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BIODEGRADATION EFFECTS OF CRUDE OIL-POLLUTED WATER USING BACTERIA ISOLATES FROM SUNFLOWER HUSK ON FISH GROWTH: PARAMETRIC OPTIMIZATION USING TAGUCHI APPROACH

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Abstract: Bacteria isolates from sunflower husk were applied for bioremediation of crude oil-contaminated water.Fish weight was monitored in bioremediated water by gravimetric method. Taguchi design matrix was employed for process parameters optimization, which include reaction temperature (20-60^{o}C), inoculums concentration (20-100 CFU/mL), crude oil concentration (50-250 mL/L), reaction time (1-5 hrs), NH_4Cl concentration (20-100 mg/L) and K_3PO_4 concentration (10-50 mg/L) for crude oil degradation and fish growth. Bacteria isolates were characterized by biochemical tests. Water samples were characterized using gas chromatograph-mass spectroscopy (GC-MS), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and carbon-hydrogen-nitrogen (CHN) analysis. Results revealed optimum crude oil degradation of 96.59 ± 0.03 % for 50 mL/L of crude oil in water supplemented with 100 CFU/mL inoculums, 10 mg/L K_3PO_4 and 10 mg/L NH_4Cl at 60^{o}C reaction temperature and 4 hrs reaction time. Also, optimum fish growth of 93.14 ± 0.04 % was achieved in 50 mL/L of crude oil in solution supplemented with 80 CFU/mL inoculums, 10 mg/L K_3PO_4 and 20 mg/L NH_4Cl at 60^{o}C reaction temperature and 5 hrs reaction time. Conclusively, characterization revealed degradation of hydrocarbons in crude oil-contaminated water from heavy into light fractions by the bacteria isolates.

Keywords: Bacteria isolates, crude oil, fish growth, optimization, polluted water

1. INTRODUCTION

Fishing contributes to the global economic growth via internal revenue generation by a nation (Williams et al., 2004, Meador & Nahrgang 2019). The number of people depending partially or totally on fisheries as a major source of income was estimated to be between 660 and 820 million of the world population (FAO 2016). The demand for fish in both developed and developing nations has been on alarming rate with annual increase of more than 2.5 percent (Brooks et al. 2019). This is because of its contribution to human health asprotein primary source. They are very rich in micronutrients (such as iron, calcium, zinc), vitamins (A, D and B12) and omega-3 fatty acids (eicosapentaenoic and docosahexaenoic) (Kumar et al., 2016). However, no fish survives without water. Thus, our water must be kept clean and safe to make it conducive for sustainable fishing activities.

Nonetheless, overdependence of world on oil as the main energy source is becoming alarming. Large volume of petroleum oil spills into the sea during exploitation, processing and transportation (Wallace et al., 2020). This pollutes the water, disrupts the ecosystem and makes the environment unbearable for aquatic lives to survive (Mundy et al., 2019). This poses havoc to the aforementioned benefits of fishing activities (Incardona 2017). Crude oil comprises complex hydrocarbons merged with other organic compounds comprising someorganometallic constituents (Jorgenson et al., 2018). Hundreds ofthousands of branched and straight chain aliphatic and aromatic hydrocarbons could be found in crude oil which are detrimental to living organisms (McConville et al., 2018). Based on this, effective means of correcting this situation must be developed.

Table 1. Previous optimization approaches for bioremediation of crude oil-contaminated water

Microorganism	Factors	Optimization Approach	References
Pseudomonas sp. sp48	Magnesium sulfate, inoculum size, glucose and Triton X-100	Plackett–Burman experimental design	Farag et al., 2018
*Pseudomonas aeruginosa*UKMP-14T, *Acinetobacter baumannii*UKMP-12Tand*Trichoderma* sp.UKMP-1M and UKMP-2M	Incubation time and crude oil concentration	Central Composite Rotatable Design	Hamzah et al., 2017
*Stenotrophomonas rhizophila*KX082814	Temperature, pH, salinity and inoculum size	Box-Behnken design	Virupakshappa et al., 2016
*Halomonas*sp. MS1	Temperature, salinity, pH, NH_4Cl conc. and $FeSO_4.7H_2O$ conc.	Taguchi experimental design L_{16}	Zavareh et al., 2016
*Rhodococcus*sp.	Initial pH, yeast conc., $K_2HPO_4.3H_2O$ conc. and NH_4Cl conc.	Plackett–Burman design	Yao et al. 2009
Marinobacterlitoralis KK1	pH, temperature, salinity and NH_4Cl	Taguchi experimental design	Moghadam et al. 2014
*Bacillus cereus, Pseudoxanthomonasmexicana, Halomonasdaqingensis*and *Parapusillimonasgranuli*	pH, yeast conc. and NaCl conc.		Singh et al., 2015
Candida tropicalis Z-04	Yeast extract, phenol, inoculum size and temperature	Central Composite Design	Zhou et al., 2011
Bacteria isolates from sunflower husk	Temperature, inoculums conc., crude oil conc., time, NH_4Cl conc., K_3PO_4 conc.	L_{25}Taguchi orthogonal array	This study

Studies have shown bioremediation approach as a novel one in tackling this menace over previously adopted methods which have proved abortive with some shortcomings (El Mahdi et al., 2016). Bioremediation is a restoration intervention technique that utilizes microorganisms (such as bacteria and fungi) and plantsto degrade harmful organic contaminants or convert them to environmentally less toxic compounds of safe levels in sludges, subsurface materials, water, soils and residues (Behera et al., 2018). The mechanism involves organisms taking in substances from the environment and utilizes them to facilitate their growth and metabolism (Chapelle, 1999). This means the optimization of the bioremediation process is very imperative in order to determine the optimum process conditions that will degrade organic contaminants (such as higher crude oil concentration) for environmental friendliness, cost effectiveness and time saving purposes (K'Oreje et al., 2020). Different optimization approaches with the use of different microorganisms for bioremediation have been applied in previous studies. Table 1 presents various optimization tools that have been applied using different microorganisms for bioremediation of crude oil-contaminated water.

Studies have shown the effectiveness of husks from sunflower for the degradation of complex hydrocarbons (Cubitto & Gentili 2015, Dzionek et al. 2016). The aim of this study is to treat crude oil-polluted water with bacteria isolates from sunflower husk and then, test its effectiveness on fish growth using gravimetric method.

