

Banana peel as substrate for α -amylase production using *Aspergillus niger* NCIM 616 and process optimization

P Radha Krishna, A K Srivastava, N K Ramaswamy, P Suprasanna* and S F D'Souza

Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

Received 8 January 2011; revised 21 July 2011; accepted 20 October 2011

Amylases are well known for applications ranging from starch and food processes industry to medical applications. In the present study, the potential of banana peel was evaluated for α -amylase production using the fungal culture of *Aspergillus niger* NCIM 616 in solid submerged (SmF) and solid state (SSF) fermentation. The effect of different parameters, such as, substrate concentration, water content, layer thickness and external salt addition was studied in terms of the amylase activity. In SmF, 25% peel concentration was found optimum for the maximal amylase activity; while in SSF, 30% water content in the 3 cm thick substrate bed was optimum. The activity was also influenced by the addition of external salts and the combination of 1% NH_4NO_3 with 0.5% CaCl_2 was found to have maximal effect. A comparative evaluation of two different fermentation systems showed 80% higher specific activity of α -amylase in SSF as compared to that in SmF, which clearly demonstrated the superiority of the SSF system. The study suggests that banana peel could be used as a potential raw material for α -amylase production.

Keywords: Agro-industrial waste, amylase, banana peel, process optimization, solid state fermentation, solid submerged fermentation

Introduction

Banana is grown extensively in the tropical and subtropical countries. At the global level, the banana production is estimated around 72.5 million metric tonnes, of which 21.77 million metric tonnes is contributed by India. Since storage and postharvest losses are critically high, considerable interest has been generated, in the recent years, for value addition to banana, such as, the production of banana juice, banana wine, banana powder, banana chips, etc. During the production of these products, the banana peel accumulates in bulk posing serious environmental problems. To prevent this, it has become necessary to develop alternative, commercial application of these agro-industrial wastes. To this endeavour, efforts have been made to use banana peel for large scale production of alcohol² and enzymes, such as, laccases³ and amylases⁴.

α -Amylase is one of the important and well-known industrial enzyme that can cause the breakdown of starch or glycogen. Use of microorganisms for the production of amylases is economical as microbes are easy to manipulate to obtain enzymes of desired

characteristics⁵. Amylases stand out as a class of enzymes useful in the food, brewing, textile, detergent and pharmaceutical industries^{6,7}. They are mainly employed for starch liquefaction, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. They are also applied during detergent production to improve cleaning effect and are also used for starch de-sizing in textile industry⁸.

Amylases can be produced either by submerged fermentation (SmF)⁹⁻¹¹ or solid state fermentation (SSF)¹² procedures. Although the conventional amylase production was carried out by SmF, SSF system offers advantages, such as, high volumetric productivity, high concentration of product with less effluent generation and utilization of simple fermentation equipments^{13,14}. Since the substrates used in SSF are mainly from agro-industrial residues, this process will also resolve the pollution problem that would arise because of their disposal^{15,16}.

A wide range of microbes, such as, bacteria and fungi, are used for the industrial production of amylases. However, fungus is preferred over bacterium for enzyme production because of its filamentous nature, which helps in its penetration through the solid substrate¹⁷. Another advantage associated, specially with *Aspergillus niger* (an acidifient mould), is the control over bacterial

*Author for correspondence:

Tel: +91-9522-25595423 ext. 3870; Fax: +91-9522-25505151

E-mail: prasanna@barc.gov.in

contamination due to its capacity to tolerate high degree of acidity ($\text{pH} < 3$)¹⁸. The present study was aimed to exploit locally available and inexpensive banana peel for amylase production using *A. niger* NCIM 616 (ATCC No. 10864). Different parameters, such as, moisture in substrate bed, substrate concentration, layer thickness and effect of external salt, were optimized for the production of amylase from banana peel either through SmF or SSF.

Materials and Methods

Fungal Culture and Inoculum Preparation

Amylase producing culture of *A. niger* NCIM 616 was used throughout the study. The medium containing starch (10 g L^{-1}), NH_4NO_3 (1 g L^{-1}), yeast extract (5 g L^{-1}), Na_2HPO_4 (3 g L^{-1}) and CaCl_2 (1 g L^{-1}) at pH 5.5 with/without 2% agar was used for the growth and maintenance of *A. niger*. The culture of *A. niger* was incubated at 37°C for 7 d and then stored at 4°C . The stored cultures were sub-cultured regularly at every 2 months to maintain viability.

