Numerical modelling of petroleum oil bioremediation by a local *Penicillium* isolate as affected with culture conditions: Application of Plackett-Burman design

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ABSTRACT

Plackett-Burman experimental design, based on numerical modelling, has been applied to evaluate the significance of culture conditions affecting petroleum oil bioremediation by newly isolated Penicillium sp. Ten variables through 13 trials were studied simultaneously, namely, ammonium sulphate, urea, potassium phosphate dibasic, casein hydrolysate, yeast extract, spore suspension concentration, sodium chloride, trace elements solution, temperature and pH, to elucidate their effect and significance on petroleum degradation percentage. Several factors affected the bioremediation process positively, while others have negative effects on oil bioremediation. Based on petroleum weight loss calculations, potassium phosphate dibasic, casein, yeast extract, pH, trace elements and spore suspension volume promoted the petroleum oil removal by Penicillium sp., whereas ammonium sulphate, urea and temperature were found to inhibit its removal from the medium. On the other hand, with respect to aliphatic hydrocarbon degradation, it was found that all other factors except potassium phosphate dibasic, pH, trace element and temperature contributed negatively in the aliphatic hydrocarbon degradation. Combination of trial number 9, which contained higher levels of casein, yeast extract, spore suspension and trace elements and low levels of all other factors represents the near optimum conditions for petroleum removal (86.64%) based on weight calculations. In addition, combination in trial number 1 which contained higher levels of ammonium sulphate, potassium phosphate dibasic, temperature and trace elements and low levels of other factors in the medium is the near optimum formulation for aliphatic hydrocarbon degradation (58.78%).

Key words: *Petroleum bioremediation, Penicillium sp., Plackett-Burman design.*

INTRODUCTION

Environmental pollution with petroleum and petrochemical derivatives has been recognized as one of the most serious problems. There has been increasing concern over the accidental spillage of petrochemical-derived hydrocarbon compounds during technological processes and transportation. Many of these hydrocarbons are considered to be a potential health hazard (Korda *et al.*, 1997). Physical treatment, using sorbing

material followed by incineration for oil removal, is one of the oldest and simplest methods used. This method proved not to be a practical method since it must be used right after the spill occurring. Whereas, chemical treatment using chemical emulsifier, proved to be expensive and cannot remove the oil absolutely from the polluted site. Bioremediation, i.e., the use of microorganisms or microbial processes to detoxify and degrade environmental contaminants, attempts to accelerate the natural degradation rates by overcoming factors that limit microbial degradation (Atlas, 1991). Conditions for biodegradation are optimized by modifying environmental factors such as pH, temperature, aeration and nutrient addition (Biostimulation) (Huesmann, 1997).

The successful application of bioremediation depends on appropriate biodegradative microbes and environmental parameters. There are few studies on microbial oil degradation in normal soils. Hydrocarbon degrading microbes have been detected (Konlechner, 1985; Kerry, 1990; Tumeo and Wolk, 1994). It has been established that bacteria, not fungi, are the major microflora of oil contaminated soils (Kerry, 1990).

Despite the huge potential of microorganisms to degrade organic compounds under favourable conditions, no single species of microorganism can degrade all the components of a petroleum oil (Korda *et al.*, 1997), and no oil-degrading "superbug" has been engineered. Currently, several organisms are known, each is usually capable to degrade one or, at least, a few petroleum components at a time. The main method that has been used for the multi-factor experimental design was the change of one variable at a time method (OVAT). This is an experimental method in which a single factor is varied, while other factors are kept fixed at a specific set of conditions. This method leads to unreliable results and wrong conclusions, and is inferior to the factorial design method (Krishnan *et al.*, 1998). Therefore, the screening design is appropriate to face the large number of cultural conditions under investigation. Plackett-Burman design (Plackett and Burman, 1946) comprises one type of a two-level screening design. It is favoured to detect the significant factors affecting the process before proceeding to the optimization stage of experimental design.

