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Characterization of Crude Alkaline β-mannosidase produced by *Bacillus* sp. 3A Isolated from Degraded Palm Kernel Cake

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Abstract

A recently isolated Bacillus *sp. 3A from degraded palm kernel cake was employed for β-mannosidase production in a 1.5% (w/v) Locust bean gum at pH 9.0. This isolate produced up to 22.62±2.3 nkat/ml of alkaline β-mannosidase. pH and temperature studies on the activity of the enzyme revealed that the alkaline β-mannosidase had optimum activity at pH 7.5 and 50°C, respectively. The enzyme retained about 80% of its activity at pH 5.5-9.0 after 30 minutes of incubation at 50°C. Investigation into the ability of the enzyme to resist thermal unfolding in the absence of substrate showed that the enzyme remained stable for 30 minutes between 30 and 70°C with a great decrease in activity noticed at temperature above 70°C. The K_m and V_{max} values for p-nitrophenyl-β-D-mannopyranoside were 0.845 mM and 42.92 nkat/mg. Partial purification by ammonium sulphate precipitation and dialysis resulted in an increased specific activity from 441.99 nkat/mg to 1,645 nkat/mg. These distinctive properties of the crude alkaline β-mannosidase from* Bacillus *sp. 3A make this enzyme attractive for industrial application.*

Keywords: Bacillus *sp. 3A, Alkaline β-mannosidase, Characterization, Locust bean gum.*

Introduction

The β-mannosidase (EC 3.2.1.25) is an enzyme that can remove the terminal mannose of Mannan polysaccharides. Mannan is a linear or branched polymer made up of a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues (Stalbrand *et al*. 1995). It is a major part of the hemicellulose fraction in softwoods and plant tissues where mannans play a structural role acting as hemicelluloses that bind cellulose (Moreira and Filho 2008; Akino *et al*. 1987). It also occurs in non-starch carbohydrate reserves in endosperm walls and vacuoles of seeds and vacuoles of vegetative tissues. Liepman *et al*. (2007) also gave evidence of an additional mannan function as a signaling molecule in plant growth and development.

Because of the hydrolytic properties of the β-mannosidase enzyme, it has found tremendous applications in transmannosylation and effective utilization of agro-industrial wastes. The transglycosylation ability of β-D-mannosidases is very useful in the field of oligosacharide synthesis as β-mannopyranoside linkage is one of the most difficult glycosidic bonds to synthesize (Ennis and Osborn 2003) because of its 1,2-cis-arrangement and the stereoelectronically disfavoured anomeric equatorial C-O linkage (Taubken and Thiem 1992). Together with β-mannanases, β-mannosidases are widely used in the saccharification of hemicellulose for further conversion to chemicals and fuels, in the treatment of coffee beans, in the production of konjac, in the hydrolysis of galactomannans used in oil and gas drilling and in the bio-bleaching of pulp and paper (Wong and

Saddler 1993; de Vries 2003). The enzyme also provides a useful tool for structural studies of stock polysaccharides having β-mannosidic linkages and for the sequencing of hetero-polysaccharides and carbohydrate moieties of numerous glycoproteins (de Vries 2003; Bhat 2000). Although there exist reports on the occurrence of β-mannosidase enzyme from various sources such as marine algae, germinating seeds, invertebrates and vertebrates, only a few reports exist on the purification and characterization of microbial β-mannosidases (Gomes *et al.* 2007). Moreover, the reports on alkaline β-mannosidase can aptly be described as limited when compared with those on other β-mannanases (Oda and Tonomura 1996) despite its biotechnological potentials. Onilude *et al.* (2012) reported the production of alkaline β-mannosidase form *Bacillus* sp. 3A using various agro-industrial residues as substrate. This present work reports the characteristics of a crude alkaline β-mannosidase produced by a *Bacillus* sp. 3A. This is necessary so as to determine its suitability for industrial purposes.

Materials and Methods

Materials and Chemicals

The *p*-nitrophenol was purchased from BDH chemicals (England, UK), Locust bean gum and *p*-Nitrophenyl β-D-mannopyranoside were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals were of analytical grade.

Microorganisms and Culture Conditions

The *Bacillus* sp. strain 3A used in this study was obtained from the Culture Collection of the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria. The isolate was sub-culture and maintained on Tryptose Soy Agar (TSA).

