Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2019.23529

144 (2019) 129–137
March

Response surface-based optimization of the biodegradation of a simulated vegetable oily ballast wastewater under temperate conditions using the Antarctic bacterium *Rhodococcus erythropolis* ADL36

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Received 24 November 2017; Accepted 26 November 2018

ABSTRACT

Discharge of vegetable oily ballast wastewater constitutes serious hazardous pollution to the environment due to its toxic effects on aquatic organisms and terrestrial animals consuming the waste. The damage is especially severe if the release of this waste occurred in temperate waters where biodegradation by existing marine microorganisms is limited due to the cold conditions. Biodegradation using cold-tolerant microorganism added to palm oil wastewater before discharge has never been studied as a method of remediation. This study aims to investigate the biodegradability of vegetable oil (palm oil) at 15°C by a cold-tolerant Antarctic bacterium under saline conditions for such purpose. The strain was cultured at different oil concentrations, temperature, pH, and inoculum size. Furthermore, the degradation of the oil was optimized using response surface methodology (RSM). Gravimetry and gas chromatography were utilized to monitor the biodegradation of the oil components. The results of the study show that maximum growth and biodegradation occur at 1% (v/v) of the oil, at 25°C, pH 6.8, and an inoculum size of 5% (v/v). The use of RSM resulted in an increase in bacterial growth of about 1 log unit. In conclusion, this study demonstrates a possible use of an Antarctic bacterium for the bioremediation of palm oil oily ballast wastewater in temperate waters.

Keywords: Palm oil; Oily ballast wastewater; *Rhodococcus erythropolis* ADL36; Bioremediation; Antarctica

1. Introduction

According to the U.S. Environmental Protection Agency, it is required by regulation that edible fats, oils, and greases (FOGs) are treated by the same safety and spill response practices that are applied to petroleum oils due to its definition under the general definition of "oil" [1]. It is evident from the literature that discharges of large volumes of FOGs into open seas, coastlines, or river bodies can be as harmful as spills of petroleum oil, resulting in environmental and economic damages [2].

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Vegetable oil spills are considered as serious contaminants when they find their way into marine environments, due to their toxicity to the marine organisms and cause damages to the ecosystem. Malaysia is one of the largest exporters of palm oil [3] with marine route as the biggest means of transport of this commodity. The transport of palm oil using tankers can result in major pollution in the event of oily ballast wastewater discharge or collision. The most recent case involved a collision of a vessel carrying 9,000 t of palm oil in Hong Kong. About 1,000 t of palm oil was spilt into the surrounding sea [4].

On the other hand, palm oil pollution in the European or temperate seas is largely due to the discharge of oily ballast wastewater from palm oil tankers and has reached the limelight as it has been suggested to be the cause of deaths of dogs and animals in England consuming the cold precipitated washed palm oil residues [5]. The rise in the number of pollution cases suspected to involve palm oil in the European waters is an alarming issue [6]. The ongoing reclassification of oil palm ballast waste as a hazardous substance by the European Union will seriously affect the cost of palm oil transport [7].

Thus, ways to remediate oily ballast wastewater containing palm oil before being discharged is an urgent demand. With the increasing problem in oily ballast wastewater containing toxic hydrocarbon and lipid wastes, the use of microorganisms as remediation agent is being developed [8,9], but this activity is still limited based on the current literature search. Currently, biological treatment for vegetable oily ballast wastewater in cold seas, especially in the European waters, is largely absent due to the difficulty in finding suitable microorganisms to treat the waste before being discharged. Therefore, this research was conducted to address the issue of oily ballast wastewater treatment using cold-tolerant microorganism. More specifically, this research aims to conduct a study on the aerobic biodegradability of palm oil under simulated oily ballast wastewater conditions. In this study, the refined commercial palm oil was selected as it is one of the main transport forms of palm oil [10]. The oil degradation capability of this bacterium will also be optimized using response surface method (RSM), which is a statistics-based optimization method that is demonstrated to efficiently optimize various processes [11–14] compared with the traditional one-factor-at-a-time (OFAT) approach. In this study, we report on the first use of an Antarctic bacterium for the biodegradation of artificial palm oil oily ballast wastewater under temperate conditions.

2. Materials and method

2.1. Rhodococcus erythropolis ADL36

The bacterium has been identified previously as a diesel-degrading bacterium isolated from Antarctica. The bacterium was identified through molecular phylogenetics of its 16S ribosomal RNA (16S rRNA) gene. The 16S rRNA gene of the bacterium has been submitted to GenBank under the accession number of KX812777. The bacterium was deposited in the Microbial Culture Collection Unit (UNiCC) of Universiti Putra Malaysia under the accession number UPMC 1205.

