Journal of Environmental Sciences, 2014; Vol. 43, No. 2 : 253-266

STABILITY AND IMMOBILIZATION OF GLUCOSE OXIDASE FROM CLADOSPORIUM OXYSPORUM

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ABSTRACT

Glucose oxidase (GO, EC: 1.1.3.4) was purified from Cladosporium oxysporum with specific activity of 28.4 units mg-1protein and 74.7-fold of purification. The optimum pH values of free and immobilized enzymes on chitosan were 6 and 7, respectively. The optimal temperatures were 30°C and 40°C for the two forms of the enzyme in the same order. GO expressed appreciable heat stability at 50, 60 and 70°C. Kd values were 11x10-2, 18x10-2 and 27x10-2. T0.5 values were 6.3, 3.85 and 2.56 min at 50°C, 60°C and 70°C, respectively. The polyols including 15 % glycerol, 15 % polyethylene glycol (PEG), 5 mM sorbitol and 5 mM mannitol provided remarkable stability against heat denaturation at 60°C. Trehalose and raffinose at 5 mM protected effectively GO from heat denaturation at 60°C.

Key words : *Glucose oxidase; Cladosporium oxysporum; Purification; Characteristics; Immobilization.*

INTRODUCTION

The study of enzymes is an important area because it exists just on the borderline where the biological and physical sciences meet (Yogananth *et al.,* 2012). GO (β-D-glucose:oxygen 1-oxidoreductase) catalyzes the oxidation of β-D-glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide (H_2O_2) (Hatzinikolaou and Macris, 1995).

GO was first isolated from the mycelia of *Aspergillus niger* and *Penicillium glaucum* by Muller (1928). GO has been produced by a variety of filamentous fungi. *Aspergillus niger* is the most common fungus utilized for the production of GO (Pluschkell *et al.,* 1996; Bhat *et al.,* 2013). Many fungal species such as *Penicillium notatum, Penicillium chrysosporium, Aspergillus niger* and *Botrytis cinerea* have the ability to produce GO (Lium et al., 1998; Bhat *et al.,* 2013).

The use of enzymes in food preservation and processing predates modern civilization. The use of enzymes in food industry also involves a range of effects including the production of food quality attributes such as flavors and fragrances and control of colour, texture, appearance besides affecting their nutritive value (Yogananth *et al.,* 2012).

GO has application in the baking industry providing slight improvements to the crumb properties in bread and croissants (Rasiah *et al.,* 2005). The most important application for GO is for the diagnostic determination of glucose using biosensor technology (Wilson and Turner, 1992; Chudobova *et al.,* 1996).

GO has also been used as an ingredient of toothpaste (Petruccioli *et al.,* 1999), for the production of gluconic acid, and as a food preservative (Pluschkell *et al.,* 1996). GO is a redox enzyme capable of oxidizing glucose using oxygen and it has been widely used for the blood glucose assay (Kuo *et al.,* 2013).

The present work aimed to investigate the activity level, purification and immobilization of intracellular *Cladosporium oxysporum* GO. Also, it aimed to compare the characteristics of the free and immobilized GO.

MATERIALS AND METHODS

Fungal source

Tomato fruit used as source for *C. oxysporum* producing intracellular GO.

Inoculum preparation

The inoculum was prepared by growing the organism on potato dextrose agar (PDA) (Vanderzant and Splittstoesser, 1992) plates for 5 days at 30°C. The conidia were harvested by covering the plates with isotonic sodium chloride solution and brushing gently with an inoculum loop. The spore suspension was filtered through double cheese cloth (mira cloth filter) to remove hyphae and conidiospores. The spores were diluted with the same solution, vertex and then counted in a haemocytometer. The spore suspension was added to the flasks at a final concentration of 107 spores/ml.

Mode of fermentation

Cultivation of *C. oxysporum* was carried out by submerged fermentation (SmF) in 250 ml Erlenmeyer flasks using the Czapek,s dox liquid medium (Eaton *et al.,* 1998) with glucose as source of carbon, autoclaved at 121℃; 15 1bs pressure for 20 min and then inoculated with prepared inoculum a final concentration of 10^7 spores/ml and incubated in the dark at 30℃ for 7 days. Samples were withdrawn at regular intervals of 24 hours and observed for GO activity.

