DETERMINATION OF THE OPTIMUM CONDITIONS FOR UREASE EXTRACTED FROM SOME LOCAL PLANTS

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ABSTRACT

In the current study, Seventeen types of plants commonly used namely (Chickpeas, Tomato, Soybean, Mustard, Baker, Lebbeck, Bean, Sesame, Male Iraqi berries, Female Iraqi berries, Indian berries, Potato, Radish, Legumes, peas, Watermelon, and Phaseolus were obtained and screened for urease activity, among these plants, Sesame was chosen with maximum enzymatic activity, and it had the highest productivity of urease enzyme (1.623 U/mg protein). The optimum extraction ratio represented by 1:10 (W: V) after 90 minutes and 0.8414U/mg protein. Sodium phosphate buffer (0.1 M, pH 7.0) was chosen as the best extraction buffer with specific activity 0.9004U/mg protein.

Keyword: Sesame, plants, urease enzyme, optimization

تحديد الظروف المثلى لأنزيم اليوريز المستخلص من بعض النباتات المحلية

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المستخلص

في هذه الدراسة تم اختبار سبعة عشر نوع نباتات مختلفة لغرض اختيار النبات الامثل كمصدر لانزيم اليوريز شملت (حمص، طماطم، فول الصويا، خردل، خباز، لبخ، فاصوليا، سمسم، توت عراقي ذكر، توت عراقي أنثى، توت هندي، بطاطا، فجل، باقلاء، بازلاء، بذور الرقي والفاصوليا). بينت النتائج ان نبات السمسم هو الأمثل من بين النباتات المنتخبه حيث امتلك اعلى فعاليه انزيميه 1.623 وحدة/ ملغم بروتين، كما أظهرت النتائج ان نسبة 10:1 (وزن: حجم) هي أفضل نسبة لاستخلاص الانزيم من النبات المنتخب بعد 90 دقيقة اذ وصلت الفعالية الانزيميه الى 0.8414وحدة /ملغم بروتين كذلك استخدم بفر فوسفات الصوديوم بتركيز 0.1 مولاري وبرقم هيدروجين 7 كأفضل دارئ للاستخلاص بفعالية 0.9004وحدة /ملغم بروتين.

كلمات مفتاحية: سمسم، نباتات، انزيم اليوريز، ظروف مثلى

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INTRODUCTION

Ureases enzymes (EC 3.5.1.5) are a nickel depending metallo enzymes that responsible for urea hydrolysis into ammonia and CO_2 (2), these enzymes are found in plants, algae, yeasts, bacterial and filamentous fungi. Fungal and plant ureases are composed of identical repetition of protein. While bacterial ureases are consist of different repetitions of 2-3 subunits of protein (24). Ureases extracted from jack beans were the first enzymes to be crystallized in laboratory and they were stilled the best characterized ureases from plants (7). Urease can be isolated from *Cajanus cajan* (29) and it could be isolated from seeds of water melon (26).Ureases have been used in many fields such as a biosensors for determining urea in human blood; diagnosis kit for urea measuring and used as a urea reducing agent in alcoholic beverages (20,23). Microbial and plant ureases appeared other biological functions as in blood platelets activation in addition to insecticidal and antifungal activity ; this confirm that urease enzyme participate in a mechanism of plant cells defense (3). Biocarbonation is considered an eco-efficient biological method for the remediation of heavy metals (1). Accordingly, as a solution, plant-derived urease enzyme (PDUE) is utilized, which accelerates the biocarbonation processes, including urea hydrolysis and heavy metal precipitation (37).The objective of this study was characterization the urease enzyme extracted from some local plants under optimum conditions.

