Purification and some properties of glucose isomerase from *Bacillus megaterium*

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Abstract

The objective of this study is to produce and purify glucose isomerase (GI) from Bacillus megaterium and to determine some of its properties. Soil sample was collected from cassava starch processing site and used immediately for bacterial isolation. Selected isolate produced the best GI activity in a preliminary test. The isolate was grown in media containing various carbon, nitrogen and metal salts for enzyme production. Among the carbon sources tested, Galactose gave the best yield (1.1 U/mg protein). Peptone was the best nitrogen source and caused the production of 0.85 U/mg protein. Of the tested metal salts, $MgSO_4.7H_2O$ caused the production of the best enzyme activity of 0.81 U/mg protein. The GI was purified by precipitation with $(NH_4)_2SO_4$ and chromatography on diethyl amino-ethyl (DEAE) – cellulose and DEAE-sephadex G-200 column. Thereafter, the enzyme activity was determined with the optimum temperature and pH at 40ºC and 6.0 respectively. The enzyme was also characterized with a molecular weight of 65kDa as determined by the SDS – PAGE analysis. The enzyme could be applied in the production of high fructose corn syrup.

Key words: Glucose isomerase, carbon sources, nitrogen sources, metal ions, enzyme purification

Introduction

Glucose isomerase (GI) (EC. 5. 3. 1.5) is considered one of the most important industrial enzymes (Sriprapundh *et al.,* 2003). The main practical application of this enzyme arose from its ability to isomerize D – glucose to D – fructose and hence, it is widely used in industry for the production of high fructose corn syrup (HFCS) which is used all over the world as an alternative to sucrose or inert sugar in the food and beverage industries (Heo *et al.,* 2008). In view of the high industrial significance, GI from various microorganisms has been studied and their catalytic and physicochemical attributes have been reviewed by Boshale *et al.,* (1996) and Hartely *et al.,* (2000). Silva *et al.,* (2006) have also noted that GI has enjoyed the largest application of the technology of immobilized enzymes over the last 40 years.

GI is an intracellular enzyme produced from a range of genera principally *Streptomyces, Bacillus, Corynebacterium* and *Arthobacter* spp. and the organisms are grown in media containing glucose and/or xylose as the free sugar. GI has also been produced from *Escherichia coli* and from *Pichia pastoris* (Angardi *et al*., 2013). Bacteria and actinomycetes were found to produce GI. *Lactobacillus brevis, Streptomyces glancescens* and *S. flavogrisens* released the enzyme. The extra cellular GI from *Candida sp.* and alkalo thermophilic *Bacillus sp.* have been purified to homogeneity by conventional purification techniques such as gel filtration, ion exchange chromatography and polyacrylamide gel electrophoresis (PAGE). *Aspergillus oryzae* has been the fungus which is reported to possess GI activity.

The high industrial application of fructose corn syrup (sweetener) motivated the current intensive researches of the various microorganisms that are capable of producing GI. High fructose syrup produced by the activity of glucose isomerase is a useful sweetener; principally in carbonated beverages. According to Lawal *et al*., (2012) fructose has found a wide market in the United States in her soft drink industries. The objective of this study is to isolate, purify and study some properties of GI produced from *Bacillus megaterium.*

Materials and Methods

Sample Collection: Soil sample was collected from a cassava processing site located at the Beach Junction, Obukpa behind the University of Nigeria, Nsukka. The sample was collected into a sterile container and transported immediately to the laboratory for microbiological analysis.

Isolation of microorganism: The soil sample was serially diluted and the dilutions were plated out on Nutrient agar (Oxoid, Ltd. UK). Pure cultures were obtained by streaking on fresh agar plates and stored at 4ºC.

Enzyme production and extraction: Bacterial cells were grown in 100 mL of medium and harvested by centrifugation at 2515 x g for 15 minutes and washed twice with 0.2 M phosphate buffer (pH 6.5). The washed cell suspension was disrupted by sonication for 10 min. using a Biologics Ultrasonic homogenizer Model 150VT (115V/60Hz). Following disruption, the mixture was centrifuged at 2515 x g for 15 minutes. The supernatant fluid was used to measure enzyme activity.

