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Screening Effective Factors in Slurry Phase Bioremediation of 2,4,6-Trinitrotoluene (TNT) Contaminated Soil

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Abstract

Soil contamination with TNT is a serious environmental hazard due to the toxic and mutagenic effects of TNT. Bioremediation is an environmentally safe method in the treatment of explosive-contaminated sites. In the present research, after selection of superior bacteria in the aqueous phase, bioremediation of TNT contaminated clay soil at 1000mg/kg was performed in slurry phase, which resulted in a maximum TNT removal of 89% after 15 days. Afterwards, the effects of operational and environmental factors were examined via the two-level fractional factorial design method (2_{IV}^{7-3}) for seven factors, i.e., glucose (2, 8g/l), yeast extract (0, 0.2g/l), $(NH_4)_2SO_4$ (0.1, 0.5g/l), Tween80 (1, 5g/l) and slurry concentrations (20, 40%w/v) as well as inoculum size (5, 10%v/v) and temperature (20, 35˚C). Among these factors, significant factors were found to be slurry, surfactant and glucose concentrations as well as inoculum size. In addition, considerable interactions were observed between glucose and the other significant factors.

Keywords: *Bioremediation, Explosive, Fractional Factorial Design, Slurry Phase, TNT*

1- Introduction

One of the major problems facing the industrialized world today is the contamination of soils with hazardous and toxic chemicals. Production of explosives as well as disposal, handling and testing activities have led to extensive contamination of soils. In particular, 2,4,6-trinitrotoluene (TNT) is most widely used as an explosive due to its low melting point, chemical and thermal stability, low sensitivity to impact, friction and high temperature and the availability of safe methods for manufacturing [1, 2]. Therefore, TNT has become a predominant contaminant at many production and training sites and could be found in soil from a trace level up to 14000mg/kg [3]. TNT is a known mutagen and can cause pancetopaenia due to bone marrow failure; also, aplastic anaemia and toxic jaundice were noted among munitions workers [4]. Due to the potential adverse

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effects of TNT on human health and environment, TNT is classified as an EPA class C with the allowable value of 16 mg/kg in soil [5, 6].

Because the cleanup of areas contaminated by this nitroaromatic explosive is now a public health concern, considerable efforts have been invested in finding economical remediation technologies [2]. Various effective technologies have been applied to treat explosive contaminated soils including incineration, chemical oxidation, alkaline hydrolysis and surfactant-enhanced washing [7-9]. However, capital and operating costs for some of these processes are high while bioremediation processes are usually cost effective and environmentally safe methods for destroying organic pollutants such as TNT [7, 10]. Bioremediation is an engineered process that utilizes the biochemical mechanisms in organisms for degradation and/or production of harmless end products [11]. Diverse bacterial strains, white rot fungi and basidiomycetes are capable in metabolizing TNT under aerobic conditions [12]. The initial steps in the catabolism of TNT by a variety of biological systems appear to involve a stepwise reduction of the nitro groups through the nitroso and hydroxylamino to the amino groups; all amino-intermediates are reported to be nonmutagenic for mammalian cells [12-14].

Solid phase bioremediations such as composting or land farming are time consuming and the required additive for these methods restricts the soil volume that could be treated [15]. In contrast, slurry phase bioremediation, where a mixture of contaminated soil, water and co-substrate is aerated, enhances mass transfer and biodegradation rate [16]. Besides, TNT could interact or make complexes with soluble humic or high molecular weight substances in soil due to its chemical structure, and this, in turn, reduces its bioavailability [17, 18]. Therefore, slurry phase bioremediation appears as a suitable technology preventing TNT binding and distribution in soil, hence improving removal efficiency compared to solid phase operation [18-20].

In the present study, the potential of some aerobic bacterial strains was evaluated for TNT removal. Furthermore, to improve the cleanup of clay soil contaminated with TNT by the selected strain, the effect of seven factors i.e., glucose, yeast and ammonium (as co-substrates), surfactant and slurry concentrations as well as inoculum size and temperature was investigated via a two-level fractional factorial design method.

