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Full Length Research Paper

Optimization of extraction parameters for trehalose from beer waste brewing yeast treated by high-intensity pulsed electric fields (PEF)

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High-intensity pulsed electric fields (PEF) was applied to treat beer waste brewing yeasts (BWBY) to improve the permeability of yeast cell membrane in order to extract trehalose. Several independent variables such as pH (3 to 7), electric field strength (0 to 50 kV/cm), pulse numbers (0 to 10) and liquid-solid ratio (20: 1 to 60: 1) were investigated. The optimal parameters were obtained by one-factor-at-a-time (OFAT) experiment and quadratic regression orthogonal design. The results showed that under the conditions of electric fields intensity of 19.97 kV/cm, pulse number of 6, and liquid-solid ratio of 30:1, the extraction rate of trehalose could reach 2.635%. In addition, the extraction efficiency of trehalose treated by PEF was compared with other two trehalose extraction methods, example microwave and ultrasound, and the efficiency of PEF was found to be 103.15 μg/s; it was 15.96 times higher than microwave and 34.08 times higher than ultrasound. This demonstrated that the PEF could be regarded as a promising technique for bio-material extraction.

Key words: High-intensity pulsed electric field (PEF), Beer waste brewing yeast (BWBY), Trehalose, 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, and they are also a source for the production of yeast extracts which enhance or impart a meaty flavor to food products (Tanguler and Erten, 2008). Moreover, the trehalose content in BWBY is quite high so that BWBY can be regarded as an important resource of trehalose (Mahmud et al., 2010; Benaroudj et al., 2001; Rúa et al., 2008) Currently, trehalose is highly valued because it can be taken as an excellent bio-protectant (Gibson et al., 2008). Meanwhile, trehalose can protect animals, plants and microorganism from severe atmospheres damages, such as nutrition deficiency, freezing, hyper-osmosis, saline stress, and dehydration, as an emergency metabolite (Voit, 2003; Streeter, 2003). Therefore, trehalose can be widely used in food, medicine, and cosmetics industries (Cesàro et al., 2008).

High-intensity 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 com-pounds (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 intensity. When pores induced are small in comparison to the membrane area and are generated with PEF treatment of low

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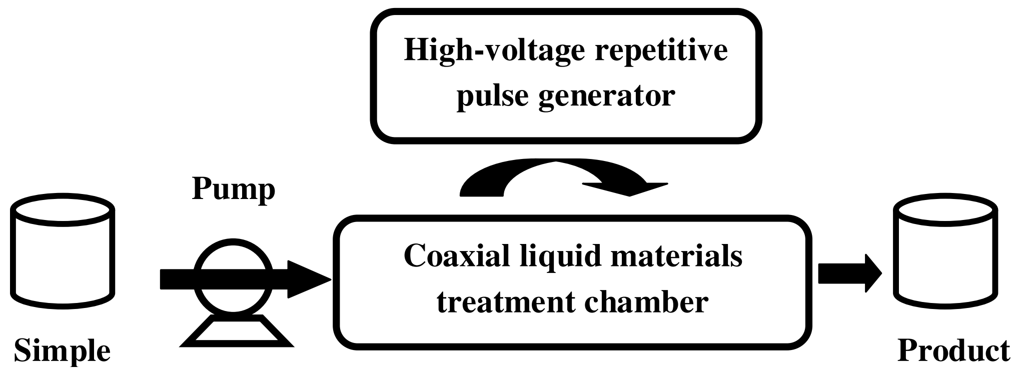


Figure 1. Schematic diagram of high intensity pulsed electric field processing apparatus.

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/or treatment time (which considers number of pulses and pulse width applied in a batch system) will promote formation of large pores and reversible permeabilization 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 so that the flavor, taste, and nutritional value could be retained (Loginova et al., 2010). At present, PEF is widely used in extraction of bio-active substances from raw materials (Zhao et al., 2008; Shynkaryk et al., 2009). Yin et al. (2006) used PEF to process *Rana temporaria chensinensis* David to extract polysaccharose, and the extraction yield was 55.59%, which is 1.77 times higher than that of enzymatic hydrolysis, and the products were pure and still kept their high activity.