2. MATERIALS AND METHODS

2.1. Samples Collection and Preparation

Sunflower seeds were collected from Addexx horticultural gardens located along Challenge, Ibadan, Oyo State, Nigeria. The seeds were busted open from the shell by manual milling. Thenthe husks were separated from the seeds by handpicking and stored in separate polythene nylon. One kilogram of sunflower husk was measured, placed in a bowl, and thoroughly washed using water. One litre of distilled water was added and mixture was allowed to ferment for 21 days at room temperature to enhance bacteria growth.

Water sample was collected from Ureje dam, Ado-Ekiti, Nigerialocated at latitude: 7°37'67.82"N and longitude: 5°18'65.87"E. Table 2 presents the chemical and physical properties of the water sample.

The properties of bonny light crude oil used for this study, which was obtained from the Nigeria Agip Oil Company, Port Harcourt, Nigeria is presented in

Table 3. Catfishes (order Cypriniformes) used to check the effectiveness of the bioremediated water were purchased from Chi-farms, Ibadan, Nigeria.

Table 2. Physico-chemical properties of water sample

Property	Value
pH	7.83
BOD (mg/L)	27.61
COD (mg/L)	36.9
Ammoniacal Nitrogen (mg/L)	0.23
Total suspended solid (mg/L)	4.44
Taste	Slightly salty
Colour	Colourless
Odour	Odourless

Table 3. Physico-chemical characteristics of crude oil sample

Crude Property	Value
API gravity	33.3
Asphaltene (wt. %)	0.41
Sulfur content (wt. %)	1.7
Nitrogen total (wt. %)	1.9
Wax content (wt. %)	0.21

2.2. Analytical Chemicals

Analytical grade chemicals were supplied byTopJ Scientific, Ado-Ekiti, Nigeria. These include Nutrient agar (lifesave biotech, USA), broth medium, sodium hydroxide, hydrochloric acid, acetone, ammoniumchloride, potassium phosphate, methanol and n-hexane.

2.3. Culturing and Isolation of Bacteria Isolates

Bacteria culture was prepared by dilution plate technique. Ten grams of fermented sunflower huskwere distilled in 90 mL of distilled water. Twenty grams of nutrient agar (peptic digest of animal tissue = 5.0 g/L, beef extract = 1.50 g/L, yeast extract = 1.50 g/L, NaCl = 5.0 g/L, agar = 15.0 g/L and final pH at 25^{o}C = 7.4 ± 0.2), a product of lifesave biotech, USA was measured and dissolved in 1000 mL of distilled water. The mixture was rigorously stirred for homogeneity while heating on a mantle to attain a constant temperature of 30^{o}C. Nine mL of diluted sunflowerhusk and 10 mL of nutrient agar were mixed together in petri dish with ten-plate dilution under aerobic condition. The mixture was placed in an incubator for tendays at 37^{o}C. Discrete colonies ofbacteria were sub-cultured and prepared stock cultures were stored at 4^{o}C for further usage.

2.4. Characterization of Bacteria Isolates from Sunflower Husk

The method described by Funke et al., (1996) was adopted for the standard biochemical tests to characterize the bacteria isolates. Morphological characterization was done by examining the rod-to-coccus transformation characteristics of the culture on nutrient agar plate at24 hours interval for 7 days. In order to know whether the isolated bacteria were Gram positive or negative, Gram staining test was conducted. Motility test was executed by observing nutrient broth culture at 28°C incubation for 6 days at 24 hours interval.

2.5. Batch Bioremediation Experiment

Batch bioremediation experiments were conducted at varying parameters (stated in Table 4) as suggested by Taguchi experimental design using Design-Expert 7.0.0 (Stat-Ease, Inc., 2005). Each batch contains a mixture of 100 mL (80 mL of water sample dosed with 5 ml each of crude oil, innoculum, ammonium chloride and potassium phosphate salts prepared at different concentrations) in 250-mL Erlenmeyer flask. The 250-mL Erlenmeyer flask was placed on a temperature controlled equipment with stirring feature (Stuart heat-stirrer SB162) for batch bioremediation process to take place at varying temperature and time. The pH of the water sample and stirring rate (130 revolutions per minute) were kept constant. The initial and final concentrations of total petroleum hydrocarbons were measured using gravimetric method prescribed by Mishra et al. (2001). Samples were extracted using n-hexane. The organic layers were pooled and dried via evaporation of solvents after which the concentration of residual TPH was recorded. Percentage of petroleum hydrocarbons degraded as a result of bacteria isolates effect after the bioremediation batch experiment was calculated using Equation 1:

$$\text{Removal efficiency } (\%) = \frac{C_o - C_f}{C_o} \times 100\% \quad (1)$$

whereC_o= TPH concentration in polluted water before bioremediation (g/kg)andC_f = final concentration of TPH after bioremediation (g/kg).

After each batch experiment, 900 mL of the water sample was added to each batch and placed in a bowl of 3000 mL capacity to make it up to approximately 1000 mL. The efficiency of the bioremediated water was then checked by examining the growth of fish in thebowl for 30 days. Gravimetric method was adopted to check the fish growth after 30 days by measuring the percentage increase in weight

using Equation 2:

$$Increase\ in\ Weight\ (\%) = \frac{W_2 - W_1}{W_1} \times 100\% \quad (2)$$

where W_1 = initial weight of fish (g) and W_2 = final weight of fish (g).

2.6. Experimental Design

In this current study, Taguchi experimental design (Rao et al., 2008, Popoola et al., 2020) was applied to optimize the biodegradation process of the crude oil contaminated water using bacteria isolates from sunflower husk. Six factors (reported in literature to have significant influence on crude oil bioremediation in aqueous solution using bacteria) namely, temperature (20, 30, 40, 50 and 60^{o}C), inoculums concentration (20, 40, 60, 80 and 100 CFU/mL), crude oil concentration (50, 100, 150, 200 and 250 mL/L), time (1, 2, 3, 4 and 5 hours), ammonium chloride concentration (20, 40, 60, 80 and 100 mg/L), potassium phosphate concentration (10, 20, 30, 40 and 50 mg/L) were studied in this experiment. An L_{25} orthogonal approach with a set of 25 experiments was applied to determine the relative effect of 6 factors which include 4 physical factors (temperature, inoculums concentration, crude oil concentration and time) and 2 nutritional factors (ammonium chloride and potassium phosphate concentrations). All factors were investigated at five levels designated as 1, 2, 3, 4 and 5 as shown in Table 4. The responses were expressed as the percent removal of crude oil by bacteria isolates and percent increase in weight of fish. The pH of the mixture was kept constant and stirring rate of temperature controlled equipment was kept constant at 130 revolutions per minute. The relation among the number of experimental test (N), process variables (P) and level (L) selected was described by Equation 3 (Dhawane et al., 2016).

$$N = (L-1)P + 1 \quad (3)$$

2.7. Characterization

Morphological examination of polluted water before and after bioremediation was executed with the aid of scanning electron microscopy (SEM-JEOL-JSM 7600F). Fourier-transform infrared spectroscopy(Nicolet iS10 FT-IR Spectrometer) was used to check the chemical bonding characteristics and surface functional groups of water samples. Gas Chromatograph-mass spectroscopy (Varian 3800/4000 GC-MS) was used to analyse the hydrocarbon fractions in the water samples. Carbon, hydrogen and nitrogen percentage of water samples were investigated using CHN analyser (Thermo finnigan FLASH EA 1242series analyser, Italy).

