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Partial purification and characterization of an inducible extracellular β-Glucosidase of *Aspergillus niger* IMI 502691

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Abstract

β-Glucosidase (EC 3.2.1.21) was produced by *Aspergillus niger* IMI 502691 using solid state fermentation of cassava root fibre. The enzyme was partially purified and characterized. The enzyme extracted using 20mM phosphate buffer pH 6.8 was concentrated to 10ml with 5M sucrose solution using dialysis membrane. It was purified from the culture medium by ion exchange chromatography on Carboxymethyl Sepharose and gel filtration chromatography on Biogel P 4. β-Glucosidase was purified 3.17 fold to give 3.68% yield relative to the total activity in the crude extract and with over 300% increase in specific activity of 106.93Umg^{-1} proteins for the partially purified protein. The enzyme exhibited decrease in total protein and total activity of 86.1% and 27.16%, respectively and optimal activity at pH 5.0 and 80°C (1h) in the absence of calcium. The β-glucosidase showed a wide range of pH and temperature of 3.0 to 7.0 and 50°C to 90°C, respectively. The enzyme was most stable at 50°C but retained above 50% of its activity for 1h at 60°C. The β-glucosidase of *Aspergillus niger* IMI 502691 was significantly activated by Sr^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, $Zn^{2+}$and Cu^{2+} tested except for Ca^{2+} which inhibited the enzyme.

Keywords: Glucosidase, chromatography, extract, activity, inhibited

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Introduction

Developing a bio-based economy is a world-wide strategy to address the global challenges of dwindling fossil energy reserve, environmental degradation and pollution associated with oil exploration, prospecting, spillage and climate change in the light of growing world population. Enzyme technology is one of the key technologies to address this complex task helping to produce sustainable raw material and energy supply from biomass while at the same time taking responsibility for the world food supply (Metzger & Huttermann, 2009).

Cellulosic biomass is essential in bio-based energy generation. It is the most abundant, cheap and renewable resource on earth (Zhang et al., 2010) whose natural degradation represents an important part of the carbon cycle within the biosphere (Bhat, 2000). Cellulase occupies a pivotal position in the success of energy generation from bio-based biomass (Arantes & Saddler, 2010). It is an enzyme complex made of exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and glucosidase (EC 3. 2. 1. 21). β- Glucosidase is a unit of the cellulase enzyme complex that acts upon β, 1-4 bonds linking two glucose or glucose-substituted molecules (Brethauer & Wyman, 2010) and

prevent cellobiose inhibition of exoglucanase and endoglucanase (Brethauer & Wyman, 2010). It is well established that cellobiose inhibits the activities of most cellobiohydrolases and endoglucanases (George et al., 2010).

Solid State Fermentation (SSF) is simple (Sreeja et al., 2006) with higher productivity (Machida, 2002). It requires less energy, less water output, less production cost and better product recovery (Priya et al., 2012). Solid State Fermentation solves the problem of environmental pollution caused by agricultural waste (Priya et al., 2012). The merits of SSF over other methods dictate the choice of the technique in this report. It offers natural potential and resembles the natural habitat of microorganisms therefore good for fungal growth (Singhania et al., 2009).

Fungi are important players in the turnover of plant biomass because they produce a broad range of degredative enzymes (Sayali et al., 2012). There are many cellulases but only a fraction has shown high enzymatic activities (Dashtbam et al., 2009). This accounts for the unabated search for efficient cellulases with efficient enzymatic activities. In this report, Aspergillus niger IMI 502691 was used for production of β-glucosidase using solid state fermentation. The enzyme was partially purified and characterized. The method employed in the production of the enzyme would help to reduce the cost of enzyme production and reduce attendant problems associated with disposal of cassava root fibre, an agricultural waste, when implemented on large scale.

Materials and Methods

Isolation and Maintenance of Microbial Inoculant.

Aspergillus niger IMI 502691 was Isolated from the soil sample collected from the cassava processing Agro-enterprise waste dump site in Anyigba, Kogi State. Spread plate technique was used for the isolation of the fungus from the soil sample. Colonies were picked and further purified by streaking. The fungus was identified by Microbial Identification Service, CABI, Europe, as Aspergillus niger IMI 502691. The pure isolate was maintained as spore in sterile potato dextrose agar (PDA, Oxoid) slant and kept at 4°C (Akinyosoye et al., 2004).

