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Production and optimization of extracellular α-amylase productivity from *Bacillus subtilis*

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A moderately thermophilic strain of *Bacillus subtilis* was isolated from agricultural soil at the National Root Crops Research Institute (NRCRI), Umudike, Umuahia, Nigeria. The organism produced a thermostable α-amylase in complex medium containing 2% (w/v) soluble starch. Peak amylase activity was obtained at 48 h of fermentation period which corresponded with the late exponential phase of the organism. Amylase activity was more strongly expressed with corn than soluble starch. Optimized medium containing 1.5% (w/v) corn starch increased amylase production to 6427±168 U/L against 4168±132 U/L obtained in the basal medium containing 2% (w/v) corn starch. The optimum temperature and pH of the enzyme were 65°C and 6.5 respectively.

Key words: *Bacillus subtilis*, amylolytic activity, submerged fermentation, α-amylase production, optimization.

INTRODUCTION

α-Amylase (EC 3.2.1.1) is a classical calcium containing enzyme that hydrolyses the endo-α-1,4 linkages of starch in a random manner, yielding glucose, maltose, maltotriose and other oligosaccharides (Senthilkumar et al., 2012). It is produced by bacterial species of *Bacillus* (Asgher et al., 2007), *Pseudomonas* (Shiau and Hung, 2003) and *Clostridium* (Kilic et al., 2005). Bacterial species such as *Bacillus subtilis* (Rajput et al., 2013), *Bacillus licheniformis* are generally preferred for the production of α-amylase because they appear to be very productive (Niazi et al., 2010). Although there are many microbial sources available for producing amylases, the capacity of *Bacillus* strains to produce large quantities of the enzymes has placed them among the most important industrial enzyme producers (Bakri et al., 2012). Increase in the production and utilization of amylases became apparent in the early 1960s when *B. subtilis* α-amylase and *Aspergillus niger* glucoamylase were used to replace acid hydrolysis in the production of dextrose from starch (Muralikrishna and Nirmala, 2005). In recent years, interest in microbial production of α-amylase has increased dramatically due to its wide spread use in food, textile, baking and detergent industries (Asgher et al., 2007). These uses have placed enormous stress on α-amylase production, stimulating the search for more diverse, efficient and cheap methods of production (Wolfgang, 2007).

Strain selection, optimization of physico-chemical parameters and medium composition are critical for improved yield of α-amylase and consequent cost

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reductions (Konsula and Liakopouloukyriakides, 2004). Utilization of low cost substrates such as agricultural and industrial by-products in production medium can make commercial production of enzymes economical (Tanyildizi and Ozer, 2011). Starch is the primary storage compound of a large number of economically important crops such as cassava, rice, corn, wheat, sweetpotato and potatoes (Hussain et al., 2013). It is an abundant source of carbohydrate (Hussain et al., 2013) and consists of amylose and amylopectin. Corn is a major cereal crop grown in tropical regions of the world. It provides major source of energy and dietary supplement among the average and low income groups in Nigeria. In Southern parts of Nigeria, corn is used in preparation of food, beverages and breakfast snacks. Among the various starches, corn, potato, sweet potato and cassava are the most abundant and relatively inexpensive (Ubalua, 2014). The use of corn starch as raw material is attractive because of the enormous postharvest losses associated with our agricultural produce and the ability to obtain high yield of corn from marginal agricultural soils in our country. The regular use of soluble starch-based fermentation media is not commercially viable for industries in the sub-Saharan Africa. For efficient commercial production effort is therefore necessary to explore cheaper substrate sources. In this study therefore, isolation and optimization of α-amylase from a moderately thermophilic strain of *B. subtilis* using these cheap native starches as carbon sources are reported.

MATERIALS AND METHODS

Sampling

Twenty (20) field soil samples from 5 different locations representing different agricultural soils at National Root Crops Research Institute (NRCRI) Umudike, Umuahia, Abia State, Nigeria were used for the analysis. Three samples from each location were collected at a distance of 200 m interval at depths of 5, 8 and 10 cm into sterile bottles with sterile hand shovel and labeled clearly. The soil samples were thereafter taken to NRCRI Microbiology laboratory. They were homogenized, mixed and sieved through a 2 mm pore size sieve (Retsch, Germany) to sieve out large debris and used for isolation and enumeration purposes (Aghamirian and Ghiasian, 2009).

