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Characterization of β-Glucosidase from *Trichoderma viride* with Cowdung as Carbon Source for Possible Industrial Use

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Abstract
β-glucosidase is an important component of the cellulase complex. It is a hydrolytic enzyme with a wide range of biotechnological applications and is specific for a wide variety of glycoside substrates .However, differences in enzyme properties depends on both the microorganism used and the mode of cultivation. Therefore, characterization of novel enzymes is essential to explore their potential biocatalytic application. This study aimed at characterization of Purified β-glucosidase in terms of optimum pH, temperature, thermostability, effect of divalent cations and kinetic properties. The enzyme had an optimum pH and a temperature of 6 and 60 ^{0}C, respectively. The enzyme was able to retain 92.98 % and 90.66 % of its activity at pH 6 and 55 ^{0}C for 1 h respectively. Effects of divalent cations (10 mM) on β-glucosidase activity was studied and the enzyme activity was found to be enhanced by Sn^{2+} (106.17 %), Ca^{2+} (102.84 %) and Mn^{2+} (105.6 %), whereas Cu^{2+} (21.31 %), Co^{2+} (48.85 %) and Hg^{2+} (28.51 %) inhibited its activity. The K_M and V_{MAX} values of the purified β-glucosidase were found to be 6.69 mM and 12.44 µmol/min respectively using salicin as a substrate.These results provide support for further studies of this enzyme and suggests its potential application for industrial use.

Keywords: β-glucosidase, Cellulose,Cow dung, *Trichoderma viride*, Industrial use, Solid waste.
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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). β -glucosidase can be produced from bacteria, fungi, plants and animals (Joo *et al.*, 2010; Han and Chen, 2008). 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). Many fungal strains secrete high amounts of β-glucosidase with *Trichoderma* spp. 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 enough for effective conversion of cellulose to glucose (Workman and Day, 1982). Other filamentous fungi that have been shown to produce β-glucosidase include *Aspergillus oryzae*, *A. niger*, *A. carbonarius,A.fumigatus,A.saccharolyticus,P enicilliumpurpurogenum*, *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).

Some industrial applications that employ the use of 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 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. 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).

Nigeria has vast agricultural land that produces a lot of agricultural wastes that can be utilized for the production of useful industrial enzymes. Enzymatic hydrolysis of such waste is one of the attractive solutions of this problematic issue that also provides an environmentally friendly means of depolymerizing cellulose. 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 carbon and nitrogen ratio in cow dung is an indication that it could be a promising feedstock for culturing microorganisms (Adegunloye *et al*., 2007). It was considered significant to characterize this enzyme through kinetic study to explore factors affecting its productivity and properties. Hence, this study aimed at characterizing β-glucosidase from *Trichoderma viride* to present its potential application for industrial use as well as provide potential solutions for managing large magnitude of solid waste.

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 1mm. The ground cow dung was stored in air tight containers until used. *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 10 ml 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 haemocytometer, 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°C for 15 min. After cooling the flask to room temperature, it was inoculated with 5 % (w/v) of *T.viride* inoculum under aseptic conditions. The culture was incubated at 30 °C 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 production. After this, the mixture was filtered using Muslin cloth and centrifuged at 10,000 x g for 20 minutes. 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 (1 % Salicin) and 0.1 ml citrate buffer (pH 4.8). The tubes containing the mixture were incubated at 50 °C for 30 min. Thereafter, 3ml 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 were expressed in terms of units per gram of cow dung (U/g).

Characterization of the partially purified β-glucosidase

Effect of temperature on activity and stability of partially purified β-glucosidase

The effect of temperature on the activity of β-glucosidase was determined by incubating the partially purified enzyme (0.1 ml) in 1 % Salicin (0.2 ml) in 10 mM citrate buffer (pH 4.8) at temperature range between 30 to 80 °C. Dinitosalicyclic acid (DNS) method was used to assay for the enzyme activity at different temperatures (Tomaz and Roche, 2002). Thermostability studies of the enzyme were conducted by preincubating the enzyme solution at 30 – 80 °C for 1 h. After the incubation, the enzyme activity was assayed using standard procedure (Bai *et al.*, 2013).