The aim of this work is to investigate the bioremediation of petroleum oil by a locally isolated *Penicillium* sp. and applying a thorough optimization process. The objective was to evaluate the effects of culture conditions, represented as media components and environmental factors, on the bioadsorption and biodegradation of petroleum oil. This is needed to develop a near optimal medium in order to enhance the bioremediation process by means of statistically designed experiments.

MATERIALS AND METHODS

Media

Basal medium: The fungal isolate was grown in the following medium to follow up the petroleum oil bioremediation process (g/l): NH_4Cl, 2.5; KH_2PO_4, 0.3; Na_2HPO_4, 0.7; $MgSO_4.7H_2O$, 0.2; NaCl, 0.2; yeast extract, 0.1 and trace element stock solution 1 ml. Crude oil is added as main carbon source in conc. of 0.5% v/v. Basal medium was modified in the Plackett-Burman experimental design as presented in Table (1).

Trace element stock solution (mg/l): $ZnCl_2$, 70; $MnCl_2.4H_2O$, 100; $CoCl_2.6H_2O$, 200; $NiCl_2.6H_2O$, 100; $CuCl_2.2H_2O$, 20; $NaMoO_4.2H_2O$, 50; $Na_2SeO_3.5H_2O$, 26; $NaVO_3.H_2O$, 10; $Na_2Wo_4.2H_2O$, 30 and HCl 25%, 1 ml.

Microorganism

The fungus used in this study was isolated from gas station soil contaminated with petroleum oil. It was purified and identified morphologically as *Penicillium* sp. Stock cultures were reserved on Sabroud medium agar slants and refrigerated at 4°C until use.

Spore suspension preparation

Slants of Sabroud Dox medium were inoculated with fungal spores and incubated for 5 days till the appearance of spores. Five ml sterile distilled water were added to each slant and the spores were scrapped off from the agar slant surface and spore suspension was stored under refrigeration.

Extraction of oil from the culture media

The oil residue after fungal growth was extracted with equal volume of chloroform. The solvent phase containing excess oil was dried using rotor vapor. The asphaltenes were removed by dissolving the extracted oil with hexane and the solid materials were removed by filtration. The oil was sent for GC analysis according to standard methods (American Public Health Association (APHA), 1998).

Oil determination

Oil concentration was determined gravemetrically using chloroform extraction method in acidified medium according to standard methods for examination of water and wastewater (APHA, 1998). Petroleum biodegradation % based on aliphatic hydrocarbon content was calculated according to the following equation:

Petroleum degradation % =

$PA_c \quad PA_s / PA_c * 100$ [1]

Where PA_c = total GC peak area of the control non-degraded petroleum sample.

PA_s = total GC peak area of the biodegraded petroleum sample.

Plackett-Burman experimental design

For screening purposes, various medium components as well as environmental factors have been evaluated. The different factors were prepared in two levels: -1 for low level and +1 for high level, based on Plackett-Burman statistical design (Plackett and Burman, 1946). Table (1) illustrates the factors under investigation as well as levels of each factor used in the experimental design. The nitrogen compounds were prepared in equimolar bases to give 0.2 M nitrogen for higher concentration (+1) and the carbon phosphorus containing compounds were prepared to give 0.04 M phosphorus for the higher level trials (+1). Petroleum oil concentration was kept constant in all trials at the level of 0.5%.

Table (1): List of variables under study and their coded levels.

Code	Variable	Values	
		-1	1
A	Ammonium Sulphate	0.132%	1.32%
B	Urea	0.06%	0.6%
C	Potassium dihydrogen phosphate	0.0544%	0.544%
D	Casein hydrolysate	0.01%	0.1%
E	Yeast extract	0.01%	0.1%
F	Spore suspension volume	1%	5%
G	Temperature	30°C	37°C
H	pH	5	7
I	Sodium chloride	0.4%	4%
J	Trace metal solution	0.2 ml	2 ml

Plackett-Burman experimental design is based on the first order model:

$Y = \beta_0 + \Sigma\beta_i x_i$ [2]

Where Y is the response (productivity or specific activity), β_0 is the model intercept and β_i is the variable estimates. This model describes no interaction among factors and is used to screen and evaluate the important factors that influence petroleum oil bioremediation and fungal growth. Ten variables were screened in twelve experiments; each variable being either medium constituent or environmental variable. All experiments were carried out in duplicates and the averages of the oil removal % by weight and degradation % based on aliphatic compounds consumption were taken as responses (Y_1 and Y_2). Variables with high confidence levels are considered significant on their effect on petroleum bioremediation.

