Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. 84 (7) 679–688 (2019)
JSCS–5218

UDC 547.562+504.4.054:582.261:
542.92.000.57
Original scientific paper

Optimization of phenol biodegradation by immobilized *Bacillus subtilis* isolated from hydrocarbons-contaminated water using the factorial design methodology

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(Received 4 December 2018, revised 8 March, accepted 11 March 2019)

Abstract: The ability of newly isolated bacteria, identified as *Bacillus subtilis* immobilized on alginate hydrogel beads, to degrade phenol was investigated under different parameters, such as phenol concentration, bead diameter and inoculums size, and was optimized using full factorial design methodology. A mathematical model that governs the degradation of phenol by the immobilized system was obtained and it fitted the experimental data very well. The model indicated that within the range of variables employed, all the parameters and their interactions influenced the biodegradation process, whereby the phenol concentration was the most significant factor. *B. subtilis* revealed a very high degradation activity and could be grown using phenol as the sole source of carbon. Phenol was degraded by the new bacteria in 8 h under the optimum conditions obtained by the desirability function: 100 mg L^{-1} phenol concentration, 3 mm beads diameter and 244.5 mg of cell dry per liter biomass size, with a desirability value of 91.25 %.

Keywords: alginate beads; biodegradation; immobilized system; phenol.

INTRODUCTION

Environmental pollution is one of the major problems and the most important in the world. The development in agriculture, energy sources, and chemical industries is necessary in order to fulfill the needs and demands of the ever-growing human population. Almost all processes employed by man for the production of goods and services lead to the production of environmental pollutants.[1,2]

Several physico–chemical methods for removing organic pollutants have been proposed, but these methods are not cost effective for large-scale applications. Nowadays, biological methods are widely used as low-cost alternative treatment methods, which offer the possibility of complete mineralization of organic compounds.[3]

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https://doi.org/10.2298/JSC181204022H

Some of these pollutants are biologically recalcitrant and inhibitory organics, which greatly reduces the ability of microorganism to biodegrade the compounds during treatment or in nature.[4,5] For this reason, the use of degradation of the pollutants by free system is not preferable.

Immobilized systems in which cells are entrapped in polymeric matrices[6] have received increasing attention in the field of contaminant treatment and may be an effective and economical technique. Immobilization provides higher cell concentrations, ease of use in continuous reactors, shorter start-up period for the bioreactor, and greater stability by protecting cells from direct exposure to toxic compounds compared with freely suspended cells and hence they can be stored for long periods without losing their degradative abilities.[6–8]

The rate and efficiency of the biodegradation of pollutants by immobilized systems depend on several factors. Among these parameters are initial concentration of pollutant, bead diameter and biomass size.[9,10]

The factorial experimental design has been used for studying, developing and optimizing a wide range of engineering systems.[11,12] Factorial designs are widely used to investigate the effects and influence of experimental factors and the interactions between them.[12] The advantages of factorial experiments include the relatively low cost, a reduced number of experiments, and increased possibilities to evaluate interactions among the variables.[12,13]

In this study, the biodegradation of phenol by immobilized system using a newly isolated bacteria strain (isolated and identified in the authors' laboratory), was optimized by application of a factorial design methodology. This mathematical method was used to determine the main and the interactive effects of different variables on the immobilized system and to predict a model that evaluates the process.

EXPERIMENTAL

Culture media

The growth medium (GM) of the strain consisted of peptone, 10.0 g L^{-1}; beef extract, 3.0 g L^{-1} and NaCl, 5.0 g L^{-1}, at a pH of 7.0, autoclaved at 121 °C for 15 min.

The mineral culture medium (MM) that contained the necessary nutrients for the growth of microorganisms was composed of: KH_2PO_4, 1.5 g L^{-1}; K_2HPO_4, 0.5 g L^{-1}; NaCl, 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}; NH_4NO_3, 3.0 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 0.02 g L^{-1} and $CaCl_2 \cdot 2H_2O$, 0.02 g L^{-1}.[8] The MM was used for the isolation and the biodegradation tests with phenol as the sole carbon and energy source. Solidified with agar, this medium (MMA) was used for preservation of the strains.

The nutrient agar (NA) contained 28 g of powdered nutrient agar dissolved in 1 L of distilled water and autoclaved at 121 °C for 15 min.

