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Activity of Glucose Isomerase from *Bacillus thuringiensis* under Different Treatments

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Bacillus thuringiensis is a Gram-positive bacterium naturally found in soil, water and grain dust, and can be cultivated in liquid, solid and semi-solid media. Glucose isomerase (EC. 5.3.1.5) catalyzes the reversible isomerization of glucose to fructose and that of xylose to xylulose. It is an important enzyme used in the industrial production of high-fructose corn syrup (HFCS). Glucose isomerase was purified from *Bacillus thuringiensis*. The final purification resulted in a considerably high yield (64.6%) with about 15.8-fold. The optimum temperature and pH were 50 °C and 7.0. The optimum glucose concentration was 8 mM. Glucose isomerase required Mn²⁺ or Ca²⁺ as cofactor. The enzyme was activated by cysteine, ascorbic acid, folic acid and sodium sulfate. The enzyme was inhibited by sodium bromide, sodium azide, sodium fluoride, sodium arsenate, glycine, tyrosine, phenylalanine, arginine, asparagine, and guanidine hydrochloride. Pyruvate, glyoxylate and 2-oxoglutarate inhibited the enzyme activity at the higher concentrations.

Introduction

Glucose isomerase enzyme was found in several bacteria which can utilize xylose as a carbon substrate for growth (Chen, 1980). The physiological function of the enzyme *in vivo* is to convert D-glucose into D-fructose (Takasaki *et al.*, 1969).

Glucose isomerase has gained more interest due to its potential applications in the biofuel industry in addition to food industry. Ethanol is the major form of biofuel, and numerous technologies have been used to improve its production (Bangrak *et al.*,

2011; Mohammadi *et al.*, 2011; Tao *et al.*, 2011).

Various microorganisms have been found to produce glucose isomerase (Deshmukh *et al.*, 1994; Belfaqui and Penninckx, 2000; Givry and Duchiron, 2007). The conversion of glucose to fructose chemically has been known to demand high pH and temperature. The possibility of producing fructose chemically from glucose has been studied by Barker *et al.* (1973).

Chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied. Thus, it cannot be used commercially. In addition, enzymatic conversion of glucose to fructose has several advantages such as (i) the reaction requirement of ambient conditions of pH and temperature, (ii) specificity of the reaction and (iii) the reaction proceeds without side products. Thus, the enzymatic conversion of glucose to fructose is better than the chemical isomerization (Anbu *et al.*, 2013).

The cost of enzyme production is an important factor in evaluation of its suitability for industrial application. One way to reduce the cost of glucose isomerase production is to reuse it several times and to recover it efficiently. Immobilization of glucose isomerase offers an excellent opportunity for its effective reuse. The largest market for glucose isomerase in the immobilized form has become of great interest (Verhoff *et al.*, 1985; Pedersen, 1993).

Several methods for immobilizing of glucose isomerase have been described. However, a few methods are economical and yield enzyme preparations with properties that are acceptable for commercial high-fructose corn syrup (HFCS) (Antrim *et al.*, 1979).

The ability of glucose isomerase to isomerize a variety of substrates including pentoses, hexoses, sugar alcohols and sugar phosphates was investigated. The enzyme was able to utilize D-ribose, L-rhamnose, L-arabinose and 2-deoxyglucose beside D-glucose and D-xylose (Chen, 1980).

The aim of the present work was to determine the activity of glucose isomerase purified from *Bacillus thuringiensis* under different treatment.

Material and Methods

Source of the Enzyme

Bacillus thuringiensis was the organism used in the isolation, purification of glucose isomerase and studying the response of the enzyme to various effectors.

Growth of *Bacillus thuringiensis*

The pure culture was inoculated into a 250 ml conical flask containing 250 ml of culture medium (peptone 1g, yeast extract 0.5 g, K₂HPO₄ 0.3g, MgSO₄.7H₂O 1g, xylose 1g, distilled water 250 ml, pH 7.0 followed by incubating at 37 °C on shaker for 24h.

