

## Optimisation of Ultrasonic-assisted Protein Extraction from Brewer's Spent Grain

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### Abstract

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Response surface methodology was employed to optimise the ultrasonic-assisted extraction of protein from brewer's spent grain. Three variables, namely the extraction time (min), ultrasonic power (W/100 ml of extractant), and solid-liquid ratio (g/100 ml) were investigated. Optimal conditions were determined and tri-dimensional response surfaces were plotted using mathematical models. The ANOVA analysis indicated that all the quantities determined, i.e. the extraction time, ultrasonic power, and solid-liquid ratio, had significant positive linear and negative quadratic effects on the protein yield. Optimum conditions for the extraction of protein were found to be: the extraction time of 81.4 min, ultrasonic power of 88.2 W/100 ml of extractant, and solid-liquid ratio of 2.0 g/100 ml. The optimal predicted protein yield obtained was 104.2 mg/g BSG while the experimental yield of protein was in agreement with the predicted value.

**Keywords:** ultrasonic-assisted extraction; protein; Brewer's spent grain; response surface methodology

Brewer's spent grain (BSG) is the major by-product of the brewing industry, representing around 85% of the total by-products generated. BSG has a high content of protein, more than 20% of protein on dry basis (MUSSATTO *et al.* 2006). BSG is of low cost and high nutritive value. The incorporation of BSG into rat diets is beneficial to intestinal digestion, alleviating both constipation and diarrhoea. These effects were attributed to the content of glutamine-rich protein, and to the high content of non-cellulosic polysaccharides and smaller amounts of  $\beta$ -glucans (MUSSATTO *et al.* 2006).

For a long time, the main application of BSG has been limited to its use as animal feed along

with its utilisation for increasing bricks porosity by its addition (RUSS *et al.* 2005), removal of Cu (II) ions from aqueous solutions (LU & GIBB 2008), and as a brewing yeast carrier (BRÁNYIK *et al.* 2004; KOPSAHELIS *et al.* 2007). The incorporation of BSG into ready-to-eat snacks was also studied (AINSWORTH *et al.* 2007; STOJCESKA *et al.* 2008). Due to the content of many beneficial components of BSG, the separation of BSG into its individual components for both food and non-food applications is of interest. This research included the valorisation of BSG to recover valuable compounds such as  $\alpha$ -tocopherol by supercritical fluid extraction (SFE) technology (FERNANDEZ *et al.* 2008), the recovery of ferulic acid from BSG

by a sequential extraction with alkali of increasing strength (MANDALARI *et al.* 2005), solubilisation of BSG carbohydrates by microwave radiation at 160°C in the presence of 0.1M HCl (MACHEINER *et al.* 2003), the extraction of ferulic and *p*-coumaric acids by alkaline hydrolysis of BSG (MUSSATTO *et al.* 2007a, b), the recovery of lignin from BSG (MUSSATTO *et al.* 2007a, b), and the production of oligosaccharides (CARVALHEIRO *et al.* 2004).

Ultrasonic-assisted extraction (UAE) has been widely employed in the extraction of valuable compounds from bio-mass since it has many advantages such as working at (or close to) ambient temperature, a higher efficiency than the conventional extraction methods, and a lower cost (the simplicity of the equipment needed with similar or better yields obtained) (ROLDÁN-GUTIÉRREZ *et al.* 2008). UAE has been studied in the extraction of biological compounds from different plant materials (HEMWIMOL *et al.* 2006; RODRIGUES & PINTO 2007; VELICKOVIC *et al.* 2008) and has been used as a low cost alternative to solvent reflux extraction of phenolic compounds from coconut shell (YANG & ZHANG 2008).

In the conventional extraction procedure, many variables such as the extraction time, extraction temperature, and solid-liquid ratio (S/L) may significantly influence the extraction efficiency. The traditional optimisation of the process was achieved by the one-factor-at-a-time approach, which is time-consuming and may ignore the interactions between variables. The response surface methodology (RSM) can overcome these limitations, since it allows accounting for the possible interaction effects between variables (BANIK & PANDEY 2008). RSM has become one of the most popular optimisation methods used in recent years and has been successfully used to model and optimise biochemical and biotechnological processes (LEE *et al.* 2000; LIYANA-PATHIRANA & SHALIDI 2005; BAŞ & BOYACI 2007; ALIAKBARIAN *et al.* 2008). It enables the evaluation of the effects of several process parameters and their interactions on the response variables based on a few sets of experiments (DIPTEE *et al.* 1989). In this study, UAE was employed in the extraction of protein from BSG while RSM was designed to investigate the relations between the main factors (extraction time, ultrasonic power, and S/L) and the yield of protein. The aim was to select the optimum conditions for the quantitative extraction of protein from BSG.

