

Application of statistical design for the optimization of microbial community of synthetic domestic wastewater

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Abstract A fractional factorial design (FFD) and a response surface methodology (RSM) were used to optimize the inoculum composition of six strains for treatment of synthetic domestic wastewater. The model predicted the highest overall specific substrate utilization rate (q) of 6.88 g TOC/(d-gVSS). The value is in accordance with the actual maximum q , and is 1.5 and 1.97 times greater than those without optimization for 4 and 6 strains respectively. Additionally, the shortest time to reach stationary phase (3.5 h) and highest maximum total organic carbon (TOC) removal efficiency (92%) were also achieved under the optimum condition. The results indicated that the FFD and RSM are powerful screening and optimizing tools for the

microbial community. The experimental approaches enhance the overall specific rate of substrate utilization as well as other biodegradation parameters.

Keywords Fractional factorial design · Central composite design · Response surface methodology · Microbial community · Specific substrate utilization rate

Introduction

The diversity of complex microbial populations and community structure are very important to ecosystem, where species richness and community evenness are considered the most important factors (Loreau et al. 2001; Wittebolle et al. 2009). High performance of a biological treatment system is often achieved through bioaugmentation of one or more strains of microorganisms. Bioaugmentation has been demonstrated to enhance the removal of many specific pollutants such as phenols, chloroaniline, chlorobenzoate, resin acid, etc. (Singer et al. 2005; Thompson et al. 2005; Damsa et al. 2007). These researches mainly focused on bioaugmentation of one strain, which has been documented well in a recent review (Fantroussi and Agathos 2005). However, there are few reports regarding the bioaugmentation of multiple strains of microorganisms. Bioaugmentation of a group of microorganisms may improve reliability of bioaugmentation (Jiang et al. 2007). The most

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important step for the newly introduced strains is to establish the positive mutualistic relationship with each other. Wittebolle et al. (2009) also confirmed that initial community evenness play a key role in preserving the functional stability of an ecosystem. However, to date, most of the related studies have focused mainly on treatment performance of bioaugmentation without considering the relationship of bacterial communities involved (Briones and Raskin 2003; Smith et al. 2003). Insights into the bacterial relationship of microbial communities are essential for improving treatment performance and stability against inhibitory compounds. However, it is very difficult to clarify the roles of each strain and the relationships among strains of a community.

Some researches used rRNA/rDNA-based molecular techniques to determine the diversity of complex microbial populations and community structure during the degradation process, especially in the field of pollutant biodegradation and biological investigations of bacterial communities (Calli et al. 2006; McMahan et al. 2001; Sekiguchi et al. 1999). These methods are powerful and accurate. However, some drawbacks such as long run times, high instrument expenses, and ignorance of the roles of each member and the interactions of the mixed microorganisms make the molecular techniques method unsuitable in some cases.

In contrast, statistical experimental design can analyze the interactions among the members of bacterial communities, and optimize community structure for improving treatment efficiency. Such a technique has been widely used in various fields such as in biochemistry for optimization of culture medium and conditions (Vohra and Satyanarayana 2002), in a microextraction for the determination of benzene, toluene, ethylbenzene and xylenes material (Gaujaca et al. 2008). However, to our knowledge, there are few reports on using the statistical method to investigate the effect of mixed culture inoculums on the performance of degrading synthetic wastewater.

The aim of this work is to apply statistical experimental design to investigate the role of each individual member and the mutualistic relations in a mixed culture consisting six bacteria during biodegradation of synthetic wastewater. We expect our experiment will facilitate better understanding of the mechanisms to construct a defined mixed culture consisting of specific microorganisms with high capability.

Materials and methods

Microorganism, nutrient medium, and solutions

Six strains of bacteria, i.e., *Agrobacterium* sp., *Bacillus* sp., *Enterobacter cloacae*, *Gordonia*, *Pseudomonas Stutzeri*, and *Pseudomonas putida* were selected based on an established mixed culture in a portable wastewater treatment system. The stock cultures were stored at 4°C. The nutrient medium contained 3 g/l of beef extract, 5 g/l of peptone, and mineral salt medium (MSM) at pH 7. The composition of MSM in g/l was KH_2PO_4 (0.42), K_2HPO_4 (0.375), $(\text{NH}_4)_2\text{SO}_4$ (0.244), NaCl (0.015), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.015), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05), and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.054). A phosphate buffer (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), and was used for diluting concentrated cells. All chemicals were analytical reagent grade.

