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Partial Purification and Characterization of Urease from Red Lentils (*Vicia lens* (L.) Coss. & Germ.)

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ABSTRACT. Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. A significant application of urease is found primarily in food, medical equipment and biosensor industries. This research aims to analyze the amino acid content of red lentil seeds and the extraction, purification, and characterization of urease from red lentils. The study started by analyzing the amino acid content in red lentil seeds using High-Performance Liquid Chromatography (HPLC). The red lentil seeds were extracted using phosphate buffer pH 7.0 and separated using centrifugal separation technique until crude extract of urease was produced. The crude extract of urease was then concentrated using acetone at varied saturation level (33, 41, 50, 60, and 67%). The fraction with the highest specific activity was then analyzed using SDS-PAGE method and characterized for its pH, incubation temperature, and substrate concentration against the urease activity. The urease activity was determined using Nessler method. The research results showed that red lentils seeds contained all essential amino acids. The highest specific activity was found in the fraction at 50% acetone saturation level (F50) and purity level 6.3 times than the crude extract. The characterization result indicated that F50 was purer than the crude extract. The optimum urease activity of crude extract and F50 was obtained at pH 7.0 and an incubation temperature of 35 °C. The K_M value of F50 was lower than crude extract. F50 has a higher affinity for binding to substrates so that the enzyme has higher efficiency in forming the products. Urease from red lentil seeds concentrated using acetone was 50% more potent as a catalyst than the crude extract. The research data will be the basis for the application of this urease.

Keywords: Acetone, characterization, partial purification, red lentil, urease

INTRODUCTION

Currently, the enzyme product issue is critical since the consumption of industrial enzyme in Indonesia is predicted to reach 2,500 tons at an import value worth around 200 billion in 2017. As high as 99 percent of the demand for industrial enzyme is met by import from such countries as China, India, Japan, and some European countries. Meanwhile, the demand for enzyme in Indonesia tends to increase by around 7.0 percent per year from 2015 to 2020 (Romadoni, 2017), Applications of enzymes in industries such as leather (Lason-Ryde et al., 2024), pulp and paper (Singh et al., 2016) textile (Madhu & Chakraborty, 2017), feed (Ojha et al., 2018) and detergent industries (Ahmed et al., 2016).

Indonesia is one of countries with abundant amount of biological wealth and biodiversity. The biological wealth and biodiversity have the potential to be utilized for developing biotechnology products, including enzyme product (biocatalyst). It is expected that enzyme production can support the independence to fulfil the national demand for enzyme. The use of enzyme which made of this biodiversity could replace chemicals. This will also make the industrial wastes decay more easily and, thus, eco-friendly (Ramadhan, 2017)

One of the enzymes that plays a role in industry is urease. The commercial applications of urease include: probes, or biosensors for detecting heavy metal ions in blood, industrial waste, food samples, and environmental monitoring systems (Pawar et al., 2022). The presence of urea in blood or urine sample analysis can also be predicted using urease (Sindi et al., 2024).

Urease is an enzyme that has an important history in the field of biochemistry, generally microbial and plant urease are the main sources of urease. Urease is a seed protein that is abundant in many plant species (Banerjee & Aggarwal, 2012). Urease has been characterized from such plants as: Jack bean (*Canavalia ensiformis*) (Saem et al., 2015), soybean (*Glycine max*) (Wiebke-Strohm et al., 2016), broad beans (*Vicia faba* L) (Bedan, 2020), *Pisum sativum* (Iyer et al., 2018), jackfruit (*Artrocarpus heterophyllus*)

(Chouhan et al., 2018), *Vicia sativa* (Sindi et al., 2024) and winged bean *(Psophocarpus tetragonolobus* (L.) DC., (Zusfahair et al., 2023). This research uses red lentils as the source of urease. Red lentil seeds are a source of macronutrients (Szedljak et al., 2019). Lentils have a protein content of 22.7% (Margier et al., 2018). The high protein content in lentils allows for the high enzyme content in red lentils. To increase the usefulness of red lentil seeds, in this research they were used as a source of urease. Considering the urease is still imported until today, therefore, obstacles in obtaining urease can be overcome, and reduce the demand for importing urease (Sindi et al., 2024).

