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Optimization of Culture Medium for Lactosucrose (^{4}G-β-D-Galactosylsucrose) Production by *Sterigmatomyces elviae* Mutant Using Statistical Analysis

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Abstract In this study, the optimization of culture medium using a *Sterigmatomyces elviae* mutant was investigated using statistical analysis to increase the cell mass and lactosucrose (^{4}G-β-D-galactosylsucrose) production. In basal medium, the cell mass and lactosucrose production were 4.12 g/l and 140.91 g/l, respectively. However, because of the low cell mass and lactosucrose production, optimization of culture medium was carried out to increase the cell mass and lactosucrose production. Culture media were optimized by the *S. elviae* mutant using analysis of variance (ANOVA) and response surface methodology (RSM). Central composite designs using RSM were utilized in this investigation. Quadratic models were obtained for cell mass and lactosucrose production. In the case of cell mass, optimal components of the medium were as follows: sucrose 1.13%, yeast extract 0.99%, bactopeptone 2.96%, and ammonium sulfate 0.40%. The predicted maximum value of cell mass was about 5.20 g/l and its experimental value was 5.08 g/l. In the case of lactosucrose production, optimal components of the medium were as follows: sucrose 0.96%, yeast extract 1.2%, bactopeptone 3.0%, and ammonium sulfate 0.48%. Then, the predicted maximum value of lactosucrose production was about 194.12 g/l and the corresponding experimental value was about 183.78 g/l. Therefore, by culturing using predicted conditions, the real cell mass and lactosucrose production increased to 23.3% and 30.42%, respectively.

Keywords: Medium optimization, response surface methodology, lactosucrose, *Sterigmatomyces elviae* mutant

Lactosucrose (^{4}G-β-D-galactosylsucrose) has fascinated attention in response to an increasing interest in so-called health foods with functional properties, such as calorie free noncariogenic food, and lipid reducing foods, and foods that improve the intestinal microflora [1–3, 8, 12, 15–18, 20, 22]. The sweetness intensity of lactosucrose, a functional and unnatural trisaccharide sweetener, is similar to that of sucrose, but it is not hydrolyzed in the human digestive system [8, 21]. It is a newly synthesized compound, an indigestible oligosaccharide produced from lactose and sucrose by β-fructofuranosidase (E.C. 3.2.1.26) or levansucrase (E.C. 2.4.1.10) from various strains such as *Bacillus* sp., *Arthrobacter* sp., and *Rahnella aquatilis* [3, 7, 8]. Fig. 1 shows the molecular structure of lactosucrose and reaction mechanisms [16]. Among lactosucrose-producing microorganisms, *Bacillus subtilis* was reported to show the highest production of lactosucrose [1, 15]. However, lactosucrose was produced by harvested free cells or immobilized cells after fermentation. Therefore, because of the small cell size, the bacterium was not suitable in a continuous process for large-scale production. Hence, in a great part of the cell immobilization process, yeast and fungi had been selected for the continuous production process [10, 11, 18, 22]. Therefore, the immobilized *Sterigmatomyces elviae* mutant was selected for lactosucrose production. However, to our knowledge, investigations have not been carried out on the optimization of culture media for lactosucrose production by the *S. elviae* mutant. Analysis of variance (ANOVA) and response surface methodology (RSM) had been the most widely used statistical techniques for bioprocess optimization and could be used to evaluate the relationship between a set of controllable experimental factors and observed results [4–6, 9, 10, 13, 14, 21]. To reduce the number of experiments and evaluate the interaction between possible parameters, we adopted a response surface methodology and used a fractional factorial design (FFD) and central composite design (CCD) to determine optimal conditions.

In this study, the optimization of culture medium using the *S. elviae* mutant was investigated using ANOVA and RSM to increase the cell mass and lactosucrose production.

