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# Biotechnological Potential of Alpha Amylase Production by *Bacillus subtilis* Using Cassava Peel Powder as a Substrate

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author EAB, who is the senior scientist, conceived, designed as well as coordinated the study, performed the statistical analysis and drafted the manuscript. Author HB participated in the design of the study, collection of the experimental materials and helped with observations and analytical procedures. Both authors read and approved the final manuscript.*

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## ABSTRACT

Cassava peels, a major agricultural waste associated with the processing of cassava tubers into value-added products such as industrial starch and derived food items including garri and foofoo, were used as raw material for the production of α-amylase. The major step of the process is solid state fermentation of the mash prepared from this by-product by *Bacillus subtilis* that was isolated from a solid municipal waste disposal site. The effects of varying durations of incubation, temperature, medium pH and substrate

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levels were characterized. The maximum α–amylase activity of 7.12 lU/ml/min was recorded in a medium containing 50g of dried cassava peel powder as substrate after 24 hours at a pH of 7.0 and temperature of 35℃. The crude α–amylase produced was confirmed by using it to hydrolyze industrial starch which yielded maltose, a demonstration that the enzyme produced can be used in different biotechnological processes. It can be concluded that the use of cassava peels as substrates for the production of α–amylase does not only add value and decrease the amount of this agro-industrial waste from the environment but also reduces the general cost of amylase production that is desired for various biotechnologically-based industrial applications.

*Keywords: α–amylase; Bacillus subtilis; cassava peels; solid state fermentation.*

## 1. INTRODUCTION

Alpha-amylase (α-1,4-glucan-4-glucanohydrolase) is a hydrolytic enzyme that hydrolyses the α-bonds of large α-linked polysaccharides such as starch and glycogen yielding glucose and maltose in the process. Presently the enzyme is one of those mostly sought after as it has great significance in biotechnology; constituting a class of industrial enzymes that controls about 25% of the world's total enzyme market [1,2].

Interestingly, α–amylase can be produced from several sources including plants, animals and microorganisms [3]. A large number of amylases derived mainly from microbes are currently available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industries. A major advantage of using microorganisms for the production of α-amylase is the economic bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes with desired characteristics. Moreover, amylases derived from microorganisms have a broad spectrum of industrial applications as they are more stable than those derived from plant and animal sources [4].

The enzyme belongs to a family of endo-amylases that can catalyze the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glucosidic bonds [3,5]. It has a three dimensional structure capable of binding to substrate and by the action of highly specific catalytic groups, promote the breakage of the glycosidic links. With functions optimal at pH 7, its single polypeptide of about 475 residues has two SH groups and four disulphide bridges with tightly bound divalent calcium ions [6,7], which are necessary for its stabilization.

Alpha-amylase has potential application in a wide variety of biotechnologically-based industrial processes such as in food, fermentation, textile, paper, detergent and pharmaceutical industries. It is a key enzyme in the conversion of starch to sugar syrups, preparation of digestive aids, and production of cyclodextrins, chocolates, cakes and fruit juices. With recent advances in biotechnology, α–amylase has equally found new applications in many other areas of human endeavour including, but not necessarily limited to, clinical, medicinal and analytical chemistry; with three other fastest growing areas of α-amylase application being in the fields of laundry and dish washing detergent production, starch saccharification and brewing and alcohol distillation industries [8].

Among the myriad of technologies currently available for the production of microbial enzymes, solid state fermentation appears to be one of the major processes for the

production of α–amylase with several advantages over submerged fermentation system. These include high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, etc. [9,10]. The process has been described as a microbial cultivation technique whereby an insoluble substrate is fermented with sufficient moisture but without free water [11]. In addition, this technique permits the use of different agricultural wastes as substrates with tremendous potential for the production of microbial enzymes.

Cassava (*Manihot esculenta* Crantz) represents one of the most important crops of the humid tropics, with a global annual production of more than 250 million tons [12]. Africa produces more than half of this global supply with the annual production from Nigeria alone (about 45 metric tons) representing more than a third of the total African output. Nigeria has consistently also been ranked as the world's largest producer of cassava since 2005 [13]. The crop has many important industrial uses including serving as raw material for the food, chemical, and pharmaceutical industries. Recently, cassava starch has equally been found to be a very promising raw material for the production of bio-ethanol and in the production of high quality biodegradable plastics [14].

