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Full Length Research Paper

Enhanced biomass production study on probiotic *Bacillus subtilis* SK09 by medium optimization using response surface methodology

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The culture conditions of lactose fermenting, spore forming probiotic *Bacillus subtilis* SK09 isolated from dairy effluent were optimized by response surface methodology to maximize the biomass production. The student's t-test of the Plackett-Burman screening design revealed that the effects of pH, ammonium citrate and peptone were more significant and these variables were further optimized using central composite design. The various effects of the variables were studied using Fisher's F-test for analysis of variance and a second order polynomial model was developed which fitted well with high statistical reliability and significance. The optimal value of significant variables was found to be pH (6.72), ammonium citrate (0.164%) and peptone (0.85%). At these optimized conditions, the maximum biomass yield was estimated to be 10.051×10^9 CFU/ml. By employing this statistical design, enhanced yield of probiotic biomass *B. subtilis* SK09 was achieved using cost-effective medium.

Key words: Probiotics, response surface methodology, central composite design, *Bacillus subtilis* SK09, lactose intolerance disorder.

INTRODUCTION

Probiotic organisms find their potential use in food and pharmaceutical industry. They have recently caught rapt attention of medical and scientific researchers as they form the part of gut micro flora which beneficially affect the host by improving the balance of its intestinal bacterial ecosystem (Audisio et al., 2001). Some common probiotics include various species of the *Lactobacillus* sp. and *Bifidobacterium* sp. which are used as live microbial feed and as pharmaceutical supplements to promote good health of mankind. The growth yield of probiotic is affected by fermentation conditions such as pH, temperature, medium formulations and others. As probiotic organisms are fastidious with respect to nutrient requirements, a rich medium is required for good growth (Rogosa et al., 1961; Aasen et al., 2000). Proper design of the

media does affect the performance of microorganisms in optimizing the biomass production (Abdul et al., 2006). The main objective of media optimization is to produce maximum yield of product or biomass per gram of substrate used (Soo et al., 2004). Statistical experimental designs minimize the error in determining the effect of parameters and also enhances finding out the optimal conditions by establishing the relationship between factors and predicted responses (Duta et al., 2006). Plackett-Burman design can be used to find the significant variables in a system and allow them to be ranked in order of importance and to decide which one is to be investigated further so as to determine the optimum values (Liu et al., 2003; Chuan et al., 2003). Response surface methods (RSM) consist of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables, which can be used to yield most of the information by a minimum number of experiments (He et al., 2009).

The experimental strain of probiotic *Bacillus subtilis* SK09 was isolated from dairy effluent of primary clarifier

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Abbreviations: RSM, Response surface methods; CCD, complete central composite design.

Table 1. Plackett Burman design for evaluation of medium components for probiotic biomass production.

Exp. No.	A	B	C	D	E	F	G	H	Biomass ($\times 10^9$ CFU ml ⁻¹)
1	-1 (0.2)	1 (2.0)	-1 (0.1)	-1 (30)	-1 (0.1)	1 (0.2)	1 (0.5)	-1 (5.5)	1.452
2	1 (2.0)	1 (2.0)	-1 (0.1)	1 (37)	1 (1.0)	-1 (0.002)	1 (0.5)	-1 (5.5)	1.425
3	1 (2.0)	1 (2.0)	-1 (0.1)	1 (37)	-1 (0.1)	-1 (0.002)	-1 (0.005)	1 (6.5)	0.324
4	1 (2.0)	1 (2.0)	1 (1.0)	-1 (30)	1 (1.0)	1 (0.2)	-1 (0.005)	-1 (5.5)	1.305
5	1 (2.0)	-1 (0.2)	-1 (0.1)	-1 (30)	1 (1.0)	1 (0.2)	1 (0.5)	1 (6.5)	1.422
6	-1 (0.2)	-1 (0.2)	1 (1.0)	1 (37)	1 (1.0)	-1 (0.002)	1 (0.5)	-1 (5.5)	1.435
7	1 (2.0)	-1 (0.2)	1 (1.0)	1 (37)	-1 (0.1)	1 (0.2)	-1 (0.005)	-1 (5.5)	1.458
8	-1 (0.2)	1 (2.0)	1 (1.0)	1 (37)	-1 (0.1)	1 (0.2)	1 (0.5)	1 (6.5)	1.471
9	-1 (0.2)	1 (2.0)	1 (1.0)	-1 (30)	1 (1.0)	-1 (0.002)	-1 (0.005)	1 (6.5)	1.128
10	-1 (0.2)	-1 (0.2)	-1 (0.1)	1 (37)	1 (1.0)	1 (0.2)	-1 (0.005)	1 (6.5)	1.176
11	-1 (0.2)	-1 (0.2)	-1 (0.1)	-1 (30)	-1 (0.1)	-1 (0.002)	-1 (0.005)	-1 (5.5)	1.264
12	1 (2.0)	-1 (0.2)	1.0 (1.0)	-1 (30)	-1 (0.1)	-1 (0.002)	1 (0.5)	1 (6.5)	1.447

