

## Original Research Article

### Purification and characterization of the glucoamylase from *Aspergillus niger*

Mohammed A. Jebor<sup>1</sup>, Zahra M. Ali<sup>2</sup>, and Baydaa A.Hassan<sup>3</sup>

<sup>1</sup>University of Babylon, college of Sciences , <sup>2</sup> University of Kufa, College of Sciences,

<sup>3</sup>University of Kufa, College of Sciences , Iraq

\*Corresponding author

#### A B S T R A C T

This study aims topurification and characterization of theglucoamylase enzyme from *Aspergillus niger*, the enzyme was purified by ammonium sulfate precipitation (80%) , dialysis and gel filtration chromatography using sephadex G-200, A trial for the purification of glucoamylase using gel filtration technique resulted in two types of glucoamylase (A& B) with specific activity of (4.195 , 21.466) respectively and terminal specific activity of glucoamylase (B) was reached (31.214 U/mg) with (10.689 folds) purification . the purified glucoamylase (A&B) had a maximum activity at pH = 8, 6.5, 40,30 °C respectively , the glucoamylase (B) was stable with pH values ranging between (6 – 6.5) and the enzyme was maintained the activity when it incubated into (10 -35) °C for 30 minutes while keep 65% of its activity in temperature 40 °C also glucoamylase (B) was stable in 30°C for ( 10-30 ) minutes , analyses of the glucoamylase (A&B) for molecular weight was carried out by PAGE and SDS-PAGE electrophoresis, which revealed 52 and 66 KDa respectively , also molecular weight of the glucoamylase (B) was achieved by gel filtration technique and was found 68 KDa this means that enzyme consisting of only one subunit , the  $K_m$  and  $V_{max}$  value of glucoamylase (B) were (2.8 mM, 9.8 mM/min ) respectively using different concentration of starch.

#### Keywords

Glucoamylase;  
*Aspergillus niger*;  
Gel filtration.

#### Introduction

Glucoamylase ( $\alpha$  -1, 4 glucan-glucohydrolases, EC 3.2.1.3) is exoenzyme of great importance for saccharification of starchy materials and other related oligosaccharides. Glucoamylase consecutively hydrolyzes 1,4-  $\alpha$ -glycosidic bonds from the non-reducing ends of starch and 1,6- $\alpha$ -glucsidic linkages in polysaccharides yielding

glucose as the end-product, which in turn serves as a feedstock for biological fermentations (Gupta, et al., 2003; Norouzian, et al., 2006). A more technical name is  $\alpha$  -(1, 4) glucanglycohydrolase where glucan is the name for a series of glucose units attached and glycohydrolase describes the breaking of the bond between two glucose units. glucoamylase

is produced by a variety of fungi but the exclusive production of this enzyme in industry have been achieved mainly by *Aspergillus niger* (Wang et al., 2006) Glucoamylase is second to proteases in worldwide distribution and sales among industrial enzymes.

This enzyme is used in the baking industry, the brewing Process, and The most important application of glucoamylase is the production of high glucosesyrups. also have various applications in major areas of food processing, animal nutrition, , fermentation biotechnology, paper making , fabric industries and in whole grain hydrolysis for the alcohol industry (Selvakumar et al.,1996; Zambare, 2010 ) on the basis of the importance of glucoamylase, the present study has been taken to purification and characterization of the enzyme.

## **Materials and Methods**

### **Inoculum preparation (Afifi et al., 2008)**

Pure culture of newly isolated *Aspergillusniger* was maintained on potato dextrose agar (PDA) slants at 4 °C, the growth (synthetic) medium was autoclaved for 15 min. after cooling the flasks, inoculum was prepared by 10 ml of sterilized distilled water was added to a sporulated 5 days old PDA slant culture, inoculum (5 ml) was added to each flask containing 150 ml of growth medium with the help of sterilized disposable syringe and flasks was incubated at 30°C on a rotary shaker at 200 rpm for 3 days.

### **Isolation of glucoamylase (Gupta, et al., 2008)**

At the end of fermentation, the mycelium was separated from the culture broth by

filtering through a filter paper (Whatman No.1), the filtrate was centrifuged at 10,000 rpm for 15 min at 10°C to remove the suspended particles, the supernatant was carefully collected and stored under refrigerated conditions as to crude enzyme for further purification steps after enzyme assay.

### **Assay of Glucoamylase**

Glucoamylase activity was assayed according to (Miller ,1959) . The colour developed was read by measuring its optical density using a spectrophotometer at 540 nm.One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose from the substrate in 1 minute under standard assay conditions.Glucose standard curve was measured according to (Muhammad et al., 2011) .

### **Protein determination**

Protein concentration was measured according to Bradford (1976).

### **Enzyme purification**

#### **Ammonium sulfate fractionation (Dennison, 2002)**

The ammonium sulfate was added in different saturation ratio (20,40,60 and 80%) to reach the optimum ratio of ammonium sulfate by adding gradually the amount of salt to each 20 ml of the crude glucoamylase enzyme in ice bath and magnetic stirrer, centrifuge the solution for 25min at 6000 rpm. Dropped the supernatant and take the precipitate and dissolved it in 5 ml distilled water and both enzyme activity and protein content were determined for each separate fraction.

### **Dialysis against buffer (El-Safey and Ammar 2002)**

The obtained ammonium sulfate precipitate (in solution) was introduced into dialysis bag against phosphate buffer at pH = 6.2. The obtained glucoamylase enzyme preparation was kept in the refrigerator at 4°C for further purification.

