Optimization of Process Variables for the Extractive Fermentation of 2,3-Butanediol by *Klebsiella oxytoca* in Aqueous Two-phase System Using Response Surface Methodology

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Aqueous two-phase (ATP) extractive fermentation is a novel technique which can overcome some of the limitations of the conventional fermentation and yield high productivities. Temperature, pH and agitation, are some of the key process parameters which play an important role in fermentation. Optimizing of the parameters for the ATP fermentation for 2,3-butanediol production was studied. Statistically designed experiments using response surface methodology was used to get more information about interactions between the parameters. A 2^3 full factorial central composite design followed by multi-stage Monte-Carlo optimization technique was employed for experimental design and analysis of the results. The optimum process conditions for the enhanced production of 2,3-butanediol in ATP system were as follows: temperature T = 31.5 °C, pH 6.7 and agitation, n = 172 rpm. Maximum average productivity of 2,3-butanediol was achieved (0.74 kg m^{-3} h^{-1}) at the optimum process conditions.

Key words:
Klebsiella oxytoca, 2,3-butanediol, aqueous two-phase system, statistical optimization, extractive fermentation

Introduction

The knowledge of limitedness of conventional fossil fuels, oil and natural gas has lead to the search for renewable sources of energy. 2,3-butanediol is a valuable chemical feedstock with heat value of 27200 kJ · kg^{-1}, which is comparable to ethanol and methanol for use as liquid fuel.[1] Its high octane rating enables its use as aviation fuel.

Conventional fermentative production of 2,3-butanediol by *Klebsiella oxytoca* has the disadvantage of product reutilization by the organism. Alternatives to overcome this problem have met with limited success. Aqueous two-phase (ATP) system has been shown to provide protective environment for biological system.[2] An effort has been made in this study to use this ATP system for the extractive fermentation of 2,3-butanediol using PEG and dextran as phase forming polymers.

Optimization of process parameters is recognized to be an essential aspect of successful fermentation.[3,4] The strong dependence of enzymatic activity and cellular maintenance requirements upon temperature[5] makes it an important quantity. Temperature exerts an important regulatory influence on the rate of metabolism. The bacterial activity rapidly reduces at temperatures below the optimum temperature.[6] The primary factor affecting the substrate utilization rate in natural system is pH. The internal environment of the living cell is believed to be approximately neutral. Most of the organism cannot tolerate pH below 4.0 or above 9.0.[7] At low (4.0) or high (9.0) pH values, acid and base can penetrate into the cells more easily because they tend to exist in undissociated form under these conditions and electrostatic force can not prevent them from entering the cells.[7,8,9] The permeated substance can upset the internal pH balance since the bacterial activity decreases as it deviates from neutral conditions. 2,3-butanediol production has been shown to be most efficient under aerobic conditions (especially at high substrate concentration) during the fermentation of five-carbon sugars. For every microbe a “critical “ dissolved oxygen level (c_{crit}) exists above which the organism’s respiration rate is independent of the oxygen concentration. The critical oxygen tension reported for *K. pneumoniae* grown on batch culture is in the range 8–15 mm Hg (760 mm Hg is equivalent to (101 293 N m^{-2}).[10] In continuous culture a slightly lower range of 2–10

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mm Hg was observed.[11] In the case of shake flask cultures the oxygen supply is indirectly a factor of agitation.

This work primarily aimed at optimizing the process variables, temperature, pH and agitation for the extractive fermentation of 2,3-butanediol in ATP system using statistical optimization technique for multivariable effect. The classical method of optimization involves varying the level of one parameter at a time over a certain range while holding the rest of the test variables constant. This single-factor-at-a-time strategy is generally time consuming and requires a large number of experiments to be carried out. Since, the effects of interactions between the variables are not considered, this method is also inadequate for full understanding of the response surface. Hence, a 2^3 full-factorial central composite design using response surface methodology,[12,13,14] was employed in this investigation.

Materials and methods

Microorganism

A 2,3-butanediol producing strain, *Klebsiella oxytoca* NRRL B199/ATCC 8724, maintained on nutrient agar slant at 4 °C, was used in this study.