Isolation, selection and purification of new bacterial strains

Samples of water contaminated by hydrocarbons were collected from the petroleum refinery situated at Hassi Massoud, south of Algeria.

The contaminated water was inoculated on agar mineral culture medium (MMA) supplemented with 100 mg L^{-1} of phenol and incubated for 7 days. The development of colonies was

monitored every day. Well-isolated colonies were subcultured on GM. This procedure was repeated several times. Phenol tolerant bacterial strains were preserved on MMA at 4 °C. The purity of the cultures was regularly checked during preservation.

Identification and characterization of the isolated strain

The genera and/or species of the most active selected strain were identified by performing Gram staining, morphological characteristics and biochemical tests (using API system tests).

Dry-cell-weight determination

Inoculums size was measured using a spectrophotometer (Shimadzu UV–Vis 1240) at 600 nm and the concentration, expressed in cell dry weight, were determined from previously established curves.

Phenol dosage

Phenol was quantified by a colorimetric method based on the condensation of 4-aminoantipyrine with phenol in the presence of an oxidizing agent, potassium ferricyanide, in an alkaline medium to give a red complex.[14,15] The absorbance was measured at 510 nm using a Shimadzu UV–Vis 1240) spectrophotometer.

Immobilization of bacteria on calcium alginate beads

First, the pure strain was cultivated into 100 mL of GM at 37 °C. Bacterial cells were then harvested at the stationary phase by centrifugation (6000 rpm at 4 °C for 10 min), washed three times with the phosphate buffer solution and suspended in sterile MM.

The bio-beads of calcium alginate were obtained by inclusion of microorganisms in sodium alginate and then application of the extrusion technique. This method involves the preparation of an aqueous solution of sodium alginate (3 mass%) in which microorganisms were incorporated.

The suspension containing the cells with the sodium alginate solution was extruded as drops into 100 mL of a 0.1 M calcium chloride solution. The formed beads were transferred into a solution of 0.2 M $CaCl_2$ and incubated at 37 °C for 2 h to allow for complete replacement of the sodium ions by calcium to ensure a better stability. After washing with sterile distilled water, the beads were stored at 4 °C for preservation.[3]

Biodegradation tests

Biodegradation tests by the immobilized system were performed in a batch bioreactor at 37 °C under aerobic conditions. A control test without bacteria allowed for verification of whether the phenomenon of adsorption or other interactions between the alginate beads and the phenol occurred. The reduced phenol was less than 2 %, which is the result of the chemical method used for the determination of phenol.

Factorial design methodology

Factorial design methodology was used to determine the main and interactive effects of different variables on the immobilized system and to predict a model to evaluate the process. The principle steps of factorial designed experiments are determination of the response that reflects the aim of the study, factors and their levels and choice of the experimental design and statistical analysis of the data. A full 2^3 factorial design was performed to evaluate the importance of three factors: the initial phenol concentration (X_1), the diameter beads (X_2), and the inoculums size expressed in dry cell weight (X_3) on the response (Y), which is the time, expressed in h, required for total phenol removal. The number of experiments conducted is considered as 2^3.

The high and low levels defined for the 2^3 factorial design are listed in Table I. The low and high levels for the factors were selected according to some preliminary experiments.

TABLE I. Factors and levels used in the factorial design

Independent variable	Coded variables level		
	Low (–1)	Center (0)	High (+1)
X_1 concentration of phenol, mg L^{-1}	100	500	900
X_2 diameter beads, mm	3	4	5
X_3 dry cell weight, mg L^{-1}	244.5	317.7	390.9

The results were analyzed with JMP® 8 software, and the main effects and interactions between factors were determined.

RESULTS AND DISCUSSION

Isolation and selection of phenol degrading strains

Ninety bacterial strains were isolated and purified from contaminated waters. Among these strains, 44 were able to degrade phenol. Of these 44 strains, only 7 started growth from the second day. The rest of the strains started development from the third day. The period of appearance of the colonies constitutes a lag phase necessary for the adaptation of these strains to the new carbon source. The faster the growth of the strain, the better its adaptation to the pollutant. For this reason, one strain taken of the seven most active strains was chosen for the study.

Identification and characterization of phenol degrading strain

The identification and characterization of the isolated bacterial was performed using preliminary analysis: morphological and cultural characteristics (Table S-I of the Supplementary material to this paper) and biochemical tests (Table S-II of the Supplementary material).

