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Full Length Research Paper

Optimization of extraction parameters for protein from beer waste brewing yeast treated by pulsed electric fields (PEF)

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In order to improve the permeability of yeast cell membrane, pulsed electric fields (PEF) was applied in extracting protein from beer waste brewing yeasts (BWBY). Through investigation of three independent variables, including electric field strength (10 to 50 kV/cm), pulse numbers (2 to 16 μs), and liquid-solid ratio (20:1 to 60:1), the optimal extraction parameters for protein were obtained by one-factor-at-a-time (OFAT) experiment and quadratic regression orthogonal design. The results showed that the extraction yield of protein could reach 2.788 ± 0.014% when electric fields intensity was chosen by 10 kV/cm, pulse number was 8, and liquid-solid ratio was of 40:1. Meanwhile, PEF method was a novel and promising method to extract protein from waste beer yeast which will benefit food and agriculture industry. The article mainly focuses on the extraction of dissoluble protein from waste beer yeasts by PEF. PEF could be considered as a highly efficient and energy saving technique to extract bio-component from raw materials.

Key words: Pulsed electric field (PEF), beer waste brewing yeast (BWBY), protein, regression model.

INTRODUCTION

Beer waste brewing yeasts (BWBY) are main by-product in beer industry, and most of them are discarded leaving a small part adding to the next batch of beer fermentation. However, yeasts are substantially beneficial in human culture, as a source for the production of yeast extracts which enhance or impart a meaty flavor to food products (Tanguler and Erten, 2008). BWBY is incorporated in complete rations at 6 of dry matter to increase crude protein from 13% (negative control) to 15%, and soybean meal is incorporated in complete rations at 12% of dry matter to increase crude protein from 13% (negative control) to 17%. (Steckley et al., 2010). Therefore, BWBY is a satisfactory source of whole protein. Moreover, the human body essential amino acids content in BWBY is quite high, especially lysine which is short in cereal protein (Sun et al., 2007) so that BWBY can be regarded as an important resource of human body essential amino acids. Currently, protein of beer is highly valued because it can be widely used in food and agriculture industry. Therefore the exploitation and utilization of extracting protein from beer yeast can not only deals with the accumulation of waste beer yeast, but also promotes development of food industry to create considerable economic benefit.

Pulsed electric field (PEF) is a novel technique that is currently used for non-thermal pasteurization of food or extraction of active ingredients from natural biomaterials (Fox et al., 2008). High electric pressure can change permeability, or irreversibly destroy the structure of cell membrane and cell wall, leading to rapid permeation of interior compounds (Heinz et al., 2003). One reason for interior compounds releasing by PEF is the pore formation. Pore formation is a dynamic process and can be reversible or irreversible depending on the treatment

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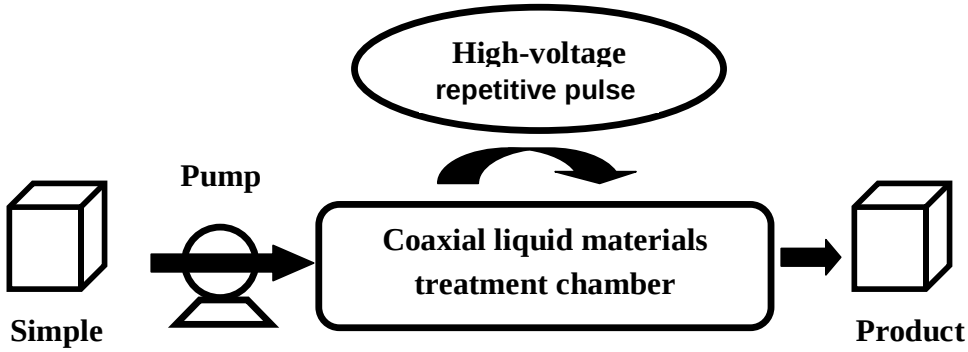

Figure 1. Schematic diagram of PEF processing apparatus.

