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Research article

Optimisation of biodegradation conditions for waste canola oil by cold-adapted *Rhodococcus* sp. AQ5-07 from Antarctica

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ABSTRACT

Background: The potential waste canola oil-degrading ability of the cold-adapted Antarctic bacterial strain *Rhodococcus* sp. AQ5-07 was evaluated. Globally, increasing waste from food industries generates serious anthropogenic environmental risks that can threaten terrestrial and aquatic organisms and communities. The removal of oils such as canola oil from the environment and wastewater using biological approaches is desirable as the thermal process of oil degradation is expensive and ineffective.
Results: Rhodococcus sp. AQ5-07 was found to have high canola oil-degrading ability. Physico-cultural conditions influencing its activity were studied using one-factor-at-a-time (OFAT) and statistical optimisation approaches. Considerable degradation (78.60%) of 3% oil was achieved by this bacterium when incubated with 1.0 g/L ammonium sulphate, 0.3 g/L yeast extract, pH 7.5 and 10% inoculum at 10°C over a 72-h incubation period. Optimisation of the medium conditions using response surface methodology (RSM) resulted in a 9.01% increase in oil degradation (87.61%) when supplemented with 3.5% canola oil, 1.05 g/L ammonium sulphate, 0.28g/L yeast extract, pH 7.5 and 10% inoculum at 12.5°C over the same incubation period. The bacterium was able to tolerate an oil concentration of up to 4.0%, after which decreased bacterial growth and oil degradation were observed.
Conclusions: These features make this strain worthy of examination for practical bioremediation of lipid-rich contaminated sites. This is the first report of any waste catering oil degradation by bacteria originating from Antarctica.
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1. Introduction

The Antarctic natural environment is often considered to be pristine. Nevertheless, studies have demonstrated major anthropogenic impacts associated with the activities of governmental research stations and the fishing and tourism industries [1,2,3,4]. Although Antarctica has no indigenous human population and only transient and small numbers of visitors relative to other continents, long-distance pollutants such as hydrocarbons and heavy metals can be detected even in remote ice-covered areas [5], whilst the very few ice-free areas are sensitive to the effects of water and soil contamination from local sources, both during routine operations and due to accidents [5,6,7]. Increasing ease of access has led to a surge in the number of both researchers and their support operations, and tourists, visiting the region. Currently, there are up to 80 research stations and facilities situated mostly in the coastal regions of the continent [1,3,8]. One of the major concerns in Antarctica is oil spillage [4,8,9].

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Fats, oils, grease and fatty acids are discharged globally into the environment in wastewater originating from kitchens, restaurants, slaughterhouses and food processing industries, as well as in accidental vegetable oil spills. Canola oil is used in food preparation in most Antarctic research stations, where the activity depends solely on the size and shape of the oil temperature together with the physiochemical and thermal properties of the oil [10,11]. In the absence of treatment, this is a source of environmental pollution similar to that of petroleum products, which share similar physical properties [12]. Such pollutants pose a serious threat to terrestrial and aquatic biota and communities [13,14]. The accumulation of lipids in wastewater also leads to serious impacts including the blocking of treatment filters and drainage systems, the occurrence of unpleasant odours and the death of aquatic organisms [15,16].

Bioremediation of lipid-contaminated sites is paramount because of the threats posed by the contamination. Without the optimum conditions in aerobic biodegradation techniques, relying on lipase-producing organisms is currently considered to be relatively inefficient and slow when applied to dense concentrations of lipid contaminants. Nevertheless, when the optimisation method is applied, this technique has been proven more viable compared to other techniques that are available [15]. Studies of lipase aerobic remediation indicate that this technique can result in better sewage quality and does not generate unpleasant odours [17]. The advantages of enzymatic biodegradation, particularly in the context of strong environmental regulation policies, are that it is eco-friendly, specific, easy to apply, disease-free, cheaper and reduces the time required. These techniques also reduce chemical oxygen demand and the amount of suspended lipid solids and do not result in undesirable colouration [18]. Kademi et al. [19] studied the biological degradation of vegetable oil in relation to its incorporation (after chemical modification) in the formulation of lubricants and greases and in the management of accidental oil spills [20,21]. Several anaerobic and aerobic microorganisms (bacteria and fungi) capable of degrading vegetable oil have been studied, particularly from the genera *Rhodococcus*, *Lactobacillus*, *Acinetobacter*, *Burkholderia, Mucor*, *Penicillium*, *Aspergillus*, *Staphylococcus*, *Serratia*, *Bacillus*, *Arthrobacter*, *Enterobacter* and *Pseudomonas* [12,15,22,23,24,25,26,27]. Most studies on vegetable oil degradation to date have centred on the use of mesophilic microorganisms (Table 1 shows the characteristics of previously isolated vegetable oil-degrading bacteria). However, currently, there is very limited information available on the potential of cold region (such as Antarctic) microorganisms to degrade vegetable oil. The discovery and exploitation of cold-adapted microorganisms with this ability would offer a step forward in the remediation of vegetable oil-contaminated sites, particularly under low temperature conditions. With specific reference to the challenge of remediation in Antarctica, it is currently prohibited to introduce non-native species of biota for such purposes under the provisions of the Environmental Protocol to the Antarctic Treaty [28].

The conventional technique for optimising vegetable oil degradation is through one-factor-at-a-time (OFAT) approach [22]. However, this method is time-consuming and laborious and overlooks the potential for interactions between different variables, leading to a danger of misinterpretation of the data obtained. These disadvantages can be overcome through the use of statistical approaches of optimisation such as the Plackett–Burman design (PBD) and response surface methodology (RSM) [29]. PBD is important in reducing the number of experiments required, by providing a means to identify the most significant parameters from the wider range available. RSM, through the use of central composite design (CCD), is used to study the effects of different parameters that influence responses by varying them simultaneously and carrying out a limited number of experiments [30]. RSM is a collection of experimental approaches, statistical interferences and mathematical techniques for exploring and constructing an estimated useful relationship between a response variable and a set of design variables.

The previously isolated strain *Rhodococcus* sp. AQ5-07, a known phenol-degrading and cold-adapted Antarctic soil bacterium [2], was tested here for its ability to degrade waste canola oil as a sole source of carbon. This bacterium is sensitive to environmental factors including temperature, pH, nitrogen source, inoculum size, oil concentration and yeast extract [22]. In addition, the intensity of metabolisation and cometabolisation of pollutants in Antarctic soil is dependent on these parameters [31]. Thus, optimisation of these parameters is required to achieve maximum canola oil degradation. The present study reports the optimisation of factors influencing growth and degradation of canola oil by this bacterial strain using both OFAT and RSM, with the aim of testing its potential as a new means of bioremediation of vegetable oil-contaminated sites in cold areas.

2. Materials and methods

2.1. Chemicals

Waste canola oil was collected from the Chilean Bernardo O'Higgins Riquelme Station, northern Antarctic Peninsula, in February 2018. Analytical grade chemicals were obtained from Fisher Scientific (Malaysia), Sigma (USA) and Merck (Darmstadt, Germany).

