academicJournals

Vol. 7(21), pp. 2682-2688, 21 May, 2013
DOI: 10.5897/AJMR2012.2482
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Purification, characterization and immobilization of glucose isomerase from *Streptomyces albaduncus*

Mahmoud Abdul Megead Yassien[1]*, Asif Ahmad Mohammad Jiman-Fatani[2] and Hani Zakarea Asfour[2]

[1]Department of Natural Products/Microbiology, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Kingdom of Saudi Arabia.
[2]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Kingdom of Saudi Arabia.

Accepted 30 April, 2013

Glucose isomerase produced from *Streptomyces albaduncus* was purified to homogeneity by ammonium sulphate precipitation, followed by ion exchange DEAE-cellulose chromatography, and finally on DEAE-Sephadex A-50 chromatography. The molecular weight of the purified enzyme was estimated to be 54 kDa by sodium dodecyl sulphate polyacrylamide gel electrophresis (SDS-PAGE). The final preparation by the purification procedure had 10.3% final activity recovery and 13.3-fold purification. The optimum pH and temperature for GI activity were 7 and 70°C, respectively. In addition, the presence of the combination of Mg^{2+} and Co^{2+} ions (5 mM) improve the GI activity. Partially purified GI enzyme was immobilized by cross-linking with gluteraldahyde, adsorption on DEAE-Cellulose, and entrapment into polyacrylamide. Immobilization of GI enzyme caused sight decrease (3-19% reduction) in the enzymatic activity as compared to that of the non-immobilized enzyme. The maximum enzymatic activity (97%) and stability (88%) was obtained in the immobilized enzyme prepared by entrapment into polyacrylamide.

Key words: Glucose isomerase, *Streptomyces albaduncus,* purification, immobilization.

INTRODUCTION

Glucose Isomerase (GI) is a microbial enzyme of immense commercial significance. The enzyme is used in large quantity for the production of high fructose corn syrup (HFCS) which is widely used in the United States and Japan as an alternative to sucrose or invert sugar in the pharmaceutical, food, and beverage industry. The production of industrially important monosaccharides, such as L-glucose, L-fructose, L-ribose, L-lyxose, D-allose, and L-galactose, by glucose isomerase has recently received much attention due to potential health and medical benefits (Joo et al., 2005; Kluskens et al., 2010). Interconversion of xylose to xylulose by GI, on the other hand, serves a nutritional requirement in saprophytic bacteria. Also, it has a potential application in the bioconversion of hemicellulose to ethanol (Borgi et al., 2004; Van Vleet and Jeffries, 2009).

Due to the expense of glucose isomerase, there is direction for using the enzyme in immobilized form that can be reused several times at minimal cost (Leang et al., 2004). Over the last decade, immobilized enzymes

*Corresponding author. E-mail: myassien61@yahoo.com. Tel: 00966-508319951. Fax: 0096602-6951696.

Abbreviations: GI, Glucose isomerase; **HFCS,** high fructose corn syrup; **PPE,** partially purified enzyme.

have primarily been used in the bio-processes for the production of food, pharmaceuticals, and other biologically-based fine products. Cross linking, physical adsorption and entrapment among the various methods for enzyme immobilization are the most suitable methods for GI immobilization (Song et al., 2011).

The present study was performed to purify the GI enzyme from *S. albaduncus* and to characterize some of its enzymatic properties. In addition, the GI enzyme in partially purified form was immobilized by different methods, and its activity and stability was evaluated to select the most suitable immobilization method.

