ENHANCED BIODEGRADATION OF PULPING EFFLUENTS BY A STATISTICAL EXPERIMENTAL DESIGN USING MICROBIAL CONSORTIA

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Statistically based experimental designs were used to construct a mixed-culture community for maximizing the chemical oxygen demand (COD) degradation of pulping effluents by the use of six different strains, i.e., *Agrobacterium* sp., *Bacillus* sp., *Enterobacter cloacae*, *Gordonia*, *Pseudomonas stutzeri*, and *Pseudomonas putida*. Significant effects of single and mixed strains on COD degradation were quantified first by applying a fractional factorial design (FFD) of experiments, and four strains were selected as the main driving factors in the process of biodegradation of effluents. Then the Steepest Ascent method was employed to approach the experimental design space, followed by an application of response surface methodology to further optimize the proportion of cell concentration for different strains in pulping effluent. A quadratic model was found to fit COD removal efficiency. Response surface analysis revealed that the optimum levels of the tested variables for the degradation of COD, and optimized cells concentrations (OD600) of four strains in mixed-culture community were 0.35 *Agrobacterium* sp., 0.38 *Bacillus* sp., 0.43 *Gordonia* sp., and 0.38 P. putid., respectively. In a confirmatory experiment, three test runs were performed by using the optimized conditions, and a COD removal efficiency of (65.3 ± 0.5)% was observed, which was in agreement with the prediction.

Keywords: Pulping effluents; Microbial consortia; Chemical oxygen demand (COD); Fractional factorial design; Response surface methodology

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INTRODUCTION

Effluents from some Chinese pulp and paper mills are highly colored and contain large amounts of organic matter, including lignin and associated bleaching by-products such as chlorophenols, dioxins, and benzodioxins, as well as other non-lignin materials such as fatty acids, carbohydrates, etc. (Nagarathnamma et al. 1999; Neto et al. 1999; Cardoso et al. 2009). In particular, chlorinated organics are present, most of them being difficult to eliminate by conventional waste water treatment processes and, therefore, accumulating in the environment (Pérez et al. 1997).

Biological treatments are often considered to be effective because they offer low treatment cost and help to avoid secondary pollution. The enzymatic systems of microorganisms play an important role in the initial depolymerization of macromolecules in effluent, including several other non-specific extracellular enzymes that catalyze lignin and chlorolignins degradation and also oxidize aromatic and halogenated compounds (Bergbauer et al. 1991). The resulting fragments with low molecular weight are ultimately metabolized in the intracellular environment to water and carbon dioxide. Research efforts have reported that different microorganisms were used to treat effluents from pulp and paper mills (Ragunathan and Swaminathan 2004; Freitas et al. 2009).

Generally, single strains have been used to degrade the organic matter in wastewater, according to most research reports (e.g. Annadurai et al. 2008; Bergbauer et al. 1991). Treatments of wastewater using mixed strains have been deeply studied by few people. The existence of synergistic among different microorganisms is a common phenomenon in the nature. Therefore, the use of microbial consortia to treat wastewater may be more efficient than using a single strain. Hisashi et al. (2000) established a specific consortium using immobilized photosynthetic bacteria, *Rhodobacter sphaeroides* S, *Rb. sphaeroides* NR-3, and *Rhodopseudomonas palustris*, fixed on ceramic media, for wastewater treatment. They found that the mixture of the three bacteria was more effective for the removal of chemical oxygen demand (COD), nitrate, phosphate, and odor compared to using a single strain. Thus, the interactions among multiple microorganisms are important factors for wastewater treatment. The traditional ‘‘one-factor-at-a-time approach” disregards the complex interactions among various microorganisms. In contrast, a statistical experimental design such as a factorial design and response surface analysis particularly fulfills this requirement. Statistical experimental design has been widely used in various fields such as biochemistry for optimizing culture medium and conditions (Annadurai et al. 2008; Chen 1996; Jiao et al. 2008; Wang and Liu 2008); however, to our knowledge, there have not been reports on using the statistical methods to investigate the effect of a mixed-culture inoculum on the treatment of pulping effluent.