Bacteria cultivation and experimental procedure

Six bacteria were activated at 30°C in the nutrient medium. The activated cells in the late exponential phase were then harvested as inoculum, respectively. The cells collected after centrifugation (6,000 rpm) for 10 min were resuspended in phosphate buffer and re-centrifuged. After cleaning, six inoculums were separately prepared by inoculating the activated six strains into the synthetic domestic wastewater to give an optical density at 600 nm (OD_{600}), cells concentrations (L), 0.1 or 0.3. In each fractional design experiment (detailed in “Statistical methods” section), each 20-ml inoculum containing $L = 0.1$ or 0.3 bacteria was added aseptically to Erlenmeyer flasks (250 ml), the final volume was 120 ml. Then the flasks were capped with cotton plugs and placed in a shaker controlled at 100 rpm and 30°C. The pH of the mixture was kept at 7.0 during the course of all experiments by adding NaHCO_3 . Samples were withdrawn every hour, and the OD_{600} of cells (L) and TOC were measured as described below.

Analysis of cells and substrates

The concentration of biomass in the sample, X (mg VSS/l), was analyzed by measuring OD at 600 nm, L using an UV/Vis spectrophotometer (Pharmaspec

UV-1700, SHIMADZU) and 1-cm path length square quartz cuvettes with the synthetic domestic wastewater as the blank. Samples with 0.1 OD corresponds to about VSS of 0.039 g/l. In the following experiment, X was analyzed by measuring L , then converted it into VSS. The samples exceeding 0.8 OD were suitably diluted with the culture medium so that the Beer–Lambert law is applied.

Total organic carbon (TOC) was analyzed on filtered samples (0.45 μm Magna nylon membrane filters, SELECTRON, USA) with a Model 100 Benchtop TOC Analyzer (Thermo Electron Corporation, USA) using the combustion/non-dispersive infrared gas analytical method. Triplicate analyses were performed for all assays.

Kinetic analysis

The specific growth rate of cell, μ (h^{-1}): with the hypotheses of small initial cell concentration and of large initial substrate concentration (S_0), μ in a batch reactor was determined according to conventional procedures for kinetic analysis from batch culture data under the assumption of first-order growth kinetics with respect to biomass concentration in the exponential phase. In order to avoid the influence of mortality, specific growth rate of cell, μ is determined by measuring the initial growth rate and is calculated as:

$$\mu = 1/X \, dX/dt = d \ln X/dt \quad (1)$$

where X is the biomass concentration (mg VSS/l). The μ value is determined based on the exponential phase of growth curve. Therefore, the specific substrate utilization rate, q (g TOC/(d-gVSS)), can be expressed as:

$$\begin{aligned} q &= \left(\frac{dS}{dt}\right) \left(\frac{1}{X}\right) = \left(\frac{1}{X}\right) \left(\frac{dX}{dt}\right) \left(\frac{dS}{dX}\right) \\ &= \left(\frac{d \ln X}{dt}\right) \left(\frac{1}{Y}\right) = \frac{\mu}{Y} \end{aligned} \quad (2)$$

where Y is the yield, biomass produced per unit mass of substrate consumed, determined as:

$$Y = \frac{X - X_0}{S_0 - S} \quad (3)$$

where X_0 and S_0 represent the initial biomass and substrate concentrations, respectively, and S is the residual wastewater concentration (mg COD/l).

Statistical methods

To obtain the optimized community constitution for biodegradation, a fractional factorial design (FFD) was employed to evaluate the preliminary significance of the factors, as well as the interactions between them. The cell concentrations measured from OD_{600} of the six microorganisms, L were the study factors and q was as the response. All factors were evaluated at two levels, low (denoted as -1) and high (denoted as $+1$). The high and low levels of L were selected to be 0.1 and 0.3 OD_{600} , respectively. A three replicate center point (level 0, $L = 0.2$) was included in the design to estimate the experimental variance and check the loss of linearity between the levels chosen for each variable (curvature check). All analyses were performed using MINITAB Software (Release 14, Minitab Inc., State College, PA).