intensity. When pores induced are smaller in comparison to the membrane area and are generated with PEF treatment of low intensities the electric breakdown is reversible (Angersbach et al., 2000). The viability of the cell is maintained and additional biosynthesis of secondary metabolites can be triggered as a response to the stress condition induced from PEF treatment. Increasing treatment intensity, by increasing electric field strength and treatment time (which considers number of pulses and pulse width applied in a batch system) will promote formation of large pores and reversible permeabilisation will turn into irreversible breakdown. Furthermore, due to its ultra-short treatment time, the quality change of food caused by heat can be reduced to the minimum, therefore, the flavor, taste, and nutritional value could be retained (Loginova et al., 2009). At present, PEF is widely used in extraction of bio-active substances from raw materials (Zhao et al., 2008; Shynkaryk et al., 2009). Published studies concern mainly egg and milk proteins. PEF treatment (from 20 to 35 kV/cm strength, 100 to 900 Hz frequency, and 2 to 8 pulse number) does not induce denaturation of diaultrafiltered egg white protein by measuring surface hydrophobicity (Jeantet et al., 1999). High-strength PEF at 31.5 kV/cm does not cause significant or permanent conformation modification of ovalbumin protein in solution and the same PEF were applied to dialysed egg white without any protein precipitation or marked alterations of gelling properties (Fernandez-Diaz et al., 2000). Effects of pulsed electric fields (PEF) treatment (0–547μs and 0 to 40 kV/cm) on physicochemical properties of soybean protein isolates (SPI) were studied. The results suggested that controlled PEF could be applied to process liquid food including soybean protein ingredient and to modify their structure and function in order to get desired products (Li et al., 2007).

At present, seldom reports have been focused on extraction of protein by PEF from BWBY. Therefore, the aim of this study was to evaluate the impact of PEF on protein extraction, and to obtain the best extraction parameters. Quadratic orthogonal design with three independent variables model is performed to establish the engineering regression model obtained the best technique parameters for protein extraction by PEF from BWBY. In this study, One-Factor-At-a-Time (OFAT) variable experiments and quadratic regression orthogonal design (QROD) were used to optimize extraction parameters for protein by PEF from BWBY which is rich in amounts of ingredients. Current study found that PEF could extract active ingredients from natural biomaterials at high efficiency. This paper using PEF technology as a rapid treatment to improve extraction yield of protein derived from BWBY is able to improve the high added value of the BWBY in medical and functional food industries.

METHODOLOGY

Materials and reagents

BWBY was supplied by Laboratory of Nutrition and Functional Food, College of Quartermaster Technology, Jilin University, Coomassie® Reagent (St. Louis, MO, USA) ,Standard sample of protein was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Tartaric acid, phosphoric acid, ethanol,and all the other materials required in the experiments were purchased from Beijing Chemical Plant (Beijing, China). All the chemicals and reagents used were of analytical grade. 0.100 g Coomassie Blue G250 was dissolved in 50 mL of 95% ethanol and mixed with 100 mL of 85% phosphoric acid ， metered volume to 1L with distilled water. γ-Globulin standard solution was made by dissolving 2.5 mg γ-globulin standard sample into 25 mL distilled water.

Instruments and equipments

High-speed freeze centrifuge (CR20B2) was produced by Hitachi Company (Hitachi Koki Co, Ltd). Visible spectrophotometer (T6 Xinyue) was produced by Beijing General Chromatography Universal Instruments Limited Company. Electronic balance (BT25S) was produced by Beijing Doric Instrument Company. Self-designed PEF system (as shown in Figure 1 consisted of a high-voltage repetitive pulse generator, a coaxial liquid materials treatment chamber, a fiber-optic temperature sensor, and a data acquisition system. The instrument could generate exponentially

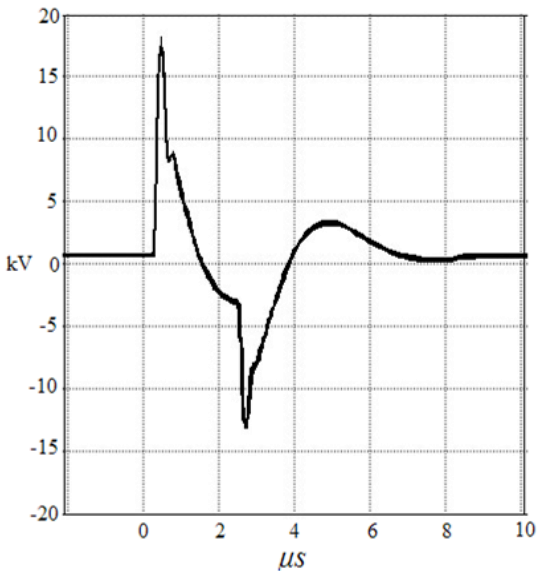

Figure 2. Form of pulsed wave.

