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Purification, characterisation and mutagenic enhancement of a thermoactive α-amylase from *Bacillus subtilis*

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Bacillus subtilis **was isolated from flour mill wastes. It produced a thermostable α-amylase in complex media containing starch. Amylase activity was optimal at the exponential phase and was more strongly expressed with sorghum, yam peel and corn starch than soluble potato starch. The enzyme was purified 24-fold to a specific activity of 2200 U mg^{-1}, with a yield of 10%. It yielded a single band when subjected to SDS-PAGE and an apparent molecular mass of 54780 was determined by mass spectrometry. The enzyme, which was optimally active at 80°C and pH 5.6, released saccharides with a polymerisation degree of 1–6 following hydrolysis of yam peel, sorghum and corn starch. Cells of *B. subtilis* were exposed to ultraviolet irradiation and N-methyl-N′-nitro-N-nitrosoguanidine. Hyperproductive mutants were obtained by these treatments.**

Keywords: α-amylase; *Bacillus subtilis*; corn; mass spectrometry; mutation; purification; sorghum; thermostable; yam peel

Introduction

α-Amylase (EC 3.2.1.1, α-1,4-glucanhydrolase) hydrolyses the endo-α-1,4 linkages of starch in a random manner bypassing the α-1,6 linkages and eventually producing mainly glucose and maltose and a limit dextrin. This enzyme is produced in copious amounts especially by the genus *Bacillus* [14,16,32], although other organisms are known to have α-amylase activity [28,29].

Thermostable α-amylase has found use in industry where it is used for the reduction of the viscosity of starch paste under high temperature conditions. The mesophile *B. licheniformis* has been reported to produce a suitable thermostable α-amylase [24,32], whereas *Bacillus subtilis* is also known to produce an α-amylase [8,9], but it is reported to be unstable over 60°C [14]. As part of a programme directed at finding new isolates for amylase from the Nigerian locality which can degrade local carbon substrates, we have recently reported on the use of cassava and yam peels to produce the enzyme in *Aspergillus* sp [33,35]. Here we report the isolation of a novel thermostable α-amylase from the mesophile *B. subtilis* which may be cultivated on cheap carbon substrates available in Nigeria.

Genetic enhancement of amylase synthesis in *Clostridium* sp [3,15] and *B. licheniformis* [21] has been reported. In all the cases N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was the mutagen of choice. In this study therefore, we also describe mutants with enhanced amylase activity obtained by exposing cells of *B. subtilis* to ultraviolet irradiation and NTG.

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Materials and methods

Isolation and maintenance of organism

The organism used in this study was isolated from flour mill wastes. Samples were collected in sealed cellophane bags and analysed immediately in the laboratory. Each sample was mixed with 0.1% peptone water in a test-tube by vortex to break up the clumps of bacteria. Serial dilutions (0.1 ml) of the suspension obtained from the interface of the column of sedimented material and the supernatant were plated on nutrient agar, NA (Oxoid, Basingstoke, UK), supplemented with 2% soluble starch (potato, BDH, Poole, UK). The agar plates were incubated at 37°C for 2–3 days. Bacterial isolates were characterised and identified based on Bergey's Manual of Systematic Bacteriology [7]. The primary criterion for the selection of amylase-producing strains was the formation of a clear zone upon addition of weak iodine solution (0.3% (w/v) I_2: 3% (w/v) KI) to the agar plates. The organism was maintained on slants of the isolation medium at 4°C and subcultured every 3 months. This organism has been deposited in the culture collection of the Department of Biological Sciences, University of Ilorin, Ilorin, Nigeria.

Inoculum preparation and amylase production

An inoculum of *B. subtilis* was first developed from a 48-h-old slant culture in 50 ml of mineral salts medium (MS), containing (g L^{-1}): KH_2PO_4, 1.5; K_2HPO_4, 2.2; $MgSO_4 \cdot 7H_2O$, 0.0025; yeast extract, 0.5; to which soluble starch, 20 g, was added. The medium pH was adjusted to 7.0 before sterilisation at 121°C for 15 min. Fermentation was carried out at 30°C on an orbital incubator (220 rpm) for 18 h. The actively growing culture (10^7 cells ml^{-1}, OD (620 nm) 0.9) was used to inoculate 50 ml MS supplemented with 2% soluble starch or various prepared starches. Samples were taken at intervals for microbiological and biochemical analysis (ie pH, biomass (OD 620 nm), and amylase synthesis). Starch concentration in the growth medium was determined by withdrawing 0.5 ml of culture

broth at intervals and adding to 2 ml of 0.1 M H_2SO_4. Iodine solution (5 ml, containing 0.3% I_2 and 3% KI) was added and the absorbance measured at 600 nm against a water/iodine blank.