Effect of pH on activity and stability of partially purified β-glucosidase

The optimum pH for β-glucosidase was determined using buffers of different pH, ranging from 3 to 10 (citrate buffer pH 3-6; Tris-HCl buffer; pH 7-9, glycine-NaOH buffer; pH 10) at 0.05M concentration after which the enzyme activity was assayed. The enzyme was placed in different pH buffers at room temperature for 1 h to check its stability at different hydrogen ion concentration. The enzyme activity was determined using standard assay procedure (Bai *et al.*, 2013).

Effect of various metal ions and EDTA on the activity of partially purified β-glucosidase

The effect of metal ions and EDTA on β-glucosidase activity was studied as: the enzyme sample was incubated with various chloride salts of these ions : Sn^{2+}, Cu^{2+}, Li^{+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+} Hg^{2+}, Mg^{2+} and EDTA at 0.01 M concentration for 30 min. Enzyme activity was then assayed as described by Tomaz and Roche (2002).

Enzyme kinetics

The K_M and V_{MAX} of the partially purified β-glucosidase were determined by Lineweaver-Burk plot (double reciprocal plot) using various concentration of Salicin (5, 10, 15, 20, 25, 30, 35, and 40mM) as described by Iqbal *et al.* (2011).

Results and Discussion

Characterization of partially purified β-glucosidase

Effect of pH and pH-stability on the activity of partially purified β-glucosidase

The activity of the enzyme was assayed using buffers of various pH (3-10) at 50 ^{0}C for 1 h. The result (Figure 1) indicated that the optimum pH of purified β-glucosidase (10.55±0.26 µmol/min) was pH 6. Further increase or decrease in pH beyond this value resulted in a decline in

β-glucosidase activity. The stability of purified β-glucosidase was also studied at different pH from 3 to 10 and the enzyme was stable at pH of 5, 6 and 7 (Figure. 2) retaining 80.85 %, 92.98 % and 76.20 % of its residual activity respectively. Most fungal β-glucosidases show optimum activity between pH 4.0 and 6.0 (Baffi *et al.*,2011;Bhatia *et al.*,2002). The optimal pH of β-glucosidase obtained in this study for *T. viride* was found to be similar to that reported for *A. nidulans* AN1804 (Liu *et al*., 2012). The result of pH stability in this study showed that the enzyme was most stable at pH 6 retaining 92.98 % of its residual activity after 1 h of incubation. A closely related result was earlier reported by Kaur *et al.* (2007) where β-glucosidase produced by *Melanocarpus* sp. MTCC 3922 was stable at pH 5.0 at 40 °C.

Additionally, it was reported that 90 % of β-glucosidase activity from *A. oryzae* remained after 17 h incubation at pH 5.0 to 7.0 at 30 ^{0}C. However, only about 70 % of the activity remained at pH 4.0 (Horii *et al.*,2009). β-glucosidase from *Penicillium funiculosum* was stable in the pH range of 2.5 to 6.0; retaining 80% of the activity after a 2 h incubation at 25^{0}C (Karboune *et al.*,2008). Xue *et al.* (2008) reported that β-glucosidase was stable at pH ranging from 5.0 to 6.8. This little variation in pH optima may be as a result of the genetic variability among different species (Irshad *et al.*, 2013).

