



Enhanced production of dimethyl phthalate-degrading strain *Bacillus* sp. QD₁₄ by optimizing fermentation medium



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ABSTRACT

Background: Integrated statistical experimental designs were applied to optimize the medium constituents for the production of a dimethyl phthalate (DMP)-degrading strain *Bacillus* sp. QD₁₄ in shake-flask cultures. A Plackett–Burman design (PBD) was applied to screen for significant factors, followed by the Steepest Ascent Method (SAM) to find the nearest region of maximum response. A Box–Behnken design (BBD) of the Response Surface Methodology (RSM) was conducted to optimize the final levels of the medium components.

Results: After the regression equation and response surface contour plots were analyzed, the concentrations of glucose, corn meal and NaCl were found to significantly influence the biomass of DMP-degrading bacteria. A combination of 22.88 g/L of glucose, 11.74 g/L of corn meal, and 10.34 g/L of NaCl was optimum for maximum biomass production of *Bacillus* sp. QD₁₄. A 57.11% enhancement of the biomass production was gained after optimization in shake-flask cultivation. The biomass production of *Bacillus* sp. QD₁₄ reached $9.13 \pm 0.29 \times 10^8$ CFU/mL, which was an excellent match for the predicted value, and the mean value of the match degree was as high as 99.30%.

Conclusion: In this work, the key factors affected by the fermentation of DMP-degrading strain *Bacillus* sp. QD₁₄ were optimized by PBD, SAM and BBD (RSM); the yield was increased by 57.11% in the conditions in our study. We propose that the conditions optimized in the study can be applied to the fermentation for commercialization production.

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1. Introduction

As one of the phthalic acid esters (PAEs), dimethyl phthalate (DMP) is widely employed in the manufacture of plastics and consumer products including plasticizers for plastics, adhesives, dope, paint, children's toys, medical devices and lubricants [1,2]. Due to the poor chemical affinity of PAEs in these products, PAEs migrate into soils [3], sediments and underground water [4,5] when discarded. A series of recent reports has shown that PAEs may act as endocrine disruptors, environmental carcinogens, teratogens and mutagens, even at low concentrations [6,7]. Therefore, PAEs are considered one of the top-priority environmental pollutants by the US Environmental Protection Agency (US EPA), the European Union and the China National Environmental Monitoring Center [4,8].

The principal methods employed to remove and eliminate environmental PAEs include photo-chemical oxidation [9] and biodegradation [2,5,10]. Due to the low rate of hydrolysis and photolysis

of PAEs [11], especially in the subsurface, metabolic breakdown of this pollutant by microorganisms may be a more feasible strategy. To achieve successful environmental degradation of PAEs, large quantities of bacterial biomass will be required. Studies on biodegradation have also demonstrated that higher cell concentrations improve degradation efficiency [12]. However, few studies have focused on the fermentation of PAE-degrading bacteria, and none have focused on the fermentation of DMP-degrading bacteria. Previous studies have focused on the identification and characterization of PAE-degrading strains [2]. Our present study is the first report on the optimization of a fermentation medium for the production of the DMP-degrading strain *Bacillus* sp. QD₁₄.

The growth of *Bacillus* and its DMP biodegradation rate are strongly influenced by medium composition. Factors include the carbon source, the nitrogen source, inorganic salts, trace elements, and growth factors [12,13,14,15]. Hence, for developing an industrial fermentation process, medium development is of the utmost importance. The single variable optimization method is not only tedious, but can also lead to misinterpretation of the results because the interactions among different variables are overlooked [16]. Statistical optimization not only allows quick screening for significant variables in a large experimental design but also teases out the roles of each component. In our study, we used a novel

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integrated statistical design that incorporated Plackett–Burman design (PBD) [17], the Steepest Ascent Method (SAM) [18] and Response Surface Methodology (RSM) [19] to optimize the medium components for the production of the DMP-degrading strain *Bacillus* sp. QD₁₄. We propose that our optimization method can be applied to the fermentation for commercialization production.

2. Materials and methods

2.1. Microorganism and medium conditions

The DMP-degrading strain *Bacillus* sp. QD₁₄ adopted in the present study was originally isolated in our laboratory from a soil sample collected in the Nenjing River (Qiqihar, China) and deposited at Qiqihar University. The strain obtained by enrichment-culture techniques with DMP was identified according to the morphology and comparison of 16SrDNA gene sequence. Cultures were maintained on nutrient agar slants containing (g/L): YE, 10.0; peptone, 5.0; NaCl, 5.0; agar, 20.0 and DMP 10.0 mg/L at 4°C and subcultured every two weeks. A standard inoculum liquid medium (Luria broth, LB) containing (g/L) YE, 10.0; peptone, 5.0; and NaCl, 5.0 and with a pH of 7.2 was inoculated by transferring a loop of microorganisms from the slant culture into 250 mL Erlenmeyer flasks which were then incubated at 37°C and agitated at 100 rpm in an orbital shaker incubator. Inoculum (10.0 mL/L) was transferred into 250 mL Erlenmeyer flasks containing 100 mL of production medium when the inoculum liquid medium contained 1.00×10^8 CFU/mL. The production medium was the same as the inoculum liquid medium and was employed as a control medium. The Erlenmeyer flasks were then incubated at 100 rpm for 48 h at 37°C. The DMP was of high-performance liquid chromatography (HPLC) grade (Sigma, USA), and all the other reagents were of analytical reagent grade and purchased from Sinopharm Chemical Reagent Co., Ltd., China.

