

## Research Article

# Optimization of Fermentation Medium for the Production of Atrazine Degrading Strain *Acinetobacter* sp. DNS<sub>32</sub> by Statistical Analysis System

Ying Zhang,<sup>1</sup> Yang Wang,<sup>1</sup> Zhi-Gang Wang,<sup>1</sup> Xi Wang,<sup>1</sup> Huo-Sheng Guo,<sup>1</sup>  
Dong-Fang Meng,<sup>1</sup> and Po-keung Wong<sup>2</sup>

<sup>1</sup> School of Resources and Environment, Northeast Agricultural University, Harbin 150030, China

<sup>2</sup> School of Life Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Correspondence should be addressed to Ying Zhang, zhangyinghr@hotmail.com and Po-keung Wong, pkwong@cuhk.edu.hk

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Statistical experimental designs provided by statistical analysis system (SAS) software were applied to optimize the fermentation medium composition for the production of atrazine-degrading *Acinetobacter* sp. DNS<sub>32</sub> in shake-flask cultures. A “Plackett-Burman Design” was employed to evaluate the effects of different components in the medium. The concentrations of corn flour, soybean flour, and K<sub>2</sub>HPO<sub>4</sub> were found to significantly influence *Acinetobacter* sp. DNS<sub>32</sub> production. The steepest ascent method was employed to determine the optimal regions of these three significant factors. Then, these three factors were optimized using central composite design of “response surface methodology.” The optimized fermentation medium composition was composed as follows (g/L): corn flour 39.49, soybean flour 25.64, CaCO<sub>3</sub> 3, K<sub>2</sub>HPO<sub>4</sub> 3.27, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2, and NaCl 0.2. The predicted and verifiable values in the medium with optimized concentration of components in shake flasks experiments were 7.079 × 10<sup>8</sup> CFU/mL and 7.194 × 10<sup>8</sup> CFU/mL, respectively. The validated model can precisely predict the growth of atrazine-degrading bacterium, *Acinetobacter* sp. DNS<sub>32</sub>.

## 1. Introduction

Atrazine is a triazine herbicide used extensively in many parts of the world to control a broad variety of weeds, primarily on corn fields [1]. Atrazine and related triazines are moderately persistent in soil [2], depending largely on soil environmental conditions [3, 4]. So atrazine has been classified by regulatory agencies as one of the major target anthropogenic pollutants requiring immediate attention and effective development of methods for decontamination of contaminated matrices [5–7]. Microbial metabolism has long been regarded as the most important mechanism of atrazine degradation in soil [8, 9]. Microbial bioaugmentation, an addition of sufficient contaminant degrading microorganisms, can be used to achieve more rapid atrazine degradation [10]. Previous studies have reported the identification, characterization of atrazine-degrading microbial strains [11–13], and the effect of cell immobilization for atrazine degradation [14]. Yet, only few studies have looked

at fermentation of atrazine-degrading microbial strains. This study, therefore, reports the optimization of fermentation medium for the production of an atrazine-degrading bacterial strain and is the first applied technology to an atrazine pollution remediation study. In order to reduce the fermentation cost, corn and soybean flour, two types of agro-industrial products, were employed as alternative substrates in this study. The other medium components were composed of CaCO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, and NaCl. The atrazine-degrading bacterium, *Acinetobacter* sp. DNS<sub>32</sub>, used in the present investigation is isolated from fresh soil. The bacterium used atrazine as sole nitrogen source (the initial concentration of atrazine is 100 mg/L), and its atrazine degradation rate reached 96.86% after 3 d. The bacterial strain in the soil samples had 20 mg/Kg atrazine and the degradation rates of the atrazine in those soil samples were above 99% within 20 d.

Plackett-Burman Design provides a fast and an effective way to screen the important factors among several variables,

TABLE 1: Two levels of the factors used in Plackett-Burman Design.