Inoculum Preparation

The inoculum used was prepared by transferring a loopful of 24-hour old culture into a 100-ml Erlenmeyer's flask containing 50 ml seed medium of composition (g/l): Soluble starch 10, Peptone 10, Yeast extract 5, NaCl 80, K_2HPO_4 1.5, and $MgSO_4$ 0.3. The seed culture medium was prepared in glycine-NaOH buffer (pH 9.0) using the modified method of Lin *et al.* (2007). Inoculation was done after sterilizing the medium at 121°C for 15 minutes. Inoculated medium was incubated at 37°C for 24 hours.

Enzyme Production

A modified medium of Jiang *et al*. (2006) containing (g/l): Locust bean gum 15, Yeast Extract 4, Peptone 8, $MgSO_4.7H_2O$ 0.6, and NaH_2PO_4 5 in glycine-NaOH buffer pH 9. 50 ml of this medium in 250 ml Erlenmeyer's flask was inoculated with a 2% (v/v) of the inoculum and incubated at 35°C. After 48 hours of incubation, the cells were removed by centrifugation at 10,000 g and 4°C for 15 minutes. The resulted supernatant was used as crude enzyme preparation.

Enzyme Assay and Protein Determination

The alkaline β-D-mannosidase activity was assayed according to the method of Hossain *et al*. (1996) using 50 μL of 1 mM *p*-nitrophenyl-β-D-mannopyranoside, 175 μL of 50 mM Phosphate buffer (pH 7.0) and 25 μL of the enzyme solution. The reaction was stopped by the addition of 350 μL of 0.2M Na_2CO_3 after incubation at 50°C for 15 minutes. The liberated *p*-nitrophenol was measured at 405 nm using Lamda 25 UV/V spectrophotometer. One nanokatal (nkat) of alkaline β-D-mannosidase activity was defined as that amount of enzyme required to catalyze the release of 1 nmol *p*-nitrophenol s^{-1} under the assay conditions.

The soluble protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard.

Determination of pH and Temperature Optima and Stabilities

The pH optimum of the alkaline β-D-mannosidase was determined by measuring its activity at pH values ranging from 3.5-11.0 using 50 mM Citrate-phosphate buffer (pH 3.5-6.0), 50 mM Sodium phosphate buffer (pH 6.5-8.0), and 50 mM Glycine-NaOH buffer (pH 8.5-11). The temperature optimum of the

enzyme was determined using the method of Jiang *et al*. (2006). The assay mixtures were incubated at different temperature ranging from 30-90°C in 50 mM Phosphate buffer at pH 7.0. The pH stability of the enzyme was determined by mixing the crude enzyme with buffers at different pH values (pH 3.5-6.0, 50 mM Citrtate-phosphate buffer; pH 6.5-8.0, 50 mM Sodium phosphate buffer and pH 8.5-11.0, 50 mM Glycine-NaOH buffer). The mixture was incubated for 30 minutes at 50°C after which the residual alkaline β-mannosidase activity was determined (Jiang *et al*. 2006). The thermal stability of the enzyme was determined by assaying for residual enzyme activity after incubation at various temperatures (30°C-90°C) for 30 minutes in 50 mM Phosphate buffer (pH 7.0).

Effect of Metal Ions and Various Reagents on Enzyme Activity

The effect of metal ions and other reagents on alkaline-β-mannosidase activity was determined by assaying for residual activity after incubating 25 μL of the crude enzyme with 175 μL of 1 mM of the various metal ions and reagents dissolved in sodium-phosphate buffer, pH 7.0 for 30 minutes at 50°C. Metal ions and reagents used include EDTA, Urea, K^{+}, Na^{+}, Ca^{+}, Fe^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ni^{+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, and Benzoic acid.

Kinetic Parameters

Kinetic parameters of alkaline β-mannosidase from *Bacillus* sp. 3A were determined using reaction mixtures containing *p*-nitrophenyl β-D mannopyranside in sodium-phosphate buffer (50 mM, pH 7.0). The release of *p*-nitrophenol was measured using different initial substrate concentrations (0.1 mM - 1 mM). All assays were performed in triplicates. Values for the maximal velocity (V_{max}) and Michaelis-Menten constant (K_m) were determined using Lineweaver-Burk double reciprocal plot.