Extraction of intracellular GO from *C. oxysporum*

The culture were filtered by Whatman no. 1 filter paper. The mycelia washed twice by 0.1 % saline solution. The mycelia were weiged. The washed mycelia were crushed in water in homogenizer at 1500 rpm for 30 min and centrifuged at 10,000 rpm for 20 min. Mycelium debris was separated and used for dry mass estimation. The filterate was precipitated with ethyl alcohol at 4℃ over night.

The precipitate was collected by centrifugation at 10,000 rpm for 15 min and pellets of each sample were dissolved in 2 ml of distilled water and refrigerated till further use. (Zia *et al.,* 2010).

Assay of GO

The assay of GO was carried out according to the method of WBC (2010).

Determination of protein content

Soluble protein content was estimated as

described by Bradford (1976). Fifty grams sample of *Cladosporium* mycelia were homogenized in 100 mM phosphate buffer pH (6.0). The extract was centrifuged for 30 min at 10,000 rpm. One ml of supernatant was added to 5 ml of $^1/_4$ diluted Coomassie Brillant Blue G - 250 and vigorously mixed. After keeping it in the dark for 15 min , the absorption of the protein in the extract at 595 nm was spectrophotometrically measured. The concentration of protein was determined from standard curve using bovine serum albumin.

Purification of GO

The purification of GO enzyme was carried out through three steps using ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Solid ammonium sulfate was added to the crude extract until it reached up 60 to 85 % saturated. Later, the desired enzyme was desalted by dialysis. A column of DEAE-(diethylaminoethyl) cellulose was prepared by the method of Sukhacheva et al, (2004). The desalted enzyme sample of 3 ml was poured on the surface of column (2x12 cm). The elution of sample was carried out with 0.1 M phosphate buffer (pH 6.0). The drop rate of eluted sample was kept constant and 10 fractions of one-ml each were collected.

A column of Sephadex G-100 (2x12 cm) was prepared by the method described by Sukhacheva *et al.* (2004). The sample having the maximum specific activity eluted with phosphate buffer during ion exchange chromatography was applied and allowed to penetrate in packed column. Elution was carried out by 0.1 M phosphate buffer (pH 6.0) at a constant drop rate. A total of 10 fractions of one-ml each were collected. The activity of the enzyme was assayed and the protein estimation was carried out as discussed above. The purity of the enzyme was verified by SDS-PAGE (Laemmli *et al.*, 1970).

Immobilization of GO on chitosan bead

Immobilization of GO was encapsulated in calcium alginate-chitosan microspheres using emulsification-internal gelatin-GO adsorption-chitosan coating method (Wang *et al.,* 2011).

Determination of the optimal pH for GO activity

The optimal pH for enzyme activity was determined over a range from 3 to 10. Sodium acetate/acetic (pH 3.0-4.0-5.0), potassium phosphate buffer (6.0-7.0), Tris-HCL buffer (8.0-9.0) and sodium bicarbonate buffer (10.0). From the results obtained a graph of enzyme activity vs. pH was plotted, and the optimum pH for glucose oxidase was subsequently determined.

Determination of the optimal temperature for GO activity

Reaction mixture was incubated at different temperatures 20, 30, 40, 50, 60 and 70°C. The optimum temperature was determined from the graph of enzyme activity against temperature. The enzyme activity was estimated by the method described above.

RESULTS

Purification of GO from *C. oxysporum*.

In this experiment GO from *C. oxysporum* was purified by ammonium sulfate (60-85 %), DEAE-Cellulose and Sephadex G-100. The results in Table 1 indicate that specific activity

was 28.4 units mg^{-1}protein with purification fold of 74.7.

Optimum pH of free and immobilized GO.

The enzyme activity of any enzyme is dependent on pH value. Therefore, it was thought of interest to study the pH profile of the purified GO from *C. oxysporum*. In fact , for such sort of study the other factors, which may affect the enzyme activity including substrate concentration and the time of incubation were fixed . So, GO activity from *C. oxysporum* was measured at a range of pH value between 3 and 10. The results in Fig. 1 indicate that the GO activity increased gradually up to optimal pH 6.0 for free GO and pH 7.0 for immobilized GO after which a gradual decrease of the activity was observed.