Inoculum was prepared by sub-culturing the fungus from the slant into liquid broth in a 25 mL conical flask with 5 mL of culture medium. A range of inoculums concentrations (0.5-5%) were screened to find the optimum concentration (1%). This was used for both SmF and SSF.

Processing of Banana Peel Substrate

The ripen Cavendish banana purchased from local market was peeled off and the pulp (fruit) was separated. The peels were cleaned by washing in tap water and were cut into small pieces followed by homogenization in a warring blender. Little amount of water [$\approx 10\%$ (W/V)] was added to facilitate the homogenization. The paste of banana peel was autoclaved to remove the microbial contamination and to inactivate the activity of polyphenoloxidase and other enzymes. Unless used, the sterilized paste of peel was stored at $2-4^\circ\text{C}$.

Extraction of Crude Amylase and Measurement of Amylase Activity

A small amount (approx. 1 g) of fermenting sample of peel was isolated under sterile condition into 1.5 mL eppendorf tube. To this, 0.5 mL of distilled water (DW) was added to extract the enzyme. Tubes were then centrifuged to collect the supernatant that was assayed for enzyme activity.

α -Amylase activity was determined following the method¹⁹. The reaction mixture consisted of 0.2 mL of

0.1% starch solution prepared in acetate buffer and enzyme extract (30 μL). The volume was made up to 0.3 mL using DW (70 μL). This mixture was incubated for 10 min at 45°C . To stop the reaction, 1 mL of 0.5 M acetic acid was added and then 0.7 mL of 0.3 mM iodine/KI reagent was added. The α -amylase activity was determined by measuring the hydrolysis of starch using iodine solution through measurement of absorbance at 700 nm. An enzyme unit is defined as the amount of starch hydrolyzed per mL of enzyme per min per g peel. Protein content of the supernatant was estimated following Lowry²⁰ method using bovine serum albumin as standard. Protein concentration was expressed in mg g^{-1} peel.

Production of α -Amylase under SmF

SmF fermentation is the process where the solid material is used as substrate in liquid medium. The SmF experiments were performed in 250 mL Erlenmeyer flasks, containing banana peel paste (20 g) with 1 mL of inoculum (culture of *A. niger* NCIM 616) and cultures were incubated at 37°C on a rotator shaker (120-150 rpm). The fermenting peel was isolated at different time points for the measurement of the α -amylase activity.

Production of α -Amylase under SSF

SSF is the process where also solid substrate is used but with minimum liquid portion in fermentation medium. The SSF was carried out in sterile Petriplates (15 cm), in which the banana peel paste of variable thickness and water content was planted under sterile condition. These plates were inoculated with 2 mL of inoculum and then incubated at 37°C . The plates were continuously aerated at $24 \text{ dm}^3 \text{ h}^{-1}$ with an air that was moistened using a bubble column filled with water at 37°C and filter sterilized using $0.2 \mu\text{m}$ hydrophobic membrane.

Optimization Studies for Production of Amylase

The growth medium containing banana peel was fermented with *A. niger* for the optimization of different parameters, such as, water content, substrate layer thickness and external salt for amylase production. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained at its optimum level in the subsequent experiments.

SmF

Effect of Peel Concentration

The optimum concentration of the peel was determined by measuring the amylase activity under

different peel concentration ranging from 10-50%. Different peel concentration was prepared independently and then inoculated with *A. niger* culture. The amylase activity was determined after 3 d of fermentation.

Effect of External Salt

At the constant peel concentration (25%), the effect of external salts was evaluated. Four different salt combinations, such as, S-1 (1.0% NH_4NO_3); S-2 (0.5% NH_4NO_3); S-3 (0.5% CaCl_2) and S-4 (1.0% NH_4NO_3 +0.5% CaCl_2) were prepared and mixed independently with the substrate peel. Substrate devoid of any salt was maintained as a control. All the sets were inoculated with the *A. niger* culture and then allowed to ferment on 37°C at 150 rpm. After 3 d, the amylase activity was determined.

SSF

Effect of Layer Thickness

The effect of different thickness of substrate layer was studied to evaluate the optimum layer thickness for the amylase production. Three different substrate beds were prepared with the thickness of 1, 3 and 5 cm. All the beds were inoculated with 1% of the viable inoculum of *A. niger* under sterile condition and then allowed to ferment for 6 d. At an interval of 24 h, samples were collected from all the sets for the measurement of the amylase activity.