Table 1
Characteristics of previously isolated vegetable oil-degrading bacteria.

Bacteria	Specialisation of the bacteria	Optimum pH & temperature	Preferred carbon source	Optimum vegetable oil concentration	Preferred nitrogen source	Kinetics of reduction	References
Immobilised *Bacillus* sp.	Olive oil	8.0, na	na	na	na	na	[52]
S. marcescens	Cooking oil	7.0; 30°C	Lactose		Tryptone	na	[44]
Acinetobacter sp. (KUL8), *Bacillus* sp. (KUL39), and *Pseudomonas* sp. (KLB1)	Olive oil	na	na	10 mL/L	na		[53]
Mixed *E. aerogenes* E13 and *Arthrobacter* sp. N3	Sunflower oil	Maintained at 6.8; 30°C	na	0.5%	Ammonium sulphate	na	[15]
Pseudomonas strain G9 and G38	Shea nut butter	7.0; 35°C	na	2%	0.5% Yeast extract	na	[25]
Bacilus spp. KRDB1 and *Staphylococcus* spp. KopB1	Vegetable oil	7.0; 28°C	na	1%	na	na	[45]
Pseudomonas sp. strain D2D3	Olive oil and animal fat	8.0; 30°C	na	1–5%	KN03, yeast extract, peptone & soytone	na	[38]
*Burkholderi*a sp. DW2–1	Salad oil, olive, sesame and beef tallow	20°C and 38°C	na	1%	na	Na	[26]
Pseudomonas aeruginosa KM110	Olive oil	7.0; 30°C	Olive oil	1%	Peptone	Na	[41]
Rhodococcus UKMP-5 M	Olive oil	5.0; 30°C		1%	Ammonium sulphate	Na	[22]

Key: na = not available.

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2.2. Microorganism and media preparation

Previously isolated *Rhodococcus* sp. AQ5-07 originally obtained from King George Island (South Shetland Islands, Antarctica) was cultured at 10°C in sterilised canola oil liquid medium. The medium used to enrich the lipid-degrading microorganism culture contained 3% canola oil and the following ingredients (g/L): 0.9 K_2HPO_4, 0.6 KH_2PO_4, 0.2 $MgSO_4$, 1.0 $(NH_4)_2SO_4$ and 0.3 yeast extract [22]. For solid medium, Tween-peptone agar was prepared containing the following ingredients (g/L): 18 agar, 10 peptone, 5 NaCl, 0.1 $CaCl_2$ and 5 mL/L Tween 80 [32]. The media were autoclaved for 20 min at 121°C. The isolate was maintained and sub-cultured every two weeks in Tween-peptone agar medium.

2.2.1. Flask culture experiments

A single loop of a freshly grown culture from a Tween-peptone agar plate was transferred to 10 mL nutrient broth medium and incubated on a rotary shaker at 150 rpm and 10°C for 24 h. About 10% (v/v) of the culture was transferred to 100 mL of the canola oil medium in 250 mL Erlenmeyer flasks in triplicate and incubated for 72 h at 150 rpm and 10°C. After 72 h, 1 mL of sample was removed and centrifuged at 12,000 rpm for 10 min, after which cell biomass was measured.

2.3. Determination of canola oil degradation by gravimetric method

The amount of residual oil was determined using a gravimetric method following Latha and Kalaivani [33] and Sihag and Pathak [34]. Fresh bacterial culture at its exponential phase was supplemented with 3% waste canola oil in 100 mL MSM media. After 72 h incubation, 1 mL was removed to measure bacterial growth by absorption in a spectrophotometer (UV mini 1240 Shimadzu, Japan) at 600 nm wavelength.

For extracting the residual oil, 10 mL of n-hexane was added to the medium and the mixture was separated using a separating funnel. Two layers were formed; the lower layer included the medium, and the upper layer included the oil. The oil from the upper layer was collected in a pre-weighed Petri dish. One millilitre of filtered residual oil was used for GC–MS analysis. The percentage of canola oil degradation was calculated using [Equation 1]].

$$\text{Canola oil } (\%) = \frac{(a-b)}{a} \times 100\% \qquad \text{[Equation 1]}$$

where a is the mass of canola oil added to the medium and b is the mass of residual canola oil.

2.4. Analytical approach (gas chromatography analysis of canola oil degradation)

Gas chromatography–mass spectrometry (GC–MS) analysis was used to detect the effects of canola oil degradation. After incubation, n-hexane was used as a solvent to extract the oil residue from each medium. The extract was allowed to dry, and the volume of each extract was adjusted to 100 mL by further addition of n-hexane. The residue was transferred to GC vials for immediate analysis or kept at 4°C if the analysis was not carried out immediately. Samples without oil inoculation served as controls.

Canola oil degradation was determined using an Agilent gas chromatograph (Hewlett-Packard, California, U.S.A), equipped with an HP 5971 MS detector for determining the composition of the present fatty acids. Separation was carried out on an Agilent (Hewlett-Packard) HP-5 fused silica capillary column (30m × 0.25 mm I.D), with film thickness of 25 μm. The interface temperature of the GC–MS was maintained at 250°C. One microliter of an unheated sample was injected and analysed at a gas flow rate of 1 mL/min. The oven temperature was initially 50°C for 5 min. The injection temperature was 250°C, under 37.1 kpa pressure.

2.5. Optimisation of growth and degradation using one-factor-at-a-time (OFAT)

Parameters including nitrogen source, temperature, inoculum size, yeast extract concentration, pH and substrate concentration were optimised. Each parameter was examined sequentially by considering the previously optimised parameters. The nitrogen sources trialled were aspartate, ammonium carbonate, ammonium chloride, ammonium sulphate, phenylalanine, acetamide, sodium nitrate and leucine. Ammonium sulphate was the best nitrogen source and thus it was subsequently tested at concentrations ranging from 0 to 1.4 g/L. Media without the addition of any nitrogen source served as control. The effect of temperature was examined across a range of 5 to 30°C. The effects of inoculum size on bacterial growth and degradation were observed across a range of 1 to 20%. pH was examined across a range of pH 5 to 9. Yeast extract concentrations ranging from 0.05 to 0.5 g/L and initial canola oil concentrations ranging from 1 to 7% were used. All experiments involving these parameters were carried out using a 72-hour incubation.