## MATERIAL AND METHODS

**Materials.** BSG (73.8% moisture, 7.6% protein, Kjeldahl N  $\times$  6.25, wet weight basis) was obtained from Zhujiang Brewery Group Co., Ltd. (Guangzhou, China). BSG was kept at  $-20^{\circ}\text{C}$  and then lyophilised (Christ, Germany). The dry BSG was ground using a grinder (Sympak, Schwarzenbek, Germany) to fine powder passing 80-mesh sieves and subsequently stored in airtight bags which were kept at  $+4^{\circ}\text{C}$  until the experiments. All other chemicals used in the experiments were of analytical grade.

**Experimental design.** The effects on  $Y$  (protein yield, mg/g) of three independent variables  $X_1$  (time, min);  $X_2$  (ultrasonic power, W/100 ml of extractant);  $X_3$  (S/L, g/100 ml) at five levels, were investigated using the central composite design (Table 1). The correspondence between the coded and uncoded values can be obtained using the following formula:

$$x_i = \frac{(X_i - X_i^0)}{\Delta X_i} \quad (1)$$

where:

$x_i$  – coded value

$X_i$  – corresponding actual value

$X_i^0$  – actual value in the centre of the domain

$\Delta X_i$  – increment of  $X_i$  corresponding to 1 unit of  $X_i$

Coded value of extraction time ( $x_1$ ), coded value of ultrasonic power ( $x_2$ ) and coded value of S/L ( $x_3$ ) were given by Eqs. (2)–(4):

$$x_1 = \frac{(X_1 - 60)}{20} \quad (2)$$

$$x_2 = \frac{(X_2 - 80)}{20} \quad (3)$$

$$x_3 = \frac{(X_3 - 3)}{1} \quad (4)$$

A 3-factor, 5-level Central Composite Rotatable Design (CCRD) was chosen to optimise the extraction conditions (Table 1). It consisted of 20 experimental points including 8 factorial points, 6 axial points, and 6 centre points (Table 2), the experiment having been carried out in a random order.

**Ultrasonic extraction.** The extraction of protein was performed by adding BSG into 100 ml of

Table 1. Independent variables and their levels in the response surface design

Independent variables	Symbol		Factor level				
	uncoded	coded	–1.68	–1	0	1	1.68
Time (min)	$X_1$	$x_1$	26.4	40	60	80	93.6
Ultrasonic power (W/100 ml of extractant)	$X_2$	$x_2$	46.4	60	80	100	113.6
Solid-liquid ratio	$X_3$	$x_3$	1.32	2	3	4	4.68

extractant in a 200 ml beaker and subjecting the mixture to an ultrasonic processor (VCX-500, Sonics and Materials, Newtown, USA) with a 13 mm high gain probe, which was controlled according to the required output ultrasonic power and time. The extraction was performed at room temperature using an integrated temperature controller, which precluded harmful overheating of the sample and guaranteed the process integrity by terminating the

Table 2. Results of the response surface analysis of the variation of the yield of protein ( $Y$ ) extracted from BSG under the coded values of time ( $x_1$ ), ultrasonic power ( $x_2$ ), and solid-liquid ratio ( $x_3$ )

Number	$x_1$	$x_2$	$x_3$	$Y$ (mg/g)
1	–1	–1	–1	44.79
2	–1	–1	1	34.37
3	–1	1	–1	65.36
4	–1	1	1	41.97
5	1	–1	–1	93.11
6	1	–1	1	82.94
7	1	1	–1	95.37
8	1	1	1	87.97
9	–1.68	0	0	41.48
10	1.68	0	0	85.51
11	0	–1.68	0	53.21
12	0	1.68	0	79.77
13	0	0	–1.68	97.33
14	0	0	1.68	50.51
15	0	0	0	90.33
16	0	0	0	84.51
17	0	0	0	82.59
18	0	0	0	89.52
19	0	0	0	90.22
20	0	0	0	92.37

ultrasound when the sample temperature reached a predetermined limit during the processing cycle. At the end of the extraction, the mixture was centrifuged (3K30, Sigma Centrifuge, Germany) at 8000 g for 10 minutes. Protein concentration in the supernatant was determined.

