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Towards efficient crude oil degradation by *Pseudomonas* sp. strain-O2: Application of Plackett-Burman design for evaluation of cultivation conditions

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Towards an efficient crude oil bioremediation, optimization study of crude oil degradation by *Pseudomonas* sp. strain-O2 was done. Preliminary experiments of crude oil degradation revealed that peptone was the optimal nitrogen source recording 73.3% removal of crude oil, in the presence of 0.5 g/L of yeast extract. The increase of phosphate ratio to 2.5 led to 80.1% removal of crude oil. To evaluate the significance of different culture conditions that affect crude oil biodegradation, Plackett-Burman factorial design was applied. Eleven variables were simultaneously examined. Among those variables, crude oil concentration was the highest positively significant variable that encourage crude oil degradation in *Pseudomonas* sp. strain-O2 affecting the degradation process, other factors namely, Na-succinate, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ showed moderate effect. While, yeast extract, inoculum concentration, agitation, K_2HPO_4 and NaCl were the lowest significant variables. Fractional factorial design experiments indicated that the pre-optimized medium showed approximately 1.5-folds increase in crude oil degradation by *Pseudomonas* sp. strain-O2.

Key words: Biodegradation, crude oil, *Pseudomonas* sp., experimental design, optimization.

INTRODUCTION

As a result of industrialization, many harmful substances including the spill of crude oil and various oil residues have been discharged into terrestrial and aquatic environments (Margesin, 2000). Dangerous accumulation of recalcitrant compounds in soil, sediments and groundwater are considered to be a potential health hazards (Korda et al., 1997). The clean-up of petroleum hydrocarbon-contaminated sites remains a priority task for restoration of the natural environment (ASTM, 1995). Considerable efforts are being spent to design cheap and feasible strategies for clean-up of contaminated sites.

Recently, bioremediation has proved to be a useful tool in removing oil (Boopathy, 2000; Ruiz et al., 2006). It attempts to accelerate the natural degradation rates by overcoming factors that limit microbial degradation (Atlas, 1991). Therefore, bioremediation technology causes the improvement of the natural capacity of microorganisms to degrade contaminants (Catallo and Portier, 1992; Atlas, 1991; Salanitro et al., 1997). Variety of bioremediation methods have been developed to support and increase the degradation activities of native microbial populations, allowing reduction in time and subsequent saving in costs. The two main approaches to bioremediation are the biostimulation and bioaugmentation (Korda et al., 1997).

On the other hand, hydrocarbon-degrading microorganisms are ubiquitous in most ecosystems where contaminants may serve as organic carbon sources (Atlas and Bartha, 1992; Margesin et al., 2000). Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders

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of spilled oil (Komukai-Nakamura et al., 1996; Ijah, 1998; Rahman et al., 2002; Head et al., 2006; da Cruz et al., 2011; Oliveira et al., 2012). Effect of various nutrients on the degradation of crude oil by different bacteria was investigated by several scientists (Gibbs, 1975; Dibble and Bartha, 1979, Wrenn et al., 1994; Oh et al., 2001; Berwick, 2004; Xu et al., 2005). Several factors, both physico-chemical and biological, affect the rate of microbial degradation of hydrocarbons in soil. Recently, growing interest in the use of several Pseudomonades during degradation of crude oil have been reported (Bosch et al., 1999; Evans et al., 2004; Wongsa et al., 2004; Meng et al., 2005; Emtiazi et al, 2005; Toledo et al., 2006; Song et al., 2006; Ueno et al., 2006; Das and Mukherjee, 2007; Mittal and Singh, 2009). However, application of statistical experimental design for optimization of crude oil degradation with *Pseudomonas* sp. was rarely investigated. Recently, medium optimization for a noval crude-oil degrading lipase from *Pseudomonas aeruginosa* SL-72 using statistical approaches for bioremediation of crude-oil was reported (Nain et al., 2012).

On the other hand, experimental design techniques present a more balanced alternative to one-variabler-at-a-time approach (OVAT) in which single factor is varied, while others are kept fixed. However, Plackett and Burman design comprise one type of two-level screening and can be constructed on the basis of fractional replication of a full factorial design (Plackett and Burman, 1947). This design is appropriate to face the large number of cultivation conditions under investigation and allow obtaining an unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations (Akhnazarova and Kafarov, 1982).

