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Determination of optimal conditions for ribonucleic acid production by *Candida tropicalis* no. 121

Huajing Ren‡, Xiaochun Chen‡, Jiaming Cao, Lei Li, Jianxin Bai, Yong Chen, Jian Xiong, and Hanjie Ying†

State Key Laboratory of Materials-Oriented Chemical Engineering, College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 210009, P. R. China
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Abstract−The experiments were based on multivariate statistical concepts, and response surface methodology (RSM) was applied to optimize the fermentation medium for the production of ribonucleic acid (RNA) by *Candida tropicalis* no. 121. The process involved the individual adjustment and optimization of various medium components at shake flask level. The two-level Plackett-Burman (PB) design was used to screen the medium components, which significantly influenced RNA production. Among seven variables, the concentrations of molasses, $ZnSO_4$, and H_3PO_4 were found to be the important factors that significantly affected RNA production (confidence levels above 95%). These factors were further optimized using a central composite design (CCD) and RSM. The optimum values for the critical components were as follows: molasses 47.21 g/L: $ZnSO_4$ 0.048 g/L; H_3PO_4 1.19 g/L. Under optimal conditions, RNA production was 2.56 g/L, which was in excellent agreement with the predicted value (2.561 g/L), and led to a 2.1-fold increase compare with that using the original medium in RNA production.

Key words: *Candida tropicalis* no. 121, Ribonucleic Acid, Ferment Production, Statistical Optimization, Response Surface Method

INTRODUCTION

Ribonucleic acid (RNA) is a macromolecular substance involved in almost all biochemical processes in organisms [1]. Researchers have focused on the degradation products of RNA, which contain 5′-nucleotides (adenosine monophosphate, uridine monophosphate, guanine monophosphate and cytidine monophosphate) in addition to ribose. 5′-Nucleotides have been used as flavor enhancers in the food industry for a long time [2]. Dietary sources of nucleotides are important for the maintenance of cellular immune response [3], and they are used as infant food additives to facilitate the development of the immune system in young children [4]. Nowadays, D-ribose is used to improve the quality of sleep and mental clarity in patients with fibromyalgia or chronic fatigue syndrome [5].

In the traditional method of medium optimization, one independent variable is studied, while the other variables are at fixed level. However, this method is complex and time consuming. Response surface methodology (RSM), a collection of statistical and mathematical techniques, has proven to be an effective method in medium optimization [6]. Recently, RSM has been applied widely in many biotechnology fields such as the optimal production of acetoin [7], uridine diphosphate N-acetylglucosamine [8], thermostable alpha-amylase [9], dairy-free emulsion [10], and substrates for indigo formation [11].

The application of this technique for optimizing medium composition for RNA production had not been reported. The optimum values for the critical components were as follows: molasses 47.21 g/L: $ZnSO_4$ 0.048 g/L; H_3PO_4 1.19 g/L.

†To whom correspondence should be addressed.
E-mail: yinghanjie@njut.edu.cn
‡These author equally contributed to this study.

METHODS

1. Microorganism and Culture Conditions

Candida tropicalis no. 121 stored in our laboratory was used for RNA production in this study. The strain was cultured on a malt juice agar slant, which contained (g/L): malt 250 and agar 20, for three days at 30 °C, and was then stored at 4 °C and subcultured every three months.

2. Inoculum Preparation and Flask Culture

Before production of the inoculum, the strain was transferred from the slant culture into an Erlenmeyer flask (500 mL) containing 30 mL seed medium. The pH of the medium was adjusted to 6.0 before autoclaving. The strain was grown at 32 °C in 50 mL of 1% $(NH_4)_2SO_4$-3% molasses broth for 24 h with shaking at 110 rpm.

The inoculum (10%, v/v) was transferred into an Erlenmeyer flask (500 mL) containing 100 mL of fermentation medium (pH 6.0) which contained (g/L): molasses 40, $(NH_4)_2SO_4$ 2, $MgSO_4$ 0.5, H_3PO_4 3.6, $ZnSO_4$ 0.06, $FeSO_4$ 0.05 and grown for 12 h at 32 °C in a rotary shaker (240 rpm). All media were autoclaved for 15 min at 121 °C.