At present, few reports have been focused on the effects of PEF on trehalose extraction from BWBY. The precise mechanism of PEF-induced changes is still not understood in depth (Soliva-Fortuny et al., 2009). Therefore, the aim of this study was to evaluate the impact of PEF on trehalose extraction, and obtain the best extraction parameters. Regression orthogonal design was performed to establish the engineering regression model to obtain the best technique parameters for trehalose extraction by PEF from BWBY.

MATERIALS AND METHODS

BWBY was supplied by Golden Hans Co. Ltd (Changchun, China). Standard sample of trehalose was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Tartaric acid, sulfuric acid, anthrone, 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. The anthrone reagent was prepared according to Leyva et al. (2008) with a little modification; 0.025 g anthrone was dissolved in 50 ml of 98% sulfuric acid, and the solution was protected from light and used within 12 h.

Instruments and equipments

Self-designed PEF system (Figure 1) consisting of a high-voltage repetitive pulse generator, a coaxial liquid material treatment chamber, a fiber-optic temperature sensor and a data acquisition system was used. The instrument could generate exponentially decaying bipolar triangle pulse waveforms with pulse duration of 2 μs. The frequency was 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 was home-made and has been described in our previous report (Yin et al., 2006).

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 as $E = Vpp /(2 \times L)$, 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 study, L was 0.1 cm, r was 0.05 cm and Q was 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 10 min under the rotate rate of 3600 rpm. The supernatant was then discarded to collect the beer yeast cell in the sediments. The beer yeast cell paste was kept under 4°C until use.

Determination of trehalose content

Sulphuric acid-anthrone colorimetry (Ferreira et al., 1997) was used to detect trehalose content at 550 ~ 750 nm and the spectrum atlas was obtained through low-velocity scanning. One milliliter of 0.1 mg/ml trehalose standard solution was mixed with 4 ml of anthrone reagent. The mixture was then kept in boiling water for 10 min, and immediately cooled down to room temperature. OD was measured at 630 nm and trehalose concentration was determined by the trehalose standard curve (Rossignol et al., 2003). The linear equation of trehalose standard curve is $A = 6.2745 \times C$ ($R^2 = 0.9984$). The yield of trehalose can be calculated according to