Table (1) : Steps of GO purification from *C. oxysporum.*

Steps	Total protein (mg)	Total activity (Umg^{-1}protein)	Specific activity (Umg^{-1}protein)	% Yield	Fold of purification
Crude extract	275	105	0.38	100	1.00
Ammonium sulfate ppt 60 – 85 %	111	96	0.86	40.3	2.26
DEAE-Cellulose	60.0	72	1.2	21.8	3.15
Sephadex G-100	1.90	54	28.4	0.69	74.7

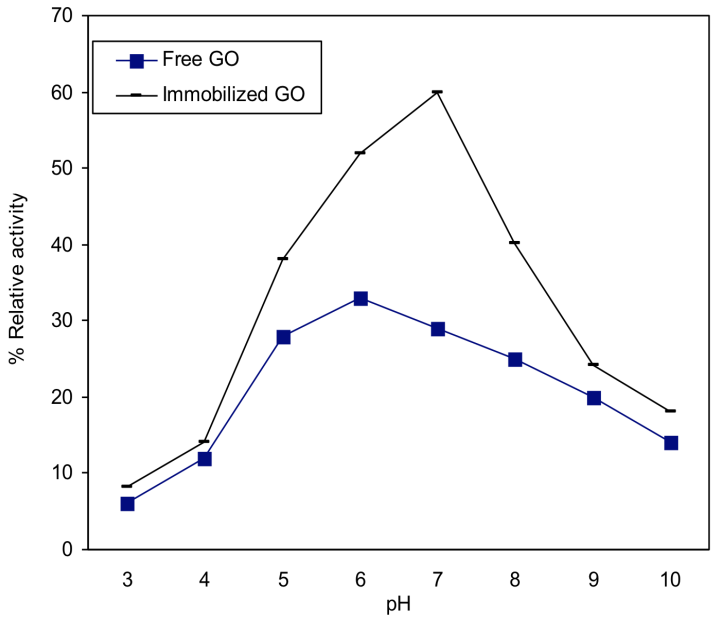

Fig. (1) : Effect of pH on activity of free and immobilized GO from *C. oxysporum.*

Optimum temperature of free and immobilized GO

The effect of temperature on free and immobilized GO from *C. oxysporum* was studied. It was observed that increasing of temperature from 20℃ concomitantly increased the activity of both free and chitosan-immobilized GO up to 40℃ and 50℃, respectively (Fig. 2). Therefore, thse two temperatures are for free and immobilized GO, respectively. Beyond 40℃ and 50℃, the free and immobilized GO displayed lower activities. Indeed the immobilized enzyme retained higher activity at 60℃ and 70℃ compared to the free enzyme. As a general observation the immobilized enzyme expressed higher activity than the free one.

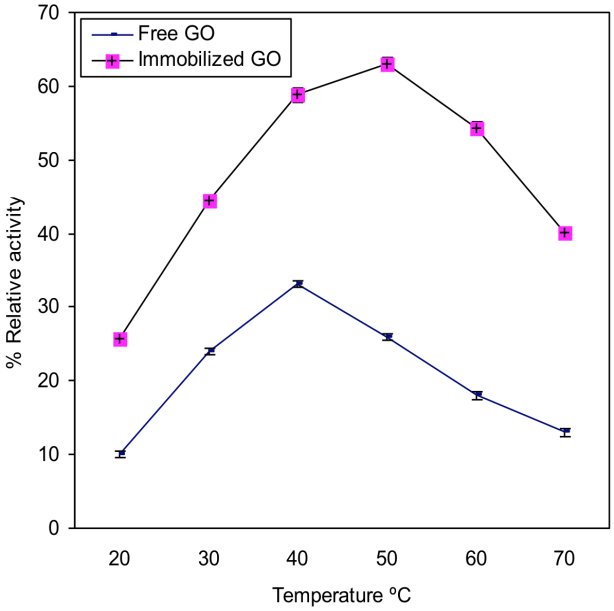

Fig. (2) : Effect of temperature on activity of free and immobilized GO from *C. oxysporum.*

Heat stability of GO

This experiment aimed to study the thermal stability of free GO from *C. oxysporum.* This was carried out at temperatures 50, 60 and 70℃. The results are shown in Fig. 3. The results indicate that GO from *C. oxysporum* was stable at 50℃ and the stability was reduced with rising of the temperature to 60℃ and 70℃.

