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Y. C. Du[ab], X. H. Long[a], Z. P. Liu[a] & H. B. Shao[abc]

[a] Jiangsu Provincial Key Laboratory of Marine Biology, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, P.R. China

[b] Key Laboratory of Coastal Biology & Biological Resources Utilization, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS), Yantai 264003, P.R. China

[c] Institute for Life Sciences, Qingdao University of Science and Technology (QUST), Qingdao 266042, P.R. China

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Optimizing medium for producing ethanol from industrial crop Jerusalem artichoke by one-step fermentation and recombinant *Saccharomyces cerevisiae*

Y. C. DU[1,2], X. H. LONG[1], Z. P. LIU[1], & H. B. SHAO[1,2,3]

[1]*Jiangsu Provincial Key Laboratory of Marine Biology, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, P.R. China;* [2]*Key Laboratory of Coastal Biology & Biological Resources Utilization, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS), Yantai 264003, P.R. China and* [3]*Institute for Life Sciences, Qingdao University of Science and Technology (QUST), Qingdao 266042, P.R. China*

Abstract
In order to obtain a high ethanol yield from the Jerusalem artichoke raw extract and reduce the fermentation cost, we have engineered a new recombinant *Saccharomyces cerevisiae* strain that could produce ex-inulinase. The response surface methodology based on Plackett–Burman and Box–Behnken design was used to optimize the medium for the ethanol production from the Jerusalem artichoke raw extracts by the recombinant strain. In the first optimization step, Plackett–Burman design was employed to select significant factors, including concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$. In the second step, the steepest ascent experiment was carried out to determine the center point with the three significant factors; the selected combinations were further optimized using the Box–Behnken design. The maximum ethanol production rate was predicted at 91.1 g/l, which was based on a medium consisting of yeast extract 9.24 g/l, inoculum 39.8 ml/l, and $MgSO_4 \cdot 7H_2O$ 0.45 g/l. In the validating experiment, the ethanol fermentation rate reached 102.1 g/l, closely matching the predicted rate.

Keywords: *RSM, optimization, Jerusalem artichoke, energy,* Saccharomyces cerevisiae*, one-step fermentation*

Introduction

Over the past decades, the huge consumption of fossil fuels resulted in high fuel prices. Fossil fuels are non-renewable, forcing a search for renewable and green energy (Haveren et al. 2008; Ghobadian 2012).

Many chemicals produced by conventional chemical routes could also be obtained from renewable resources by biotechnological processes (Ragauskas et al. 2006; Lozada et al. 2011). The first important factor to be taken into account is substrate cost. Bioethanol, as one of the best substitutes for fossil fuels, has attracted increasing interest (Cardona & Sanchez 2007; Konopka et al. 2010). It has huge potential because of its environmental and renewable advantages (Sticklen 2008; Martellos et al. 2011). Using microorganisms to produce bioethanol may reduce production costs.

Currently, bioethanol is produced mostly from sugar or starch (Bai et al. 2008; Zhang et al. 2010). However, Jerusalem artichoke is one of the most suitable materials for ethanol production because it contains nearly 20% of carbohydrates, 70–90% of which is inulin. Inulin is a natural polysaccharide consisting of linear chains of fructose residues linked by β-2,1 bonds and terminated by a sucrose residue (Yu et al. 2009; Marchetti et al. 2012). Importantly, Jerusalem artichoke can grow on poor land (Ma et al. 2011; Zhang et al. 2012). With these advantages, Jerusalem artichoke can be used as a cheap substrate for bioethanol production in addition to its current uses as single-cell oil or oligofructose syrup (Zhao et al. 2010; Erdal et al. 2011; Rewald et al. 2011).

Traditionally, Jerusalem artichoke tubers had to be hydrolyzed to fructose and glucose by inulinase or acids before fermentation (Copetta et al. 2011), called

Correspondence: X. H. Long and Z. P. Liu, Jiangsu Provincial Key Laboratory of Marine Biology, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, P.R. China. Email: longxiaohua@njau.edu.cn; zhaopuliu@163.com; H. B. Shao, Institute of Life Sciences, Qingdao University of Science and Technology (QUST), Qingdao 266042, P.R. China. Email: shaohongbochu@126.com

two-step ethanol fermentation, which was time consuming and costly. In order to reduce the fermentation cost, one-step ethanol fermentation was investigated (Gao et al. 2010; Lim et al. 2011). In our previous study, we have successfully made a recombinant *Saccharomyces cerevisiae* strain that can produce ex-inulinase (Wang et al. 2011; Raimondo et al. 2012), and inulinase can hydrolyze effectively Jerusalem artichoke tubers (Singh & Gill 2006; Braglia et al. 2011).