2- Materials and methods

2.1- Microorganism and inoculum

Microorganisms selected for this study are efficient strains for aromatic hydrocarbon degradation. *Pseudomonas putida* (PTCC 1694), *P. aeroginosa* (PTCC 1310) and *Alcaligenes faecalis* (PTCC 1624) were obtained from the Persian Type Culture Collection and *P. sp.* previously isolated bacterium from oil contaminated soil. To prepare bacterial inoculum, each strain was cultivated for 24h in nutrient rich medium (g $liter^{-1}$: peptone, 10; NaCl, 10; and yeast extract, 5) at 30°C and 200rpm. After centrifugation at 4000rpm for 20min, the cells were separated, washed and re-suspended in physiological serum to reach an optical density of 1 at 600nm.

2.2- Media

The mineral salts medium used in the aqueous and slurry phase contained (g liter^{-1}): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4$, 0.1; NaCl, 0.1 and $(NH_4)_2SO_4$, 0.25. Also, all bacterial culture media were supplemented by 3ml per liter trace salts solution (611mg H_3BO_3, 389mg $MnCl_2$, 56mg each of $CuSO_4.5H_2O$, $Al_2(Cl)_3.6H_2O$, $NiSO_4.6H_2O$, $CoCl_2.6H_2O$ and 28mg each of $SnCl_2$ and KI per liter).

2.3- Soil

A clean clay soil (Kaolin) with fine particle size and known chemical composition was used in this research. According to the artificial soil contamination procedure [21], TNT dissolved in organic solvent (acetone) was transferred into dried and sieved soil and was evenly distributed to obtain a final TNT concentration of 1000mg/kg in soil matrix.

2.4- Experimentation procedures

Primary experiments were carried out in water phase containing 100mg/l TNT (water solubility of TNT), for identification of the superior organism in TNT removal. All cultures were supplemented with glucose and yeast extract at (g/l): 5 and 0.1, respectively. Also, each culture flask was inoculated at 5 (%v/v) and incubated at 30°C and 200rpm.

In the next part, capability of the superior organism was examined in the slurry phase bioremediation of TNT contaminated soil at 1000mg/kg. Slurry culture at 25 (%w/v) was supplemented by glucose (5g/l), yeast extract (0.1g/l) and Tween80 (1g/l) as a non ionic surfactant for promotion of enzyme release and enhancement of TNT desorption from soil [22]. This experiment was performed at 30°C and 100rpm.

Lastly, the influence of environmental factors on TNT removal was evaluated in the slurry phase and a statistical design of experiments was conducted to identify the influence of seven important factors, i.e., slurry concentration [22], temperature [22, 23], microbial community [17], co-substrates concentrations i.e., glucose [23-25], yeast extract, $(NH_4)_2SO_4$ [25-27] and Tween80 as a surfactant [28, 29].

2.5- Statistical design approach

When a relatively large number of independent factors ($k \geq 7$) affect the system response, the application of statistical screening experiments is obligatory to avoid enormous time consumption for trials. Fractional Factorial Design (FFD) is a definite part of full factorial experiments and the design matrix is based on two-level factor variations. This design includes 2^{k-p} runs ($1/2^p$ fraction of the 2^k design), where p is the number of linear effects confounded with interaction effects [30]. The resolution of fractional factorial design is determined based on the chosen design generators. For instance, every main effect in resolution *IV* design (2_{IV}^{k-p}) is aliased by three-factor and higher interactions, whereas two-factor interactions are aliased with each other and with higher-order interactions [31].

In the current work, a 2_{IV}^{7-3} fractional factorial design plus 3 replicates of center points was used to evaluate the combined effect of seven efficient factors on TNT removal in slurry phase. Table 1 presents the real and coded value of experimental variables at the low, central and high levels.

Table 1. Coded and actual values of factors in 2_{IV}^{7-3} fractional factorial design.