MATERIALS AND METHODS

Organisms, medium and growth

S. albaduncus is a high GI producer strain isolated in our previous study from West Area of Saudi Arabia (Yassien and Jiman-Fatani, 2011). Microbial growth and enzyme production was carried out in a 14L laboratory glass fermentor (Bioflo 110, New Brunswick Scientific Co., Inc., NJ, USA) containing 8 L working volume as described by Yassien and Jiman-Fatani (2012). The fermentation medium consists of 1% D-xylose, 2% corn steep liquor, 1% yeast extract, 0.1% $MgSO_4$, 0.1% ammonium phosphate, 0.01% DL-isoleucine, pH adjusted to 7. Spores from 4 to 5-day-old cultures on starch nitrate agar (20 g soluble starch, 2.0 g KNO_3, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 3.0 g $CaCO_3$, 0.01 g $FeSO_4$, 0.01 g $MnCl_2$, 0.01 g $ZnSo_4$, 20 g agar per liter) were harvested in sterile normal saline. The final count in the spore suspension was adjusted to about 10^5 CFU/ml, as determined by the standard viable count technique. The spore suspension was used as an inoculum to give a final count of 10^3 CFU/ml in the fermentation medium. The fermentation was operated at 30°C for 108 h, using batch mode without pH control.

Determination of microbial production of glucose isomerase

Quantitative determination of the GI activities was carried out as described by Bok et al. (1984). Briefly, an aliquot of the culture broth obtained after fermentation was sonified on ice for 10 min at 22 volts output power by using sonicator (Soniprep 150 plus, UK). Then, a 0.5 ml of each sonified culture broth was mixed with 5 ml of maleate buffer-salts solution containing 1% glucose and incubated in a water bath at 65°C for 60 min. The mixture was cooled in ice to stop the enzymatic reaction. The amount of fructose in the prepared mixture was determined as described by Kulka (1956). One unit of GI activity (U) is defined as the amount of enzyme that produced 1 µmol of D-fructose per min under the assay conditions. Specific GI production is expressed as units of enzyme activity per gram of dry cells.

Purification of glucose isomerase enzyme

Preparation of crude enzyme

The microbial cells were harvested from the 5 days old culture by centrifugation at 12,000 rpm for 30 min and washed with water twice. The cells were suspended in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1% cetylpyridinum chloride, and the suspension was autolyzed at 40°C. After 6 h, the autolyzate was centrifuged and filtered. The filtrate was used as a crude enzyme preparation (Chen and Anderson, 1979).

Purification of the crude enzyme

Unless otherwise indicated, all steps of the purification were carried out at 4°C. Solid ammonium sulfate was added to the crude enzyme preparation to give 70% saturation, and the precipitate was discarded. More ammonium sulfate was added to the supernatant to give 90% saturation. The precipitate was collected, dissolved in 0.05 M sodium phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer. The dialysate was centrifuged at 12,000 xg for 10 min. After dialysis and centrifugation, the obtained supernatant is considered as partially purified enzyme (PPE). The collected supernatant was applied to a column (2.5 x 16 cm) of DEAE-cellulose (Sigma Aldrich) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl (0 to 1 M NaCl) at a flow rate of 50 ml/h. About 2.0 ml fraction was collected in different tubes. The active fractions (F-24 to -32) were collected, dialyzed overnight with the same buffer, and centrifuged at 12,000 xg for 10 min. The supernatant was applied to a column of DEAE-Sephadex A-50 (1.5 by 20 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer containing a linear gradient of NaCl (0 to 1 M NaCl) at a flow rate of 30 ml/h. About 1.0 ml fraction was collected in different tubes. The active fractions (F-12 to -16) were collected, dialyzed overnight against same buffer, and the homogeneity of the purified enzyme and its molecular mass were measured by SDS-polyacrylamide gel electrophoresis.

Methods of immobilization of GI enzymes

All the chemicals used in the immobilization processes are from Sigma Aldrich.

Cross-linking using gluteraldahyde

An aqueous gluteraldahyde solution was added to the PPE preparation to give a final concentration of 2.5%, in a 50 ml glass beaker, with sufficient stirring to thoroughly intermix both. The pH of the mixture was adjusted to 7.0 with careful addition of 1 N NaOH. The mixture was left at room temperature for 2 h, with constant stirring with a glass rod for the first 30 min. The coherent mass obtained was broken up by mild stirring, washed with distilled water, recovered by centrifugation at 2000 xg for 15 min. The collected sediment was air dried and used to evaluate the enzymatic activity (Tyagi et al., 1999).