3. Analytical Method

The cells were harvested by centrifugation at 8,000 rpm for 15 min, and then washed into a 10 mL centrifugal tube with distilled water and perchloric acid (PCA). The tubes were then placed into a boiling water bath for 15 min, immediately cooled under running tap water and centrifuged at 8,000 rpm for 15 min. The supernatant, which was diluted 500 times was used for the determination of RNA. The absorbance was read at 320 nm.

RNA concentration was calculated as follows: the absorbance was read at 320 nm; 1 unit of absorbance was taken as equal to 33.3

μg RNA/mL [12].

EXPERIMENTAL DESIGN AND DATA ANALYSIS

1. Plackett-Burman Design

Different fermentation components were investigated using the PB experimental design, and three important experimental factors were found to significantly affect RNA production. A PB design can rapidly screen multiple factors and identify influential factors [13]. Elements related to the level of choice are very important for the experimental results [14]. Each variable was represented at two levels (Table 1), high and low, which were denoted by "+1" and "−1", respectively. In the experiments, the variables, which were significant at 95% confidence level (P<0.05), were considered to influence RNA production significantly and were further optimized by a CCD. An 11-run PB design (Table 2) was used [15], and the experimental responses were analyzed by the method of least squares to fit the following first-order model:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_6X_6+\beta_7X_7 \quad (1)$$

Where Y was the predicted response, β_0, β_1, β_2, β_3, β_4, β_5, β_6, and β_7 were the regression coefficients, and X_1, X_2, X_3, X_4, X_5, X_6, and X_7 were the coded levels of the independent variables.

2. Path of Steepest Ascent Method

The steepest ascent was generated by the first-order empirical equation obtained by the PB design. The steps along the path were proportional to the regression coefficients β_i. Experiments were performed along the steepest ascent path until the response showed no further increase. The point obtained was used as the center point for further optimization [16].

Table 1. Levels of the factors tested in the PB design

Factor	Level of factors		
	+1	0	−1
Molasses (X_1, g/L)	45	40	35
$(NH_4)_2SO_4$ (X_2, g/L)	2.3	2.0	1.7
$MgSO_4$ (X_3, g/L)	0.7	0.5	0.3
$ZnSO_4$ (X_4, g/L)	0.08	0.06	0.04
$FeSO_4$ (X_5, g/L)	0.08	0.05	0.02
H_3PO_4 (X_6, g/L)	5.4	3.6	1.8
pH (X_7)	6.5	6.0	5.5

Table 2. PB design variables (in coded levels) for RNA production as response

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	RNA (g/L)	
								Observed	Predicted
1	−1	−1	1	1	−1	−1	1	1.17	1.17
2	−1	−1	−1	1	1	1	−1	1.06	1.07
3	1	−1	−1	−1	−1	1	1	1.28	1.29
4	−1	1	1	−1	−1	1	−1	1.01	1.01
5	1	1	−1	1	−1	−1	−1	1.26	1.25
6	1	1	1	1	1	1	1	1.20	1.19
7	−1	1	−1	−1	1	−1	1	1.26	1.27
8	1	−1	1	−1	1	−1	−1	1.50	1.51
9	0	0	0	0	0	0	0	1.22	1.22
10	0	0	0	0	0	0	0	1.29	1.22
11	0	0	0	0	0	0	0	1.23	1.22

3. Central Composite Design and Response Surface Methodology

The three most significant factors (molasses, $ZnSO_4$ and H_3PO_4) led to optimization using a CCD to enhance RNA production . The three independent factors were observed at five different levels (−1.68, −1, 0, +1, +1.68). A set of 16 experiments was carried out. All the variables were taken at a central-coded value considered as zero. To validate the optimization of medium composition, triplicates were used for each experimental design to confirm the results from the response surface analysis.

The behavior of the system was explained by the following second-order polynomial equation:

$$Y=\beta_0+\Sigma\beta_iX_i+X\beta_{ii}X_{ii}^2+\Sigma\beta_{ij}X_iX_j,\ i=1, 2, \ldots, k \quad (2)$$

Where Y was the predicted response, β_0 was the intercept, X_i and X_j were the coded independent factors, β_i was the linear coefficient, β_{ii} was the quadratic coefficient and β_{ij} was the interaction coefficient.

