



Plackett-Burman and Response Surface Methodology for Optimization of Oily Wastewater Bioremediation by *Aspergillus sp.*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Plackett-Burman is beneficial design not only in determining the significant variables of bioremediation, but also in optimization of these variables. In this study, Plackett-Burman (PB) experimental model had been applied to assess the significant of some nutritional and environmental condition affecting oily wastewater bioremediation by *Aspergillus niger*. Eleven variables through twelve assays were planned, namely; Temperature, pH, sucrose, KH_2PO_4 , NaCl, MgSO_4 , Na_2HPO_4 , NH_4Cl , NaNO_3 , urea and spore suspension to explain their effects on remediation of water contaminated with oil. The degradation process was enhanced based on oil and grease experiment. pH, sucrose, KH_2PO_4 , NaCl, Na_2HPO_4 and urea were recognized as the positive factors that stimulate the degradation. On the other hand, other variables affected negatively on remediation process. The regression coefficient ($R^2 = 0.99$) ensure the adequate integrity of the model. It was showed that sucrose (A), NaCl (B) and KH_2PO_4 (C) give high removal of oily wastewater when approaching to +1, on the other hand, the temperature (D) give high removal of oily wastewater when approaching to -1. (B) had positive effects on oily wastewater degradation,

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whereas C and D had negative effects. The factor with confidence level above 95% is considered as significant parameter. It was clear that variable B was the chief factor, while variables A, C, D, with levels below 95%, were considered insignificant.

Keywords: Plackett- burman design; bioremediation; *Aspergillus niger*; optimization.

1. INTRODUCTION

Industrial wastewater includes some major pollutants that affect negatively on public health as petroleum hydrocarbon, heavy metals and dyes. Diesel oil that polluted wastewater has been considered as one of the most concerned pollution sources. Oily wastewater produced from different sources such as crude oil production, automotive garage, oil refinery, petrochemical industry, metal processing, lubricant and car washing. These sources serve as the major contributor to the environmental problems especially in soil and water. Wastewater contains some toxic constituents such as petroleum hydrocarbons, phenols, polyaromatic hydrocarbons which are inhibitory to animal and plant growth and also are mutagenic and carcinogen to human being [1]. Hydrocarbons have an effect on aquatic life, coastal environment and habitats as coral reefs and the marine organisms in addition to causing damage and death to agricultural crops [2]. As concern for effect of hydrocarbon contaminated wastewater continues to grow, so are the different technologies that continue to emerge to remediate contaminated site. One of these technologies is bioremediation. Bioremediation of oily wastewater is treatment technology that use of microorganisms or their enzymes to reduce the concentration or toxicity of hydrocarbon contaminants into less toxic forms [3]. Microorganisms as fungi and bacteria may be indigenous to contaminated area or they are isolated from other area and brought to the hydrocarbon contaminated area [4]. According to Atlas [5], the bioremediation process can be occurred at site, less expensive, less required energy and can be used with other physical or chemical treatment method.

Fungi have several advantages over bacteria. Due to their hyphal growth mode, they can form mycelial networks, which they can use to transport water, nutrients and electron acceptors within mycelia. Unlike bacteria they can also grow through air-filled pores and penetrate soil aggregates. Furthermore, because these produced fungal enzymes have low specificity

they are able to degrade a wide range of organic compounds and mixtures of various chemicals [6].

The capability of microorganisms to degrade contaminants and growth of cells are strongly affected by nutritional and ecological factors such as carbon sources, nitrogen sources, inorganic salts, temperature, and pH. These experiments may take several times and consume large amounts of chemicals for achieving optimization. Therefore, it is essential to design suitable process for maximizing the removal efficiency of diesel oil by *Aspergillus niger*. Plackett-Burman design provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter [7]. In the present study, a Plackett-Burman model has been employed to determine the weighty factors and optimize degradation. The main aim of the present study is to determine the optimum concentrations of some nutritional and environmental parameters affecting diesel oil remediation from industrial wastewater using *Aspergillus niger*.

