Optimization of Liquid Fermentation Process for Improved Exo-Polysaccharides Production by Kombucha ZJU1

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Abstract: In order to improve exo-polysaccharides production by Kombucha ZJU1, the optimization of liquid fermentation process for cell growth and Exo-Polysaccharide (EPS) production by Kombucha ZJU1 was conducted with Response Surface Methodology (RSM). A Fractional Factorial Design (FFD) and Central Composite Design (CCD) were applied to optimize the main factors that affect EPS production during fermentation. Both the concentration of brown sugar and inoculation level significantly affected EPS production by Kombucha ZJU1 through FFD experiment. The optimized fermentation process was derived from the predicted model using central composite design as follows: 280 g/L brown sugar, 4 g/L Pu-erh tea, 1 g/L KH_2PO_4 and 1 g/L $(NH_4)_2 SO_4$, initial pH unadjusted, the culture medium was inoculated with 1.95% (v/v) Kombucha ZJU1, the cell age was 5 days, then incubated at a temperature of 30±1°C for 6 days on the rotary shaker. The predicted maximal EPS concentration was 2.66 g/L using this optimized fermentation process, which was of value to further work.

Keywords: Exo-Polysaccharide (EPS), fermentation process optimization, kombucha, Response Surface Methodology (RSM)

INTRODUCTION

Kombucha, also named as tea fungus, can be traced back to the Qin Dynasty in ancient China. As a mixed microorganism system of yeasts, acetic acid bacteria and lactic acid bacteria (Liu *et al*., 1996; Mayser *et al*., 1995), kombucha is generally used to prepare fermented sugared tea with a sour and sweet flavor. Kombucha is composed of two portions: a floating cellulosic pellicle layer and the sour liquid broth (Chen and Liu, 2000). The pellicle of kombucha looks like the skin of jellyfish, thus it is called "sea treasure" or "stomach treasure" in some areas of China, because kombucha tea can help digestion, clean up stomach and intestines; other health benefits include: preventing and treating of diabetes, constipation, hemorrhoids, hypertension, hyperlipidemia, atherosclerosis and other cardiovascular diseases, avoiding calculus, detoxification, cancer prevention, regulating body immunity, etc (Sreeramulu *et al*., 2000; Allen, 1998; Chu and Chen, 2006; Dufresne and Farnworth, 2000; Jayabalan *et al*., 2008). The proposed beneficial effects of kombucha are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during fermentation (Vijayaraghavan *et al*., 2000).

Polysaccharides from plants, microorganism (fungi and bacteria), algae and animals were reported to have various bioactivities such as immuno-modulating, antitumor, antivirus, antioxidant, lowering blood sugar, etc (Yang and Zhang, 2009; Xie *et al*., 2008; Kiyohara *et al*., 2006; Mao *et al*., 2008). Kombucha Exo-Polysaccharides (EPS) may have similar bioactivities to fungal polysaccharides from mushrooms, *Ganoderma*, *Poria cocos*, etc., (Zhang *et al*., 2011; Saltarelli *et al*., 2009; Pillai *et al*., 2008). At present, the studies on kombucha mainly focused on microbial compositions and interactions (Mayser *et al*., 1995; Liu *et al*., 1996; Yang *et al*., 2010), fermentation conditions (Loncar *et al*., 2006), antimicrobial protein, antioxidative activity of the fermentation broth (Chu and Chen, 2006) and so on. However, there are fewer studies involving into the exo-polysaccharides produced by kombucha. As a consequence, it was highly interesting to keep the research on the submerged cultivation of EPS produced by this special microbial system.

Multiple factors and their interactions can be optimized and evaluated simultaneously with Response Surface Methodology (RSM). RSM can not only reduce the trial times but also identify the optimal conditions for the multiple factors system quickly and effectively, which has been widely used in the optimization of biological reaction process (Vohra and Satyanarayana, 2002; Ma *et al*., 2010; Firatligil-Durmus and Evranuz, 2010; Yin *et al*., 2010). To the best of our knowledge, no report has been documented concerning liquid culture medium and fermentation conditions for EPS

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production by kombucha. In this study, thus, RSM was utilized to optimize the liquid fermentation process for the production of EPS by Kombucha ZJU1.