and characterized. This isolate was proven for its ability to ferment lactose as well as sporulate under stress environment (Sreekumar and Soundarajan, 2010 a, b). The biomass of this novel strain could be well suited as an oral supplement for lactose intolerance disorder therapy. Hence, the present study is aimed at optimizing culture conditions for enhanced biomass production of probiotic *B. subtilis* SK09 strain. A total of 20 experimental runs were carried out, according to a three level fractional factorial design and the effect of each parameter on the biomass concentration was statistically analyzed and optimized by using response surface plots.

MATERIALS AND METHODS

Microorganism

A probiotic microbial isolate of *B. subtilis* SK09 was used for the present investigation. The strain was selectively isolated from the dairy effluent of primary clarifier tank, Aavin Dairy farm, Chennai, India. It was grown at 37°C for 48 h and maintained on nutrient agar (M001, HiMedia, India) slants at 4°C and was sub-cultured at four weeks intervals.

Culture propagation

The inoculum was prepared by transferring a loopful of slant culture to a 100 ml Erlenmeyer flask containing 20 ml sterile nutrient medium at pH 7.0; it was incubated statically at 37°C for 20 h. For the experimental studies, 0.5% of inoculum were transferred to 1000 ml flasks containing 200 ml of sterile medium of experimental compositions (Table 1) and incubated at 37°C and 160 rpm.

Experimental design for media screening

As per Plackett-Burman design, the factors such as lactose (A), peptone (B), glucose (C), temperature (D), ammonium citrate (E), beef extract (F), sodium acetate (G) and pH (H) were considered for screening purpose. Potassium dihydrogen phosphate (I), sodium chloride (J) and sodium sulphate (K) were also prepared at the

same levels as unassigned variables. The Plackett-Burman design in 12 runs was used (Table 1) for the experiment containing 11 factors and the biomass yield in each run was determined by taking samples aseptically after 24 h and counted on a colony counter for the amount of viable bacterial cells (CFU) in 1 ml of suspension.

In order to determine the optimum level of each key independent variable, a complete central composite design (CCD) had been adopted, which allows the estimation of a full quadratic model for each response (Adinarayana and Ilaiah, 2002; Beg et al., 2002; Kristo et al., 2003; Chang et al., 2006). The CCD in 20 runs was used (Table 2) for the experiment containing 3 factors at 5 levels each (Raji et al., 2009). The mathematical relationship of the independent variable and the responses were calculated using the second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 X_1 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{22} X_2 X_2 + \beta_{23} X_2 X_3 + \beta_{33} X_3 X_3 \quad (1)$$

Where Y is the response; β_0 is the intercept; $\beta_1, \beta_2, \beta_3$ are linear coefficients; $\beta_{11}, \beta_{22}, \beta_{33}$ are squared coefficients; $\beta_{12}, \beta_{13}, \beta_{23}$ are interaction coefficients. The analysis of variance (ANOVA) table was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The effect of the independent variables on biomass concentration was represented via response surface plots. These response curves were then used to predict the levels of the factors required to obtain an optimum culture condition (Pham et al., 1998; Kwak et al., 2006).