A full factorial design for the six bacteria (factors) requires 64 (2^6) experiments to evaluate the whole set of main effects as well as all interaction effects. The main effects and the lower-order interactions, however, are usually the most significant terms. In this work, a 2^{6-2} fractional factorial design consisting of 16 factorial runs along with three experiments at the center of the design for the analysis of curvature in each replicate was performed as a screening process to identify the significant factors and interactions.

Once critical factors were identified via screening and significant gross curvature has been detected in the design space, the central composite design (a response surface methodology (RSD) based on a highly fractionalized three-level factorial design) was proceeded to obtain a quadratic model, consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate the pure process variability and reassess gross curvature, with the overall specific TOC degradation rate as response. The central composite design used was generated by MINITAB. According to this design, 30 experiments were conducted containing six replicates at the center point for estimating the purely experimental uncertainty variance in triplicates. Each variable was designated as ± 2 , ± 1 , and 0. The significant variables indicated by the Pareto chart (obtained from multiple linear regression and analysis of variance) were optimized using a central composition design. A quadratic equation that correlates the variables and the response was obtained, and the critical points of

this equation were estimated through this mathematical relationship.

Results and discussion

Screening design

The characterization of individual strains has been presented in our previous published work (Chen et al. 2009). The screening design was to identify the major effects and mutual biodegradation effects of six strains of bacteria, i.e., *Agrobacterium* sp. (R_1), *Bacillus* sp. (R_2), *E. cloacae* (R_3), *Gordonia* (R_4), *P. Stutzeri* (R_5), *P. putida* (R_6). Table 1 shows the design, along with the results of three replicates of FFD for the overall specific rate of substrate utilization q (gTOC/(d-gVSS)). The q varied considerably from 1.92 to 5.36 g TOC/(d-gVSS) under different composition of bacteria in the cultivation. It suggests

that these variables significantly affected bacterial growth and TOC degradation. A three-replicate center point (level 0, $L = 0.2$) was included in the design to estimate the experimental variance and check the loss of linearity between the levels chosen for each variable (curvature check). The order in which the experiments were performed was selected at random, making this a completely randomized design.

The experimental values of q in the triplicate runs of the FFD were used for statistical analysis of the design. The mathematical correlation between analytical response and the variables was performed by a linear regression and curvature check. Figure 1 shows the Pareto chart.

From Fig. 1, only *Gordonia* (R_4) produced significant positive effect on q as a single strain, whereas the other strains were not significant or had negative effects on their own within the levels and conditions tested. In contrast, the positive interaction among

Table 1 Experimental design and results of the three replicates of the overall specific substrate utilization rate

Run	Coded variables						q (g TOC/(d-gVSS))
	X_1 <i>Agrobacterium</i>	X_2 <i>Bacillus</i>	X_3 <i>E. cloacae</i>	X_4 <i>Gordonia</i>	X_5 <i>P. stutzeri</i>	X_6 <i>P. putida</i>	
N_1	1	-1	-1	-1	1	-1	1.92 ± 0.10
N_2	1	1	-1	-1	-1	1	2.70 ± 0.14
N_3	-1	1	-1	1	1	-1	5.12 ± 0.23
N_4	1	1	1	-1	1	-1	3.12 ± 0.09
N_5	1	1	-1	1	-1	-1	3.12 ± 0.14
N_6	-1	1	-1	-1	1	1	4.06 ± 0.32
N_7	-1	-1	1	-1	1	1	2.86 ± 0.23
N_8	1	-1	1	1	-1	-1	5.36 ± 0.21
N_9	-1	1	1	1	-1	1	3.12 ± 0.21
N_{10}	-1	-1	-1	1	-1	1	3.54 ± 0.16
N_{11}	1	-1	-1	1	1	1	4.16 ± 0.21
N_{12}	-1	-1	-1	-1	-1	-1	4.55 ± 0.21
N_{13}	1	-1	1	-1	-1	1	3.28 ± 0.17
N_{14}	-1	1	1	-1	-1	-1	2.39 ± 0.07
N_{15}	-1	-1	1	1	1	-1	3.61 ± 0.12
N_{16}	1	1	1	1	1	1	5.12 ± 0.27
N_{17}	0	0	0	0	0	0	3.68
N_{18}	0	0	0	0	0	0	3.53
N_{19}	0	0	0	0	0	0	3.59