Pulping effluents contain a mass of organic contaminants; COD is often used as the principal parameter to reflect the degree of organic pollution in wastewater (Kim et al. 2000), and serves as one of the most important parameters to characterize pulping effluents. In the present study, six strains were selected to understand their roles in organic removal and how they interact with each other. Fractional factorial design (FFD) and response surface methodology (RSM) were employed to build models to investigate the interplay of six bacterial strains for treating pulping effluent, and to select optimum conditions for enhanced wastewater treatment. A microbial consortium was constructed to treat pulping effluent and achieved a good result, which showed the synergistic effect among different microorganisms. The study will be helpful for the further improvement of wastewater biodegradation.

MATERIALS AND METHODS

Effluent Source

Black liquor and bleach effluent from the processing stage with chlorine dioxide were obtained in the laboratory utilizing *E. globules* as raw material and the kraft pulping process.

Microorganisms

Six strains of bacteria, i.e., *Agrobacterium* sp., *Bacillus* sp., *E. cloacae*, *Gordonia sp.*, *P. stutzeri*, and *P. putid* were used to prepare the inoculum for degrading pulping effluents.

Growth Medium

The nutrient medium contained 3 g/L of beef extract, 5 g/L of peptone, and mineral salt medium (MSM), and the pH value was adjusted to 7.0. The composition of MSM (g/L) was: KH_2PO_4 (0.42), K_2HPO_4 (0.375), $(NH_4)_2SO_4$ (0.244), NaCl (0.015), $CaCl_2{\cdot}2H_2O$ (0.015), $MgSO_4{\cdot}7H_2O$ (0.05), and $FeCl_3{\cdot}6H_2O$ (0.054). A phosphate buffer solution (PBS) (pH 7) was prepared by dissolving NaCl (8 g/L), KCl (0.2 g/L), K_2HPO_4 (1.15 g/L), and KH_2PO_4 (0.2 g/L) in deionized water (Millipore, Milli-Q). The PBS was used for diluting the cell concentration in solutions.

Bacteria Cultivation and Biodegradation Experiments

To recover the activity of the stock culture, one loop of each of the six bacteria from the culture-contained agar was separately transferred to 20 mL of the nutrient medium in a glass flask. Each bacterium was activated at 30^{o}C. These activated cells were harvested as inocula in the late exponential phase, respectively. The cells collected after centrifugation (10000 rpm for 5 min) were resuspended in the PBS and then centrifuged again. After cleaning, for biodegradation experiments according to FFD, six inocula were separately prepared by inoculating the six activated strains into the pulping effluents to give an initial optical density at 600 nm (OD_{600}) of 0.30±0.01. Then each 50mL inoculum containing 0 or 0.3 OD_{600} of bacteria (according to the fractional experimental design detailed in Tables 1 and 2) was added aseptically to Erlenmeyer flasks (500 mL) yielding a final volume of 300 mL. After inoculation, the Erlenmeyer flasks were capped with cotton plugs and placed in a shaker controlled at 150 rpm and 30^{o}C. The pH of the mixture was adjusted to 7.0. Samples were withdrawn every 2 hours, and the OD_{600} of cells and COD were measured as described below. Each experiment was stopped when there was no further increase of OD_{600}.

Table 1. Coded and Real Values of Independent Variables in the FFD

Variable	Bacteria species	Applied level of cells density (OD_{600})		
		-1 (low)	0	+1 (high)
X_1	*Agrobacterium* sp.	0	0.15	0.3
X_2	*Bacillus* sp.	0	0.15	0.3
X_3	*E. cloacae*	0	0.15	0.3
X_4	*Gordonia sp.*	0	0.15	0.3
X_5	*P. stutzeri*	0	0.15	0.3
X_6	*P. putid*	0	0.15	0.3