2.8. Statistical Analysis

The statistical analysis of experimental data was executed via Analysis of Variance (ANOVA). An ANOVA test reveals the significance level of experiment results. The significance of developed model equations was examined using F-value and *p* value. The percentage contribution of each of the significant process parameters on the bioremediation process was investigated using sum of square values. The correlation coefficient (R^2) value measures the degree of intimacy between experimental and predicted values.

3. RESULTS AND DISCUSSION

3.1. Taguchi Model and Statistical Analysis

Table 5 presents the experimental and predicted values (in percentages) of crude oil degraded and fish growth after bioremediation of contaminated crude oil water at different experimental runs.

Table 4. Description of examined process variables at different levels

Variable	Description	Unit	Type	Level				
				1	2	3	4	5
T	Temperature	^{o}C	Factor	20	30	40	50	60
I	Inoculumsconcentration	CFU/mL	Factor	20	40	60	80	100
C	Crude oil concentration	mL/L	Factor	50	100	150	200	250
t	Time	hr	Factor	1	2	3	4	5
A	Ammonium chloride conc.	mg/L	Factor	20	40	60	80	100
P	Potassium phosphate conc.	mg/L	Factor	10	20	30	40	50
D	Degraded crude oil	%	Response					
F	Fish growth	%	Response					

The result revealed experimental run 7 to have highest percentage (89.26%) of crude oil degraded in the contaminated water using bacteria isolates from sunflower husk at temperature, inoculums concentration, crude oil concentration, time, ammonium chloride concentration and potassium phosphate concentration of 60°C, 40 CFU/mL, 50 mL/L, 5 hrs, 80 mg/L and 30 mg/L respectively. Under this condition, highest percentage of fish growth (64.37%) was also recorded. The regression model equations relating the independent process parameters with percent crude oil degraded and fish growth were developed using Taguchi approach and are presented respectively as Equations 4 and 5.

The values indicated in the square bracket represent corresponding model terms as stated in Table 4. Tables 6 and 7 present the analysis of variance (ANOVA) results for the fit of crude oil degraded and fish growth from the Taguchi orthogonal design array. The models were highly significant and adequate for

$$\textit{Crude Oil Degraded}\ (\%) = +50.25 - 18.87T[1] - 8.27T[2] - 1.76T[3] + 8.03T[4] - 6.35I[1] + 0.98I[2] - 1.15I[3] + 2.25I[4] + 9.30C[1] - 2.70C[2] - 1.13C[3] - 0.89C[4] - 6.78t[1] - 0.049t[2] + 2.12t[3] + 1.15t[4] + 0.75P[1] - 3.33P[2] + 0.40P[3] - 3.30P[4] \quad (4)$$

$$\textit{Fish Growth}\ (\%) = +41.40 - 15.44T[1] - 1.69T[2] - 1.20T[3] + 3.69T[4] - 1.19I[1] - 2.19I[2] - 2.48I[3] - 0.79I[4] + 7.36C[1] - 2.67C[2] + 1.63C[3] + 2.08C[4] - 8.02t[1] + 0.38t[2] + 2.68t[3] + 4.41t[4] - 1.79A[1] - 0.81A[2] + 1.07A[3] + 6.27A[4] \quad (5)$$

Table 5. Taguchi experimental design array, experimental and predicted responses

	Bioremediation process variables at levels						Crude oil degraded (%)		Fish growth (%)	
Run	T (°C)	I (CFU/mL)	C (mL/L)	t (hr)	A (mg/L)	P (mg/L)	Experimental	Predicted	Experimental	Predicted
1	40 [3]	60 [3]	250 [5]	2 [2]	80 [4]	10 [1]	47.37	43.46	35.48	35.96
2	20 [1]	60 [3]	150 [3]	3 [3]	60 [3]	30 [3]	31.11	31.60	25.22	28.86
3	50 [4]	20 [1]	200 [4]	2 [2]	100 [5]	30 [3]	48.88	51.38	37.99	41.63
4	60 [5]	20 [1]	250 [5]	4 [4]	60 [3]	20 [2]	57.53	58.02	48.64	51.93
5	20 [1]	80 [4]	200 [4]	4 [4]	80 [4]	40 [4]	34.48	30.57	38.59	37.93
6	40 [3]	80 [4]	50 [1]	3 [3]	100 [5]	20 [2]	56.32	58.82	41.43	44.72
7	60 [5]	40 [2]	50 [1]	5 [5]	80 [4]	30 [3]	89.26	85.35	64.37	68.01
8	40 [3]	20 [1]	150 [3]	5 [5]	40 [2]	40 [4]	41.91	41.26	41.02	40.36
9	60 [5]	100 [5]	200 [4]	3 [3]	40 [2]	10 [1]	78.04	77.39	66.15	66.63
10	30 [2]	40 [2]	150 [3]	4 [4]	100 [5]	10 [1]	41.23	43.73	38.34	38.82
11	60 [5]	80 [4]	150 [3]	2 [2]	20 [1]	50 [5]	76.11	77.68	62.22	60.47
12	30 [2]	20 [1]	100 [2]	3 [3]	80 [4]	50 [5]	44.44	40.53	51.55	49.80
13	40 [3]	40 [2]	200 [4]	1 [1]	60 [3]	50 [5]	46.78	47.27	39.89	38.14
14	20 [1]	20 [1]	50 [1]	1 [1]	20 [1]	10 [1]	26.73	28.30	21.84	22.32
15	30 [2]	100 [5]	50 [1]	2 [2]	60 [3]	40 [4]	51.72	52.21	55.83	55.17
16	30 [2]	60 [3]	200 [4]	5 [5]	20 [1]	20 [2]	38.59	40.16	34.76	38.05
17	20 [1]	40 [2]	100 [2]	2 [2]	40 [2]	20 [2]	26.92	26.27	17.38	18.67
18	40 [3]	100 [5]	100 [2]	4 [4]	20 [1]	30 [3]	50.04	51.61	43.15	45.79
19	30 [2]	80 [4]	250 [5]	1 [1]	40 [2]	30 [3]	33.93	33.28	18.04	21.68
20	50 [4]	40 [2]	250 [5]	3 [3]	20 [1]	40 [4]	51.93	53.50	36.04	35.38
21	50 [4]	60 [3]	50 [1]	4 [4]	40 [2]	50 [5]	73.71	73.06	60.32	59.57
22	50 [4]	100 [5]	150 [3]	1 [1]	80 [4]	20 [2]	55.22	51.31	48.33	51.62
23	20 [1]	100 [5]	250 [5]	5 [5]	100 [5]	50 [5]	37.64	40.14	26.75	24.00
24	60 [5]	60 [3]	100 [2]	1 [1]	100 [5]	40 [4]	54.69	57.19	38.8	38.14
25	50 [4]	80 [4]	100 [2]	5 [5]	60 [3]	10 [1]	61.64	62.13	42.75	43.23

