

# EXPRESSION AND CHARACTERIZATION OF RECOMBINANT $\beta$ -GLUCOSIDASES FROM *ASPERGILLUS NIDULANS* AN2227.

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## ABSTRACT

Recombinant  $\beta$ -glucosidase (EC 3.2.1.21) from *Aspergillus nidulans* AN2227 was expressed using Buffered Methanol Complex Medium (BMMY). Purification was conducted using ammonium sulphate precipitation and anion exchange chromatography on DEAE-Sephadex A-50 column. The enzyme was purified 2.58 fold from the crude extract.  $\beta$ -glucosidase was purified to electrophoretic homogeneity and had a relative molecular weight of 100 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme had an optimum pH and temperature of 6.0 and 40 °C respectively. The most striking characteristics of this enzyme are the dramatically broad pH and temperature profile. The enzyme also had a  $K_m$  of 0.42 mM for 4-Nitrophenyl- $\beta$ -D-glucopyranoside (pNPG). The activity of the enzyme was inhibited by HgCl<sub>2</sub> and slightly activated by CoCl<sub>2</sub>, FeCl<sub>3</sub>, CaCl<sub>2</sub>, FeCl<sub>2</sub> and ZnCl<sub>2</sub> suggesting that the enzyme may not be a metalloprotein and therefore does not require metal ions for optimum activity.

**Keywords:** *Aspergillus nidulans*, Cellulase,  $\beta$ -glucosidase, p-nitrophenol- $\beta$ -glucopyranoside.

## INTRODUCTION

The breakdown of lignocellulosic waste by microbes is usually accomplished by the combined effort of several enzymes, the most outstanding of which are the cellulases produced by a number of microorganisms (Sukumaran *et al.* 2005). Cellulase producing microbes find application in biofuel industries and constitute a major group of industrial enzymes. Cellulolytic microbes generally do not use lipids or proteins as source of energy for growth but primarily degrade carbohydrates and use its product as source of energy (Lynd *et al.* 2002).

The conversion of cellulolytic materials into fermentable sugars requires the action of endoglucanase, exoglucanase and  $\beta$ -glucosidase which work together to hydrolyse cellulose (Del Pozo *et al.* 2012; Zafar *et al.* 2011; Onyike *et al.* 2008). Endoglucanases attack amorphous sites of the cellulose, randomly yielding oligosaccharides of various lengths, while exoglucanases attack crystalline cellulose from both reducing and non-reducing ends generating glucose or cellobiose as major products (Lynd *et al.* 2002). Both endoglucanases and exoglucanases hydrolyse  $\beta$ -1, 4-glycosidic bonds.  $\beta$ -glucosidases finally hydrolyse cellobiose and other oligosaccharides into

glucose.  $\beta$ -glucosidases are essential components of the cellulase system and are important in the complete enzymatic breakdown of cellulose to glucose. The catalysis of cellobiose is important since the accumulation of cellobiose creates feed-back inhibition.  $\beta$ -glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al.* 2002).

Enzyme yield normally depends on factors like temperature, pH, carbon sources and incubation periods (Gautam *et al.* 2011). The pH and temperature of a solution is an important factor that influences enzyme production and activity. For a successful fermentation process, investigation is required to establish the optimum conditions for enzyme production. The enzymatic hydrolysis of cellulose by cellulolytic microorganisms has been documented as an attainable substitute for the conversion of lignocellulosic material into biofuel (Lynd *et al.* 2005; Lowe *et al.* 1987). Although fungi are the main cellulase enzyme producing microorganisms, bacteria also produce cellulase activity (Tomme *et al.* 1988).

Fungal and bacterial strains possessing cellulose degrading enzymes have been isolated and screened for many years (Ram *et al.* 2014). Examples of some fungi that have been studied for  $\beta$ -glucosidase production include *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus saccharolyticus*, *Trichoderma koningii*, *Trichoderma reesei*, *Neosartorya sicheri* and *Fusarium solani* (Sorensen *et al.* 2013). *Trichoderma reesei* has strong cellulose degrading properties and its cellulase systems have been widely studied (Tiwari *et al.* 2013). A number of bacteria such as *Bacillus subtilis* (Bagudo *et al.* 2014) and some *Clostridium* species (Menendez *et al.* 2015) have also been reported for their cellulolytic activities. Previous research has shown that the cost of enzyme production is associated with the specific cellulase producing microbial strains (Chahal *et al.* 1992, Duff and Murray 1996, Reczey *et al.* 1996, Omojasola and Jilani 2008).

