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PURIFICATION OF β-GLUCOSIDASE PRODUCED FROM *Trichoderma viride* USING COW DUNG AS CARBON SOURCE

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ABSTRACT
β-glucosidases have characteristics of biotechnological interest and have thus become important industrial enzymes.In this study, β-glucosidase produced by *Trichoderma viride* from cow dung was subjected to a three step purification process involving ammonium sulphate precipitation, gel filtration by Sephadex G-100 and ion exchange chromatography by DEAE-Sephadex A-25. The elution profile on Sephadex G-100 resulted in a single broad peak (fractions 9-21) which had a yield of 3.7% and a purification fold of 4.29 with a specific activity of 25.70 µmol/min/mg proteins while the elution profile on DEAE-Sephadex A-25 resulted in a single broad peak (fraction 8-14) which had a yield of 2.76% and a purification fold of 22.14 with a specific activity of 132.41µmol/min/mg of protein. The purified enzyme was obtained as a single band and had a molecular mass of 51.8 kDa on SDS-PAGE. This results provide support for further studies of this enzyme towards revealing its potential biotechnological applications.

Keywords: β-glucosidase, Purification, Cow dung, Enzyme activity *Trichoderma viride.*

INTRODUCTION

β-glucosidase (EC 3.2.1.21) is a component of cellulase complex and has a synergistic action on the degradation of cellulose with endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) (Del pozo *et al.*, 2012). The highest proportion of municipal and plant wastes are made up of celluloses and represent a major source of raw materials and renewable energy (Jatinder *et al.*,2007). Some of the industrial applications that employ the use β-glucosidase include biofuel production, cassava detoxification, food and feed processing (Obilie *et al.*, 2004; Coughlan, 1985) Cellulases are currently the third largest industrial enzymes being sold at commercial level worldwide. The global enzyme market was expected to have reached $4.4 billion by 2015 and the highest sales of enzymes occurred in the leather market, followed by the bioethanol market (Binod *et al.*, 2013). Due to the expanding applications of cellulases in bioenergy and other sectors, the market of cellulases is expected to expand to become the largest in the nearest future. β-glucosidase can be produced from bacteria, fungi, plants and animals (Joo *et al.*,2010; Han and Chen, 2008). Many fungal strains secrete higher amounts of β-glucosidase with *Trichoderma* sp. being the most potent. Although, the cellulolytic enzymes from *Trichoderma reesei* have been investigated thoroughly (Saloheimo *et al.*,1997; Arja *et al.*,2004), the quantity of β-glucosidase secreted by *T. reesei* is not a enough for effective conversion of cellulose to glucose (Sorensen *et al.*, 2013). Other filamentous fungi that have been shown to produce β-glucosidase include *Aspergillus oryzae, A. niger, A. carbonarius, A. fumigatus, A. saccharolyticus, Penicillium purpurogenum, Trichoderma koningi*, *T. viride* and *Fusarium solani* (Brumbauer *et al.*, 2000; Tsao *et al.*, 2000; Dhake and Patil, 2005; Zhang *et al.*, 2007; Irshad *et al.*, 2013; Sorensen *et al.*, 2013).

The ability of fungi to grow on surfaces of various substrates and penetrate into their inter-particle spaces makes them one of the best adapted species in the use of agro-residues (Viniegra-Gonzalez and Favela-Torres, 2006). Several lignocellulosic substrates, such as sugarcane bagasse, wheat straw, rice straw, saw dust, corn cobs, corn stover, banana peels, rice husk and orange peel (Iqbal *et al.*,2011a; Iqbal *et al.*,2011b; Irshad *et al.*,2013) have been utilized as potential substrates for β-glucosidase production. The bioprocessing of these low cost feedstocks can help reduce environmental pollution as well as production cost of raw materials.