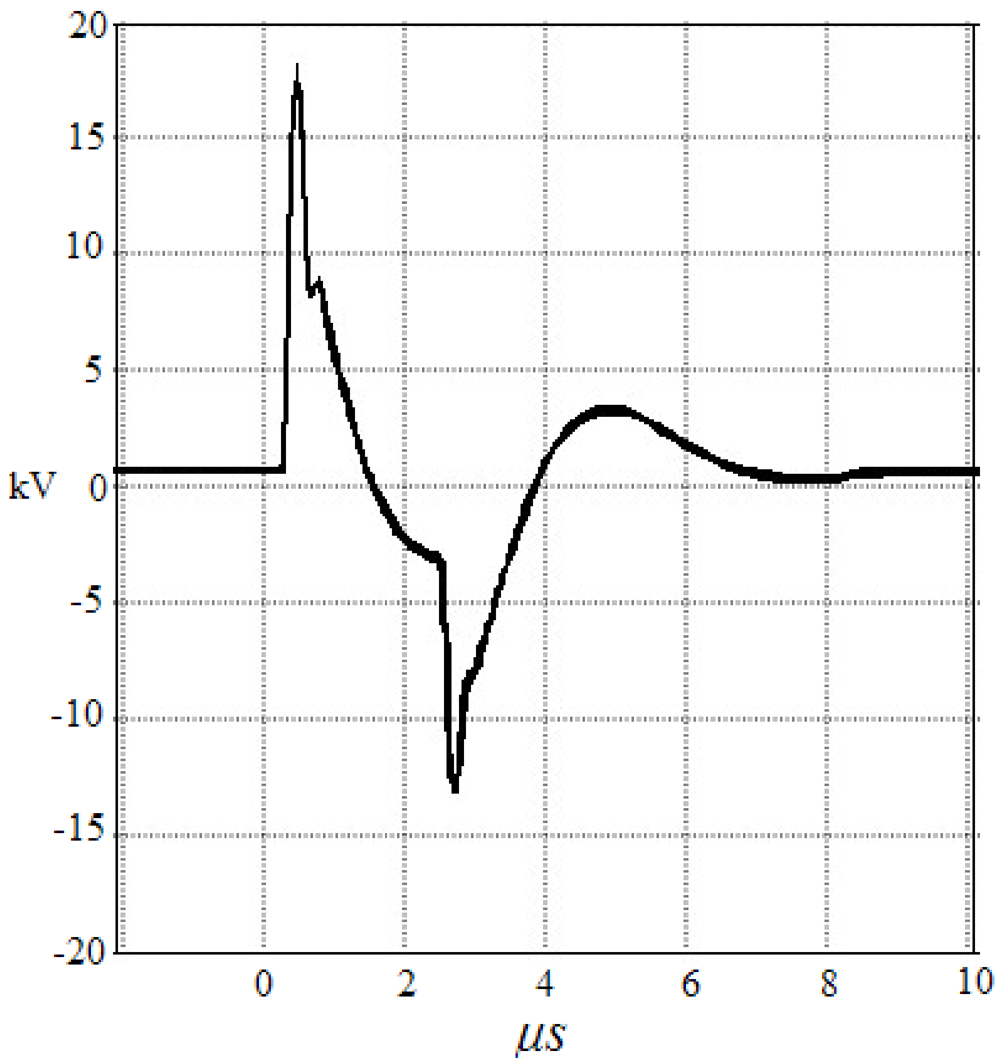


Figure 2. Form of pulsed wave.

Equation 1, and extraction efficiency of trehalose from WBY can be calculated according to Equation 2 as follows:

$$\text{Trehalose yield (\%)} = \frac{\text{Trehalose content in supernatant (mg)}}{\text{Weight of dry yeast paste(mg)}} \times 100\% \quad (1)$$

$$\text{Extraction rate } ((\mu g/s) = \frac{\text{Trehalose content in supernatant } (\mu g)}{\text{Extraction time (s)}} \times 100\ \% \quad (2)$$

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

Yeast cell pastes were dissolved with deionized water into desirable liquid-solid ration 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), pH value (3, 5 and 7), pulse number (0, 2, 4, 6, 8 and 10) and liquid-solid ratio (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. Finally, trehalose 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 trehalose yield. This experiment was carried out in random order to minimize the effects of unexpected variability in the observed responses. In total eleven runs were required to cover all possible combinations

Table1. Independent variables and levels of quadratic regression orthogonal design.

Z_j (X_j)	Z_1 Electric field intensity (kV/cm)	Z_2 Pulse number (n)	Z_3 liquid-solid ratio (ml :g)
$Z_{0j}+\Delta_j$ (1)	30	8	40:1
Z_{0j} (0)	20	6	30:1
$Z_{0j}-\Delta_j$ (-1)	10	4	20:1
Δ_j	10	2	10:1
Code transformation	$X_1=\frac{Z_1-20}{100}$	$X_2=\frac{Z_2-6}{2}$	$X_3=\frac{Z_3-30}{10}$

To investigate the effects of electric field intensity (10 to 30 kV/cm), pulse number (4 to 8 n) and liquid-solid ratio (20:1 to 40:1 ml :g) on the trehalose 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 trehalose yield. The settings for the independent variables were displayed with low and high values.

of factors levels. Finally, the regression equation model was established to determine the relationship between trehalose yield and three independent variables.

Comparison of three trehalose extraction methods

The efficiencies on trehalose extraction from BWBY of Ultrasound technique, microwave technique, as well as PEF treatment were compared. The specific treatment procedures were illustrated as follows.

Ultrasound technique

5.00 ± 0.01 g of yeast cell paste was weighed accurately and then dissolved with deionized water to 200 ml. The solution was treated by ultrasound cell crusher (power: 600W, time: 20 min), and then cooled down to room temperature after boiled water bath for 6 h. Finally, the sample was centrifuged and the supernatant was taken to detect trehalose content.

Microwave technique

5.00 ± 0.01 g of yeast cell paste was weighed accurately, dissolved with deionized water to 150 ml. The solution was treated by Microwave (power: 600W, temperature: 80°C, time: 3 min), boiled for 3.5 h, and finally centrifuged. The supernatant liquid was then taken to detect trehalose content.

PEF technique

5.00 ± 0.01 g of yeast cell paste was weighed accurately, dissolved with deionized water to a volume of 150 ml. The solution was treated by PEF (pulse number of 6, electric field intensity of 19.97 kV/cm and time of 6 min) and then centrifuged. The clear supernatant liquid was taken to detect trehalose content.

Statistical analysis

ANOVA was performed using 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 difference was determined with $P < 0.05$.