The main aim of this work was to investigate the possible improvement of crude oil degradation by *Pseudomonas* sp. strain-O2 and to evaluate the influence of different cultivation condition on efficiency of crude oil degradation. Preliminary controlled experiments were conducted to address the most effective nitrogen source and its optimal concentration, yeast extract, $MgSO_4.7H_2O$ and the level of phosphate salts that might affect crude oil degradation. To determine the significance among other physical and nutritional requirement, fractional factor design namely; Plackett-Burman experimental design was applied and the significant variables were determined.

MATERIALS AND METHODS

Microorganism and cultivation medium

Bacterial strain used in this study, *Pseudomonas* sp. strain-O2 was isolated and identified as previously mentioned (Mostafa et al., 2012). The bacterium was grown on minimal salts medium (MSM), it was the modified medium of Ijah (1998) with the following composition, (g/L): yeast extract, 0.5; NaCl, 0.5; $(NH_4)_2S0_4$, 2; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 5; KH_2PO_4, 2 and trace elements (with the following composition, (g/L): $FeSO_4$, 5; H_3BO_4, 0.025; $CuSO_4.5H_2O$, 0.005; KI, 0.005; $CoSO_4$, 0.3; $MnSO_4.4H_2O$, 3; $ZnSO_4.7H_2O$, 5; $NaMoO_4$, 0.012, and distilled water up to 1 L), 0.1 mL.

Monitoring of crude oil biodegradation

Biodegradation of crude oil was investigated by cultivation of the bacterium in 250-mL Erlenmeyer flasks containing 100 mL sterile MSM amended with 0.92% (w/v) crude oil as sole carbon source. 1.5 mL of active inoculum, prepared from 24 h preculture of the bacterial strain grown on nutrient broth medium, was used for inoculation. All flasks were incubated at 30°C under shake conditions at 120 rpm. At the end of 4 days incubation period, flasks were analyzed for the residual crude oil as described below.

Extraction of crude oil

For extraction of the residual crude oil remaining at the end of cultivation period, each flask was acidified with 5 mL H_2SO_4 (1:1) pH 2; then extracted three times by 60 mL methylene chloride in a 250 mL separating funnel, the organic layer was drained through a funnel containing anhydrous sodium sulfate into a 50 mL boiling rounded flask; then moved to a rotary evaporator at about 40°C to reduce the volume of the extract to 1 mL. The residual oil was transferred to a pre-weighed 2 mL vial for gravimetric analysis, where the amount of crude oil remaining was determined and the percent of crude oil removal was calculated.

Influence of medium composition on crude oil degradation

A series of preliminary on-variable-at-a-time (OVAT) experiments were carried out to provide information for determination of settings of variables that might be used during experimental design. The effect of different N-sources was firstly investigated, nitrogen sources used (2 g/L) were; peptone, ammonium sulfate, ammonium chloride or sodium nitrate. Other tested conditions included; different levels of peptone (2 to 5 g/L), inoculum concentration (0.5 to 2% v/v), yeast extract (0 to 2.5 g/L), $MgSO_4.7H_2O$ (0.1 to 0.5 g/L) and phosphate salts K_2HPO_4 and KH_2PO_4 (1- to 5-folds) the basal concentration (2:0.8 g/L).

Plackett-Burman design

Plackett-Burman experimental design most commonly used for screening purpose was applied to evaluate the significance of various medium components as well as environmental factors affecting crude oil degradation by *Pseudomonas* sp strain-O2. The different factors were prepared in two levels: -1 for low level and +1 for high level, based on Plackett-Burman statistical matrix design, which is a fraction of a two-level factorial design and allows the investigation of n-1 variables in at least n-experiments (Plackett and Burman, 1947). Eleven independent variables (Table 1) were screened in 14 combinations according to the design shown in Table 2. All trials were performed in triplicate and the average of observation was considered as the final result. The main effect of each variable was calculated simply as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (-1) of that factor. Plackett-Burman experimental design is based on the first order model (equation 1):

$$Y = \beta_0 + \sum \beta_i x_i \qquad (1)$$

Where Y is the predicted response (% removal of crude oil), β_0, β_i are constant coefficients, and x_i is the coded independent variables estimates or factors.

Analysis of data

The data of crude oil degradation was statistically analyzed.

Table 1. Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting crude oil degradation by Pseudomonas sp. strain-O2.

