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ORIGINAL PAPER

Statistical medium optimization and biodegradative capacity of *Ralstonia eutropha* toward *p*-nitrophenol

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Abstract The effect of *p*-nitrophenol (PNP) concentration with or without glucose and yeast extract on the growth and biodegradative capacity of *Ralstonia eutropha* was examined. The chemical constituents of the culture medium were modeled using a response surface methodology. The experiments were performed according to the central composite design arrangement considering PNP, glucose and yeast extract as the selected variables whose influences on the degradation was evaluated (shaking in reciprocal mode, temperature of 30°C, pH 7 and test time of about 9 h). Quadratic polynomial regression equations were used to quantitatively explain variations between and within the models (responses: the biodegradation capacity and the biomass formation). The coefficient of determination was high ($R^2_{\text{adjusted}} = 0.9783$), indicating the constructed polynomial model for PNP biodegradative capacity explains the variation between the regressors fairly well. A PNP removal efficiency of 74.5% occurred within 9 h (15 mg/L as the initial concentration of PNP with use of yeast extract at 0.5 g/L).

Keywords *Ralstonia eutropha* · Biodegradation · *p*-nitrophenol · Central composite design · Yeast extract · Glucose

Abbreviations

PNP	*p*-nitrophenol
LC	Liquid culture
RSM	Response surface methodology
CCD	Central composite design
NAs	Nitroaromatics
NADPH	Nicotinamide adenine dinucleotide phosphate

Introduction

Environmental pollutants pose a real danger to human health, mainly due to their carcinogenic and mutagenic potential. The wide distribution of nitroaromatic compounds in the chemical and processing industries has intensified the study of the degradation and removal of these substances from the environment. The use of microbial metabolic potential for the removal of pollutants has been shown to be a safe and economically efficient method as compared to other available physicochemical techniques (Shen

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et al. 2009; Schmidt et al. 1987). *p*-nitrophenol (PNP) takes priority in the lists of organic pollutants outlined by the United States Environmental Protection Agency (limitation <10 ng/L in natural waters) (Wan et al. 2007). The diverse group of phenolic derivatives originating from chemical substitution in the molecular structure is indicative of the activity of different enzymes that have been appearing throughout biological evolution. As a result of this evolution, the capability of the microbe(s) in biotreating organic pollutants has been extended (Kulkarni and Chaudhari 2006). However, the specificity of these identified microbial enzymes toward the refractory substrates would be widely different (Bhushan et al. 2000a, b; Gemini et al. 2005; Heitkamp et al. 1990; Shinozaki et al. 2002). *Ralstonia eutropha* (formerly named *Alcaligenes eutrophus*) is a Gram-negative bacterium recognized for its efficiency in degrading a wide variety of aromatic pollutants, especially nitroaromatics (NAs) (Muller and Babel 1996). Four specific molecular mechanisms have been suggested for the mineralization of homocyclic NAs by microbial systems: an initial oxygenation reaction and production of NO_2^-; an initial reduction, yielding aromatic amines; a complete reductive reaction with removal of the NO_2^- group; or a partial reduction of the nitro group, yielding hydroxyl amines, followed by replacement reactions. Some bacteria that are able to use NAs as a source of nitrogen by oxygenolytic removal of the nitro group may not completely degrade these compounds; oxidative removal of NO_2^- from PNP, yielding nitrate, has been shown in various bacteria such as *R. eutropha* (Marvin-Sikkema and Bont 1994; Ecker et al. 1992). However, degradation of NAs by several microbes can occur by initially reducing the nitro group to an amine through the use of nitroreductase, which requires an NADPH coenzyme. This reduction reaction proceeds via a nitroso group and a hydroxyamino group. Further degradation of the resulting amino-aromatic product yields ammonium and catechol. The mineralization is continued by a ring-cleavage reaction (Marvin-Sikkema and Bont 1994; Heitkamp et al. 1990).

Response Surface Methodology (RSM) is a sequential statistical procedure that allows one to make a decision regarding the vicinity of the optimum response by performing a series of experiments and measurements (Sharma et al. 2009). Central composite design (CCD), which is frequently used in RSM, shows major gains over the classical approach in terms of the information gained and the accuracy of the conducted experiment (Ahmadi et al. 2006). Obtaining informative results depends on finding effects between key parameters of the particular process and the joint effects of these controllable variables. To achieve this, residual plots of the CCD are used to check the uniformity of the error distribution and the adequacy of the proposed model (Xiarchos et al. 2008). The regression model obtained from the least squares technique provides a reasonable explanation for the effect of each individual factor and for all possible relationships that exist between the dependent and independent variables (Vining 2003).

The objectives of the present work were mainly focused on the development and evaluation of a statistical RSM model to explain the quantitative and qualitative relationships that may exist between some of the chemical constituents selected (i.e., independent variables) and on the preparation of a culture medium to study the growth and degradative ability of *R. eutropha* toward PNP (i.e., dependent or response variables). In CCD modeling, the selection of the ranges for these chemicals needs to be precise; otherwise, the optimal condition for the biodegradation, in terms of the medium composition, cannot be found inside the experimental region through the analyses of statistics and contour plots. The results are discussed in terms of the secondary substrate utilization effect on the degradative ability of *R. eutropha.*

Materials and methods

Microorganism and cultivation medium

Ralstonia eutropha was supplied by Japan Collection of Microorganisms (JCM) (accession number of the strain was JCM 20644 in 2007). The liquid culture medium (LC) was prepared according to the description given elsewhere; Table 1 shows the necessary details (Muller et al. 1999).

Chemicals

PNP was provided by Kanto Chemical Company (Japan). All chemicals used in this study were of analytical grade with the highest purities.

