EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF BETA-GLUCOSIDASE FROM APRICOT DEFATTED SEEDS POWDER

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ABSTRACT

The objective of this study, is characterization of partially purified beta-glucosidase (BGS) extracted from apricot defatted seeds powder. The enzyme was extracted using sodium phosphate solution (0.2M, pH 7) containing 2% sodium chloride with mixing ratio of (1:5 solid: liquid) for extraction period of 3 hours at 4C. The crude extract was precipitated by ammonium sulfate at 20-70% saturation, dialyzed then concentrated. The specific activity of the enzyme, folds of purification and the enzymatic yield were 5.45 units/mg, 7.8 fold and 84.5%, respectively. The results showed that the optimum conditions for the enzyme activity were at pH 5.5 and 6.5, the optimum temperature for the enzyme's activity was 40°C and 55°C, while the thermal stability of the enzyme was between 25-45°C when the enzyme retained more than 90% of its activity over 30 minutes incubation at the optimum pH for activity, and retained 88% of its activity at 50° C. The results also revealed the inhibitory and activator effects of minerals and chemical compounds on the BGS activity.

Key words: Plant β-D-glucoside glucohydrolase, apricot kernel, inhibitors.
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استخلاص وتنقية وتوصيف جزئي لأنزيم بيتا كلوكوسيديز من مسحوق بذور المشمش المزالة الزيت

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المستخلص

هدفت الدراسة الحالية الى توصيف أنزيم بيتا كلوكوسيديز المنقى جزئيا والمستخلص من مسحوق بذور المشمش المزالة الزيت. تم استخلاص الانزيم باستخدام محلول فوسفات الصوديوم(0.2M ، 7 pH) الحاوي على 2% كلوريد الصوديوم بنسب خلط (5: 1) وفترة استخلاص 3 ساعة عند درجة حرارة 4 مئوي . ثم تم ترسيب المستخلص الخام بواسطة ملح كبريتات الامونيوم عند نسبه تشبع بين 20–70%. تم اجراء الديلزة ثم تركيز الانزيم لتصبح الفعالية النوعية للانزيم وعدد مرات التنقية والحصيلة الانزيمية 5.45 وحدة/ملغم ، 7.8و 84.5% على التوالي. أظهرت النتائج ان الظروف المثلى لعمل انزيم بيتا كلوكوسيديز هي عندالرقم الهيدروجيني 5.5 ,6.5 وكانت درجة الحرارة المثلى لفعالية الإنزيم 40مْ و55ْ م .أما الثبات الحراري للإنزيم فقد كان بين 45–25ْ م لدى حضنها لمدة 30 دقيقة عند الرقم الهيدروجيني الأمثل للفعالية إذ احتفظ الإنزيم بأكثر من 90% من فعاليته كما احتفظ بـ 88%من فعاليته عند 50 م، كما أشارت النتائج الى تأثير المنشط والمثبط لبعض الايونات الفلزية وبعض المركبات الكيميائية على فعالية الانزيم.

الكلمات الافتتاحية: بيتا – دي –كلوكوسيدكلكوهايدروليز النباتي، نوى المشمش، المثبطات.
البحث جزء من أطروحة الدكتوراه للباحث الاول