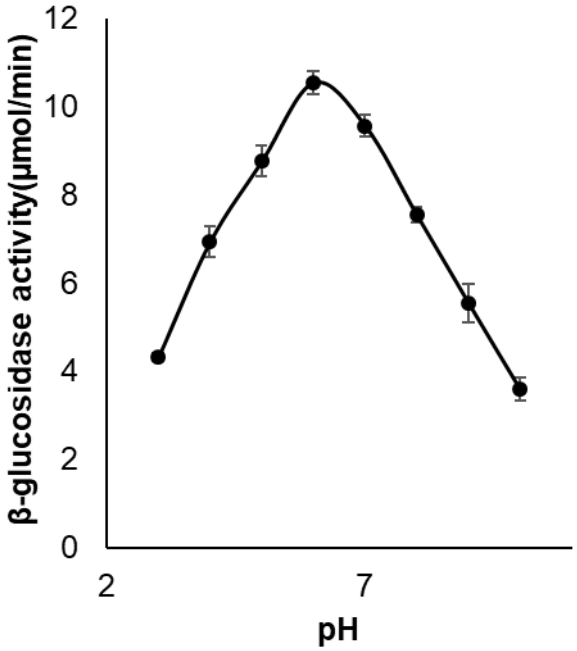

Figure 1: Effect of pH on the activity of the partially purified β-glucosidase

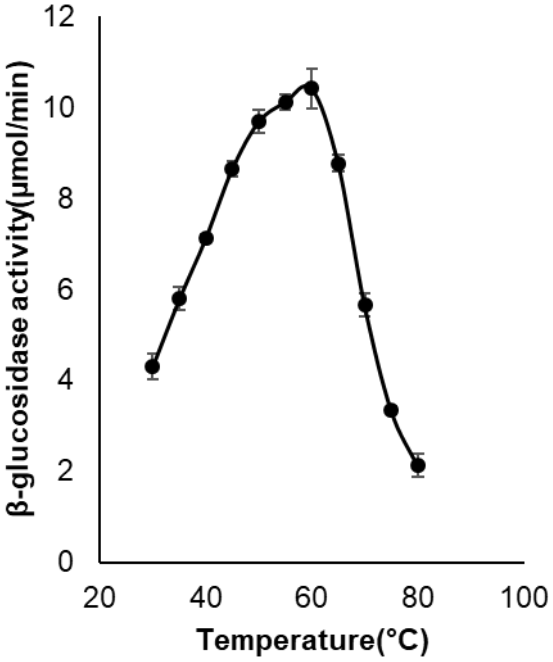

Figure 3: Effect of Temperature on partially purified β-glucosidase activity

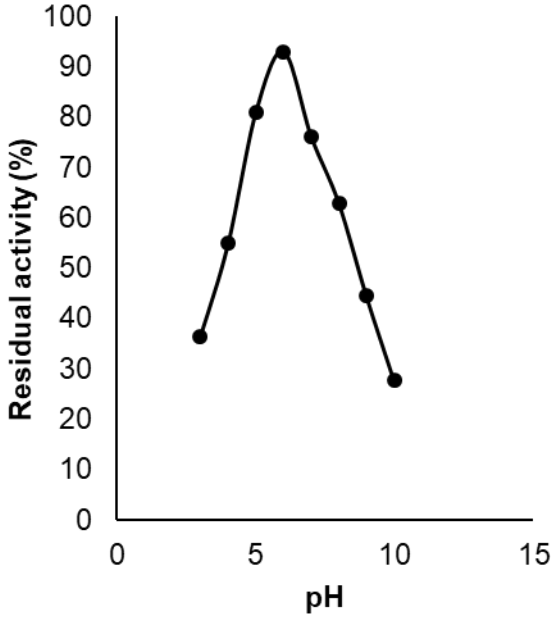

Figure 2: Effect of pH Stability on partially purified β-glucosidase activity

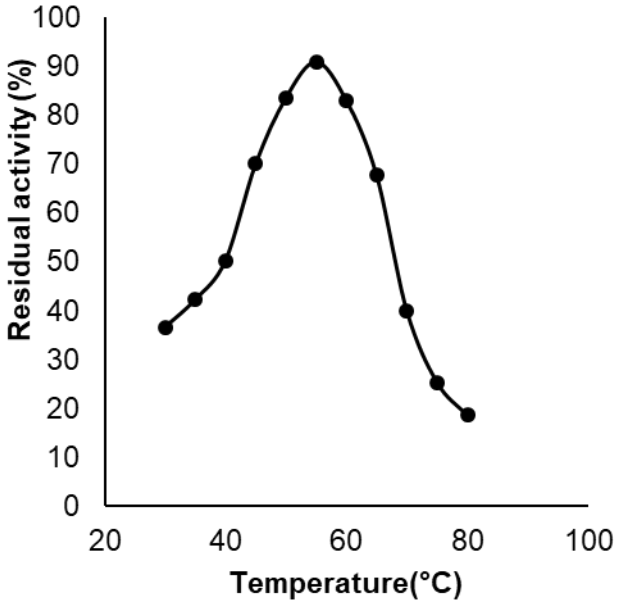

Figure 4: Effect of Temperature stability on partially purified β-glucosidase activity