Table 1 Culture medium used in the present study

Minerals	Concentration (mg/L)
KH_2PO_4	310
K_2HPO_4	310
NH_4Cl	760
$MgSO_4.7H_2O$	71.2
$ZnSO_4.7H_2O$	0.44
$MnSO_4{\cdot}H_2O$	0.61
$CuSO_4{\cdot}5H_2O$	0.78
$NaMoO_4{\cdot}2H_2O$	0.25
$Fe(SO_4)_2{\cdot}7H_2O$	4.98

Biodegradation studies

A LC medium containing PNP at different specified initial concentration was used in the biodegradation study. To test the effects of the supplementary substrate(s) on the degradation trend, glucose and yeast extract were added to the LC medium (sterilization of the medium was done in an autoclave at 121°C for 15 min). The ranges for these three compounds were selected according to the results obtained from the preliminary experiments. Fifty milliliters of sterile medium consisting of LC medium, PNP (5, 15 and 25 mg/L) (A), glucose (0, 0.5 and 1 g/L) (B) and yeast extract (0, 0.5 and 1 g/L) (C) was inoculated in a 250 mL Erlenmeyer flask and incubated in a reciprocal shaker at 30°C for 9 h. Table 2 shows the ranges used in this study. In each experiment, *R. eutropha* cells, grown aerobically in a reciprocal shaker at 30°C and pH 7.0 for 20 h were used as an inoculum after acclimation in PNP. The cell suspension was adjusted to an initial optical density of 0.1 at 546 nm. The time of complete degradation, when no PNP was detected by high performance liquid chromatography (HPLC analysis), was recorded to obtain the bacterium performance in the degradation experiments.

A three-level, three-factor CCD (face-centered cube CCD) was employed in this biodegradation study. The arrangement of this design is shown in Table 2. A set of 17 experimental runs, including 3 replicates at the center points, was performed in such a way that development of the appropriate empirical equations using the experimental data became possible (second-order polynomial multiple regression equations):

Table 2 Design matrix for a face-centered cube CCD ($\alpha = 1$, three-level, three-factor)

Experiment number	Coded level of variables			Actual level of variables			Responses			
	x_1	x_2	x_3	PNP (mg/L)	G (g/L)	YE (g/L)	$\hat{Y}_{D-PNP}$		$\hat{Y}_{OD_{546}}$	
							Actual	predicted	Actual	predicted
1	−1	−1	−1	5	0	0	67	73.53	0.1	0.125
2	+1	−1	−1	25	0	0	2	0.63	0.098	0.105
3	−1	+1	−1	5	1	0	86	81.03	0.099	0.125
4	+1	+1	−1	25	1	0	5	8.13	0.098	0.105
5	−1	−1	+1	5	0	1	97	93.43	0.415	0.441
6	+1	−1	+1	25	0	1	61	57.53	0.65	0.629
7	−1	+1	+1	5	1	1	100	100.93	0.474	0.441
8	+1	+1	+1	25	1	1	64	65.03	0.65	0.629
9	$-\alpha$	0	0	5	0.5	0.5	84	85.08	0.199	0.163
10	α	0	0	25	0.5	0.5	30	30.68	0.21	0.247
11	0	$-\alpha$	0	15	0	0.5	74.5	76.38	0.146	0.13
12	0	α	0	15	1	0.5	84	83.88	0.161	0.13
13	0	0	$-\alpha$	15	0.5	0	50	46.68	0.11	0.04
14	0	0	α	15	0.5	1	80	85.08	0.38	0.46
15	0	0	0	15	0.5	0.5	74.3	71.93	0.1	0.13
16	0	0	0	15	0.5	0.5	74	71.93	0.16	0.13
17	0	0	0	15	0.5	0.5	71	71.93	0.1	0.13

$$\hat{Y} = \beta_0 + \sum_{i=0}^{k} \beta_i x_i + \sum_{i=0}^{k} \beta_{ii} x_i^2 + \sum_{i=0}^{k} \sum_{j=0}^{k} \beta_{ij} x_i x_j,$$

where $\hat{Y}$ is the predicted response, k is the number of independent variables, and β_0, β_i, β_{ii}, β_{ij} are a set of regression coefficients that indicate the model constant, main effect(s), quadratic terms and interaction terms, respectively. The arrangement in Table 2 shows that the first eight treatment combinations form a 2^3 factorial design, the next six treatment combinations are referred to as the axial runs because they lie on the axes defined by the design variables, and the last three treatment combinations represent the contour runs. The "Design Expert" (version 7) and Statistica (version 7) software packages were used for regression and graphical analyses of the data.

Analytical methods

PNP quantification was performed by a high performance liquid chromatograph (HPLC system Shimadzu) with a UV detector (280 nm). The column was an Inertsil ODS-3V (150 × 4.6 mm I.D.), and the elution was acetonitrile (A) and 100 mM $NaClO_4$ (B) (pH adjustment 'pH 2.5' performed with $HClO_4$). The gradient elution program was as follows: A/B = 30/70 for 25 min, 60/40 for 10 min and 60/40 for the rest of the time. The analysis was done on the cell-free supernatants which obtained after centrifugation at 12,000 rpm for 20 min. Cell growth measurements were performed spectrophotometrically at 546 nm (Schenzle et al. 1997).