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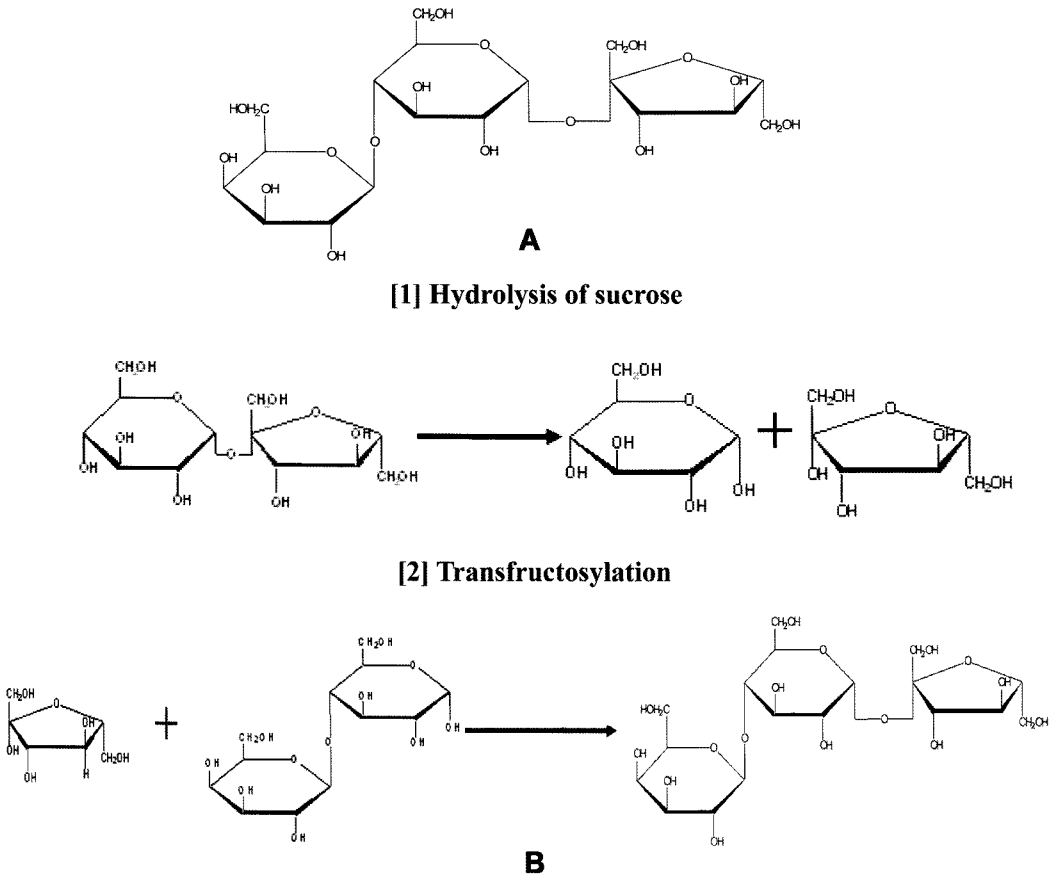

Fig. 1. Structure of lactosucrose (**A**) and reaction mechanisms of lactosucrose production (**B**).

MATERIAL AND METHODS

Microorganisms and Culture Maintenance

The microorganism used in this study was a *Sterigmatomyces elviae* mutant, which was obtained through NTG mutagenesis of *Sterigmatomyces elviae* ATCC 18894. The microorganism was subcultured on a sucrose and yeast extract agar plate. The basal medium consisted of 1% sucrose, 1% yeast extract, 1% bacto-peptone, 0.5% ammonium sulfate, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0 before autoclaving. Main cultures were carried out for 90 h at 30°C, and 150 rpm in a rotary shaking incubator.

Experimental Design

Fractional factorial designs (FFD) of 2^{n-1} were carried out. This design uses two levels of each factor from screening experiments to determine an optimal concentration of medium components to a linear approximation. In the first one, to identify and eliminate parameters, FFD was performed in order to study the effects of sucrose, yeast extract, bacto-peptone, $(NH_4)_2SO_4$, and K_2HPO_4 with 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$. The independent variables and their levels are shown in Table 1.

RSM consists of a set of experimental techniques that evaluate the relations between a cluster of controlled experimental factors and measured responses, according to one or more selected criteria [4–6, 9, 10, 13, 14, 21]. A central composite design (CCD) is basically constituted by a factorial part with two levels, and by axial or a four start point ($\alpha=(2^n)^{1/4}$) of each factor for the intermediate level for the other factors and six replicates at the center point. Factorial points represent the variance design for the first-order model or first order plus coupled interaction. CCD is a design that was developed aiming to find the maximum or minimum response point. The resulting 20 or 30 experiments were used to optimize the culture media for the cell mass and lactosucrose production by the *S. elviae* mutant. When developing the regression equation, test variables were coded according to the following equation:

$$x_i=(X_i-X_0)/\Delta X \qquad i=1, 2, 3 \ldots, j \qquad (1)$$

where x_i is the independent variable coded (dimensionless) value, X_i is the independent variable real value, X_0 is the

Table 1. Real and coded values of the factors for analysis of variance (ANOVA) and response surface methodology (RSM).

