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Combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean

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Abstract

The individual effect of two different enzymes—protease and cellulase—on oil and protein extraction yields combined with other process parameters—enzyme concentration, time of hydrolysis, particle size and solid-to-liquid ratio—was evaluated by Response Surface Methodology. The selection of the enzymes for the study was based on preliminary experiments that showed higher increments in the extraction yield with the use of the two enzymes when compared to hemicellulase and pectinase. The levels of the quantitative parameters studied were: i) enzyme concentration: 0.1, 0.45, 2 w/w %; ii) liquid-to-solid ratio: 0.05, 0.125, 0.2; iii) mean particle size: 212.5, 449.5, 855 μm; iv) time of hydrolysis: 30; 60; 120 min. Experimental data for both oil and protein extraction yields obtained with and without enzymes correlated very well with process parameters ($P < 0.0001$), resulting in models with high coefficient of determination for oil and protein extraction yields ($r^2 = 0.9570$ and $r^2 = 0.9807$, respectively). The use of protease resulted in significantly higher yields over the control (protein yield increased from 27.8 to 66.2%, oil yield increased from 41.8 to 58.7%) only when heat treated flour was used, or when non-heat treated flour with large particle sizes was used in the extraction. The yields of protein and oil from non-heat treated material in general decreased slightly with the use of enzymes. © 2001 Published by Elsevier Science Inc.

Keywords: Aqueous enzymatic extraction; Soybean protein; Soybean oil; Response surface methodology; Protease; Cellulase

1. Introduction

Industrial processes for the extraction of edible oil from oilseeds generally involve 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 intensification in the search for environmentally cleaner alternative technologies for oil extraction. In the case of soybean, an aqueous process can potentially result in: (1) the simultaneous production of edible oil and protein isolate or concentrate in the same process and (2) lower protein damage during extraction. It is worth noting that aqueous enzymatic extraction can also be applied to other processes involving soybean, for instance, the production of soymilk, high nutritional value soy beverages (containing protein hydrolysate), and soy protein concentrates (isolate or hydrolysate). A detailed investigation of the relationship between processing conditions and product quality is therefore necessary.

It has been reported that the low extraction yields of aqueous processes can be overcome by using enzymes that hydrolyse the structural polysaccharides forming the cell wall of oilseeds, or that hydrolyse the proteins which form the cell and lipid body membranes [4–6]. Rosenthal et al. [7,8] have reviewed the main aspects relating to aqueous and enzymatic processes for oil extraction [7], and have also studied the mechanisms involved in non-enzymatic aqueous extraction of oil and protein from soybean [8]. These studies reported that the disruption of the cell wall of soybean cotyledon during milling operation resulted in an increase in oil and protein extraction yields. Further, these studies also showed that the conditions that favoured protein extraction (pH, temperature, particle size, agitation rate) also favoured

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oil extraction. This can be explained on the basis of the observation that lipid bodies enmeshed in the cytoplasm, which is predominantly composed by protein, are themselves surrounded by proteinaceous membranes (mainly oleosins) [9–12].

In principle, it should therefore be possible to achieve an improvement in oil yield by using enzymes, especially, proteases. Even though the use of certain proteases and cellulases have been reported to improve the yield of aqueous processes [4,13–20], a detailed examination of the use of enzymes in relation to other operational parameters has not yet been undertaken. This study aims to establish how process variables influence extraction yield especially when cell-wall degrading enzymes (mainly protease and cellulase) are added to aqueous extraction media.

2. Materials

2.1. Soybean

Soybean flour was purchased from Spillers Premier Products (Cambridge, England). Two different types were used in the experiments: a whole full-fat non-heat treated flour, and another which had been previously subjected to heat treatment (at 121°C for 8 min) to inactivate deteriorative enzymes and anti-nutritional factors present in the grain, before dehulling and grinding.

2.2. Enzymes

Four different types of enzymes—cellulase, hemicellulase, pectinase and protease, the first three operating under acidic conditions, and the latter, under basic conditions—were selected on the basis of the chemical composition of soybean; these are described below:

2.2.1. Cellulase

Cellulase purchased from Sigma (commercial code n^o C2415), was produced from *Aspergillus niger*. The declared activity at pH 5.0 and 37°C (which is around the optimum conditions for the enzyme activity) was 5.1 unit/mg of solids. It may be noted that one unit of cellulase activity corresponds to the one which releases 1 μ-mol of D-glucose from cellulose per hour.

2.2.2. Hemicellulase

This was also obtained from Sigma (commercial code n^o H2125), and its activity at pH 5 and 37°C was stated to be 0.026 unit/mg of solids. In this case, the activity is defined as that which releases 1 μ-mol of D-galactose per hour.

2.2.3. Pectinase

The pectinase used in the study, produced from *Aspergillus niger* by Novo Nordisk and commercially named Pectinex ultra sp, had a declared activity of 26,000 galacturonic acid/ml, measured at pH 3.5 and 20°C, which also coincides with the optimum conditions for maximum enzyme activity.

2.2.4. Protease

The protease used in the experiments, Alcalase 2.4L (Novo Nordisk), had a declared activity of 2.4 AU/g (AU = Anson Unit), which is equivalent to 2,736 I.U. (international standard unit) when soya isolate at pH 8.0 and 50°C was used as substrate. One I.U. of proteolytic activity is defined as that which can cleave one micromole peptide bond per minute (initial reaction rate). The optimum activity for alcalase has been found to be under the following conditions: pH between 8.0–9.0 and temperature between 50–60°C, depending on the substrate [13].