$X_i = (L_i - 0.2)/0.1$; $i = 1, 2, 3, 4, 5, 6$, indicate six strains respectively; L_i is the actual level of cells concentration (OD_{600})

Results are averages and the standard deviations from three replicates

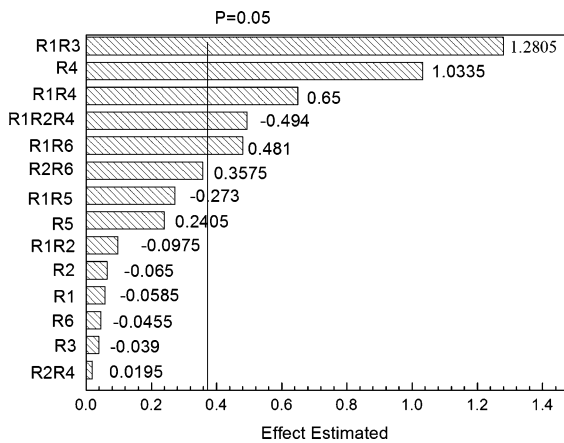


Fig. 1 Fractional factorial design Pareto graph. Response is the overall specific substrate utilization rate (q)

some strains should be given consideration due to its synergistic effects. The composition of *Agrobacterium* sp. and *E. cloacae* (R_1R_3) produces the largest effect on q . Other interactions among the strains, such as *Agrobacterium* sp., and *Gordonia* (R_1R_4), *Agrobacterium* sp., *Bacillus* sp and *Gordonia* ($R_1R_2R_4$) and *Agrobacterium* sp., *P. putida* (R_1R_6), were also significant based on a 5% level of significance. The curvature P value of 0.965 indicated that the mean are not significantly different from the average of the center points and the average of the factorial points. As a result, the optimum point should be near or within the experimental design space.

Base on the above results, a more detailed study using a response surface method, central composition design, was conducted with strains R_1 , R_3 , R_4 , R_6 , considering their significant impact on q . Strains R_2 and R_5 , which did not have significant, were excluded.

Optimization with central composite design and response surface analysis

The objective of this set of experiment is to build a second-order (quadratic) model for the response variable (q). The four main effects variables, *Agrobacterium* sp. (R_1), *E. cloacae* (R_3), *Gordonia* (R_4) and *P. putida* (R_6), were further investigated using a response surface design within the design space introduced in the FFD.

RSD is appropriate when the optimal region for running the process has been identified. A central

composite design with eight-star points and five replicates at the center point for each of four factors was carried out to further optimize the microbial community. To make the design rotatable, the axial distance at the higher star points was assigned a value of 2, while the axial distances of these factors at the lower star point were assigned to be -2 . Table 2 showed the experimental design and results.

The coefficients, t test and P value for the linear, quadratic and interaction terms are shown in Table 3. The results of Analysis of Variance (ANOVA) are shown in Table 4.

The four variables tested expressed strong linear effect on the response ($P = 0.001$) (Tables 3, 4), whereas *Agrobacterium* (R_1) displayed negative effect while *E. cloacae* (R_3), *Gordonia* (R_4) and *P. putida* (R_6) showed positive effect. The four strains all presented significant negative quadric effect with P value of 0.000 on response.

Significant negative interactions were noted between *Agrobacterium* and *Gordonia* (R_1R_4), *E. cloacae* and *Gordonia* (R_3R_4), *Gordonia* and *P. putida* (R_4R_6) based on a 5% level of significance (Table 3). The significant negative effect implied that the specific substrate utilization rate initially increased to the maximum, and finally decreased at the even higher levels of variables. The results were different from our FFD experiments (“Screening design” section), which showed that significant interactions between *Agrobacterium* sp. and *E. cloacae* (R_1R_3), *Agrobacterium* sp. and *Gordonia* (R_1R_4), *Agrobacterium* sp., *Bacillus* sp. and *Gordonia* ($R_1R_2R_4$) and *Agrobacterium* sp. and *P. putida* (R_1R_6), were significant. This can be explained by the fact that FFD experiments were performed with six strains while RSD was with four strains, the actual interaction between strains may be more complicated than that we have reported here.

