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Application of the Response Surface Methodology for the Optimization of the Aqueous Enzymatic Extraction of *Pistacia Khinjuk* Oil

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ABSTRACT: Aqueous enzymatic extraction of oil from pistacia khinjuk was performed using cellulase. The central composite design was used to optimize the parameters that are significant to the process. The influence of three regressors on the percentage of oil recovery from seed was evaluated using second-order polynomial multiple regression model. Analysis of variance showed a high coefficient of determination (R^2) value of 0.99 indicating a satisfactory adjustment of the regression model to the experimental data. The positive sign for the coefficient of the interaction between pH and reaction time on the response indicated that a simultaneous increase in the pH and the reaction time led to an increase in oil recovery percentage. The optimum condition are as follows: temperature of 60 °C, pH of 9, reaction time of 20 h and oil extraction recovery of 74.93%.

Keywords: *Cellulase, Central Composite, Design and Response Surface Methodology, Enzymatic Extraction, Pistacia khinjuk, Seed Oil.*

Introduction

Pistacia is a genus of flowering plants belonging to the family Anacardiaceae (Shuraki and Sedgley, 1994), that comprises 11 species (Zohary, 1996). Among them, Pistacia vera L., Pistacia atlantica subsp. mutica (Fisch. & C. A. Mey.) Rech. f. (Pistacia mutica), and Pistacia khinjuk Stocks, are the native species to Iran (Razavi, 2006), that only P. vera has economical importance and its cultivation, as a traditional nut crop, extends to the dry land areas of the country. P. vera and P. khinjuk are the most primitive species and also postulated that P. khinjuk was directly descended from P. vera (Zohary, 1996) as a bridge to other Pistacia species

Iran is the world's largest producer of Pistacia spp., with over 44% of the world production. Most of the production is from orchards that account for 53% of world planted, but there are a few places, such as in the Zagros Mountains, where wild pistachio persists in natural and extensively managed (i.e., semi-natural) stands (Razavi, 2006). They are the most important types of pistachio and for this reason, Iran is known as the origin of pistachios. Therefore the Pistacia khinjuk seed would be as a novel source of the plant oil for the pharmaceutical industries.The oil obtained from P. khinjuk seed showed an antihelminthic effect against

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protoscoleces of E. granulosus and anti-echinococcal activity. The oil from plant seeds is conventionally extracted either by mechanical pressing or solvent extraction (Mani *et al*., 2007; Shojaei *et al*., 2011). Mechanical pressing is a very inefficient process with low oil recovery.

In spite of its higher efficiency in the range of 90–98%, solvent extraction (SE) suffers a poor quality protein in oil cake (meal), and it requires high investment and energy requirements. The commercial solvent for SE process is hexane that is listed among the hazardous air pollutants associated with neurological and respiratory disorders with prolonged exposure (the International Standard Organization permits only 50 ppm residual hexane in the oil seed meal)(Sharma *et al*., 2001). Hence, there is a need to explore a safe and efficient oil extraction alternative process.