Table 2. Experimental Designs and the Results of the FFD

Run	Coded variables						
	X_1 *Agrobacterium*	X_2 *Bacillus*	X_3 *E.cloacae*	X_4 *Gordonia*	X_5 *P.stutzeri*	X_6 *P.putid*	R_{max} (%)
1	-1	1	1	-1	-1	-1	21.2
2	-1	1	1	1	-1	1	60.9
3	1	-1	-1	1	1	1	40.3
4	-1	-1	-1	1	-1	1	35.8
5	1	1	-1	-1	-1	1	50.0
6	1	1	-1	1	-1	-1	46.3
7	1	-1	1	-1	-1	1	50.9
8	-1	-1	1	1	1	-1	35.8
9	-1	-1	-1	-1	-1	-1	12.8
10	-1	-1	1	-1	1	1	25.9
11	-1	1	-1	-1	1	1	37.5
12	1	1	1	1	1	1	62.5
13	1	1	1	-1	1	-1	32.6
14	1	-1	-1	-1	1	-1	34.6
15	-1	1	-1	1	1	-1	52.8
16	1	-1	1	1	-1	-1	48.5
17	0	0	0	0	0	0	45.8
18	0	0	0	0	0	0	51.2
19	0	0	0	0	0	0	47.6

Determination of Bacterial Growth and COD

The concentration of cells in the samples was analyzed by measuring OD_{600} using an Agilent-8453 UV/VIS spectrophotometer with the distilled water as the blank. Chemical oxygen demand (COD) values of samples were measured by the dichromate method (Association of Official Analytical Chemists, 1990).

Experimental Design

In order to identify which bacteria have a significant effect on degradation of COD in pulping effluent, the first optimization step was developed with FFD. Six strains of bacteria, as have been mentioned, were used as factors, and the maximum COD removal efficiency (R_{max}) was used as the response in the factorial designs. A two-level, 1/4 fractional design with sixteen runs was employed to evaluate the individual and combined effects of the six different strains. The range of the coded level for six factors is listed in Table 1. Fractional and full factorial design data were regressed by Minitab software to obtain the first-order polynomial,

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j \qquad (1)$$

where Y = predicted response; x_i and x_j are input variables; β_0 is a constant; β_i is a linear coefficient; and β_{ij} is a cross-product coefficient.

The regression models can be applied in screening important experimental parameters. In the screening tests, the magnitude and sign of the regression constants can be used to identify the significance of the variables on responses. If the coefficient is relatively large, it has a more significant effect on the response as compared to the small ones. Furthermore, the variable with a positive-fitted constant is helpful to the response, while the one with negative coefficient has inhibitory effects on the response. We can identify the most effective ingredients by the regression models.

Frequently, the initial estimate of the optimum operating conditions for the system will be far from the actual optimum. In such circumstances, the objective is to move rapidly to the general vicinity of the optimum. The method of the Steepest Ascent is a procedure for moving sequentially along the Steepest Ascent path which is constructed according to the coefficients of the model. Further studies involved experiments carried out along the path of Steepest Ascent in order to attain the maximum increase of responses.

The objective of the second experiment is to develop an empirical model of the process and to obtain a more precise estimate of the optimum conditions for the factors involved (Montgomery 1997) by using central composite design. In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed. The quadratic model was used to predict the optimal point based on the following Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j + \sum_{j=1}^{n} \beta_j {x_j}^2 \tag{2}$$

where Y = predicted response; x_i and x_j are input variables; β_0 is a constant; β_i is a linear coefficient; β_{ij} is a crossproduct coefficient; and β_j is a quadratic coefficient.

The effects on COD degradation caused by individual strains or by their combinations were evaluated by T statistics and analysis of variance (ANOVA). The levels of experimental factors and the selected runs are discussed in later sections. A significance level of 5% was used as the criterion to reject the null hypothesis. All analyses were performed using the Minitab software.

RESULTS AND DISCUSSION

Screening the Principal Factors for COD Degradation by Fractional Factorial Design

The factorial design enables the identification of bacteria that play a significant role in the degradation of chemical oxygen demand. The screening experiments were designed to evaluate the impact of six factors, *Agrobacterium* sp., *Bacillus* sp., *E. cloacae*, *Gordonia sp.*, *P. stutzeri*, and *P. putid* on COD degradation. The maximum COD removal efficiency, R_{max}, was measured after inoculation with different strains or strain combinations. The fractional design experiments were performed at two levels of inoculated concentrations. The variables considered for the design are listed in Table 1. A two-level fractional factorial design was conducted to screen out the bacterial strains that can enhance the degradation of COD in pulping effluents. Runs of center points were included in the matrix, and statistical analysis was used to identify the effect of each variable on COD degradation.