Gel filtration chromatography technique was measured according to (El-Safey and Ammar 2002) . using sodium phosphate buffer (0.2M, pH = 6.2) , at flow rate 9 ml/hour (3ml for each fraction ), the protein fractions was measured at 280 nm

### **Characterization of glucoamylase (Afifi et al., 2008)**

#### **Optimal pH for enzyme activity**

The starch (substrate) was prepared with different pH ranges (4,4.5 , 5 , 5.5 ,6 ,6.5, 7 , 8 , 9 ).

#### **Optimal pH for enzyme stability**

The pure enzyme samples were incubated 1:1(V:V) in different pH ranges (4, 4.5, 5 ,5.5, 6, 6.5 ,7 , 7.5, 8 , 9) for 30 minutes at room temperature , then the samples were transferred freezing bath , the activity of samples were estimated using optimal pH for activity and then the relation was drawn between remaining activity and pH ranges.

#### **Optimal temperature for enzyme activity**

The starch (substrate) was prepared in tubes ,the tubes were incubated in different temperatures (10, 20, 25 ,30 ,35,40 , 50) °C to 30 min for mixing , then the pure enzyme was added and incubated

for 10 min to start the reaction , then the activity was estimated and the relation was drawn between enzyme activity and different temperatures .

#### **Optimal temperature for enzyme stability**

#### **Using different temperatures at the same time (30 minutes)**

The pure enzyme was incubated in tubes in different temperatures (10, 20, 25, 30, 35, 40, 50)°C to 30 min then the tubes were transferred freezing bath, then the starch (substrate) was added the tubes were incubated in water bath for 10 minutes the activity of enzyme was estimated and the relation was drawn between remaining activity and different temperatures.

#### **Using the same temperature at different times**

The pure enzyme was incubated in tubes in 30°C to different times (10, 20 , 30, 40, 50, 60) min, then the starch (substrate) was added the tubes were incubated in water bath for 10 minutes ,the activity of enzyme was estimated and the relation was drawn between remaining activity and different times.

#### **Determination of the kinetic parameters**

( $K_m$  and  $V_{max}$ ) the values of  $K_m$  and  $V_{max}$  were determined by plotting Lineweaver–Burk plot (Segel,1976).

Determination of the Molecular weight includes two methods:

Gel filtration technique was measured according to (Jing et al., 2005).

## **Polyacrylamide gel electrophoresis technique (Laemmli, 1970)**

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS - PAGE (12.5 % polyacrylamide). using Phosphorylase (97 KDa), BSA (67 KDa, ovalbumin (43 KDa), pepsine (34 KDa), trypsin (23 KDa), lysozyme (14.4 KDa) as molecular weight standards (molecular weight marker kit S7 were from Sigma Chemical Co., USA the gel was stained with 0.25% Coomassie Brilliant Blue G- 250

## **Results and Discussion**

### **Glucoamylase purification**

#### **Precipitation with ammonium sulfate**

The ammonium sulfate used in different saturation ratios (20 ,40 ,60 , and 80 )% , then the 80% ratio was selected as best ratio for precipitate the crude extract of enzyme , when the specific activity reached to ( 3.626 U/mg ) , with a purification fold (1.241) and yield (0.38) % as shown in table (1) , while the other saturation ratios (20 ,40 ,60) gave low titer of specific activity (2.580, 2.982, 3.135U/mg) respectively Figure (1). the ammonium sulfate was used in enzyme precipitation because it high soluble and cheap compared with the other salts, unaffected in pH and enzyme stability. The concentration by ammonium sulfate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to precipitate it. The other results of (Mervat, 2012) when she purified glucoamylase from Marine Endophytic *Aspergillus* sp. JAN-25 used ammonium sulfate to precipitate glucoamylase with 70 % saturation ratio, the specific activity

was (47.64 U/mg) protein with purification fold (2.75) .

#### **Dialysis against phosphate buffer**

The obtained ammonium sulfate precipitate was introduced into dialysis bag over night against ( 0.2 M) phosphate buffer at (pH= 6.2) , then the specific activity reached to ( 4.137U/mg ) , with a purification fold (1.416) and yield (0.31) % as shown in table (1) the other results of (Patel , et al., 2005) when he purified glucoamylase from *Aspergillus oryzae*. Used dialysis bag against (0.1M) acetate buffer at (pH= 5) , the specific activity was (51.09 U/mg) protein with purification fold (7.14) .