Partial Purification of the Alkaline β-Mannosidase

The alkaline β-mannosidase enzyme was partially purified using the ammonium sulphate precipitation method. The culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove the bacterial cells. The extract was dispensed into clean Erlenmeyer flask and treated with solid ammonium sulphate to bring 0-50% and 50-100% saturation, respectively. The mixture for each batch of percentage saturation was stirred continuously to completely dissolve the ammonium sulphate. On dissolution, the set-up was kept at 4°C for 24 hours after which it was centrifuged at 10,000 rpm for 15 minutes at 4°C and filtered to obtain the precipitate. The supernatant was treated to the next batch of precipitation. At the end, the precipitates from each batch were pulled and re-suspended in a buffer (0.05 M Phosphate buffer, pH 7.0) of initial volume of culture filtrate and dialyzed in a tubular cellulose membrane against two liters of the same buffer for 24 hours at 4°C. Enzyme activity and protein content were determined at each stage of partial purification.

Statistical Analysis

All experiments were carried out in triplicates (at least) and the results presented are mean and standard deviation values. Separation of means was done using Duncan Multiple Range Test (Duncan 1955).

Results and Discussion

Bacillus sp. 3A was positive for the productions of alkaline β-mannosidase when cultured in 1.5% Locust bean gum in submerge fermentation giving an enzyme titer of 22.62 ± 2.32 nkat/ml.

Optimum pH and pH Stability

Investigation into the optimum pH of activity of the alkaline β-mannosidase of *Bacillus* sp. 3A revealed that it was active over a wide range of pH (5.5 - 8.5). However, pH 7.5 appears to be the optimum pH for the activity of this crude enzyme (Fig. 1a). Similar pH values have also been reported for β-mannosidase of alkalophilic *Bacillus* sp. AM001 (Akino *et al.* 1987) and halostable β-mannosidase of *Bacillus* sp. NN (Wainø and Ingvorsen 1999). Reduction in the activity of alkaline β-mannosidase of *Bacillus* sp. 3A beyond pH 7.5 could be as a result of the

changes in the state of acidic or basic amino acids in the protein caused by the measure of hydrogen ion concentration (Zittan 1981). Results of the pH stability studies of alkaline β-mannosidase produced by *Bacillus* sp.3A showed that the enzyme was most stable at pH 8.0 (having 100% activity (100%) at this pH after 30 minutes of incubation at 50°C (Fig. 1b). The good stability of the enzyme under alkaline conditions may be explained by the ability of the bacterium to grow under alkaline conditions.

This stability under alkaline condition is advantageous as it may be used together with other hemicellulolytic enzymes for application in kraft pulping (Kansoh and Nagieb 2004), detergent industry (Bettiol and Showell 2002) and hydraulic fracturing of oil well (McCutchen *et al.* 1996) which are usually done at high pH levels.

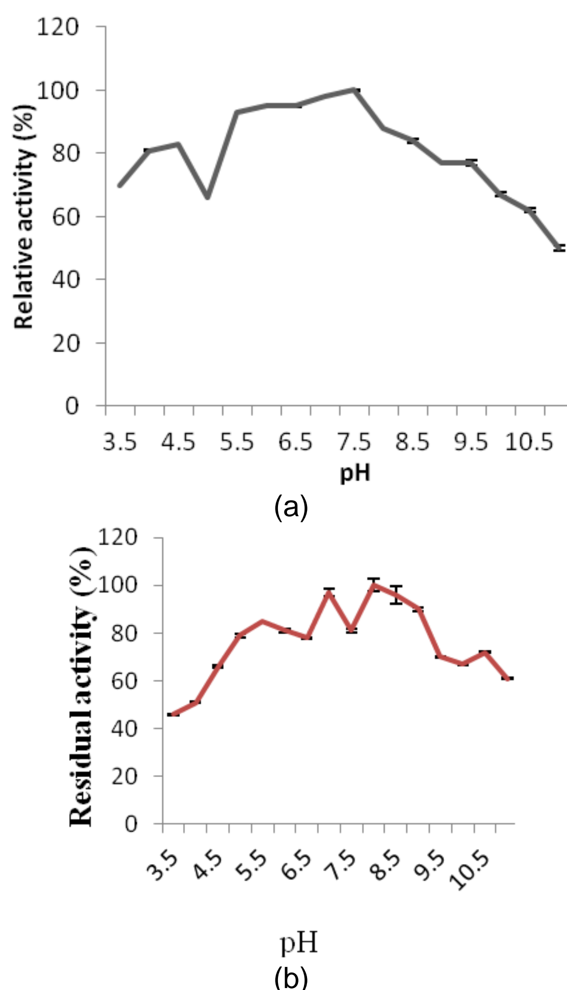

Fig. 1. Effect of different pH levels on the activity (a) and stability (b) of alkaline β-mannosidase from *Bacillus sp.* 3A produced in 1.5%w/v Locust bean gum.