RESULTS AND DISCUSSION

Effects of independent variables on trehalose extraction through OFAT experiment

Effects of electric field intensity and pH value on trehalose yield are shown in Figure 3. Increasing the pH value from 3 to 7 significantly increased the trehalose yield ($P < 0.05$), of which the best value was obtained from pH value of 7. When the pH value was set at 7, compared with the control group (E = 0 kV/cm), the trehalose yield reached a higher rate of 2.658% at electric field intensity of 20 kV/cm. Moreover, the sample with pH value of 7 showed the significantly higher trehalose yield than pH 3 and 5 ($P < 0.05$). As for the effect of electric field intensity, no dramatic variances of trehalose yield were observed when it increased from 0 to 30 kV/cm within all three groups of samples with different pH values. Moreover, when the electric field intensity increased from 30 to 40 kV/cm; the trehalose yield of the sample with pH value of 3 dropped significantly. Increasing treatment intensity by increasing electric field strength and/or treatment time will promote formation of large pores and reversible permeabilization will turn into irreversible breakdown. However, some reports showed that critical electric field strength to induce membrane permeabilization 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). Therefore, trehalose yield may already have reached a high value at electric field intensity of 10 kV/cm, hence a higher intensity above 10 kV/cm caused insignificant variance on trehalose yield.

Effects of pulse number on trehalose yield are shown in Figure 4. When the pulse number increased from 0 to 6,

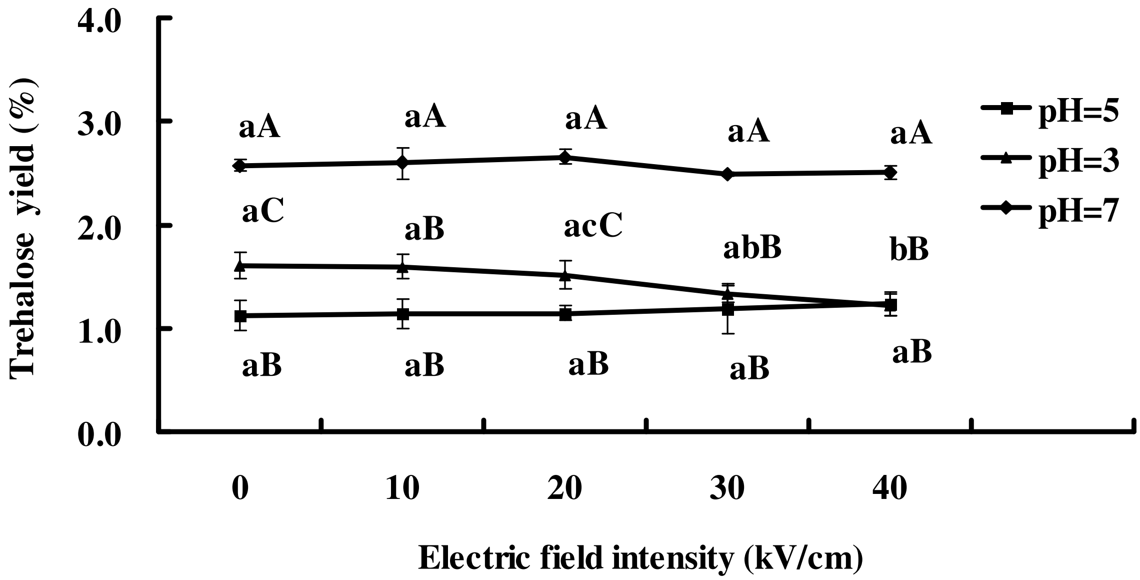


Figure 3. Effects of electric fields intensity and pH on trehalose yield.
To evaluate the effect of pH and electric fields intensity BWBY on the trehalose yield, different pH of 3, 5 and 7, and electric field intensity of 10, 20, 30 and 40 kV/cm were investigated at liquid-solid ratio of 40:1 and pulse number of 6. Different lowercase letters indicate that the variances of trehalose yield are significant (P < 0.05) under different electric field intensity with the same pH value. While different capital letters donate that the variance of trehalose yield are significant with different pH value under the same electric field intensity (*P < 0.05*).