The traditional "single-factor experiment" technique used for optimizing a multivariable system is time consuming and often misses the alternative effects between the components. Recently, many new experimental design methods have been employed in bioprocess optimization (Xiong et al. 2007; Chen et al. 2011; Lim et al. 2011). As a model, response surface methodology (RSM), consisting of mathematical and statistical techniques, was widely used to study the effects of several variables and to seek the optimum conditions for a multivariable system (Francis et al. 2003; Kalavrouziotis & Koukoulakis 2012). This method has been successfully applied to optimize alcoholic fermentation (Paseephol et al. 2007; Yang et al. 2012). A detailed account of this technique has been outlined (Bezerra et al. 2008; Peterson & Soberon 2012). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model (Myers et al. 2004; Manolaki & Papastergiadou 2012).

The object of this study was to optimize the fermentation medium for the Jerusalem artichoke raw extract. Hence, Plackett–Burman design and Box–Behnken design were used to identify the effects of important factors influencing ethanol production.

Materials and methods

Jerusalem artichoke raw extract

Jerusalem artichoke was harvested at maturity from Dafeng in China at the end of November. The tubers were washed in cold water to remove the soil, cut into pieces, heated at 80°C in an oven until constant weight, and ground to mash. The raw extract was obtained at 80°C with excess water followed by filtration through gauze. The total sugars comprised 26% of the raw extract according to the phenol–sulfuric acid method (Chow & Landhäusser 2004).

Yeast strain and medium

The chosen yeast was recombinant *S. cerevisiae 6525* with the ex-inulinase gene from *Penicillium janthinellum*. The medium to produce ex-inulinase was yeast – peptone– inulin (YPI) containing (in g/l) yeast extract, 10; peptone, 20; and inulin, 20. All the cultures were sterilized at 115°C for 20 min before use.

Pre-cultivation

An aliquot of 100 µl recombinant *S. cerevisiae* inoculum was added to 100 ml YPI medium, at 30°C and 180 rpm, for 72 h to produce enough ex-inulinase. The proper amount of this suspension was then used as inoculum to 100 ml of fermentation medium in 250-ml flasks followed by cultivation on a rotary shaker. The fermentation medium composition was varied based on Plackett–Burman and Box–Behnken designs (Assab et al. 2011).

Analytical methods

The ethanol solution was separated from fermentation broth by centrifugation. The ethanol concentration was measured by Agilent GC-7890A (Chicago, USA) gas chromatography with a flame ionization detector and a 30-m INNOWAX column (0.32 mm inside diameter, 0.25 µm wall thickness). The chromatography was run at 280° oven temperature and 200° injection temperature using N_2 as carrier gas and H_2 as flaming gas.

Experimental design

Single factor level confirmation. Factors including pH, peptone, yeast extract, temperature, inoculum, NaCl and $MgSO_4 \cdot 7H_2O$ concentration, and fermentation time in producing ethanol from Jerusalem artichoke were examined in our experiments. The level of each factor was estimated by the preliminary single-factor experiments. All experiments were run in triplicate, and the average values were used.

Plackett–Burman design. The Plackett–Burman design was employed to select the important factors affecting the ethanol yield. The Plackett–Burman design is an efficient technique for the optimization of media components. Such design is based on an assumption that there were no interactions among the factors tested regarding variables under consideration. Following the single-factor experiments, a high and a low level of selected factors were chosen (Table I) for the design; 12 trials were run and the average values were adopted. The experiment was designed by Minitab16 statistical software.

Steepest ascent method. The path of steepest ascent is a process of moving sequentially to the optimal region by following a direction of the maximum increase in the response. Based on the experiments using the Plackett–Burman design, the optimal level of each selected factor was achieved using the steepest ascent method.

Table I. Screening of various media variables using Placket–Burman design.