Optimum Temperature and Thermal Stability

As shown in Fig. 2a, alkaline β-mannosidase of *Bacillus* sp. 3A has optimal activity at 50°C. Below this temperature, enzyme activity was found to increase with an increase in temperature (Fig. 2a). Above the optimum temperature, the enzyme was found to decrease with a corresponding increase in temperature. Optimum β-mannosidase activity at high temperatures has also been reported in literatures: Akino *et al.* (1987) observed 55ºC as optimum for β-mannosidase produced by alkalophilic *Bacillus sp.* AM001 and 50°C-60°C for β-mannosidase from *Bacillus* sp. KK01 (Hossain *et al.* 1996). Decrease in β-mannosidase activity at temperatures beyond the optimum could be due to the weakening of the intermolecular forces responsible of the preservation of its three-dimensional structure (Misset 1993). With respect to thermal stability which is an enzyme's ability to resist thermal unfolding in the absence of its substrate, this alkaline β-mannosidase in this present investigation retained 100% of its activity at 60°C after 30 minutes of incubation (Fig. 2b).

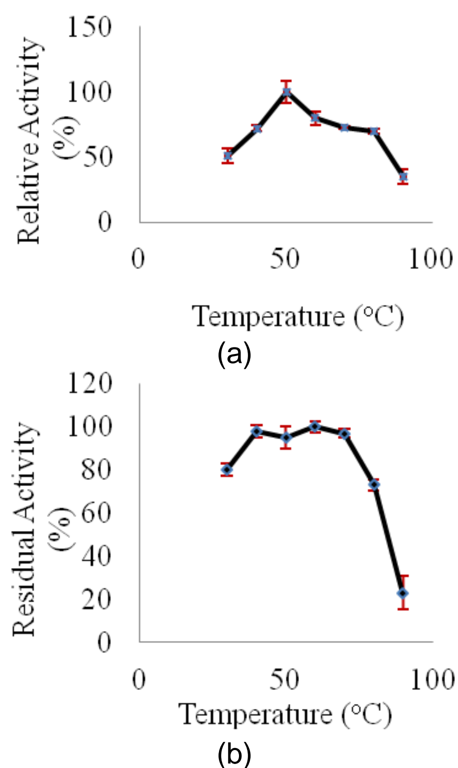

Fig. 2. Effect of temperature on the activity (a) and stability (b) of alkaline β-mannosidase from *Bacillus sp.* 3A produced in 1% Locust bean gum.

The thermo-stability of the crude alkaline β-mannosidase makes this enzyme attractive for use in industrial processes that make use of β-mannosidase at elevated temperatures.

Effect of Various Metal Ions and Chemical Inhibitors

Studies on the effect of various metal ions and other chemical reagents on the activity of the alkaline β-mannosidase produced by *Bacillus* sp. 3A as presented in Fig. 3a,b revealed that the alkaline-β-mannosidase activity was strongly activated by Fe^{3+} (200%), Zn^{2+} (100%) and Ni^{2+} (50%); and was inhibited by Na^{2+} (75%), Ca^{2+} (20%) and K^{2+} (50%). Mn^{2+}, Mg^{2+}, Fe^{2+}, and Cu^{2+} all had no effect on the activity of the enzyme (Fig. 3a). As shown in Fig. 3b, all the inhibitors studied varied in their inhibitive ability: Urea (50%), EDTA (30%), $HgCl_2$ (30%) and Benzoic acid (40%).