Cow dung is one of the most abundant and unexploited resource for β-glucosidase production. It contains 35.4 % cellulose, 32.6 % hemicelluloses, 13.3 % ash, and 1.4 % nitrogen (Misra *et al.*, 2003). The nitrogen and carbon ratio in cow dung is an indication that it could be a promising feedstock for culturing microorganisms (Adegunloye *et al.*, 2007). This study was aimed at purifying β-glucosidase produced by *Trichoderma viride.*

MATERIALS AND METHODS

Sample collection

Cow dung was obtained from Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. It was air-dried for seven (7) days at room temperature and finely ground using mortar and pestle to have a uniform particle size of 1 mm. The ground cow dung was stored in air tight containers before further use. *Trichoderma viride* was obtained from the culture stock of Department of Microbiology, Ahmadu Bello University, Zaria.

Microbial inoculum preparation.

Seven-day-old potato dextrose agar slant containing *Trichoderma viride* was used to prepare the inoculum culture. In this study, the spore suspension was prepared by aseptic addition of 10ml of sterile distilled water into the culture slant. Conidial clumps were broken using a sterile glass rod. The tube was shaken to make homogenous mixture of the suspension which was then counted using a hemocytometer, and the inoculum concentration of 1 x 10^6 spores per ml was prepared as described by Irfan *et al.* (2014).

β-glucosidase Production by Solid State Fermentation

Eight grams (8g) of the substrate (cow dung) was transferred into a 250-mL Erlenmeyer flask and the moisture content was maintained at 60 % (w/v). The content was mixed thoroughly and autoclaved at 121 ℃ for 15 mins. After cooling the flask to room temperature, it was inoculated with 5% (w/v) of *T. viride* inoculum under aseptic conditions. The culture was then incubated at 30 ℃ for 7 days, and after incubation, 80 ml of distilled water was added to the fermented substrate. This was placed in an orbital shaker at 150 rpm for 30 min. for enzyme extraction. After this, the mixture was filtered using Muslin cloth and centrifuged at 10,000 x g for 20 min. The supernatant obtained was used to assay for the activity of enzyme (Irfan *et al*, 2014).

β-glucosidase assay

The enzyme activity was assayed using the method of Tomaz and Roche (2002). The reaction mixture consisted of 0.1 ml enzyme solution, 0.2 ml substrate (1 % Salicin) and 0.1 ml citrate buffer (pH 4.8). The tubes containing the mixture were incubated at 50 ℃ for 30 min. Thereafter, 3 ml of Dinitrosalicylic acid (DNS) was added into each tube and then transferred into a water bath set at 100 ^{0}C for 15 min. The tubes were allowed to cool and absorbance was taken at 540 nm. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1 µmol of glucose from the substrate per minute under standard assay conditions. The results are expressed in terms of units per gram of cow dung (U/g).

Protein assay

Protein content of the culture supernatant was assayed according to Bradford method (1976) using bovine serum albumin (BSA) as a standard. To 0.25 ml of culture supernatant, 1.35 ml of distilled water was added and mixed; then 0.4 ml of Bradford reagent was added to the reaction mixture and incubated for 30 min at room temperature. The absorbance was read at 595 nm and the amount of protein in the sample was determined with reference to the protein standard curve.

Partial purification of extracellular β-glucosidase

Ammonium sulphate precipitation

Crude enzyme supernatant was precipitated by adding ammonium sulphate at 90 % saturation level. After addition, the enzyme solution was left for 24 h at 4 ^{0}C and the protein precipitate was collected by centrifugation at 8000 xg for 15 min at 4 ^{0}C and then resuspended in 20 ml of 0.05 M citrate buffer (pH 4.8) to get the concentrated enzyme suspension (Swangkeaw *et al.*, 2009).

Gel filtration chromatography

The concentrated enzyme sample was purified on sephadex G-100 (Sigma, USA) column (1.5 x 50 cm). The sephadex column was equilibrated with 0.05 M citrate buffer, pH 4.8. The enzyme sample was loaded on the sephadex G-100 and eluted with the same buffer. The flow rate was maintained at 0.5 ml/min. Up to 28 fractions were collected each of 2 mL and both the enzyme activity and protein concentration were determined for each fraction. The fractions with higher enzyme activity were pooled together for the next purification step (Bai *et al.*, 2013).