	Factor	Low level (−1)	High level (+1)
A	pH	5	6.5
B	Peptone (g/l)	20	40
C	Yeast extract (g/l)	10	30
D	Temperature (°C)	28	32
E	Inoculum (ml/l)	50	150
F	NaCl (g/l)	1	2
G	$MgSO_4 \cdot 7H_2O$ (g/l)	0.7	1
H	Fermentation time (h)	72	108

Box–Behnken design and RSM. Based on the steepest ascent experiment, Box–Behnken design for three independent variables, each at three levels with three replicates at the center points was employed. A 2^3 full factorial design leading to 17 sets of experiments was used to develop a statistical model for ethanol production. Three assays at the center point were carried out to estimate the error involved in the experiment and also to ascertain whether there was any curvature in the response surface. The ethanol productivity was taken as the dependent variable or response. For statistical calculations, the variables X_1, X_2, and X_3 were coded according to

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i}, \quad i = 1, 2, 3, \ldots, k$$

where X_i is the coded value of an independent variable, x_i is the real value of an independent variable, X_{cp} is the real value of an independent variable at the center point, and ΔX_i is a step change of real value of the variable. The experimental was designed by Design Expert 8.0 software.

Results

Screening of significant nutrients using Placket–Burman design

Based on the previous studies, a total of eight components were analyzed for their effect on ethanol yield using Placket–Burman design. In the design, each row represents an experiment and each column an independent variable. The high and low values were chosen based on the previous study (Table I). The Placket–Burman design for 12 trials assessing ethanol production is given in Table II. The analysis of variance (ANOVA) and the significance of each variable were determined by *t*-test (Table III). The liner regression

Table II. The levels (−1 = low and 1 = high, see Table I) of each variable in Placket–Burman design and corresponding response in terms of ethanol production.

Run	pH	Peptone (g/l)	Yeast extract (g/l)	Temperature (°C)	Inoculum (ml/l)	NaCl (g/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	Fermentation time (h)	Ethanol (g/l)
1	−1	1	1	1	−1	1	1	−1	73.4
2	1	−1	−1	−1	1	1	1	−1	65.8
3	1	−1	1	1	−1	1	−1	−1	73.6
4	1	1	−1	1	1	−1	1	−1	68.7
5	−1	1	1	−1	1	−1	−1	−1	69.8
6	−1	−1	1	1	1	−1	1	1	63.9
7	1	1	−1	1	−1	−1	−1	1	81.5
8	−1	1	−1	−1	−1	1	1	1	76.7
9	1	1	1	−1	1	1	−1	1	65.4
10	−1	−1	−1	−1	−1	−1	−1	−1	76.8
11	−1	−1	−1	1	1	1	−1	1	77.3
12	1	−1	1	−1	−1	−1	1	1	67.9

Table III. Effect of each variable on the ethanol production and the statistical analysis in the one-step fermentation of the raw Jerusalem artichoke extract using *S. cerevisiae-C3*.

Factor	−1	+1	Effect	Coefficient	*T* value	*P* value
pH	5	6.5	−2.500	−1.250	−2.01	0.138
Peptone (g/l)	20	40	1.700	0.850	1.37	0.266
Yeast extract (g/l)	10	30	−5.467	−2.733	−4.39	0.022
Temperature (°C)	28	32	2.667	1.333	2.14	0.122
Inoculum (ml/l)	50	150	−6.500	−3.250	−5.22	0.014
NaCl (g/l)	1	2	0.600	0.300	0.48	0.663
$MgSO_4 \cdot 7H_2O$ (g/l)	0.7	1	−4.667	−2.333	−3.75	0.033
Fermentation time (h)	72	96	0.767	0.383	0.62	0.582

$R^2 = 96.0\%$; $R^2(\text{adj}) = 85.3\%$.

coefficient R^2 was 0.960 and the adjusted R^2 was 0.854, indicating that the model was well supported.

The path of steepest ascent

The aforementioned results indicated that only yeast extract, inoculum volume, and $MgSO_4 \cdot 7H_2O$ concentrations significantly influenced ethanol production. Moreover, a decrease in concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ concentrations exerted positive effects on the production of ethanol (Table III).

To search for the optimal concentrations of these three factors (with other factors fixed), the path of the steepest ascent method was employed (Table IV). The yield of ethanol was highest when the concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ were 9.0 g/l, 40 ml/l, and 0.45 g/l, respectively, suggesting that these were optimum concentrations for ethanol production under the given conditions.