RESULTS AND DISCUSSION

The data on probiotic biomass yield was subjected to statistical analysis using MINITAB 15.0 software to estimate t-value, p-value and confidence level. The student's t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. In this work, variables with highest confidence levels were considered as most significant on probiotic biomass production. On the basis of the confidence level reported in Table 3, pH (confidence level = 74.5%), ammonium citrate (confidence level = 71.7%) and peptone (confidence level = 69.4%) were found as the most important

Table 2. Central Composite Design for optimization of medium components for probiotic biomass production.

Exp. No.	Variable			Biomass ($\times 10^9$ CFU/ml)	
	pH (X_1)	Ammonium citrate % (X_2)	Peptone % (X_3)	Experimental	Predicted
1	5.5	0.002	0.100	4.331	4.302
2	6.5	0.002	0.100	5.614	4.878
3	5.5	0.200	0.100	7.245	5.228
4	6.5	0.200	0.100	5.622	5.001
5	5.5	0.002	1.000	1.953	2.922
6	6.5	0.002	1.000	8.119	10.484
7	5.5	0.200	1.000	2.583	3.668
8	6.5	0.200	1.000	10.050	10.427
9	5.12	0.101	0.550	1.456	1.618
10	6.84	0.101	0.550	8.442	7.785
11	6.0	0.000	0.550	9.473	8.112
12	6.0	0.267	0.550	7.977	8.843
13	6.0	0.101	0.000	1.141	3.331
14	6.0	0.101	1.306	9.418	6.733
15	6.0	0.101	0.550	8.938	9.478
16	6.0	0.101	0.550	9.607	9.478
17	6.0	0.101	0.550	9.749	9.478
18	6.0	0.101	0.550	9.536	9.478
19	6.0	0.101	0.550	9.654	9.478
20	6.0	0.101	0.550	9.300	9.478

Table 3. Estimated effects and coefficients for analysis of PB design for probiotic biomass production.

Variable	Coefficient	t-value	p-value	Confidence level (%)
Constant	1.2757	-0.63	0.593	40.7
A	-0.0455	-1.27	0.333	66.7
B	-0.0915	1.36	0.306	69.4
C	0.0985	-0.84	0.489	51.1
D	-0.0608	0.55	0.639	36.1
E	0.0395	1.45	0.283	71.7
F	0.105	1.24	0.314	68.6
G	0.0765	-1.19	0.357	64.3
H	-0.0857	-1.58	0.255	74.5

variables. These key variables were selected for further optimization using RSM.

The student's t-test and F-test were performed using MINITAB 15.0 software for experimental results reported for CCD in Table 4. The coefficients t and p values for linear, quadratic and combined effects are given in the Table 5, at 95% significance level. The p-values were used as a tool to check the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between the variable. The smaller p-value indicates more significance in the corresponding coefficient. It was observed that the coefficient for overall effect of the variables had high significance ($p = 0.000$)

on probiotic biomass production. The individual effect of pH ($p = 0.002$), quadratic effect of pH ($p = 0.003$) and peptone ($p = 0.005$) and the interaction effect of pH versus peptone were also found to have high influence on probiotic biomass production. It was also found from the student's t-test that there was no or less interaction effect between the variables on probiotic biomass production.

The effects of the independent variables on biomass concentration were represented via response surface plots (Pham et al., 1998; Kwak et al., 2006). The graphical representations of the interaction effect of the variables called the contour plots were developed using

Table 4. Estimated regression coefficients of second order polynomial model for optimization of probiotic biomass production.

Factor	Coefficient	Estimated coefficient	Standard deviation	t- value	p-value
	β_0	9.478	0.686	13.800	0.000
X_1	β_1	1.833	0.455	4.024	0.002
X_2	β_2	0.217	0.455	0.477	0.644
X_3	β_3	1.011	0.455	2.220	0.051
X_1^2	β_{11}	-1.688	0.443	-3.807	0.003
X_2^2	β_{22}	-0.353	0.443	-0.797	0.444
X_3^2	β_{33}	-1.571	0.443	-3.543	0.005
X_1X_2	β_{12}	-0.200	0.595	-0.337	0.743
X_1X_3	β_{13}	1.746	0.595	2.934	0.015
X_2X_3	β_{23}	-0.045	0.595	-0.076	0.941

Table 5. Analysis of variance (ANOVA) for second order polynomial model for probiotic biomass production.