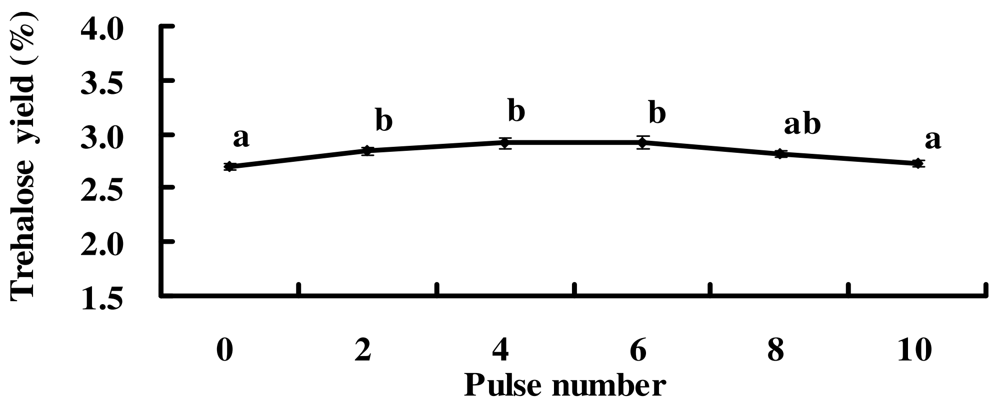


Figure 4. Effects of pulse number on trehalose yield.
To evaluate the effect of pulse number of BWBY on the trehalose yield, different pulse number of 0, 2, 4, 6, 8 and 10 was investigated at pH value of 7, electric field intensity of 20 kV/cm, and liquid-solid ratio of 40 : 1. Different lowercase letters donate that variance of samples are significant *(P < 0.05)*.

trehalose yield increased significantly *(P< 0.05)*, and reached the highest value of 2.921% when the pulse number was 6. However, with the increase in pulse number from 6 to 10, trehalose 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, thus leading to the electric potential difference of permeability and decomposition of cell structures. Therefore, more and more pores could be formed in cell membrane so that 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

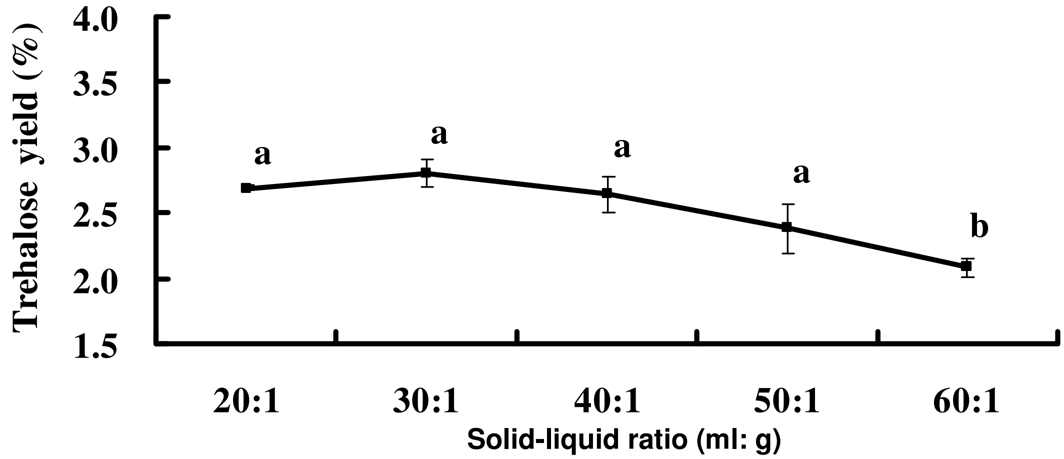


Figure 5. Effects of liquid-solid ratio on trehalose yield.
To evaluate the effect of liquid-solid ratio of BWBY on the trehalose yield, different liquid-solid ratio of 20 : 1, 30 : 1, 40 : 1, 50 : 1 and 60 : 1 was investigated at pH value of 7, electric field intensity of 20 kV/cm, pulse number of 6. Different lowercase letters donate that variance of samples are significant *(P<0.05)*.

themselves and accelerate chemical reaction. Besides, more pulse number would lead to higher resonance vibration energy of materials and large swing of resonance vibration. Hence, the chemical reaction would rapidly occur (Yin and He, 2008). How-ever, when the energy provided by PEF exceeded the critical limit, no effects were observed. Therefore, severe PEF treatment was not recommended.