Isolation of *Bacillus* colonies from the soil

Isolation and enumeration of the *Bacillus* were performed using dilution method with nutrient agar (Oxoid, Basingstoke, UK) medium. Ten (10) grams of each of the soil samples were suspended in 90 ml of 0.85% normal saline (pH 7.0) and shaken vigorously at 150 rpm and 18°C for 1 h according to Agrawal and Agrawal (2013). The resulting slurries were serially diluted with 0.85% normal saline. A 0.1 ml of the 10^{-4} dilution was spread plated in triplicate on nutrient agar (NA) medium. Cultures were incubated at 37±2°C for 24 h. Each colony was assayed for morphological, physiological and biochemical characteristics and were further compared with standard descriptions given in Bergey's Manual of Determinative Bacteriology. Purified isolates were screened for starch hydrolysis as described by Chu (2007). This was done with NA containing 1% soluble starch (potato, BDH, Poole, UK). Amylolytic activity was demonstrated by streaking the isolates on the NA. Incubation was at 37°C for 24 h before flooding with iodine solution. *In situ* enzyme production was observed with the clearing of zone of whitish appearance in the area surrounding the isolate.

Spore stain test

A dried heat-fixed bacterial film on a clean slide was placed over a beaker of boiling water, with the bacterial film upper most. After condensation of water droplets on the underside of the slide for several seconds, the slide was flooded with a 5% aqueous solution of malachite green and was left for 1 min to act while the water continued to boil. The slide was further rinsed in running cold water and treated with 0.5% safranine for 30 s. It was again rinsed in running cold water and dried. When viewed under microscope, the spores appeared green, while the vegetative bacilli appeared red. Lipid granules were unstained.

Extraction of starch milk

Cassava, sweet potato, corn and potato starches were prepared as already described by Ubalua et al., 2014; Ubalua, 2014. The starches were sterilized in a hot air oven at 175°C for 40 min with the powder depth not exceeding 1 cm according to El- Tayeb et al. (2007) before substituting in the amylase producing medium.

Growth profile and amylase induction from *B. subtilis*

250 ml Erlenmeyer flasks were used for α-amylase production by *B. subtilis*. Each contained 50 ml of the culture medium with the following composition: Starch (1.0%); yeast extract (0.5%); $CaCl_2$ (0.02%); NaCl (0.1%); and $MgSO_4$ (0.1%) (Lily et al., 2012). The pH of the medium was adjusted to 7 with 1 N H_2SO_4 and/or 1 N NaOH before sterilization at 121°C for 15 min. An inoculum was prepared from a slope of *B. subtilis* by growing the organism for 24 h in a 250-ml Erlenmeyer flask containing 50 ml of the medium at 30°C in an orbital incubator (200 rpm) for 24 h. The growing cells were centrifuged at 2000 rpm and an aliquot (0.5 ml) of the suspension were inoculated into three 250 ml Erlenmeyer flasks containing 50 ml each of the medium supplemented with 1% (w/v) soluble starch or the native starches. Fermentation was for 48 h at 200 rpm while samples were taken at intervals for analysis (biomass (OD 600 nm), and amylase production. Extents of starch depletion in the media were determined by withdrawing 0.5 ml of the culture broth at intervals and testing with aqueous iodine.

Optimization of media components for α-amylase production

Various carbon sources (Cassava, sweetpotato, corn, potato), nitrogen sources (groundnut cake, soybean cake, $(NH_4)_2S0_4$, NH_4N0_3, coconut cake, peptone) and metal ions (in chloride and sulphate forms) were varied in different concentrations in the basal medium one at a time. Other medium ingredients in the basal medium were kept constant when one component was varied. Optimum concentration of the carbon sources, nitrogen sources and metal ions were deduced by determining the α-amylase activity of the cell free extract after 48 h of incubation (Poddar et al., 2012).