Results and discussion

Figure 1 shows the results obtained by performing some experiments to determine the PNP degradative capability of *R. eutropha* with and without the supplementary substrates, namely, glucose and yeast extract. Nitroaromatic compounds are characterized as energy deficient substrates for bacterial growth. In fact, microbes have evolved complex metabolic strategies to tolerate such different environmental conditions. As an example, the adaptive responses of *R. eutropha* to feast and famine conditions were studied using sources of carbon and energy: phenol (200 mg/L) as the poor substrate and pyruvate (2,000 mg/L) plus yeast extract (1,000 mg/L) as the growth-rich substrate (Muller et al. 1999). In the present work, the following substrates were included in the LC medium with the inoculum size of 0.1 $OD_{546\ nm}$: PNP as the sole source of carbon, nitrogen and energy (8 and 13 mg/L) (poor substrate) or PNP in combination with either glucose or yeast extract (500 mg/L) (growth rich substrate). PNP at 8 and 13 mg/L was utilized within 50–150 h with a low amount of biomass (Fig. 1). The biodegradation enhancement of a low concentration of PNP is defined as the simultaneous use of PNP with other organic compound(s) that can readily be metabolized by the test microorganism (Schmidt et al. 1987; Simkins and Alexander 1984). This secondary substrate utilization event has ecological significance, especially when one

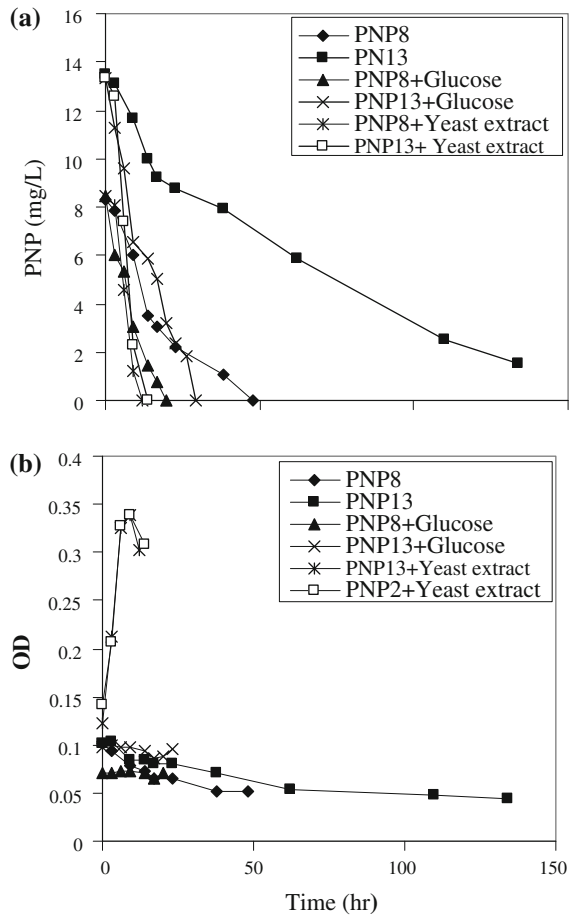

Fig. 1 PNP at two different initial concentrations (8 and 13 mg/L) was used to prepare the culture medium while either glucose or yeast extract had been added as the supplementary substrate (**a**). Trend of OD_{546} as an index for the growth determination of *R. eutropha* (**b**)

focuses on the carbon-limited situation of the natural environment. For instance, studies on the effect of glucose (20 μg/mL) on mineralization of PNP (1 μg/mL) by Pseudomonas sp. showed that there is a threshold concentration for the test pollutant relative to the supplementary substrate. The degradation occurred within 15 h, although glucose at that level did not affect PNP degradation at 10 ng/mL (Schmidt et al. 1987). The threshold level refers to the concentration below which the substance (i.e., PNP) does not meet the requirement for the maintenance of cellular energy and/or the pollutant concentration is too low to induce the necessary degradative type of enzymes (Schmidt et al. 1987). The results in Fig. 1 are in agreement with the findings of other researchers (Muller et al. 1999; Schmidt et al. 1987). The inhibitory effect of phenol on *R. eutropha* growth has been shown to occur through the cell membrane fluidity (phenol would normally be localized in the lipid part of the membrane). Phenol hydroxylase as the major membrane site for phenol (substrate) inhibition expresses sensitivity to hydrophobic stress, which affects the integrity of the cell membrane (Leonard and Lindley 1999; Muller et al. 1999). Biodegradation of 3-methyl-4-nitrophenol by Ralstonia sp. SJ98 occurred through the formation of the hydroxylated intermediate (Bhushan et al. 2000a, b). The ability of *P. putida* to degrade phenols is found to be related to an increasing degree of saturation of the cell membrane fatty acids (Keweloh and Heipieper 1996). The cis-to-trans fatty acid conversion increases the orderly structure of the cell membrane, and the fluidity thus decreases (Kulkarni and Chaudhari 2006). In the present work, 5 mg/L was the lowest level of PNP at which *R. eutropha* growth was observed, and a fivefold increase was the highest concentration selected for the RSM study. Development of the culture medium in this work became possible by using RSM, and the quantitative data was described by solving multivariate equations to obtain the optimum condition.

Biodegradative capacity of *R. eutropha* toward PNP using the RSM technique

Table 2 presents results obtained from the experiments performed according to the CCD arrangement. Considering the effects of individual factors and the interactions between two factors on a particular response, Eq. 1 takes the following form:

$$\hat{Y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

where the regression coefficients (i.e., the main effect (β_i), quadratic (β_{ii}) and two-factor interactions (β_{ij})) were estimated from the experimental results. The experimental results obtained were statistically treated using ANOVA to assess the goodness of fit. The quadratic model appears to fit the measured data for both of the responses due to the high coefficient of determination R^2 computed from the experimental data. The results of the ANOVA are presented in Table 3; the predicted models for Y_{OD} and $Y_{D\text{-}PNP}$ were significant by the F-test at the 5% confidence level. Besides the model term in each one of these two responses, the terms that were insignificant in the original model were removed from the equation without eliminating those terms that needed to be in place to support the hierarchy of the model. A new ANOVA was then performed for the reduced model. The terms in the model were considered to be statistically significant when the value of 'Prob > F' was less than 0.05. The non-significant value of lack of fit (>0.05) is also indicative of the validity of this constructed quadratic model in the present work (neither of the two models exhibited lack of fit). A reduced form of the quadratic regression model was suggested by using coded values from the data estimation:

$$\hat{Y}_{D-PNP} = 71.93 - 27.20x_1 + 3.75x_2 + 19.20x_3 + 9.25x_1x_3 - 14.05x_1^2 + 8.20x_2^2 - 6.05x_3^2 \quad (2)$$

$$\hat{Y}_{OD_{546}} = 0.13 + 0.042x_1 + 0.21x_3 + 0.052x_1x_3 + 0.075x_1^2 + 0.12x_3^2. \quad (3)$$