Model F value is calculated as ratio of mean square regression and mean square residual. The high F value and the associated low P values indicated that the strong significance of the model predictors based on the experimental results. The four variables tested expressed strong effect on the regression (Table 4). There is high significance in the linear, square and interaction terms existed by the four strains (Table 4), indicating that the degradation of synthetic domestic wastewater was primarily controlled by four selected strains.

Table 2 Central composite design with four variables and the corresponding response (the overall specific substrate utilization rate)

Run	Coded variables				Overall specific substrate utilization rate q (g TOC/(d-gVSS))
	F_1 <i>Agrobacterium</i> sp.	F_3 <i>E. cloacae</i>	F_4 <i>Gordonia</i>	F_6 <i>P. putida</i>	
M_1	-1	-1	-1	-1	0.450 ± 0.075
M_2	1	-1	-1	-1	1.800 ± 0.028
M_3	-1	1	-1	-1	1.470 ± 0.012
M_4	1	1	-1	-1	1.800 ± 0.032
M_5	-1	-1	1	-1	4.800 ± 0.039
M_6	1	-1	1	-1	1.650 ± 0.042
M_7	-1	1	1	-1	5.580 ± 0.043
M_8	1	1	1	-1	0.390 ± 0.010
M_1	-1	-1	-1	1	1.290 ± 0.021
M_2	1	-1	-1	1	1.800 ± 0.016
M_3	-1	1	-1	1	3.150 ± 0.032
M_4	1	1	-1	1	4.080 ± 0.022
M_5	-1	-1	1	1	3.900 ± 0.035
M_6	1	-1	1	1	1.350 ± 0.067
M_7	-1	1	1	1	3.960 ± 0.024
M_8	1	1	1	1	0.975 ± 0.052
M_7	-2	0	0	0	4.665 ± 0.017
M_8	2	0	0	0	2.445 ± 0.016
M_9	0	-2	0	0	0.675 ± 0.009
M_{10}	0	2	0	0	1.635 ± 0.038
M_{11}	0	0	-2	0	1.470 ± 0.046
M_{12}	0	0	2	0	2.535 ± 0.021
M_{13}	0	0	0	-2	1.785 ± 0.021
M_{14}	0	0	0	2	3.165 ± 0.052
M_{15}	0	0	0	0	5.745
M_{16}	0	0	0	0	5.535
M_{17}	0	0	0	0	5.650
M_{18}	0	0	0	0	5.535
M_{19}	0	0	0	0	5.435
M_{20}	0	0	0	0	5.100

$F_i = (L_i - 0.2)/0.1$; $i = 1, 3, 4, 6$, indicate four strains respectively; L_i is the actual level of cells concentration (OD₆₀₀)

The averages and the standard deviations are from three replicates

By applying the multiple regression analysis on the experimental data, a second-order polynomial model in coded unit explains the role of each variable and their second-order interactions in q .

$$\begin{aligned}
 q = & 5.47 - 0.63L_1 + 0.26L_3 + 0.37L_4 + 0.22L_6 \\
 & - 0.47L_1^2 - 1.07L_3^2 - 0.86L_4^2 - 0.74L_6^2 \\
 & - 0.19L_1L_3 - 1.06L_1L_4 + 0.16L_1L_6 - 0.37L_3L_4 \\
 & + 0.21L_3L_6 - 0.44L_4L_6 \tag{4}
 \end{aligned}$$

where L_i is cells concentration, $i = 1, 3, 4, 6$ for 4 strains respectively. After neglecting the insignificant terms based on 5% level of significance, it can be expressed as following:

$$\begin{aligned}
 q = & 5.47 - 0.63L_1 + 0.26L_3 + 0.37L_4 \\
 & + 0.22L_6 - 0.47L_1^2 - 1.07L_3^2 - 0.86L_4^2 \\
 & - 0.74L_6^2 - 1.06L_1L_4 - 0.37L_3L_4 \\
 & - 0.44L_4L_6 \tag{5}
 \end{aligned}$$

Table 3 Estimated effects and corresponding coefficients of the overall specific substrate utilization rate, q

