CHARACTERIZATION OF UREASE EXTRACTED UNDER THE OPTIMUM CONDITIONS FROM SOME LOCAL PLANT SEEDS

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ABSTRACT

This study was aimed to determine the optimum conditions of urease extracted from different plant sources and evaluate its biochemical properties. Eighteen types of plants commonly used namely Tomato, Chickpea, Sesame, Mustard, Lebbeck, Watermelon, Soybean, Cotton, Male Iraqi berries, Female Iraqi berries, Indian berries, Pumpkin, Muskmelon, Legumes, Cowpea, Radish, Pisum and Phaseolus were taken and tested for the presence and activity of urease. Cotton (*Gossypium barbadense*) was selected as the plant source with highest specific enzymatic activity among them (2.51 U/mg protein) when extracted the enzyme after ground seeds using 0.15 M of tris base buffer pH 8.5 at a ratio 1:10 (w:v) for 15 min. Also the results showed that urease was stable in pH 8, and has the highest activity at 37 ^{o}C at 60 min of reaction time. .

Keyword: Cotton, plants, enzyme, extraction

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تحديد الظروف المثلى لاستخلاص انزيم اليوريز من بذور بعض النباتات المحلية

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المستخلص

هدفت الدراسة الحالية الى تحديد الظروف المثلى لاستخلاص انزيم اليوريز من مصادر نباتية مختلفة وتقييم خواصه الكيموحيوية، حيث تم اختبار ثمانية عشر نوعاً من النباتات شائعة الاستخدام لغرض اختيار النبات الامثل كمصدر لانزيم اليوريز وشملت (الطماطم، الحمص، السمسم، الخردل، اللبخ، البطيخ، فول الصويا، القطن، توت عراقي ذكر، توت عراقي انثى، توت هندي، القرع، الرقي ، باقلاء، لوبيا، الفجل ، بازلا، والفاصوليا). بينت النتائج ان نبات القطن هو الامثل من بين النباتات المنتخبة حيث أمتلك أعلى فعالية انزيمية 2.51 وحدة/ملغم بروتين عند استخلاص الانزيم من البذور المطحونة باستخدام 0.15 مولاري من محلول دارئ الترس برقم هيدروجيني 8.5 عند نسبة 10:1 (وزن: حجم) لمدة 15 دقيقة. كما أوضحت النتائج أن اليوريز كان مستقراً برقم هيدروجيني 8 وله أعلى نشاط عند 37 درجة مئوية خلال 60 دقيقه من فترة الحضانه.

كلمات مفتاحية: القطن، نباتات، انزيم، استخلاص

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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 (3), these enzymes are found in plants, algae, yeasts and filamentous fungi. Fungal and plant ureases are homo-oligomeric proteins (composed of identical repetition of protein). While bacterial ureases are consist of different repetitions of 2-3 subunits of protein (19). The high amino acid sequence similarity among all ureases indicates that all ureases are variants of the same enzyme and are likely to possess similar tertiary structures and catalytic mechanisms (21). This conclusion is supported by the available biochemical and structural data obtained for the best-characterized ureases, e.g., from jack bean and *Klebsiella aerogenes* (15).The presence of urease in plants was first reported from the leaves of a legume, soybean (*Glycine max*), later it was isolated and purified from several other plant sources such as jack bean (*Canavalia ensiformis*), pigeonpea (*Cajanus cajan*), watermelon (*Citrullus vulgaris*), and mulberry (*Morus alba*) (12). This enzyme take place in catalyst reactions of hydroxyurea, dihydroxyurea and semicarbazides as well as it uses urea as substrate and converts it to ammonia and carbon dioxide (16). In addition, the enzyme decomposes urea formed from arginase that is found in seed germination (34). Urease is also important in human bodies due to the fact that many urinary tract and gastroduodenal diseases. Including cancer are related in some ways to this enzyme (28). The increased need in finding proper ways to remove urea from different environments brought great attractions in the biotechnology field (25). The purpose of this study is to determination the optimum condition for extraction the urease from some local plants and assessment some of its biochemical properties.

MATERIALS AND METHODS

Plants: The plants used in this research were readily accessible on the market in the area. Namely, Tomato (*Salanum lvcopersicum*), Soybean (*Glycine max*), Chickpeas (*Cicer arietinum*), Watermelon (*Citrullus lanatus*), Been (*Phaseolus vulgaris*), Cotton (*Gossypium barbadense*), Muskmelon (*Cucumis melo*), Male and Female Iraqi berries (*Berberis vulgaris*), Indian berries (*Cordia dichotoma*), Pumpkin (*Cucurbita pepo*), Cowpea (*Vigna*), Mustard (*Sinapis*), Lebbeck (*Albizia lebback*), Sesame (*Sesameum indicum*), Radish (*Raphanus satvus*), Legumes (*Fabaceae*), and Peas (*Pisum sativum*) and used as a source of plants materials for measuring the activity of the urease enzyme.