Starch preparation from sorghum, yam peel and corn

Sorghum (*Sorghum biocolor*), yam (*Dioscorea esculenta*) peel and corn (*Zea mays*) starches were prepared as already stated [35].

Assay of amylase activity

Amylase activity was quantitatively assayed in 1 ml containing 0.5 ml of 1% (w/v) soluble starch (BDH), 0.4 ml of 0.02 M phosphate buffer (pH 6.0) and 0.1 ml of appropriately diluted enzyme solution. After 5 min at 25°C, reducing sugars were determined by the method of Bernfeld [4]. The activity of one unit (U) of enzyme was defined as that amount liberating 1 μmol of reducing sugar (with maltose as standard) per min under the assay conditions. Specific activity was expressed as units per mg of protein after the concentration of protein in the enzyme solution was determined by the method of Bradford [5].

Purification of α-amylase

For the preparation of large amounts of the enzyme, bacteria were cultivated for 24 h in a series of 1-L Erlenmeyer flasks each containing 200 ml of mineral salts medium supplemented with soluble starch, and the cultures combined. All steps of purification were carried out at 4°C.

Step I: The fermented broth was centrifuged at 12 000 $\times$ *g* for 30 min to remove bacterial cells. The supernatant was concentrated by a tangential flow device with a 10-kDa cutoff membrane (Filtron Technology Corp, Northborough, MA, USA). The peristaltic pump used for this operation was a Watson-Marlow 603 U (Falmouth, UK), at a crossflow rate of 500 ml min^{-1} and a filtrate flow of 19 ml min^{-1} with a back pressure of 12–20 psi. The concentrated supernatant was fractionated with ammonium sulphate (20–60% saturation) and allowed to stand overnight with gentle mixing. The precipitate formed was collected by centrifugation (12 000 $\times$ *g*, 30 min) and stored in a minimal volume of 3 M ammonium sulphate dissolved in 0.05 M K_2HPO_4.

Step II: For ion-exchange chromatography, the partially purified enzyme was dissolved and dialysed overnight against 0.02 M Tris-HCl buffer (pH 8.0). The enzyme sample was then applied to a column of DEAE-Sephadex A-50 anion exchanger gel (2 $\times$ 50 cm) that was previously equilibrated with 0.02 M Tris-HCl buffer (pH 8.0). The column was eluted (18 ml h^{-1}) with a linear NaCl concentration gradient from 0–1.0 M in the same buffer. The amylase fractions were pooled, concentrated using a Vivaspin centrifugal concentrator (Vivascience Ltd, Lincoln, UK), and subjected to further purification by gel filtration.

Step III: The enzyme obtained from ion exchange chromatography was loaded onto an Ultragel ACA 34 (BioWhittaker, Wokingham, UK) column (2 $\times$ 50 cm) that was previously equilibrated with 0.02 M Tris-HCl buffer (pH 8.0). Active fractions were pooled, concentrated by ultrafiltration, and used to study the properties of the enzyme.

Homogeneity test

The homogeneity of the purified enzyme was determined by slab gel electrophoresis using Laemmli buffers [19]. The purified α-amylase was mixed in a solution containing 1.0% (w/v) SDS and 1.0% (v/v) mercaptoethanol. Denaturation was for 5 min at 100°C before electrophoresis. Protein was stained with Coomasie brilliant blue R-250 (Sigma, Poole, UK).

Estimation of relative molecular weight

The native molecular mass of the α-amylase was determined by the method of Andrews [1] using a column (2 $\times$ 50 cm) of Ultragel ACA 34 equilibrated with 0.02 M phosphate buffer (pH 8.0). The flow rate of the column was maintained at 15 ml h^{-1}. The void volume was determined with blue dextran and the column was calibrated with myoglobin (MW 17 000), egg albumen (MW 45 000), bovine serum albumin (MW 67 000) and lactate dehydrogenase (MW 140 000), as marker proteins. Parallel tests were carried out using PAGE in the presence of SDS, and mass spectrometry.