Effect of Water Content

In order to evaluate the effect of initial moisture content in the bed, different peel homogenates were prepared containing variable water content (15, 20, 30, 35, 40 & 50%). The initial moisture level was adjusted in the substrate with the addition of sterile DW to attain the required level before spreading the layer onto the tray. All the substrate beds with different water content but with the constant layer thickness (5 cm) were inoculated separately with the *A. niger* culture and then α -amylase activity was determined at the end of 3d.

Effect of External Salt

For studying the effect of external salt supplementation, the substrate bed was independently mixed with various salt combination (same as in SmF) and then spread onto the tray. The 5 cm thickness and 30% water content was maintained for all the salt combination. The *A. niger* culture was inoculated in all the sets and the amylase activity was measured at the end of 3d.

Results and Discussion

In recent past, great interest has been shown for value addition to agro-industrial biowastes in order to develop industrial products, such as, enzymes, organic acids, mushrooms, flavour and aroma compounds, pigments, polysaccharides, hormones and animal feeds¹⁵. The selection of an ideal agro-biotech waste for enzyme production in a SSF process depends upon several factors related to availability and cost of the substrate material and, thus, involves screening of several agro-industrial residues^{15,16}. In the present study, we have studied the potential of banana peel to be used as a substrate for the production of amylase from *A. niger* through SmF and SSF.

Banana peel contains high concentration of carbohydrates (59%) and crude fibre (31.7%), which makes it a good source of carbohydrates. Most of the carbohydrates are in the form of starch²⁸. Apart from this, peel also contains minerals like calcium (19.2 mg/g) and potassium (78.1 mg/g)²⁸. Since calcium is required for α -amylase activity, banana peel makes a good substrate for α -amylase production. In the present study, we have tested the potential of banana peel as a substrate for the production of amylase from *A. niger* through SmF and SSF. The main difference between these two types of solid fermentations is the moisture level. In SmF the peel concentration was less and the water level was more, and it was performed in conical flasks on continuous shaking condition; whereas in the SSF, the moisture level was very less and this was done in glass petriplates and on stable platform with surface aeration without shaking.

Initial substrate and moisture content are the crucial factors that determine the enzyme production^{17,21,22}. Thus, different substrate concentrations and water contents in fermentation media were evaluated to establish their optimum level that can support the better growth of organism and better substrate utilization leading to the maximum enzyme production. *A. niger* was found to require considerably lower moisture levels, which could be due to the greater water holding capacity of banana peel substrate. The optimum peel concentration for SmF (Fig. 1) and water content for SSF (Fig. 2A) for the maximum amylase production were found to be 25 and 30%, respectively. Under this moisture content, the swelling of substrate was found appropriate and, therefore, their utilization was facilitated by the microorganisms. Further, the inoculum level was also found to be an important

factor for the production of amylase. The 1% inoculum for the peel paste was found optimum for both SmF (v/v) and SSF (v/w) for the amylase production. The higher inoculum concentration (beyond 1%) results in increased moisture content and excess unabsorbed liquid acts as an additional diffusion barrier, which ultimately decreases the

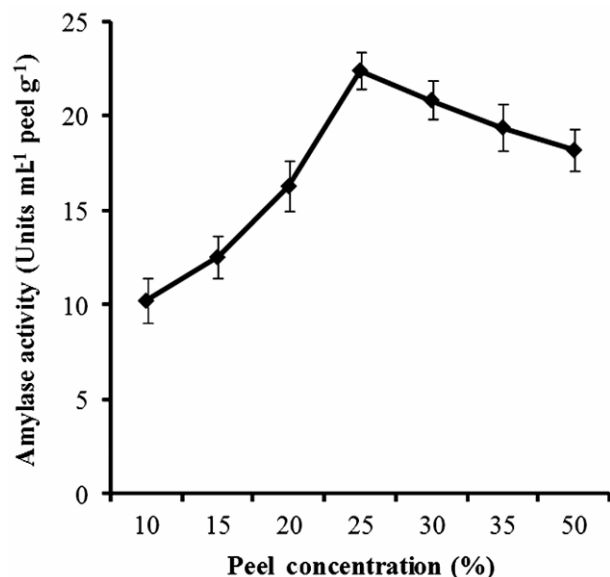


Fig. 1—Effect of different peel concentration on the amylase activity in SmF. [The values represent the mean of six technical and three biological replicates ($\pm$ SD); Differences in the mean values were found to be statistically significant at P , 0.01 (one-way ANOVA)]

growth of the organism as well as the enzyme production. The increased moisture level also causes the loosening of particle structure and decreases the porosity of the substrate, which negatively affects the aeration in the substrate²³.