The statistical parameters obtained from performing an ANOVA on the reduced models for the two responses are given in Table 3C. The coefficient of variance (CV) for PNP biodegradation efficiency equaled 6.46% (where CV is the ratio of the standard error of the estimate to the mean value of the experimentally measured response *100). The CV is an indicator of the reproducibility of the suggested model, and the constructed regression model can be considered reproducible because CV was less than 10% (Ahmadi et al. 2006). The adequate precision value as a measure of the 'signal-to noise ratio' was greater than 4 and any noise 'background' to the

Table 3 Analysis of variance (ANOVA) for the fitted (reduced) quadratic polynomial model for optimization: PNP biodegradation and biomass formation (OD_{546}) for *R. eutropha* (A), the regression coefficients (B) and statistical parameters obtained for the reduced model (C)

Source of variation	$\hat{Y}_{D-PNP}$				$\hat{Y}_{OD}$			
	df	SS	*F*	Prob > *F*	df	SS	*F*	Prob > *F*
A								
Model[+]	7	12,812.16	103.93	<0.0001	5	0.58	58.74	<0.0001
x_1	1	7,398.40	420.10	<0.0001	1	0.018	8.90	0.0124
x_2	1	140.63	7.99	0.0199	–	–	–	–
x_3	1	3,686.40	209.32	<0.0001	1	0.43	216.01	<0.0001
x_1^2	1	528.84	30.03	0.0004	1	0.017	8.58	0.0137
x_2^2	1	180.188	10.23	0.0109	–	–	–	–
x_3^2	1	98.04	5.57	0.0426	1	0.04	20.40	0.0009
x_1x_2	–	–	–	–	–	–	–	–
x_1x_3	1	684.50	38.87	0.0002	1	0.021	10.86	0.0071
x_2x_3	–	–	–	–	–	–	–	–
Residual	9	158.50			11	0.022		
Lack of fit[a]	7	151.84	6.51	0.1395	9	0.019	1.79	0.41
Pure error	2	6.66			2	2.400E-003		
Corrected total	16	12970.66			16	0.6		
R^2		0.9878				0.9639		
$R^2_{adjusted}$		0.9783				0.9475		

Coefficient	Dependent variable	
	$\hat{Y}_{D-PNP}$	$\hat{Y}_{OD}$
B		
β_0	71.93	0.13
β_1	−27.20	0.042
β_2	3.75	–
β_3	19.20	0.21
β_{11}	−14.05	0.075
β_{22}	8.20	–
β_{33}	−6.05	0.12
β_{12}	–	–
β_{13}	9.25	0.052
β_{23}	–	–

Parameter	$\hat{Y}_{D-PNP}$	$\hat{Y}_{OD_{546}}$
C		
Standard deviation (SD)	4.2	0.044
Coefficient of variance (CV%)	6.46	18.19
Mean	64.93	0.24
Adequate precision	34.841	22.029

[+] Significant at *P* value < 5%

[a] Not significant

system can be disregarded (adequate precision for the $\hat{Y}_{D-PNP} = 34.84$ and this statistical parameter for $\hat{Y}_{OD_{546}} = 22.029$). The predicted R^2, 0.9456, is in agreement with the adjusted R^2, 0.9783. The predicted residual sum of squares (PRESS) statistic indicates how a particular model fits each point in the design; the PRESS statistic was 7.05E + 002 and 0.058 for $\hat{Y}_{D-PNP}$ and $\hat{Y}_{OD_{546}}$, respectively.

Two-dimensional lines or three-dimensional surfaces properly display the constructed model, treating