(a) Coded values of ANOVA

		Coded values		
	Symbol	−1	0	+1
Sucrose	A	1%	10%	19%
Yeast extract	B	1%	2%	3%
Bacto-peptone	C	1%	2%	3%
$(NH_4)_2SO_4$	D	0.5%	1%	1.5%
K_2HPO_4	E	0.3%	0.5%	0.7%

▶ 1% KH_2PO_4 and 0.5% $MgSO_4 \cdot 7H_2O$ were fixed.

(b) Coded values of RSM

		Coded value				
	Symbol	−2	−1	0	+1	+2
Sucrose	X_1	0.5%	0.75%	1.0%	1.25%	1.5%
Yeast Extract	X_2	0.5%	0.75%	1.0%	1.25%	1.5%
Bacto-peptone	X_3	1.0%	2.0%	3.0%	4.0%	5.0%
$(NH_4)_2SO_4$	X_4	0.3%	0.4%	0.5%	0.6%	0.7%

independent variable real value at the center point, and ΔX is the step change value. The behavior of the system was explained by the following second-degree polynomial equation:

$$y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii}x_i^2+\Sigma\beta_{ij}X_iX_j \quad (2)$$

where y is the predicted response, X_i and X_j are input variables that influence the response variable Y, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the ijth interaction coefficient. A CCD for four independent variables, each at five levels with eight star points and six replicates at the center points, was employed to fit a second-order polynomial model; 30 experiments were required for this procedure [19–21]. The SAS 9.1 package program was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. The maximum values of the cell mass and lactosucrose production were taken as the response of the design experiments.

Production of Lactosucrose

After cultivation, cells were harvested from culture broth by centrifugation and were then washed with distilled water several times. One g of free cells (wet cell weight) was added to 10 ml of a sucrose and lactose mixed solution (25% and 25%, w/v) and allowed to produce lactosucrose at 100 rpm in a water bath for 15 h at 50°C.

Determination of the Cell Mass and Quantification of Lactosucrose

Cell mass (dry cell weight) was calculated using the relationship between absorbance at 660 nm and DCW. One ml of culture broth was transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 5 min. The cell pellet was washed three times by suspending it in distilled water and recentrifuging. Finally, the absorbance of the resuspended cell pellet was measured at 660 nm using a spectrophotometer. Reaction products were analyzed by

Table 2. Experimental design and results for ANOVA.

Run	A	B	C	D	E	Predicted value of cell mass (g/l)	Experimental value of cell mass (g/l)	Predicted value of lactosucrose production (g/l)	Experimental value of lactosucrose production (g/l)
1	−	−	−	−	+	3.20	3.20	140.67	140.67
2	+	−	−	−	−	2.14	2.14	115.32	115.32
3	−	+	−	−	−	3.65	3.65	147.44	147.44
4	+	+	−	−	+	2.56	2.56	126.27	126.27
5	−	−	+	−	−	4.47	4.47	164.36	164.36
6	+	−	+	−	+	1.78	1.78	107.28	107.28
7	−	+	+	−	+	3.97	3.97	156.95	156.95
8	+	+	+	−	−	1.60	1.60	104.93	104.93
9	−	−	−	+	−	2.52	2.52	121.60	121.60
10	+	−	−	+	+	1.36	1.36	94.30	94.30
11	−	+	−	+	+	3.69	3.69	150.09	150.09
12	+	+	−	+	−	1.76	1.76	101.67	101.67
13	−	−	+	+	+	3.58	3.58	145.92	145.92
14	+	−	+	+	−	1.29	1.29	87.26	87.26
15	−	+	+	+	−	3.10	3.10	134.88	134.88
16	+	+	+	+	+	1.67	1.67	110.63	110.63
17	0	0	0	0	0	1.984	1.57	111.10	113.95
18	0	0	0	0	0	1.984	2.26	111.10	109.31
19	0	0	0	0	0	1.984	1.96	111.10	114.78
20	0	0	0	0	0	1.984	2.15	111.10	106.36