Aqueous enzymatic extractions could serve as a potential alternative in oil industries due to high specificity and low operating temperatures. These are the reasons that make the enzyme process more economical for oil extraction processes (Rosenthal *et al*., 1996). The enzymes break down the cell structure of plants. The cell wall of plants consists mainly of pectic substances, cellulose, hemicelluloses, lignin and protein, whereas lipid bodies are enveloped in a lipoprotein layer. Enzymes like cellulase, himicellulase and pectinase break down the cell, while proteases permeabilize the liposome membrane and facilitate oil release from the oil body (Fullbrook, 1983; Rosenthal *et al*., 1996). Aqueous enzymatic oil extraction is based on simultaneous isolation of oil and protein from the oil seed by dispersing finely ground seed in water and separating the dispersion by centrifugation into oil, solid, and aqueous phases. Dobozi et al. reported treatment of mustard seeds with cellulolytic enzymes resulted in an increase (20–30%) in oil yield (Dobozi *et al*., 1988). Optimization of the enzymatic treatment during aqueous oil extraction with cellulases from sunflower seeds has been reported by Sineiroa et al. (Sineiro *et al*., 1998). Latifa et al reported oil and protein extraction from sesame seeds during an enzyme-assisted aqueous extraction process (Latif and Anwar, 2011). Extraction of oil from watermelon seeds by aqueous enzymatic extraction method has been studied by Sui et al. and obtained the optimum parameters form single-factor experiments and response surface methodology (Xiaonan et al., 2011). Extraction of oil from peanut with a ultrasonic-assisted aqueous enzymatic extraction by response surface method has been studied (Li *et al*., 2011a). Najafian et al found that oil extraction from olive can be enhanced by enzymatic hydrolysis and demonstrated that pre extraction enzyme digestion increases cellular degradation and significantly increases oil recovery upon extraction (Najafian *et al*., 2009). An aqueous enzymatic extraction of peanut oil and protein has been studied by Jian et al (Jiang *et al*., 2010). Hadj-Taieb et al studied the effect of enzymatic formulation on Tunisian olive oil extraction yields (Hadj-Taieb *et al.*, 2012). Optimization of the aqueous enzymatic extraction of pine kernel oil by response surface methodology and extraction of olive oil using enzymatic formulations during malaxation has also been reported (Yang *et al*., 2011).

Previous studies have not been carried out on the aqueous enzymatic extraction of oil from Pistacia khinjuk. This study was conducted to optimize the process parameters (temperature, reaction time and pH) of enzymatic extraction of oil from the Pistacia khinjuk by cellulase with RSM, and to find the optimum operating conditions that maximize the oil recovery.

Materials and Methods

Pistacia Khinjuk seeds were purchased from the local market in Iran. The seeds

wrapped in plastic bags and stored at 4°C until use. Seeds were ground and screened to select the fraction size. All the chemicals used were from Merck (Darmstadt, Germany) or Sigma–Aldrich (Buchs, Switzerland) Chemical Companies. Cellulase preparation from *Aspergillus niger* was obtained from Sigma Chemical Company.

- *Aqueous extraction of Pistacia Khinjuk*

Pistacia Khinjuk was dispersed in distilled water to prepare a slurry at a ratio of 1:6 w/v using a flask. The pH of slurry was adjusted to the desired value with 0.1 N NaOH or 0.1 N HCl, and was stirred by a magnetic stirrer at 250 rpm for 30 min. The enzymes were added at different concentrations, and the samples were incubated at differant temperatures, times, with controlled speed of mixing. The samples were then incubated at constant temperatures. A shaker-incubator (DK-S1060, DAIKI SCIENCE CO.) was used for temperature-controlled shaking of the sample solutions, followed by centrifugation (10000g, 30 °C) for 20 min (MIKRO 200, HETTICH) yielding three distinct phases (i) an oil phase, (ii) creamy phase and (iii) aqueous phase. The upper oil layer was separated and weighed. Oil recovery was expressed relative to that obtained by Soxhlet extraction with hexane.

$$\%\ \text{oil recovery} = \frac{weight\ of\ oil\ extracted \times 100}{\text{total weight of oil estimated by soxhlet method}}$$

The total amount of extracted oil was determined with Soxhlet apparatus following the standard AOAC standard procedure (Horowitz, 1984). All experiments were repeated three times to render mistakes during experiments.

- *Experimental design*

As shown in Table 1, a CCD in the form of the 2^3 full factorial design was used, in which three independent variables were converted to dimensionless ones (x_1, x_2, x_3,), with the coded values at 3 levels: −1, 0, +1. The arrangement of CCD as shown in Table 2 was in such a way that allows the development of the appropriate empirical equations (second order polynomial multiple regression equations) (Lapin, 1997; Tomaino *et al*., 2010):

The predicted response (y) was therefore correlated to the set of regression coefficients (β): the intercept (β_0), linear (β_1, β_2, β_3), interaction (β_{12}, β_{13}, β_{23}) and quadratic coefficients (β_{11}, β_{22}, β_{33}). The "Design expert" (Trial version 5) was used for regression and graphical analyses of the obtained data.