Associated with the agro-industrial processing of the cassava tuber are the peels, which are a major agricultural waste product that contribute significantly to environmental pollution. It constitutes about 20 - 35% by weight of the original tuber, especially during hand peeling [15], and is found to contain about 41.8% carbohydrate, 4.8% protein, 1.25% ether extract, 4.2% total ash and 21.1% crude fibre [16] on a dry weight basis. Cassava peels also contain phytates and those derived from some cultivars have a high concentration of cyanogenic glycosides, which makes them unsuitable for direct consumption both as animal feed and by humans.

It has been estimated that over 450,000 metric tons of cassava peels are discarded annually from cassava processing plants across different parts of Nigeria alone [17]. Despite the large amounts produced, this rich organic material has received very little attention as a low-cost by-product and is usually discarded and allowed to rot without any value addition. The peels could serve as a potentially valuable resource for industrial exploitation in the production of several value-added products such as organic acids, flavour and aroma compounds, and microbial enzymes that can be applied for various industrial processes or for use as food or feed after an appropriate biological treatment. Consequently, the current study was designed to characterize different parameters that could be optimized for α-amylase production by *Bacillus subtilis* (a gram positive, rod shaped bacterium) under solid substrate fermentation using an inexpensive and abundantly available agro-residue as substrate, thereby reducing the cost of enzyme production through biotechnological means.

## 2. MATERIALS AND METHODS

### 2.1 Raw Material and Substrate Preparation

Cassava peels were collected from an agro-industrial processing factory in Boje, Boki Local Government Area of Cross River State, Nigeria. The peels were washed thoroughly under running tap water to remove soil and dirt. The outer brownish scales were removed and discarded while what was left of the peel was dried and pulverized into a fine powder using a blending machine.

## 2.2 Preparation of Inoculum and Culture Conditions

*Bacillus subtilis* was isolated from a soil sample taken from a municipal solid waste disposal site in Calabar, Cross River State, Nigeria and cultured on a nutrient agar slant containing 0.05% peptone, 0.3% yeast extract, 0.05% NaCl, 1.5% agar (w/v) and distilled water at a pH of 7.0. The cultures were maintained in an incubator at 35℃ in the Tissue Culture Laboratory at the Department of Genetics & Biotechnology, University of Calabar, Calabar, Nigeria.

A few bacterial colonies were isolated from the nutrient agar slant and transferred aseptically to a 500ml Erlenmeyer flask containing 100ml of pre-sterilized inoculum medium supplemented with glucose (2g), yeast extract (0.3g), peptone (0.05g), NaCl (1.5g), $Na_2HPO_4.2H_2O$ (1.1g), $NaH_2PO_4.2H_2O$ (0.61g), KCl (0.3g), and $MgSO_4.7H_2O$ (0.01g) in a laminar air-flow bench. The flask was maintained on a shaker (120rpm) at 37℃ for 24 hours and the homogenous spore suspension was used as inoculum thereafter according to Kokab et al. [18].

## 2.3 Fermentation of Substrate

Varying concentrations of the dried cassava peel powder (as substrate) were weighed out in quintuplets into 500ml Erlenmeyer flasks, to which were added about 100ml of sterile distilled water to moisten the mash. The pH of the medium was adjusted to 7.0 with HCl and sterilized in an autoclave for 15 minutes at 121℃. The substrate was allowed to cool after which 5ml of the inoculum was added to each flask in the laminar air-flow bench with a sterilized pipette. The flasks containing cassava mash and the bacterial inoculum were incubated at 35℃ in an oven for 24 hours. During this period, all flasks were gently shaken after every 12hours for uniform mixing of the substrate and microorganism.

## 2.4 Optimization of Process Parameters

The growth medium of cassava peel powder was fermented with *B. subtilis* for optimization of different parameters for α-amylase production.

### 2.4.1 Incubation period

Growth medium containing 50g of cassava peel powder was incubated at varying durations (12, 24, 36, 48 and 60 hours) at 35℃ and pH of 7.0.

### 2.4.2 pH of medium

The pH of the cassava peel powder medium was adjusted at different levels to 3, 4, 5, 6, 7 and 8 before inoculation and incubated at 35℃ for 24 hours.