prediction as F-values of 19.98 and 12.82 were obtained for % crude oil degraded and fish growth respectively. Nevertheless, a Prob > F (*p* value) less than 0.05 implies significance level of model terms. In this current study, the most significant model terms are temperature (*T*), inoculums concentration (*I*), crude oil concentration (*C*), time (*t*) and potassium phosphate concentration (*P*) for crude oil degraded and; temperature (*T*), inoculums concentration (*I*), crude oil concentration (*C*), time (*t*) and ammonium chloride concentration (*A*) for fish growth.

The estimated values of the percent contribution of individual significant process parameters, calculated using Equation 6, are included in Tables 6 and 7.Of all the variables examined, temperature (37.07%), inoculums concentration (17.52%), crude oil concentration (23.53%), time (13.98%) and potassium phosphate concentration (7.90%) contributed to percent crude oil degraded in solution using bacteria isolates from sunflower husk as presented in Table 6. Also, temperature (44.15%), inoculums concentration (15.5%), crude oil concentration (22.49%), time (10.32%) and ammonium chloride concentration (7.54%) contributed to percent fish growth as presented in Table 7.

$$Percentage\ contribution = \left[\frac{SS_i}{\sum SS_i}\right] \times 100\% \left(i \neq 0\right) \quad (6)$$

where SS_i = sum of square of significant parameter.

3.2. Parameter Interaction Effect on Crude Oil Degradation

Figure 1presents the 3D surface plot showing interaction effects of temperature and inoculums concentration (Fig. 1a), temperature and crude oil concentration(Fig. 1b), temperature and time (Fig. 1c), temperature and ammonium chloride concentration (Fig. 1d), temperature and potassium phosphate concentration (Fig. 1e), inoculums concentration and crude oil concentration (Fig. 1f), inoculums concentration and time (Fig. 1g), inoculums concentration and ammonium chloride concentration (Fig. 1h),inoculums concentration and potassium phosphate concentration (Fig. 1i), crude oil

Table 6. Analysis of variance for crude oil degraded

Source	Sum of Squares	df	Mean Square	F-Value	p-value		% Contribution
					Prob > F		
Model	6139.82	20	306.99	19.98	0.019	significant	
T	2179.65	4	1159.91	37.70	0.002		37.07
I	1030.06	4	582.52	12.68	0.018		17.52
C	1383.69	4	845.92	18.74	0.008		23.53
t	822.17	4	330.54	12.62	0.019		13.98
P	464.24	4	96.06	8.15	0.044		7.90
Residual	123.08	4	30.77				
Cor Total	6262.90	24					
R^2	0.9803						
Adj R^2	0.9745						
Pred. R^2	0.9211						
Adeq Precision	11.62						

Table 7. Analysis of variance for fish growth

Source	Sum of Squares	df	Mean Square	F-Value	p-value		% Contribution
					Prob > F		
Model	4124.26	20	206.21	12.82	0.021	significant	
T	1953.23	4	1088.31	36.69	0.005		44.15
I	685.80	4	471.45	10.81	0.041		15.50
C	994.86	4	773.72	19.98	0.016		22.49
t	456.74	4	314.18	12.30	0.029		10.32
A	333.62	4	83.41	6.95	0.039		7.54
Residual	351.54	4	87.88				
Cor Total	4475.79	24					
R^2	0.9615						
Adj R^2	0.9421						
Pred. R^2	0.908						
Adeq Precision	8.59						

concentration and time (Fig. 1j), crude oil concentration and ammonium chloride concentration (Fig. 1k), crude oil concentration and potassium phosphate concentration (Fig. 1l), time and ammonium chloride concentration (Fig. 1m), time and potassium phosphate concentration (Fig. 1n), ammonium chloride concentration and potassium phosphate concentration (Fig. 1o), on bioremediation of crude oil-contaminated water using bacteria isolates from sunflower husk. All the examined factors at different levels showed high level of interactive effects on the bioremediation process. Two independent variables were plotted against each other while the remaining variables were kept constant at Level 1. The percentage of crude oil degraded from aqueous solution using the bacteria isolates from sunflower husk was greater than 50% in each of the plots. This suggests that the bacterial isolates have the potential to degrade crude oil-contaminated water.

3.3. Optimization of Bioremediation of Crude oil-Contaminated Water and Fish Growth

The optimization of process parameters was executed to determine the favorable operation conditions at which optimum crude oil could be degraded in the contaminated water using bacteria isolates from sunflower husk. Also, optimum conditions at which maximum fish growth could be achieved were also determined. Among the six independent variables examined, the ammonium chloride concentration and potassium phosphate concentration were found to be insignificant for the optimization of crude oil degradation and fish growth respectively. Thus, each of the process parameter was kept aside separately for each of the optimization process while other five variables were optimized.

At optimum values of 60^{o}C reaction temperature, 100 CFU/mL inoculums concentration, 50 mL/L crude oil concentration, 4 hrs reaction time and 10 mg/L potassium phosphate concentration, maximum crude oil degradation of 94.78 % in solution was predicted by the model (Equation 4). The predicted crude oil degraded (in percentage) was validated such that the ammonium chloride concentration was kept at a minimum value of 10 mg/L. Bioremediation reaction tests were then conducted in triplicate using the optimal reaction conditions and average percentage of crude oil degradation of 96.59 ± 0.03 % was achieved.