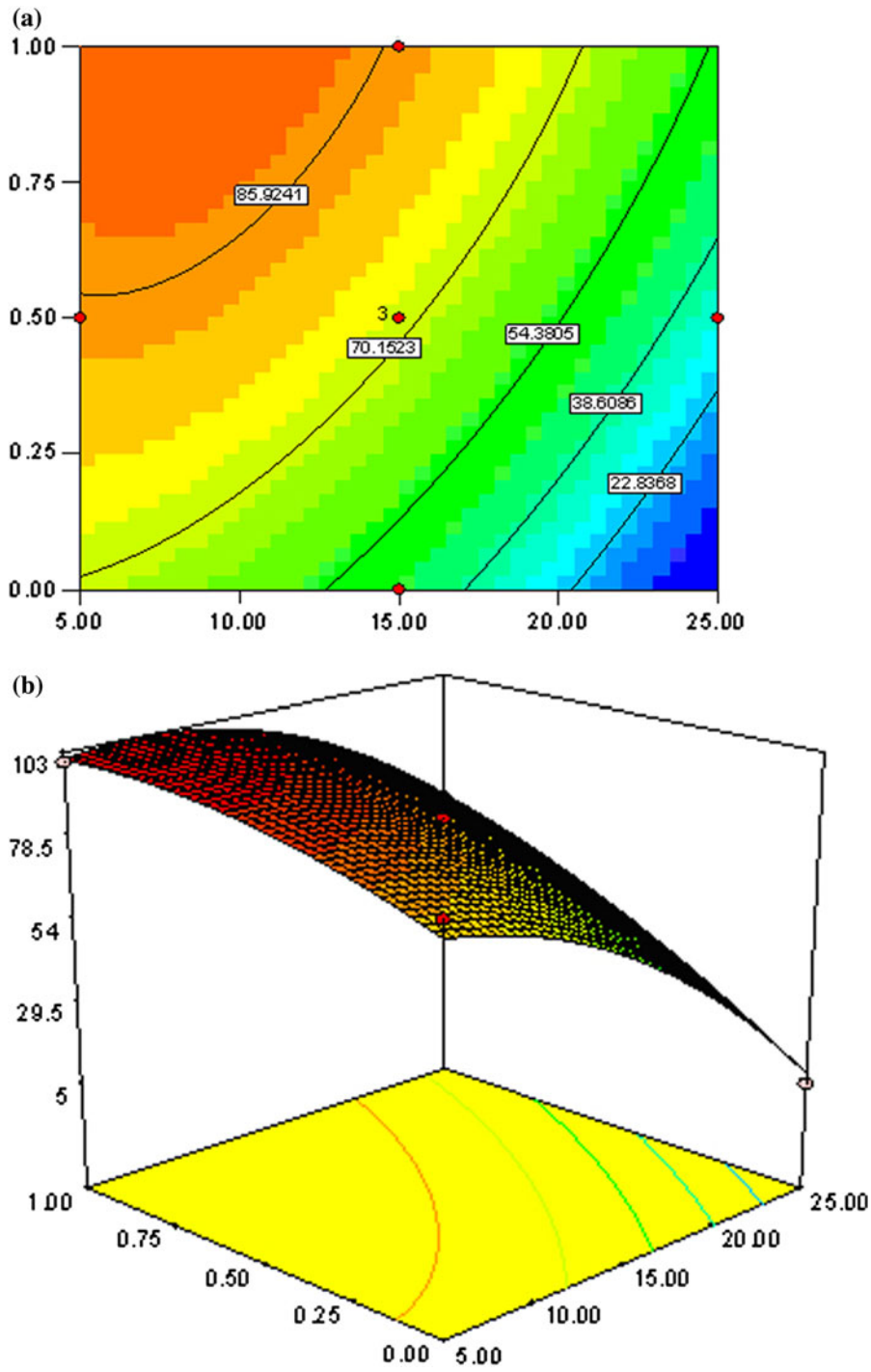

Fig. 2 2D contour plot and 3D response surface curve of the constructed model equation predicting degradation of PNP by *R. eutropha*; effect of PNP concentration and yeast extract level were considered when glucose was held constant at its center point (0.5 g/L)

the experimental observations (responses) as a function of two independent variables while the third variable is held constant. All of these views are essential to show the model interpolating behavior (Baranyi and Roberts 1995). Figure 2 shows a 2D contour plot and a 3D response surface curve for variation in the biodegradation of PNP as a function of PNP concentration and yeast extract level used in the medium preparation. Interactions (PNP and yeast extract) would be identified from the circular or elliptical nature of the contour plots (x_1x_3 = statistically significant terms, see Table 3). The 3D response surface plot in Fig. 1b is convex in nature, indicating that there were well-defined PNP and yeast extract concentrations that achieved the maximum level of PNP biodegradation when glucose was kept at the middle level (0.5 g/L). Figure 3a is a 2D plot with yeast extract concentration graphed on the vertical axis and amount of PNP on the horizontal axis, while glucose level as the third factor was held constant at its middle level. Each axis on the plot represents an increasing factor level. The OD_{546} is read similarly to a topographical land map where the contours represent different values for OD_{546} by which the amount of the biomass formed has been determined. The maximum of 0.525 OD_{546} falls at about 0.9–1 g/L yeast extract and at 22–23 mg/L PNP. For better visualization, these data were plotted in three dimensions (Fig. 3b). The effect of increasing the level of yeast extract on the increasing trend of OD_{546} was different from that of the PNP levels. An enhancement of the biodegradation due to the simultaneous use of PNP with the readily degradable compounds was observed in the present work; yeast extract was more effective than glucose. Efficient use of ammonium sulfate as the nitrogen source with PNP by *Rhodococcus* sp. has been reported (Gemini et al. 2005), and those results are in agreement with the results reported in the present work. The study with *Rhodococcus* sp. showed that in the presence of ammonium sulfate, the amount of nitrite released into the test medium was stoichiometric; although, in the absence of this nitrogen source, the amount of released nitrite decreased. Nitro-group removal of PNP enhances the sensitivity of the aromatic ring to biodegradation (Gemini et al. 2005). Moreover, rapid degradation of PNP (20 and 50 mg/L) by *Psudomonas putida* was observed in the presence of glucose (400 mg/L). Although, out of 300 mg/L PNP, only 19% of the pollutant was degraded, and glucose did not positively affect PNP degradation at high concentrations (Kulkarni and Chaudhari 2006). In the present work, the degradation percentage of PNP at 25 mg/L in the presence of glucose (1 g/L) was 8%, while the degradation decreased to $\leq$1% when glucose was absent in the LC medium. When only yeast extract was used as the supplementary substrate, a considerable increase in PNP degradation (57%) was observed (Table 2).

Moreover, the appearance of a red color in the present work was observed during PNP degradation when glucose and yeast extract were included in the LC medium. Conversion of NAs to nitrocatechols before removing the nitro group as nitrite has been reported for several microorganisms. For instance, the conversion of PNP to *p*-nitrocatechol has been proposed as the initial step in the degradation by *Flavobacterium* sp. (Raymond and Alexander 1971). Figure 4 gives a brief description of the oxidative removal of the nitro group of PNP via the formation of *p*-nitrocatechol (Marvin-Sikkema and Bont 1994; Samanta et al. 2000; Wan et al. 2007).

