African Journal of Biotechnology Vol. 8 (8), pp. 1583-1590, 20 April, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Optimization of biomass-producing conditions of *Micrococcus* sp. S-11 for L-cysteine production

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Accepted 23 March, 2009

Micrococcus **S-11 isolated from sediments could transform racemic 2-Amino-Δ^2-thiazoline-4-carboxylic acid (ATC) into L-cysteine. The optimal carbon and nitrogen source for its biomass production were glucose and urea. The optimal culture conditions for biomass production were investigated through statistical experiment design and data analysis. A screening test was first conducted on ten process variables using a Plackett–Burman design, from which three parameters including glucose, urea and rotational speed were chosen as significant ones influencing biomass production. Then these three variables were optimized by Box-behnken experimental design and response surface methodology, and a multinomial equation was constructed to describe the correlation between the biomass production and the three tested variables. By solving to this equation, the predicted maximum biomass was obtained at 11.30 g/L when the culture conditions were glucose 21.98 g/L, urea 4.75 g/L and rotational speed 124 rpm. The validation experiments were carried out under the optimal conditions, from which the average biomass obtained was 11.26 g/L close to the predicted biomass (11.30 g/L), which was 80.7% higher than the one 6.23 g/L obtained under the initial conditions. The results from validation experiments verified the accuracy of the model in terms of depicting the biomass production of** ***Micrococcus*** **sp. S-11.**

Key words: L-cysteine, 2-Amino-Δ^2-thiazoline-4-carboxylic acid (ATC), biomass, Plackett-Burman design, Box-behnken design, response surface methodology.

INTRODUCTION

L-Cysteine is a unique sulfhydryl-containing amino acid, which is widely applied in many fields such as food additives, pharmaceutical industry, feedstuff and cosmetic additives. Traditionally, industrial production of L-cysteine has depended on acid or alkali hydrolysis of hair. However, this technology suffers from a low yield, objectionable odors, and intractable wastes. Recently, a bioconversion of DL-2-amino-Δ^2-thiazolin-4-carbonic acid (ATC) to produce L-cysteine (Sano and Mitsugi, 1978; Ki Moon Pae et al., 1992; Ryu et al., 1997; Yamamoto et al., 2001) has been developed, which has the advantage of low energy requirement and a possibility of more than 90% molar yield. Some bacteria in the genus *Pseudomonas* have potent activities of asymmetrical hydrolysis of DL-ATC to form L-cysteine have been isolated (Sano et al., 1977, 1979; Sano and Mitsugi, 1978; Ryu and Shin, 1991; Tamura et al., 1998). Up to now, all the isolated L-cysteine-producing strains referred in literatures with high hydrolytic activity of DL-ATC exclusively belong to the genus *Pseudomonas*. In our previous work, a germ *Micrococcus* sp. S-11 with comparable hydrolytic activity in comparison with the *Pseudomonas* in producing L-cysteine from DL-ATC was obtained. The amazing performance in the production of L-cysteine makes *Micrococcus* sp. S-11 a promising L-cysteine producer.

With respect to enzymatic production of L-cysteine from DL-ATC, a number of studies have been performed on been performed on the strains screening and genes clone (Tamura et al., 1998; Ohmachi et al., 2002, 2004; Shiba et al., 2002; Tashima et al., 2006; Yu et al., 2006). However, as we know there is no literature concerning the statistical optimization on the conditions of L-cysteine