HPLC (YOUNG-LIN Instrument Co. Ltd., Korea) using a ZOBAX NH_2 column (150×6.0 mm, Agilent Technologies, Inc., U.S.A.) and a refractive index detector (YOUNG-LIN Instrument Co. Ltd., Korea). The column temperature was maintained constant at 50°C. As a mobile phase, 70% (v/v) acetonitrile and 30% (v/v) water were used as the mobile phase at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Analysis of Variance (ANOVA)

Because information of medium components was not known for cell mass and lactosucrose production, a fractional factorial design (FFD) was used to determine the influence of medium components on the cultivation of a lactosucrose, producing microorganism. Among the existing components of the culture medium, sucrose should have a higher cell activity for lactosucrose production than other carbon sources (data not shown). Therefore, each component of the culture medium such as sucrose, yeast extract, bacto-peptone, $(NH_4)_2SO_4$, and K_2HPO_4 was chosen on the basis of these preliminary experiments. Table 2 shows the experimental designs and results for ANOVA. The relative magnitude of the effect of different factors could be obtained by the analysis of variance (ANOVA). An ANOVA analysis was operated for estimating the error variance for the factor effects on the microorganism. Its results are shown in Table 3. The value of the determination coefficient (R^2= 0.984 and 0.995), being a measure of the goodness of fit of the model, indicates 98.4% of the variability in the response by the model. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of the experiment. Here, a lower value of CV (=12.15%, 3.24%) indicates a high reliability of factors

Table 3. Analysis of variance (ANOVA) for the selected model and statistical analysis of factors connected with cell mass and lactosucrose production.

(a) Analysis of variance for cell mass

Source	Sum of squares	Degrees of freedom	Mean square	F-value	*P*>F
Model	17.223	16	1.0764	11.59	0.0353
Error	0.279	3	0.093		
Corrected total	17.501	19			

Coefficient of variation (CV)=12.15%, Coefficient of determination (R^2)=0.984.

(b) Analysis of variance for lactosucrose production

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P>F
Model	9,099.490	16	568.718	36.02	0.0065
Error	47.365	3	15.788		
Corrected total	9,146.855	19			

Coefficient of variation (CV)=3.24%, Coefficient of determination (R^2)=0.995.

(c) Statistical analysis of factors connected with cell mass and lactosucrose production

	Cell weight (g/l)		Production (g/l)	
Factor	F value	*P* value	F value	*P* value
A	72.77	0.0029	215.72	0.0006
B	1.68	0.2862	12.92	0.0369
C	1.27	0.3417	12.26	0.0395
D	13.59	0.0346	54.96	0.0051
E	0.17	0.7074	0.76	0.4480
A*B	0.14	0.7330	2.19	0.2351
A*C	8.01	0.0662	19.83	0.0211
A*D	0.16	0.7155	0.06	0.8176
A*E	0.03	0.8804	0.10	0.7743
B*C	7.26	0.0741	10.70	0.0467
B*D	0.94	0.4044	6.09	0.0903
B*E	3.76	0.1479	12.58	0.0382
C*D	0.00	0.9741	0.27	0.6414
C*E	0.01	0.9343	0.04	0.8599
D*E	2.85	0.1902	12.16	0.0399

in the experiment. F-values of these models were 11.59 and 36.02, respectively. They show that the statistical significance level was proven to be the influencing results for cell mass and lactosucrose production. According to this analysis, the F-values for all regressions were high and it indicates high efficiency of factors, and a low *P*-value (>0.5) proved to be considerably variable and statistically significant. Sucrose and ammonium sulfate of the highest F-values were the most influential factors in medium components for cell mass and lactosucrose production. However, except for sucrose and ammonium sulfate, the relative level of F-values was low compared with those of sucrose and ammonium sulfate. In addition, an interaction of factors also existed. However, because of the low level, the effect of cell mass and lactosucrose production was little. Specifically, variance analysis [Table 3(c)] showed that K_2HPO_4 concentration (Factor E), as well as its interactions, have had low statistical significance. Therefore, this factor was eliminated on the CCD design.