Factor	Low level (-1)	High level (+1)
Corn flour concentration (g/L) ( $X_1$ )	40.000	50.000
Soybean flour concentration (g/L) ( $X_2$ )	24.000	34.000
CaCO <sub>3</sub> concentration (g/L) ( $X_3$ )	3.000	5.000
K <sub>2</sub> HPO <sub>4</sub> concentration (g/L) ( $X_4$ )	2.000	4.000
MgSO <sub>4</sub> ·7H <sub>2</sub> O concentration (g/L) ( $X_5$ )	0.200	0.400
NaCl concentration (g/L) ( $X_6$ )	0.200	0.400
Dummy variable ( $X_7$ )	—	—

thereby, saving time and not only maintaining but also convincing information from each parameter [15]. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analyzing problems which involved a response of interest influenced by several variables and the objective is to optimize this response [16, 17]. Plackett-Burman Design and RSM have been successfully used to optimize some bioprocesses using the software of design expert [18–20]. Although the software provided by design expert is good, its module function is less powerful than the statistical software provided statistical analysis system (SAS). Moreover, few studies have employed SAS software. Thus, in this study, experimental designs provided by SAS software were used for the optimization of fermentation medium for atrazine-degrading bacterium, *Acinetobacter* sp. DNS<sub>32</sub>.

The aim of this work was to define the optimized composition of fermentation medium for atrazine-degrading bacterium, by experimental design components (i.e., Plackett-Burman Design and RSM) of SAS software.

## 2. Materials and Methods

**2.1. Bacterial Strain.** From the corn-planted soil (0–10 cm) under long-term applying atrazine in Heilongjiang Province, one bacterial strain named DNS<sub>32</sub> obtained by enrichment culture has been identified according to the morphology and comparison of 16SrDNA gene sequence (*Acinetobacter* sp.TM1.6). The bacterial isolate was then named *Acinetobacter* sp. DNS<sub>32</sub>. The stock culture was maintained on agar slant containing (g/L): glucose 3, K<sub>2</sub>HPO<sub>4</sub> 1.6, KH<sub>2</sub>PO<sub>4</sub> 0.4, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2, NaCl 0.1, atrazine 0.1, and agar 20, cultured for four days at 30°C, then stored at 4°C and subcultured every month.

**2.2. Fermentation Medium and Flask Culture.** The components of fermentation medium were: corn flour, soybean flour, CaCO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, and NaCl. The concentrations of components were adjusted according to the experimental design. For the production of the inoculum, one loopful of the bacterial culture was transferred from a slant culture into an Erlenmeyer flask (50 mL) containing 20 mL LB (Luria-Bertani) medium. The pH of the medium was adjusted to 7.0. The flask was incubated in a shaking incubator at 30°C and 130 rpm for 12 h. Subculture was conducted by inoculating 100  $\mu$ L overnight (~12 h) bacterial

culture in a 150 mL Erlenmeyer flask containing 50 mL of the medium each. The fermentation cultures were grown at 30°C on a rotary shaker incubator at 130 rpm for 24 h, and the initial pH was adjusted to 7.0 with 1 mol/L NaOH.

### 2.3. Experimental Methods and Analysis

**2.3.1. Plackett-Burman Design.** The importance of 6 components of fermentation culture medium was investigated using a Plackett-Burman Design. Plackett-Burman Design was introduced in this study as a first optimization step to identify the factors that have significant effects on the strain DNS<sub>32</sub> production. Hadamard matrix was employed in Plackett-Burman Design. Based on Plackett-Burman factorial design, each variable was examined in two levels: -1 for low level and +1 for high level [21]. This design was used to screen and evaluate the important factor(s) that influence the response of 6 assigned factors and one dummy factor to estimate test error in 8 experimental designs. All experiments were performed in duplicate and the average of the biomass of atrazine-degrading bacterium was taken as the response. The factors included in the screening experiment and their settings are given in Table 1.