2.2. Cultural medium

The culture medium was designed to mimic bioremediation under marine conditions. A modified artificial seawater/minimal salt medium [15] supplemented with commercial palm oil (1% v/v) as the sole source of carbon was utilized in this work. The medium (1 L of distilled water; pH 7.5) consists of a standard artificial seawater-based medium [15] supplemented with 4 g/L of NH_4CL, 0.2 g $MgSO_4{\cdot}7H_2O$, and 0.01 g $FeSO_4{\cdot}7H_2O$ and buffered using the phosphate buffer system consisting of 1.2 g KH_2PO_4 and 1.8 g/L K_2HPO_4. For solid media, 15 g of agar was added before autoclaving. The medium was also supplemented with 0.1% of trace elements ($MnSO_4{\cdot}7H_2O$ 0.1 g/L, $CuCl_2$ 0.025 g/L, $NaB_4O_7{\cdot}10H_2O$ 0.025 g/L, $Co(NO_3)_2{\cdot}6H_2O$ 0.025 g/L, ZnCl 0.025 g/L, NH_4NO_3 0.01 g/L, and $(NH_4)_6MO_7O_2{\cdot}H_2O$ 0.025 g/L).

2.3. Growth and biodegradation of crude oil

Biodegradation experiment was carried out at various temperatures (10°C–35°C), substrate concentrations (palm oil 0.5%–10%), and pH (5.8–8). The effect of inoculum concentration was studied from 1% to 5% (v/v). To study the effects of inoculum, the bacterium was grown aerobically at 15°C on an orbital shaker (150 rpm) for 7 d. The bacterium was harvested through centrifugation at 10,000 g for 10 min and the pellet resuspended in artificial seawater-based medium to an optical density of 1.0 measured at 600 nm. The effect of pH was studied by varying the composition of KH_2PO_4 and K_2HPO_4 according to a standard method [16]. All the experiments were performed in triplicate. Growth is determined as CFU/mL.

2.4. Gas chromatography-mass spectrometry analysis

In order to monitor the degradation of oil palm, a Gas chromatography-mass spectrometry (GC-MS) analysis was conducted. After the incubation period of 7 d, the remaining oil residue was extracted with 20 mL of *n*-hexane in a separating funnel to remove cellular material. The residues were transferred to tarred vials, and the volume of each extract was adjusted to 100 mL by further adding *n*-hexane. The vials were kept at 4°C until the GC analysis. The fatty acid samples were esterified before injection [17]. Un-inoculated control was incubated to monitor substrate degradation. The GC (GCMS-QP2010 Shimadzu Corporation, Kyoto, Japan) was equipped with the column ZB-5MS (30 m × 0.25 mm ID × 0.25 μm film thickness). One microliter of the sample was injected. The oven temperature was initially held at 50.0°C for 5 min. The injection temperature was carried out at 300.00°C and a pressure of 26.7 kPa, with a flow of 17.4 mL/min and a linear velocity of 30.0 cm/s.

2.5. Determination of oil degradation via gravimetry

The rate of oil degradation was studied according to a standard method [18]. After 7 d of inoculation, *n*-hexane was used to extract the oil residue in each medium. A suitable aliquot of the sample was first acidified to pH 2.0 and then placed in a separatory funnel. Then 30 mL of *n*-hexane was added to the funnel and shaken vigorously for several minutes with occasional venting in a fume hood. The mixture was left to separate the organic from the aqueous layer. Approximately 10 g of anhydrous Na_2SO_4 was placed in a filter funnel and rinsed with a small amount of *n*-hexane. A clean boiling flask containing 3–5 boiling chips was placed

for 2 h in an oven at 105°C–115°C to dry the flask and chips. The flask was cooled in a desiccator to room temperature and then weighed. The *n*-hexane layer (upper layer) from the separatory funnel was drained through the Na_2SO_4 and into the pre-weighed boiling flask. The flask was placed in a water bath set at 70°C and connected to a distilled to remove the *n*-hexane. The residue was allowed to dehydrate. The flask was cooled to room temperature, and the dry residue was weighed using a 0.1 mg accurate microbalance. The rate of oil degradation was evaluated from the difference in the average dry weight. The oil degradation rate was calculated as follows:

$$\text{Degradation rate}\% = \frac{\text{Weight of the control} - \text{The weight of residue}}{\text{The weight of the control}} \times 100 \quad (1)$$

2.6. Optimization using statistical approach

2.6.1. Plackett–Burman factorial design

In order to identify significant factors required for high growth and degradation, screening of the factors was performed using Plackett–Burman factorial design (PBFD). The effects of four parameters, namely, substrate concentration, temperature, pH, and inoculum size on the growth of the bacterium were measured. The parameters were studied at two levels of high and low ranges. The Design Expert software 6.0.6 generated 12 required experiments. The experiments were conducted in triplicates, and the response (CFU/mL) was recorded. The statistical significant effect of each factor on the bacterial growth was determined by calculating the *P*-value of each factor.

