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# Production of Alpha Amylase from *Bacillus subtilis:* Effects of Enzyme Hydrolysis on Starches

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*The enzyme alpha ( $\alpha$ ) amylase was produced by laboratory fermentation of the bacterium Bacillus subtilis in a broth consisting of lactose (4.75% or 7.85%), soya meal, yeast extract, sodium caseinate and magnesium sulphate at pH 7.0 for 24 hrs or 48 hrs. Enzyme production was most favoured with lactose (4.75%) at 37°C, pH 7.0 - 8.0 after 48 hr fermentation. Partial purification of enzyme was by precipitation with dehydrated alcohol at 4°C. The activity of the enzyme on the rate of starch hydrolysis was indicated spectrophotometrically by the decrease in iodine-staining capacity. Bacterial enzymic activity ranged from approximately 344 amylase units in 24 hr fermentation (14.3 amylase units/hr) to 741-909 amylase units in 48 hr fermentation (15.4 - 18.9 amylase units/hr) on hydrolysis of corn, potato, tapioca and arrowroot starches. Higher sugar intermediates (limit dextrins) and maltose were revealed by liquid chromatography on starch hydrolysis at 60°C for 10 mins at pH 6.0.*

## 1. Introduction

The bioprocessing of starches into malto-oligosaccharides is gaining importance because of their uses in food, pharmaceutical and chemical industries.[1] Alpha-amylase and glucoamylase (amyloglucosidase) have been reported to be probably the most used enzymes in the starch industry.[2] Thermostable and thermolabile $\alpha$ -amylases have been widely used industrially for starch liquefaction.[3] Alpha-amylases find occasional use in sugar-refining since small quantities of starch can occasionally occur in sugarcane.[2] Among the commercial amylases, the enzyme from *Bacillus subtilis* has the largest output and is most widely used.[4] Microorganisms suitable for use in the production of enzymes should possess a number of useful attributes such as easy and rapid growth in large fermenters on comparatively cheap and simple nutrients without the need for inducers. A high yield of enzyme should be obtained in a form that is easy to isolate, purify and concentrate without the formation of toxic or immunogenic metabolites.[2] Purification of an $\alpha$ -Amylase from a crude enzyme preparation has been carried out by liquid-liquid reversed micellar extraction. After a full forward and backward extraction cycle, the $\alpha$ -amylase was purified about 1.5 fold with 85% recovery.[5] Research has been done to demonstrate the possibility of a high-producing thermostabile $\alpha$ -Amylase using inexpensive raw materials and glucose as a main carbon source by *Bacillus licheniformis*.[6]

Most starch granules are composed of a mixture of two polymers, an essentially linear polysaccharide called amylose and a highly branched polysaccharide called amylopectin.[7] Amylose constitutes about 20 - 30% (w/w) of most starches and consists of long chains of $\alpha$ -D-

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glucopyranosyl residues which are joined by their 1 and 4 positions. Amylopectin constitutes about 70 - 80% (w/w) of most starches. The glucose units of the amylopectin are joined by $\alpha$-1-4 glycosidic links, but some 4 - 5% of the glucose units are also involved $\alpha$ 1-6 links.[8] The amylose/ amylopectin ratio of maize (*Zea mays*), tapioca (*Manihot esculenta*), potato (*Solanum tuberosum*) and arrowroot (*Maranta arundinacea*) are 26/74, 17/83, 24/76 and 21/79 respectively.[9] Alpha ($\alpha$) amylase is an endo-enzyme that cleaves both amylose and amylopectin molecules, internally producing oligosaccharides.[9] Alpha ($\alpha$) amylase can hydrolyse $\alpha$ (1-4) glucan links located in the inner region of starch and the $\alpha$ (1-6) linkages of the outer branches of the amylopectin to yield D-glucose, a small amount of maltose and a resistant 'core' called a limit dextrin.[10] Starch obtained from corn, potatoes, cassava and wheat accounted for 99% of the world production.[11] Limited research has been done on the hydrolysis of tropical starches such as tapioca and arrowroot in the production of high sugar intermediates to be applied in food processing industry. Therefore, the purpose of this work was to produce $\alpha$-amylase from the bacterium *Bacillus subtilis* using suitable culture conditions, and to compare the ability of the enzyme to hydrolyse tropical starches such as tapioca, and arrowroot and commonly used corn and potato starches in the production of high sugar intermediates and maltose.