MATERIAL AND METHODS

Plants: The plants used throughout this study were locally available in the market. Namely, Chickpeas (*Cicer arietinum*), Tomato (*Solanum lycopersicum*), Soybean (*Glycine max*), Mustard (*Sinapis*), Baker (*Urginea maritime*), Lebbeck (*Albizia lebbeck*), Bean (*Phaseolus vulgaris*), Sesame (*Sesameum indicum*), Male Iraqi berries , Female Iraqi berries, Indian berries, Potato (*Solanum tuberosum*), Radish (*Raphanus satvus*), Legumes (*Fabaceae*), Peas (*Pisum sativum*), Watermelon (*Citrullus lanatus*), and Phaseolus (*Phaseolus vulgaris*) were used as a source of material to screen for urease enzyme activity.

Extraction and recovery of urease enzyme

One gram of each plant was homogenizing separately with 10 ml of 0.02 M of phosphate buffer pH 7.0; the mixing was done using mortar for 15min at room temperature. The slurry was centrifuged at 10000 rpm for 15 min and filtered through a Whatman No.1 filter paper for removing any cell debris that remains in the preparation (12). The clear supernatant obtained represented the crude extract and was assayed for the urease enzyme.

Estimation of the standard curve of NH_4Cl

In order to prepare NH_4Cl standard curve for the urease assay, serial concentrations (100-500 µM) from a stock solution of NH_4Cl (0.5 mM) were prepared in triplicate. The standard curve of NH_4Cl was plotted between the ammonium chloride concentration (µM) and the corresponding absorbance of standard ammonium chloride at 625 nm, as shown in (Fig. 1).

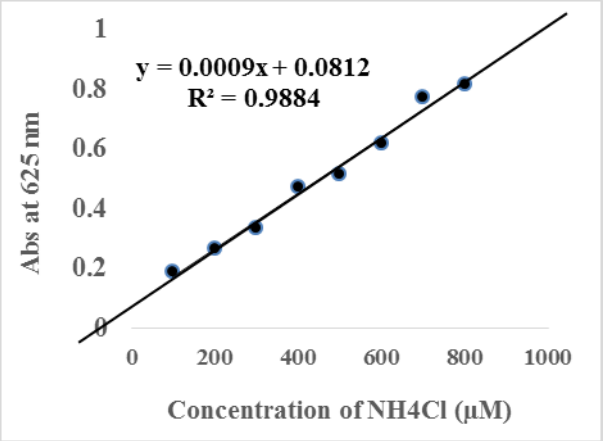

Figure1. Ammonium chloride standard curve

Determination urease Assay

The urease activity was measured according to modified Berthelot reaction (15), which depends on the standard curve of NH4Cl throughout liberated ammonia by the enzyme. All glassware sterilized should be washed with warm dilute hydrochloric acid and rinsed thoroughly with distilled and de-ionized water. The test reaction combination contained 1 ml of plant seed extract, 1 ml of 500 mM urea prepared in (100 mM, pH 6.8) of phosphate buffer and finally 0.8 ml of same buffer, the mixture was incubated for an hour at 37°C in a water bath. The process was stopped by heating at 80°C for 5 minutes. Black sample was prepared as a test sample except heated the plant seed extract before adding to reaction

mixture. Determine the ammonia concentration was assess by adding 1 ml of the reaction mixture with 10 ml of Berthelot reagents [5 ml from 0.01 M of reagent A (5gm of phenol and 0.02 gm of sodium nitroprusside) with 5 ml from 0.01 M of reagent B (2.5gm of sodium hydroxide with 8.4 ml of sodium hypochlorite) in 500 ml distilled water for each, for 1 hr. in a water bath at 37°C. Urease activity was detected by measuring the absorbance increase at 625 nm. Enzymatic activity unit known as the amount of enzyme liberated one µmole from ammonia in one minute under optimum conditions as follows:

$$Urease\ Activity = \frac{\frac{Ab}{Slope}}{T \times C}$$

Where:

$\frac{Ab}{slop}$: is the Concentration of ammonia,

T: is the Time of reaction, 60 min.

C: is the Constant, (14)

Protein concentration, was measured according to the method described by Bradford (4).