Symbols	Factors	Levels		
		-1	0	1
A	Glucose (g/l)	2	5	8
B	$(NH_4)_2SO_4$ (g/l)	0.1	0.3	0.5
C	Tween80 (g/l)	1	3	5
D	Slurry concentration (%w/v)	20	30	40
E	Temperature (˚C)	20	27.5	35
F	Yeast extract (g/l)	0	0.1	0.2
G	Inoculum size (%v/v)	5	7.5	10

A set of 19 experiments was conducted in triplicate according to the scheme mentioned in Table 2. All experiments were carried out at shaking speed of 100rpm and Design-Expert Software (Stat-Ease, Version 7.1.4) was employed for statistical analyses.

2.6- Analyses

Residual TNT was analyzed by a sensitive colorimetric assay developed by Jenkins [33]. Briefly, a medium size pellet of KOH and 0.2g anhydrous Na_2SO_3 were added to 5 ml of a sample extracted by acetone and after agitation for color development of the reaction product (Janwsky anion). Finally, the absorbance of the filtrate was read at 540nm.

3- Results and discussion

3.1- Selection of a superior bacterium

To compare the potential of four bacterial strains in TNT removal, primary experiments were performed in the aqueous phase and the percentages of TNT removal and reaction rate constants were determined for each bacterial strain as given in Table 2.

The reaction rate constant ($k_{obs,\ TNT}$) was calculated by plotting $-\ln(TNT_t/TNT_0)$ versus time and finding the slope as given in Eq. (1):

$$-\ln\frac{(TNT_t)}{(TNT_0)} = k_{obs,TNT}.t \qquad (1)$$

where TNT_0 and TNT_t (mg/l) are TNT concentrations at time 0 and t, respectively. The percentage of TNT removal (R_{TNT}) was calculated as given in Eq. (2):

$$R_{TNT} = \frac{TNT_0 - TNT_t}{TNT_0} \times 100\% \qquad (2)$$

P. putida resulted in 94.38% TNT removal during 48hr of incubation and the highest reaction rate constant of $0.063h^{-1}$ was obtained for this microorganism. *A. faecalis*, *P. aeroginosa* and *P* sp., were also capable of TNT transformation, albeit to a lesser removal rate ($k_{obs,\ TNT} < 0.032h^{-1}$). So, *P. putida* was found to be the superior bacterium for TNT transformation among these strains and therefore was used for further experiments in the slurry phase.

Table 2. TNT removal and reaction rate constant during 48h of incubation in aqueous phase.

Bacterial strains	Reaction rate constant (h^{-1})	TNT removal (%w/v)
Pseudomonas putida	0.063	94.38
P. aeroginosa	0.028	73.4
P. sp.	0.022	64.4
Alcaligenes faecalis	0.032	78.9

3.2- Soil bioremediation in slurry phase using *P. putida*

Results in Fig. 1 show that TNT removal is mostly growth-related and *P. putida* can remove 89% TNT from clay soil in the slurry phase during the 15 days of incubation. It should be mentioned that a 3.24% TNT removal was observed in the control experiments (without inoculum) which might be a result of physical or chemical elimination or experimental error. This result confirms that *P. putida* is a high potential organism for TNT removal in slurry phase bioremediation of clay contaminated soil. Hence, this strain was further used in screening experiments.

3.3- Screening effective factors in slurry phase

To make bioremediation more applicable in large scale cleanup processes, the operational and environmental factors for stable and efficient operation must be clearly defined. Thus, we have attempted to identify the most efficient environmental factors for TNT removal in slurry phase. The statistical design consisted of 16 experiments plus 3 replications in the center points for investigation of the response curvature. The amount of TNT removal (%) after 15 days of incubation was taken as the system response. The experimental conditions and the corresponding TNT removal (average of the three replicates) for all tests are summarized in Table 3. Results show 68.1-92.3% TNT removal by changing the factor levels.