2. MATERIALS AND METHODS

2.1 Media Preparation

The enrichment procedure as described by Nwachukwu [8] was used in the estimation of hydrocarbon utilizes. A minimal salt broth containing 2.0 g of Na₂HPO₄, 0.17 g of K₂SO₄, 4.0 g of NH₄NO₃, 0.53 g of KH₂PO₄, 0.10 g of MgSO₄. 7H₂O and 5.0 g of agar – agar dissolved in 1000 ml of distilled water was prepared. The solution was sterilized by autoclaving. Diesel oily wastewater is added as main carbon source in concentration of 0.5% v/v.

2.2 Isolation and Identification of Fungi

Plates were incubated at room temperature (29° – 31°C) for 15 days. The fungus used in this study was isolated from minimal salt broth medium. It was purified and identified morphologically as *Aspergillus niger*.

2.3 Spore Suspension Preparation

Aspergillus niger was grown on Sabouraud dextrose agar plates at 37°C for 96-hrs and their homogenous spore suspension was prepared in the Sabouraud dextrose broth. Spores from the fungal colonies of 96-hrs cultures in the plates were picked up by an applicator and transferred to sterile physiological saline in a tube or harvested by adding 1.0 ml of sterile physiological saline directly to the plates and recovering back the spore suspension into screw cap tubes. The tubes were vortexed gently for 3 to 4 min at room temperature to prepare a homogeneous suspension of the spores. The spores were counted by using haemocytometer and the number was adjusted to 1×10^8 spores/ml

2.4 Plackett-Burman Experiment

Every variable was prepared from stock solution to obtain accurate results. The different factors were prepared in three levels: -1 for low concentration, 0 for medium concentration and +1 for high concentration, depending on Plackett-Burman modeling design [9]. A control experiment was made for every assay in this design as the same manner of experiment without spore suspension. Table 1 illustrates the factors under investigation and their levels that used in this model. Oily wastewater concentration was kept constant in all trails at the level of 0.5%.

2.5 Analytical Determination of Oil Hydrocarbons

Oil concentration was determined gravimetrically using petroleum ether extraction method in acidified medium according to standard methods for examination of water and wastewater [10]. Petroleum biodegradation % based on oil and Grease content. It was calculated according to the following equation:

$$\text{Petroleum degradation \%} = \frac{\text{control} - \text{fungal}}{\text{control}} * 100 \quad [1]$$

Where *control*= amount of oil and grease without spore suspension. *Fungal*= amount of oil and grease with spore suspension.

2.6 Plackett-Burman Experimental Design

For screening purpose, various medium components have been evaluated using Plackett-Burman (PB) statistical design [9]. The different factors were prepared in two levels: -1

for low level, 0 for medium level and +1 for high level. (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 compounds that containing carbon and phosphorus were prepared to give 0.04 M phosphorus for the higher level assays (+1). Petroleum oil concentration was kept constant in all assays at the level of 0.5%. The PB experimental design is based on the first order model (2):

$$Y = a_0 + \sum_{i=1}^n a_i x_i \quad (1)$$

Where Y is the response (productivity or specific activity), a_0 is the model intercept and a_i is the variable estimates. This model describes no interaction among factors and is used to evaluate the important factors that influence petroleum oil bioremediation and fungal growth. Eleven variables were screened in twelve experiments; each variable being either medium constituent or environmental variable. Variables with high confidence levels are considered significant on their effect on petroleum bioremediation.