Preparation of Inocula

The inocula were prepared by cultivation of the pure isolate on sterile PDA in a Petri dish at room temperature (28°C) for 7 days. The fungal spores were scraped off with sterile cotton bud and suspended in 10ml sterile distilled water containing a drop of tween 80. The resultant spore suspension was standardized after counting with haemocytometre and adjusted with sterile distilled water to approximately 10^7 spores ml^{-1}. The spore suspension (0.5ml) was used as inoculum.

Source and preparation of solid substrate

Cassava root fibre was sourced from the Cassava Processing Agro-enterprise in Anyigba, Kogi State. It was sundried for 4h, and then oven dried at 80°C for 1h (Singh et al., 2010). The dried sample was cooled and ground with clean wooden pestle and mortar. The pulverized cassava root fibre, of mixed particle sizes, was stored in a clean, dry, airtight polyethene bag at room temperature (28°C), until required.

Time course for β- Glucosidase Production

Five grammes of cassava root fibre homogenate were soaked in triplicate 250ml Erlenmeyer flasks each containing 20ml of basal medium made up of % (w/v) $CaCl_2.2H_2O$, 0.05g, $MgSO_4 \cdot 7H_2O$, 0.05g; KH_2PO_4, 0.3g; NH_4NO_3, 0.3g and Tween 20, 0.5% (v/v). The flasks were autoclaved at 121°C for 15min and cooled. The spore suspension (0.5ml) was use to inoculate each of the buffered sterile solid substrate in each Erlenmeyer flask. The inoculated flasks were hand shaken vigorously then incubated at room temperature (28°C) for 5 days in stationary condition. Biomass changes, pH and β-glucosidase activities were monitored daily. Two flasks were harvested per day to determine the monitored parametres.

Production and extraction of β-glucosidase enzyme

Triplicate Erlenmeyer flasks containing the content used for time-course were incubated at 28°C for 5 days. Fifty millilitres of 20mM phosphate buffer (pH 6.8) was added to each flask prepared under enzyme production. The mixtures were shaken for 30min at 28°C and 140 rpm on a rotary shaker. The flasks contents were filtered through a folded, sterile nylon scarf to separate mycelia and spores from the filtrate. The filtrate was further centrifuged at 10,000 x g for 15 min at 4°C using Beckman J2 21 M/E refrigerated centrifuge (Rani et al., 1994). The supernatant was separated and collected as crude enzyme.

Enzyme Assay

The crude enzyme solution (0.2 ml) was added to 0.2 ml of 1% (w/v) salicin in 20 mM phosphate buffer, pH 6.8. A negative control experiment was set up with the same protocol except that the enzyme was replaced with 0.2 ml 20 mM phosphate buffer (pH 6.8). A blank reaction mixture made up of 0.4 ml 20 mM phosphate buffer (pH 6.8) was also set up. Each reaction mixture was incubated in a water bath at 40°C for 10 min. This was followed by the determination of the reducing power of the resulting sugar with the aid of 3, 5 dinitrosalicylic acid (DNSA) reagent (Miller, 1959). DNS acid reagent (0.4 ml) was added to each test tube to stop the reaction. The tubes were allowed to stay in boiling water for 15 min, followed by cooling in running tap water to ambient temperature. The absorbance was read with Spectronic 21 spectrophotometer at 540 nm wavelength. The unit of enzyme activities was defined as those activities shown by an amount of reducing sugar equivalent to 1.0 mg glucose when incubated for 10 min at 40°C with 0.2 ml enzyme substrate dissolved in 20 mM phosphate buffer at pH 6.8.

The net absorbance (A_{540}): value of the enzyme blanks are subtracted from the value for the analyzed sample and value of the substrate (Gusakov et al., 2011).

$$A_{540} = (A_{test} \quad A_{blank}) \quad (A_{control} \quad A_{blank})$$

Where A_{540} = net absorbance

A_{test} = absorbance of test reaction mixture

$A_{control}$ = Absorbance of the control reaction mixture

A_{blank} = Absorbance of the blank reaction mixture

The result was recorded as the mean of triplicate readings + or standard deviation

Estimation of protein concentration

Soluble protein concentration in the culture filtrates were measured by the dye binding method of Bradford (Bradford, 1976) with reference to a standard concentration curve for bovine serum albumin (BSA).