2.2. Degradation experiments

The minimum salt medium (MSM) used in the degradation experiments contained (g/L): K₂HPO₄, 0.5; KH₂PO₄, 0.2; NH₄NO₃, 1.0; MgSO₄·7H₂O, 0.4; CaCl₂, 0.1; FeCl₂, 0.01; NaCl, 1.0; MnCl₂·4H₂O, 0.01 and ZnCl₂, 0.01. The initial pH of the MSM was adjusted to 7.2 with sterile 1.0 mol/L NaOH or HCl. The medium was then sterilized by autoclaving for 25 min at 121°C.

Biodegradation of DMP (100 mg/L) by *Bacillus* sp. QD₁₄ was studied in 100 mL of sterilized MSM in 500 mL glass flasks incubated at 37°C on a rotary shaker operated at 150 rpm in the dark. At 8 h intervals, 2 mL of sample was withdrawn and preserved at -20°C for optical density measurements and gas chromatogram analysis. All experiments were performed in triplicate. Samples and sterile controls (non-inoculated MSM) were periodically analyzed in similar way.

The concentration of DMP was determined with an Agilent 7820A gas chromatogram (GC) equipped with an FID detector and HP-5 capillary column (0.32 mm × 30 m × 0.25 μm). The conditions were as follows: carrier gas, high pure nitrogen gas (1 mL/min); FID detector, 280°C; injector temperature, 250°C; injection volume, 1 μL. The column was maintained at 60°C for 5 min and then increased to 270°C over a 10 min period with an increase rate of 30°C/min.

The microbial biomass in the culture flasks was determined spectrophotometrically by measuring optical density at 600 nm (OD₆₀₀) in a UV–VIS spectrophotometer (Persee T9, Purkinje General Instrument Co., Ltd., China).

2.3. Fermentation process

Batch experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of liquid fermentation medium. The compositions of the fermentation medium were glucose, wheat bran, corn meal,

KH₂PO₄, MgSO₄·7H₂O, FeSO₄, CaCl₂, NaCl and riboflavin. The concentrations of components were adjusted according to the experimental designs in Table 1, Table 2 and Table 3. An inoculum (10.0 mL/L) that contained 1.00×10^8 CFU/mL was transferred from the LB medium into each fermentation medium, and fermentation cultures were grown at 100 rpm for 48 h at 37°C. The initial pH of the media was adjusted to 7.2 with sterile 1.0 mol/L NaOH. All flasks were heat sterilized by autoclaving at 121°C and 103 kPa for 15 min prior to inoculation in the shaking incubator.

2.4. Optimization of the production of the DMP-degrading strain *Bacillus* sp. QD₁₄

2.4.1. Identifying the significant variables with Plackett–Burman design

The PBD is a two-factorial design, which identifies the critical physical–chemical parameters required for elevating the biomass of DMP-degrading bacteria by screening n variables in $n + 1$ experiments. All the variables were investigated at two widely spaced intervals specified as negative values (low level, -1) and positive values (high level, +1) [17,20]. The details of the experimental design matrix and experimental results obtained for the screening of the variables are shown in Table 1. Two dummy variables, whose levels did not change in the design, were introduced to estimate the standard error of the population. Each row represents a trial, and each column represents an independent (assigned) or dummy (unassigned) variable. All experiments were performed in triplicate and analyzed with ‘Minitab’ software (Version 16.1.0, Minitab Co., USA). The effects of individual parameters on the bacterial biomass were determined by [Equation 1]:

$$E(X_i) = \frac{(\sum M_i^+ - \sum M_i^-)}{N} \quad [\text{Equation 1}]$$

where $E(X_i)$ is the concentration effect of the variables tested in the study, M_i^+ and M_i^- represent the responses (the biomass of *Bacillus* sp. QD₁₄) in trials in which the parameter was at its higher and lower levels, respectively. N is the total number of trials, which was equal to 12.

2.4.2. Optimal region of the significant variables by the Steepest Ascent Method

Experiments for each response were conducted along the path of SAM with defined intervals by stepwise increasing or decreasing the concentrations of variables, which were determined according to the coefficients of [Equation 3]. The design and experimental results obtained are shown in Table 2. The path starts from the design center of the PBD, fully stretches outside the design space, and ends when no further improvement in the response can be achieved. The paths of X_1 , X_3 and X_7 for the biomass begin at 15.00, 7.50 and 6.00 (g/L), with a step (Δ) of 2.50, 1.00 and 1.00 (g/L), respectively. While a maximum value was found, the point would be close to the optimal parameters and could be applied as a center point in the subsequent optimization design [18,20].

2.4.3. Optimization of the significant variables by applying Response Surface Methodology

The Box–Behnken Design (BBD) approach was used to determine the optimum levels of three critical independent variables for increasing the biomass production of *Bacillus* sp. QD₁₄: glucose, corn meal and NaCl. The experimental plan consisted of 17 trials, and each independent variable in the design was studied at three different levels, low (-1), medium (0) and high (+1) [19]. The experimental design employed for the study is shown in Table 3. All the experiments were performed in triplicate and the average of the

Table 1
Experimental design matrix and results of PBD.