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production by not only *Pseudomonas*, especially, but also *Micrococcus*. The nutritional and physical conditions of cultivation have strong influence on the productivity of the bioprocess. Optimization of fermentation or biotransformation process has always been paid much attention due to its crucial role in both biomass and enzyme production. But the conventional optimization method, one-variable-at-a-time, is laborious and time-consuming, moreover, it fails to predict response under untested sets of variables and depict the frequent interactions occurring between two or more variables and to guarantee the determination of the optimal conditions (Wang and Lu, 2005; Nikerel et al., 2006). On the contrary, Statistic experimental design and analysis methods are time-saving and reliable, which minimize the error in determining the effect of parameters (Song et al., 2007; Jo et al., 2008; Xu et al., 2008). One of such method is the combination of Plackett-Burman design and response surface methodology, which has been frequently applied in bioprocess optimization (Chen et al., 2005; Liu and Wu, 2007; Lotfy et al., 2007; Gangadharan et al., 2008; Kammoun et al., 2008). The productions of biomass and enzymes involved in L-cysteine production by *Pseudomonas* have different nutrient requirements; moreover the enzymes were induced by adding DL-ATC during culture process (Tamura, 2001). We supposed that *Micrococcus* sp. S-11 might have similar characteristics. Therefore, the L-cysteine production would be strikingly enhanced through a two-stage process strategy, with the first stage under optimal conditions for biomass and the second for enzymes productions, respectively.

In present study, the optimization of its biomass production was conducted using statistic experimental design and analysis method. A screening test was first conducted using a Plackett–Burman design to rapidly identify the significant variables from a ten-variables system, and then three significant variables were further investigated by Box-behnken design in details, lastly the experimental results were analyzed and the optimal levels of the important variables were determined by using response surface methodology. Comparing to original culture conditions, the optimal conditions strongly enhanced biomass production. The optimization of enzyme production will be discussed using statistic experimental design and analysis method.

MATERIALS AND METHODS

Microbe, medium and cultivation

Micrococcus sp.S-11 was isolated from sediments which were sampled from DL-ATC-producing workshop and store in our lab.

The basal biomass-producing medium consisted of 15 g glucose, 3 g urea, 1.5 g NaCl, 3 g K_2HPO_4, 0.12 g $MnSO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$ and 1000 ml distilled water. The pH was adjusted to 7.0. For preparation of the inoculum, *Micrococcus* sp. S-11 was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 50 ml basal medium and incubated at 35℃ on a rotary shaker at 130 rpm for 20 h.

All the experiments were accomplished in 250 ml flask containing 50 ml medium. Each of the flasks was inoculated with 10% (v/v) the inoculum.

Biomass measurement

The biomass of *Micrococcus* sp. S-11 grown under different conditions was expressed as dry cell weight. The cultivation broth was centrifuged (8000 g × 15 min) at 4℃ and washed twice with distilled water to get rid of the residual medium, and then freeze-dried to constant weight at -40℃ for 24 h.

Experimental designs and data analysis

Plackett-Burman experimental design and analysis: In present study, Plackett-Burman design was chosen to find out the key variables influencing the biomass of *Micrococcus* sp. S-11. Plackett–Burman experimental design (Plackett and Burman, 1946) is a two level design (high and low) and offers the screening of a large number of variables (*n*) with a small number of experiments (*n*+1) (Bie et al., 2005; Li et al., 2006; Pan et al., 2008). A Plackett–Burman experimental design was formulated for 10 variables as follows: glucose, urea, K_2HPO_4, $MnSO_4$, and $FeSO4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, NaCl concentration, initial cultural pH, rotational speed and cultural temperature. Each variable was investigated at two levels, high (+1) and low (-1). A design of a total of twelve experiments was generated by using the Minitab. version 14.13 (Minitab. Inc., USA) and response was measured in terms of biomass in Table 1. All the experiments were done in triplicate and the average of biomass production obtained was taken as the response. The effect of individual variable on biomass production was calculated by the following equation:

$$E_i = \frac{(\sum M_{i+} - \sum M_{i-})}{N} \quad (1)$$

Where E_i is the effect of variable *i* under study, M_{i+} and M_{i-} are responses (biomass) of trials at which the variable was at its higher and lower levels, respectively, and *N* is the total number of trials. The effects of different variables were determined and shown in Table 2.

Box-behnken design and response surface methodology: The significant variables identified from the screening experiment were optimized using Box-behnken design (for experimental design) and response surface methodology (for analysis of the optimal levels), while the other variables of non-significance were fixed at constant level (same as the initial medium). In developing the regression equation, the relation between the coded values and actual values are described as the following equation:

$$X_i = \frac{\left(A_i - A_{i0}\right)}{\Delta A_i} \quad (2)$$

Where X_i is the coded value of the *i*th variable, A_i the actual value of the *i*th variable, A_{io} the actual value of the *i*th variable at the center point and ΔA_i is the step change value of the *i*th variable.