Term	Coefficient	T	P
Constant	5.474	31.547	0.000
R_1	-0.633	-7.388	0.000
R_3	0.262	3.056	0.009
R_4	0.371	4.325	0.001
R_6	0.222	2.589	0.021
R_1^2	-0.469	-5.845	0.000
R_3^2	-1.069	-13.330	0.000
R_4^2	-0.857	-10.687	0.000
R_6^2	-0.739	-9.213	0.000
$R_1 * R_3$	-0.192	-1.831	0.088
$R_1 * R_4$	-1.062	-10.120	0.000
$R_1 * R_6$	0.160	1.527	0.149
$R_3 * R_4$	-0.372	-3.546	0.003
$R_3 * R_6$	0.205	1.956	0.071
$R_4 * R_6$	-0.440	-4.189	0.001

Table 4 Analysis of variance for the overall specific substrate utilization rate, q

Source	DF	SS	Adj MS	F	P
Blocks	1	0.1591	0.1591	0.90	0.358
Regression	14	93.8931	6.7066	38.05	0.000
Linear	4	15.7444	3.9361	22.33	0.000
Square	4	53.1106	13.2776	75.33	0.000
Interaction	6	25.0381	4.1730	23.67	0.000
Residual error	14	2.4677	0.1763		
Lack-of-fit	10	2.3807	0.2381	10.94	0.017
Pure error	4	0.0870	0.0218		
Total	29	96.5199			

$$S = 0.4198, R^2 = 97.4\%, R_{adj}^2 = 94.7\%$$

SS , sum of squares; DF , degree of freedom; MS , mean square

The results by RSD showed that the coefficient of determination (R^2) was 97.4%, which ensured satisfactory adjustment of the quadratic model to the experimental data and indicated that 97.4% of the variability in the response variables could be explained by the second-order polynomial prediction equation given in Eq. 4. The value of adjusted determination coefficient (R_{adj}^2) was calculated to be 94.7%, which is also very high, indicating a high significance of the model. Lack of fit P value of 0.017 and low pure error demonstrated the experiments

conducted were precise and reliable within the range of variables employed.

Response surface plot provides a method to predict the yields of active substances 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 two-dimensional contour plot and three-dimensional response surface from the calculated response surface in the overall specific substrate utilization rate was plotted in Fig. 2, which further explains the results of statistical analyses. Each subplot presented the effect of two variables on q , while the other two variables were held at zero level.

The circular contour plots of response surfaces suggest that the interaction is negligible between the corresponding variables. The significant perfect interaction between the independent variables can be shown when elliptical or saddled contours are obtained (Muralidhar et al. 2001). The 2-D contour plots of R_1R_4 , R_3R_4 and R_4R_6 had remarkable interaction, and the 3-D response surface curves of R_3R_4 and R_4R_6 were convex in nature (Fig. 2), which suggested that there were optimized concentrations of $R_3R_4R_6$ strains. However, there were not convex in R_1R_4 response surface curve. At low *Gordonia* (R_4) concentration, q increased as increase in *Agrobacterium* (R_1) concentration, and decrease at high *Gordonia* (R_4) concentration.

The optimum concentrations of the variables were calculated from the data obtained using RSM system. The optimal values of each test component in coded units were as follows: $R_1 = -2.00$, $R_3 = -0.054$, $R_4 = 1.63$ and $R_6 = -0.63$. Their corresponding L values were $L_1 = 0$, $L_3 = 0.146$, $L_4 = 0.363$ and $L_6 = 0.137$. At this point, the model predicted q_{max} of 6.88 g TOC/(d-gVSS), and the composite desirability is 0.81.

Validation of the experimental design

To understand the composite effects of the six strains of bacteria on the TOC degradation performance and to confirm the model adequacy for predicting the maximum overall specific rate of substrate utilization, additional three batch comparison experiments were performed.

In each experiment, six inoculums were separately prepared by inoculating the activated six strains into