The standard curve estimation of NH_4Cl

To create the NH_4Cl standard curve for the urease test, successive concentrations (100-500µM) were made in triplicate from a stock solution of NH_4Cl (0.5 mM). For one hour in a water bath at 37 °C, one milliliter of each NH_4Cl concentration was added separately to 10 ml of berthelote reagents [5 ml from 0.01 M of reagent A (5 gm of phenol and 0.02 gm of sodium nitroprusside) with 5 ml from 0.01 M of reagent B (2.5 gm of sodium hydroxide with 8.4 ml of sodium hypochlorite) in 500 ml as shows in fig. (1), the NH_4Cl standard curve was drawn between the ammonium chloride chloride concentration (µM) and the matching absorbance of standard ammonium chloride at 625nm (31).

Urease assay determination

Activity of urease enzyme has been determined using a modified Berthelot reaction (2) that relies on NH_4Cl standard curve across the ammonia released by enzyme. The sterilized glassware must wash in dilute hydrochloric acid and thoroughly cleaned by de-ionized and distilled water. The test reaction mixture was containing 0.8 ml of 100 mM phosphate buffer pH 6.8 with 1 ml of 500 mM urea prepared in same buffer and 1 ml of plant seeds extract, the mixture was incubated for one hour in a water bath at 37 ^{o}C. The reaction was halted by heating at 80 ^{o}C for 5 minutes to stop reaction. Urease activity was measured by measuring the absorbance rise at 625 nm after adding 10 ml of Berthelot's reagent for one hour in water bath at 37 °C to determine ammonia concentration. The quantity of enzyme released one mole from ammonia (1 min) under ideal conditions is known "an enzymatic activity unit" as follows, also Bradford's method was used to determine the concentration of protein (5).

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

Where:

$=\frac{Ab}{slope}$: is the concentration of ammonia,

T: is the time of reaction, 60 min

C: is the constant, (18)

Urease extraction under optimum condition

Plant sources: The seeds of eighteen plants were crushed and extracted by using 0.02 M of phosphate buffer pH 7.0. One gram of each plant seeds was mixing separately with 10 ml (w:v) of buffer solution using mortar for 15min at room temperature. Centrifugation at 10000 rpm for 15 min and filtered through filter paper. The filter was taken to determine the enzyme activity, protein concentration, and specific activity (1, 13, 14).

Extraction buffer: For urease extraction, cotton seeds was homogenized with various buffers for 15 minutes at 30°C. 0.02 M sodium acetate buffer (pH 4, 4.5, 5, 5.5, and 6), 0.02 M sodium phosphate buffer (pH 6.5, 7, and 7.5), and 0.02 M tris-based buffer are the buffers used (pH 8, 8.5 and 9). Each experiment measured enzyme activity, protein concentration, and specific activity (1, 14).

Concentration of extraction buffer

The concentration course for extraction was (0.01, 0.02, 0.05, 0.1, 0.15, and 0.2) M of tris base buffer by mortar, in order to identify the optimal concentration of extraction buffer. Filtered using filter paper after centrifugation at 10000 rpm for 15 minutes. The protein concentration, enzyme activity, and specific activity of the supernatant were all determined (1, 14).

Extraction ratio: Different ratios of 0.15 M tris base buffer have been used to determine the best urease extraction ratio from cotton seeds include 1:5, 1:10, 1:15, 1:20, 1:25 and 1:30 (w:v). The optimum urease extraction ratio was determined by mixing 1 gm of cotton seeds with each extraction ratio for 15 minutes individually. After that, it was centrifuged for 15 minutes at 10000 rpm then filtered by filter paper. The specific activity, enzyme activity, and protein concentration were all measured (1, 14).

Extraction time: The time course for extraction of the urease enzyme was (5, 15, 30, 60, 90, and 120) minutes by mortar, followed by centrifugation at 10000 rpm for 15 minutes and filtering through filter paper to identify the best extraction time. The enzyme activity, protein content, and specific activity were all measured in the filtrate (1, 14).

Characterization of Urease

Urease stability affected by pH

At a ratio of (1:1), equal amount of urease enzyme was combined with buffers at varying pH (4, 5, 6, 7, 8, 9, and 10) and incubated inside a water bath at 37 C for about 15 minutes. The samples were immediately placed in an ice bath, and the residual activity percentage has been calculated (32).

Urease stability affected by temperature

Urease was maintained for 15 minutes at temperatures ranging from 25 to 60 ^{o}C, then cooled in an ice bath to determine the enzyme's residual activity percent (33).

Urease activity affected by temperature

Urease activity was determined in different range of temperature include (25, 30, 37, 40, 50, 55 and 60) ˚C for 60 min., urease activity was estimated and the optimal temperature for urease activity was determined by plotting the relationship between enzyme activity and temperature degrees.

Effect the reaction time on enzyme activity

For determination of optimum reaction period, enzyme assay was carried out in a water bath at different reaction time include (15, 30, 60, 90 and 120) minutes at optimum temperature and pH.