Adsorption on DEAE-Cellulose

This process was carried out as described by Chen and Anderson (1979). Suspension of 500 mg (dry weight) of DEAE-Cellulose in 0.05 M sodium phosphate buffer (pH 7.0) was stirred gently at 4°C. The PPE preparations was added and stirred for 30 min at the same temperature. The enzyme-support complex obtained was washed by the same buffer, allowed to dry, and then assayed for enzyme activity.

Entrapment into polyacrylamide

The GI enzyme in the PPE preparation was entrapped into polyacrylamide by free-radical cross-linking polymerization of acrylamide in aqueous solutions. The acrylamide (2.85 g),

Table 1. Purification steps of GI enzyme from *S. albaduncus* culture.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	2728	4736	1.7	100	1.00
Ammonium sulfate precipitation (70 to 90%)	342	2682	7.8	56.6	4.6
DEAE-Cellulose	122	1486	12.2	31.4	7.2
DEAE-Sephadex A-50	22	498	22.6	10.5	13.3

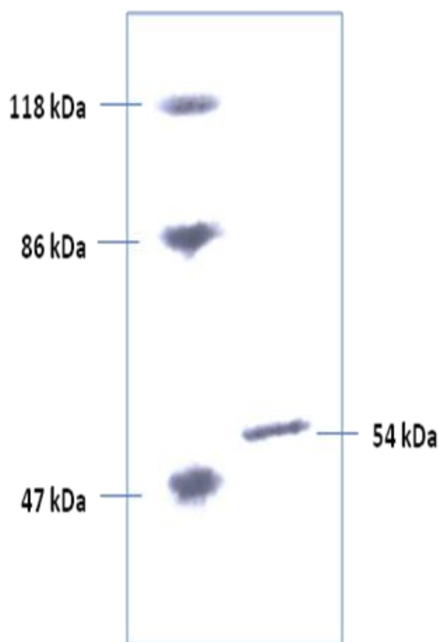

Figure 1. SDS-PAGE of purified GI (20 µg) produced by *S. albaduncus*. Lane 1: Molecular mass standards (β-galactosidase, 118 kDa; Bovine serum albumin, 86 kDa; ovalbumine, 47 kDa). Lane 2: purified GI enzyme produced by *S. albaduncus.*

ammonium persulfate (10 mg) and bisacrylamide (0.15 g) were dissolved in distilled water (20 ml). After the addition of TEMED (1 ml) and enzyme preparation (1 ml), the solution was poured into a flat bottom Petri dish. Polyacrylamide hydrogels were cut into equal size cubes (1 to 2 mm). Gel cubes were washed thoroughly three times with distilled water and used to evaluate the activity of the immobilized enzyme (Demirel et al., 2006).

In each experiment**,** the activity yield of the immobilized enzymatic preparation was calculated with respect to the original activity before immobilization. Each experiment was carried out in triplicate. The immobilized preparation was recovered after the first assay and reused for a total of 7 times at 48 h intervals.

Statistical analysis

Statistical significance between means was tested by analysis of variance and student t-test using Instat-ANOVA software. The differences between means were considered statistically significant when the test yielded a value $P<0.05$.

RESULTS

Purification of the GI enzyme

The purification of GI enzyme is summarized in Table 1. The enzyme was purified to homogeneity by ammonium sulfate precipitation, DEAE-cellulose chromatography, and finally ion exchange chromatography on DEAE-Sephadex A-50. The final preparation had 10.5% activity recovery and approximately 13.3-fold purification. The purified GI enzyme had a single protein band on SDS-PAGE with approximate relative molecular mass 54 kDa (Figure 1).

Effects of different factors on the activity of the GI enzyme

Effect of temperature

To observe the effects of temperature on the GI activity, the reaction mixture with the purified enzyme sample was incubated at various incubation temperatures (50 to 90°C) for 60 min and the produced fructose was quantitatively determined as described before. According to the obtained results (Figure 2), the maximum enzymatic activity (85 to 100%) was obtained at a temperature range of 65 to 75°C, with optimum temperature at 70°C.