2.7 Screening of Significant Medium Variables by PB Design

The PB design was applied to obtain the estimates of the different culture determinants for petroleum removal by *Aspergillus niger*. A polynomial model for oily wastewater removal% was developed by using the estimated coefficients (coded units) and given in Eq. (2).

$$Y = 56.66 + 0.61A + 3.96B - 9.43C - 1.66D + 25.81E - 5.09F - 6.40G - 3.03H + 3.22J - 2.93K - 2.5L \quad (2)$$

2.8 Optimization of Significant Nutritional and Environmental Parameters

Based on growth of *Aspergillus niger* in the preliminary experiment, four variables (Temperature, KH_2PO_4 , sucrose and NaCl) were selected as the various nutritional and environmental parameters for Plackett-Burman design in this study. The concentrations for the different variables were selected according to some preliminary experiments and given in Eq. (3).

$$Y = 57.4 + 1.41A + 6.15B - 0.58C - 0.15D + 5.68AB + 1.72AC + 12.37AD - 0.16BC - 9.03BD - 4.95CD - 4.89ABD - 3.58ACD - 6.61BCD \quad (3)$$

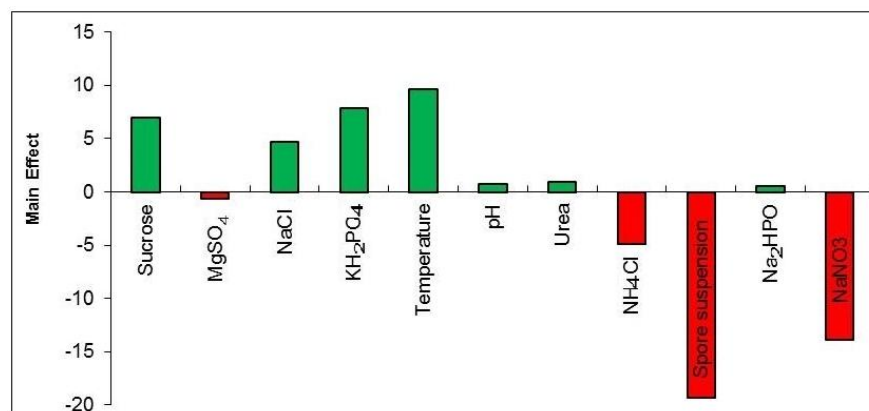
Table 1. List of different variables under study and their coded levels

Factor	Factor name	unit	Low level (-1)	Medium level (0)	High level (+1)
A	Sucrose	g/L	0.1	2	4
B	MgSO ₄		0.1	0.5	0.9
C	NaCl		0.1	0.4	0.8
D	KH ₂ PO ₄		0.2	0.6	1.2
E	pH		3	5	7
F	Temperature	°C	18	20	30
G	Urea	g/L	0.1	0.5	0.8
H	NH ₄ Cl		0.1	0.4	0.8
I	Spore suspension	%	0.2	0.3	0.53
J	Na ₂ HPO ₄	g/L	0.2	0.4	0.8
K	NaNO ₃		0.1	0.3	0.9

3. RESULTS AND DISCUSSION

Eleven variables were chosen in PB design that resembles the most important nutritional and environmental factors affecting oily wastewater bioremediation using fungal isolate Table 1. Twelve assays were made as stated by PB design and the response, oily wastewater remediation was obtained as given in Table 2. The data listed in Table 2 showed a wide variation in oily wastewater degradation, from 7.6 to 96.3, in the 12 assays. The variation suggested that process optimization was important for improving the bioremediation efficiency. Fig. 1 shows that pH, sucrose, KH₂PO₄, NaCl, Na₂HPO₄ and urea were recognized as the positive factors that stimulate the degradation. Other variables affected negatively on diesel oily remediation process. Fig. 2 shows the ranking of variable evaluations in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate (independent on its contribution, either positive or negative) and is an appropriate technique to show the results of a PB model [11]. The highest positive