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	p-value
Model	9	155.114	17.234	6.08	0.005
Linear	3	60.534	20.178	7.12	0.008
Square	3	69.836	23.278	8.21	0.005
Interaction	3	24.744	8.248	2.91	0.087
Residual error	10	28.358	2.835		
Total sum of squares	19	183.473			

MINITAB 15.0 software and interaction between any two variables on probiotic biomass production was studied by keeping other variable constant at their middle values. The elliptical or circular shapes of the response surface plots, indicates the interactions between the variables as significant or not, respectively. In our study, circular shape of the contour plots of peptone versus ammonium citrate (Figure 1), peptone versus pH (Figure 2) and ammonium citrate versus pH (Figure 3) indicated that there was no significant influence interaction effect between these set of variables on probiotic *B. subtilis* SK09 biomass production.

Based on the response from CCD, the value for correlation coefficient was determined using regression analysis and was found to be (R^2) 0.902. A higher value of the correlation coefficient signifies a good correlation between the independent variables and probiotic biomass production. A second order polynomial equation was then fitted to the data by a multiple regression procedure. The equation resulted in an empirical model that relates the measured response to the independent variables of the experiment.

Analysis of variance (ANOVA) was used to test the significance and adequacy of the second order polynomial model. The ANOVA of the model is shown in Table 5 at 95% confidence level which was used to evaluate the adequacy of the fitted model. The Fisher F-test with a very low probability value for response

demonstrated a high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The R-squared value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of one represents the ideal case at which 100% of the variation in the observed value can be explained by the model (Siti et al., 2006). In this study, the ANOVA of the regression model demonstrates that the model is highly significant, this is evident from the calculated F-value (F-model = 6.08) and probability value (P = 0.005). It is evident that the linear (p = 0.008) and quadratic effect (p = 0.005) of the variables had greater influence on probiotic biomass production. There was no significant influence (p = 0.087) on probiotic biomass production due to the interaction effect of the variables.

$$Y_1 = 9.47 + 20.2X_1 + 0.03X_2 + 0.8X_3 - 1.62X_1^2 - 0.29X_2^2 - 1.5X_3^2 - 0.5X_1X_2 + 1.4X_1X_3 + 0.2X_2X_3 \quad (2)$$

The regression in Equation (2) was solved by response optimizer in MINITAB 15.0 software for optimum value of the variables for maximum probiotic biomass production. The optimum value of the variables in actual unit was predicted as pH (6.72), ammonium citrate (0.16%) and peptone (0.85%) with the predicted maximum probiotic biomass yield of 10.051×10^9 CFU/ml.