Effect of pH

For studying the effects of pH on the GI activity, the pH of the reaction mixture was adjusted at different values (pH 5 to 9) and incubated at 70°C for 60 min. The produced fructose was quantitatively determined as described before. As shown in Figure 3, a promising enzymatic activity (84 to 100% of the highest activity) was obtained over the pH range 6.5 to 8, while the optimum pH was 7.

Effect of metal ions

Regarding the effects of metal ions, the presence of Mg^{2+} or Co^{2+} (5 mM) in the reaction mixture enhanced the

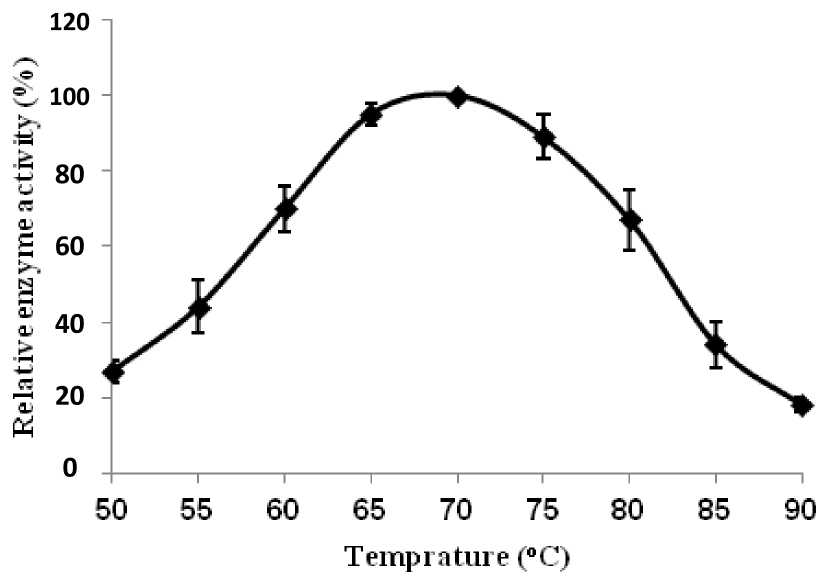

Figure 2. Effects of temperature on the GI activity.
The enzymatic reaction mixture was incubated at different temperatures (50 to 90°C) for 60 min and the produced fructose was quantitatively determined and compared with the maximum value.

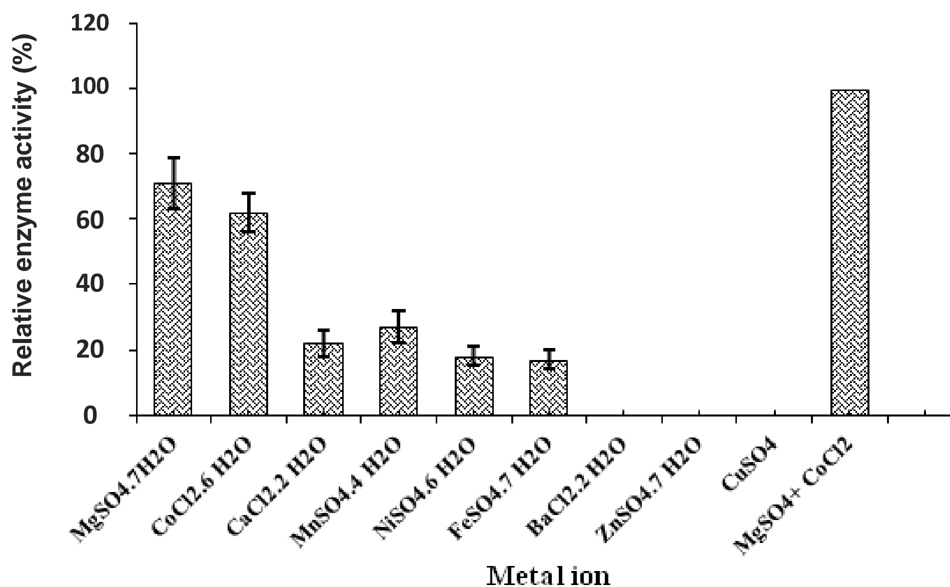

Figure 3. Effects of pH on the GI activity.
The pH of enzymatic reaction mixture was adjusted to different pH (5 to 9) and incubated at 70°C for 60 min. The produced fructose was quantitatively determined and compared with the maximum value.

enzymatic activity and the combination of the two metal ions induced the highest enzymatic activity. On the other hand, weak GI activity was observed in the presence of Ca^{2+}, Mn^{2+}, Ni^{2+} and Fe^{2+} (17 to 27% of the maximum activity). While the enzymatic activity was inhibited in the presence of Ba^{2+}, Zn^{2+} and Cu^{2+} (Figure 4).