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Results and Discussion

- *Effect of hydrolysis time on oil recovery*

Enzymatic extraction of oil from Pistacia Khinjuk seeds were subjected to different durations of incubation (4-24hr). The effect of different incubation period on the recovery oil from Pistacia seed is given in Fig.1. The results show that the oil recovery increased with prolonged enzymatic extraction time and after 16 hours the increase was not significant. Oil recovery of 70% was observed within the first 16 hours of incubation with slight increase of 1.3% afterwards. Similar observations have been reported in literature (Li *et al*., 2012; Sharma *et al*., 2001; Yang *et al*., 2011). It has also been reported that the optimal duration of the enzymatic extraction process was about 18 h (Sharma *et al*., 2001).Sharma *et al* showed that the minimum incubation time to achieve maximum oil recovery from rice bran by enzyme-assisted aqueous extraction was about 18 h (Li *et al*., 2012).

Table 1. Independent variables and their levels for the central composite design used in the present study

Variable	Symbol	Coded variable levels		
		−1	0	1
Temperature	x_1	40	50	60
pH	x_2	4	6.5	9
Extraction time	x_3	4	12	20

Table 2. Arrangement of the Central Composite Design for the three independent variables used in the present study along with the theoretically predicted values for the four response variables. The actual values are also given in the parenthesis

Experiments no.	Temperature (x_1)	pH (x_2)	Reaction time (x_3)	oil recovery	
				Experimental	Predicted
1	-1	-1	-1	16.10	16.23
2	1	-1	-1	20.05	21.09
3	-1	1	-1	19.69	21.06
4	1	1	-1	24.91	25.92
5	-1	-1	1	24.52	24.34
6	1	-1	1	29.94	29.20
7	-1	1	1	69.94	69.65
8	1	1	1	74.93	74.51
9	-1	0	0	29.35	29.43
10	1	0	0	35.11	34.29
11	0	-1	0	28.01	28.41
12	0	1	0	54.95	53.48
13	0	0	-1	15.30	11.99
14	0	0	1	38.03	40.34
15	0	0	0	30.22	31.86
16	0	0	0	31.08	31.86

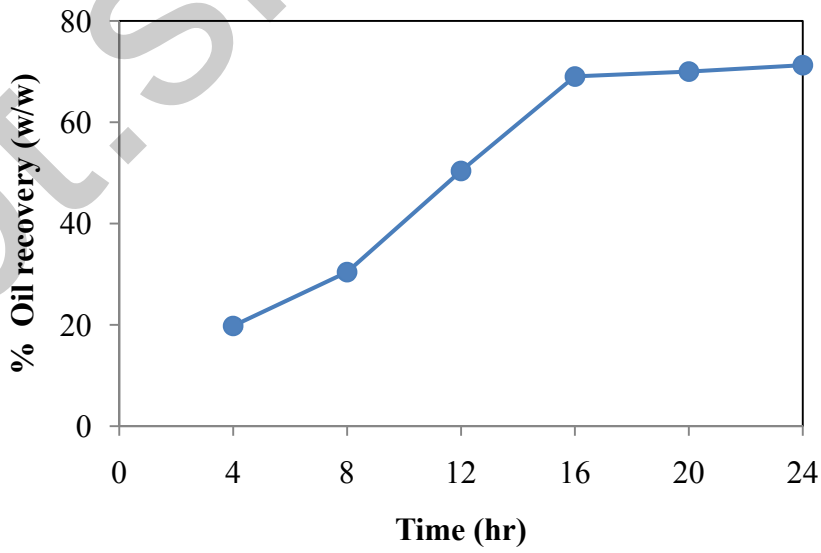

Fig. 1. Effect of incubation time on the oil recovery by enzymatic aqueous extraction of Pistacia khinjuk (pH 8, 50°C, 80rpm)

- *Influence of pH on oil recovery*

In order to assess the effect of pH on oil recovery, the enzymatic extraction was carried out at different pH values in the range of 4-9 by adding the desired amount of 0.1N HCl or 0.1 N NaOH into the slurry. The mixture was then incubated overnight at 50°C with constant mixing at 80 rpm. As shown in Figure 2 the oil recovery with aqueous enzymatic extraction by cellulase increased along with the increase in pH. Sharma et al reported that the optimum value of pH to achieve maximum oil recovery from peanut seeds by protease was in the range of 7-10 (Sharma *et al*., 2001). similar result was reported by Jian et al that found optimum pH of 9.5 for aqueous enzymatic extraction of peanut oil (Jiang *et al*., 2010).