Determination of the Optimal Concentrations of Medium Components Using Response Surface Methodology

The experimental and predicted values of free cells with respect to lactosucrose production by the *S. elviae* mutant harvested after 72 h culture are given in Table 4. Using multiple regression analysis, the following second-order polynomial equation was found to explain the cell mass and lactosucrose production:

$$Y_1=4.868+0.663X_1+0.0183X_2-0.0075X_3+0.070X_4 -0.3331X_{11}-0.2243X_{22}-0.2219X_{33}-0.1981X_{44} -0.065X_{12}-0.0388X_{13}-0.0713X_{14}-0.145X_{23} -0.0725X_{24}+0.0788X_{34} \quad (3)$$

$$Y_2=189.202-6.31X_1+9.246X_2-0.726X_3-6.54X_4 -11.08X_{11}-5.654X_{22}-14.407X_{33}-9.13X_{44}+3.79X_{12} -4.96X_{13}+1.012X_{14}+0.6X_{23}+1.4X_{24}+4.725X_{34} \quad (4)$$

where X_1=coded value of sucrose concentration, X_2=coded value of yeast extract concentration, X_3=coded value of

Table 4. Experimental design and results for response surface methodology (RSM).

Run	Sucrose	Yeast extract	Bacto-peptone	$(NH_4)_2SO_4$	Predicted value of cell mass (g/l)	Experimental value of cell mass (g/l)	Predicted value of lactosucrose production (g/l)	Experimental value of lactosucrose production (g/l)
1	-1	-1	-1	-1	2.833	2.56	159.824	160.950
2	1	-1	-1	-1	4.510	4.66	147.524	148.656
3	-1	1	-1	-1	3.435	3.45	166.736	167.851
4	1	1	-1	-1	4.851	4.82	169.592	170.729
5	-1	-1	1	-1	3.028	2.99	157.642	158.774
6	1	-1	1	-1	4.550	4.49	125.498	126.620
7	-1	1	1	-1	3.050	2.84	166.958	168.085
8	1	1	1	-1	4.311	4.37	149.971	151.194
9	-1	-1	-1	1	3.103	3.02	132.469	133.592
10	1	-1	-1	1	4.495	4.45	124.218	125.346
11	-1	1	-1	1	3.415	3.22	144.982	146.117
12	1	1	-1	1	4.546	4.56	151.887	153.016
13	-1	-1	1	1	3.613	3.39	149.188	150.311
14	1	-1	1	1	4.850	4.81	121.093	122.227
15	-1	1	1	1	3.344	3.17	164.105	165.230
16	1	1	1	1	4.321	4.34	151.166	152.295
17	-2	0	0	0	2.209	2.66	175.968	171.477
18	2	0	0	0	4.863	4.69	158.124	153.620
19	0	-2	0	0	3.934	4.10	164.823	160.324
20	0	2	0	0	4.007	4.12	190.972	186.474
21	0	0	-2	0	3.996	4.08	161.421	156.923
22	0	0	2	0	3.966	4.16	159.369	154.871
23	0	0	0	-2	3.936	3.99	180.193	175.693
24	0	0	0	2	4.216	4.44	161.698	157.200
25	0	0	0	0	4.868	4.79	189.201	189.505
26	0	0	0	0	4.868	4.99	189.201	191.847
27	0	0	0	0	4.868	4.94	189.201	192.513
28	0	0	0	0	4.868	4.91	189.201	186.940
29	0	0	0	0	4.868	4.86	189.201	195.277
30	0	0	0	0	4.868	4.72	189.201	197.168

