *et al.* 2010). Industrial waste products in developing countries have become a problem creating environmental effluence, and have not been fully utilized (Dashtban *et al.* 2009).

Synergistically, a cellulose-degrading enzyme system contains three enzyme types that work together to break cellulose down into glucose and extra monosaccharides (Gori and Malana 2010). These three enzymes work in collaboration and can be divided into the following: 1) glucoside glucohydrolases, 2) exoglucanases, and 3) endoglucanase (Brijwani *et al.* 2010; Singhanian *et al.* 2010). The cellulase enzyme complex is responsible for converting cellulose into oligosaccharides and ultimately into glucose subunits (Gori and Malana 2010). Currently, cellulase is extensively used in biotechnological research, particularly as an animal feed intake enhancer, to increase digestibility in juice extraction, as a detergent enzyme, and in the agricultural industry (Nagendran *et al.* 2009; Singhanian *et al.* 2010; Sun *et al.* 2010; Yin *et al.* 2010; Iqbal *et al.* 2013).

To determine the factors responsible for the synthesis, production, purification, and physicochemical properties of the endoglucanase, it is necessary to purify and describe endoglucanase, using kinetic studies. For this purpose, the investigation and optimization of pH, temperature, substrate concentration, and inhibitor/enhancers are essential. So far, there is none or few reports available on detergent compatibility features, although a huge amount of research investigations has already been documented on cellulases, particularly endoglucanase production and characterization from a wider spectrum of fungal and bacterial strains. However, to the best of our knowledge, the detergent compatibility and de-staining characteristics of this indigenous endoglucanase from *T. harzianum* are described for the first time, in this study. The aim of the present study was to purify endoglucanase from *T. harzianum* and explore various factors for existing possible and potential application for different industrial products particularly as an effective additive for the detergent industry.

## EXPERIMENTAL

### Materials and Methods

#### *Chemicals and substrate*

All the utilized chemicals were of analytical grade. A lignocellulosic agro-industrial residue (wheat straw) was collected from agriculture research farms from the University of Agriculture, Faisalabad (UAF) in Pakistan. The wheat straw was dried, powdered into 40 mm mesh size, and kept in polyethylene bags to prevent the development of moisture.

#### *Micro-organism and inoculum development*

The fungal culture of *T. harzianum* for endoglucanase production was obtained from the enzyme biotechnology laboratory (EBL) of the Department of Biochemistry, University of Agriculture, Faisalabad. The appropriate inoculation conditions were

developed by growing *T. harzianum* in Vogel's nutrient medium (Vogel 1956), supplemented with trace elements for extraordinary growth. The trace elements solution was prepared using 5.0 g of  $C_6H_8O_7 \cdot H_2O$ , 1.0 g of  $Fe(NH_4)_2 \cdot 6H_2O$ , 5.0 g of  $ZnSO_4 \cdot 7H_2O$ , 50 mg of  $MnSO_4 \cdot H_2O$ , 250.0 mg of  $CuSO_4 \cdot 5H_2O$ , 50.0 mg of  $Na_2MoO_4 \cdot 2H_2O$ , 50.0 mg of  $H_3PO_3$ , and up to 100 mL of  $dH_2O$  (Aslam *et al.* 2010). The medium was sterilized at 121 °C and 15.0 lbs/inch<sup>2</sup> for 15 min. Afterward, the media was cooled down and loopful spores of *T. harzianum* were transported under hygienic conditions. To obtain efficient and accurate results, the inoculated flask was left for five days on an orbital shaker at 30 °C and 180 rpm.

#### *Production and extraction of endoglucanase*

*T. harzianum* was used under optimized fermentation conditions (2% HCl pretreated wheat straw; a temperature of 35 °C; pH = 5.5; a moisture content of 40%; an inoculum size of 10%; a substrate concentration of 3%, and a fermentation time period of 7 days) to yield endoglucanase. After the stipulated fermentation time, endoglucanase was isolated by adding a citrate buffer (0.05 M of pH 4.8) (Iqbal *et al.* 2010). The extracted contents were filtered and treated with citrate buffer thrice. The collected filtrate was centrifuged at  $10,000 \times g$  (4 °C) for 15 min, and the carefully obtained supernatant was used to determine enzyme activity for purification purposes.