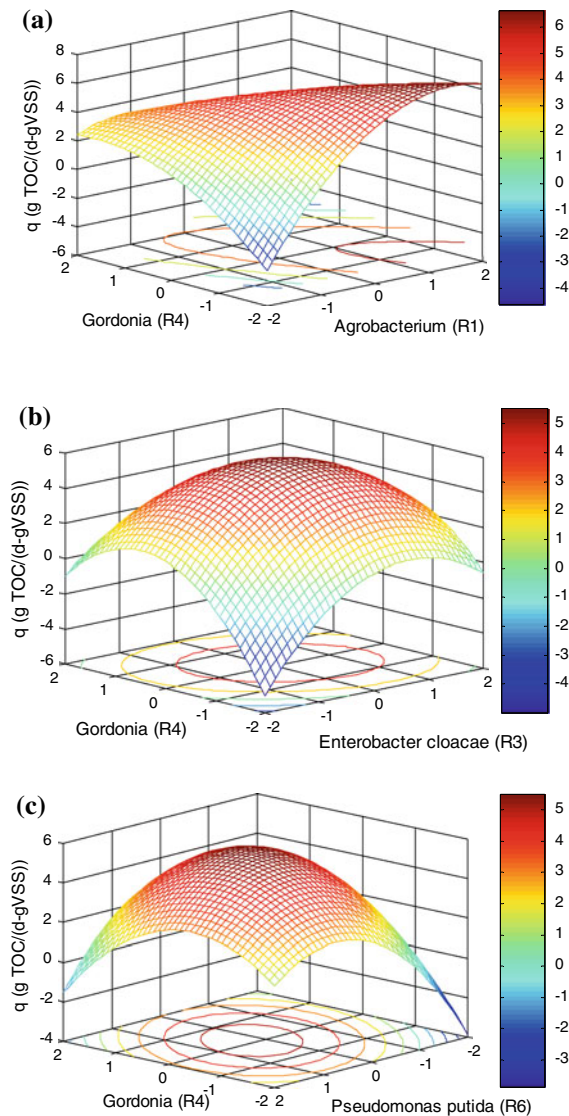


Fig. 2 Two-dimensional (2-D) contour plot and three-dimensional (3-D) response surface curve predicted by the full quadratic model of **a** *Agrobacterium* sp. and *Gordonia* (R_1R_4), **b** *E. cloacae* and *Gordonia* (R_3R_4), and **c** *Gordonia* and *P. putida* (R_4R_6)

the synthetic domestic wastewater to give the specific applied level of cell concentration L , OD_{600} (Run I: $L_1 = 0$, $L_3 = 0.146$, $L_4 = 0.363$ and $L_6 = 0.137$; Run II: $L_1 = 0$, $L_3 = L_4 = L_6 = 0.162$; Run III: $L_1 = L_2 = L_3 = L_4 = L_5 = L_6 = 0.162$). Then each 30-ml (four strains) or 20-ml (six strains) inoculums containing specific cell concentration was added aseptically to Erlenmeyer flasks (250 ml), which made all three batch experiments have the same

initial cell concentration ($L_{\text{initial}} = 0.162$) and the same volume (120 ml). The first batch experiment was performed with the optimal OD_{600} values of the selected three strains, the strain concentration are the same in the second (four strains) and third (six strains) control experiments.

Although the model was developed using q as the response, some other parameters such as the initial cell density (L_{initial}), the time to reach stationary phase (i.e., at stable TOC concentration, T_s), the maximum total organic carbon (TOC) removal efficiency (R_{max}) and the yield (Y) were observed to evaluate the biodegradation process. The experimental results are showed in Table 5.

It is found that although L_{initial} of the three batch experiments were the same (0.162), the shortest T_s (3.5 h) value, the highest R_{max} (92%) and q (7.13 ± 0.12 g TOC/(d-gVSS)) were achieved by the optimum composite of three strains (Run I).

The three replicates with the optimal concentration composition of three strains (Run I) yielded an average q of 7.13 ± 0.12 g TOC/(d-gVSS), which was closer to the predicted response (6.88 g TOC/(d-gVSS)). The value was 1.5 and 1.97 times greater than those microbial communities of four (Run II) and six strains (Run III) without optimization. Therefore, statistical analysis and the application of optimal conditions enhanced the overall specific rate of substrate utilization as well as other biodegradation parameters.

Interestingly, the q and R_{max} of both three and four strains (Run I, II) were higher than those of six strains (Run III). That implied that the biodegradation process inoculated with $R_3R_4R_6$ performed better than that composed of six strains. This suggests that the optimizing the inoculums is important for improving treatment performance.