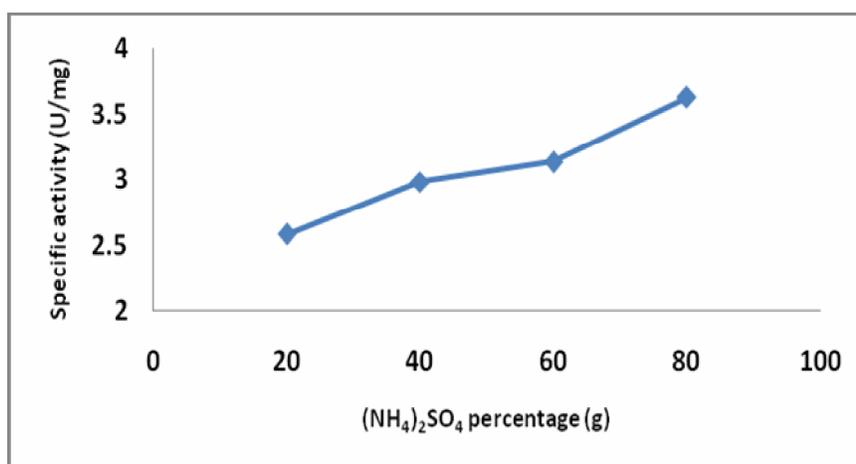
#### **Gel filtration chromatography**

The enzyme solution produced from dialysis was passed through gel filtration using (sephadex G-200) column (2×40 cm) that equilibrated with ( 0.2 M) phosphate buffer at (pH= 6.2) , the fractions were collected from column and measured at 280 nm absorbency. two peak of protein with two peak of enzymatic activity were appeared Figure (2) , in the first step of gel filtration the specific activity was the first peak of protein (4.195 U/mg) with purification fold (1.436) while to the second peak of protein (21.466 U/mg) with purification fold (7.351) , then the second peak in second step of gel filtration the specific activity reached ( 31.214 U/mg) with purification fold (10.689) shown in table (1) . the results of this study were higher than the results recorded by (Imran, et al., 2012) when they got specific activity (8.72 U/mg) with purification fold (5.73) when they purified glucoamylase from *Aspergillus niger* by using sephadex G-100.

**Table.1** The purification steps of glucoamylase from *Aspergillus niger*

Purification steps	Volume (ml)	Activity (U/ml)	Total activity(U)	Protien con.(mg/ml)	Specific activity(U/mg)	Fold	Yeild %
Crude enzyme	20	2.412	48.24	0.826	2.920	1	100
Ammonium sulfate precipitation (80)%	10	1.864	18.64	0.514	3.626	1.241	0.38
Dialysis against phosphate buffer	10	1.535	15.35	0.371	4.137	1.416	0.31
Gel filtration (first step)	<b>Peak(A)</b>	5	1.074	5.37	0.256	4.195	1.436
	<b>Peak(B)</b>	5	0.966	4.83	0.045	21.466	7.351
Gel filtration (Second step)	5	0.874	4.37	0.028	31.214	10.689	0.09

**Figure.1** Effect of different Ammoniumsulphate percentage on the purification of glucoamylase from *A.niger*



**Characterization of Glucoamylase**

**The optimum pH for enzyme activity**

The figure(3) shown the increasing the activity of glucoamylase (A and B) purified from *Aspergillusniger* with increasing the pH until reach to maximum activity of glucoamylase(A) (0.253U/ml) in pH = 8 then it began to decreased in higher pH values (0.062 ,0.033 U/ml ) in pH= 9,10 respectively, while the

maximum activity of glucoamylase (B) ( 0.511U/ml) in pH = 6.5 then it began to decreased (0.087,0.057 ,0.029 U/ml ) in pH = 8, 9,10 respectively using starch as a substrate of enzyme. this study was agreed with (Mariana, et al., 2000).

When they shown the highest activity of glucoamylase purified from the thermophilic fungus *Scytalidium thermophilum* occurred at the pH=6.5, the change in the pH of the reaction mixture

results in the change in the ionic character of the amino and carboxylic acid components of the enzyme. this in turn affects both the catalytic site and conformational status of the enzyme thereby altering its activity (Prakash, et al., 2009).

### **The optimum pH for enzyme stability**

To study the effect of optimum pH for enzyme stability the glucoamylase(B) solution was incubated with different buffers pH values ranging between (4.5 - 9) , for 30 minutes at room temperature , then measured the remaining activity.

The figure (4) illustrates the optimum pH for glucoamylase (B) stability ranging between (6 - 6.5) and the stability was decreased in extreme acidic and alkaline pH. The enzyme was kept 90% of activity in pH= 5.5 while the activity was decline in pH= 4.5, in pH= 8 and in pH =9 to 60%, 25%, 7% respectively . the effect at in pH on the enzyme stability is belong to the effect of pH in enzyme structure lead to denaturation of enzyme molecule or change the ionic state of active site ,also it effect on secondary and tertiary of enzyme structure lead to losing the activity in buffers that far away from optimum pH (Segel, 1976) .several researches pointed to the optimum pH of glucoamylase stability, (Mervat , 2012) showed that the optimum pH of glucoamylase stability purified from Marine Endophytic *Aspergillus* sp. JAN-25 ranging between (4–6.5).

### **The optimum temperature of enzyme activity**

To determine the optimum temperature of glucoamylase (A&B) activity purified from *Aspergillus niger*, the enzyme

reaction was done in different range of temperature (10 - 60) °C , and the results shown in figure (5) increasing the activity of glucoamylase (A and B) with increasing the temperature until reached to maximum activity of glucoamylase (A) (0.243U/ml) in 40°C then it began to decreased in higher temperature values (0.034, 0.026 U/ml) in 50, 60 °C respectively , while the maximum activity of glucoamylase (B) (0.703 U/ml) in 30°C then it began to decreased (0.431 ,0.065 , 0.016 U/ml) in 40, 50, 60°C respectively. This study was agreed with (Nouadri et al., 2010) when they shown the highest activity of glucoamylase purified from *Penicillium camemberti*PL21 was in 30°C.

