Kor. J. Microbiol. Biotechnol.
Vol. 31, No. 1, 83–89 (2003)

Statistical Optimization for Biodegradation of 2,4-Dichlorophenoxyacetic Acid by Soil Isolated Bacterium

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2,4-Dichlorophenoxyacetic acid (2,4-D) as a widely used herbicide has caused serious environmental problems because of its difficult decomposition in nature. We isolated the strain capable of metabolizing 2,4-D as sole carbon and energy source by an enrichment culture technique from the 2,4-D contaminated soil collected at orchard in Gwangju, Korea. This strain was identified tentatively as *Aeromonas* sp. NOH2. With this strain, we established the response surface methodology (Box-Behnken Design) to optimize the principle parameters for maximizing biodegradation of 2,4-D such as culture pH, temperature, and nutrient concentration in liquid batch culture. The ranges of parameters were obtained from preliminary works done at our laboratory and chosen as 5.5, 6.5, and 7.5 for pH, 25, 30, and 35°C for temperature, and 5, 20, and 35 g/l nutrient concentration. Initial concentration of 2,4-D was 500 ppm and nutrient source was tryptic soy broth. The experimental data were significantly fitted to a second order polynomial equation using multiple regression. The most important parameter influencing 2,4-D degradation and biomass production was nutrient concentration. For 2,4-D degradation, the optimum values of pH and temperature, and nutrient concentration were obtained at pH (6.5), temperature (31.8 to 32.1°C), and nutrient concentration (29.6 to 30.1.0 g/l).

Key words: 2,4-dichlorophenoxyacetic acid, response surface methodology, *Aeromonas* sp. optimization, Box-Behnken

The pollution of soil and water by chlorinated aromatic compounds used as herbicides, pesticides, preservatives, solvents, and lubricants is a problem of increasing environmental concern [13]. Of these compounds, 2,4-dichlorophenolacetic acid (2,4-D) is a widely used herbicide. It is leached easily from soils and sediment components, being frequently detected in surface waters and ground water [1]. This compound causes serious environmental problems because it is difficult to be decomposed in nature. The toxicity of 2,4-D has been reported that the exposure of human causes acute congestion of all organs, degenerative changes in the ganglionic cells of the central nerve system, and Hodgkins disease [10,15]. The 2,4-D is also a carcinogen causing soft-tissue sarcoma in humans and malignant lymphoma in digs [10].

Since 2,4-D is a recent origin compound, the characteristics of catabolism of this compound are sure of an interesting field. The degradation of 2,4-D by microorganisms has been studied intensively at the biochemical, genetic, and ecological level [17]. However, there are a few reports about the process optimization of 2,4-D degradation, even so, the most have been focused on soil-oriented places [3, 12,16]. In general, process optimization can be approached either empirically or statistical methods. The latter gives more complete information for process optimization because experimental design techniques can provide statistical models showing the relative influence of various factors. In our study, response surface methodology was applied to optimize abiotic factors; culture pH, temp, and nutrient concentration for degradation of 2,4-D in the liquid batch culture.

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Materials and Methods

Bacterial Isolation and Culture Conditions

The strains capable of metabolizing 2,4-D as sole carbon and energy source were isolated by an enrichment culture technique. The soil samples were collected several different plots of the orchard located at Gwang-Ju city in Korea. Samples (ca. 1 g) were aseptically transferred into 250 ml flasks containing 50 ml of sterilized mineral medium. Mineral medium consisted of 4 g KH_2PO_4, 1 g Na_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$. $7H_2O$, 0.003 g $FeSO_4$. $7H_2O$, 0.003 g $ZnSO_4.7H_2O$, 0.1 g $MnSO_4.H_2O$, 0.1 g $MnSO_4.H_2O$, and 0.025 g $CaCl_2$ per liter of deionized water and adjusted to pH 7.0. Five ml aliquots from each soil suspension were transferred to new 250 ml culture flasks containing 45 ml of sterile mineral medium containing 500 ppm 2,4-D. Flasks were incubated on shaker with culture conditions of 30°C and 200 rpm. After growth for 48 h, 5 ml from each culture was again transferred to a fresh medium. This work was repeated until consistent growth observed. The culture purity was determined by streaking the culture suspension on tryptic soy agar (TSB; Difco Laboratories, Detroit, USA). Pure cultures were isolated by repeated streaking the cultures onto Petri-dish containing mineral medium agar plus 500 ppm 2,4-D.