Thin layer chromatography

Sugars formed from starch by the enzyme were determined by TLC using a two-step solvent system with TLC on sheets of silica gel 60 W (Merck, Darmstadt, Germany). The sugar products were developed firstly with a solvent of n-butanol-ethanol-water (5:3:2) and then with n-butanol-isopropanol-water (10:5:4). Sugars were located by spraying with aniline-diphenylamine-phosphoric acid [23].

Mutagenic improvement of Bacillus subtilis

A modification of the method of Cutting and vander Horn [10] was employed for the mutagenic treatment of cells of *B. subtilis*. All procedures during and following irradiation were carried out in yellow light or in the dark to avoid photoreactivation.

Step I. Ultraviolet (UV) irradiation of cells

Bacillus subtilis cells at mid-logarithmic phase (10^7 cells ml^{-1}, OD (620 nm) 0.9) were harvested. The culture was divided into 5-ml portions and pelleted at 10 000 $\times$ *g* for 20 min. Each cell pellet was resuspended in 0.5-ml volumes of 0.1 M $MgSO_4 \cdot 7H_2O$ by vortex to break up clumps of bacteria. Serial dilutions of one of these samples (0.1 ml) was plated on NA plus 2% soluble starch as an unirradiated control. The remaining cultures were transferred to sterile glass Petri dishes for irradiation and usually diluted 1000-fold. Irradiation was performed with a Philips 15 W TUV tube. The Petri dishes with samples were uncovered and placed under the lamp at a 20-cm distance from the UV source and irradiated with gentle shaking over a six-fold time scale (30 s–3 min). Each sample was serially diluted immediately after UV treatment and plated on NA plus 2% soluble starch for determination of survival rates. The plates were incubated overnight at 30°C.

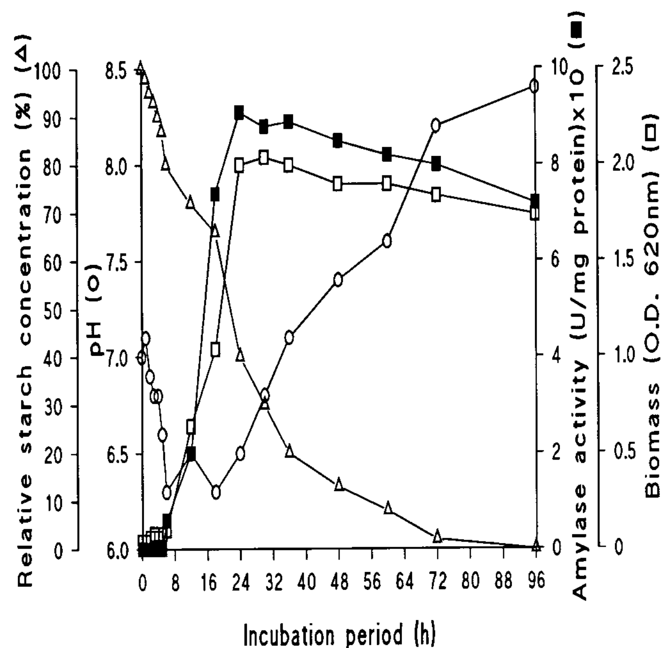

Figure 1 Time course of growth and amylase synthesis by *Bacillus subtilis* in mineral salts medium supplemented with soluble starch as carbon source (pH, ○; biomass, □; amylase activity, ■; starch concentration, △).

Step II. Test for amylase activity

At the end of the incubation period, the resultant colonies were tested for amylase activity, normally with iodine solution. Amylase-positive mutants were isolated onto fresh plates of MS plus 2% soluble starch and screened for maximum amylase production in MS broth medium supplemented with 2% soluble starch.

Step III. N-methyl-N′-nitro-N-nitrosoguanidine (NTG) treatment

Fresh overnight cultures of promising mutants obtained from UV irradiation were inoculated into 25 ml of MS plus 2% soluble starch and incubated at 30°C and 220 rpm until the mid-logarithmic phase. The cells were pelleted at 10000 × *g* for 20 min and washed with warm 0.01 M sodium citrate plus 0.15 M NaCl (SC), pH 7.0. The cells were resuspended in 10 ml of warm SC containing routinely either 160 μg ml^{-1} or 200 μg ml^{-1} NTG, freshly prepared. Incubation was at 30°C with gentle shaking for 1 h. At the end of treatment, cells (10^3 dilution) were washed twice with warm MS and resuspended in 50 ml of warm MS plus soluble starch, and incubated at 30°C for 15 h. The cells were pelleted and the pellets washed three times with warm SC and resuspended in 10 ml SC. To estimate cell survival, an aliquot of the cells resuspended in SC was taken before and after mutagenesis, serial diluted and plated onto NA plus 2% soluble starch. Amylase-positive mutants isolated onto fresh NA plus soluble starch were screened for maximum amylase production in MS as outlined above.