Determination of o4ptimum condition for urease extraction

Type of plant material: Chickpea, Tomato, soybean, Mustard, Baker, Lebbeck, bean, Sesame, Male Iraqi berries, Female Iraqi berries, Indian berries, Potato, Radish, Iegumes, Peas, Watermelon, and Phaseolus were extracted by using 0.02 M of phosphate buffer pH 7.0. One gm of each plant was mixing separately with 10 ml (w : v) of buffer solution using mortar for 15 min at room temperature. Centrifugation at 10000 rpm for 15 min and filtered through filter paper. The filtrate was taken to determine the enzyme activity, protein concentration, and specific activity (13).

Type of extraction buffer

Sesame was homogenized with different types of buffers for 15 min at 30^{o}C for urease extraction. These buffers are 0.02 M sodium acetate buffer (pH 4, 5 and 6), 0.02 M sodium phosphate buffer (pH 6.5, 7 and 7.5) and 0.02 M Tris-based buffer (pH 8, 8.5 and 9). The enzyme activity, protein concentration, and specific activity were assayed in each experiment (13).

Concentration of extraction buffer

In order to determine the optimum concentration of extraction buffer, the concentration course for extraction was (0.02, 0.05, 0.1, 0.2, 0.35, 0.5, 0.75, and 1 M) by mortar. Centrifugation at 10000 rpm for 15 min and filtered through a filter paper. The supernatant was analyzed to determine the enzyme activity, protein concentration, and specific activity (13).

Extraction ratio: Different ratios of buffers were selected to determine the best Sesame ratio to extract the enzyme included 1:5, 1:10, 1:15, 1:20, 1:25, and 1:30 (w:v) by mixing 1gm of Sesame with each extraction ratio for 15 min separately. Then centrifugation at 10000 rpm for 15 min and filtered through a filter paper. The enzyme activity, protein concentration, and specific activity were obtained (13).

Extraction time: In order to determine the optimum extraction time for the urease enzyme, the time course for extraction was (5, 10, 30, 60, 90, and 120) min by mortar, then centrifugation at 10000 rpm for 15 min and filtered through a filter paper. The filtrate was taken for the determination of the enzyme activity, protein concentration, and specific activity (13).

Characterization of Urease: Effect of temperature on urease activity: Urease activity was determined in different range of temperature include (20, 30, 37, 50, 60, 70, 80and 90) ^{O}C for 60 min., urease activity was estimated and the relationship between temperature and enzyme activity was plotted to determine the optimal temperature of urease activity.

Effect of temperature on urease stability

Urease was incubated at different temperatures ranged between 20-90˚C for 30 min. followed by incubation in ice bath, remaining activity% of enzyme was estimated.

Effect pH on Urease stability

Equal volume from urease enzyme was mixed with the buffers at different pH (5, 6, 7, 8, 9, and 10) as prepared at a ratio of (1:1), and the mixture was incubated in a water bath for 30 min at 80˚C. The samples were transferred directly to the ice bath, and then the remaining activity % was estimated.

Effect of the substrate concentrations on urease activity

Table 1. Effect of urea (substrate) of enzyme activity

Tube	1	2	3	4	5	6
Bromothymol blue (ml)	1	1	1	1	1	1
2% urea solution (ml)	2	1.5	1	0.8	0.5	0.2
Distilled water (ml)	0	0.5	1	1.2	1.5	1.8

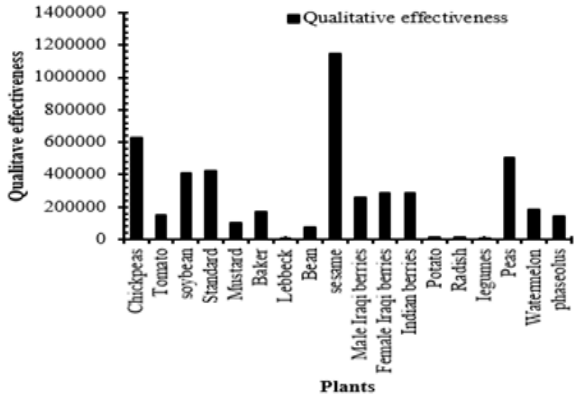

Figure 2. Effect the types of plant material on urease extraction using phosphate buffer pH 7, M 0.02 at 30˚C and for 15 min