RESULTS AND DISCUSSION

Urease extraction under optimum conditions: For maximum enzyme extraction, several bioprocess parameters that impact urease extraction from various plants seeds were adjusted. The extraction of urease is affected by a variety of parameters, including plant sources, extraction duration, buffer type, and extraction ratio, among others. As a result, optimizing these conditions can assist minimize extraction costs and increase urease enzyme extraction.

Types of plants sources

Eighteen species of plants seeds were used to assess the effects of plant type on enzyme extraction using 0.02 M of phosphate buffer and pH 7.0, and the findings revealed that urease extraction was the maximum in cotton seeds followed with Pumpkin and cowpea

seeds, the specific activity was reached to 1.36, 1.14, 1.05 U/mg respectively fig. (2). The variation between the plant sources in urease content may be due to the genetic variation, the type and sources of plants and the environment conditions of cultivation, such as, temperature, pH (6). Bedan (4) extracted urease enzyme from *Vicia faba* seeds and the activity was reached to 33.3 U/ml, while others extracted urease from germinating *Pisum sativum* L. seeds and precipitated the enzyme using acetone and gave enzyme activity 190 U/g (9).

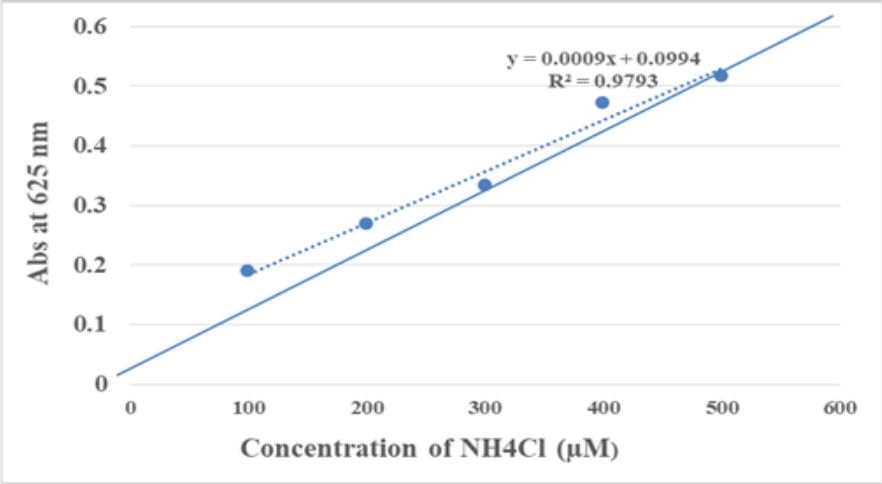

Figure 1. Ammonium chloride standard curve

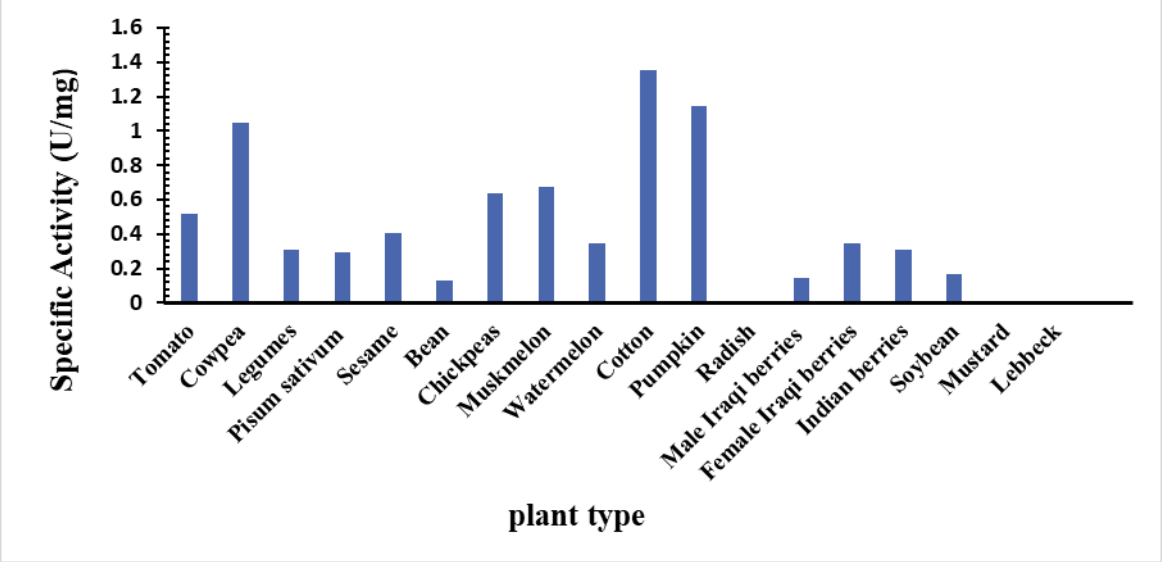

Figure 2. Effect plant sources on urease extraction using 0.02 M phosphate buffer pH 7.0 for 15 min at 30°C