Culture medium

The glucose/mineral salts medium[15,16] had the following composition ($kg \cdot m^{-3}$): Glucose 50.0, EDTA (disodium salts) 0.51, $(NH_4)_2SO_4$ 7.2, $(NH_4)_2HPO_4$ 6.0, KOH 0.45, $MgSO_4 \cdot 7H_2O$ 0.3; $CaCl_2 \cdot 2H_2O$ 0.09, $FeSO_4 \cdot 7H_2O$ 0.0225, $ZnSO_4 \cdot 7H_2O$ 0.0075, $MnSO_4 \cdot 4H_2O$ 0.00375. For Aqueous two-phase fermentation, in addition to the above components, w = 8 % Dextran 40 000 and 9.2 % PEG 6000, which was observed to be the optimum phase composition,[17] were used.

Experimental methods

For all cases, fermentations were carried out in 250 ml Earlenmeyer flask with 100 ml working volume. The flasks were inoculated with 18 hour, φ = 2.5 % (≅ 0.0625 kg m^{-3}) inoculum for fermentation in ATP system[17] and kept in a temperature and agitation controlled rotary shaker (Yorco Instruments, India). The temperature, initial pH and agitation were varied on the basis of statistical experimental design. The range and the levels of the process variables under study are given in Table 1. The samples were drawn at regular intervals and analyzed for 2,3-butanediol, cell concentration and glucose concentration.

Table 1 – *Experimental range and levels of the independent variables (temperature, pH and agitation)*

Dependent variable	$-\alpha$	–1	0	+1	$+\alpha$
Temperature, °C	13.18	20	30	40	46.82
pH	3.636	5	7	9	10.364
Agitation, rpm	129.546	150	180	210	230.45

The analytical methods

2,3-butanediol was analyzed in a gas chromatograph (NUCON Engineers, India) equipped with a flame ionization detector, using a Poropak Q column under isothermal conditions (oven temperature 190 °C, injector and detector temperatures 300 °C). Nitrogen gas (flow rate 35 ml min^{-1}) was used as carrier gas.

Residual glucose was analyzed by Dinitrosalicylic method as described by *Miller*.[18]

Dry cell mass concentration was estimated by measuring the optical density of the sample at 600 nm in a spectrophotometer (UV-visible spectrophotometer, Shimadzu Corporation, Japan) and by its correlation with the dry cell weight, obtained gravimetrically.

Statistical optimization technique

A full-factorial design,[12,14] which includes all possible combinations for each of the factors, is a powerful tool for understanding complex processes. The full-factorial central composite design consists of (a) a complete 2^k factorial design, where k (= 3) is the number of test variables, (b) n_0 center points ($n_0 > 1$) and (c) two axial points on the axis of each design variables at a distance of α ($\alpha = 2^k/4$, = 1.6828 for $k = 3$) from the design center.[13] Hence, the total number of design points is $N = 2^k + 2k + n_0$ and these data are fitted in a second order polynomial model. An orthogonal 2^3 full-factorial central composite design[19] with six replicates ($n_0 = 6$) at the central point, all in duplicates, resulting in total of 20 experiments, were used to optimize the chosen key variables for maximizing 2,3-butanediol average productivity. The levels of parameters that have effect on average productivity are given in Table 1. The variables are coded according to the equation 1.

$$X_i = \frac{x_i - x_0}{\Delta x_i} \qquad (1)$$

Where X_i, x_i and x_0 are the coded value, uncoded value and the value at the center point respectively of the ith test variable and Δx_i is the step change value. The full experimental design in coded and uncoded form is given in Table 2. The