### 2.4.3 Substrate concentration

Erlenmeyer flasks containing different substrate levels (20 -100g) were inoculated and incubated for 24 hours at pH 7 and 35℃.

### 2.4.4 Incubation temperature

Solid state fermentation media of cassava peel powder (50g) were inoculated and incubated at pH 7.0 under different temperatures (between 20 and 60℃) for 24 hours.

### 2.4.5 Preparation of amylase

Alpha-amylase was extracted from the solid state fermentation medium using a simple contact method according to Ramesh and Lonsane [19]. After a specified incubation period, 100ml of phosphate buffer at pH 6.9 was added into each experimental flask. The flasks were shaken at 150rpm for 30 minutes and the culture filtered through Whatman filter paper (No. 1). The filtrate was also passed through a 0.45 mm Millipore filter and centrifuged at 1000rpm for 10 minutes at 10℃ to obtain a clear supernatant, which was carefully collected and used as enzyme preparation and source for the enzyme assay analysis.

### 2.4.6 Enzyme assay

Alpha-amylase activity was determined by the spectrophotometric method described by Bernfeld [20]. In an assay mixture containing enzyme extract, industrial starch as substrate and 3,5-dinitrosalicylic acid (DNSA) as coupling reagent. One unit of α-amylase activity was defined as the number of micromoles of maltose liberated by 1ml of enzyme solution per minute, under assay conditions of pH 7 and incubation temperature of 37℃ with phosphate buffer solution.

The spectrophotometer was adjusted at 540nm and 25℃. A maltose standard curve was prepared using maltose stock solution as follows: 9 maltose dilutions were prepared ranging from 0.1 to 0.9 g/ml and two blank tubes with reagent grade water only were included. One (1) ml of DNSA colour reagent was added and the tubes were incubated in a boiling water bath for 5 minutes and cooled to room temperature. One hundred (100) ml of reagent grade water was added to each tube and mixed properly and the spectrophotometric reading of the mixture was obtained.

### 2.4.7 Estimation of amylase activity

The maltose test was undertaken as a confirmation for the presence of reducing sugar after enzyme extract was used to hydrolyze starch using Benedict's solution.

Enzyme activity was determined using the formula:

$$\text{Units/mg} = \frac{\text{Micromoles maltose released}}{\text{3 minutes x mg enzyme in reacting mixture}}$$

### 2.4.8 Data collection and statistical analysis

Data collected was based on the following parameters: incubation period, substrate level, pH of medium and incubation temperature. Data collected were subjected to analysis of variance test (ANOVA) while significant means were separated using least significant differences (LSD).

## 3. RESULTS

The results of a series of tests conducted to optimize different parameters for the production of α-amylase are summarized below.

### 3.1 Substrate Characteristics

*B. subtilis* showed good growth on the cassava peel powder medium containing 80ml of distilled water (80%). Cassava peel powder was also a good medium for α-amylase production.

### 3.2 Effect of Substrate Concentration

Fermentation medium containing 20, 30, 40, 50, 60, 70, 80, 90 and 100g, respectively, of cassava peel powder was sterilized, inoculated with 5ml of inoculum suspension containing *B. subtilis* at a temperature of 35℃ for 24 hours at pH of 7.0. It was observed that 50g cassava peel powder yielded the highest mean α-amylase activity of 6.81 lU/ml/min with the enzyme extract harvested at 24 hours (see Fig. 1).

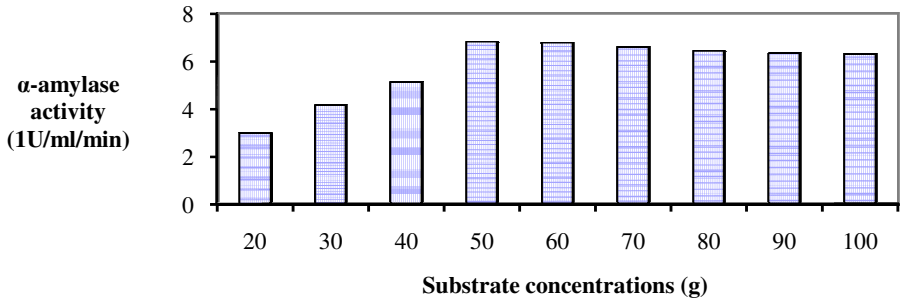


**Fig. 1. Mean α–amylase activity for varying substrate concentrations**

### 3.3 Effect of Incubation Duration

The mean values for α-amylase activity derived from duplicate flasks containing 50g of cassava peel powder that had been autoclaved, allowed to cool down and inoculated with 5ml of inoculum suspension containing *B. subtilis* at a temperature of 35℃ for 12, 24, 36, 48 and 60 hours, respectively, are presented in Fig. 2. The maximum α-amylase activity of 6.56 1U/ml/min was observed with the enzyme extract harvested at 24 hours at a pH of 7.0 and temperature of 35℃.