#### *Enzyme activity and protein contents*

The enzyme activity of the collected supernatants was measured using the method of Iqbal *et al.* (2011). The Bradford (1976) assay was applied to determine the protein contents of the crude and purified enzyme using bovine serum albumin (BSA) as a standard.

### **Purification Parameters of Endoglucanase**

#### *Partial purification*

The maximum clarity of *T. harzianum*-produced crude extract was achieved after centrifugation ( $10,000 \times g$  for 15 min at 4 °C). Briefly, the crude enzyme was subjected to ammonium sulfate precipitation overnight at 4 °C to attain 50% saturation, and was subsequently centrifuged at  $10,000 \times g$  for 15 min at 4 °C to collect the resultant precipitate. Following that, the obtained pellets were disposed of, and the supernatant was subjected to ammonium sulfate precipitation overnight at 4 °C to achieve 80% saturation at 4 °C overnight, followed by centrifugation as previously described. The obtained pellets were thawed in a 0.2 M Tris-HCl buffer of pH 8, and dialysis was carried out against  $dH_2O$  to eliminate the ammonium sulfate after certain changes of water. As described earlier, the enzyme activity and protein contents were measured before and after dialysis. The desalted endoglucanase was carried out for additional purification studies (Ahmed *et al.* 2015).

#### *Gel filtration chromatography*

Gel filtration chromatography was carried out for the further purification of desalted endoglucanase. A Sephadex-G 100 (Sigma-Aldrich, USA) with specifications of 120-cm height and 2.0-cm interior diameter (Sharma *et al.* 2006) was used. Twenty fractions measuring 1 mL each were collected at a flow rate of  $0.5 \text{ mL min}^{-1}$  and analyzed for protein content and the determination of enzyme activity using the methodology of Iqbal *et al.* (2011) and Bradford (1976).

### *SDS–PAGE for the determination of molecular weight*

The molecular weight of purified endoglucanase was determined using the sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS–PAGE) technique of Laemmli (1970). The standard protein marker (21 to 116 kDA) was purchased from Sigma (USA) to compare the molecular weights of endoglucanase after the purification steps.

### **Characterization of Purified Endoglucanase**

The characterization of the purified endoglucanase enzyme was performed using kinetic studies. The effect of various factors such as pH range (4 to 6), incubation temperature (30 to 60 °C), different concentrations (100 to 1000 µM) of purified endoglucanase enzyme as a substrate, and activators and inhibitors (EDTA, SDS, Co<sup>2+</sup>, Mn<sup>2+</sup>, and Hg<sup>2+</sup>) on *T. harzianum*-produced endoglucanase was studied.

The effect of pH was investigated using different buffers ((sodium phosphate, pH 7 and 8), (0.2 M citrate phosphate, pH 4 to 6), (carbonate buffer, pH 9 and 10)) on purified endoglucanase. The effect of different incubation temperature (30 to 60 °C) on the activity of purified endoglucanase enzyme was investigated. The former enzyme assay was applied after an incubation period (15 min) of purified endoglucanase under controlled temperature conditions.

To determine the Michaelis-Menten kinetic constants  $K_M$  and  $V_{max}$ , various concentrations ranging from 100 to 1000 µM of purified endoglucanase enzyme was used. The obtained results for enzyme activities were plotted as a graph, and the y-axis represented enzyme activity (U/mL), while the x-axis represented the concentration of the substrate. Different activators and inhibitors (100 µL) were incubated at 50 °C with extracted purified endoglucanase enzyme for 15 min and subjected to assay protocol using carboxymethyl cellulose as a substrate. The standard assay conditions were applied to observe the enzyme activities of each sample, the purified endoglucanase enzyme was taken as a substrate and incubated for 15 minutes, and the absorbency was measured on a spectrophotometer at a wavelength of 540 nm.