Further optimization of the nutrients using Box–Behnken design

Experiments were carried out to further examine the optimal concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ based on Box–Behnken design (Table V). After analysis, the regression model was given as where R_1 was the ethanol yield (g/l), and A, B, and C were the concentrations of yeast extract (g/l), inoculum volume (ml/l), and $MgSO_4 \cdot 7H_2O$ (g/l), respectively. The ANOVA of the quadratic regression model demonstrated that Equation (1) was a highly significant model, as was evident from the Fisher's F-test [(Pmodel $> F$) $= 0.0500$] (Table VI).

The model goodness of fit was checked by calculating the determination coefficient

Table IV. The experimental design of the steepest ascent method and corresponding ethanol yield.

Run	Yeast extract (g/l)	Inoculum (mL/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	Ethanol yield (g/l)
1	10	50	0.7	74.4
2	9.8	48	0.65	72.6
3	9.6	46	0.60	73.7
4	9.4	44	0.55	76.4
5	9.2	42	0.50	81.5
6	9.0	40	0.45	82.5
7	8.8	38	0.40	79.0
8	8.6	36	0.35	78.1
9	8.4	34	0.30	78.3
10	8.2	32	0.25	71.4

Table VI. ANOVA for the ethanol production according to the response surface quadratic model.

Source	Sum of squares	df	Mean square	F value	P-value Prob $> F$
Model	539.18	9	59.91	52.70	<0.0001
A – yeast extract	2.64	1	2.64	2.33	0.1710
B – inoculum	0.72	1	0.72	0.63	0.4523
C – $MgSO_4$	0.045	1	0.045	0.040	0.8480
AB	4.84	1	4.84	4.26	0.0780
AC	7.29	1	7.29	6.41	0.0391
BC	2.89	1	2.89	2.54	0.1549
A^2	180.09	1	180.09	158.41	<0.0001
B^2	90.65	1	90.65	79.74	<0.0001
C^2	196.99	1	196.99	173.28	<0.0001
Cor. total	547.14	16			

Table V. The level of each variable and corresponding production of ethanol: Box–Behnken design.

	Coded variable level			Real variable level			Ethanol	
Run	Yeast extract	Inoculum	$MgSO_4 \cdot 7H_2O$	Yeast extract (g/l)	Inoculum (ml/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	Predicted (g/l)	Experimental (g/l)
1	0	0	0	9	40	0.45	93.7	92.2
2	0	0	0	9	40	0.45	93.7	94.4
3	0	0	0	9	40	0.45	93.7	94.8
4	−1	−1	−1	8.6	40	0.4	79.4	79.6
5	0	0	0	9	40	0.45	93.7	94.7
6	0	0	−1	9	42.5	0.4	82.6	83.0
7	0	0	1	9	37.5	0.5	83.4	83.1
8	−1	−1	0	8.6	37.5	0.45	82.3	82.2
9	0	0	0	9	40	0.45	93.7	92.3
10	1	1	1	9.4	40	0.5	78.5	78.3
11	1	1	0	9.4	37.5	0.45	83.4	83.8
12	−1	−1	0	8.6	42. 5	0.45	83.9	83.4
13	0	−1	−1	9	37.5	0.4	81.6	81.5
14	1	0	−1	9.4	40	0.4	81.0	80.6
15	0	1	1	9	42. 5	0.5	81.2	81.2
16	1	1	0	9.4	42. 5	0.45	80.5	80.6
17	−1	0	1	8.6	40	0.5	82.3	82.7