Label six test tubes from 1 to 6, then use graduated pipettes to add the following volumes from bromothymol blue solution, 2% urea solution and distilled water in tubes 1 to 6 as follow: Mix the contents of each of the six test tubes well. Place them in a water bath at 35°C for 5 minutes. Thereafter add 0.5 ml of the enzyme extract into each of the tubes. And start the stopwatch immediately. Shake thoroughly to mix the contents of the tubes and quickly place them back in the water bath (Table.1).

RESULTS AND DISCUSSION

Optimum conditions for urease extraction

Different bioprocess conditions that affect urease extraction from some plants were optimized for maximum enzyme production. A large number of factors affect the extraction of urease, such as types of plant material, types of buffer, extraction ratio and extraction time, etc. Hence, optimization of these conditions helps to reduce extraction cost and to obtain a high yield of urease enzyme.

Type of plant material

The influence of plant type on the enzyme extraction was determined by Seventeen types of plants commonly used chickpea, Tomato, soybean, Mustard, Baker, Lebbeck, bean, Sesame, Male Iraqi berries, Female Iraqi berries, Indian berries, Potato, Radish, Iegumes, Peas, Watermelon and phaseolus by using 0.02 M of phosphate buffer pH 7.0, it was observed from the results that urease extraction was found to be maximum in Sesame followed with chickpea and peas the specific activity was reached to1.623, 0.8928 and 0.7321 U/mg respectively (Fig.2). El-Hefnawy (6) founded that the specific activity of urease extracted from *Pisum Sativum* L. seeds was reached to 0.19 U/mg protein.

Type of extraction buffer

The specific activity of urease was estimated after extraction using different buffers. The results were illustrated in (Fig. 3). These results show that sodium phosphate buffer (0.02 M, pH 7.0) was the best extraction buffer with specific activity 1.0636 U/mg protein. While other buffer with different pH were given low specific activity 0.4472, 0.5532, 0.5895, 0.8797, 0.7049, 0.5017, 0.45 and 0.3682 U/mg. pH effected of enzymatic extraction by the fact that protein structure of an enzyme molecule is influenced by the acidity and alkalinity of the solution because of the differences in ionization state of various amino acid residues through changing the charge state of the solute. If the pH of the solution is such that a particular molecule carries no net electric charge, the solute often has minimal solubility and precipitates out of the solution (25).The pH of the enzyme environment affects the activity of the enzyme in several ways. Firstly each enzyme has its own optimum pH, at which the maximum enzyme activity, but the enzyme is stable within certain limits under and above the optimum. Secondly, enzyme stability is influenced by environmental pH, at extremes acidity or alkalinity, the enzyme may be denatured. Thirdly, the reaction mixture pH may affect the association of substrate with the enzyme (10). A buffer solution can protect the integrity of the proteins while separating them from other integrated cell components, the pH balance of the buffer must correspond with that of the cell in vivo (8). There are many studies that used different buffers with different pH values for urease extraction from

different sources, (27) pointed to the extraction of urease from *Proteus mirabilis* by phosphate buffer (20 Mm, pH 7.5).

