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Statistical factor-screening and optimization in slurry phase bioremediation of 2,4,6-trinitrotoluene contaminated soil

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ABSTRACT

Since slurry phase bioremediation is a promising treatment for recalcitrant compounds such as 2,4,6-trinitrotoluene (TNT), a statistical study was conducted for the first time to optimize TNT removal (TR) in slurry phase. Fractional factorial design method, 2_{IV}^{7-3}, was firstly adopted and four out of the seven examined factors were screened as effective. Subsequently, central composite design and response surface methodology were employed to model and optimize TR within 15 days. A quadratic model ($R^2 = 0.9415$) was obtained, by which the optimal values of 6.25 g/L glucose, 4.92 g/L Tween80, 20.23% (w/v) slurry concentration and 5.75% (v/v) inoculum size were estimated. Validation experiments at optimal factor levels resulted in 95.2% TR, showing a good agreement with model prediction of 96.1%. Additionally, the effect of aeration rate (0–4 vvm) on TR was investigated in a 1-liter bioreactor. Maximum TR of 95% was achieved at 3 vvm within 9 days, while reaching the same removal level in flasks needed 15 days. This reveals that improved oxygen supply in bioreactor significantly reduces bioremediation time in comparison with shake flasks.

1. Introduction

Nowadays, contamination of ecosystems originating from agricultural, industrial, and military facilities has become one of the important issues. One of the serious environmental concerns is currently the vast scale of soil pollution with explosive compounds during production, ordnance demilitarization, demolition procedures, and destruction of outdated and faulty ammunition [1,2]. These hazardous and toxic chemicals may penetrate the soil by surface runoff or leaching into ground water, resulting in contamination of streams and aquifers [3]. Among such compounds, 2,4,6-trinitrotoluene (TNT) is a predominant contaminant at ammunition plants, testing facilities and military zones. The amount of TNT in contaminated soils may vary from trace levels up to 14,000 mg/kg-soil, which is close to its explosive level [4]. TNT is considered to be toxic for a wide range of aquatic organisms [5,6], terrestrial species [7], mammals [6] and human monocytes [8]. Exposure to TNT can increase the incidence of aplastic anemia, liver damage, dermatitis, ocular disorders and gastrointestinal distress [9,10]. TNT is therefore classified as an EPA class C (possible human carcinogen) with a drinking water equivalent level of 20 mg/L and a lifetime health advisory level of 2 mg/L [11,12].

Due to the risks associated with TNT, considerable efforts have been made to implement effective remediation strategies in explosive-contaminated sites. Various physicochemical techniques such as incineration, chemical oxidation, alkaline hydrolysis [13] and surfactant-enhanced washing [14] have been used in this regard. However, most of these methods are expensive and may cause serious harm to ecosystems [13]. Over the last few decades, exploitation of microorganisms for degrading and co-metabolizing explosive materials in water and soil has received wide attention by many researchers. Bioremediation has emerged as an economic and environmentally safe alternative for cleaning explosive-contaminated sites [15].

Various microorganisms have been reported to be competent in metabolizing TNT, e.g. certain strains of gram-negative bacteria such as *Acinetobacter*, *Alcaligenes*, *Cytophaga*, *Klebsiella*, *Pseudomonas* and *Sphingomonas*, gram-positive bacteria such as *Arthrobacter*, *Bacillus*, *Corynebacterium*, and *Micrococcus* [16,17], white rot fungi and basidiomycetes under aerobic conditions [18] as well as anaerobic bacteria of two genera including sulfate-reducing and methane-producing [16]. With regard to TNT metabolism in bacteria, reports exist on TNT biotransformation to isomers of amino-derivatives such as 4-amino-2,6-dinitrotoluene (4-ADNT), 2-amino-4,6-dinitrotoluene nitro (2-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT) via reductive pathways [16] as well as oxygenolytic metabolism after elimination of nitro group during the formation of π-Meisenheimer complex [17].

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Table 1
The 2^{7-3}_{IV} FFD screening experiments and the corresponding system responses.

Trial	Glucose (g/L) *A*	NH_4Cl (g/L) *B*	Tween80 (g/L) *C*	Slurry con. (% (w/v)) *D*	Temperature (°C) *E*	Yeast extract (g/L) *F*	Inoculum size (% (v/v)) *G*	TR (%)
1	2	0.1	5	20	35	0.2	10	90.5
2	8	0.1	5	20	20	0.2	5	80.1
3	8	0.1	5	20	35	0	10	92.3
4	2	0.1	5	40	35	0	5	82.9
5	2	0.1	1	40	20	0.2	10	68.1
6	8	0.5	1	20	20	0.2	10	90.4
7	2	0.5	1	40	35	0	10	71.6
8	8	0.1	1	40	35	0.2	5	79.5
9	8	0.5	5	40	35	0.2	10	86.5
10	2	0.5	5	40	20	0.2	5	84.1
11	8	0.5	5	20	35	0	5	91.3
12	2	0.5	1	20	35	0.2	5	89.7
13	8	0.5	1	40	20	0	5	78.1
14	2	0.1	1	20	20	0	5	90.4
15	2	0.5	5	20	20	0	10	91.0
16	8	0.1	5	40	20	0	10	83.6
17	5	0.3	3	30	27.5	0.1	7.5	85.6
18	5	0.3	3	30	27.5	0.1	7.5	89.7
19	5	0.3	3	30	27.5	0.1	7.5	88.3