Effect of temperature and its stability on the activity of the partially purified β-glucosidase

Figure 3 showed an increase in β-glucosidase activity as the temperature increased and the maximum activity of 10.42 ±0.43 µmol/min was achieved at 60 ^{0}C. There was no statistical difference ($p>0.05$) in β-glucosidase activity between 45 and 50 ^{0}C and a sharp decline in the enzyme activity was observed at 65 ^{0}C (Figure 3). The stability of the purified β-glucosidase was studied and the enzyme was stable below 60 ^{0}C. The enzyme retained 90.96 % of its residual activity at an optimum temperature of 55 ^{0}C after 1 h of incubation. The enzyme became unstable when the temperature was above 60 ^{0}C. At 75 ^{0}C, only 25 % of the enzyme activity was retained after incubating for 1 h (Figure 4). Thus, temperature plays an important role in expressing the activity of biological systems and has great influence on the production of end products (Chandel *et al.*,2013). Similarly, other β-glucosidases purified from *Thermomyces lanuginosus* (Lin *et al.*,1999), *Trichoderma reesei* (Chen *et al.*,1992), *Aspergillus japonicus* (Claudia *et al.*,2000), *Penicillium pinophilum* (Joo *et al.*,2010), *Humicola insolens* (Souza *et al.*,2010) and *Trichoderma viride* (Irshad *et al.*,2013) had optimum temperature of 60 ^{0}C. In case of thermostability, Marques *et al.* (2003) purified and characterized β-glucosidase from *T. viride* and the enzyme was stable at 55 ^{0}C for 46 h and at 80 ^{0}C only 20 % of the enzyme activity was retained after a 30 min incubation. However, Irshad *et al.* (2013) found that purified β-glucosidase from *T. viride* was stable at 60 ^{0}C without losing much of its original activity after 1 h of incubation.

High thermostability is a desirable property for an enzyme to be used for bioconversion processes where increased reaction rates and reduced contamination are achieved by employing high reaction temperatures (Asgher and Iqbal, 2011). A wide range of industrial applications require relatively high thermostability as an attractive and desirable characteristic of an enzyme (Iqbal *et al.*, 2011). Thermostability of β-glucosidase from *T. viride* suggests its potential for industrial applications such as food processing and biofuel production.

Effect of various metal ions and EDTA on β-glucosidase activity

Figure 5 showed that Sn^{2+}, Ca^{2+}, and Mn^{2+} with relative activities of 106.17 %, 102.84 % and 105.06 % respectively significantly ($p<0.05$) enhanced β-glucosidase activity; while Hg^{2+}, Co^{2+} and Cu^{2+} with relative activities of 28.51 %, 48.85 % and 21.31 % strongly inhibited β-glucosidase activity. Thus, Li^{+} and Ca^{2+} with relative activities of 94.95 % and 102.84 % had no significant ($p>0.05$) difference on β-glucosidase activity when compared with the control. Also, Zn^{2+}, Fe^{2+}, Fe^{3+} and EDTA with relative activities of 89.9 %, 73.05 %, 83.72 % and 89.32 % slightly reduced β-glucosidase activity. In this study, the effect of metal ions and EDTA on β-glucosidase activity showed that the β-glucosidase activity was neither stimulated by Li^{+}, Zn^{2+} Fe^{2+} and Fe^{3+} (each at 0.0l mM), nor activated by EDTA at (0.0l mM) (Figure 5). These findings are in agreement with other studies where β-glucosidases from other fungal species were activated by Mn^{2+}, Mg^{2+} (Chen *et al.*,2012), Sn^{2+}, Ca^{2+}, Li^{+} (Bai *et al.*,2013) and strongly inhibited by Hg^{2+}, Cu^{2+} (Bhatti *et al.*,2013), Pb^{2+} and Cd^{2+} (Ma *et al.*,2011). The sensitivity of β-glucosidases to mercuric chloride ($HgCl_2$) suggests that SH-groups may be involved in the enzyme catalyzed reaction mechanism. This is because heavy metals like $HgCl_2$ would selectively react with –SH residues of the enzyme thereby inactivating it (Onyike *et al*. 2008).