K_d and $t_{0.5}$ of GO from *C. oxysporum.*

The K_d and $t_{0.5}$ of GO purified from *C. oxysporum* was determined and the results are recorded in Table 2. The Kd increased with increasing temperature from 50℃ to 70℃, whereas the t0.5 value decreased with increasing the temperature and reached down 2.56 min at 70℃.

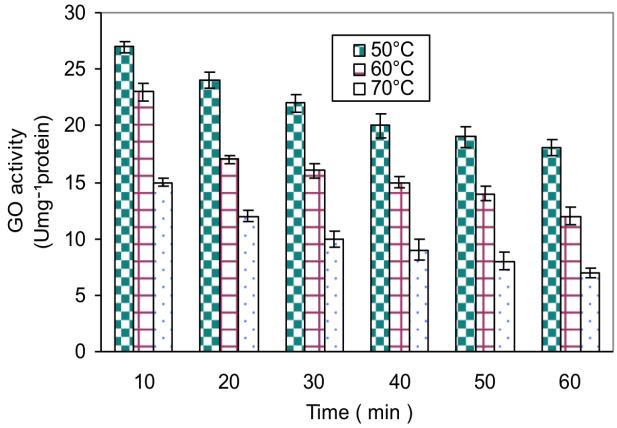

Fig. (3) : Heat stability of free GO from C. oxysporum at various temperatures.

Table (2) : K_d and $t_{0.5}$ of free GO from *C. oxysporum*.

Temperature (°C)	$K_d/min^{-1}x10^{-2}$	$T_{0.5}$ (min)
50	11	6.3
60	18	3.85
70	27	2.56

k_d (first order rate constant of denaturation) is determined from $t_{0.5}$ (half-life)=0.693/k_d.

Effect of polyols on stability of GO at 60°C

In this experiment the stability of GO from *C. oxysporum* at 60°C was investigated in presence of some additives including 15 % glycerol, 15 % PEG, 5 mM sorbitol and 5 mM mannitol.

The results in Fig. 4 show that sorbitol offered higher stability to the enzyme at 60°C compared to the stability of the enzyme in presence of other additives. The other three compounds protected the enzyme from heat denaturation and they can be arranged as glycerol > PEG > mannitol.

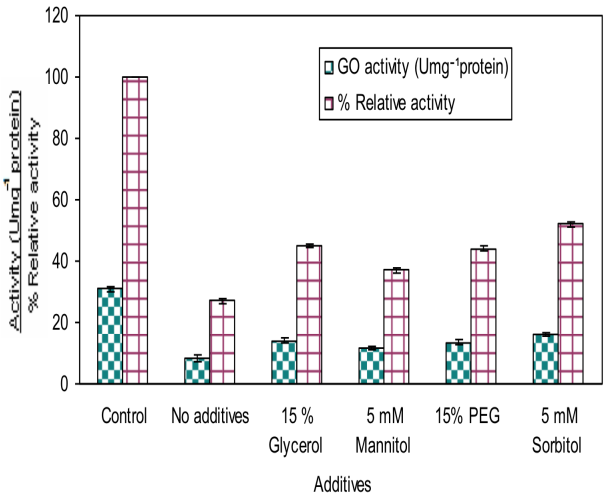

Fig. (4) : Effect of polyols at 60°C on GO activity from *C. oxysporum.*

Effect of trehalose and raffinose on stability of GO at 60°C

This experiment aimed to study heat stability of GO from *C. oxysporum* at 60°C in presence of some stabilitizers of protein. The compounds tested were trehalose and raffinose. These compounds were tested at 5 mM for various time intervals throughout 100 min. The results in Fig. 5 show that trehalose and raffinose protected the enzyme from heat denaturation at 60°C particularly trehlose which was better stabilizer than raffinose throughout the exprimental period.