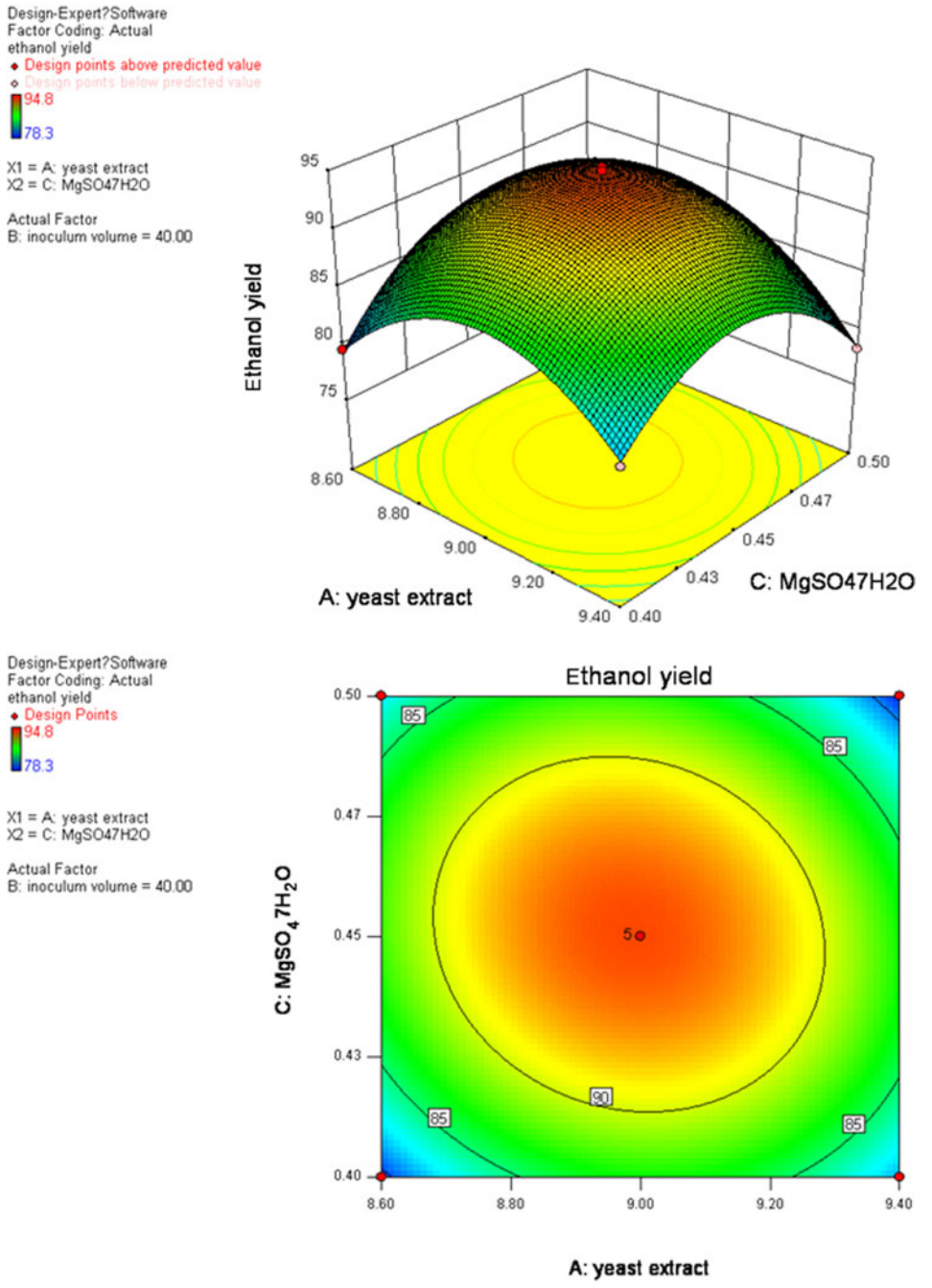

Figure 1. The response surface plot and the corresponding contour plot showing the effects of yeast extract and $MgSO_4 \cdot 7H_2O$ concentrations on ethanol production by recombinant *S. cerevisiae* (inoculum volume = 40 ml/l).

($R^2 = 0.985$), which indicated that only 1.5% of the total variation was not explained by the model. The value of the adjusted determination coefficient (Adj$R^2 = 0.967$) also indicated high significance of the model. In addition, a relatively low coefficient of variation (CV = 1.25%) indicated good precision and reliability of the experiments. The interaction between concentrations of yeast extract and $MgSO_4 \cdot 7H_2O$ was more significant than the interaction between concentrations of yeast extract and inoculum or inoculum and $MgSO_4 \cdot 7H_2O$ (Table VI).