Type of extraction buffer

After extracting urease using various buffers, the specific activity of urease was calculated, and the results were show in fig. (3). The best extraction buffer was Tris-based buffer (0.02 M, pH 8.5), which had a specific activity of 1.62 Unit/mg protein. An appropriate buffer solution added to a protein mixture during the extraction process can help improve the stability of protein molecules as these molecules are subjected to various forces designed to isolate them for study. A buffer solution can protect the integrity of the proteins while separating them from other integrated cell components, the pH of enzymatic extraction is affected by the fact that solution alkalinity and acidity alter the protein structure of an enzyme molecule owing to changes in ionization state of specific amino acid residues caused by changing the charge state of the solute (10). The solute precipitates out of the solution also has little solubility when pH value of solution is that same to a specific molecule holds no net electric charge

(20). Many research has employed various buffers and various pH values to extract urease from various sources; Hussein *et al*., (14) found the sodium acetate buffer (0.2 M, pH 5.0) was the best extraction buffer of urease from chick pea with specific activity 1460 U/mg protein .

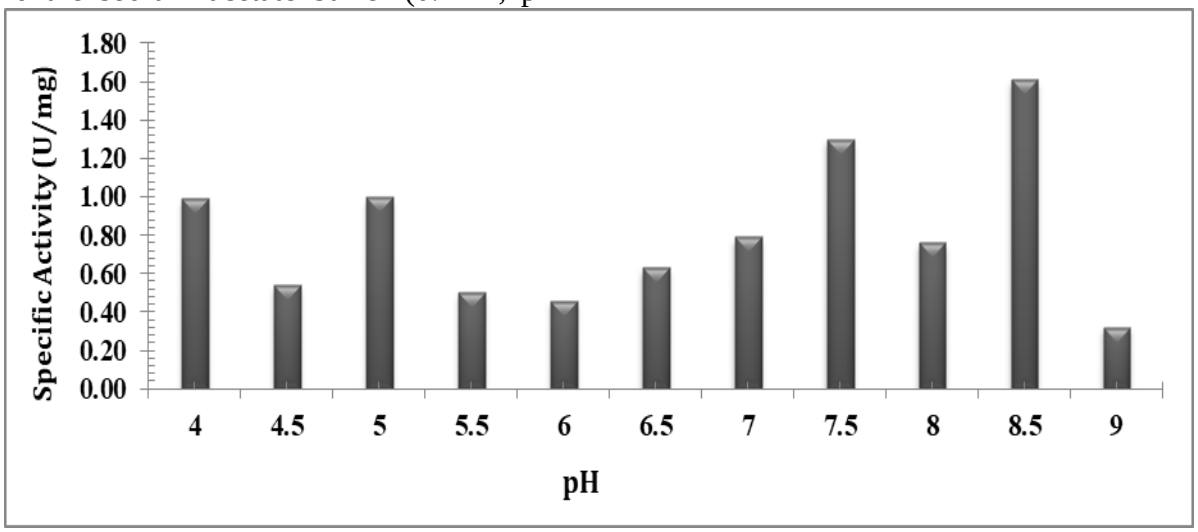

Figure 3. Urease extraction affected by the types of buffers for 15 min from Cotton seeds at 30^{o}C

Concentration of tris base buffer

Six concentration of Tris-based were chosen to determine the best concentration of this buffer using to extract the urease from cotton (*gossypium*) seeds include (0.01, 0.02, 0.05, 0.1, 0.15 and 0.2) M in pH 8.5. From the results in fig. (4), show the highest specific activity was measured for crude extract in 0.15 M, it was reached to 2.44 U/mg protein, compared to the lower specific activity in 0.01 M reached to 1.12 U/mg protein. Also the specific activity was low at 0.02, 0.05, 0.1 and 0.2 M is reached to 1.33, 1.30, 2.33 and 2.15 U/mg protein respectively. It has been found that the use of high concentrations of buffer in the extraction can adversely affect the activity of the urease, and the reason may be due to the presence of an abundance of ionic groups that complicate the work of the enzyme activation. The other study by (18) found the maximum specific activity of urease extraction from *Pisum sativum* seeds at pH 7.5, 0.2 M sodium phosphate buffer.