Nevertheless, at optimum values of 60 ^{o}C reaction temperature, 80 CFU/mL inoculums concentration, 50 mL/L crude oil concentration, 5 hrs reaction time and 20 mg/L ammonium chloride concentration, maximum fish growth of 91.63 % was predicted by the model (Equation 5). The predicted fish growth (in percentage) was validated such that the potassium phosphate concentration was kept at a minimum value of 10 mg/L. Bioremediation reaction tests were then conducted in triplicate to examine the effect on fish growth using the optimal reaction conditions and average percentage of fish growth of 93.14 ± 0.04 % was achieved.

These are strong indications that the developed models areefficient to predict the process responses accurately as the experimental values of the crude oil degradation and fish growth were verymuch closer to theirrespective predicted values.

3.4. Characterization

3.4.1. Morphological Behavior and Biochemical Tests of Bacteria Isolates

Morphological examination and biochemical tests were done to characterize the bacteria isolates from sunflower husk. Transformation of rod-coccus to short irregular-shaped rod was observed on nutrient agar after 19 hours of incubation. Nonetheless, the short irregular-shaped rod changed to coccoid shape after further incubation for 4 days. The younger cultures revealed Gram-negative rods while older cultures are Gram-positive coccoid in nature. Culture isolates responded positively to citrate, oxidase, 5-keto-gluconate, inositol and sorbitol tests (Lennox et al., 2019) and negatively to D-arabitol, mannose, erythritol, sucrose, glucose and lactose (Zhang et al., 2018). They also exhibited negative response to methyl red and produced blue-green pigment (Zouboulis et al., 2004).

3.4.2. Gas Chromatograph-Mass Spectroscopy (GC-MS)

Gas chromatograph-mass spectroscopy (GC-MS) analysis was conducted in order to affirm the crude oil biodegrading efficiency of bacteria isolates from sunflower husk. The result of the crude oil-contaminated water was compared before and after the bioremediation process using the optimum process conditions predicted by the Taguchi optimization design matrix. In this current study, complex mixtures of hydrocarbons with high molecular weight at different retention times were revealed by the crude oil-contaminated water before the bioremediation process (Table 8). After the biodegradation process, new hydrocarbon compounds with less molecular weight and complexity were formed at different retention times (Table 9). The bacteria isolate from sunflower husk were able to biodegrade the crude oil fractions in solution with higher preference for the alkane compounds having intermediate carbon chains.

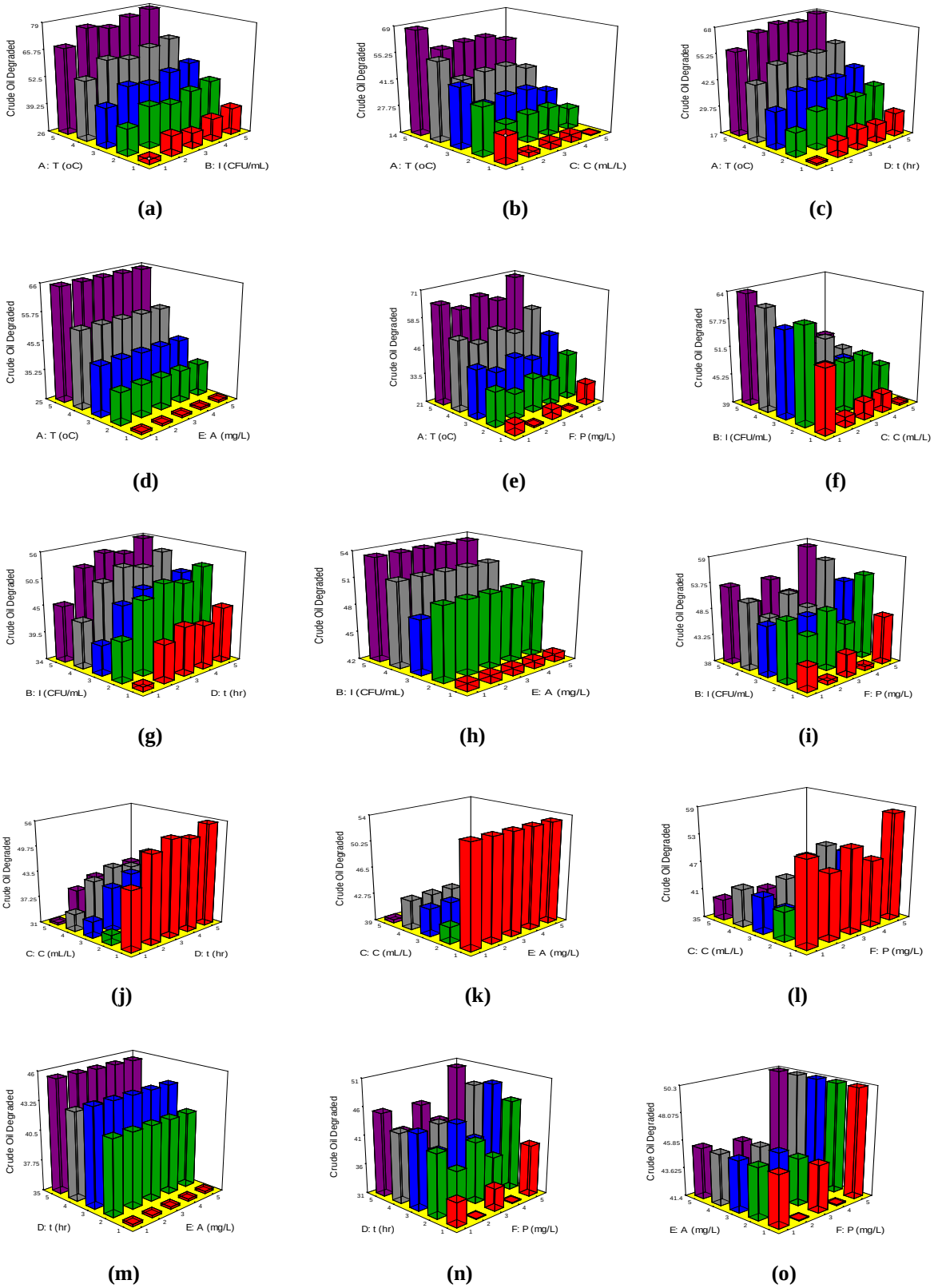


Figure 1. The 3D surface plot showing interaction effects of (a) temperature and inoculums concentration, (b) temperature and crude oil concentration, (c) temperature andtime, (d) temperature and ammonium chloride concentration, (e) temperature and potassium phosphate concentration, (f) inoculums concentration and crude oil concentration, (g) inoculums concentration and time, (h) inoculums concentration and ammonium chloride concentration,(i) inoculums concentration and potassium phosphate concentration,(j) crude oil concentration and time,(k) crude oil concentration and ammonium chloride concentration,(l) crude oil concentration and potassium phosphate concentration, (m) time and

ammonium chloride concentration, (n) time and potassium phosphate concentration, (o) ammonium chloride concentration and potassium phosphate concentration, on bioremediation of crude oil-contaminated water using bacteria isolates from sunflower husk.