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INTRODUCTION

β-Glucosidase, which is also known as β-D-glucoside glucohydrolase (E.C 3.2.1.21), is a group of enzymes that hydrolyses a variety of glycosides, including aryl- and alkyl- β-D-glucoside and ρ-nitrophenyl-β-D-glucoside, and disaccharides such as cellobiose. β -Glucosidases are the essential part of cellulase system and catalyze the last and final step in cellulose hydrolysis. Cellulase enzymes hydrolyze the cellulose to produce cellobiose and other short oligosaccharides which are finally hydrolyzed to glucose by β -glucosidase (8,17,22). β -Glucosidases cleave the glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate. Most β -glucosidases that have been characterized (i.e., GH1, GH3 and GH30 family enzymes) are retaining enzymes, and they perform catalysis in two steps, glycosylation and deglycosylation, during first glycosylation step, a conserved glutamate residue acts as nucleophile and attacks on the glycosidic bonds or cellobiose and other oligosaccharides formed by the hydrolytic action of other enzyme of cellulase system. This results into the formation of an enzyme-substrate intermediate complex, during second step called deglycosylation, an another conserved glutamate residue activates a water molecule present in the proximity by general acid/base catalyst reaction and now this activated water molecule acts on the intermediate complex to release the free glucose residue (18,20). β-Glucosidases have dual activity, namely the cleavage and synthesis of glycosidic bonds, and both play an important role in biotechnological processes, including the production of biofuel and ethanol from cellulosic agricultural wastes and synthesis of useful β -glucosides (25). These enzymes are employed in industry for hydrolysis of bitter compounds during juice extraction and liberation of aroma from wine grapes, also used to enhance the flavor in tea and fruit juice (13,30). β -Glucosidases play an important role in flavor liberation from glucosylated (b-glucosides conjugated) precursors in fruits and other plant tissues (23). Cleavage of phenolic and phytoestrogen glucosides from fruits and vegetables is also carried out by applying this enzyme to extract medicinally important compounds and to enhance the quality of beverages (28). Most of the β -glucosidase to date have been isolated and purified from awide range of plants (roots of a plant Orobanche minor (26), Brassica oleracea (4), Citrus sinensis var (9), Muscat of Bornova grape (31). Many techniques were used to purify β- glucosidase to either homogeneity or partial purification, Verma *et al*.,(33) were able to achieve partial purification of β- glucosidase from (*Rauvolfafia serpentina*) by two steps with ammonium sulphate precipitation (30-70%) and ion exchange chromatography, while Asic *et al.,* (2) conducted a partial purification for Mushroom β- glucosidase by two steps of a ammonium sulphate precipitation (40-80%) and Gel filtration chromatography with using Sepharose-4B-L-tyrosine-1-napthylamine column. Dikshit and Tallapragada. (12) in their results were capable of purifying β-glucosidase from the *Monascus sanguineus* partial purification with ammonium sulphate precipitation) (25-80%). The pH optimum for BGS plant is typically between 5 and 6.5 (23). In Prunus domestica seeds (11), olive (32), and apricot (5), the temperature optimum for BGS has been reported to be 55°C, 40 and 45C, respectively. Apricot seeds are by - products of the apricot processing industry. The kernel of apricot seeds contains approximately 50-150 μmol/g (dry weight basis) potentially toxic cyanogenic glycosides, mainly amygdaline and prunasin (1). Therefore, it is worthwhile to explore the kernel of apricot for its oil, enzymes, and related products. The seeds (kernel) of the apricot have been shown to contain hydroxynitrile lyase and B-glucosidase (3,5). They were chosen to take advantage of these wastes in the extraction of B-glucosidase enzyme. In previous study the optimum conditions for extraction and purification of B-glucosidase (BGS) from locally cultivated apricot seeds was identified (29). This study aimed to identify the optimal pH and temperature for activity and stability of the partially purified BGS, and investigate the effect of some metal ions and chemical compounds on the enzyme's activity.

MATERIALS AND METHODS

1- Extraction of the enzyme: The extraction of the enzyme carried out according to Han *et*

al. (15) method using sodium phosphate buffer solution at a concentration of 0.2 molar and pH 7.0 containing 2% sodium chloride in a ratio of (1: 5) (solid: liquid) and the mixture was stirred for 3 hours. Using a magnetic stirrer, large parts filtered by Watt man filter No.1 and centrifuged at 6000 x g for 20 minutes to remove the coarse parts. The supernatant was designated as crude extract, then its volume, enzymatic activity, and protein concentration were determined and the specific activity was calculated

2- Enzymatic activity estimation

A 0.5 ml aliquot of the sample was added to 0.5 ml of reaction mixture citrate phosphate buffer (0.1M, pH 5.2) and 0.5 ml of substrate (0.63mM p-nitrophenyl –β-D-glucopyranoside (pNPG) in 0.1mM citrate phosphate buffer, pH 5.2) and incubated at 37 °C for 30 min. A 0.5 ml of 0.4M Na_2CO_3 was added after incubation to terminate the reaction, and samples were centrifuged at 15490 ×g for 15 min. The absorbance at 410 nm was used to determine the amount of p-nitrophenyl produced. One unit of BGS enzyme activity was defined as the amount of enzyme required to cause a 0.01 change in absorbance under experiment conditions, according to the method described by (6).