2.6.2. Central composite design experiments

The central composite design (CCD) was applied for the optimization of palm oil degradation using *Rhodococcus erythropolis* ADL36. From the Plackett–Burman analysis, three significant experimental factors were chosen for the present studies, namely, pH, temperature, and substrate (palm oil) concentration. The variables optimized were (i) temperature in the range of 20°C–30°C, (ii) oil concentration of 2%–6% (v/v), and (iii) inoculum concentration of 2%–5% (v/v). A 2^3 factorial central composite experimental designs leading to a set of 20 experimental runs were used to optimize the palm oil degradation.

3. Results and discussion

3.1. Optimization of bacterial growth on palm oil using OFAT

OFAT is a conventional method that is based on changing one independent variable while at the same time fitting others to a certain range. This method allows individual effects of medium constituents and conditions of the process to be viewed graphically. Thus, it is considered to be a simple method [19]. However, some of its disadvantages are the interfaces between optimized components are usually disregarded, and it is time consuming and expensive since a large number of variables is involved [11,12]. OFAT is a classical method of optimizing bacterial growth, and the results obtained are useful for further optimization using design of experiment (DOE)-based methods for comparative purposes and as a basis for process optimization. The effect of various parameters such as pH, temperature, bacterial inoculum, and palm oil concentration on bacterial growth was first optimized through OFAT (Figs. 1(a)–1(d)).

The optimum concentration of palm oil supporting growth was 1% (v/v). At the highest concentration of palm oil tested (10% v/v), there was no bacterial growth (Fig. 1(a)). The highest degradation was also observed at 1% (v/v) of palm oil, with 57% degradation through gravimetry, and negligible degradation was observed at 10% (v/v) of palm oil (Fig. 2). At high concentrations, the oil may be toxic to the bacteria thereby decreasing growth and degradation. Antimicrobial activities can be exerted by certain fatty acid esters as reported by Shon *et al.* [20] where they observed a dramatic decrease of bacterial growth by 10% (v/v) of olive oil. In addition, the resulting free fatty acids and fatty acid salts from lipid degradation can inhibit the lipases of bacteria, and this was observed in the lipid degradation by *Pseudomonas fragi* and *Pseudomonas aeruginosa* [21].

The bacterium grew optimally in between the pHs of 6.8 and 7.2 (Fig. 1(b)), indicating that the bacterium is a neutrophilic microorganism. The effect of inoculum size indicates that the best concentration of bacterial inoculation occurred between 1% and 4% (v/v), and higher inoculant concentrations did not add further benefits to bacterial growth (Fig. 1(c)). At the maximum optimal concentration of palm oil (4% v/v), the optimal temperature that supported the growth of *Rhodococcus erythropolis* was between 20°C and 25°C (Fig. 1(d)). At the highest temperature tested, which was 30°C, growth was not affected initially but after day 2 of incubation a dramatic drop in bacterial number was recorded, and this is not surprising as the bacterium is a psychrotolerant bacterium isolated from Antarctica. At the lowest temperature tested (10°C), the viscosity of the oil increases and this delays the onset of biodegradation. The bacterium was able to grow on palm oil at a subtropical temperature of 15°C, a feat that will make it useful for the bacterium to be applied as a remediation agent for ballast wastewater in the temperate region such as in the Atlantic where palm oil shipping is an important route. Palm oil pollution occurring in the Atlantic Ocean in winter often results in clumping of the palm oil into nonbiodegradable form. Palm oil clumps are the most common form that wash onto the beaches of England [5]. Temperatures from 15°C and 25°C favor the growth of the bacterium, and a linear increase in bacterial growth was observed. At the highest temperature tested (30°C), a decrease in the growth was observed after day 2 of incubation probably as a result of enzyme denaturation since the bacterium is a psychrotolerant microorganism. Further possible improvements to the bacterial growth were explored using the DOE methods, Plackett–Burman, and RSM.

3.2. Statistical optimizations

3.2.1. Selection of significant variables by Plackett–Burman design

A Plackett–Burman design is used as a prescreening tool to find the most significant independent factors among multiple factors [18]. The four independent variables studied in OFAT, namely, oil concentration, initial pH, temperature,