Runs	Levels	Variables ^a											Biomass ($\times 10^8$ CFU/mL)	
		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Observed	Predicted
	+1	20.00 ^b	10.00	10.00	4.50	1.50	0.90	9.00	1.50	0.30	–	–		
	-1	10.00	5.00	5.00	1.50	0.50	0.30	3.00	0.50	0.10	–	–		
1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	+1	6.83 ± 0.12	6.85
2	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	7.33 ± 0.25	7.30
3	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1	+1	7.00 ± 0.26	7.02
4	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	-1	+1	6.50 ± 0.20	6.53
5	+1	+1	+1	-1	+1	+1	-1	-1	+1	-1	-1	-1	7.13 ± 0.21	7.12
6	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	7.13 ± 0.06	7.10
7	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	-1	+1	7.57 ± 0.15	7.60
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	6.43 ± 0.21	6.42
9	-1	+1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	7.57 ± 0.21	7.55
10	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1	+1	-1	7.67 ± 0.25	7.63
11	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	7.20 ± 0.10	7.22
12	+1	-1	+1	-1	-1	-1	+1	+1	+1	+1	-1	+1	7.87 ± 0.25	7.90

^a X_1 , glucose; X_2 , wheat bran; X_3 , corn meal; X_4 , KH_2PO_4 ; X_5 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; X_6 , FeSO_4 ; X_7 , NaCl; X_8 , CaCl_2 ; X_9 , riboflavin; X_{10} and X_{11} were dummy variables.

^b Units: g/L.

biomass production obtained was taken as the dependent variable or response (Y).

The data obtained from RSM (BBD) were subjected to analysis of variance (ANOVA). The results of RSM were used to fit a second order polynomial, [Equation 2], in the 'Design Expert' software (Version 8.0.5, State-Ease Inc., USA) statistical package. The general form of the second order polynomial equation is:

$$Y_i = \alpha_0 + \sum \alpha_i x_i + \sum \alpha_{ii} x_i^2 + \sum \alpha_{ij} x_i x_j \quad [\text{Equation 2}]$$

where Y_i is the predicted response, i and j are from 1 to the number of variables (n), α_0 is the offset term, α_i is the i th linear coefficients, α_{ii} is the i th quadratic coefficient, α_{ij} is the ij th interaction coefficient, and x_i and x_j are the levels of the independent variables that influence the response variable Y . Statistical significance of the model equation was determined by Fisher's test (F -value), and the proportion of variance explained by the model was obtained by calculating the multiple coefficients of determination R -squared (R^2) value.

2.4.4. Analytical method of the bacterial biomass production

The biomass of *Bacillus* sp. QD₁₄ was determined by counting viable cells with the 'Drop count method' of Miles and Misra [21]. Decimal serial dilutions of fermentation solution were prepared in sterile water. The fermentation solution (0.1 mL) was dropped onto 3–4 day-old agar plates and incubated at 37°C for 48 h. The composition

Table 2
Experiment design and results of the SAM for the biomass of *Bacillus* sp. QD₁₄.

Runs	Step change value	Variables ^b			Biomass ($\times 10^8$ CFU/mL)
		X_1	X_3	X_7	
1	X	15.00 ^c	7.50	6.00	7.37 ± 0.21
–	Δ^a	2.50	1.00	1.00	–
2	X + 1 Δ	17.50	8.50	7.00	7.77 ± 0.15
3	X + 2 Δ	20.00	9.50	8.00	8.10 ± 0.10
4	X + 3 Δ	22.50	10.50	9.00	8.53 ± 0.21
5	X + 4 Δ	25.00	11.50	10.00	9.23 ± 0.25
6	X + 5 Δ	27.50	12.50	11.00	8.60 ± 0.30
7	X + 6 Δ	30.00	13.50	12.00	8.33 ± 0.15
8	X + 7 Δ	32.50	14.50	13.00	8.17 ± 0.29
9	X + 8 Δ	35.00	15.50	14.00	8.03 ± 0.12

^a A step of the Steepest Ascent Method.

^b X_1 , glucose; X_3 , corn meal; X_7 , NaCl.

^c Units: g/L.

of the agar plate was the same with the nutrient agar slopes. In addition, the biomass of the bacterium was calculated as colony forming units (CFU) per mL. Dilutions with less than 10 or more than 150 colonies were discarded [22]. Three plates were taken each time for sampling. Each data point is shown as an average with an error bar (mean ± SD, $n = 3$).

3. Results and discussion

3.1. Degradation of DMP by *Bacillus* sp. QD₁₄

The biodegradation efficiency of DMP by *Bacillus* sp. QD₁₄ was investigated in MSM in the dark. As shown in Fig. 1, *Bacillus* sp. QD₁₄ could degrade DMP completely in 64 h time, and more than 90% of DMP was degraded after a 48 h incubation. The cell concentration reached a maximum of $\text{OD}_{600} = 0.54$ at 56 h. A significant positive correlation was found between the degradation efficiency of DMP and

Table 3
Experimental design matrix and results of BBD for the biomass of *Bacillus* sp. QD₁₄.

