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Statistical Optimization for Succinic Acid Production from *E. Coli* in a Cost-Effective Medium

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Abstract Response surface methodology (RSM) was employed for optimization of medium components and cultural parameters in cost effective cane molasses based medium for attaining high yield of succinic acid. The important factors obtained by "one-variable-at-a-time-approach" (cane molasses, corn steep liquor, sodium carbonate, and inoculum density) were further optimized by RSM. The optimum values of the parameters obtained through RSM (cane molasses 12.5%, corn steep liquor 7.5%, and sodium carbonate 25 mM) led to almost double yield of succinic acid (15.2 g/l in 36 h) as against "one-variable-at-a-time-approach" (7.1 g/l in 36 h) in 500-ml anaerobic bottles containing 300-ml cane molasses based medium. Subsequently, in 10-l bioreactor succinic acid production from *Escherichia coli* was further improved to 26.2 g/l in 30 h under conditions optimized through RSM. This fermentation-derived succinic acid will definitely help in replacing existing environmentally hazardous and cost-intensive chemical methods for the production of succinic acid.

Keywords Response surface methodology · Succinic acid · Cane molasses · Corn steep liquor · *Escherichia coli*

Introduction

Succinic acid, a member of C_4 dicarboxylic acid, is an important organic acid used in drug compounds production, food processing, and cosmetics [1–3]. Besides these, it is also used as green feedstock for manufacture of synthetic resins, biodegradable polymers, and as an intermediate chemical in the production of numerous products with large market potential, such as 1,4-butanediol, tetrahydrofuran, adipic acid, γ-butyrolactone, *n*-methyl pyrrolidinone, and 2-pyrrolidinone [4–10].

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Succinic acid is currently chemically produced by hydrolyzing petroleum products, which is associated with certain environmental hazards leading scientists to develop biological processes for its continuous production [11–14]. This is because it is a common intermediate in the metabolic pathway of several anaerobic microorganisms [15]. Efforts are being made worldwide to develop low-cost fermentation processes using renewable resources such as agricultural, dairy, and industrial waste products, so as to replace current processes using petroleum as a feedstock [16].

Any fermentation process is significantly influenced by various physical and chemical parameters. Chemically defined medium permits the determination of specific requirements for growth and product formation by systematically adding or eliminating chemical components from the formulation, with minimal complicated medium interactions [17]. Optimization through response surface methodology (RSM) is now widely used to evaluate and understand the interactions between different physiological and nutritional parameters [18–21]. This technique is an empirical modeling technique devoted to the evaluation of relations existing within a group of controlled experimental factors and observed results of one or more selected criteria [22]. It includes factorial design and regression analysis, which helps in evaluating the effective factors and building blocks to study interactions and select optimum conditions of variables for a desired response [23, 24].

Cane molasses, the by-product of sugar refinery process, containing 45–50% sugars, is the most economical source of carbohydrate for various industrial fermentations [25]. Therefore, in the present investigation, an attempt was made to optimize the cultural conditions for maximizing the production of succinic acid from *Escherichia coli* in an inexpensive cane molasses based medium using statistical approaches. Furthermore, a feasibility of large-scale production was attempted in a laboratory bioreactor. This is the first report on use of statistical methods for optimization of succinic acid production in cane molasses based medium.

Materials and Methods

Source of Strain

Strain of *E. coli* was isolated from rumen of buffalo and was identified as *E. coli* M87049 using 16S rRNA sequencing carried out at MIDILABS, USA. For growth and maintenance, the strain was grown in 500-ml sealed bottles containing 300-ml of the medium containing (g/l): glucose, 20; peptone, 10; yeast extract, 5.0; K_2HPO_4, 3.0; NaCl, 1.0; $(NH_4)_2SO_4$, 1.0; $CaCl_2{\cdot}2H_2O$, 0.2; $MgCl_2{\cdot}6H_2O$, 0.2; and Na_2CO_3, 1.0. An indicator resazurin (1.0 g/l) was added in the medium to ensure anaerobic conditions. This indicator turns colorless to pink if oxygen is present in the medium. The medium was sterilized (15 min, at 121°C) in bottles sealed with butyl rubber bungs with N_2 headspace. To the sterilized medium, a few drops of 1 N sulfuric acid was added aseptically to adjust the pH to 6.5. The N_2 headspace was replaced by CO_2, and $Na_2S{\cdot}9H_2O$ (0.02 g/l) was added to remove traces of dissolved oxygen [8, 26]. The reduced medium was inoculated with 2% (*v*/*v*) seed inoculum having OD of 0.6 at 660 nm (cell count=4.9×10^9 cfu/ml) and incubated at 39±1°C for 24 h under static conditions with intermittent gentle shaking [27].