The chemotaxis ability of *Ralstonia* sp. as a motile bacterium is well documented and has been shown to occur through three molecular mechanisms (Pandey et al. 2002; Andreev et al. 1994). By use of a specialized type of transporter, a chemical item can be transferred (Stanier et al. 1976). The affinity of the synthesized cellular transporter depends mainly on the concentration of the test chemical item outside of the cell, i.e., a high affinity transporter is usually identified by its low k_m value while a high k_m value is indicative of a low affinity transporter (Fristedt et al. 1999). More work is needed to isolate the PNP-transporter in *R. eutropha* as related to the different PNP concentrations with or without a secondary substrate.

Residual plots

The modeling process focuses on obtaining a good generalization property (good performance of the model upon confrontation with new data) (Geeraerd et al. 2004). Polynomial model types are known to be flexible (high value for adjusted R^2), and this characteristic has made polynomial regression models very popular, while RSM models are applied in a variety of situations. A high R^2 value does not

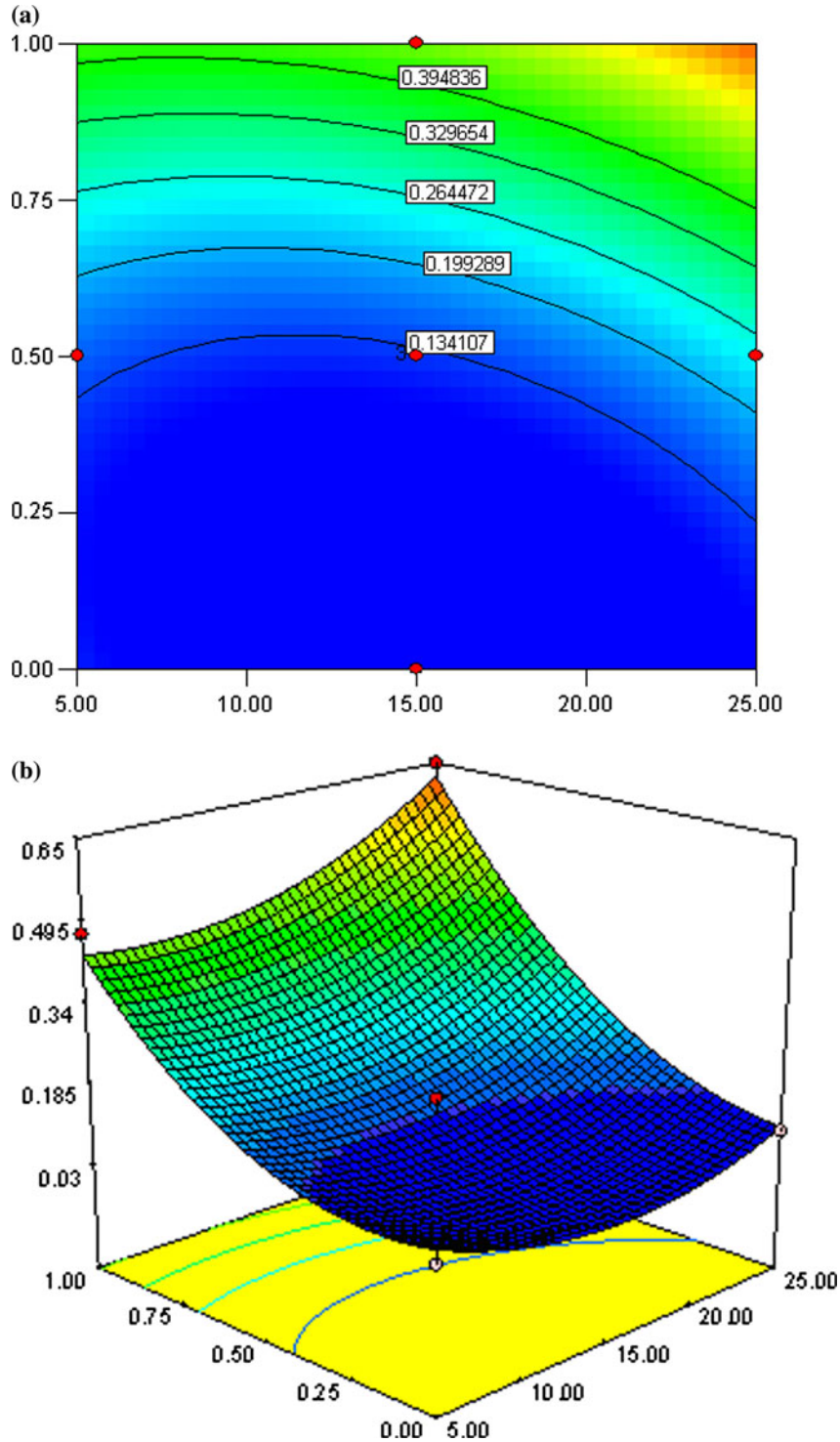

Fig. 3 2D contour plot and 3D response surface curve predicting OD_{546} as an index for the growth of *R. eutropha* in PNP biodegradation studies, from the constructed model equation; effect of PNP and glucose concentration were considered when yeast extract level was held constant at its center point (0.5 g/L)