Immobilization of the GI enzyme

The activity yield of the different immobilized preparations was relatively evaluated as compared to that of the original non immobilized PPE preparation. According to the obtained results, the relative activities of the immo-

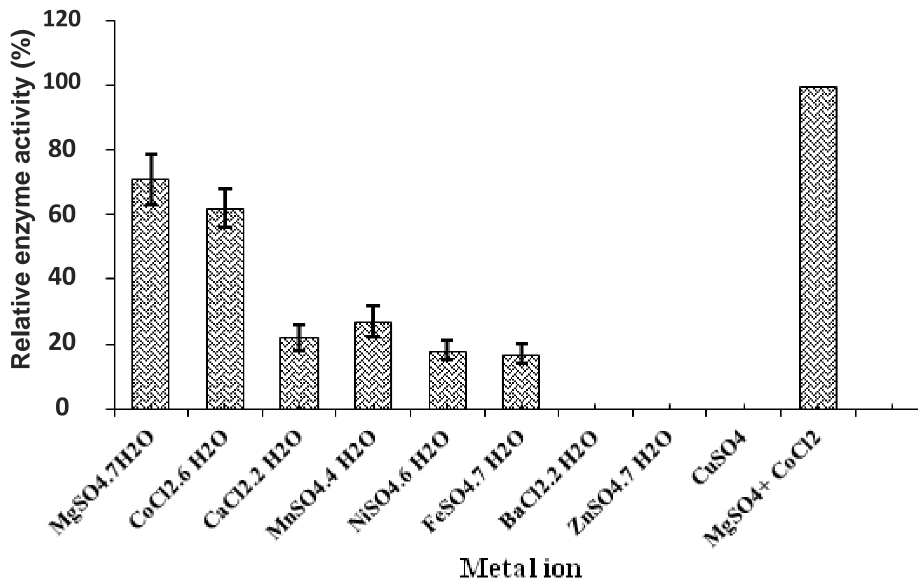

Figure 4. Effects of different metal ions on the GI activity. Different types of metal ions were added to the reaction mixture at concentration of 5 mM. The reaction mixture was adjusted to pH 7 and incubating at 70°C for 60 min. The produced fructose was quantitatively determined and compared with the maximum value.