2.6. Statistical optimisation (data analysis with RSM and CCD)

Optimisation was carried out in two stages using RSM. PBD was first used to screen the significant parameters that affected waste canola oil degradation, which were then further optimised using CCD. The same parameters investigated using OFAT were studied using RSM. The major factors selected were ammonium sulphate concentration (0.7–1.4 g/L), temperature (5–20°C), pH (6–8.5), yeast extract concentration (0.05–5 g/L) and waste canola oil concentration (1–6%). A total of 12 experiments were conducted on the PBD with 30 different experiments for CCD. All experiments were conducted in triplicate, and the mean of the degradation percentage was used as the response variable, *Y*. The final RSM predicted response was further validated experimentally. A second-order model was used to describe the correlation between the independent variables and the response [Equation 2]]:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1 + \beta_{22} X_2 \\ & + \beta_{33} X_3 + \beta_{44} X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\ & + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \end{aligned} \qquad \text{[Equation 2]}$$

where Y is the predicted response parameter and β_0, β_1, β_2, β_3, β_4, β_{11}, β_{22}, β_{33}, β_{44}, β_{12}, β_{13}, β_{23}, β_{24} and β_{14} are constant regression coefficients of the model, in which β_0 is the intercept term, β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients, β_{12}, β_{13}, β_{23}, β_{24}, and β_{14} are the interaction coefficients and β_1, β_2, β_3 and β_4 are the linear coefficients. X_1, X_2, X_3 and X_4 are the independent parameters. Parameter permutations (X_1, X_2) show the interaction between the variables [35,36,37].

2.7. Statistical analysis

All experiments were carried out in triplicate, and data are presented as a mean ± standard deviation. One-way ANOVA (95% confidence interval) in SPSS statistics V. 24 software package (SPSS Inc., Chicago, Illinois, USA) was used to compare results within treatment groups, and Tukey's test was used to conduct *post hoc* pairwise tests if significant differences were obtained. The experimental design and analysis of variance (ANOVA) in RSM were conducted using Design Expert software version 6.0.8. Any $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

The bacterial strain *Rhodococcus* sp. AQ5-07 was tested for its ability to degrade high concentrations of waste canola oil. Bacterial growth and

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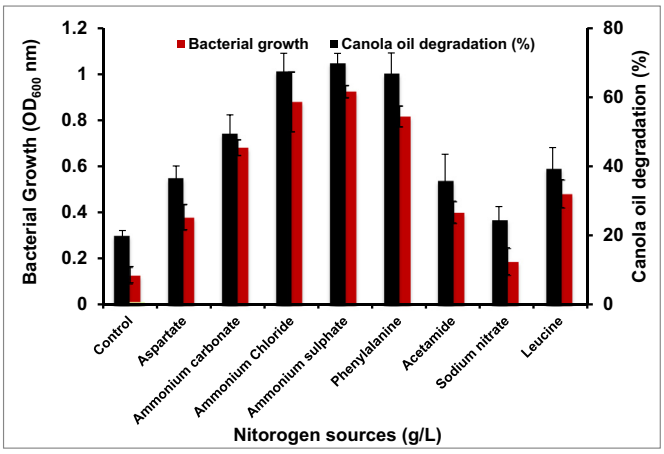

Fig. 1. The effect of nitrogen source on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 15°C, 0.1 yeast extract and pH 7 after 72 h incubation. Error bars represent standard deviation, $n = 3$.

oil degradation were optimised using the OFAT approach and CCD using RSM.

3.1. Optimisation using OFAT

3.1.1. Effect of nitrogen source

Other than the carbon source, the nitrogen source is one of the most important elements influencing bacterial growth. Different nitrogen sources may have inhibitory or stimulatory effects on bacterial growth and oil degradation. Out of the eight organic and inorganic nitrogen sources tested here, *Rhodococcus* sp. AQ5-07 exhibited maximum growth and oil degradation when ammonium sulphate, ammonium chloride or phenylalanine were used, with the best performance observed when using ammonium sulphate (Fig. 1). One-way ANOVA showed significant differences between the nitrogen sources in terms of bacterial growth and oil degradation (F (8, 18) = 69.13, $p < 0.001$ and F (8, 18) = 42.16, $p < 0.001$, respectively), although *post hoc* tests showed that there were no significant differences between the mean values for ammonium sulphate (M = 0.9237, SD = 0.0265), (M = 69.78, SD = 2.95), ammonium chloride (M = 0.8793, SD = 0.1294), (M = 67.36, SD = 5.39) and phenylalanine (M = 0.8167, SD = 0.0448), (M = 66.73, SD = 6.11). There were significant differences between ammonium sulphate and the remaining nitrogen sources tested, including the control (all $p < 0.001$).

Nitrogen is essential for metabolism and growth of any microorganism and is required for RNA, DNA and protein synthesis. The results of the effects of different nitrogen sources obtained here differ from those reported by Shon et al. [38] who, in a study of the bacterium *Pseudomonas* sp. strain D2D3, found that ammonium sulphate and ammonium chloride decreased bacterial growth and vegetable oil degradation, whilst yeast extract and potassium nitrate increased them. Much less growth and oil degradation were observed in the absence of a nitrogen source, thus verifying the importance of an appropriate nitrogen source being provided.

3.1.2. Effect of ammonium sulphate concentration

The effects of different ammonium sulphate concentrations on bacterial growth and waste canola oil degradation were investigated, indicating that 1.0 g/L was the optimum concentration (Fig. 2). At a concentration lower than the optimum, the rate of canola oil degradation decreased, presumably due to insufficient ammonium sulphate being available to sustain the replication rate of the bacterium. This might be caused by the higher pH due to the ammonia concentration in the media. Excessive ammonia causes the environment to become more alkaline and therefore decreases the degradation rate. Analysis of variance for bacterial growth and oil degradation identified significant differences between the ammonium sulphate concentrations (F (7, 16) = 18.21, $p < 0.001$ and F (7, 16) = 25.45, $p < 0.001$). However, in *post hoc* tests, the degradation achieved at 0.8 g/L (M = 59.97, SD = 5.93), 1.0 g/L (M = 69.22, SD = 5.34), 1.2 g/L (M = 66.09, SD = 4.09) and 1.4 g/L (M = 57.28, SD = 5.49) did not differ significantly. A similar observation applied to bacterial growth, although significant differences were obtained between the optimum concentration (1.0 g/L) and the remaining concentrations ($p < 0.001$). This result is similar to the study of Nagarajan et al. [22], who reported optimum oil degradation with 1.0 g/L ammonium sulphate, but differs from that of Rodriguez-Mateus et al. [39], who described 3.0 g/L ammonium sulphate as the optimum for oil and grease degradation by *Candida* and *Bacillus* species.

3.1.3. Effect of temperature

Temperature is one of the essential physical factors affecting the survival and growth of microorganisms. Any specific enzyme-mediated degradation process will have an optimum temperature [40]. The optimum temperature for bacterial growth and oil degradation measured here was 10–15°C, above which temperature the rates declined (Fig. 3). The patterns of bacterial growth and degradation were typical of a psychrotolerant organism, that is, maximum at low temperature, but continuing at temperatures above 20–25°C. One-way