Runs	Levels	Variables ^a			Biomass ($\times 10^8$ CFU/mL)	
		X_1	X_3	X_7	Observed	Predicted
	+1	30.00 ^b	13.50	12.00		
	0	25.00	11.50	10.00		
	-1	20.00	9.50	8.00		
1	0	-1	+1		8.60 ± 0.20	8.60
2	0	-1	-1		8.40 ± 0.30	8.38
3	-1	0	-1		8.53 ± 0.31	8.57
4	-1	-1	0		8.77 ± 0.25	8.74
5	0	+1	+1		8.60 ± 0.10	8.63
6	0	0	0		9.17 ± 0.21	9.12
7	+1	0	-1		8.67 ± 0.23	8.65
8	+1	0	+1		8.40 ± 0.17	8.38
9	0	0	0		9.10 ± 0.20	9.12
10	-1	+1	0		8.90 ± 0.30	8.88
11	0	0	0		9.17 ± 0.06	9.12
12	0	+1	-1		8.70 ± 0.10	8.70
13	1	+1	0		8.63 ± 0.15	8.66
14	-1	0	+1		8.97 ± 0.38	8.99
15	0	0	0		9.10 ± 0.17	9.12
16	0	0	0		9.07 ± 0.35	9.12
17	+1	-1	0		8.40 ± 0.10	8.45

^a X_1 , glucose; X_3 , corn meal; X_7 , NaCl.

^b Units: g/L.

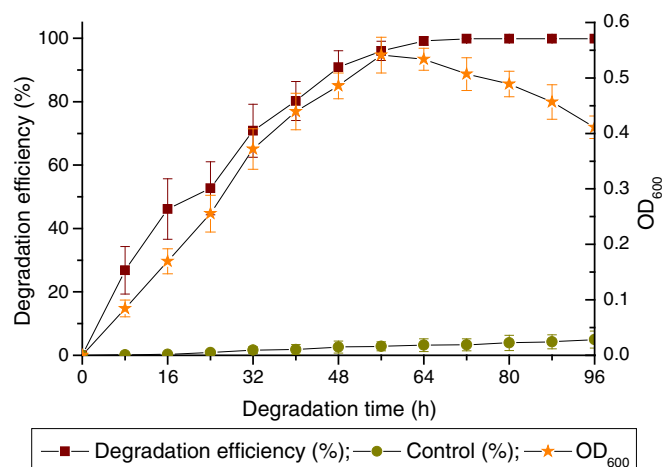


Fig. 1. Bacterial growth and DMP degradation by *Bacillus* sp. QD₁₄.

the cell concentration of *Bacillus* sp. QD₁₄. Previous studies have shown that factors such as cell concentration, pH and temperature affect the degradation of PAEs by bacterium. Higher cell concentration led to higher PAE degradation efficiency. The degradation of PAE by bacteria is generally caused by the production of an esterase [23]. It is reasonable to assume that more esterase produced by larger numbers of cells degrades DMP more efficiently.

3.2. Screening for significant variables by employing a Plackett–Burman design

This experiment was conducted in 12 runs to study the effect of the screened variables. Table 1 shows the results of the screening experiments. The results of statistical analyses of the responses are shown in Table 4 (part of the data is not shown). The *F*-value of the model, 58.65, indicated that the model was significant (the *p* values < 0.05 indicated that the model terms were significant). The coefficient *R*² (0.9962) of the models for the biomass production and the *R*-Adj (0.9792) indicated that the data variability could be fully explained by the models.

A variable with a confidence level above 95% is considered a significant parameter. Accordingly, *X*₁, *X*₃ and *X*₇ were significant terms. The other tested factors did not result in significant variations in biomass production. Based on our ANOVA results, a first-order polynomial equation was derived, which represented the biomass production of the *Bacillus* sp. QD₁₄ as an equation of nine independent variables:

$$Y = 5.25 + 0.0483X_1 + 0.00778X_2 + 0.0544X_3 + 0.0204X_4 - 0.0278X_5 - 0.0278X_6 + 0.105X_7 - 0.05X_8 + 0.75X_9. \quad [\text{Equation 3}]$$

Table 4

Regression coefficients and associated statistical analysis of the model for PBD.

Source	Coefficient	Sum of squares	Degree of freedom	Mean square	<i>F</i> -value	Prob > <i>F</i>
Model	4.936 (constant)	2.19935	9	0.24437	58.65	0.017*
<i>X</i> ₁	0.0483	0.70083	1	0.70083	168.20	0.006*
<i>X</i> ₃	0.0544	0.22231	1	0.22231	53.36	0.018*
<i>X</i> ₇	0.126	1.18231	1	1.18231	283.76	0.004*
Residual	–	0.00833	2	0.00417	–	–
Total	–	2.20769	11	–	–	–

*R*² = 0.9962, Adjusted *R*² = 0.9792, Predicted *R*² = 0.8641.

* Statistically significant at the 95% confidence level (*p* < 0.05).

When the concentration effect value of the experimental variable is negative, the variable is associated with greater biomass production at lower concentrations. Conversely, when the experimental variable is positive, the variable is associated with greater biomass production at higher concentrations. According to the coefficients of [Equation 3], *X*₁, *X*₂, *X*₃, *X*₄, *X*₇ and *X*₉ led to greater biomass production with higher concentrations, while *X*₅, *X*₆ and *X*₈ showed the inverse effect. Thus, higher concentrations of the three significant parameters *X*₁, *X*₃ and *X*₇ were selected for the further optimized fermentation medium to achieve a maximum response region by SAM.

