Production of alkaline β-mannosidase by *Bacillus* sp. 3A in Solid State Fermentation using different Agro Wastes

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Abstract: Production of alkaline β-mannosidase by *Bacillus* sp. 3A in solid state fermentation using five different agro waste materials was investigated. All the substrates investigated supported the growth of *Bacillus* sp. 3A and enzyme production at different levels. Orange peels supported the highest production of 20.74±0.62 nkat/ml followed by plantain peels (15.57±1.56 nkat/ml). Sugar cane pulp and potato peels stimulated β-mannosidase production of 7.68±0.15 nkat/ml and 11.67±0.09 nkat/ml respectively while the lowest β-mannosidase titre of 5.74±2.09 nkat/ml was recorded in mango peels. Further optimization studies using the best three substrates (Orange, plantain and potato peels) revealed that a % moisture content of 110% stimulated highest enzyme titre in all the three substrates. An inoculum density of 6% and incubation period of 120 h were found to be optimum for highest β-mannosidase by the organism in the substrates while an incubation temperature of 35 ºC supported maximum β-mannosidase production all the three substrates investigated. The addition of different nitrogen sources to the complex carbon sources revealed that ammonium nitrate at 6% (w/v) supported the maximum enzyme accumulation of 21.11±0.01 nkat/ml in plantain peels while 6% (w/v) soy bean meal stimulated 24.08±0.2 nkat/ml and 25.88±0.3 nkat/ml in potato and orange peels respectively.
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Key words: *Bacillus* sp. 3A, agro waste, solid state fermentation

Introduction

β-mannosidase (E.C. 3.2.1.25; β-1, 4-D-mannoside manohydrolase) is an enzyme which catalyses the hydrolysis of mannose units from the non-reducing end of mannosides present in mannan polymers (Bauer *et al*., 1996). Mannan, a major part of the hemicellulose fraction in softwoods and plant tissues, where it plays a structural role, is made up of mannose or a combination of glucose and mannose residues (Stalbrand *et al*., 1995; Moreira and Filo, 2008).

β-mannosidase have stimulated considerable research interests due to their transmannosylation ability which makes them very useful in the field of oligosacharide synthesis as well as effective utilization of agro-industrial waste. The β-mannosidase enzyme have also been reported to be useful 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 biobleaching of pulp and paper (Wong and Saddler, 1993; de Vries, 2003).

Although the production of β-mannosidase have been reported to occur in many bacteria, yeasts, fungi, marine algae, germinating seeds, invertebrates and vertebrates (Gomes *et al.,* 2007), microbial sources appears to the most preferred due to low cost, high production rate and readily controlled conditions. Furthermore, the lignocellulosic have been reported a suitable substrate for microbial production of β-mannosidase due to rich carbon content and readily availability (Malherbe and Cloete, 2003).

However, due to the high demand for β-mannosidase enzymes by the various industries, there is the need to screen for, new β-mannosidase-producing organisms as well as optimize the production of the β-mannosidase enzyme by the isolated organisms. This will go a long way to meet the high β-mannosidase-demand of the various industries in Nigeria. This present research work aims at investigating the production of alkaline β-mannosidase in solid state fermentation using different agro waste.

Materials and Methods

Sample procurement

Orange, Mango, Plantain peels and Sugar cane pulp were obtained from a retail fruit seller at Bodija Market with Ibadan metropolis and transported to the Laboratory in clean polythene back while Potato peels was obtained from Spices Kitchen in Abadina, University of Ibadan.

p-nitrophenol was purchased from BDH, 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.

Microorganism and culture conditions

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

Pre-inoculum preparation

This was done by transferring a loopful of a 24 h old culture of the organism into a 100 ml Erlenmeyer's flask containing sterile 20 ml seed medium of composition (g/l): Soluble starch 10, Peptone 10, Yeast extract 5, NaCl 80, K_2HPO_4 1.5, $MgSO_4$ 0.3, prepared in glycine-NaOH buffer (pH 9.0) using the modified method of (Lin *et al.,* 2007). The medium was incubated at 37ºC for 24 h.