The optimum time required for the maximum enzyme production was studied. The initial water content was adjusted to 30% in SSF and amylase activity was measured at different time periods. The enzyme activity was found increased in a time dependent manner and the maximum activity was observed on 3rd d of growth (Fig. 2B). This indicates that enzyme production was dependent upon the growth phase of the organism²⁴. The decrease in production of enzyme after 3d could be attributed to the decrease in the growth rate of organism, which could have been due to the limitation in the availability of nutrient resources. The production of the secondary metabolites in the stationary phase of growth might also contribute to decrease the enzyme activity.

In SSF, the different thickness of substrate layer was employed to monitor its effect on the enzyme production. The layer thickness of 3 cm was found optimum for maximum enzyme production. The sub (1 cm)/supra (5 cm) optimal thickness resulted in the lower activity (Fig. 2B). This could be explained because the increase in the layer thickness could reduce the microbial respiration/aeration and, therefore, resulted in poor growth. The increase in the

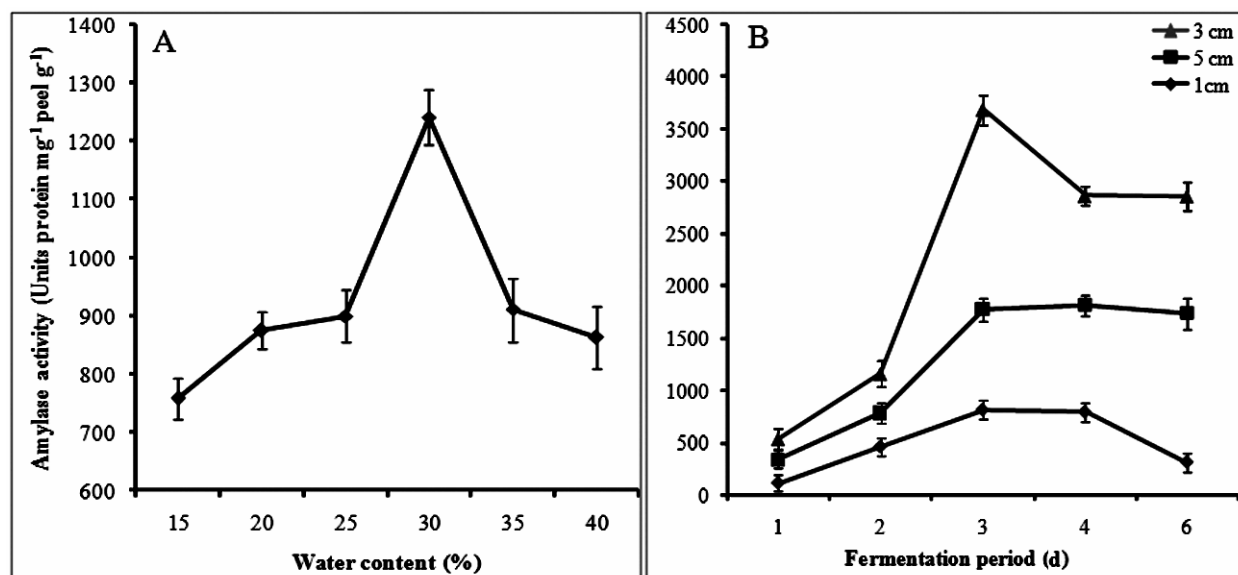


Fig. 2 (A & B)—Effect of different water content (A) and substrate layer thickness (B) on the amylase activity in SSF. [The values represent the mean of six technical and three biological replicates ($\pm$ SD); Differences in the mean values were found to be statistically significant at P , 0.01 (one-way ANOVA)]

substrate thickness could also decrease the diffusion of the product which could decrease the enzyme activity due to the feedback inhibition. On the contrary, the lower thickness might not be able to support the complete growth of fungal mycelia²⁵.