**2.3.2. Path of Steepest Ascent.** The direction of steepest ascent is the direction in which the response increased most rapidly. The direction of steepest ascent was parallel to the contour line of response curve of (1), and the low level of Plackett-Burman Design was identified as the center point of steepest ascent path. Experiments were performed along the steepest ascent path until the response did not increase anymore. This point would be near to the optimal point and could be used as center point to optimize [22].

**2.3.3. Central Composite Design.** A central composite design (CCD) was employed to optimize the three most significant factors (corn flour, soybean flour, and K<sub>2</sub>HPO<sub>4</sub>) screened by Plackett-Burman Design. The CCD is one of the most commonly used response surface designs for fitting second-order models. A central composite design consists of  $F$  factorial points,  $2k$  axial points ( $\pm\alpha$ ), and  $nc$  center points. The factorial portion is used for fitting all linear and interaction terms. The axial points provide additional levels of the factor for purposes of estimation of the quadratic terms [23]. According to the central composite design, three independent factors, namely, corn flour concentration ( $X_1$ ),

TABLE 2: Coded values for each factor of the central composite design.

Coded values of the experimental factors	$X_1$ : corn flour concentration (g/L)	$X_2$ : soybean flour concentration (g/L)	$X_3$ : $K_2HPO_4$ concentration (g/L)
-1.682	23.180	7.180	1.318
-1	30.000	14.000	2.000
0	40.000	24.000	3.000
+1	50.000	34.000	4.000
+1.682	56.800	40.800	4.680

TABLE 3: Experimental design and results of the Plackett-Burman Design.

Run	$X_1$	$X_2$	$X_3$	$X_4$	$X_5$	$X_6$	$X_7$	Biomass ( $\log_{10}$ CFU/mL)
1	+1	+1	+1	-1	+1	-1	-1	8.561
2	-1	+1	+1	+1	-1	+1	-1	8.781
3	-1	-1	+1	+1	+1	-1	+1	8.895
4	+1	-1	-1	+1	+1	+1	-1	8.722
5	-1	+1	-1	-1	+1	+1	+1	8.683
6	+1	-1	+1	-1	-1	+1	+1	8.627
7	+1	+1	-1	+1	-1	-1	+1	8.690
8	-1	-1	-1	-1	-1	-1	-1	8.868

soybean flour concentration ( $X_2$ ), and  $K_2HPO_4$  concentration ( $X_3$ ) were studied at five different levels (coded: -1.682, -1, 0, +1, and +1.682) with six repetitions at the central point and two replicates at axial and factorial points (Table 2).

2.3.4. *Statistical Analysis.* SAS release 8.01 for Windows (Institute Incorporation, Cary, NC, USA) was employed for the experimental designs and regression analysis of the experimental results.

2.4. *Analytical Method.* The biomass production of the bacterium was determined by measuring the total biomass using the method of Miles and Misra [24]. Decimal serial dilutions of culture solution were prepared using sterile water. 100  $\mu$ L were dropped onto 3-4 d agar plates and then incubated at 30°C for 2-3 d. The agar plate composition was as same as in stock slant. The biomass of atrazine-degrading *Acinetobacter* sp. DNS<sub>32</sub> was calculated as  $\log_{10}$  colony forming units per mL. Dilutions with less than 10 or more than 130 colonies were discarded [25].

### 3. Results and Discussion

3.1. *Plackett-Burman Design.* Plackett-Burman Design was used as a screening method to determine which of the 6 components of the fermentation medium significantly affect the biomass production of the bacterium. The experimental results with the Plackett-Burman Design are shown in Table 3. These are tested simultaneously by shifting factors from a low value (-1) to a high value (+1). The experimental results were interpreted based on the partition of the overall effect of all the factors to the response into individual factor effect. This partition has been made statistically. When