3. Methods

3.1. Extraction and enzymatic reaction

The extraction was carried out in batch mode using a laboratory scale (11 vessel) micro fermenter unit (H 500 series iii modular fermenter). The process involved two steps. In the case of enzymes operating in the acidic pH range (cellulase, hemicellulase, and pectinase) the enzyme was added to the 500 ml suspension soya flour/distilled water in the flask; the pH was set at the optimum value; and the impeller speed and the temperature were fixed at 200 rpm and 50°C, respectively. The enzyme was allowed to act at this pH for 1 hour on the basis of previous studies [18–20]. After carrying out the enzymatic reaction, the pH and agitation rate were adjusted to the values selected, as follows. In the case of exploratory experiments undertaken to evaluate the independent effect of each enzyme, the conditions selected for the extraction were: pH = 8.0; extraction time = 15 min; and agitation rate = 2000 rpm. For the experiments evaluating the combined effect of enzymes and other parameters: the pH was 9.0; the extraction time was 1 hour; and the agitation rate was 2000 rpm. The higher pH was chosen with the aim of obtaining a higher extraction yield without risk of producing toxic compounds (particularly lysinoalanine). The lower agitation rate during enzyme action enabled the enzymes to be evaluated, without letting agitation rate influence the process. In the case of protease, the enzyme was added after an hour of extraction, and there was no need to change the pH since this coincided with that for maximum enzymatic activity. The quantities of enzyme used in the experiments evaluating the effects of the individual parameters were: cellulase: 0.1% (w/w), equivalent to 1000 unit/100 g of soybean flour; hemicellulase: 3.85% (w/w), equivalent to 100 unit/100 g of soybean flour; pectinase: 2% (v/w); and protease: 3% (v/w).

3.2. Solid-liquid separation

Following extraction, the suspension was centrifuged under 1519 × *g* (or 3000 rpm) for 15 min at 20°C in a Sorvall RC5C centrifuge. The supernatant was removed, and the precipitate was weighed, mixed, sampled for moisture content determination, and freeze-dried. Analyses of total solids, oil and protein were carried out on the residue, and the extracted amounts of the components were calculated from the difference between the amounts originally present in the flour and the amount remaining in the residue after extraction. Extraction yields were expressed as the percentage of the component extracted in relation to the amount originally present in the flour.

3.3. Degree of protein hydrolysis

The degree of protein hydrolysis (DH) is defined as the percentage of peptide bonds cleaved, as follows:

$$DH = (h/h_{tot}) \times 100\%$$

where h is the hydrolysis equivalent or the amount of peptide cleaved during the hydrolysis (in meqv peptide bonds per gram protein) and h_{tot} is the total amount of peptide bonds determined from an amino acid assay, and assumed to be 7.8 meqv/g for soy protein [13].

The value of h in the experiments was calculated on the basis of a standard curve relating h for soya protein for different degrees of hydrolysis with the leucine amino equivalents (LEU-NH_2 eqv) determined by the TNBS (trinitrobenzenesulphonic acid) method [13]. The LEU-NH_2 eqv in the experiments was also determined by the TNBS method, using another standard curve relating absorbance and LEU-NH_2 eqv, and using a 1500 mM L-leucine solution as standard [13].

3.4. Combined effect of enzymes and other aqueous process parameters

The combined effect of the enzyme type and concentration, time of hydrolysis, particle size and solid-to-liquid ratio, on oil and protein extraction yields was evaluated by Response Surface Methodology. This experiment involved 5 factors, one qualitative (enzyme type) and four quantitative (particle size, solid-to-liquid ratio, enzyme concentration, time of hydrolysis). The actual and coded levels of the independent variables used in the experimental design are shown in Table 1. Preliminary studies showed that the relationship between oil extraction yield and particle size is approximately logarithmic [21]. Given that factors appearing in three equally spaced levels give the best estimates of the second-order model parameters, the particle sizes were chosen in such way that their logarithms were approximately in 3 equally spaced levels. Similarly, by arguing that the kinetics of the enzymatic hydrolysis is logarithmic in the protease [16], it was decided that an equidistant logarithmic spacing could be also tried between the actual levels for the time of hydrolysis and enzyme concentration. A second-degree polynomial model was fitted for each enzyme, but, in this case, involving four factors:

Table 1
Coded and real levels of the independent variables used in the design to estimate the oil yield in the enzymatic aqueous extraction process

Independent variables	Factor level		
	−1	0	+1
Particle size (μm)	212.5	449.5	855
Liquid-to-solid ratio	0.05	0.125	0.2
Enzyme concentration (%)	0.1	0.45	2
Time of hydrolysis (min)	30	60	120

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2$$

where Y represents the estimated dependent variable; x_1, x_2, x_3 and x_4 are the levels of the independent variables; β_0 is the intercept term; β_1, β_2, β_3 and β_4 are the linear terms; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction terms; and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic terms of the factors. A modified central composite design ($\alpha = 1$) with enzymes appearing only at two levels was employed. The 2-level factorial of the central composite design employed a half fraction of the 2^5 factorial. The defining contrast to select this interaction was the five-factor interaction. The design was completed by adding a replicate of axial points for the quantitative factors, plus 4 centre points for each enzyme. Therefore the complete design involved a total of 40 experimental data points.

The mathematical transformation of any actual level of particle size, solid-to-liquid ratio, enzyme concentration and time of hydrolysis into the coded level can be obtained, respectively, by the following expressions:

$$P = \frac{\ln p_i - 5.93}{0.806}$$

$$R = \frac{R_i - 0.125}{0.075}$$

$$T = \frac{\ln t_i - 4.09}{0.69}$$

and

$$C = \frac{\ln c_i + 0.8}{1.5}$$

where P, R, C and T are the coded values and p_i, R_i, c_i and t_i are the actual values for particle size, solid-to-liquid ratio,

concentration of enzyme and time of enzymatic hydrolysis, respectively.

With regard to other process variables, the following conditions were set: 1) extraction conditions: combined weight of oilseed flour and aqueous medium = 350 g, temperature = 50°C, extraction time = 1 h, pH = 9 and agitation rate = 200 rpm; 2) centrifugation conditions for solid-liquid separation: 1519 × *g* for 15 min.

3.5. Non-enzymatic process

In order to evaluate the effect of the process parameters—particle size and solid-to-liquid ratio—in the non-enzymatic process, a complete 3^2 factorial design (in this case, a central composite design with $\alpha = 1$) was employed, and the central point was repeated twice to assist in the evaluation of the experimental error and to enhance the precision of the model. This resulted in an experimental design consisting of 10 runs. The same levels of the particle size and solid-to-liquid ratio as described in the previous design were used in this case.

3.6. Overall effect of enzymes and other aqueous process parameters

A joint statistical analysis of the two sets of experiments—(1) without using enzymes and (2) using two enzymes (protease and cellulase)—was carried out to evaluate the effect of the enzymes on the extraction in relation to the other aqueous process parameters (particle size and solid-to-liquid-ratio). In order to perform a joint statistical analysis of both experiments, a "dummy" factor was introduced, which gathered together to all the factors that are in any way connected with the enzymes—i.e. the nature of the enzyme, the enzyme concentration and the time of hydrolysis. Thus, the dummy factor could assume two different levels: 1, for enzyme related runs; and −1, for non-enzyme related runs.