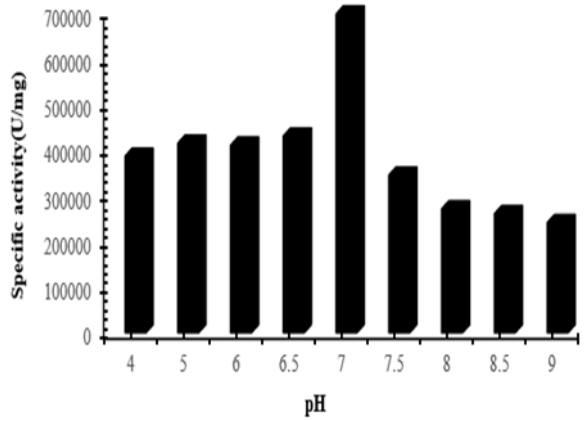

Figure 3. Effect the types of buffers on urease extraction from Sesame at 30˚C for 15 min

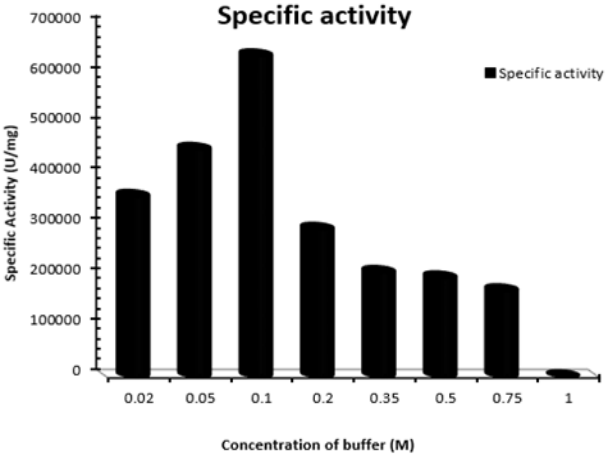

Figure 4. Effect the Concentration of buffers on urease extraction from Sesame at 30˚C for 15 min

Concentration of sodium phosphate buffer

Eight concentration of sodium phosphate were chosen to determine the best concentration of this buffer using to extract the urease from sesame include (0.02, 0.05, 0.1, 0.2, 0.35, 0.5, 0.75 and 1) M in pH 7 (Fig.4). The highest specific activity was measured for crude extract in 0.1 M; it was reached to 0.9004 U/mg protein, compared to the lower specific activity in 1M reached to 0.1342 U/mg protein. Also the specific activity was low at 0.02, 0.05, 0.2, 0.35, 0.5, and 0.75M, it reached to 0.5061, 0.6381, 0.4138, 0.2927, 0.2537 and 0.2041 U/mg protein respectively. It has been found that the use of high concentrations of the buffer used in the extraction can adversely affect the activity of the enzyme urease, and the reason may be due to the presence of an abundance of ionic groups that complicate the work of the enzyme activation (22). The other study by (16) found the maximum specific activity of urease extraction from *Canavalia ensiformis* at pH=7.5, 0.2M sodium phosphate buffer.

Extraction ratio

Six extraction ratio were chosen 1:5, 1:10, 1:15, 1:20, 1:25 and 1:30 (w:v) to determine the best extraction ratio of urease by using sodium phosphate(0.1 M, pH 7.0). The highest specific activity was measured for crude extract at 1:10 ratio, it was reached to 0.8414 U/mg protein, while other ratio were gave the following specific activities 0.3541, 0.3013, 0.1886, 0.297 and 0.4362 U/mg protein respectively (Fig.5). Variation in native extract ratio can result in a variable amount of herbal material used in an extract. In some instances, variation in the equivalent dry weight of a herb used in herbal preparation. Where a large proportion of extractable material is obtained from a herbal material, the native extract ratio will be low. For example, a low native extract ratio of 1:20indicates that 50 percent of the extractable matter obtained from the herb is represented in the final extract. However, when only a small amount of extractable material is obtained using a particular extraction profile, the native extract ratio will be high e.g. a native extract ratio of 1:20 indicates that only 5 percent of extractable components are obtained (5). The reason for the difference in the extraction ratios is due to the source and quantity of the enzyme, and that an increase in the extraction solution may lead to a decrease in the specific activity due to a decrease in the enzymatic activity resulting from a decrease in the speed of complex formation (17).There are many studies that use different extraction ratio buffer solution, (13) found the best ratio of urease extraction by using sodium phosphate buffer 0.2M, the highest specific activity was at 1:8 ratio, it reached to 1988 U/mg protein.