The pathway of DMP biodegradation by the bacterium could be described as follows: first, DMP is rapidly hydrolyzed by an esterase to metabolites such as phthalic acid (PA), after which PA is metabolized to PCA by phthalate dioxygenase, which enters the tricarboxylic acid cycle (TCA) and is finally oxidized to carbon dioxide and water [2,24, 25]. Thus, bacterial concentration and enzyme activity are equally critical in the DMP biodegradation process. Meanwhile, there is a positive correlation between bacterial biomass and metabolites within a certain bacterium concentration range [26]. Based on these principles, the optimization of biomass production was selected as the main focus of study. To improve degradation ability, the bacterial concentration was increased. Another reason that led us to focus on the biomass production rather than the activity of an enzyme was that the activity of an enzyme would be affected by many different ions and growth factors [27].

Various ions have a significant impact on bacterial growth, including Na⁺, Ca²⁺, Mg²⁺, Fe²⁺, Cu²⁺ and PO₄³⁻ [28]. Hence, KH₂PO₄, MgSO₄·7H₂O, FeSO₄, NaCl and CaCl₂ were chosen as mineral salts and trace elements added to the fermentation medium [14,29]. Riboflavin serves as a precursor for the synthesis of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are utilized as electron acceptors for many oxidoreductases [30]. Thus, minute quantities of riboflavin were added into the medium as a growth-promoting factor. The PBD results demonstrated that only carbon and nitrogen nutrient sources and Na⁺ were significant variables that influenced the bacterium concentration in the fermentation.

3.3. Determining the optimal region by applying the Steepest Ascent Method

Given the PBD results and the aforementioned linear model [Equation 3], glucose, corn meal and NaCl were the three significant variables for the response. The higher the concentration of glucose, corn meal, and NaCl, the greater the biomass production of the bacterium. Therefore, the SAM followed a path in which *X*₁, *X*₃ and *X*₇ would increase with defined intervals. The experimental results are shown in Table 2. The highest response was achieved in experiment 5 when *X*₁, *X*₃ and *X*₇ were 25.0 g/L, 11.5 g/L and 10.0 g/L, respectively, and this optimal point was chosen as the basis for further optimization.

As shown in Table 2, the biomass production did not increase further with higher concentrations of the three variables. For glucose, the growth of *Bacillus* sp. QD₁₄ was inhibited at concentrations of glucose higher than 25.0 g/L and could tolerate this glucose concentration limit. This phenomenon is known as the Crabtree effect [31], which states that the growth of a bacterium is different in the presence of different glucose concentrations. The bacterial biomass decreases when the glucose concentration exceeds the limit. Consequently, the bacterial yield could not be further improved by increasing the glucose concentration from 25.0 g/L to 35.0 g/L. Although corn meal contains large amounts of trace elements and growth factors that are beneficial for the growth of bacterium at higher concentrations, excessive corn meal in the fermentation medium also results in decreased water activity (*α*_w), which may make the cells less adaptable [32]. The growth of DMP-degrading bacteria is also different in the presence of Na salt. Some strains isolated from the South China

Table 5
ANOVA of the quadratic model for BBD.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob > F
Model	1.22	9	0.14	67.27	<0.0001**
X ₁	0.14	1	0.14	70.55	<0.0001**
X ₃	0.056	1	0.056	27.56	0.0012*
X ₇	0.443	1	0.443	4.41	0.0739
X ₁ X ₃	0.124	1	0.124	1.24	0.3022
X ₁ X ₇	0.12	1	0.12	60.77	0.0001**
X ₃ X ₇	0.022	1	0.022	11.16	0.0124*
X ₁ ²	0.15	1	0.15	74.74	<0.0001**
X ₃ ²	0.28	1	0.28	136.71	<0.0001**
X ₇ ²	0.35	1	0.35	174.65	<0.0001**
Residual	0.014	7	0.1	–	–
Lack of fit	0.304	3	0.101	1.02	0.4726
Pure error	0.398	4	0.1	–	–
Cor total	1.23	16	–	–	–

R² = 0.9886, Adj R² = 0.9739, Pred R² = 0.9107, C.V. % = 0.51.

* Statistically significant at the 95% confidence level ($p < 0.05$).

** Statistically significant at the 99.9% confidence level ($p < 0.001$).

Sea can degrade DMP effectively when grown in the salinity range of 0–10.0 g/L, but the lag phase also increased with increasing salinity [10]. Higher salinity levels also reduced bacterial growth rates, resulting in longer degradation times. The highest concentration of NaCl the bacteria could tolerate was 40.0 g/L [33]. In the present study, our optimal concentration was 10.0 g/L. The most probable reason is that higher salt concentrations in the sea eventually lead to some degree of tolerance or dependence.

3.4. Optimization of the significant variables by applying Response Surface Methodology

BBD was employed not only to study the interactions among the three significant variables (glucose, corn meal and NaCl) but also to determine their optimal levels. The other variables in the study were maintained at a constant level that gave maximal biomass in the PBD experiments. The coefficients of the first-order polynomial equation derived from PBD experiments represented the effects of influential experiment variables that were positively or negatively correlated with responses (biomass production) [34]. According to

the coefficients of [Equation 3], the variables X₂, X₄ and X₉ were positively correlated with biomass production, which indicated that higher levels were beneficial. Thus, the wheat bran, KH₂PO₄ and riboflavin concentrations were fixed at higher levels (+1) (10.0, 4.5 and 0.3 g/L) while MgSO₄·7H₂O, FeSO₄ and CaCl₂ concentrations were fixed at lower levels (-1) (0.5, 0.3 and 0.5 g/L).