Effects of liquid-solid ratio on trehalose yield are shown in Figure 5. 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 30:1, the trehalose yield first slightly increased from 2.690 to 2.805%, and then gradually declined to 2.379% when the liquid-solid ratio reached to 50 : 1. Also, when the liquid-solid ratio increased from 50 : 1 to 60 : 1, trehalose yield significantly dropped to 2.081% in the end *(P<0.05)*. This demonstrated that it is only when the high-voltage pulse worked on the proper number of surface area of membrane that it could exhibit the best pore formation ability, leading to the more trehalose being released (Bouzraraand and Vorobiev, 2003). Therefore, the liquid-solid ratio of 30 : 1 was chosen as the most suitable condition for PEF treatment.

Establishment of regression model of trehalose 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: 2 to 8), and liquid-solid ratio (X_3: 10:1 to 40:1), on trehalose 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 1. The regression co-efficients are summarized in Table 2. The final mathematical model can be expressed by the following quadratic equation,

$$Y = 2.330 + 0.165\ X_1^2 + 0.165\ X_2^2 + 0.165\ X_3^2$$

Where, Y is the trehalose yield from BWBY(%); X_1 is the electric field intensity (kV/cm); X_2 is the pulsed number (n); X_3 is the liquid-solid ratio (ml : g).

By analyzing the data in Table 2, significant tests of coefficient and equation, and model fitting were assayed. Statistic analyses results of independent variables and their interaction are shown in Table 2. The results indicate that three independent variables (electric field intensity, pulse number, and solid- liquid ratio) all had significant effects on trehalose yield at the significant level of $P = 0.1$. From Table 2, we could tell that the sum of deviation squares (*S)* was 0.180, and sum of residual squares (S_R) was 0.0687. Moreover, static variables could be calculated according to equation below, and $F = 6.116419 > F_{0.05}\ (3,7) = 4.35$, which meant the regression model of coding space was significant at the level of α of *0.05*.

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 experi-mental error, lack of fit test was performed, and the results showed that static variables S_{lf} and S_e were 0.0575 and 0.0112 respectively. According to the Equation $F_{lf} = (S_{if} / f_{if}) / (S_e / f_e)$, $F_{lf} = 2.0588 < F_{0.25} = 3.28$, which meant the regression model does not lack fitness. The regression equation of nature space was obtained by

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

Number	x_0	x_1	x_2	x_3	x_1^2	x_2^2	x_3^2	y
1	1	1	1	1	0.314	0.314	0.314	2.300
2	1	1	1	-1	0.314	0.314	0.314	2.229
3	1	1	-1	1	0.314	0.314	0.314	2.161
4	1	1	-1	-1	0.314	0.314	0.314	2.227
5	1	-1	1	1	0.314	0.314	0.314	2.311
6	1	-1	1	-1	0.314	0.314	0.314	2.207
7	1	-1	-1	1	0.314	0.314	0.314	2.253
8	1	-1	-1	-1	0.314	0.314	0.314	2.260
9	1	0	0	0	-0.686	-0.686	-0.686	2.510
10	1	0	0	0	-0.686	-0.686	-0.686	2.647
11	1	0	0	0	-0.686	-0.686	-0.686	2.527
Dj	11	8	8	8	2.201	2.201	2.201	
Bj	25.632	-0.113	0.146	0.103	0.364	0.364	0.364	
bj	2.330	-0.014	0.018	0.013	0.165	0.165	0.165	
Sj	59.728	0.002	0.003	0.001	0.060	0.060	0.060	
Fj		0.283	0.476	0.237	10.742	10.742	10.742	
αj					0.1	0.1	0.1	

In this study, a quadratic regression orthogonal design 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 trehalose from the BWBY were determined as follows: an electric fields intensity of 19.97 kV/cm, a pulse number of 6, and a liquid-solid ratio of 30:1. The extraction rate of trehalose was 2.635%.

replacing the code by transformation equation as shown in Table 1, the final equation of natural space was expressed as: $Y=4.534 + 0.00166\ Z_1^2 + 0.00166\ Z_2^2 + 0.00166\ Z_3^2 - 0.066\ Z_1 - 0.0198\ Z_2 - 0.099\ Z_3$, where Z_1 is electric field intensity (kV/cm); Z_2 is pulsed number (n); Z_3 is Liquid-solid ratio (ml :g).