Ion exchange chromatography

The β-glucosidase active fraction obtained following the Gel filteration was loaded onto a DEAE-Sephadex A-25 column (1.5 cm x 50 cm) pre-equilibrated with phosphate buffer (0.05 M, pH 7.0). Elution of column was carried out using stepwise gradient ranging from 0.05 to 1.0 M of sodium chloride (NaCl) in 0.05 M citrate phosphate buffer (pH 7.0) with a flow rate of 0.5 ml/min. β-glucosidase activity and protein concentration were determined by standard assay procedure (Bai *et al*., 2013).

SDS-PAGE for molecular weight determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12 % separating and 5 % stacking gel according to the method of Laemmli (1970) to determine the molecular weight of partially purified β-glucosidase. To 100 µL of protein sample, 50 µL of sample buffer (0.05 % bromophenol blue, 5 % β-mercaptoethanol, 10 % glycerol, and 1 % SDS in 0.25 M Tris– HCl buffer; pH 6.8) was added and boiled in a water bath for 5 min, cooled at room temperature and loaded onto the gel. Electrophoresis was performed at room temperature for 2.5 h at 100 volt. Thereafter, the gel was placed in isopropanol fixing solution [10 %(v/v) acetic acid, 25 % (v/v) isopropanol, 65 % double distilled H_2O]

for 20 minutes followed by washing with three changes of distilled water at every 30 min interval. The protein bands were visualized by staining with Coomassie Brilliant Blue G-250 and destaining was done again with distilled water which was kept overnight at room temperature. The molecular weight of the purified β-glucosidase was determined in comparison with standard protein marker (12- 150 kDa; Sigma, USA).

Partial purification of β-glucosidase
Purification table

The results of the partial purification of β-glucosidase from *T. viride* was presented in Table 1. Crude β-glucosidase had a specific activity of 5.98 µmol/min/mg protein, but when subjected to three purification steps, the specific enzyme activity increased to 132.41 µmol/min/mg protein with a total yield of 2.76 % and purification fold of 22.14 as shown in Table 1.

RESULTS AND DISCUSSION

Table 1: Purification Table of partially purified β-glucosidase from *T.viride*

Purification Step	Total enzyme Activity (µmol/min)	Total Protein Concentration (mg)	Specific Activity (µmol/min/mg protein)	Purification fold	Yield (%)
Crude enzyme	277.80	46.40	5.98	1.00	100
$(NH_4)_2SO_4$ precipitation	119.50	15.92	7.50	1.25	43.01
Sephadex G-100	10.28	0.40	25.70	4.29	3.70
DEAE Sephadex A-25	7.68	0.06	132.41	22.14	2.76

Gel filtration on Sephadex G-100

The elution profile (Figure 1) resulted in single broad peak (fractions 9-21) which had a yield of 3.7% and a purification fold of 4.29 with a specific activity of 25.70 µmol/min/mg protein. These active fractions (9-21) were pooled together for the next purification step.