Inoculum preparation

The 2% (v/v) of the inoculated medium above was transferred into 100 ml Erlenmeyer's flask containing fresh sterile 20 ml of the enzyme production medium containing (g/l): Locust bean gum 15, Yeast extract 4, Peptone 8, $MgSO_4 \cdot 7H_2O$ 0.6, and NaH_2PO_4 5 in glycine-NaOH buffer pH 9 (modified method of Jiang *et al.,* 2006). Incubation was done for 48hrs of cultivation at 35 ºC.

Solid State Fermentation

The substrates used were prepared as described by Onilude *et al*. (2011). One grams of the each of the pre-treated substrate was mixed with a buffered (glycine-NaOH buffer, pH 9.0) basal solution containing (g/l): Yeast extract 4, Peptone 8, $MgSO_4.7H_2O$ 0.6, and NaH_2PO_4 5- to a moisture content of the 50% in a 150 ml Erlenmeyer's flask. The whole content of the flasks were autoclaved at 121 ºC for 10 min to prevent the substrate from denaturing. The flasks and their contents were allowed to cool to ambient temperature. The cooled substrates were then inoculated 2% inoculum level and incubated at 35 ºC for 72 h in a static mode.

Extraction of alkaline β-mannosidase

This was done using modified method of Meenakshi *et al.* (2010) by adding sterile distilled water at 1:10 (w/v) to the fermented medium. The resultant solution was mixed using a shaker at room temperature (27±2 ºC) for 30 min at 150 rpm agitation speed. The mixture was filtered through a Whatman filter paper No. 1 and then the soluble protein further concentrated in the supernatant by centrifuging the filtrate at 10,000 rpm for 15 min at 4 ºC.

Determination of alkaline β-Mannosidase activity

The alkaline β-D-Mannosidase activity was assayed according to the method of Hossain *et al.* (1996) using 50 µL of 1mM *p*-nitrophenyl-β-D-mannopyranoside, 17 5µL 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 0.2 M Na_2CO_3 after incubation at 50 ºC for 15 min. The liberated *p*-nitrophenol was measured at 405 nm using Lamda 25 UV/Visible Spectrophotometer. One nanokatal (nkat) of alkaline β-D-mannosidase activity was defined as that amount of enzyme required to catalyse the release of 1 nmol *p*-nitrophenol s^{-1} under the assay conditions. The enzyme solution was used in the place of enzyme substrate for blank preparation.

Effect of moisture content on enzyme production

The effect of the moisture content of the substrates on the β-mannosidase production was determined at five different moisture content levels (50, 70, 90, 110 and 130 %) in each of the substrate. This was carried out by the addition of appropriate amount of basal medium to the substrates prior to sterilization. Thereafter, the set up was inoculated with 2% (v/w) inoculums and incubated for 72 h at 35 ºC. The pH of basal medium was kept at 9.0 with glycine-NaOH buffer.

Effect of inoculum density

Four different inoculum densities (2, 4, 6, and 8 %,) were tested at 50 % moisture content. The pH of the substrates was adjusted to pH 9.0 using glycine-NaOH buffer solution. Cultures were incubated separately at 35 ºC for 72 h after which the enzyme produced was extracted and the titre determined as previously described.

Effect of incubation time

The time course of fermentation on alkaline β-mannosidase production was studied by extracting and assaying for the amount of β-mannosidase produced at 48 h, 72 h, 96 h, 120 h, and 144 h in each of substrates (at 50% moisture content and 35 ºC incubation) differently.

Effect of the initial pH of the fermentation substrate

The effect of the initial pH of the fermentation medium on the production of the alkaline β-mannosidase was done by adjusting the moisture content of substrate with buffered basal medium of

varying pH (5.0, 6.0, 7.0, 8.0, and 9.0). Sterilization was done at 121 ºC for 10min, after which inoculation was done with 2% (v/w) of the inoculum level. The cultures were incubated at 35 ºC for 72 h after which alkaline β-mannosidase was extracted and activity determined.

Effect of Temperature

Four different incubation temperatures; 30 ºC, 35 ºC, 40 ºC, 45 ºC were used to cultivate the cultures in the different substrates for alkaline β-mannosidase. Incubation was done for 72 h after which the amount of enzyme produced was assayed.