The fitted response for the regression model mentioned earlier was plotted in Figures 1–3. Three-dimensional graphs were generated for the pair-wise combinations (with the third factor being kept at an optimum level for ethanol production). The predicted maximum ethanol production (91.1 g/l) derived from RSM regression was obtained when the initial concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ were 9.24 g/l, 39.8 ml/l, and 0.45 g/l, respectively. In this paper, the concentration of yeast extract was confirmed to be 9.24 g/l based on Box–Behnken design (Figures 1 and 2).

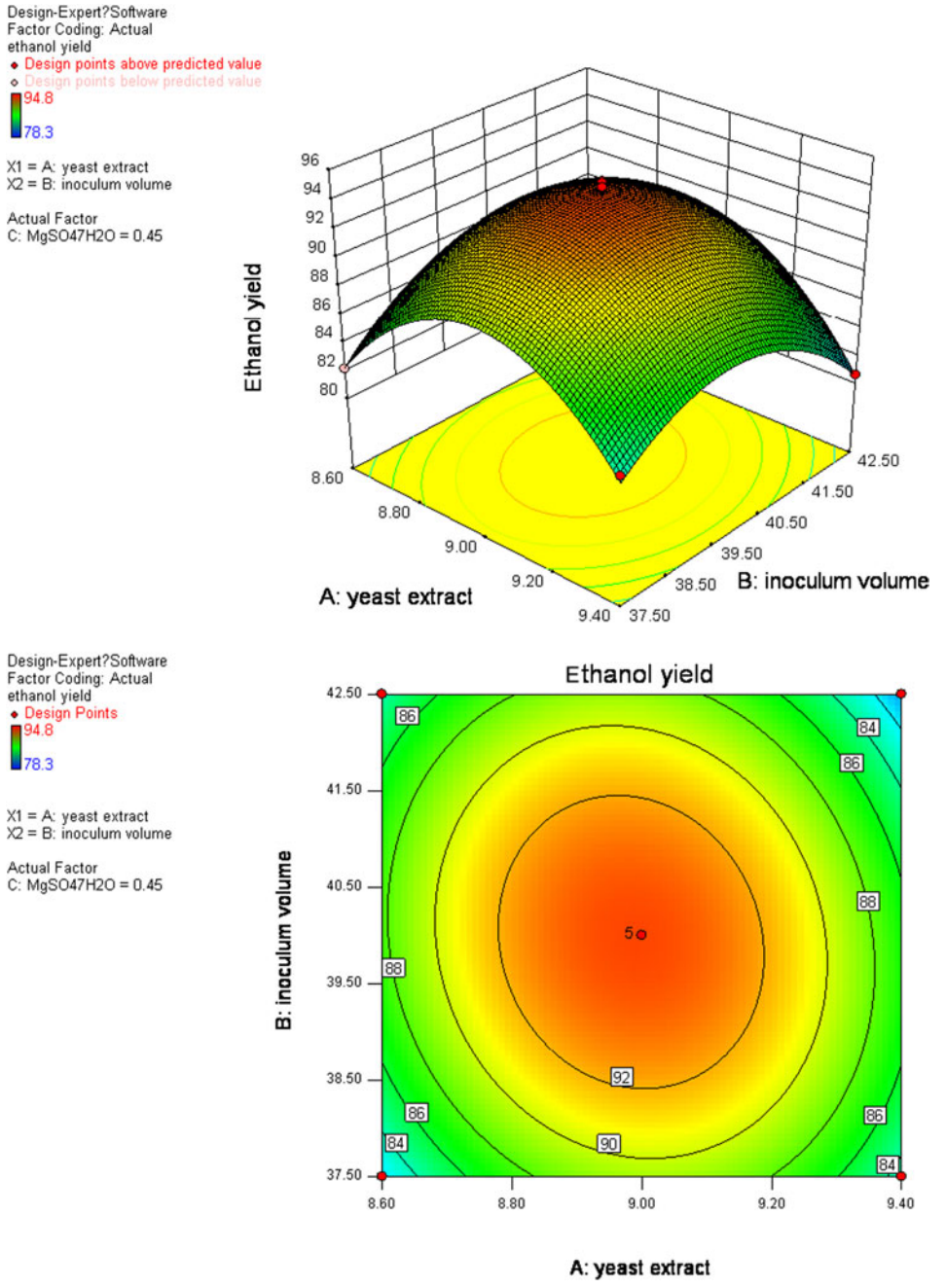

Figure 2. The response surface plot and the corresponding contour plot showing the effects of yeast extract and inoculum concentrations on ethanol production by recombinant *S. cerevisiae* ($MgSO_4 \cdot 7H_2O$ = 0.45 g/l).