The use of red lentil seeds as a source of urease has never been done. In this study, urease was isolated from red lentil seeds. The crude urease extract obtained needs to be partially purified using acetone. This is because acetone has high selectivity, resulting in high specific activity (Weber et al., 2008). In addition, purifying enzyme using cold acetone gives higher activity than such methods as ammonium sulphate, ethyl alcohol and ultra filtration (Abed, 2010). (Bedan, 2020) has extracted urease from *Vicia faba* L. using water solution at 1:3 ratio (b/v). The crude extract showed an enzyme activity of 33.3 U/mL, while the crude extract purified with acetone had an activity of 74.8 U/mL. Urease was extracted from watermelon and partially purified using 44% acetone (Javadi et al., 2018). Urease has been extracted from jack bean meal using 60% acetone (Pawar et al., 2022). The crude extract of long bean seeds was fractionated gradually using acetone with acetone concentrations of 20, 40, 60, 80% (Zusfahair, et al. 2018)

To support the independence of national enzyme needs, it is necessary to extract urease from red lentil seeds. The enzyme produced from the extraction process is a crude extract. The crude extract of enzyme was then partially purified using acetone. The characteristics of the urease obtained were determined, including the effect of pH, incubation temperature, substrate concentration on urease activity and protein analysis using electrophoresis. This was done to provide insight into various aspects of enzymes.

EXPERIMENTAL SECTION

Tools and Materials

The tools used in this research were those commonly used in any biochemistry laboratory, incubator, freezer (LG), analytical balance (O'Haus), centrifugator (O'Haus), and spectrophotometer UV-Vis (Shimadzu UV-1800), a set of electrophoresis equipment and High-Performance Liquid Chromatography (HPLC) (Knauer). The materials used in this research were red lentil seeds, ammonium sulphate, Nessler's reagent, urea, casein, acetone, Na_2HPO_4, NaH_2PO_4, Na_2CO_3, NaOH, $CuSO_4$, Na(K)-Tartrat, Folin-Ciocalteu reagent, acetic acid, $C_6H_8O_7.H_2O$, $Na_3C_6H_5O_7.2H_2O$, HCl, $BaCl_2.2H_2O$, and $ZnCl_2.H_2O$. All chemicals used herein were purchased from Merck, Germany.

Research Procedure

Determining the red lentil plants

The red lentil plants were determined at the Environmental Laboratory, Biology Faculty, Jenderal Soedirman University under an acceptance test letter number 043/HP.1.1/V/2023.

Determining the standard curve

The standard curve of ammonium sulphate was determined based on the method carried out by (Zusfahair et al., 2023). Using Lowry's method (Lowry et al., 1951), the absorbance of casein standard curve was measured at 750 nm wavelength.

Amino acid analysis

Amino acid analysis was carried out using HPLC at the UGM Integrated Research and Testing Laboratory with certificate number: 00670.01/VI/UNI/ LPPT/2024.

Urease extraction from red lentil seeds

Enzyme extraction from red lentil seeds was carried out according to the method in (Zusfahair, et al. 2018). A total of 20 grams of dry red lentil seeds were weighed and then soaked in distilled water for 6 hours. The soaked red lentil seeds are then drained and crushed using a mortar and pestle which has been stored in the freezer for 12 hours. The ground red lentil seeds were then suspended in 60 mL of 0.2 M phosphate buffer pH 7 and stirred with a stirrer for 30 minutes until two layers were produced, namely the filtrate and suspension. The filtrate was separated using muslin cloth. The obtained filtrate was centrifuged at 7,000 rpm at 4 °C for 15 minutes. The obtained supernatant was used as a crude extract. The resulting crude extract was determined for activity, protein content and specific activity and fractionated using acetone.

Enzyme extraction from red lentil seeds was carried out according to the method in Zusfahair, et al. (2018). As many as 20 g of dried red lentil seeds were weighed and then submerged in distilled water for 6 hours. The red lentil seeds that had been submerged were then drained and ground using mortar and pestle that had been saved in a freezer for 12 hours. The ground red lentil seeds were then suspended into 60 mL buffer phosphate 0.2 M pH 7 and stirred using a stirrer for 30 minutes until 2 layers, namely filtrate and suspension, were produced. The filtrate was separated using muslin cloth. The obtained filtrate was centrifuged at 7,000 rpm and 4 °C for 15 minutes. The obtained supernatant was used as crude extract. The produced crude extract was determined for its activity, protein content and specific activity and fractionated using acetone.