Enzyme Production and Extraction

Bacterial cells grown in 250 ml of medium described above were harvested by centrifugation at 3000 g for 15 min and then washed twice with 0.2 M phosphate buffer (pH 7.0). After washing the cell suspension was treated with 5% Triton X-100 for 10 min to disrupt the cell membrane. Following disruption, the mixture was centrifuged at 3000 g for 15 min. The resulting supernatant was used to measure the enzyme activity.

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Enzyme Assay

One ml of the prepared enzyme solution was added to a test tube and incubated with 2 ml of 100 mM phosphate buffer (pH 7.0) containing 1% glucose for 25 min followed. The tubes were then kept in iced bath for 30 min. The formed fructose was determined by the method of Kulka (1956). Aliquot (0.5 ml) of the reaction mixture was mixed with 1.5 ml distilled water. Then 6 ml of ketose reagent [1:1 ratio of A (0.05 g resorcinol in 100 ml ethanol) and B [0.216 g $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 1000 ml HCl solution] was added. The tube containing the mixture was placed in a water bath at 80°C for 30 min. The tube was then cooled in ice water and the absorbance measured spectrophotometrically at 480 nm. Calibration curve with D-fructose (0–200 $\mu\text{g}/\text{ml}$) was prepared. One unit of activity was defined as the amount of enzyme that released 1 μg fructose per min under the assay condition.

Purification of Glucose Isomerase

The obtained crude extract of glucose isomerase was precipitated with 40 % - 80 %) ammonium sulphate. It was kept at 4°C overnight then centrifuged at 6000 g for 20 min. The pellet was collected and then dissolved in 100 mM phosphate buffer (pH 7.5). Ammonium sulphate precipitated samples were tested for enzyme activity and protein then were further subjected to gel filtration-using Sephadex G-200.

Protein Content

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Influence of Temperature and pH on Enzyme

Activities of glucose isomerase activity was determined under standard assay conditions

at different temperatures 20-70 °C. The optimal pH value was determined at pH values ranging from 2.0 to 9.0.

The Effect of Metal Ions

The effect of metal ions Zn^{+2} , Cd^{+2} , Mn^{+2} , Co^{+2} , Ca^{+2} and Na^+ on the enzyme activity at 5 mM was studied. They were assessed by including each cation individually in the reaction mixture of the enzyme 50°C for 30 min and then assaying the residual enzymatic activity under standard conditions.

The Effect of Anions

Various anions namely arsenate, azide, bromide, fluoride and sulfate were tested as sodium salts. They were tested at 10 mM in the reaction mixture.

Effect of Amino and 2-oxo Acids

The effect of some amino acids including glycine, tyrosine, phenylalanine, arginine, asparagine and cysteine was investigated at 10 mM in the reaction medium. The 2-oxo acids pyruvate, 2-oxo-glutarate and glyoxalate were tested in the reaction medium at various concentrations (5, 10, 15, 20, 25 and 30 mM).

Results and Discussion

Purification of Glucose Isomerase

Glucose isomerase was purified with ammonium sulphate and Sephadex G-150. The specific activity, yield and the fold of purification were calculated. The results are shown in Table 1. These results indicate that the obtained specific activity was 42.8 units mg^{-1} protein. The yield was 64.6 % with 15.8-fold of purification. Similar results were reported for the enzyme from *Arthrobacter nicotiane* (Sapunova et al.,

2010) where the enzyme was purified with 16-fold. Also the enzyme was purified with 42.8 units mg protein from *Streptomyces* SP. CH7 (Chanitnun and Pinphanichakarn, 2012). Glucose isomerases produced by *Thermoanaerobacter* strain B6A and *Clostridium* thermosulfurogenes strain 4B were purified 10-11-fold (Lee and Zeikus, 1991).