3- Protein concentration

Bradford method was used to determine the protein concentration using Bovine Serum Albumin (BSA) as standard protein (7).

4- Enzyme purification

Ammonium sulfate saturation (20, 40, 60, and 70) % were set up to precipitate the crude enzyme. The crude extract precipitated after about 4 hrs. Subsequently, the mix was centrifuged at 10,000 x g for 30 min, carefully the supernatant poured off and the pellets dissolved in appropriate volume of 0.2 M sodium phosphate buffer at pH 7. Whole process achieved in at 4C. The ammonium sulfate precipitated enzyme was dialyzed against sodium phosphate buffer (pH7, 0.2 M) for 24 hours with three changes of dialysis buffer. Then it was concentrated and the enzyme activity and protein concentration were determined and the specific activity was calculated.

Characterization of the partially purified BGS enzyme : Determination of the optimum pH for the BGS activity: The optimum pH for the activity of the enzyme was determined using sodium citrate buffer solution of (0.2 M) with pH values ranging from 3.5-5.5, sodium phosphate buffer solution with pH value ranging between 6.0-7.5, and Tris-HCL buffer solution with a pH value (8-9) to prepare the substrate solution. The reaction carried out by incubating the substrate with enzyme for each pH (3.5-9.0) in test tubes for 30 minutes in a water bath at 37 ° C, then it was cooled directly in an ice bath to stop the enzymatic reaction and the enzymatic activity determined as explained previously and then the relationship between the enzyme activity and the pH values is plotted.

Determination of the optimal pH for BGS enzyme stability: A particular volume of the pure enzyme was incubated with an equal volume of buffer solutions with different pH values (3.5-9.0.) in test tubes for 30 minutes in water bath at 37 ° C, then cooled directly in an ice bath and the activity was measured. The remaining enzyme activity was calculated as a percentage of the activity out of the highest activity. The relationship between the remaining enzyme activity versus the pH values was plotted to determine the optimal pH for the enzyme stability.

Determination of the optimal temperature for enzyme activity: BGS activity was tested after incubation of the reaction mixture (substrate and enzyme) under a range of temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65) °C. Then, the BGS activity was estimated for each temperature.

Determination of the thermal stability of the enzyme: The enzyme was incubated in a water bath at different temperatures degrees ranging from 25-65 ° C for 15 minutes, then the tubes were cooled directly in an ice bath and the reaction solution was added to them (with the optimum pH). The residual enzymatic activity was estimated and the relationship between the remaining activity and different temperatures degrees was estimated.

Determination of the effect of metal ions and some activators and inhibitory reagents on BGS enzyme activity: A certain volume of partially purified enzyme was incubated with an equal volume of chemical solutions (NaCl, potassium chloride, calcium chloride, copper

sulfate, urea, Ethylen diamine tetraacetic acid (EDTA), L-cysteine, Mercaptoethano at a concentration of 1 and 5 mM) in test tubes for 30 minutes in a water bath at 37°C, then immediately cooled in an ice bath, and then the remaining enzymatic activity was estimated as a percentage (%) of the activity of the untreated enzyme.