Optimization of physical parameters for growth and α-amylase production

Microbial growth progression and α-amylase production were

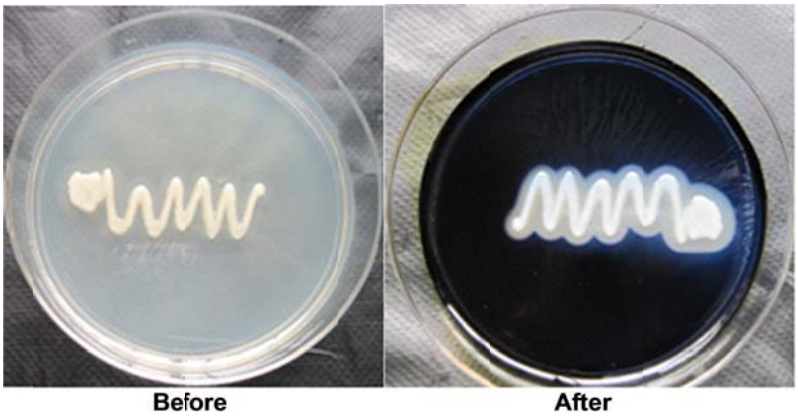

Plate 1. Isolate CNS_3 (*Bacillus subtilis*), with zone of clearing.

assessed at different temperatures after inoculating the broth medium and incubating on a shaker at 200 rpm for 48 h. Similarly, media pH was also varied with the addition of 1 N H_2SO_4 and/or 1 N NaOH before sterilization at 121°C for 15 min. Media of different pH were separately inoculated with *B. subtilis* and were incubated for 48 h in rotary shaker at 37°C to determine optimum pH for microbial growth and α-amylase production (Poddar et al., 2012).

Analysis of data

All experiments were carried out in triplicate and data subjected to statistical analysis using Gen Stat. Discovery Edition 3, HP1 1ES UK.

RESULTS AND DISCUSSION

Native microorganisms growing in the agricultural soils were isolated. All of the soil samples showed varied values of colony forming units per gram of soil (CFU g/1 = 1-6x10^3). Out of the 13 isolates observed, isolate CNS_3 demonstrated highest starch hydrolyzing ability by showing area of clear zone along the line of streaking after 24 h incubation and flooding with iodine solution (Plate 1). The bacterial isolate on nutrient agar medium was creamy brown and dull in appearance. Gram staining revealed, a Gram-positive rod, blunt ended, singly and in pairs. Motility test was positive. The isolate was positive for catalase, oxidase, Voges-Proskauer and nitrate reduction tests (Table 1). The isolate was able to utilize glucose and mannitol with gas production. It also liquefied gelatin and starch indicating the presence of hydrolytic enzyme. The isolate was negative to indole and urease tests. The results obtained were confirmed with Bergy's manual of determinative bacteriology and based on the isolates ability to hydrolyze starch and spore production it was therefore assigned to the phenotypic group 11 of the genus *Bacillus* (*B. subtilis*) group.

The patterns of α-amylase production, as well as biomass, and starch depletion were monitored. At the onset of the experiment, adaptation, growth and multiplication of the organism were slow. This might not be unconnected with the adaptation of the organism to its new environment and the time required for the organism to hydrolyze starch in the medium to utilizable units. The implication is that this may be accomplished by the consistent secretion of a low basal level of hydrolyzing enzymes (Chu, 2007). Moreover, it appears that the biosynthesis of the α-amylase by *B. subtilis* is growth-related since the enzyme in this organism is primarily produced during the exponential phase (Ubalua, 2014). Soluble starch and starches from different agricultural origin (corn, sweet potato, cassava and potato) were examined for growth and amylase production by *B. subtilis*. The organism expressed activity in the growth media containing 2% (w/v) of all the starches as the sole carbon source. In all the media, highest amylase activity was observed during the late exponential phase of growth of the organism. All the starch media produced variable amylase activities at 2% (w/v) concentration as sole carbon source (Table 2). Comparing the performances of all the starch media, corn starch produced the highest amylase activity of 4168±132 U/L as compared to 2759±146 and 3988±126 U/L for cassava and soluble starch, respectively (Table 2). The recorded maximum amylase activity was higher than the values obtained for *B. subtilis* (0.6 U/mL) by Ajayi and Fagade (2003, 2006), but lower than 6.24, 72 U/mL obtained for *B. subtilis* by Ajayi and Fagade (2008) and Asgher et al. (2007), respectively. The observed variability in α-amylase yield has been reported to be strain specific and/or due to the source of the bacterium (Mirakabadi et al., 2012). The present result therefore corroborated the report of Ajayi and Fagade (2003, 2006) on the yield performance of corn starch on α-amylase production and the attestation that starch-rich substrates may prove useful as cheap alternative sources of carbon and energy source for α-amylase production (Prakash and Jaiswal, 2010). Carbon sources and their compositions have been variously reported as a factor that influences amylase production