Table 8. Hydrocarbon fractions in crude oil-contaminated water before bioremediation

Retention Time (mins)	**Formula**	**Compound**
3.37	2-Nitropentane	$C_5H_{11}NO_2$
4.71	1-Nonadecene	C_7H_8
5.94	Cyclopentane, 1-acetyl-1,2-epoxy-	$C_7H_{10}O_2$
7.72	Heptadecane	C_8H_{10}
8.01	17-Pentatriacontene	C_8H_{16}
9.74	Docosane	$C_{10}H_{22}$
10.66	Cyclotriacontane	$C_{11}H_{24}$
12.93	Tetradecane	$C_{14}H_{30}$
13.18	Octadecane	$C_{18}H_{38}$
15.93	2-Methylnonadecane	$C_{20}H_{42}$
16.24	n-Heneicosane	$C_{21}H_{44}$
19.86	9-Octylheptadecane	$C_{25}H_{52}$

Table 9. Hydrocarbon fractions in crude oil-contaminated water after bioremediation at optimum predicted conditions

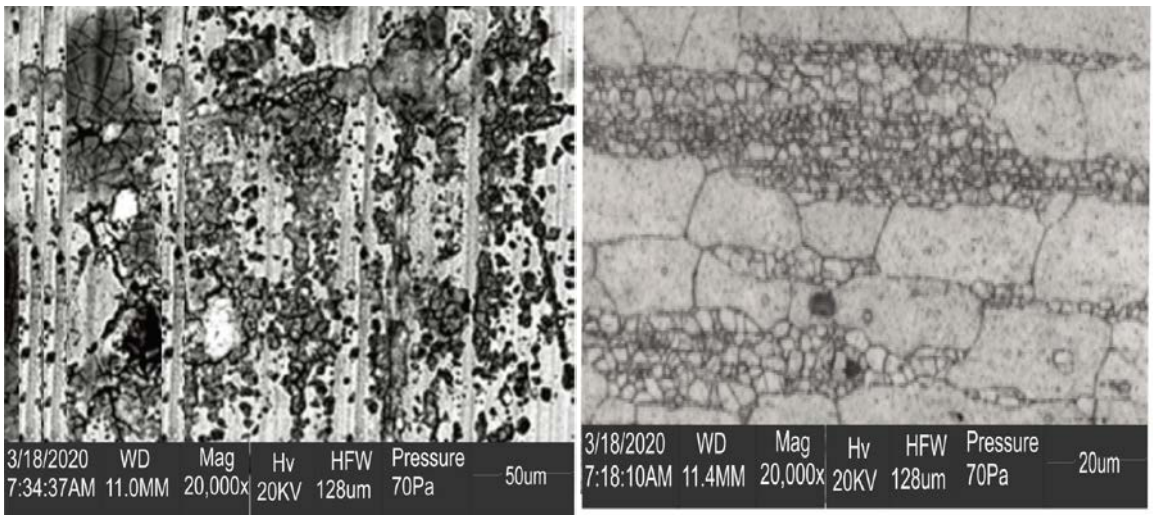
Retention Time (mins)	Formula	Compound
4.46	2-Pentanone	$C_6H_{12}O$
6.91	Nitrohexane	$C_6H_{13}NO_2$
7.17	Toluene	C_7H_8
7.77	4-Methyl-3-pentenoic acid	$C_6H_{10}O_2$
8.35	2-Methyl-pentene	C_7H_{14}
8.94	o-Xylene	C_8H_{10}
10.13	2-Hexanecarboxylic acid	$C_7H_{14}O_2$
10.89	Cyclohexane, ethyl-	C_8H_{16}
16.18	Tetramethylhexadecane	$C_{20}H_{42}$

3.4.3. Scanning Electron Microscopy (SEM)

The SEM micrographs of the crude oil-contaminated water before and after the biodegradation using bacteria isolates from sunflower husk at optimum predicted process parameters by Taguchi array are presented in Figures 2a and 2b respectively. The water surface was covered with densely-arranged and agglomerated crude oil binded together by strong forces (Fig. 2a). A change in crude oil-contaminated water surface morphology was observed after biodegradation using the bacteria isolates (Fig. 2b). A clear water surface with relatively equal patternbinded by weak van der Waals forces was displayed. Nevertheless, the crude oil on the water surface looks more healthier and bigger in diameter (≈ 50 μm) before being biodegraded by bacteria isolates (Fig. 2a) whereas, a reduction in diameter (≈ 20 μm) was observed (Fig. 2b) under the same magnification (20,000×) after the biodegradation process. These exhibitions affirm the efficacy of the bacteria isolates to degrade crude oil in contaminated water.

3.4.4. Fourier-Transform Infrared Spectroscopy (FT-IR)

he FTIR spectra of crude oil-contaminated water beforeand afterbiodegradation at optimum predicted conditions using bacteria isolates from sunflower husk are depicted in Figure 3. In both, absorption bands were observed around 3430-3442 cm^{-1}, 2938-3007 cm^{-1}, 2342-2357 cm^{-1}, 1745-1758 cm^{-1}, 1658-1689 cm^{-1}, 1310-1348 cm^{-1} and 703 -919 cm^{-1} which correspond to O-H group stretching, stretching of C-H present in alkane, weak C≡C stretching of alkyne, strong C=O stretching of cyclopentanone, weak C=C stretching of alkene, O-H bending of phenol and strong C=C bending of alkene respectively. However, new broad bands were noticed after the biodegradation of the crude oil-contaminated water at around 3676-3693 cm^{-1}, 2831-2847 cm^{-1} and 1988-1996 cm^{-1} which correspond to sharp O-H stretching of alcohol, mediumC-H stretchingof aldehyde and weak C-H bending of aromatic compound respectively.