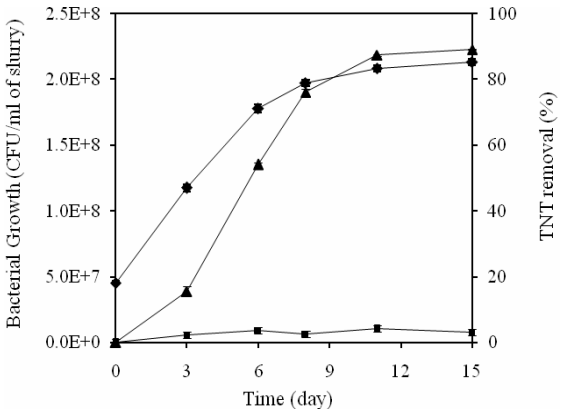

Figure 1. Slurry phase bioremediation of TNT contaminated soil at initial concentration of 1000mgTNT/kg soil, TNT removal with (▲) and without (■) inoculum and bacterial growth (♦).

Table 3. The coded values for 2_{IV}^{7-3} FFD experiments and the results for TNT removal.

Runs	Design matrix based on coded level							TNT removal (%)
	A	*B*	*C*	*D*	*E*	*F*	*G*	
1	-1	-1	1	-1	1	1	1	90.5
2	1	-1	1	-1	-1	1	-1	80.1
3	1	-1	1	-1	1	-1	1	92.3
4	-1	-1	1	1	1	-1	-1	82.9
5	-1	-1	-1	1	-1	1	1	68.1
6	1	1	-1	-1	-1	1	1	90.4
7	-1	1	-1	1	1	-1	1	71.6
8	1	-1	-1	1	1	1	-1	79.5
9	1	1	1	1	1	1	1	86.5
10	-1	1	1	1	-1	1	-1	84.1
11	1	1	1	-1	1	-1	-1	91.3
12	-1	1	-1	-1	1	1	-1	89.7
13	1	1	-1	1	-1	-1	-1	78.1
14	-1	-1	-1	-1	-1	-1	-1	90.4
15	-1	1	1	-1	-1	-1	1	91
16	1	-1	1	1	-1	-1	1	83.6
17	0	0	0	0	0	0	0	85.6
18	0	0	0	0	0	0	0	89.7
19	0	0	0	0	0	0	0	88.3

In order to determine the factors that have the greatest influence over the system response, the various components of variance analyses (ANOVA) were calculated and are shown in Table 4. Based on the *P*-values of each factor, glucose (*A*), Tween80 (*C*), slurry concentration (*D*) and inoculum size (*G*) had highly significant effects, whereas $(NH_4)_2SO_4$ (*B*), temperature (*E*) and yeast extract (*F*) were found to be ineffective on TNT removal at the 0.05 significance level.

The Pareto chart shown in Fig. 2 confirms the above mentioned results and indicates that among the 7 evaluated factors, slurry concentration (*D*) and surfactant level (*C*) are the most effective factors on decontamination of TNT in slurry phase. Also, the most efficient interaction was observed between inoculum size and glucose concentration (*AG*).

Table 4. ANOVA results for all incorporated effects.

Source	SS	DF	MS	F-	*P*-	Significance
Model	545.50	15	36.37	73.72	0.013	*
A	38.13	1	38.13	70.18	0.014	*
B	1.05	1	1.05	1.93	0.299	
C	93.61	1	93.61	172.28	0.006	**
D	489.52	1	489.5	900.95	0.001	**
E	5.88	1	5.88	10.82	0.081	
F	0.016	1	0.016	0.029	0.880	
G	13.88	1	13.88	25.54	0.037	*
AB	1.27	1	1.27	2.33	0.266	
AC	20.93	1	20.93	38.52	0.025	*
AD	19.58	1	19.58	36.04	0.027	*
AE	3.15	1	3.15	5.80	0.138	
AF	0.96	1	0.96	1.75	0.317	
AG	102.52	1	102.5	188.68	0.005	**
BD	3.90	1	3.90	7.18	0.116	
ABD	1.89	1	1.89	3.48	0.203	
Pure error	1.09	2	0.543			
Curvature	36.68	1	36.68	67.51	0.014	*
Total	834.03	18				