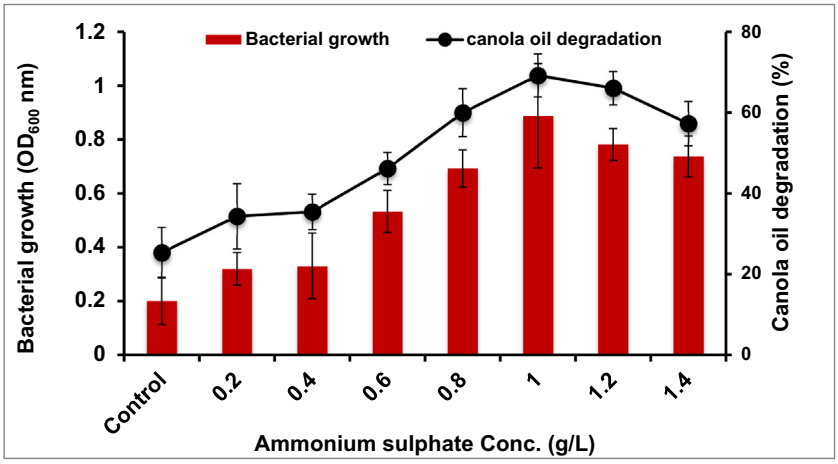

Fig. 2. The effect of ammonium sulphate concentrations on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 15°C, 0.1 yeast extract and pH 7 after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

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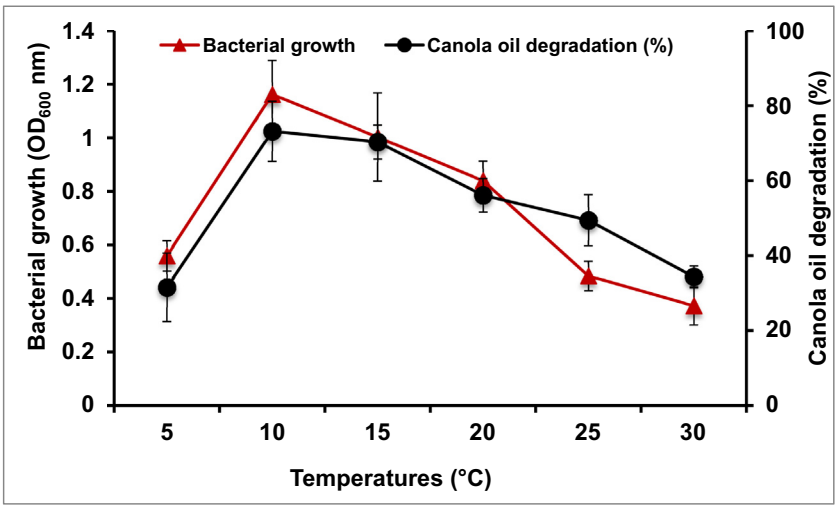

Fig. 3. The effects of temperature on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 1.0 $(NH_4)_2SO_4$, 0.1 yeast extract and pH 7 after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

ANOVA identified a significant overall difference between the temperature classes in both growth and degradation (F (5, 12) = 29.51, $p < 0.001$ and F (5, 12) = 22.79, $p < 0.001$). *Post hoc* comparisons indicated that there were no significant differences between the degradation and bacterial growth achieved at 10°C (M = 73.18, SD = 8.02), 15°C (M = 70.34, SD = 4.47) and 20°C (M = 56.12, SD = 4.45). At 5°C, there were significant decreases in growth and degradation of the oil (31.54%), which may be due to changes in enzyme conformation away from the optimum temperature. There were significant differences between 5°C and other tested temperatures with the exception of 30°C ($p < 0.001$).

Other studies of (temperate) bacteria have found that the optimum temperatures for olive oil degradation were between 27 and 30°C, as the study organism was a mesophilic bacteria [22]. Mobarak-Qamsari et al. [41] and Čipinytė et al. [15] also reported an optimum degradation temperature of 30°C for *Pseudomonas aeruginosa* KM110 and a mixed culture of *Enterobacter aerogenes* E13 and *Arthrobacter* sp. N3. Metabolic activity in microorganisms involves enzymes that are prone to irreversible destruction of protein structure usually at high temperature.

3.1.4. Effect of inoculum size

The number of bacteria loaded in the medium affects the acclimatisation of the cell and the enzyme levels synthesised to serve cell metabolism [42]. The effect of inoculum size was studied over a range of 1–20% (v/v). Bacterial growth and oil degradation were optimum with inoculum sizes of 8–10% (v/v), after which there was a decline (Fig. 4). The effectiveness of the enzyme was reduced to 51.45% and 38.47% degradation at 15% and 20% inoculum, respectively. One-way ANOVA identified overall significant differences in bacterial growth and oil degradation between different inoculum sizes (F (7, 16) = 8.49, $p < 0.001$ and F (7, 16) = 15.99, $p < 0.001$, respectively). *Post hoc* comparisons indicated that there were no significant differences between the degradation achieved with 4% (M = 55.04, SD = 2.66), 6% (M = 63.54, SD = 4.00), 8% (M = 72.33, SD = 4.63), 10% (M = 73.09, SD = 4.59) and 15% (M = 56.70, SD = 5.46) inoculum sizes. With 1% inoculum, the degradation rate of *Rhodococcus* sp. AQ5-07 was significantly lower (31.45%).

Nagarajan et al. [22] reported a rapid increase in oil degradation at 6% inoculum size, though this effect reduced with larger inoculum size, likely as a result of insufficient total dissolved oxygen available to the cells, combined with nutrient depletion [43].

3.1.5. Effect of yeast extract concentration

Yeast extract is rich in amino acids, minerals and vitamins, which are necessary for bacterial growth and function. The effects of yeast extract concentration on bacterial growth and oil degradation were studied.

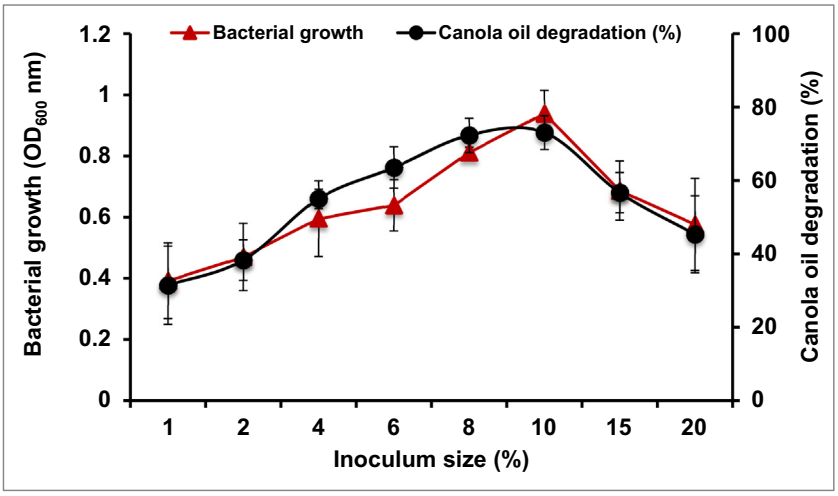

Fig. 4. The effects of inoculum size on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 10°C, 1.0 $(NH_4)_2SO_4$, 0.1 yeast extract and pH 7 after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