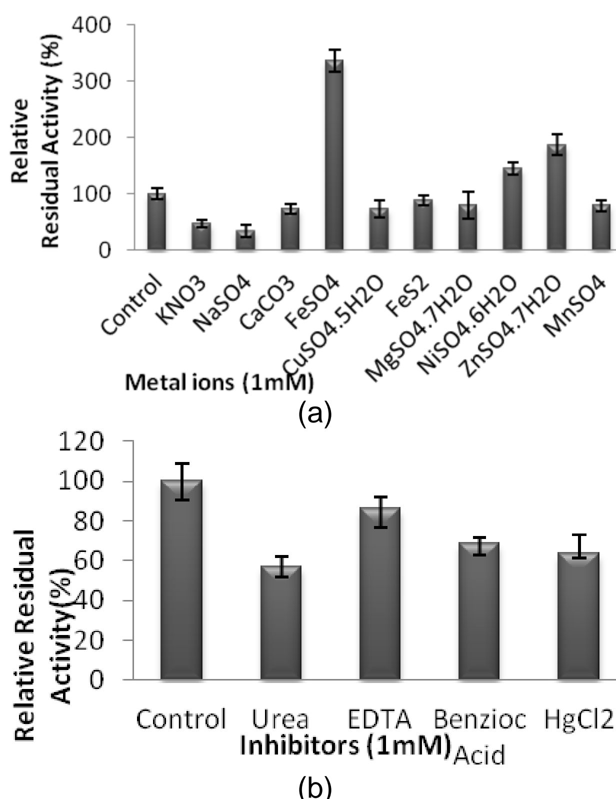

Fig. 3. Effect of metal ions (a) and inhibitors (b) on the activity of alkaline β-mannosidase from *Bacillus* sp. 3A.

Interestingly, none of these compounds could completely inhibit the activity of alkaline β-mannosidase of *Bacillus* sp. 3A, thus confirming the assertion of Fujinami and Fujisawa (2010) that alkaline enzymes often show activities in a broad pH range, they are thermo-stable and tolerant to oxidants/inhibitors. Inhibition of this β-mannosidase by EDTA is suggestive of the dependence of this enzyme on divalent ion for its catalytic activity as they affects the catalytic activity of the enzyme as it relates to divalent cofactors. The effect of metal ions on enzyme activity may be relevant when considering the use of substrate with high salt content.

Kinetic Parameters

Kinetic parameters studied revealed that, in the presence of sufficient substrate, alkaline β-mannosidase of *Bacillus* sp. 3A had a maximum velocity (V_{max}) of 42.92 nkat/mg. Also, at 0.845 mM concentration of the substrate *p*-Nitrophenyl β-D-mannopyranoside, alkaline β-mannosidase converted the substrate to product at half its maximal velocity (K_m). K_m of 0.30 mM and V_{max} of 500 nkat/mg were recorded for *Aspergillus niger* (Ademark *et al.* 2001). Oda and Tonomura (1996) recorded 91.7 U/mg protein for V_{max} and 0.2 mmol/L for K_m for the β-mannosidase of the yeast *Trichosporon* cutaneum JCM 2947. Also, 1.1 mM and 61 nkat/mg were recorded for the K_m and V_{max} of *Thermoascus aurantiacus* (Gomes *et al.* 2007), while 2.4 mM and 50 U/mg were noted, respectively, as the K_m and V_{max} *Aplysia fasciata* - a marine anaspidean by Andreotti *et al.* (2005). From the above values, there exist great variations in the kinetic parameters of β-mannosidase of different sources.

Partial Purification of Alkaline β-Mannosidase

After 3.7-fold purification level, 4.5% activity was recovered resulting in an increase in specific activity from 441.99 nkat/mg to 2645.16 nkat/mg (Table 1).

Table 1. Purification of alkaline β-mannosidase from the culture filtrate of *Bacillus sp.*3A grown on Locust bean gum at 35°C.

Purification steps	Volume (ml)	Total enzyme activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification factor	Recovery (%)
Crude filtrate	100	2,209.95	5	441.99	1	100
Ammonium sulphate	100	1,864.8	2.4	777.00	1.76	84.38
Dialysis	15	98.7	0.06	1,645.16	3.7	4.5

Conclusion

Conclusively, this works presents the characteristics of the crude β-mannosidase from *Bacillus* sp. 3A as showing high thermo-stability and good pH stability at alkaline pH range. These properties make this enzyme of great potential importance in industrial processes such as kraft pulping, detergent industry, hydraulic fracturing of oil well which make use of β-mannosidase at high pH values.

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