Code	Variable	Value	
		-1	+1
X_1	Inoculum concentration	0.5	1.5
X_2	Ammonium sulfate (g/L)	0.5	2
X_3	Peptone (g/L)	2	5
X_4	Yeast Extract (g/L)	0	5
X_5	$MgSO_4.7H_2O$ (g/L)	0.2	0.5
X_6	K_2HPO_4 (g/L)	2	5
X_7	KH_2PO_4 (g/L)	0.8	2
X_8	NaCl (g/L)	5	2
X_9	Agitation (rpm)	60	120
X_{10}	Oil Concentration (g/L)	0.5	1.5
X_{11}	Na-succinate (g/L)	0.5	2

Essential Experimental Design free software (Steppan et al., 2000) was used for data analysis and determination of coefficients. Factors having highest t-value and confidence level over 95% were considered to be highly significant on crude oil degradation.

RESULTS

Influence of medium composition by OVAT

Nitrogen, phosphate and sulfate levels

To provide information on the variable levels that might be used in experimental design study, a series of preliminary OVAT experiments were carried out. At the end of each experiment, cells were separated and the residual crude oil was estimated. Results in Figure 1 indicated that peptone was the optimal nitrogen source recording 73.3% removal of crude oil. Inorganic nitrogen sources such as; ammonium sulfate and sodium nitrate showed positive significance on the growth and crude oil degradation and recorded 71.1 and 69.6% removal of crude oil, respectively. Also, 2 g/L was the optimal peptone concentration, any further increase in concentration led to reduction in crude oil degradation efficiency (Table 3). On the other hand, to determine the most suitable level of yeast extract, MSM was amended with different concentrations of yeast extract ranging from 0 to 2.5 g/L. Results in Table 3 indicated that 0.5 g/L was the optimal concentration that led to approximately 83% removal of crude oil. Approximately, 60.4 and 52.9% removal of crude oil concentration was recorded when yeast extract was increased to 1 or 2.5 g/L, respectively.

Furthermore, one of the crucial chemical constituents in the medium that affect crude oil degradation is the phosphate salt. Results in Table 4 indicated that the increase of phosphate ratio to 2.5 led to 80.1% removal of amount of crude oil. Further increase or decrease in phosphate salt ratio led to decrease in oil degradation efficiency and hence in percent removal of crude oil. Indeed, the optimal concentration of $MgSO_4.7H_2O$ was 0.5 g/L recording approximately 60% removal of the crude oil (data not shown).

It is known that the concentration of the bacterial inoculum plays an important role in determining the efficiency of crude oil degradation. Results revealed that 1.5% (v/v) was the optimal inoculum concentration that led to approximately 80% removal of crude oil (data not shown).

Evaluation of cultivation condition by FFD

Fractional factorial design (FFD) is a kind of experimental design that enables researchers to evaluate the most significant factors affecting the process with the least number of trials. Plackett-Burman design is a FFD, which succeeds in ranking factors from different categories to enable better understanding of the medium effects, eleven factors were studied through the application of Plackett-Burman design. Evaluation of process variables was carried out according to the experimental matrix presented in Table 2, where the residual crude oil estimated and the percent of crude oil removal was the measured response. Variation in crude oil degradation expressed as % of crude oil removal (73 - 96.6%) is shown in Table 2. Results collectively showed that the highest crude oil removal of 96.6 was obtained in the combination number 9, while the lowest crude oil removal was obtained in combination numbers 6. Statistical analysis of these data revealed that the value of determination coefficient R2, that measures the goodness of the model fitting, is >0.99. This indicates that less than 1% of the total variations are not explained by the model, which ensures the good adjustment of the model.

Moreover, the main effects of the examined variables on degradation of crude oil were calculated and illustrated graphically in Figure 2a.

On analysis of regression coefficients and *t*-value (Table 5), it was clear that mainly crude oil concentration, together with $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4 and Na-succinate were found to be the most significant variables that encourage crude oil degradation in *Pseudomonas* sp. strain-O2. Whereas, bacterial inoculum, yeast extract concentration, agitation and NaCl were the lowest significant variables that discourage crude oil degradation.

The determination coefficient represents the quality of fitting the polynomial model, which can be represented as follows:

$$Y_{\%\ removal} = 11.70 - 2.88X_1 + 0.7X_2 + 0.07X_3 - 4.16X_4 + 1.71X_5 - 0.46X_6 + 0.85X_7 - 0.29X_8 - 0.54X_9 + 6.11X_{10} + 0.52X_{11}$$

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or

Table 2. Plackett-Burman experimental design for evaluation of factors affecting crude oil degradation by *Pseudomonas* sp. strain-O2.