decaying bipolar triangle pulse waveforms with pulse duration of 2 μs. The frequency is adjustable, ranging from 1000 to 3000 Hz. The bipolar pulse waveform and input voltage to the treatment chamber can be displayed on a digital oscillograph as shown in Figure 2. The system can process fluid samples continuously for the mass production. The generator is home-made and has been described in our previous report (Lin et al., 2012, 2011; Jin et al., 2011). The pulse number (*C*) can be calculated by the equation of $C = L\pi r^2 f / Q$, while the electric field intensity is expressed in $E = Vpp /(2 \times L)$, where f is the frequency (Hz), *L* is the length of electrode (cm), *r* is the radius of electrode (cm), *Q* is the flow velocity (mL/s) of sample, *E* is the electric field intensity (kV/cm), and *Vpp* is the input voltage shown on the oscillograph. In this paper, *L* is 1.5 cm; *r* is 0.05 cm; *Q* is 0.4 mL/s, other parameters were changed with the experimental condition.

Pre-treatment of BWBY

BWBY paste was sieved into a particle size of 200 μm (mean diameter), and then dissolved with deionized water into 8% yeast suspension. The suspension was mixed with 5% of tartrate, and kept still for 30 min. The mixture was vortexed and then centrifuged for 10min under the rotate rate of 3600 rpm. The supernatant was discarded to collect the beer yeast cell in the sediments. The beer yeast cell paste was kept under 4°C until use.

Determination of protein content

The method of coomassie brilliant blue G250 used to obtain standard reagent. Coomassic Brilliant Blue G250 is a kind of dye solution. As soon as the dye solution is mixed with a protein sample, the absorbance of the mixture can be measured (Scdmak and Grossbcrg, 2005; Bradford, 2009). 0.1 ml of each standard or unknown sample (10, 20, 30, 40, 50 and 60 μl of 0.1 g/ml γ-globulin standard solution) was pipette into appropriately labeled test tubes and 5.0 ml of the Coomassie® Reagent added to each tube and mix well. After that, samples were incubated for 10 min at room temperature (RT). And then the average 595 nm reading for the Blank replicates was subtract from the 595 nm readings of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average Blank-corrected 595 nm reading for each body surface area (BSA) standard versus its concentration in μg/ml, and the standard curve was use to determine the protein concentration of each unknown sample. The density of protein can be calculated according to the following equation:

$$\text{Protein density (mg/mL)} = \frac{C \times n}{V_T} \times 1000$$

where *C* is the value from calibration curve (μg); V_T is the volume of sample solution (1.0 ml); n is diluted times. Regression equations was Y = 09382 X + 0.0183 (R^2 = 0.9956).

Optimization of protein extraction by PEF through one-factor-at-a-time experiment

Yeast cell pastes were dissolved with deionized water into desirable liquid-solid ratio before PEF treatment. Based on our preliminary study, four independent variables were investigated through one-factor-at-a-time (OFAT) experiment, including electric field intensity (10, 20, 30, 40, and 50 kV/cm), pulse number (0, 2, 4, 6, 8, 10,12,14 and 16), and liquid-solid ration (20: 1, 30:1, 40:1, 50:1, and 60:1). After the PEF treatment, the sample was centrifuged for 10 min under the rotate rate of 4200 rpm. Protein content in suspension was tested.

Optimization of extraction parameters by orthogonal quadratic design

Based on the primarily OFAT experiment, three independent variables at four levels (4^3) were adopted for orthogonal quadratic design. This type of designs is very important to study the effect of several factors, since each replication of the experiment contains all possible combinations of the levels of the factors (Wai et al., 2010). Three independent variables studied were the electric field intensity (Z_1), pulse number (Z_2), and liquid-solid ratio (Z_3), which were coded into X_1, X_2 and X_3, respectively according to the coding equations shown in Table 1. The dependent variable Y was the protein yield. This experiment was carried out in random order to minimize the effects of unexpected variability in the observed responses. Totally eleven runs were required to cover all possible combinations of factors levels. Finally, the regression equation model could be established to determine the relationship between protein yield and three independent variables.

Statistical analysis

ANOVA was performed to use the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The significances of the regression coefficients were also tested by F-test. The quality of the fitness of the polynomial model equation was expressed by the coefficient of determination R^2. All experiments were in triplicates and the means of three data sets were presented. The significant different was determined with $p < 0.05$.