RESULTS AND DISCUSSION

Evaluation and optimization of petroleum removal from fungal cultures.

Application of Plackett-Burman experimental design:

The Plackett-Burman design was applied to obtain the first estimates of the different culture determinants for petroleum removal by *Penicillium sp.* Table (2) illustrates the highest petroleum removal percentage of 86.64% that was obtained in the combination number 9. The least removal percentage was obtained at the combination number 2 with a value of 53.68%. Additionally, it can be said that the variability created in the petroleum bioremediation results in the different trials reflects the importance of studying the effect of different variables (either nutritional or environmental) on this microbiological process.

Table (2): Plackett-Burman experimental design for evaluating the effect of different nutritional and environmental categories on oil bioremediation.

Trial	$(NH4)2SO4$	Urea	KH_2PO_4	Casein	Yeast extract	Spore suspension	Temperature	pH	NaCl	Trace metal	Petroleum[1] removal %	Petroleum[2] removal %
1	1	-1	1	-1	-1	-1	1	1	-1	1	72.80	58.78
2	1	1	-1	1	-1	-1	-1	1	1	-1	53.68	33.75
3	-1	1	1	-1	1	-1	-1	-1	1	1	66.48	32.56
4	1	-1	1	1	-1	1	-1	-1	1	1	68.68	28.17
5	1	1	-1	1	1	-1	1	-1	-1	1	62.66	28.91
6	1	1	1	-1	1	1	-1	1	-1	-1	81.54	30.84
7	-1	1	1	1	-1	1	1	-1	-1	-1	77.46	45.18
8	-1	-1	1	1	1	-1	1	1	1	-1	63.80	35.70
9	-1	-1	-1	1	1	1	-1	1	-1	1	86.64	37.53
10	1	-1	-1	-1	1	1	1	-1	1	-1	54.36	27.23
11	-1	1	-1	-1	-1	1	1	1	1	1	58.72	32.21
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	74.26	33.33

[1] *Petroleum removal % based on weight*
[2] *Petroleum removal % based on GC analysis of aliphatic hydrocarbon*

Statistical analysis of these data revealed that the value of determination coefficient R^2, that measures the goodness of model fitting, is > 0.99. This indicates that less than 1% of the total variations is not explained by the model, which ensures the good adjustment of the model (in equation 2) to experimentalize the results.

The factors tested in this design contributed differently on petroleum bioremediation by the *Penicillium sp.*, which means that some of them affected positively and others negatively. Fig. (1) shows that potassium phosphate dibasic, casein, yeast extract, spore suspension concentration, pH and trace elements promoted petroleum removal by *Penicillium sp.* While, ammonium sulphate, urea, temperature and sodium chloride inhibited the oil bioremediation process. These results reflect the importance of phosphorus containing compounds for the bioremediation process as reported by Prince *et al.* (1994). The addition of yeast extract and casein to the medium components was also in favour of bioremediation since the fungi require a primary growth substrate to co-oxidize hydrocarbon compounds as postulated by Korda *et al.* (1997). Keeping the pH level near the alkaline range is favourable for bioremediation (Norris, 1994) and this is in accordance with our results. Trace metal solution contains many metal ions that may favour fungal enzymatic activity and others that may inhibit it. The net result of the partial effect created by the trace metals used in this design was favouring petroleum bioremediation by the *Penicillium* strain used in the present study. On the other hand, high temperature and salinity inhibit the capacity of the fungus to remove and degrade petroleum oil. This is an indication on the unsuitability of the fungus for removal of oil spills from marine environment and at higher temperatures.

