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ORIGINAL PAPER

Enzyme-Assisted Aqueous Extraction of Kalahari Melon Seed Oil: Optimization Using Response Surface Methodology

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Abstract Enzymatic extraction of oil from Kalahari melon seeds was investigated and evaluated by response surface methodology (RSM). Two commercial protease enzyme products were used separately: Neutrase® 0.8 L and Flavourzyme® 1000 L from Novozymes (Bagsvaerd, Denmark). RSM was applied to model and optimize the reaction conditions namely concentration of enzyme (20–50 g kg^{-1} of seed mass), initial pH of mixture (pH 5–9), incubation temperature (40–60 °C), and incubation time (12–36 h). Well fitting models were successfully established for both enzymes: Neutrase 0.8 L (R^2 = 0.9410) and Flavourzyme 1000 L (R^2 = 0.9574) through multiple linear regressions with backward elimination. Incubation time was the most significant reaction factor on oil yield for both enzymes. The optimal conditions for Neutrase 0.8 L were: an enzyme concentration of 25 g kg^{-1}, an initial pH of 7, a temperature at 58 °C and an incubation time of 31 h with constant shaking at 100 rpm. Centrifuging the mixture at 8,000*g* for 20 min separated the oil with a recovery of 68.58 ± 3.39%. The optimal conditions for Flavourzyme 1000 L were enzyme concentration of 21 g kg^{-1}, initial pH of 6, temperature at 50 °C and incubation time of 36 h. These optimum conditions yielded a 71.55 ± 1.28% oil recovery.

Keywords Enzyme-assisted aqueous extraction · Kalahari melon seed oil · Optimization · Response surface methodology · Central composite design (CCD) · Neutrase 0.8 L · Flavourzyme 1000 L

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Introduction

Industrial processes for the extraction of edible oil from oilseeds generally involves a solvent extraction step, sometimes preceded by pressing. Safety considerations on the use of organic solvents prompted attempts in the past to develop aqueous extraction but these were unsuccessful mainly due to the low oil yields [1–3]. Recently, interest in aqueous extraction processes has been revived due to need for environmentally cleaner alternative technologies for oil extraction.

Application of aqueous enzymatic in oil extraction is undoubtedly an emerging technology in the fats and oil industry since it offers many advantages compared to conventional extraction. For instance, it eliminates solvent consumption, which reportedly may also lower investment costs [4, 5] and energy requirements [4]. Also, it enables simultaneous recovery of oil and protein from most oilseeds and the process yields oil of good quality complying with Codex specifications [6]. The need for further degumming operations is reduced and the process allows ready removal of some toxins or antinutritional compounds from certain oilseeds [6]. In this sense, some of the needs triggering technology innovation in the oil extraction such

as cost savings, environmental and safety concerns, and nutrition issues seem to be achievable by successful development of aqueous enzyme-based processes.

The economics of enzyme-assisted aqueous oil extraction have been previously compared with solvent-based extraction, which involves a high capital cost to install [7]. It was determined that (1) if market rates for product oil are high, the enzyme-assisted oil extraction process can compete favorably with the conventional approach; and (2) if immobilized (reusable) forms of enzyme are used, recycling the enzyme can considerably reduce the cost.