The effect of the predictor variables was evaluated by performing the *F* test from the analysis of variance. The goodness of fit of the model was also evaluated by the analysis of variance and graph of residuals, where the residuals are the differences between the values predicted from the models and the observed (experimental) values.

SAS software (version 6.12 for windows) was used for statistical analyses and also for response surface and contour plots.

4. Analytical methods

4.1. Major chemical composition analysis

The protein content in solid and liquid samples was determined by the Kjeldahl method, described by AOAC [22]. The oil content in the solid sample was determined by the Soxhlet method [19]. The oil content in the liquid phase was determined by acid extraction following the Werner-Schmidt method [23]. The total solids content was determined by drying a sample at 100°C up to constant weight, also described by the AOAC [22].

Table 2
Percentage composition of the raw materials used in the study

Component	Raw material		
	Non-heat treated flour	Heat treated flour	Soya grain
Protein[1]	38.01 ± 1.33	39.15 ± 0.87	3.24 ± 0.66
Oil[1]	19.97 ± 1.46	21.72 ± 1.23	17.75 ±0.76
Cellulose[1]	5.25 ± 0.89	3.13 ± 0.76	8.92 ± 1.28
Hemicellulose[1]	14.51 ± 2.33	13.51 ± 1.45	18.06 ± 2.42
Lignin[1]	2.68 ± 0.63	1.77 ± 0.44	4.71 ± 0.58
Ash[1]	1.08 ± 0.21	0.23 ± 0.03	0.32 ± 0.18
Soluble carbohydrate plus pectin and starch[2]	10.75	13.68	4.78
Moisture[1]	7.75 ± 0.32	6.81 ± 0.43	7.22 ± 0.29

[1] Mean of three determinations
[2] Calculated as the difference to 100%

The composition of the fibre fraction in terms of cellulose, hemicellulose and lignin was determined using the Acid Detergent Fibre (ADF) and Neutral Detergent Fibre (NDF) methods, according to AOAC [22], and using a Fibretec System M to carry out the analyses. The treatment with boiling detergent solution (NDF) dissolves all the components of the sample, except hemicellulose, lignin, cellulose and ash. The treatment with boiling acid detergent solution (ADF) dissolves all the substances, except lignin, cellulose and ash. Thus, the hemicellulose content is obtained from the difference between the NDF and the ADF determinations. The lignin content is obtained by treating the ADF with potassium permanganate, which dissolves lignin. The cellulose content is obtained by placing the remaining material in muffle oven at 500°C for 3 hours, where the cellulose is burnt up. The residue remaining corresponds to the amount of ash in the sample.

All the analytical determinations were done in triplicate.

5. Results

The chemical composition of the raw materials used in the experiments is shown in Table 2. All the fibre components were present in higher proportions in the non heat-treated flour compared to the heat-treated one. This is certainly due to the dehulling operation, which was carried out in the preparation of the latter. In consequence, the relative amounts of protein, oil and the carbohydrate plus pectin fractions were higher in the heat-treated flour.

In the case of soya grains (used mainly in the experiments where different levels of flour particle size were required), the higher relative amount of the fibre components must be mainly due to differences in the varieties, as compared with the soybean flours used in the study.

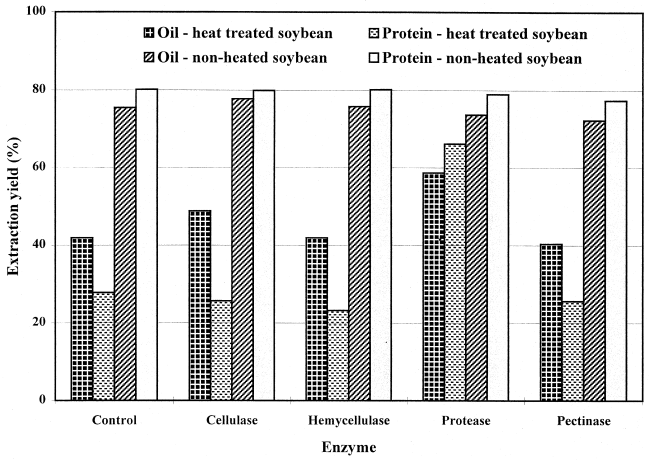

Fig. 1. Effect of enzymes on extraction yield using both flour subjected or not to heat treatment (Enzymatic treatment: 1 h at 200 rpm and optimum enzymatic conditions; extraction conditions: 15 min at 2,000 rpm; pH 8.0; 50°C).

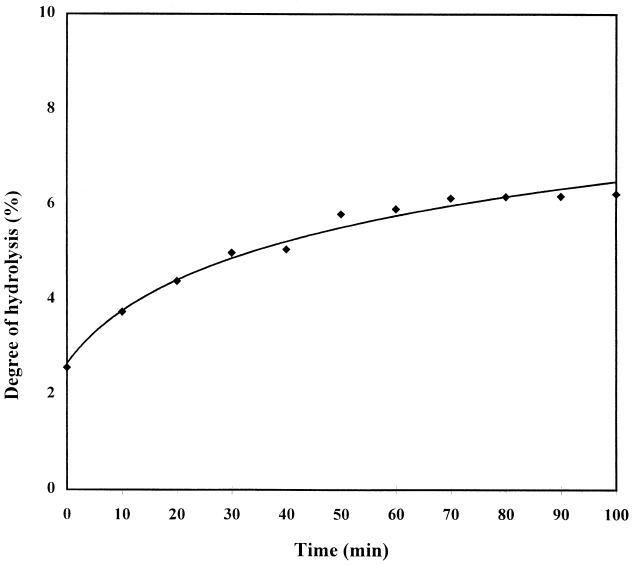

Fig. 2. Degree of hydrolysis (%) with proteolytic reaction for non-heat treated material.

5.1. Effect of enzymes on extraction yields—preliminary evaluation

The effect of different enzymes on protein and oil extraction, when the heat and non-heat treated soybean flour was used in the extraction, is shown in Fig. 1. It is clear by comparing the controls from the experiments carried out with the two materials—i.e., experiments carried out without enzyme using the same extraction conditions—that the extraction yields obtained with the heat-treated material were much lower than those obtained with the non heat treated material. It is also possible to see that the increases that happened due to the action of the enzymes were in general very small and almost negligible in comparison to the controls. The main exemption was the considerable increase obtained with protease when heat-treated material was used.