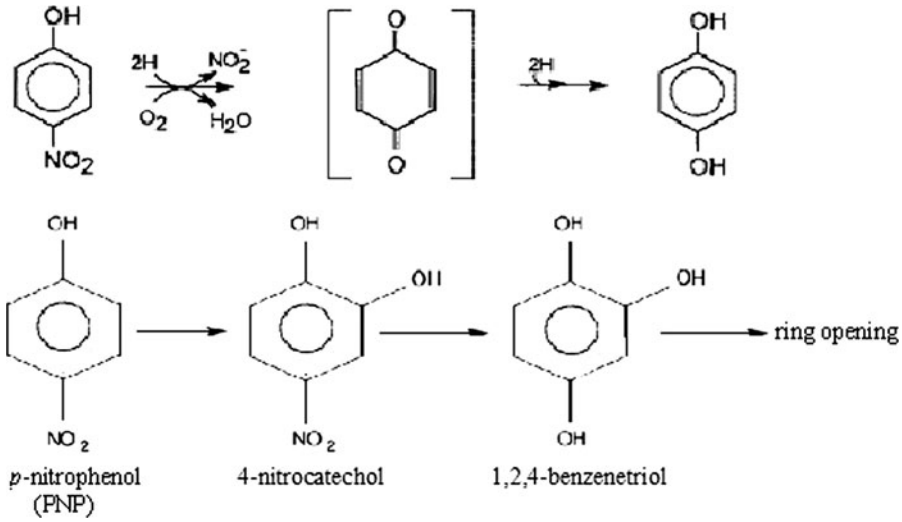

Fig. 4 Enzymatic degradation of PNP according to the suggested pathways in the literature (Marvin-Sikkema and Bont 1994; Wan et al. 2007)

necessarily mean the model corresponds to a real situation. Another weakness involves the possibility of obtaining an unrealistic curvature in the design space. To overcome these weaknesses, a general approach in statistical works is to test the model prediction following the model estimation (Lapin 1997). Weak points in a constructed model reveal themselves through systematic patterns in the residuals (difference between experimental observation and the predicted responses) (Vining 2003). Interpretation of the residual plots relevant to PNP biodegradation would help to assess the strengths of the estimated model. Improvement in model building through predictive microbiology has been extensively reviewed (Geeraerd et al. 2004). By incorporating microbial knowledge into polynomial modeling, it has become possible to take a novel approach in this important area in microbiology (Mc Meekin et al. 2002).

The residuals provide a measure of the quality of the data analysis (the extent of the model prediction would be checked through the information about lack of fit, which is contained in the residuals). The emphasis in the use of residual plots is usually on the use of some simple graphical techniques, which make it possible to detect and explain the departures from the assumptions used in developing the regression equations (Lapin 1997). Figure 5 presents residual plots that were drawn based on the data analysis of the PNP biodegradation: the pattern of the straight line in the normal plot of the residuals implies the following: (a) A normal distribution of the errors and adequacy of the least squares technique was confirmed; the data supported the constructed model. Residuals against the predicted values of the response show an almost equal scattering pattern above and below the axis; (b) The predicted versus measured values for the PNP biodegradation also fits the data very well; (c) The residuals versus the run order of the experiments according to the design of the experiment (for instance, considering CCD with three factors at three levels) is helpful to show random scattering of the residuals around zero; and (d) This informative approach implies that the constructed model(s) are adequate, and the assumptions that the errors are normally distributed and are independent of each other has been recognized (Vining 2003).

Optimization

Two responses of interest were studied in the present work: the culture medium constituents need to be optimized not only in terms of the biodegradation rate of the test pollutant by *R. eutropha*, but also in terms of which is indicative of the bacterium's ability to express the relevant enzyme(s) to properly utilize PNP. A desirability function as a basic approach in

Fig. 5 Residual plots obtained based on the data analysis of PNP biodegradation by *R. eutropha*. Plots of the PNP biodegradation are given under heading (**A**), while the plots of OD_{546} are shown under heading (**B**). Normal probability plot of residuals (**a**), residual versus the predicted values of the response (**b**), predicted values against the actual (experimental) values of the response (**c**) and residuals versus the run number (experiments' number) (**d**)