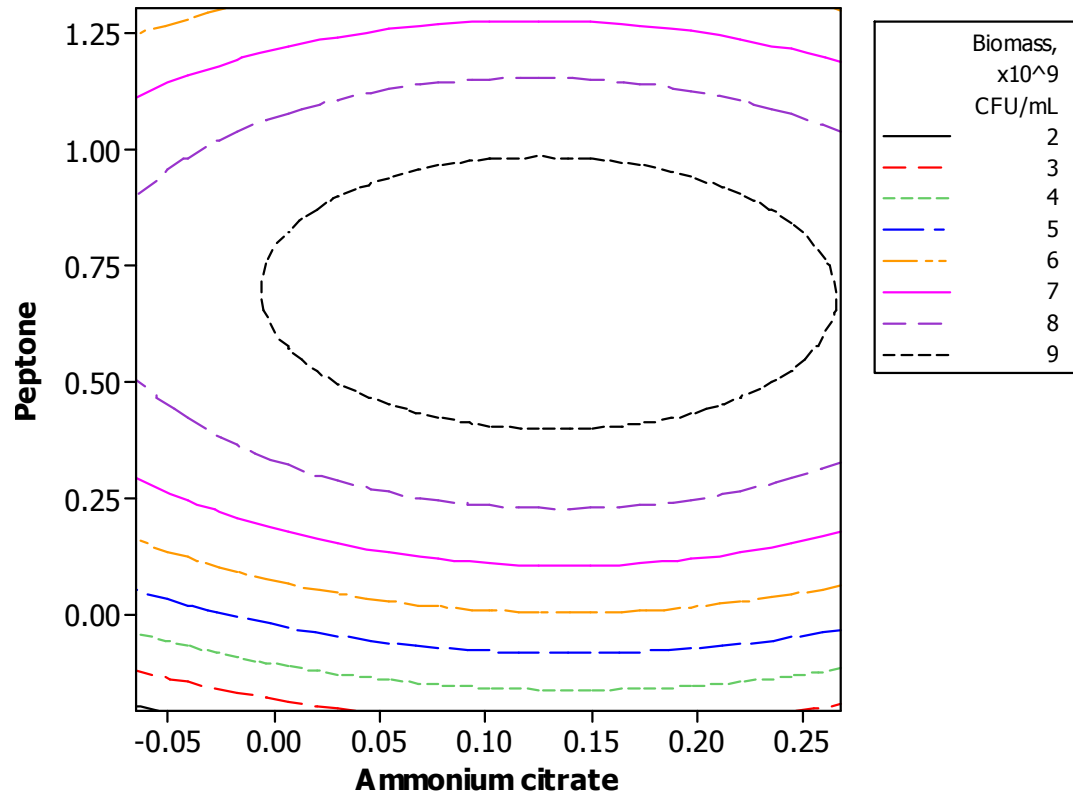


Figure 1. Contour plot showing the interaction effect of peptone and ammonium citrate on probiotic biomass production.

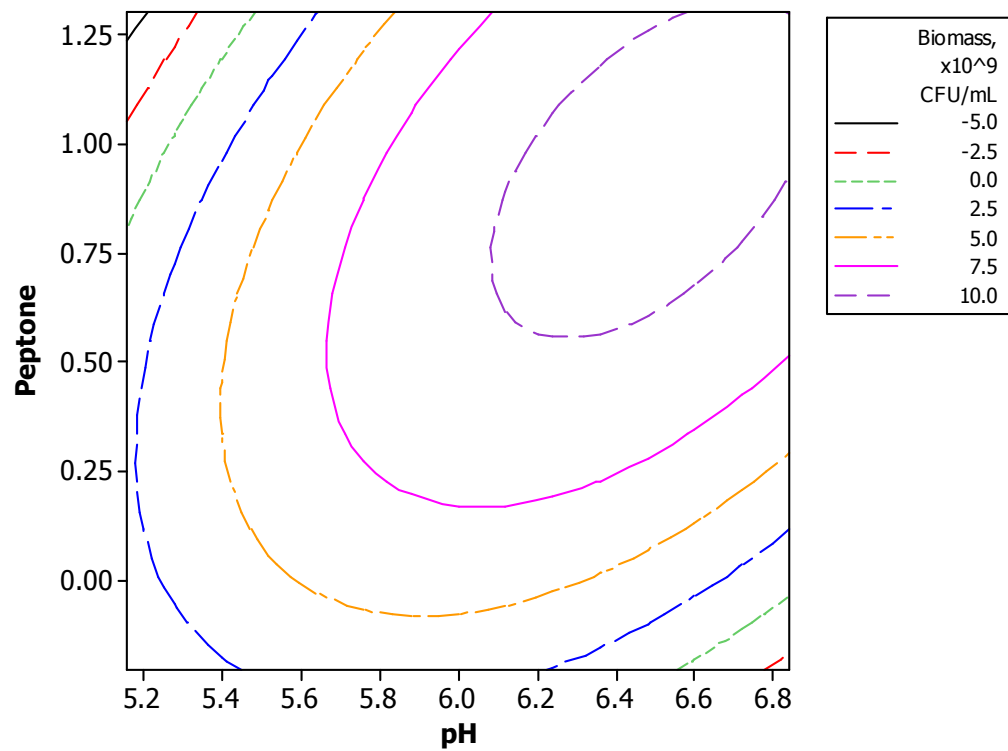


Figure 2. Contour plot showing the interaction effect of peptone and pH on probiotic biomass production.