Effect of the addition of different nitrogen sources

This was done as described Darah and Omar (2010). The nitrogen sources investigated were Urea, Peptone, Yeast Extract, Casein, Corn Steep Liquor, and Soy bean Meal, KNO_3 and NH_4NO_3. These were added to each of the solid substrate at the concentration of 4% (w/w). Moisture content was kept at 50%, pH 9.0 and incubation was done at 35 ºC for 72 h. After the measurement of the amount of the enzyme caused to be produced by each nitrogen source, the concentration of the best performing nitrogen was varied so as to determine its optimum production concentration.

Statistical Analysis

All experiment was carried out in triplicates and the results obtained were subjected to analysis of variance using ANOVA, and separation of means was carried out by Duncan's multiple range test (Duncan 1955).

Results and Discussion

Effect of different agro waste as carbon sources

All the substrates used (potato peels, plantain peels, orange peels, mango peels and orange peels) were able to support the growth and stimulate the production β-mannosidase by the *Bacillus* sp. 3A. However, the results of the effect of different carbon sources on β-mannosidase production presented in Fig. 1 revealed that the substrate varied in their ability to induce alkaline β-mannosidase production. Orange peels induced the production of this enzyme more with enzyme activity of 20.27±0.62 nkat/ml. This was followed by plantain peels with 15.57±2.09nkat/ml and potato peels (11.67±0.09 nkat/ml) while least β-mannosidase titre was recorded in Mango peel (5.74±23.02 nkat/ml). Various agro industrial residues have been reported to support microbial growth and metabolite production at different levels (Gomes *et al*. 2007). This variation could be attributed to the nature of cellulose or hemicellulose, presence of some components (activator or inhibitors) in these materials and variations in the substrate accessibility (Mabrouk and El-Ahwany, 2008). Variation in this study could also be attributed to variation is the amount of mannan constituents of the different substrates (Gomes *et al*., 2007).

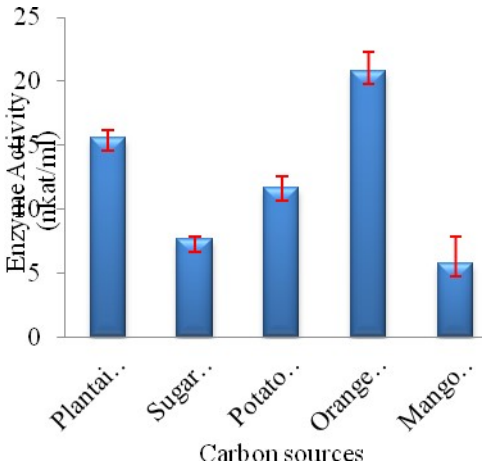

Figure 1: Effect of some complex carbon sources on alkaline β-mannosidase production by *Bacillus sp.*3A in solid state fermentation

Effect of moisture content on enzyme production

As shown in Figure 2, an increase moisture content of the fermentation orange peel 50% to 70% led to a corresponding increase in alkaline β-mannosidase production from 5.03±2.09 nkat/ml to 9.77±1.41 nkat/ml and 9.91±0.45 nkat/ml at 90% reaching an optimum of 25.98±0.22 nkat/ml at 110% moisture content. Thereafter, an increase in moisture content of the substrate led to a decrease in alkaline β-mannosidase titre. Similar trend was also observed for plantain and potato peels (Figure 2). The % moisture content of the fermentation medium has been described as one of the several factors that affect microbial growth and metabolite production (Darah and Omar, 2010; Sadhya *et al.,* 2005). Low enzyme concentration at reduced moisture content could be due to reduced solubility of nutrient of substrate low grade of swelling and water tenseness (Ong *et al.,* 2004). While low alkaline β-mannosidase concentration above the optimum could be attributed to reduction in porosity leading to oxygen transfer limitations (Onilude *et al*., 2011).