Kalahari melon (*Citrullus lanatus*) is an important source of water in the Kalahari during dry months of the year when no surface water is available. In the Kalahari region, the fresh Kalahari fruits are used as a stock feed in times of drought [8]. Seeds are roasted and ground into meal, a nutritious food with a pleasant nutty taste. In addition, leaves and young fruits are utilized as green vegetables. The medicinal uses of Kalahari melon have been reported and these include treatment of worms, renal stones, dropsy, alcohol poisoning, and diabetes, and as a purgative and emetic. In West Africa, there is a sizeable industry in the production of seeds from Kalahari melon, with annual production figures in the range of 250–300,000 tonnes of seed [8]. Conventionally, Kalahari melon seeds are deoiled by cold pressed ($T < 60$ °C) and no solvents or chemicals are used in Africa. Kalahari melon seed oil is particularly interesting to the cosmetic industry where it is used by a number of prominent European cosmetics companies for moisturizing, regenerating and restructuring skin-care formulations. Oil of Kalahari melon seeds is also used as cooking oil in some countries in West Africa and the Middle East [9].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. It also has important applications in design, development and formulation of new products, as well as improvement of existing product designs [10]. Response surface models may involve main effects and interactions or have quadratic and possibly cubic terms to account for curvature. It has been successfully utilized to optimize the enzymatic process for extracting oil and protein from rice bran [11]. Conventional methods (such as one factor at one time) have been applied previously to evaluate the use of enzymes to enhance oil recovery during aqueous extraction of oil [12]. However, these methods require a large number of experiments to describe the effect of individual factor, are time consuming, and no statistical method can be established to distinguish the interaction effects from main effects. Thus, the aim of this study was to optimize the oil recovery from Kalahari melon seed (*C. lanatus*) by enzymatic extraction. In order to obtain basic technological information for the enzymatic extraction process, the performance of the extraction operation through the response surface approach was used.

Materials and Methods

Materials

Kalahari melon (*C. lanatus*) seeds were obtained from Namibia. Two protease enzymes (Flavourzyme® 1000 L and Neutrase® 0.8 L) were provided by Novozymes (Bagsvaerd, Denmark). The enzyme activity unit for Flavourzyme 1000 L and Neutrase 0.8 L are 1000 leucine amino peptidase units per gram (LAPU/g) and 0.8 Anson units per gram (AU/g), respectively.

Methods

Experimental Design

RSM with central composite design (CCD) was employed to investigate the effect of enzyme-assisted aqueous extraction on oil yield from Kalahari melon seeds. Four independent parameters namely enzyme concentration, initial pH of mixture, temperature, and incubation time at three different levels each, were employed. The parameters chosen and their levels were based on preliminary experiments carried out in our laboratory. The experimental plan was designed and the results obtained were analyzed using Design Expert version 6.0 (Stat-Ease Inc., Minneapolis, MN) software to build and evaluate models and to plot the four-dimensional response surface curves. For this study, a total of 30 experiments was carried out. The experimental design consisted of sixteen (2^4) factorial points, eight extra points (star points) to form a faced-centered CCD and six replicates for the center point. Optimization was performed using a faced CCD with an alpha value of ± 1.00 for four factors. The experiments were run in random order to minimize the effects of unexpected variability in the observed responses due to extraneous factors.

Aqueous Enzymatic Oil Extraction from Kalahari Melon Seed

Kalahari melon seeds were ground in a blender (MX-291-N, National, Selangor, Malaysia). The seeds were ground in large quantities and kept in a cold room (−20 °C) prior to suspension preparation. The ground seeds used in the extraction experiments were between 0.6 and 1.0 mm. The suspension was prepared with powdered Kalahari melon seeds (5 g) in 30 mL distilled water. Flavourzyme 1000 L or Neutrase 0.8 L was added after the pH of the suspension

was adjusted. The suspension was then incubated at the desired temperature with constant shaking at 100 rpm for the specified duration. The stir rate of 100 rpm was determined experimentally because it produced an oil-rich phase that was not emulsified. The upper oil phase was collected after centrifugation at 8,000*g* for 20 min and weighed.

Enzyme concentration, initial pH of mixture, incubation temperature and incubation time were varied from 20–50 g kg^{-1}, pH 5–9, 40–60 °C, and 12–36 h, respectively. Table 1 shows the matrix of faced CCD for Flavourzyme 1000 L and Neutrase 0.8 L. Oil recovery was calculated as the percentage oil (w/w) obtained with respect to the total oil present in the Kalahari melon seed:

$$\% \text{ Oil recovery, } Y = \frac{\text{Weight of oil extracted} \times 100}{\text{Total weight of oil estimated by Soxhlet method}} \quad (1)$$

The total amount of Kalahari melon seed oil was determined by solvent extraction using petroleum ether in a Soxhlet apparatus following the standard AOCS [13] procedure and was found to be 304.9 ± 8.0 g kg^{-1} of Kalahari melon seed. By using the optimizer function of Design Expert, optimized reaction conditions were generated. In order to confirm the predicted results of the optimized model, experiments using the reaction conditions representing this optimum point were performed in triplicate.