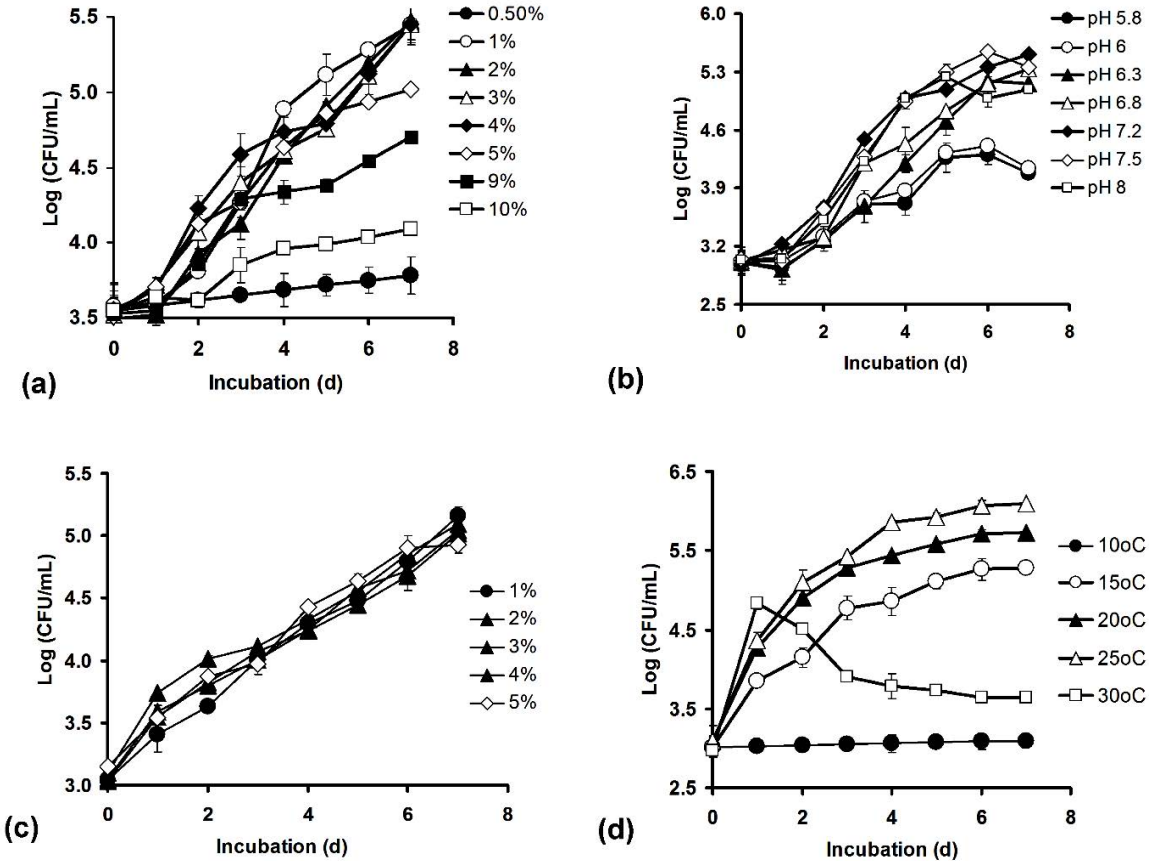

Fig. 1. The effects of palm oil (% v/v) (a), pH (b), % bacterial inoculum (v/v) (c), and temperature (d) on the growth of *Rhodococcus erythropolis* ADL36 on palm oil. Error bars represent mean ± standard deviation (n = 3).

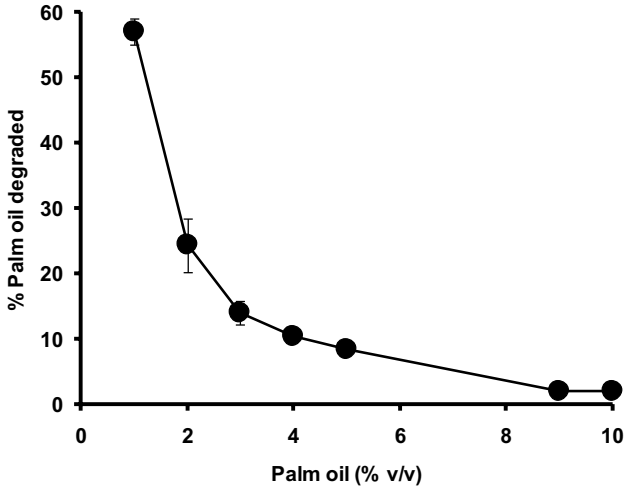

Fig. 2. The degradation of refined palm oil by *Rhodococcus erythropolis* ADL36 measured through gravimetry. Error bars represent mean ± standard deviation (n = 3).

and inoculum, were chosen to be evaluated on the biodegradation of palm oil. The advantage of statistical optimization technique is that the main effects of independent variables as studied using the Plackett–Burman method can lead to a better yield of response [19]. The codes and levels of the independent variables for the Plackett–Burman design are shown in Table 1.

Twelve sets of experiments were designed by the software Design Expert 6.0. The design conditions and corresponding responses (CFU/mL) obtained are presented in Table 2. Three factors, namely, temperature, oil concentration, and inoculum size were the most significant factors while pH was not a significant factor in supporting growth on palm oil.

The significance of each factor was determined by the P-value (Table 3). The lesser the P-value, the more significant are the factors [19]. Factors one, two, and four were the most significant factors having a P-value of less than 0.05 ($P < 0.05$). Analysis of variance (ANOVA) for the model is shown in Table 3. The F-value of 13.48 specifies that the model terms are significant, and an R^2 value of 0.9183 implies that the correlation is significant.