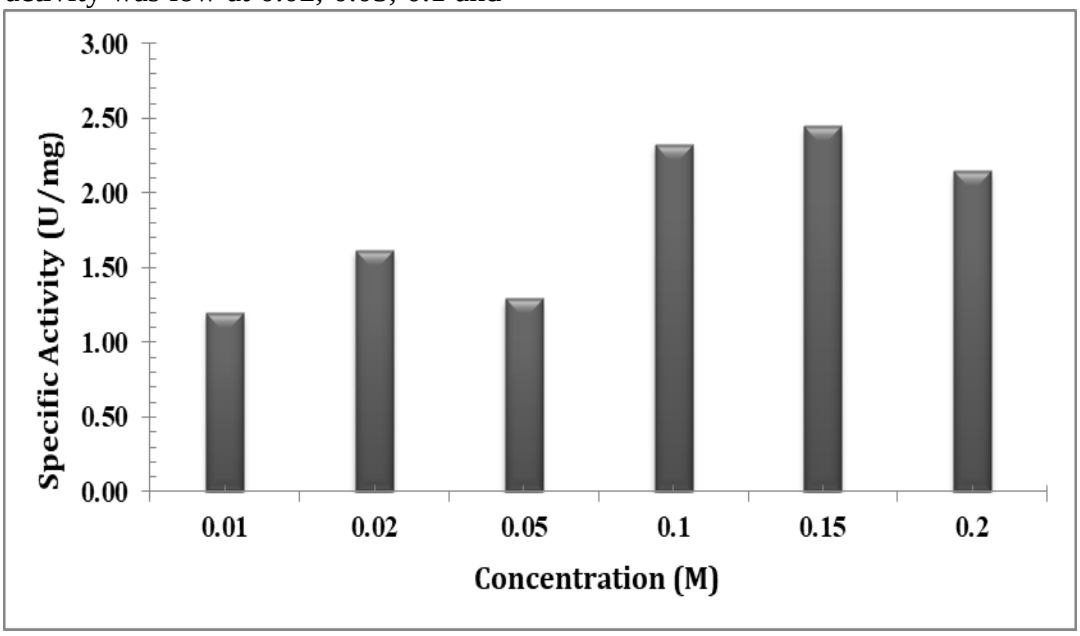

Figure 4. Urease extraction from cotton seeds affected by concentration of tris base buffer for 15 min from at 30

Extraction ratio

To identify the optimal urease extraction ratio using Tris based (0.02 M, pH 8.5), six extraction ratios were chosen: 1:5, 1:10, 1:15, 1:20, 1:25, and 1:30 (w: v). The results in fig. (5) show the maximum specific activity was recorded for crude extract at a 1:10 ratio, which was 2.4 U/mg protein, whereas other ratios yielded specific activities of 2.19, 0.64, 1.59, 0.77, and 0.88 U/mg protein. The amount of herbal material utilized in an extract might vary depending on the native extract ratio. Variation in the equivalent dry weight of a plant used in herbal preparation in various cases. The native extract ratio will be low when a substantial fraction of extractable material is recovered from a herbal source. A low native extract ratio of 1:20 means that the final extract contains 50% of the extractable materials collected from the plant. The native extract ratio will be high if just a little amount of extractable material is collected using a certain extraction profile, for example, a native extract ratio of 1:20 implies that only 5% of extractable components be obtained (8). 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 (24). here are many studies that use different extraction ratio of buffer solution, Hussein *et al.*, (14) found the best ratio of urease extraction by using 0.2M sodium phosphate buffer and the highest specific activity was at 1:8 ratio, it reached to 1988 U/mg protein.

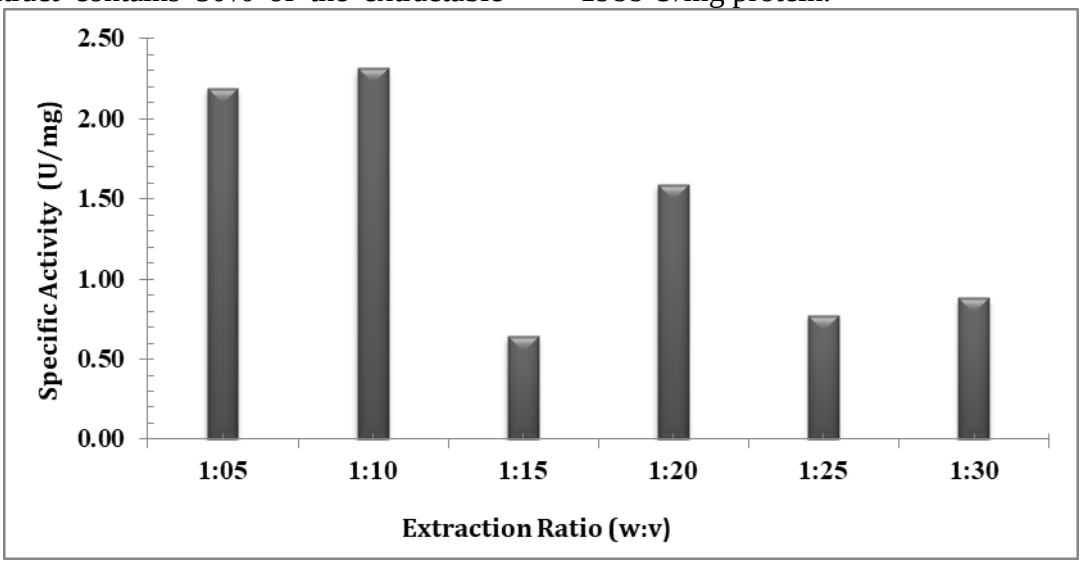

Figure 5. Urease extraction from cotton seeds affected by extraction ratio using 0.15 M of tris base buffer for 15 min at 30^{o}C

Extraction Time

Using Tris-based extraction (0.02 M, pH 8.5) and six extraction periods (5, 15, 30, 60, 90, and 120 minutes) were used to identify the optimal urease extraction time. After 15 minutes, crude extract had the maximum specific activity of 2.51 U/mg protein, compared to the lowest specific activity after 5, 30, 60, 90 and 120 min 1.70, 2.04, 1.28, 1.30 and 2.4 U/mg protein respectively (Fig. 6). It was found necessary to determine the optimal time period due to the difference of the extraction of urease 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 (23). The highest specific activity was measured for urease crude extract after 15 min, it was reached to 1988.5 U/mg protein (14).