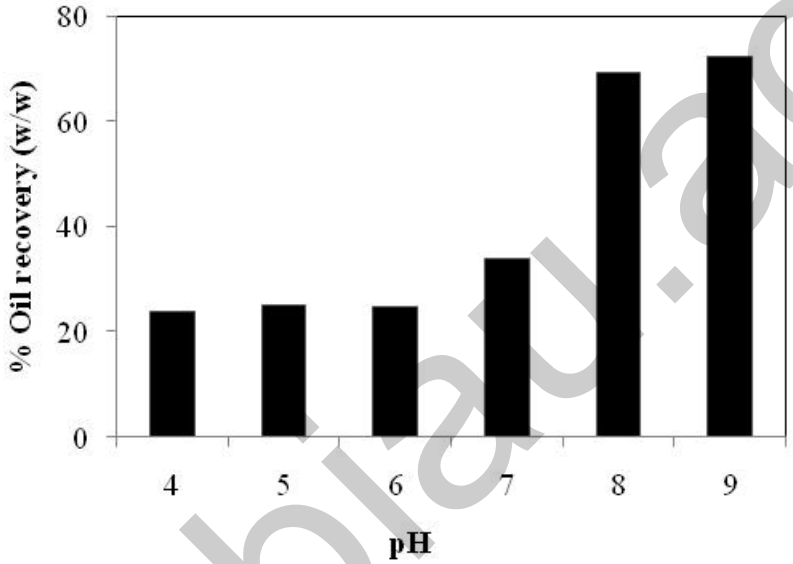

Fig. 2. Effect of pH on the enzymatic oil extraction from Pistacia khinjuk.
The enzyme mixture was then incubated overnight at 50°C with constant mixing at 80 rpm

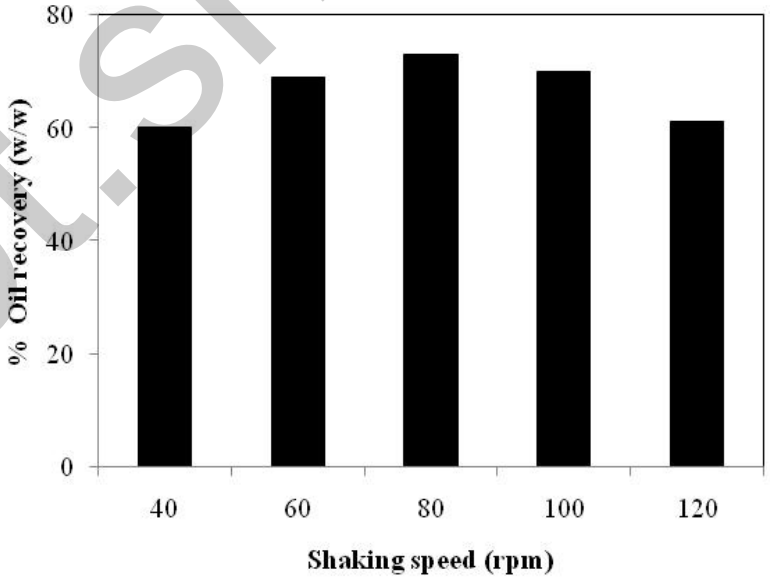

Fig. 3. Effect of mixing speed on the enzymatic oil extraction from Pistacia khinjuk by aqueous Enzyme Extraction (pH 8, overnight incubation at 50°C)

- *Effect of mixing speed on enzymatic oil recovery*

Mixing speed has been identified as another parameter that could significantly affect the oil recovery via enzymatic extraction. The effect of mixing speed on oil recovery from *P. Khinjuk* was studied in the range of 40-120 rpm. Figure 3 shows the effect of mixing speed on the oil recovery. Increasing the mixing speed from 40 to 80 rpm led to an increase in the oil recovery. It was observed that the oil obtained at 80 rpm was clear, whereas increased more than 80 rpm oil recovery was reduced. Increasing the speed led to the formation of a clearly visible emulsion of the oil layer at the top of decanter. Sharma *et al* also found decreasing in mixing speed led to a decrease in oil recovery from peanut seeds (Sharma *et al*., 2002).