A full factorial design for the six factors that requires 64 (2^6) experiments provides sufficient information to evaluate the whole set of main effects as well as interaction effects. The main effects and the lower-order interactions, however, are usually the most significant terms (Montgomery et al. 2007). In this work, a 2^{6-2} fractional factorial design consisting of 16 factorial runs was performed to reduce the overall number of experiments. The order in which the experiments were performed was randomized. The selected set of experiments and the measured R_{max} are listed in Table. 2.

Figure 1 is a normal probability plot of the effects of different strain combinations on R_{max}. The effects that lie along the normal probability line are negligible (round points), whereas significant effects are those square points far from the normal probability line, and these effects play important roles on the COD degradation.

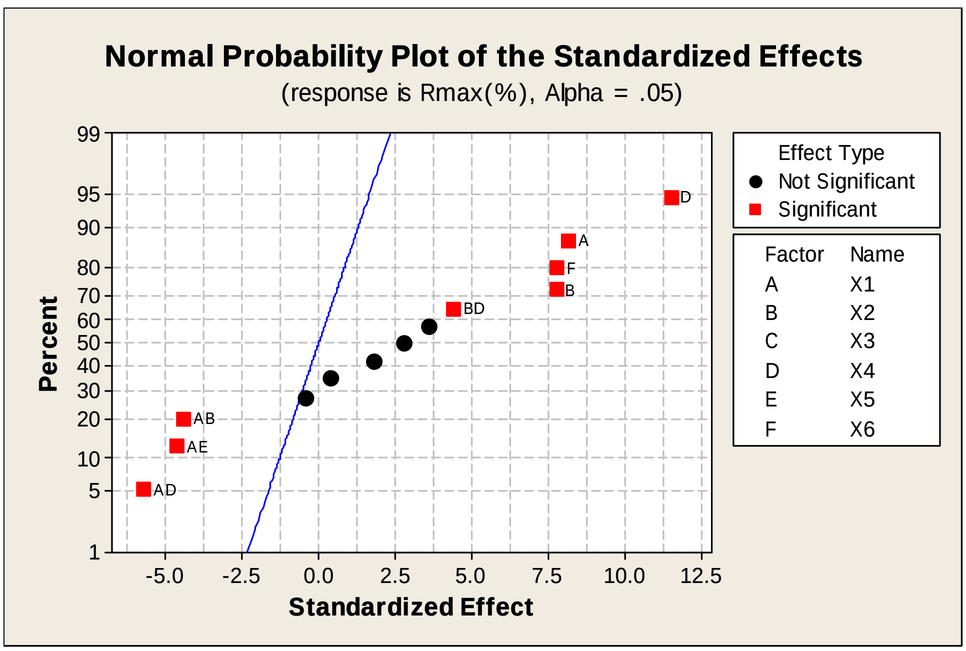

Fig. 1. Normal probability plot of the effects of different strain combinations on the COD removal efficiency (R_{max})

It can be seen from Fig. 1, for single strains, *Agrobacterium* sp. (X_1, p=0.015), *Bacillus* sp. (X_2, p=0.016), *Gordonia sp.* (X_4, p=0.007), and *P. putid* (X_6, p=0.016) showed significant enhancement of the COD degradation, while the other strains did not produce significant effects on their own within the levels tested. In addition, there were four significant interaction terms in the model, X_2X_4 (p=0.047), X_1X_2 (p=0.048), X_1X_4 (p=0.029), and X_1X_5 (p=0.044), respectively. Only X_2X_4 was positive among all the significant interaction terms, while others were negative for R_{max}. By applying multiple regression analysis on the experimental data and neglecting the insignificant terms based on the 5% level of significance, a first-order model equation was obtained from FFD:

$$R_{max} = 40.54 + 5.20X_1 + 4.94X_2 + 7.34X_4 + 4.96X_6 + 2.82X_2X_4$$
$$- 2.81X_1X_2 - 3.64X_1X_4 - 2.94X_1X_5 \quad (3)$$

Equation 3 demonstrates that X_1, X_2, X_4, and X_6 were responsible for most of the observed COD degradation. There was only one significant interaction term (X_2X_4) that had a positive effect on R_{max}. Table 3 shows the analysis of variance (ANOVA) of the empirical model (Because our work is the first report, there is no basis of comparison with other researchers). The ANOVA evaluates the significance of the main effects and interaction terms on R_{max}. The results showed a coefficient of determination (R-sq) of 99.55%, indicating a satisfactory adjustment of the model to the experimental data. The statistical significance of the empirical model was checked by an F-test. The higher F-value and reduced main effects than those of the two-way interactions indicate that certain single strains contributed more significantly to the response than the significant two-way interaction terms.