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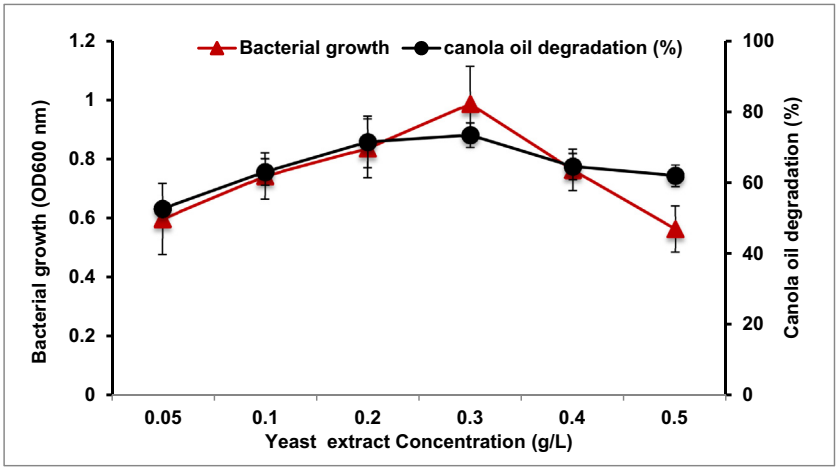

Fig. 5. The effect of yeast extract concentration on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 10°C, 1.0 $(NH_4)_2SO_4$, 10% (v/v) inoculum size and pH 7 after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

The data obtained indicated that optimum bacterial growth and oil degradation occurred at 0.3 g/L (Fig. 5). One-way ANOVA showed that the effect of different yeast concentrations on growth and oil degradation were significant ($F(5, 12) = 7.60$, $p < 0.002$ and $F(5, 12) = 8.37$, $p < 0.001$, respectively). *Post hoc* tests indicated that there was a significant difference in the degradation achieved at 0.3 g/L (M = 73.34, SD = 3.47) and 0.05 g/L (M = 52.63, SD = 1.85) yeast extract concentrations ($p = 0.004$). There was also a significant difference in the growth of the bacteria achieved at 0.2 g/L and 0.5 g/L ($p = 0.046$). Other than these, the remaining pairwise comparisons identified no significant differences.

3.1.6. Effect of initial pH

pH is an essential parameter that affects the growth and metabolism of all microbes [40]. The effects of pH on bacterial growth and canola oil degradation were studied at different initial pH values ranging from pH 5 to 9, and an optimum pH of 7.5 was identified (Fig. 6). One-way ANOVA indicted that the effects of pH on oil degradation and bacterial growth were significant ($F(9, 20) = 7.35$, $p < 0.001$ and $F(9, 20) = 21.70$, $p < 0.001$, respectively). Degradation at the optimum pH 7.5 (M = 75.02, SD = 13.81) differed significantly from the other tested pH levels excluding pH 7 (M = 70.23, SD = 4.47) in *post hoc* comparisons.

This isolate performed best under neutral or slightly basic pH conditions, with performance dropping off rapidly at more alkaline pH values. In contrast, Nagarajan et al. [22] reported that optimum vegetable oil degradation occurred at a pH of 5 (acidic). Other studies have reported that the optimum pH for hydrocarbon degradation for individual and mixed colonies was at neutral values [25,44,45,46]. In addition, studies have demonstrated that the suitable pH range for bioremediation under controlled environment and field conditions is from 6.5 to 7.5 and that the growth of hydrocarbon-degrading bacteria is best supported by pH of around 7 [46], which is consistent with the present study.

3.1.7. Effect of substrate concentration

At high concentrations, some substrates can be toxic to microorganisms [47]. Any potential biodegradation strain should have the ability to survive in and degrade a high level of canola oil. The effects of different waste canola oil initial concentrations (1–7% (v/v)) on bacterial growth rate and oil degradation were therefore studied. Optimum growth, and oil degradation of 78.60% after 72 h incubation was obtained at an initial oil concentration of 3% (Fig. 7). Growth and degradation reduced rapidly at initial oil concentration above 4%. One-way ANOVA identified overall significant differences in growth and oil degradation between initial concentration classes ($F(6, 14) = 41.07$, p

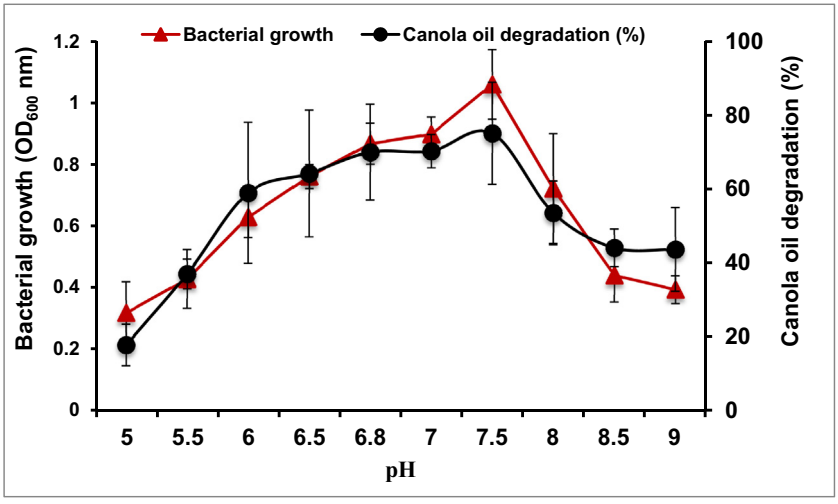

Fig. 6. The effect of initial pH on bacterial waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 10°C, 1.0 $(NH_4)_2SO_4$, 10% (v/v) inoculum size and 0.3 yeast extract after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

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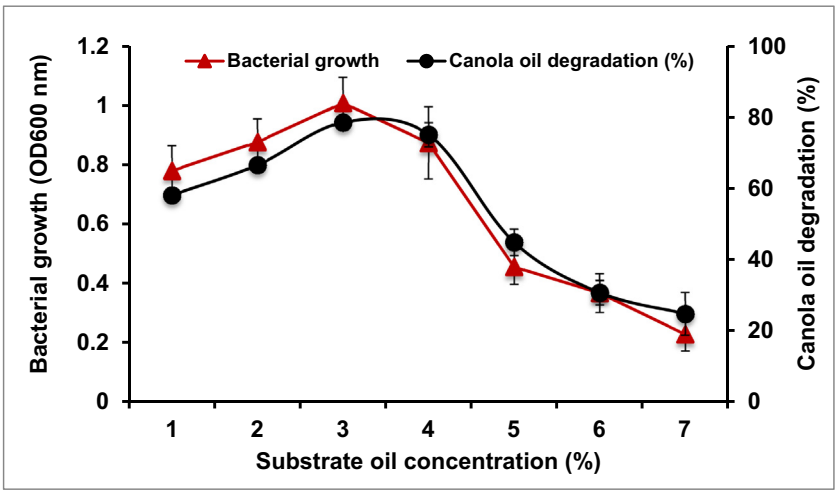

Fig. 7. The effect of substrate concentration on bacterial growth and waste canola oil degradation by *Rhodococcus* sp. AQ5-07 at 10°C, 1.0 $(NH_4)_2SO_4$, 10% (v/v) inoculum size, 0.3 yeast extract and pH 7.5 after 72 h incubation. Error bars represent mean ± standard deviation, $n = 3$.