Effect of Temperature on Glucose Isomerase

This experiment was carried out to find out the optimal temperature for glucose isomerase. Therefore, the enzyme activity was measured at various temperatures 20, 30, 40, 50, 60 and 70°C. The results in Fig. 1 show that the enzyme activity increased continuously by increasing the temperature until it reached 50°C, after which there was a continuous decline and reached 16.8 units mg^{-1} protein at 70°C. This result is in harmony with the finding of Nwokoro (2015) for the enzyme *Bacillus licheniformis*. The enzyme from *Streptomyces* sp. had maximal activity at 85°C (Chanitnun and Pinphanichakarn, 2012).

Effect of pH on Glucose Isomerase Activity

The enzyme activity was measured at various pH values (2, 3, 4, 5, 6, 7, 8 and 9). The results in Fig. 2 show that there was a continuous increase in the enzyme activity with increasing the pH up to pH 7.0 where the enzyme activity was 33.4 units mg^{-1} protein after which the activity reduced continuously at pH 8.0 and pH 9.0. Therefore, it is apparent that the pH optimum for glucose isomerase in the present study was 7.0 which is in agreement with that reported for the enzyme from *Streptomyces* sp. CH7 (Chanitnun and Pinphanichakarn, 2012). However, lower

optimum pH (6.0) for glucose isomerase from *Bacillus licheniformis* was reported by Nwokoro (2015).

Effect of Glucose on Glucose Isomerase Activity

This experiment aimed to investigate the effect of various glucose concentrations (2, 4, 6, 8 and 10 mM) on the activity of glucose isomerase. The results in Fig. 3 show that there was continuous increase in glucose isomerase activity with increasing the substrate concentrations up to 8 mM where the activity was 21.4 units mg^{-1} protein. However, at 10 mM little decline in the activity of the enzyme was observed where the activity was 21.2 units mg^{-1} protein.

Effect of Some Vitamins on Glucose Isomerase Activity

This experiment aimed to study the effect of two vitamins ascorbic acid and folic acid on the enzyme activity. Each vitamin was tested at both 1 and 2 mM. The results in Fig. 4 show that both vitamins activated the enzyme with different rates. Folic acid was better activator than ascorbic acid at the two tested concentrations. The enzyme was activated by 14% and 38% in presence of 1 mM and 2 mM folic acid, respectively. It was reported that these two vitamins increased paraoxonase, arylesterase, creatine kinase, lactic dehydrogenase, and alkaline phosphatase activities (Gursu *et al.*, 2004).

Effect of some Cations on Glucose Isomerase Activity

This experiment aimed to investigate the effect of cations including Zn^{2+} , Cd^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} and Na^+ on the enzyme activity. The examined cations were in the form of sulfate salt. Each was tested at 5 mM. The results in Fig. 5 indicate that the cations

Zn^{2+} , Cd^{2+} and Co^{2+} inhibited the activity. Na^+ did not show any effect, however Mn^{2+} and Ca^{2+} . There have been some reports that glucose isomerases require divalent metal ions such as Mg^{2+} and Mn^{2+} as cofactors

(Lehmacher and Bisswanger, 1990; Lama *et al.*, 2001). Nwokoro (2015) reported that the best enzyme activity from *Bacillus licheniformis* was observed in the presence of Mg^{2+} .

Table.1 Summary of Purification of Glucose Isomerase

Purification (fold)	Yield (%)	Specific activity (U/mg protein)	Total activity (U)	Total protein (mg)	Purification step
1	100	2.71	129.9	47.9	Crude extract
9.7	77.1	26.4	100.2	3.8	Ammonium sulphate (40 % - 80 %))
15.8	64.5	42.8	83.9	1.96	Sephadex G-150