Table 1. Microscopic and biochemical characteristics of the bacterial isolates.

Parameter	Isolates			
	CNS_1	**CNS_2**	**CNS_3**	**CNS_4**
Colony features	Pigmented, smooth butter-like glistening surface	Large, rough and shinny colonies	Dull and creamy brown	Swarming with distinct odour
Microscopy				
Gram reaction	+	-	+	-
Motility	-	+	+	+
Cell arrangement	Cocci, singly and regular in clusters	Rod shaped	Blunt ended, singly and in pairs	Straight rod, coccoid and irregular in chains
Spore stain test	-	-	+	-
Biochemical reaction				
Catalase	+	+	+	-
Oxidase	-	+	+	ND
Indole	-	ND	-	+
Voges-Proskauer	+	ND	+	+
NO_3	+	+	+	+
Carbohydrate utilization				
Glucose	+a	+a	+a	+g
Lactose	+a	-	ND	-
Maltose	+a	-	ND	-
Mannitol	+a	+a	+a	-
Urease	+	+	-	+
Hydrogen sulphide	-	-	ND	+
Hydrolysis of:				
Starch	-	-	+	+
Gelatin	+	+	+	+
Identity	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Proteus mirabilis*

+ = Positive; - = negative; ND = not done, +a = positive with acid; +g = positive with gas.

Table 2. Production of α-amylase from *Bacillus subtilis* grown on starches from different agricultural substrates.

Starch (2%)	Protein content (mg)	Enzyme activity U/L)	Optical density (660 nm)	Specific activity (U/mg)
Cassava	219±18^b	2759±146^b	0.62±0.01^a	12.60±2.4^b
Corn	485±26^a	4168±132^a	0.71±0.02^a	8.59±1.3^c
Sweet potato	488±44^a	1622±98^b	0.34±0.04^c	3.23±1.0^d
Potato	82±7.1^c	622±15^c	0.42±0.01^b	7.59±1.0^c
Soluble starch	203±12^b	3988±126^a	0.65±0.13^a	19.65±7.1^a
LSD (0.05)	4.554	4.080	0.047	0.556

Results represent mean values for three replications for each treatment. Same letters are not significantly different at p>0.05.

(Nurullah, 2011; Esfahanibolandbalaie et al., 2008). Several authors have reported that the differences in the amylose, amylopectin composition and lipid contents of the starches could account for the variability in amylase

Table 3. Production of α-amylase from *Bacillus subtilis* grown on nitrogen sources.

Nitrogen source (2%)	Protein content (mg)	Enzyme activity (U/L)	Optical density (660 nm)	Specific activity (U/mg)
Soybean cake	384±37.5[b]	6126±166[b]	0.54±0.03[b]	15.95±2.0[b]
$(NH_4)_2S0_4$	264±11.5[c]	3526±164[c]	0.43±0.05[c]	13.36±0.6[c]
Groundnut cake	420±32[b]	7280.7±144[a]	0.63±0.06[a]	17.3±0.6[a]
NH_4N0_3	584±35.5[a]	8024±198[a]	0.78±0.07[a]	13.73±0.5[c]
Coconut cake	128±12.5[d]	456.7±34.5[c]	0.34±0.03[d]	3.57±1.0[d]
Peptone	461±71.5[a]	7643.3±213[a]	0.65±0.13[a]	16.58±1.2[a]
Yeast extract	352±8.5[b]	6218.7±188[b]	0.57±0.14[b]	17.67±1.6[a]
LSD (0.05)	4.894	4.649	0.046	0.161