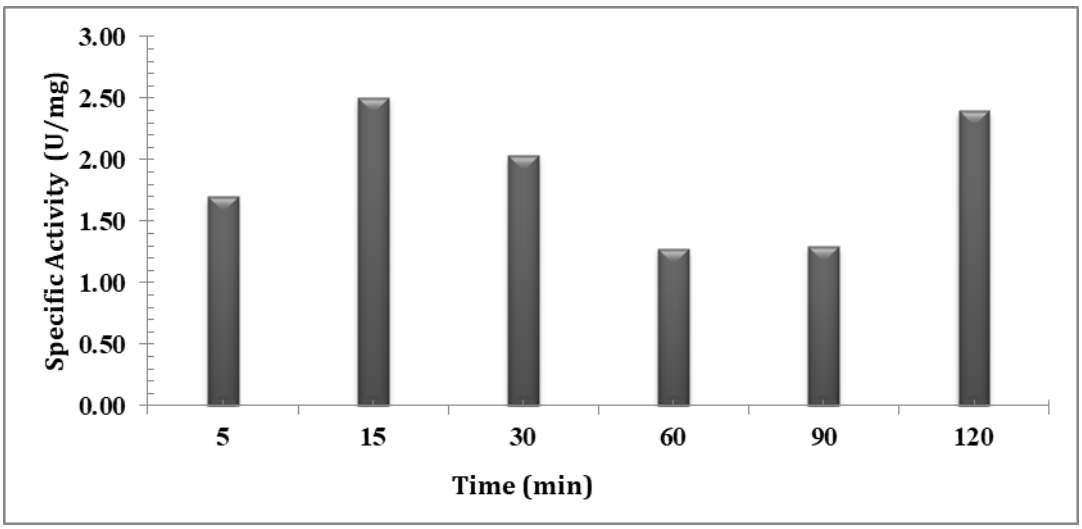

Figure 6. Urease extraction from cotton seeds affected by extraction time at 30°C using 0.15 M of tris base buffer

Characterization of Urease

Urease stability affected by pH: According to the results shows in fig. (7), the enzyme preserved a significant portion of its activity in pH value of 8 and around 58.22, 68.36, 71.91, and 71.29 % in pH 4.0, 5.0, 6.0, and 7.0, respectively. Also, the residual activities in pH 9.0 and 10 were 84.36 and 81.6 %, respectively, as the activity dropped away from the optimal pH values. At acidic pH, the enzyme activity was very low. The results might lead to the conclusion that the urease enzyme is more stable at pH around neutral. In general, the effect of pH stability on enzyme structure, which leads to denaturing the enzyme molecule or 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 loss of activity in buffers solutions that are far from optimal pH (26). In addition, sufficiently acidic or basic solutions can cause permanent denaturation of most enzymes, the efficacy of the active side of the enzyme in building the enzyme substrate complex is affected by the pH value of the environment. Changes in pH cause ionization levels in enzymes and substrates to fluctuate, which affects activity. In the urea degradation process, this causes the enzyme-substrate interaction to be at its peak, resulting in the formation of the product (30).