Fig.1 Effect of Temperature on Glucose Isomerase Activity

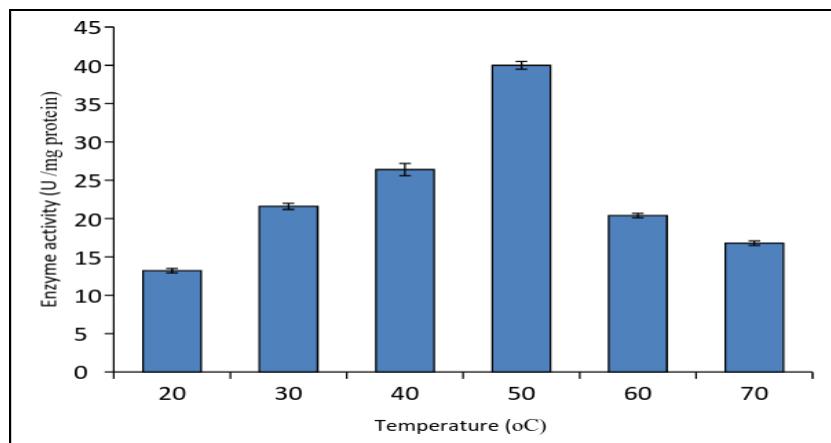


Fig.2 Effect of pH on Glucose Isomerase Activity

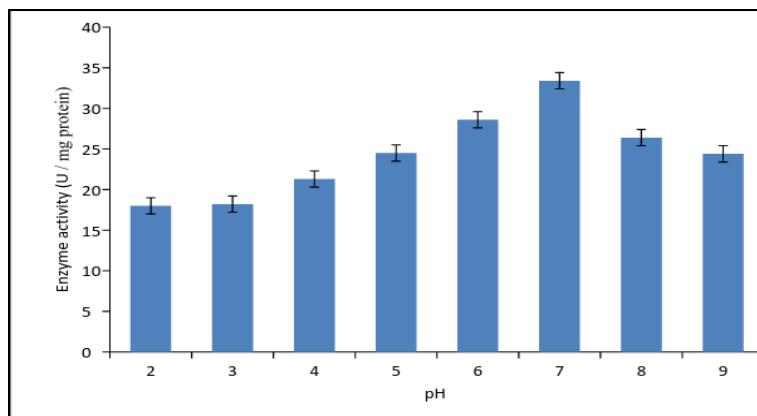


Fig.3 Effect of Various Concentrations of Glucose Isomerase on the Enzyme Activity