Enzyme purification

All operations were carried out at 4°C.

Concentration of crude enzyme solution.

The culture filtrate (80 ml) of *Aspergillus niger* IMI 502691 was concentrated by dialyzing 6h against three changes of 5 M sucrose solution using dialysis membrane (Sigma). The β-glucosidase activities of the dialysate were determined as described earlier. So also was the protein content.

Ion Exchange Chromatography on CM-Sepharose Fast Flow (FF).

Exchange column was packed with carboxymethyl sepharose resin (1.8 x 16.2 cm) in 0.1 M phosphate buffer (pH 6.8). The resin was equilibrated with 100 ml 0.1 M phosphate buffer, pH 6.8. Dialyzed enzyme solution (7.4 ml) was loaded into the column and eluted with 100 ml 0.1 M phosphate buffer, pH 6.8 followed by linear NaCl gradient from 0.0 - 0.5 M in phosphate buffer. Thirty-five 10 ml fractions were collected at a flow rate of 1.43 ml min^{-1}during the elution. All fractions (1-35) were assayed spectrophotometrically for protein by measuring absorbance at 280 nm with a Biophotometer (Eppendorf). β-Glucosidase activity was equally assayed. Active fractions were pooled together.

Gel Filtration Chromatography on Biogel P4

Gel filtration column was packed with Biogel P4 resin (2.5 x 65 cm) in deionized water. The resin

was equilibrated with 200 ml of 0.1 M phosphate buffer (pH 6.8). The dialysate (4.5 ml) of the positive fractions for β- glucosidase from ion-exchange chromatography was loaded on the gel filtration column. The enzyme elution was carried out with 0.1 M phosphate buffer (pH 6.8) and 35 fractions (10 ml) were collected at a flow rate of 0.2 ml min^{-1}. The fractions were analyzed for protein content and β- glucosidase activities.

Enzyme Characterization

Effect of temperature on β-glucosidase activities.

The partially purified enzyme solution was incubated in 16 test tubes, each containing 0.2 ml of diluted (1:4) partially purified enzyme solution with 0.2 ml of salicin 1% (w/v) in 20 mM phosphate buffer (pH 6.8) in a water bath at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C, respectively for 10 min. The reaction was stopped by adding 0.4 ml DNSA reagent and allowed to stand in boiling water for 15 min. Different concentrations of glucose (0 1 $mgml^{-1}$) were prepared. To 0.2 ml glucose concentration was added 0.2ml 20 mM phosphate buffer (pH 6.8) and 0.4 ml DNSA reagent. The tubes were heated in boiling water for 15 min. The absorbance of the glucose tubes and other tubes were read with Spectronic 21 spectrophotometer at 540 nm. Glucose standard curve was established with the plot of glucose concentration against absorbance from which units of activity were read.

Effect of temperature on β-glucosidase stability.

Diluted (1:4) partially purified enzyme solution (0.2 ml) was incubated at different temperatures (40°C, 50°C, 60°C, 70°C, 80°C and 90°C) for 1h. The reaction tubes were promptly cooled. The control and reaction tubes (residual) enzyme activity were determined as described above.

Effect of pH on β-glucosidase activities.

The pH activity profiles of endoglucanase was determined by incubating 0.2 ml of the diluted (1:4) purified enzyme with 0.2 ml of 1 % (w/v) salicin prepared in buffers of different pH values (3.0 to 10.0) at 40°C for 10 min (Shruti et al., 2013). Buffers used were: 0.1 M citrate buffer for pH 3 and 4; 0.2 M sodium acetate buffer for pH 6, 7 and 8; 0.2 M carbonate bicarbonate buffer for pH 9 and 10. The reactions were stopped by the addition of 0.4 ml of 3,5-dinitrosalicylic acid reagent and the β-glucosidase activity was determined and expressed as percentages (relative activities) of the initial enzyme activities.