< 0.001 and F (6, 14) $= 117.03$, $p < 0.001$, respectively). *Post hoc* comparisons showed that there was no significant difference between degradation achieved at 3% (M = 78.59, SD = 1.23) and 4% (M = 75.21, SD = 3.36) initial concentrations. However, there were significant differences between the optimum 3% and the remaining concentrations tested ($p < 0.001$). *Post hoc* comparisons for bacterial growth were also carried out using Tukey's test. There was no significant difference in growth between 2 and 3% and 3 and 4% initial concentrations, but 3% differed from all other concentrations significantly ($p < 0.001$). The data obtained indicate that at concentrations above 4%, canola oil is toxic to this bacterial strain, with reduced growth and degradation. Shon et al. [38] similarly reported high olive oil degradation at initial concentrations of 0.1% to 5%, which then drastically reduced at an initial concentration of 10%. Danikuu and Sowley [25] also reported optimum shea nut butter degradation at 2% initial concentration, whilst several other studies have reported optimum degradation at 1% initial oil degradation using different types of oil [26,41,35].

3.2. Plackett–Burman design

In PBD, the major effects have a complex relationship with two parameter interactions. Thus, these designs should be used to study major effects where it is thought that two-way interactions are negligible. PBD was used to screen and select the significant parameters affecting waste canola oil degradation. The approach can be applied to a number of independent parameters in one experiment [48]. The ranges used for each of the independent parameters selected were chosen based on the ranges used in OFAT, and a total of 12 experiments were conducted in PBD using state parameters (Table 2). Significant parameters identified were then further optimised using CCD.

PBD is useful for fitting first order models (which identify linear effects) and can give an indication of the existence of second-order curvature effects when the design includes centre points [35,49]. Comparing PBD and full factorial design, in the latter there would be five parameters with two levels for each parameter. In this case, a total of 32 different experiments would have to be conducted to characterise the response. In PBD, a total of 12 different experiments were required with the five parameters, saving time, resources and energy, and the response is larger when compared with full factorial design.

The model F value of 100.98 confirms that the model was significant (Table 3). The model revealed that, of the parameters used, only temperature was not a significant parameter for *Rhodococcus* sp. AQ5-07 for waste canola oil degradation. This is consistent with the report of Salihu et al. [49], which showed that olive oil, $(NH_4)_2SO_4$ and sucrose, amongst others, are the most significant parameters influencing lipase production by *Aspergillus niger* using shea butter cake. Another report on enantioselective lipase

Table 2
Experimental design, and percentage oil degradation achieved, in 12 experimental runs of Placket–Burman Design applied to waste canola oil degradation after 72 h incubation.

Run	A	B	C	D	E	Degradation (%)
1	6	1.4	0.05	8.5	20	54.20
2	1	0.7	0.50	8.5	20	48.23
3	1	0.7	0.05	8.5	20	49.30
4	1	1.4	0.50	6.5	20	69.20
5	1	1.4	0.50	8.5	5.0	48.30
6	6	1.4	0.50	6.5	20	57.20
7	6	0.7	0.50	8.5	5.0	70.30
8	6	1.4	0.05	8.5	5	54.20
9	1	0.7	0.05	6.5	5	60.50
10	1	1.4	0.05	6.5	5	55.30
11	6	0.7	0.05	6.5	20	64.41
12	6	0.7	0.50	6.5	5	67.30

A: canola oil concentration (%), B: nitrogen source concentration (ammonium sulphate) (g/L), C: yeast extract concentration (g/L), D: pH, E: temperature (°C).

Table 3
Analysis of variance (ANOVA) for canola oil degradation from PBD.

Source	Sum of squares	DF	Mean square	*F* value	*p* value
Model	710.54	9	78.95	100.98	0.0098**
A – canola oil concentration	85.48	1	85.48	109.33	0.0090**
B – nitrogen source	151.73	1	151.73	194.07	0.0051**
C – yeast extract	58.79	1	58.79	75.19	0.0130*
D – pH	50.042	1	50.04	64.01	0.0153*
E – temperature	4.12	1	4.19	5.27	0.1486
AB	80.88	1	80.88	103.45	0.0095**
AD	215.40	1	215.40	275.52	0.0036**
BC	22.75	1	22.75	29.10	0.0327*
CD	18.09	1	18.09	23.14	0.0406*
Residual	1.56	2	0.78		
Cor Total	712.10	11			
Std. Dev.	0.88		R-Squared	0.9978	
Mean	58.20		Adj R-Squared	0.9879	
C.V.	1.52		Pred R-Squared	0.9061	
PRESS	66.89		Adeq Precision	27.97	

* $p < 0.05$.
** $p < 0.01$.

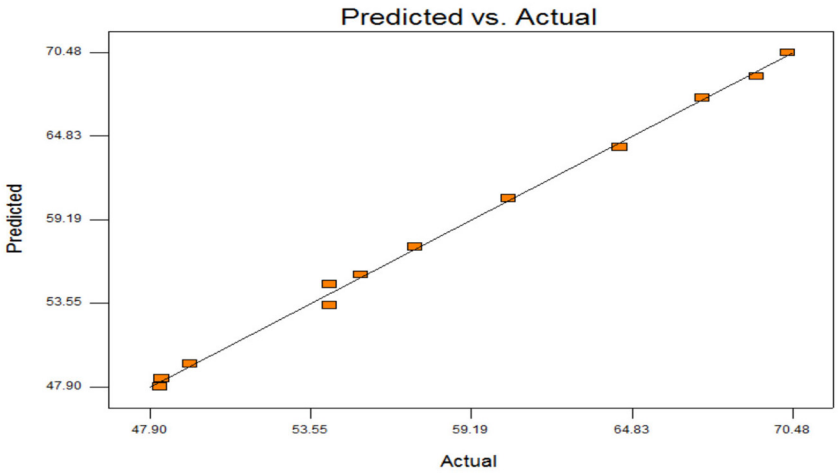

Fig. 8. Relationship between actual and predicted values of canola oil degradation for *Rhodococcus* sp. AQ5-07 in PBD.

production by *A. niger* strain AC54 showed that KH_2PO_4, NaH_2PO_4, peptone, glucose and yeast extract concentration were the most significant parameters when using wheat bran as a substrate [50]. Hence, these four parameters were used in designing CCD in the current study. Fig. 8 shows the close relationship between the actual and the predicted value obtained in the PBD, which can be expressed as [Equation 3]:

$$Y = 115.48 - 11.21A - 4.67B + 40.85C - 8.15D + 0.10D - 3.85AB - 2.20AD + 22.71BC - 7.09CD. \quad \text{[Equation 3]}$$

3.2.1. Central composite design (CCD)

The significant parameters identified in PBD were further optimised using RSM. A total of 30 different experiments were conducted, with the design and responses shown in Table 4. The R^2 value of the model was 0.9422, which is close to 1, and thus signifies that there is 94.22% models behaviour can be interpreted, whilst only about 5.78% of the full variance cannot be explained by the model. An R^2 value very close to 1 indicates a good fit to the data. Hence, the model provides useful clarification of the relationships between the response variable and the independent variables.