Reproducibility of results

The results of most experiments are the means of three independent readings. It should be noted that in mass spectrometry, we were unable to get consistently the same peak patterns and values reported in a later Figure. The difference in molecular weights, however, were small (less than 1%).

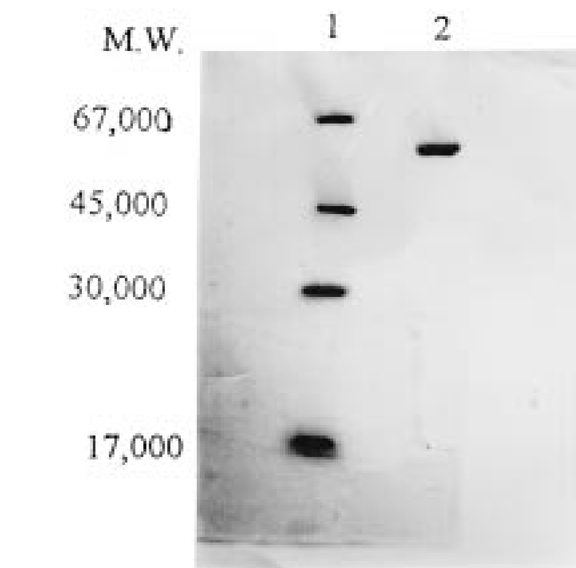

Figure 2 Gel electrophoretic pattern of purified α-amylase from *Bacillus subtilis*. Standard proteins used were: myoglobin (MW 17000); carbonic anhydrase (MW 30000); egg albumen (MW 45000); and bovine serum albumin (MW 67000). Lane 1: marker proteins; lane 2: α-amylase sample (picture of plate was reproduced with Biovision Imaging Technology, BioGene Ltd, Kimbolton, UK).

Results and discussion

Selection of amylase-producing bacteria

About 63 isolates were plated individually onto NA-soluble starch medium. Three colonies with large halos upon treatment with iodine solution were selected for amylase production in nutrient broth medium in shaking culture. Finally the particular isolate which produced the most thermostable amylase was chosen. Physiological and biochemical tests were carried out on the bacterium as described in Bergey's Manual of Systematic Bacteriology [7]. Colonies appeared circular, translucent, butyrous and wrinkled on NA. Pigmentation was pink-white and spores were ellipsoidal and central which did not distend the sporangium. The organism was aerobic and rod-shaped (0.7–0.8 μm width; 1.8–3.0 μm length), Gram-positive and motile. Citrate was assimilated and it was positive by the Voges–Proskauer test but negative by the methyl red test. Catalase was produced, casein hydrolysed and gelatine liquefied. It reduced nitrate to nitrite and the pH in Voges–Proskauer broth was 7.2. The organism grew at 30–45°C at a pH range of 5.5–10.0 and an NaCl concentration of 0–5%. Acid was produced from glucose, arabinose, xylose, mannitol, but no gas production was observed. Starch was hydrolysed. According to these data and using the scheme of Bergey's Manual of Systematic Bacteriology [7], this organism was identified as *B. subtilis*.

Growth and amylase synthesis by B. subtilis on soluble starch

Bacillus subtilis grew well in mineral salts medium supplemented with soluble starch (Figure 1), and reached the

Table 1 Purification of α-amylase from *Bacillus subtilis*

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} of protein)	Yield (%)	Purification
Culture supernatant	2200	90420	990	91.3	100	1
Tangential flow ultrafiltration	650	84630	682.5	124	93.6	1.4
20–60% $(NH_4)_2SO_4$ precipitation and dialysis	108	51408	194.4	264.4	56.9	2.9
Ion-exchange chromatography on DEAE-Sephadex A-50	84	24024	33.6	715	26.6	7.8
Gel filtration on Ultragel ACA 34	36	8820	4.0	2205	10	24.1

Details of purification procedures are contained in Materials and methods.