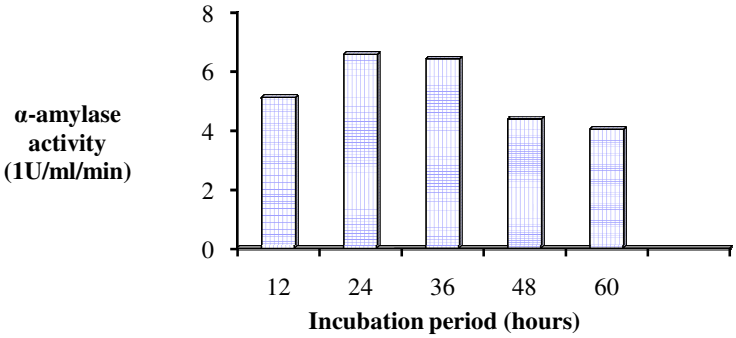


**Fig. 2. Mean α–amylase activity for varying durations of incubation**

## 3.4 Effect of Initial pH on Enzyme Production

The effect of pH on amylase activity produced by *B. subtilis* was studied by varying the pH from 3.0 to 8.0. The results are depicted in Fig. 3 and indicate that with an increase in pH from 3 to 7, the activities of amylase increased until reaching a maximum value which was followed by a gradual decrease thereafter at pH 8. It is thus obvious that a pH of 7 was found to be best for α–amylase activity with the highest mean enzyme activity of 6.94 IU/ml/min observed in the fermentation medium (50g) at a temperature of 35℃ after 24 hours of incubation (see Fig. 3).

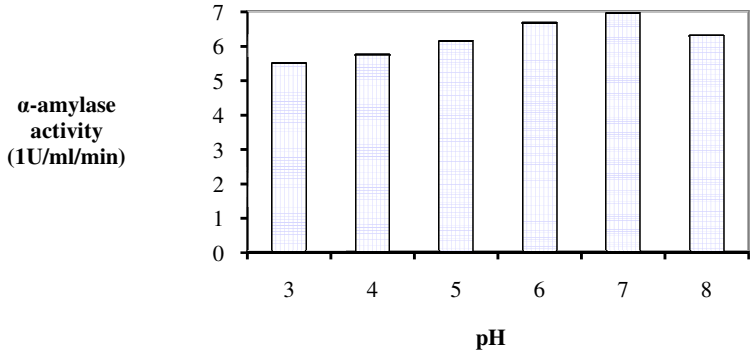


**Fig. 3. Mean α–amylase activity for varying levels of pH in the medium**

## 3.5 Effect of Incubation Temperature on Enzyme Production

Incubation temperature not only influences the growth of microorganisms but also their biological activities. Consequently, the effect of temperature on α–amylase production by *B. subtilis* was evaluated by varying the temperature from 20℃ to 60℃ and the results are presented in Fig. 4.

In the current study, the α–amylase produced by *B. subtilis* showed considerable enzyme activity at the range of 20℃ to 60℃. It was observed that enzyme activities were low at temperatures below 30℃ but showed a gradual increase at temperatures up to 35℃ before declining thereafter. Fig. 4 shows that with cassava peels as substrates, the enzyme activity increased up to 5.19 IU/ml/min at 35℃ but dropped tremendously from 4.56 to 3.68 IU/ml/min at 45℃ and 60℃, respectively. Overall, the highest α–amylase activity was verified at 35℃, corresponding to 7.12 IU/ml/min in a medium containing 50g of substrate that was incubated for 24 hours at pH of 7.0.

## 3.6 Maltose Test

Test for maltose gave a positive result of orange coloured precipitate when varying concentrations of starch were hydrolyzed by 1ml of crude α–amylase produced. The standard curve obtained from spectrophotometric absorbance of maltose is given in Fig. 5.