**Determination of protein.** The protein was quantitatively analysed using Bradford method (BRADFORD 1976). One milliliter of the diluted sample was placed in a test-tube. Five milliliters of coomassie brilliant blue solution was added and the resulting mixture was stirred and allowed to stand for 1 hour. The absorbance of the mixed sample was measured at 595 nm on an UV-vis spectrophotometer (Cary 50, Varian, Palo Alto, USA), using bovine serum albumin (BSA) as the standard. The protein yield was defined as the protein quantity extracted from 1 g of BSG (on dry basis).

**Data analysis.** The data from the central composite design were analysed by multiple regressions to fit the following quadratic polynomial model.

$$y = b_0 + \sum_{i=1}^3 b_i x_i + \sum_{i=1}^3 b_{ii} x_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 b_{ij} x_i x_j \quad (5)$$

where:

$b_0, b_i, b_{ii}, b_{ij}$  – constant regression coefficients of the model  
 $x_i, x_j$  – independent variables

The data analyses were performed using the Statistical Analysis System (SAS, version 8.0, SAS Institute Inc., USA). The SAS was used to generate the response surfaces and contour plots while holding one variable constant in the second-order polynomial model.

## RESULTS AND DISCUSSION

### Effects of the process variables on the protein yield

In a preliminary experiment, a 4-factor, 5-level CCRD was carried out for the screening of the ex-