the value of the concentration effect,  $E_{(X_i)}$ , of the tested variable is positive, the conclusion is that the influence of the concerning variable is greater at a high concentration tested, and when negative, this means that the influence of the given variable is greater at a low concentration [26]. The variable with confidence level above 90% is considered a significant factor. Regarding to the effects of the variables, it is apparent from Table 4 that the influence of corn flour concentration, soybean flour concentration,  $CaCO_3$  concentration,  $MgSO_4 \cdot 7H_2O$  concentration, and NaCl concentration is greater on the biomass of atrazine degrading strain DNS<sub>32</sub> at a low concentration and that  $K_2HPO_4$  concentration has a greater effect at a high concentration. The confidence levels of concentrations of  $CaCO_3$ ,  $MgSO_4 \cdot 7H_2O$ , and NaCl were less than 90% and hence was considered to be insignificant. Thus, low concentrations of these variables were employed in the optimized fermentation culture medium. Only the concentrations of corn flour, soybean flour, and  $K_2HPO_4$  had confidence levels greater than 90% and were considered to significantly influence the biomass production of the bacterium. The concentrations of corn flour, soybean flour, and  $K_2HPO_4$  were selected for further optimization to achieve a maximum response.

3.2. *The Path of Steepest Ascent.* Based on the results of the Plackett-Burman Design, it could be known that concentrations of corn flour ( $X_1$ ), soybean flour ( $X_2$ ), and  $K_2HPO_4$  ( $X_4$ ) were significant factors.  $E_{(X_1)}$  and  $E_{(X_2)}$  were positive, while  $E_{(X_4)}$  was negative; this means that increasing  $X_1$  and  $X_2$  while decreasing  $X_4$  had positive effect on the biomass of the bacterium. Experimental design of the steepest ascent and corresponding results showed that the biomass of the bacterium had a growing trend before rising to a maximum of the run four and then declined. The other factors were

TABLE 4: The effect of variables in the Plackett-Burman Design and associated statistical tests.

Factor	$E_{(X_i)}$	$t$ -value	$P >  t $
Corn flour concentration (g/L) ( $X_1$ )	-0.0783	-16.963	0.0375
Soybean flour concentration(g/L) ( $X_2$ )	-0.0486	-10.800	0.0588
CaCO <sub>3</sub> concentration (g/L) ( $X_3$ )	-0.0122	-2.634	0.231
K <sub>2</sub> HPO <sub>4</sub> concentration (g/L) ( $X_4$ )	0.0438	9.485	0.0669
MgSO <sub>4</sub> ·7H <sub>2</sub> O concentration (g/L) ( $X_5$ )	-0.0130	-2.824	0.217
NaCl concentration (g/L) ( $X_6$ )	-0.0250	-5.405	0.116

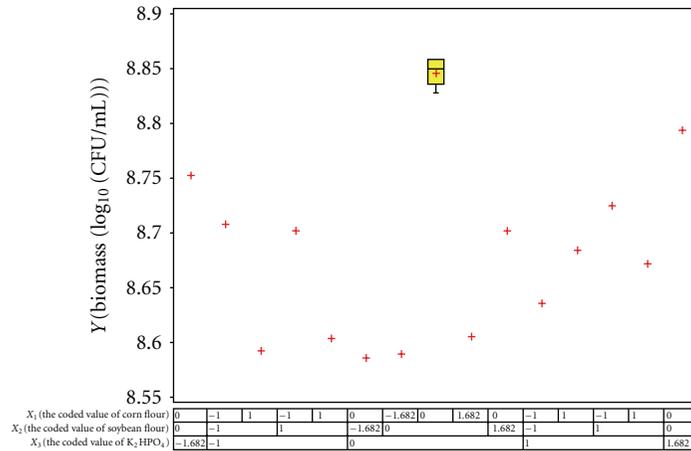


FIGURE 1: Boxplot of results of the central composite design.

fixed at low level (-1). It suggested that this point was near the region of maximum response. So this point was chosen for further optimization.