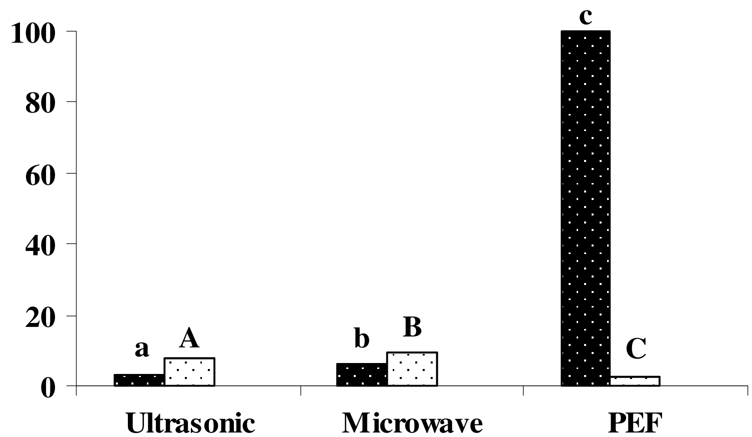
Furthermore, the best parameters for trehalose extraction by PEF are determined as electric field intensity of 19.97 kV/cm, pulse number of 6 and a liquid-solid ratio of 30:1. In order to validate the accuracy of regression model, the trehalose yield were tested three times under the optimal conditions, and reached to 2.639, 2.646 and 2.620% respectively with average of 2.635%. Meanwhile, the predicted results, according to the models for the trehalose yield were close to the observed experimental responses. The results therefore indicated that the PEF treatment under the best conditions can effectively facilitate trehalose extraction from WBY.

Comparison of three techniques for trehalose extraction

The trehalose yield treated by different extraction methods are shown in Figure 6. The results show that the trehalose yield of 2.635% by PEF was the lowest compared to 9.24% by microwave and 7.72% by ultrasound, respectively. However, due to the ultra-short treatment time, PEF had the highest extraction efficiency. The extraction rates of trehalose from WBY were 103.15 µg/s by PEF, 3.03µg/s by ultrasound and 6.46µg/s by microwave, respectively. The extraction rate of PEF was significantly higher than other two methods extraction *(P<0.01)* by 15.96 times than for microwave, and 34.08 times than ultrasound. Therefore, as a non-thermal food processing technology, PEF could be applied to extract trehalose from BWBY as a quite efficient novel method.

Conclusion

In the present study, PEF was successfully applied to extract trehalose from WBY and the parameters of PEF treatment were obtained. It is non-thermal, fast, efficient and has no negative effect. Engineering regression model of extracting trehalose from waste beer yeast by PEF was designed to obtain the optimization parameters, The optimal conditions to obtain the highest trehalose yield were determined as follows: An electric fields intensity of 19.97 kV/cm, a pulse number of 6 and a liquid-solid ratio of 30:1. With these conditions, the extraction rate of trehalose could reach 2.635%, while the extraction efficiency of trehalose treated by PEF could reach 103.15 µg/s. Therefore, we conclude that PEF represents a valuable alternative to the traditional extraction methods (example ultrasound and microwave techniques) for the efficient extraction of trehalose from BWBY. However, further work is needed to identify the specific mechanism



	Ultrasonic	Microwave	PEF
■ Extraction Efficiency of trehalose （μg/s)	3.03	6.46	103.15
□ Trehalose yield (%)	7.72	9.24	2.635
Extraction time (min)	380	213	6

Figure 6. Comparison of three extraction methods.
Different lowercase letters indicate that the variances of trehalose extraction rate are significant *(P < 0.05)* treated by different techniques, while the different capital letters indicate that the variances of trehalose yield are significant treated by different methods *(P < 0.05)*. The applied ultrasound treatment conditions were: a liquid-solid ratio of 40:1, a ultrasound power of 600 W, a treatment time of 20 min, and a boiling time of 6 h. The applied microwave treatment conditions were: a liquid-solid ratio of 30 : 1, a microwave power of 600 W, a treatment time 3 min, a temperature of 80℃, and a boiling time of 3.5 h. The applied PEF treatment conditions were: a liquid-solid ratio of 30 : 1, a pulse number of 6, an electric field intensity of 19.97 kV/cm and a treatment time of 6 min.

of PEF activity on yeast cell membrane.

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Abbreviations

PEF, High-intensity pulsed electric fields; **BWBY,** beer waste brewing yeasts; **OFAT,**
one-factor-at-a-time.

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