Table 3. Analysis of Variance for R_{max}(%)

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Main effects	6	2128.20	2128.20	354.70	54.87	0.018
2-Way Interactions	7	711.19	711.19	101.60	15.72	0.061
Residual error	2	12.93	12.93	6.46		
Total	15	2852.32				

S=2.54245; R-Sq=99.55%; R-Sq (adj) = 96.60%; DF: degree of freedom; SS: sum of squares; MS: mean sum of square.

The results of the t-test for variance between averages of observation of two-level experiments and center points showed that the difference was not significant (p =0.3606). Results indicated that the optimum point was outside the domain of the experiment. Experiments using the Steepest Ascent path approach were necessary to reach the optimum domain of the maximal response.

Steepest Ascent Experiment and Analysis for COD Degradation

The direction of steepest ascent path can be determined by Eq. (3) and the regression results. From the first-order model Eq. (3), it was predicted that increasing the concentrations of *Agrobacterium* sp. (X_1), *Bacillus* sp. (X_2), *Gordonia sp.* (X_4), and *P. putid* (X_6) should improve the degradation of COD. However, increasing X_1, X_2, and X_4 simultaneously resulted in enhancement of two significant interaction terms (X_1 X_2 and X_1 X_4), which are negative for COD degradation. Thus, four strains (X_1, X_2, X_4, and X_6) were selected for the steepest ascent experiment, and the path of the steepest ascent was aimed at optimizing the cell concentrations of X_1, X_2, X_4, X_6 in order to enhance COD removal efficiency (R_{max}). Table 4 illustrates the experimental design and responses of the steepest ascent path experiments. The center point of the fractional factorial design is considered to be the origin of the path. Regarding the results from the path of steepest ascent, it is clearly seen that the R_{max} showed a maximum 64.8% at run six. After the sixth step on the path, further experimentation did not increase R_{max}. This result indicated that the experiment was approximating the neighborhood of the optimum R_{max}. Consequently, the combination of the sixth step was chosen for further optimization.

Table 4. Experimental Design and Response of the Steepest Ascent Path Experiments

Run	Variables (OD_{600})				R_{max} (%)
	Agrobacterium sp. X_1	*Bacillus* sp. X_2	*Gordonia sp.* X_4	*P. putid* X_6	
1	0.15	0.15	0.15	0.15	42.7
2	0.19	0.18	0.20	0.18	48.3
3	0.23	0.21	0.25	0.21	46.6
4	0.27	0.24	0.30	0.24	50.7
5	0.31	0.27	0.35	0.27	58.6
6	0.35	0.30	0.40	0.30	64.8
7	0.39	0.33	0.45	0.33	58.5
8	0.43	0.36	0.50	0.36	61.4

Optimization by Response Surface Methodology

Response surface methodology (RSM) has been a popular and effective method to solve multivariate problems and optimize several responses in many types of experiment (Dale et al. 2007; Jeong et al. 2007; Korbahti et al. 2007). RSM is appropriate when the optimal region for running the process has been identified. Based on the identification of variables by the 2-level fractional factorial, a central composite design was developed for variables significantly affecting COD degradation in each isolate. The central composition design of four variables that are coded along with R_{max} as responses is presented in Table. 5.