Experiment	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Oil removal (%)
1	1	-1	-1	-1	1	1	1	-1	1	1	-1	80.7
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	92.3
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	90.0
4	0	0	0	0	0	0	0	0	0	0	0	88.0
5	1	-1	1	1	-1	1	-1	-1	-1	1	1	91.5
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	73.0
7	1	1	-1	1	1	-1	1	-1	-1	-1	1	96.4
8	1	1	-1	1	-1	-1	-1	1	1	1	-1	92.4
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	96.6
10	1	-1	1	-1	-1	-1	1	1	1	-1	1	94.7
11	-1	-1	1	1	1	-1	1	1	-1	1	-1	81.4
12	0	0	0	0	0	0	0	0	0	0	0	88.0
13	-1	1	-1	-1	-1	1	1	1	-1	1	1	75.0
14	-1	1	1	1	-1	1	1	-1	1	-1	1	96.3

Variables coded as follows: X_1, inoculum concentration; X_2, ammonium sulfate; X_3, peptone; X_4, yeast extract; X_5, , $MgSO_4.7H_2O$; X_6, K_2HPO_4; X_7, KH_2PO_4; X_8, NaCl; X_9, Agitation; X_{10}, Oil concentration; X_{11}, Na-succinate.

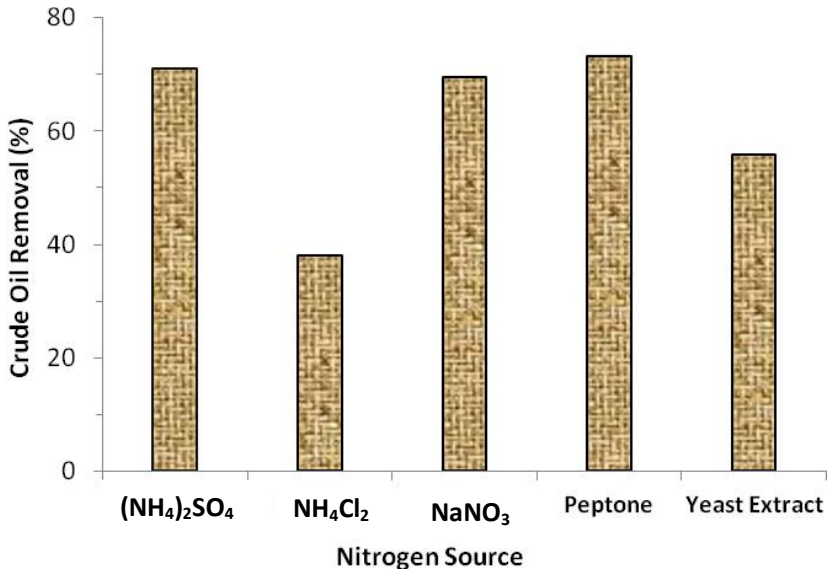

Figure 1. Effect of different nitrogen sources on degradation of crude oil by *Pseudomonas* sp. strain-O2.

physical factor) or sign (whether contributes positively or negatively). Interestingly, Figure 2b shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and is a convenient way to view the results of Plackett-Burman design (Strobel and Sullivan, 1999).

DISCUSSION

Given the environmental importance of bacteria, especially Pseudomonades, in degradation of crude oil and their application in bioremediation and namely, bioaugmentation and biostimulation, the influence of different growth parameters on degradation process was closely examined. In a series of controlled OVAT experiments, optimal concentrations of medium constituents were closely investigated. Preliminary investigations indicated that peptone is the optimal organic nitrogen source. Supplementation of the medium with yeast extract enhanced growth and % removal of crude oil from the culture medium reflecting its importance as nitrogen and vitamin source. The use of yeast extract as nitrogen as

Table 3. Effect of different peptone and yeast extract concentrations on crude oil degradation by Pseudomonas sp. strain-O2.

Peptone concentration (g/L)	Extracted crude oil (mg)	Crude oil removal (%)	Yeast extract concentration (g/L)	Extracted crude oil (mg)	Crude oil removal (%)
2	16.3	82.3	0	18.0	80.1
3	28.9	68.6	0.5	15.6	83.0
4	37.9	58.8	1.0	36.4	60.4
5	21.3	76.8	2.5	43.3	52.9

Table 4. Effect of different phosphate ratios on crude oil degradation by *Pseudomonas* sp. strain-O2.