Results and Discussion

It is important to point out that the extraction of oil from Kalahari melon seed by an aqueous process absolutely requires an enzyme, as no oil was released in the control experiments without an enzyme. This may be due in part to the size of ground seeds used in the extraction experiments being between 0.6 and 1.0 mm, not sufficiently small to release the oil without an enzyme. Kalahari melon seeds have a high oil content, which makes it difficult to reduce their size. Further grinding was avoided in order to prevent adhesion of particles and to avoid sieving [14]. Efficient grinding which breaks down the walls of the oil-containing cell is considered essential [15, 16]. Smaller particle size allows not only the easier diffusion of water-soluble components, thereby disintegrating the original structure and facilitating oil release, but also enhances enzyme diffusion rates which can then more easily act on the substrates.

Table 1 shows the experimental data and the observed response values with different combinations of enzyme concentration (g kg^{-1}) (X_1), initial pH of mixture (X_2), incubation temperature (°C) (X_3) and incubation time (h) (X_4) for aqueous enzymatic oil extraction by Neutrase 0.8 L and Flavourzyme 1000 L. The yield is based on the amount of lipid extracted using solvent extraction. The oil recovery for aqueous enzymatic extraction was lower (<80%) than solvent extraction. This is consistent with the yields obtained from other nuts [11, 12]. This yield would be acceptable for an industrial process.

Model Fitting

The models were found to agree with the data at the probability level of 99%. The accuracy of the models was evaluated by coefficient of determination (R^2 and adjusted R^2 values). Adjusted R^2 is a measure of the amount of variation with respect to the mean explained by the model. Values of R^2 and adjusted R^2 for the enzyme Neutrase 0.8 L and Flavourzyme 1000 L were 0.9410 and 0.9144, 0.9574 and 0.9412, respectively. The results indicated that the models explain 94% and 96% of the variability for enzyme-assisted aqueous extraction by Neutrase 0.8 L and Flavourzyme 1000 L, respectively. Analysis of variance (Tables 2 and 3) also shows that the regression models for aqueous enzymatic oil extraction by Neutrase 0.8 L and Flavourzyme 1000 L were statistically good with a significance level of $P < 0.0001$ and the models had no significant ($P > 0.05$) lack of fit. The *F*-ratio in this table is the ratio of the mean square error to the pure error obtained from the replicates at the design center. The significance of the *F*-value depends on the number of degrees of freedom (DF) in the model, and is shown in the *P*-value column (95% confidence level).

Neutrase 0.8 L-assisted Aqueous Extraction

Table 4 shows that aqueous enzymatic oil extraction by Neutrase 0.8 L was positively affected by all four reaction parameters. Among all parameters, the incubation time (X_4) most strongly affected the aqueous enzymatic oil extraction, followed by concentration of enzyme (X_1), initial pH of mixture (X_2) and incubation temperature (X_3).

Although incubation temperature (X_3) had an insignificant ($P > 0.05$) effect on oil recovery, it was not removed by backward elimination in order to maintain the hierarchy of the model. In addition, the quadratic terms for initial pH (X_2^2) of mixture and incubation time (X_4^2) had negative effects on oil recovery.

Table 4 shows that among the three significant ($P < 0.05$) interaction parameters, interaction between temperature and time (X_3X_4) had the greatest effect on oil recovery. Response surface was generated based on the second-order equation after backward elimination:

Table 1 Experimental data and the observed response values with different combinations of enzyme concentration (g kg^{-1}) (X_1), initial pH of mixture (X_2), incubation temperature (°C) (X_3) and incubation time (h) (X_4) for aqueous enzymatic oil extraction by Neutrase 0.8 L and Flavourzyme 1000 L