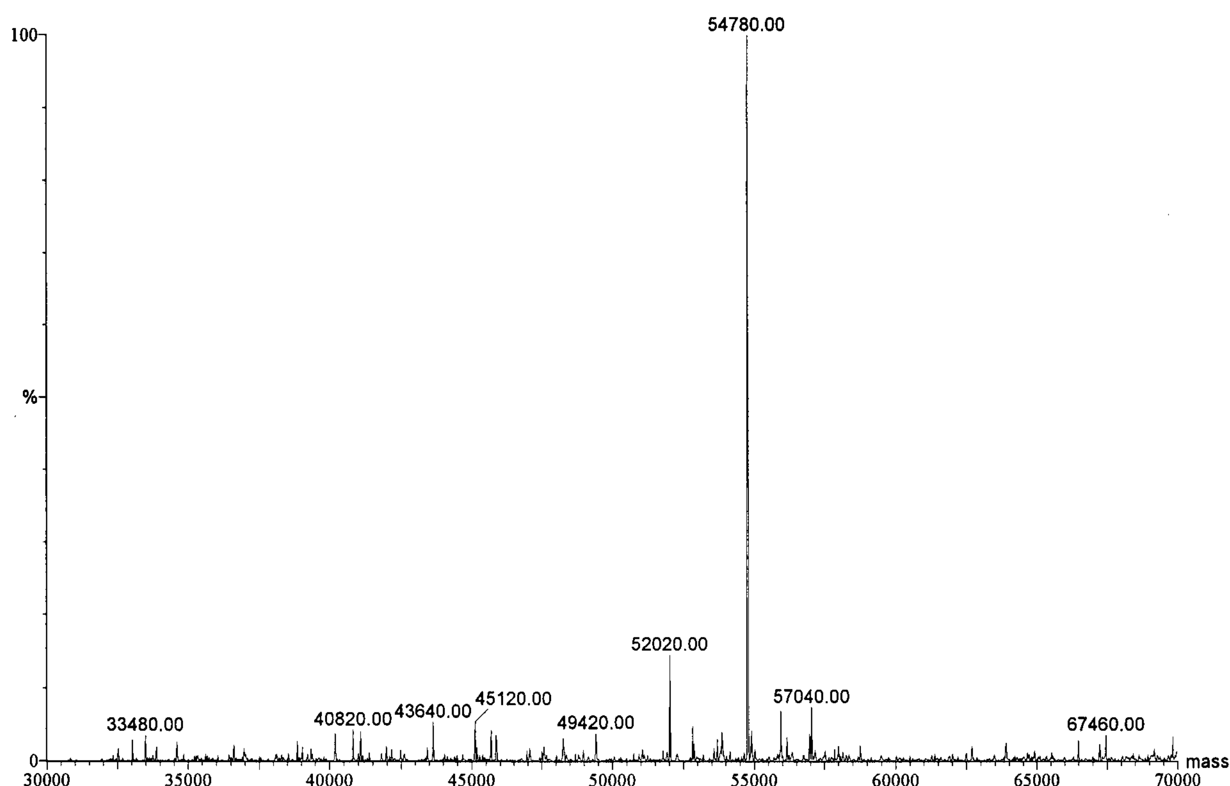

Figure 3 Estimation of the molecular weight of purified α-amylase from *Bacillus subtilis* by electrospray mass spectrometry. A dialysed sample was lyophilised and then dissolved in 50 : 50 acetonitrile (ACN) : water, containing a final concentration of 1% formic acid. The carrier solvent was 50 : 50 ACN : water at a final flow rate of 20 μl min^{-1}. The sample was analysed on a Fisons Instrument VG Biotech (Loughborough, UK). The source temperature was 65°C. Scans were made across a m/z range of 650 : 1550. Data handling used was the Masslynx Maximum Entropy Algorithm.

stationary phase after 30 h. Growth was paralleled by the production of α-amylase, and the activity of the enzyme peaked at 91 U mg^{-1}. The growth of *B. subtilis* on starch showed a long lag phase that corresponded to a dramatic increase in amylase activity. We tried to eliminate this lag phase in amylase activity by serial passaging of the organism in an identical medium until the mid-exponential phase, before inoculation into the production flask. The lag phase was reduced considerably by this method but not removed. Starch is a complex polysaccharide and unless it is broken down into smaller, more soluble compounds, it cannot penetrate the cell walls. The long lag phase is possibly a reflection of the time required to hydrolyse the starch to metabolizable subunits [3]. It is suggested that this may be achieved by the continual secretion of a low basal level of hydrolysing enzymes [12,30]. The biosynthesis of α-amylase by *B. subtilis* appeared to be growth-related since the enzyme in this organism is primarily produced during the exponential phase. This observation is similar to the pattern of amylase synthesis by *Clostridium acetobutylicum* [3].