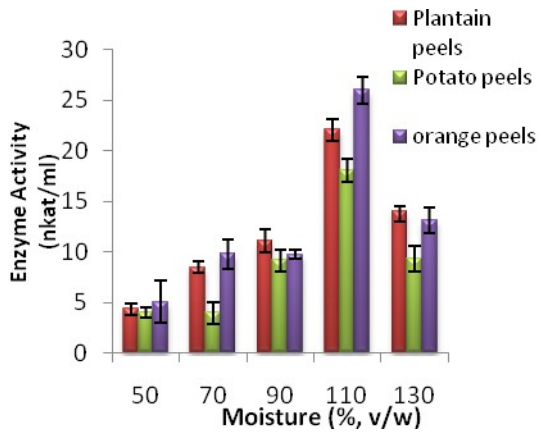

Figure 2: Effect of moisture content of the different agro wastes on alkaline β-mannosidase production by *Bacillus* sp.3A in solid state fermentation.

Effect of Inoculum density

The result of the effect of different inoculum sizes on β-mannosidase production is presented in Figure 3. There seem to be variation in the amount of inoculum producing the optimum β-mannosidase concentration with regards to the different substrates. While the highest β-mannosidase concentration of 16.33±2.47 nkat/ml and 22.12±0.15 nkat/ml was reached with a 6% (v/w) inoculum density in both potato and orange peels respectively, a 4% inoculum density gave the highest enzyme concentration of 11.46±0.21 nkat/ml in plantain peels. A balanced correlation between biomass proliferation and presence of nutrients has been reported to be an important factor in the synthesis of enzyme to a maximal level (Darah and Omar, 2010). Low enzyme titre below the optimum inoculum density could be as a result of the fact there is low density of the bacterial mass to utilize the available nutrients for enzyme synthesis while a reduction in enzyme titre at above the optimum inoculum density could be due to the competition for nutrients, resulting in a decrease of the metabolic activity of the organism (Ramachandran *et al,*. 2004).

Effect of incubation time on alkaline β-mannosidase

As represented in Figure 4, in orange peels, there was an increase in enzyme titre from 3.63±2.10 nkat/ml to 12.3±1.91 nkat/ml as the time of incubation increases from 48 h to 72 h and further to 18.09±1.09 nkat/ml at 120 h before reaching an optimum of 26.36±0.15 nkat/ml at 120 h. Thereafter, a decrease in enzyme accumulation was observed with an increase in time of incubation. Similar trends were also observed in plantain and potato peels substrate (Figure 4). Low enzyme accumulation after 120 h period of incubation has been attributed to the release of total protease due to autolysis of cells, which may have initiated the degradation of mannanase.

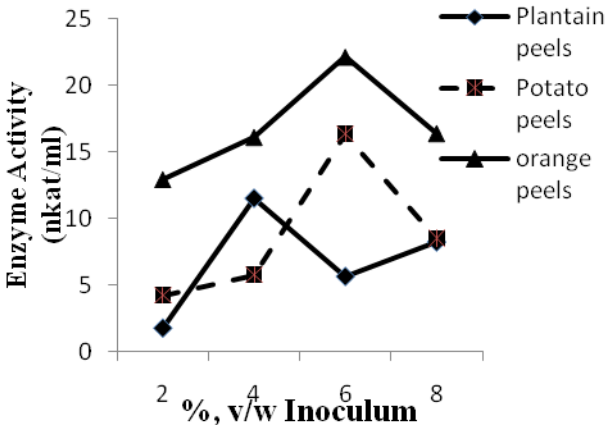

Figure 3: Effect of different inoculum densities on alkaline β-mannosidase production during the solid state fermentation of different agro waste by *Bacillus* sp.3A.

Effect of initial pH of the fermentation substrates on β-mannosidase

The results of the effect of initial pH of the fermentation substrate on β-mannosidase production production as presented in Figure 5 revealed in orange peels, there was an initial increase in β-mannosidase accumulation from 2.82±0.91 at pH 5.0 to an optimum concentration of 17.71±1.06 at pH 6.0. Beyond 6.0, a decrease in enzyme titre was observed from 17.71±1.06 at 6.0 to 5.58±0.78 at 7.0. However, at pH 8.0, a further rise in enzyme concentration was observed again (10.55±1.98). Similar trend was also observed for potato peels. However, the optimum pH supporting highest enzyme production was found to be pH 5.0 for plantain peels (Figure 5). Pandey (1992) reported that the pH of the fermentation medium is an important factor in solid state fermentation as its affects the metabolic activities of the organism which in turns affect its growth and metabolite production. Variation in pH values for optimum enzyme production (in the different substrate studied) in this study could be as result of the fact the complex media contain substances such as salts, which can inhibit cellular growth and even

some microbial enzymes (Singh and Bhermi 2008). Furthermore, a an alternating enzyme titre observed in this study for each of the substrate investigated might be due to the fact that the complex medium might posses some buffering properties which is resisting a change in pH of the fermentation substrate as a result of microbial activities.