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

*Significant (*P*-value <0.05)

**Highly significant (*P*-value <0.01)

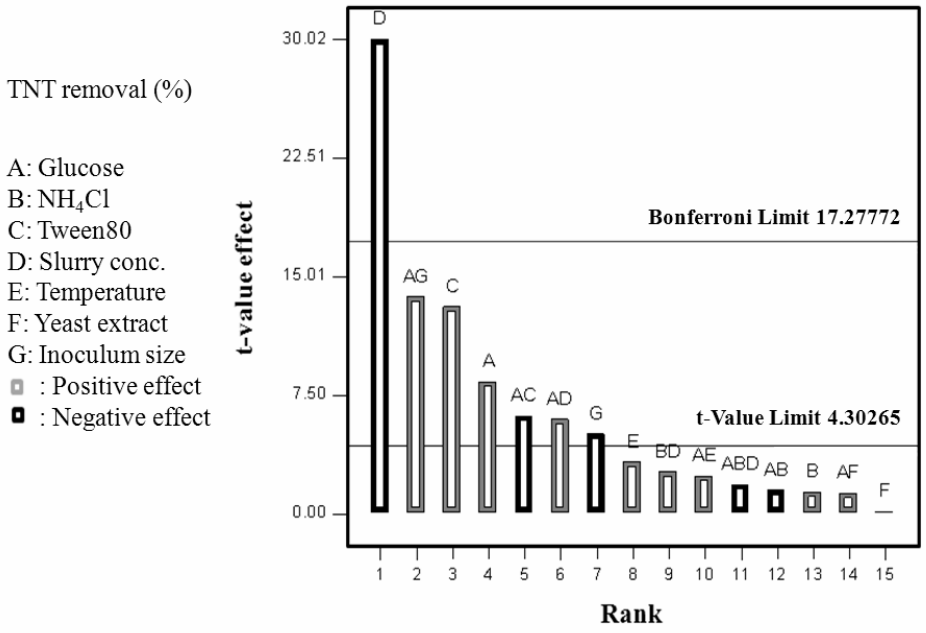

Figure 2. Pareto chart for fractional factorial design (2_{IV}^{7-3}) for seven factors affecting TNT removal in slurry phase by P. putida.

Regression analysis was performed on the final results and a first order regression model which represents the TNT removal as a function of the independent variables was obtained as given in Eq. (3).

$$TNT\ Removal(\%) = 118.148 - 3.549A - 12.375B + 2.162C - 0.8975D - 0.0018E - 3.75F - 2.06G + 1.25AB - 0.191AC - 0.19722AD + 0.0198AE + 0.8128AF + 0.3378AG + 0.533BD - 0.0572ABD \quad (3)$$

ANOVA results confirmed that the first order regression model is significant with a *P*-value of 0.013. However, there exists curvature with a *P*-value of 0.014, which implies that an increase in factor levels and order of regression model would be necessary for process optimization.

3.4- Effect of slurry concentration

Fig. (3-a) shows the negative effect of slurry concentration on TNT removal and the highest elimination was observed at the lowest level (20 (%w/v)). The interaction effect between slurry and glucose concentration in Fig. (3-b) indicates a much larger change in TNT removal at low slurry rather than a high slurry concentration. This result is in accordance with the fact that mass transfer is limited under lower water activity and is in agreement with the published data of Park and co-workers in which TNT removal decreases at soil slurry concentrations over 30% [22].

