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Determination of The Optimum Conditions for Urease Extraction from Chickpea Seeds Using (Design Expert Software)

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Abstract

This study aimed to utilise response surface methodology (RSM) to determine the optimal conditions for extracting urease from a plant source and evaluate its biochemical properties. Chickpea seeds were selected as the source of urease. Results showed that chickpea seeds had a high enzyme activity (3.99 U/mg protein) under the best extraction conditions of pH 7.99, tris base buffer concentration of 0.2, time of 60 minutes and ratio of 1:13 (g: mL). When a second-order polynomial model was used, the predicted correlation coefficient (pred. R_2) was 0.8477, the adjusted correlation coefficient (adj. R_2) was 0.9333 and the correlation values (R_2) were 0.9665, indicating that the predicted models and experimental values agreed very well. The method has the potential to enhance the cost-effectiveness of enzyme production through large-scale manufacturing and offers an economical alternative to other techniques.

***Keyword**: Chickpeas; plants; enzyme; extraction; RSM*

1. Introduction

Ureases (EC 3.5.1.5) are metalloenzymes that depend on nickel and break down urea into CO_2 and ammonia [1]. These enzymes are present in various organisms, including plants, algae, yeasts and filamentous fungi. Ureases from plants and fungi are made up of identical protein units, while bacterial ureases are made up of complicated repetitions of two or three subunits of varying sizes [2]. Ureases are applied in numerous fields, such as biosensors for measuring urea in human blood, diagnostic kits for urea measurement and urea-reducing agents in alcoholic beverages [3]. Plant and microbial ureases have other biological functions, such as activating blood platelets, killing insects and killing fungi, which suggest that they are involved in the defence mechanisms of plant cells [4]. The crystallisation of urease enzymes extracted from jack beans marked a pioneering achievement in laboratory studies, and these enzymes remain the most extensively studied plant ureases. Other studies isolated seed urease from watermelon and *Cajanus cajan* [2]. A number of variables, including environmental conditions (such as temperature and pH), type of enzyme used and substrate, influence the rate of urea hydrolysis. Temperature and pH have been investigated for their effect on urease activity [5]. Research of urease activity at various temperatures discovered that it was most effective between 35 °C and 50 °C; at 60 °C, the enzyme retained more than 80% of its activity [6]. Jack bean urease was found to be most active at 65 °C. Biotechnology has gained increasing interest because of the growing need for efficient urea removal technologies in various contexts [7]. Urea is used in treatment of industrial wastes, manufacture of alcoholic beverages, haemolysis and life support systems in space missions. Bacterial ureases have complicated repeats of two or three subunits of varying sizes, while plant and fungal ureases are homo-

oligomeric and contain identical protein repeats [8]. The efficiency of enzyme function is frequently highly dependent on its basic structure and local environment. Anything in the surrounding environment that alters the enzyme's conformation or blocks its active site can affect its function [7]. Through a series of tests conducted using the Design Expert tool, the present study aims to identify the ideal conditions for urease enzyme extraction from chickpea seeds. We will use central composite design (CCD) to establish experimental circumstances and build a statistical model to forecast extraction conditions. The effect of process variables on responses will be assessed using ANOVA. We will then conduct experiments to confirm the optimal extraction conditions predicted. The methodology will establish a connection between a desired response or outcome and a set of process variables by applying statistical methods. Response surface methodology (RSM) requires simple and effective experimental designs [9] and may fix problems with linear and nonlinear multivariate regression while decreasing the number of experimental trials.

2. Materials and Methods

Chickpeas were hand-picked from an Iraqi market. Experiments were carried out using high-grade chemical reagents acquired from trustworthy vendors. The following chemicals were purchased from Hi-Media Co.: sodium nitroprusside, tris base, tris HCl ($C_4H_{11}NO_3$), and urea (CH_4N_2O). Coomassie Brilliant Blue G-250, sodium acetate (CH_3COONa) and sodium hydroxide (NaOH) were procured from BDH. Sodium dibasic and sodium dihydrogen phosphate ($NaH_2PO_4.12H_2O$) were provided by CDH, and ammonium chloride (NH4Cl) was supplied by Sigma. Sodium hypochlorite (NaClO) was bought from Sehat Company, and phenol was obtained from Thomas Baker Co. These chemical reagents were meticulously chosen to guarantee the precision and dependability of the experimental processes and were commercially available and of highest possible purity.