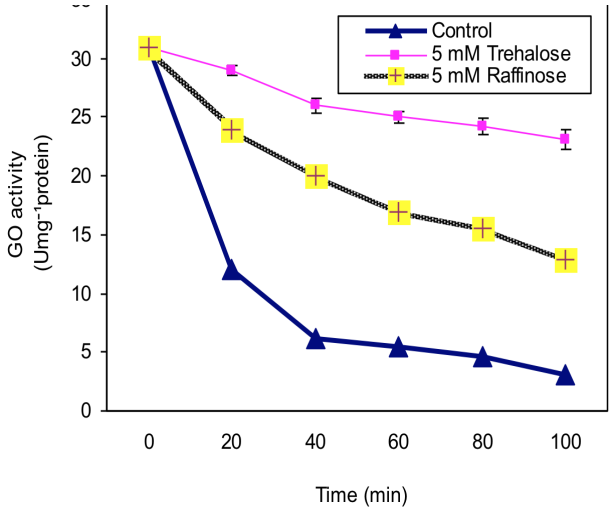

Fig. (5) : Effect of trehalose and raffinose at 60°C on stability of GO from *C. oxysporum.*

DISCUSSION

The GO from *C. oxysporum* was purified to 28.4 units mg^{-1} protein with 47.7-fold of purification. The purification of GO in the present investigation was supported with the single band of SDS-PAGE. The specific activity of GO from *Aspergillus flavus* and *Fusarium sp*. was 18 units mg-1protein (Bhat *et al.,* 2013). The specific activity of GO from *Geotrichum sp.* was 7.3 units mg^{-1} protein which is smaller than that observed in the present investigation. However, the purification fold was 8.3 (Hwang *et al.,* 2011) which is lower than that of the present work. GO was also purified from *Phanerochaete chrysosporium* (Kelly and Reddy , 1986) with lower specific activitiy of 4.2 units mg^{-1} protein. Higher specific activity 59 and 280 units mg-1protein were recorded for the enzyme from *Aspergillus niger* (Zia *et al.,* 2012) and *Penicillium notatum* (Bhatti and Saleem, 2009).

The optimal pH value for the free GO from *C. oxysporum* was 6.0. The pH of free GO was found to be 5.0 (Saravanan *et al.,* 2010), 5.4 (Bhatti and Saleem, 2009) and 6.5 (Owalude and Odebunmi, 2007) from various sources. However, the pH optimum of the immobilized GO in the present work was 7.0. Optimum pH was 6.0 for CMC-immobilized GO by Ahmed *et al.* (2001). These authors reported that immobilized GO was more stable at this pH than its native counterpart suggesting that the attached polymer chain can protect the active enzyme conformation at alkaline pH and avoids protein unfolding.

A shift of pH optimum of GO on immobilization is well known properties of many immobilized enzymes (Davis and Burns, 1992; Rogaiski *et al.,* 1999). The fixed position of immobilized enzyme on the surface of the carrier is thought to prevent extensive distortion of the native conformation of the enzyme protein caused by external pH (Pye and Chance, 1976; Davis and Burns, 1992).

Since the characteristics of ionizable side chains of amino acids depend on the pH value. The enzyme activity usually varies with pH changes. At extreme pH, the tertiary structure of the protein may be disrupted and the protein is denatured. Even at moderate pH values where the tertiary structure is not disrupted the enzyme activity may depend on the degree of ionization of certain amino acid chains and the pH profile of an enzyme may suggest the identity of those residues (Palmer, 1985).

Furthermore, the results show that the pH curve of GO is more or less bell-shaped. Frequently, such curve reflects the deprotenization of an ionizable substrate or amino-acyl side chain in the enzyme. Usually, if the pH profile of the enzyme activity appeared as bell-shaped, it means the presence of two amino acid residues in the active site giving a narrow pH optimum. However, if it appeared as a plateau it indicates one important amino acid residue in the active site (Price and Stevens, 1982).

In general, the behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution can have a different pH optimal value from the same enzyme when immobilized on a solid matrix. Depending on the surface and residual changes on the solid matrix and the nature

of the bound enzyme, the pH value in the immediate vicinity of the enzyme molecule may change, thus causing a shift in the pH optimum of the enzyme activity (Palmer, 1995).