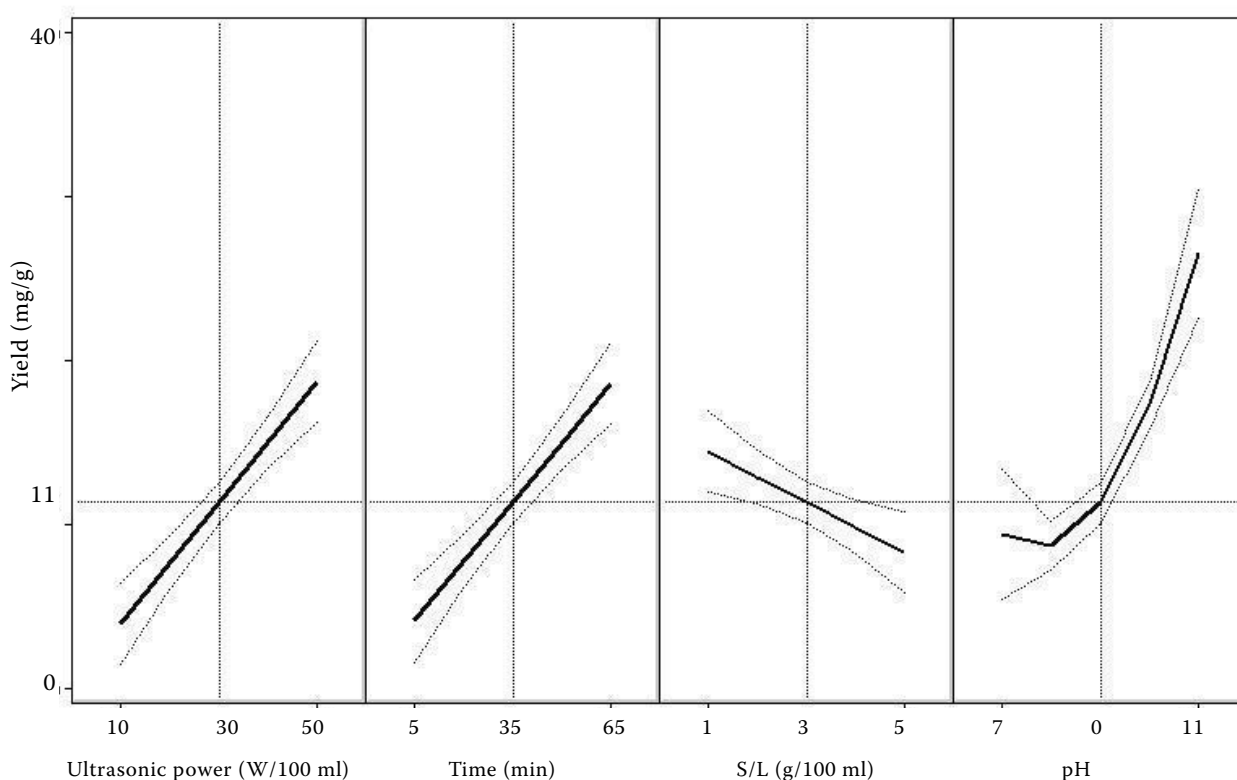


Figure 1. The prediction profile of the 4-factor, 5-level central composite rotatable design experiment

traction parameters. A total of four variables were analysed with regard to their effects on the protein yield. Four extraction parameters (extraction time, ultrasonic power, S/L, and pH value of extractant) were studied. The influence of the variables on the protein yield was indicated in Figure 1. It was found that the protein yield increased with the extraction time, ultrasonic power, and pH value of the extractant while it decreased with the increasing S/L. The significance of each variable was determined using the *F*-test and *P* value. The corresponding variables would be more significant if the *P* value became smaller (WANG *et al.* 2007). All of these four parameters were found to have significant effects on protein extraction as evidenced by their *P* values ( $< 0.05$ , significant at 5% level) obtained from the regression analysis (data not shown). To ensure the stability of protein under high pH conditions, sodium carbonate buffer with pH 10 was selected as the extractant and the effects of other three parameters were further studied.

The experimental conditions and corresponding responses are shown in Table 2. Regression analysis of the experimental data (Table 3) showed that the extraction time, ultrasonic power, and S/L had significant positive linear effects on the protein

yield. Our of the three parameters, the extraction time was found to have the highest impact on the protein yield as given by the highest linear effect ( $X_1$ ,  $P < 0.0001$ ), followed by S/L ( $X_3$ ,  $P = 0.0009$ ) and ultrasonic power ( $X_2$ ,  $P = 0.0172$ ). These extraction parameters also showed significant negative quadratic effects on the protein yield indicating that the protein extraction yield increased as the levels of these factors increased, and decreased as the levels of these factors increased above certain values. Table 3 also indicates that the interaction between all variables was not significant.

### Fitting the model

A response surface regression analysis was carried out to fit mathematical models to the experimental data aiming at finding the optimal region for the protein yield. The respective equation was given as follows:

$$Y = -197.7220 + 3.4357X_1 + 3.2452X_2 + 18.9889X_3 - 0.0211X_1^2 - 0.0184X_2^2 - 4.7528X_3^2 \quad (6)$$

Only those terms which had significant effects on protein extraction were included in the model

Table 3. Estimated regression model of the relationship between the response variables (protein yield) and independent variables (extraction time,  $X_1$ ; ultrasonic power,  $X_2$ ; and solid-liquid ratio of  $X_3$ )

Source	DF	SS	MS	F	P
$X_1$	1	4465.4540	4465.4540	77.3062	< 0.0001 <sup>+</sup>
$X_2$	1	470.1362	470.1362	8.1390	0.0172 <sup>+</sup>
$X_3$	1	1239.7870	1239.7870	21.4633	0.0009 <sup>+</sup>
$X_1^2$	1	1026.2280	1026.2280	17.7661	0.0018 <sup>+</sup>
$X_2^2$	1	784.8375	784.8375	13.5872	0.0042 <sup>+</sup>
$X_3^2$	1	325.5370	325.5370	5.6357	0.0390 <sup>+</sup>
$X_1 * X_2$	1	54.4968	54.4968	0.9435	0.3543
$X_1 * X_3$	1	32.9672	32.9672	0.5707	0.4674
$X_2 * X_3$	1	13.0050	13.0050	0.2251	0.6453