The effect of different combination of external salts, such as, NH_4NO_3 and CaCl_2 , was studied to know their impact on amylase production. Ammonium nitrate was chosen because of its ability to serve as a cheap source of nitrogen for *Aspergillus* species²⁶. Since, the amylase activity is regulated by calcium²⁷, the effect of external calcium chloride was also studied. For all the combinations, the amylase activity in SSF was found higher than that in SmF. In both SmF and SSF, the maximum enzyme activity was observed when ammonium nitrate (1%) was used along with calcium chloride (0.5%) (Fig. 3). However, the enzyme activity in SSF was 80% higher as compared to that in SmF. The lower enzyme activity in SmF was found to be associated with the increased production of non-specific proteins (data not shown). Thus, the comparative analysis of SmF and SSF data clearly suggest that SSF can be applied as a method of choice for the amylase production. Apart from this, another advantage of using the SSF for agro-industrial waste is its unique

buffering capacity which rules out the need of any pH adjustment of the substrate¹⁴. In view of this, the initial and final pH (after different periods of SSF) of the substrates was also analysed and good buffering capacity was recorded as there were only marginal or no changes in the pH values (data not shown). This further advocates the potential advantage of using banana peel for the production of amylase, as it does not require any extra effort to control pH of the medium.

In summary, the present work suggests that banana peel could be employed as a promising substrate for the production of amylase by *A. niger*. Further, SSF is a better choice compared to SmF for amylase production and it can be employed with 3 cm substrate layer thickness and 30% water content for optimal production of enzyme with in short fermentation period of 3 d. The addition of external salt is also found beneficial for increasing the enzyme production.

References

- 1 Surendranathan K K, Post-harvest biotechnology of fruits with special reference to banana—Perspective and scope, *Indian J Biotechnol*, 4 (2005) 39-46.
- 2 Tewari H K, Marwaha S S & Rupal K, Ethanol from banana peels, *Agric Wastes*, 16 (1986) 135-146.
- 3 Johann F O, Jose L, Herrera T & Couto S R, Banana skin: A novel waste for laccase production by *Trametes pubescens* under solid-state conditions. Application to synthetic dye decolouration, *Dyes Pigm*, 75 (2007) 32-37.
- 4 Shaista K, Asghar M, Rehman K, Asad M J & Adedeyo O, Bio-processing of banana peel for alpha amylase production by *Bacillus subtilis*, *Int J Agric Biol*, 5 (2003) 36-39.
- 5 Aiyer P V, Amylases and their applications, *Afr J Biotechnol*, 4 (2005) 1525-1529.
- 6 Rosell C M, Haros M, Escrivá C & Benedito D C, Experimental approach to optimize the use of alpha-amylases in breadmaking, *J Agric Food Chem*, 49(2001) 2973-2977.
- 7 Pandey A, Nigam P, Soccol C R, Soccol V T, Singh D *et al*, Advances in microbial amylases, *Biotechnol Appl Biochem*, 31 (2000) 135-152.
- 8 Lonsane B K & Ramesh M V, Production of fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis, *Adv Appl Microbiol*, 35 (1990) 1-56.
- 9 Aguilar C N, Torres E F, Gonzalez G V & Augur C, Culture conditions dictate protease and tannase production in submerged and solid-state cultures of *Aspergillus niger* NCIM Aa-20, *Appl Biochem Biotechnol*, 103 (2002) 407-414.
- 10 Bose K & Das D, Thermostable alpha-amylase production using *Bacillus licheniformis* NRRLB 14368, *Indian J Exp Biol*, 34 (1996) 1279-1282.
- 11 Egas M C, Da Costa M S, Cowan D A & Pires E M, Extracellular alpha-amylase from *Thermus filiformis* Ork A2: Purification and biochemical characterization, *Extremophiles*, 2 (1998) 23-32.