### **Industrial Application**

#### *Detergent compatibility of endoglucanase*

Four different locally produced detergent powders (Ariel, Bonus, Surf Excel, and Wheel) were purchased and used in normal settings to study the compatibility efficacy towards endoglucanase. Different quantities of detergents as prescribed on the sachets were used for the solution preparation. Purified enzyme solution (0.5%) was prepared in a phosphate buffer solution of pH 6 and used as a substrate. A reaction mixture containing detergent solution (1.10 mL), substrate solution (3.0 mL), and endoglucanase enzyme (0.9 mL) was incubated for 15 min at 50 °C. Enzyme assay was carried out after the incubation period as mentioned previously. The control group contained substrate and detergent solution only to compare the mixture solution.

#### *De-staining ability of endoglucanase*

Two pieces (10 × 10 cm) of white cloth were stained with locally available permanent blue ink. The Color Index System of the ink used was mainly comprised on copper phthalocyanine (as a colorant pigment with molecular formula and weight as C<sub>32</sub>H<sub>16</sub>CuN<sub>8</sub> and 576.069 g/mol, respectively). Both pieces were then dipped into purified enzyme-supplemented detergent solution and detergent solution without enzymes. The de-

staining ability of purified endoglucanase was observed after the incubation period (10 to 15 min) at 50 °C and after being washed twice with water.

### Statistical Analysis

All experimental data were evaluated statistically and the results were elaborated as mean  $\pm$  standard error (S.E.) (Steel *et al.* 1997).

## RESULTS AND DISCUSSION

### Production of Endoglucanase

*T. harzianum* was cultivated under optimized fermentation conditions, and the medium was supplemented with 2% HCl pretreated growth supportive substrate wheat straw. A maximum endoglucanase activity of  $480 \pm 4.22$  U/mL was obtained when the substrate was inoculated using 10% inoculum size and 3% substrate concentration with pH 5.5 at 35 °C for the stipulated fermentation time period (Iqbal *et al.* 2010). Particle size is considered to be the most critical factor among other growth factors, and greatly influenced the growth and enzyme yields of micro-organisms (Zadrazil and Puniya 1995). The previous literature illustrates that some low-cost agro-industrial substrates (molasses, rice straw, wheat bran, and flour) have a significant effect on production and maximally enhance enzyme yield (Mehta *et al.* 2006; Sen *et al.* 2009). Ojumu *et al.* (2003) found that saw dust, corn cob, and bagasse agro substrates treated with 3% HCl increase cellulase activity at a maximum level.

It has been revealed that the hydrolysis rate and maximum yield of cellulose depends on substrate concentration (Raghavarao *et al.* 2003). Inoculum size affects endoglucanase and enzyme activity; and Omojasola and Jilani (2009) found maximum activity with 8% inoculum size. The early lag phase was influenced by the size of the inoculum; a smaller inoculum size reduced the lag phase, while a larger size lowered the production of endoglucanase enzyme by increasing the moisture content by a substantial amount (Swelim *et al.* 2010).

### Extraction and Purification of Endoglucanase

After the stipulated fermentation period, 0.05 M of a citrate buffer of pH 4.8 was added to the crude endoglucanase extract from the fermented biomass. The extracted contents were washed three times with citrate buffer after filtration. The filtrate was subjected to centrifugation at  $10,000 \times g$  for 10 to 15 min at 4 °C, and a crude enzyme containing a clear supernatant was achieved. The extracted clear supernatant showed the endoglucanase activity of 96,000 U/200 mL and a specific activity of 55.17 U/mg. For further purification, the supernatant was subjected to ammonium sulfate precipitation in two different fractions.

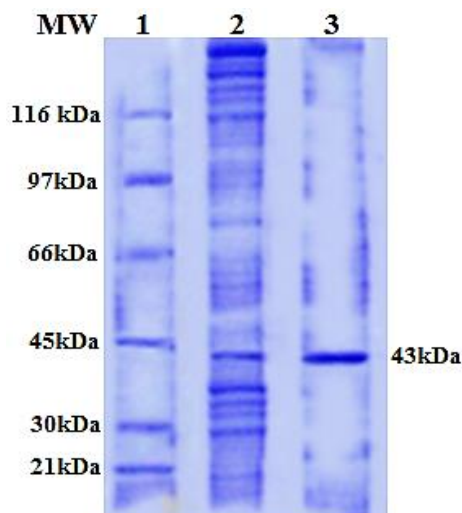
The specific activity (59.32 U/mg) and 1.07-fold purification was found after precipitation of 80% saturation of crude endoglucanase enzyme. The removal of extra salt was performed by dissolving the precipitates in 0.2 M Tris-HCl buffer of pH 8 and dialyzed against dH<sub>2</sub>O four times every 6 h. The known volume of the partially purified endoglucanase for further purification was loaded on a Sephadex-G-100 column for gel filtration chromatography. The specific activity of 101.05 U/mg and 1.83-fold purification was achieved with a yield of 2.00% after gel filtration chromatography (Table 1).