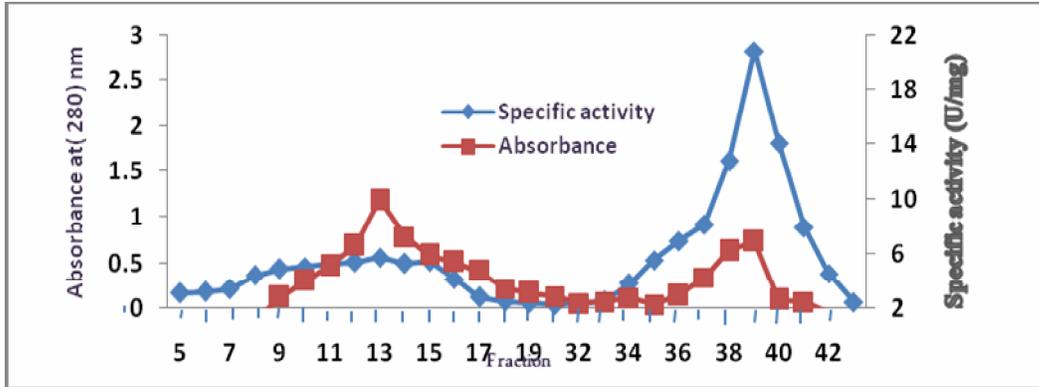
### **The optimum temperature of enzyme stability**

#### **Using different temperatures at the same time (30 minutes)**

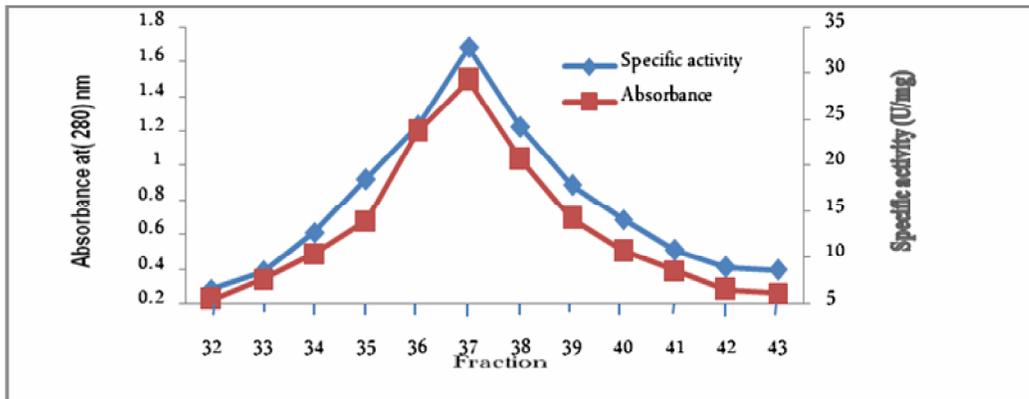
The figure(6) shown the results of incubation of enzyme with different temperature ranging between (10 -60 ) °C for 30 minutes , the enzyme was maintained the activity when it incubated into (10 -35) °C . while keep 65% of its activity in temperature 40 °C , while its keep only 13.48% in 60 °C . this study was agreed with ( Nguyen, et al., 2002) when they found the optimum temperature of glucoamylase stability purified from thermophilic fungus *Thermomyces lanuginosus*strain ATCC 34626 ranging between (20 -35) °C for 30 minutes.

The differences of thermostability values and inhibition period of enzyme depend on the type of substrate, ionic strength of buffer, molecular weight of enzyme, and the enzyme source (Nessel & Mader, 1977).

**Figure.2** Gel filtration chromatography using sephadex G- 200 column with dimensions (2×40 cm) for purification the glucoamylase from *Aspergillus niger* equilibrated with phosphate buffer (0.2 M , pH = 6.2) , flow rate 9 ml/hour and fraction volume 3 ml. (A) = the first step , (B) = the second step .

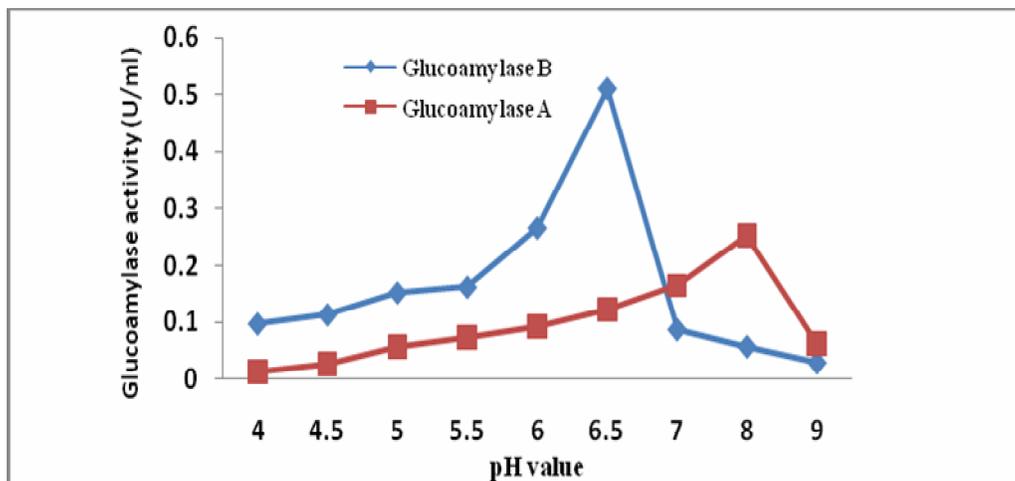


(A)