For inoculum preparation, several loopfuls of colonies from the stock cultures were aseptically transferred into culture tubes (25×150 mm) containing 10 ml of mineral medium and TSB containing 500 ppm of 2,4-D, respectively. The culture tubes were incubated in a shaker at 30°C and 200 rpm for 48 h. Five ml of culture was transferred into 250 ml culture flasks containing 45 ml of mineral medium containing 500 ppm 2,4-D and 0.5% TSB (w/v).

Identification of selected bacteria

The bacteria showing to degrade prominently 2,4 D was selected finally and identified by the API 20NE identification system (Analytab Products, Plainview, USA). These results indicated that isolated strain was *Aeromonas* sp. and designated as *Aeromonas* sp. NOH2.

Experimental design

We established a Box-Behnken Design to optimize the parameters such as culture pH, temperature, and nutrient concentration for biodegradation of 2,4-D [2]. This optimization process basically involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response, and checking the adequacy of the model. Suppose we code the levels in standardized units so that the values taken by each of the three variables X_1 X_2, and X_3 are -1, 0, and +1. Coded values were obtained by the following formula:

$$Z = (X - X^{o})/\Delta X$$

Where Z is the coded values, X is the corresponding natural value, X^{o} is the natural value in the center of the domain, and ΔX is the increment of X corresponding to one unit of Z. For each factor, three levels were given and a second order was proposed. Data were analyzed by multiple regressions to fit the following second order equation:

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=j=1}^{k} B_{ij} X_i X_j$$

Where, Y = predicted response, it can be observed that three variables are involved and hence k takes the value 3. Thus, by substituting the value 3 for k, the equation becomes:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{12}X_2X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2,$$

Where, Y is predicted response, X_1, X_2, and X_3 are input variables (pH, temperature, and nutrient concentration); B_0 is a constant; B_1, B_2, and B_3 are linear coefficients; B_{12}, B_{13}, and B_{23} are cross product coefficients; B_{11}, B_{22}, and B_{33} are quadratic coefficients. The low, middle, and high levels of each variable, namely pH, temperature, and nutrient concentration that are coded as -1, 0, and +1 respectively are given in Table 2. Design Expert (Couresy: Stat-ease Inc., Statistics Made Easy, Minneapolis, USA) was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The goodness of fit of the model was given by the coefficient of determination R^2.

Experimental set up

The low, middle, and high levels for variables were based on a prior screening done at our laboratory and accordingly, 5.5, 6.5, and 7.5 were chosen for the variable X_1 (pH); 25, 30, 35°C for X_2 (temperature), and 5, 20, 35 g/l for X_3 (nutrient concentration) as shown in Table 2. Complex media TSB was used for nutrient source. Initial concentration of 2,4-D was 500 ppm. The fermentation was carried out in 250 ml culture flasks containing 50 ml of medium with agitation speed of 200 rpm for 48 h.

Analysis

The growth of *Aeromonas* sp. strain NOH2 was determined by measuring the biomass, following the method of Otts and Day (1987). Values of optical density at 660 nm were converted into the weight of dried biomass; an absorbance of 4.0 was equal to 4.52 mg/ml dry weight of bacteria. To extract 2,4-D, cells were removed by centrifuging the culture broth. The supernatant was acidified to pH 2.5 with 5% (v/v) aqueous HCl and extracted with ethyl acetate. The ethyl acetate layer was transferred to clean amber vials equipped with teflon-lined caps and stored at 20°C until analyzed. The 2,4-D was assayed by a spectrophotometer at 280 nm. The amount of 2,4-D was estimated by a calibration curve.