4. Statistical Analysis

Statistica 8.0 was used for the experimental designs and regression analysis of the experimental data. The significance of the data was evaluated by variance analysis (ANOVA). 3D surface plots were drawn to show the effects of independent variables on the response. The variance explained by the model was shown by the multiple determination coefficient (R^2) value. The statistical significance of the model equation was evaluated by the F-test for ANOVA. The significance of the regression coefficients was determined by a t-test for ANOVA.

RESULTS AND DISCUSSION

1. Selection of the Carbon Sources

Generally, a carbon source is indispensable for microorganism growth in most fermentation processes. In this work, a series of experiments was carried out to study the effects of various carbon sources

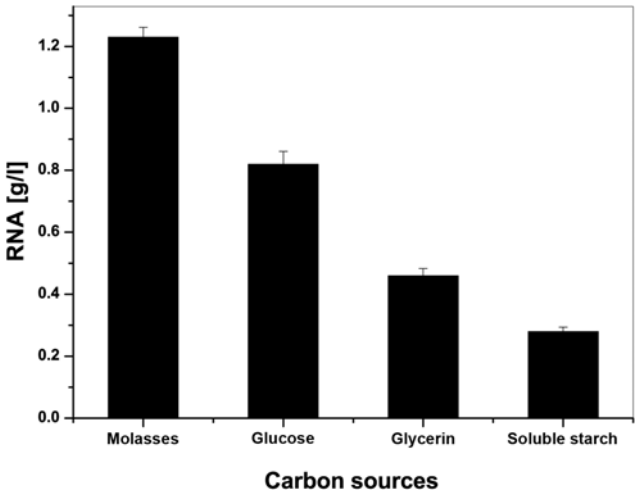

Fig. 1. Effect of different carbon sources on RNA production.

on the production of RNA by *C. tropicalis* no. 121, such as molasses, glucose, soluble starch and glycerin. Fig. 1 shows that molasses had an obvious advantage as the carbon source over the other sugars when the RNA concentration reached 1.23 g/L. Molasses produced by agro-industrial processes contains abundant sugars, amino acids, organic acids, inorganic compounds, and vitamins, and has often been used as a carbon source in microorganism fermentation due to its low cost [17,18]. Thus, molasses as the carbon source was used in subsequent optimization steps.

2. Effect of Phosphate

Phosphorus (P) as nutrient is an essential macronutrient for biological growth and development [19]. Phosphate ion is precursor for PRPP synthesis, and sufficient supplying of PRPP is the prerequisite to high-level production of nucleotides, and PRPP synthesis is the rate-limiting step of nucleotide production [20]. Our results demonstrate that H_3PO_4 is very useful for RNA production, which is optimized by RSM. Fig. 2 shows that H_3PO_4 had an obvious advantage on the RNA concentration.

3. Effect of Zn^{2+}

Zinc, copper and manganese ions are very interesting because they have a positive effect on the respiratory activity and the growth rate of *yeast*, *Saccharomyces cerevisiae*. In *Saccharomyces cerevisiae*, CuZnSOD and MnSOD are encoded by the nuclear genes *SOD1* and *SOD2*, respectively [21]. Yeast, CuZnSOD has also been found to reside in the cytosol and mitochondrial intermembrane space [22].

However, Enzyme secretion is strongly inhibited by the presence of Hg^{2+}, Cu^{2+}, and Co^{2+} in the medium and to some extent by Zn^{2+} and Ni^{+} [23]. In our study, $ZnSO_4$ is very useful for RNA production, which is optimized by RSM. Fig. 3 shows that $ZnSO_4$ had an obvious advantage on the RNA concentration.