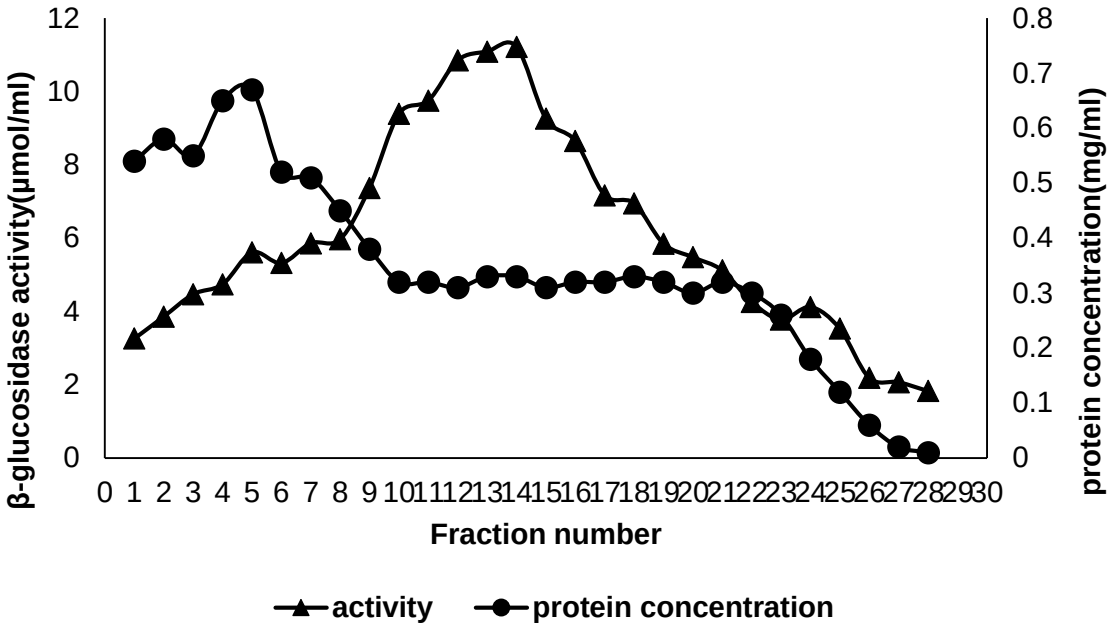

Figure 1: Elution Profile of β-glucosidase from *T.viride* on Sephadex G-100 Column Chromatography (1.5x50cm).The elution profile of gel filtration on sephadex G-100 showed highest β-glucosidase activity at fraction 14 with an enzyme activity of 11.22 µmol/min and protein concentration of 0.33 mg.

Ion Exchange Chromatography on DEAE-Sephadex A-25

Figure 2. Showed the elution profile of β-glucosidase on DEAE sephadex A-25 which resulted in a single broad peak (fraction 8-14). This peak had a yield of 2.76 % and a purification fold of 22.14 with a specific activity of 132.41µmol/min/mg of protein. The seven active fractions (8 – 14) that showed highest β-glucosidase activity were pooled and stored at 4 ^{0}C for further use. From the result of this study, there was an increase in the purification fold from 1 to 22.14 (Table 1), while the specific activity also increased from 5.84 µmol/min/mg to 132.41 µmol/min/mg. The increased purification fold and specific activity of the crude β-glucosidase after the three purification stages could be as a result of removal of other unwanted interacting components of the enzyme (Hiol *et al.*,2000). Auta *et al.* (2016) reported 2.58 purification fold with 16.67 % yield of β-glucosidase form *Aspergillus nidulans* AN 2227; while Kaur *et al*. (2007) reported 4.06 purification fold with 15.89 % yield of β-glucosidase isolated from *Melanocarpus* sp MTCC 3922. Also, Chauve *et al.* (2010) purified β-glucosidase from two fungal species with a yield of 95 % and purification fold of 53 using anion exchange chromatography. Irshad *et al.* (2013) purified an extracellular β-glucosidase from *T. viride* by ammonium sulphate precipitation and Sephadex G-100 gel filtration chromatography and the enzyme was purified 5.1 fold with a yield of 8.1 %.