Table 5 Degradation of the verification experimentation

Run	L_{initial}	OD_{600}	T_s (h)	R_{max} (%)	Y	q (gTOC/ (d-gVSS))
I	0.162 ± 0.03		3.5	92	0.743	7.13 ± 0.12
II	0.162 ± 0.05		5	89	0.642	4.69 ± 0.17
III	0.162 ± 0.04		5.1	79	0.679	3.62 ± 0.15

* L_{initial} the initial cell density, T_s the time required for getting stable phase of TOC degradation, R_{max} the maximum TOC removal efficiency, Y the yield, biomass produced per unit mass of substrate consumed and q the specific substrate utilization rate

The good agreement between the predicted and experimental results validated the model and reflected the existence of an optimal point. The results derived from this study demonstrate that the FFD and RSD are powerful tools for elucidating the individual factors and synergistic relationship among multiple strains and optimizing the composition microbial community in bioaugmentation treatment.

Conclusions

Statistics-based experimental design methods proved to be valuable tools in optimizing the strains composition for synthetic domestic wastewater degradation. FFD was employed in the first step to screen out the significant factors affecting substances degradation by six strains of microorganisms. Central composite designs and response surface analysis revealed that *Agrobacterium* sp., *E. cloacae*, *Gordonia* and *P. putida* had the most significant linear, square and interaction terms effects on the response, q . The optimum values of each strains concentration were $L_1 = 0$, $L_3 = 0.146$, $L_4 = 0.363$ and $L_6 = 0.137$. At this point, the model predicted q of 6.88 g TOC/(d-gVSS). The actual q is 7.13 ± 0.12 g TOC/(d-gVSS), which was 1.5 and 1.97 times higher than those without optimization. The results derived from this study demonstrated that the FFD and RSM are powerful tools for optimizing individual factors.

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References

Briones A, Raskin L (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr Opin Biotechnol* 14:270–276

Calli AB, Mertoglu B, Roest AK, Inanc B (2006) Comparison of long-term performances and final microbial compositions of anaerobic reactors treating landfill leachate. *Bioresour Technol* 97:641–647

Chen Y, Lin C-J, Gavin J, Shiyu F, Zhan H (2009) Enhancing biodegradation of wastewater by microbial consortia with fractional factorial design. *J Hazard Mater* 171:948–953

Damsa RI, Patonb G, Killham K (2007) Bioaugmentation of pentachlorophenol in soil and hydroponic systems. *Int Biodeterior Biodegrad* 60:171–177

Fantroussi SE, Agathos SN (2005) Is bioaugmentation a feasible strategy for pollutant removal and site remediation. *Curr Opin Microbiol* 8:268–275

Gaujaca A, Elissandro SED, Navickieneb S, Ferreirac SLC, Dóreab HS (2008) Multivariate optimization of a solid phase microextraction-headspace procedure for the determination of benzene, toluene, ethylbenzene and xylenes in effluent samples from a waste treatment plant A. *J Chromatogr A* 1203:99–104

Jiang H-L, Maszenan AM, Tay JH (2007) Bioaugmentation and coexistence of two functionally similar bacterial strains in aerobic granules. *Appl Microbiol Biotechnol* 75:1191–1200

Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime JP, Hector A, Hooper DU, Huston MA, Raffaelli D, Schmid B, Tilman D, Wardle DA (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294:804–808

McMahon K, Stroot PG, Mackie RI, Raskin L (2001) Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—II: microbial population dynamics. *Water Res* 35:1817–1827

Muralidhar RV, Chirumamila RR, Marchant R, Nigam P (2001) A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. *Biochem Eng J* 9:17–23

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H (1999) Fluorescence in situ using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. *Appl Environ Microbiol* 65:1280–1288

Singer AC, Van Der Gast CJ, Thompson LP (2005) Perspectives and vision for strain selection in bioaugmentation. *Trends Biotechnol* 23:74–77

Smith NR, Yu Z, Mohn WW (2003) Stability of the bacterial community in a pulp mill effluent treatment system during normal operation and a system shutdown. *Water Res* 37:4873–4884

Thompson LP, van der Gast CJ, Ciric L, Singer AC (2005) Bioaugmentation for bioremediation: the challenge of strain selection. *Environ Microbiol* 7:909–915

Vohra A, Satyanarayana T (2002) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochem* 37:999–1004

Wittebolle L, Marzorati M, Clement L, Balloi A, Daffonchio D, Heylen K, De Vos P, Verstraete W, Boon N (2009) Initial community evenness favours functionality under selective stress. *Nature* 458:623–626