K2HPO4 : KH2PO4 (g/L)	Fold	Extracted crude oil (mg)	Crude oil removal (%)
2 : 0.8	1	46.2	50.6
4 : 1.6	2	29.5	49.8
5 : 2	2.5	18.3	80.1
6 : 2.4	3	23.6	74.3
7 : 2.8	3.5	23.2	74.8
9 : 3.6	4.5	35.2	61.7
10 : 4	5	37.7	59.0

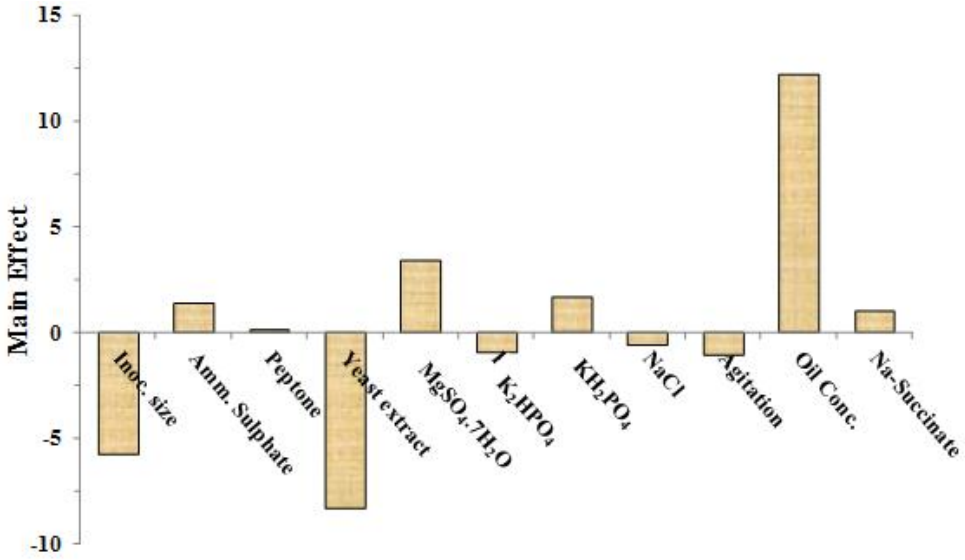

Figure 2a. Effect of different factors on crude oil degradation by *Pseudomonas* sp. strain-O2 as screened with Plackett-Burman design.

well as vitamin source during hydrocarbon and petroleum degradation by many organisms was reported (Lemos et al., 2002; Singh et al., 2005). Interestingly, increase in yeast extract concentration from 0.5 to 2.5 g/L led to approximately 20% reduction in crude oil removal. In concordance, results of Plackett-Burman experiments revealed that the increase in yeast extract concentration negatively affected crude oil degradation. On the other hand, one of the crucial chemical constituents in the medium that affect crude oil degradation is the phosphate salt, especially due to its suggested importance in indirect control of pH and providing the organism with the required phosphate ions necessary for energy and ATP production. Indeed, buffering effect of phosphate salts during degradation of crude oil was reported by Emtiazi et al., (2005). This finding supported by the results of fractional factorial design which indicated that phosphate ion (KH_2PO_4), contributed positively to crude oil biode-

Table 5. Statistical analysis of data obtained from Plackett-Burman design showing coefficient values, t- and P-values of each variable.

Variable	Statistical analyse			
	Coefficient	*t*-Stat	P-value	Coefficient level (%)
X_1	-2.88	9.98	0.009	98.4
X_2	0.70	-2.43	0.135	04.0
X_3	0.07	-0.24	0.828	99.8
X_4	-4.16	14.3	0.004	99.4
X_5	1.71	-5.91	0.027	93.5
X_6	-0.46	1.59	0.251	96.0
X_7	0.85	-2.95	0.098	89.0
X_8	-0.28	0.99	0.424	99.2
X_9	-0.53	2.00	0.183	95.6
X_{10}	6.11	-21.1	0.002	96.1
X_{11}	0.52	-1.54	0.261	48.4