End-Product Analysis

Concentrations of succinic acid and left over sugars were analyzed on HPLC (Shimadzu RID 10A, LC-10AD pump, CTO-10AS column oven, Tokyo, Japan) equipped with an ion

exchange column (Aminex HPX-87H, 300 mm×7.8 mm, Hercules, CA) with a column temperature of 50°C using 5 mM H_2SO_4 as a mobile phase with a flow rate of 0.6 ml min^{-1} [26–28].

Optimization by Applying RSM

The chemically defined medium optimized earlier by "one-variable-at-a-time-approach" for succinic acid production [28] was used for further optimization by applying RSM of Central Composite Design (CCD). The levels of four independent variables [cane molasses (*A*), corn steep liquor (*B*), Na_2CO_3 (*C*), and inoculum density (*D*)] chosen for this study were optimized by RSM. The statistical software package "Design Expert 6.0", Stat-Ease, Inc., Minnaepolis, USA was used to analyze the experimental design. Each factor in the design was studied at five different levels ($-\alpha$, −1, 0, +1, $+\alpha$ (Table 1). A set of 32 experiments was performed. All the variables were taken at a central-coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form is listed in Table 2. Upon completion of experiment, the succinic acid production was taken as dependent variable or response (*Y*).

Statistical Analysis and Modelling

The data obtained from RSM on succinic acid production was subjected to analysis of variance (ANOVA), appropriate to the design of the experiment. A second-order polynomial equation was then fitted to the data by multiple regression procedure resulting in an empirical model that related the response measured to the independent variables of the experiment. For a four-factor system, the model equation is—

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

where *Y* is the predicted response; β_0, intercept; β_1, β_2, β_3, β_4, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficient; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34}, interaction coefficients.

Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination (R^2). Responses of the selected variables were analyzed using three-dimensional plots. All experiments were performed in triplicates, and the data represents a mean of three.

Table 1 Levels of the four independent variables (factors) used in RSM.

Variables	Units	Coded value	Range of levels				
			$-\alpha$	−1	0	+1	$+\alpha$
Cane molasses	%	*A*	10.0	11.25	12.5	13.75	15.0
Corn steep liquor	%	*B*	5.0	6.25	7.5	8.75	10.0
Na_2CO_3	mM	*C*	20.0	22.5	25.0	27.5	30.0
Inoculum density	%	*D*	4.0	4.5	5.0	5.5	6.0

Table 2 Central composite design of the variables with succinic acid production as response.