RESULTS AND DISCUSSION

Enzyme extraction

Enzyme extraction and concentration: Results of the βeta- glucosidase extraction from the apricot seed by sodium phosphate buffer 0.2 M at pH 7 at 4 c, showed a higher enzymatic activity of 1.6 units/ml, protein concentration 2.3 mg/ml, specific activity 0.69 units/mg, total activity 272 units and yield 100% (Table 1). Figure 1 shows the enzyme activity through ammonium sulfate precipitation of crude enzyme extract. It has been noticed that the enzyme precipitated out between 20 - 70% of ammonium sulfate saturation and the highest activity for the precipitated fraction were achieved at 65% saturation. It was clear that as the enzyme activity increased in precipitated fraction, it is decreased in supernatant fractions. The enzyme units and the specific activity in this step was 16 U/ml and 5.01 U/mg respectively. After dialysis step the enzymatic activity was 18.4 U/ml. Bhalla *et al*., (5) reported that the beta glucosidase can be isolated in the 20-70% saturation using fractional precipitation with ammonium sulfate. Another study demonstrated that beta glucosidase precipitated out between 40- 80% of ammonium sulfate saturation (2).

Table 1. Purification steps of beta glucosidase from apricot seeds defatted powder

Purification step	Enzymatic activity (U/ml)	Volume (ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	No. of folds	Yield %
Crude extract	1.6	170	2.3	0.69	272	1	100
Ammonium Sulfate 20-70%	16	15	3.19	5.01	240	7.2	88.2
Dialysis (Concentrated)	18.4	12.5	3.1	5.93	230	8.5	84.5

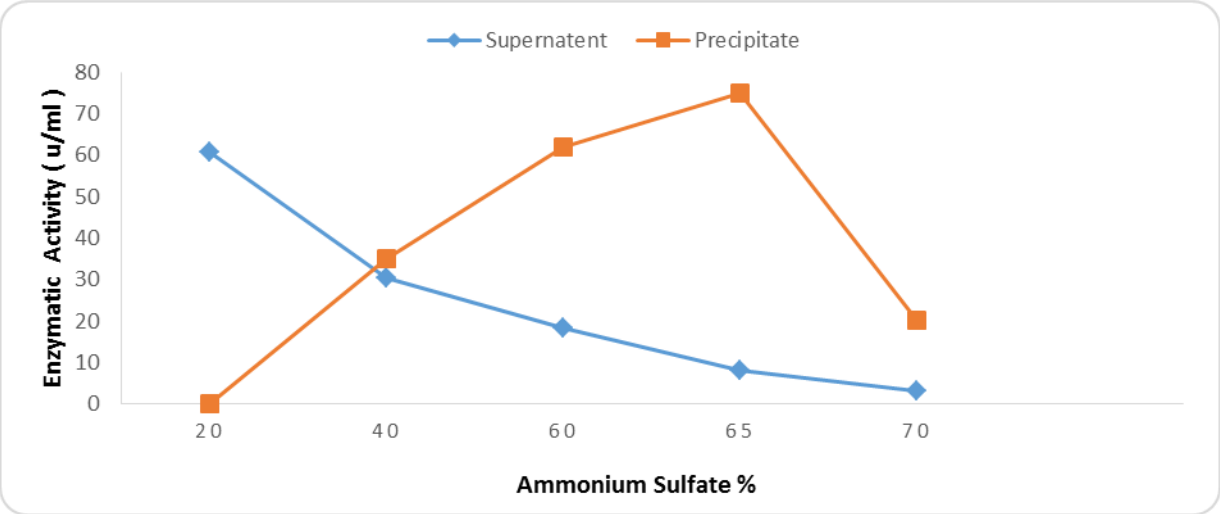


Figure 1. Ammonium sulfate salt Precipitation of beta glucosidase from Apricot defatted seeds powder

Characterization of the purified enzyme

The optimum pH for beta glucosidase activity : Figure (2) shows the pH profile of the beta glucosidase activity (pH range 3.5 – 8). It has been noticed that the optimum pH for the activity of the enzyme was (5.5,6.5), these two values might be attributed to the presence of isozymes because of the partial purification. The enzymatic activity decrease remarkably at extreme values of pH (below 4 and above 7). This may be due to the effect of the pH changes on the ionic properties of the enzyme molecule and other components of the reaction medium. As a result, there is a change in the composition of the three-fold stacked shape to a more random composition, meaning a

change in the natural state of the enzyme occurs. This may weaken the activity of the enzyme to the extent that the enzyme completely loses its effectiveness. The effectiveness of the enzyme may also decrease due to a change in the shape, or arrangement of the molecules of the base material. As a result, the tendency of the enzyme to interact with the base material will decrease, which leads to a decrease in the enzymatic activity (21,27). The results of the current study were close to the results of other related studies about beta glucosidase from different sources. It was mentioned (11) that the optimum pH for the activity of beta glucosidase extracted from peach seeds was 5.5. Velázquez-Palmero etal, (32) found that optimum activity of olives beta glucosidase was at pH 6.5. Whereas, (Yan et al., (34) mentioned that the optimal pH for the Paecilomyces Bainier) beta glucosidase was 3.5.