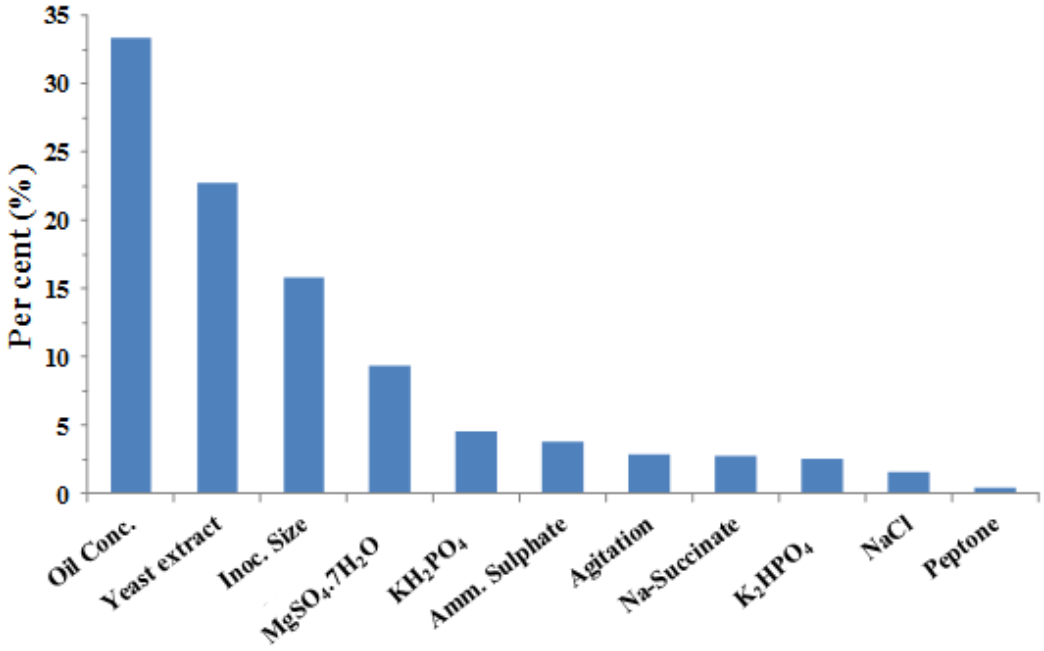

Figure 2b. Pareto chart rationalizing the effect of each variable on crude oil degradation by *Pseudomonas* sp. strain-O2.

gradation and showed high significant effect reflected by the p-value (0.098). Furthermore, supplementation of fermentation medium with inorganic nitrogen source such as $(NH_4)_2SO_4$ contributed positively to crude oil degradation, consequently can be used instead of other organic nitrogen sources. In accordance, Nain (2012) reported that ammonium and phosphate ions contributed positively to production of crude oil degrading lipase from *P. aeruginosa* SL-72 during application of Plackett-Burman experimental design. The use of inorganic nitrogen sources, such as ammonium chloride and potassium nitrate, was preferably used during crude oil degradation (Wrenn et al., 1994). Many scientists reported the importance of amendment of biodegradation medium with phosphate and inorganic nitrogen fertilizers (Westlake et al., 1978; Jobson et al., 1974; Piehler and Paerl, 1996; Emtiazi et al., 2005; Margesin et al., 2007). Furthermore, it was clear that the degradation efficiency was positively affected by the amount of crude oil due to the increase in the amount of C-source (p-value= 0.002). Similar finding was reported by Margesin et al. (2007) and Xu et al. (2005). Addition of Na-succinate as co-substrate to enhance crude oil degradation resulted in a slightly positive effect. Simple co-substrates were reported to

positively affect the rate of hydrocarbon degradation. Significant increase in degradation of crude oil as well as the saturated branched hydrocarbon (squalane) by addition of glycerol, rhamnolipid or Na-succinate was reported (Berekaa and Steinbuchel, 2000; Meng et al., 2005). Successful degradation of crude oil PAHs by co-metabolism was reported (Arun et al., 2011). Whereas, the other variables namely, agitation, NaCl and K_2HPO_4 were the lowest significant variables affecting crude oil degradation. Therefore, might be dropped in further optimization experiments.

The results of this study collectively revealed the possible optimization of crude oil degradation by *Pseudomonas* sp. strain-O2 through improvement of chemical and environmental parameters. Preliminary experiments gave an idea about the setting of some variables that might be used in experimental design. Plackett-Burman design showed that the most significant variables encourage crude oil degradation. Furthermore, the design succeeded to rank factors from different categories to enable better understanding of the medium effect. It is worthwhile to further optimize the significant variables determined in the present study to attain maximum crude oil degradation by applying other suitable statistical designs.

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