Regression analysis was performed to fit the response function with the experimental data, and a second-order polynomial model (Eq. (4)) in coded units explains the role of each variable and their second-order interactions in COD degradation.

$$R_{max} = 63.138 + 0.317X_1 + 2.592X_2 + 2.125X_4 + 1.408X_6 - 2.294X_1^2 - 1.381X_2^2$$

$$- 1.619X_4^2 - 1.119X_6^2 + 0.600X_1X_2 + 1.063X_1X_4 - 0.088X_1X_6$$

$$+ 1.038X_2X_4 + 1.238X_2X_6 + 0.075X_4X_6 \quad (4)$$

where X_i is cell concentration in coded unit, i = 1, 2, 4, 6 for the four strains, respectively.

Table 5. Central Composition Design of Four Variables in Coded Form along with R_{max} as Response Values

Run	X_1	X_2	X_4	X_6	R_{max} %
1	-1	1	-1	1	56.7
2	1	1	-1	-1	52.4
3	-1	1	1	-1	57.5
4	-1	-1	1	-1	51.4
5	1	1	1	-1	60.3
6	1	-1	-1	1	50.2
7	-1	1	-1	-1	53.7
8	1	1	-1	1	60.5
9	-1	-1	-1	-1	51.1
10	-1	-1	1	1	54.2
11	1	-1	-1	-1	51.6
12	1	-1	1	-1	56.4
13	1	-1	1	1	55.4
14	1	1	1	1	68.5
15	-1	1	1	1	62.7
16	-1	-1	-1	1	55.4
17	-2	0	0	0	55.8
18	0	-2	0	0	54.3
19	0	0	-2	0	53.2
20	0	0	0	-2	58.1
21	2	0	0	0	53.3
22	0	2	0	0	62.1
23	0	0	2	0	61.3
24	0	0	0	2	60.4
25	0	0	0	0	61.4
26	0	0	0	0	63.7
27	0	0	0	0	62.6
28	0	0	0	0	65.1
29	0	0	0	0	61.2
30	0	0	0	0	63.6

$X_1 = (x_1\text{-}0.35)/0.05$; $X_2 = (x_2\text{-}0.30)/0.05$; $X_4 = (x_4\text{-}0.40)/0.05$; $X_6 = (x_6\text{-}0.30)/0.05$. x_i (i = 1, 2, 4, 6) indicate the actual level of cells concentration (OD_{600}) for four strains, respectively.

Table 6 shows the results of the second-order response surface model in the form of analysis of variance (ANOVA). The Fisher's *F*-test with a very low probability value ($p < 0.001$) indicated that the model was highly significant. The fitness of the model was examined by the determination coefficient (R-sq = 0.9178), which implied that the sample variation of more than 91% was attributed to the variables and less than 9% of the total variance could not be explained by the model. The adjusted determination coefficient (Adj R-sq = 0.8297) was also satisfactory to confirm the significance of the model. It can be seen that linear and quadric terms had a significant effect on R_{max}, but their interaction was insignificant. The model also showed statistically insignificant lack of fit (p = 0.240), so the model was suitable to describe the response of the experiment pertaining to COD degradation.

Table 6. Analysis of Variances (ANOVA) for the COD degradation in Coded Level Variables

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*p*
Regression	14	607.08	607.08	43.36	10.98	< 0.001
Linear	4	319.59	319.59	79.90	20.24	< 0.001
Square	4	221.73	221.73	55.43	14.04	< 0.001
Interaction	6	65.76	65.76	10.96	2.78	0.054
Residual Error	14	55.27	55.27	3.95		
Lack-of-Fit	10	46.60	46.60	4.66	2.15	0.240
Pure Error	4	8.67	8.67	2.17		
Total	29	672.35				

S=1.9869; R-Sq=91.78%; R-Sq (adj) = 82.97%; DF: degree of freedom; SS: sum of squares; MS: mean sum of square.

The significance of each coefficient was determined by the Student's t-test and *p*-value, which are listed in Table. 7. The larger the magnitude of t-test and smaller the *p*-value, the more significant is the corresponding coefficient.