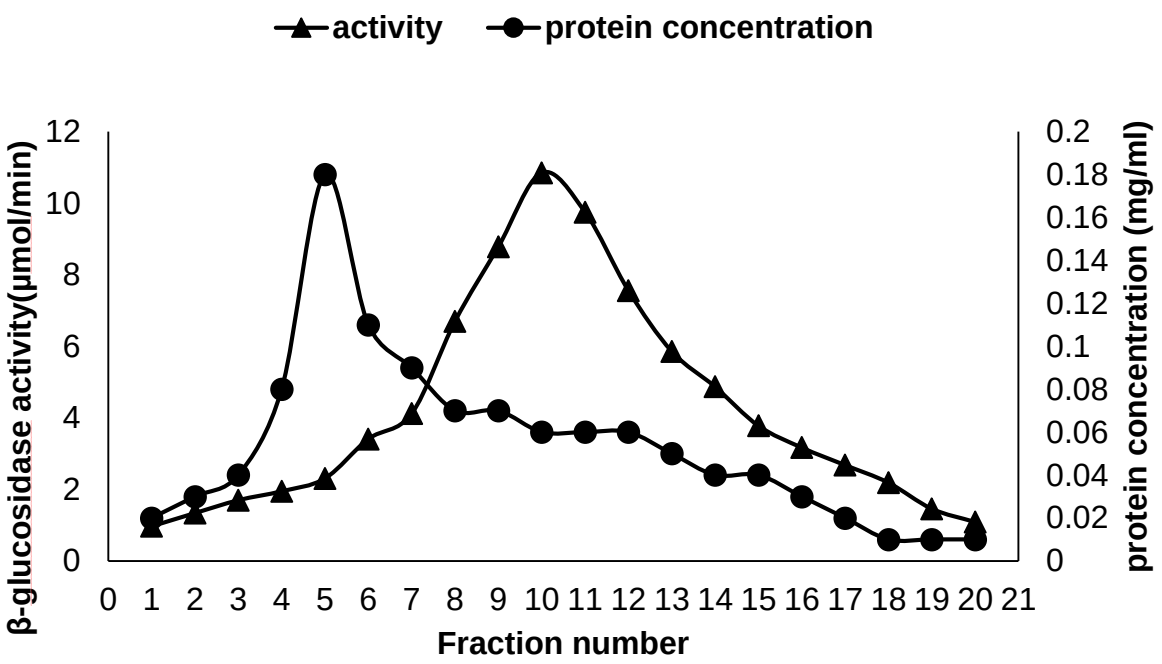

Figure 2: Elution Profile of β-glucosidase from *T. viride* on DEAE Sephadex A – 25 Column Chromatography (1.5x50cm).

Determination of molecular weight of β-glucosidase by SDS-PAGE

Figure 3 indicates that the purified β-glucosidase had a single band corresponding to 51.8 kDa on SDS-PAGE relative to the standard molecular weight markers suggesting the enzyme is a monomer. Irshad *et al.* (2013) reported that β-glucosidase from *T. viride* was purified to homogeneity by SDS-PAGE with a molecular mass of 68 kDa; while Saleem *et al.* (2009) reported the purification of β-glucosidase from *Bacillus* sp. with a molecular mass of 46 kDa as determined by SDS-PAGE. The difference in the molecular weight of β-glucosidase in different organisms could be due to the presence of multiple forms and glycosylation of the enzyme (Narasimha *et al.*, 2016).

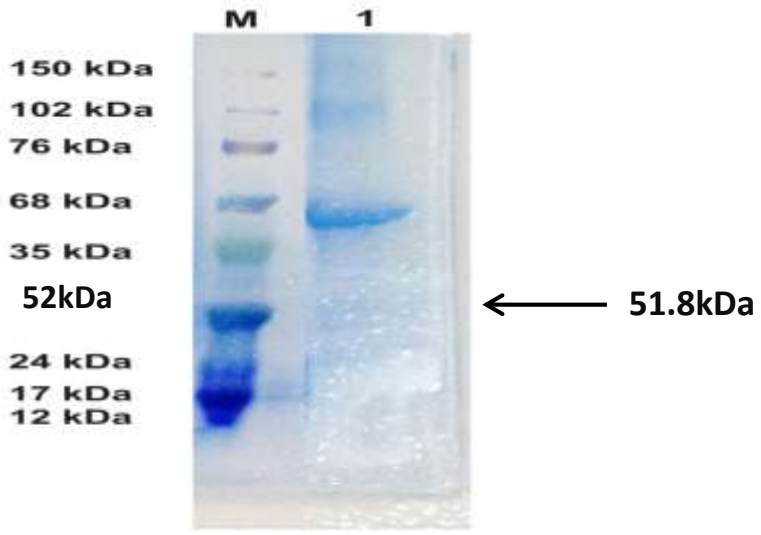

Figure 3: SDS-PAGE of partially purified β-glucosidase from *T. viride;* Lane M, Standard Protein Markers with molecular weights in kDa; Lane 1, purified β-glucosidase

CONCLUSION

The produced β-glucosidase by *Trichoderma viride* was partially purified 22 fold using a 3 step purification process (Ammonium Sulphate Precipitation; Gel-filtration and Ion Exchange chromatography) and had a molecular weight of 51.8 kDa based on SDS-PAGE

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