The identification of the strain by the preliminary analysis confirmed that the strain was *B. subtilis* species.

Biodegradation of phenol by the immobilized bacteria

Factorial design methodology. The design matrix of the coded values for factors and the response Y, measured in each factorial experiment, is shown in Table II. The response represents the time for total degradation.

The data listed in Table II indicate a wide variation in the response Y, from 8 to 103, in the 10 trials.

Application of factorial design methodology as an optimization technique requires the selections of a model at the beginning. The model employed for 2^3 factorial designs with interaction is represented by Eq. (1):[16–18]

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + \\ + a_{123}X_1X_2X_3 \quad (1)$$

TABLE II. Experimental values for the total time of degradation

Experiment number	X_1	X_2	X_3	Y (Response)
1	–1	–1	–1	08
2	+1	–1	–1	78
3	–1	+1	–1	09
4	+1	+1	–1	75
5	–1	–1	+1	10.5
6	+1	–1	+1	77
7	–1	+1	+1	13
8	+1	+1	+1	103
9	0	0	0	46
10	0	0	0	46

In Eq. (1), Y is the estimated response that represents the time of total degradation, a_0 is the independent coefficient (a constant term), a_i (i = 1, 2, 3) are the linear coefficients for the variables: phenol concentration, bead diameter and inoculums size, respectively, a_{ij} represents the coefficients of the interaction parameters X_i and X_j with $i < j$. The coefficients a_i, a_{ij} and a_{ijk} were determined using JMP® 8 software.[17,19] Substitution of coefficient a_i, a_{ij} and a_{ijk} in Eq. (1) by their values, the mathematical model selected is the following:

$$Y = 46.55 + 36.5625X_1 + 3.3125X_2 + 4.187\,X_3 + 2.4375X_1X_2 + \\ + 2.5625X_1X_3 + 3.8125X_2X_3 + 3.4375X_1X_2X_3 \quad (2)$$

Eq. (2) showed how the experimental variables and their interactions influence the time of total degradation.[13,20] The phenol concentration (X_1) had the greatest effect on Y, followed by the inoculum size, bead diameter–inoculum size interaction (X_2X_3), phenol concentration–bead diameter–inoculum size interaction ($X_1X_2X_3$), bead diameter (X_2) and phenol concentration–bead diameter interaction (X_1X_2).

According to the obtained model, all parameters had a positive effect on the response. The positive sign shows that there is a direct relation between the parameter and the dependent variable. Thus, an increase in the phenol concentration, bead diameter or inoculums size increases the time of total degradation.

The predicted values *vs*. the experimental values of the time of total degradation of phenol are shown in Fig. 1. The model presented a high square correlation coefficient (R^2) of 99.993 % and an adjusted square correlation coefficient (R^2 adj) of 99.9697 %, which confirmed that the chosen of 2^3 factorial design with the interaction model was found to be adequate for the prediction within the range of variables employed.[18,21–23]

The Student's t-*test*

The significance of the regression coefficients was determined by applying a Student's *t*-test (Table III).[13,16]

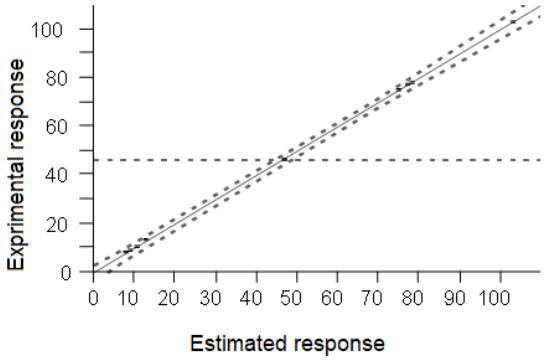

Fig. 1. Comparison of the experimental and the estimated responses at the levels of the variable.