A similar inhibitory effect was reported for Hg^{2+} on β-glucosidase from *A. niger* (Galas and Romanowska, 1997), *Clostridium paradoxa* (Lucas *et al.*, 2000*), C. resinae* (Oh *et al.*, 1999) and *Xylaria regalis* (Wei *et al.*, 1996). It has been known that β-glucosidase from *Cladosporium fulvum* and *Melanocarpus* sp. MTCC 3922 was inhibited strongly by Cu^{2+} (Zhao *et al.*, 2009; Kaur *et al.*, 2007). The increase in enzyme activity in the presence of these metal ions could be as a result of their effect as co-factors; where they enhance the catalytic efficiency of the

enzymes. The response of these ions to certain amino acid residues in the active site of the protein could cause a conformational change in favour of higher activity of the enzyme (Olajuyigbe *et al.*, 2016). The effect of EDTA (a chelating agent) on β-glucosidase activity indicates that the enzyme did not depend on a metallic co-factor at its active site. This study showed that β-glucosidase activity is slightly enhanced in the presence of some metal ions tested indicating that β-glucosidase may not be a metalloprotein and therefore does not depend soley on metal ions for optimum activity (Pei *et al*. 2012; Krishna, 2005). The slight activation by Sn^{2+}, Ca^{2+}, and Mn^{2+} may be explained by stabilization of the enzyme. This result is in agreement with the report of Meko'o *et al*. (2012), where 1 % EDTA had no effect on the activity of β-glucosidases expressed from *Penicillium pastoris*, indicating that β-glucosidases are not metalloproteins.