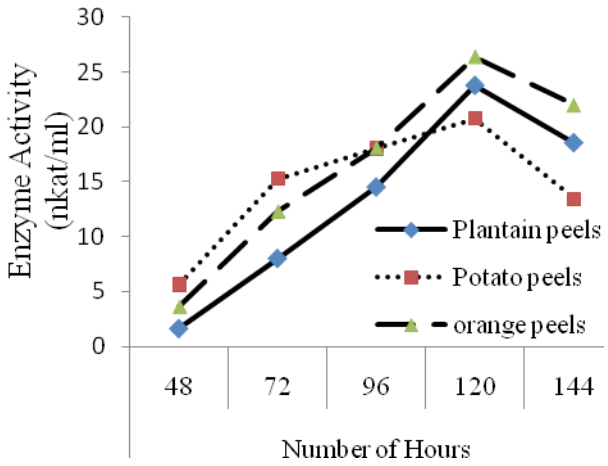

Figure 4: Effect of different incubation time on alkaline β-mannosidase production by *Bacillus* sp.3A during the solid state fermentation of different agro wastes.

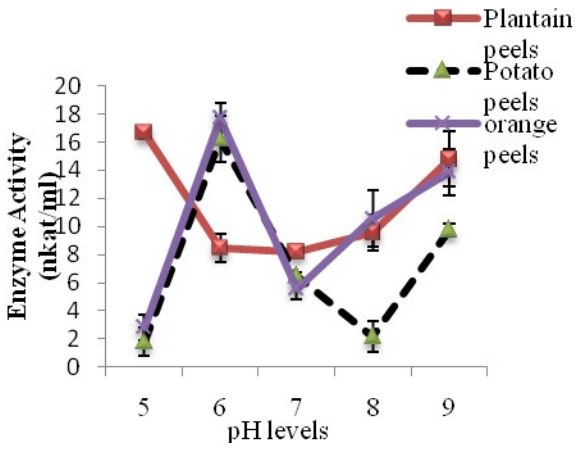

Figure 5: Effect of different pH levels on alkaline β-mannosidase production by *Bacillus* sp.3A during the solid state fermentation of different agro wastes.

Effect of temperature on alkaline production

Figure 6 shows the results of the effect of different incubation temperatures on the production of β-mannosidase in the different complex media. For orange peels, maximum β-mannosidase titre of 25.04±2.12 nkat/ml, was recorded at 35 ºC while, the minimum titre of 4.56±1.02 nkat/ml was recorded at 45 ºC. The same trend was also observed for potato and plantain peels. Temperature of 35 ºC optimum for enzyme production by this Bacillus sp 3A is in the range of temperature of 25 ºC to 40 ºC optimum reported for enzyme production by mesophilic organisms in solid state fermentation (Darah and Omar, 2010). Highest enzyme production at 35 ºC could be as its affects the metabolic activities of the organism.

Effect of different nitrogen sources on production of the enzyme

Figure 7 shows the results of the addition of different nitrogen sources to the different fermentation substrate on enzyme production. In plantain peels, addition of ammonium nitrate stimulated the highest alkaline β-mannosidase titer of 18.88±0.91 nkat/ml while the lowest titer of 2.18±1.94 nkat/ml was stimulated by corn steep liquor. In potato and orange peels however, the highest enzyme titre of 15.78±1.25 nkat/ml and 25.99±1.03 nkat/ml respectively was recorded with the addition of soy bean meal preparation while the lowest accumulation of 0.23±0.27 nkat/ml was recorded in potato peels by the addition of corn steep liquor and 1.77±2.09 nkat/ml in orange peels by the addition of KNO_3.