**3.3. Screened Medium Constituents Optimization with Central Composite Design.** Central composite design (CCD) was employed to determine the optimal levels of the three selected factors. The responses of biomass production of the bacterium as a function of the concentrations of corn flour, soybean flour, and K<sub>2</sub>HPO<sub>4</sub> were evaluated. The respective low and high levels with the coded levels for the factors are defined in Table 2. The other factors were fixed at low level (-1). In total, 20 experimental runs with different combinations of three factors were performed and their responses are presented in Figure 1. Based on regression analysis of the experimental results, the following second-order polynomial equation was written:

$$\begin{aligned}
 Y = & 8.846 - 0.0140X_1 + 0.0202X_2 + 0.0131X_3 \\
 & - 0.0870X_1^2 - 0.0107X_1X_2 + 0.0262X_1X_3 \\
 & - 0.0706X_2^2 + 0.00893X_2X_3 - 0.0250X_3^2,
 \end{aligned} \quad (1)$$

where  $Y$  was the predicted response,  $X_1$ ,  $X_2$ , and  $X_3$  were coded values of corn flour, soybean flour, and K<sub>2</sub>HPO<sub>4</sub> concentrations, respectively.

The statistical significance of (1) was evaluated by  $F$ -test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 5. The analysis of

variance performed on the model demonstrates that the model is statistically valid ( $F = 25.598$ , and  $P < 0.0001$ ). The regression equation obtained after ANOVA indicated a  $R^2$  value of 95.84% which was consistency with the adjusted  $R^2$  of 92.1%. Normally, the closer  $R^2$  is to 1, the stronger the model and the better it predicts the response [27]. This ensured a satisfactory adjustment of the theoretical responses to the experimental data by this model. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared [28]. A lower value of CV (0.324) showed a better precision and reliability of the experiments conducted [29]. Both linear and quadratic terms were significant at the 5% level. Consequently, the model equation (1) was selected in this optimization work.

The significance of the regression coefficients was tested by a  $t$ -test. The Student's  $t$ -distribution, the corresponding  $P$  value, and the parameter estimate are presented in Table 6. The  $P$  values are employed to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the  $P$  values, the bigger is the significance of the corresponding coefficient [30]. Table 6 shows that among the independent factors, linear term of  $X_2$  (soybean flour concentration) had a significant effect on the biomass production of the bacterium. The positive coefficient of  $X_2$  shows a linear effect to increase biomass. The quadratic term of the three variables and the interaction between  $X_1$  and  $X_3$  also had a significant effect. Notably,  $X_1^2$  and  $X_2^2$  ( $P < 0.0001$ ), as part of the quadratic term,

TABLE 5: ANOVA for the second-order polynomial model.

Source	Degree of freedom	Sum of squares	Mean square	F-value	P > F
Linear	3	0.0106	—	4.420	0.0318
Quadratic	3	0.167	—	69.430	<0.0001
Cross product	3	0.00706	—	2.940	0.0854
Total model	9	0.184	0.0205	25.598	<0.0001
Total error	10	0.008	0.0008	—	—

$R^2 = 95.8\%$ ; Adj.  $R^2 = 92.1\%$ ; CV = 0.324.

TABLE 6: Effect estimates for the second-order polynomial model.

Model term	Degree of freedom	Estimate	Standard error	t-value	P >  t
Intercept	1	8.846	0.0115	766.830	<0.0001 <sup>a</sup>
$X_1$	1	-0.0140	0.00765	-1.831	0.0970
$X_2$	1	0.0202	0.00765	2.640	0.0247 <sup>a</sup>
$X_3$	1	0.0131	0.00765	1.714	0.1172
$X_1^2$	1	-0.0870	0.00745	-11.679	<0.0001 <sup>a</sup>
$X_2^2$	1	-0.0706	0.00745	-9.470	<0.0001 <sup>a</sup>
$X_3^2$	1	-0.0250	0.00745	-3.357	0.0073 <sup>a</sup>
$X_1X_2$	1	-0.0107	0.0100	-1.066	0.3115
$X_1X_3$	1	0.0262	0.0100	2.624	0.0254 <sup>a</sup>
$X_2X_3$	1	0.00893	0.0100	0.893	0.3929

<sup>a</sup>Significant at 5% level ( $P < 0.05$ ).