2.1 Sample Preparation

A locally accessible cultivar of spherical, light-coloured chickpeas called Kabuli was sourced from Türkiye and used in the experiment to evaluate urease activity. Pozzolana buffer solution, which had a concentration of 0.02 M and a pH of 7.0, was used to crush and extract chickpea seeds. A 10 mL of the buffer solution was used to collect 1 gram of plant seeds after leaving the sample at room temperature for 15 minutes.

2.2 Urease Assay

Urease activity was measured using a modified Berthelot reaction [10]. The amount of ammonia released was measured using the NH_4Cl standard curve. Glassware were sterilised by washing in a solution of warm, diluted hydrochloric acid and rinsed well with distilled or deionised water. About 1 mL of the seed extract was mixed with 1 mL of 500 mM urea solution containing phosphate buffer (100 mM, pH 6.8) and 0.8 mL of the same buffer. The mixture was placed in a water bath and incubated at 37 °C for 1 h. The reaction was terminated by applying heat at 80 °C for 5 minutes. A test sample, referred to as the ‘black sample’, was prepared. The plant seed extract was heated before adding to the reaction mixture. The reaction mixture (1 mL) was mixed with 10 mL of Berthelot reagent to determine ammonia concentration. The Berthelot reagent consisted of 5 mL of 0.01 M reagent A (containing 5 g of phenol and 0.02 g of sodium nitroprusside) and 5 mL of 0.01 M reagent B (containing 2.5 g of sodium hydroxide and 8.4 mL of sodium hypochlorite). The mixture was then diluted in 500 mL of distilled water and incubated in a water bath at 37 °C for 1 hour. Urease activity was assessed by quantifying the increase in absorbance at a wavelength of 625 nm. An enzymatic activity unit is the amount of enzyme liberated from ammonia within 1 minute under the optimum conditions. The concentration of protein was determined using Bradford’s technique [11].

Urease Activity =Ab/slope/(T×C)

where:

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

T: is the time of reaction (60 min)

C: is a constant [12]

2.3 Urease Extraction

In a mortar, 1 g of plant was mixed with 10 mL of phosphate buffer at pH 7.0 for 15 minutes at room temperature to achieve a homogenous mixture. The mixture was centrifugation at 10,000 RPM for 15 minutes and filtered through Whatman No. 1 filter paper (0.1 mm thick, Chinese manufacturer) to remove all cellular debris from the slurry. Protein content, enzyme activity and specific activity of the supernatant were measured.

2.3.1 Extraction Buffer

The sample with the maximum activity, as indicated during preliminary screening, was selected to investigate the effect of the buffer used for extraction. The selected plant sample was homogenised for 15 minutes at 30 °C by using various buffers:
At pH 4, 5 and 6, 0.2 M sodium acetate; at pH 7, 0.2 M phosphate-buffered saline; and at pH 8 and 9, 0.2 M Tris-base. We examined protein content, enzyme activity and any significant interactions in triplicate.

2.3.2 Concentration of Extraction Buffer

Extraction was carried out using different concentrations (0.085 M to 0.2 M) of tris base buffer, sodium phosphate buffer, and sodium acetate buffer to determine the appropriate concentration for extraction.

2.3.3 Extraction Ratio

Extraction ratio was optimised using different buffer ratios, ranging from 1:5 to 1:30 (w/v). In brief, 1 g of chickpeas were mixed with the appropriate extraction ratio followed by centrifugation at 10,000 rpm for 10 minutes and filtration using Whatman No. 1 filter paper. The resulting clear supernatant represented the crude extract, which was then assayed for urease inhibitor activity.