The correlation between the response (biomass) and the three variables were fitted to a predictive quadratic polynomial equation as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (3)$$

Table 1. Plackett–Burman design for screening of significant variables on biomass production of *Micrococcus* sp. S-11.

Run order	Glucose (g/L)	Urea (g/L)	K_2HPO_4 (g/L)	$MnSO_4$ (g/L)	$FeSO_4·7H_2O$ (g/L)	$MgSO_4·7H_2O$ (g/L)	NaCl (g/L)	pH	Rotational speed (rpm)	Temperature (℃)	Biomass (g/L)
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	
1	15(-)	3 (-)	3 (-)	0.15 (+)	0.0125 (+)	0.625 (+)	2.5 (-)	8(+)	130(+)	25(-)	6.19
2	20(+)	4.5 (+)	3 (-)	0.15 (+)	0.0125 (+)	0.5 (-)	5(+)	7(-)	110(-)	25(-)	9.54
3	15(-)	4.5 (+)	3.75 (+)	0.15 (+)	0.01 (-)	0.625 (+)	5(+)	7(-)	130 (+)	25(-)	8.34
4	20(+)	3 (-)	3 (-)	0.12 (-)	0.0125 (+)	0.625 (+)	5(+)	7(-)	130 (+)	35(+)	8.96
5	20(+)	3 (-)	3.75 (+)	0.12 (-)	0.01 (-)	0.5 (-)	5(+)	8(+)	130 (+)	25(-)	8.99
6	20(+)	4.5 (+)	3 (-)	0.15 (+)	0.01 (-)	0.5 (-)	2.5 (-)	8(+)	130 (+)	35(+)	10.94
7	15(-)	3 (-)	3 (-)	0.12 (-)	0.01 (-)	0.5 (-)	2.5 (-)	7(-)	110(-)	25(-)	4.91
8	15(-)	3 (-)	3.75 (+)	0.15 (+)	0.0125 (+)	0.5 (-)	5(+)	8(+)	110(-)	35(+)	4.93
9	20(+)	4.5 (+)	3.75 (+)	0.12 (-)	0.0125 (+)	0.625 (+)	2.5 (-)	8(+)	110(-)	25(-)	9.49
10	20(+)	3 (-)	3.75 (+)	0.15 (+)	0.01 (-)	0.625 (+)	2.5 (-)	7(-)	110(-)	35(+)	7.52
11	15(-)	4.5 (+)	3.75 (+)	0.12 (-)	0.0125 (+)	0.5 (-)	2.5 (-)	7(-)	130 (+)	35(+)	8.36
12	15(-)	4.5 (+)	3 (-)	0.12 (-)	0.01 (-)	0.625 (+)	5(+)	8(+)	110(-)	35(+)	7.54

(+) Indicates the high level and (-) indicates the low level.

Where Y is predicted response, β_0 the constant term, β_i the linear coefficients, β_{ii} the squared coefficients and β_{ij} the interaction coefficients.

The levels of the significant variables and the interaction effects between these variables were optimized by Box–Behnken methodology. The experimental plan consisted of 15 trials and the significant variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study is shown in Table 3. All the experiments were done in triplicate and the average of biomass production obtained was taken as the response (Y).