Fractionation using acetone

Fractionation is carried out according to the method found in (Sabilla & Susanti, 2019). The crude urease extract was aliquoted into 5 parts, each with

absolute acetone (5 °C) added little by little with an absolute acetone concentration (ratio of the volume of crude extract to the volume of absolute acetone) of 33% (10:5), 41% (10: 7), 50% (10:10), 60% (10:15), and 67% (10:20) were stirred slowly in an ice bath using a magnetic stirrer. The mixture was left for 12 hours in the freezer to maximize settling. The precipitated enzyme was separated by centrifugation at a speed of 7,000 rpm at a temperature of 4 °C for 15 minutes. The supernatant was separated from the precipitate. The remaining acetone that is still attached to the surface of the sediment is removed by airing it or absorbing it using sterilized filter paper. The precipitate resulting from each fraction was dissolved in 10 mL of 0.2 M pH 7.0 phosphate buffer so that the resulting suspensions were F33, F41, F50, F60 and F67. Each fraction obtained was determined for urease activity, protein content, and specific activity. The fraction that had the highest specific activity was characterized including the effect of variations in pH, incubation temperature and substrate concentration on the urease activity of red lentils. Protein fraction analysis using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) method was carried out at the Biotechnology Study Center, Gadjah Mada University, Yogyakarta.

Urease activity test

Urease activity was determined by the method in (Tetiker & Ertan, 2017); (Sabilla & Susanti, 2019) .The urease activity test was carried out by adding 1 mL of 12,000 ppm urea solution into a sample test tube and then adding 1.95 mL of 0.2 M pH 7 phosphate buffer and 0.05 mL of enzyme solution. The control test tube was filled with 1 mL of 12,000 ppm urea solution and 2 mL of 0.2 M phosphate buffer pH 7. The sample and control test tubes were incubated for 15 minutes at 35 °C. The reaction tube was cooled in the refrigerator for 5 minutes, then heated at 80 ºC for 2 minutes. Both tubes were centrifuged for 15 minutes at room temperature at a speed of 500 rpm. 1.5 mL of the supernatant formed was taken then 0.25 mL of Nessler's reagent was added. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 500 nm. Determination of the urease activity curve of red lentils was carried out using the ammonium sulfate standard curve (Zusfahair et al., 2023). One unit of activity was expressed as the amount of ammoniac amonia formed in 1 µgram per mL per minute of the urea hydrolysis by urease in the sample.

Characterization of urease of red lentil seeds

The urease was characterized in the crude extract and fraction with the highest specific activity (optimum fraction). The influence of pH, temperature and substrate concentration variations on urease activity was tested the same way as the activity test, yet it was carried out at pH 5, 6, 7, 8, and 9 variations. The incubation temperature variations used were 30, 35, 40, 45, and 50 °C. The urea substrate concentration variations used were 6,000, 9,000, 12,000, 15,000 and 18,000 ppm. The obtained data were then used to determine the value of Michaelis-Menten constant (K_M).

RESULTS AND DISCUSSION

Determination of Red Lentil Plant

The determination result was as follows: Family: Fabaceae; Genus: *Vicia;* Species: *Vicia lens* (L.) Coss. & Germ. Red lentils are shown in **Figure 1.**

Standard Curve

The regression equation obtained for the standard curve of ammonium sulphate was y = 0.0397x–0.0126 at r value of 0.9993. The linear regression equation obtained for the standard curve of casein was y = 2.054x–0.0365 at r value of 0.9991. The regression equation obtained is used to determine the concentration of ammonia produced.

Analysis of Red Lentil Seeds' Amino Acid

The amino acid was analyzed using High-Performance Liquid Chromatography (HPLC). The result of analysis can be seen **Table 1.**

Figure 1. Red lentils (Source: personal photo)

Table 1. Result of analysis of red lentil seeds' amino acid.