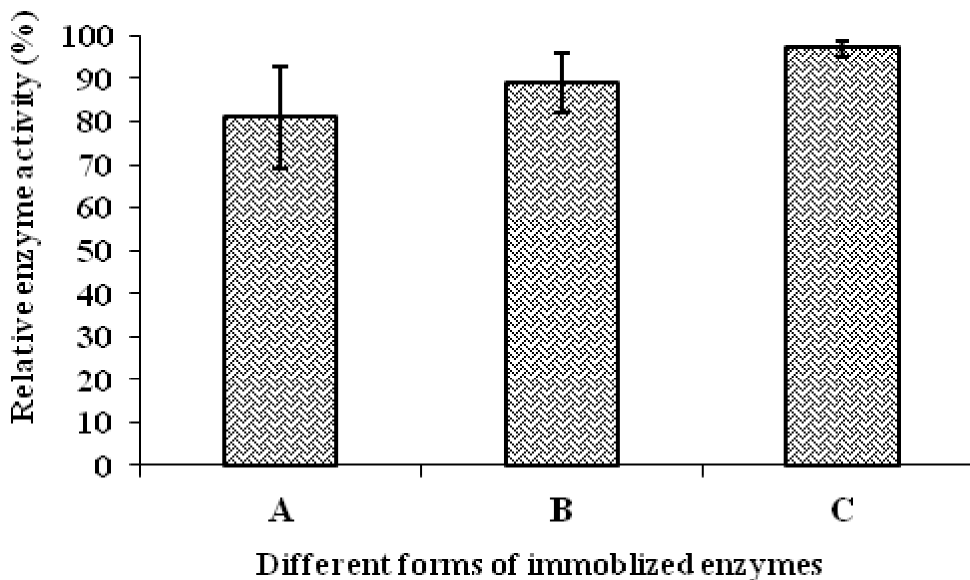

Figure 5. Relative enzymatic activities of the different forms of immobilized GI enzyme as compared to that of the original non immobilized PPE preparations. A: cross-linking with gluteraldehyde; B: adsorption on DEAE-Cellulose; C: entrapment into polyacrylamide.

bilized preparations by cross-linking with gluteraldahyde, adsorption on DEAE Cellulose, and entrapment into polycrylamide were 81, 89 and 97%, respectively (Figure 5).

Immobilized GI preparations were stored at +4°C, and their enzymatic activities were measured periodically over duration of 14 days. Upon 14 days of storage, the preserved activity of the immobilized preparations by cross-linking, adsorption on DEAE-Cellulose, and entrapment into polycrylamide were 65, 78 and 88%, respectively, as compared to the original activity (Table 2).

Table 2. Relative enzymatic activity of different forms of immobilized GI enzyme along a duration of 14 days.

The duration time (days)	The relative enzymatic activity (%)		
	Cross-linkage by gluteraldahyde	Adsorption on DEAE-cellulose	Entrapment into polyacrylamide
0	100	100	100
2	91	94	96
4	83	89	94
6	78	85	92
8	73	82	90
10	69	80	89
12	67	79	88
14	65	78	88

DISCUSSION

The effective catalytic properties of GI enzymes have already prompted their introduction into several industrial products and processes (Kirk et al., 2002).

The efficiency of production of GI from *Streptomyces* species has been documented previously (Abdul-Rahman et al., 2011; Bhasin and Modi, 2012). A high GI-producer *Streptomyces* isolate, identified as *Streptomyces albaduncus*, was selected for the present studies (Yassien and Jiman-Fatani, 2011). To optimize the activity of the GI enzyme produced by the selected strain, it is essential to study the effect of some factors on the purified enzyme. Preparation of the purified GI enzyme from culture supernatant of *S. albaduncus* was started by ammonium sulphate precipitation, followed by ion exchange DEAE-cellulose chromatography, and finally on DEAE-Sephadex A-50 chromatography. The enzyme homogeneity in the final product was confirmed by formation of single protein band of estimated molecular weight 54 kDa in the SDS-PAGE. Kawai et al. (1994) purified GI enzymes from culture supernatant of *Bifidobacterium adolescent* with estimated molecular weight of 53 kDa, while the GI enzyme produced by *Streptomyces flavogriseus* has a smaller molecular weight of 43 kDa (Chen and Anderson, 1979).

The GI catalytic activity may be affected by some factors such as temperature, pH, and metal ions. The optimum temperature of GI activity may be ranged from 60 to 80°C (Bhosale et al., 1996). In the present study, the optimum temperature of the enzymatic activity of GI produced by *S. albaduncus* is 70°C. The same optimum temperature was reported by Chen and Anderson (1979). However, higher optimum temperature (80°C) for GI activity was observed in the study of Azin et al. (1997) and Srivastava et al. (2010). On the other hand, lower temperature (60°C) was the optimum for the activity of GI produced by *Lactobacillus xylosus* (Kawai et al., 1994).

A suitable GI activity can be obtained over a pH range from 6 to 8 (Kawai et al., 1994). In the present study, a promising enzyme activity was observed over the range of 6.5 to 8, with optimum level at pH 7 which is in agreement with that reported by Kawai et al. (1994). While, slight alkaline condition, pH 7.5 and 8, are the optimum for the activity of GI enzyme produced by *Streptomyces flavogriseus* and *Streptomyces phaeochromogenes* NRRL B-3559, respectively (Standberg and Smiley, 1971; Chen and Anderson, 1979).