RESULTS AND DISCUSSION

Screening the significant variables on biomass production

In order to determine the appropriate harvest time for biomass, growth curve of *Micrococcus* sp. S-11 was determined (not shown). The cells in the batch culture experienced a rapid growth period of 6^{th} - 18^{th} h and then entered the stationary phase and the death phase followed at the 26^{th} h. So the culture broth at 20^{th} h was chosen for biomass assay of *Micrococcus* sp. S-11. Effects of different carbon sources and nitrogen sources on biomass production of *Micrococcus* sp. S-11 were investigated. The results (not shown) showed that the optimal carbon source was glucose and the optimal nitrogen sources urea and yeast extract. Urea was chosen as the carbon source because of its low cost. So glucose and urea were chosen as the carbon source and nitrogen source of the basal biomass-producing medium, respectively. The relative significance of the studied variables was analyzed through Equation 1 (Table 2). When the sign of the effect E_i of the tested variable is positive, the biomass production is greater at a high level of the parameter, and when negative, the biomass production is greater at a low level of the parameter. According to the analysis of the experimental data, all variables except K_2HPO_4, $MnSO_4$ and $FeSO_4·7H_2O$ with the tested range had a positive effect on biomass. Out of the ten tested variables, only glucose, urea and rotational speed had a significant effect on biomass at a confidence level above 95%. Therefore, these three variables were regarded as the significant variables and further investigated in the following optimization experiments. The other variables had confidence levels below 95% and hence, were considered to be insignificant. The three significant variables had positive influence on biomass production which indicated the biomass production would be enhance with these variables at their high level. In other words, the level above 20 g/L for glucose, 4.5 g/L for urea and 130 rpm for rotational speed should be investigated in Box-behnken design so that the optimal response locate in the studied range of each variables.

Table 2. Effect analysis of variables on responses from the Plackett–Burman design.

Term	Effect	Coef.	SE Coef.	T	P	Significance
X1	2.528	1.264	0.0475	26.61	0.024	*
X2	2.118	1.059	0.0475	22.30	0.029	*
X3	-0.075	-0.038	0.0475	-0.79	0.575	
X4	-0.132	-0.066	0.0475	-1.39	0.398	
X5	-0.128	-0.064	0.0475	-1.35	0.406	
X6	0.062	0.031	0.0475	0.65	0.633	
X7	0. 148	0.0874	0.0475	1.56	0.363	
X8	0.075	0.038	0.0475	0.79	0.575	
X9	1.308	0.654	0.0475	13.77	0.046	*
X10	0.132	0.066	0.0475	1.39	0.398	

*Significant at $P < 0.05$

Table 3. Results and design table of Box-behnken on biomass.

Run order	Glucose (g/L)	Urea (g/L)	Rotational speed (rpm)	Biomass (g/L)
	X1	X2	X9	
1	20(0)	4.5(0)	130(0)	10.98
2	20(0)	3.0(-)	150(+)	9.21
3	15(-)	6.0(+)	130(0)	7.36
4	25(+)	4.5(0)	110(-)	10.53
5	25(+)	4.5(0)	150(+)	8.07
6	15(-)	4.5(0)	110(-)	6.34
7	20(0)	6.0(+)	150(+)	9.17
8	25(+)	6.0(+)	130(0)	9.46
9	15(-)	3.0(-)	130(0)	6.09
10	20(0)	4.5(0)	130(0)	10.90
11	25(+)	3.0(-)	130(0)	9.66
12	20(0)	3.0(-)	110(-)	8.78
13	20(0)	6.0(+)	110(-)	10.01
14	15(-)	4.5(0)	150(+)	6.72
15	20(0)	4.5(0)	130(0)	11.01

(+) Indicates the high level, (0) indicates the medium level and (-) indicates the low level.

Optimization of significant variables on biomass production

The three significant variables (glucose, urea and rotational speed) were further explored using Box-behnken design, while the other variables which had been tested as non-significance were fixed at the low level (same as in basal medium). Table 3 listed the experimental design and results. The regression equation obtained after the analysis of variance gave the response (biomass) as a function of three significant variables. A quadratic model was attempted to fit the data by least-squares, and all terms regardless of their significance were included in the following equation:

$$Y = -86.46 + 4.626X_1 + 5.198X_2 + 0.549X_9 - 0.084X_1^2 - 0.321X_2^2 - 0.002X_9^2 - 0.049X_1X_2 - 0.06X_1X_9 - 0.009X_2X_9 \quad (4)$$

Where Y is the predicted biomass production, X1, X2, and X9 are the coded values of glucose concentration, urea concentration and rotational speed, respectively.