Magnesium enhanced ethanol production as shown in Figures 1 and 3. However, proper concentration of $MgSO_4 \cdot 7H_2O$ was also important for the cell growth because low Mg concentration could cause deficiency and high concentrations could lead to cell water imbalance and cell growth inhibition, followed by a decrease in ethanol production.

Experimental validation and time-course of fermentation

The time-course of ethanol production in the optimized medium was monitored for 156 h (Figure 4). After approximately 12 h, the cell growth increased strongly, whereas the production of ethanol increased only after 48 h of cultivation. The ethanol production reached a maximum (102.1 g/l) after 84 h of cultivation in the optimized medium. Due to a decrease in cell growth after 84 h or a decrease in sugar concentration, ethanol production decreased after 96 h.

The maximal ethanol production (102.1 g/l) in the optimized medium was similar to the value predicted by the model, indicating the model adequacy. The final values and components of medium optimized with RSM were pH 5.75, peptone 30 g/l, yeast extract

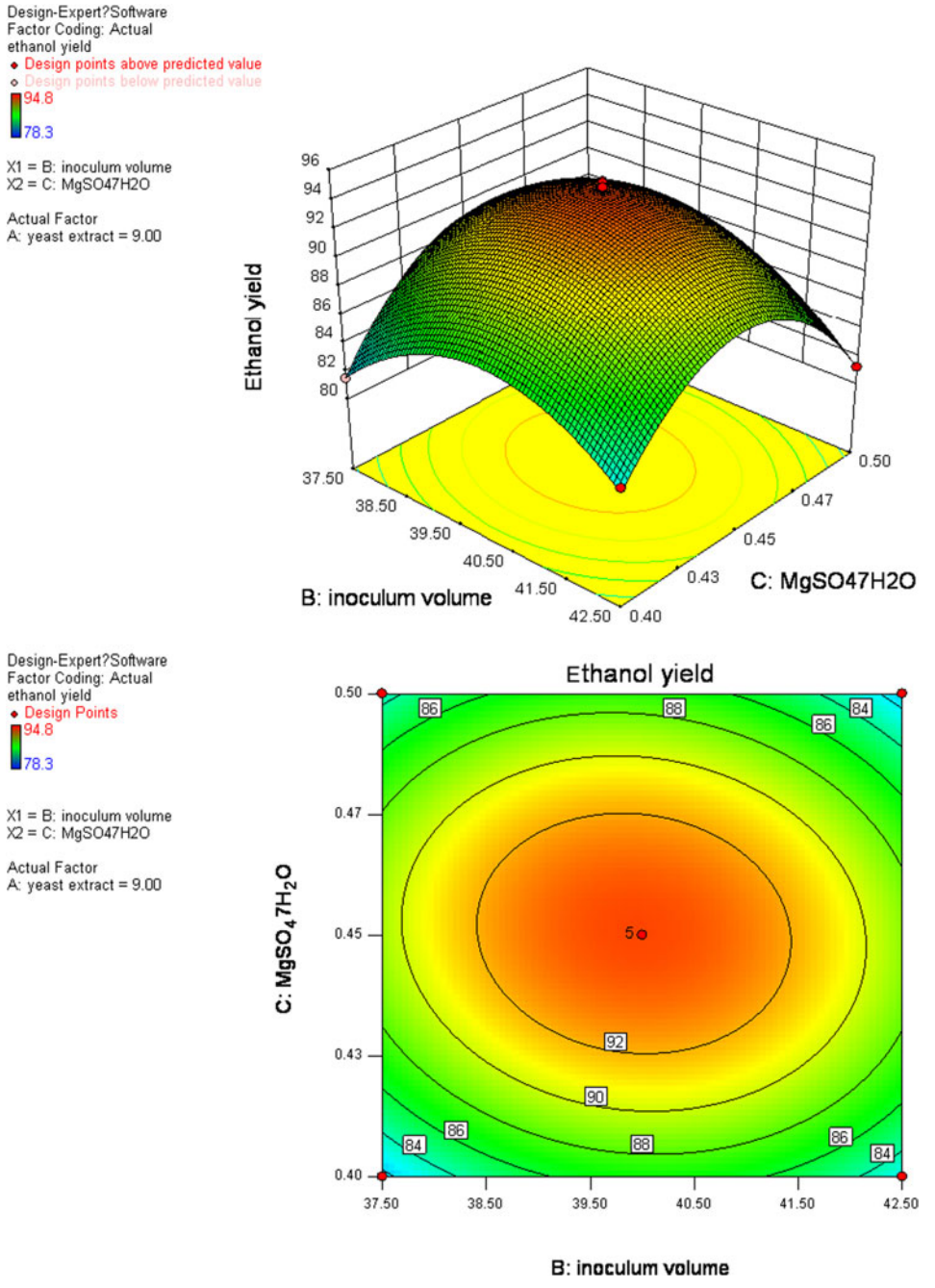

Figure 3. The response surface plot and the corresponding contour plot showing the effects of inoculum and $MgSO_4 \cdot 7H_2O$ concentrations on ethanol production by recombinant *S. cerevisiae* (yeast extract = 9 g/l).

9.24 g/l, temperature 30°C, inoculum 39.8 ml/l, NaCl 1.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.45 g/l, and fermentation time 84 h.

Discussion

The analysis of nutrients screening test showed that concentrations of yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ had significant influence on the ethanol production. The optimum combinations of these three were further analyzed. In the fermentation, the raw Jerusalem artichoke extract acted as a main carbon source for the *S. cerevisiae* inoculum in the fermentation culture, yeast extract provided nitrogen, and $MgSO_4 \cdot 7H_2O$ was a mineral nutrient probably involved in a catalytic action of inulinase in the ethanol production.