## 2. Materials and Methods

### 2.1 Bacillus subtilus

*Bacillus subtilis* B20 strain (not modified genetically) was obtained from the University of Birmingham, UK and was subcultured onto agar slants containing malt extract (40.0g/l), yeast extract (20.0g/l), and bacto agar (20.0g/l) at pH 7.0 and allowed to grow at 37°C for 48 hrs. The growth of the organism was observed for 6 months on the slants and also on acidified potato dextrose agar acidified with 10% tartaric acid[12] (PDA, Oxoid Hampshire, England). Biochemical tests such as gram-staining, hydrolysis on starch agar plates using iodine as an indicator and liquefaction on gelatin were performed to aid in the identification of the bacterium.[13] *Bacillus subtilis* was identified on its morphological and physiological characteristics. Bacteria were gram-positive and rod-shaped. Growth on agar plates was rough and wrinkled, creamy white and translucent. On agar slants, growth was abundant, flat and finely spread. There was positive hydrolysis of gelatin and starch and growth in broth at 37°C by the bacterium. The culture was stored at 4°C subcultured every 2 weeks.

### 2.2 Production of $\alpha$ - amylase

Using the experimental technique,[13] a standard lactose medium consisting of lactose 78.3 g/l; soya 10.0 g/l; yeast extract 15.6 g/l; sodium caseinate 6.6 g/l, and magnesium sulphate 0.4 g/l adjusted to pH 7.0 was used for the growth of the inoculum and for fermentation. The lactose was used as a carbon source for growth of *B. subtilis*. To a sterile medium (30 ml), two (2) loopfuls of cells were introduced and incubated at 37°C for 18 hrs. Culture used as inoculum had an absorbance of >0.05 at a wavelength of 600 nm. Inoculum (1 ml) was transferred to a shake flask containing sterile media (30 ml) and agitated continuously at a speed of 200 strokes per minute.

Several flasks were inoculated for fermentation. Treatment A was conducted over 24-hrs, treatment B for 48 hrs (7.85%) and treatment C for 48 hrs using an adjusted lactose concentration of 4.75%. A larger scale treatment D was performed for 48 hrs using 90 ml of sterile medium of a 4.75% lactose with 3 ml of inoculum. The pH of each fermentation broth was measured (**Table 1**). Absorbance measurements (1/50 dilution) of the broths were made at 600 nm. The samples were stored at -18°C for further work on the assay of the enzyme produced during fermentation.

Enzyme was partially purified.[14] Fermentation broth was centrifuged (5000 rpm) at 4°C for 5 mins. The clear supernatant was decanted and the volume of the broth measured and placed on a reciprocating shaker (200 strokes/ min), and an equal volume of dehydrated alcohol at 4°C was slowly added, then agitated for 5 mins and allowed to stand for precipitation (10 - 15 mins). The process was repeated with three sets of volume of alcohol added to the broth. The final precipitate was filtered through Whatman filter paper #2 and allowed to dry at 25°C. The partial purified enzyme appeared as a cream-coloured paste.

### 2.3 Analysis of Fermentation Broth

All samples were diluted (1 ml broth in 49 mls distilled water) and the absorbance was measured at wavelength 600 nm, with deionised water as a reference on a spectrophotometer (Perkin Elmer Model). The dry mass was measured after harvesting the bacteria for 24 hrs, diluting 1/50 and centrifuging at 1000 rpm for 10 mins at 5°C (Bench Top General Purpose, Centrifuge, Model HT, International Equipment Company, Boston, USA). The cells were dried at 100°C to a constant weight. Total carbohydrate was estimated using Devar's method and the protein content in the fermentation broth was estimated by the Biuret method using albumin a protein standards as described by Thayanithy (1993).[13]

### 2.4 Starch Standard Solutions

Starch standard solutions (1%) were prepared using starches from corn, potato, tapioca and arrowroot (Analar grade, Sigma Chemical Company Limited, St. Louis, USA). Soluble starch (10.0 g) was slurried in 50 mls of deionised water and transferred to 650 mls of vigorously boiling water. Boiling was allowed to continue for 2 mins followed by quenching with 200 ml of cold deionised water and immersed in a cold water bath so as to attain 29 - 30°C. Toluene (0.5 ml) was added to the solution and made up to 1000 ml with deionised water. Toulene served as an activating group and would react fast in all electrophilic substitutions. The solution was used within three days of preparation.