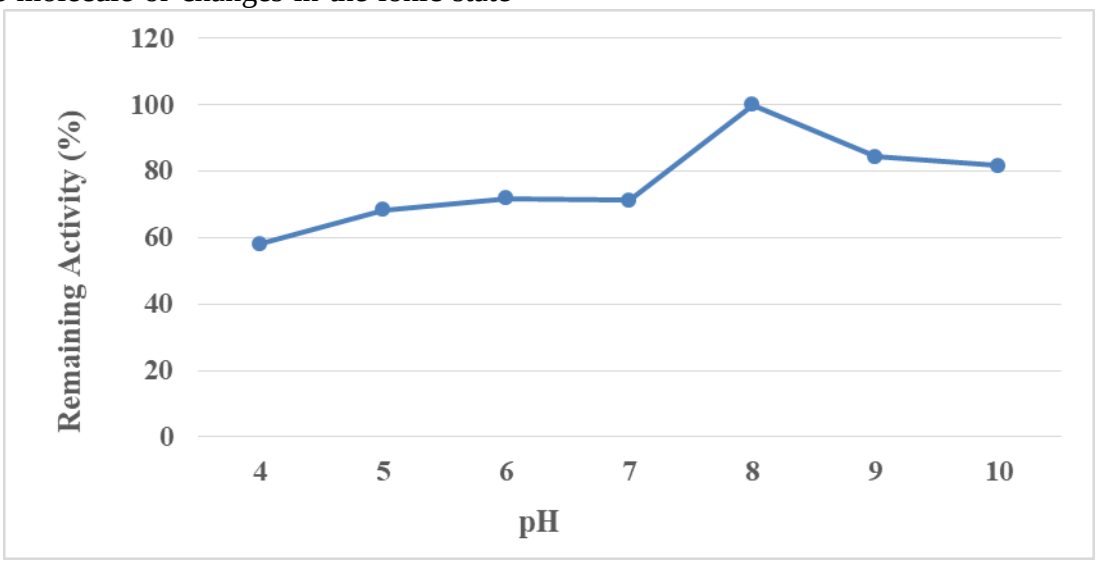

Figure 7. Urease remaining activity changes with different pH values

Urease activity affected by temperature

Urease activity was measured for 60 minutes at various temperatures of 25, 30, 37, 40, 50, 55, and 60 °C. Urease activity increased at 37 °C, reaching 2.01 U/ml, then decreased depends on the increasing temperature about more than 37 °C, reaching a minimum of 0.583 U/ml at 60 °C as shown in (Fig. 8) also

below 37 °C, it was reduced too much. The enzymatic reactions have been affected by the temperature in a number of different ways, including enzyme-substrate affinity, pH, and system ionization (26, 29). The study indicate that reaction speed increased until it reached 37 °C, while above 40 °C it began to deteriorate. This could be responsible for the increase in the collision between the enzymatic molecules able to share the reaction with the substrate as just a way of increase the molecules' movement energy, so even though enzymatic activity reduces at temperatures above 40 °C due to changes in the active sites and denatured of protein structure, resulting in a loss of enzymatic activity (17). The kinetic energy of molecules increases as the temperature increase, resulting in a faster rate of reaction. When the temperature was raised even higher, the enzyme molecules broke through the energy barrier. This causes both hydrophobic hydrogen bonds that keep the enzyme's three-dimensional structure intact to break down (7). While EL-Hefnawy *et al.*, (9) was found the maximum activity of urease enzyme from *Pisum sativum* L. seeds seeds has been obtained at 40 °C.

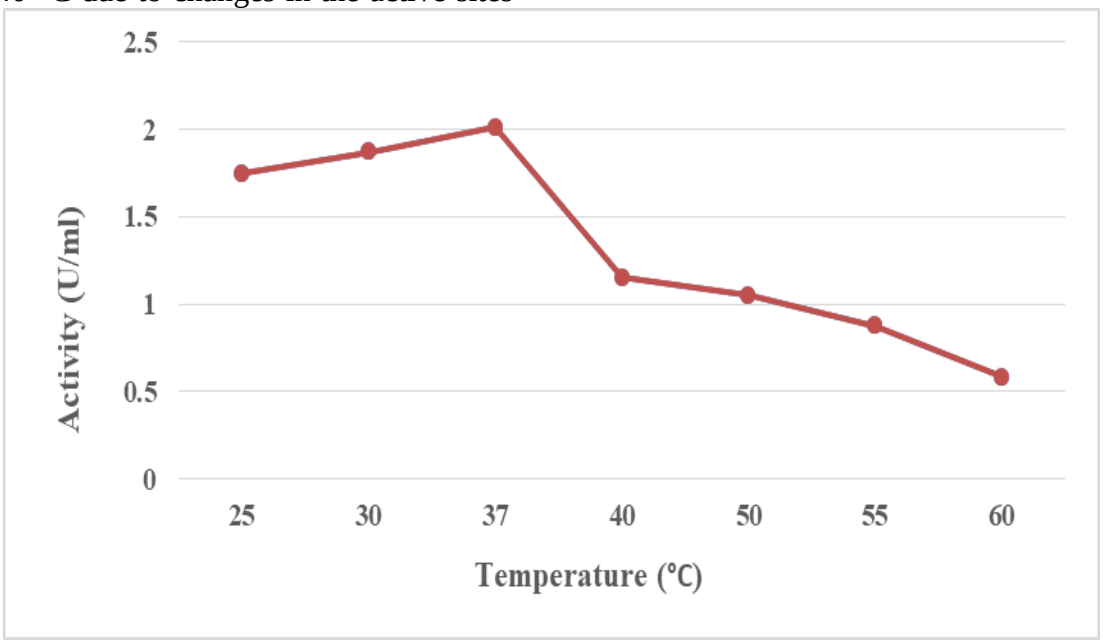

Figure 8. Effect different temperature values on urease activity

Stability of urease at various temperatures

The stability of urease at various temperatures has been assessed by incubation the enzyme at several temperatures ranging between (25 to 60 °C) then measuring residual activity after 15 minutes at 37˚C. According to the results in fig. (9), show the enzyme maintained its activity at temperatures ranging from 25 to 37 °C, after which the activity began to decrease with temperature increase. Significant drop in stability was resulted in lower temperatures in addition to, the enzyme preserved 71.38 percent of its initial activity with temperatures of 60 °C, while the remaining urease activity was 78.78% at 25 °C. Temperatures up or below the optimal temperature for any enzymatic activity will limit the reaction rate dramatically. The catalytic activity of enzymes results from a precise and highly ordered tertiary structure. The tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds. The activity was decreased of collagenase at temperatures above 45 °C due to its susceptibility to high temperatures (11). A high temperature will increase the enzyme and substrate collision and this is offset by the denaturation (27). While at 60 °C, (18) had the highest stability of urease enzyme from *pisum sativum* seeds.