The optimal temperatures of free and immobilized GO were 40℃ and 50℃, respectively. The optimal temperature for free GO from *Penicillium notatum* was 45℃ (Bhatti and Saleem, 2009) which is more or less comparable to our results. Petruccioli *et al.* (1994) reported optimal temperature for free GO at 30℃ from *Penicillium variable*. The increase in optimum temperature of immobilized GO in the present work may be caused by the changing enzyme conformational structure upon immobilization (Deere *et al.,* 2002). Also, Sari et al. (2012) reported that the optimal temperatures for free and immobilized GO were 60℃ and 70℃, respectively at pH 7.0. However, they showed that at pH 4.0 the optimal temperature for free GO was 40℃.

Changing in the temperature of the protein alter the degree of vibrational motion and diffusion of protein molecules that affects on protein composition and aggregation (Mansour *et al.,* 2010; El-Fallal *et al.,* 2013).

The activity of free GO declined at 50℃, 60℃ and 70℃ due to denaturation of enzyme protein. These results are in agreement with those reported by Kretavicius *et al.* (2010). Thermal denaturation of GO proceeds mainly through the destabilization of ionic and hydrophobic interactions and the breakage of hydrogen bonds and van der Waals forces, leading to conformational changes in tertiary structure that inactivate the enzyme (Gouda *et al.,* 2003; Zoldak *et al.,* 2004).

Generally, increasing the temperature causes an increase in the inherent energy of the system and more molecules obtain the necessary activation for the reaction to take place. However, there comes a point where the increase of the reaction due to the effect of temperature on the activation of molecules is equal to the decrease of reaction rate due to the destruction of the protein tertiary structure (Zoldac *et al.,* 2004). At this point the activity is a maximum and this temperature is often known as the optimum temperature. Thus, any chemical reaction whether exothermic or endothermic must overcome the activation energy in order to take place. It is known that the greater the activation energy, the more the heat which supports a successful reaction. An enzyme may lower the overall activation energy of the reaction to take place (Whnn, 1984).

Studying the themostability of free GO indicated that the activity of the enzyme was reduced at temperatures over the optimum for each individual enzyme and that $t_{0.5}$ of inactivation was decreased with increasing the temperature. From the practical stand point, the thermal inactivation is by far the most important mode of enzyme inactivation. Enzymes are usually quite stable at the temperature ambient for the organism from which they are obtained and lose their activity when the temperature is increased to a significantly higher level. There is still much uncertainly about the mechanism of thermal inactivation of enzymes. The first step in enzyme thermoinactivation is partial unfolding of the protein molecule (Palmer, 1985).

Polyols increased the thermal stability of

GO. The high capability of the polyols such as PEG, mannitol and sorbitol to form hydrogen bonds should play the most important role in stabilization of enzyme against thermal stress and increasing the degree of organization of water molecules (Norikos *et al.,* 1999). It was reported that PEG binds to the free amino group situated in the amino acid side chains of the protein particulary lysine (Matsuyama *et al.,* 1991; Norikos *et al.,* 1999).

The increase in the thermal stability by adding PEG was probably due to the reinforcement of the hydrophobic interactions among non polar amino acids inside the enzyme molecule and thus increased their resistance to inactivation since it has been reported that polyhydrophobic alcohols modify the structure of water or strengthen hydrophobic interactions among non polar amino acids inside the protein molecule.

It has been suggested that glycerol exercises its protective effect by inhibiting unfolding (Gekko and Timasheff, 1981). Also, glycerol is known to lead to the preferential hydration of several proteins and increases their transition temperature (Tiwari and Bhat, 2006). Generally, the stabilizing effect of additives for the enzymes is not an absolute effect valid for all enzymes, but it depends on the nature of the enzyme, its hydrophobic character and on the degree of interaction with the additive (Lozano *et al.,* 1994; Christiansen and Nielsen, 2002).

The role of the polyols in enzyme stabilization is as a water-structure marker, which depresses the hydratation of the protein. The polyol molecules are preferentially excluded from the surface layer of the protein molecule and the water shell around the protein molecule is preserved, so that the conformation of the protein becomes more rigid (Longo and Combes, 1999). Generally, polyols have the ability to maintain solvophobic interaction and have the capability to form hydrogen bonds that play the key role in supporting the native conformation of the protein and aid in protein stabilization (Lozano *et al.,* 1994).