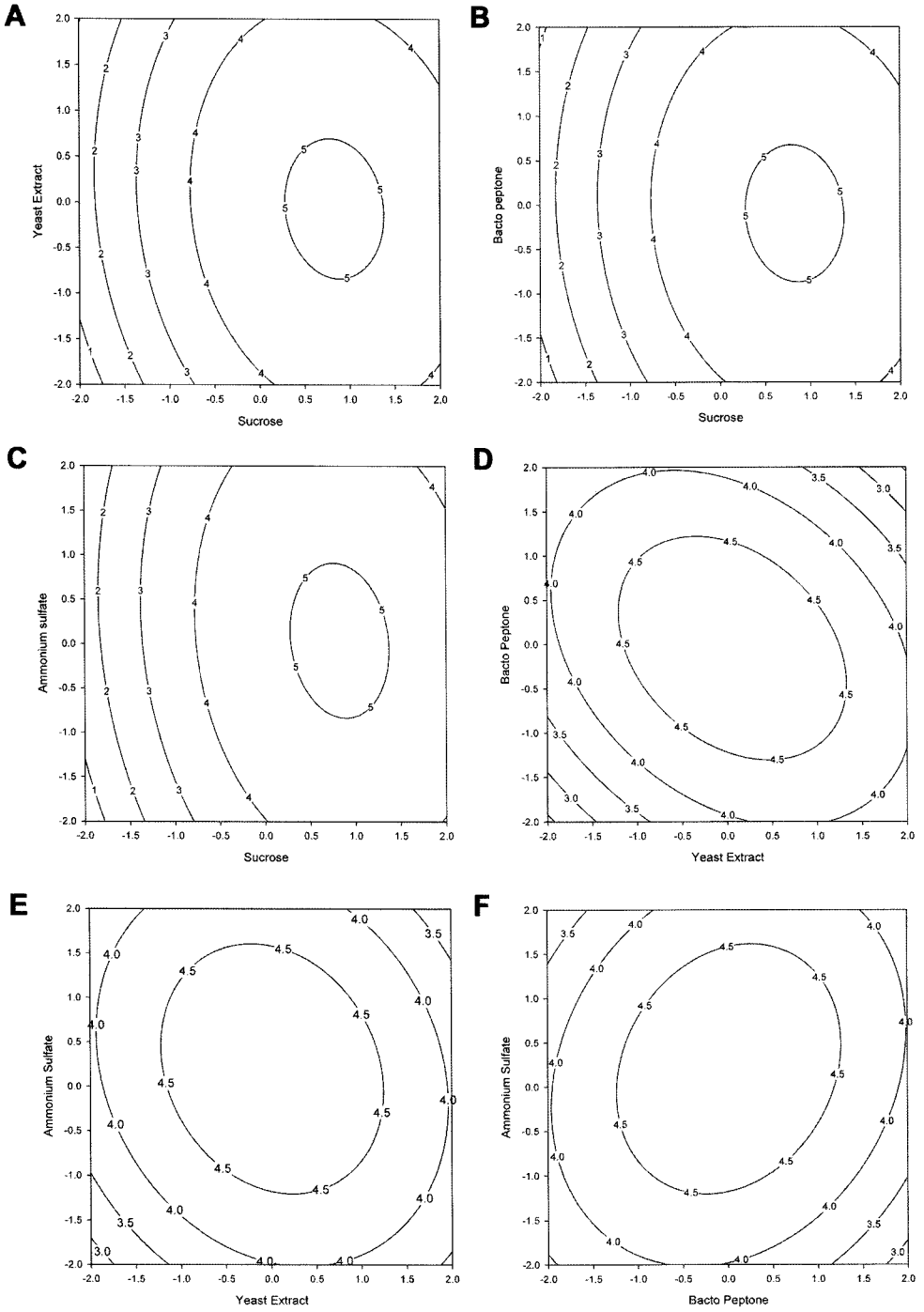

Fig. 2. Isoresponse contour plots showing the effect of medium components on cell mass production.
A. Sucrose and yeast extract. **B.** Sucrose and bacto-peptone. **C.** Sucrose and ammonium sulfate. **E.** Yeast extract and bacto-peptone. **E.** Yeast extract and ammonium sulfate. **F.** Bacto-peptone and ammonium sulfate.

bacto-peptone, X_4=coded value of ammonium sulfate, Y_1=cell mass, and Y_2=lactosucrose production.

Response surface plots provide a method to predicting responses for different test values of variables, and the contours of the plots help to identify the type of interactions between test variables. The three-dimensional mesh and two-dimensional contour plots on cell mass and lactosucrose production obtained from the calculated response surface are presented in Figs. 2 and 3. Each 3D-mesh and 2D-contour curve represents an infinite number of combinations of two test variables, with the other two maintained at their respective zero levels. The maximum predicted value is