(a) **(b)**

Figure 2. SEM micrographs of crude oil-contaminated water (a) before (b) afterdegradation at optimum predicted conditions using bacteria isolates from sunflower husk

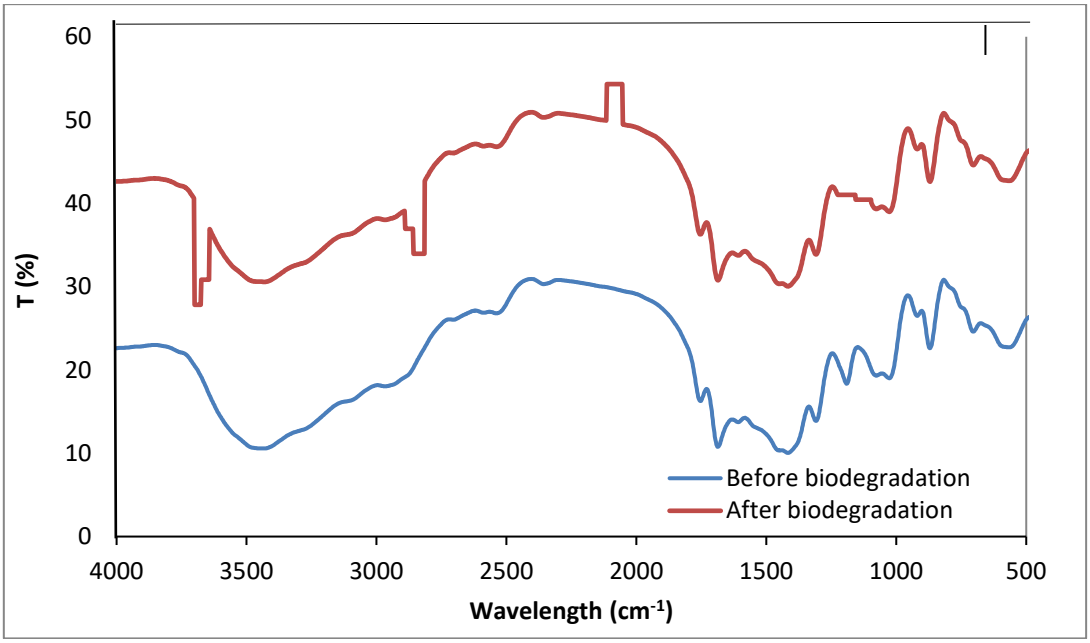


Figure 3. FTIR spectra of crude oil-contaminated water beforeand afterbiodegradation at optimum predicted conditions using bacteria isolates from sunflower husk

Table 10. CHN analysis result of crude oil-contaminated water beforeand afterbiodegradation

Water Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)
Crude oil-contaminated	69.73	21.64	2.19
Bioremediated	18.11	3.18	0.88

3.4.5. Carbon-Hydrogen-Nitrogen (CHN) Analysis

Table 10 presents the result of carbon-hydrogen-nitrogen analysis for the crude oil-contaminated water beforeand afterbiodegradation. The percentage of carbon and hydrogen content of the crude oil-contaminated water (C = 69.73%, H = 21.64%) was observed to be higher than the biodegraded contaminated water (C = 18.11%, H = 3.18%). However, presence of nitrogen (2.19 %) in the crude oil-contaminated water resulted from trace constituent of the crude oil. A residual nitrogen percentage of 0.88 % was recorded after the bioremediation.

4. CONCLUSION

The current study investigated the factors influencing the removal of crude oil from aqueous solution by bacteria isolates from fermented sunflower husk. The efficacy of bioremediated water was checked via application for fish growth monitoring. Six factors at five levels were examined

using an L_{25} orthogonal approach with a set of 25 experiments. Subsequently, statistical analysis using ANOVA and further optimization using Taguchi design matrix were applied. Successively, the developed model equations were found as effective tools in predicting the percentage of crude oil degraded and fish growth as experimental and predicted values were in good agreement. Optimum crude oil degradation of 96.59 ± 0.03 % in solution was achieved when 50 mL/L of crude oil in solution was supplemented with 100 CFU/mL inoculums, 10 mg/L potassium phosphate and 10 mg/L ammonium chloride at 60ºC reaction temperature and 4 hrs reaction time. Also, optimum fish growth of 93.14 ± 0.04% was achieved in water adjusted according to experimental design with parameters: 50 mL/L of crude oil in solution supplemented with 80 CFU/mL inoculums, 10 mg/L potassium phosphate and 20 mg/L ammonium chloride at 60ºC reaction temperature and 5 hrs reaction time. Characterization revealed degradation of complex mixtures of hydrocarbons in crude oil-contaminated water into low molecular weight hydrocarbons by the action of the bacteria isolates. In conclusion, bacteria isolates from sunflower husk could effectively degrade crude oil in solution.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Behera, B.K., Das A, Sarkar, D.J., Weerathunge, P., Parida, P.K., Das, B.K., Thavamani, P., Ramanathan, R. & Bansal, V. 2018. *Polycyclic aromatic hydrocarbons (PAHs) in inland aquatic ecosystems: perils and remedies through biosensors and bioremediation.* Environ. Pollut., 241, 212-233.

Brooks, B.W. & Conkle, J.L. 2019. *Commentary: perspectives on aquaculture, urbanization and water quality.* Comp. Biochem. Physiol. C Toxicol. Pharmacol., 217, 1-4.

Chapelle, F.H. 1999. *Bioremediation of petroleum hydrocarbon-contaminated ground water: the perspectives of history and hydrology.* Groundwater, 37(1), 122-132.

Cubitto, M.A. & Gentili, A.R. 2015. *Bioremediation of crude oil-contaminated soil by immobilized bacteria on an agro-industrial waste-sunflower seed husks.* Biorem J, 19, 277-86.

Dhawane, S.H., Kumar, T. & Halder, G. 2016. *Biodiesel synthesis from Hevea braziliensis oil employing carbon supported heterogeneous catalyst: optimization by Taguchi method.* Renew Energy, 89, 506-514.

Dzionek, A., Wojcieszyńska, D. & Guzik, U. 2016. *Natural carriers in bioremediation: A review.* Electron J Biotechn., 23, 28-36.

El Mahdi, A.M., Aziz, H.A., Amr, S.S.A., Sh, N. & Nassar, E.H.N. 2016. Isolation and characterization of *Pseudomonas* sp. NAF1 and its application in biodegradation of crude oil. *Environ. Earth Sci*., 75, 1-11.

FAO 2016. *The State of World Fisheries and Aquaculture 2016: Contributing to Food Security and Nutrition for All*, p. 200. Rome.