Enzyme assay: Enzyme solution (1 mL) in test tubes were incubated with 2 mL of phosphate buffer (pH 6.5) containing 1% glucose at 30±2°C for 60 minutes followed by keeping the tubes in iced bath for 30 min. The amount of fructose formed was

determined by the method of Kulka (1956). Briefly 0.5 mL of the reaction mixture in a test tube was added to 1.5 mL distilled water. Then 6 mL of ketose reagent [1:1 ratio of A (0.05g resorcinol in 100 mL ethanol] and B [0.216 g $FeNH_4$ $(S0_4)_2$. $12H_2O$ in 1000 mL HCl solution] was added. The content of the tube was mixed and immersed in a water bath (Kotterman, Bremen, Germany) at 80°C for 40 min. The tube was cooled in ice water and the absorbance measured in a Spectrumlab 23 A spectrophotometer at 480 nm. The absorbance was used to prepare a calibration curve with D- fructose (0-200 µg/mL). One unit of activity was defined as the amount of enzyme that released 1 µg of fructose per min. under the assay conditions.

Effect of carbon substrates on enzyme production: The basal medium for bacterial growth contained different carbon sources as indicated plus 0.5% yeast extract, 0.5% peptone in 0.2 M phosphate buffer (pH 6.5). The culture medium was inoculated with 1.5 x 10^7 colony forming units of *Bacillus megaterium* and incubated for 24 h in a Gallenkamp orbital incubator at 35 ^oC followed by enzyme extraction.

Effect of glucose concentration on glucose isomerase production: The effect of glucose concentration on the activity of glucose isomerase was determined by varying the concentrations of glucose from 0.1 – 1.2 (mg/mL) contained in the basal medium and incubated at a temperature of 35^oC for 24 h.

Effect of nitrogen substrates on enzyme production: Culture media contained different nitrogen sources as indicated plus 1% glucose in 0.2 M phosphate buffer (pH 6.5). The culture media were inoculated with 1.5 x 10^7 colony forming units of *Bacillus megaterium* and incubated for 24 h in a Gallenkamp orbital incubator at 35 ^oC followed by enzyme extraction.

Partial purification of the enzyme: The culture supernatant obtained after enzyme production and extraction was pooled and dialyzed overnight against 0.2M phosphate buffer (pH 6.5). Ammonium sulphate was added to the crude enzyme extract to 45% saturation, incubated for 8 hours with gentle mixing. The solution was centrifuged at 2515 x g for 15 min. and the supernatant was subjected to further stepwise precipitation with ammonium sulphate to 65% and 75% saturation followed by centrifugation.

Effect of metal ions on enzyme production: The culture media contained different metal ions plus 1% glucose, 0.5% yeast extract, 0.5% peptone in 0.2 M phosphate buffer (pH 6.5). The culture media were inoculated with 1.5 x 10^7 colony forming units of *Bacillus megaterium* and incubated for 24 h in a Gallenkamp orbital incubator at 35 ^oC followed by enzyme extraction.

The influence of pH on enzyme activity: The effect of pH on activity of partially purified enzyme was determined by using buffer solutions of different pH (Phthalate-NaOH buffer pH 4.0-5.5, and Tris-Maleate buffer 6.0 to 8.5) for enzyme assay. The buffers were used at a concentration of 0.1 Mol/L. The pH activity profile of the enzyme was determined by incubating 0.5 mL of the enzyme contained in test tubes with 0.5 mL of 1 % (w/v) glucose prepared in buffers of different pH values (4.0-8.5) at 35^oC for 2 h. The reaction was stopped by placing the tubes in iced water and the enzyme activities were determined.