### 2.5 Reaction Substrate Solutions

To 250 mls starch standard solution, 150 mls of acetate buffer (0.1 M, pH 6.0), and 31.2 mls of 0.5 M calcium chloride were added. The mixture was stirred and adjusted to a pH 6.0 ± 0.02 with 1 M sodium hydroxide and diluted to 500 ml with deionised water. Approximately 1 g sample was diluted with 0.0025 M calcium chloride solution containing 0.25 units of activity /ml. Aliquots of 10 ml were pipetted into test tubes (replicates and a blank x 200 nm) and placed in a water bath at 60°C and allowed to equilibrate for 10 mins. To 1.0 ml of solution, 1.0 ml of 1.0 M HCL was added and diluted with approximately 50 ml distilled water. The reaction tubes were removed from the water bath approximately 15 secs prior to the 10-min period for incubation.

### 2.6 Enzyme Activity

An assay of enzyme activity was determined by the method of the Enzyme Biosystems Ltd., USA[15]. Commercial food grade ∞ -amylase, referred to as G-zyme G995 was obtained from *Bacillus stearothermophilus*. Enzyme activity was determined by the rate of hydrolysis of starch as reflected in the rate of decrease in iodine-staining capacity measured spectrophotometrically. One unit of bacterial ∞ -amylase activity is the amount of enzyme required to hydrolyse 10mg of starch per minute under specified conditions. A comparison of enzymic activity was made using a commercial bacterial ∞ -amylase referred to as (G-zyme G995) and experimental ∞ -amylase from *B. subtilis*. The iodine-staining capacity of each broth sample was determined by pipetting 3.0 ml of 0.05% iodine solution and diluting to 100 ml with distilled water. The peak absorbance which remained constant for 30 - 40 secs was measured at 620 nm after 3 mins on addition of iodine solution for samples and after 5 mins for the blank.

Calculations: units/g or ml

$$\frac{\text{(blank abs-sample abs) (50mg starch) (dilution vol)}}{\text{(blank abs) (10min) (10mg/min) (sample size g or ml)}} = 1 \text{ unit}$$

abs-absorbance

### 2.7 Sugar Analysis of Starch Hydrolysates

The samples used for assay of enzymic activity were used to determine the type of sugars formed on hydrolysis of starches at incubated at 60°C for 10 mins at pH 6.0. Liquid chromatography using a Waer Sugar-Pak Column (model 1090, Hewlett Packard, Corvallis, USA), was used to separate monosaccharides and disaccharides according to molecular weight. This column has been used for the analysis of sugar products and process streams in beet, sugarcane and starch hydrolysis processing plants. The reference standards were dextrin, melitriose (raffinose), sucrose, maltose, glucose and fructose. The hydrolysed samples were filtered through 0.45 $\mu$ filter paper. Water was used as the mobile phase at a flow rate 0.5 ml/m. Samples were injected at 75°C.

### 2.8 Viscometric Technique

This depends on the decrease in viscosity of the substrate after the action of amylase breaks down the large polysaccharide molecules into smaller fragments. Viscosity measurements before and after the incubation period have been shown to be proportional to the amylase activity.[13]

## 3.0 Results and Discussion

### 3.1 Selection of Fermentation Medium

The most favourable conditions for fermentation of the *B. subtilis* were pH 7.0 at 37°C for 48 hrs with continuous agitation (**Table 1**). The 48-hr fermentation broth (treatments B and C) had a higher protein content and enzyme activity ($P<0.05$) when compared to the 24-hr fermentation broth (treatment A). There were no differences in analyses of fermentation broth ($P>0.05$) between treatment B (7.85% residual lactose) and treatment C (4.75% residual lactose), thus it was more economical to use treatment C with the lower lactose percentage for further experimental work.