Results represent mean values for three replications for each treatment. Same letters are not significantly different at p>0.05.

production (Chavez et al., 2004; Gomes et al., 2005). Corn starch has approximately 28% amylose, 72% amylopectin and 6.0% lipids while cassava starch has approximately 17% amylose, 83% amylopectin and 0.1% lipids (Tester et al., 2004). Cruz et al. (1997) posited that the ability of an organism to produce amylolytic enzymes that liberate reducing units is higher when the carbon source is more of amylose rather than amylopectin, and that corn amylose is a better inducer of amylase than its homologue from potatoes. Typical growth and enzyme production profiles in the media containing different nitrogen sources at 2% concentration is as summarized in Table 3. All the nitrogen sources yielded α-amylase in the following decreasing order (in U/L): NH_4NO_3 (8024±198)>peptone (7643.3±213)>groundnut cake (7280.7±144)>yeast extract (6218.7±188)>soybean cake (6126±166)>$(NH_4)_2SO_4$ (3526±164)> coconut cake (456.7±34.5) (Table 3). Nurulla (2011) reported 560, 797, 798, 800 and 800 U/mL at 1% concentration for growth medium containing peptone, ammonium nitrate, ammonium chloride, ammonium sulphate and sodium nitrate, respectively.

In contrast, 7643±213, 8024±182, 3526±164 U/L were obtained for peptone, ammonium nitrate and ammonium sulphate at 2% concentration in this study. The oilseed cakes (soybean and groundnut) remarkably enhanced production of extracellular α-amylase (Table 3). However, the degree of enhancement was relative to the nature and the concentration of the soybean and groundnut cakes (Ramachandran et al., 2006).

Optimization studies and growth kinetics

Growth and enzyme production profiles in the media containing different carbon sources showed that α-amylase production was highest in the corn starch medium as compared to other carbon sources (Table 2). Gradual increase in the concentrations of carbon and nitrogen sources resulted in the corresponding increase in both growth kinetics and α-amylase production. Under this growth condition, corn and soluble starch media induced a maximum α-amylase activities of 6427±168 and 5982±167 U/L against 4168±132 and 3988±126 U/L produced in the basal media respectively (Figure 1 and Table 2). Similarly, 7856±306 and 8203±353 U/L were produced in soybean cake and NH_4NO_3 media respectively (Figure 2) against 6126±166, 8024±198 U/L in the basal media (Table 3). Beyond 1.5% concentration of starch or nitrogen source, growth and activity gradually declined. Lily et al. (2012) achieved optimum α-amylase production with 1% peptone against 1.5% observed in this study. At lower concentration, groundnut seed cake induced additive effect while beyond 1.5% concentration adverse effect was observed. Defatted coconut meal was observed to be ineffective at low concentrations and retarded at concentrations above 1.5%. Groundnut cake has been credited with a well-balanced composition of nutrients that are essential for α-amylase production in contrast to coconut cake (Ramachandran et al., 2006). Previous reports by various authors also reported an optimum α-amylase production with 2% starch concentration (Qader et al., 2006; Lily et al., 2012) in contrast to 1.5% starch concentration obtained in this study. However, Yasser et al. (2013) reported a concentration of 1.25% starch for optimum α-amylase production against 1.5% obtained from the present study.

It is worthy to mention here that starches are characterized by a wide degree of variation in their susceptibility to enzymatic attack. The susceptibility and mode of enzyme action depends on both the botanic origin of the starch, granule size and enzyme(s) involved (Satish and Aniruddha, 2007; Aderibigbe et al., 2009). Starch granules from different groups, including cereals (corn), roots (cassava, sweetpotato) and tubers (potato) are attacked preferentially (Aderibigbe et al., 2009). Nadir et al. (2010) reported that starches that naturally show a porous surface, as in corn, are prone to attack more easily than those with a smooth surface as in tapioca starch and that small starch granules are hydrolyzed