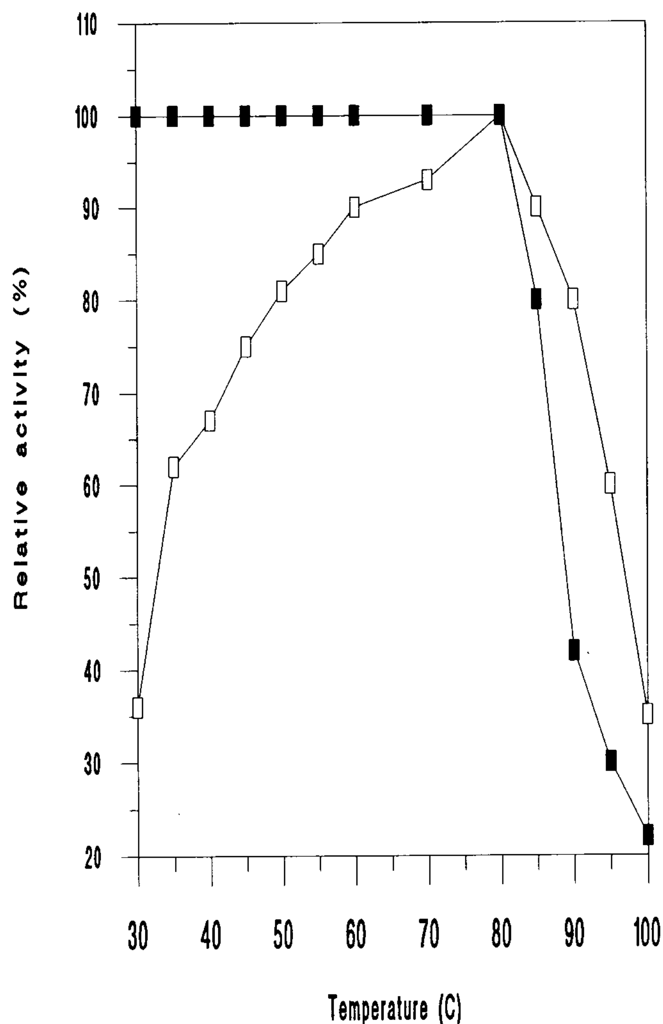

Figure 4 Effect of temperature on the activity (□) and stability (■) of purified α-amylase from *Bacillus subtilis*.

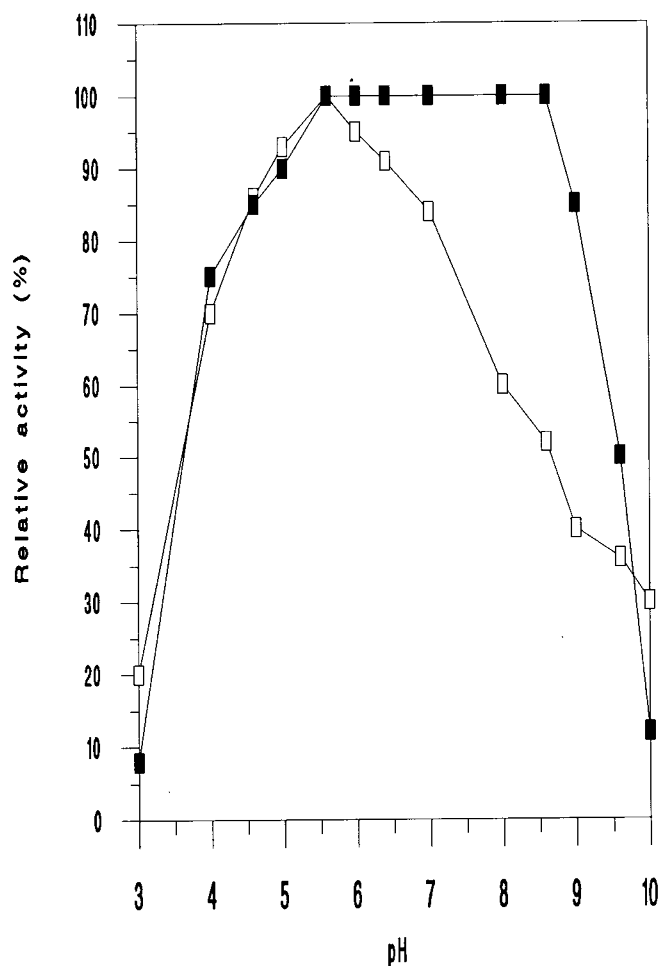

Figure 5 Effect of pH on the activity (□) and stability (■) of purified α-amylase from *Bacillus subtilis*.