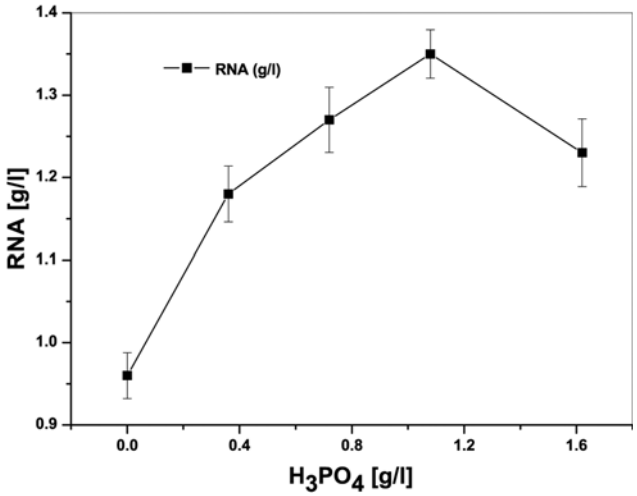

Fig. 2. Effect of different amount of H_3PO_4 on RNA production.

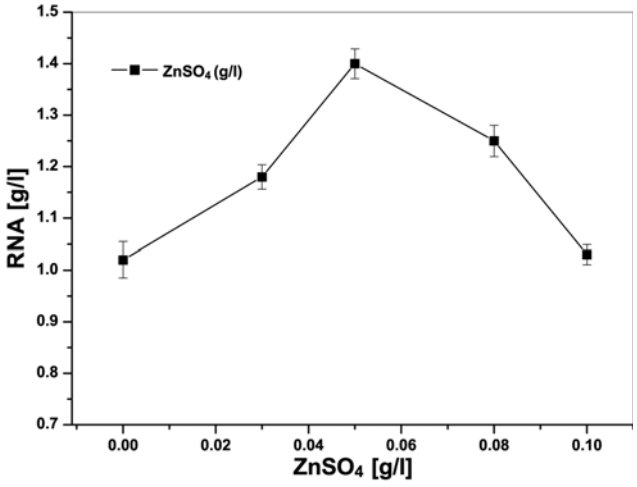

Fig. 3. Effect of different amount of $ZnSO_4$ on RNA production.

4. Plackett-Burman Design

The PB design was employed to identify which variables have important effects on RNA production. The medium for RNA production contained molasses, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4$, $FeSO_4$, and H_3PO_4. Table 2 shows the PB experimental design for 11 trials with two levels for each variable. Three controls were used to estimate the experimental error and check the adequacy of the first-order model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB experiment:

$$Y=1.22+0.09X_1-0.04X_2+0.002X_3-0.05X_4+0.04X_5-0.08X_6+0.01X_7 \quad (3)$$

The regression coefficients and determination coefficient (R^2) for the linear regression model of RNA production are shown in Table 3. The model was significant ($P<0.05$) and $R^2=0.9694$, indicating that 96.94% of the variability in the response could be explained by the model. Based on the statistical analysis, the factors which had the greatest impact on the production of RNA were identified as X_1 (molasses), X_4 ($ZnSO_4$) and X_6 (H_3PO_4). These variables were ranked as follows: molasses>H_3PO_4>$ZnSO_4$. It can be seen from Table 3 that the other variables had confidence levels below 95% and hence were considered insignificant.

Thus molasses, $ZnSO_4$ and H_3PO_4 were selected for further optimization to achieve a maximum response.

5. The Path of Steepest Ascent

Based on the first-order model equation obtained above and regression results, the concentrations of molasses, $ZnSO_4$ and H_3PO_4 were considered to be significant variables. The coefficient of X_1 was positive, while the coefficients of X_4 and X_6 were negative, which meant that increasing the concentration of X_1 and decreasing the concentrations of X_4 and X_6 had a positive effect on RNA production. The path of the steepest ascent was determined to identify the

Table 3. Regression results of the PB design

Factor	Coefficient	T-value	*P*-value
Intercept	1.2244	100.2880	0.000002[a]
X_1	0.0910	6.3567	0.007877[a]
X_2	−0.0358	−2.4973	0.087919
X_3	0.0020	0.1397	0.897744
X_4	−0.0458	−3.1958	0.049491[a]
X_5	0.0370	2.5846	0.081456
X_6	−0.0798	−5.5708	0.011416[a]
X_7	0.0103	0.7160	0.525694

$R^2=0.9694$

[a]Statistically significant at 95% of confidence level

Table 4. Experimental design and results of the steepest ascent path

Run	Molasses (g/L)	$ZnSO_4$ (g/L)	H_3PO_4 (g/L)	RNA (g/L)
Origin	40	0.06	3.6	1.20
1	42.57	0.055	2.79	1.38
2	45.14	0.05	1.98	1.41
3	47.71	0.045	1.17	1.48
4	50.28	0.04	0.36	1.32
5	52.85	0.035	0	1.11
6	55.42	0.03	0	1.09

correct direction of the changing variables. The experimental design of the steepest ascent and the corresponding results are shown in Table 4. The concentrations of the other factors were fixed at zero as shown in Table 1. The maximum RNA yield was obtained in run 4, which meant that the point was near the region of maximum RNA production response. Therefore, this point was chosen as the center point of the CCD for further optimization.