DF – degree of freedom; SS – sum of square; MS – mean square; F – F-statistics test to determine significance; p – probability value (the same in Table 4)

regression Eq. (6). In general, proceeding with exploration and optimisation using a fitted response surface may produce misleading results unless the model exhibits an adequate fit. This makes the checking of the model adequacy essential (LIU *et al.* 2008). Analysis of variance (ANOVA) gives the validity of the model and can explain whether this model adequately fits the variation observed in protein extracted at the designed extraction level. If the F-test for the model is significant at the 5% level ( $P < 0.05$ ), then the model fits and can adequately explain the variation observed. If the F-test for the lack of fit is significant ( $P < 0.05$ ), then a more complicated model is required to accommodate the data. ANOVA for the model predicted for this experiment is given in Table 4. The coefficient of determination ( $R^2$ ) of the predicted model was 92.17%, suggesting that 92.17% of the variations could be explained by the fitted model. The probability ( $P$ ) value of the regression model significance was less than 0.0001. Therefore, the model adequately represented the real relationship between the parameters chosen. The

predicted model seems to represent reasonably the values observed.

The regression model Eq. (6) allowed the prediction of the effects of the three parameters on the protein yield. The relationship between the independent and dependent variables is illustrated in tri-dimensional representation of the response surfaces and two-dimensional contour plots generated by the model for protein (Figures 2–4). Two variables were depicted in tri-dimensional surface plots while the third variable remained constant.

The response surface and contour plot in Figure 2 shows the interaction between S/L and extraction time, indicating that a lower S/L led to a higher yield of protein when the ultrasonic power was about 80 W/100 ml of extractant. The protein yield increased with the increase of ultrasonic power both in the high and low S/L systems (Figure 3). Both the extraction time and ultrasonic power displayed a significantly quadratic effect on the protein yield in the response surface and contour plots given in Figures 2 and 3. A higher yield of

Table 4. ANOVA of the second-order predictive regression model on the yield of protein

Source	DF	SS	MS	F	P
Regression	9	8087.0800	898.5645	15.5560	< 0.0001
Residual error	10	577.6323	57.7632		
Total	19	8664.7130			
$R^2$			92.17%		