TABLE III. Estimated regression coefficients for the time of total degradation

Parameter	Estimate coefficients	Standard error	*t*-value	*P*-value[a]
Constant	46.55	0.194454	239.39	<.0001*
X_1	36.5625	0.217407	168.18	<.0001*
X_2	3.3125	0.217407	15.24	0.0043*
X_3	4.1875	0.217407	19.26	0.0027*
X_1X_2	2.4375	0.217407	11.21	0.0079*
X_1X_3	2.5625	0.217407	11.79	0.0071*
X_2X_3	3.8125	0.217407	17.54	0.0032*
$X_1X_2X_3$	3.4375	0.217407	15.81	0.0040*

[a]*P*-value < 0.05

The Student's *t*-test and *P*-value were used to determine the significance of the regression coefficients of the parameters. With the 95 % confidence level and two degrees of freedom, the value of *t*-critic is equal to 2.92. The coefficient of the regression is statistically significant if the corresponding *t*-value is higher than 2.92. It was observed from Table III that the linear effects of phenol concentration, bead diameter, and inoculum size and the interaction effect between these factors were significant. The values for each effect are shown in the Pareto chart by the horizontal columns (Fig. 3).

The importance of the data can also be judged by their *P*-value, which is the probability value that is used to determine the statistically significant effects in the model, with values closer to zero denoting greater significance. For the 95 % confidence level, the *P*-value should be less than or equal to 0.05 for the effect to be considered statistically significant.[24,25] Hence, the final model is given in Eq. (3):

$$Y=46.55+36.5625\,X_1 + 3.3125\,X_2 +4.1875\,X_3 + 2.4375\,X_1X_2 +2.5625X_1X_3 + 3.8125\,X_2X_3 +3.4375X_1X_2X_3 \quad (3)$$

Interaction plots

The plots of the interaction effects are shown in Fig. 2. An interaction is effective when the change in the response from low to high levels of a factor is dependent on the level of a second factor, *i.e.,* when the lines do not run parallel.[13] Thus, significant interactions between the parameters could be concluded.

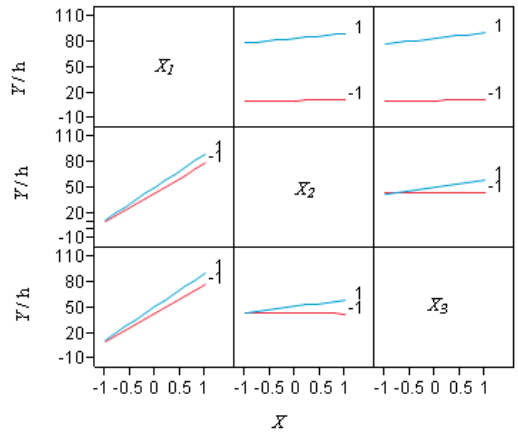

Fig. 2. Interaction plot for the time of total degradation (X_1: phenol concentration, mg L^{-1}; X_2: diameter of beads, mm; X_3: dry-cell weight, mg L^{-1}).

The standardized Pareto chart (Fig. 3) depicts the main effect of the independent variables and interactions on the time of total degradation. The length of each bar in the graph indicates the effect of these factors and the level of their effects on the response.[26,27]

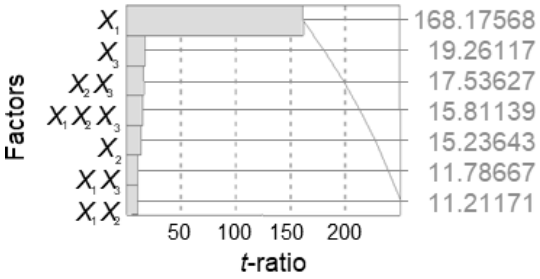

Fig.3. Pareto chart of the standardized effects.

This graphic (Fig. 3) shows both the magnitude and the importance of the effects (variables and interactions).[26]

According to Fig. 3, it could be inferred that the main factors (X_1, X_2 and X_3) and their interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) were significant at the 0.05 level. The concentration of phenol represents the most significant effect on the time of total degradation.

Analysis of variance (ANOVA)

The adequacy of the model was checked using analysis of variance (ANOVA), as shown in Table IV.[18,23]

TABLE IV. Analysis of variance (ANOVA)

Source	Sum of squares	*DF*	Mean square	*F*-value	*t-F*
Model	11233.469	7	1604.78	4244.5	19.4
Residual (error)	0.756	2	0.38	*Prob* > *F*	
Correlation total	11234.225	9		0.0002	

The *F*-value of the model obtained (4244.5) was higher than the tabular value *t-F* ($F_{0.05,5,4}$ = 19.4), suggesting that the model is highly significant.