RESULTS AND DISCUSSION

Effects of independent variables on protein extraction through OFAT experiment

5 g clean yeast mud was dissolved with distilled water

Table 1. Independent variables and levels of quadratic regression orthogonal design.

Z_j (X_j)	Z_1 Electric field intensity (kV/cm)	Z_2 Pulse number	Z_3 Liquid- solid ratio (ml : g)
$Z_{0j}+\Delta_j$ (1)	30	8	50:1
Z_{0j} (0)	20	6	40:1
$Z_{0j}-\Delta_j$ (-1)	10	4	30:1
Δ_j	10	2	10:1
$X_j = (Z_j - Z_{0j}) / \Delta_j$	$X_1 = (Z_1 - 20)/10$	$X_2 = (Z_2 - 6)/2$	$X_3 = (Z_3 - 40)/10$

The independent variables and their levels in the quadratic regression orthogonal design are shown. To investigate the effects of Electric field intensity (10 to 30 kV/cm), Pulse number (4 to 8) and liquid-solid ratio (30:1 to 50:1) on the protein yield from BWBY, a quadratic regression orthogonal design with three independent variables was applied in this study to determine the response pattern and then to establish a model for the optimization of the PEF treatment. The three independent variables were coded as X_1, X_2 and X_3 representing Electric field intensity, Pulse number and liquid-solid ratio, respectively, while the dependent variable was the protein yield. The settings for the independent variables were displayed with low and high values.

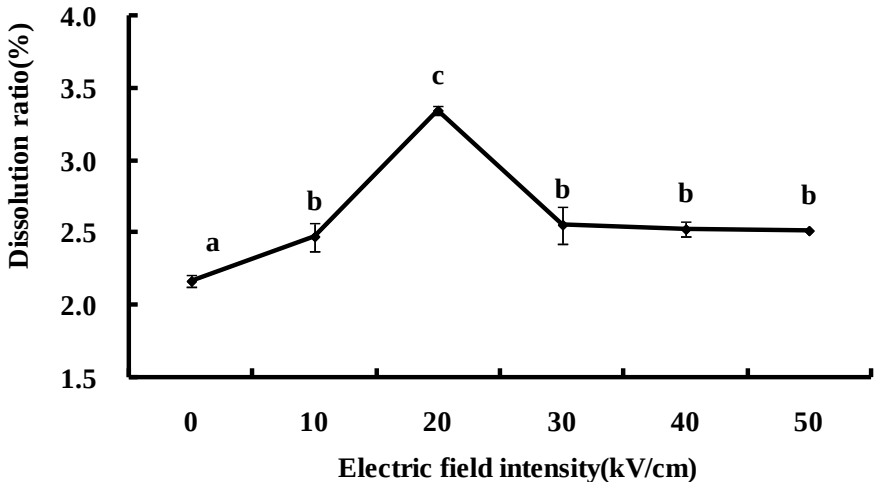

Figure 3. Effects of field strength on dissolution ratio of dissoluble protein. Different lowercase letters mean that variance of two samples are significant *(P < 0.05).*

reaching to liquid-solid ratio 40:1. Yeast solution was treated by PEF as pulse number was 6. Effects of electric field intensity on protein extraction were showed in Figure 3. No dramatic variances of protein yield were observed when electric field intensity increased from 0 to 20 kV/cm and dissolution ratio of protein increasing from 2.17 ± 0.021% to 3.34 ± 0.050%. It is obvious that PEF intensity has a direct influence on dissolution of protein. The higher PEF intensity, the higher dissolution ratio of protein was from BWBY. It is generally accepted that PEF exerts its effect primarily by causing the membranes of cell destroyed and protein denatured. Under high electric field strength, more solvent will enter into the cell and more compounds can permeate the cell membrane easily. Besides, different electric field strength between the inner and the exterior of the cell makes electropolar perforation of cell membrane (Sun et al., 2001). Therefore, increasing PEF intensity could increase the dissolution ratio of protein. However, extraction of protein dropped from 3.34 ± 0.050% to 2.52 ± 0.052%, when electric field intensity increased from 20 to 50 kV/cm. By increasing electric field strength and treatment time, increasing treatment intensity will promote formation of large pores and reversible permeabilisation will turn into irreversible breakdown. However, some reports showed that critical electric field strength to induce membrane permeabilisation is dependent on cell geometry and size, in the range of 1 to 2 kV/cm for plant cells (cell size 40 to 200 mm) and in the range of 12 to 20 kV/cm for microorganisms (cell size 1 to 10 mm) (Heinz et al., 2002). When PEF intensity was 20 kV/cm, dissolution of protein was the highest. As in this research, PEF intensity of 20 kV/cm could improve the dissolution ratio of protein from BWBY significantly ($p < 0.05$).