Effect of pH on β-glucosidase stability.

The pH stability of β-Glucosidase was determined by incubating 0.2 ml of the enzyme with 0.2 ml of the various buffers (pH 3.0 to 10.0), prepared as described above, for 1h at room temperature (28°C). Thereafter, 0.2ml of 1% (w/v) of salicin solution in 20mM phosphate buffer (pH 6.8) was added and the mixture re-incubated for 10 min at 40°C. The reaction was stopped by the addition of 0.4 ml DNSA reagent to each tube and allowed to stay in boiling water for 15 min. β-Glucosidase activity was then determined and expressed as percentage (residual activities) of initial enzyme activities.

Effect of metal ions on β-glucosidase activities.

A number of metal ions (Ca^{2+},Sr^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, $Zn^{2+}$and Cu^{2+}) were tested for their effects on β-glucosidase activity. The reaction mixture was made up of 0.2 ml of enzyme solution, 0.2 ml of 5 mM solution of metal salt in 20 mM phosphate buffer and 0.2 ml 1% (w/v) salicin in 20 mM phosphate buffer (pH 6.8). The reaction tubes were incubated at 40°C for 10 min. Reaction stopped by boiling with 0.4ml DNS for 10 min. β-Glucosidase activities were determined and expressed as percentage (relative activities) of the initial enzyme activities.

Results

Aspergillus niger IMI 502691 β-Glucosidase had maximum enzyme yield at day 4 corresponding to a culture pH of 2.290 as shown in Figure 1. In the time course for the production of β-glucosidase by *Aspergillus niger* IMI 502691 growth was accompanied with rapid increase in microbial mass.

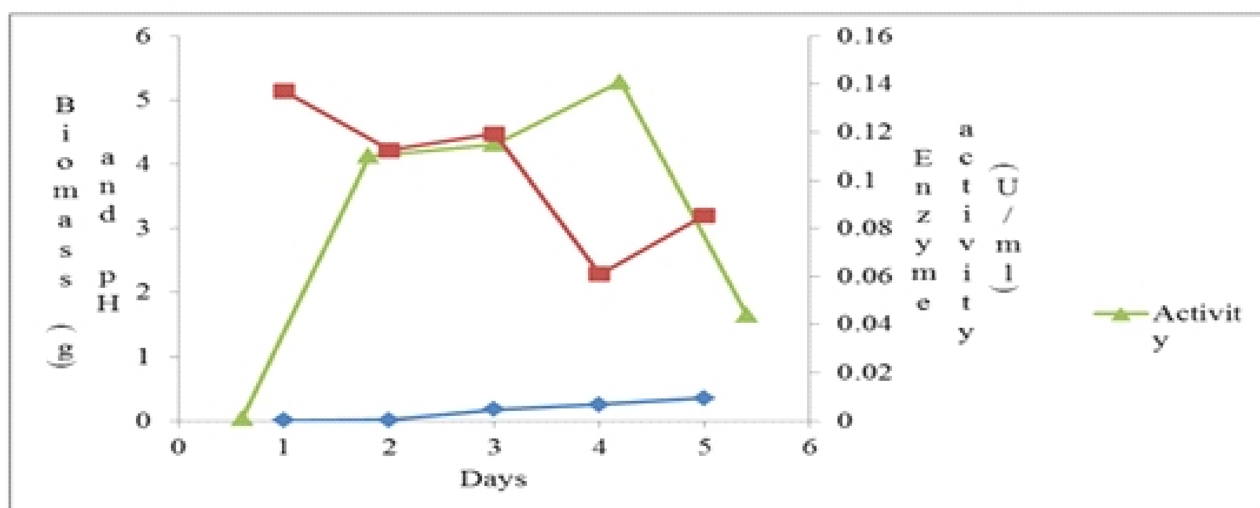

Figure 1: Time-course of β-Glucosidase Production by *Aspergillus niger* IMI 502691 in 5-Day Solid Substrate Fermentation of Cassava Root Fiber with Basal Medium: $CaCl_2.2H_2O$ 0.05; $(NH_4)_2SO_4$, 0.6; $MgSO_4.7H_2O$, 0.05; KH_2PO_4, 0.3; NH_4NO_3, 0.3%(w/v) and Tween20, 0.5%(v/v).