Farag, S., Soliman, N.A. & Abdel-Fattah, Y.R. 2018. *Statistical optimization of crude oil bio-degradation by a local marine bacterium isolate Pseudomonas* sp. sp48. J. Genet. Eng. Biotechn, 16, 409-420. https://doi.org/10.1016/j.jgeb.2018.01.001

Funke, G., Hutson, R.A., Bernard, K.A., Pfyffer, G.E., Wauters, G. & Collins, M.D. 1996. *Isolation of Arthrobacter spp. from clinical specimens and description of Arthrobacter cumminsii sp. nov. and Arthrobacter woluwensis sp.* J. Clin. Microbiol., 34(10), 2356-2363.

Hamzah, A., Manikan, V. & Abd-Aziz, N.F. 2017. *Biodegradation of Tapis Crude Oil using Consortium of Bacteria and Fungi: Optimization of Crude Oil Concentration and Duration of Incubation by Response Surface Methodology.* Sains Malays., 46(1), 43-50.

Incardona, J.P. 2017. *Molecular mechanisms of crude oil developmental toxicity in fish.* Arch. Environ. Contam. Toxicol., 73, 19-32.

Jorgenson, Z.G., Thomas, L.M., Elliott, S.M., Cavallin, J.E., Randolph, E.C., Choy, S.J., Alvarez, D.A., Banda, J.A., Gefell, D.J., Lee, K.E., Furlong, E.T. & Schoenfuss, H.L. (2018) *Contaminants of emerging concern presence and adverse effects in fish: A case study in the Laurentian Great Lakes.* Environ. Pollut., 236, 718-733.

K'Oreje, K.O., Okoth, M., Van Langenhove, H. & Demeestere, K. 2020. *Occurrence and treatment of contaminants of emerging concern in the African aquatic environment: literature review and a look ahead.* J. Environ. Manag., 254, 109752.

Kumar, D. & Asolekar, S.R. 2016. *Significance of natural treatment systems to enhance reuse of treated effluent: a critical assessment.* Ecol. Eng., 94, 225-237.

Lennox, J.A., Asitok, A., John, G.E. & Etim, B.T. 2019. *Characterization of products from sawdust biodegradation using selected microbial culture isolated from it.* Afr. J. Biotechnol., 18(29), 857-864.

McConville, M.M., Roberts, J.P., Boulais, M., Woodal, B.I., Butler, J.D., Redman, A.D., Parkerton, T.P., Arnold, R.W., Guyomarch, J., LeFloch, S., Bytingsvik, J., Camus, L., Volety, A. & Brander, S.M. 2018. *The sensitivity of a deep-sea fish species (Anoplopoma fimbria) to oil-associated aromatic compounds, dispersant, and Alaskan North Slope crude oil.* Environ. Toxicol. Chem., 37, 2210-2221.

Meador, J.P. & Nahrgang, J. 2019. *Characterizing crude oil toxicity to early-life stage fish based on a complex mixture: Are we making unsupported assumptions?.*

Environ. Sci. Technol., 53, 11080-11092.

Mishra, S., Jyot, J., Kuhad, R.C. & Lal, B. 2001. *Evaluation of innoculum addition to stimulate in-situ bioremediation of oily-sludge contaminated soil.* Appl. Environ. Microbiol., 67(4), 1675-1681.

Moghadam, S.M., Ebrahimipour, G., Abtahi, B., Khazaei, N. & Karbasi, N. 2014. *Statistical Optimization of Crude Oil Biodegradation by Marinobacter sp. Isolated from Qeshm Island, Iran.* Iran J Biotechnol, 12 (1), 35-41.

Mundy, L.J., Bilodeau, J.C., Schock, D.M., Thomas, P.J., Blais, J.M. & Pauli, B.D. 2019. *Using wood frog (Lithobates sylvaticus) tadpoles and semipermeable membrane devices to monitor polycyclic aromatic compounds in boreal wetlands in the oil sands region of northern Alberta, Canada.* Chemosphere, 214, 148-157.

Popoola, L.T., Yusuff, A.S., Adeoye, B.K. & Aderibigbe, T.A. 2020. *Cd(II) biosorption using bacterial isolates from sawdust: optimization via orthogonal array Taguchi method.* Water SA, 46(4), 627-637.

Rao, R.S., Kumar, C.G., Prakasham, R.S. & Hobbs, P.J. 2008. *The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal.* Biotechnol. J., 3, 510-523.

Singh, P., Parmar, D. & Pandya, A. 2015. *Parametric optimization of media for crude oil degradation bacteria isolated from crude oil contaminated site.* Int J Curr Microbiol App Sci, 4(2), 322-328.

Stat-Ease, Inc. 2005. Design-Expert (design of experiment software system), version 7.0.0. www.statease.com.

Virupakshappa, P.K.S., Krishnaswamy, M.B., Mishra, G. & Ameenuddin, M.M. 2017. *Optimization of crude oil and PAHs degradation by Stenotrophomonas rhizophila KX 082814 Strainthrough Response Surface Methodology using Box-Behnken Design.* Biotechnol. Res. Int., Vol. 2016, Article ID 4769542, 1-13.

Wallace, S.J., De Solla, S.R., Head, J, Hodson, P.V., Parrott, J.L., Thomas, P.J. & Langlois, V.S. 2020. *Polycyclic aromatic compounds (PACs) in the Canadian environment: exposure and effects on wildlife.* Environ. Pollut., 114863.

Williams, M.J., Nandeesha, M.C. & Choo, P.S. 2004. *Changing traditions: first global look at the gender dimensions of fisheries.* 7th Asian Fisheries Forum, 1–2 December 2004. Penang, Malaysia, WorldFish Center.

Yao, Y., Lv, Z., Min, H., Lv, Z. & Jiao, H. 2009. *Isolation, identification and characterization of a novel Rhodococcus* sp. *strain in biodegradation of tetrahydrofuran and its medium optimization using sequential statistics-based experimental designs.* Bioresour Technol., 100, 2762–2769.

Zavareh, M.S.H., Ebrahimipour, G., Moghadam, M.S., Fakhari, J. & Abdoli, T. 2016. *Bioremediation of Crude Oil using Bacterium from the Coastal Sediments of Kish Island, Iran.* Iran J Public Health, 45(5), 670-679.

Zhang, H., Krafft, T., Gao, D., Zheng, G. & Cai, L. 2018. *Lignocellulose biodegradation in the biodrying process of sewage sludge and sawdust.* Dry Technol., 36(3), 316-324.

Zhou, J., Yu, X., Ding, C., Wang, Z. & Zhou, Q. 2011. *Optimization of phenol degradation by Candida tropicalis Z-04 using Plackett-Burman design and response surface methodology.* J Environ Sci (China), 23 (1), 22-30.

Zouboulis, A., Loukidou, M. & Matis, K. 2004. *Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils.* Process Biochem., 39, 909-916.

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