All intermediates in TNT metabolism are less cytotoxic than TNT as evaluated in the human hyphoblastic cell lines V79 and TK6 where none was found mutagenic for mammalian cells [19]. Rarely has TNT mineralization been reported to occur by microorganisms and where observed it has been under ligninolytic conditions [16,17].

Solid phase bioremediation methods such as composting [20–22] and land farming [23,24] have been used to treat TNT-contaminated soils. However, the major disadvantage of such methods is the prolonged incubation time due to the limited mass transfer in solid phase. Therefore, slurry phase bioremediation, where a mixture of contaminated soil, water and co-substrates is treated under controlled mixing and aeration conditions, has been used as an alternative to solve this problem [25]. This technique has been applied to TNT-contaminated soils by several researchers [24,26–31] and higher removal efficiencies have been observed in slurry phase bioremediation as compared to solid phase [27,29,31].

The effect of various factors on TNT bioremediation has been previously studied. This includes supplemental carbon and nitrogen sources [31–35], microorganism [36], pH and temperature [32,36] in aqueous phase as well as slurry concentration, agitation speed [28] and surfactant [29,37] in slurry phase. Nevertheless, a one at a time approach was used to look at the effect of each factor in slurry phase and therefore the possible interactions among the effective factors have not yet been assessed. This necessitates applying a systematic optimization approach to slurry phase bioremediation of TNT-contaminated soils.

When a system is affected by a large number of independent factors, experimental design methods are commonly used to systematically determine the effective factors and their interactions as well as to model and optimize the whole system [38–40]. To avoid prolonged testing time, preliminary statistical screening experiments are usually conducted to identify the effective factors [41–43]. A combination of statistical and mathematical techniques is then employed to model the system response and predict the optimal level of effective factors [38]. Although statistical modeling and optimization have formerly been used for biodegradation of some contaminants in aqueous [44–46] and solid [47–49] phases, no statistical optimization has yet been applied to slurry phase bioremediation of TNT-contaminated soils. The aims of the present study are hence to statistically optimize slurry phase TR in shake flasks and further to evaluate the effect of aeration rate on TR in slurry bioreactor.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The microorganism used in this study, *Pseudomonas putida* (PTCC 1694), was obtained from Persian Type Culture Collection. This strain had previously revealed a high potential for TR in comparison with other degrading strains examined [50]. For inoculum preparation, bacterium was first cultivated in 250-mL flasks containing 50 mL of nutrient rich medium (containing g/L: peptone, 10; NaCl, 10; and yeast extract, 5) at 28 °C and 200 rpm. Subsequently, the mid-log phase culture (10^8 CFU/mL) was centrifuged at 4000 rpm for 20 min. The cells were then re-suspended in saline solution (8.5 g/L NaCl) and cell suspension at OD_{600} equal to 1 was used as inoculum. In shake flask experiments, a range of inoculum sizes was utilized according to Tables 1–3. Bioreactor was inoculated at the optimal size predicted by RSM in shake flask experiments.

2.2. Culture media

Mineral salts medium (MSM) containing (g/L-distilled water) K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4$, 0.1; and NaCl, 0.1 and 3 mL per liter of trace salts solution (TSS) was used in all experiments. TSS contained (mg/L-distilled water) H_3BO_3, 611; $MnCl_2$, 389; $CuSO_4{\cdot}5H_2O$, 56; $Al_2(Cl)_3{\cdot}6H_2O$, 56; $NiSO_4{\cdot}6H_2O$, 56; $CoCl_2{\cdot}6H_2O$, 56; $SnCl_2$, 28; and KI, 28. Culture medium was also supplemented by glucose, Tween80, NH_4Cl and yeast extract in screening and optimization shake flask experiments at concentrations given in Tables 1–3.

2.3. Soil preparation

A clean soil (Kaolin) with fine particle size (≈5 μm) obtained from Iran China Clay Industries Corporation (ICCIC) was used to artificially prepare TNT-contaminated soil at a final TNT concentration of 1000 mg/kg-soil. In order to obtain an even distribution of TNT in soil matrix, TNT was first dissolved in acetone at 1000 mg/L and then 1 mL of the resulting solution was added to 1 g of dried soil followed by mixing (FINEPCR, Finevortex Mixer) for 10 min [51]. After evaporation of the surplus acetone at room temperature, the treated soil was aged for 2 weeks and then sterilized at 120 °C for 20 min.

Table 2
Coded and natural values of factors in CCD matrix used in optimization experiments.