**Table 1.** Purification Summary of Endoglucanase Produced from *T. harzianum* under Optimum Fermentation Conditions

Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Enzyme	200	96,000	1740	55.17	1.00	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitation	30	13,050	220	59.32	1.07	13.59
Dialysis	25	7875	115	68.45	1.24	8.20
Sephadex-G-100	12	1920	19	101.05	1.83	2.00

### SDS-PAGE

The molecular weight of purified endoglucanase enzyme was determined using SDS-PAGE containing 12% resolving and 5% stacking gel, and an evident standardized monomeric protein of 43 kDa was found on SDS-PAGE when compared with the molecular weight marker (Fig. 1). The previous reports regarding *Trichoderma* sp.-produced endoglucanase showed a single band of molecular weight on the gel ranging between approximately 25 and 50 kDa (Quiroz-Castaneda *et al.* 2009). The present study identified that *T. harzianum* with a molecular weight of 43 kDa and just one subunit of endoglucanase enzyme was found on SDS-PAGE. In comparison with the other fungal species produced, the endoglucanase enzyme showed the same range of molecular weight, including *Trichoderma viride* (38 to 58 kDa) (Irshad *et al.* 2012) and *Aspergillus* sp. (31.2 kDa) (Olama *et al.* 1993). It has also been illustrated that different species including *A. saitoi*, *T. viride*, and *Aspergillus* produced endoglucanase enzyme containing one subunit (Olama *et al.* 1993; Irshad *et al.* 2012).



**Fig. 1.** Molecular mass determination of purified endoglucanase produced by SDS-PAGE (Lane MW=standard molecular weights marker; Lane 1=standard protein markers (116 kDa  $\beta$ -Galactosidase; 97 kDa Phosphorylase B; 66 kDa albumin; 45 kDa ovalbumin; 30 kDa carbonic anhydrase; and 21 kDa trypsin inhibitor); Lane 2= endoglucanase crude extract; Lane 3=Purified endoglucanase (43 kDa))

### Effect of pH and Temperature on Endoglucanase Activity

The results indicated that the purified endoglucanase was entirely stable within the range of pH 5 to 8. At a pH of 6, the purified enzyme showed a maximum activity of 195 U/mL, while *Mucor circinelloides* at a pH of 4.0 to 7.0 (Saha 2004) and *Bacillus circulans* at a pH of 4.5 to 7.0 (Kim 1995) showed less enzymatic activity, whereas further increases in pH of 6 showed a decreasing trend in the activity of endoglucanase. The optimum temperature was found to be 50 °C for purified endoglucanase. Figure 3 depicts the effect of temperature on enzyme activity. The increase in temperature from 50 °C caused a rapid loss of enzyme activity. For a variety of commercial applications, thermal stability at high temperatures and specific characteristics can increase the attractiveness of an enzyme (Beg and Gupta 2003; Joo *et al.* 2003; Haddar *et al.* 2009).