Seventeen experimental runs were carried out with different combinations of the three significant variables. The results for biomass production are presented in Table 3. The second-order polynomial model for biomass production is shown in [Equation 4] (in coded value) and [Equation 5] (in actual value):

$$Y = 9.12 - 0.13X_1 + 0.083X_3 + 0.033X_7 + 0.025X_1X_3 - 0.18X_1X_7 - 0.075X_3X_7 - 0.19X_1^2 - 0.26X_3^2 - 0.29X_7^2 \quad [\text{Equation 4}]$$

$$Y = -17.088 + 0.498C_{\text{glucose}} + 1.638C_{\text{corn meal}} + 2.116C_{\text{NaCl}} + 0.124C_{\text{glucose}}C_{\text{corn meal}} - 0.0175C_{\text{glucose}}C_{\text{NaCl}} - 0.0188C_{\text{corn meal}}C_{\text{NaCl}} - 0.377C_{\text{glucose}}^2 - 0.0640C_{\text{corn meal}}^2 - 0.0723C_{\text{NaCl}}^2 \quad [\text{Equation 5}]$$

where Y is the predicted biomass production ($\times 10^8$ CFU/mL) and X₁, X₃ and X₇ are coded values for glucose, corn meal and NaCl concentrations, respectively.

The adequacy of the model was checked using ANOVA and tested using F-test. The results are shown in Table 5. The Model F-value of 67.27 implies that the model is significant and the 'Lack of Fit' F-value is 1.02 which indicates the 'Lack of Fit' is not significant. A coefficient of determination (R² value) closer to 1 indicates that there is a better correlation between observed and predicted values [35]. In the present study, R² was 0.9886, indicating suitable agreement between experimental and predicted values. The predicted R² of 0.9107 and the adjusted R² of 0.9739 were also consistent. Here, the coefficient of variation (CV) indicates the degree of precision with which the experiments were compared. A higher reliability of the experiment is usually indicated by a lower value of CV [36]. In the present study, the CV of the model was 0.51%, reflecting the high precision and reliability of the experiments. All the statistical results of the models show high accuracy and general applicability of the second-order polynomial

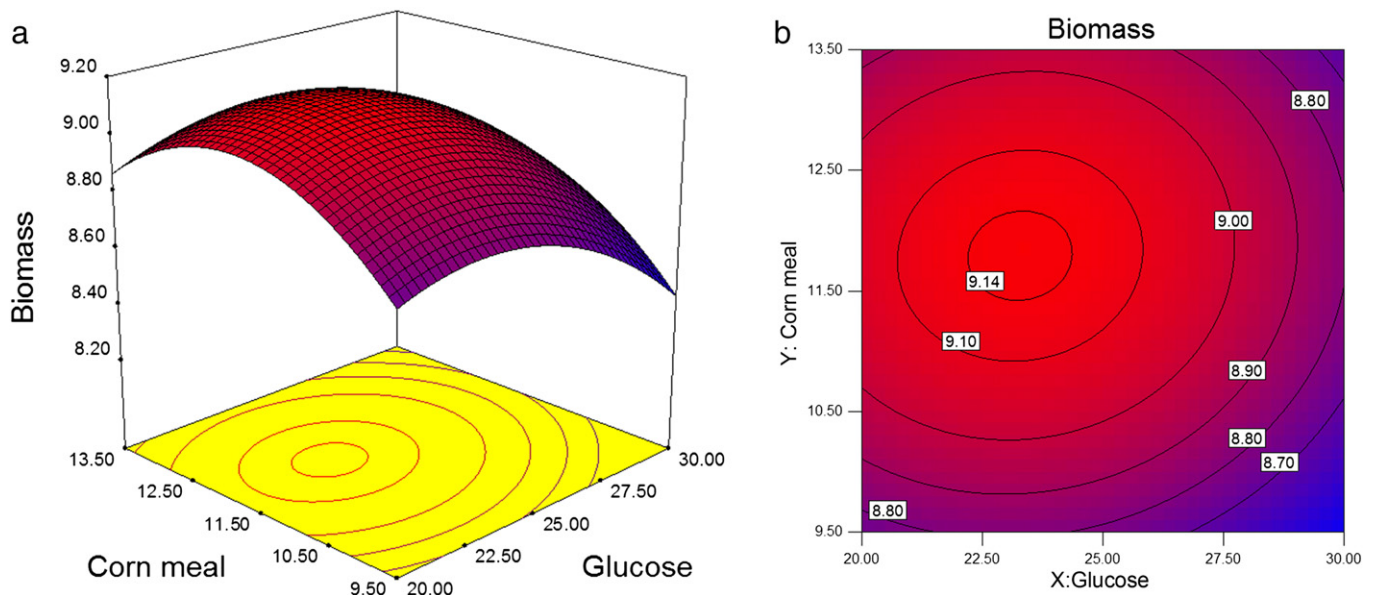


Fig. 2. Response surface (a) and contour plots (b) for the effect of glucose and corn meal on biomass production.