Table 2 – *Coded and uncoded full factorial central composite design.*

Experiment number	Temperature		pH		Agitation	
	code	value/°C	code	value	code	value/rpm
1	–1	20	–1	5	–1	150
2	+1	40	–1	5	–1	150
3	–1	20	+1	9	–1	150
4	+1	40	+1	9	–1	150
5	–1	20	–1	5	+1	210
6	+1	40	–1	5	+1	210
7	–1	20	+1	9	+1	210
8	+1	40	+1	9	+1	210
9	–1.682	13.18	0	7	0	180
10	+1.682	46.82	0	7	0	180
11	0	30	–1.682	3.636	0	180
12	0	30	+1.682	10.364	0	180
13	0	30	0	7	–1.682	129.55
14	0	30	0	7	+1.682	230.45
16	0	30	0	7	0	180
15	0	30	0	7	0	180
17	0	30	0	7	0	180
18	0	30	0	7	0	180
19	0	30	0	7	0	180
20	0	30	0	7	0	180

behavior of this system was explained by the following second-degree polynomial equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (2)$$

Where β_0, β_i (i = 1, 2, 3), β_{ii} (i = 1, 2, 3) and β_{ij} (i = 1, 2, 3; j = 1, 2, 3 and $i \neq j$) are constants for the offset term, linear effects, quadratic effects and interactions effects, respectively. X_i (i = 1, 2, 3) and X_j (j = 1, 2, 3) ($i \neq j$) are the process parameters and Y is the response variable. A statistical program package, Design Expert (Start-Ease Inc., Minneapolis, MN) was used for regression analysis of the data obtained and to estimate the coefficient of multivariate equation. The graphical representation of these equations are called response surfaces, which was used to describe the individual and cumulative effects of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response.[13] The correlation measures for the estimation of the regression equation are the multiple correlation coefficient R and the determination coefficient R^2. ANOVA (Analysis of Variance) was used to test the significance and adequacy of the model.

The F value (Fisher's variance ratio, Sr^2/Se^2) was calculated from ANOVA. F values much higher than unity indicate that the factors explain adequately the variation in the data about its mean and effects of estimated factors are true.

The Student-t-distribution and the corresponding probability values (P values) indicate the significance of each of the coefficient, which in turn governs the patterns of interactions between the variables. The smaller the value of P, the more significant is the corresponding coefficient.[13]

Results and discussion

Since the two-phase forming polymers, PEG and dextran are also organic compounds, preliminary experiments were carried out to see if they could be utilized by *K.oxytoca* as glucose substitutes. Absence of cell growth even up to 72 hours in the production medium with either PEG or dextran as the carbon source proved that they were not utilized by the organism. Similar observations were made by several investigators with various organisms.[20]

Optimization of temperature, pH and agitation using response surface methodology

Effect of physical parameters like temperature, pH and agitation on 2,3-butanediol fermentation were studied by many workers in different situations.[11,21,22] However, such studies have not been attempted for extractive fermentation in ATP system. Since, these three variables have combined role on the fermentation process, it was impractical to study their individual effects keeping other variables at constant levels.

Response surface methodology is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental facts and observed values. It requires a prior knowledge of the process to achieve a statistical model.[6,12,19,23,24,25] The average 2,3-butanediol productivity at each fermentation run is summarized and listed in Table 3. The experimental data were fitted to a quadratic model involving all the process parameters and their interaction terms using the same experimental design software. The regression equation obtained for average maximum productivity of 2,3-butanediol as a function of the temperature, pH and agitation is given as follows (equation 3).

Table 3 – *Experimental and theoretical predicted values for average maximum productivity of 2,3-butanediol fermentation*

Experiment number	Experimental value	Predicted value
1	0.42	0.4510
2	0.55	0.5442
3	0.42	0.4629
4	0.52	0.5311
5	0.43	0.4621
6	0.55	0.5504
7	0.35	0.3990
8	0.45	0.4622
9	0.35	0.2787
10	0.40	0.4102
11	0.60	0.5865
12	0.57	0.5224
13	0.064	0.6137
14	0.60	0.5651
15	0.70	0.7151
16	0.72	0.7151
17	0.70	0.7151
18	0.73	0.7151
19	0.72	0.7151
20	0.71	0.7151

$$\begin{gathered} Y = 0.715077 + 0.039105\,X_1 - \\ - 0.019072\,X_2 - 0.014448\,X_3 - \\ - 0.131008\,X_1^2 - 0.056780\,X_2^2 - \\ - 0.044426\,X_3^2 - 0.006250\,X_1X_2 - \\ - 0.00125\,X_1X_3 - 0.018750\,X_2X_3 \end{gathered} \qquad (3)$$