Results and Discussion

About 10 bacteria capable of utilizing 2,4-D as a sole carbon and energy source on mineral medium agar plates were preliminary isolated. Four strains that showed notable difference in their morphology on trytic soy and mineral media agar plate were selected and designated as NOH1, 2, 3, and 4, respectively. Finally we choose NOH2 that showed the fastest and balanced growth at relatively higher 2.4-D concentration. NOH2 strain was tentatively identified it as *Aeromonas* sp. based on its biochemical characteristics (Table 1). *Aeromonas* sp, a nonspore forming, Gram negative that was also catalase and oxidative positive, is first strain in microbial groups that are capable of utilizing 2.4-D as the sole carbon and energy sources. Previous studies have reported numerous microorganisms that undergo the degradation of 2,4-D. *Alcaligenes eutrophus* [5,6], *Pseudomonas putida* [4,7], *Pseudomonas cepecia* [8], and *Sphingomonas* sp. [9] have been extensively studied for mechanism of 2,4-D degradation at molecular gene level.

With *Aeromonas* sp. NOH2, we investigated to optimize the conditions for 2,4-D degradation, aiming at applying it to the slurry phase bioremedation. Several criteria for bioremediation of 2,4-D was considered in the selection of parameters such as the pH, temperature, and nutrient concentration in or on site application (Table 2). With the objective of understanding the effects of major parameters pH, temperature, and nutrient concentration on biomass production and 2,4-D degradation, the present experiments were performed by a statistically designed experimental plan (Box-Behnken). The model was evaluated the effect of each independent variable on a response either singly or in combination with other variables. Coded levels were established based on preliminary experiments and prior information. Degree of degradation of 2,4-D and biomass production and were dependent variables. The experimental design and the values of responses of two dependent variables against pH, temperature, and nutrient concentration were listed in

Table 1. Taxonomic characteristics of *Aeromonas* sp. strain NOH2.

Characteristics	Description
Gram stain	–
Enzymatic reaction	
Catalase	+
Oxidase	+
Reduction of nitrate	+
Urease	+
β-glucosidase	+
Protease	+
β-galactosidase	–
Arginine dihydrolase	–
Indole production	+
Glucose acidification	+
Carbon assimilation	
Glucose, arabinose, mannose, N-acetyl-glucosamine	+
maltose, gluconate, malate, citrate, phenyl-acetate	
Caprate, adipate	–

+ Positive reaction. - Negative reaction.

Table 2. The experimental design and resulting responses for Box-Benken design response surface analysis.

Samples	Variables			pH	Temp. (C°)	Nutrient (g/l)	Biomass (g/l)	Degradation (%)
	X_1	X_2	X_3					
1	-1	-1	0	5.5	25	20.0	2.40	27.1
2	1	-1	0	7.5	25	20.0	3.20	35.9
3	-1	1	0	5.5	35	20.0	3.48	38.9
4	1	1	0	7.5	35	20.0	7.89	58.9
5	-1	0	-1	5.5	30	5.0	1.92	1.93
6	1	0	-1	7.5	30	5.0	2.10	6.90
7	-1	0	1	5.5	30	35.0	4.70	56.7
8	1	0	1	7.5	30	35.0	5.89	46.7
9	0	-1	-1	6.5	25	5.0	1.20	8.14
10	0	1	-1	6.5	35	5.0	2.70	6.70
11	0	-1	1	6.5	25	35.0	3.50	48.0
12	0	1	1	6.5	35	35.0	6.10	68.0
13	0	0	0	6.5	30	20.0	6.28	59.7
14	0	0	0	6.5	30	20.0	5.73	62.7
15	0	0	0	6.5	30	20.0	6.52	61.3
16	0	0	0	6.5	30	20.0	5.54	55.5
17	0	0	0	6.5	30	20.0	5.84	54.2