Bauer *et al.* (2006) have already reported the successful cloning and expression of a number of plant and fungal cellulases using a *Pichia pastoris* expression system. Bauer *et al.* (2006) reported the production of five putative  $\beta$ -glucosidase enzymes from *A. nidulans* with the accession numbers AN227, AN2612, AN0712,

AN1551 and AN1804. The recombinant *Pichia* clones of these accessions carrying the  $\beta$ -glucosidase genes from *A. nidulans* are deposited in the Fungal Genetic Stock Centre (FGSC) USA. In this study, the clones were obtained and the recombinant  $\beta$ -glucosidase production was studied.

Protein purification involves the concentration of the protein, separation of the different proteins using chromatographic techniques and performing SDS-PAGE analysis to verify the presence of the protein (Ward and Swiatek 2009). Several researchers have successfully produced and purified  $\beta$ -glucosidases from fungal and bacterial sources using *Pichia pastoris* (Kaur *et al.* 2007; Meko'o *et al.* 2012; Bauer *et al.* 2006; Chen *et al.* 2011; Chang *et al.* 2012; Sorensen *et al.* 2013). Similarly, Chen *et al.* (2011) expressed a 76 kDa  $\beta$ -glucosidase from *Trichoderma reesei* using *Pichia pastoris*. Under the control of methanol-inducible alcohol oxidase (AOX) promoter and using *Saccharomyces cerevisiae* secretory signal peptide ( $\alpha$ -factor), the recombinant  $\beta$ -glucosidase was expressed and secreted into the culture medium. A hetero karyon 28, derived through protoplast fusion between *Aspergillus nidulans* and *Aspergillus tubingensis* (Da18), has been subjected to cyclic mutagenesis by Kaur *et al.* (2014) followed by selection on increasing levels of 2-deoxy glucose (2-DG) as selection marker. The derived deregulated cellulase hyper producing mutant 64, when compared to fusant 28, produced 7.8 folds  $\beta$ -glucosidase under shake cultures.

The goal of this work is focused on the expression and characterisation of recombinant  $\beta$ -glucosidases from *A. nidulans* AN2227.

## MATERIALS

Bovine Serum Albumin (BSA), p-Nitrophenol (pNP), p-Nitrophenol  $\beta$ -D glucopyranoside (pNPG), Ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), DEAE-Sephadex A-50 and Bradford Reagent were all obtained from Sigma-Aldrich United Kingdom. Dipotassium hydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>), Potassium phosphate (K<sub>3</sub>PO<sub>4</sub>), Disodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) and Sodium di- hydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>) were purchased from Acros organics, UK. Methanol was supplied by Fisher Scientific, Loughborough UK. Clones were obtained from the Fungal Genetics Stock Centre (FGSC) USA. Sterling™ rapid silver stain was obtained from Geneflow UK. Buffers and chemicals used are of analytical grade.

## METHODS

*Pichia pastoris* clone carrying *A. nidulans*  $\beta$ -glucosidase gene in pPICZ vectors (*A. nidulans* AN2227) that exhibited satisfactory levels of expression of recombinant  $\beta$ -glucosidase (Bauer *et al.* 2006) were obtained from the Fungal Genetics Stock Centre (FGSC) USA. Yeast Potatoes Dextrose YPD agar (1% yeast extract, 2% peptone, 2% glucose and 15% agar) was prepared according to the *Pichia* expression system manual from Invitrogen and were used to maintained clones which were stored at 4°C. All media were prepared according to the Invitrogen manuals (EasySelect™ *Pichia* Expression Kit user guide Cat. no. K1710-01 and *P. pastoris* Fermentation Process Guidelines, 2010) and were sterilized by autoclave on liquid cycle at 121 °C for 15 minutes.

## Small scale cultivation of *A. nidulans* AN2227 $\beta$ -glucosidase clones

The experiment was carried out to determine protein production yield and activity of the recombinant  $\beta$ -glucosidases which are reported by Bauer *et al.* (2006) as putative  $\beta$ -glucosidase enzymes from *A. nidulans* and are *Pichia* clones using pPICZ expression system. The engineered *A. nidulans* AN2227 was inoculated into 25 ml of BMGY medium in a 250 ml flask. The cell cultures were incubated on an orbital shaker at 30 °C and 250 rpm for 16 - 18 hours to grow in the repressive medium before induction. When the culture concentration reached an OD<sub>600</sub> = 3, the cells were harvested by centrifugation (5000 rpm for 5 minutes) at room temperature. The cell pellet was re-suspended in 90 ml Buffered Methanol Complex Medium (BMMY - Yeast extract 1%, Peptone 2%, 100 mM potassium phosphate (pH 6.0), YNB 1.34%, Biotin 4 x 10<sup>-5</sup>%, Methanol 0.5%) medium until OD<sub>600</sub> = 1 to induce expression. The culture was incubated in an orbital shaker at 150 rpm and 30 °C for 4 days. The growth and induction took place in 1 litre flasks and the cell culture was induced every 24 hour after the start of cultivation with methanol to 0.5% of the total volume of the culture to compensate for methanol loss caused by evaporation from medium and uptake by cells.