MATERIALS AND METHODS

Materials: The research work was finish at Zhejiang University in May, 2012. Kombucha ZJU1 was preserved in the Food and Biotechnology Laboratory of Zhejiang University, China and was maintained in sugared tea at 4°C after several generations of activation.

Brown sugar and Pu-erh tea were bought from the local market of Hangzhou. All reagents used in the experiment were of analytical grade.

The basic culture medium was composed of 50 g/L brown sugar, 4 g/L Pu-erh tea, 1 g/L KH_2PO_4 and 1 g/L $(NH_4)_2 SO_4$. Fermentation medium (100 mL) in an erlenmeyer flask (250 mL) was inoculated with 20% (v/v) Kombucha ZJU1, the cell age of which was 5 days, then incubated at a temperature of 30±1°C for 6 days on the rotary shaker (150 r/min). The liquid fermentation process was performed according to the experimental design as follows.

Experimental design: In order to identify which component (s) of the medium or fermentation condition (s) has a significant effect on EPS production, Fractional Factorial Design (FFD) was developed as the first optimization step with Design-Expert software v. 6.0 (Stat-Ease, Inc., Minneapolis, MN, USA). Three major components in fermentation medium (brown sugar, Pu-erh tea and $(NH_4)_2 SO_4$), cell age and inoculation level were selected as factors in the factorial design. If certain high-order interactions can reasonably be assumed negligible, information on the main effects and low-order interactions may be obtained by performing only a fraction of the complete factorial experiment. For a moderately large number of factors, smaller fractions of the 2^k designs are frequently useful (Montgomery, 1997). The range of the coded level for the five factors was listed in Table 1. The initial estimate of the optimum operating conditions will be frequently far from the actual optimum. On this occasion, the steepest ascent method is a procedure for moving sequentially along the path of steepest ascent to reach the general vicinity of the optimum (Jiao *et al.*, 2008). In order to attain the maximum increase of responses, further studies involved experiments are carried out along the path of steepest ascent.

In the second experiment, Central Composite Design (CCD) is used to develop an empirical model of the process and to obtain a more precise estimate of the optimum conditions for the factors involved (Montgomery, 1997). A central composite design with five coded levels was performed to describe the nature of response surface in the optimum region. This design

Table 1: Factors and coded data of fractional factorial design

Factors/levels	-1	0	+1
Brown sugar(x_1, g/L)	20	50	80
Pu-erh tea (x_2, g/L)	1	4	7
$(NH_4)_2 SO_4$ (x_3, g/L)	0.50	1	1.50
Cell age (x_4, days)	3	5	7
Inoculation level (x_5, %)	10	20	30

was made up of a full 2^2 factorial design (two factors) with its four cube points, augmented with five replications of center points and four-star points. To make the design rotated, 1.414 was chosen to be the axial distance (Montgomery, 1997). The optimal point could be predicted by the quadratic model based on the following equation Eq. (1):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \qquad (1)$$

where,

Y = The value of response variable
$x_i, x_j (i \neq j)$ = The levels of the independent variables
b_0 = The intercept
$b_i, b_{ij} (i \neq j), b_{ii}$ = The linear, cross product and quadratic coefficients, respectively

Preparation method: Tea leaves (Pu-erh tea) were added to hot water (85°C), kept at 85°C for 10 min in the water bath and filtered through an 11 cm qualitative filter study (Whatman-Xinhua Filter Study Co., Hangzhou, China). Various concentrations of carbon source (brown sugar), nitrogen source ($(NH_4)_2 SO_4$) and KH_2PO_4 were dissolved in the filtrate when it was cooled down to 60°C and then sterilized at 121°C (0.12 MPa) for 20 min. After that, Kombucha ZJU1 of different inoculation levels and cell ages was inoculated to the culture medium described above after cooling to room temperature and incubated at 30±1°C for 6 days on the rotary shaker (150 r/min).

The fermentation broth of Kombucha ZJU1 was filtered and then anhydrous ethanol was added under vigorous agitation condition until the absolute ethanol concentration reached 90% and left at 4°C for 1 h. The mixture was centrifuged at 1,000×g for 20 min and the supernatant was discarded, then the resulting sediment was treated with 80% (v/v) ethanol and centrifuged as above to get the sediment; the above procedures were repeated for 3-4 times. Consequently, crude EPS was precipitated and then dissolved with distilled water after ethanol volatilization to prepare for the estimation of EPS concentration.