2.3.4 Extraction Time

Optimal extraction time was determined by varying it from 5 to 120 minutes.

2.4 Experimental Design

The experimental data obtained from CCD were analysed and fitted to a second-order polynomial model, as represented by the equation:

$$Y = \beta_0 + \sum \beta i X i + \sum \beta ii X i^2 + \sum \sum \beta ij X i X j \quad \ldots (1)$$

where Y represents the expected response, β_0 is the intercept parameter, β_i and βii are linear and quadratic coefficients and β_{ij} represents the interaction coefficients. X_i and X_j denote the coded values of the independent factors [13]. The ranges for each parameter were carefully selected to assess their effects on the efficiency of urease extraction.

Design Expert software, which incorporated RSM and CCD, was utilised to simulate the experiments and determine the optimal conditions. A total of 25 individual experiments were simulated, and a second-order polynomial relationship was derived using RSM for subjective evaluation of urease output. The operating ranges for each parameter can be found in Table 1.

Table 1,
Applied values and variables for urease extraction

Factors	Units	Low (−1)	High (+1)
pH	-----	4	9
Concentration	mg/L	0.085	0.2
Extraction ratio	mg mL^{-1}	1:5	1:30
Extraction time	min	5	120

2. 5 Design Expert Modelling –RSM-CCD

RSM provided excellent statistical tools for experimental design and data interpretation to optimise the process. RSM reveals the optimal condition under which a high yield of product that meets specifications is produced at the lowest cost.
The present design of the experiment excludes the CCD model, which was previously incorporated into the theoretical framework [14]. The model was generated using a linear regression approach, with the inputs as coadded values [15]. We employed ANOVA to determine how autonomous factors in regression affect the dependent variable. ANOVA is a statistical method used to differentiate systematic factors from other factors and random factors to explain the observed aggregate variability within a data set. Systematic factors, as opposed to random factors, affect the offered data set statistically [16].

3. Results and Discussion

3.1 Optimisation Results

After experimenting with several variables, such as extraction time, ratio, buffer and concentration, the optimum conditions for urease extraction from chickpeas were determined. The specific activity was 3.99 U/mg, and the best conditions for extraction were a water-to-solid ratio of 1:13 for 1 hour in 0.2 M Tris-base buffer. Urease extraction from various sources has been the subject of prior research into various buffers and pH values. According to Hussein et al. [17], the optimal buffer for urease extraction from chickpeas was sodium acetate buffer (0.2 M, pH 5.0), which led to an enzyme with a specific activity of 1460 U/mg. Enzyme extractability can vary depending on source combination and other interfering factors and is crucial to establish the optimal extraction period. Contaminants should be removed before extraction to obtain a stable protein extract [18].

3.2 ANOVA

Table 3 shows the outcomes of the four variables tested by ANOVA. The chosen regression models fitted the data well, with an R2 value of 0.9665. A strong agreement was found between the adjusted R2 (R2ad) value of 0.9419 and the anticipated R2 value of 0.8477, with a skewness of less than 0.2. A and an extremely high signal-to-noise ratio, as shown by an appropriate precision (AP) value of 17.204; hence, the models employed were statistically significant and accurate in describing the experimental data [19]. Table 3 shows no significance in the misfit F values (0.5431, 0.7765), thereby adding credence to the significance and correctness of the quadratic model. With a CV% of 13.34%, the model was quite accurate and dependable for predicting experimental outcomes [20]. The quadratic models were used to determine the optimal conditions for urease extraction when the AP ratio exceeds four and the modified coefficient of determination is more than the 80% threshold. The right factor levels should be selected to obtain values closer to the average theoretical system efficiency [20].