### 3.2 Selection of Amylase Measurement Method

The viscometric 'falling drop' method for the assay of enzymic activity[13] which was based on the viscosity reduction of gelled starch resulted in poor reproducibility of results as

**TABLE 1:** *Analysis of Fermentation Broth*

| Analysis | A[1] | B[2] | C[3] | D[4] |
|---|---|---|---|---|
| pH | 7.24a | 7.19a | 7.43a | 7.37a |
| Absorbance of 1/50 dil at 600 nm | 0.11b | 0.28a | 0.31a | 0.25a |
| Residual lactose (%) | 5.75a | 3.25b | 1.69b | 2.00b |
| Dry wt (%) | 3.69a | 2.47b | 1.60b | 1.99b |
| Protein (%) of broth | 4.42b | 9.63a | 11.13a | 9.10a |
| Protein (%), purified enzyme | 22.67b | 50.09a | 52.50a | 45.00a |
| Purified enzyme wt (%) of broth | 8.00a | 8.79a | 8.43a | 6.00a |
| Activity of Fermentation Broth (Bacterial Amylase Units) | | | | |
| (A) Soluble starch | | 76.25b | 105.40b | 141.20a |
| (B) Corn starch | | 96.70a | 95.95a | - |
| (C) Potato starch | | 107.50a | 111.64a | - |
| (D) Tapioca | | 105.85a | 105.85a | 110.60a |
| (E) Arrowroot | | 109.75a | 108.65a | - |
| Average bacterial activity | | 99.21a | 105.49a | 125.45a |
| Activity of Fermentation Broth (Bacterial Amylase Units) | | | | |
| (A) Soluble starch | 386.63c | 599.95b | 742.85b | 1045.00a |
| (B) Corn starch | 285.90b | 661.35a | 867.70a | - |
| (C) Potato starch | 399.00b | 891.50a | 1061.80a | - |
| (D) Tapioca | 353.23b | 774.53a | 945.40a | 863.40 |
| (E) Arrowroot | 296.00a | 780.00a | 926.54a | - |
| Average enzyme activity | 344.15b | 741.47a | 908.86a | 954.20a |
| Ratio of increased enzyme activity | 14.47a | 7.47b | 8.61b | 7.59b |

Means with different letters in rows are different ($P<0.05$)
[1] Fermentation (Treatment) A (24-hr) (average of 3 readings)
[2] Fermentation (Treatment) B (48-hr) with 7.85% lactose (average of 3 readings)
[3] Fermentation (Treatment) C (48-hr) with 4.75% lactose (average of 2 readings)
[4] Fermentation (Treatment) D large-scale fermentation (average of 3 readings)

the factors of temperature, starch volumes, length and thickness of tubes influenced the 'drop time'. The iodometric assay of enzyme activity, as indicated by a decrease in iodine-staining capacity upon hydrolysis of starches using the Enzyme Biosystems Method (1993)[15] was found to produce more reliable results when compared to the viscometric 'falling drop' method.

### 3.3 Purification of $\alpha$-Amylase

**Table 1** shows that the percentage of protein in the fermentation broth increased approximately 5-fold by purification. Activity of purified enzyme was approximately 14.47 times higher than activity of enzyme in fermentation broth (Treatment A) and 7.47 and 8.61 times that of Treatments B and C respectively. Activity of the purified enzyme of 24-hr fermentation (Treatment A) broth had a range of 296 - 399 amylase units when compared to a maximum activity of 891 - 1062 amylase units in the 48-hr fermentation broth (Treatments B and C). The activity of the commercial enzyme, G-zyme, 995 had an activity rate of approximately 4000 amylase units. There was more protein in the commercial $\alpha$-amylase enzyme obtained from *B. stearothermophilus* thus providing more amino acids as active sites for catalytic activity than experimental *B. subtilis*. Also, possibly, there was little or no inhibition of enzyme but this experiment did not test for this activity.