The significant terms identified by Plackett–Burman were used for further optimization exercise using RSM. The nonsignificant factor pH was included at the optimum level achieved during OFAT. The model equation fitted by regression analysis is given by

$$\text{Response} = +5.11 - 0.21 \times A + 0.19 \times B - 0.089 \times C + 0.20 \times D \quad (2)$$

Table 1
Coded and actual values of significant factors used in Plackett–Burman factorial design

Factors	Low actual	High actual	Low coded	High coded
A oil concentration (%)	1.00	6.00	–1.000	1.000
B Temperature (°C)	15.00	30.00	–1.000	1.000
C pH	6.00	7.50	–1.000	1.000
D Inoculum size (%)	2.00	6.00	–1.000	1.000

3.2.2. Response surface methodology

The CCD was employed to study the optimum concentration of the three most significant factors obtained by Plackett–Burman in the form of the coded and actual value of each independent factor (Table 4). Twenty experiments were designed by the Design Expert software 6.0 with six replicates of midpoints, which are useful to determine the experimental error (Table 5).

The regression equation and the determination coefficient (R^2) were assessed to test for the model fitness (Table 6). The model *F*-value of 270.63 is an indication that the model was significant. There is only a 0.01% chance that a higher "model *F*-value" could be obtained as a result of noise alone. It was observed that the model had a low probability value (<0.0001) and a lack of the fit test of 0.2252 (nonsignificant) implying that the model was fit [11–13]. The model exhibited a good coefficient of determination ($R^2 = 0.9959$).

The reliability of the experiments can be further established with a low coefficient of variance (CV) value [19]. The CV of 3.1% for this study is an indication of the reliability of experiments performed. The significance of regression for the coefficients was considered. In this case, *A*, *B*, *C*, A^2, B^2, C^2, *AB*, *BC*, and *AC* were the significant model terms (Table 6). Hence, statistical analysis of the experimental data revealed that all the three factors (oil concentration, temperature, and inoculum size) had a significant effect on growth on oil palm throughout the study. The comparative closeness of the actual CFU/mL in all the replicates of midpoints (Table 5) obtained (runs 3,7,8,11,13, and 19) further supported the reliability of the model.

Table 3
Analysis of ANOVA for the model of factors in Placket–Burman

Source	Sum of squares	Mean square	*F*-value	*P*-value
Model	1.51	0.30	13.48	0.0033[a]
A	0.54	0.54	23.98	0.0027[a]
B	0.44	0.44	19.66	0.0044[a]
C	0.052	0.052	2.30	0.1805[b]
D	0.47	0.47	20.70	0.0039[a]

Note: [a]denotes significance and [b]nonsignificant.

Table 4
Coded and actual values of significant factors used in central composite design (CCD)

Factors	Low actual	High actual	Low coded	High coded
A: oil concentration (%)	2.00	6.00	−1.000	1.000
B: temperature (°C)	15.00	30.00	−1.000	1.000
C: inoculum size (%)	2.00	6.00	−1.000	1.000

The fitness of data into the selected model was examined through diagnostic model plots (Figs. 3(a)–(d)). The plots are important particularly in the assessment of data error that differs from model predictions, which aids in assessing and improving model adequacy [19]. The plot of actual versus predicted CFU obtained from the experiment (Fig. 3(a)) revealed a close relationship between the actual and predicted values as the data points assembled close to the line that divides the plot into equal halves (45°). The adequacy of the model was further verified by plotting the predicted values and studentized residuals (Fig. 3(b)). Studentized residues are variation between the predicted value and actual responses obtained

Table 2
Experimental design matrix of Plackett–Burman and the response

Runs	*A*: oil concentration	*B*: temperature	*C*: PH	*D*: inoculum concentration	Response CFU/mL
1	6.00	15.00	6.50	6.00	4.95
2	1.00	30.00	7.50	6.00	5.42
3	6.00	30.00	7.50	6.00	5.35
4	6.00	15.00	7.50	2.00	4.20
5	1.00	30.00	6.50	2.00	5.30
6	6.00	30.00	6.50	2.00	5.00
7	1.00	30.00	7.50	2.00	5.36
8	1.00	15.00	7.50	6.00	5.30
9	1.00	15.00	6.50	6.00	5.44
10	6.00	15.00	7.50	2.00	4.50
11	6.00	30.00	6.50	6.00	5.20
12	1.00	15.00	6.50	2.00	4.99

Table 5
CCD experimental matrix generated by Design Expert and corresponding responses (actual and predicted)