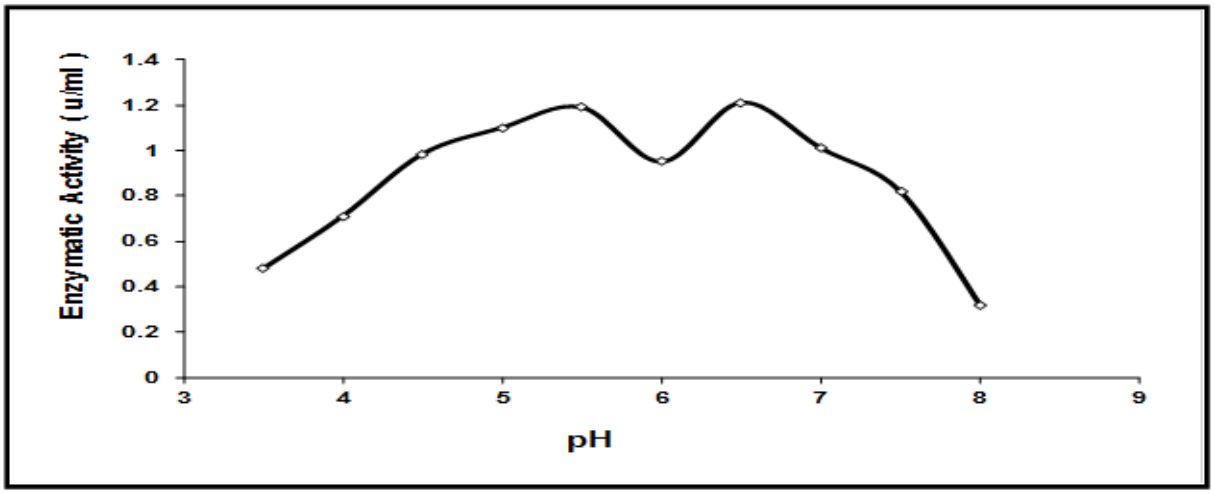
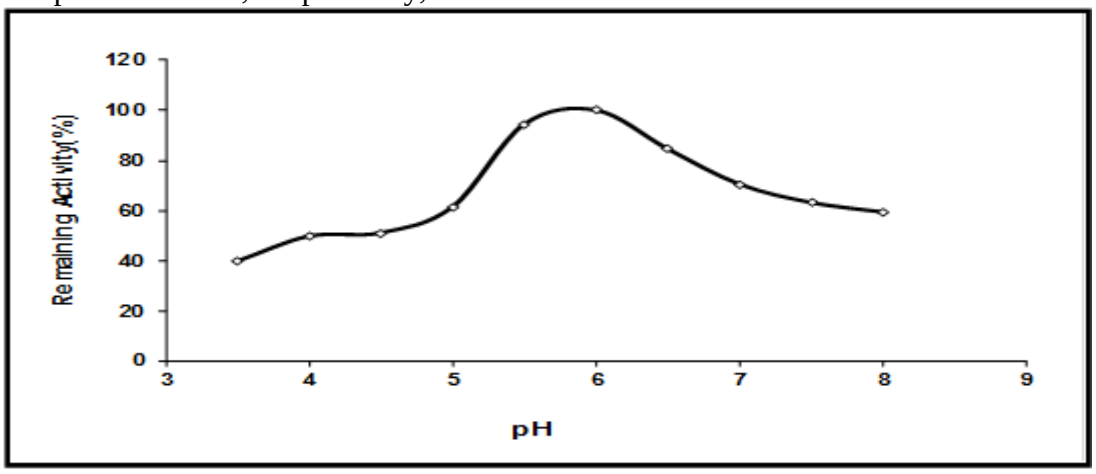