Actual parameter values					Oil recovery (%)	
Run	X_1, Enzyme concentration	X_2, pH	X_3, Temperature	X_4, Time	Neutrase 0.8 L	Flavourzyme 1000 L
1	35	7	50	24	68.88	67.62
2	35	7	50	24	67.59	66.23
3	50	9	40	12	61.18	54.27
4	20	5	40	36	51.53	61.16
5	50	7	50	24	70.90	68.60
6	35	7	40	24	66.52	59.63
7	50	5	40	36	60.61	64.56
8	20	9	60	36	68.80	65.61
9	50	5	40	12	59.86	59.30
10	35	9	50	24	65.67	67.79
11	50	5	60	36	65.18	59.97
12	35	7	50	24	67.44	69.70
13	50	5	60	12	48.39	55.69
14	20	9	60	12	44.52	52.04
15	20	5	40	12	48.57	57.46
16	20	5	60	12	46.22	52.83
17	20	9	40	12	45.06	50.49
18	50	9	60	12	54.46	58.85
19	35	7	50	24	66.91	69.71
20	50	9	40	36	60.69	65.16
21	20	9	40	36	62.60	64.56
22	35	7	50	24	64.15	67.94
23	20	5	60	36	64.78	63.41
24	35	5	50	24	60.49	66.55
25	50	9	60	36	64.58	66.52
26	35	7	50	24	70.57	65.96
27	35	7	50	12	54.72	62.44
28	35	7	50	36	69.12	71.55
29	35	7	60	24	68.27	59.62
30	20	7	50	24	64.97	66.49

Table 2 Analysis of variance for the response surface quadratic model for aqueous enzymatic oil extraction by Neutrase 0.8 L

Source	Sum of squares	Degree of freedom	Mean square	F-value	$P > F$[a]
Oil recovery[b] model	1794.66	9	199.41	35.42	<0.0001
Residual	112.61	20	5.63		
Lack of fit	89.74	15	5.9	1.31	0.4105
Pure error	22.86	5	4.57		
Total	1907.27	29			
Coefficient of variation = 3.88%, $R^2 = 0.9410$					

[a] Defined by Eq. 1

[b] $P < 0.05$ indicates the statistical significance

$$Y = 67.68 + 2.71X_1 + 1.22X_2 + 0.48X_3 + 5.83X_4 - 4.90X_2^2 - 6.06X_4^2 - 1.64X_1X_3 - 2.26X_1X_4 + 3.06X_3X_4 \quad (2)$$

Flavourzyme 1000 L-assisted Aqueous Extraction

Among the four major reaction parameters, concentration of enzyme Flavourzyme 1000 L (X_1) and incubation time (X_4) had significantly ($P < 0.05$) positive effects on oil recovery, with incubation time (X_4) having the greatest effect (Table 4). Although initial pH of mixture (X_2) and incubation temperature (X_3) had an insignificant ($P > 0.05$) effect on oil recovery, they were not removed by backward elimination in order to maintain the hierarchy of the model.

Table 3 Analysis of variance for response surface quadratic model for aqueous enzymatic oil extraction by Flavourzyme 1000 L

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	$P > F$[a]
Oil recovery[b] model	900.57	8	112.57	59.03	<0.0001
Residual	40.05	21	1.91		
Lack of fit	26.91	16	1.68	0.64	0.7732
Pure error	13.14	5	2.63		
Total	940.62	29			
Coefficient of variation = 2.20%, $R^2 = 0.9574$					

[a] Defined by Eq. 1

[b] $P < 0.05$ indicates the statistical significance

Table 4 Regression coefficients and *P*-values for aqueous enzymatic oil extraction by Neutrase 0.8 L and Flavourzyme 1000 L after backward elimination

Neutrase 0.8 L			Flavourzyme 1000 L	
Variables[a]	Regression coefficients	*P*-values[b]	Regression coefficients	*P*-values[b]
Intercept	67.68	<0.0001	67.55	<0.0001
X_1	2.71	<0.0001	1.05	0.0041
X_2	1.22	0.0415	0.24	0.4650
X_3	0.48	0.4041	−0.11	0.7299
X_4	5.83	<0.0001	4.40	<0.0001
X_1^2	–	–	–	–
X_2^2	−4.90	0.0010	–	–
X_3^2	–	–	−8.04	<0.0001
X_4^2	−6.06	0.0001	–	–
X_1X_2	–	0.7767	–	–
X_1X_3	−1.64	0.0118	–	–
X_1X_4	−2.26	0.0011	−0.86	0.0207
X_2X_3	–	–	1.20	0.0070
X_2X_4	–	–	1.40	0.0023
X_3X_4	3.06	<0.0001	–	–