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Purification of β-glucosidase

Carboxymethyl Sepharose Fast Flow (FF) Ion Exchange Chromatography: The elution profile of protein and β-glucosidase on CM-Sepharose fast flow (FF) ion exchange chromatography is shown in Figure 2. The elution pattern in β-glucosidase demonstrated a single major peak of activity in fractions 3 to 5.

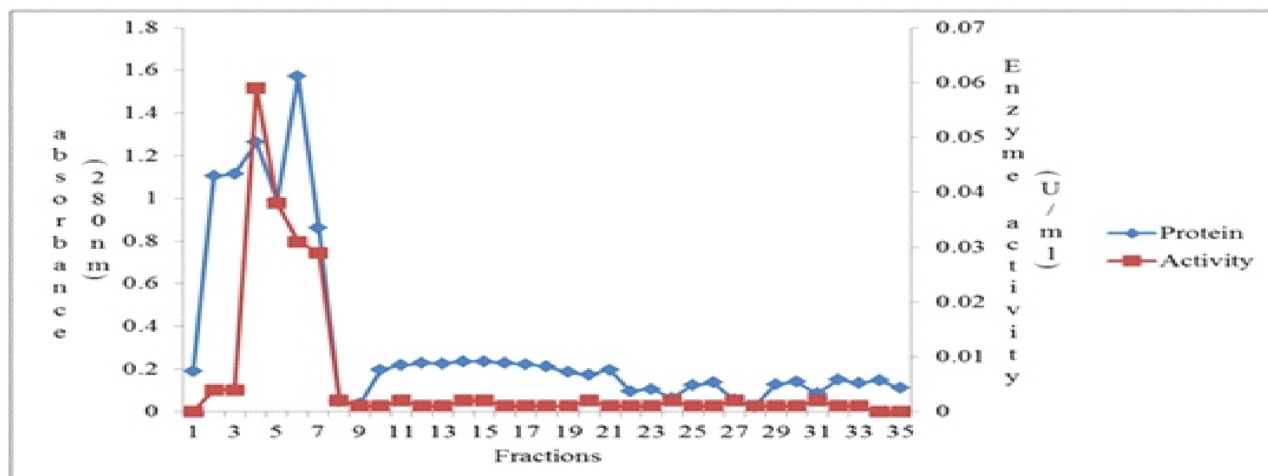

Figure 2: Elution Profile of *Aspergillus niger* IMI 502691 β-Glucosidase Activity on CM-Sepharose Fast Flow (FF) Ion Exchange Chromatography.

Biogel P4 gel filtration chromatography: The elution pattern of β- glucosidase on Biogel P4 gel filtration chromatography is shown in Figure 3, major peak of β- glucosidase activity occurred in fractions 6 to 8.