Extraction Time

Six extraction periods were chosen (5, 10, 30, 60, 90 and 120) min to determine the best extraction time of urease by using sodium phosphate (0.1 M, pH 7.0). The highest specific activity was measured for crude extract after 90 min, it was reached to 1.1097

U/mg protein, compared to the lower of specific activity after 5, 10, 30, 60 and 120 min0.5232, 0.5536, 0.9615, 1.0549 and 0.8241U/mg protein respectively (Fig.6). It was found necessary to determine the optimal time period due to the difference of the extractor of the urease enzyme from one source to another due to the difference of the materials present in that source and interfering with the enzyme and that the process of removing the impurities leads to obtaining a protein extract with high stability towards decomposition (28). The results were higher compared to founding of (33) when they blended the yellow lupine for 120 min.

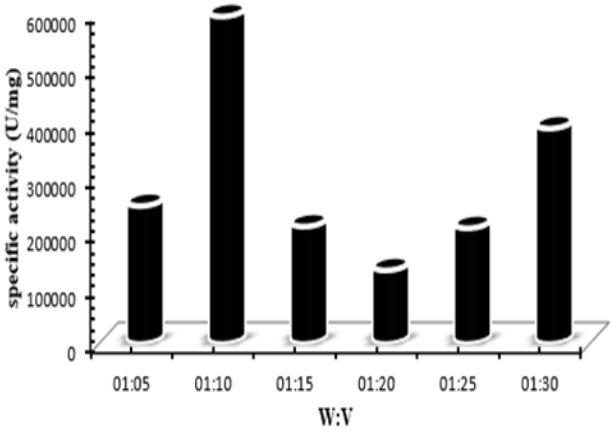

Figure 5. Effect of extraction ratio on urease extraction from Sesame at 30˚C for 15 min

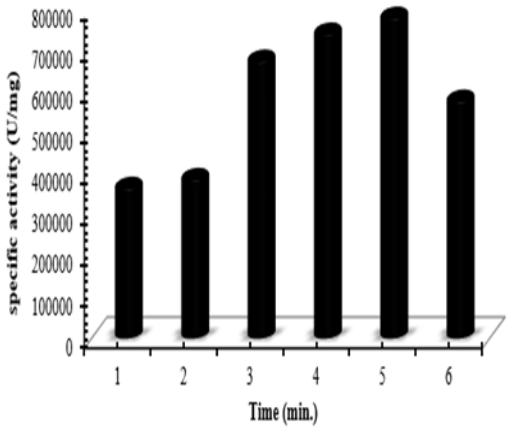

Figure 6. Effect of extraction time on urease extraction from Sesame at 30˚C for 15 min

Characterization of Urease

Effect of temperature on Urease activity

Urease activity was estimated at different range of temperature include ranging from 20,30,37,50,60,70,80 and 90°C for 60 min. The results in fig. (7) indicated an increase in the activity at 37°C, the urease activity reached to0.7845 U/ml, then the activity was declined with increasing temperature up to 37 °C with a minimum activity (0.6261 U/ml) observed at 90 °C. However, urease activity was decreased too below 37 °C. The temperature has an influence on the enzymatic reaction in different ways, such as pH, enzyme-substrate affinity, and ionization of prosthetic group of the system (34). The results showed an increase in reactions speed until it reached 37˚C then began to decline over 40˚C, this may due to the increase of the clash between the enzymatic molecules sharing in the reaction with the substrate as a result of increasing the movement energy of the molecules, whereas the decline in the enzymatic activity by temperatures over 40^0C is a result of the denaturation of protein structure and changes in the active sites which leads to loss of the enzyme activity (18). The kinetic energy of molecules increases with an increase in temperature, which results in speeding up the rate of reaction. When the temperature was further increased, the molecules of the enzyme exceed the barrier of energy. This causes the breakage of hydrogen and hydrophobic bonds that are responsible for maintaining the 3D structure of the enzyme (21, 27). This study agreed with (6). The complete assays of enzyme were incubated at different temperatures from 10 to 80°C for 10 minute. Results showed that urease had an optimum temperature at 40°C, while Iyer *et al.*, (14) was found the highest activity of urease enzyme from *pisum sativum* seeds at 40 ˚C.