Crude enzyme samples (1.5 ml) were withdrawn from the culture broth after 0, 6, 12, 24, 48, 72 and 96 hours and filter sterilized using syringe filter (0.22  $\mu$ m) to separate mycelial cells from culture filtrates.

## Crude enzyme production

The fermentation broth was centrifuged at 8000 rpm for 10 minutes to remove the cells, and the crude  $\beta$ -glucosidase in the culture supernatant was precipitated by adding solid ammonium sulphate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 60% saturation. The solution was kept overnight at 0 – 4 °C. The precipitated proteins were collected by centrifugation at 26000 rpm for 20 minutes. The resultant pellet was re-dissolved in approximately 20 ml of 20 mM phosphate buffer (pH 7.0) and dialyzed against phosphate buffer for 2 days with 3 changes of buffer. The insoluble proteins were removed by centrifugation at 8000 rpm for 10 minutes.

The dialysis tubing (Sigma-Aldrich D-9777) with average flat width of 25 mm was initially treated by washing in running water for 3 – 4 hours to remove glycerin. This was followed by treatment with a 0.3% sodium sulfide solution at 80 °C for 1 minute to remove sulfur compounds. The tube was then washed with hot water (60 °C) for 2 minutes followed by acidification with 0.2% sulfuric acid. Finally, the dialysis tubing was thoroughly washed with hot water to remove the acid following the manufacturer's instructions.

## Enzyme assay

$\beta$ -glucosidase catalyses the hydrolysis of p-nitrophenol- $\beta$ -glucoside to p-nitrophenol and glucose. Under alkaline conditions, the p-nitrophenolate anion absorbs light at 400 - 450 nm. The amount of enzyme present is therefore determined by measuring the amount of p-nitrophenolate anion produced in the reaction.  $\beta$ -Glucosidase was assayed using p-nitrophenyl-  $\beta$ -D-glucopyranoside (pNPG) as substrate as specified in Ghose and Bisaria (1987). Appropriately diluted enzyme samples of 25  $\mu$ l were incubated with 25  $\mu$ l of 10 mM pNPG in citrate buffer (0.05 M, pH 4.8) and 50  $\mu$ l of citrate buffer (0.05 M, pH 4.8) at 40 °C for 15 minutes. The reaction was terminated by adding 100  $\mu$ l of 0.2

M Na<sub>2</sub>CO<sub>3</sub> solution. Appropriate blanks devoid of enzyme or substrate were also run in parallel to the enzyme assay. The colour developed due to liberation of p-Nitrophenol (pNP) was read in a spectrophotometer (Biochrom spec Libra S4. Serial no. – 105773, Biochrom Ltd, Cambridge England) at 405 nm and the amount of pNP liberated was calculated by comparing the reading corrected for blanks against a standard curve generated using varying concentrations of pNP.

One unit of β-glucosidase activity was defined as the amount of enzyme needed to liberate 1 μM of p-Nitrophenol (pNP) per minute under the standard assay conditions.

Each experiment was repeated three times and mean values taken. Standard error of mean (SEM) were calculated using Microsoft excel.

#### Protein estimation

The protein concentration was determined using the Bradford method (Bradford 1976). Five milliliters of the dye reagent was added to 100 μl of appropriately diluted enzyme sample and was incubated for 5 minutes after which the absorbance was measured at 595 nm in a spectrophotometer (Biochrom spec Libra S4. Serial no. – 105773, Biochrom Ltd, Cambridge England). The amount of protein was determined by extrapolating from a bovine serum albumin (BSA) standard curve, constructed by using a solution containing 1 mg/ml BSA. Bradford protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to proteins occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

#### Enzyme purification

##### Anion-exchange chromatography

Five milliliter of the dialyzed sample was loaded onto the DEAE-Sephadex A-50 column equilibrated with 20 mM phosphate buffer (pH 7). The column was eluted with a NaCl (0.05M – 0.75 M) in 20 mM phosphate buffer at pH 7.0 during washing. The activity of β-glucosidase in each fraction was determined. The active fractions were pooled together, concentrated using polyethylene glycol 1500 (BDH) and dialyzed extensively against phosphate buffer. The homogeneity of the purified β-glucosidase was checked by SDS-PAGE as described below.