Figure 2. The optimal pH for the activity of the partially purified beta glucosidase from apricot defatted seeds powder

Optimal pH for beta glucosidase stability

Figure. 3 represents the stability of the experimental beta glucosidase at different pH values. The enzyme stability was noticed at pH ranges from5 .5 – 6.5, as the enzyme retained more than 80% of its activity at pH ranged between (5.5 - 6.5) and retained 70.5% of the its total activity at pH 7, 63% at pH 7.5 and 59% at pH 8. The same figure showed that the enzyme retained only 39.7% and 49.7% of its activity at pH 3.5 and 4, respectively, these results were in agreement with Yu *et al*., (35) findings that for apple seeds beta glucosidase stability pH values ranged between (5 - 9). Besic *et al*. (4) stated that the optimum pH for the stability of beta-glucosidase enzyme purified from Cabbage (Brassica oleracea) was at a range of 4-7. Gong *et al*., (14) reported that the optimum pH for the stability of beta-glucosidase enzyme produced from Aspergillus niger was in the range of 3-8.



3. The optimal pH for the stability of the partially purified beta glucosidase from apricot defatted seeds powder

The optimal temperature for activity

The effect of temperature on the activity of the partially purified beta-glucosidase enzyme was studied by conducting the enzymatic reaction at different temperatures that ranged between (25-65 °C) and with a difference of 5 degrees between one treatment and another. The results showed that there was an increase in enzyme activity with increasing temperature, and the enzyme activity reached its highest levels at 40°C and 55°C, then gradually decreased when the temperature increased after this point. This could be attributed to the increase in contact between the enzyme molecules and the substrate due to the increase in the kinetic energy of the molecules (27). The enzymatic activity decreases due to a change in the nature of the enzyme or the substrate or both, which in turn leads to a decrease in enzyme activity and reaction rate. reaction decreases, and the heat may affect the base material itself and become not subject to the enzyme (21). Similar findings were reported by other related studies. Velazquez-Palmero *et al*., 2017 (32), observed that optimum temperature for the activity of Olive Fruit BGS was at 40 °C. Ünal *et al*.,(31) reported that optimal temperature for activity of Muscat of Bornova grape BGS was at 55°C. Bhalla *et al*., (5) demonstrated that optimum temperature for activity of BGS produced from seeds of Wild apricot (Prunus armeniaca L.) was at 37 °C. While Besic *et a*l., ,(4) found that Brassica oleracea BGS had an optimum temperature for its activity at 30°C. The reason for the difference in the optimum temperatures of the enzyme in different studies is due to the difference in the source of the enzyme, the working conditions and the extent of the purity of the enzyme.