Design factors	Levels				
	$+\alpha$	+1	0	−1	$-\alpha$
A: glucose conc. (g/L)	10.25	8	5.75	3.5	1.25
C: Tween80 conc. (g/L)	6.5	5	3.5	2	0.5
D: slurry conc. (% (w/v))	42.5	30	25	20	12.5
G: inoculum size (% (v/v))	12.5	10	7.5	5	2.5

2.4. Experiments

Three sets of experiments in shake flasks were conducted in 100-mL Erlenmeyer flasks with working capacity of 20 mL in triplicate at 100 rpm and 30 °C for 15 days. This was followed by slurry bioreactor experiments at optimal factor levels.

2.4.1. Screening experiments

Screening experiments were performed based on a fractional factorial design (FFD) method to identify the effective factors on TR among seven factors each at two levels, i.e. glucose (2, 8 g/L), yeast extract (0, 0.2 g/L), NH_4Cl (0.1, 0.5 g/L), Tween80 (1, 5 g/L), slurry concentration (20, 40% (w/v)), inoculum size (5, 10% (v/v)) and temperature (20, 35 °C). Table 1 gives the details of each trial. System response was then calculated using Eq. (1) and it was reported as the average of triplicates.

$$\text{TR}\ (\%) = Y = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where C_i and C_f (mg/kg-soil) are the initial and final TNT concentrations, respectively.

2.4.2. Optimization experiments

Optimization experiments were designed based on central composite design (CCD) method using the four screened factors. The coded levels and natural values of factors in this design space are presented in Table 2. The CCD matrix is given in Table 3.

2.4.3. Validation experiments

To check the validation of model predictions, a set of shake flask experiments at optimal factor levels was run and the experimental and predicted TRs were compared.

2.4.4. Bioreactor experiments

The effect of aeration rate on TR was also investigated in a 1-liter laboratory-scale slurry bioreactor (see Fig. 1) with a working volume of 500 mL. Bioreactor experiments were performed under 0, 1, 2, 3, and 4 vvm aeration rates at ambient temperature (27 ± 1 °C). To avoid evaporative culture loss, bioreactor was supplied with saturated air by passing air through a humidifier. To examine the probable non-biological TNT elimination, bioreactor was also operated under 2 vvm aeration rate without inoculation (control).

Table 3
The CCD matrix used in optimization experiments and the experimental and predicted TRs.

Trial	Coded levels				TR (%)	
	A	*C*	*D*	*G*	Experimental	Predicted
1	1	1	−1	1	91.8	94.4
2	−1	−1	−1	−1	88.5	87.9
3	1	1	1	1	83.8	83.0
4	−1	1	1	1	78.4	77.4
5	1	1	1	−1	86.1	86.5
6	−1	1	−1	−1	92.9	93.8
7	−1	−1	−1	1	83.6	84.6
8	−1	1	−1	1	86.2	85.1
9	1	−1	−1	−1	84.2	86.6
10	−1	1	1	−1	91.1	90.1
11	−1	−1	1	1	80.0	80.3
12	1	−1	1	1	84.0	84.5
13	−1	−1	1	−1	88.7	87.6
14	1	−1	1	−1	83.0	82.6
15	1	−1	−1	1	92.9	92.5
16	1	1	−1	−1	93.6	93.9
17	$-\alpha$	0	0	0	72.4	76.6
18	0	$-\alpha$	0	0	89.8	88.9
19	0	0	$-\alpha$	0	94.3	92.7
20	0	0	0	$-\alpha$	90.0	90.6
21	α	0	0	0	86.2	85.0
22	0	α	0	0	92.5	93.3
23	0	0	α	0	78.6	81.0
24	0	0	0	α	84.4	83.8
25	0	0	0	0	92.1	92.2
26	0	0	0	0	93.3	92.2
27	0	0	0	0	92.1	92.2
28	0	0	0	0	92.1	92.2
29	0	0	0	0	91.9	92.2
30	0	0	0	0	91.1	92.2
31	0	0	0	0	89.1	92.2

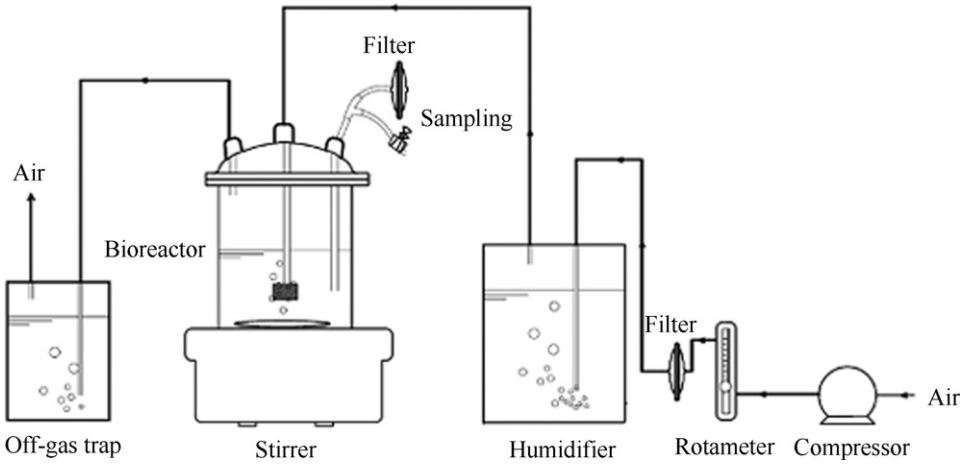

Fig. 1. Schematic diagram of the laboratory-scale slurry bioreactor set up.