##### Determination of purity and molecular mass

Purity and molecular mass of the partially purified β-glucosidase was determined by SDS-PAGE using an appropriate molecular mass marker. The gel was stained using Sterling rapid silver stain following the manufacturer's instructions. After staining, photos of the bands were digitally captured.

##### Effect of pH on β-glucosidase activity

The effect of pH on the enzyme activity was studied within the pH 3.0 – 10.0 ranges: 10 mM citrate buffer, pH 3.0 – 6.0; 10 mM phosphate buffer, pH 7.0 – 8.0; 10 mM glycine/NaOH buffer, pH 9.0 – 10.0. β-glucosidase activity was then assayed at each pH level. A graph of the β-glucosidase activity versus pH was drawn to determine optimum pH.

##### Effect of temperature on β-glucosidase activity

The effect of temperature on β-glucosidase activity was followed between 20 and 90°C at the optimum pH established. A graph of β-glucosidase activity versus temperature was plotted to determine optimum temperature.

##### Kinetic Constants (K<sub>m</sub> and V<sub>max</sub>)

The substrate concentration was varied over the range of 0.01 – 0.14 g/ml of pNPG. β-glucosidase activity was conducted using p-nitrophenyl-β-Dglucopyranoside (pNPG) as substrate as described by Ghose and Bisaria (1987). The kinetic constants, K<sub>m</sub> and V<sub>max</sub> for the enzyme were determined from a Lineweaver-Burk plot (double – reciprocal plot).

##### Effect of Cations on β-glucosidase Activity

Exactly 25 μl of the enzyme and 5 μl of 20 mM of the cations (MgCl<sub>2</sub>, CoCl<sub>2</sub>, FeCl<sub>3</sub>, CaCl<sub>2</sub>, FeCl<sub>2</sub> and HgCl<sub>2</sub>) were incubated with 25 μl of pNPG and the enzyme activity was then assayed spectrophotometrically.

## RESULTS

##### Small scale expression of recombinant β-glucosidase

Figure 1 shows the changes in crude protein concentration and time course of β-glucosidase activity in Buffered Methanol Complex Medium (BMMY). β-glucosidase protein concentration increased until it reached optimum of 0.56 mg/ml after 48 hours and the concentration subsequently declined. The activity of the enzyme on p-nitrophenyl-β-Dglucopyranoside (pNPG) as substrate, had an optimum activity of 0.54 μmole/ml/min on the second day (48<sup>th</sup> hour) and subsequently declined (Figure 1)

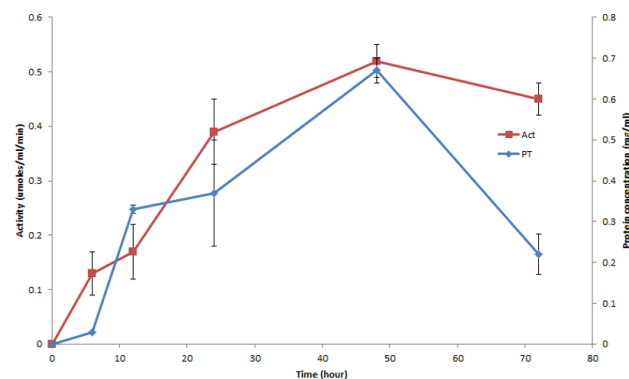


Fig. 1: Time course for β-glucosidase expression and activity by *A. nidulans* AN2227

Act: activity, PT: Total protein. Error bars represent the mean ± SEM (n = 3)

##### Ammonium sulphate precipitation

A pilot run conducted to establish the appropriate percentage of ammonium sulphate saturation to precipitate β-glucosidase enzyme showed that the enzyme suspension was precipitated between 40 – 80%. At this stage of β-glucosidase enzyme purification, a 1.02 fold purification was achieved over the crude extract (Table 1).

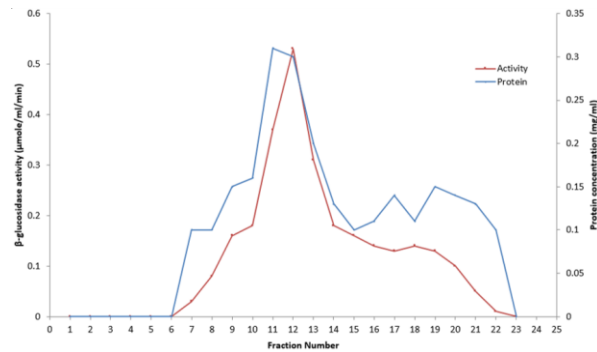
**Table 1:** Purification profile of  $\beta$ -glucosidase