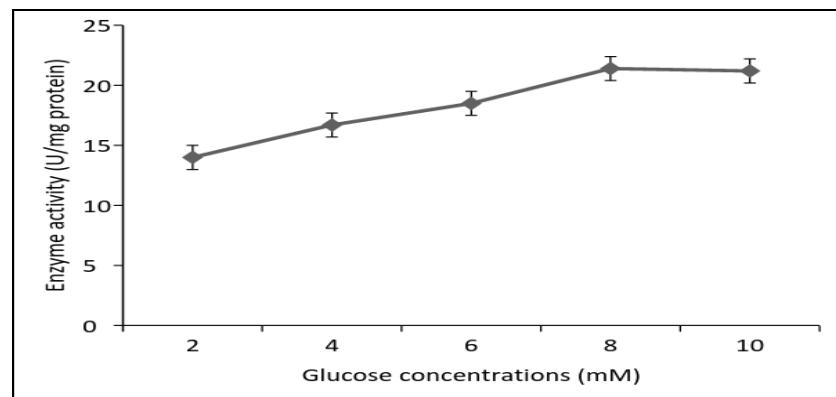


Fig.4 Effect of some Vitamins on the Rnzyme Activity

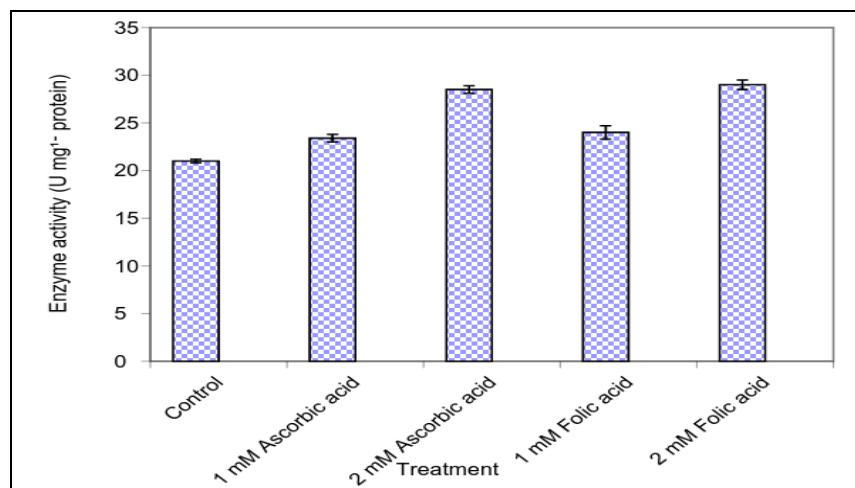


Fig.5 Effect of Various Cations on Enzyme Activity

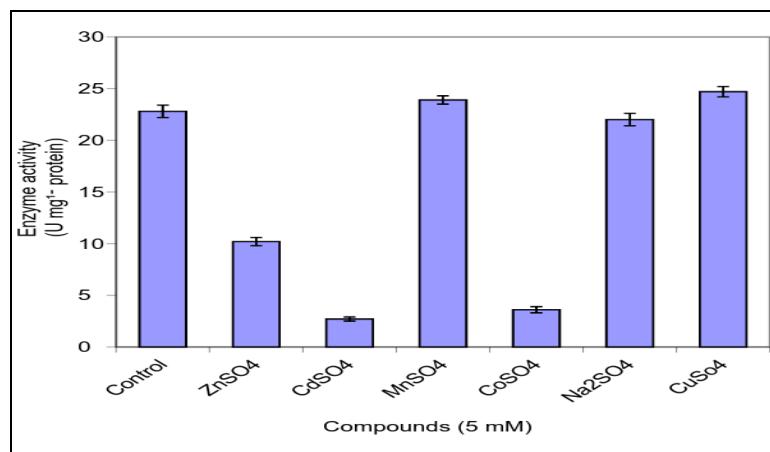


Fig.6 Effect of Ca+2 ion on Glucose Isomerase Enzyme Activity

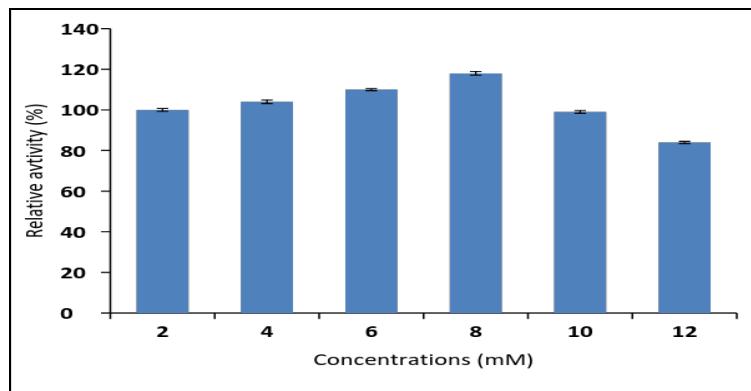