The fermentation broth of Kombucha ZJU1 was centrifuged at 9,000×g for 15 min and Dry Cell Weight (DCW) of Kombucha ZJU1 was measured after repeated washing with deionized water and dried to constant weight at 90°C EPS concentration was determined by phenol-sulfuric acid method (Dubois *et al.*, 1945). The calibration curve for EPS was

generated by linear regression of OD_{490} values against the absolute weights of glucose. It showed good linearity between OD_{490} (y) against reduced absolute weights of glucose (x, mg) over the calibration ranges ($y = 6.3002x-0.0013$, $r^2 = 0.9959$). The determination method of EPS concentration by Kombucha ZJU1 was the same as that for construction of the calibration curve. Thus, EPS concentration (presented by glucose) could be calculated with OD_{490} value and the calibration curves obtained above.

Data analysis: Design-Expert software v. 6.0 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for the regression analysis of experimental data obtained. The quality of the fit of polynomial model equation was expressed by the determination coefficient (R^2 value) and its statistical significance was checked by *F*-test (Montgomery, 1991). The significance of the regression coefficient was tested by *t*-test.

RESULTS AND DISCUSSION

Screening the principal factors for EPS production with fractional factorial design: The fractional factorial design enables the identification of medium components and cultivation conditions that play a significant role in cell growth and EPS production. The screening experiments were designed to evaluate the impacts of these five factors, cell age, inoculation level and concentrations of brown sugar, Pu-erh tea and $(NH_4)_2 SO_4$ on the production of EPS by Kombucha ZJU1. The independent variables and levels investigated in this study were presented in Table 1. The two-level FFD and its results were given in Table 2. The EPS concentration varied widely from 0.33 to 1.24 g/L under different levels of medium components and cultivation conditions. The concentration of brown sugar (x_1) ($p<0.05$) and inoculation level (x_5) ($p<0.05$) were found to be more significant on EPS production (Y_1) and biomass of Kombucha ZJU1 (Y_2) than other three factors. The main effects of the concentration of brown sugar (x_1) and the inoculation level (x_5) were positive (x_1) and negative (x_5), respectively. Other factors were insignificant on EPS production. There was no evidence of any interactions among the five factors investigated. Sufficient carbon source of the culture medium and appropriate inoculation level of Kombucha ZJU1 both have conspicuous influence on metabolic product (EPS) accumulation of this microbial system.

Multiple regression techniques were also applied to analyze the effects of these factors on EPS accumulation. The values of the regression coefficients were calculated and a first-order equation Eq. (2) was attained with a R^2 value of 0.8587, indicating that 86%

Table 2: Designs and results of fractional factorial experiment[a]

Runs	X_1	X_2	X_3	X_4	X_5	Y_1/EPS (g/L)	Y_2DC W (g/L)
1	-1	+1	-1	+1	+1	0.57	2.07
2	-1	-1	-1	+1	-1	0.77	2.27
3	+1	-1	-1	+1	+1	0.71	4.18
4	0	0	0	0	0	0.83	4.03
5	+1	-1	+1	-1	+1	0.82	4.29
6	-1	+1	+1	+1	-1	0.63	2.50
7	+1	+1	+1	-1	-1	1.24	5.72
8	0	0	0	0	0	0.81	3.71
9	-1	+1	-1	-1	-1	0.72	2.56
10	0	0	0	0	0	0.88	3.62
11	-1	+1	+1	-1	+1	0.57	2.24
12	-1	-1	+1	+1	+1	0.49	1.93
13	-1	-1	+1	-1	-1	0.34	2.54
14	+1	-1	-1	-1	-1	0.75	5.70
15	0	0	0	0	0	0.53	3.79
16	+1	+1	-1	+1	-1	0.47	5.29
17	-1	-1	-1	-1	+1	0.33	1.99
18	+1	+1	-1	-1	+1	0.43	4.49
19	+1	-1	+1	+1	-1	0.83	5.97
20	+1	+1	+1	+1	+1	0.56	4.35