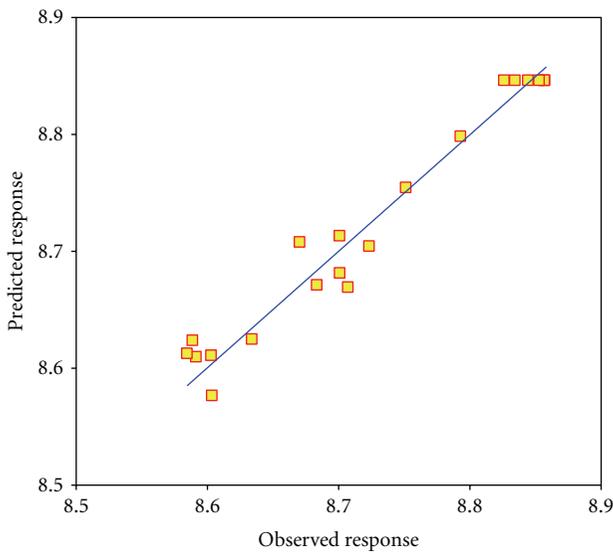


FIGURE 2: Observed responses predicted responses under the central composite design.

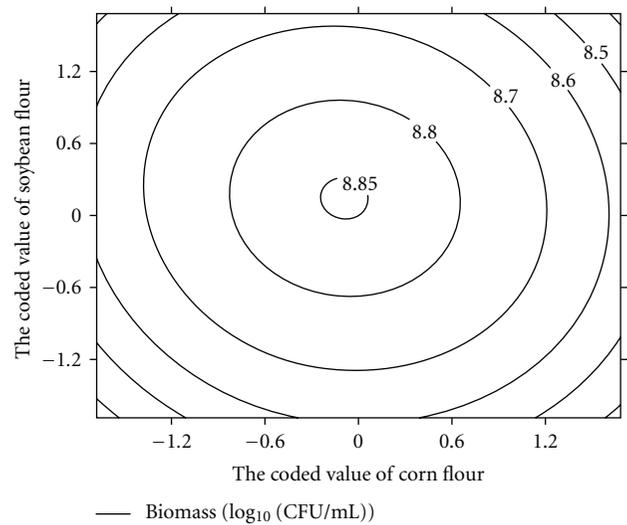


FIGURE 3: Contour plot for DNS<sub>32</sub> strain production as a function of  $X_1$  (corn flour concentration) and  $X_2$  (soybean concentration) when the coded value of  $X_3$  ( $K_2HPO_4$  flour concentration) was fixed at 0.

were considerably higher than other effects demonstrating that these are the most significant factors affecting biomass production of the bacterium.

The regression model can be applied to predict future observations on the response corresponding to particular values of the regression factors. Actual values, depicted in Figure 2, are the observed response data for a run arranged by central composite design, and the predicted values assess

the models and are generated by using the approximating functions. The value of correlation coefficient of  $R^2$  was evaluated as 0.9584. This proves the predicted responses from the second-order polynomial equation are consistent with the observed ones.

The quadratic polynomial equations to experimental data (1) can be described by the response surface plots that explain the interactions of medium components and the