The influence of temperature on enzyme activity: The influence of temperature on partially purified enzyme activity was studied by incubating 0.5 mL of the enzyme solution contained in test tube and 0.5 mL of 1 % glucose solution prepared in 0.2 M phosphate buffer (pH 6.5) for 1 h at various temperatures (40, 50, 60, 70, 80 and 90^oC) in a thermostatic water bath (Kottermann, Bremen, Germany). The reaction was stopped by placing the tubes in iced water and the enzyme activities were determined.

Gel Filtration column Chromatography: Enzyme sample was applied to a gel filtration column containing a chromatographic matrix of defined pore sizes. Sample was eluted with an aqueous sodium phosphate buffer (pH 6.5), collected as individual chromatographic fractions and analyzed separately. Sephadex G – 200 (10 g) was suspended in 350 ml, 0.5M of sodium phosphate buffer (pH 6.5). It was stirred gently with a glass rod. The mixture was allowed to stand aseptically, followed by decantation of undissolved supernatant. It was then re-dissolved and allowed to stand for seven hours prior to packing. The column was mounted vertically on a retort stand. The gel suspension (150 mL) was poured into the column to the required height (64.2cm). After the column had been packed to the desired bed height, 3 mL of the sodium phosphate buffer (same pH) was carefully layered on top of the gel. The gel packed column was washed for several times with buffer using drip syringe. At the end of the washing, a column bed height of 62.8cm was achieved. Glucose isomerase dialysate (30 mL) was applied to the packed column using a Pasteur pipette. Then 20 mL of sodium phosphate buffer (same pH) was layered gently on top of the gel and allowed to elute. The enzyme was collected using tube collector. At the end of the elution phase, fractions were collected and assayed.

Sodium dodocyl sulphate//Poly acrylamide gel electrophoresis Analysis: SDS/PAGE was performed as described by Laemmli (1970). The gel casting apparatus was loaded with the resolving gel solution and topped with distilled water. The comb for the stacking gel solution was inserted followed by stacking gel solution preparation. The gel solution was gently mixed, and stacking gel solution (10% polyacrylamide) loaded carefully not to introduce air bubbles around the comb. Stacking gel was allowed to polymerize, glass and gel sandwich were removed from the casting base. The sandwich was placed into the electrophoresis tank, taking note of the terminals. Comb was removed from the gel and loaded with sample. The bottom of the

electrophoresis tank with electrophoresis buffer, 0.01 tris (hydroxymethyl) aminomethane-phosphate was filled to the level marked on the tank and connected to power supply at 4mA. The gel was stained by washing off the glass plates with 500 ml of the gel-fixing (12.5 % trichloroacatic acid) solution and soaked in the fixing solution for 1 h. At the end of this time, the solution was removed by aspiration and covered with 400 mL of the Coomasie blue G-250 (Bromophenol blue) stain, and stained at room temperature for 3 – 4 h with gentle agitation. After this, the destaining solutions (Bromo-phenol blue) was applied and destaining process continued until the protein band was seen without background staining of the gel.

Assay procedures: Protein content was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin (Sigma-Aldrich) as a standard. Fructose concentration was determined by the method of Kulka (1956) using 50-200 µg fructose as standard.

Results and discussion

The isolate selected for this study was identified as *Bacillus megaterium* based on the taxonomic descriptions described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

To study the effect of different carbon sources on enzyme production, xylose, sorbitol, galactose, sucrose and lactose were used as carbon sources. Galactose caused the production of the highest GI activity (1.1 U/mg protein) followed by sorbitol (0.88 U/mg protein). Lower enzyme activities were observed with other carbon sources namely, xylose, sucrose and lactose (Fig. 1). This result suggests that the type of carbon source in the production media influenced the production of GI by *Bacillus megaterium.* Xylose was a good source of carbon for GI production by many bacteria which isomerized xylose in addition to glucose (Pandicurai *et al.,* 2011). The presence of D-xylose significantly increased the enzyme productivity by *Streptomyces* spp (Wong *et al*., 1991).