Number	Test Parameters	Content (μg/g)
1	L-Aspartic Acid	4316.36
2	L-Glutamic Acid	5800.88
3	L-Asparagine	0
4	L-Histidine	1432.14
5	L-Serine	870.76
6	L-Glutamin	0
7	L-Threonine + L- Glycine	2484.78
8	L-Arginine	1374.75
9	L-Alanine	1601.79
10	L-Tyrosine	708.58
11	L-Thryptophan + L-Methionine	1374.76
12	L-Valine	1571.85
13	L-Phenylalanine	2063.37
14	L-Isoleucine	1382.23
15	L-Leucine	2771.95
16	L-Lycine	2856.783

To add scientific information about red lentil seeds, this study conducted an analysis of the amino acid content. Based on the analysis result of amino acid in Table I red lentil seeds contained all of the essential acid needed by body, namely: L-Lycine, L-Leucine, L-Threonine, L-Phenylalanine, L-Valine, L-Histidine, , L-Isoleucine, L-Thryptophan, and L-Methionine. Treonin was the amino acid existing in the active side of urease of *Staphylococcus xylosus* (Jose et al., 1994). According to (Kappaun et al., 2018), the active site of urease, other than two nickel atoms, consisting of one carbamylated lysine, four histidine, and one aspartate residue. The amino acid on the active site was found in the red lentil seeds.

Urease Activity

Urease is the enzyme that catalyzes the hydrolysis reaction of urea to produce ammonia and carbamate. The latter eventually decomposes spontaneously into the second molecules of ammonia and bicarbonate (**Figure 2**). The urease reaction was based on Hausinger et al. (Jabri et al., 1995). In the first step of this hypothesis, urea would bind in a mono-dentate mode in the active site of urease by coordinating to the least coordinatively saturated Ni(1) via its carbonyl oxygen atom, completing a tetra coordination environment for this ion and causing polarization of the carbonyl group. This interaction is aided by αHis219 NεH acting as a H-bonding donor to the urea O atom. In the second step, a hydroxide ion, identified as the Wat-1 solvent molecule bound to Ni(2), was proposed to act as the nucleophile for the reaction by attacking the carbonyl C atom of urea with the formation of a tetrahedral intermediate (**Figure 3**). In the final step, the tetrahedral intermediate was assumed to decompose, with the participation of an unidentified generic acid that was previously proposed to protonate a C_{urea}-NH_2 group.

Extraction of Urease from Red Lentil Seeds

The extraction aimed to get the enzyme out of the plants' tissue cells (Pramono et al., 2018). The urease was extracted from red lentil seeds by submerging the red lentil seeds in distilled water. As they were being submerged, an imbibition processs, where water was absorbed by the dried seeds, occured. This water absorption made the seeds inflate and enlarge (Pratantie et al., 2021). The submerged red lentil seeds were then drained and ground using mortar and pestle. The grinding process was done to break the cell tissues and walls, allowing the cell contents to get out of it (Tazkiah et al., 2019). During the grinding process, the temperature should be kept cold to maintain the enzyme from being denatured, i.e., by keeping the mortar and pestle in a freezer before being used and working in an air-conditioned room. The ground red lentil seeds were then suspended into phosphate buffer 0.2 M pH 7 and stirrred in an ice bath using a magnetic stirrer. The filtrate was separated using muslin cloth. The obtained filtrate was centrifuged until sediment and supernatant were acquired. The acquired supernatant was used as crude extract. The produced crude extract was then tested for its activity, protein content and difractionation using acetone.

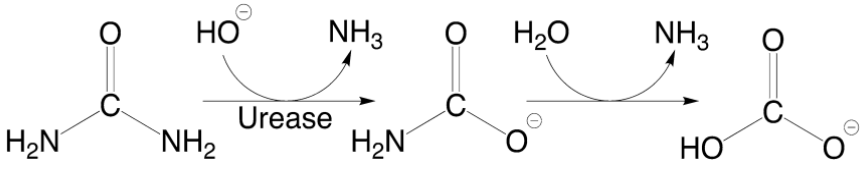

Figure 2. The enzymatic stage for urea hydrolysis (Mazzei et al., 2020)