[a]: X1 is the coded level of brown sugar concentration, X_2 is the coded level of Pu-erh tea concentration, X_3 is the coded level of $(NH_4)_2 SO_4$ concentration, X_4 is the coded level of cell age, X_5 is the coded level of inoculation level of Kombucha ZJU1; Results are averages from three replicates

of the variability could be explained by this model. This ensured a satisfactory adjustment of the first-order model to the experimental data. The F-value (3.869) implied the significance of the model and meant that there was only a 4.18% chance that a model could occur due to noise. The results of t-test for variance between averages of observation of two-level experiments and center points showed that the difference was not significant ($p>0.05$), indicating that optimum point was outside the current experimental domain, so it was necessary to reach optimal domain of the maximal response using the path design of steepest ascent:

$$Y_1 = 0.64+0.087x_1+0.009375x_2+0.046x_3-0.011x_4-0.079x_5-0.061x_1x_2+0.091x_1x_3-0.073x_1x_4+0.056x_2x_3-0.081x_2x_4-0.047x_3x_4 \qquad (2)$$

The steepest ascent experiment and analysis: The steepest ascent can be used to determine the next set of experiments. A sequence of specifically spaced locations along the path were then selected to form a set of experiments. In order to improve EPS production, the concentration of brown sugar (x_1) was increased and the inoculation level (x_5) was decreased. The other factors were fixed at zero level. The design and results of the steepest ascent experiment were shown in Table 3. EPS production increased when the brown sugar concentration was increasing and the inoculation level was reducing in some way. Yet it could not be improved by further experiments after the seventh step on the path. The highest amount of EPS and biomass of Kombucha ZJU1 were simultaneously attained in the

Table 3: Design and results of the steepest ascent experiment[b]

Runs	x_1 (g/L)	x_5 (%)	Y_1/EPS (g/L)	Y_2/DCW (g/L)
1	50	20	0.88	4.05
2	80	17	0.90	4.96
3	110	14	1.16	6.86
4	140	11	1.05	7.68
5	170	8	1.20	8.53
6	200	5	1.47	8.56
7	230	2	1.75	9.67
8	250	1	1.49	8.90
9	270	0.5	1.62	8.67

[b]: x_1 = The actual level of brown sugar concentration, x_5 = The actual level of inoculation level of Kombucha ZJU1; Results are averages from three replicates

Table 4: Factors and levels of central composite design[c]

Factors/levels	-1.414	-1	0	+1	+1.414
x_1(g/L)	159.30	180	230	280	300.70
x_5 (%)	1.2900	1.50	2.00	2.50	2.7100

[c]: x_1 = The actual level of brown sugar concentration, x_5 = The actual level of inoculation level of Kombucha ZJU1

Table 5: Design and results of central composite experiment[d]

Runs	X_1	X_5	Y_1/EPS (g/L)	Y_2/DCW (g/L)
1	+1	-1	2.18	9.92
2	-1.414	0	1.40	8.55
3	-1	+1	1.62	9.02
4	0	+1.414	1.61	9.31
5	-1	-1	1.26	8.94
6	0	-1.414	1.84	11.24
7	+1	+1	2.30	10.50
8	+1.414	0	3.07	11.08
9	0	0	2.08	10.46
10	0	0	2.02	11.40
11	0	0	2.31	10.91
12	0	0	2.45	11.13
13	0	0	1.96	11.31

[d]: X_1 = (x_1-230) /50, X_5 = (x_5-2) /0.5, X_1 = The coded level of brown sugar concentration, X_5 = The coded level of inoculation level of Kombucha ZJU1, x_1 = The actual level of brown sugar concentration, x_5 = The actual level of inoculation level of Kombucha ZJU1; Results are averages from three replicates

seventh step, which indicated that the experiment was approximating the vicinity of the optimum response.

Therefore, the combination of the seventh step was selected as the center point of CCD.