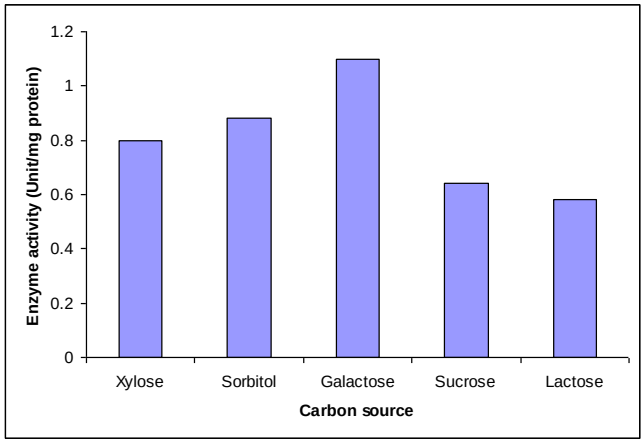

Fig 1: Effects of carbon sources on glucose isomerase activity

The optimum enzyme activity of GI was achieved at 0.5 mg/mL glucose, and after which further increases in glucose concentration caused decrease in GI production (Fig. 2). This confirms that an increase in the concentration above the optimum concentration lead to further decreases in enzyme activity. Kwakman and Postma (1994) reported that the presence of high glucose concentrations in microbial growth media caused reduction in the specific activities of the enzymes involved in the catabolism of other carbon sources. This could be attributed to the repressor effect of high concentrations of glucose on GI synthesis.

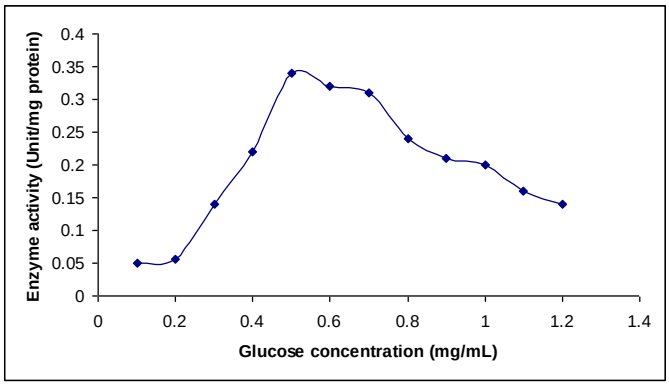

Fig. 2: Effects of glucose concentration on glucose isomerase activity

Organic and inorganic nitrogen sources namely, yeast extract, casein, peptone, ammonium chloride and ammonium sulphate were used for enzyme production. Of the nitrogen sources tested, peptone caused the production of the highest GI activity (0.85 U/mg protein) followed by casein (0.82 U/mg protein). Yeast extract, $(NH_4)_2SO_4$ and NH_4Cl caused the production of less GI activities (Fig. 3). The effects of nitrogen substrates on GI production by microorganisms have been reported (Yassien and Jiman-Fatani 2012). Aiyer (2004) reported ammonium dihydrogen phosphate to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than other tested nitrogen sources. Peptone, tryptone and yeast extract supported maximum growth and enzyme production by *Streptomyces thermonitrificans* and organic nitrogen sources were observed to support the highest GI production when compared to inorganic nitrogen sources (Deshmukh *et al*., 1994). Yassien and Jiman-Fatani (2012) reported that a combination of corn steep liquor (2%) and yeast extract (1%) resulted in an increase in GI production by about 3 to 4 times higher than other nitrogen sources tested.