GI enzymes require different types of metal ions such as Mg^{2+}, Co^{2+}, or Mn^{2+} or a combination of these cations to enhance their catalytic activities. Although both Mg^{2+} and Co^{2+} are essential for the activity, they play differential roles. While Mg^{2+} is superior to Co^{2+} as an activator, Co^{2+} is responsible for stabilization of the enzyme by holding the ordered conformation, especially the quaternary structure of the enzyme (Gaikwad et al., 1992; Bhosale et al., 1996). In the present study, the maximum enzyme activity of GI produced by *S. albaduncus* was observed in the presence of the combination of Mg^{2+} and Co^{2+} ions (5 mM). The reducing effects of some metal ions such as Ag^{1+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Ca^{2+} on the GI activity observed in the present study was also reported by other investigators Chen and Anderson (1979), Smith et al. (1991) and Kawai et al. (1994).

Because enzymes are biocatalysts that promote the rate of reactions without being themselves consumed, they may be used repeatedly as long as they remain active. Therefore, there is an incentive to use enzymes in an immobilized form so that they may be retained in a biochemical reactor to catalyze further the subsequent feed and allow operation in a continuous mode and consequently lowers cost of its use (Cabral, 2001). Also, immobilizations of enzymes of partially purified preparations are sometimes preferred to avoid costly purification processes or to increase catalytic stability (Tanino et al., 2010).

In the present study, immobilization of GI from the PPE preparations of *S. albaduncus* was carried out by three different methods; cross-linking with gluteraldahyde, adsorption on DEAE-Cellulose, and entrapment into poly-

crylamide. The efficiency of these three immobilization methods was confirmed previously (Bhosale et al., 1996; Cabral, 2001; Demirel et al., 2006). In the present study, the obtained results revealed that the most suitable GI immobilization process is the entrapment into polyacrylamide, by which the immobilization process did not significantly ($P < 0.05$) reduced the enzymatic activity as compared with that of the non immobilized form. Enzymes in such immobilization process is physically bound within the matrices of the gel network by forming the gel in the presence of the enzyme. Physical or chemical alteration of the enzyme appears to be much less with this treatment as compared to the other two immobilization methods that may employ an interaction between the enzyme and insoluble carrier (Standberg and Smiley, 1971). The results obtained in the present report can be used as initial step for production of GI enzyme from *S. albaduncus* at industrial level and improve its efficiency through enzyme immobilization.

ACKNOWLEDGEMENTS

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah; under grant No (1432 /166 /223).The authors, therefore, acknowledge with thanks DSR technical and financial support.

REFERENCES

Abdul-Rahman N, Hussain MA, Hasan M, Jahim JM (2011). Mathematical modeling of fructose production by immobilized glucose isomerase as a function of temperature and pH variations. Afr. J. Biotech. 10(14):2766-2779.

Azin M, Moazami N, Nemat-Gorgani M (1997). Production, purification and immobilization of glucose isomerase from *Streptomyces olivochromogenes* PTCC 1547. World J. Microbiol. Biotechnol. 13:597-598.

Bhasin S, Modi HA (2012). Optimization of fermentation medium for the production of glucose isomerase using *Streptomyces sp. SB-P1.* Biotech. Res. Int. Article ID 874152, 1-10.

Bhosale SH, Rao MB, Deshpande VV (1996). Molecular and industrial aspects of glucose isomerase. Microbiol. Rev. 60:280-300.

Bok SH, Seidman M, Wopat PW (1984). Selective isolation of acidophilic *Streptomyces* strains for glucose isomerase production. Appl. Environ. Microbiol. 47:1213-1215.

Borgi MA, Srih-Belguith K, Ben Ali M, Mezghani M, Tranier S, Haser R, Bejar S (2004). Glucose isomerase of the *Streptomyces* sp. SK strain: purification, sequence analysis and implication of alanine 103 residue in the enzyme thermostability and acidotolerance. Biochimie 86(8):561-8.