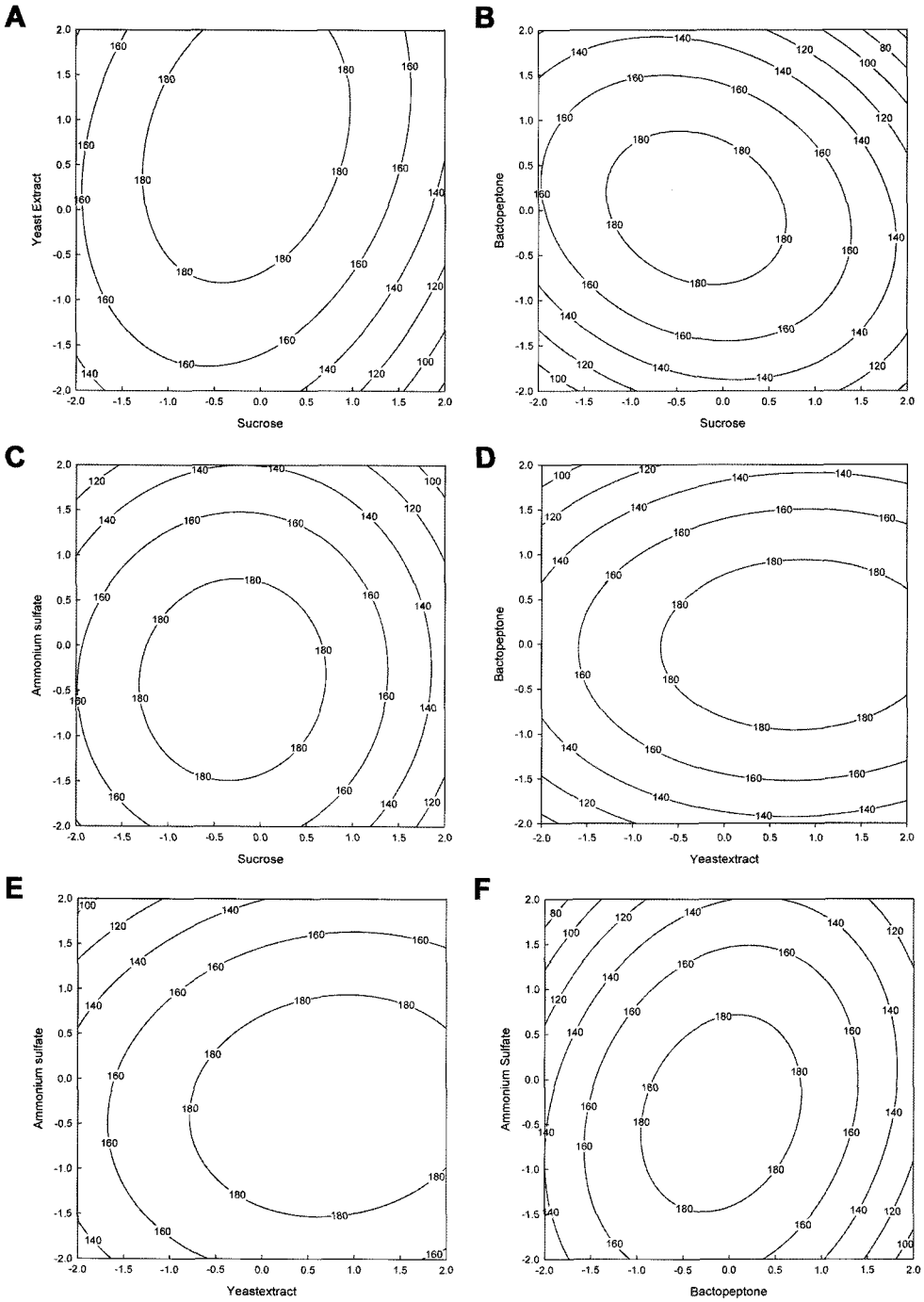

Fig. 3. Isoresponse contour plots showing the effect of medium components on lactosucrose production.
A. Sucrose and yeast extract. **B.** Sucrose and bacto-peptone. **C.** Sucrose and ammonium sulfate. **D.** Yeast extract and bacto-peptone. **E.** Yeast extract and ammonium sulfate. **F.** Bacto-peptone and ammonium sulfate.

indicated by the surface confined within the smallest ellipse in the 3D-mesh and 2D-contour diagrams. The 3D-mesh and 2D-contour plots show a significant interaction between sucrose, yeast extract, bacto-peptone, and ammonium sulfate, and also that the optimal sucrose coded value is 0.5–1.5. Yeast extract showed a moderate interaction with bacto-peptone and almost no interaction with ammonium sulfate, as is evident from the relatively circular nature of the contour plots and an optimal coded value of –0.5–0.5. The optimum point of the two factors and maximum values of the model on the cell mass and lactosucrose production could be obtained as Table 5. The optimal

Table 5. The optimal point of the four factors and maximum values of the model on cell mass and lactosucrose production.