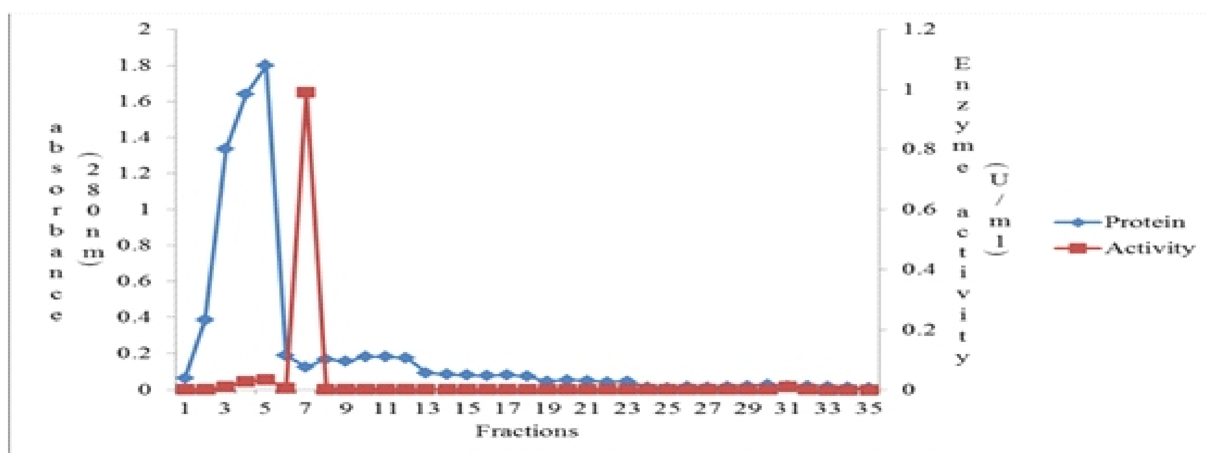

Figure 3: Elution Profile of *Aspergillus niger* IMI 502691 β-Glucosidase Activity on Biogel P 4 Gel Filtration Chromatography.

β- Glucosidase Purification Summary

The purification summary of β- glucosidase is shown in Table 1. β-Glucosidase of *Aspergillus niger* IMI 502691 was purified 3.17 fold to give 3.68% yield relative to the total activity of the crude filtrate and a final specific activity of 106.93 Umg^{-}protein.

Table 1: Purification Summary of *Aspergillus niger* IMI 502691 β-Glucosidase.

Purification Step	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (Umg-¹)	Yield of activity (%)	Purification (fold)
Crude enzyme	80	13332.40	395.44	33.72	100	1.0
Dialysis in 5M sucrose	10	1556.21	33.71	46.16.	11.67	1.37
Ion Exchange chromatography in CM-Sepharose	7.4	983.18	12.90	76.22	7.37	2.26
Gel filtration on Biogel P 4	4.5	490.81	4.59	106.93	3.68	3.17

Characterization of β-glucosidase

Effect of temperature on the activities of *Aspergillus niger* IMI 502691 was optimal at 80°C as shown in Figure 4. β-Glucosidase remained stable at 50°C and retained above 50% of its initial activity at 60°C for 1 h (Figure 4).

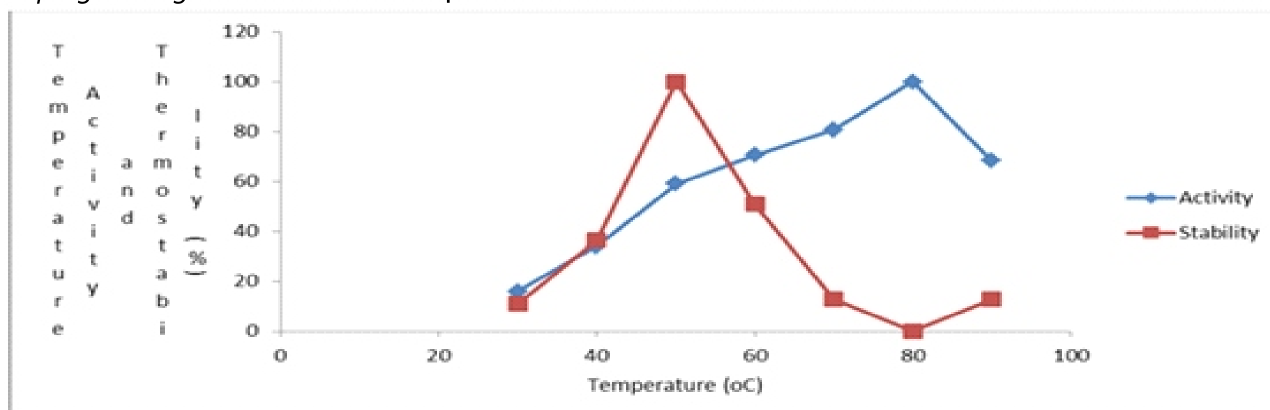

Figure 4: Temperature Activity and Thermostability Profiles of *Aspergillus niger* IMI 502691 β-Glucosidase at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C.

The results of the effect of pH on *Aspergillus* spp. IMI 502691 β-glucosidase activity and stability is shown in Figure 5. β- Glucosidase activity was optimal at pH 5.0 and was optimally stable at this pH (Figure 5). It retained at least 55.5% of its relative activity at pH 3.0 to 9.0 for 1 h.