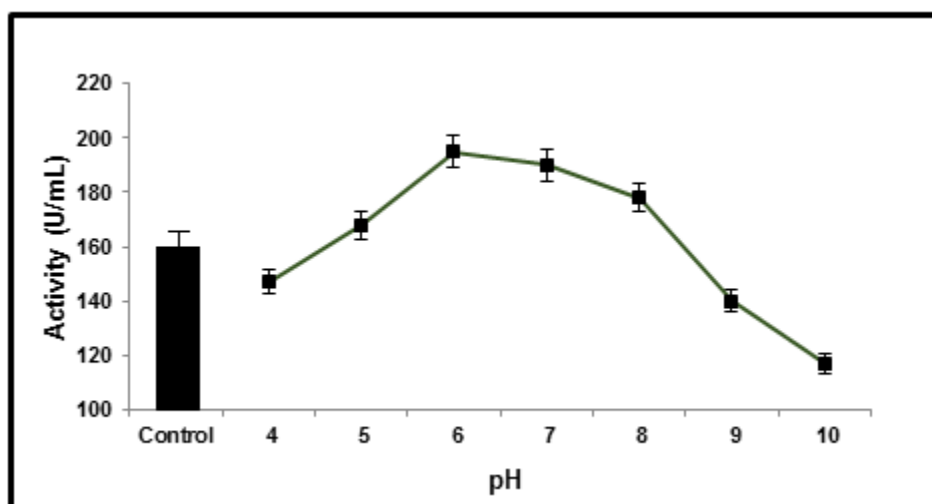


Fig. 2. Effect of varying pH values on purified endoglucanase activity

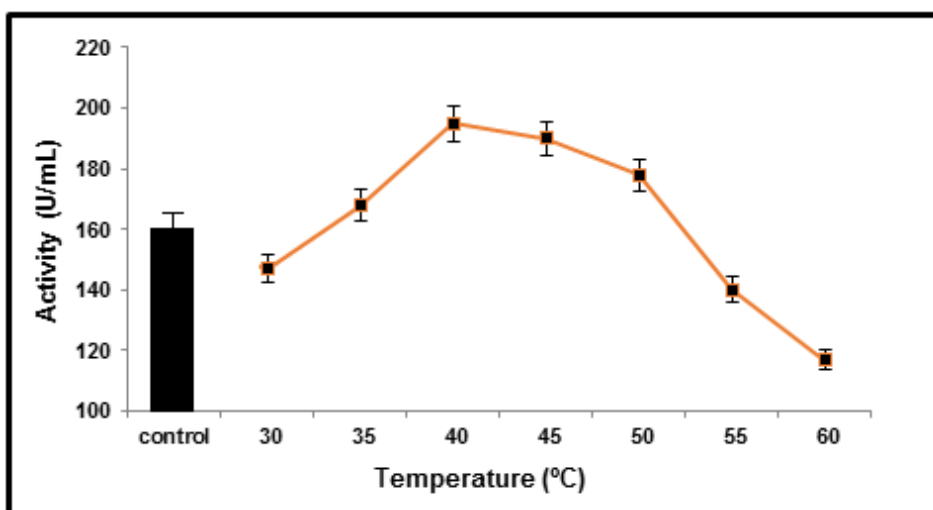
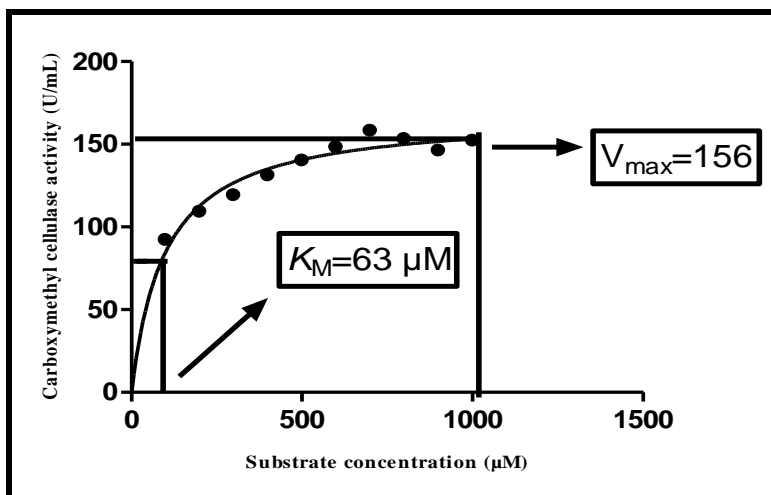