Effect of different carbon sources on amylase synthesis

Various carbon sources at 2% concentration were tried as replacements for commercial soluble starch. Bacteria were first serially passaged, normally in the respective media until the mid-exponential phase before final inoculation. All the carbon sources supported good growth of the organism and the growth curves were similar to that obtained in the soluble starch medium (Figure 1). The amylase production (in U mg^{-1}) was in the order of sorghum (216) > corn starch (200) > yam peel (176) > soluble starch (91) > maltose (52) > glucose (38). The superiority of amylase activity with complex substrates has been earlier reported [6,35]. Among the artificial media, the rate of amylase synthesis was greater when soluble starch rather than maltose or glucose was the sole source of carbon. Repression of amylase synthesis by glucose in *Aspergillus niger* and *Clostridium* sp has previously been reported [2,11,25].

After a 72–96 h incubation period, the starches were completely exhausted. This is an indication of the easy degradability of the starches, and the high amylase activity with the cheap local carbon substrates suggests possible utilisation of these substrates in industrial fermentation processes.

Purification of α-amylase

The culture filtrate of *B. subtilis* grown in mineral salts supplemented with soluble starch was concentrated by ultrafiltration, precipitated with ammonium sulphate and applied to a DEAE-Sephadex column. The elution pattern showed a peak of α-amylase activity (data not presented) which was heterogeneous by SDS-PAGE analysis. Therefore, fractions of α-amylase activity were combined, concentrated and fractionated on an Ultragel ACA 34 column. A summary of the purification is given in Table 1. The purified α-amylase was adjudged to be homogenous based on two criteria: protein and activity profiles coincided in the gel filtration (data not presented); and a single band was obtained when the purified enzyme was subjected to SDS-PAGE (Figure 2).

Relative molecular weight

The molecular weight of the enzyme determined by SDS-PAGE was estimated to be 56000 and the value determined by gel permeation chromatography (data not presented) was estimated to be 54000. Confirmation was obtained by mass

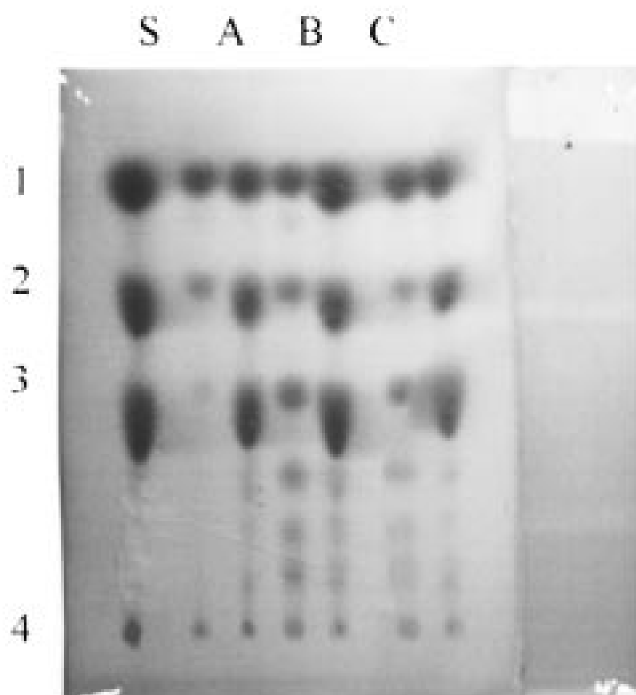

Figure 6 Thin layer chromatogram of hydrolysate products produced by the α-amylase of *Bacillus subtilis*. S: Standard sugars; 1: glucose; 2: maltose; 3: maltotriose; 4: dextrin. Substrates, A: yam peel starch; B: corn starch; C: sorghum starch. The first and second lanes for each substrates were after 10 and 30 min incubation, respectively (picture of plate was reproduced with Biovision Imaging Technology (BioGene Ltd, Kimbolton, UK)).

spectrometric analysis (Figure 3) which showed a prominent peak with an estimated molecular weight of 54 780. This molecular weight value is dissimilar to that reported by Hayashida *et al* [14] and more like the one described by Marco *et al* [20]. Interestingly there have been discrepancies in the molecular weight values of α-amylase from *B. subtilis* determined by gel electrophoresis and amino acid sequences, respectively. Marco *et al* [20] estimated the molecular weight of α-amylase from *B. subtilis* at 48 000 by gel electrophoresis, while a corresponding value of approximately 58 000 was calculated from the derived amino acid sequence. Similarly, the molecular weight of α-amylase from *B. subtilis* Marburg was reported to be 68 000 by amino acid sequence [13], and 59 000 by gel electrophoresis [34].