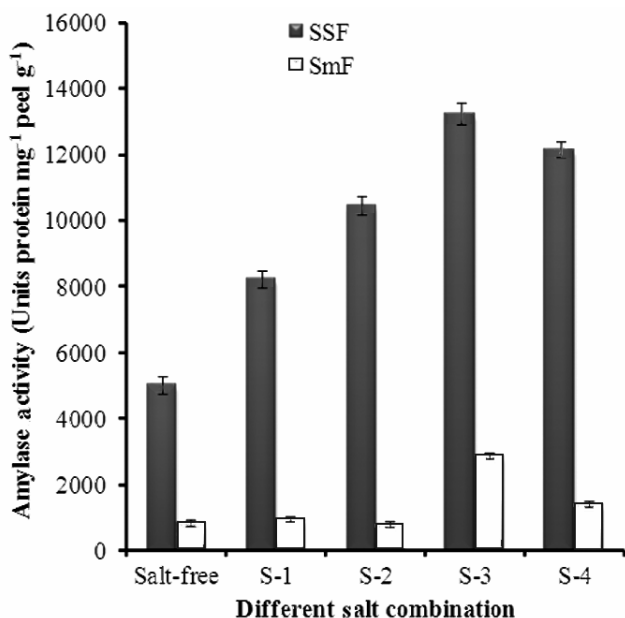


Fig. 3—Effect of external salt on the amylase activity in SmF/SSF: S-1, 1% NH_4NO_3 ; S-2, 0.5% NH_4NO_3 ; S-3, 0.5% CaCl_2 ; & S-4, 0.1% NH_4NO_3 +0.5% CaCl_2 . [The values represent the mean of six technical and three biological replicates ($\pm$ SD); Differences in the mean values were found to be statistically significant at P, 0.01 (one-way ANOVA)]

- 12 Viswanathan P & Surlikar N R., Production of alpha-amylase with *Aspergillus flavus* on Amaranthus grains by solid-state fermentation, *J Basic Microbiol*, 41 (2001) 57-64.
- 13 Pandey A, Selvakumar P, Soccol C R & Nigam P, Solid state fermentation for the production of industrial enzymes, *Curr Sci*, 77 (1999) 149-162.
- 14 Pandey A, Soccol C R & Mitchell D, New developments in solid-state fermentation, I: Bioprocesses and applications, *Process Biochem*, 35 (2000) 1153-1169.
- 15 Pandey A, Soccol C R, Nigam P & Soccol V T, Biotechnological potential of agro-industrial residues I: Sugarcane bagasse, *Bioresour Technol*, 74 (2000) 69-80.
- 16 Pandey A, Soccol C R, Nigam P, Soccol V T, Vandenberg L *et al*, Biotechnological potential of agro-industrial residues II: Cassava bagasse, *Bioresour Technol*, 74 (2000) 81-87.
- 17 Ramachandran S, Patel A K, Nampoothiri K M, Francis F, Nagy V *et al*, Coconut oil cake—A potential raw material for the production of α -amylase, *Bioresour Technol*, 93 (2004) 169-174.
- 18 Raimbault M, Fermentation en milieu solide: Croissance de champignons filamenteux sur substrats amylacés, *ORSTOM-Paris*, 127 (1983) p 291.
- 19 Machiah J P & Vakil U K, Isolation and partial characterization of α -amylase components evolved during early wheat germination, *J Biosci*, 6 (1984) 47-59.
- 20 Lowry O H, Roenbrough N J, Farr A L & Randal E J, Protein measurement with the folin phenol reagent, *J Biol Chem*, 193 (1951) 265-275.
- 21 Ramesh M V & Lonsane B K, Critical importance of moisture content of the medium in alpha amylase production by *Bacillus licheniformis* M 27 in a solid state fermentation system, *Appl. Microbial. Biotechnol.* 33 (1990) 501-505.
- 22 Pandey A, Ashakumary L, Selvakumar P & Vijayalakshmi K S, Influence of water activity on growth and activity of *Aspergillus niger* NCIM 616 for glucoamylase production in solid state fermentation, *World J Microbiol Biotechnol*, 10 (1994) 485-486.
- 23 Lonsane B K, Ghildyal N P, Budiatman S & Ramakrishna S V, Engineering aspects of solid state fermentation, *Enzyme Microb Technol*, 7 (1985) 258-265.
- 24 Sudo S, Ishikawa T, Sato K & Oba T, Comparison of acid stable α -amylase production by *Aspergillus kawachii* in solid-state and submerged cultures, *J Ferment Bioeng*, 77 (1994) 484-489.
- 25 Yovita S P, Rahardjo, Weber F J, Haemers S, Tramper J *et al*, Aerial mycelia of *Aspergillus oryzae* accelerate α -amylase production in a model solid-state fermentation system, *Enzyme Microb Technol*, 36 (2005) 900-902.
- 26 Kundu A K, Das S & Gupta T K, Influence of culture and nutritional conditions on the production of amylase by the submerged culture of *Aspergillus oryzae*, *J Ferment Technol*, 51 (1973) 142-150.
- 27 Haki G D & Rakshit S K, Developments in industrially important thermostable enzymes: A review, *Bioresour Technol*, 89 (2003) 17-34.
- 28 Anhwange B A, Ugye T J & Nyiaatagher T D, Chemical composition of *Musa Sapientum* (Banana) peels, *EJEAF Che*, 8 (2009) 437-442.