Estimation of optimal design conditions by the method of desirability function.

The optimization by the desirability function improved the performance of the analytical process and discovering the conditions at which the best response is obtained.[28]

The optimum condition for the degradation of phenol by the immobilized process was obtained from the desirability plot (Fig. 4).

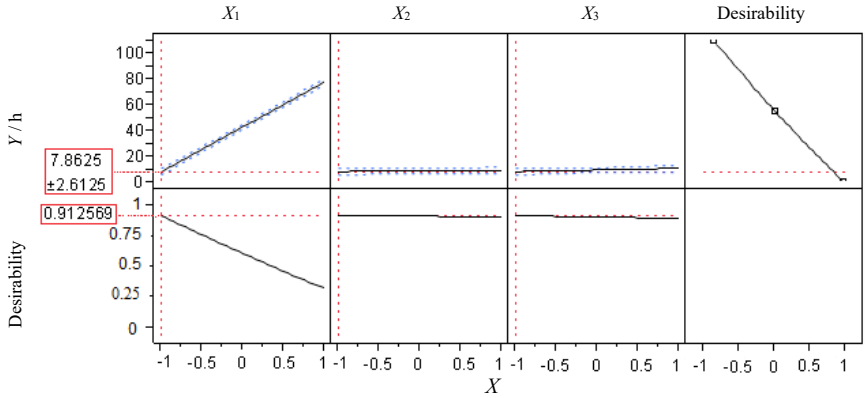

Fig. 4. Desirability functions for the optimization of the response (X_1: phenol concentration, mg L^{-1}); X_2: bead diameter, mm; X_3: dry-cell weight, mg L^{-1}).

The best combination of factor settings for achieving the optimum response (minimum time of total degradation) was found to be phenol concentration 100 mg L^{-1}, bead diameter 3 mm and inoculums size 244.5 mg L^{-1} for the predicted response of 7.86 h and a desirability value of 91.25 % (Fig. 4).

CONCLUSIONS

In conclusion, a new bacterial strain capable of degrading phenol was isolated from hydrocarbons contaminated water, collected from the petroleum refin-

ery, situated in the south of Algeria. It was identified as *B. subtilis* species based on morphological and cultural characteristics and biochemical tests

The *B. subtilis* species presented a very high activity for phenol biodegradation. The best time of complete degradation by immobilized cells determined using factorial design methodology was 7.86 h, which corresponds to the optimum conditions: phenol concentration 100 mg L^{-1}, bead diameter 3 mm and biomass size 244.5 mg L^{-1}. The concentration of phenol represented the most significant effect on the total time of degradation.

Full factorial design methodology can be an efficient method for testing the effect of operating conditions and optimize the degradation of phenol by the studied immobilized process.

SUPPLEMENTARY MATERIAL

Additional data are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

ОПТИМИЗАЦИЈА БИОДЕГРАДАЦИЈЕ ФЕНОЛА ИМОБИЛИЗОВАНОМ БАКТЕРИЈОМ *Bacillus subtilis* ИЗОЛОВАНОМ ИЗ ВОДЕ КОНТАМИНИРАНЕ УГЉОВОДОНИЦИМА ПРИМЕНОМ МЕТОДЕ ФАКТОРИЈАЛНОГ ДИЗАЈНА

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Испитана је примена новоизоловане бактерије *Bacillus subtilis*, имобилизоване на честицама алгинатног хидрогела, за деградацију фенола, варирајући концентрацију фенола, пречник честица и величину инокулума, а за оптимизацију је коришћена метода потпуног факторијалног дизајна. Израђен је математички модел разлагања фенола имобилизованим системом и он се добро уклапао са експерименталним резултатима. Према моделу, све промењиве које су тестиране, као и њихов примењени опсег, су утицале на процес биодеградације, а концентрација фенола је била најутицајнији фактор. Показано је да *B. subtilis* испољава јаку деградациону активност и да може расти користећи фенол као извор угљеника. Ова бактерија разлаже фенол у року од 8 h са 91,25 % ефикасности, под следећим оптималним условима: концентрација фенола 100 mg L^{-1}, пречник честица 3 mm и 244,5 mg сувих ћелија по литру биомасе.

(Примљено 4. децембра 2018, ревидирано 8. марта, прихваћено 11. марта 2019)

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