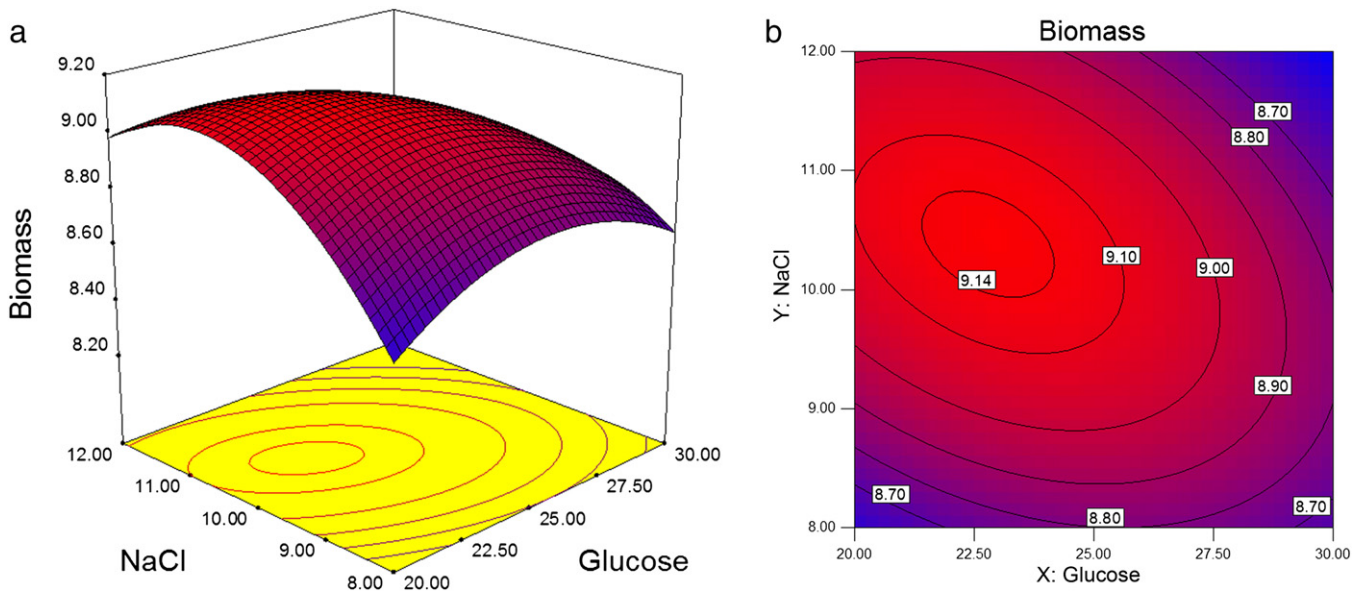


Fig. 3. Response surface (a) and contour plots (b) for the effect of glucose and NaCl on biomass production.

equations, and they are adequate to describe the responses observed in the experiments.

The values of 'Prob > F' are employed to check the significance of each model term which, in turn, are of vital importance for understanding the pattern of mutual interactions between the experiment variables. Values of 'Prob > F' less than 0.001 indicate which model terms are the most significant. Table 5 shows that the linear terms of the independent variables including glucose and corn meal exert a significant effect on the biomass production of bacterium. According to [Equation 4], the positive coefficient of X_2 and the negative coefficient of X_1 showed a linear effect on biomass production. The quadratic terms of the three variables and the interaction between X_1 and X_7 , X_3 and X_7 also had significant effects. Notably, X_1^2 , X_3^2 , X_7^2 and X_1X_7 ($p < 0.001$) were higher than the other effects, which demonstrated that these are the most significant variables influencing biomass production.

Fig. 2 shows the response surface plots and corresponding contour plots for the biomass production generated by the predicted model. Biomass production increased significantly until the concentration of corn meal reached 11.79 g/L, and then it decreased. The effect of glucose on biomass production was also sensitive in the designed range, yielding the p -value (< 0.0001) in Table 5. According to the response curves in Fig. 3, the most significant interaction between glucose and NaCl could be explained reasonably by the elliptical shape of the contour plot, with p -value (0.0001). Biomass production was also linearly increased with NaCl concentrations from 8.00 g/L to 10.38 g/L, and then decreased. In Fig. 4, the biomass value began to fall when corn meal or NaCl were higher than 11.81 g/L or 10.07 g/L, respectively, indicating a maximum predicted value of biomass production.

Based on these graphs, the maximal biomass production of 9.149×10^8 CFU/mL could be observed at 23.29 g/L of glucose and