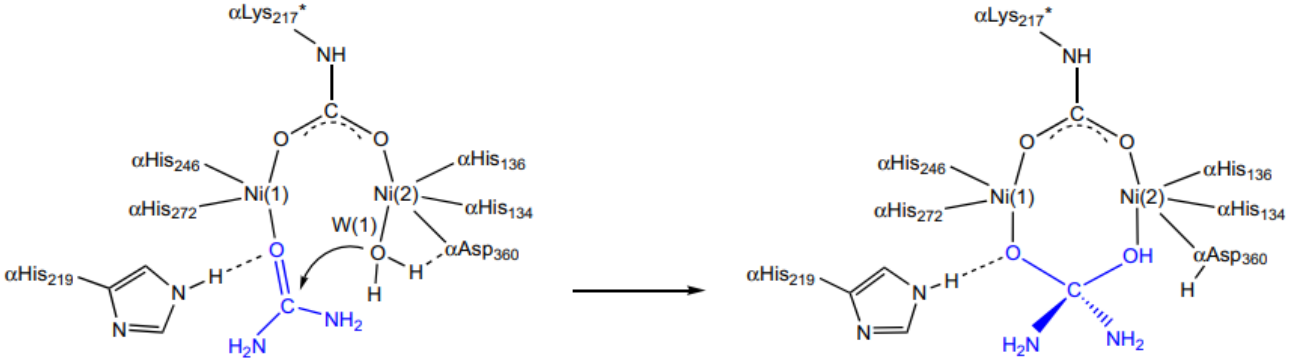

Figure 3. Urease reaction mechanism as per Hausinger et al (Mazzei et al., 2020).

Fractionation Using Acetone

The enzyme extract had not completely consisted of the desired protein enzyme; thus, a purification was needed to separate it from impurity. Fractionation enzyme is the process of separating enzyme from other protein mixture. The enzyme was commonly fractionated using organic solvent. Since its dielectric constant was lower than water, the organic solvent increase the attraction between protein molecules, and the aggregate was formed until the particles reached a macroscopic proportion and sedimented (Trentini et al., 2015).

Acetone is an effective organic solvent in the sedimentation process of enzyme. The acetone was fractionated to sediment the enzyme solution until enzyme sediment was produced in each fraction. The enzyme sedimentation in the fraction occurred thanks to the difference in solubility in acetone solvent.

Acetone is an organic compound capable of breaking the enzyme. Thus, the acetone to be used should be cold and added slowly. The strength of enzyme fractionation using acetone as compared to ammonium sulphate was that acetone fractionation did not require a dialysis treatment process to remove the remaining salts (Zusfahair, et al., 2018). The data on partial purification of urease from red lentils are shown in **Table 2**.

Based on the research results as shown in **Table 2**, after each fraction was partially purified, their specific activity increased. This increased specific activity indicated that the obtained enzyme got purer and purer. The fraction with the highest specific activity was FA 50 at a specific activity of 7.444 ± 0.015 U/mg. This fraction had a purity 6.3 times greater than in its crude extract. The acetone fractions had lower protein content than in the crude extract. This was because the acetone fractions had gone through a separation process that reduced the non-enzyme protein mixed in the enzyme (Zusfahair, et al., 2018). These data were supported by the result of enzyme analysis using SDS PAGE electrophoresis in **Figure 4.** The data in **Figure 4** showed that the amount of protein band in F50 was fewer than the crude extract. This showed that F50 was purer than the crude extract. The protein with a greater band thickness indicated that the protein had higher concentration (Machsun & Zulaika, 2017).

Characterization of Urease from Red Lentil Seeds

Effect of pH variation on urease activity

The effect of pH variation on urease activity of the crude extract and F50 can be seen in **Figure 5**. Based on the curve in **Figure 5**, the optimum urease activity was obtained in pH 7 with a value of the urease activity from crude extract being 28.212 U/mL ± 0.176 and the urease activity from F50 being 37.168 ± 0.077 U/mL. Similar result was also obtained in the urease from asparagus bean (*Vigna unguiculata ssp sesquipedalis* L.) seeds that had pH (Zusfahair, et al. 2018). Some other studies reported that the urease from pea (*Pisum Sativum* L.) seeds had an optimum pH 7.5 (El-Hefnawy et al., 2014), sword bean (*Canavalia gladiata*) seeds had an optimum pH 7.5 (Saem et al., 2015), and the urease from *Vicia faba* L. had an optimum pH 8 (Bedan, 2020).