5 g of clean yeast mud was dissolved with distilled water to desirable liquid-solid of 40:1. Yeast solution was

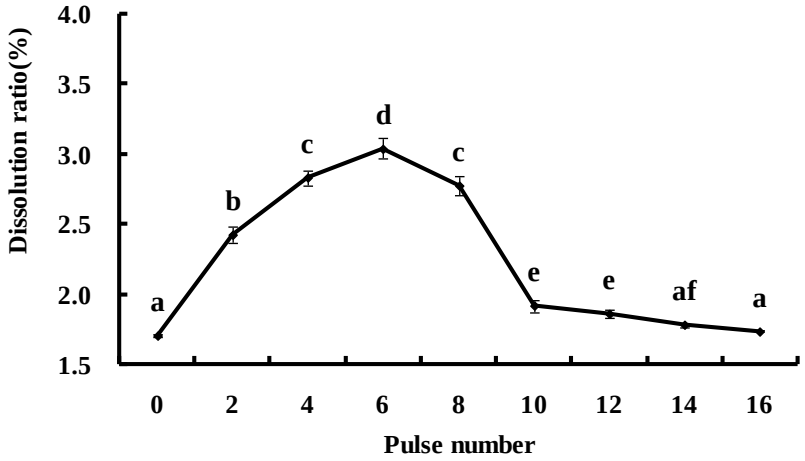

Figure 4. Effects of pulse number on protein yield. To evaluate the effect of pulse number of BWBY on the protein yield, different pulse number of 0, 2, 4, 6, 8,10,12,14 and 16 was investigated at electric field intensity of 20 kV/cm, and liquid-solid ratio of 40:1. Different lowercase letters donate that variance of samples are significant *(p < 0.05)*.

treated by PEF as electric field intensity was 30 kV/cm. Effects of pulse number on protein yield are showed in Figure 4. When the pulse number increased from 2 to 6, protein yield increased significantly *(p < 0.05)*, and reached from 1.70 ± 0.014% to the highest value of 3.04 ± 0.039% when the pulse number is 6. However, with the increase in pulse number from 6 to 16, protein yield gradually declined *(p < 0.05)*. Therefore, pulse number of 6 was determined as the best condition. According to some researches, PEF could greatly promoted physical reaction from the yeast suspension. There are mainly two reasons for the phenomenon. First, PEF could accumulate the charge in the two peaks of cells, which could lead to the electric potential difference of permeability and decomposition of cell structures. Therefore, more and more pores could be formed in cell membrane. As a result, the permeability of cell membrane is improved. Secondly and the most importantly, PEF could produce the pulsed that could stimulate resonant effects to provide more energy for raw materials. The material was treated by PEF with electric pulse. The narrow pulse would excite material to self-frequency oscillation that is resonance vibration. Therefore, materials would produce huge energy by themselves and accelerate chemical reaction. Besides, more pulse number would lead to higher resonance vibration energy of materials and large swing of resonance vibration. The chemical reaction would rapidly occur (Yin and He, 2008). However, when the energy provided by PEF exceeded the critical limit, no effects were observed. Therefore, severe PEF treatment was not recommended.

5 g of clean yeast mud was dissolved with distilled water to different liquid-solid ratio. Yeast solution was treated by PEF under conditions of intensity 30 kV/cm and pulse number 6. Effects of liquid-solid ratio on protein yield are showed in Figure 4. According to some research, solid–liquid extraction is an important unit operation to recover the soluble matter from different biological materials (Belghiti and Vorobiev, 2004). The results in Figure 5 indicated that with the increase of liquid-solid ratio from 20:1 to 40:1, the protein yield first slightly increased from 1.03 ± 0.014 to 1.69 ± 0.016%, when the liquid-solid ratio reached to 50:1. When the liquid-solid ratio increased from 50:1 to 60:1, protein yield significantly dropped to 1.59 ± 0.016% in the end *(p < 0.05)*. It could demonstrate that only when the high-voltage pulse worked on the proper number of surface area of membrane, it could exhibit the best pore formation ability, leading to the more protein being released (Bouzraraand and Vorobiev, 2003). Therefore, the liquid-solid ratio of 50:1 should be chosen as the most suitable condition for PEF treatment.