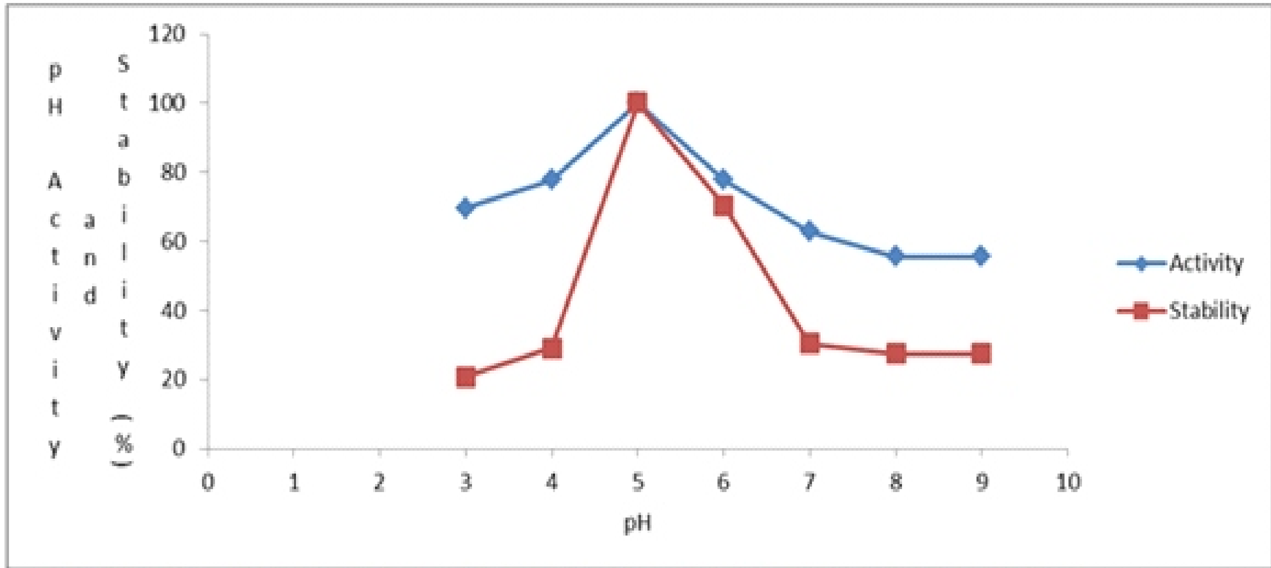

Figure 5: Effect of pH on Activity and Stability of *Aspergillus niger* IMI 502691 β-Glucosidase.

Figure 6 shows the effect of metal ions on β-glucosidase. Stimulation of β-glucosidase was achieved by all the metal ions tested except for calcium which was inhibitory relative to the initial reaction without the tested metals.

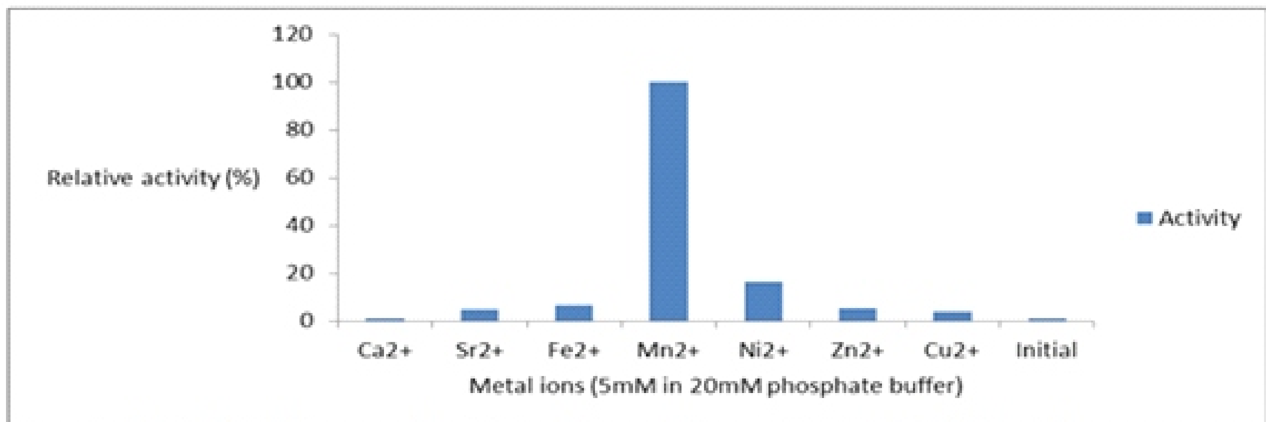

Figure 6: Effect of Metal Ions on the Activity of *Aspergillus niger* IMI 502691β-Glucosida