- *Central composite design*

In this work, the relationship between percentage of oil recovery and three controllable factors (namely temperature, pH and extraction time) were studied. A CCD is shown in Table 3 allows the development of mathematical equations where each response variable y is assessed as a function of temperature (x_1), pH (x_2) and extraction time (x_3) and calculated as the sum of a constant, three first-order effects (terms in x_1, x_2 and x_3), three interaction effects (terms in x_1x_2, x_1x_3 and x_2x_3) and three second-order effects (x_1^2, x_2^2 and x_3^2) as expressed in Eq. (1). The results obtained were then analyzed by ANOVA to assess the "goodness of fit". The models for oil recovery is significant as determined via the F-test at the 5% confindence level (Prob > F < 0.05). Only the statistically significant terms were included in the model. β_{12}, β_{13} and β_{11} coefficient were non-significant.The following final empirical model (equations in terms of actual parameters for the regressors) was used to quantitatively describe the effects of temperature, pH and extraction time on the characterization of aqueous enzymatic oil extraction:

$$\begin{aligned}\%\ \text{oil reovery} = {} & 53.92 + 0.253X_1 \\ & - 20.07X_2 + 0.574X_3 \\ & + 0.512X_2X_3 + 1.458X_2^2 \\ & - 0.089X_3^2\end{aligned}$$

The coefficient of multiple determinations (R^2) is 0.994 that indicate a satisfactory adjustment of the quadratic model to the experimental data. Therefore, the empirical equations are adequate to represent the relationship between the variables.

As shown in Table 4, the coefficient of variance (CV) has been found to be 5.11 %. The CV is (the ratio of the standard error of estimate to the mean value of the observed response) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if its CV is less than 10% (Vining, 2003). This model has high R^2 value and shows no lack of fit. By applying diagnostic plots such as normal probability plot of residual, plot of residuals versus predicted values independence and randomness of the residuals were satisfied. The adequate precision value is a measure of the "signal to noise ratio" and was found to be 54.76, that indicates an adequate signal (see Table 4). A ratio >4 is desirable (Vining, 2003). The predicted models can be used to navigate the space defined by the CCD.

The relative contribution of each factor to the dependent variable (oil recovery) was directly measured by the respective coefficient in the fitted model. A positive sign for the coefficients (β_1, β_2 and β_3) in the fitted models indicates that the level of oil recovery increased with increase in the levels of factors. The greatest coefficient (β_3 = 14.13) in the fitted model reveals the high sensitivity of the time in enzymatic extraction. On the other hand, the lowest β_1s was obtained that indicates that the oil recovery is not significantly affected by the temperature.