Table 7. Estimated Effects and Coefficients of the Empirical Model for R_{max}

Term	Coefficient	Error	*T*	*p*
Constant	63.14	0.821	76.88	< 0.001
X_1	0.317	0.406	0.78	0.448
X_2	2.592	0.406	6.39	< 0.001
X_4	2.125	0.406	5.24	< 0.001
X_6	1.408	0.406	3.47	0.004
X_1*X_1	-2.294	0.379	-6.05	< 0.001
X_2*X_2	-1.381	0.379	-3.64	0.003
X_4*X_4	-1.619	0.379	-4.27	0.001
X_6*X_6	-1.119	0.379	-2.95	0.011
X_1*X_2	0.6	0.497	1.21	0.247
X_1*X_4	1.063	0.497	2.14	0.051
X_1*X_6	-0.088	0.497	-0.18	0.863
X_2*X_4	1.038	0.497	2.09	0.055
X_2*X_6	1.238	0.497	2.5	0.026
X_4*X_6	0.075	0.497	0.15	0.882

The parameter estimates and the corresponding *p*-values suggest that, among the independent variables, X_2, X_4, and X_6 expressed strong linear effects on the response ($p < 0.01$), whereas X_1 was insignificant (p=0.448). All the quadric terms of these four variables also had a significant effect, but were negative for the response. Only an interaction (X_2X_6) among the four variables was found to contribute to the response at a significant level (p=0.026). In this case, X_2, X_4, X_6, X_1^2, X_2^2, X_4^2, X_6^2, and X_2X_6 were significant model terms, respectively. After the neglect of insignificant terms, the model Eq. (4) was modified to a reduced fitted model Eq. (5):

$$Y=63.138 + 0.317 X_1 + 2.592 X_2 + 2.125 X_4 + 1.408 X_6 - 2.294 X_1^2 - 1.381 X_2^2 - 1.619 X_4^2 - 1.119 X_6^2 + 1.238 X_2 X_6 \quad (5)$$

The 2D contour plot and 3D response surface curve are generally the graphical representation of the regression equation (Wang and Liu 2008; Li et al. 2007, 2009). Response surface plotting provides a method to predict COD removal efficiency for different values of the test variables, and the contours of the plot are helpful in identification of the type of interactions between test variables. The circular contour plots of response surfaces suggest that the interaction is negligible between the corresponding variables. Instead, elliptical or saddle contour plots indicate the significance of the interactions between the corresponding variables. It can be seen from Equation 5 that only X_2 X_6 was significant for COD degradation among all interactions. The 2D contour plot and 3D response surface curve of X_2 and X_6 are shown in Fig. 2, which presents the effect of two variables on the COD degradation, while another two variables were held at zero level of RSM. From the response surface plots, it is easy and convenient to understand the interactions between two variables and also to locate their optimum levels. It can be seen from Fig. 2, an elliptical 2D contour plot of X_2 *(Bacillus sp.)* and *X6 (P. putid)*, that a remarkable interaction of two variables took place with respect to COD degradation, which is consistent with the results of statistical analysis (Table. 7). The 3D response surface curve was convex in nature, suggesting that there were well-defined cell concentrations of *Bacillus* sp. and *P. putid*.

Validation of the Experimental Design

The optimum concentrations of the variables were calculated from the data obtained. The optimal levels of each test variables in coded units were as follows: X_1=0.068, X_2=1.632, X_4=0.656, and X_6=1.527. Their actual values were 0.35 (X_1), 0.38 (X_2), 0.43 (X_4), and 0.38 (X_6), respectively. The model predicted that the R_{max} of COD in the pulping effluent could reach 66.7% by using the above optimized concentrations of the variables. To confirm these results, test runs were performed by using the optimized conditions, and a mean value of (65.3 ± 0.5)% (N=5) was obtained. The good correlation between these two results confirmed the validity of the response model. Thus, the optimum combination of four strains for COD degradation should consist of 0.35 (OD_{600}) *Agrobacterium* sp., 0.38 *Bacillus* sp., 0.43 *Gordonia sp.*, and 0.38 *P. putid*.