The analysis of variance for response surface quadratic model was summarized in Table 4. The linear and square terms have significant effect on the response with the low *P*-values of less than 0.005. And the interactive terms except the interaction between urea concentration and

Table 4. Estimated regression coefficients for biomass.

Term	Coefficients	Standard error	T	P
Constant	-86.46	6.370	-13.572	0.000
X1	4.626	0.278	16.614	0.000
X2	5.198	0.822	6.325	0.001
X9	0.549	0.066	8.318	0.000
X1*X1	-0.084	0.006	-15.054	0.000
X2*X2	-0.321	0.062	-5.175	0.004
X9*X9	-0.002	0.000	-6.807	0.001
X1*X2	-0.049	0.018	-2.743	0.041
X1*X9	-0.06	0.001	-5.299	0.003
X2*X9	-0.009	0.004	-2.370	0.064
R-Sq = 99.1% R-Sq(adj) = 97.5%				

X1: Glucose, X2: urea, X9: rotational speed.

rotational speed also have a significant effect on biomass production. The determination coefficient R^2 value of 99.1% indicates the excellent agreement between the predicted and experimental values of response and suggests the mathematical model is quite reliable for depicting biomass production of *Micrococcus* sp. S-11. The value of adjusted R^2 (97.5%) suggested that the total variation of 97.5% for biomass was attributed to the independent variables and only 2.5% of the total variation cannot be explained by the model.

By solving the Equation (4), the optimal values of the tested variables in uncoded units were glucose 21.98 g/L, urea 4.75 g/L and rotational speed 124 rpm. Under these conditions, the maximum predicted biomass of *Micrococcus* sp. S-11 was obtained at 11.3 g/L.

The contour curves and corresponding response surface curves are graphic representations of the regression equation. The interaction of two variables and their effects on the response, with the third variable at optimal level, were plotted in Figures 1 and 2 using the mentioned earlier software Minitab. The contour curves directly exhibit whether the interactions exist between the variables, if the contour lines are parallel with either of axes, no interaction exists between these two variables, or else the interaction does exist. Elliptic contours in Figure 1 indicated that there were striking interactions between the variables. And the summits of the response surface of biomass production suggested that the optimal conditions lied inside the tested range of the variables. In order to obtain biomass production above 11.0 g/L, the ranges of the tested variables were as follows: glucose concentration 20.0 - 23.5 g/L, urea concentration 3.75 - 5.75 g/L and rotational speed 112 – 135 rpm. According to the analysis above, it was concluded that the maximum yield of biomass occurred at low level of rotational speed (below level 0) and medium level of urea concentration (around level 0) and high level of glucose concentration (between level 0 and 1). When glucose concentration increased from 15 to 22 g/L, urea concentration from 3 to 4.75 g/L and rotational speed from 110 to 122 rpm, the biomass had an obvious rise to its peak and then declined a little if the three variables continued increasing (Figure 2). Strong shearing strength might account for the remarkable decrease of biomass with the further rise of rotational speed.

Validation of the mathematic model

The cultivation of *Micrococcus* sp. S-11 under both initial and optimal conditions was conducted in triplicate. The average biomass obtained under optimized conditions was 11.26 g/L very close to the predicted maximum biomass (11.30 g/L), 80.7% higher than the one 6.23 g/L obtained under initial conditions. The coherence between the experimental and estimated responses verifies the existence of maximum points and the accuracy of model in terms of depicting the biomass production of *Micrococcus* sp. S-11.