2.5. Analytical methods

2.5.1. TNT analysis

Analysis of TNT-contaminated soil was accomplished by the sensitive colorimetric method of CRREL/Jenkins developed by Jenkins and co-workers at Cold Regions Research and Engineering Laboratory [52–54]. Firstly, TNT was extracted from dried slurry sample by addition of acetone as solvent (5 mL acetone/g dried slurry) followed by severe mixing for 15 min. After separation of soil via settling, the extract was filtered by Whatman paper (541-grade). To the filtrate were then added a medium size pellet of KOH and 0.1 g anhydrous Na_2SO_3 followed by 2 min agitation for color development. After filtration of un-dissolved reagents, the absorbance of red-colored Janowsky anion was measured against the reagent blank at 540 nm on a Metertech-SP8001 spectrophotometer [54]. Eight standard TNT-acetone solutions at 0.1–15 mg/L were used to obtain the calibration curve.

2.5.2. Bacterial growth in slurry bioreactor

Cell growth in bioreactor was evaluated using the standard total plate count method [27]. Firstly, 1 mL of slurry sample was mixed with 9 mL sterile phosphate buffer and stirred for 2 min to detach the bacteria from soil matrix. Then, serial dilutions of the treated samples were carried out in the range of 10^{-4}–10^{-8} and 0.1 mL of each diluted sample was spread onto nutrient agar plates (as duplicate). The colonies on each plate were counted after 48 h of incubation at 30 °C and the average of the two measurements was reported as bacterial growth in colony forming units per milliliter of slurry (CFU/mL-slurry).

2.6. Experimental design approaches

2.6.1. Fractional factorial design

The screening experiments in this study was carried out based on FFD method which is a definite part of full factorial design matrix with two-level factor variations including 2^{k-p} runs ($1/2^p$ fraction of the 2^k design), where k and p are the number of independent factors and linear effects confounded with interaction effects, respectively [39]. Having $k = 7$ and $p = 3$ as well as adopting resolution *IV*, overall 19 trials were designed including the 3 replicates used at center points to examine the response curvature.

2.6.2. Response surface methodology

Response surface methodology (RSM) consists of a group of empirical techniques to study the relationship between a cluster of controlled experimental factors and the measured response. Experimental design matrix used in this study was specified according to CCD method with the total number of $2^k + n_\alpha + n_0$ trials, where k, n_α and n_0 are the number of independent variables, axial points and replications at center point, respectively. The distance of axial points from center of design (α) equals to $2^{k/4}$ [38,39]. In this study, $k = 4$, $n_\alpha = 8$, $n_0 = 7$ and $\alpha = 2$ resulted in 31 trials. By using experimental results, a quadratic polynomial model was then employed to predict the system response as given in Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_i X_i^2 + \sum_{i=1}^{k}\sum_{i=2}^{k}\beta_{ij} X_i X_j + e \quad (2)$$

where Y = system response, X_i = un-coded independent variables, β_0 = offset term, β_i = linear effect, β_{ii} = squared effect, β_{ij} = interaction effect and e = random error. The optimal response and the corresponding optimal factors were obtained by solving the regression equation [55].

2.6.3. Software

The design of experiments, analysis of variance (ANOVA) as well as regression and graphical analyses were accomplished using Design Expert software (Stat-Ease, Version 8.0.3).

3. Results and discussion

3.1. Screening experiments

The values of TR for the 19 trials in screening experiments are presented in the last column of Table 1. Variations from 68.1 to 92.3% can be observed in experimental results. In order to study the effect of each factor on system response, ANOVA results are given in Table 4. Setting a confidence level of 95% for factor screening, slurry (D), Tween80 (C) and glucose (A) concentrations as well as inoculum size (G) with P-values of 0.001, 0.006, 0.014 and 0.037, respectively, are identified as the most influential factors on TR in slurry phase. Other factors with P-value > 0.05 are considered insignificant. Besides, ANOVA results show that the curvature is significant (P-value = 0.0145 < 0.05) which confirms that the design space is not linear.

Previous studies have shown that carbon and nitrogen sources are essential for TNT metabolism [31–35] since cultures deficient in these two sources could not grow [31]. Boopathy et al. [31] and Kalafut et al. [35] have shown that NH_4Cl can serve as a suitable nitrogen source in aqueous phase. Moreover, yeast extract has been used as a growth enhancer of inoculated organism [36,56]. Among co-substrates examined in this study, glucose was found to have

Table 4
ANOVA results for all incorporated effects in screening experiments.