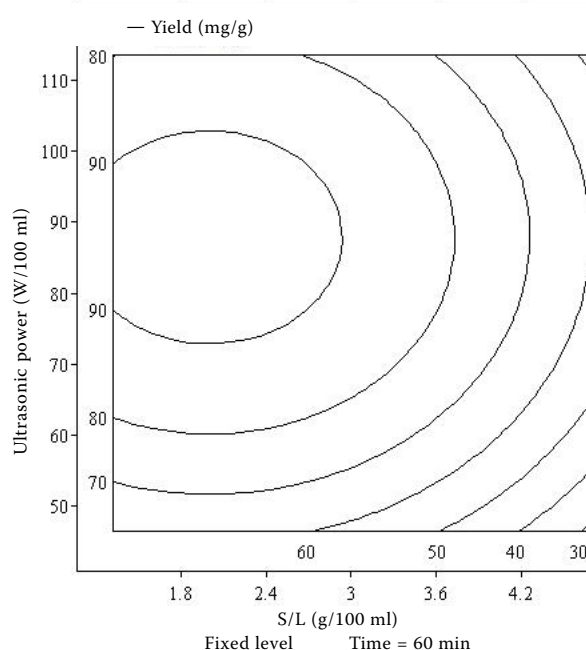
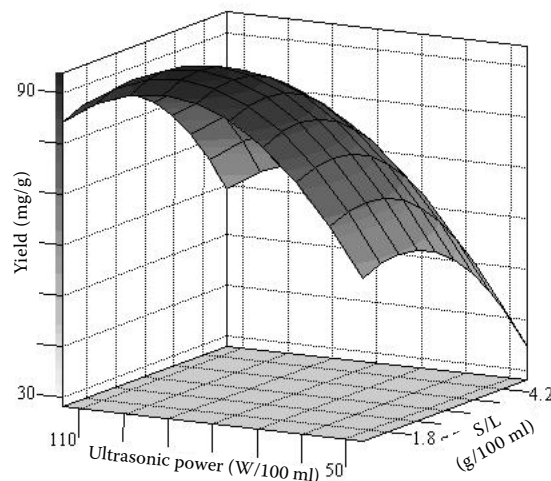
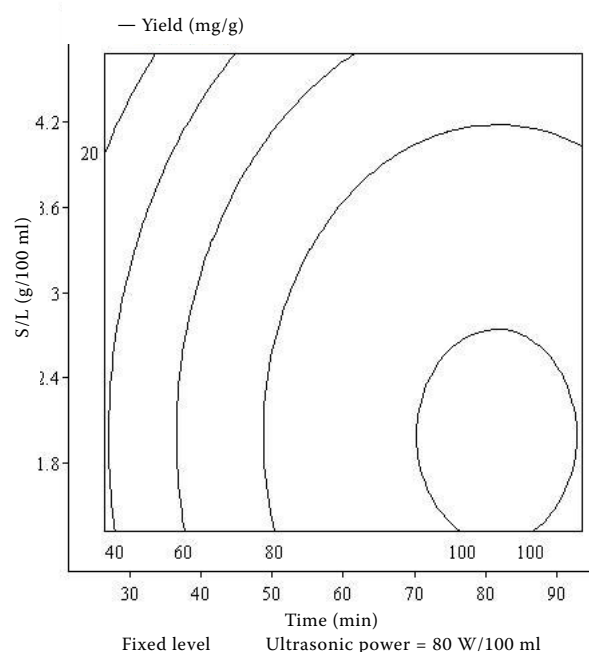
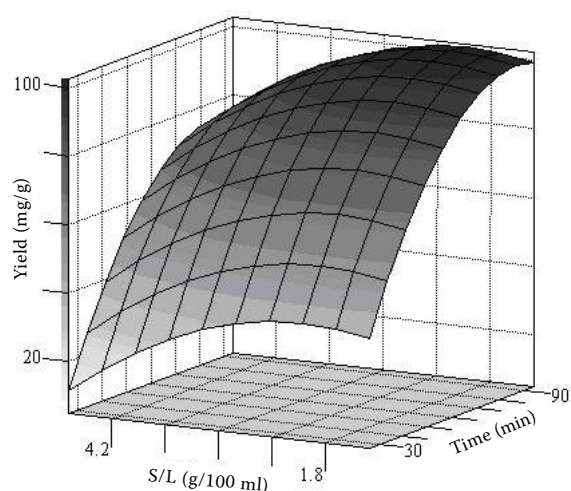


Figure 2. Response surface and contour plots for the effects of extraction time and solid-liquid ratio at ultrasonic power of 80 W/100 ml of extractant on the yield (mg/g) of dried BSG

Figure 3. Response surface and contour plots for the effects of ultrasonic power and solid-liquid ratio at constant extraction time (60 min) on the yield (mg/g) of dried BSG

protein was observed in a lower S/L system than in a higher S/L system. It was due to the relatively higher protein content in the solution with a higher amount of the liquid than that in a higher S/L system when the extraction reached equilibrium.

Figure 4 depicts the effect of the extraction time and ultrasonic power on the protein yield. The extraction time demonstrated a linear increase of the response when the time of extraction was lower than 80 minutes. The mass transfer controls the solvent extraction of any component from the plant matrix; when the solvent saturates with the extracted com-

pound, the concentration gradient becomes null and the phenomena stops. In the ultrasonic-assisted extraction of proteins from BSG, the mass transfer stopped after 80 min and the process could be interrupted. Sonication led to an increase in the mass transfer process and this effect reached maximum at a short sonication time, which was verified with phenolic compounds extraction from coconut shell powder (RODRIGUES *et al.* 2008). The ultrasonically assisted extraction mechanism involves two main types of physical phenomena: cavitations produced in the solvent by the passage of an ultrasonic wave,