Run	Factor 1 *A*: oil concentration (%)	Factor 2 *B*: temperature (°C)	Factor 3 *C*: inoculum size (%)	Actual value CFU/mL	Predicted value
1	7.36	22.50	4.00	6.01	6.03
2	4.00	22.50	2.32	6.30	6.32
3	4.00	22.50	4.00	6.65	6.62
4	4.00	35.11	4.00	6.15	6.15
5	2.00	15.00	3.00	6.21	6.20
6	6.00	30.00	3.00	6.19	6.18
7	4.00	22.50	4.00	6.61	6.62
8	4.00	22.50	4.00	6.61	6.62
9	2.00	30.00	3.00	6.12	6.12
10	4.00	22.50	5.68	6.20	6.21
11	4.00	22.50	4.00	6.62	6.62
12	0.64	22.50	4.00	6.14	6.14
13	4.00	22.50	4.00	6.62	6.62
14	2.00	15.00	5.00	6.13	6.12
15	6.00	15.00	3.00	6.13	6.11
16	4.00	9.89	4.00	6.01	6.04
17	6.00	15.00	5.00	5.94	5.92
18	2.00	30.00	5.00	6.17	6.17
19	4.00	22.50	4.00	6.60	6.62
20	6.00	30.00	5.00	6.15	6.14

Table 6
Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	Mean square	*F*-value	Probability > *F*	
Model	1.10	0.12	270.63	<0.0001	Significant
A	0.014	0.014	31.17	0.0002	
B	0.015	0.015	33.60	0.0002	
C	0.013	0.013	29.70	0.0003	
A^2	0.50	0.50	1,114.43	<0.0001	
B^2	0.49	0.49	1,093.45	<0.0001	
C^2	0.23	0.23	498.88	<0.0001	
AB	0.013	0.013	28.32	0.0003	
AC	5.00E-003	5.00E-03	11.06	0.0077	
BC	9.800E-003	9.800E-003	21.68	0.0009	
Lack of fit			2.05	0.2252	Not significant

from the model. The plot of normal probability demonstrates slight or no abnormality in the experimental data (Fig. 3(c)). An outlier plot (Fig. 3(d)) visualizes the distantly standout standard deviation of actual response from the rest of the data. No outlier was evident from the plot as all the data fall between 3.5 and −3.5.

Visualization of the optimum growth (CFU/mL) of all the factors required for maximum growth is presented through 3-dimensional (3D) responses and contour plots (Figs. 4–6). The plots are of utmost importance in determining the growth at zero or intermediate levels of different combinations of independent factors before performing a real experiment [22,23]. The 3D response plot shows that there is an interaction between each pair of factors.

The result of the experiment revealed that a high CFU/mL was attained at the midpoints of the runs (3, 7, 8, 11, 13, and 19). In the current study, it was observed that with an increase in temperature and inoculum size, maximum growth was attained at 4% of the oil (temperature, oil concentration, and inoculum size had a significant effect on the bacterial growth in palm oil). A significant improvement of bacterial growth ($p < 0.05$) was obtained after RSM exercise compared with OFAT (Table 7), indicating the use of RSM was successful.

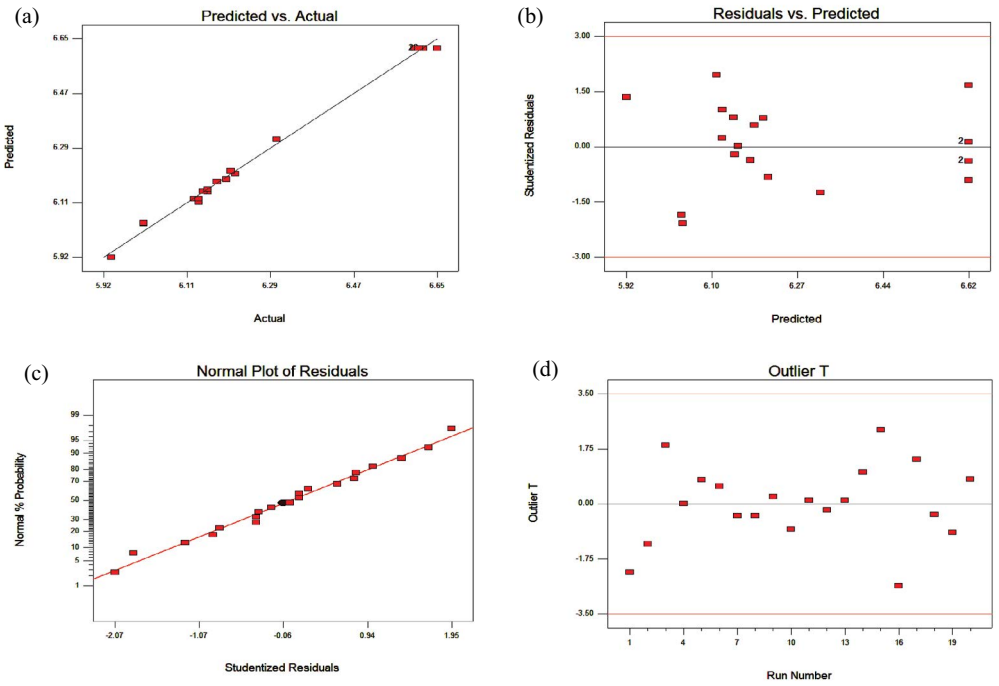

Fig. 3. Model diagnostic plots: (a) predicted versus actual, (b) studentized residue versus predicted, (c) normal plots of residue, and (d) outlier *T* versus run.