Source	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value	Significance
A	38.13	1	38.13	70.18	0.0140	S5[d]
B	1.05	1	1.05	1.93	0.2989	
C	93.61	1	93.61	172.28	0.0058	S5
D	489.52	1	489.52	900.95	0.0011	S5
E	5.88	1	5.88	10.82	0.0813	
F	0.016	1	0.016	0.029	0.88009	
G	13.88	1	13.88	25.54	0.0370	S5
AB	1.27	1	1.27	2.33	0.2665	
AC	20.93	1	20.93	38.52	0.0250	S5
AD	19.58	1	19.58	36.04	0.0266	S5
AE	3.15	1	3.15	5.80	0.1377	
AF	0.96	1	0.96	1.75	0.3169	
AG	102.52	1	102.52	188.68	0.0053	S5
BD	3.90	1	3.90	7.18	0.1156	
ABD	1.89	1	1.89	3.48	0.2031	
Pure error	1.09	2	0.543			
Curvature	36.68	1	36.68	67.51	0.0145	S5
Total	834.03	18				

[a] Sum of square of errors.
[b] Degree of freedom.
[c] Mean square of errors.
[d] Significant at level of 5%.

significant effect on TR, while both NH_4Cl and yeast extract had insignificant effect. This can be explained by the fact that the lower level of NH_4Cl (0.1 g/L) used in this investigation could sufficiently support bacterial growth in presence of glucose (2, 8 g/L) and therefore yeast extract was found redundant.

The observed insignificant effect of temperature on TR (20–35 °C) is an evidence of efficient bioremediation at ambient temperature (27 ± 1 °C) which is in agreement with the results reported by Boopathy et al. [31]. This is an advantage since there is no need to rigorous heating/cooling operations, which makes the system economically favorable.

Since ANOVA results led to ruling out the insignificant factors, optimization was further conducted based on slurry, Tween80 and glucose concentrations as well as inoculum size.

3.2. Optimization experiments

The results of the 31 trials are illustrated in Table 3, which shows TR variations from 76.6 to a maximum of 94.4% in this design space. These results are utilized to develop a model and visualize the effect of factors in 3D surface plots by means of RSM. Subsequently, the optimal factor levels and TR were estimated by using this model.

3.2.1. Model generation and analysis

Using multiple regression analysis, it was found that TR in slurry phase optimization experiments can be well expressed as a function of the four screened variables by means of a second order polynomial model (Eq. (3)):

$$\begin{aligned} Y = {} & 18.02396 + 4.46424\,A + 6.60713\,C + 3.57807\,D + 3.27499\,G \\ & + 0.10222\,AC - 0.081444\,AD + 0.40744\,AG - 0.11517\,CD \\ & - 0.36000\,CG - 0.079900\,DG - 0.46763\,A^2 + 0.12606\,C^2 \\ & - 0.053795\,D^2 - 0.20298\,G^2 \end{aligned} \quad (3)$$

where Y is the predicted value of TR and A, C, D and G are the un-coded levels of glucose, Tween80, slurry concentration and inoculum size, respectively.

Tables 5 and 6 give the various ANOVA components for optimization experiments. The lack of fit of results (P-value > 0.05) reveals that the quadratic model is statistically significant and hence it can be used for further analyses. The inconsistency of variables and fitness values is also evaluated using the multiple coefficient of determination, $R^2 = 0.942$, which shows an excellent correlation between the predicted and experimental results. In other words, more than 94.2% of the variance is attributable to variables and only 5.8% of the total variance cannot be explained by this model. As it can be seen in Table 6, Fisher's F-test ($F_{model} = 18.39 > F_{14,16,\alpha=0.05} = 2.37$) yields a very low probability value (P-value < 0.0001) which indicates that the model is highly significant.

Regarding the significance of each term in Eq. (3), glucose, Tween80 and slurry concentrations show significant linear effects (P-value < 0.05). Moreover, all mutual interaction effects are significant except glucose–Tween80 (*AC*) and Tween80–slurry (*CD*) concentrations. With respect to the quadratic terms, apart from Tween80, all factors represent a negative effect on TR.

3.2.2. Three dimensional response plots

To investigate the individual and cumulative effects of glucose, Tween80 and slurry concentration as well as inoculum size on TR, 3D plots are depicted in Fig. 2. The surface plots are generated for the pair-wise combination of factors with significant mutual effects (see Section 3.2.1) while other factors are set at their middle levels.

Fig. 2a indicates a significant interaction between glucose concentration and inoculum size. Increasing glucose concentration up to 6 g/L has a positive effect on TR through the entire inoculum range. However, it shows a negative effect above 6 g/L and at lower inoculum values. Fig. 2a also shows that maximum TR occurs at 4.5–6 g/L glucose and 5–7% (v/v) inoculum values.