Specific Activity Equation: **the predicted model shown in Equation (2).**

$$\text{Specific Activity} = -0.490676 + 0.903056X_1 - 4.37723X_2 + 0.044360X_3 - 0.000018X_4 + 0.795855X_1X_2 - 0.000345X_1X_4 - 0.070835X_2X_3 - 0.012132X_2X_4 + 0.000117X_3X_4 - 0.065062X_1^2 + 27.28951X_2^2 - 0.001405X_3^2 \quad \ldots(2)$$

where X_1: pH of solution X_2: concentration of solution (M) X_3: ratio (weight: volume) X_4: extraction time (min)

This equation can predict responses (specific activity) to different levels of each factor by expressing the levels in terms of the actual factors. Original units at which the factors were measured were used when specifying the amounts of each element.

3.3 Design expert modelling –RSM-CCD

Table 2 shows the experimental conditions and the results obtained from 25 experiments according to RSM.

Table 2
Design experiment program for maximum enzyme urease extraction

A:pH	B:C0 M	C:Ratio	D:Time min.	Specific Activity
4	0.01	30	120	.362
4	0.085	15	58	2.3
4	0.2	30	5	2.53
4	0.2	30	85	2.62
4	0.085	15	58	2.45
4	0.2	5	120	2.68
4.93	0.01	5	5	2.71
5.3	0.01	24	5	2.61
5.38	0.15	5	5	3.11
5.5	0.01	5	110	2.5
5.78	0.13	21	120	2.78
5.78	0.13	21	120	3.2

5.88	0.13	21	9.6	3.25
6.88	0.09	6.3	63	2.58
6.95	0.086	30	60	2.8
6.95	0.086	30	60	3.13
7.05	0.2	15	58	4
7.05	0.2	15	58	3.89
7.05	0.2	15	58	3.95
9	0.14	5	110	3.5
9	0.01	5	28	2.4
9	0.01	23	110	2.66
9	0.2	30	120	3.21
9	0.01	23	28	2.66
9	0.15	24	5	3.42

Table 3,
ANOVA results for response surface of quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.62	12	0.4683	28.88	< 0.0001	significant
A-pH	1.13	1	1.13	69.74	< 0.0001	
B-C0	2.47	1	2.47	152.24	< 0.0001	
C-Ratio	0.0485	1	0.0485	2.99	0.1094	
D-Time	0.0879	1	0.0879	5.42	0.0382	
AB	0.2819	1	0.2819	17.39	0.0013	
AD	0.0181	1	0.0181	1.12	0.3112	
BC	0.0533	1	0.0533	3.29	0.0949	
BD	0.0363	1	0.0363	2.24	0.1604	
CD	0.0541	1	0.0541	3.34	0.0927	
A^2	0.6789	1	0.6789	41.88	< 0.0001	
B^2	0.2768	1	0.2768	17.08	0.0014	
C^2	0.1959	1	0.1959	12.08	0.0046	
Residual	0.1945	12	0.0162			
Lack of Fit	0.0840	7	0.0120	0.5431	0.7765	not significant
Pure Error	0.1105	5	0.0221			
Cor Total	5.81	24				

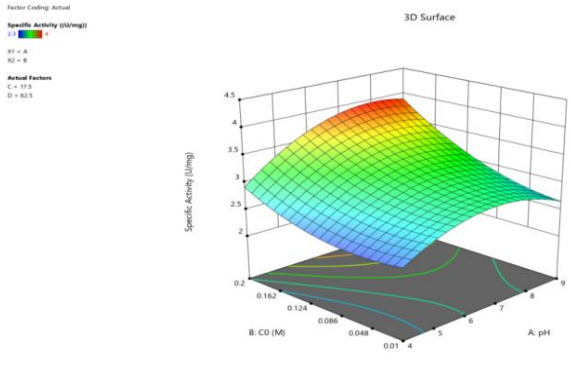
R^2 = 0.9665, $R^2_{adjusted}$ = 0.9419, $R_{predicted}$ =0.8477 ,AP =17.204, CV = 13.34%