Optimization of the significant factors with central composite design and response surface analysis: As the optimal region for running the process had been identified, a central composite design was adopted to further optimize the accumulation of EPS. Levels of the two principal factors (the concentration of brown sugar and the inoculation level) in central composite experiment were illustrated in Table 4. In Table 5 the design and its experimental results were presented. Quadratic regression analysis was performed to analyze the experimental data. The statistical significance of the second-order model equation was checked by F-test. The R^2 value (0.8682) was calculated, indicating that 87% of the variability in the response could be explained by the second-order polynomial prediction equation given below Eq. (3). The results of Analysis of Variance for the production of EPS were shown in Table 6. The p-value of 0.0055 (p<0.01) obtained for the significant variables confirmed the suitability of the model equation to describe the response of the experiment pertaining to EPS production. The p-value of 0.3407 (p>0.05) indicated that the lack of fit test was not significant and the fitting degree of Eq. (3) was rather good. As a result, the reliability of this model was high. In addition, the influence of brown sugar concentration on EPS production had reached an extremely significant level (p<0.01). Similarly, the results of Analysis of Variance for the biomass of Kombucha ZJU1 were shown in Table 7:

$$Y1 = 2.16+0.50x_1+0.019x_5+0.00055x_1^2-0.25x_5^2-0.061x_1x_5 \qquad (3)$$

The response surface graphs of brown sugar concentration and inoculation level against EPS

Table 6: Results of ANOVA for the production of EPS by kombucha ZJU1

Source	S.S.	DF	M.S.	F-value	p-value	
Model	2.430	5	0.490	9.220	0.0055	Significant
X_1	1.960	1	1.960	37.21	0.0005	
X_5	0.003	1	0.003	0.058	0.8172	
X_1^2	$2.104×10^{-6}$	1	$2.104×10^{-6}$	$3.993×10^{-5}$	0.9951	
X_5^2	0.440	1	0.440	8.390	0.0231	
X_1X_5	0.015	1	0.015	0.280	0.6124	
Residual	0.370	7	0.053			
Lack of fit	0.200	3	0.065	1.510	0.3407	Not significant
Pure error	0.170	4	0.043			

Table 7: Results of ANOVA for the biomass of kombucha ZJU1

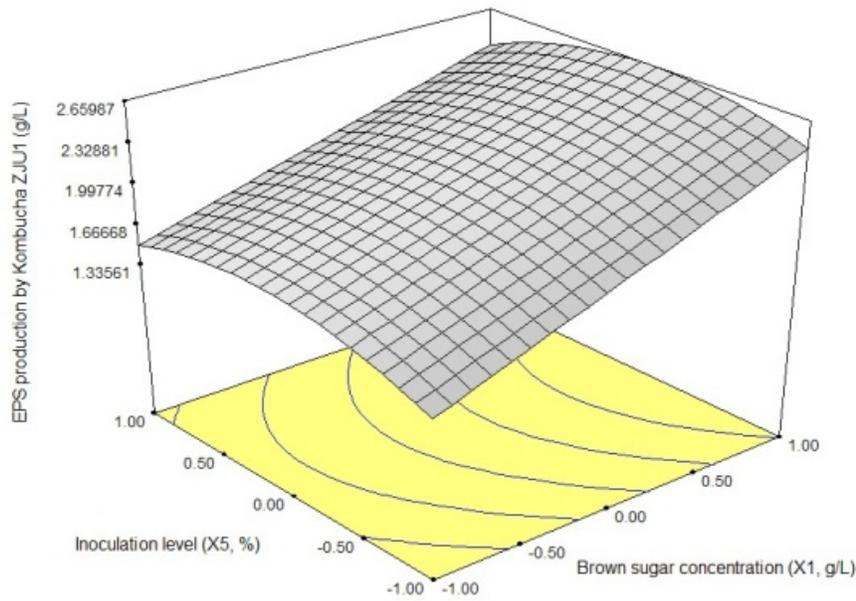| Source	S.S.	df	M.S.	F-value	p-value	
Model	9.96	5	1.99	5.450	0.0232	Significant
X_1	4.56	1	4.56	12.46	0.0096	
X_5	0.54	1	0.54	1.460	0.2657	
X_1^2	3.67	1	3.67	10.02	0.0158	
X_5^2	1.71	1	1.71	4.680	0.0673	
X_1X_5	0.063	1	0.063	0.170	0.6917	
Residual	2.56	7	0.37			
Lack of fit	2.00	3	0.67	4.720	0.0839	Not significant
Pure error	0.56	4	0.14			