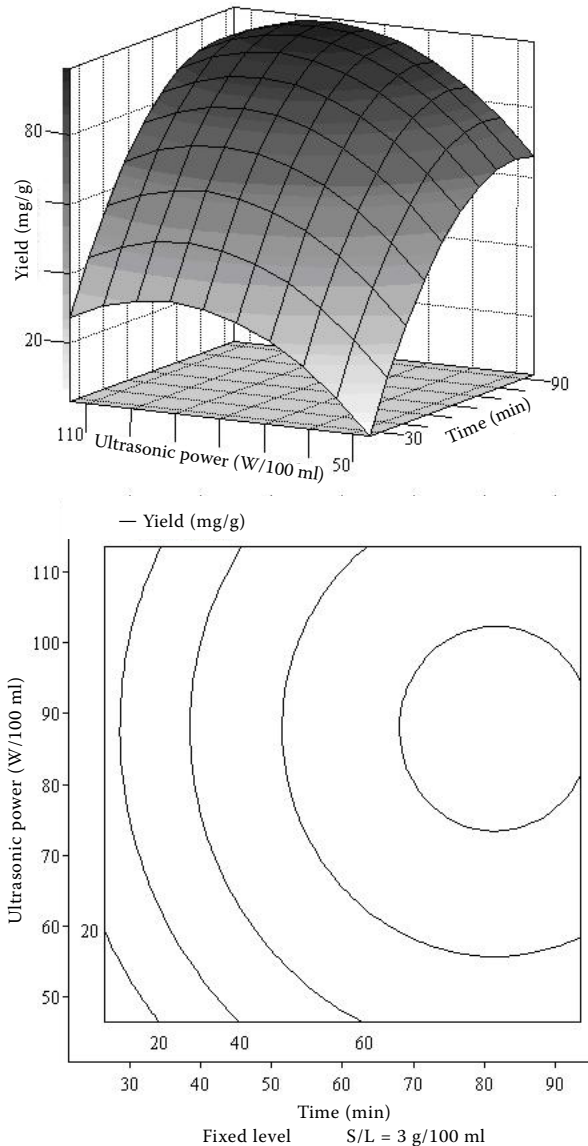


Figure 4. Response surface and contour plots for the effects of ultrasonic power and extraction time at constant solid-liquid ratio (3 g/100 ml min) on the yield (mg/g) of dried BSG

and diffusion through the cell walls (Li *et al.* 2007). It needed a little longer time to reach the extraction balance in the case of BSG. This might be because BSG had been extracted in the brewing process and the majority of the remaining protein was of a low solubility.

The prediction obtained by SAS showed that the ultrasonic power, extraction time, and S/L had positive linear effects and negative quadratic effects on the protein yield. In this case, the partial derivative of Eq. (6) is zero when the yield of protein from BSG reaches maximal value. The following three equations can be constructed:

$$3.4357 - 0.0422X_1 = 0 \quad (7)$$

$$3.2452 - 0.0368X_2 = 0 \quad (8)$$

$$18.9889 - 9.5056X_3 = 0 \quad (9)$$

Using Eqs. (7–9) the following results can be obtained:

$$X_1 = 81.4 \quad X_2 = 88.2 \quad X_3 = 2.0$$

This indicated that the maximal protein yield from BSG could be obtained at the extraction time of 81.4 min, ultrasonic power of 88.2 W/100 ml of extractant, and S/L of 2.0 g/100 ml. The predicted maximal yield of 104.2 mg/g BSG could be obtained from Eq. (6) and the optimised conditions.

### Validation of the Model

An amount of  $96.4 \pm 3.5$  mg/g ( $n = 3$ ) of protein was obtained in a control experiment carried out under the optimised operating condition (extraction time 81.4 min, ultrasonic power 98.2 W/100 ml of extractant, and S/L 2 g/100 ml). The experimental yield of protein was in agreement with the predicted value.

### CONCLUSIONS

Response surface methodology was successfully used to optimise the extraction parameters for the extraction of protein from BSG. Three parameters (extraction time, ultrasonic power, and S/L) were tested using Central Composite Rotatable Design experiment. The three parameters tested showed significant linear and quadratic effects on the yield of protein while no interaction between the three parameters was observed.

The optimal predicted protein yield of 104.2 mg from 1 g of dried BSG was obtained under the optimum conditions of the extraction time of 82.4 min, ultrasonic power of 88.2 W/100 ml of extractant, and S/L of 2.0 g/100 ml. The application of ultrasonic-assisted extraction of BSG dramatically improved the yield. The experimental yield agreed closely with the predicted yield under optimised conditions.

If the conditions were to be applied to a full-scale process, the effect of the downstream process costs should be included, for example the cost of

removing large volumes of water. The optimum ultrasonic power would require scaling-up according to the volume processed.

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