[a] Enzyme concentration (X_1), pH of mixture (X_2), incubation temperature (X_3) and incubation time (X_4)

[b] $P < 0.05$ indicates the statistical significance

The quadratic terms for temperature (X_3^2) also had negative effect on oil recovery. Lower temperatures resulted in lower extraction yields, but higher temperatures affected enzyme stability. Among the three significant ($P < 0.05$) interaction parameters, interaction between pH and incubation time (X_2X_4) had the greatest effect on oil recovery (Table 4). The response surface was generated based on the second-order equation after backward elimination:

$$Y = 67.55 + 1.05X_1 + 0.24X_2 - 0.11X_3 + 4.40X_4 - 8.04X_3^2 - 0.86X_1X_4 + 1.20X_2X_3 + 1.40X_2X_4 \quad (3)$$

Comparison of Neutrase 0.8 L- and Flavourzyme 1000 L-assisted Aqueous Extractions

By using the optimizer function of Design Expert, optimized reaction conditions were generated. The optimum point (70.97%) was produced with an enzyme concentration of 25 g kg^{-1}, an initial pH of 7, a temperature at 58 °C and an incubation time of 31 h.

In order to confirm the predicted results of the optimized model, experiments using the reaction conditions representing this optimum point were performed in triplicate. A validation experiment showed that the predicted value for enzyme Neutrase 0.8 L was 70.97 while the actual experimental result was 68.58 ± 3.39%. However, it must be noted that the reaction conditions for both predicted and actual data were slightly different and the difference is attributed to experimental error. A chi-square test was performed to verify the adequacies of the models established. Chi-square test showed that there were no significant ($P > 0.05$) differences between the observed and predicted values for Neutrase 0.8 L-assisted aqueous enzymatic extraction. The chi-square values for Neutrase 0.8 L-assisted aqueous enzymatic extraction (0.25) was much smaller than the cut off point (5.99) at α-0.05 and df-2.

For Flavourzyme 1000 L, an optimum point (71.14%) was produced with an enzyme concentration of 21 g kg^{-1}, an initial pH of 6, a temperature at 50 °C and an incubation time of 36 h. A value of 71.55 ± 1.28% ($n = 3$) was obtained. The good correlation between these two results verified the validity of the response model and the existence of an optimal point. A chi-square test showed that there were no significant differences ($P > 0.05$) between the predicted and observed values for Flavourzyme 1000 L-assisted aqueous enzymatic extraction. The chi-square value for Flavourzyme 1000 L (0.07) was smaller than the cut off point (5.99) at α-0.05 and df-2. Therefore, both models were adequate for predicting the oil recovery by Neutrase 0.8 L- and Flavourzyme 1000 L-assisted aqueous enzymatic extraction with high accuracy.

Decreasing the shaking speed led to a decrease in oil recovery. Increasing the speed led to emulsification and reduced the amount of clear oil obtained at the top [12]. The shaking speed 100 rpm was chosen in the above experiments because the oil droplets were bigger and could be clearly seen at the top of the reaction mixture, making it easier to recover and therefore, more oil was obtained. Increasing the speed to 120 rpm and beyond led to the formation of a clearly visible emulsion in the oil layer. The main difficulty found in the aqueous extraction process was the formation of an emulsion. Therefore, to increase the recovered yield in the aqueous extraction process, emulsion formation must be avoided. The oil released after

enzymatic reaction was recovered by centrifugation. The centrifuged suspension was separated into liquid and solid phases. The liquid phase consisted of three layers (free oil, emulsion and syrup of dissolved carbohydrates and proteins). The solid phase consisted of an upper layer of sedimented proteins and a lower layer of undestroyed cell debris [17].

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