S. number	Coded levels				Succinic acid production (g/l)	
	Cane molasses	Corn steep liquor	Na_2CO_3	Inoculom density	Observed	predicted
1	−1	+1	−1	−1	11.4	11.32
2	+1	−1	−1	+1	11.6	11.59
3	+1	+1	−1	+1	11.4	11.42
4	−1	−1	+1	−1	11.6	11.35
5	−1	+1	+1	−1	10.6	10.51
6	−1	−1	−1	+1	11.46	11.33
7	0	0	$-\alpha$	0	12.78	13.17
8	−1	−1	−1	−1	11.4	11.12
9	−1	+1	+1	+1	11.21	11.14
10	+1	−1	−1	−1	11.9	11.74
11	0	0	0	+1	15	15.16
12	+1	−1	+1	+1	13.33	13.18
13	+1	+1	−1	−1	12.84	12.52
14	+1	−1	+1	−1	11.9	11.96
15	0	0	0	0	15.07	15.16
16	0	0	0	$-\alpha$	9.1	9.54
17	0	0	0	0	15.1	15.16
18	0	0	0	0	15.14	15.16
19	+1	+1	+1	+1	11.8	11.97
20	0	0	0	0	15.11	15.16
21	0	$+\alpha$	0	0	12.24	12.39
22	−1	+1	−1	+1	10.75	10.59
23	0	0	$+\alpha$	0	14	13.94
24	−1	−1	+1	+1	12.7	12.92
25	0	$-\alpha$	0	0	13.21	13.40
26	+1	+1	+1	−1	11.8	11.70
27	$+\alpha$	0	0	0	11	11.08
28	0	0	0	0	15.08	15.16
29	$-\alpha$	0	0	0	9.37	9.63
30	0	0	0	0	15.13	15.16
31	0	0	0	$+\alpha$	10.13	10.02
32	0	0	0	0	15.11	15.16

Succinic Acid Production

Anaerobic bottles (500-ml) containing 300-ml of chemically defined medium (g/l): cane molasses (variable); corn steep liquor (variable); yeast extract, 2.5; K_2HPO_4, 3.0; NaCl, 1.0; $(NH_4)_2SO_4$, 1.0; $CaCl_2{\cdot}2H_2O$, 0.2; $MgCl_2{\cdot}6H_2O$, 0.2; and Na_2CO_3 (variable) were inoculated with bacterial inoculum ($OD_{660\ nm}$) (variable) and incubated in an incubator shaker at 39°C for 36 h and agitated at 150 rev/min. The culture was harvested by centrifugation at 10,000 rpm for 10 min at 4°C, and the supernatant was used to estimate succinic acid on high performance liquid chromatography (HPLC).

The bacterial strain was also cultivated in 10-l bioreactor (Bioflow IV, New Brunswick Scientific Inc. Co. USA) containing 7.5 l of the medium optimized by RSM. The optimized medium (pH 6.5) was sterilized at 121°C in situ for 15 min. Sugar was sterilized separately and was mixed aseptically with other components of the medium in the bioreactor. The

medium was inoculated with appropriate amount of inoculum, and fermentation was carried out at 39°C. CO_2 was sparged continuously into the medium at 0.5 volume of air per unit volume of the medium (vvm) during the fermentation run. The pH of the medium was maintained at 6.5 using 1 N $NaOH/H_2SO_4$. Samples were withdrawn at regular intervals of 6 h until 48 h and analyzed for succinic acid production. Foaming was controlled by the addition of silicon antifoam agent (obtained from Central Drug House, India; 0.1 ml of 50% *v*/*v* prepared in distilled water). Fermentation parameters such as temperature, pH, CO_2 were continuously monitored using microprocessor-controlled probes.

Validation of the Model

The model was validated by considering different permutation and combination of medium components, selected within the model range so as to fit the second-order polynomial equation. Six sets of experiments were generated and carried out.

Results

On the basis of "one-variable-at-a-time-approach", four factors [10% (*v*/*v*) cane molasses, 10% (*v*/*v*) corn steep liquor, 20 mM sodium carbonate, and 4% (*v*/*v*) inoculum density] had maximum influence on succinic acid production. The result of CCD experiments for studying the effect of four independent variables are presented along with the predicted and observed responses in Table 2. Regression analysis of the experimental data obtained after ANOVA resulted in the following second-order polynomial equation (in terms of coded factors).

$$Y = +15.16 + 0.36*A - 0.25*B + 0.19*C + 0.12*D - 1.20*A^2 - 0.57*B^2 - 0.40*C^2 - 1.34*D^2 + 0.14*A*B - 004*A*C - 0.089*A*D - 0.26*B*C - 0.24*B*D + 0.34*C*D$$

where Y is the succinic acid produced as a function of cane molasses (A), corn steep liquor (B), sodium carbonate (C), and inoculum density (D).

The coefficient of determination (R^2) was calculated as 0.98 for succinic acid production (Table 3), indicating that the statistical model can explain 98% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger

Table 3 ANOVA for response surface model.