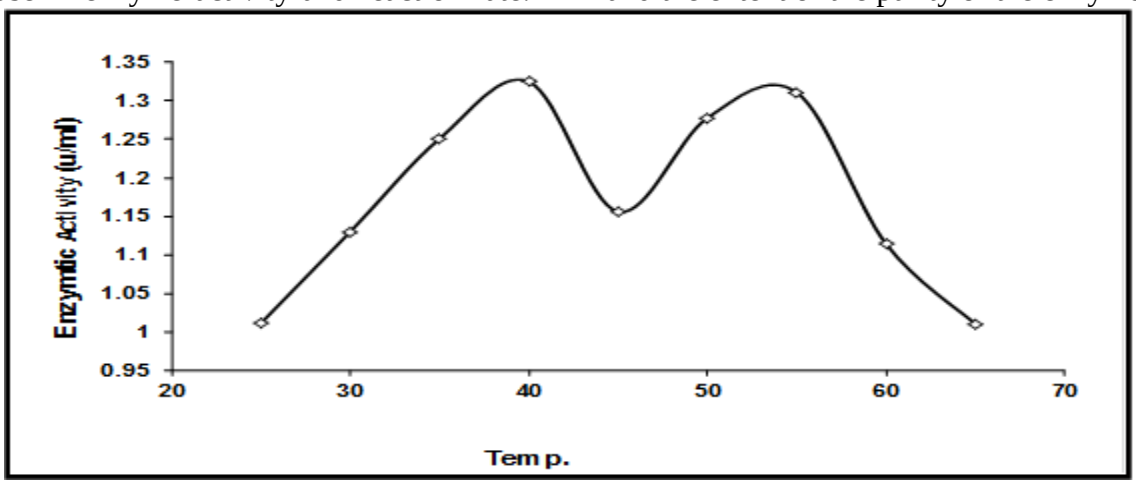


Figure 4. The optimal temperature for activity of the partially purified beta glucosidase from apricot defatted seed powder

Determination of the thermal stability of BGS the enzyme: Figure (5) shows the thermal stability of the partially purified beta-glucosidase as incubated for 15 minutes at temperatures ranging between (25-65° C) and with a difference of 5 degrees between each treatment. It was observed that the enzyme retained more than 90% of its entire activity when incubated at temperatures ranged between 25-45° C for 30 minutes and 88.8% of its effectiveness at a temperature of 50° C, and the BGS activity continued to decrease as the temperature increased above 50C. As the high temperature leads to an increase in the thermal inactivation speed of the enzymes, therefore, the enzymes are kept at low temperatures to maintain their stability (24). Since the enzyme under study retained approximately 70% of its effectiveness over 30 min. incubation at a 65 °C, this qualifies it for use in Multiple industrial fields.The results of this study were consistent with what was indicated by other studies that dealt with the effect of temperature on the stability of the beta-glucosidase enzyme, as chen *et al*., (10) stated that the beta-glucosidase enzyme produced from *Penicillium decumbens* retained its full effectiveness when incubated at temperatures ranging from 20-55 C. Dikshit and Tallapragada (12) found that the beta-glucosidase enzyme produced from Monascus sanguineus was stable at temperatures ranging from 20-60° C.

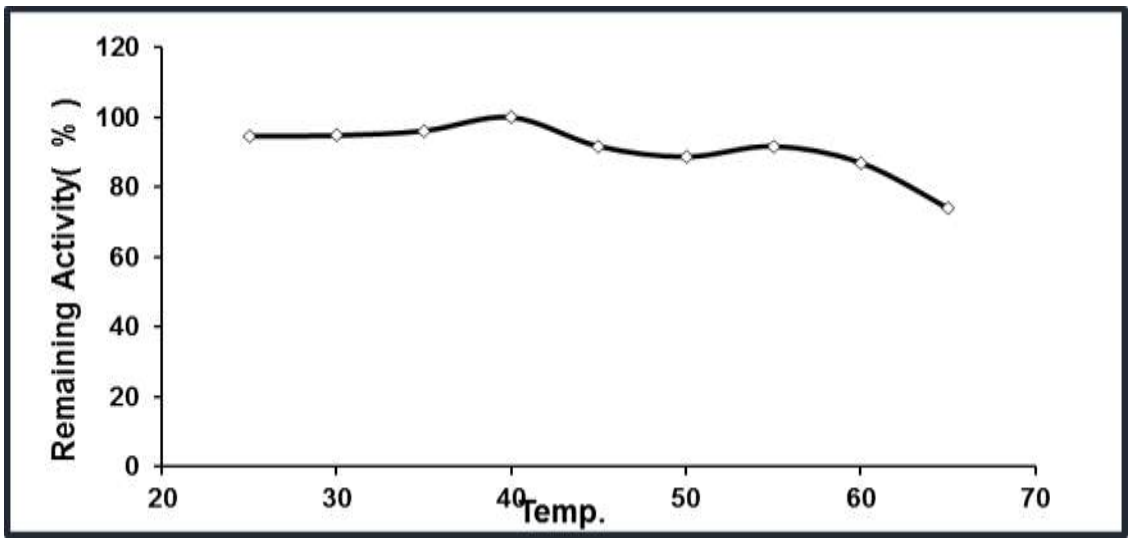


Figure 5. The optimal temperature for stability of the partially purified beta glucosidase from apricot defatted seed powder