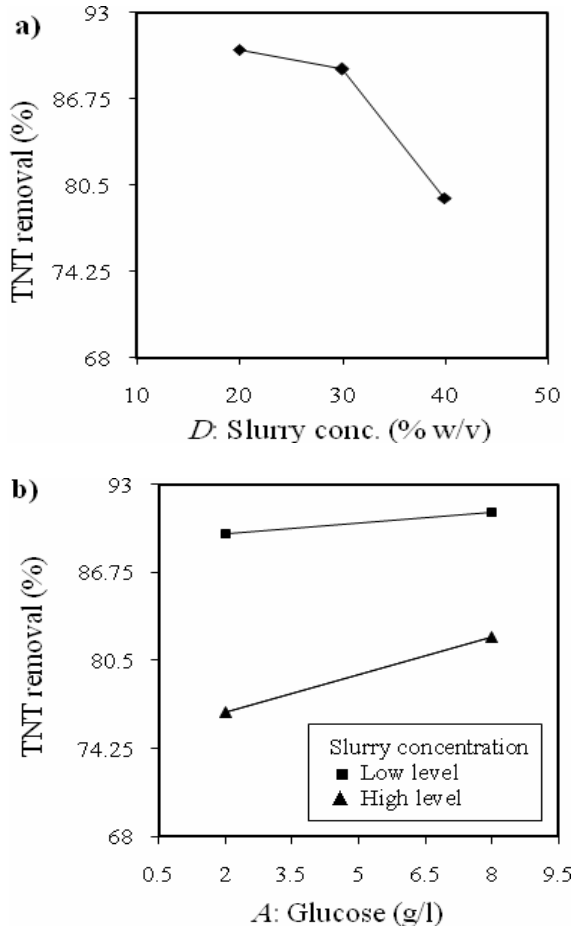

Figure 3. Effect of slurry concentration (a) and its interaction with glucose concentration (b) during TNT removal in slurry phase

3.5- Effect of Tween80

This study shows that Tween80 had a statistically significant effect on TNT removal. Boopathy and Manning [23] and Popesku and co-workers [18] demonstrated that the addition of Tween80 as a nonionic surfactant increases TNT removal efficiency. Basically, water solubility, desorption and ultimately the bioavailability of TNT increases in the presence of surfactant. Also, surfactant can modify microbial plasma membrane permeability and promote enzyme release from cells [34]. According to Fig. (4-a), TNT removal is enhanced by increasing Tween80 level up to 3g/l, however, the addition of more surfactant decreases this positive effect. The surfactant-glucose interaction in Fig. (4-b) shows that the positive effect of glucose in the lower level of Tween80 (0.1%) is higher than its upper level (0.5%). Therefore, the addition of Tween80 up to 0.3% stimulates TNT removal, but the toxic effect of surfactant in the upper level inhibits microbial function and subsequently enzyme release.

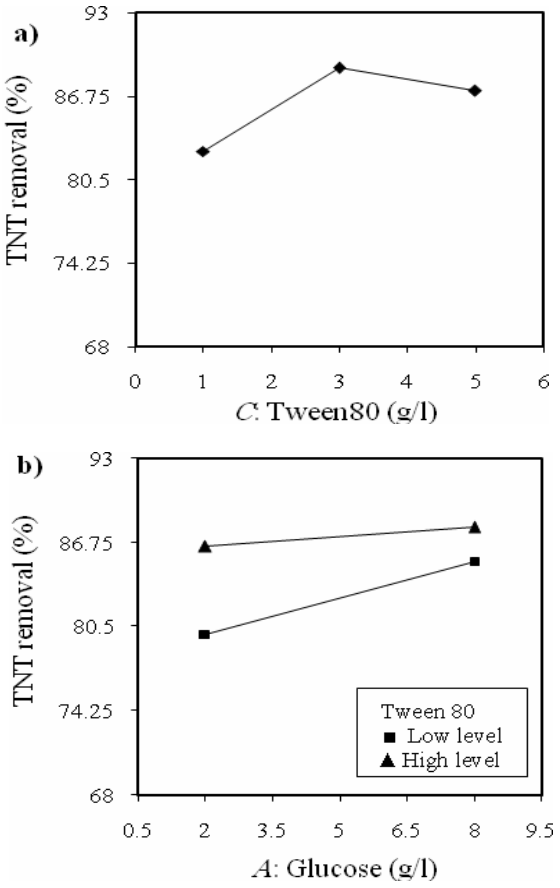

Figure 4. Effect of Tween80 concentration (a) and its interaction with glucose concentration (b) during TNT removal in slurry phase.