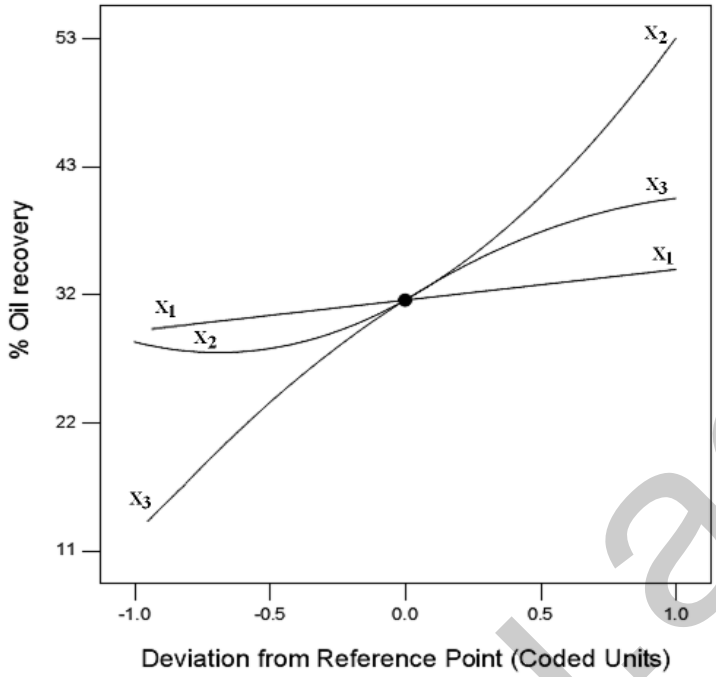

Fig. 4. Perturbation graph showing the effect of each of the independent variables on oil recovery while keeping other variables at their respective mid-point levels. (x1) Temperature, (x2) pH and (x3) Reaction time.

Table 3. Regression coefficients of the second order polynomial model for response variables

Factor	Estimated Coefficient	
	Coded Factors	Actual Factors
Constant	31.77**	54.23**
Linear		
Temperature (x_1) (^oC)	2.53*	0.304*
pH (x_2)	12.58**	-20.35**
Reaction time (x_3) (h)	14.13**	0.465**
Interaction		
$x_1 \times x_2$	0.10	4.192
$x_1 \times x_3$	0.15	1.936
$x_2 \times x_3$	10.24**	0.512**
Quadratic		
Temperature (x_1) (^oC)	-0.10	-1.011
pH (x_2)	9.15**	1.464**
Reaction time (x_3) (h)	-5.67*	-0.089*

* $p<0.05$

** $p<0.0001$

Table 4. Analysis of variance (ANOVA) of RSM for enzymatic extraction

Source	SS[a]	DF[b]	MS[c]	F-value	Prob>F
Model	4734.8	6	789.13	263.04	< 0.0001
Residual	27	9	3.00		
Lack of Fit	26.63	8	3.33	9.00	
Pure Error	0.37	1	0.37		
Total	4761.77	15			
R^2	0.994			Adj-R^2	0.990
C.V%	5.11			PRESS	84.22

[a] SS, sum of squares
[b] DF, degree of freedom
[c] MS, mean square

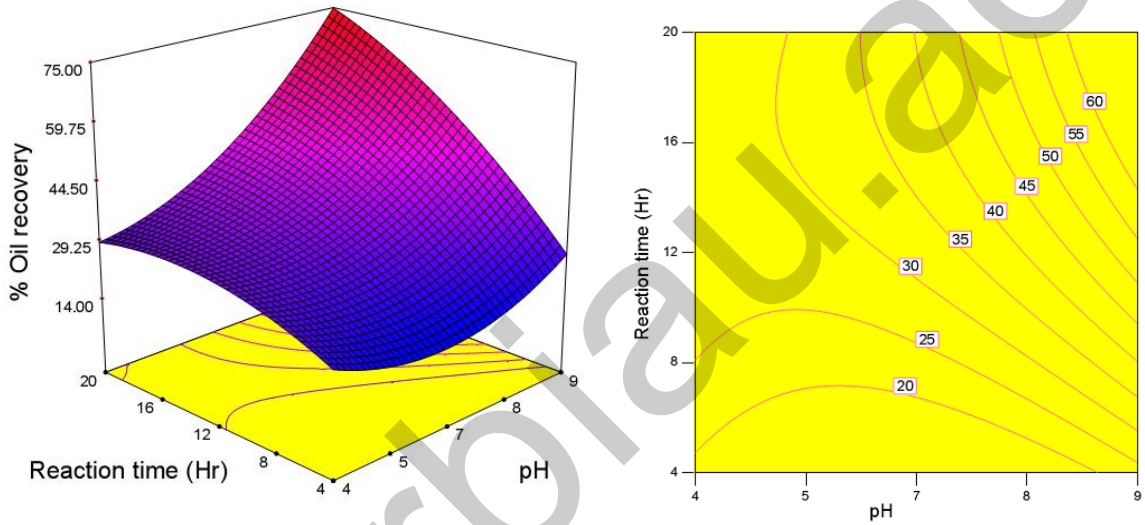

Fig. 5. Response surface and contour plots for the effect of reaction time and pH on the oil recovery, (Temperature=60 °C)