Where X_1, X_2 and X_3 represent coded values of temperature, pH and agitation respectively, and Y is the response variable (average maximum productivity of 2,3-butanediol). By solving equation 3 analytically the optimum coded value for temperature was found to be 0.1563 and the corresponding uncoded value was calculated to be 31.5 °C for fermentation in ATP system. The optimum temperature for the bacterial production of 2,3-butanediol is generally reported to be in the range 30–37 °C. Optimum temperature for diol production with glucose and sucrose as substrates were observed to be 34 °C[21] and 35–37 °C,[15] respectively, in conventional system.

The coded and uncoded value for pH was obtained as –0.1545 and 6.7 respectively in the present study solving the model equation 3. *Jansen* et al.[22] investigated the effect of pH on both growth and butanediol production over a pH range of 4.8 to 5.6 in batch fermentation taking xylose as the substrate, using *K.oxytoca* in conventional system. The maximum specific growth rate occurred at pH 5.2. The butanediol yield was not strongly affected over the range of pH 4.4 to 5.8, but appeared to reach a peak value between pH 5.2 and 5.6. Above pH 6.0, the yield decreased. *Harrison* and *Pirt*[11] used *K.aerogenes* in chemostat culture in conventional system with pH control and demonstrated that at pH 6.2 butanediol production was favored over acetate production.

By solving the objective function, the optimum coded value for agitation was found to be –0.1327 and the corresponding uncoded value was calculated to be 176 rpm. This value was little higher then conventional fermentation (160 rpm was found to be optimum in shake flask study for butanediol fermentation using *K.oxytoca* as the microorganism by *Sivakumar*[4]). In ATP extractive fermentation the cells remain bonded in the dextran rich bottom phase and dextran, being a long chain polymer, imparts viscosity to the medium that causes resistance to the transport process, particularly gas transport.

Sablayrolles and *Goma*[16] reported that 2,3-butanediol production by *Aerobacter aerogenes* NRRL

B 199 grown on glucose requires an optimal rate of aeration for obtaining 2,3-butanediol. In the absence of air, *A. aerogenes* NRRL B199 growth and production are weak. Agitation-aeration is necessary for production of the biomass, but an excess of oxygen proves to be toxic with regard to metabolite production. Oxygen is limiting substrate with regard to growth and an inhibitor with regard to the specific metabolite productivity.

The analysis of variance (ANOVA) of the quadratic regression model demonstrates that the model is highly significant (Table 4), as is evident from Fisher F test ($F_{\text{model}} = 20.99$) and a very low failure probability ($P = 0.0001$). Moreover, the computed F value ($F_{0.01(9,10)} = 20.99$) was much grater than the tabular F value ($F_{0.01(9,10)} = 5.26$) at the 1 % level, indicating that the treatment differences are highly significant. The correlation measures for the estimation of the regression equation 3 are the multiple correlation coefficient (R) and determination coefficient (R^2). The closer the value of R to 1, the better is the correlation between the observed value and the predicted values. Here the value of R (= 0.9745) indicates a high degree of correlation between the observed and the predicted values. The value of the determination coefficient (R^2 = 0.9497) being a measure of goodness of fit to the model, indicates that only about 5 % of the total variations are not explained by the model. The adjusted R^2 value (0.9045) is also very high, making the model very significant. The coefficient of variation (CV) indicates the degree of precision with which the treatment is compared. Usually, the higher the value of CV, the lower is the reliability of the experiment. Here the low value of CV (=7.38 %) indicates a greater reliability of the experiments performed. The parameter estimates and the corresponding P values (Table 5) suggest that, among the test variables, linear and quadratic effect of temperature and pH are highly significant. However, the quadratic effect of agitation was more pronounced than linear effect. These observations can be interpreted as a consequence of proportional relationship between the variables and 2,3-butanediol fermentations. The mutual effect of pH and agitation was found to be most significant and other interactions were negligible.