	Total protein (mg)	Total activity ( $\mu$ mole/ml/min)	Specific activity ( $\mu$ mole/ml/min/mg protein)	Purification (fold)	Yield (%)
Culture filtrate (crude)	0.31	0.66	2.13	1	100
$(\text{NH}_4)_2\text{SO}_4$ precipitation	0.17	0.37	2.17	1.02	56.06
Dialysis	0.03	0.12	4.00	1.88	18.18
DEAE-Sephadex A-50	0.02	0.11	5.50	2.58	16.67

After ammonium sulphate precipitation, the  $\beta$ -glucosidases were dialyzed against 20 mM phosphate buffer (pH 7.0) with a 1.88 fold purification over the crude extract.

**Anion-exchange chromatography**

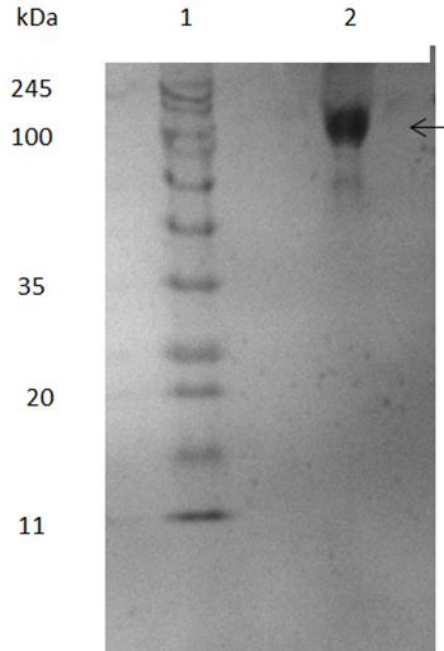
Figure 2 shows the anion exchange chromatography elution profile of  $\beta$ -glucosidase. The elution profile resulted in a single broad peak (fraction 9 – 19). The active fractions (9 – 15) were pulled together and dialysed against the phosphate buffer. The collated fractions produced a final purification of 2.58 fold over the crude extract.



**Fig. 2:** Elution profile of  $\beta$ -glucosidase from DEAE-Sephadex A-50 anion exchange column

**Determination of purity and molecular mass**

The purity and molecular mass of the partially purified  $\beta$ -glucosidases was determined by SDS-PAGE and stained using Sterling rapid silver stain. Figure 3 shows the purified enzyme moved homogenously as a single band on the polyacrylamide gel. The  $\beta$ -glucosidase enzyme moved at the same speed corresponding to an estimated molecular weight of 100 kDa as S6-0024 BLUeye prestained protein ladder (Tris-Glycine 4 – 20%) with an estimated concentration of 300 ng.

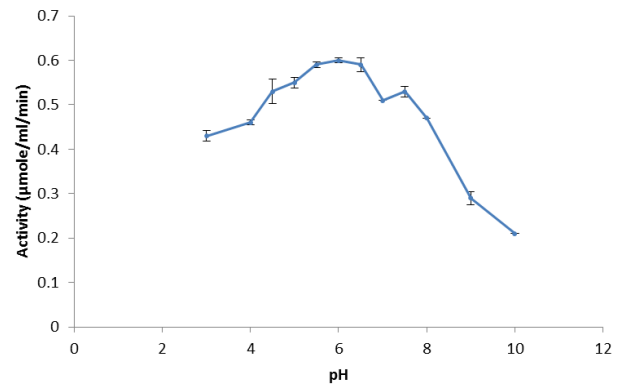


**Fig. 3:** SDS-PAGE of  $\beta$ -glucosidase from *A. nidulans* AN2227.

Lane 1: molecular weight standards. Lane 2: purified enzyme. The gel was stained with Sterling rapid silver stain.

**Effect of pH on  $\beta$ -glucosidase activity**

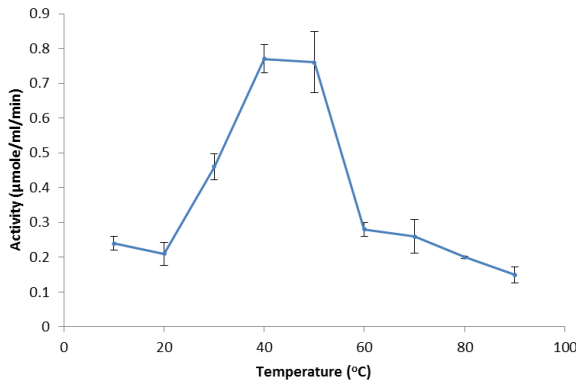
Figure 4 shows the pH profile of  $\beta$ -glucosidase from *A. nidulans* AN2227. The effect of pH on the enzyme activity was studied within the pH 3.0 – 10.0 ranges. The enzyme had strikingly broad pH range and were most active at a pH 6.0. The activity decreased markedly beyond pH 8.0.