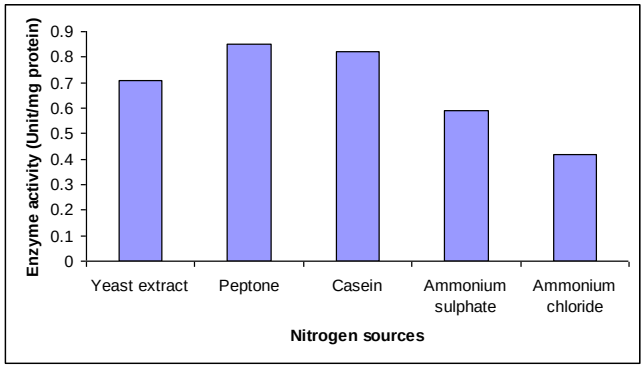

Fig. 3: Effects of nitrogen sources on glucose isomerase activity

Among the tested metal salts, $MgSO_4.7H_20$ gave the best result with 0.81 U/mg protein, more than $CoCl_2.6H_20$ (0.65 U/mg protein), $FeSO_4$ (0.24 U/mg protein), $CuSO_4$ (0.11 U/mg protein) and $ZnSO_4$ (0.2 U/mg protein) (Fig. 4). It is obvious from this result that Mg^{2+} and Co^{2+} enhanced the activity of GI while Zn^{2+}, Fe^{2+} and Cu^{2+} inhibited the enzyme. GI typically requires the presence of divalent metal cations such as Mg^{2+} or Co^{2+} as essential cofactors for their catalytic activity (Whitlow *et al*., 1991). Kasumi *et al.,*(1980) found that Co^{2+} gave only 56% reaction activity as compared to Mg^{2+}, but Ryu e*t al*., (1977) reported that the addition of Co^{2+} into a culture medium stimulated the formation of GI. Treatment of purified enzyme with EDTA resulted in an almost complete loss of enzyme activity but the activity was restored by the addition of metal ions (Kitada *et al*., 1989).

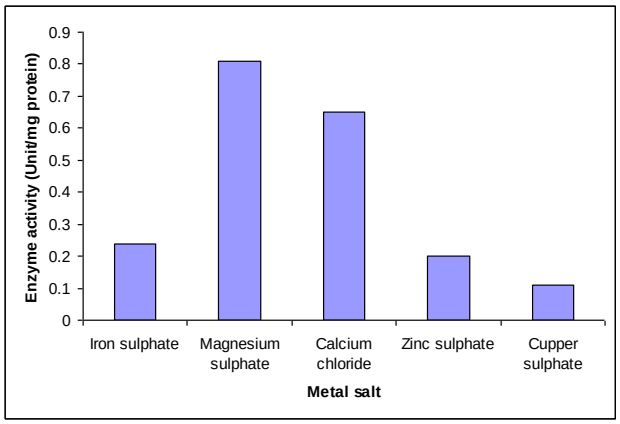

Fig. 4: Effects of metal ions on glucose isomerase activity

The pH of the reaction mixture was adjusted to different values (pH 4.0 to 8.5). The highest enzyme activity was obtained at the pH 6.0, while the optimum pH ranges from 5.5 – 8.0 (Fig. 5). The results obtained were in line with the results of Pandicurai (2011) in which the glucose isomerase produced by *Enterobacter agglomerans* had optimum pH at 6.0. Ryu e*t al*., (1977) showed pH 8.3 to be optimal for glucose isomerase production while Danno (1970), Chen *et al.*, (1979) and Yassien and Jiman-Fatani (2012) reported pH optima at 7.0 with the following microorganisms: *Bacillus coagulans, Streptomyces flavogriseus* and *Streptomyces albaduncus* respectively. Lawal *at al.,* (2012) reported pH optimum at 10.0 with *Bacillus* spp. The optimum pH for GI production was in the range between pH 7.0-9.0 (Lee and Zeikus 1991). The optimum pH for GI production was slightly acidic, pH 6.9 for *Streptomyces* species (Dhungel *et al.*, 2007).