Fig. 3. Effect of different temperatures on purified endoglucanase activity

### Effect of Substrate Concentration: Determination of $K_M$ and $V_{max}$

A hyperbolic curve was obtained with  $K_M$  and  $V_{max}$  values, as shown in Fig. 4. The purified endoglucanase produced from *T. harzianum* indicated the catalytic values of  $K_M$  (63  $\mu$ M) and  $V_{max}$  (156 U/mL), respectively. An enzyme with low  $K_M$  has a greater affinity

for its substrate. Previous studies reported that different fungal species have various ranges of  $K_M$  and  $V_{max}$ . Ekperigin (2007) found that *Branhamella* and *A. anitratus* species can be used at values of 0.32 and 2.54 mM as a substrate for cellobiose, and at values of 4.97 and 7.90 mg/mL for CMC substrate using the same species. *Pseudomonas fluorescens* showed a  $K_M$  value of 3.6 mg/mL and *Trichoderma reesei* 1.1 mM, as stated by Bakare *et al.* (2005) and Cascalheira and Queiroz (1999), respectively. The  $K_M$  value reported in the present study for endoglucanase obtained from *T. harzianum* was lower than the value obtained for *Branhamella* sp. and showed a higher affinity for its substrate, whereas it was only slightly higher than that reported for *A. anitratus*.



**Fig. 4.** Determination of  $K_M$  and  $V_{max}$  for purified endoglucanase through Michaelis-Menten kinetics

### Effect of Various Activators and Inhibitors

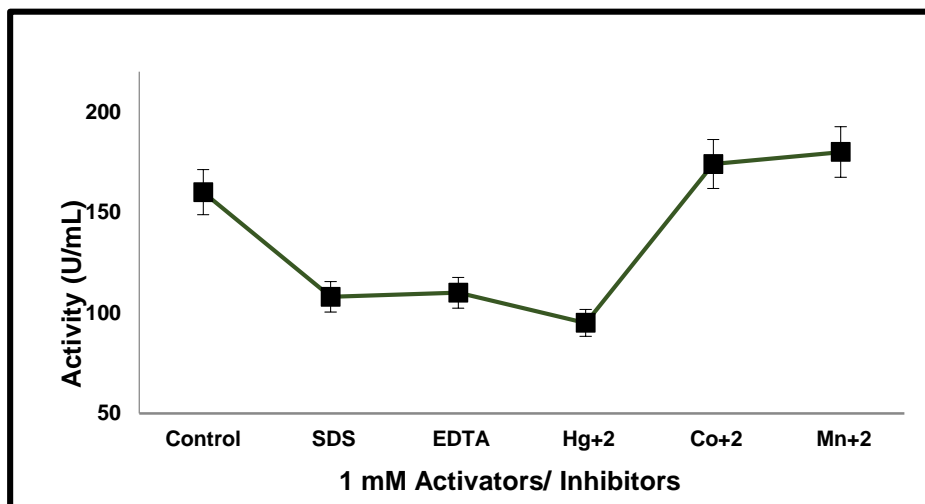
Figure 5 illustrates the inhibition and activation of various metal compounds. Ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate, and mercury ( $Hg^{2+}$ ) inhibited the activity of purified endoglucanase, while  $Co^{2+}$  and  $Mn^{2+}$  enhanced enzyme activity when compared with the control. The cellulase enzyme produced from *Pseudomonas fluorescens* showed an inhibitory effect on enzyme activities when incubated with EDTA (Bakare *et al.* 2005). Our results have high similarity to those found for *Catharanthus roseus* (Sanwal 1999). Saha (2004) and Lucas *et al.* (2001) reported that the activities of enzymes produced by *Mucor circinelloides* and *Chalara paradoxa* were greatly enhanced when incubated with  $Co^{2+}$  and  $Mn^{2+}$ .