The mutual effects of slurry concentration with glucose and inoculum size are depicted in Fig. 2b and c, respectively. At low slurry concentrations, glucose and inoculum size have more significant positive effects on TR. Maximum TR corresponds to 20–22% (w/v) slurry concentration within 6–7 g/L glucose (see Fig. 2b) or 5–8% (v/v) inoculum size (see Fig. 2c). This is due to the positive effect of water content on oxygen supply, nutritional availability and thus bacterial growth and enzyme production [57,58]. The effect of slurry concentration on TR was previously investigated using a one at a time approach by Park et al. [27] where they observed maximum TR at 10–30% (w/v) while negative effect was found at 30–50% (w/v). Comparison of our obtained optimal value of slurry concentration with their results reveals that statistical optimization can lead to more accurate optimal factor values as it considers factor interactions.

Table 5
Model coefficients and regression analysis.

Term	Coefficient	MS[a]	*F*-value	*P*-value	Significance
Intercept	18.02396				
A	4.46424	27.01	9.86	0.0063	S1[b]
C	6.60713	29.04	10.6	0.005	S1
D	3.57807	203.47	74.3	<0.0001	S1
G	3.27499	10.05	3.12	0.0965	
AC	0.10222	1.9	0.70	0.4166	
AD	−0.081444	13.43	4.9	0.0416	S5[c]
AG	0.40744	134.68	41.80	<0.0001	S1
CD	−0.11517	11.94	4.36	0.0532	
CG	−0.36000	29.16	10.65	0.0049	S1
DG	−0.079900	15.96	5.83	0.0281	S5
A^2	−0.46763	160.26	58.52	<0.0001	S1
C^2	0.12606	2.3	0.84	0.3730	
D^2	−0.053795	51.72	18.89	0.0005	S1
G^2	−0.20298	46.02	16.81	0.0008	S1

[a] Mean square of errors.
[b] Significant at level of 1%.
[c] Significant at level of 5%.

Table 6
ANOVA results for the fitted second-order polynomial model.

Source of variation	SS[a]	DF[b]	MS[c]	*F*-value	*P*-value	Significance
Model	705.53	14	50.37	18.39	<0.0001	S5[d]
Lack of fit	33.23	10	3.32	1.88	0.2263	N[e]
Pure error	10.59	6	1.76			
Residual	43.82	16	2.74			
Total	749.05	30				
R^2 = 0.9415; Adj R^2 = 0.8903						

[a] Sum of square of errors.
[b] Degree of freedom.
[c] Mean square of errors.
[d] Significant at level of 5%.
[e] Not significant.

Fig. 2d shows that Tween80 concentration has a positive effect on TR through the entire inoculum range and this effect is more noticeable at values greater than 7% (v/v). Maximum TR is achieved at surfactant concentration of about 5 g/L and inoculum sizes of 5–6.5% (v/v). Boopathy [37] showed that stepwise addition of Tween80 into slurry up to 5 g/L enhanced TNT desorption from soil and this in turn improved bioavailability of TNT for microbial degradation. Taha et al. [14] examined the effect of Tween80 on TNT desorption in slurry phase and concluded that due to the competition of negatively charged soil surfaces and surfactant, more than 1 g/L of surfactant is required for efficient TNT desorption. Additionally, the effect of Tween80 on modification of plasma membrane permeability as well as production and transportation rates of membrane-associated enzymes [59,60] were studied. Hodgson et al. [61] reported the positive role of Tween80 on enzymatic activities and the rate of TR in aqueous phase. Moreover, comparison of TR in aqueous phase with/without Tween80 by Popesku et al. [34] resulted in complete TR in a shorter time in presence of surfactant.

3.2.3. Optimal TNT removal and validation experiments

The quadratic model presented in Section 3.2.1 was used for optimizing TR in slurry phase. This model predicts that the optimal values of 6.25 g/L glucose, 4.92 g/L Tween80, 5.75% (v/v) inoculation and slurry concentration of 22.23% (w/v) result in a maximum TR of 96.1%. To validate model predictions, further experiments were performed at optimal factor levels which led to 95.2% TR within 15 days. Comparison of the experimental and predicted TRs shows that this model can suitably predict the effect of screened factor on TR in slurry phase. In order to enhance TR efficiency, these optimal values were used as a basis for further studies in bioreactor.

3.3. Bioreactor experiments

A preliminary bioreactor experiment was carried out at 2 vvm aeration rate for 15 days which resulted in complete TR. The maximum bacterial growth of 2.7×10^8 CFU/mL-slurry was obtained at 9th day as shown in Fig. 3. Bioreactor control experiment showed only a 5.3% TR within 15 days which could be attributed to non-biological TR (see Fig. 3).