**Fig. 4:** Effect of pH on  $\beta$ -glucosidase activity. Error bars represent the mean  $\pm$  SEM (n = 3).

**Effect of temperature on  $\beta$ -glucosidase activity**

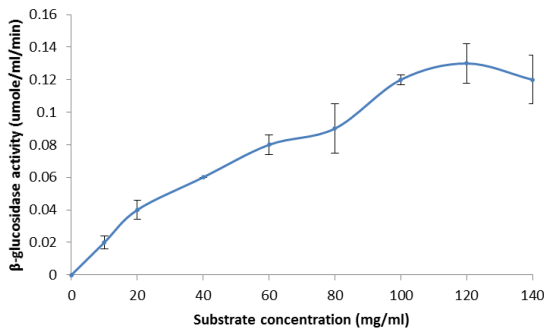
Figure 5 shows the temperature profile of  $\beta$ -glucosidase activity which was followed between 20 and 90°C using the optimum pH of 6.0. The enzyme was optimally active at 40 °C but the activity however dropped sharply above 50 °C.



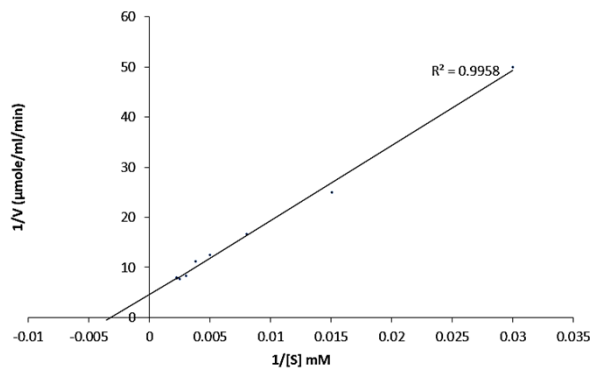
**Fig. 5:** Effect of temperature on β-glucosidase activity  
 Error bars represent the mean ± SEM (n = 3).

**Kinetic Constants ( $K_m$  and  $V_{max}$ )**

The effect of substrate concentration in this study was varied over the range of 0.01 – 0.14 g/ml of pNPG.  $K_m$  and  $V_{max}$  for the hydrolysis of pNPG by β-glucosidases was determined and data analyzed from Lineweaver-Burk plots. The optimum pH of 6.0 and temperature of 40 °C was used for the enzymes. Figure 6 shows the effect of substrate concentration on reaction velocity at constant enzyme concentration and the double reciprocal plot of β-glucosidase activity. The enzyme activity increased with substrate concentration until all the enzyme was saturated at a substrate concentration of 100 mg/ml.



**Fig. 6:** Effect of substrate concentration on β-glucosidase activity  
 Error bars represent the mean ± SEM (n = 3).

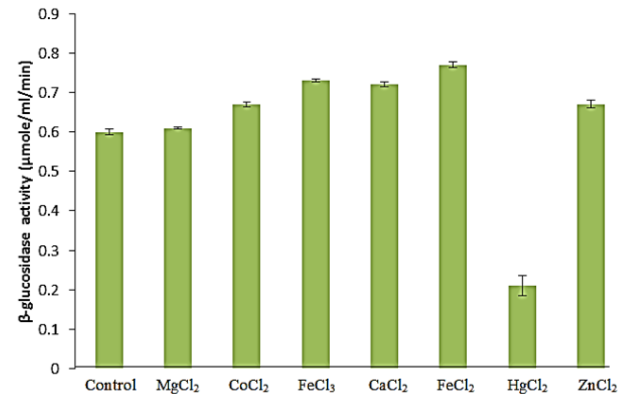


**Fig. 7:** Double reciprocal plot of β-glucosidase activity  
 Points are mean values of triplicate

The curve was characterized by a first order and zero order kinetics with a rectangular hyperbola shape. Figure 7 shows the double reciprocal plot of β-glucosidase activity. The maximum velocity ( $V_{max}$ ) and the Michaelis-Menten constant ( $K_m$ ) of purified β-glucosidase for pNPG were 0.20 µmole/ml/min and 0.42 mM respectively.