significant variable is KH₂PO₄, while spore suspension is the highest negative significant variable. Abd El Hamid [6] shows that spore suspension had a highest contribution while pH had low contribution for the growth of *Pencillium sp.* Hegazy et al. [12] showed that sodium dihydrogen phosphate, temperature, sodium chloride inhibited the growth of oil bioremediation. Fig. 3 shows the model validation, a comparison was held between estimated and predicted results. The linearity of correlation is an evidence of the excellent agreement between experimental and predicted data. According to positive effects of selected variables on oily wastewater bioremediation, optimization of selected variables was chosen as in Table 3. Zhou et al. [13] stated that inoculum concentration was vital element for the xenobiotic compounds degradation. A similar result was observed by Ghanem et al. [14] for the chloroxyleneol degradation by *Aspergillus niger*. Nitrogen sources like yeast extract has been proved to support a quick growth of cells and metabolites biosynthesis, as well as extracellular enzymes production.

**Fig. 1. Effect of different culture conditions on diesel oil removal %**

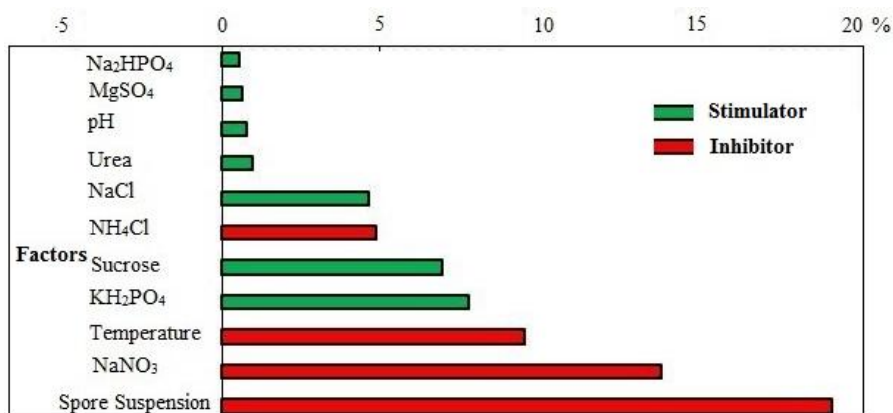


Fig. 2. Pareto plot for PB parameter estimates

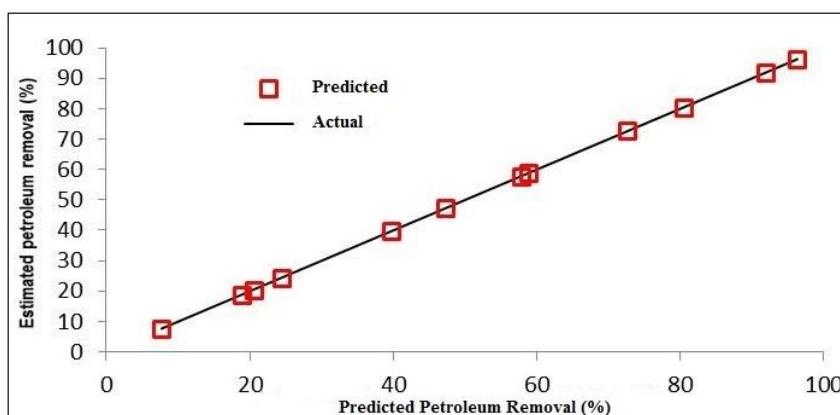


Fig. 3. The relationship between predicted and actual value

Table 2. PB experimental design for evaluating the effect of different nutritional and environmental categories on oil bioremediation

