Electronic Journal of Biotechnology 18 (2015) 244–251

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Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Enhanced production of dimethyl phthalate-degrading strain *Bacillus* sp. QD_{14} by optimizing fermentation medium

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ARTICLE INFO

Article history:
Received 15 November 2014
Accepted 20 February 2015
Available online 11 April 2015

Keywords:
Bacillus
Box–Behnken design
Dimethyl phthalate
Medium optimization
Plackett–Burman design

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. QD_{14} 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. QD_{14}. A 57.11% enhancement of the biomass production was gained after optimization in shake-flask cultivation. The biomass production of *Bacillus* sp. QD_{14} 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. QD_{14} 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.

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 disrupters, 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. QD_{14}.

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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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.03.013

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. QD_{14}. 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. QD_{14} 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): K_2HPO_4, 0.5; KH_2PO_4, 0.2; NH_4NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2$, 0.1; $FeCl_2$, 0.01; NaCl, 1.0; $MnCl_2 \cdot 4H_2O$, 0.01 and $ZnCl_2$, 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. QD_{14} 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 (OD_{600}) 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, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, $CaCl_2$, 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. QD_{14}

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]:

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

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. QD_{14}) 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. QD_{14}: 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