Discussion

In the time-course of β-glucosidase production from *Aspergillus niger* IMI 502691, reduced culture pH condition between day 3 and day 4 (pH 4.47 to pH 2.29) gave a corresponding increase in the β-glucosidase activity with optimal activity being observed at day 4 (Figure 1). The reduction in β-glucosidase activity between day 4 and day 5 in contrast recorded biomass accumulation between day 4 and day 5 an indication of the fact that biomass production is not synonymous with enzyme production as shown in Figure 1.The change in pH has more pronounced effect on biomass production after day 4.

The elution patterns of proteins on CM-Sepharose fast flow ion exchange chromatography showed single peak of β-glucosidase activity. Recovery of the fractions led to 7.37% yield relative to the overall activity and 2.26 fold purification for β-glucosidase. On Biogel P4 gel filtration, recovery of fractions led to 3.17 fold purification for β-glucosidase to give 3.68% yield relative to the total activity in the crude extract with a final specific activity of 106.93 Umg^{-1} proteins.

Although the total protein decreased by 86.1% after the partial purification of β-glucosidase, the specific activity increased by over 300%. This is an indication of effective purification of the β-glucosidase enzyme.

β-glucosidase optimal activity was observed at 80°C while more than 50% of the activity was retained between 50°C 90°C . The enzyme optimal stability temperature for 1hr was recorded at 50°C.

However, the enzyme was thermostable for 1h between 45°C 65°C retaining more than 50% of the relative enzyme activity. Peshin and Mathur (1999) found that the β-glucosidase of *Aspergillus niger* strain 322 remained stable during 30 minutes incubation at 50°C, retaining 100% activity for that period and temperature. The thermostability of *A. niger* IMI 502691 β-glucosidase over 45°C 65°C and ability to retain 100% activity for up to 1 h at 50°C places the enzyme of the fungus at a good advantage for industrial use. The thermostability of β-glucosidase in this report is superior to that of *Ruminococcus albus* F-40 which lost 20% of the Initial activity after 10min at 37°C (Ohmiya & Shiniuzu, 1988) and β-glucosidase of *Robillarda* spp. Y-20 which were stable below 50°C (Uzile & Sasaki, 1987) but less to a highly thermostable β-glucosidase with a half-life of 460min at 70°C from *Talaromyces emersonile* reported by McHale and Coughlan (1981).

Partially purified β-glucosidase of *Aspergillus niger* IMI 502691 activity increased from pH 3.0 to pH 5.0 with optimal activity recorded at pH 5.0 The enzyme was optimally stable at pH 5.0. Kaur et al. (2007) reported that *Melanocarpus* spp. ATTCC 3922 with an optimal glucosidase activity at temperature and pH of 60°C and 6.0 respectively retained only 45% activity at 60°C and pH 6.0 after only 30 minutes. The β-glucosidase in this report exhibited 100% relative activity and optimal stability at pH 5.0.

Aspergillus niger IMI 502691 β-glucosidase was strongly enhanced by all the bivalent metal ion tested, with Mn^{2+} having the most stimulatory effect and Fe^{2+} with the least stimulatory effect on the enzyme activity. Studies have shown that supplementing divalent or trivalent cations is one strategy to increase enzyme reaction efficiency. Inhibitory effect of Cu^{2+} and Fe^{2+} on β-glucosidase has been reported (Harnpicharnchai et al., 2009). Stimulatory effects of Mg^{2+}, $Mn^{2+}$and Zn^{2+} on β-glucosidase have been reported (Chem et al., 2012). This work provides a useful novel β-glucosidase which displayed favourable industrial properties: high thermostability and broad pH activity range (pH 4.0^{-}pH 6.0).

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