Conclusion

The optimal conditions for biomass production by *Micrococcus* sp. S-11 were investigated using statistical experimental design and analysis methods. Three variables including glucose concentration, urea concentration and rotational speed significantly influencing the biomass production of *Micrococcus* sp. S-11 were screened out from ten process variables using a Plackett–Burman experimental design. Then these three variables were optimized using a Box-behnken design of experiments and response surface methodology. A multinomial equation was constructed to describe the correlation of biomass production and the three tested variables. According to the equation, the optimal conditions were glucose 21.98 g/L, urea 4.75 g/L and rotational speed 124 rpm, under which the maximum bio-

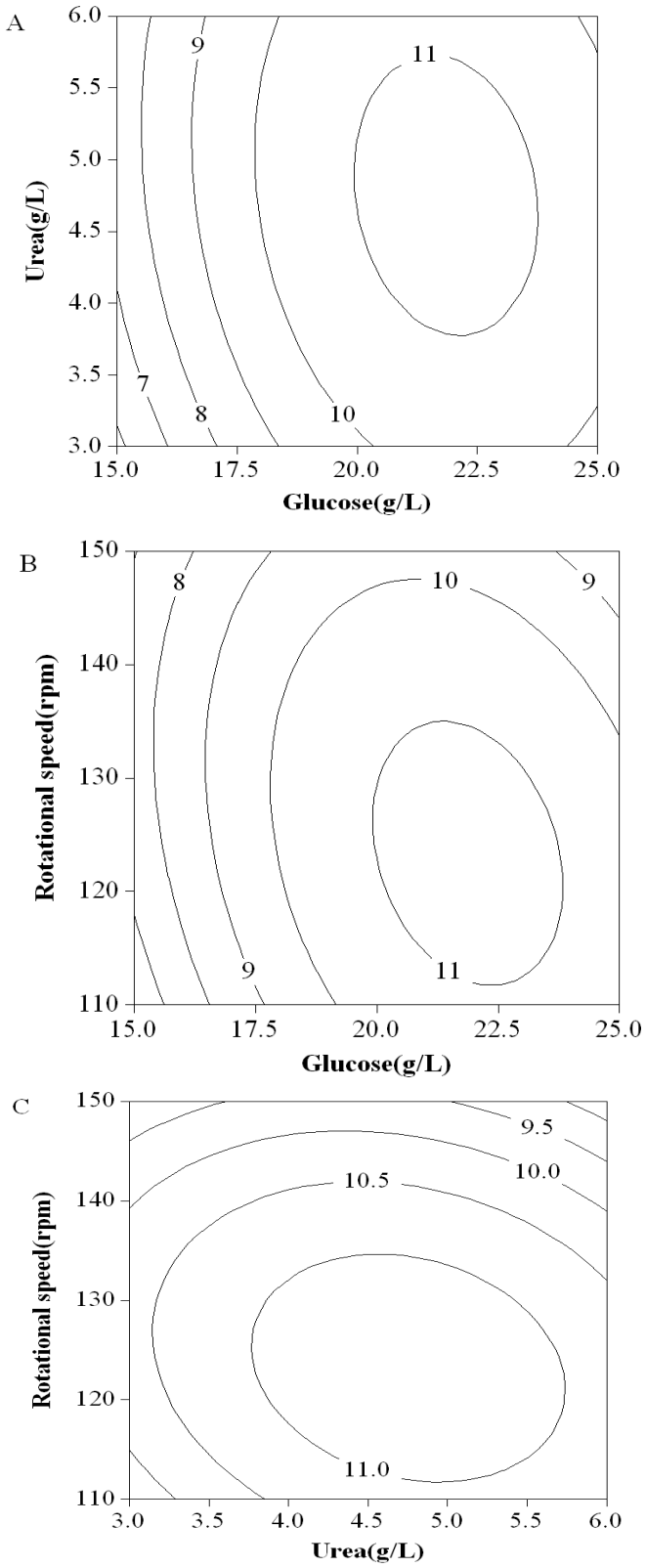

Figure 1. Contour plots of the biomass production by Micrococcus sp. S-11, (A) glucose concentration vs urea concentration at optimal rotational speed; (B) glucose concentration vs rotational speed at optimal urea concentration; (C) urea concentration vs rotational speed at optimal glucose concentration.