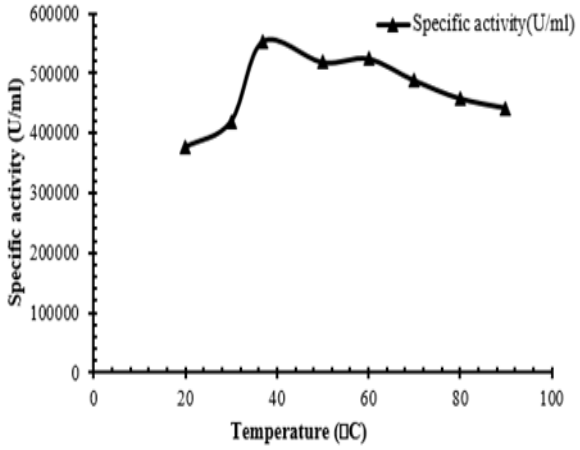

Figure 7. Effect different temperature value (20-90) ˚C on urease activity

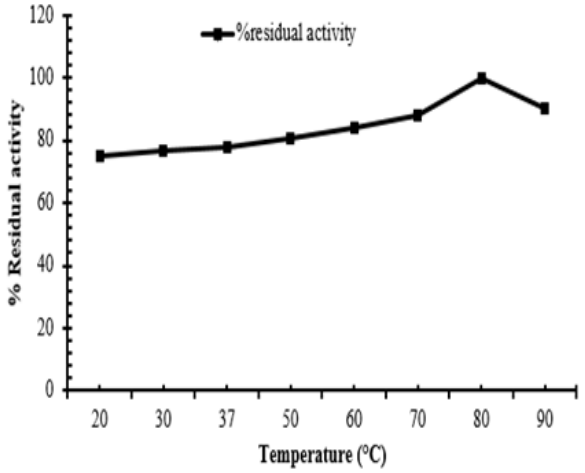

Figure 8. Effect different temperature value (20-90) ˚C on urease stability

Urease stability at different temperatures

The urease stability at different temperatures was evaluated by incubating the enzyme at different temperatures varying from 20°C to 90°C and assaying esidual activity after 60 min at 37°C. From the results presented in fig.(8), it was found that the enzyme was maintained its activity at temperatures ranging between 50-70°C, then the activity began to increase with increasing temperature, although at 80°C, about 100% of the activity remained. Lower temperatures showed a sharp decrease in instability, the enzyme retained 76.76% of the initial activity at 30°C, whereas, at 20°C remaining urease activity reached to 74.94%.Generally, for any enzymatic reaction, temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds (35). The decreased activity of collagenase at temperatures above 45°C is due to its susceptibility to high temperatures (11). While Karmali (16) was found the highest stability of urease enzyme from *pisum sativum* seeds at 60 ˚C.