The stronger effect of protease compared to the other enzymes can be attributed to the production of peptides and amino acids by protein hydrolysis, which have a greater higher solubility than the original heat treated protein. This effect allows not only the extraction of the nitrogenous compounds of proteinaceous origin, but also oil with an overall increase in the total solids extracted. Thus, the higher oil extraction can be explained by the solubilisation and hydrolysis of proteins, which possibly causes a breakdown in the protein network characteristic of the cotyledon cells cytoplasm, and in the protein (oleosin) based membranes that surround the lipid bodies, so liberating the oil [9–12].

None of the enzymes appear to increase extraction yields in the case of non heat-treated material. Although the protease slightly improves yields, the enhancement values are much lower than those obtained with heat-treated material. This can be attributed to the much lower rates of hydrolysis of non-denatured soy proteins, in comparison with the denatured substrate [13]. Moreover, the degree of hydrolysis that can be accomplished with the former is much higher [13]. However, the yield increment obtained with the enzyme using the heat treated material is not enough to achieve levels of extraction obtained in the case of the non-heat treated material, even when the enzymes were not used in the latter case, as already mentioned.

The kinetic extraction curve using alcalase with the non-heat treated soya flour, expressed in terms of degree of protein hydrolysis (DH) *versus* time, is shown in Fig. 2. The DH obtained after 1 h of reaction was around 6 and the curve apparently tended to a relatively lower value in comparison with earlier data reported for denatured/insolubilised substrates (such as soya isolate obtained by acid precipitation and soya protein insolubilised with hot aqueous ethanol) [13]. In these cases, DH can reach values as high as 8.5 and 7, respectively, after 1 hour of enzymatic reaction, and around 12 and 10 after 150 min that results in a proportionally higher increase in the solubility of nitrogenous compounds, as is the case with the heat denatured protein. However, as previously pointed out, the increase in solubility of the nitrogenous compounds in the case of heat treated material was negligible in comparison with the decrease in protein solubility caused by heat treatment itself, which led to significantly lower extraction yields in the case of the heat treated material.

5.2. Effect of protease and cellulase on oil and protein extraction yields in relation to other parameters

The experimental data resulting from each combination of the variable levels used in the 50-run-experimental design—i.e., 40 runs for experiments with enzyme and 10 runs for non-enzymatic process—are given in Table 3. The pre-

Table 3
Observed and predicted values for different levels of experimental design

Run	Enzyme	P	R	C	T	Oil extraction yield		Protein yield	
						Observed	Predicted	Observed	Predicted
1	Protease	−1	−1	−1	−1	74.64	74.66	87.12	85.34
2	Protease	0	0	−1	0	57.58	60.41	80.55	83.46
3	Protease	0	0	0	−1	60.83	58.35	63.68	67.41
4	Protease	0	0	1	0	25.31	24.64	53.50	53.80
5	Protease	1	1	−1	−1	68.97	71.82	85.16	85.08
6	Protease	0	−1	0	0	36.80	39.86	58.83	61.33
7	Protease	−1	0	0	0	34.80	39.57	54.98	59.04
8	Protease	0	1	0	0	38.52	37.62	59.79	60.50
9	Protease	−1	1	−1	1	52.54	49.26	68.72	67.82
10	Protease	0	0	0	0	43.03	41.99	63.50	60.90
11	Protease	0	0	0	0	39.79	41.99	63.20	60.90
12	Protease	0	0	0	0	49.95	41.99	66.30	60.90
13	Protease	−1	1	1	−1	32.52	31.90	63.80	62.49
14	Protease	1	−1	−1	1	53.65	51.23	71.81	71.68
15	Protease	1	−1	1	−1	36.16	36.39	64.52	63.99
16	Protease	−1	−1	1	1	18.52	17.63	31.30	31.23
17	Protease	0	0	0	0	41.68	41.99	60.21	60.90
18	Protease	0	0	0	1	34.05	38.68	44.78	44.26
19	Protease	1	1	1	1	16.03	17.98	31.95	33.56
20	Protease	1	0	0	0	43.17	40.56	61.74	60.89
21	Cellulase	1	1	1	−1	21.14	20.39	53.42	53.15
22	Cellulase	0	0	0	0	27.33	27.86	53.96	55.31
23	Cellulase	0	0	0	0	25.23	27.86	59.43	55.31
24	Cellulase	−1	1	1	1	25.54	25.57	30.59	30.82
25	Cellulase	1	−1	−1	−1	43.50	46.70	79.05	79.61
26	Cellulase	0	1	0	0	26.67	24.65	54.17	52.84
27	Cellulase	0	0	0	1	34.08	36.27	46.31	47.59
28	Cellulase	0	0	0	−1	40.46	35.35	74.90	72.99
29	Cellulase	1	0	0	0	29.53	28.27	54.30	55.31
30	Cellulase	0	0	0	0	28.33	27.86	54.66	55.31
31	Cellulase	0	0	−1	0	42.87	37.97	64.96	63.23
32	Cellulase	−1	−1	−1	1	40.92	39.89	47.24	47.03
33	Cellulase	0	0	0	0	21.78	27.86	51.32	55.31
34	Cellulase	1	−1	1	1	23.45	27.10	30.31	29.37
35	Cellulase	0	−1	0	0	25.61	24.70	50.73	51.43
36	Cellulase	−1	−1	1	−1	19.88	18.96	51.85	51.73
37	Cellulase	−1	1	−1	−1	37.09	40.67	75.12	76.85
38	Cellulase	−1	0	0	0	28.21	26.54	54.89	53.25
39	Cellulase	0	0	1	0	15.72	17.70	39.39	40.49
40	Cellulase	1	1	−1	1	42.69	41.84	52.92	52.56
41	None	−1	1	None	None	55.88	59.43	67.34	68.04
42	None	−1	0	None	None	59.83	59.55	78.02	76.34
43	None	0	−1	None	None	52.84	49.82	78.31	74.56
44	None	0	0	None	None	38.72	38.61	57.11	59.50
45	None	0	1	None	None	36.90	40.26	48.14	50.04
46	None	1	1	None	None	35.19	28.28	38.85	36.25
47	None	−1	−1	None	None	75.80	72.53	898.25	90.23
48	None	1	−1	None	None	28.02	34.31	60.33	63.09
49	None	0	0	None	None	38.84	38.61	60.04	59.50
50	None	1	0	None	None	24.25	24.87	47.04	46.87

dicted values from complete response surface analysis models for both variables are also given in the same table.