Model terms	Values
Standard deviation	0.29
Mean	12.53
R^2	0.98
Adj R^2	0.97
Pred R^2	0.94
Adeq precision	27.85
Model F-value	86.44
Lack of fit F-Value	1.25
PRESS	6.14
Coefficient of variance	2.35

the model and the better it predicts the response [30]. The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. Adequate precision measures signal to noise ratio. An adequate precision of 27.85 for succinic acid production was recorded. The predicted R^2 of 0.94 is in reasonable agreement with the adjusted R^2 of 0.97. This indicated a good agreement between the experimental and predicted values for succinic acid production.

The Model *F*-value of 86.44 for succinic acid production and values of PROB > *F* (<0.05) demonstrated a high significance for the regression model. For succinic acid production, *A*, *B*, *C*, A^2, B^2, C^2, D^2, *BC*, *BD*, *CD* were significant model terms. The "Lack of Fit *F*-value" of 1.25 implied that lack of fit is insignificant relative to pure error, which indicated that the model was suitable to represent the experimental data. Also, the PROB value was equal to 0.0001 and the model was significant. The predicted sum of squares (PRESS), which is a measure of how particular model fits each point in the design, was 6.14. The model was found to be significant for production within the range of variables employed.

The coded model was used to generate three-dimensional response surface curves and contour presentations to understand the interaction of medium components and the optimum concentration of each component required for maximum succinic acid production. ANOVA showed that factor *D* (inoculum density) was insignificant and *A* (cane molasses concentration), *B* (corn steep liquor concentration), and *C* (sodium carbonate concentration) were significant. Therefore, three response surfaces were obtained by considering all the possible combinations. Figure 1 depicts three-dimensional diagram and a contour plot of calculated response surface from the interaction between cane molasses and corn steep liquor while keeping all other variables at their 'O' level. A linear increase in succinic acid production was observed when cane molasses concentration was increased up to 12.5% (*v*/*v*), and thereafter, it declined sharply. Cane molasses is a by-product of the sugar industry; therefore, using it as a sole source of carbon makes the medium inexpensive. In addition to large amounts of sugars [ca 50% (sucrose 33.5%, invert sugar 21.2%)], molasses contain a small amount of nitrogenous substances (0.4–1.5%), vitamins such as thiamine, riboflavin,

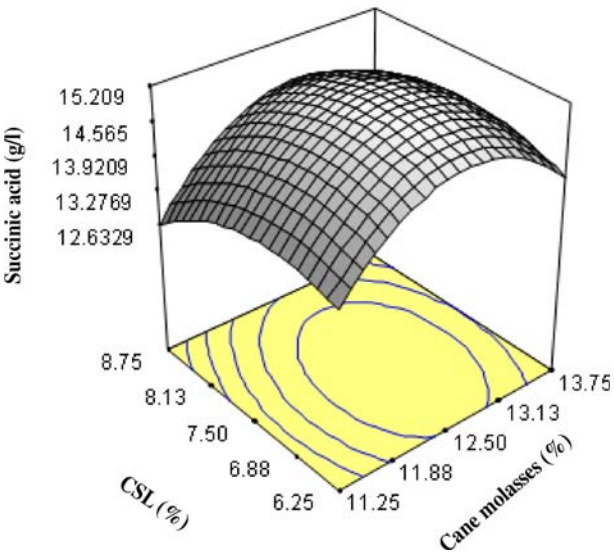

Fig. 1 Response surface curve of succinic acid production showing interaction between cane molasses and CSL at constant sodium carbonate (25 mM) concentration