Subsequently, the effect of various aeration rates on bacterial growth and TR was compared after 9 days, the results of which are shown in Fig. 4. Both bacterial growth and TR were positively affected by aeration rate from 0 up to 3 vvm, whereas both decreased at 4 vvm. This may be due to the formation of large bubbles via coalescence and hence lower mass transfer coefficient, which in turn can lead to reductions in bacterial growth and TR. A similar trend was observed by Partovinia et al. [62] who investigated the effect of aeration rate on bioremediation efficiency in slurry bioreactor. Park et al. [32] also performed a set of 1-liter slurry bioreactor experiments to study the effect of agitation speed (0–100 rpm) on TR with initial TNT concentration of 1000 mg/kg-soil and 30% (w/v) slurry concentration. They used a medium containing supplemental sources (i.e. corn steep liquor, Tween80 and NH_4Cl) at optimal concentrations which were obtained in previous aqueous phase experiments [27]. By increasing agitation speeds up to 40 rpm, TR increased, however it reduced at speeds higher than 60 rpm. This trend is comparable to our results as agitation usually mimics aeration in bioreactors.

Fig. 4 shows a maximum TR of 95% after 9 days which is equal to a daily TR of about 100 mg/kg-soil. Park and co-workers reported the value of 50 mg/kg-soil which led to complete TR after 20 days in a 1-liter slurry bioreactor [32]. They reported 55% removal after

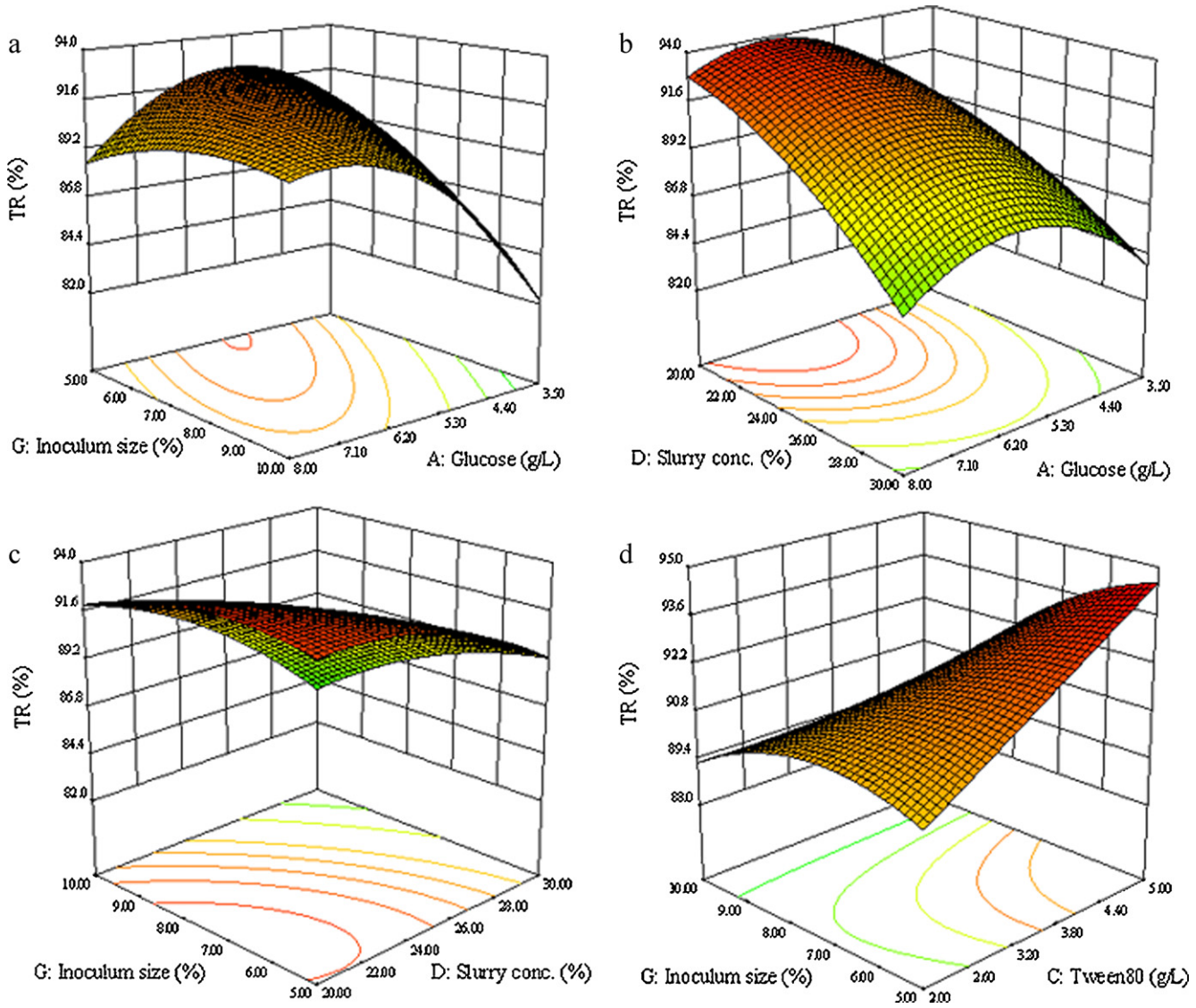

Fig. 2. 3D surface plot of TR as functions of (a) *A* and *G*, (b) *A* and *D*, (c) *G* and *D*, and (d) *G* and *C*. Glucose conc.: *A*; Tween80 conc.: *C*; slurry conc.: *D*; inoculum size: *G*.