Fig. 1: Response surface plot of brown sugar concentration and inoculation level against the production of EPS by kombucha ZJU1
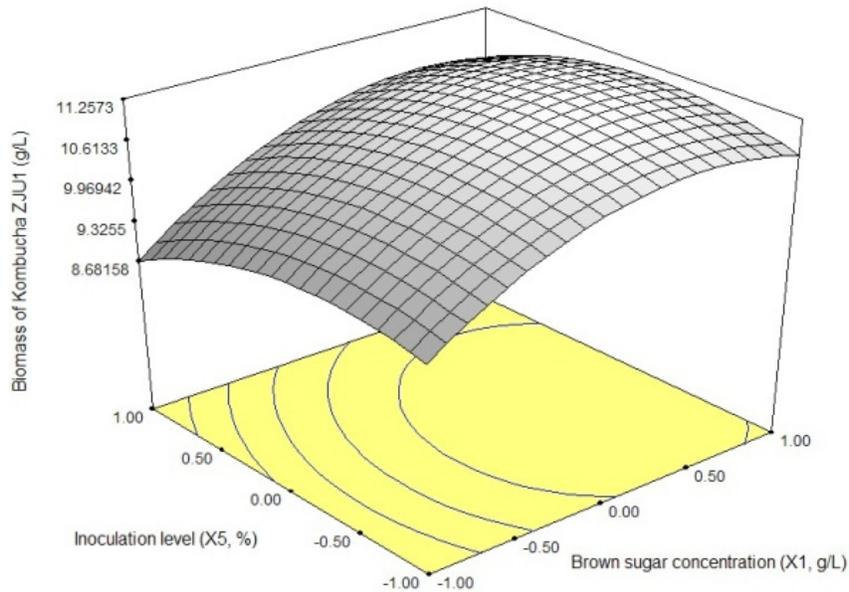

Fig. 2: Response surface plot of brown sugar concentration and inoculation level against the biomass of kombucha ZJU1

production and biomass of Kombucha ZJU1 were plotted in Fig. 1 and 2. Moreover, the contour plots of Fig. 3 and 4 also depicted the two principal factors against EPS production and biomass of Kombucha ZJU1. The results of statistical analyses could be further explained by these plots.

The EPS production and biomass of Kombucha ZJU1 simultaneously reached the maximums at a combination of coded level of 1.00 (X_1) and -0.11 (X_5) and the actual uncoded values were 280 g/L (x_1) and

1.95% (x_5), respectively. At this point, the model predicted the maximum responses of 2.66 g/L (Y_1) and 11.08 g/L (Y_2).