3.6- Effect of inoculum size

Fig. (5-a) shows the effect of inoculum size on TNT removal. The maximum TNT elimination was obtained at 7-10 (%v/v)

inoculation. These results are in agreement with the published data by Popesku and co-workers where the highest extent of bioremediation was achieved with the largest inoculum size [17]. Major interaction effect was observed between glucose and inoculum size in Fig. (5-b). Accordingly, TNT removal depends on inoculum size and in a high level of inoculation (10 (%v/v)), an increase in glucose concentration led to a greater removal of TNT.

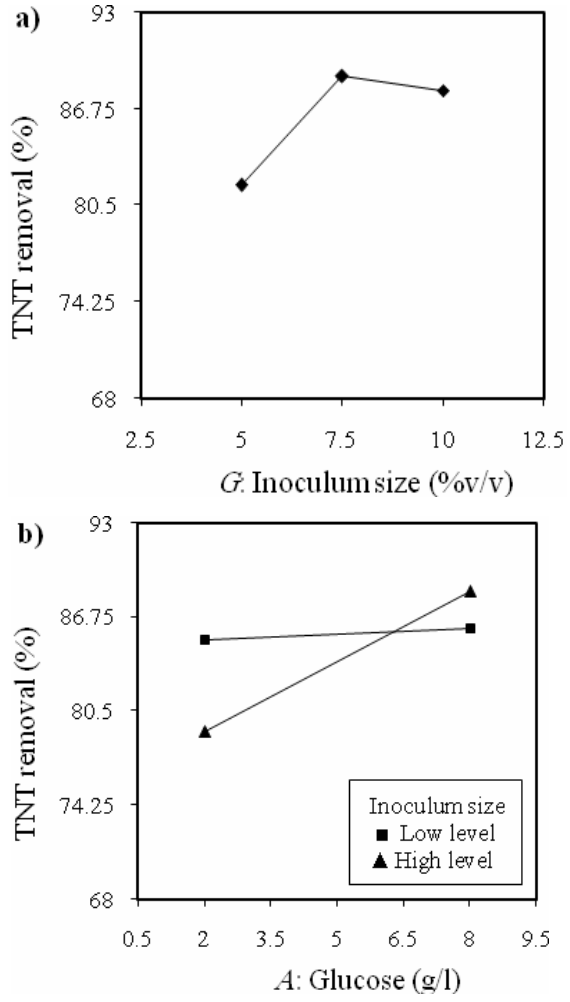

Figure 5. Effect of inoculum size (a) and its interaction with glucose concentration (b) during TNT removal in slurry phase.

In contrast to the significant effect of slurry, Tween80 and glucose concentrations, as well as inoculum size on TNT removal, the effects of temperature (*E*), ammonium sulfate (*B*), yeast extract (*F*) and all interactions involving these factors are negligible. Similarly, Boopathy and co-workers have shown an insignificant effect of temperature on TNT removal in the range of 20-30˚C [23]. The fact that TNT removal is temperature independent is an advantage for *P. putida* and makes slurry phase bioremediation of TNT economically beneficial.

4- Conclusions

Due to the toxic properties of TNT in soil, considerable efforts have been put forward in approaching a successful TNT treatment system. In this study, after selection of *P. putida* as a superior bacterial strain for TNT removal, bioremediation of clay soil contaminated with TNT was tested in slurry phase, resulting in more than 89% removal of this explosive during a period of 15 days. To screen the important factors in slurry phase bioremediation of TNT, seven factors were assessed by fractional factorial design, out of which 4 factors i.e., surfactant, glucose and slurry concentrations in addition to inoculum size were found effective. Temperature and other co-substrate levels (yeast extract and $(NH_4)_2SO_4$) were found to have an insignificant effect on TNT elimination. In addition, the first order regression model was proved to be unsatisfactory due to the significant curvature noticed by using the three center points. Therefore, other statistical methods such as response surface methodology (RSM) are suggested to be used to model the impact of effective factors on TNT removal in slurry phase.

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