Assay	Sucrose	MgSO ₄	NaCl	KH ₂ PO ₄	Temperature	pH	Urea	NH ₄ Cl	Spore suspension	Na ₂ HPO ₄	NaNO ₃	Oil removal %
1	+1	-1	1	-1	-1	-1	1	1	-1	-1	-1	7.69
2	+1	-1	-1	+1	-1	-1	-1	1	+1	-1	-1	39.69
3	-1	-1	+1	-1	-1	-1	-1	-1	+1	+1	+1	20.52
4	+1	-1	+1	+1	-1	-1	-1	-1	-1	+1	-1	72.64
5	+1	+1	-1	-1	-1	-1	-1	-1	-1	-1	+1	80.51
6	+1	-1	-1	-1	+1	1	-1	+1	-1	-1	-1	91.8
7	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	-1	47.29
8	-1	-1	+1	1	+1	-1	+1	+1	-1	+1	-1	96.32
9	-1	-1	-1	+1	+1	-1	-1	+1	+1	-1	-1	18.88
10	+1	-1	-1	-1	-1	-1	+1	-1	-1	-1	-1	57.85
11	-1	+1	-1	-1	-1	+1	+1	-1	-1	-1	+1	24.38
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	58.82
13	0	0	0	0	0	0	0	0	0	0	0	58.82

Table 3. Plackett-Burman design for optimization of selected variables

RUN	Sucrose	NaCl	KH ₂ PO ₄	Temperature
	A	B	C	D
1	-1	-1	-1	-1
2	-1	1	-1	1
3	1	1	1	1
4	1	1	-1	-1
5	1	1	-1	1
6	-1	1	1	-1
7	-1	-1	-1	1
8	-1	1	1	1
9	1	-1	1	-1
10	1	-1	-1	1
11	-1	-1	1	-1
12	1	1	1	-1
13	1	-1	1	1
14	-1	1	-1	-1
15	1	-1	-1	-1
16	-1	-1	1	1

Table 4. Effects of the variables and statistical analysis of the Plackett-Burman design

Source	Sum of squares	df	Square	F- value	p-value Prob > F
Model	6639.01	13.00	510.69	134.44	0.01
A-A	31.71	1.00	31.71	8.35	0.10
B-B	605.08	1.00	605.08	159.29	0.01
C-C	5.44	1.00	5.44	1.43	0.35
D-D	0.34	1.00	0.34	0.09	0.79
AB	515.33	1.00	515.33	135.66	0.01
AC	47.36	1.00	47.36	12.47	0.07
AD	2449.67	1.00	2449.67	644.89	0.00
BC	0.39	1.00	0.39	0.10	0.78
BD	1305.39	1.00	1305.39	343.65	0.00
CD	392.28	1.00	392.28	103.27	0.01
ABD	382.08	1.00	382.08	100.59	0.01
ACD	205.47	1.00	205.47	54.09	0.02
BCD	698.49	1.00	698.49	183.88	0.01
Residual	7.60	2.00	3.80		

C.V. % -3.40; R²:0.9268; adjusted R²: 0.9914

Low concentration of ammonium sulfate leads to highly degradation of naphthalene in validated tests. The kinetics of ammonium uptake is first order and its specific uptake rate and microbial growth increase as the ammonium ion concentration increases. However, growth and ammonium uptake level reach a maximum and then decrease with the increase in aqueous ammonium ion concentration related to some possible inhibition mechanism [15]. Table 4 shows that B value had significant as positive effects on oily wastewater degradation, whereas C, D had negative effects. The variable with confidence level above 95% is considered as significant parameter. It was clear that variable B was the significant factor, while variables A, C, D

with confidence levels below 95%, were reflected an insignificant value. The statistical significance of the model was checked by F-test and the results were presented in Table 4. The R² values (multiple correlation coefficients) closer to 1 denoted high agreement between the experimental and predicted responses and indicate that the mathematical model is very reliable in the present study. The coefficient of variation (CV) indicated the degree of accuracy in the comparison of experiments. A lower reliability of the experiment is usually indicated by high value of CV; in the present case, the low value of CV (3.40) indicated that experiments conducted were precise and reliable. ANOVA analysis for oily wastewater degradation by