Table 2 Table of partial purification of urease from red lentil seeds

Phase	Activity (Unit/mL)	Protein (mg/mL)	Specific activity (Unit/mg)	Purity
Crude extract	32.443 ± 0.067	27.482 ± 0.077	1.181 ± 0.002	1.0
F33	33.630 ± 0.205	4.817 ± 0.012	6.982 ± 0.025	5.9
F41	35.824 ± 0.216	4.862 ± 0.007	7.368 ± 0.035	6.2
F50	36.966 ± 0.103	4.966 ± 0.022	7.444 ± 0.015	6.3
F60	34.794 ± 0.067	5.343 ± 0.032	6.513 ± 0.026	5.5

F67	32.667 ± 0.169	5.606 ± 0.022	5.828 ± 0.007	4.9

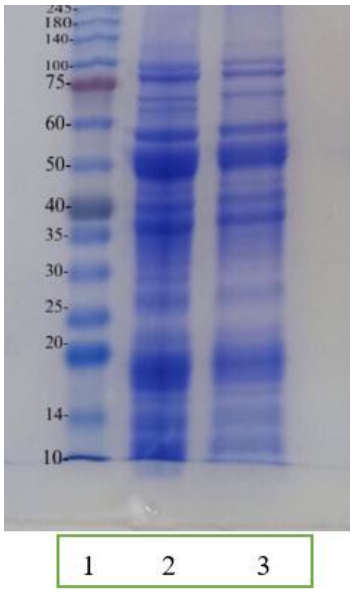

Figure 4. Analysis of protein fraction using SDS PAGE. 1= Marker; 2 = Crude extract of urease enzyme from red lentils; 3 = Urease enzyme from red lentils fractionated with 50% (F50)The correlation curve between pH variations and urease activity of crude extract from red lentils and urease from red lentils fractionated with 50% acetone (F50)

The urease activity from red lentil seeds was optimum at pH 7. At this optimum pH, the enzyme conformation was on an ideal condition, allowing the enzyme and substrate to interact maximally to change the enzyme-substrate complex into a product (Habibie et al., 2014). This result might show that any pH under 7.0 had an poor effect on the urease, decreasing its activity. The change in reaction media pH could affect the amino acid ionization occurring in the active site of urease (Singh et al., 2017).

Effect of incubation temperature variations on urease activity

The effect of incubation temperature variations on urease activity of the crude extract and F50 can be seen in **Figure 6.** Based on the curve in **Figure 6**, the optimum urease activity of crude extract and F50 was obtained at incubation temperature of 35 ºC with the value of urease activity of crude extract being 29.018 ± 0.201 U/mL and urease activity of the optimum fraction being 37.526 ± 0.039 U/mL. This result was similar to (Zusfahair et al., 2023) who found that the optimum incubation temperature of urease from winged bean seeds was 35 ºC. (El-Hefnawy et al., 2014) managed to isolate urease from peas (*Pisum sativum* L.) and obtained an optimum incubation temperature at 40 ºC.

An increase in incubation temperature led to an increase in the kinetic energy of enzyme molecule and in turn increase the reaction rate. An incubation temperature exceeding the optimum temperature would break the hydrophobic bond and the hydrogen which played a role in maintaining the enzyme's functional structure, and hence decreased the enzyme activity (Singh et al., 2017).

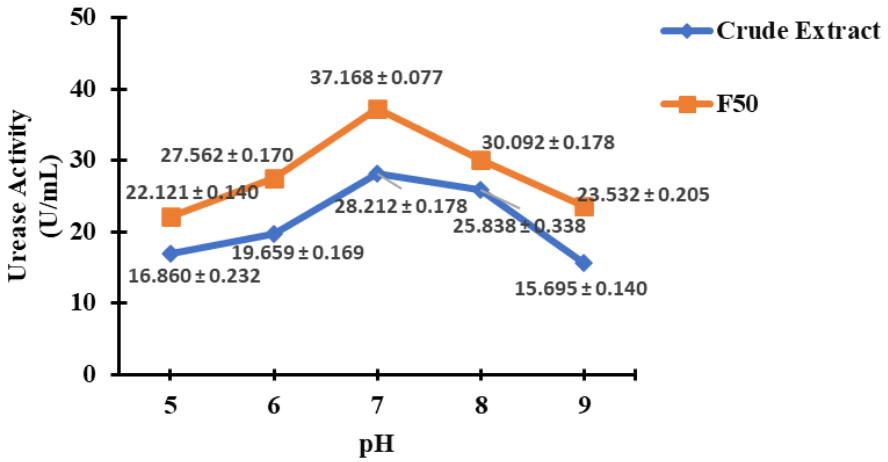

Figure 5. The Correlation curve between pH variations and urease activity of crude extract of red lentils and urease from red lentils fractionated with 50% acetone (F50).