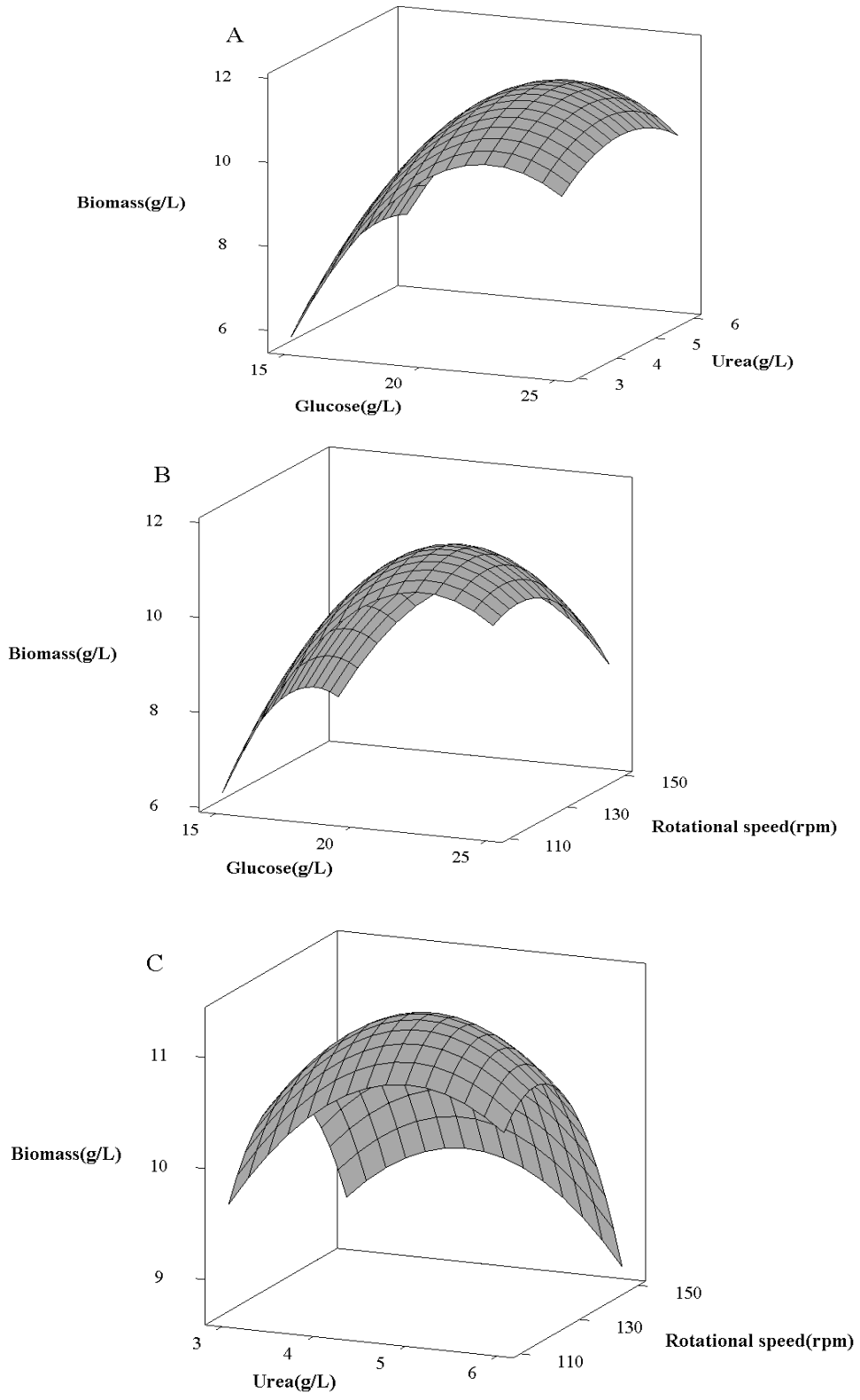

Figure 2. Surface plots of the biomass production by *Micrococcus* sp. S-11, (A) glucose concentration vs urea concentration at optimal rotational speed; (B) glucose concentration vs rotational speed at optimal urea concentration; (C) urea concentration vs rotational speed at optimal glucose concentration.

mass was obtained. The results from verified experiments validated the accuracy of model in terms of depicting the biomass production of *Micrococcus* sp. S-11.

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