Aspergillus niger showed that the regression model was significant and the lack of fit was insignificant. When the model results are better, it would explain the variability between the experimental and the model predicted values. The graphical representations of the regression model, named the response surface plots and their corresponding contour plots were obtained by Design-Expert software and are obtainable in Fig. 4. Here, each response surface plot represented the effect of two independent variables, holding the other factors at zero levels. The shape of the corresponding contour plot showed whether or not the mutual interaction among the independent factors is significant as in Fig. 5. It was showed that A, B and C give good optimizing conditions for remediation of oily wastewater when approaching to +1, and the same with D when approaching to -1. Zahed et al. [16] suggests that increasing phosphorus and nitrogen concentration can increase n-alkane removal. The optimized predicted removal was

obtained at a phosphorus concentration of 20 mg/L at approximately 24 days. Due to dominating interaction effects of time and phosphorus, higher, levels of these variables increase biodegradation up to 23 days. Optimum levels of nutrient are both economically and ecologically important: high nutrient concentrations may cause eutrophication and harmful algal blooms (HABs) in the aquatic ecosystems [17]. In this study, results imply that it can be a good degrader in presence of significant nutritional and environmental parameters within a very short period of time as Fig. 6. The effect of the selected parameters interaction on oily wastewater bioremediation by *Aspergillus niger* was investigated by plotting the response surface curves against any two independent variables while keeping the other independent variable at constant level. They provided data about the interaction between two factors and allowed an easy interpretation of the results and prediction of the best standards.

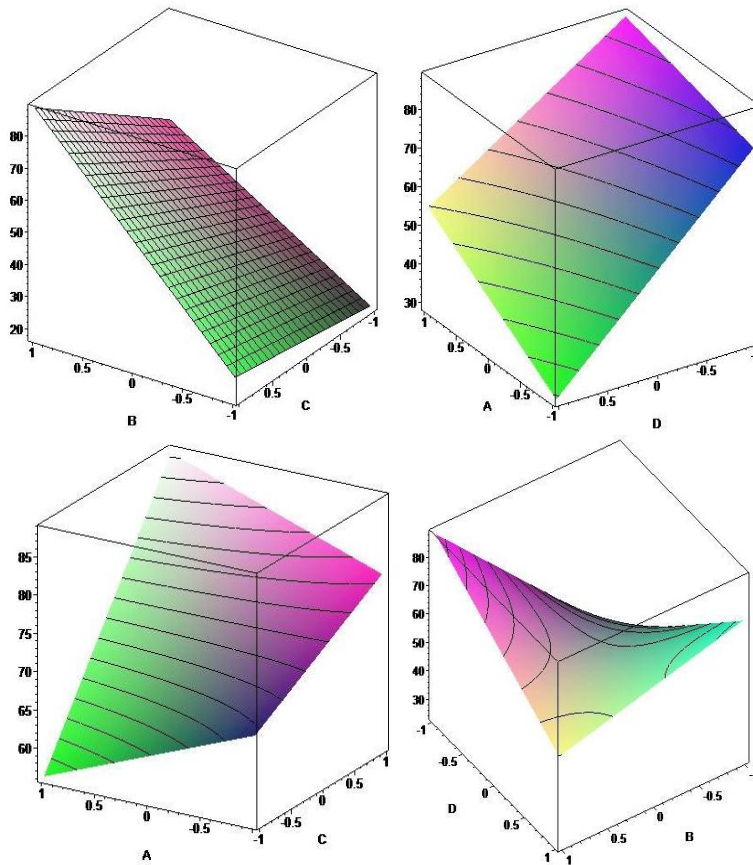


Fig. 4. Three-dimensional response surface plots and two-dimensional contour plots for degradation by *Aspergillus niger* showing variable interactions of: (A) Sucrose; (B) sodium chloride; (C) KH_2PO_4 and (D) Temperature