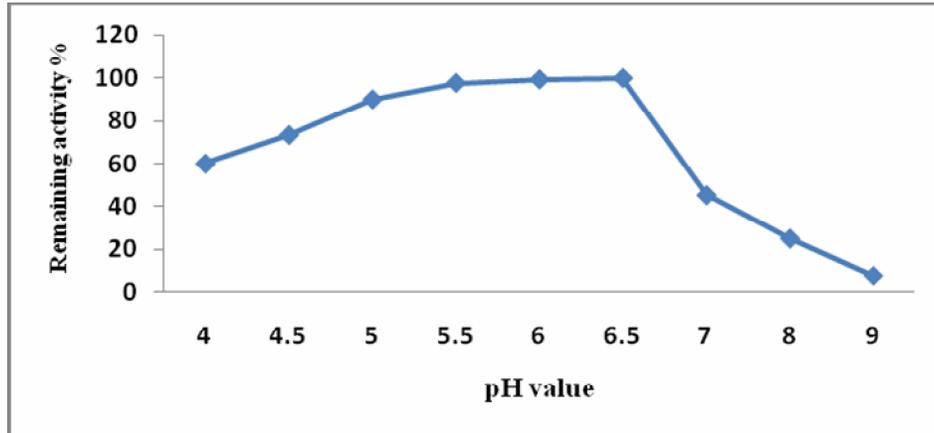


(B)

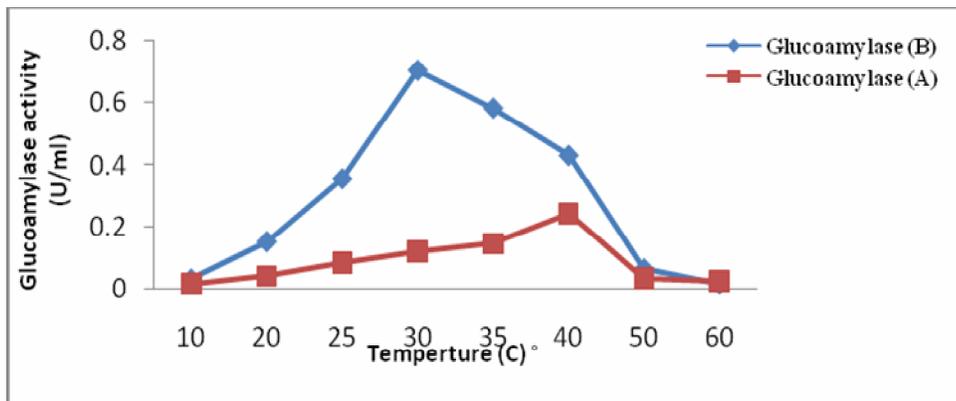
**Figure.3** Effect of different pH on the activity of purified glucoamylase (A &B ) from *A.niger*



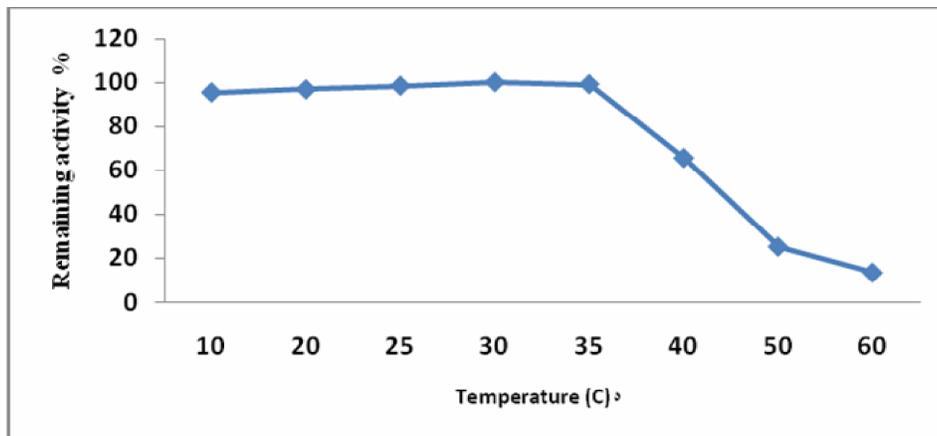
**Figure.4** Effect of pH on the stability of purified glucoamylase ( B) from *A.niger*.



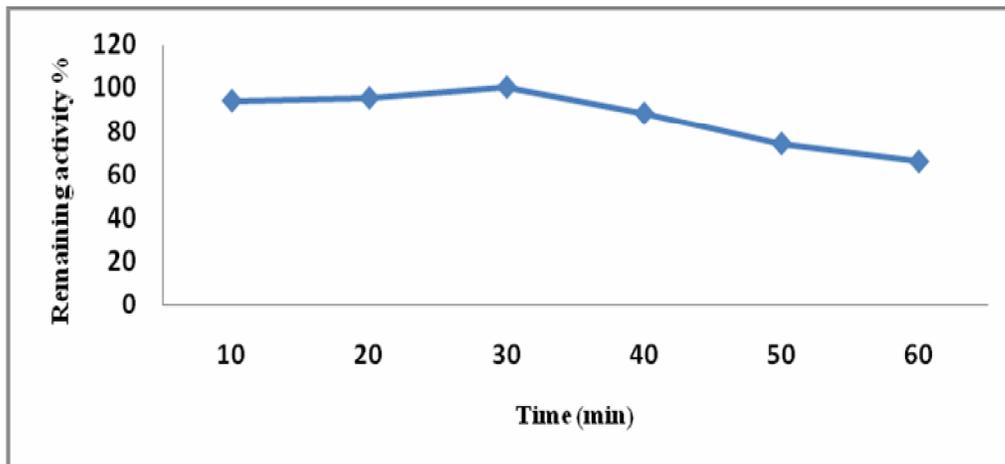
**Figure.5** Effect of different temperature on the activity of purified glucoamylase (A&B) from *A.niger*