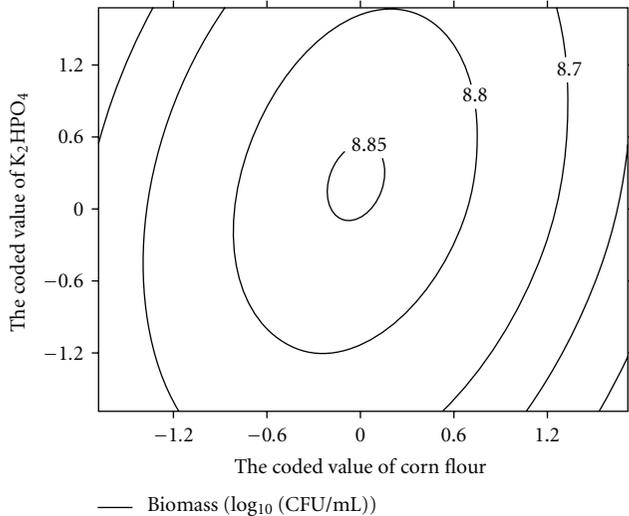


FIGURE 4: Contour plot for DNS<sub>32</sub> strain production as a function of  $X_1$  (corn flour concentration) and  $X_3$  ( $K_2HPO_4$  flour concentration) when the coded value of  $X_2$  (soybean concentration) was fixed at 0.

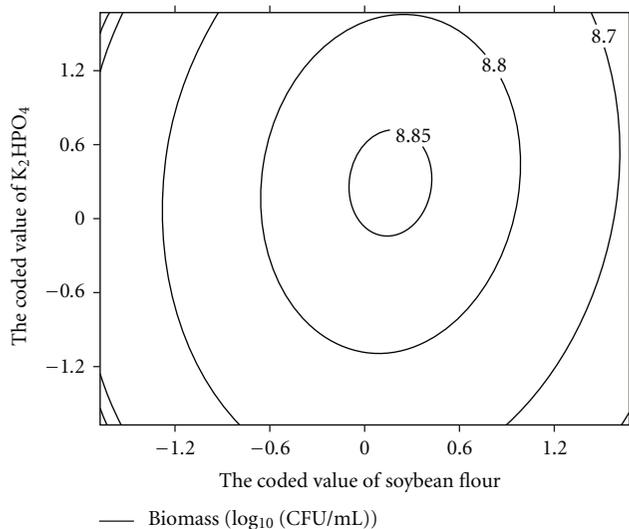


FIGURE 5: Contour plot for DNS<sub>32</sub> strain production as a function of  $X_2$  (soybean concentration) and  $X_3$  ( $K_2HPO_4$  flour concentration) when the coded value of  $X_1$  (corn flour concentration) was fixed at 0.

optimum concentration of each component required for the biomass of atrazine degrading strain DNS<sub>32</sub> as a function of two factors at a time, maintaining the other factors fixed at level zero. These respective contour plots (Figures 3, 4, and 5) showed a visual interpretation of the interaction between two variables and facilitate the location of optimum experimental conditions. The optimal values of the selected factors in their respective coded values were  $X_1 = -0.0506$ ,  $X_2 = 0.164$ , and  $X_3 = 0.265$ . At these values, the actual concentrations of corn flour, soybean flour, and  $K_2HPO_4$  were 39.494, 25.638, and 3.265 g/L, respectively. The maximum predicted value of the common logarithm of biomass obtained was 8.850 (Biomass

of atrazine degrading strain DNS<sub>32</sub> is  $7.079 \times 10^8$  colony forming unit (CFU)/mL).

To check the model adequacy for predicting the maximum response, triplicate sets of experiments under the optimized medium composition were performed. The mean experimental value of the common logarithm of biomass was 8.857 (Biomass of the bacterium is  $7.194 \times 10^8$  CFU/mL), this value excellently matches the predicted value. This behavior shows a good predicting pattern of the model to the experimental data, confirming the validity and adequacy of the models.

#### 4. Conclusions

Statistical optimization of fermentation medium components for the production of atrazine-degrading bacterium, *Acinetobacter* sp. DNS<sub>32</sub>, had been proved to be a valuable tool for the strain production. These experimental designs can convert the process variable correlations into mathematical model that predicts where the response is likely to be identified. It can be concluded that the optimized medium composition was (g/L): corn flour 39.494, soybean flour 25.638,  $CaCO_3$  3,  $K_2HPO_4$  3.265,  $MgSO_4 \cdot 7H_2O$  0.2, and NaCl 0.2. The predicted and verifiable values were  $7.079 \times 10^8$  and  $7.194 \times 10^8$  CFU/mL, respectively. The model validation proved a good consistence between the experimental results and predicted response.

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