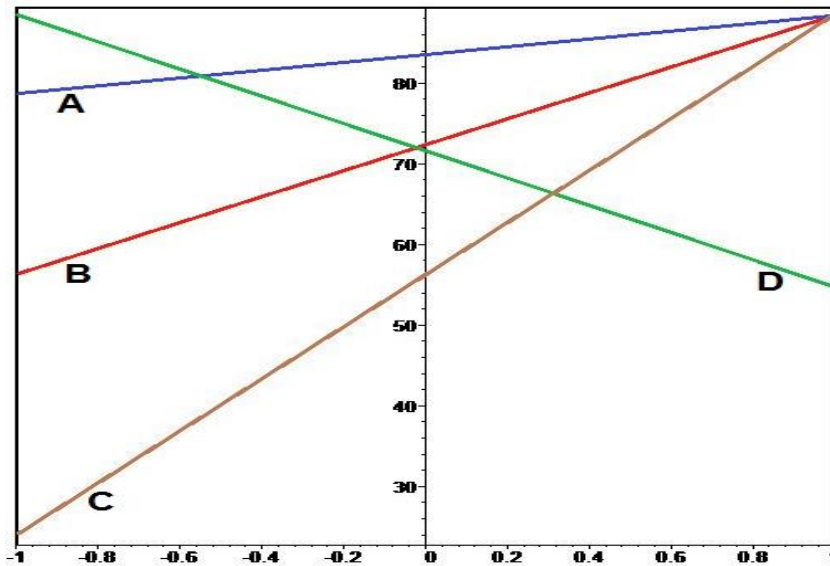


Fig. 5. Positive and negative effects of selected variables on oily wastewater removal

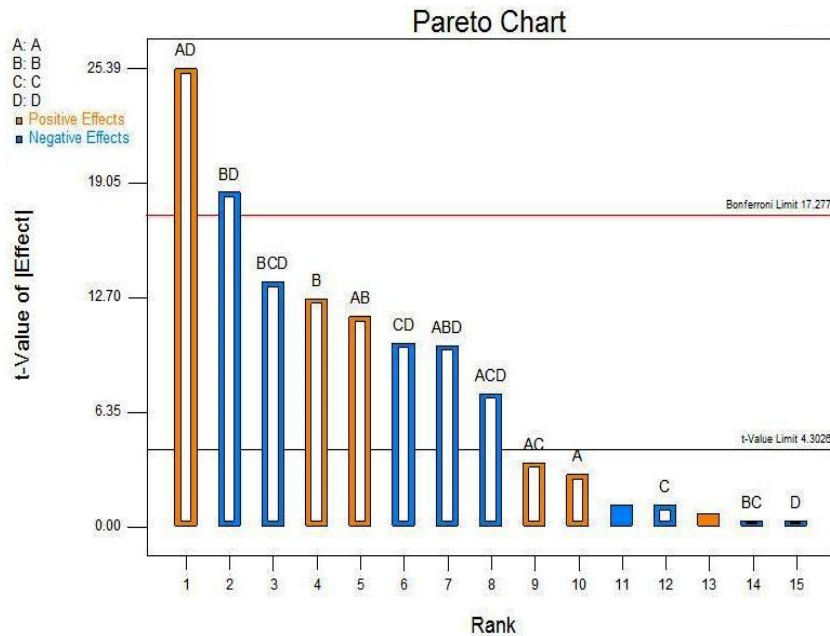


Fig. 6. Relationship between t-value of effect and various parameters

4. CONCLUSION

Bioremediation is an efficient and low cost method for reducing different pollutants i.e. oily wastewater. Fungi especially were used in this research. This present study showed the ability of *Aspergillus niger* in degrading oily wastewater under different conditions. Optimum conditions that achieved in this research encourage using of

this isolate in the remediation of high-strength of oily wastewater discharges. Different parameters approved the ability for remediation optimization conditions like MgSO₄, urea, pH, sucrose, temperature, Na₂HPO₄, KH₂PO₄ and NaCl. On the other hand, some other parameters are inhibitors as NH₄Cl, spore suspension and NaNO₃. This study showed that the response surface methodology was suitable design to

enhance the culture conditions for obtaining the maximum bioremediation of oily wastewater. This method may be modified for more removal of toxic environmental pollutants in different industrial aspects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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