Yeast extract was reported to be the best nitrogen source for ethanol production by *S. cerevisiae* in submerged fermentation. Carbon compounds are the sources of carbon skeleton and energy for microbial cells. Jerusalem artichoke is not only a significant carbon source, but also an important reducer in ethanol production by recombinant *S. cerevisiae*

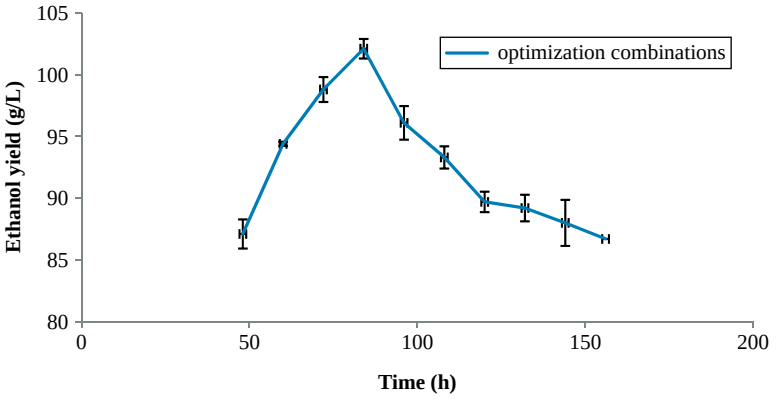

Figure 4. Time-dependent fermentation of raw Jerusalem artichoke extract by recombinant *S. cerevisiae* to produce ethanol using the optimized medium.

(Xiong et al. 2007). In addition, the Jerusalem artichoke raw extract provides nitrogen, vitamins, and other nutrients for ethanol production by recombinant *S. cerevisiae*. However, high concentration of Jerusalem artichoke tuber extract can lead to catabolic repression as only proper sugar concentration could stimulate yeast growth and high concentrations can be inhibitory. So, when the sugar concentration was fixed, the inoculum volume was important. Powchinda et al. stated that up to a critical amount, the increase in inoculum concentration increases ethanol production due to better utilization of the sugars. However, a high inoculum volume can adversely affect ethanol yield by decreasing the viability of yeast population and causing inadequate development of biomass.

Conclusions

After the optimization using RSM, yeast extract, inoculum, and $MgSO_4 \cdot 7H_2O$ were found to be important factors for the fermentation. Under the optimized conditions, ethanol yield of 102.1 g/l was reached using the recombinant *S. cerevisiae*, which corresponded well with the predicted maximum ethanol yield of 91.1 g/l derived from RSM regression. The optimization performed here can be used for industrial production of ethanol in the future.

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