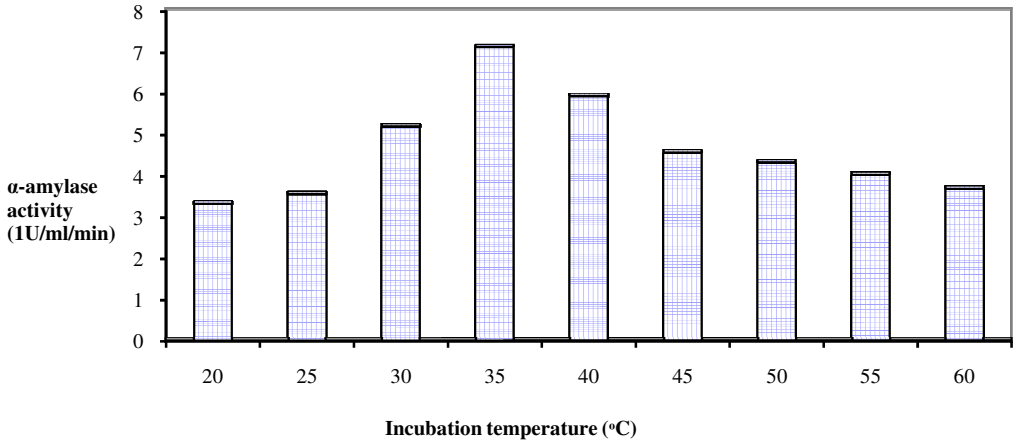


**Fig. 4. Effect of varying incubation temperatures on mean α–amylase activity**

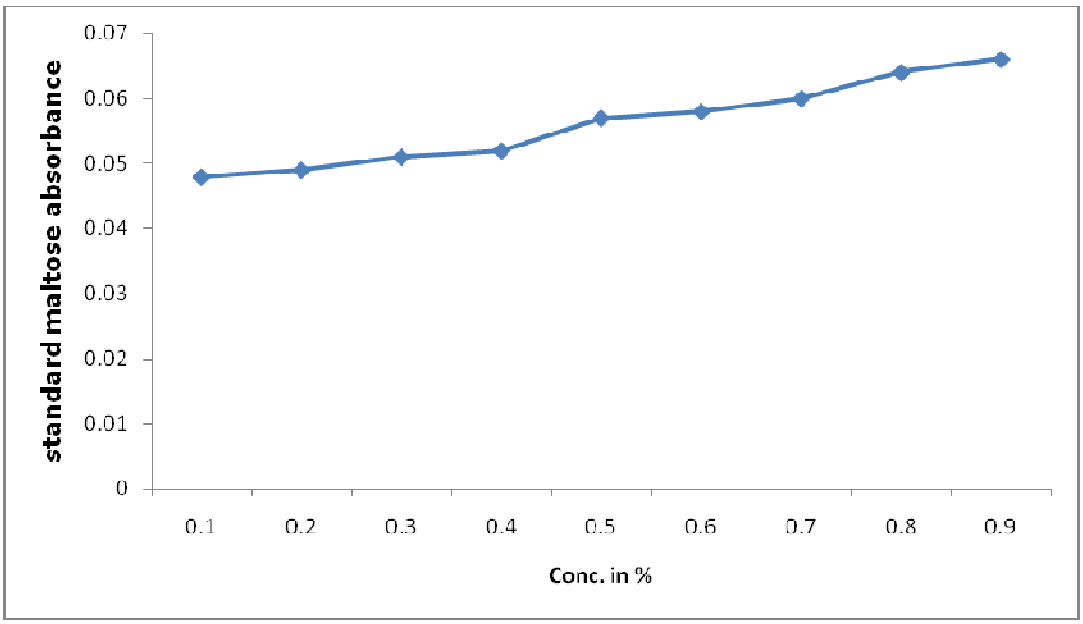


**Fig. 5. Maltose standard curve**

## 4. DISCUSSION

There has been an increasing trend towards more efficient utilization of the by-products from agro-industrial processing plants such as corn cob, tomato pomace, citrus pulp, sugarcane bagasse, banana peels, cassava peels, etc., which hitherto have posed a serious environmental problem in their disposal. In most cases these materials are dried and burnt, producing a strong offensive smell or emit harmful gases that increase global warming or they are simply allowed to decompose, a process that is not environmentally friendly as it often results in surface water pollution. In recent years, however, several biotechnological

processes, especially in the area of microbial fermentation technology, have been developed that utilize these agricultural wastes as a major source of raw materials for the production of bulk chemicals and value-added industrial products including ethanol, organic acids, enzymes, amino acids, etc.