Effect pH on Urease stability

From the results in fig. (9), it was noticed that pH ranged between 6-7 were the optimum for urease stability; the enzyme was retained 100 % of its activity in pH 6.0 while retained 56.66 % of its activity in pH 5.0 and about 67.77% and 41.85% at pH 7.0 and 8.0 respectively. The activity was decreased away either side of the optimum pH values; the residual activities 35.185% and 30.185% at pH 9, and 10, respectively. The enzyme activity was very low at acidic and alkaline pH. The results may donate a conclusion that Urease enzyme is more stable in nearly neutral and neutral pH. In general, this lowering activity at pH values away from the optimum condition may be due to the effect of pH stability in enzyme structure, which lead to denaturing the enzyme molecule or to changes in the ionic state of the enzyme active site, as well as its effect on the secondary and tertiary structure of the enzyme which leads to losing the activity in buffers solution that far away from optimal pH (32). Additionally, most enzymes may undergo irreversible denaturation in high acidic or basic solutions (36). The pH value of the environment affects the effectiveness of the active side of the enzyme in forming the enzyme-substrate complex. Changes in pH lead to changes in ionization levels in enzymes or substrates that affect activity. This causes the interaction between enzyme and substrate to be maximal in urea degradation process and formed the product. The enzyme has an active site with certain groups acting as a catalyst in the formation of the enzyme substrate complex (30). Changes in pH lead to changes in ionization levels in enzymes or substrates that affect activity and the changing in optimal pH because of the electrostatic interactions effected by the carrier microenvironment (34, 38). Found the optimum pH value of urease stability isolated from Jack bean (Canavalia) was at pH 7.4 (13).

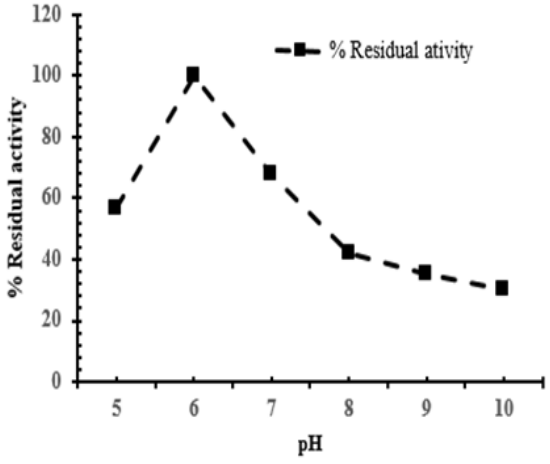

Figure 9. Effect of different pH values in urease stability

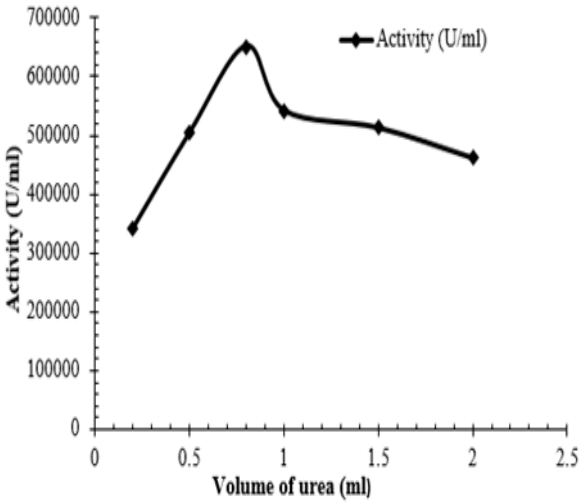

Figure 10. Effect different substrate concentration on urease activity

Effect substrate concentration

Several volumes of bromothymol blue, 2% urea solution, and distilled water in six tube and adding 1 ml of enzyme to all tube equally, were studied as possible substrate for urease enzyme activity. The highest activity was measured for crude extraction at urea solution equal to 0.8 ml, bromothymol blue (1) ml, and distilled water (1.2) ml and reached to 0.9226 U/ml, compared with the other ratios followed by urea solution(2) ml it was reached to 0.6535 U/ml, urea solution (1.5) ml, it was reached to 0.7261 U/ml, urea solution (1) ml, it was reached to 0.7678 U/ml, urea solution (0.5) ml, it was reached to 0.7142 U/ml and the lower activity of enzyme at urea solution (0.2) ml, it was reached to 0.4845 U/ml. The optimum value of substrate concentration, where the urease activity has the largest value, The decrease in the activity could be explained by substrate inhibition at higher urea concentrations. The rate of hydrolysis of urea increases with increasing urea concentration until reaching a maximum, beyond that hydrolysis activity starts to decrease (19, 31).

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