Table 5 – *Least square fit and the parameter estimation*

Variables	Parameter estimate	Standard error	t Value	Probability > $\|t\|$
Intercept	0.715077	0.016737	42.72	—
X_1	0.039105	0.011104	3.522	0.0055
X_2	–0.019070	0.011104	–1.717	0.1167
X_3	–0.014448	0.011105	–1.301	0.2224
$X_1 X_1$	–0.131008	0.010808	–12.12	0.0001
$X_2 X_2$	–0.056780	0.010808	–5.253	0.0004
$X_3 X_3$	–0.044426	0.010811	–4.109	0.0021
$X_1 X_2$	–0.006250	0.014509	–0.4308	0.6758
$X_1 X_3$	–0.001250	0.014509	–0.0862	0.9330
$X_2 X_3$	–0.018750	0.014509	–1.292	0.2253

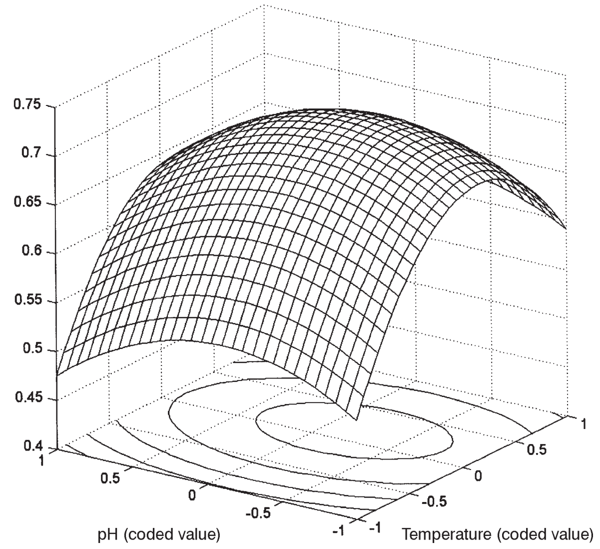

Fig. 1 – *Response surface plot for temperature vs. pH (3D diagram).*

This data analysis also substantiates the inference that can be drawn from the contour plots (Fig. 1 to 3, in 3D form). The interactions amongst temperature, pH and agitation, are quite prominent from the elliptical nature of the respective contour plots. The figures also suggest the optimum range of the process variables (temperature, pH and agitation). The factor effect function plot (Fig. 4) was used to

Table 4 – *Analysis of variance (ANOVA)*

Some of variations	Some of squares	Degree of freedom	Mean square	F value	Probability > F
Model	0.318214	9	0.035357	20.99	0.0001
Error	0.016841	10	0.001684		
Corrected total	0.335055	19			