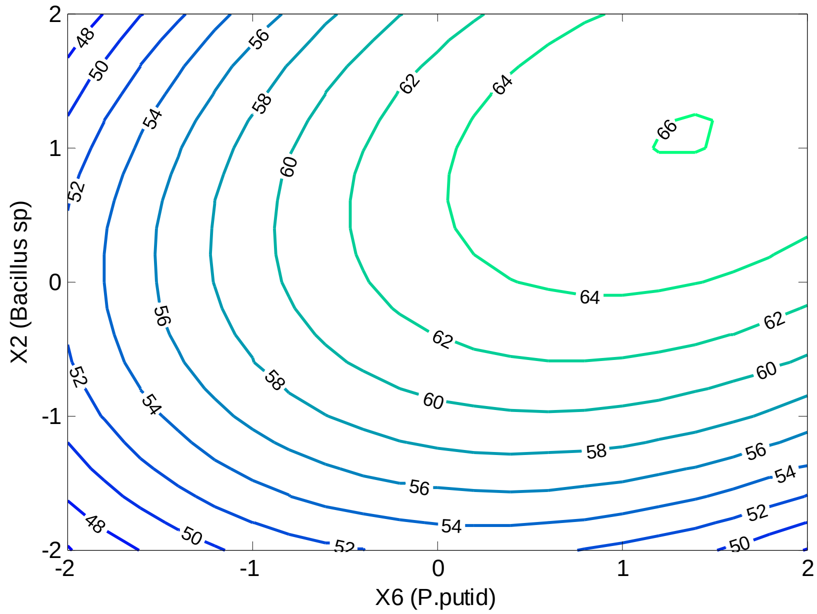

Fig. 2(a). 2D contour plot of *Bacillus* sp. and *P. putid* predicted by the full quadratic model

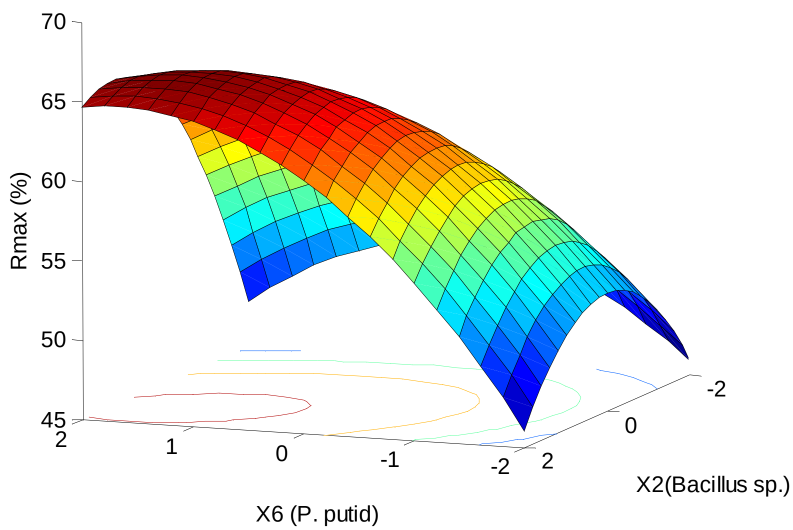

Fig. 2(b). 3D response surface curve of *Bacillus* sp. and *P. putid* predicted by the full quadratic model

CONCLUSIONS

Six strains of microorganisms, i.e., *Agrobacterium* sp., *Bacillus* sp., *E. cloacae*, *Gordonia sp.*, *P. stutzeri*, and *P. putid* were investigated for their roles in the degradation of chemical oxygen demand (COD) in pulping effluents. A fractional factorial design (FFD) of experiment was employed in the first step to select factors in an effort to construct a mixed-culture community that yields optimal treatment performance for COD degradation in pulping effluents, and four strains were selected for further study. Then the method of Steepest Ascent was used to approach the optimum conditions within the experimental design space. Central composite designs and response surface analysis introduced in the last steps were useful to determine the optimum levels of the factors that significantly influenced COD removal efficiency. The optimized cell concentrations (OD_{600}) of four strains were as follows: 0.35 *Agrobacterium* sp., 0.38 *Bacillus* sp., 0.43 *Gordonia sp.*, and 0.38 *P. putid*. The validity of the model was proven by fitting the values of the variables in the second-order equation and by carrying out the experiment at those values of the variables, and test runs showed that (65.3 ± 0.5)% of COD removal efficiency was close to predicted value (66.7%). These results reveal that statistical experimental design including fractional factorial design (FFD) and response surface methodology (RSM) are useful for biological screening and for characterizing the interplay among different strains, and can be used to enhance the remediation of pulping effluents.

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