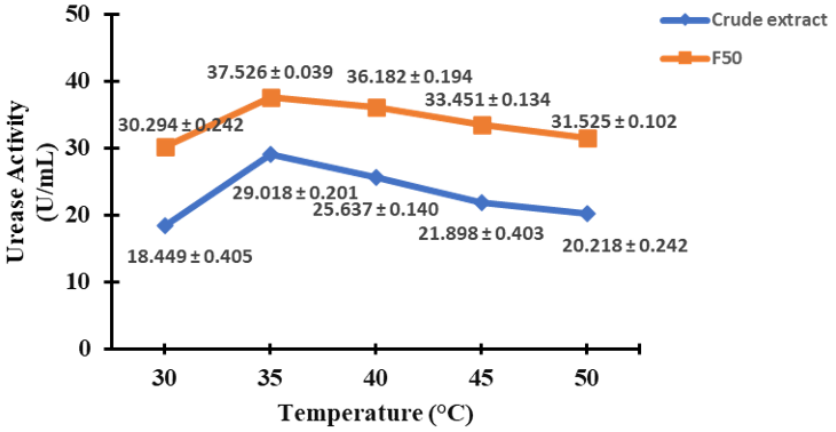

Figure 6. The correlation curve between incubation temperature variations and urease activity of crude extract of red lentils and urease from red lentils fractionated with 50% acetone (F50).

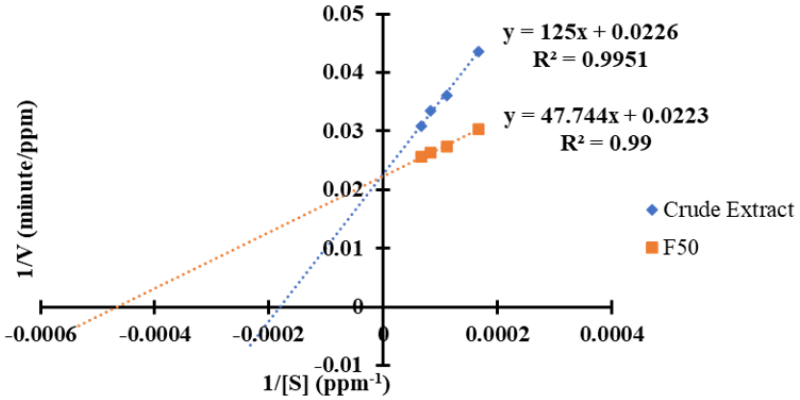

Figure 7. The correlation curve between 1/[S] and 1/V based on Lineweaver-Burk equation

Determining the values of V_{max} and K_M

The values of K_M could be determined using Lineweaver-Burk equation by plotting the value of 1/[S] and 1/V. The value [S] indicated the substrate concentration, and the value V showed the enzyme's reaction rate which was equated with the urease activity. The correlation curve between 1/[S] and 1/V can be seen in **Figure 7.**

Based on the regression value obtained in **Figure 7**, the obtained value of K_M was 92.18 mM in the crude extract and 35.68 mM in F50. The obtained value of K_M in F50 was less than in the crude extract. This meant that F50 had higher affinity to bind with the substrate, and thus the enzyme had higher efficiency in forming the product. The Km value for urease extracted from germinated *Pisum sativum* was found to be 500 mM. This value indicates a low affinity of the substrate to urease. The Km value ranges from 19–476 mM for the soybean seed urease, depending on the buffer system of the solution (Das et al., 2002)

CONCLUSIONS

Based on these results, it could be concluded that: red lentil seeds contained all essential amino acids: L-Histidine, L-Threonine, L-Thryptophan, L-Methionine, L-Valine, L-Phenylalanine, L-Leucine, L-Lycine, and L-Isoleucine. The urease of crude extract from red lentil seeds had been fractionated using acetone to obtain the highest specific activity in a fraction at acetone saturation level of 50% (F50) and purity level of 6.3 times the enzyme's crude extract. The urease of crude extract and F50 from red lentils had an optimum activity at pH 7, incubation temperature of 35 °C, and K_M value of 92.18 mM in crude extract and 35.68 mM in F50.

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