**Effect of Cations on β-glucosidase Activity**

In this study, the effect of cations on the purified β-glucosidases was conducted using 20 mM of MgCl<sub>2</sub>, CoCl<sub>2</sub>, FeCl<sub>3</sub>, CaCl<sub>2</sub>, FeCl<sub>2</sub> and HgCl<sub>2</sub>. Figure 8 showed that the enzyme activity was slightly activated by CoCl<sub>2</sub>, FeCl<sub>3</sub>, CaCl<sub>2</sub>, FeCl<sub>2</sub> and ZnCl<sub>2</sub> and deactivated by HgCl<sub>2</sub>. MgCl<sub>2</sub> had minimal effect on the enzyme activities.



**Fig. 8:** Effect of cations on *A. nidulans* AN2227 β-glucosidase  
 Error bars represent the mean ± SEM (n = 3).

**DISCUSSION**

In this study, β-glucosidases were purified from *A. nidulans* AN2227 clone (Bauer *et al.* 2006). The expression of the enzyme was monitored by screening its activity. Although the crude β-glucosidase enzyme produced were found to reach optimum enzyme expression and activity of 0.52 µmole/ml/min, a lag period was observed before the enzyme activity reached the peak value on the second day following methanol induction. The lag period suggests that there was adsorption of the methanol and as the hydrolysis proceeded, a portion of the adsorbed enzymes was gradually released into the reaction supernatant as suggested by Lee *et al.* (1994). β-glucosidases from *A. nidulans* AN2227 was successfully expressed and the enzymes was used for biochemical characterization using pNPG as a substrate.

A purification of 1.02 fold was achieved for β-glucosidase after ammonium sulphate precipitation. This result is expected because ammonium sulphate has been reported to be the best protein precipitant without having effect on the biological activity of the enzyme (Onyike *et al.* 2008). Ammonium sulphate also has high solubility characteristics and changes of temperature have little or no effect on its solubility (Dixon and Webb 1964). Dialysis produced a purification of 1.88. The increase in purification is due to desalting and the removal of low molecular weight compound.

The anion exchange chromatography separation of the enzymes by elution on DEAE-sephadex A-50 columns with linear gradient NaCl (0.05 M – 1.0 M) showed a single broad peak suggesting the presence of a single active protein in the eluate. The purified

enzyme preparation produced the final purification of 2.58 fold with 16.67 % recovery of enzyme activity. Kaur *et al.* (2007) reported 4.06 fold purification with 15.89 % recovery activity of  $\beta$ -glucosidase isolated from *Melanocarpus sp* MTCC 3922. The difference in the purification fold and percentage yield of  $\beta$ -glucosidase activity in this work and that reported by Kaur *et al.* (2007) is probably due to the difference in the source of the enzymes. The overall specific activity of  $\beta$ -glucosidases in this studies was improved from 2.13 to 5.50  $\mu$ mole/ml/min/mg protein indicating that sephadex A-50 column chromatography yielded effectively pure enzyme.

Judged by the SDS-PAGE data (Figure 3), the purified  $\beta$ -glucosidases was homogeneous. The molecular weight of the  $\beta$ -glucosidases was estimated by SDS-PAGE to be 100 kDa. Lima *et al.* (2013) purified a monomeric  $\beta$ -glucosidase from *A. niger* (AnBgl1) with an apparent molecular weight of 116 kDa. The molecular weight of 100 kDa for *A. nidulans* AN2227  $\beta$ -glucosidase correlate with 102 kDa molecular weight reported by Kaur *et al.* (2007) for  $\beta$ -glucosidase from *Melanocarpus sp* MTCC 3922, and the molecular weight of 90 – 91 kDa is also reported (Meko'o *et al.* 2012; Liu *et al.* 2012) for recombinant  $\beta$ -glucosidase from *Pichia pastoris*. Bai *et al.* (2013) also reported a molecular weight of 126 kDa for  $\beta$ -glucosidase isolated from *Penicillium simplicissimum*-11. Though some  $\beta$ -glucosidases have a simple monomeric structures with around 35 kDa molecular weight, others have dimeric structure with molecular weight of 146 kDa or even trimeric structures with over 450 kDa (Mehdi *et al.* 2009).

Previous studies on enzyme production have shown that the initial pH of a medium has an influence in enzyme production and vary from species to species (Shaikh *et al.* 2013; Gautam *et al.* 2011; Chandel *et al.* 2013). The maximum activities of  $\beta$ -glucosidases in this studies was observed at pH 6.0. The enzyme had a broad acidic pH range and were not active in the extreme alkaline pH. The optimal pH of 6.0 for the enzyme is similar to pH 6.0 reported for *A. nidulans* AN1804  $\beta$ -glucosidase (Bauer *et al.* 2006; Liu *et al.* 2012). A pH of 4.4 – 5.2 was also reported for  $\beta$ -glucosidases isolated from *Penicillium simplicissimum*-11 (Bai *et al.* 2013). The pH optimum for  $\beta$ -glucosidase reported in this work is also in agreement with those reported for most fungal sources which are near pH 6.0 (Wei *et al.* 1996; Bhat *et al.* 1993; Bauer *et al.* 2006; Kaur *et al.* 2007). Reports have also shown that maximum activity at an acidic pH is a common property of cellulases from fungal sources (Alam *et al.* 2004).