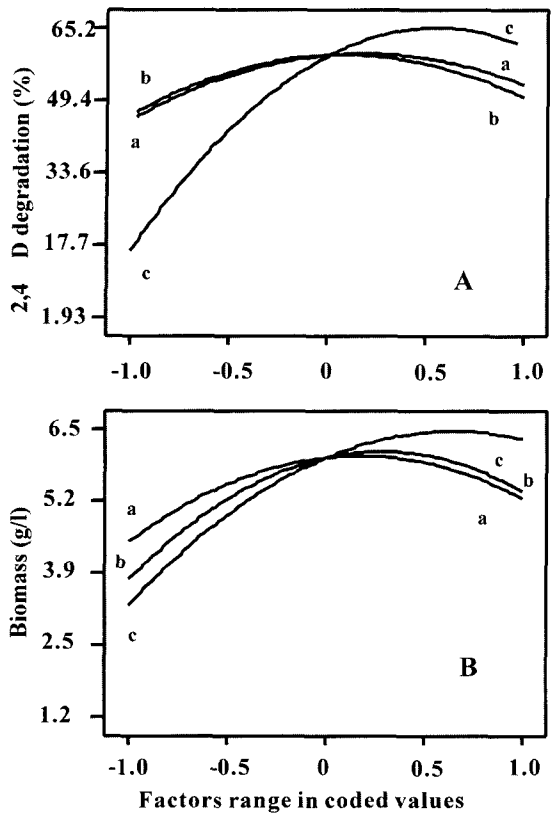

Fig. 4. Contour plot representing 2,4-D degradation (%) (A) and biomass production (B) with reference to each variable. a, b, and c indicate the pH, temperature, and nutrient concentration, respectively.

a relatively more alkaline pH 6.8 and 7.0, respectively. The shapes of the response surfaces, circular or elliptical, indicate that the interaction between the variables is significant or not. The elliptical nature of the contour plots among the Fig. 1 A, B, and C indicates that the mutual interactions between these set of variables has a significant effect on the degradation of 2,4-D. A maximum biomass was obtained at pH 7.5, 35°C, and 20% nutrient concentration (Table 2), but maximum biomass (6.13 to 6.65) by statistically valued by Design-Expert was at pH (6.7 to 6.8), temperature (31.8°C), and nutrient concentration (30.5 to 31.0%) as shown in the Fig. 2A, B, and C.

The trace plot was used to assess their effect of each parameter graphically. At the coded values -1, 0, +1, the effect of pH, temperature, and nutrient concentration on the biomass production and 2,4-D degradation has been pronounced as Fig. 4 A for 2,4-D degradation and B for biomass production. With respect to 2,4-D degradation, changes in pH and temperature have not been a significant criterion compared to that in nutrient concentration. Drastically change of 2,4-D degradation and biomass production was observed at coded values of nutrient concentration from 1 to -1. This indicates that nutrient concentration is most significant parameters for both increases. Meanwhile, a slight change was found observed at coded value 0 of pH and temperature.

Acknowledgment

This work was supported by a Korean Research Foundation Grant (YOO316).

국문초록

토양 분리 박테리아에 의한 2,4-Dichlorophenoxyacetic산의 분해 최적화

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2,4-D는 자연에서 잘 분해되지않아 환경에 대하여 심각하게 문제를 일으키지만 널리 사용되고 있는 제초제이다. 이 연구는 광주광역시 인근 지역 과수원에서 분리한 균주 중 가장 높은 2,4-D 분해 활성을 보이는 균주를 선정하여, 동정한 결과 *Aeromonas* sp.로 판명된 균주를 가지고 표면 반응법(Response Surface Methodology)을 이용하여 2,4-D 분해를 최대화하기 위한 중요한 변수들인, pH, 온도, 영양분의 농도들의 최적조건을 고찰하였다. 2,4-D분해에 많은 영향을 미치는 변수는 영양분의 농도로 밝혀 졌으며, 통계 분석결과 2,4-D분해 최적 조건은 pH 6.5, 온도 31.8-32.1°C, 영양분 29.6-30.1 g/l로 밝혀 졌다.

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(Received Feb. 27, 2002/Accepted Dec. 7, 2002)