### Commercial Application

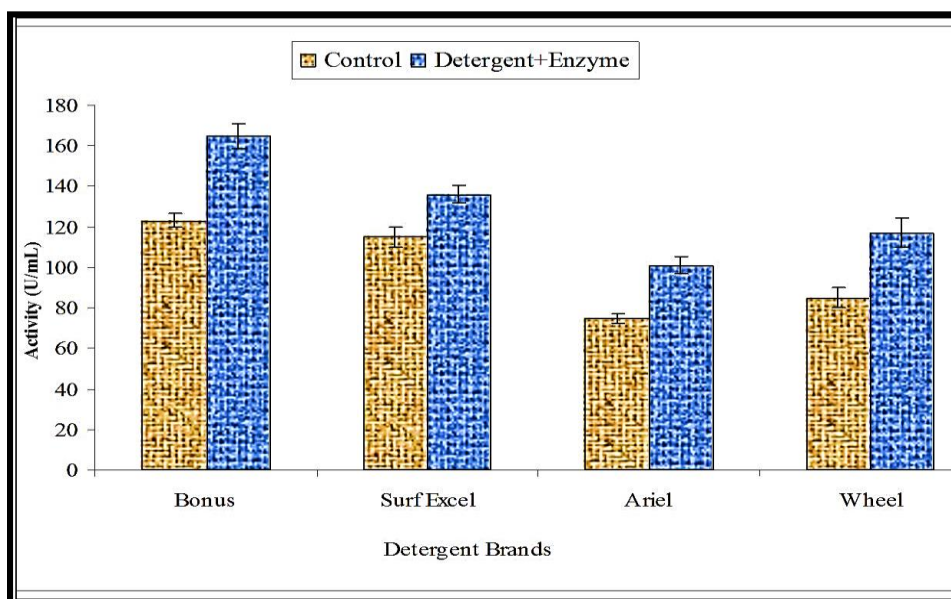
#### *Detergent compatibility*

The purified enzyme was used for de-staining a permanent ink-stain on white cloth. A detergent solution of locally available detergents was mixed with extracted purified enzyme and incubated at 50 °C to test the detergent compatibility. Bonus and Surf Excel revealed a maximum compatibility at 50 °C (Fig. 6). The control sample showed very low values of enzyme activity compared with the endoglucanase-appended solution. The results obtained validate the compatibility of endoglucanase enzyme with detergents and suggest its possible applications in the detergent industry.





**Fig. 5.** Effect of various activators and inhibitors on purified endoglucanase activity



**Fig. 6.** Detergent compatibility of purified endoglucanase with local detergent brands

#### *De-staining ability of endoglucanase*

The incubation of the enzyme at 50 °C with detergent solutions revealed its maximum compatibility. The cloth containing the ink-stain was dipped into the mixed solution, while one piece of ink-stained cloth was dipped in the detergent-only solution. Figure 7 shows that the enzyme-mixed detergent solution completely removed the ink-stain present on the white cloth, while the detergent-only solution left the mark. It was also observed that the addition of endoglucanase improved the fabric's quality by finishing and reducing dullness, as compared with detergent solution without endoglucanase supplement. This suggests that endoglucanase may be useful to the detergent and laundry industries as a suitable additive to detergents for improved washing and maintenance of the fabric quality.



**Fig. 7.** De-staining ability of purified endoglucanase: Sample 1 was treated with detergent-only solution, presenting yellowish ink stain retained on it, while Sample 2 was treated with detergent solution with the addition of purified endoglucanase enzyme, displaying the complete elimination of the ink stain as compared with the control sample.

## CONCLUSIONS

1. Purified endoglucanase revealed its maximum activity at  $\text{pH} = 6$  and a temperature of  $50\text{ }^{\circ}\text{C}$ , and possessed a molecular weight of 43 kDa. The maximum enzyme activity ( $V_{\text{max}}$ ) and  $K_M$  values were observed to be 156 U/mL and 63  $\mu\text{M}$ , respectively.
2. The *T. harzianum* endoglucanase exhibited the highest substrate affinity and specific activity. Detergent compatibility enhanced the washing maintenance of the fabric quality; therefore, it can be concluded that it can be useful for industrial purposes, and particularly for the detergent industry.

## ACKNOWLEDGEMENTS

Muhammad Azhar Hussain and Muhammad Tahir Naveed are thankfully acknowledged for providing technical expertise and collaborative help for the present study.

## CONFLICT OF INTEREST STATEMENT

The authors are happy to declare that we do not have any conflict of interest in any capacity.

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Article submitted: January 22, 2016; Peer review completed: Peer review completed: May 9, 2016; Revised version received: June 3, 2016; Published: June 15, 2016.  
DOI: 10.15376/biores.11.3.6393-6406