The results of the second-order response surface model for canola oil degradation are summarised in Table 5. The terms A, C, A^2, B^2, C^2, D^2 and BC were significant. The lack of fit term was not significant, signifying that the model is accurate without significant noise. Manogaran et al. [37] and Ibrahim et al. [35] similarly reported no significant lack of fit, concluding that the model was an excellent fit.

Table 4
Experimental design and result of CCD on waste canola oil degradation after 72 h incubation.

Std	Run	A	B	C	D	Actual value (%)	Predicted value (%)
26	1	3.5	1.05	0.275	7.5	81.54	87.61
14	2	6	0.7	0.5	8.5	63.95	64.05
19	3	3.5	0.35	0.275	7.5	40.39	43.51
28	4	3.5	1.05	0.275	7.5	93.54	87.61
6	5	6	0.7	0.5	6.5	58.63	63.90
29	6	3.5	1.05	0.275	7.5	88.54	87.61
20	7	3.5	1.75	0.275	7.5	57.45	54.37
23	8	3.5	1.05	0.275	5.5	31.64	30.18
21	9	3.5	1.05	-0.175	7.5	36.37	39.04
16	10	6	1.4	0.5	8.5	32.36	43.19
27	11	3.5	1.05	0.275	7.5	92.54	87.61
12	12	6	1.4	0.05	8.5	46.18	47.84
8	13	6	1.4	0.5	6.5	58.92	58.53
15	14	1	1.4	0.5	8.5	44.17	32.70
22	15	3.5	1.05	0.725	7.5	59.64	57.01
3	16	1	1.4	0.05	6.5	42.30	36.33
1	17	1	0.7	0.05	6.5	9.62	4.61
9	18	1	0.7	0.05	8.5	21.85	16.37
10	19	6	0.7	0.05	8.5	42.09	41.18
4	20	6	1.4	0.05	6.5	64.04	68.20
11	21	1	1.4	0.05	8.5	32.04	32.59
18	22	8.5	1.05	0.275	7.5	78.35	65.16
13	23	1	0.7	0.5	8.5	42.33	44.00
5	24	1	0.7	0.5	6.5	34.76	27.23
17	25	-1.5	1.05	0.275	7.5	0.00	13.23
24	26	3.5	1.05	0.275	9.5	25.10	26.60
25	27	3.5	1.05	0.275	7.5	81.54	87.61
7	28	1	1.4	0.5	6.5	24.68	31.42
2	29	6	0.7	0.05	6.5	40.45	46.05
30	30	3.5	1.05	0.275	7.5	87.95	87.61

A: canola oil concentration (%), B: nitrogen source concentration (ammonium sulphate) (g/L), C: yeast extract concentration (g/L) and D: Ph.

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Table 5
Analysis of variance (ANOVA) for canola oil degradation for CCD.

Source	Sum of squares	DF	Mean square	*F* value	*p* value
Model	17,052.47	14	1218.03	17.47	<0.0001***
A – canola oil concentration	4044.83	1	4044.83	58.00	<0.0001***
B – nitrogen source	176.75	1	176.75	2.53	0.1322
C – yeast extract	483.93	1	483.93	6.94	0.0188*
D – pH	19.28	1	19.28	0.28	0.6067
A^2	4017.96	1	4017.96	57.61	<0.0001***
B^2	2563.21	1	2563.21	36.75	<0.0001***
C^2	2685.96	1	2685.96	38.51	<0.0001***
D^2	6011.59	1	6011.59	86.20	<0.0001***
AB	91.44	1	91.44	1.31	0.2701
AC	22.63	1	22.63	0.32	0.5773
AD	276.31	1	276.31	3.96	0.0651
BC	757.49	1	757.49	10.86	0.0049**
BD	239.86	1	239.86	3.44	0.0834
CD	25.18	1	25.18	0.36	0.5569
Residual	1046.07	15	69.74		
Lack of fit	911.93	10	91.19	3.40	0.0946
Pure error	134.14	5	26.83		
Cor total	18,098.54	29			
Std. dev.	8.35		R-Squared	0.9422	
Mean	50.43		Adj R-Squared	0.8883	
C.V.	16.56		Pred R-Squared	0.6991	
PRESS	5445.89		Adeq Precision	14.05	

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Fig. 9 shows the strong correlation between the actual and the predicted values obtained in the CCD. The equations describing these in terms of actual and coded factors are [Equation 5] and [Equation 6], respectively:

$$Y = -1052.29 + 34.67A + 290.03B + 184.80C + 237.07D - 1.94A^2 - 78.91B^2 - 195.47C^2 - 14.80D^2 - 2.73AB - 2.11AC - 1.66AD - 87.37BC - 11.06BD + 5.58CD \quad \text{[Equation 5]}$$

$$Y = 87.61 + 12.98A + 2.71B + 4.49C - 0.90D - 12.10A^2 - 9.67B^2 - 9.90C^2 - 14.80D^2 - 2.39AB - 1.19AC - 4.16AD - 6.88BC - 3.87BD + 1.25CD \quad \text{[Equation 6]}$$

The 3D response surfaces and their respective contour plots in CCD show the interaction between two parameters whilst keeping other parameters constant [51] (Fig. 10). This visualisation helps to understand the interaction between two factors and pinpoint the

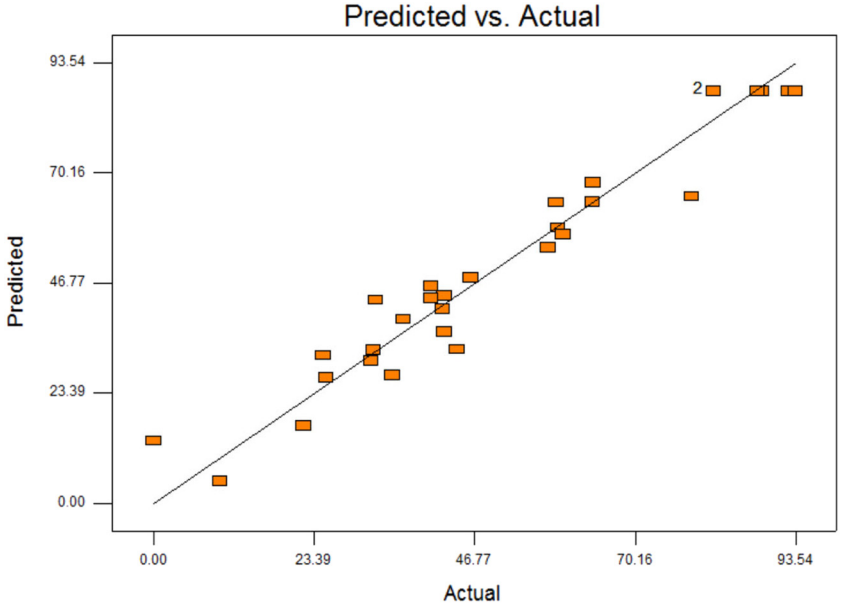

Fig. 9. Relationship between actual and predicted value for *Rhodococcus* sp. AQ5-07 canola oil degradation in CCD.