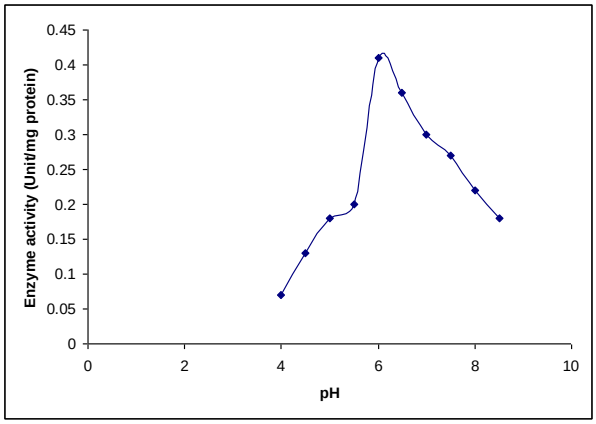

Fig. 5: Effects of pH on glucose isomerase activity

The best temperature for the enzyme production was 40ºC (Fig. 6). The result indicated that the bacterium is mesophilic. Optimal enzyme activity of glucose isomerase was observed at 42-43 ºC by Ryu e*t al*., (1977) while Yassien and Jiman-Fatani (2012) reported a suitable temperature for microbial growth and GI production in the range of 25-35 ºC for *Streptomyces albaduncus.*

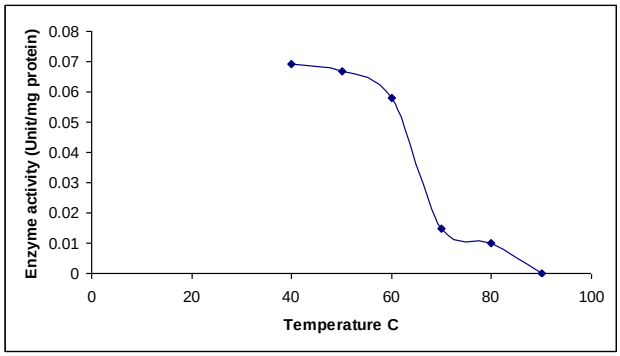

Fig 6: Effects of temperature on glucose isomerase activity

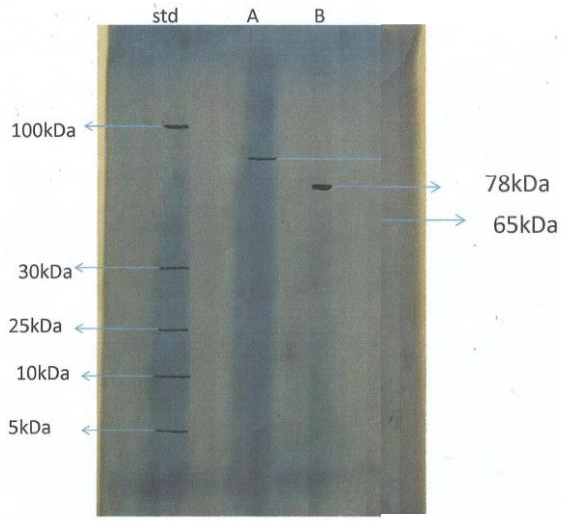

Fig. 7: SDS- PAGE analysis: Lane std is the standard, lane A represent molecular weight of partially purified enzyme; Lane B represent enzyme purified 100-fold

Purification of the glucose isomerase enzyme from culture supernatant of through ammonium sulphate precipitation, DEAE – cellulose chromatography, DEAE – sephadex G – 200 and SDS – PAGE analysis confirmed the molecular weight of the enzyme as 65kDa (Fig 7).

Conclusion

A soil bacterium, *Bacillus megaterium* was isolated and used to produce glucose isomerase (GI) in liquid culture. The bacterium produced the best GI among the other organisms tested and was therefore selected for further work. Among the carbon sources tested, Galactose caused the production of the best enzyme activity (1.1 U/mg protein). Peptone was the best nitrogen source and caused the production of 0.85 U/mg protein. Of the tested metal salts, $MgSO_4.7H_2O$ produced the best result (0.81 U/mg protein). The GI was purified by precipitation with $(NH_4)_2SO_4$ and chromatography on diethyl amino-ethyl (DEAE) – cellulose and DEAE-sephadex G-200 column. Thereafter, the enzyme activity was then determined with the optimum temperature and pH at 40ºC and 6.0 respectively. The enzyme was also characterized with a molecular weight of 65kDa as determined by the SDS – PAGE analysis. The enzyme could be applied in the production of high fructose corn syrup.

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