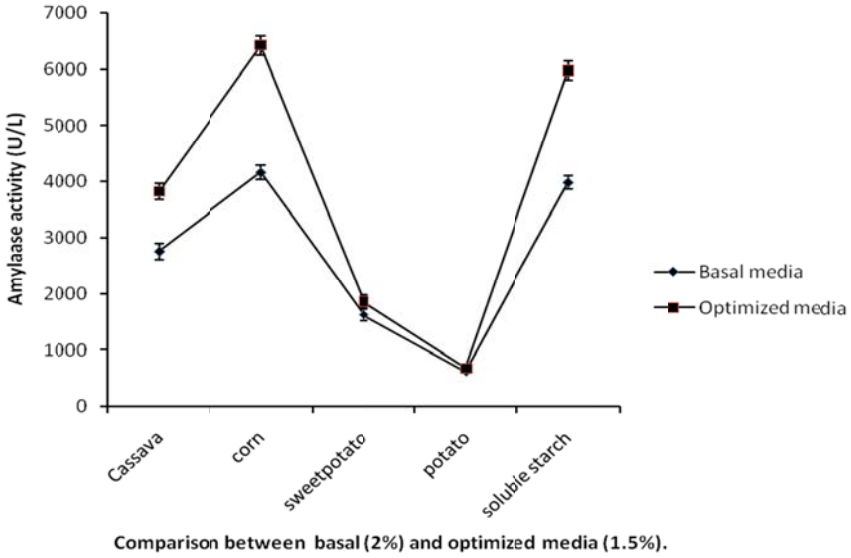

Figure 1. Effect of carbon source on α-amylase production.

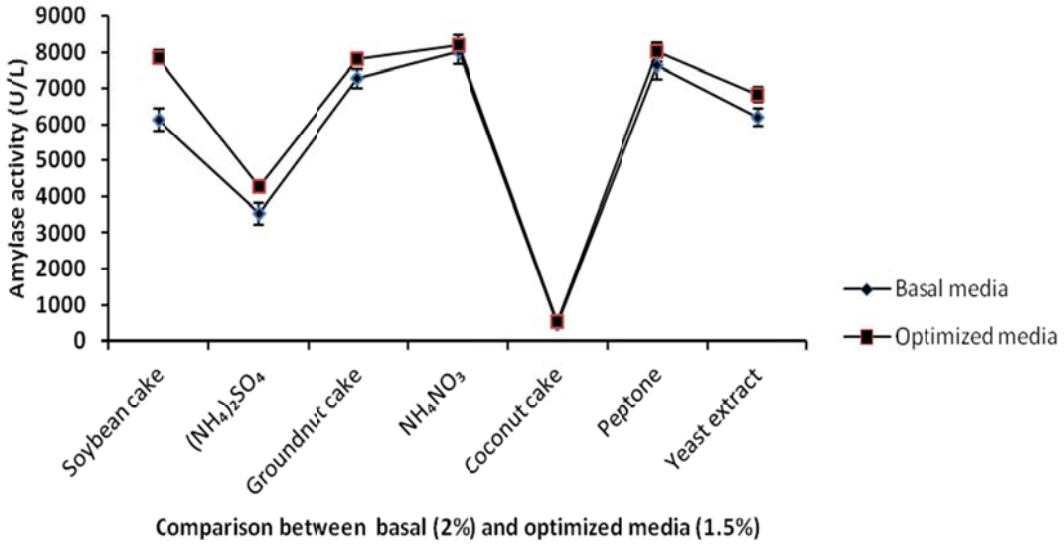

Figure 2. Effect of nitrogen source on α-amylase production.

more than larger ones. On the contrary, potato starch granules diameter is almost three times higher than tapioca and corn starches (Satish and Aniruddha, 2007). According to Aderibigbe et al. (2009), starches that are rapidly digested with enzymes, such as corn and wheat starch, have surfaces that are readily attacked, with the formation of canals. The enzyme(s) penetrates into starch granules through natural pores and disrupts the inside part of the starch. In contrast, sweetpotato and potato starch may not have natural pores or deep channels (Aderibigbe et al., 2009). Therefore, the high susceptibility of corn starch to enzymatic degradation compared to that of sweetpotato and potato observed in this study might be due to the presence of natural pores on the corn starch and its minute granular size. This observation is consistent with the findings of Satish and Aniruddha (2007), who reported that cereal starches are generally less resistant to enzymatic attack as compared to non-cereal starches and that pores present on starch surfaces could become centers of enzymatic attack.