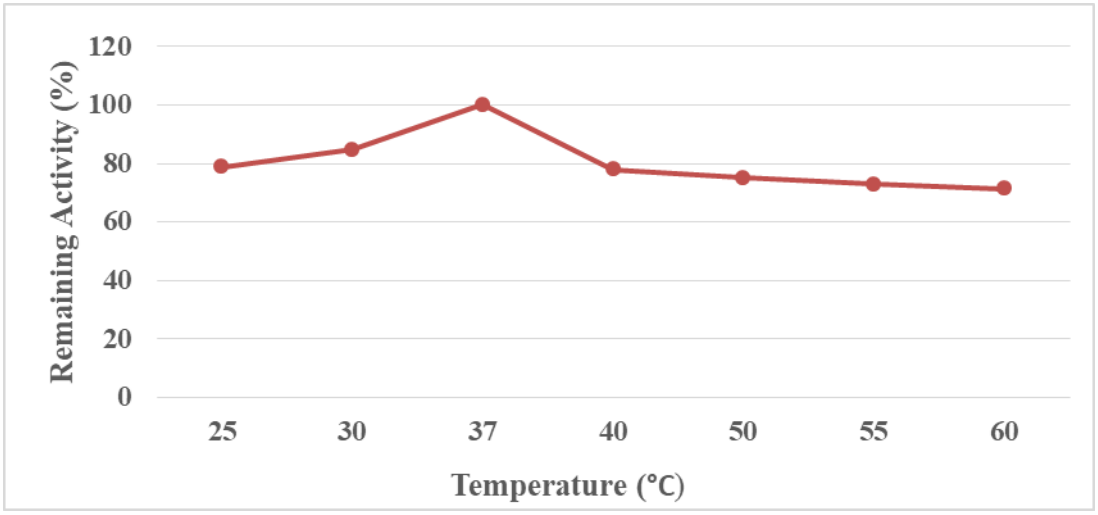

Figure 9. Effect different temperature values on urease stability Effect of reaction time on enzyme activity

The data on the influence of enzymatic reaction time on activity of urease from cotton seeds can be see in fig (10). It was observed that, the enzyme exhibited its maximum activity at 60 min of reaction time at 1.683 U/ml. Also the activity began to decrease gradually reaching the time of 120 min where the enzymatic activity reached to 1.302 U/ml. The highest activity shows that the 60 minute enzymatic reaction time is the optimum reaction time of urease enzyme from cotton seeds. The urease from cotton seeds catalyzes the reaction of urea into ammonia. The longer the enzymatic reaction time, the more ammonia is produced. The resulting ammonia is suspected to increase the reaction pH and makes the enzyme activity decrease (22).

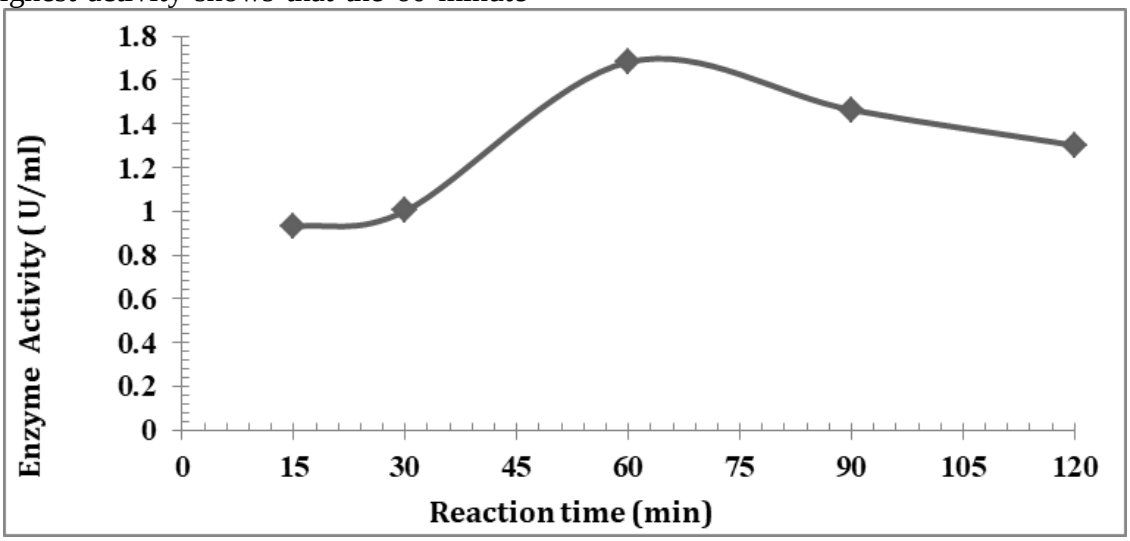

Figure. 10 Effect of the reaction time on urease activity at optimum pH and temperature

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