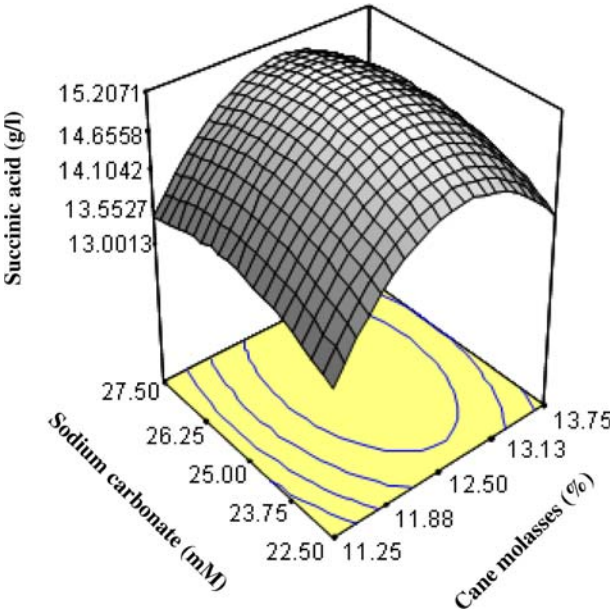

Fig. 2 Response surface curve of succinic acid production showing interaction between cane molasses and sodium carbonate at constant CSL concentration

pyridoxine, folic acid, biotin, pentothenic acid, and trace elements [31]. These compounds are also essential for the production of succinic acid. Increasing the cane molasses concentration beyond 12.5% (*v*/*v*) led to decline in succinic acid production. This might be because succinic acid production was subjected to catabolite repression by cane molasses (>12.5%).

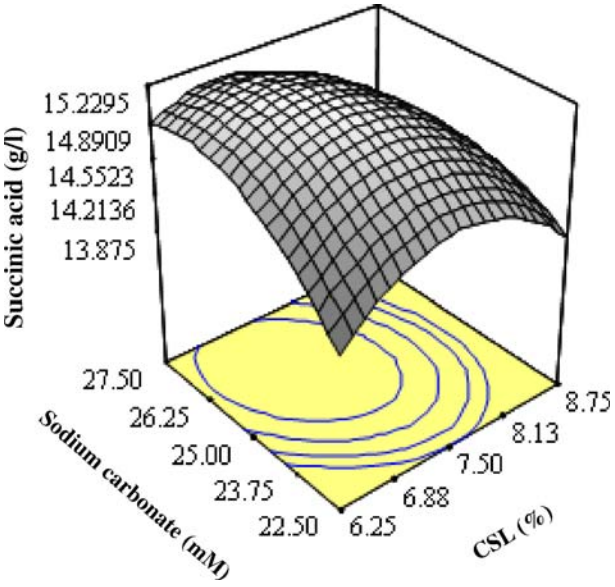

Fig. 3 Response surface curve of succinic acid production showing interaction between CSL and sodium carbonate at constant cane molasses concentration

Table 4 Validation of CCD using different levels of cane molasses, corn step liquor, and sodium carbonate at constant inoculum density.

S. number	Cane molasses (%)	Corn steep liquor (%)	Sodium carbonate (mM)	Inoculum Density (%)	Succinic acid yield (g/l)	
					Predicted	Observed
1	12.5	7.5	25	5	15.2	15.14
2	11.25	8.25	25	5	14.03	14.2
3	11	7.5	27	5	13	12.7
4	13.75	7.5	23	5	13.2	12.8
5	12.5	8.5	22.5	5	13	13.1
6	12.5	8.5	27	5	12.8	12.5

When the level of corn steep liquor concentration was decreased from 10 to 7.5% (*v*/*v*), an increase in the succinic acid production was recorded. At the 'O' level of corn steep liquor, the response between sodium carbonate and cane molasses indicated that a higher concentration of sodium carbonate (25 mM) was desirable with 12.5% (*v*/*v*) cane molasses (Fig. 2). The response surface was mainly used to find out the optima of the variables for which the response was maximized. An interaction between the remaining two parameters (sodium carbonate and corn steep liquor) at constant cane molasses concentration and inoculum density of 5% resulted in maximum succinic acid yield of 15.2 g/l in 36 h at 25 mM Na_2CO_3 and 7.5% (*v*/*v*) CSL (Fig. 3). From Figs. 1, 2, and 3 cane molasses (12.5% *v*/*v*), corn steep liquor (7.5% *v*/*v*), sodium carbonate (25 mM) and inoculum density (5%) were adequate for attaining a maximum succinic acid titre (15.2 g/l).