Cabral JMS (2001). Biotransformations. In: Ratledge C and Kristiansen B (eds) Basic Biotechnology, Cambridge University Press, Cambridge. pp. 471-501.

Chen WP, Anderson AW (1979). Purification, immobilization, and some properties of glucose isomerase from *Streptomyces flavogriseus.* Appl. Environ. Microbiol. 38:1111–1119.

Demirel G, Ozcetin G, Sahin F, Tumturk H, Aksoy S, Hasirci N (2006). Semi-interpenetrating polymer networks (IPNs) for entrapment of glucose isomerase. React. Funct. Polym. 66:389–394.

Gaikwad SM, Rao MB, Deshpande VV (1992). D-Glucose/xylose isomerase from Streptomyces. Differential roles of magnesium and cobalt ions. Enzyme Microb. Technol. 14:317-320.

Joo GJ, Shin S, Heo GY, Kim YM, Rhee IK (2005). Molecular cloning and expression of a thermostable xylose (Glucose) isomerase gene, xylA, from Streptomyces chibaensis. J. Microbiol. 43(1):34-7.

Kawai Y, Konishi K, Horitsu H, Sakurai H, Takamizawa K, Suzuki T, Kawai K (1994). Purification and characterization of D-xylose isomerase from *Bifidobacterium adolescentis.* Biosci. Biotech. Biochem. 58(4):691-691.

Kirk O, Borchert TV, Fuglsang CC (2002). Industrial enzyme applications. Curr. Opin. Biotecnol. 13:345–351.

Kluskens LD, Zeilstra J, Geerling AC, de Vos WM, van der Oost J (2010). Molecular characterization of the glucose isomerase from the thermophilic bacterium Fervidobacterium gondwanense. Environ. Technol. 31(10):1083-90.

Kulka RG (1956). Colorimetric estimation of ketopentoses and ketohexoses. Biochem. J. 63:542–548.

Leang K, Takada G, Fukai Y, Morimoto K, Granström TB, Izumori K (2004). Novel reactions of L-rhamnose isomerase from *Pseudomonas stutzeri* and its relation with D-xylose isomerase via substrate specificity. Biochim. Biophys. Acta. 1674(1):68-77.

Smith CA, Rangarajan M, Hartley BS (1991). D-Xylose (D-glucose) isomerase from *Arthrobacter* strain NRRL B3728. Biochem. J. 277:255–261.

Song YS, Kim JE, Park C, Kim SW (2011). Enhancement of glucose isomerase activity by pretreatment with substrates prior to immobilization. Korean J. Chem. Eng. 28(4):1096-1100.

Srivastava P, Shukla S, Choubey SK, Gomase VS (2010). Isolation, purification, characterization of glucose isomerase enzyme form *Streptomyces* species isolated from Parbhani Region. J. Enzyme Res. 1(1):1-10.

Standberg GW, Smiley KL (1971). Free and Immobilized Glucose Isomerase from *Streptomyces phaeochromogenes.* Appl. Microbiol. 21(4):588-593.

Tanino T, Hotta A , Ito T, Ishii J, Yamada R, Hasunuma T, Ogino C, Ohmura N, Ohshima T, Kondo A (2010). Construction of a xylose-metabolizing yeast by genome integration of xylose isomerase gene and investigation of the effect of xylitol on fermentation. Appl. Microbiol. Biotechnol. 88(5):1215-21.

Tyagi R, Batra R, Gupta MN (1999). Amorphous enzyme aggregates: Stability toward heat and aqueous-organic cosolvent mixtures. Enzyme Microb. Technol. 24:348–354.

Van Vleet JH, Jeffries TW (2009). Yeast metabolic engineering for hemicellulosic ethanol production. Curr. Opin. Biotechnol. 20(3):300-6.

Yassien MAM, Jiman-Fatani AM (2011). Production of glucose isomerase by *Streptomyces* species isolated from West Area of Saudi Arabia. Arab J. Lab. Med. 37(3):635-644.

Yassien MAM, Jiman-Fatani AM (2012). Optimization of glucose isomerase production by *Streptomyces albaduncus.* Afr. J. Microbiol. Res. 6(12):2976-2984.