The analyses of variance of oil and protein extraction are given in Tables 4 and 5, respectively. *Enz*, *Pi*, *Ri*, *Ci* and *Ti* shown in the tables are the terms related to the independent variables, coded variables for the type of enzyme, particle size, solid-to-liquid ratio, enzyme concentration, and time of hydrolysis, where $Pi = \ln(p_i)$, $C_i = \ln(c_i)$, $Ti = \ln(t_i)$, and p_i, R_i, c_i and t_i are actual levels of particle size (μm), solid-to-liquid ratio, enzyme concentration (%) and time of hydrolysis (min), respectively. The high F values combined with the high coefficients of determination ($r^2 = 0.9570$ for oil extraction yield and $r^2 = 0.9807$ for protein yield) attest the goodness of fit of the model in both

Table 4
Analysis of variance for oil yield using data from enzymatic and non-enzymatic aqueous extraction process

Source	Freedom degree	Sum of Square	Mean Square	F value	P-value
Model	28	9922.48	354.37	16.71	0.0001
DUMMY	(1)	533.05	533.05	25.14	0.0001
ENZ[1](DUMMY)	(1)	1670.65	1670.65	78.79	0.0001
Ci[2](DUMMY)	(1)	9.29	9.29	0.44	0.5152
Ti[3](DUMMY)	(1)	6.52	6.52	0.31	0.5851
Pi[4]	(1)	5678.18	5678.18	267.79	0.0001
Ri[5]	(1)	576.32	576.32	27.18	0.0001
Ci*Ci(DUMMY)	(1)	18.68	18.68	0.88	0.3586
Ti*Ti(DUMMY)	(1)	1.09	1.09	0.05	0.8227
Pi*Pi	(1)	69.52	69.52	3.28	0.0845
Ri*Ri	(1)	354.95	354.95	16.74	0.0005
Pi*DUMMY	(1)	51.26	51.26	2.42	0.1349
Ri*DUMMY	(1)	0.04	0.04	0.00	0.9648
Ci*ENZDUMMY)	(1)	0.69	0.69	0.03	0.8588
Ti*ENZ(DUMMY)	(1)	6.01	6.01	0.28	0.6002
Pi*ENZ(DUMMY)	(1)	300.31	300.31	14.16	0.0011
Ri*ENZ(DUMMY)	(1)	529.83	529.83	24.99	0.0001
Ci*Ti(DUMMY)	(1)	0.17	0.17	0.01	0.9290
Ci*Pi(DUMMY)	(1)	0.68	0.68	0.03	0.8595
Ci*Ri(DUMMY)	(1)	3.37	3.37	0.16	0.6943
Ti*Pi(DUMMY)	(1)	4.66	4.66	0.22	0.6438
Ti*Ri(DUMMY)	(1)	13.54	13.54	0.64	0.4331
Pi*Ri	(1)	62.41	62.41	2.94	0.1009
Pi*Pi*DUMMY	(1)	16.93	16.93	0.80	0.3817
Ri*Ri*DUMMY	(1)	1.04	1.04	0.05	0.8268
Ci*Ci*ENZ(DUMMY)	(1)	10.41	10.41	0.49	0.4912
Ti*Ti*ENZ(DUMMY)	(1)	0.25	0.25	0.01	0.9141
Pi*Pi*ENZ(DUMMY)	(1)	0.04	0.04	0.00	0.9660
Ri*Ri*ENZ(DUMMY)	(1)	2.58	2.58	0.12	0.7309
Error	21	445.27	21.20		
Total	49	10367.75			

[1] Type of enzyme; [2] ln(concentration of enzyme); [3] ln(time of enzymatic hydrolysis); [4] ln(particle size); [5] solid-to-liquid ratio

Table 5
Analysis of variance for protein extraction yield using data from enzymatic and non-enzymatic aqueous extraction process

Source	Freedom degree	Sum of Square	Mean Square	F value	P-value
Model	28	9935.60	354.84	38.25	0.0001
DUMMY	(1)	166.95	166.95	18.00	0.0004
ENZ[1](DUMMY)	(1)	608.01	608.01	65.54	0.0001
Ci[2](DUMMY)	(1)	19.15	19.15	2.06	0.1655
Ti[3](DUMMY)	(1)	0.41	0.41	0.04	0.8346
Pi[4]	(1)	4722.58	4722.58	509.06	0.0001
Ri[5]	(1)	3846.69	3846.69	414.64	0.0001
Ci*Ci(DUMMY)	(1)	4.95	4.95	0.48	0.4940
Ti*Ti(DUMMY)	(1)	4.02	4.02	0.43	0.5175
Pi*Pi	(1)	40.48	40.48	4.36	0.0491
Ri*Ri	(1)	5.23	5.23	0.56	0.4609
Pi*DUMMY	(1)	12.27	12.27	1.32	0.2630
Ri*DUMMY	(1)	0.07	0.07	0.01	0.9303
Ci*ENZDUMMY)	(1)	0.05	0.05	0.01	0.9393
Ti*ENZ(DUMMY)	(1)	6.27	6.27	0.68	0.4202
Pi*ENZ(DUMMY)	(1)	59.72	59.72	6.44	0.0192
Ri*ENZ(DUMMY)	(1)	6.33	6.33	0.68	0.4181
Ci*Ti(DUMMY)	(1)	115.35	115.35	12.43	0.0020
Ci*Pi(DUMMY)	(1)	4.08	4.08	0.44	0.5144
Ci*Ri(DUMMY)	(1)	1.49	1.49	0.16	0.6928
Ti*Pi(DUMMY)	(1)	1.61	1.61	0.17	0.6809
Ti*Ri(DUMMY)	(1)	4.58	4.58	0.49	0.4900
Pi*Ri	(1)	27.03	27.03	2.91	0.1026
Pi*Pi*DUMMY	(1)	0.47	0.47	0.05	0.8235
Ri*Ri*DUMMY	(1)	12.92	12.92	1.39	0.2511
Ci*Ci*ENZ(DUMMY)	(1)	18.05	18.05	1.95	0.1776
Ti*Ti*ENZ(DUMMY)	(1)	20.89	20.89	2.25	0.1483
Pi*Pi*ENZ(DUMMY)	(1)	97.92	97.92	10.56	0.0038
Ri*Ri*ENZ(DUMMY)	(1)	128.45	128.45	13.85	0.0013
Error	21	194.82	9.28		
Total	49	10130.42			

[1] Type of enzyme; [2] ln(concentration of enzyme); [3] ln(time of enzymatic hydrolysis); [4] ln(particle size); [5] solid-to-liquid ratio

cases. The plots of predicted and experimental values resulting from oil and protein extraction (Figs. 3a and 3b, respectively) also show the consistency of the empirical models, which was confirmed by the lack of any prevalent trend in the graphs of residuals [21].