Establishment of regression model of protein extraction by PEF

According to the results of OFAT optimization, ternary quadratic orthogonal regression model was employed to study effects of three independent variables, including electric field intensity (X_1:10 to 30), pulse number (X_2:4 to 8), and liquid-solid ratio (X_3: 30:1 to 50:1), on protein yield in an effort to obtain an optimized PEF treatment and the coefficients of its second-order polynomial equation. A total amount of 11 experiments were conducted covering a wide range of the variables and levels, as shown in Table 2. The regression coefficients are summarized in

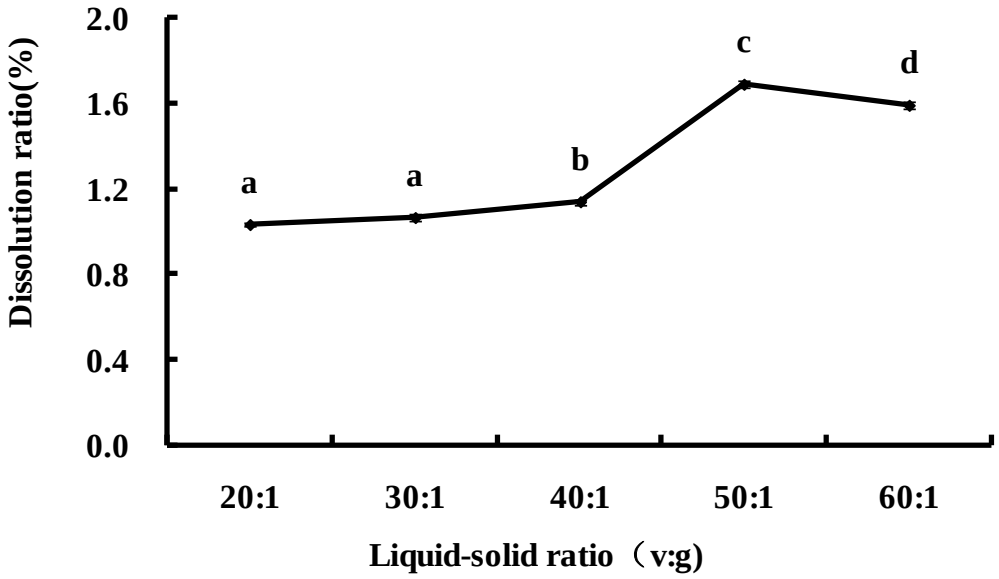

Figure 5. Effects of liquid-solid ratio on trehalose yield. To evaluate the effect of liquid-solid ratio of BWBY on the protein yield, different liquid-solid ratio of 20:1, 30:1, 40:1, 50:1 and 60:1 was investigated at electric field intensity of 20 kV/cm, pulse number of 6. Different lowercase letters donate that variance of samples are significant *($p < 0.05$)*.

Table 2. Design and results of quadratic regression orthogonal design.

No.	X_0	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	$Y_i \pm$ RSD
1	1	1	1	1	1	1	1	2.571 ± 0.030
2	1	1	1	-1	1	-1	-1	1.789 ± 0.024
3	1	1	-1	1	-1	1	-1	2.072 ± 0.042
4	1	1	-1	-1	-1	-1	1	1.714 ± 0.027
5	1	-1	1	1	-1	-1	1	2.788 ± 0.014
6	1	-1	1	-1	-1	1	-1	1.838 ± 0.013
7	1	-1	-1	1	1	-1	-1	2.685 ± 0.033
8	1	-1	-1	-1	1	1	1	1.741 ± 0.046
9	1	0	0	0	0	0	0	2.112 ± 0.012
10	1	0	0	0	0	0	0	2.236 ± 0.002
11	1	0	0	0	0	0	0	2.062 ± 0.011

Table 3. Results of variance of regression model.