6. Central Composite Design and Response Surface Methodology

A highly significant quadratic polynomial obtained by CCD was very useful in determining the optimal concentration of the medium components which had a significant effect on RNA production. The levels of the components chosen were set based on the previous data analysis. Each variable was studied at five coded levels (−1.68, −1, 0, +1, +1.68) shown in Table 5. The other variables were taken at a central-coded value of zero as shown in Table 1. Experimental design and results are shown in Table 6. The screened variables were expressed by the following fitted second-order polynomial equation from the regression results:

$$Y=2.56-0.02A+0.01B+0.06C-0.09A^2-0.10B^2-0.10C^2 +0.007AB-0.02AC+0.06BC \quad (4)$$

Where Y was the predicted response, A, B and C were coded values of the molasses, $ZnSO_4$ and H_3PO_4 concentrations, respectively.

Statistical significance of the model equation was checked by the F-test, and the proportion of variance explained by the model was obtained by the multiple coefficient of determination (R^2). The R^2 value was a measure of the goodness of fit of the model. In this experiment, R^2 was calculated to be 89%. The inputs of the test data set were presented to the model for prediction, and the model's predictive results were compared with the actual output values. R^2 values depict the percentage of response variability accounted for by the model [24,25]. The plot of predicted values versus experimental values, shown in Fig. 4, indicated that all the predicted values of the RSM model were close to the experimental values.

The regression coefficients were determined by a *t*-test. The re-

Table 5. Levels of the factors tested in the central composite design

Factor	Levels of factors				
	+1.68	+1	0	−1	−1.68
Molasses (A, g/L)	52.75	50.71	47.71	44.71	42.67
$ZnSO_4$ (B, g/L)	0.079	0.065	0.045	0.025	0.011
H_3PO_4 (C, g/L)	1.26	1.22	1.17	1.12	1.08

Table 6. Experimental design and results of the central composite design

Run	A (molasses)	B ($ZnSO_4$)	C (H_3PO_4)	RNA(g/L)	
				Observed	Predicted
1	−1	−1	−1	2.09	2.27
2	1	1	−1	2.20	2.17
3	1	−1	−1	2.33	2.25
4	0	1.68	0	2.23	2.29
5	−1.68	0	0	2.33	2.34
6	0	0	0	2.51	2.56
7	1	−1	1	2.18	2.21
8	0	0	0	2.56	2.56
9	1.68	0	0	2.25	2.27
10	0	0	−1.68	2.06	2.18
11	−1	1	−1	2.19	2.15
12	−1	−1	1	2.38	2.31
13	−1	1	1	2.45	2.43
14	0	−1.68	0	2.20	2.26
15	1	1	1	2.42	2.37
16	0	0	1.68	2.40	2.38

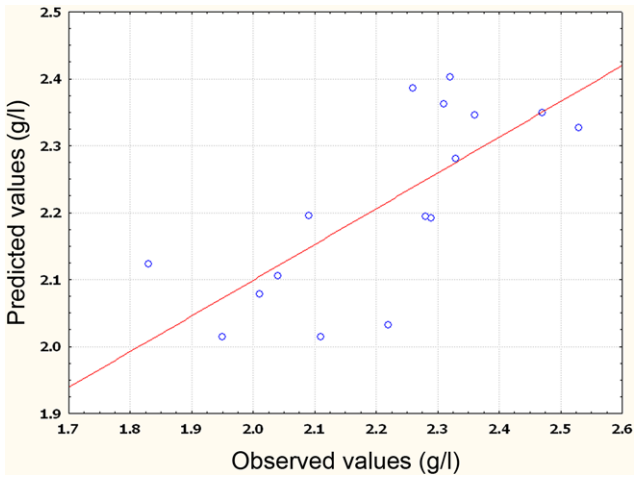

Fig. 4. Comparison between the observed values and the predicted values of RSM model: zero error is shown as a thin line.