Temperature also plays an important role in expressing the activity of biological system and has great influence on the production of end product (Chandel *et al.* 2013). The effect of temperature on the activity of the purified  $\beta$ -glucosidases on pNPG was also analyzed in this studies. The maximum activity of  $\beta$ -glucosidase was observed at 40 °C. The lower value of 40 °C for  $\beta$ -glucosidase in this studies agreed with the value of 40 °C reported for  $\beta$ -glucosidase from the thermophilic fungus, *Humicola insolens* (Moreira *et al.* 2010) but lower than that reported for most  $\beta$ -glucosidases from *Aspergillus niger* KCCM 11239 (Chang *et al.* 2012) with an optimum temperature of 70 °C. However, the most striking characteristics of these enzymes are the dramatically broad pH and temperature profiles.

The  $K_m$  is a means of characterizing an enzyme's affinity for a substrate. A low  $K_m$  value means that the enzyme has a high affinity for the substrate, as a little substrate is enough to run the

reaction at half its speed (Kaur *et al.* 2007).  $\beta$ -glucosidase from *A. nidulans* AN2227 had a low  $K_m$  value of 0.42 mM for pNPG indicating higher affinity of the enzyme for pNPG substrate. The  $K_m$  value obtained in this study is higher than the  $K_m$  of 0.117 mM, 0.116 mM, 0.057 mM and 0.3 mM reported for  $\beta$ -glucosidase from *Fomitopsis palustris*, *Humicola insolens*, *Penicillium funiculosum* NCL1 and *Phoma sp* KCTC11825BP respectively (Yoon *et al.* 2008; Choi *et al.* 2011; Ramani *et al.* 2012; Bhatti *et al.* 2013); and it is lower than the  $K_m$  value of 3.11 mM, 1.9 mM, 2.5 mM and 2.67 mM for  $\beta$ -glucosidases isolated from *Aspergillus niger* NRRL 599, *Aspergillus saccharolyticus*, *Aspergillus terreus* NRRL 265 and *Trichoderma koningii* AS3.2774 respectively (Lin *et al.* 1999; Elshafei *et al.* 2011; Zahoor *et al.* 2011; Sorensen *et al.* 2012).

These studies showed that the enzyme activities are slightly enhanced in the presence of almost all the cations tested indicating that  $\beta$ -glucosidase may not be a metalloprotein and therefore does not require metal ions for optimum activity (Pei *et al.* 2012). The slight activation by  $\text{CoCl}_2$ ,  $\text{FeCl}_3$ ,  $\text{CaCl}_2$ ,  $\text{FeCl}_2$  and  $\text{ZnCl}_2$  may be explained by stabilization of the enzyme. Only  $\text{HgCl}_2$  caused 25.93% loss in activity of the enzyme indicating inhibition. This result is in agreement with the report of Meko'o *et al.* (2012), where they reported that 1% EDTA (a chelating agent) did not affect the activity of  $\beta$ -glucosidases expressed from *P. pastoris*, indicating that  $\beta$ -glucosidases are not metalloproteins. The sensitivity of  $\beta$ -glucosidase enzymes to mercuric chloride ( $\text{HgCl}_2$ ) suggests that SH-groups may be involved in the enzyme catalyzed reaction mechanism. This is because heavy metals like  $\text{HgCl}_2$  would selectively react with –SH residues of the enzyme thereby inactivating it (Dixon and Webb 1964; Onyike *et al.* 2008).

## Conclusion

A characterization of *A. nidulans*  $\beta$ -glucosidase expressed from an already sub-cloned pPICZ recombinant plasmid was attempted. To the best of our knowledge, this report is the first on the purification, expression and characterization of  $\beta$ -glucosidase from *A. nidulans* AN2227 obtained from FGSC USA. In this study, an extracellular  $\beta$ -glucosidase enzyme was purified to electrophoretic homogeneity from the crude extract. The characteristics of the recombinant  $\beta$ -glucosidases expressed from *P. pastoris* X33 were also described, with high-level expression. The study suggest that the protein may be present in monomeric form, with the enzyme in particular having a good pH and temperature stability making it a good candidate for cellulose hydrolysis.

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