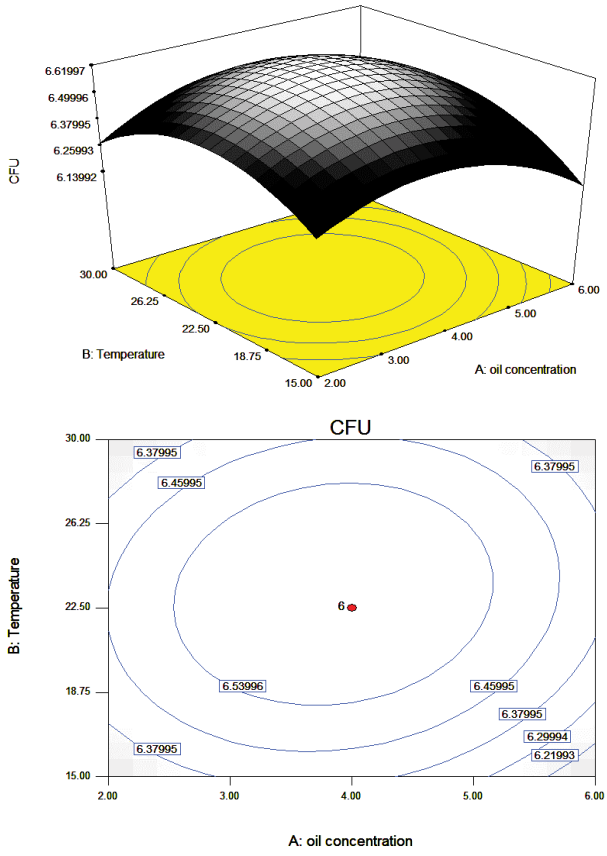

Fig. 4. 3D and 2D surface response views showing the interaction between oil concentration and temperature.

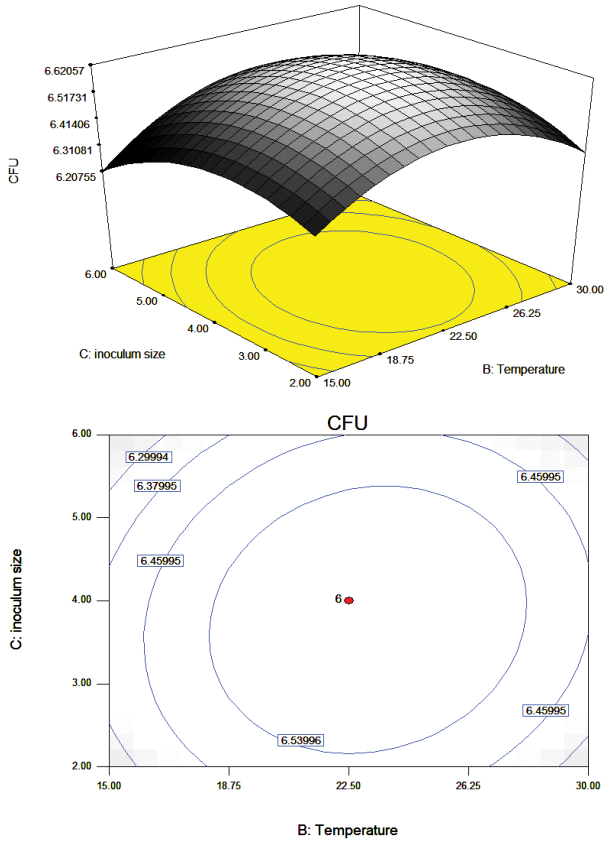

Fig. 5. 3D and 2D surface response views showing the interaction between temperature and inoculum size.