Table 7. Regression results of the central composite design

Factor	Coefficient	*P*-value
Intercept	2.529977	
A	−0.008384	0.705140
B	−0.074573	0.027208[a]
C	0.024020	0.296638
A^2	−0.099019	0.007665[a]
B^2	0.087541	0.006140[a]
C^2	−0.097201	0.009703[a]
AB	−0.007500	0.794818
AC	−0.060000	0.072545
BC	0.042500	0.174315

[a]Statistically significant at 95% of confidence level

gression coefficients and corresponding P-values were used to check the significance of each coefficient. This showed that the smaller the P-value, the bigger the significance of the corresponding coefficient. Table 7 shows that C (H_3PO_4) had a significant effect on RNA production, and A (molasses) and B ($ZnSO_4$) were found to have no significance. The quadratic terms for the three factors were found to be significant effects.

Three-dimensional response surface curves were plotted to explain the interaction between two factors for maximum RNA production (Figs. 5-7). The 3D plots and the shapes of the contour plots show the interaction between two variables. The maximum RNA yield was obtained when the non-coded values of the test variables were as follows: molasses 47.21 g/L; $ZnSO_4$ 0.048 g/L; H_3PO_4 1.19 g/L. The predicted maximum RNA production corresponding to these values was 2.56 g/L, which increased by 2.1-fold when compared with that using the original culture conditions.

7. Experimental Validation of the Optimized Conditions

Validation experiments were performed to verify the accuracy of the models. The results showed that the predicted values were in accordance with the experimental results. The model validation was

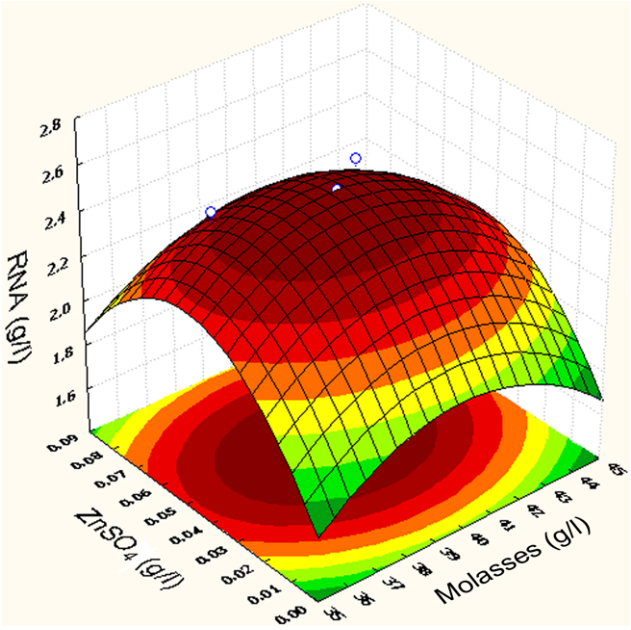

Fig. 5. Response surface curve for RNA production by *Candida tropicalis* no. 121 as a function of molasses and $ZnSO_4$ concentrations, when H_3PO_4 concentration was maintained at 1.19 g/L.