Temperature of fermentation medium is of essence and influence of temperature on amylase production is related to the growth of the organism (Ubalua, 2014). At low temperature of about 10°C, α-amylase activity was low, but

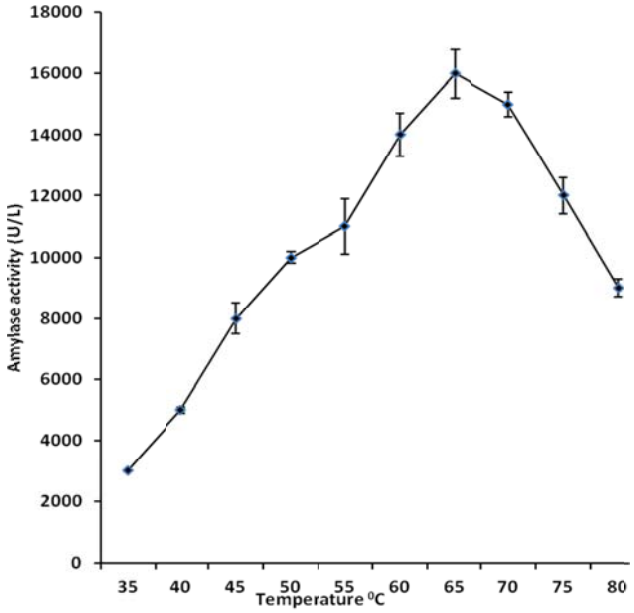

Figure 3. Effect of temperature on α-amylase production.

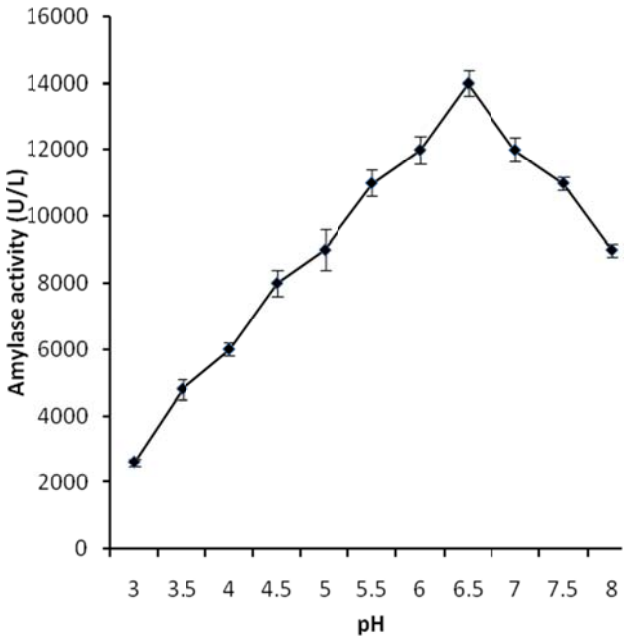

Figure 4. Effect of pH on α-amylase production.