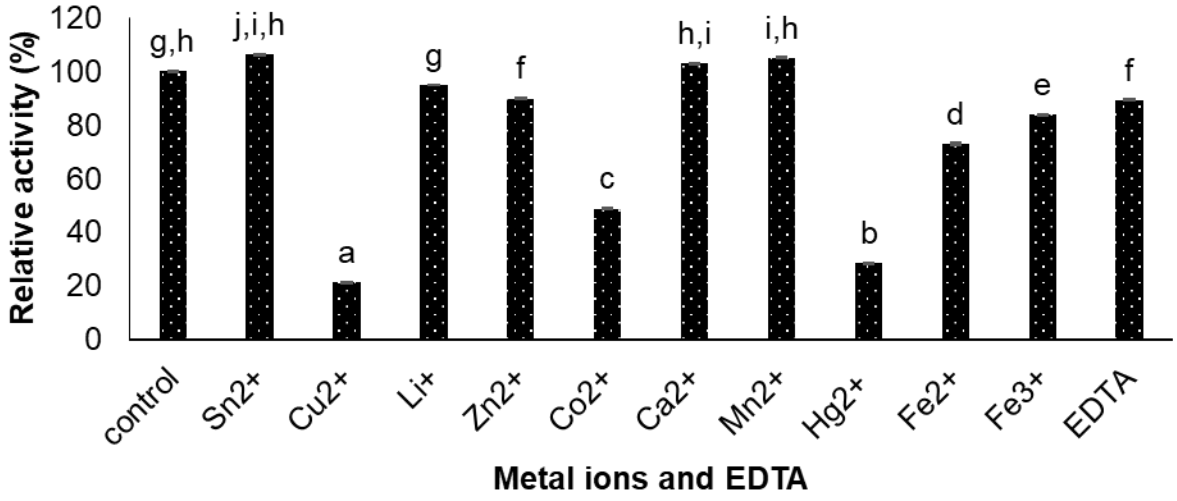

Figure 5: Effect of various Metal ions and EDTA on β-glucosidase Activity

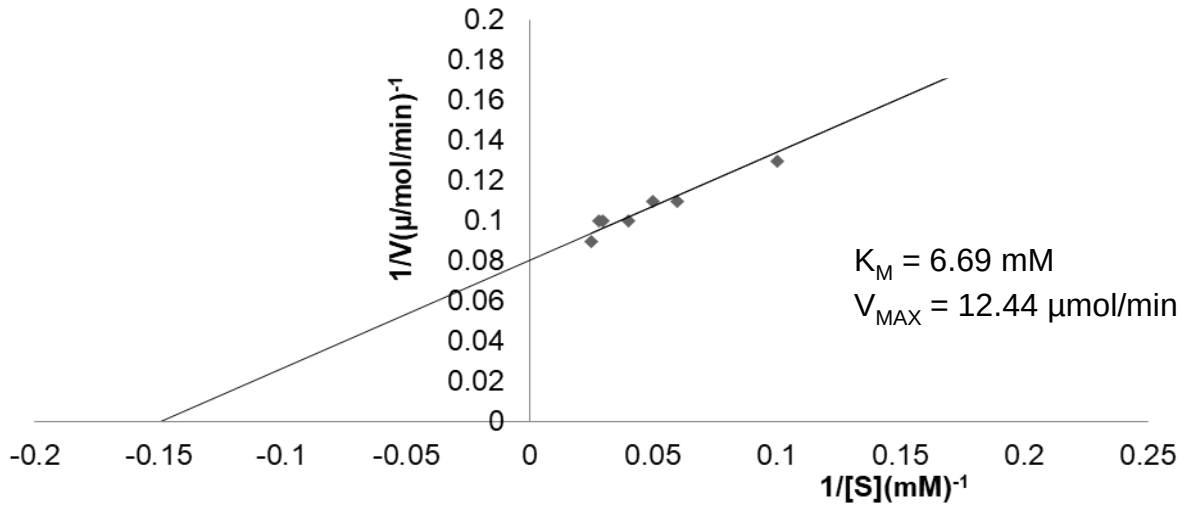

Figure 6: Lineweaver-burk plot of β-glucosidase activity with salicin as substrate

Enzyme kinetics

The kinetic parameters K_M and V_{MAX} of partially purified β-glucosidase was estimated by Lineweaver-Burk plot using various concentrations (5 – 40 mM) of salicin as a substrate. Results (Figure 6) revealed that K_M and V_{MAX} values of β-glucosidase were 6.69 mM and 12.44 µmol/min respectively. 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 (Kaur *et al.,* 2007). Narasimha *et al.* (2016) reported that the K_M and V_{MAX} of β-glucosidase from *A. niger* was found to be 8 mM and 166 µmol/min/mg of protein respectively. In case of β-glucosidase from *Trichoderma koningii* AS3.2774, K_M value of 2.67 mM was reported (Liang *et al.,* 2010). The K_M values of β-glucosidase for salicin from *A. terreus* NRRL 265 and *A. glaucus* were 5.5 mM and 2.58 mM respectively (Elshafei *et al.*,2011; Chen *et al.,*2011). The difference in K_M value of the presently purified β-glucosidase from *Trichoderma viride* and other reported fungal species may be as a result of the genetic variability among different species (Iqbal *et al.,* 2011).

Conclusion

The purified β-glucosidase had an optimum pH and temperature of 6 and 60 ^{0}C respectively. The enzyme was stable and retained 92.98 % of its activity at pH 6 and thermostable at 55 ^{0}C. The enzyme has no specific metal requirement and EDTA did not inhibit the enzyme activity indicating that β-glucosidase from *T. viride* is not a metalloenzyme. Kinetic study of the enzyme revealed that it had a K_M and V_{MAX} values of 6.69 mM and 12.44 µmol/min respectively. The pH and thermostability of β-glucosidase produced from cow dung by *T. viride* suggest its potential for wide industrial applications.

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