	Cell weight (g/l)				Production (g/l)			
	Sucrose	Yeast extract	Bacto-peptone	$(NH_4)_2SO_4$	Sucrose	Yeast extract	Bacto-peptone	$(NH_4)_2SO_4$
Optimum point	1.13%	0.99%	2.96%	0.40%	0.97%	1.13%	3.02%	0.477%
Coded value	0.504	-0.039	-0.040	-0.003	-0.12	0.51	-0.02	-0.23
Maximum values	5.20 (g/l)				194.12 (g/l)			

points giving the maximum cell mass were 1.13% (w/v) (X_1=0.504) sucrose, 0.99% (w/v) (X_2=−0.039) yeast extract, 2.96% (w/v) (X_3=−0.040) bacto-peptone, and 0.40% (w/v) (X_4= −0.003) $(NH_4)_2SO_4$, respectively, and the predicted maximum value of cell mass was 5.20 g/l. If no interaction exists between the variables, the contour plots presented as straight or circular. Otherwise, the contour plots were inclined at different angles depending on the effects of variables [10]. In Fig. 2, most of the contour plots were circular. Therefore, interactions were presented very low. Specifically, when the sucrose concentration was changed, the cell mass changed rapidly (Figs. 2A, 2B, and 2C). However, in the case of other components, gradual changes were presented (Figs. 2D, 2E, and 2F). Therefore, sucrose was found to be the most influential factor in cell mass. In the case of lactosucrose production (Fig. 3), since the contour plots are circular, it can be inferred that low interaction exists between the sucrose, yeast extract, bacto-peptone, and ammonium sulfate. Most the of contour plots were inclined steeply. Therefore, each factor presented a higher independent effect than interaction. These results were similar to ANOVA and proved that each statistical analysis such as ANOVA and RSM had high accuracy [10]. The optimal points giving the maximum lactosucrose production were 0.96% (w/v) (X_1=−0.16) sucrose, 1.2% (w/v) (X_2=0.8) yeast extract, 3.0% (w/v) (X_3=0) bacto-peptone, and 0.47% (w/v) (X_4=−0.28) $(NH_4)_2SO_4$, respectively, and the predicted maximum value for lactosucrose production was 194.12 g/l, as shown in Table 5. The corresponding experiment values of cell mass and lactosucrose production were 5.08 g/l and 183.78 g/l, respectively. In basal medium, cell mass and lactosucrose production were 4.12 g/l and 140.91 g/l, respectively. Therefore, cell mass and lactosucrose production increased about 23.3% and 30.42% when the strain was grown in the optimized medium as compared with the initial basal medium; the results are shown in Fig. 4.

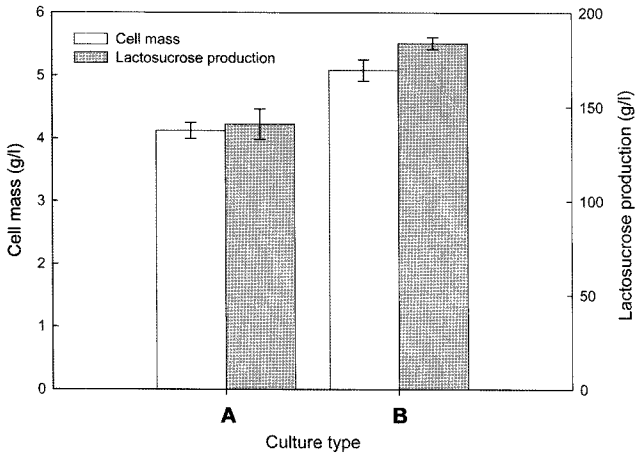

Fig. 4. Comparison of cell mass and lactosucrose production in different culture medium.
A. Before culture medium optimization. **B.** After optimization.

It has been suggested that RSM can be used as a potential tool for optimizing factors showing ambiguous correlations. Cell mass and lactosucrose production were increased about 23.3% and 30.42% in cultures of *S. elviae* mutant by using ANOVA and RSM, and they also showed the presence of interactions between medium components by analysis of the variables. Therefore, the process variables were optimized for cell mass and lactosucrose production using ANOVA and RSM. Overall, from the results of variables optimization, it suggests that process optimization by statistical analysis has potential for application to industrial lactosucrose production.

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