as the medium temperature was increased from 10 to 40°C, α-amylase activity gradually increased. But at 65°C, optimum enzyme production was achieved. Above 65°C amylase production progressively decreased (Figure 3). This observation is supported by the reports of some authors that higher temperature may inactivate the expression of gene in enzymes responsible for starch degradation (Samantha et al., 2013). Thus, temperature is another critical parameter that has to be controlled and varied from organism to organism. The present experiments recorded an optimum temperature of 65°C (Figure 3) which is within the range of the findings of many authors in the literature (Gupta et al., 2003; Gomes et al., 2005). In summary, the optimum temperature of 65°C for α-amylase with its broad pH range (6.0-8.0) for activity suggests that the enzyme may have potential for application in the brewing industry. The culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). *B. subtilis* isolated in this study exhibited optimum growth and activity at pH 6.5 (Figure 4). Although, below and above pH 6.5, amylase activity gradually dropped. Gupta et al. (2003) had earlier reported that most starch degrading bacterial strains thrives at a pH range of 6.0-7.0 for growth and enzyme production. Cordeira et al. (2002) also reported an optimum pH of 7.5, with a broad active range of pH 5.5-10.0 for *Bacillus* species. Haq et al. (2005) and Tanyildizi et al. (2005) also stated that bacterial cultures such as *B. subtilis* and *B. licheniformis*, requires an initial pH of 7.0 for optimum yield of α-amylase, suggesting that the obtained optimum pH of 6.5 falls within the reported pH values in the literature. The results on the effect of metal ions on the amylase activity are as shown in Figure 5. Enhancement of the enzyme production was observed in the medium containing 0.003% (w/v) Ca^{2+} while that of Mn^{2+} and Zn^{2+} ions inhibited the activity of the enzyme (Figure 5). Lily et al. (2012) obtained maximum α-amylase production with 0.02% $CaCl_2$ while 0.003% was used in this present study. This is a confirmation of previous reports which states that α-amylases are mostly metalloenzymes and requires calcium ions for activity, structural integrity and stability (Varalakshmi et al., 2007; Michelin et al., 2010). In addition, Nurulla (2011) noted that some metal salts like $CuSO_4$ and $ZnSO_4$ decreases enzyme production probably as a result of pH changes associated with their use in the medium. These assertions were corroborated by the observations of Kiran and Chandra (2008) that $ZnSO_4$ is potentially inhibitory in the production of α-amylase by *Bacillus* sp. K-12. Thus, the enhanced activity of the amylase in the presence of Ca^{2+} ions could be ascribed to their interaction with negatively charged amino acid residues including aspartic and glutamic acids, thereby conferring stabilization of enzyme conformation (Linden et al., 2003).

Conclusion

The study showed that corn and cassava starches could serve as an alternative to soluble starch in the production of α-amylase by the newly isolated *B. subtilis*. The results also demonstrated that the newly produced enzyme could degrade native starches although their rates were

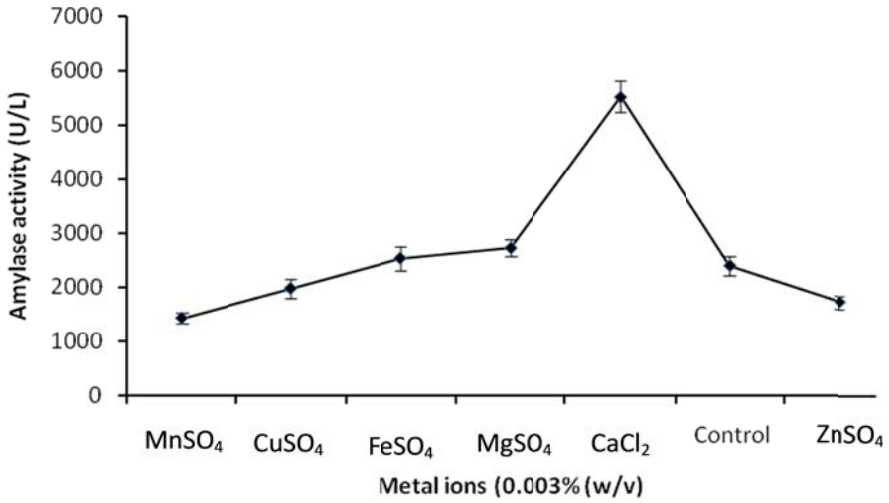

Figure 5. Effect of metal ions on α-amylase production.

somewhat lower than that for corn starch, especially potato. The starch degradation was influenced by not only the enzyme and the native starch characteristics but also by the degradation condition. The susceptibility was likely affected by the botanical origin, nature of the granule surface, amylose content and other compound granules (Satish and Aniruddha, 2007; Aderibigbe et al., 2009). Thus, the effective production of enhanced α-amylase by the corn starch medium as compared to soluble starch medium informs its appropriateness for development of industrial production of α-amylase, using sustainable and affordable natural carbon sources.

Conflict of interest

The author(s) have not declared any conflict of interests.

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