Effect of temperature and pH on activity and stability

The enzyme was equilibrated at different temperatures for 10 min at pH 6.0 before assay at the same temperature for 5 min. The α-amylase displayed a peak of activity at 80°C (Figure 4). The test for thermostability was conducted in thin-walled test-tubes at different temperatures for 30 min according to the method described by Kocchar and Dua [17]. Thereafter, the tubes were promptly chilled in ice and the residual activity assayed at 25°C as described earlier. The enzyme retained more than 50% of its activity at 85°C (Figure 4). The optimal temperature for activity and stability of the α-amylase was quite high and comparable with other thermostable amylases [16,32]. The activity and stability of our sample were higher than those reported for *B. subtilis* 65 [14]. The reason for this high thermostability in our enzyme sample is not yet clear.

The pH activity profile approximates to a bell-shaped curve with an optimum of 5.6. To determine the pH stability, the enzyme was incubated in the following buffers: McIlvaine citrate-phosphate buffer (0.02 M, pH 2.8–7.6), Sorensen phosphate buffer (0.02 M, pH 7.8), and Clark borate (0.02 M, pH 8.0–10.0) for 24 h at 4°C as described by Hayashida *et al* [14]. The activity was assayed normally at pH 6.0. The enzyme was stable between pH 5.2 and 8.6. At lower and higher pH values of 3.0 and 10.0, about 90% of the activity was lost (Figure 5). The pH activity profile of the enzyme is in agreement with the characteristic single pH peaks shown by most α-amylases [28,29]. Morgan and Priest [26] and Ramachandra *et al* [31], however, reported the possession of two pH optima for the thermostable α-amylases of *Aspergillus niger* and *B. licheniformis*, respectively.

Thin layer chromatography of digests demonstrated the production of glucose, maltose and maltotriose including some small amounts of G4–G6 from starch prepared from several sources (Figure 6). The results were consistent with the action of an endoenzyme which released saccharides with a polymerisation degree of 1–6 [14]. The simultaneous liberation of all the maltooligosaccharides indicated a random attack of the substrate chain by an α-amylase. On longer incubation, glucose, maltose and maltotriose were the end products of hydrolysis (data not presented). Similar results were reported for α-amylase of *B. subtilis* [22] and *C. acetobutylicum* [29]. This suggests that the enzyme is of the saccharifying type and contrasts with the liquefying α-amylase of *B. amyloliquefaciens* and *B. licheniformis* which produces predominantly maltosaccharides during starch hydrolysis [18,22,27].

Mutagenesis of cells of Bacillus subtilis

After UV treatment of the parental strain, 41 isolates were screened and one isolate which showed a relative increase in α-amylase production of 135% was selected for mutation with NTG. A further 38 isolates were obtained from this mutant and 25 showed enhancement in α-amylase activity with one of the isolates exhibiting a relative increase in α-amylase production of 167%. However, there was an observed variability in amylase activity of the mutants (ranging from about 6–124 U mg^{-1} and 59–206 U mg^{-1} respectively, after UV and NTG treatments). Thus using two-step mutation, α-amylase activity was improved by as much as about 200% in several of the mutants when compared to the parental strain. This clearly suggests that mutation played a role in the process of amylase synthesis and secretion [21]. A preliminary study with one of these hyperproducers showed that thermostability of the α-amylase was maintained which suggests that mutation could have occurred in the regulatory [36] rather than in the structural gene but this must await further investigation.

The *B. subtilis* strain isolated in this study (which produces saccharifying α-amylase) and *B. licheniformis* (which produces liquefying α-amylase) could be jointly used for the efficient conversion of locally produced starch to simple sugars in industry. Furthermore, this two-step

mutation technique developed to obtain mutants with enhanced overall amylase production is interesting in that the process is simple, effective and economic. This merits consideration in future mutagenic studies, especially if such studies are being conducted in the underdeveloped countries where cost is a major factor.

Acknowledgements

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