In the case of oil extraction, variables which turned out to be significant were: the "dummy" variable, which compares mean oil yields with and without enzyme; the effect of enzyme, which is nested in each level of the "dummy" variable (since for the level −1 of "dummy" there is no enzyme, this effect compares the 2 enzymes in the other level—i.e., +1—of "dummy"); the linear terms for particle size and solid-to-liquid ratio; the quadratic term for the ratio; and the interaction of both these terms with enzyme. The factors that turned out to be significant at 10% were the quadratic term for the particle size and the interaction between particle size and ratio. The interaction between particle size and dummy was the only significant factor at 15% significance. The most significant term in the analysis of variance is the linear effect of particle size, as evident from the value of sum of squares. The significance of the term related to the "dummy" can be understood by comparing the lower predicted mean value of oil yield obtained with enzymes (37.0%) and without enzyme (44.6%), mainly due to the detrimental effect of the cellulase. The strong effect of the enzyme type is evident from the mean value of the oil-extracted yield using each enzyme, which was 43.4% for the protease and 30.5% for the cellulase. To obtain a simplified model for interpretation, all non-significant terms were eliminated except those which were present in significant interactive terms. The simplified models had $r^2 = 0.9450$, indicating that the model explains the results for oil extraction yield as well as the complete models. The resulting second-degree polynomials for oil extraction (Y_o) are:
a) for non-enzymatic process:

$$Y_o = 38.6 - 17.3P - 4.8R + 3.6P^2 + 6.4R^2 + 1.8PR$$

a) for process using protease:

$$Y_o = 41.3 - 17.9P - 9.8R - 1.4P^2 + 4.6R^2 + 1.8PR$$

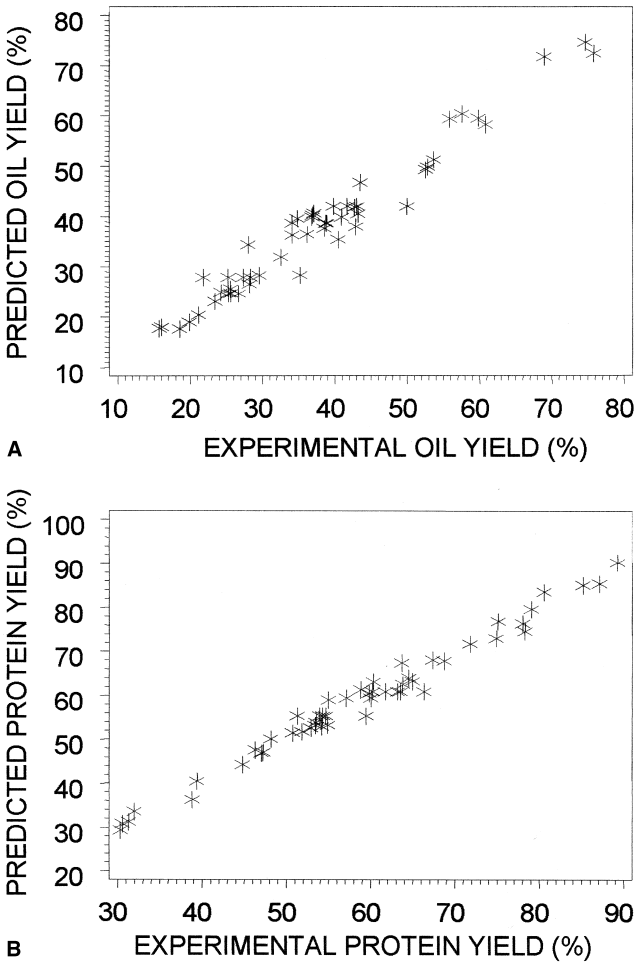

Fig. 3A. Graph of predicted values *versus* experimental values for oil yield
3B. Graph of predicted values *versus* experimental values for protein yield.

b) for process using cellulase:

$$Y_o = 27.4 - 10.1P + 0.5R - 1.4P^2 + 6.6R^2 + 1.8PR$$

The response surface obtained for the non-enzymatic process using simplified model is shown in Fig. 4a, together with its contour plot (4b). The plots for protease and cellulase are also shown in the same figure (Figs. 4c and 4d, respectively). Although the general trends shown by the curves are similar, the lower yields obtained from the cellulase are also evident.

For protein extraction, the factors that turned out to be significant at 5% from the analysis which combined the experimental data with and without enzymes were: "dummy," enzyme, the linear effect of particle size and ratio, the quadratic effect of particle size, the interaction between particle size and enzyme, concentration and time of hydrolysis, quadratic effect of particle size and enzyme, and quadratic effect of ratio and enzyme; at 15% of significance the interaction between particle size and ratio, and quadratic effect of time of hydrolysis and enzyme; at 20% of significance the linear effect of concentration of enzyme, and the interaction between the quadratic effect of concentration and enzyme. The reduced models in the case of protein extraction yield gave $r^2 = 0.9796$, indicating that it explains the response variable as well as the complete models. The resulting models for protein extraction yield (Y_p), obtained by using only the significant variables for refitting, are:

a) for non-enzymatic process:

$$Y_p = 59.5 - 14.7P - 12.3R + 2.1P^2 + 2.8R^2 - 1.2PR$$

b) for protease:

$$Y_p = 60.9 - 14.8P - 11.6R + 0.9C - 0.4T + 7.7P^2 - 5.1R^2 + 0.9C^2 - 2.7CT - 1.2PR$$

c) for cellulase:

$$Y_p = 55.3 - 11.4P - 12.7R + C + 0.7T - 3.4P^2 + 5R^2 - C^2 - 3.2T^2 - 2.7CT - 1.2PR$$

where $P = (\ln(p_i) - 5.93)/0.806$, $R = (R_i - 0.125)/0.075$, $C = (\ln(c_i) + 0.8)/15$ and $t = (\ln(t_i) - 4.09)/0.695$, and p_i, R_i, c_i and t_i are the actual values of particle size, solid-to-liquid ratio, concentration of enzyme and time of enzymatic hydrolysis.