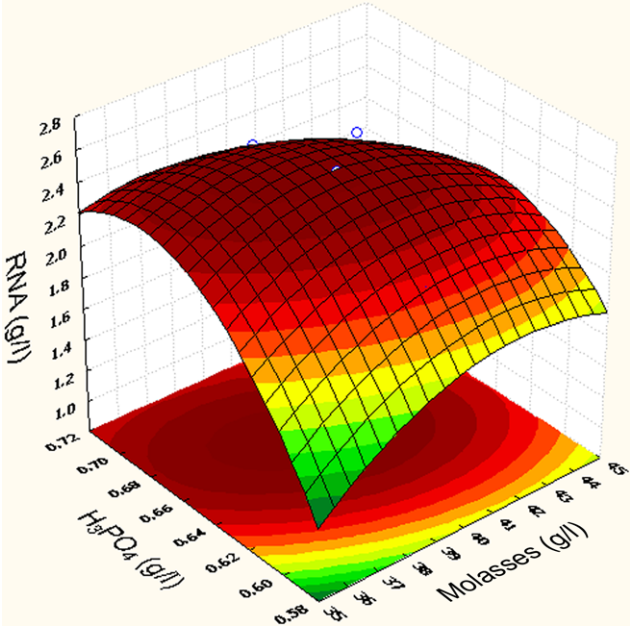

Fig. 6. Response surface curve for RNA production by *Candida tropicalis* no. 121 as a function of molasses and H_3PO_4 concentrations, when $ZnSO_4$ concentration was maintained at 0.045 g/L.

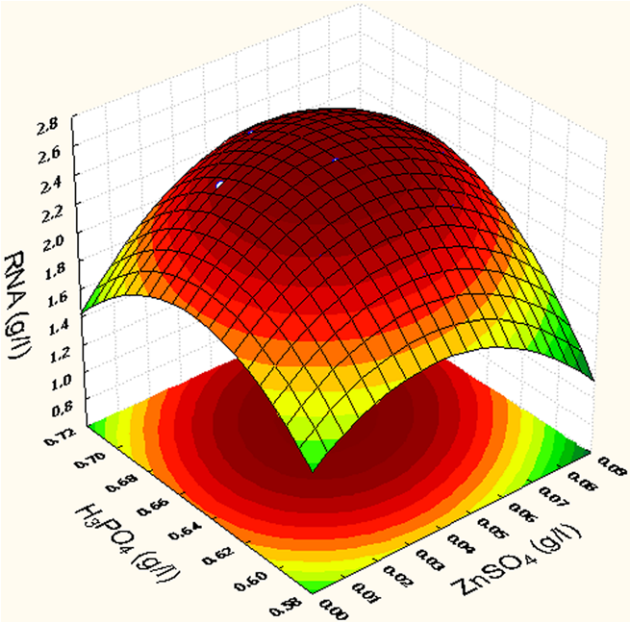

Fig. 7. Response surface curve for RNA production by *Candida tropicalis* no. 121 as a function of $ZnSO_4$ and H_3PO_4 concentrations, when molasses concentration was maintained at 47.71 g/L.

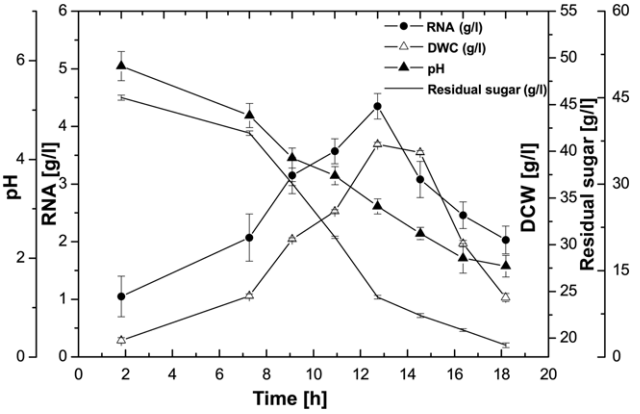

Fig. 8. Time course of batch fermentation in 30 L fermenter for RNA production by *Candida tropicalis* no. 121 under the optimized composition.

done in a shake-flask culture. The RNA concentration was 2.56 g/L, which was in excellent agreement with the predicted value (2.561 g/L).

The feasibility of the regression models was confirmed in a 30 L fermenter under optimum medium composition. Fig. 7 shows the batch profile of cell growth and RNA production and pH and residual sugar. Under these optimum variables, the RNA concentration reached 4.35 g/L.

CONCLUSION

The application of statistical design for the screening and optimization of culture conditions allowed quick identification of the important factors and the interaction between these factors. The final optimal medium composition was (g/L): molasses, 47.21; $ZnSO_4$, 0.048; H_3PO_4, 1.19; $(NH_4)_2SO_4$, 2; $MgSO_4$, 0.5; $FeSO_4$, 0.05 and pH 6.0, which resulted in a 2.1-fold increase in RNA production compared with the original medium. The optimum culture composition obtained in these experiments provides a basis for further study using large scale batch fermentation for RNA production.

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