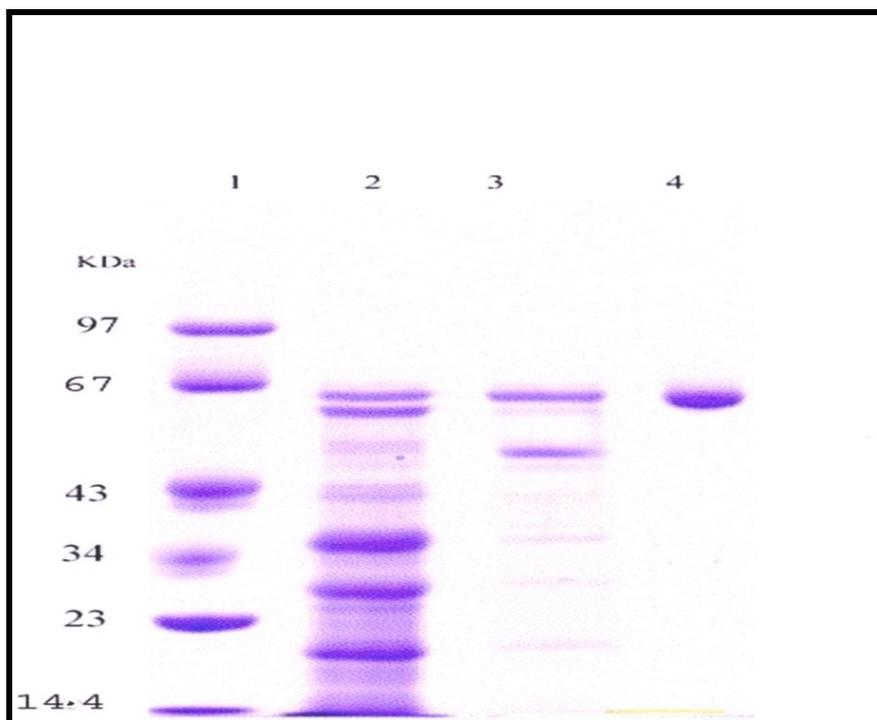
**Figure.6** Effect of different incubation temperature on the stability of purified glucoamylase ( B) from *A.niger*



**Figure.7** Effect of different incubation time on the stability of purified glucoamylase (B) from *A.niger*



**Figure.8** The polyacrylamide gel electrophoresis of the glucoamylase from *Aspergillusniger* orange fruits under denaturing conditions . Lane (1) standard molecular Weight markers, lane( 2 ) crude extract enzyme , lane( 3 ) purified glucoamylase (A&B) produced from the first step of gel filtration, lane(4) purified glucoamylase (B) produced from the second step of gel filtration.



### Using the same temperature at different times

The figure.7 shown the results of incubation of enzyme in 30°C for different times (10, 20, 30, 40, 50, 60) minutes, the enzyme was maintained the activity when it incubated in this temperature for (10 - 30) minutes. While keep 87%, 73%, 65 % of its activity after 40, 50, 60 minutes respectively . several researches pointed to the optimum temperature of glucoamylase stability at different times , (Nguyen, et al., 2002) found that the optimum temperature of glucoamylase stability purified from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626 was 70 °C for ( 20- 30) min.

### Determination of Molecular weight of enzyme

#### Polyacrylamide gel electrophoresis technique

In order to investigate the purity of the glucoamylase , which was purified from *Aspergillus niger* orange fruits , polyacrylamide gel electrophoresis under denaturing and non – denaturing conditions with concentration 12.5 % , the electrophoresis involve three samples , the first sample was crude enzyme extract, the second sample was the first step of gel filtration , the third sample was the second step of gel filtration, when the gel is immersed in Coomassie brilliant blue G-250, several protein bands seemed with different molecular weight along the gel in crude extract sample, while two bands appeared in the second sample, while only one band appeared in the third sample as shown in figure (8&9).

The appearance of many protein bands along the gel is imputed to that crude

extract contains large number from different proteins with different molecular weights , the second sample was gave two bands , this means that two proteins with two molecular weights of approximately 66 & 52 kDa are found, the third sample was gave only single protein band with molecular weight of approximately 66 kDa, the results come closer to (Imran, et al., 2012) when they found the molecular weight of the glucoamylase isolated from *Aspergillus niger* approximate 60 kDa when its mobility relative to those of standard proteins on SDS-PAGE.

#### Gel filtration technique

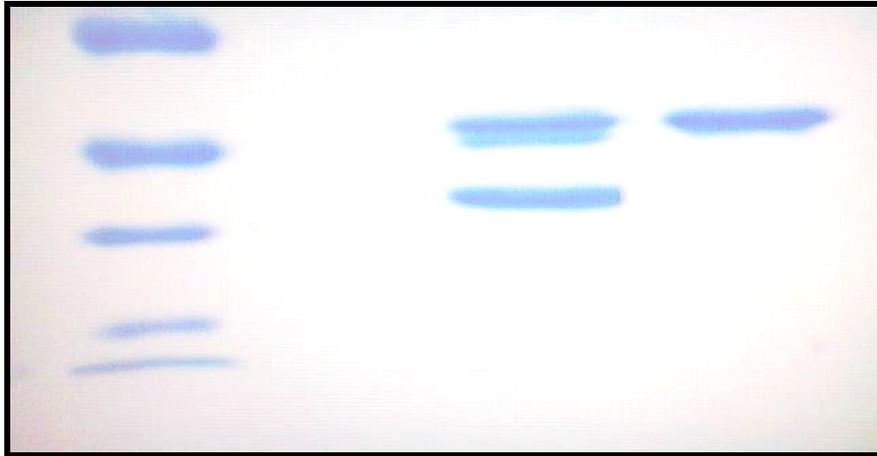
The figure (9) shown the results of gel filtration of the glucoamylase (B) purified from *A. niger* orange fruits, the molecular weight of enzyme was determined by the relation was drawn between the elution volume ( $V_e$ ) of the each standard protein to elution volume of the the blue dextran - 2000 ( $V_e/V_o$ ) and Log (molecular weight) of the each standard protein and found to be 68 kDa, this means the purified glucoamylase (B) have only single subunit.