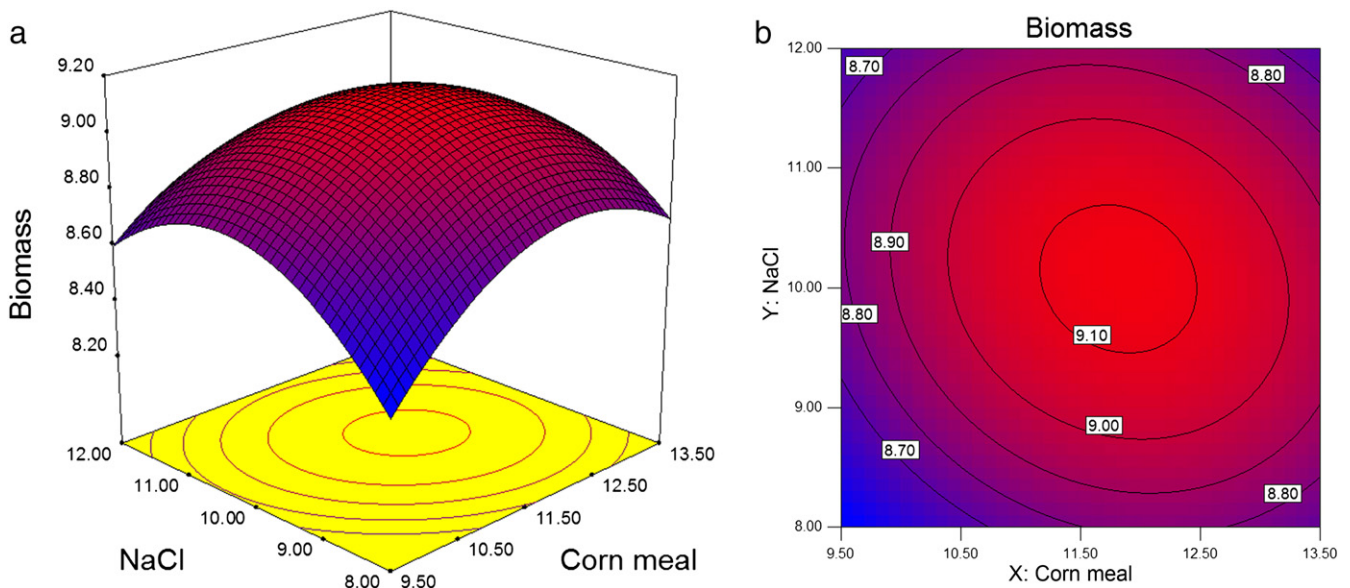


Fig. 4. Response surface (a) and contour plots (b) for the effect of corn meal and NaCl on biomass production.

11.79 g/L of corn meal, while NaCl concentration was held at the zero level (10.00 g/L) (Fig. 2); the highest biomass production of 9.153×10^8 CFU/mL could be observed at 22.80 g/L of glucose and 10.38 g/L of NaCl, and 11.50 g/L of corn meal (Fig. 3). With 11.82 g/L of corn meal and 10.07 g/L of NaCl, biomass production reached its highest value of 9.127×10^8 CFU/mL with glucose held at 25.00 g/L (Fig. 4). According to [Equation 5], we predicted that a maximum biomass production of 9.156×10^8 CFU/mL could be achieved at 22.88 g/L of glucose, 11.74 g/L of corn meal and 10.34 g/L of NaCl. The coded values of X_1 , X_3 and X_7 were -0.424, 0.118 and 0.170, respectively.

3.5. Validation of the experimental model

Our experimental conditions are presented in Table 6, along with the responses obtained. The observed biomass production, $8.60 \pm 0.10 \times 10^8$ CFU/mL, achieved a 100.00% match degree compared with the predicted values (run No. 6). Moreover, the mean value of biomass production was $8.75 \pm 0.18 \times 10^8$ CFU/mL, which was in good accordance with the predicted value of 8.81×10^8 CFU/mL (the mean match degree: 99.30%). The model showed good agreement with the experimental data, which confirmed the validity and adequacy of the model.

In conclusion, the optimal combined levels of three significant variables were obtained through BBD. Biomass production peaked at $9.13 \pm 0.29 \times 10^8$ CFU/mL with optimal medium composition, which approximately equaled the predicted value of 9.16×10^8 CFU/mL and was 57.11% higher than the biomass obtained from the initial production medium ($5.83 \pm 0.12 \times 10^8$ CFU/mL).

4. Conclusions

We conducted an investigation of the optimal medium components for liquid fermentation of the DMP-degrading strain *Bacillus* sp. QD₁₄ in shake-flask culture. A highly efficient optimization method that incorporated Plackett–Burman Design, the Steepest Ascent Method and Response Surface Methodology (BBD) was developed and utilized. Biomass production was significantly influenced by glucose, corn meal and NaCl.

The predicted values were in excellent agreement with the experimental values in validation experiments, which confirmed the accuracy of the model. The resulting fermentation biomass was 57.11% higher than the biomass obtained from the initial production medium. Furthermore, the optimization of the medium resulted in a reduced cost of medium constituents. Our optimization method proved to be effective and relatively simple and saved both time and materials.

Table 6
Validation of the model and results of the confirmatory experiments.

Runs	Variables ^a			Biomass ($\times 10^8$ CFU/mL)		Match degree (%) ^c
	X_1	X_3	X_7	Observed	Predicted	
1	20.00 ^b	11.50	8.00	8.50 ± 0.10	8.57	99.18
2	20.00	11.50	10.00	8.93 ± 0.32	9.06	98.60
3	25.00	13.50	10.00	8.87 ± 0.15	8.95	99.07
4	25.00	9.50	10.00	8.77 ± 0.15	8.78	99.85
5	30.00	13.50	8.00	8.47 ± 0.12	8.58	98.68
6	25.00	9.50	12.00	8.60 ± 0.10	8.60	100.00
7	22.88	11.74	10.34	9.13 ± 0.29	9.16	99.71
Ave	–	–	–	8.75 ± 0.18	8.81	99.30

^a X_1 , glucose; X_3 , corn meal; X_7 , NaCl.

^b Units g/L.

^c Match degree (%) = (observed value / predicted value) \times 100%.

Conflict of Interest Statement

No conflict of interest exists in the submission of this manuscript, and the manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejbt.2015.03.013>.

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