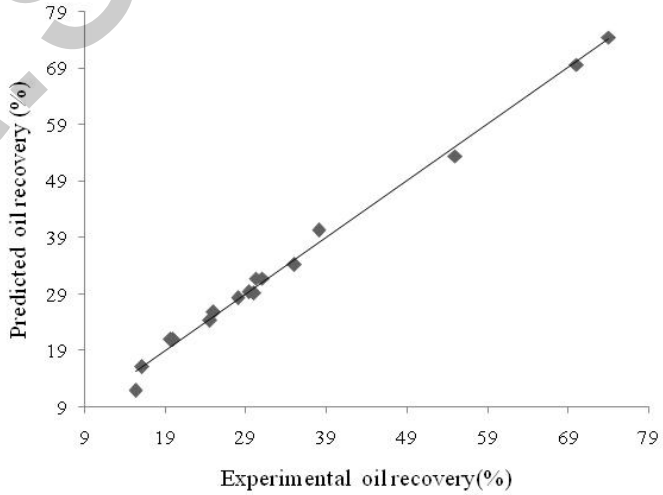

Fig. 6. Parity plot for the experimental and predicted value of oil recovery (%)

The perturbation plot, Fig. 4 shows the effects when all factors at the center point in the design space are compared. The perturbation plot assists the comparison of the effects of all factors at a particular point in the design space, when the factor curvature is sharper, the effect of factor is more important to the response. The plot was obtained for 50°C temperature, pH of 6.5 and reaction time of 12 hours. In order to gain a better understanding of the results, the predicted model is presented in Fig. 5 as the three-dimensional response surface plots contour plots. The effect of pH and reaction time on the oil recovery at fixed temperature at 60°C (the center point of this experiment) was shown in Fig 5. There was significant interaction between pH and reaction time. The pH has a slightly positive effect on the oil recovery. Similar result was also reported by Jiang et al. that found pH significantly affected the peanut oil recovery using enzymatic extraction (Jiang *et al*., 2010). In addition, reaction time positively affected the oil recovery throughout the experiment. In the models for the oil recovery, x_3 was identified as the major regressor variable affecting the responses (greatest coefficients, β_3=14.13) and oil recovery increases almost linearly as reaction approaches its peak (at 20hr). Studies concerned with the oil recovery using enzymatic extraction indicated that there was significant interaction between temperature and reaction time. Generally, reaction time has a positive effect on the oil yield (Li et al., 2011b).

Fig. 6 shows that the response of the oil recovery was very sensitive to the reaction time, followed by pH, and finally, by temperature. For a model to be reliable, the response should be predicted with a reasonable accuracy by the model when compared with the experimental data .Fig.6 compares the experimental oil recovery with the predicted values obtained from the model. The figure indicated good agreements between the experimental and predicted values of oil recovery. Second-order polynomial models obtained in this study were utilized for each response in order to determine the specified optimum conditions. By applying the method of desirability function, the optimum oil recovery condition was determined as follows: temperature of 60 °C, pH of 9 and a reaction time of 20 h. At this point, extraction of oil recovery was calculated at 74.65%.

Conclusion

In this work the enzymatic Extraction of oil from *Pistacia khinjuk* seed by cellulase was studied using CCD and RSM. The following conclusions could be made:

- There is a quadratic correlation between oil recovery and temperature. This is evidenced when oil recovery reaches its peak at the center point (60 °C) followed by a steady decrease afterward. With respect to pH, the oil recovery increased as the pH of aqueous solution was increased from 4 to 9.
- With respect to time, oil recovery increased as reaction time progressed from 4 to 20h.
- Optimum conditions for process could be achieved by setting temperature at 60°C, pH at 9 and reaction time of 20 hours.

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