This study was agreed with (Muhammed, et al., 2011) when they found the molecular weight of glucoamylase purified from *Trichoderma harzianum* was 70 KDa using gel filtration and polyacrylamide gel electrophoresis thereby glucoamylase consist of single subunit.

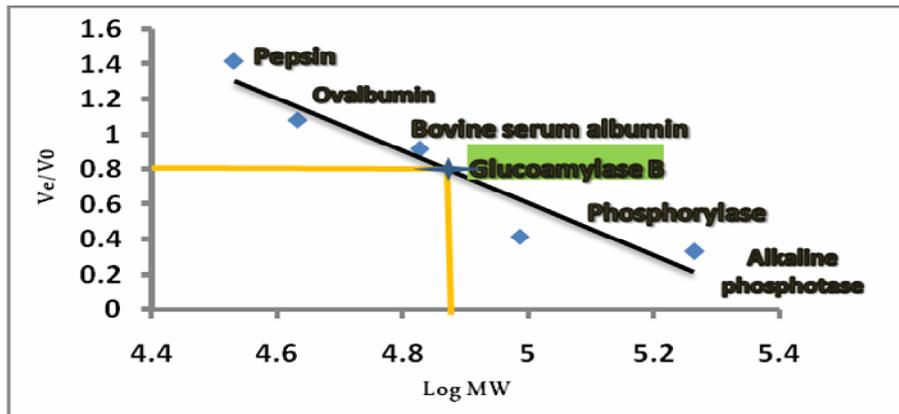
#### Determination of $K_m$ and $V_{max}$ values of glucoamylase (B)

The values of Michalis – menten constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) of glucoamylase (B) purified from *Aspergillus niger* were estimated by using the plotting Lineweaver-Burk plot which

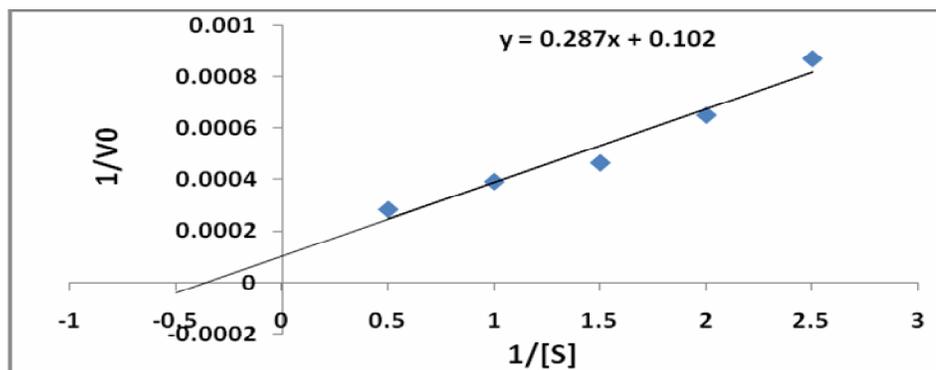
**Figure.9** The polyacrylamide gel electrophoresis of the glucoamylase from *Aspergillus niger* orange fruits under non - denaturing conditions. Lane (1) crude extract enzyme, lane(2) purified glucoamylase (A&B) produced from the first step of gel filtration, lane(3) purified glucoamylase(B) produced from the second step of gel filtration



**Figure.9** Determination of the molecular weight of glucoamylase (B) Purified from *A.niger* using gel filtration by (Sephadex G- 200) column.



**Figure.10** The relationship between substrate concentration (starch) and velocity of glucoamylase (B) purified from *A. niger*.



express the relationship between reaction velocity and substrate concentration, the values of  $K_m$  and  $V_{max}$  were (2.8 mM, 9.8 mM/min) respectively using the starch as the substrate of enzyme. The results come closer to (Nguyen, et al., 2002) when they found the values of  $K_m$  and  $V_{max}$  of the glucoamylase purified from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626 were (2.4 mM, 9.6 U/ml/min) respectively using the starch as the substrate of enzyme