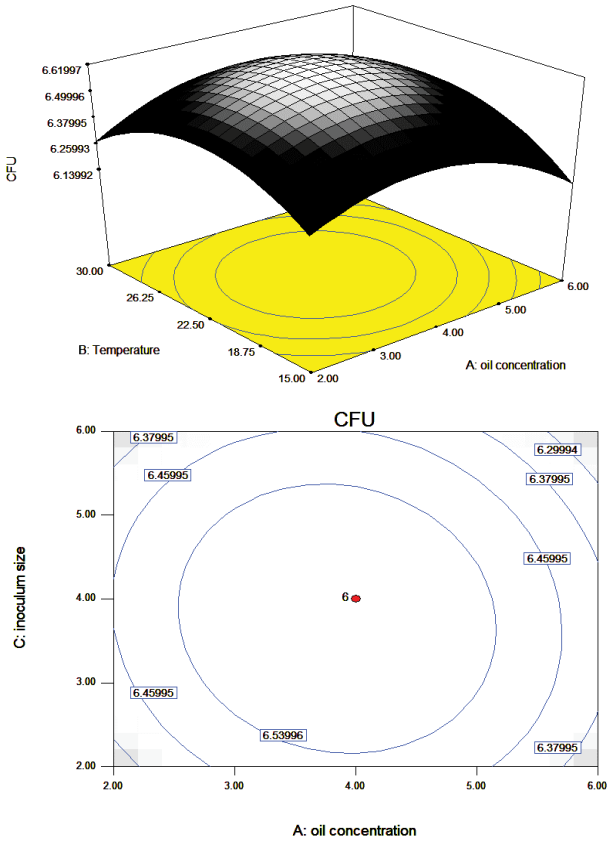

Fig. 6. 3D and 2D surface response views showing the interaction between oil concentration and inoculum size.

Table 7
Growth on palm oil (4% v/v) using OFAT and RSM

CFU of 4% oil for OFAT after 7 d of incubation	CFU of 4% oil after RSM after 5 d of incubation
Log CFU/mL 5.64 ± 0.12	Log CFU/mL 6.62 ± 0.14

The model equation fitted by regression analysis for bacterial growth on palm oil is given by

$$\text{Response} = +6.62 - 0.032 \times A + 0.033 \times B - 0.031 \times C - 0.19 \times A^2 - 0.19 \times B^2 - 0.13 \times C^2 + 0.040 \times AB - 0.025 \times AC + 0.035 \times BC \quad (3)$$

The GC-MS profile of the palm oil residue at 1% v/v after 7 d of incubation (Fig. 7(b)) compared with the uninoculated control (Fig. 7(a)) showed a dramatic reduction of the palm oil components such as palmitic, stearic, oleic, and linoleic acids. A literature search showed that most of the works on bacterial biodegradation of palm oil focus on treating palm oil mill effluent and very limited to almost no work carried out on the biodegradation of palm oil under high salinity and at low-temperature conditions.

4. Conclusion

The current problems faced by palm oil exporting countries especially when exporting palm oil to the temperate regions is the oily ballast wastewater discharge that has contributed to the ongoing ecological issue. A green

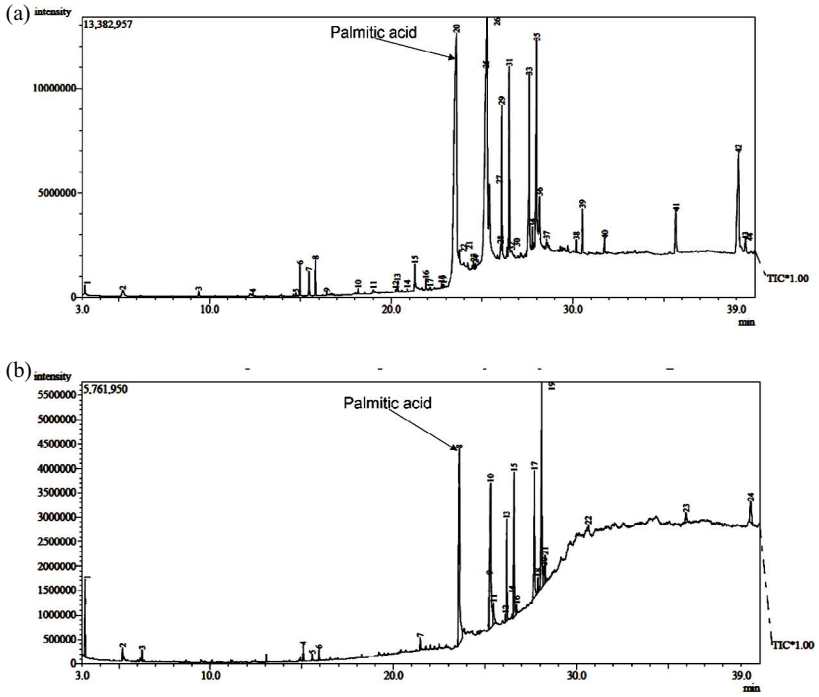

Fig. 7. GC-MS profiles of palm oil extracted from the aqueous phase of the medium after 7 d of incubation with 1% of palm oil (v/v) as a substrate without (a) and with bacterial inoculation (b).

approach is being sought, and this study has the potential to solve this problem through the lipid degradation activity of an Antarctic bacterium that is capable of remediating palm oil-containing ballast wastewater under temperate conditions. The optimization of growth on palm oil has been successfully carried out using OFAT and RSM with the latter significantly improving the performance of the bacterium. This study is the first of its kind to provide a solution to an ongoing problem, and the results obtained in this work indicate a promising solution.

Acknowledgement

This project was supported by Putra-IPS fund received from Universiti Putra Malaysia under the Grant Number 9486100 and the Ministry of Science, Technology and Innovation (MOSTI) grant (GA006-2014FL).

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