Validation of the model was carried out in 300-ml medium contained in 500-ml anaerobic bottles under conditions predicted by the model. The results of random set of six experiments (Table 4) clearly showed that experimental values were found to be very close to the predicted values, and hence, the model was successfully validated. Validation of the statistical model and regression equation was performed by taking *A* (12.5% *v*/*v*), *B* (7.5% *v*/*v*), and *C* (25 mM) in the experiment. The predicted response for succinic acid production was 15.2 g/l, while the actual (experimental) response was 15.14 g/l, thus proving the validity.

Succinic Acid Production in a 10-l Bioreactor

Succinic acid production was also carried out in a 10-l bioreactor, using conditions obtained as optimum through RSM. The medium was inoculated with 5% of inoculum obtained as

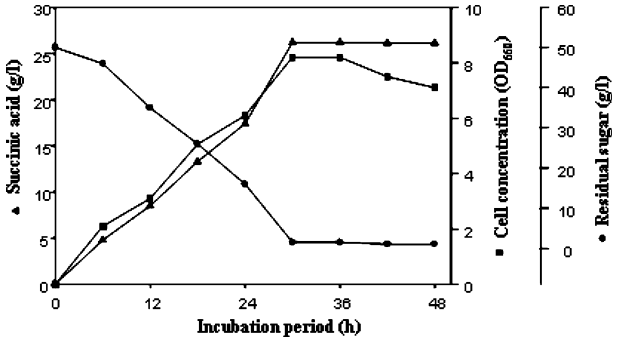

Fig. 4 Growth and succinic acid production by *E. coli* in a 10-l bioreactor under conditions optimized by RSM. *filled triangles*: succinic acid (g/l); *filled squares*: cell concentration (OD_{660}); *filled circles*: residual sugar (g/l)

optimum through RSM. During the course of the experiment, it was found that 200 rpm is the best for maximum succinic acid production in the bioreactor (data not provided). Therefore, fermentor was run at 200 rpm. During the run, the production was initiated at 6 h and reached a peak (26.2 g/l) within 30 h as compared to that of 36 h in anaerobic bottles. Carbohydrate source was almost fully utilized in 30 h, and after that, no increase in the production of acid was observed (Fig. 4). This enhancement in succinic acid production could be attributed to maintenance of level of CO_2 available and pH of the medium to 6.5, which otherwise decreases due to the formation of acids. Improvement in product yield is expected in the fermentor compared to that in bottles because of better control of process parameters in the former [32]. Fermentation studies indicated that succinic acid production was regulated by the level of available CO_2 and culture pH [33]. Vander Werf [34] reported that the production of succinate as a fermentation product requires CO_2 fixation. Samuelov et al. [26, 35] reported that CO_2 concentration regulates the level of key enzymes of the PEP carboxykinase pathway in *A. succiniciproducens*. High levels of CO_2 stimulated PEP carboxykinase levels, whereas alcohol dehydrogenase and lactate dehydrogenases were significantly decreased. Consequently, CO_2 functions as an electron acceptor and alters the flux of PEP, which metabolizes to pyruvate and lactate/ethanol at low CO_2 levels but makes succinate at high CO_2 concentration [7]. pH is also an important parameter that effects both growth and acid production. Samuelov et al. [26] reported that in an anaerobic bacterium, *A. succiniciproducens*, maximum succinic acid was produced at pH 6.2. On increasing the pH to 7.2, the succinic acid yield was decreased [36].

Conclusion

To formulate cost effective and economical medium, industrial by-products like molasses and CSL are being used for the production of microbial products. RSM helped in minimizing the cost of succinic acid production in anaerobic bottles by enhancing the succinic acid titres. The organism secreted 26.2 g/l of succinic acid in 10-l bioreactor, suggesting a good scope for scale up of succinic acid production. The fermentation time was further reduced from 36 to 30 h in the bioreactor, and thus, making the process more economical. This fermentation-derived succinic acid will enable us to replace existing environmentally hazardous and cost-intensive chemical methods for the production of succinic acid.

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