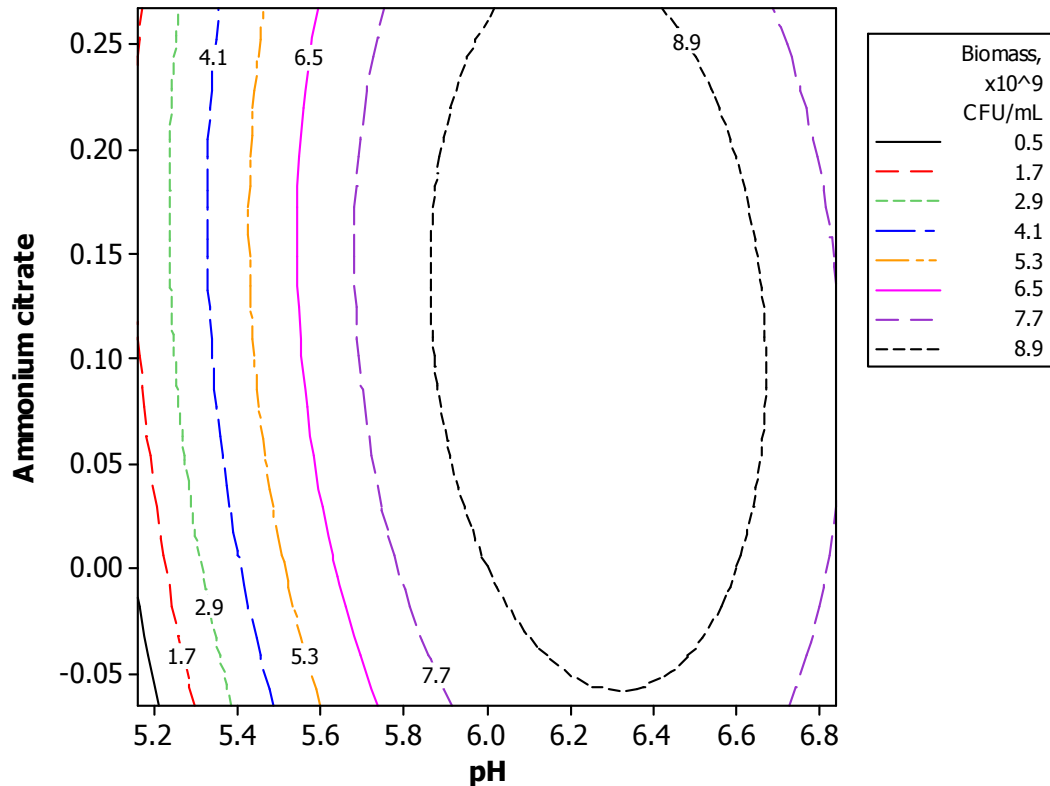


Figure 3. Contour plot showing the interaction effect of ammonium citrate and pH on probiotic biomass production.

Conclusion

The experimental trial to optimize the culturing conditions for the enhanced production of *B. subtilis* SK09 biomass was the successful use of statistical methods such as Plackett-Burman design and CCD. The pH, ammonium citrate and peptone were found to significantly enhance the probiotic biomass yield. These significant variables were further optimized using CCD and second order polynomial model was well fitted to represent the effect of these three variables. The predicted optimum value of the variables was found to be pH (6.72), ammonium citrate (0.164%) and peptone (0.85%) with the maximum probiotic *B. subtilis* SK09 biomass yield of 10.051×10^9 CFU/ml. Based on the predicted optimum values, confirmation experiment studies were conducted in triplicate and the average yield of probiotic *B. subtilis* SK09 biomass was 10.297×10^9 CFU/ml which was obviously in close relation with the model prediction and hence the model was successfully validated.

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