9 days compared to 95% obtained in this study. This increase can be attributed to the systematic optimization approach used in this work, which considers all interactions in contrast to their one factor at a time investigations. Besides, they used 1 g/L Tween80 in slurry phase TR which was optimal for aqueous phase [32]. This contradicts the previous studies suggesting more than 1 g/L surfactant for TNT desorption from soil in slurry phase [14,37]. In aqueous phase, Tween80 affects permeability of cell membrane [59–61], while both TNT desorption form soil and membrane permeability

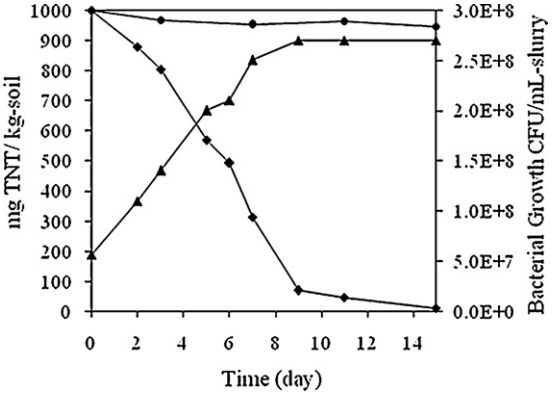

Fig. 3. Bioremediation of TNT-contaminated soil at 2 vvm aeration rate in slurry bioreactor. Bacterial growth (▲); TNT concentration with (♦) and without (●) inoculum.

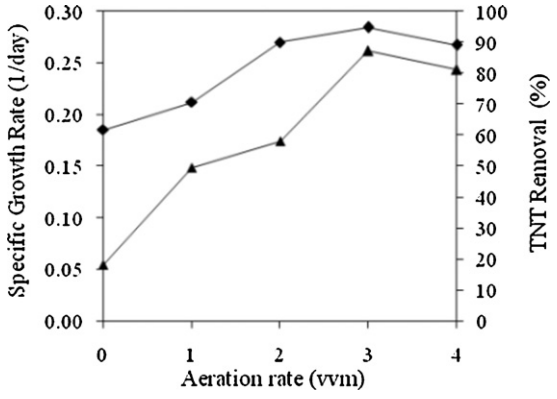

Fig. 4. Effect of aeration rate on bacterial growth (♦) and TR (▲) at 9th day in slurry bioreactor.

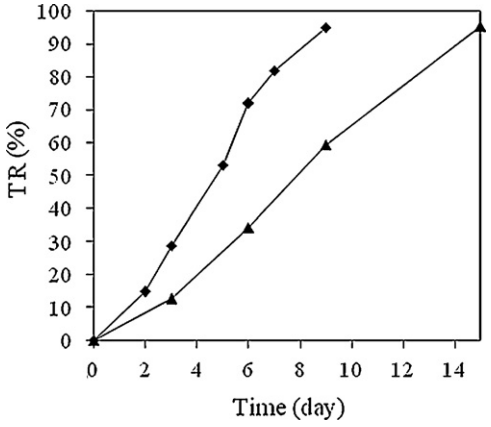

Fig. 5. Comparison of TR in slurry bioreactor at 3 vvm aeration rate (♦) and validation shake flask (▲) experiments.

are affected by Tween80 in slurry phase [14,37] and hence distinct Tween80 concentrations are required in each case.

Fig. 5 compares the time courses of TR in shake flask and bioreactor experiments (at 3 vvm aeration rate) under optimal factor levels. It can be seen that bioremediation time is much shorter in slurry bioreactor, with 95% TR achievable at 6 days earlier (9 days in bioreactor compared to 15 days in shake flasks). This shortened bioremediation time in slurry bioreactor is a direct consequence of improved oxygen availability.

4. Conclusion

Slurry phase bioremediation of TNT-contaminated soil was studied via statistical design of experiments in order to identify the effective factors and optimize TR. Glucose, Tween80, slurry concentrations and inoculum size were identified as significant factors on TR by performing experiments based on FFD method. Using CCD and RSM techniques, it was found that the effect of these factors on TR in shake flask experiments can be adequately expressed by a second order polynomial model. Additionally, the predicted optimal TR obtained by this model was satisfactorily validated experimentally. It was also concluded that the two distinct functions of Tween80 in slurry phase, i.e. TNT desorption from soil and the transport across cell membrane make it necessary to perform optimization in slurry phase rather than using optimal values of Tween80 in aqueous phase where surfactant only affects cell membrane permeability. It was finally shown that exploitation of slurry bioreactor with an appropriate aeration rate resulted in shortened bioremediation time or enhanced TR compared to shake flask.

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