Effect of some chemicals on the activity of beta-glucosidase enzyme: Table (2) shows the effect of some chemicals on the activity of beta-glucosidase enzyme, which included incubating the enzyme with (sodium chloride, potassium chloride, calcium chloride, copper sulfate, urea, ethylene diamine tetra acetic acid (EDTA), L-cysteine, Mercaptoethanol at a concentration of (1 and 5 mM) at 37ºC for 30 minutes, then the remaining enzyme activity was measured. The used salt solutions showed different effects on the activity of the enzyme under study. The addition of heavy metal salts such as copper sulfate at a concentration of 1 mM and 5 mM resulted in a loss of most of the enzyme activity as the remaining enzymatic activity is droped to 14% and 8%, thus copper sulfate is a strong inhibitor for the enzyme. Similar findings were reported by other related studies. Yu *et al*., ((35), mentioned to an inhibition of beta-glucosidase enzyme extracted from apple and almond seeds in the presence of copper sulfate at a concentration of 5 mM. The remaining enzyme activity was 0.8% and 0.3% respectively. Gong *et al*., (14) found that copper sulfate inhibits 80% of the activity of beta-glucosidase enzyme extracted from *Aspergillus niger* mold. Kaur *et al*., (16) mentioned that copper sulfate inhibits about 38% of the activity of beta-glucosidase enzyme extracted from *Melanocarpus* .=====whereas potassium chloride and calcium chloride had a slight effect in inhibiting the activity of the enzyme. The percentage of the remaining activity was 94% and 90.4 after treating with 1 mM and 85.4% and 86% in case of 5 mM respectively. These results were close to what was mentioned by Yu *et al*., (35) a slight reduction was noticed in beta-glucosidase enzyme extracted from apple and almond seeds activity in the presence of potassium chloride. The remaining enzymatic activity reached 91.5% and 84.1% and reached 80.2 and 81.8 in the presence of calcium chloride. Dikshit and Tallapragada (12) indicated that there was 14.8% inhibition in the presence of calcium chloride and 21% in the presence of potassium chloride of beta-glucosidase enzyme extracted from *Monascus sanguineus*. Both sodium chloride and mercaptoethanol showed an activating effect on the beta-glucosidase activity, where the enzymatic activity after incubating the enzyme with solutions of these substances and at the concentrations referred to above reached 111%, 100%, 109 and 102, respectively. Schroder *et al*., (28) indicated that the beta-glucosidase enzyme extracted from hydrothermal spring metagenome was activated in the presence of sodium chloride at an 5mM concentration. The enzymatic activity reached 104% but the enzyme activity was not affected by the presence of the same salt at a concentration of 1 mM, as the enzyme retained its full activity. We infer from the above results that metal ions have an effect on enzyme activity that varies according to their types and concentrations, and the effect of ions often increases with the increase in their concentration and some heavy metal ions have the effect of a non-competitive inhibitor of the enzyme (Nelson and Freeman, 19). Using

EDTA (a chelating agent) at an 1mM concentration drived the enzyme to lose its activity, and this confirms that the enzyme belongs to the group of metallo enzymes), which need a metal as an enzyme co-factor. The chelating agents form complexes with ions of metal and remove them from the active site, which leads to an inhibition in the activity of the enzyme. The same table shows that Urea and Cysteine presence had no effect on the BGS activity, as the enzyme retained its entire activity. Schroder et al., (28) indicated that beta-glucosidase enzyme extracted from hydrothermal spring metagenome was not affected by the presence of urea.

Table 2. Effects of chemical reagents on the activity of partially purified beta glucosidase from apricot defatted seeds powder

Reagent	Remaining Activity %	
	(1Mm)	(5Mm)
None	100	100
$CaCl_2$	90.4	86
KCl	94	85.4
NaCl	111	100
EDTA	78	72.5
Mercaptoethanol	109	102
L-Cysteine	100	100
$CuSo_4$	14	8
Urea	100	100

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