3.4 Interactive effects of two variables

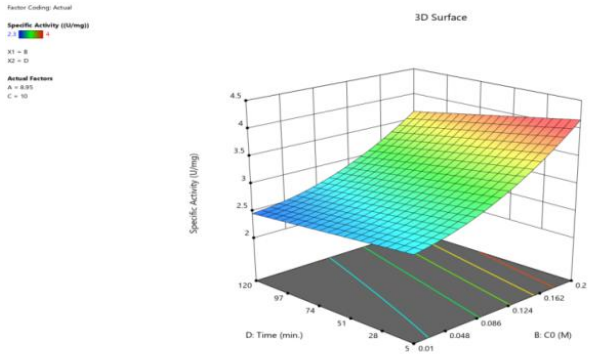
The interaction effects of buffer and concentration on urease activity are depicted in Figure 3a. The findings of this study demonstrate that within the pH range of 7–9 and with concentrations ranging from 0.162–0.2 M, there was a notable increase in urease activity. Specifically, the specific activity reached a value of 3.99 U/mg. The pH of the enzyme environment plays a significant role in enzyme activity. The pH of the environment affects stability, while each enzyme has an ideal pH range where it performs at its best. In addition to the pH of the reaction mixture that influenced substrate–enzyme interactions, the enzyme was denatured by extremely acidic or alkaline conditions [21]. Selecting a buffer solution whose pH is similar to the cell's in vivo pH is critical to maintain protein integrity [22]. Urease has been extracted from many sources by using different buffers and pH levels. An earlier work [23] extracted urease from *Proteus mirabilis* by using 20 mM phosphate buffer with a pH of 7.5.

The combination of the extraction ratio (10–15, w/v) and buffer (pH 7–9) is illustrated in Figure 3B. The amount of material extracted from the herb is proportional to the initial extract percentage. The proportion of the extractable components in the final extract is directly proportional to the percentage of the original extract; conversely, a low original extract percentage suggests a low percentage of extractable components. The source and amount of an enzyme determine its extraction rate. Increasing the volume of the extraction solution may reduce specific enzyme activity because of slow extract production [23]. The results showed a specific enzyme activity of 0.988 U/mg after extraction using 1:8 ratio and sodium phosphate buffer (0.2 M) [17].
The interactions between concentration (0.162–0.2 M) and extraction time (5–28) are shown in Figure 3c.

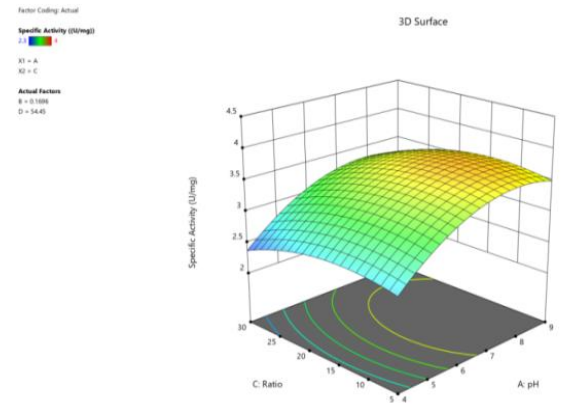
The maximum urease activity (3.99 U/mg) was achieved after extraction for 1 hour, which indicated that this time period was ideal for enzyme production. High concentrations of extraction buffer can damage urease activity because it contains too many ionic groups that can prevent enzymes from working [24]. Another study found that 0.2 M sodium phosphate buffer (pH 7.5) was the best value to extract urease from *Cannavalia enciforme*. In Figure 3E, the ideal extraction conditions were a ratio of 10–15 and an extraction time of 5–28 min. The optimal extraction time for each source of urease enzyme should be determined because of variations in materials present and their potential interferences with the enzyme. Moreover, contaminants should be eliminated because they generate a protein extract with a high resistance to decomposition [18]. The present results were higher than those obtained in another work that blended yellow lupine for 120 minutes [25].
Figure 4a demonstrates the precision of the model by indicating the close relationship between the forecast derived from the RSM model and the empirical data. Figure 4b presents the optimal condition, which includes a pH of 7.99, a buffer concentration of 0.2 M, an extraction ratio of 1:13 and an extraction time of 60 minutes, resulting in a desirability factor of 0.825. These conditions represent the most favourable combination for urease extraction.