### 3.4 Components of Starch Analysis

The various percentages of sugars resulted on enzymatic hydrolysis of soluble starches of corn, potato, tapioca and arrowroot are shown in **Table 2**. The components isolated by liquid chromatography were mainly single- and multiple-branched (limit dextrins), thus indicating the incomplete or partial starch hydrolysis at 60°C for 10 min at pH 6.0. Higher temperature and longer application of heat on starch hydrolysis may have resulted in the production of more sugars. The formation of 'dextrin' in soluble starches of Treatment C was higher, 54.91 (**Table 2**) than for commercial starches (43.17 - 43.77; **Table 3**) on enzymatic hydrolysis with $\alpha$-amylase. Generally, 'limit dextrin' was higher in

**TABLE 2:** *Products from Hydrolysis of Soluble Starch*

| Enzyme | Dextrin | Limit Dextrin | Melitriose | Maltose |
|---|---|---|---|---|
| A[1] | 55.66 | 41.09 | 2.31 | 0.99 |
| B[2] | 55.16 | 41.44 | 2.35 | 1.06 |
| C[3] | 54.91 | 41.43 | 2.50 | 1.16 |
| D[4] | 53.20 | 43.99 | 2.01 | 0.78 |
| G-zyme[5] | 51.39 | 44.85 | 2.37 | 1.38 |

Means were not different (P>0.05) in columns
[1] Fermentation (Treatment) A (24-hr) (average of 3 determinations)
[2] Fermentation (Treatment) B (48-hr) with 7.85% lactose (average of 2 determinations)
[3] Fermentation (Treatment) C (48-hr) with 4.75% lactose (average of 2 determinations)
[4] Fermentation (Treatment) D (48-hr) larger scale determinations (average of 2 determinations)
[5] Commercial enzyme, G-zyme from Enzyme BioSystems
Starch hydrolysis performed at 60°C., pH 6.0 for 10 mins.

**TABLE 3:** *Products from Hydrolysis of Commercial Starches*

| Type of Starches | Enzyme | Dextrin | Limit Dextrin | Melitroise | Maltose |
|---|---|---|---|---|---|
| Corn | B[2] | 43.18 | 50.11 | 4.42 | 2.25 |
| Corn | C[3] | 43.17 | 50.39 | 4.35 | 2.07 |
| Potato | B[2] | 41.86 | 50.12 | 5.18 | 2.84 |
| Potato | C[3] | 43.23 | 50.78 | 4.07 | 1.94 |
| Tapioca | B[2] | 42.96 | 50.07 | 4.80 | 2.17 |
| Tapioca | C[3] | 43.77 | 49.06 | 4.79 | 2.38 |
| Tapioca | D[4] | 39.18 | 54.32 | 4.43 | 2.05 |
| Tapioca | G-syme[5] | 37.43 | 53.02 | 5.83 | 3.60 |

Means were not different (P>0.05) in columns
[2] Fermentation (Treatment) B (48-hr) with 7.85% lactose
[3] Fermentation (Treatment) C (48-hr) with 4.75% lactose
[4] Fermentation (Treatment) D (48-hr) in a larger scale fermentation
[5] Commercial enzyme, G-enzyme from Enzyme Biosystems

commercial starches (49.06 - 50.39) than in soluble starches of Treatment C (41.43). The percentages of sugars formed on hydrolysis of experimental soluble starches were lower (**Table 2**), when compared to the commercial starches with **Table 3**. The soluble starches were more resistant to enzyme action, which may be attributed to the higher percentage of amylopectin when compared to the other starches.

## 4.0 Conclusion

The most favourable condition for enzyme production of ∞-amylase by *Bacillus subtilis* was by fermentation of lactose medium (4.75% or 7.85%) for 48 hrs at 37°C at pH 7.0. Purified enzyme showed an enzymatic activity of approximately 800 amylase units, which was capable of hydrolysing a number of starches to produce higher carbohydrate intermediates.

Further work is recommended on the kinetics of the enzyme, as influenced by initial temperature and pH, on the enzyme activity and on hydrolysis of starches. Commercial production of ∞-amylase has the potential of being applied in the hydrolysis of starches in the sugarcane industry, in the production of liquid syrup and in designing textiles.

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