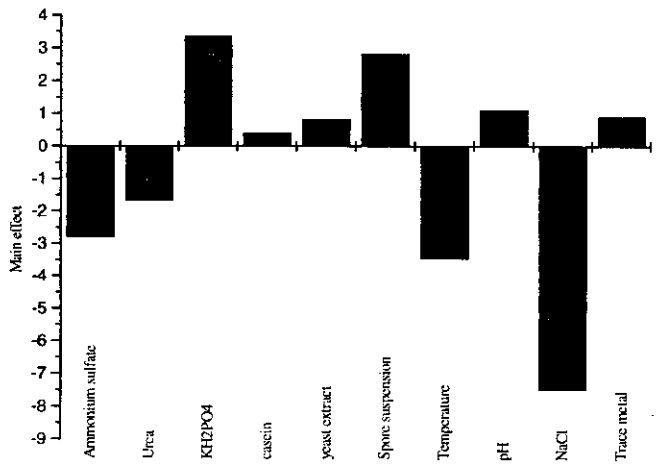

Fig. (1): Effects of different culture conditions on petroleum oil removal (based on weight) by Penicillium sp. in Plackett-Burman experimental design.

Fig. (2) shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate (independent on its contribution, either postive or negative) and is a convenient way to view the results of a Plackett-Burman design

(Strobel and Sullivan, 1999). The highest negative significant variable is sodium chloride (98.8%), while potassium phosphate dibasic is the highest positive significant variable (97.2%).

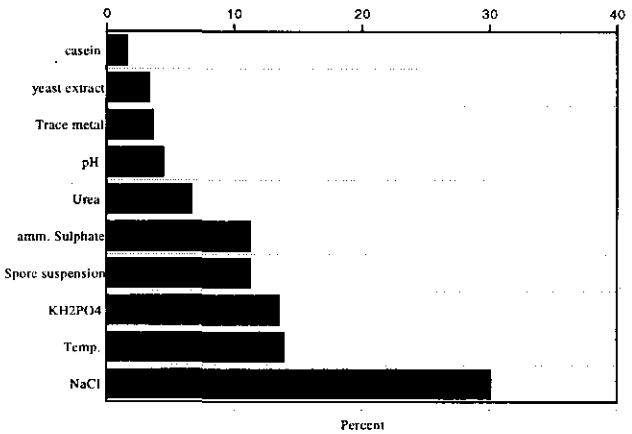

Fig. (2): Pareto plot for Plackett-Burman parameter estimates.

To examine the model validation, a comparison was held between estimated and predicted results as shown in Fig. (3). The linearity of correlation is an evidence of the excellent agreement between experimental and predicted data. The created model (as in equation 1) could be used to predict the response (petroleum removal percentage) when using different culture conditions.

Another response has been also examined based on GC analyses of the different trials to determine the factors affecting aliphatic chain degradation by the fungal isolate. Fig. (4) represents the main effects created by different variables, where K_2HPO_4, temperature and pH increase oil degradation (based on aliphatic chain) as their levels increase. All other factors in the study were found to contribute negatively.

These results seem to be realistic, as the importance of phosphorus containing compounds in bioremediation of oil is well known as reported by Prince *et al.* (1994). Increasing the temperature in the bioremediation medium increases the availability of oil to the degrading fungal isolate, and increases the rate of oxidation processes by dissolving more oxygen into culture. Keeping the pH level near the alkaline range is favourable for bioremediation and this is in accordance to the results obtained by Margesin *et al.* (2000).

On the model level, the $R^2 = 0.8$, which although is not that high as the coefficient of determination of the residual oil response determined by weight, but it is good enough to explain the variabilities of the data.