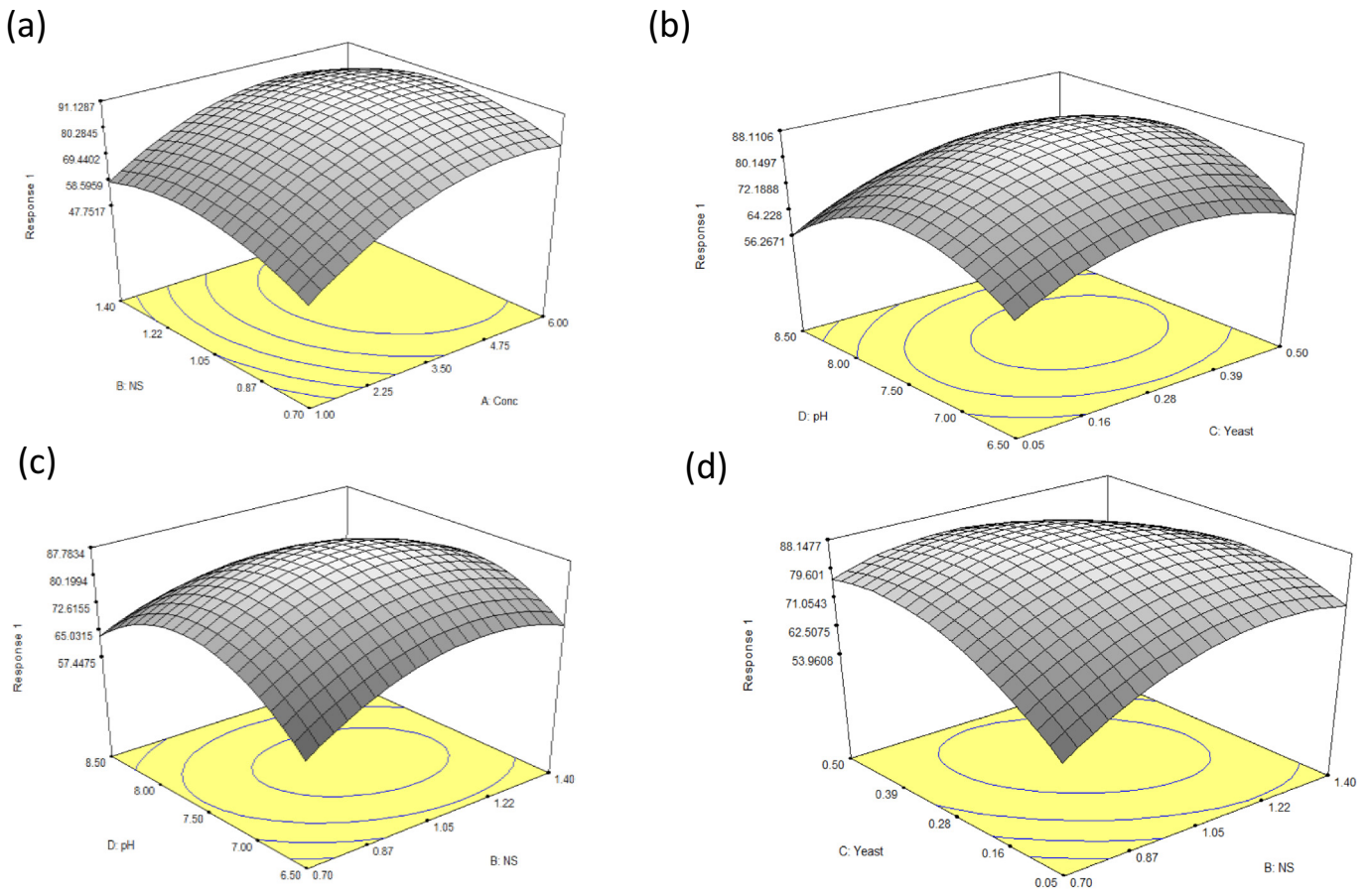

Fig. 10. Three-dimensional response surface plots for waste canola oil degradation showing the interactive effects between (a) nitrogen source and canola oil concentration (b) pH and yeast extract concentration nitrogen source (c) pH and nitrogen source and (d) yeast extract and nitrogen source concentration.

optimum level of each parameter. The interaction between nitrogen source (ammonium sulphate) and canola oil concentration showed an important effect. As the oil concentration increased, the ability of strain AQ5-07 to degrade the oil reduced. A similar effect was seen with ammonium sulphate concentration. The best degradation ability is indicated at 1.05 g/L ammonium sulphate and 3.5% initial canola oil concentration (Fig. 10a). There was also a strong interaction between pH and yeast extract concentration (Fig. 10b). The interactions between pH and nitrogen source concentration (Fig. 10c), and yeast extract and nitrogen source concentrations (Fig. 10d) were the main factors influencing canola oil degradation.

3.2.2. Validation of experiment

Based on the RSM-CCD results, the optimum conditions for canola oil degradation were 3.5% initial canola oil concentration, pH 7.5, yeast extract concentration of 0.28g/L and ammonium sulphate concentration of 1.05 g/L, whilst the other parameter (temperature, 10°C) was kept constant based on OFAT because it was not significant based on the PBD analysis. RSM predicted a degradation rate of 87.61%. An experiment was then carried out using the optimal parameters identified above in order to verify the result estimated by CCD. Comparison between the prediction generated from RSM and that obtained experimentally ($M = 81.67$, $SD = 4.54$) showed no significant difference in canola oil degradation percentage between the experimental and predicted values ($t(2) = 2.19$, $p = 0.160$).

Comparison between OFAT and RSM revealed higher degradation using RSM (Table 6), with the latter achieving about 9% greater maximum oil degradation. Other factors in terms of concentrations used showed distinct optimum levels in the two approaches (Table 6).

Table 6
Comparison of optimum conditions and results obtained between OFAT and RSM.

Conditions	OFAT	RSM
Canola oil (%)	3.00	3.50
Nitrogen source (g/L)	1.00	1.05
Yeast extract (g/L)	0.30	0.28
pH	7.5	7.5
Temperature (°C)	10.00	12.50
Degradation (%)	78.60	87.61

4. Conclusions

This study confirmed the efficient degradation of waste canola oil by the bacterial strain *Rhodococcus* sp. AQ5-07 originally isolated from Antarctica. The optimum canola oil degradation rates achieved were at initial conditions of 3% oil concentration, 1.0 g/L ammonium sulphate concentration, 0.3 g/L yeast extract concentration, pH 7.5 and a temperature of 10°C. The strain can tolerate up to 4.0% canola oil. There was a close relationship between the optimal parameters identified in OFAT and RSM. OFAT maximum degradation was 78.60%, whilst that in RSM was 87.61%. According to these results, the biological method could be a valuable alternative to the thermal method of oil degradation for the treatment of wastewater and environment.

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Conflict of interests

The authors declare no conflicts of interest.

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