The current study was developed with this objective in mind and had two major findings of immense significance. First, cassava peels that are usually considered as a problematic agricultural waste were utilized as raw material for the production of α-amylase. Second, it was possible to isolate a strain of *B. subtilis* from a municipal waste dump site that could be utilized for the production of an industrial enzyme. Taken together, these results are fascinating as they do not only serve as proof that cassava peels can be utilized as raw material for optimizing the production of α-amylase but also uniquely addresses the issue of environmental pollution arising from their disposal.

In order to achieve these objectives, however, several conditions as outlined herein needed to be optimized. For example, optimization of the incubation duration was seen to be critical for the growth of the bacterial cells with significant alpha amylase productivity. An increase in the duration from 12 to 24 hours resulted in an increase in enzyme productivity. While enzyme activity was found to be low after 12 hours of incubation, probably due to the fact that the bacterial cells had just entered the lag phase of growth where they acclimatized with the environment, enzyme productivity gradually increased with an increase in the incubation duration from 12 to 24 hours, which was optimal perhaps on account of the fact that this was when the bacterial cells had entered the late exponential phase of growth [21]. Enzyme productivity remarkably declined following extension of the duration to 36, 48 and 60 hours, respectively, as illustrated in Fig. 1, possibly on account of the depletion of nutrients, accumulation of enzymatic by-products such as toxins and inhibitors [22,23].

Enzyme activity also increased in the fermentation media containing between 20g and 50g of substrate. There was no need to increase the amount of substrate beyond this level as this resulted in a decrease in enzyme activity (see Fig. 2). This result is in agreement with that of Shaista et al. [24], which yielded a maximum enzyme activity with 50g of banana peel medium at 24 hours, pH of 7.0 and 35°C while Krishna and Chandrasekaren [25], reported maximum amylase production by *B. substilis* using 10g of banana stalk medium moistened to 70% with mineral salts. Taken together, these results could be attributed to the fact that the levels of substrate used in these studies contained pretty high levels of nutrients, which may have been essential and adequate for *B. substilis* to produce alpha amylase.

There was also an increase in enzyme activity as the medium pH was increased from 4.0 to 7.0, which appeared to be the optimum. Of course, this is not surprising as different organisms have different pH optima and a decrease or increase on either side of the optimum results in poor microbial growth. It is thus possible that this pH range was the optimum for the *B. substilis* used in the current study, as also demonstrated by Terui [26] and Shaista et al. [24], who reported pH of 6.8 and 7.0, respectively, as being optimum for the production of α–amylase by *B. subtilis*.

A decrease or increase in incubation temperature causes a reduction in enzyme production by *B. subtilis* as seen in Fig. 4. The optimum temperature for the production of α–amylase from cassava peel powder was 35°C as also reported by Krishna and Chandraskaren [25] and Shaista et al. [24]. In itself, this is not surprising as a lower temperature would perhaps reduce the activities of the bacterium while higher temperatures would definitely lead to its inactivation.

## 5. CONCLUSION

In general, 50g cassava peel powder as substrate at an incubation temperature of 35℃, initial pH of 7, and incubation duration of 24 hours were found to be the optimal conditions for the biosynthesis of α-amylase in the culture medium used in the current study. It can thus be concluded that *B. subtilis*, a bacterial strain derived from a municipal solid waste disposal site can be used to produce α-amylase, an enzyme with several biotechnological applications in the food, pharmaceutical and medical industries. Apart from being a low cost substrate and good medium for the production of α-amylase, the use of cassava peels can be considered as a significant way to reduce enzyme production cost. Besides, it is noteworthy that the process will, no doubt, have a major environmental impact as it helps to resolve the problem associated with the disposal of cassava wastes. This will not only create a safe and friendly environment, especially in major cassava processing regions of the world, but could also be regarded as a biological resource of great economic importance.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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