Variance source	Square sum (SS)	Degree of freedom (df)	Mean square (MS)	F_{value}	α
X_1	0.102	1	0.102	12.616	0.10
X_2	0.075	1	0.075	9.24	0.10
X_3	1.151	1	1.151	142.075	0.01
X_1X_2	0.018	1	0.018	2.163	0.25
X_1X_3	0.071	1	0.071	8.783	0.10
X_2X_3	0.023	1	0.023	2.852	0.25
S	1.44	6	$F_{0.25}(1,1) = 5.83$	$F_{0.1}(1,1) = 39.1$	$F_{0.01}(1,1) = 4052$

p-Values were used as a tool to check the significance of each coefficient. The smaller the *p*-value, the more significant was the corresponding coefficient. (Muralidhar et al., 2007).

Table 3. The final mathematical model can be expressed by the following quadratic equation:

$$Y=2.146 - 0.113X_1 + 0.09673X_2 + 0.379X_3 + 0.047X_1X_2 - 0.071X_1X_3 + 0.054X_2X_3$$

where, Y is the protein yield from BWBY (%); X_1 is the electric field intensity (kV / cm); X_2 is the pulsed number; X_3 is the liquid-solid ratio. By analyzing the data in Table 2, significant tests of coefficient and equation, and model fitting were assayed. Table 2 shows the experimental design and the response of the extract yield of protein from the BWBY obtained for each of the experiments. In this study, QROD with three independent variables was used to determine the response pattern and, then, to establish a model for the optimization of the PEF treatment. Experimental design, data analysis and quadratic model building were accomplished by optimal design and analysis of experiments. The optimal conditions to obtain the maximum extract yield of protein from the BWBY were determined as follows: an electric fields intensity of 10 kV/cm, a pulse number of 6, and a liquid-solid ratio of 50:1, the extraction rate of protein could reach to 2.788 ± 0.014%. Statistic analysis results of independent variables and their interaction were showed in Table 3. The results showed that three independent variables (electric field intensity, pulse number, and liquid-solid ratio) all had significant effects on protein yield at the significant level of $p = 0.1$. From Table 3, we could tell that the sum of deviation squares (S) is 1.36499 ($f = 4$), and sum of residual squares (S_R) is 0.11337 ($f_R = 6$). Moreover, static variables could be calculated and $F_j = (S_j / f_j) / (S_R / f_R) = 18.06032 > F_{0.01}(4,6) = 9.148301$, which meant the regression model of coding space was significant at the level of α of 0.01. In general, the validity of the model can be judged by lack of fit to check the adequacy of a regression model (Stalikas et al., 2009). In order to estimate the experimental error, lack of fit test was performed, and the results showed that static variables S_{lf} and S_e were 0.09717 and 0.0162, as f_{lf} and f_e were 4 and 2 respectively. Then:

$$F_{lf} = (S_{lf} / f_{lf}) / (S_e / f_e) = 2.998451 < F_{0.25}(4, 2) = 3.232051$$

which meant the regression model does not lack of fit. The regression equation of nature space was obtained by replacing the code by transformation equation showed in Table 2, the final equation of natural space was expressed as:

$$Y = 0.51881 + 0.01Z_1 + 0.048365Z_2 + 0.0521Z_3 - 0.00071Z_1Z_3$$

where, Z_1 is electricity field intensity (kV / cm), Z_2 is pulse number, Z_3 is liquid-solid ratio. The best parameters for protein extraction by PEF are determined as electric field intensity of 19.97 kV/cm, pulse number of 6, and the liquid-solid ratio of 50:1. And the protein yields reached 2.788 ± 0.014%. Meanwhile, the predicted results, according to the models for the protein yield, were close to the observed experimental responses. The results showed that the PEF treatment under the best conditions can effectively facilitate protein extraction from BWBY.

Conclusion

The PEF technique was performed for extraction of protein from BWBY in order to increase the yield extraction. It is non-thermal, fast, and efficient and has non - negative effect. Based on single-factor experiments, QROD was used to estimate and optimize the experimental variables in terms of the electric field intensity (kV/cm), ratio of liquid to raw material, and pulse duration. A desirable quadratic polynomial mathematical model was obtained with the following optimal extraction conditions for protein extraction: electric field intensity of 10 kV/cm, ratio of liquid to raw material 50:1, and pulse duration 8. Under these conditions, the validated experimental yield of protein was 2.788 ± 0.014%. Therefore, we concluded that PEF represents a valuable alternative to the traditional extraction methods (for example, ultrasound and microwave techniques) for the efficient extraction of protein from BWBY. However, further work is needed to identify the specific mechanism of PEF working on yeast cell membrane.

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