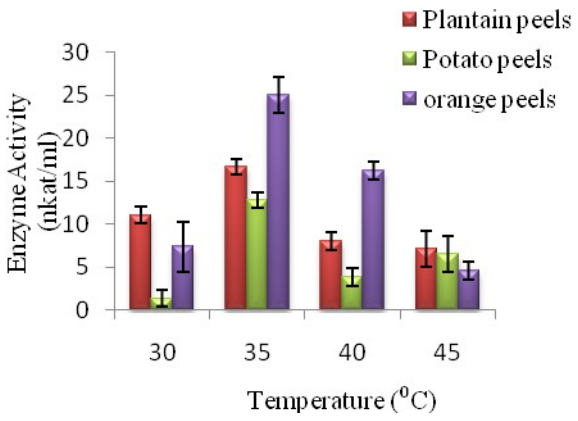

Figure 6: Effect of different incubation temperatures (ºC) on alkaline β-mannosidase production by *Bacillus* sp.3A during the solid state fermentation of different agro wastes.

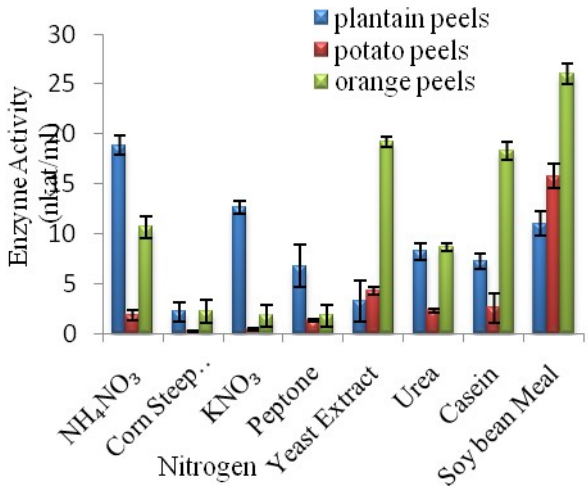

Figure 7: Effect of the addition of different nitrogen source on alkaline β-mannosidase production by *Bacillus* sp.3A during the solid state fermentation of different agro wastes.

Since ammonium nitrate did well for plantain peels and soybean meal supported high enzyme production with potato and orange peels, effect of the various concentrations of ammonium nitrate and soybean meal ranging from 2-10% (w/w) were studied. As shown in Figure 8, all the two nitrogen sources investigated viz-a-viz ammonium nitrate and soy bean meal supported maximum enzyme production at 6% (w/w). Ammonium nitrate stimulated β-mannosidase titre of 21.11±0.01 nkat/ml for plantain peels, while soy bean meal stimulated 24.08±0.2 nkat/ml and 25.88±0.3 nkat/ml in potato and orange peels respectively. Lower or higher concentrations of these nitrogen sources outside the opitimal resulted in reduced alkaline β-mannosidase production (Fig 8).

This study presented the production of alkaline β-mannosidase by *Bacillus* sp. 3A in solid state fermentation of orange peels, plantain peels and potato peels. The results of this study presented orange peels as a potential suitable substrate for the production of alkaline β-mannosidase by this organism in solid state fermentation. This is of particular importance when considering the production of β-mannosidase enzyme using cheap and readily available agro wastes as alternative sources. However, a further study of the activity of the enzyme is required to further determine the suitability of the produced enzyme for industrial applications.

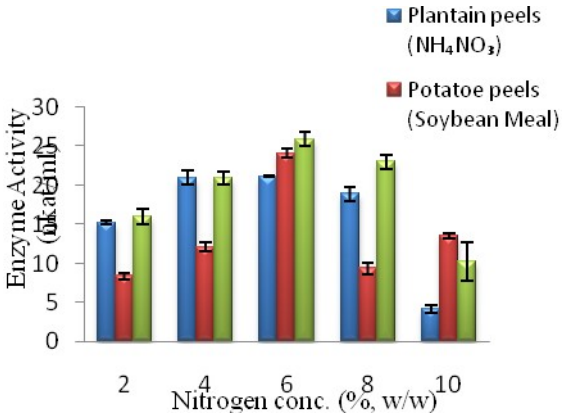

Figure 8: Effect of the addition of different nitrogen source on alkaline β-mannosidase production by *Bacillus* sp.3A during the solid state fermentation of different agro wastes.

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