Validation of the optimized results: To confirm these results, verified experiments were performed under the optimized liquid fermentation process attained from the above study and mean values of 2.73±0.11 g/L (N = 3) (Y_1) and 11.53±0.02 g/L (N = 3) (Y_2) were obtained. The predicted response results and the actual tested
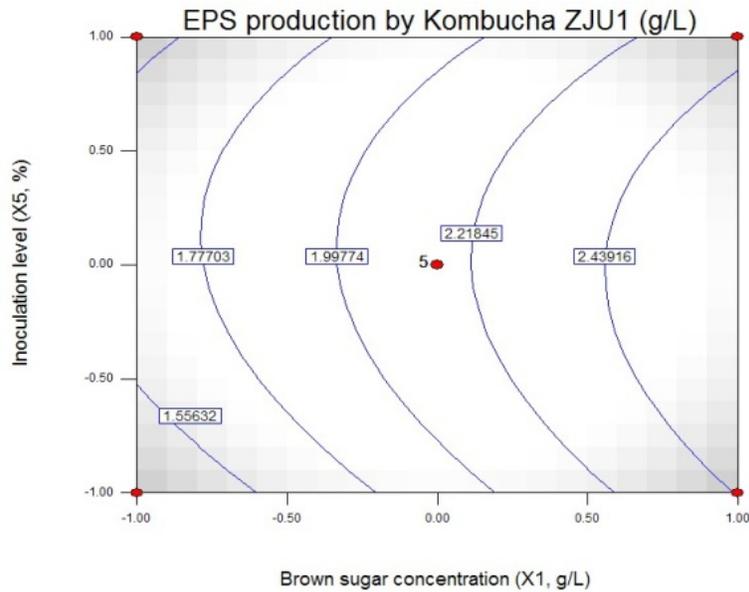
Fig. 3: Contour plot of brown sugar concentration and inoculation level against the production of EPS by kombucha ZJU1
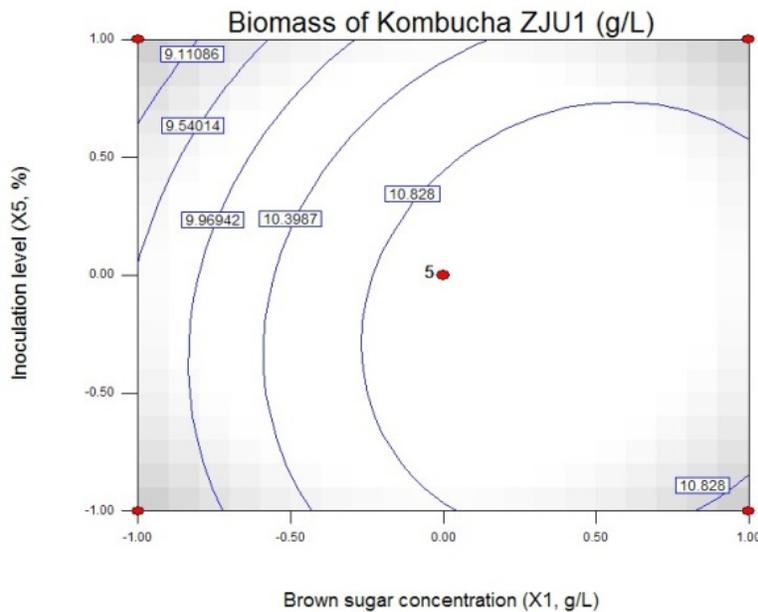

Fig. 4: Contour plot of brown sugar concentration and inoculation level against the biomass of kombucha ZJU1

ones were in high agreement, which proved the validity of this response model. Finally, the optimum fermentation process for EPS production was as follows: 280 g/L brown sugar, 4 g/L Pu-erh tea, 1 g/L KH_2PO_4 and 1 g/L $(NH_4)_2 SO_4$, initial pH unadjusted, the culture medium was inoculated with 1.95% (v/v) Kombucha ZJU1, the cell age was 5 days, then incubated at a temperature of 30±1°C for 6 days on the rotary shaker (150 r/min).

Kefir is a fermented milk beverage that originated in Eastern Europe. It can be considered a probiotic resource for a variety of health claims. The starter of kefir is kefir grain, which is also a microbial symbiont like kombucha (Yang *et al.*, 2010). Studies have been carried out to investigate the properties of crude EPS produced by Streptococcus thermophilus LFG isolated from Kefir in goat milk yogurt (Lim and Lee, 2009) as well as physical characteristics of EPS produced by Lactobacillus plantarum KF5 isolated from Tibet Kefir (Wang *et al.*, 2010). It merits more attention that Kombucha ZJU1 EPS is produced by which microorganism (s) in the symbiotic culture, too.

Kefiran, an exo-polysaccharide obtained from kefir grains, has the ability to form a new biodegradable edible food film (Ghasemlou *et al.*, 2011). Lee *et al.* (2011) reported that a polysaccharide from kefir had more than a 97% inhibition effect against human rotavirus infection in MA-104 cells. Two fractions of Tibetan Kefir EPS have exhibited immune regulation activities in mice (Meng and Zhang, 2009). Equally, the health promoting properties and potential applications of EPS produced by Kombucha ZJU1 need further research.

CONCLUSION

In conclusion, response surface methodology technique was demonstrated to be effective and reliable in establishing optimum liquid fermentation process to achieve the highest EPS production and cell growth of Kombucha ZJU1. By means of validation experiments, the accuracy of this model was proved. The maximal EPS concentration (2.66 g/L) attained under the optimum fermentation process was remarkably higher than that of the original fermentation process (0.43 g/L). The optimum fermentation process developed in this work can be used to produce EPS by Kombucha ZJU1 for the future work on structural characterization and bioactivities assessment.

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