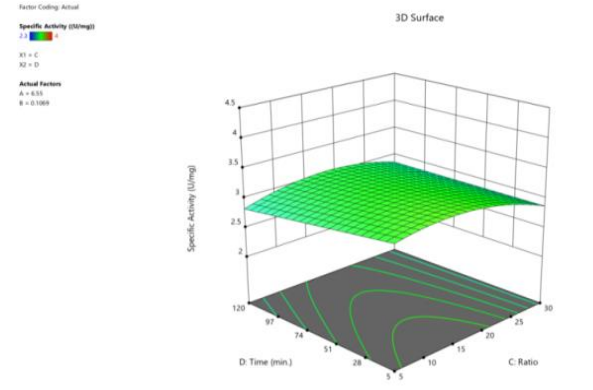
(A)



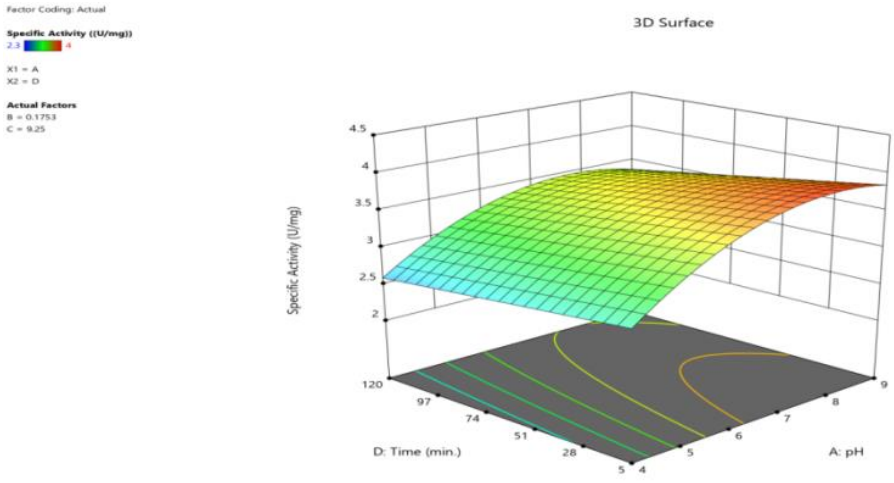
(B)



(C)



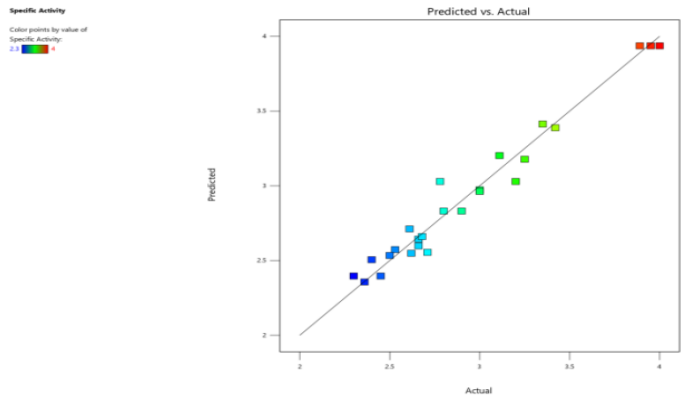
(D)



(E)

Fig. 1 3D surface plots for multiple interactive effects on enzyme extraction

(a)



(b)