## References

- Afifi, AF.,Kamel , E .M. , Khalil, A.A., Fouaad, E., Fazxi, M. , Housery, M . 2008. Purification and characterization of  $\alpha$ -amylase from *Penicillium olsonii* under the effect some Antioxidant Vitamins, Global J. Biotechnol.Biochem. 3(1): 14-21.
- Berka, R.M., Dunn-coleman, N. , Ward, M. 1992 . Industrial enzymes from *Aspergillus* species. In *Aspergillus: Biology and Applications*. Bennets, J.W., &Klich, M.A. (Edn.). Oxford, Heinemann , pp., 155-202
- Biesebeke, R. E. , Record, N. van ,Biezen, M. , Heerikhuisen, A. ,Franken, P. J. Punt, C. A. van den Hondel .2005 . Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates. *Appl. Microbiol. Biotechnol.*, 69: 44-50.
- Bradford, M. M., 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry* 72: 248-254.
- Cherry, HM.,Hossain T, Anwar, MN., 2004. Extracellular glucoamylase from the isolate *Aspergillusfumigatus*. *Pak. J. Biol. Sci.* 11: 1988- 1992.
- Clarke , A.J. and Svensson , B. 1984 b. The role of tryptophanyl residues in the function of *Aspergillus niger* glucoamylase G1 and G2. *Carlesberg Res. Commun.* 49,111-122.
- Dennison , C. 2002. A guide to protein isolation. Kluwer Academic Publishers. New York. ISBN: 0-306-46868-9.
- El-Safey EM,Ammar MS 2002.  $\alpha$ -amylase production using Nile Hyacinth under solid-state fermentation (SSF) conditions. *Int. Conf. for Develop. and the Environ.* In the Arab world march 26-28, pp. 101-113, Assuit Univ. Center for Environmental studies, Assiut,Egypt.
- Fumiaki , S.,Masaynki , O. , Hiroyuki , N. , Haruhide , M. , Atsuo , K. 2005 . Glucoamylase originating from *Schwanninmyces occidentalis* typical  $\alpha$ glucosidase . *Biosci .Biotechnol .Biochem .* , 69 (10) , 1905- 1913 .
- Gupta, R. P.,Gigras, H. , Mohapatra, V.K., Goswami, B. , Chauhan, 2003 . Microbial  $\alpha$ -amylases: a biotechnological perspective. *Process Biochem.* 38: 1599-1616.
- Imran, M . , Muhammad, J. A., Muhammad, G., Rahmatullah, Q., Hina, G., Nazish, M. and Arshad, N. C. 2012. Glucoamylase production from *Aspergillus niger* by using solid state fermentation process, *Pak. J. Bot.*, 44(6): 2103-2110 .
- Jing C, Duo-Chuan, L. , Yu-Qin, Z . , Qing-Xin , Z. 2005. Purification and characterization of a thermostable glucoamylase from *Chaetomium thermophilum* . *J. Gen. Appl. Microbiol.*, 51: 175–181.
- Laemmli, UK., 1970 . Cleavage of structural protein during the assembly of the heat of bacteriophage T4.*Nature.* 227: 680 – 685 .
- Mariana , C. , Hector, F. T., Joao, A. J., Lewis, J. G., Jose, C. R. and Maria, D. T. M. P. 2000 .Glucoamylase activity from the thermophilic fungus *Scytalidium thermophilum*. *Biochemical and regulatory properties,*

- J. Basic Microbiol. 40 : 2, 83–92 .
- Mervat , M. A. 2012 . Production of Glucoamylase by Marine Endophytic *Aspergillus* sp. JAN-25 under Optimized Solid-state Fermentation Conditions on Agro Residues , Australian Journal of Basic and Applied Sciences, 6 (4): 41-54, , ISSN 1991-817 .
- Miller, G.L., 1959 . Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31: 426-429.
- Muhammad, E., Hassan, EL., and Mohamed, B. J. 2007 . Optimization of growth and extracellular glucoamylase production by *Candida famata* isolate. African Journal of Biotechnology 6 (22): 2590-2595.
- Muhammad, J. A. , Muhammad, G., N. M., Nyla, J., Saqib, H., Hadri, M. I., Zahid A. and Dawood, A. 2011. Hyper production of glucoamylase by *Aspergillus niger* through the process of chemical mutagenesis. International Journal of the Physical Sciences Vol. 6(26), pp. 6179-6190.
- Nagisetty, D., Padmini, S. Bhattacharya, A. D. and Subbaramiah S. R . 2012 . Solid-State Fermentation and Characterization of  $\alpha$ -Amylase from a Rhizospheric Isolate of *Aspergillus flavus* associated with *Mangifera indica* , Annals of Biological Research, , 3 (8):4082-4090.
- Nessel, A. and Mader , M. 1977. Physiological Significance of the isoenzyme groups of peroxidase from Tobacco demonstrated by Biochemical properties.
- Nguyena, Q. D., Judit, M. , Rezessy-Szabó, Marc C., Ingeborg S., Ágoston H. 2002 . Purification and characterisation of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626. Enzyme and Microbial Technology 31 : 345–352.
- Norouzian, D., A. Akbarzadeh, J.M. Scharer, M.M. Young, 2006 . Fungal glucoamylases. Res. Rev. Paper, 24: 80-85.
- Nouadri , T., Zahia, M., Djekrif, D. S. and Bennamoun L. 2010. Purification and characterization of the glucoamylase isolated from *Penicillium camemberti* PL21. African Journal of Biochemistry Research Vol. 4(6), pp. 155-162.
- Pandey, A . , Nigam P, Soccol, R., Soccol, T. , Singh . D ., Mohan, R. 2000. Advances in microbial amylases (Review). Biotechnol. Appl. Biochem., 31: 135-152.
- Patel , A.K., Napoothiri, K.M. Szakacs, G., Pandey , A. 2005 . Partial Purification and characterization of the glucoamylase from *Aspergillus oryzae* using spent- brewing grains. Indian Journal of Biotechnology. Vol. 4 . pp 336 – 341 .
- Segel, I. H. 1976. Biochemical Calculation, Jon Wiley and Sons. Inc. New York.
- Selvakumar, P., Ashakumary, L., Helen, A. and Pandey, A. 1996. Biotechnology Unit, Regional Research Laboratory, Council of Scientific and Industrial Research, Trivandrum, India, 11: 47-96.
- Wang, X.J., Bai, J.G. , Liang, Y.X. 2006 . Optimization of multienzyme production by two mixed strains in solid-state fermentation. Appl. Microbiol. Biotechnol., 73: 533-540.
- Zambare, V. 2010 . Solid State Fermentation of *Aspergillus oryzae* for Glucoamylase Production on Agro residues Int J Life Sci4 : 16 - 25.