Fig.7 Effect of Anions on Enzyme Activity

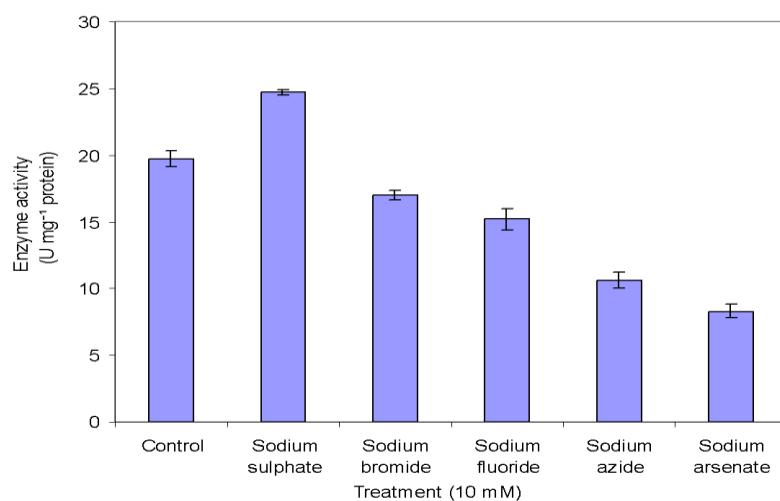


Fig.8 Effect of Amino Acids on Enzyme Activity

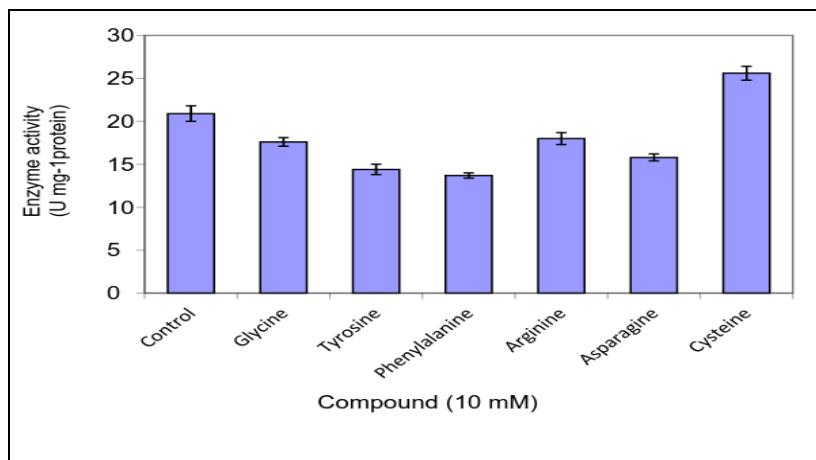


Fig.9 Effect of Guanidine HCl on Enzyme Activity

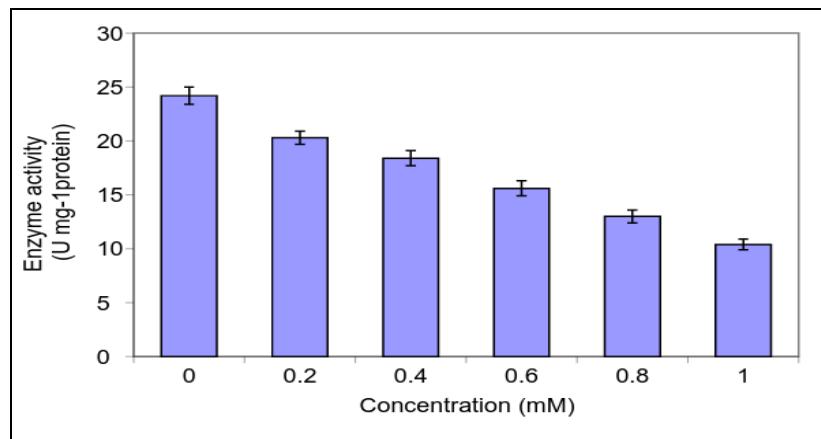
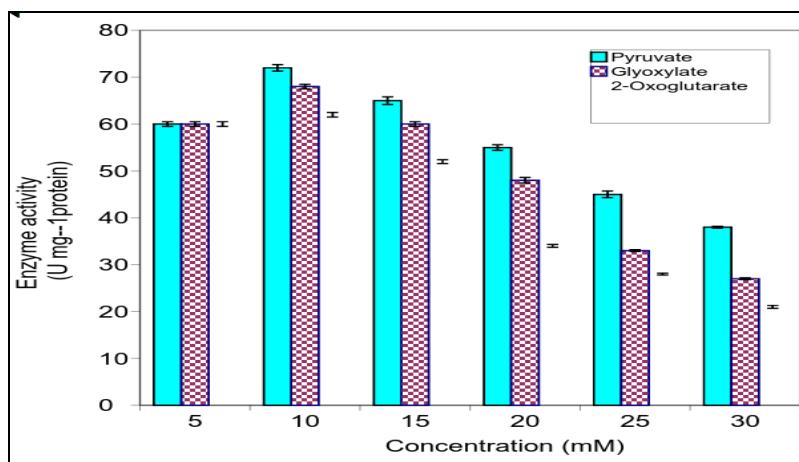