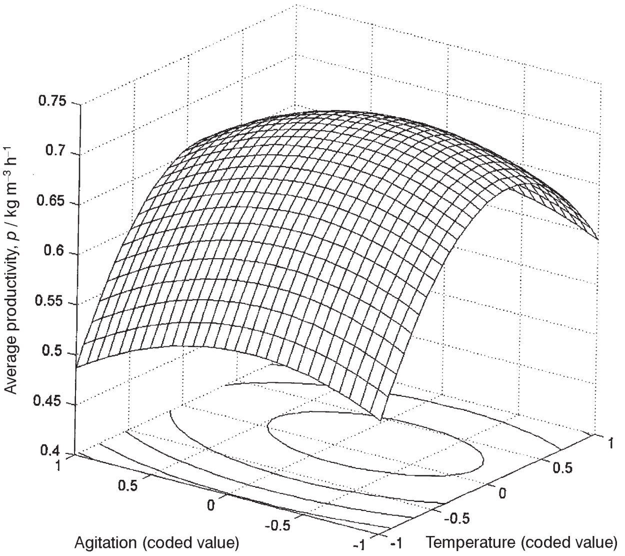

Fig. 2 – *Response surface plot for temperature vs. agitation (3D diagram)*

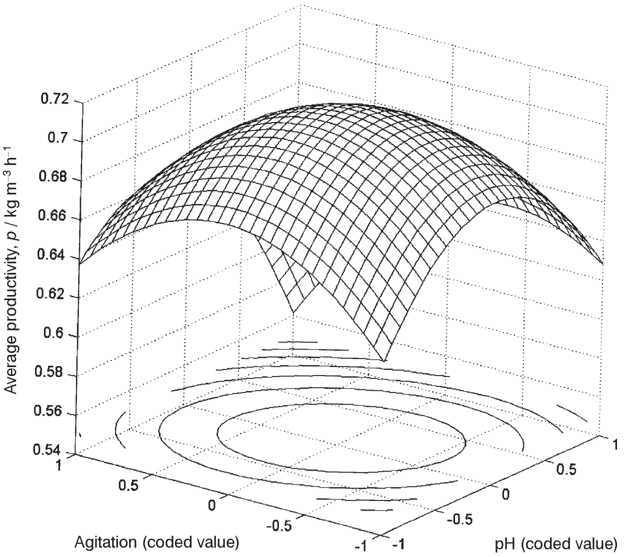

Fig. 3 – *Response surface plot for pH vs. agitation (3D diagram)*

assess the effect of each factor graphically. The effect of function of a certain factor is a function that describes how the response moves as the level of the factor changes, when the other factors are fixed at their optimum levels. From the trace plot it can be observed that each of the three variables used in the present study has its individual effect on 2,3-butanediol productivity by *K.oxytoca* in ATP system. This is also to be noted from the Figure 4 that over the range of temperature (20 °C to 40 °C) the average productivity changed in a wide range, which was not the case for pH and agitation. This clearly indicates that keeping pH and agitation at the optimum level a change in temperature affects the process more severely then done otherwise. The optimum values were found by solving the regression equation analytically. The highest 2,3-butanediol productivity that can be achieved, according to the model prediction under the optimal experimental conditions, is 0.72 kg m^{-3} h^{-1}. The experimental results indicated a productivity of about 0.74 kg m^{-3} h^{-1} under the optimal process conditions. This confirms the closeness of the model to the experimental results.

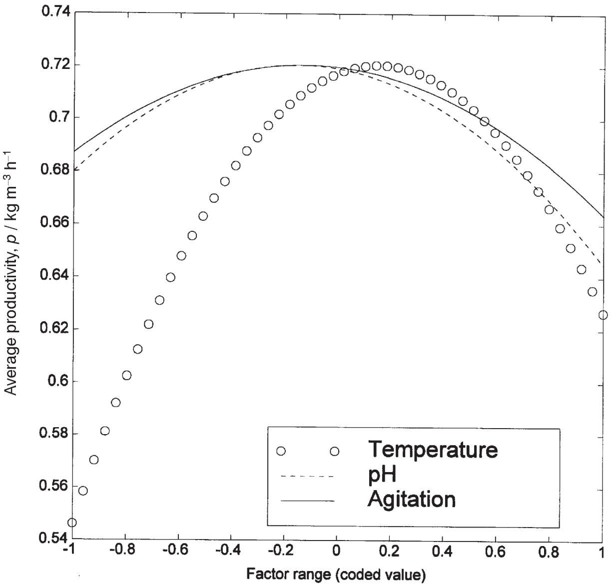

Fig. 4 – *Factor plot representing the individual variable effect on average productivity of 2,3-butanediol*

Conclusions

Fermentation of 2,3-butanediol using ATP system was successful and it was found that the organism could not utilize PEG or dextran as substitute of carbon source. The response surface methodology was adopted to optimize the process parameters like temperature, pH and agitation for the extractive fermentation using ATP system. The statistical analyses and the closeness of the experimental results and model predictions highlight the reliability of the regression model. In the experimental range there is no significant influence of initial pH and the agitation on the average productivity.

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