Trehalose and raffinose partially protected the pure GO from heat denaturation at 60°C. Xie and Timasheff (1997) showed that trehalose, in solution, stabilized ribonuclease A during exposure to high temperatures. Kaushik and Bhat (2003) by analyzing the thermal stability of ribonuclease A, lysozyme and cytochrome C in the presence of trehalose concuded that trehalose is an exceptional protein stabilizer. Trehalose was predicated to function as a universal protein stabilizer as it was able to stabilize a wide range of proteins used in the study. There are different opinions on how sugars offer protection to proteins. One such opinion is preferential hydration. This refers to the accumulation of water near the protein (Arakawa, 2002). Polar groups on the surface of proteins are bound to water molecules hence proteins are hydrated in aquous solution. If this hydration is maintained in the presence of sugar (cosolute), a difference in the concentration of the sugars develops between the bulk solution and the vicinity of the protein, resulting in preferential hydration where excess water accumulates near the protein. This leads to cosolute-induced stabilization (Arakawa, 2002).

Timasheff (1992, 1993) explained protein stabilization by sugars differently. It was

shown that when sugar levels increase in bulk water with protein there is competition for available water, this competition leads to less water surrounding the protein. The solvation layer around the protein becomes reduced. The protein becomes more compact and stabilized, being less susceptible to heat and dehydration. Whatever the mechanism involved, trehalose provides exceptional stability to a variety of proteins therefore there is a great demand for this sugar. It is currently being mass produced from starch (Chaen, 1997) and is being used extensively in the comestic, pharmaceutical, medicinal and food industries. Trehalose can be synthesized from glucose, maltodextrins (polymers of glucose) or maltose.

In conclusion, thermostability of GO from *C. oxysporum* was increased in precence of polyols, raffinose and trehalose. In fact, this is of good value for application of GO in the industrial purposes.

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Received on 10 / 12 / 2013

الملخص العربى

ثبات وتسكين إنزيم أوكسيديز الجلوكوز من فطر الكلادوسبوريوم أوكسيسبوروم

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تم تنقية إنزيم أوكسيديز الجلوكوز من (EC١,١.٣,٤) من فطر الكلادوسبوريوم أوكسيسبوروم بنشاط نوعى مقداره ٢٨,٤ وحده لكل ميلليجرام بروتين. كانت قيم الرقم الهيدروجينى للإنزيم الحر ٦ بينما للإنزيم المثبت ٧. كانت درجات الحرارة المثلى للإنزيم الحر والمثبت هى ٤٠ و ٥٠ درجة مئوية بالتتابع. أظهر إنزيم أوكسيديز الجلوكوز ثباتاً ملحوظاً عند درجات الحرارة ٥٠ و ٦٠ و ٧٠ درجة مئوية. كانت قيم ثابت التحلل الحرارى عند درجات الحرارة ٥٠ و ٦٠ و ٧٠ درجة مئوية هى ١١ × ١٠ $^{-٢}$ و ١٨ × ١٠ $^{-٢}$ و ٢٧ × ١٠ $^{-٢}$ كانت قيم فترة نصف العمر عند نفس درجات الحرارة الثلاث هى ٦,٣ و ٣,٨٥ و ٢,٥٦ دقيقة. أدت إضافة البولى أول والمشتملة على ١٥٪ سوربيتول و ١٥٪ بولى إيثيلين جلايكول و ٥ ميللى مول سوربيتول و ٥ ميللى مول مانيتول إلى إعطاء الإنزيم ثباتاً حرارياً ملحوظاً عند درجة حرارة ٦٠ درجة مئوية. كذلك وجد أن إضافة التريهالوز والرافينوز بتركيز ٥ ميللى مول إلى حماية الإنزيم من التحلل الحرارى عند ٦٠ درجة مئوية.

JOESE 5

STABILITY AND IMMOBILIZATION OF GLUCOSE OXIDASE FROM CLADOSPORIUM OXYSPORUM

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Reprint

from

Journal of Environmental Sciences, 2014; Vol. 43, No. 2 : 253- 266

http://env.mans.edu.eg/journals/jes

P-ISSN 1110-192X
e-ISSN 2090-9233