Fig.10 Effect of 2-oxo acids on Enzyme Activity



Effect of Ca^{2+} ions on Glucose Isomerase Activity

This experiment aimed to study the effect of various concentrations of Ca^{2+} on glucose isomerase activity. Ca^{2+} was tested at various concentrations of CaCl_2 (2, 4, 6, 8, 10 and 12 mM). The results of these experiments are shown in Fig. 6. These results indicate that there was continuous increase in the relative activity at the concentrations 2, 4, 6 and 8 mM CaCl_2 where the relative activities were 100%, 104%, 110% and 118%, respectively. The reason for the increase in the enzyme

activity by Ca^{2+} may be due to the stabilization of the enzyme in its active conformation rather than or it is involved in the catalytic reaction (Strongin, 1978; El-Shora and Abo-Kassem, 2001).

Effect of some Anions on Glucose Isomerase Activity

The effect of various anions on glucose isomerase was investigated. The anions were arsenate, azide, bromide, fluoride and sulfate. All anions are used as sodium salts. They were tested at 10 mM in the reaction mixture. The results in Fig. 7 show that all

the tested anions inhibited the enzyme activity with different rates except sulfate was activator. The activity in presence of sodium sulphate was 24.7 Umg⁻¹protein which was higher than that of the control value (19.7 Umg⁻¹protein). Arsenate inhibited other enzymes (El-Shora and Lila, 2011; EL-Shora and Abd El-Gawad, 2014) Arsenate inhibition for enzymes is believed to be due to its ability to bind protein thiols (Hu *et al.*, 1998). Bromide and fluoride inhibited other enzymes (El-Shora and Metwally, 2009).

Effect of Amino Acids on Glucose Isomerase Activity

The effect of various amino acids on the enzyme activity was studied. These amino acids were glycine, tyrosine, phenylalanine, arginine, asparagine and cysteine. Each amino acid was tested at 10 mM in the reaction mixture. The results in Fig. 8 indicate that all tested amino acids inhibited the enzyme activity with various rates with exception of cysteine which activated the enzyme. The activity in presence of cysteine was 25.6 units mg⁻¹protein which was higher than that recorded for the control (20.9 units mg⁻¹protein). Cysteine activated other enzymes such as tyrosinase (El-Shora and Hegazy, 2014). These results reveal that SH-group of glucose isomerase may be protected during the incubation time or cysteine activated the enzyme through lowering its K_m for the substrate.

Guanidine-HCl (Gdn-HCl) on Glucose Isomerase Activity

Effect of Gdn-HCl on the enzyme activity was investigated using different concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 mM in the reaction mixture. The results in Fig. 9 show that increasing the Gdn-HCl concentration resulted in inhibition of the

glucose isomerase activity. The reduction in the activity reached 10.4 units mg⁻¹protein at 1.0 mM compared to that of the control values (24.2 units mg⁻¹protein). Gdn-HCl could be ionized to Gdn and Cl ions in aqueous solution as being a salt and then possibly masked the positively and negatively charged amino acids of the enzyme. Therefore, Gdn-HCl contained a strong ionic strength at high concentration and hence disrupted the enzyme and destabilized electrostatic interactions or reduced stability (Muzammil, 2000).

Effect of 2-oxo acids on Glucose Isomerase Activity

This experiment was carried out to investigate the effect of various keto acids namely pyruvate, glyoxylate and 2-oxoglutarate in the reaction medium glucose isomerase. These compounds were tested at various concentrations 5, 10, 15, 20, 25 and 30 mM. The results in Fig. 10 indicate that the three compounds activated the enzyme at the lower concentration (5 and 10 mM) but the activity was inhibited by the higher concentrations.

In conclusion, glucose isomerase was purified with appreciable activity from *Bacillus thuringiensis* which can be used as a source for the enzyme.

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