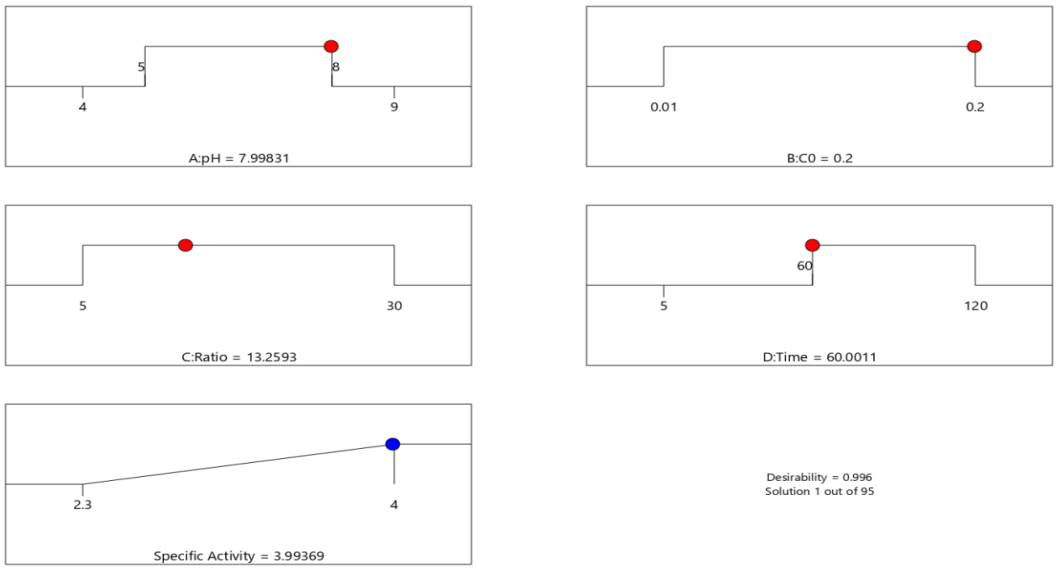


Fig. 2 (a) Actual experimental data in comparison with the projected data; (b) results of optimisation analysed using the desirability function.

4. Conclusion

This study mainly aimed to ascertain the most effective method for isolating urease from chickpea seeds. RSM and CCD were used to implement the experimental design. Urease obtained from the seeds of chickpea plants had a high enzyme activity (3.99 U/mg protein) when extracted under the optimal conditions of pH of 7.99, a tris base buffer concentration of 0.2, an extraction time of 60 minutes and a ratio of 1:13 (gm: ml). The experimental data demonstrate a superior level of quality. By employing a second-order polynomial model, our analysis yielded a forecasted correlation coefficient (pred. R_2) of 0.8477, an adjusted correlation coefficient (adj. R_2) of 0.9333 and a correlation value (R_2) of 0.9665. This finding confirms a strong agreement between the anticipated theoretical models and the empirical evidence obtained through experimentation. This method has potential for cost-effective enzyme synthesis through large-scale industrial production and a financially viable alternative to existing methods.

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تحديد الظروف المثلى لاستخلاص اليورياز من بذور الحمص باستخدام Design Expert (Software).

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المستخلص

هدفت هذه الدراسة الى التحقق من منهجيه سطح الاستجابة (RSM)لتقيم الظروف المثلى لاستخلاص انزيم اليوريز من مصدر نباتي وتقيم خواصه الكيموحيوية حيث تم اختيار بذور الحمص كمصدر لانزيم اليوريز. اظهرت النتائج ان بذور الحمص امتلك اعلى فعالية انزيمه 3.99 وحدة /ملغم بروتين عند افضل الظروف استخلاص على التوالي (برقم هايدروجيني 7.99, تركيز 0.2 من المخزن المؤقت لقاعدة تريس, زمن 60 دقيقه, نسبه 1:13(غرام:مل).وبين ان البيانات التجريبه هي افضل تركيبها للنموذج متعدد الحدود من المرتبة الثانية مع عامل الارتباط المتوقع (R_2 .pred)هو 0.8477,معامل الارتباط المعدل (R_2 .Adj)هو 0.9333وقيم الارتباط R_2 0.9665وهذا يدل على ان النماذج المتوقعة والقيم التجريبية هي في اتفاق جيد.وهذه الطريقة واعده ويمكن الاستفادة منها في المستقبل ,إضافة إلى ذلك ، تحسين فعالية تكلفة هذه الإنزيمات من خلال الإنتاج الضخم ، مما يجعلها خيارًا أكثر جدوى من الناحية الاقتصادية مقارنة بالطرق الأخرى.