The response surfaces for protein extraction for the non-enzymatic process using simplified models is shown in Fig. 5a, together with its contour plot (Fig. 5b) and the ones for the processes using protease (Fig. 5c) and cellulase (Fig. 5d). Again the general trends of the three graphs are the same, and also comparable to the ones obtained for oil extraction. Also the levels of protein extraction for cellulase resulted in lower yields than those obtained for aqueous process and protease, as expected, which is reflected in the smaller predicted mean values for cellulase (54.0%) compared to the process without enzyme and with protease (61.8%). On the other hand, the protease had an overall positive effect on protein yield, which did not generally happen for oil extraction. Thus, although there is a clear connection between the oil and protein extraction yields, as confirmed once again by the lower levels of oil and protein extraction with cellulase, the higher protein yields that could be obtained with protease were not accompanied by higher oil yields.

One possible explanation for the much lower average protein yield for the process carried out with cellulase in comparison to those carried out without enzyme or with protease, is that the optimum pH for the cellulase activity (pH 5) coincides with the isoelectric point (pI) for the soybean protein [22]. Although the pH was increased after the enzymatic reaction to a value of high protein solubility (pH 9), it seems that part of the protein still remained insoluble at that pH, decreasing the overall protein yield. This also affected the oil yield, as can be seen from the

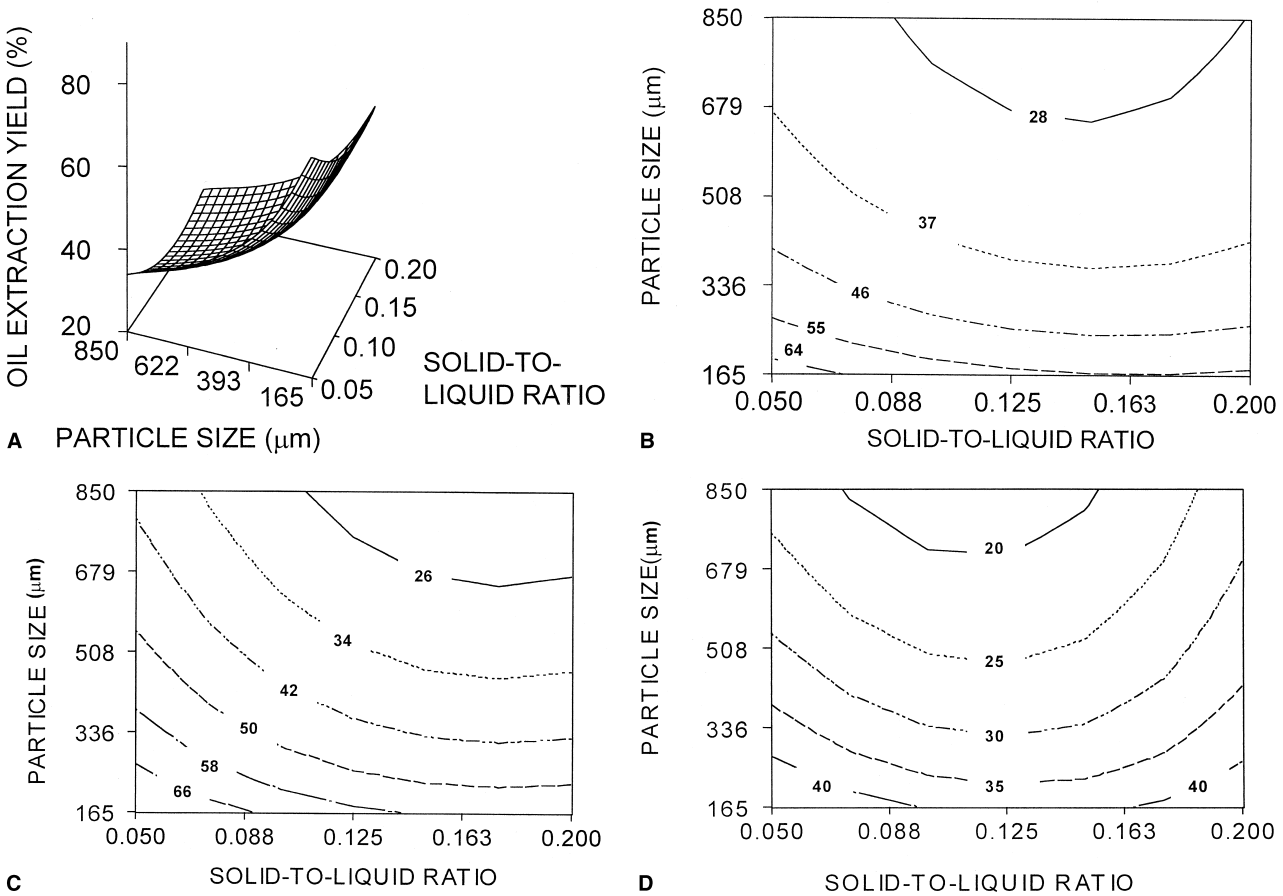

Fig. 4A. Response surface plot for oil yield (%) resulting from the non-enzymatic process, at varying particle size (μm) and solid-to-liquid ratio. 4B. Contour plot for oil yield (%) resulting from the non-enzymatic process, at varying particle size (μm) and solid-to-liquid ratio. 4C. Contour plot for oil yield (%) resulting from the process using protease, at varying particle size (μm) and solid-to-liquid ratio (time of enzymatic action = 60 min; enzyme concentration = 0.45 (% v/v)). 4D. Contour plot for oil yield (%) resulting from the process using cellulase, at varying particle size (μm) and solid-to-liquid ratio (time of enzymatic action = 60 min; enzyme concentration = 0.45 (% w/v)).

lower average oil yield (30.5%) resulting from the process with cellulase in comparison to the processes carried out without enzyme and with protease (43.4%).

It is clear from the analysis that the enzymes used do not have a strong influence on oil extraction, although protease does influence the protein yield. This conclusion is in marked contrast with studies reported earlier where the enzymes seemed to appreciably enhance the oil extraction yields in particular. These studies did not seem to have considered the dominant role of the particle size in the oil extraction and their conclusions are based sometimes on studies where the particle size was possibly not controlled [8,13]. This clearly highlights the need for a statistical design of experiments to determine the role of all process parameters.