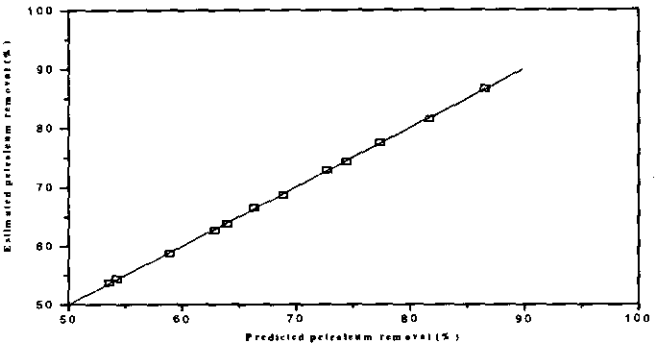

Fig. (3): Correlation between predicted results (calculated from the model) and estimated results (experimental) to show the model validation.

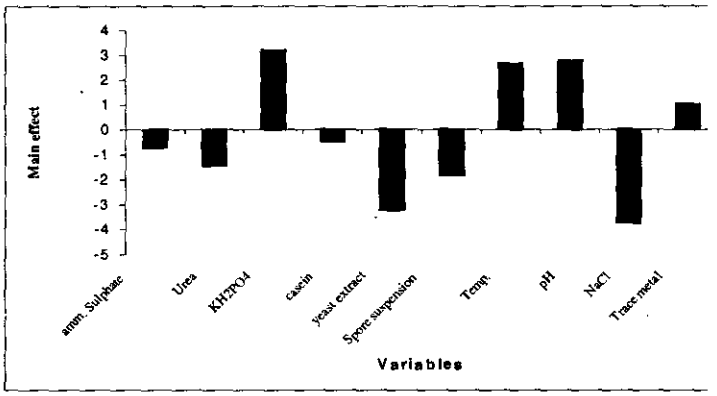

Fig. (4): Effects of different culture conditions on petroleum oil degradation (based on GC analysis) by Penicillium sp. in Plackett-Burman experimental design.

It is worthwhile to mention that Plackett-Burman design is useful not only in evaluating the significance of some variables on the bioprocess, but also in comparing between different categories, in which is difficult to compare between their effects in conventional experiments, and hence maintain a comprehensive evaluation of the overall process.

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الملخص العربى

النمذجة العددية لعملية تكسير زيت البترول بواسطة عزله البنيسيليوم المحلية باستخدام تصميم بلاكت بيرمان الاحصائى

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نموذج بلاكت بيرمان هو نظام احصائى تم استخدامه فى هذه الدراسة لتحديد أمثل الطرق للتحلل البيولوجى لزيت البترول بواسطة عزله محلية من فطر البنسيليوم. من خلال هذا النموذج تمت دراسة عشرة متغيرات خلال أثنتى عشرة محاولة فى نفس الوقت لتحديد انسب الظروف لإزالة التلوث البترولى فى الوسط قبل وبعد إجراء التجربة وكذلك تركيز الهيدروكربونات الاليفاتية المحسوبة باستخدام التحليل الكروماتوجرافى الغازى بعد التجربة.

فى الحالة الأولى وجد أن كل من فوسفات البوتاسيوم ثنائى القاعدية، الكازين، مستخلص الخميرة، تركيز الفطر فى الوسط، الأس الهيدروجينى، وتركيز المعادن الثقيلة له تأثير فعال فى زيادة إزالة زيت البترول من الوسط. على النقيض وجد أن كبريتات الأمونيوم، اليوريا، درجة الحرارة، كلوريد الصوديوم لهم تأثير مثبط لعملية الإزالة. على أساس تركيز الهيدروكربونات الاليفاتية وجد أنه باستثناء فوسفات البوتاسيوم ثنائى القاعدية, درجة الحرارة, الأس الهيدروجينى, تركيز المعادن الثقيلة فإن باقى المتغيرات تؤثر سلبيا على التحلل البيولوجى للهيدروكربونات الاليفاتية. كما وجد أن أنسب الظروف المواتية لإزالة الزيت البترولى هى المتمثلة فى التجربة رقم (٩) بنسبة إزالة ٨٦,٦٤% بينما كانت التجربة رقم (١) تمثل أنسب الظروف للتحلل البيولوجى للهيدروكربونات الاليفاتية بنسبة تحلل ٥٨,٧٨%.