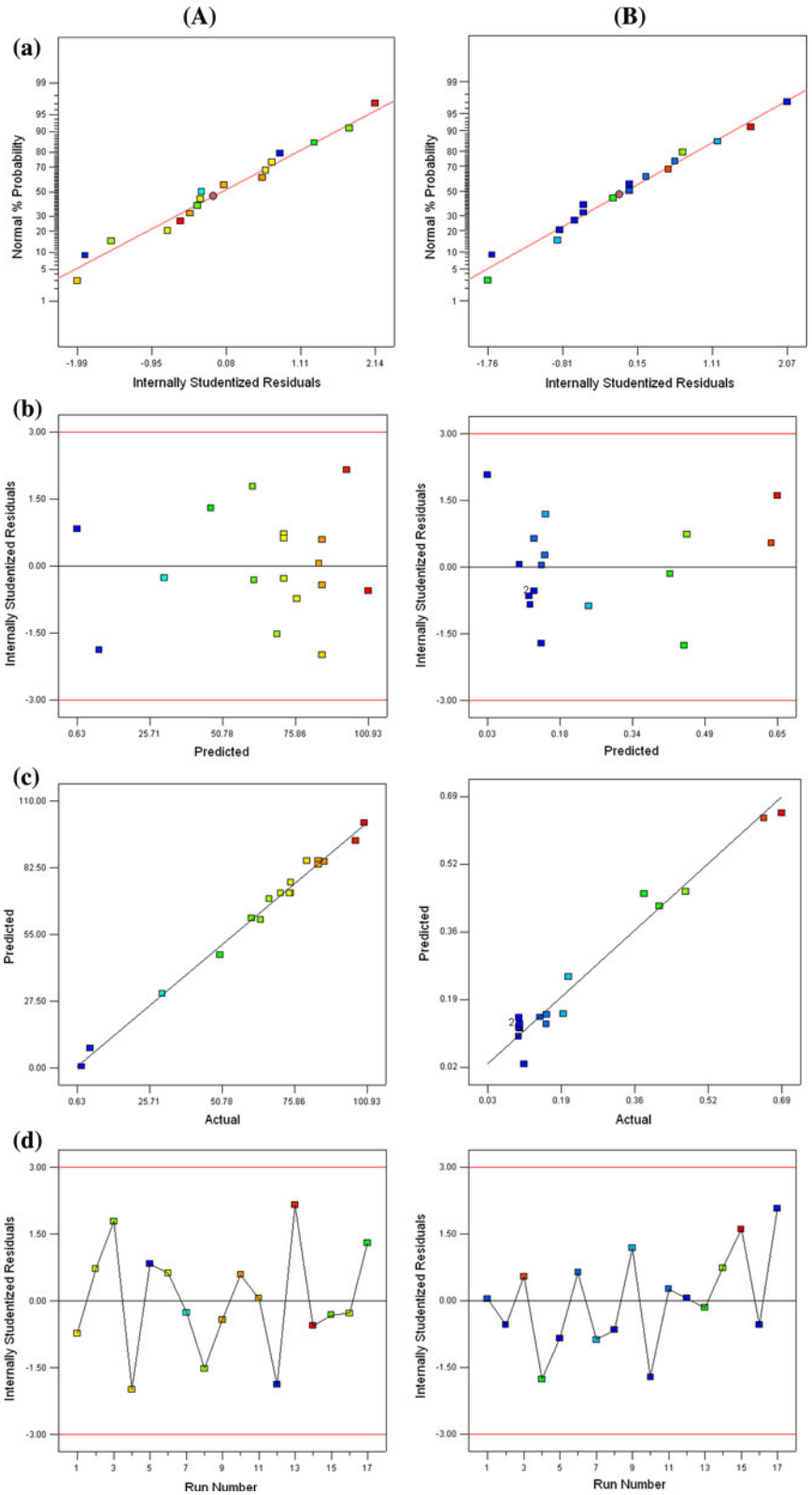
(A)
(B)
(a)
Normal % Probability
Internally Studentized Residuals
(b)
Internally Studentized Residuals
Predicted
(c)
Predicted
Actual
(d)
Internally Studentized Residuals
Run Number

Table 4 Medium optimization criteria used in the present study (A) and optimal solution (predicted values) obtained using the optimization function of the Design Expert software (B)

Independent variables and responses		Limits				Criterion used for selection
		Lower		Upper		
A						
x_1		5		25		Maximize
x_2		0		1		Minimize
x_3		0		1		Minimize
$\hat{Y}_{D-PNP}$		2		100		Maximize
$\hat{Y}_{OD}$		0.098		0.65		Is in range
Experiment number	x_1 (mg/L)	x_2 (g/L)	x_3 (g/L)	$\hat{Y}_{D-PNP}$	$\hat{Y}_{OD}$	Desirability
B						
15	19	0	0.33	54.9285	0.09799	0.709
5	18.57	0	0.34	57.2776	0.09800	0.708
7	19.95	0	0.41	55.2277	0.135154	0.698
12	6.92	0	0	71.3986	0.09799	0.51
16	24.26	0	0.003	7.71	0.09799	0.48

statistical design is usually used to give an overall measure for the 'goodness' of a specific setting in experimental design (Vining 2003). In defining the desirability, one suggestion is as follows:

$$d = \begin{cases} 0 & \text{for} \quad \widehat{Y} < Y_L \\ \frac{\hat{Y}-Y_L}{Y_T-Y_L} & \text{for} \quad Y_L \le \hat{Y} \le Y_T \\ \frac{Y_u-\hat{Y}}{Y_u-Y_T} & \text{for} \quad Y_T \le \hat{Y} \le Y_u \\ 0 & \text{for} \quad \hat{Y} > Y_u \end{cases}$$

for a desirability scale ranging from 0, which indicates completely undesirable for the particular test variable (dependent and independent), to 1—fully desirable. Y_L and Y_u are the lowest and largest possible values, respectively, that have any desirability, while Y_T is the specific target value for the test variable (Vining 2003).

Use of an energy deficient substrate (toxic pollutant) for a particular microbe in the culture medium has an impact on the use of the other nutrient ingredients, especially when one monitors the trend of the biodegradation of the test pollutant. The optimal conditions for the biodegradation of PNP by *R. eutropha* were predicted using the optimization function of the Design Expert software. The criterion was selected on the basis of reaching maximum PNP biodegradation while biomass formation was considered to be within the correct range (Table 4A). Table 4B presents the optimal solutions. The results show that Experiment 4 is the experiment of choice: PNP at 19 mg/L, with yeast extract at about 1/3 of the amount of the initial level and no glucose. Medium optimization is only one part of the biodegradation study, and by providing more information about the mechanism of the microbial action (i.e., PNP biodegradation), roper decisions about the culture medium preparation could be established.

Conclusion

Response surface methodology was employed for optimization of the chemical constituents used in this study of growth medium preparation for *R. eutropha* to assess the PNP biodegradative capability of the bacterium under some defined conditions. The existence of interaction(s) between the factors of interest and how they affected the response growth medium in terms of its constituents was studied, and regression was used to create a model. The optimum concentrations of the constituents for the biodegradation were as follows: 19 mg/L, 0 and 0.33 g/L for PNP, glucose and yeast extract, respectively.

The removal efficiency of PNP by *R. eutropha* under these conditions was about 55%. The enhancement effect(s) of glucose and yeast extract (with

emphasize on the latter) on PNP degradation by the test bacterium has been described. More work is needed to differentiate between the sources of nitrogen and carbon relative to the appropriate range of concentration for each of these secondary substrates. Using a statistical design for the media development, it was possible to explain both qualitatively and quantitatively the degradation behavior of *R. eutropha* towards PNP.

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