The role of enzymes must not only be evaluated on the basis of the extraction yields. Their effects on the stability of the resulting oil-in-water emulsion must also be considered. For instance if enzymes can result in less stable emulsions, even a marginal loss in extraction yield can be offset by an easier separation. It is therefore necessary to take an integrated approach to assess the role of enzymes in the process. Moreover, in the specific case of protease, the higher protein yield and the simultaneous production of protein hydrolysate, with a high commercial value, must also be considered.

6. Concluding remarks

From the enzymes evaluated, protease was the only one that resulted in a definite increase in oil and protein extraction from soybean, under defined circumstances—i.e., for large particle sizes or when heat-treated flour was used. Although protease produced higher yields when heat-treated material was used, the resulting values were lower than the ones obtained for non-heat treated material without using enzymes. Analysis of the experimental data using Response Surface Methodology showed that extraction yields obtained with protease and cellulase correlated very well with process parameters. While the extraction levels for protease were comparable with non-enzymatic process, the treatment with cellulase led to lower extraction levels, possibly due to the adverse effect of the pH set to carry out the treatment. Operational parameters and enzymes must be evaluated not only in

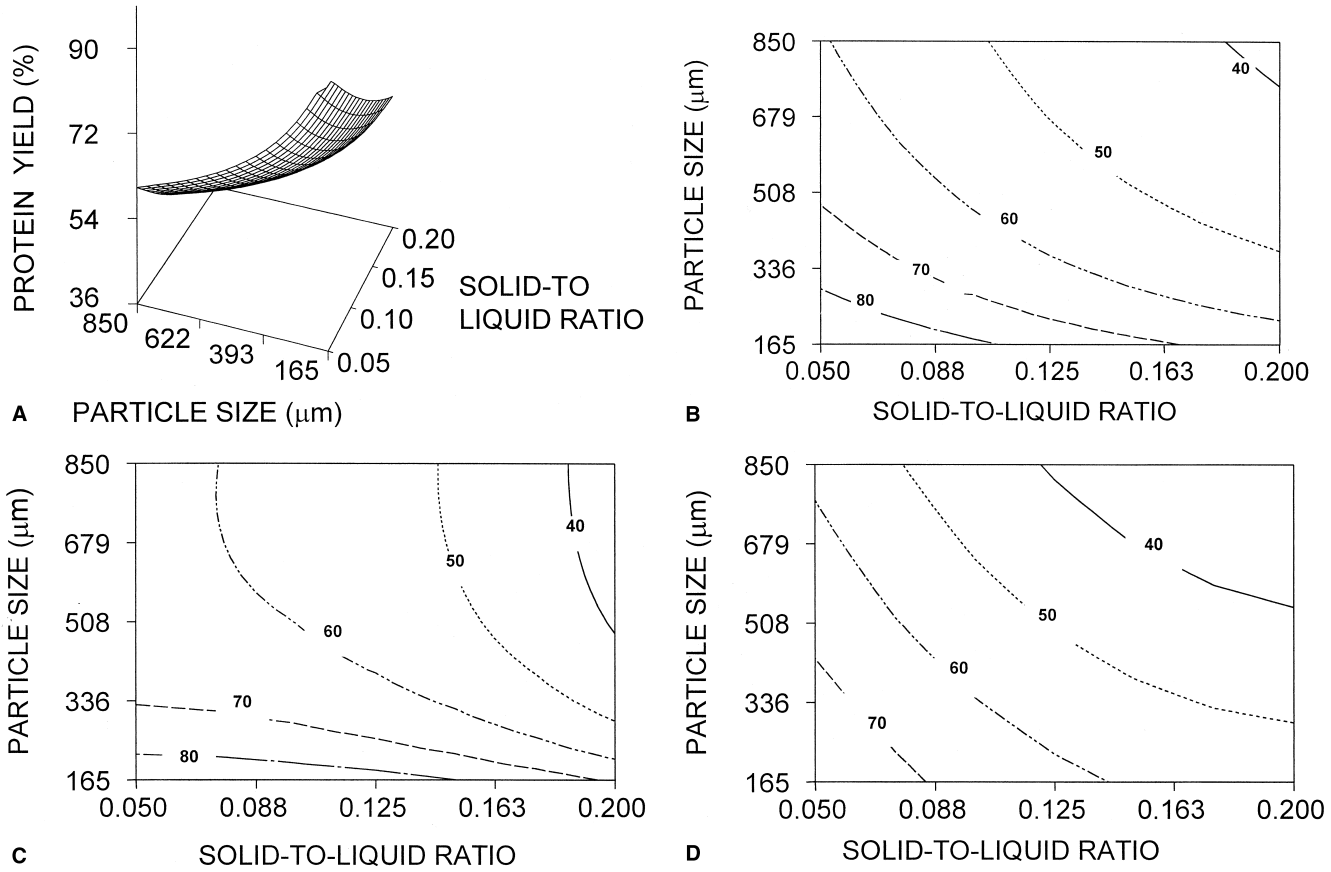

Fig. 5A. Response surface plot for protein yield (%) from non-enzymatic process, at varying particle size (μm) and solid-to-liquid ratio. 5B. Contour plot for protein yield (%) from non-enzymatic process, at varying particle size (μm) and solid-to-liquid ratio. 5C. Contour plot for protein yield (%) from process using protease, at varying particle size (μm) and solid-to-liquid ratio (time of enzymatic action = 60 min; enzyme concentration = 0.45 (% v/v)). 5D. Contour plot for protein yield (%) from process using cellulase, at varying particle size (μm) and solid-to-liquid ratio (time of enzymatic action = 60 min; enzyme concentration = 0.45 (% w/v)).

terms of the extraction yields, but also in terms of their effect on emulsion stability.

In spite of the limited increase in the extraction yield, the use of protease can be associated with a very interesting possibility of simultaneously extracting oil and producing a protein hydrolysate in the same step. Further this strategy can also be applied to the production of products where separation into individual components is not necessary, for instance, the production of high nutritional value soy beverages containing previously hydrolysed protein. The application of enzymatic extraction in different processes depends on a proper understanding of the factors influencing extraction. This study has elucidated both individual and interactive effects of process parameters, which is a key step in process optimisation.

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