Use of Taguchi design for optimization of diesel-oil biodegradation using consortium of *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas balearica* isolated from tarball in Terengganu Beach, Malaysia

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Abstract
A consortium of bacteria capable of decomposing oily hydrocarbons was isolated from tarballs on the beaches of Terengganu, Malaysia, and classified as *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas balearica*. The Taguchi design was used to optimize the biodegradation of diesel using these bacteria as a consortium. The highest biodegradation of diesel-oil in the experimental tests was 93.6%, and the individual n-alkanes decomposed 87.6—97.6% over 30 days. Optimal settings were inoculum size of 2.5 mL (1.248 OD600nm); 12% (v/v) the initial diesel-oil in a minimal salt medium of pH 7.0, 30.0 gL−1 NaCl and 2.0 gL−1 NH4NO3 concentration, incubated at 42 °C temperature and 150 rpm agitation speed. Parameters significantly improved diesel-oil removal by consortium as shown by the model determination coefficient (R2 = 90.89%; P < 0.001) with a synergistic effect of agitation speed significantly contributing 81.03%. Taguchi design determined the optimal settings for the parameters under study, which significantly improved diesel-oil removal by consortium. This can be used to design a novel bioremediation strategy that can achieve optimal decontamination of oil pollution in a shorter time.

Highlights
- Hydrocarbon-degraders in Tarball were isolated and identified by their 16S rRNA gene sequence as *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas balearica*.
- Taguchi method was applied to optimize effects of parameters such as initial diesel concentration, salinity (NaCl concentration), nitrate (NH4NO3) concentration, pH, temperature, agitation speed and inoculum size on diesel-oil removal in 30 days.
- Maximum diesel-oil biodegradation by experimental runs was 93.6% with individual n-alkanes degraded between 87.6% – 97.6% in 30 days
- Optimal settings were 2.5 mL (1.248 OD600nm) inoculum size; 12% (v/v) initial diesel-oil in MSM media with 7.0 pH, 30.0 gL−1 NaCl and 2.0 gL−1 NH4NO3 concentration, incubated at 42 °C temperature and 150 rpm agitation speed
- Parameters significantly improved diesel-oil removal by this consortium as indicated by model determination coefficient (R2 = 90.89%; P < 0.001) with synergistic effect of agitation speed significantly contributing 81.03%
- Taguchi design established optimal settings of investigated parameters that produced significant improvement on diesel-oil removal by consortium

Keywords Bacteria consortium · Biodegradation · Diesel-oil · Optimization · Taguchi design · Tarball
Introduction

Marine ecosystems are affected by oil spills from the production, exploration, refining, and transportation of crude oil [27]. The oil spill breaks down into slicks, accumulates debris, and aggregates to form dark, foam-like balls called tarballs [115]. Tarballs contain several recalcitrant hydrocarbons, so they remain in the marine environment and eventually settle in coastal areas. Tarballs are part of the oil pollutants that were considered to be among the most common causes of environmental degradation in the modern world [79]. Petroleum hydrocarbons penetrate marine ecosystems as oil spills. Spilled oil decomposes into oil slicks and disperses several distances from the original source [12]. Oil slicks disperse into the marine environment and lose their volatile hydrocarbons due to various atmospheric processes such as photooxidation, biodegradation, emulsification, dissolution, and evaporation. They accumulate debris and merge to form dark, foam-like, soft, oily balls called Tarballs [79]. Tarballs settle in beaches and coastal areas a few miles from their origin [101]. They contain several recalcitrant hydrocarbons that can be used as biological markers to trace their original source [96]. The concentration of trapped Tarball on the beaches indicates marine hydrocarbon pollution in the region [13, 53, 60]. Oil pollution causes economic losses due to the harmful effects of hydrocarbon toxicity on humans, fishing, aquaculture, and tourism [108]. Foods and water contaminated with hydrocarbons are unhealthy for humans because they can cause anaemia, cancer, and death if ingested [89]. Oil spills in the marine environment are initially decomposed by photooxidation, resulting in the release of more toxic by-products that are soluble, pseudo-soluble, or insoluble. These by-products pose a significant risk to marine organisms, especially in the early stages of their life forms [15, 19]. The molecular weight of oil and its solubility influence their final forms and effects on marine life [41, 88]. The marine environment is rich in a variety of organisms such as fish, sea otters, sea turtles, invertebrates, crustaceans, mollusks, starfish, and zooplankton. The effects of these organisms on macro (tarballs) or micro droplets in oil or its by-products give rise to antioxidant protection in fish. Mussels, crustaceans, and crabs are polluted due to the slightest oil spill in shallow waters [82]. An uptake of only 0.1 mgL$^-1$ oily hydrocarbons can destroy the eggs of fish, mussels, and several marine life forms [81]. Oil pollution harms marine life, including birds, marine organisms, fish, and plants, affecting the food chain and biodiversity. Primary producers of the marine ecosystem are also killed by oil, threatening the existence of other marine organisms [109]. The eggs of some fish, birds, or reptiles thin out after prolonged exposure to the oil. Seagrass and algae are contaminated and cause long-term effects on the ecosystem [3].

The entry of hydrocarbons into the marine ecosystem promotes the growth of native hydrocarbon-degrading bacteria that can be used to purify hydrocarbon contaminants during bioremediation [25, 109]. Bioremediation strategies are designed to isolate and characterize hydrocarbon decomposers by providing optimal growth nutrients and environmental conditions (pH, temperature, salinity, dissolved oxygen) conducive to the decomposition of pollutants through their metabolic activity [11, 109]. This technique can safely restore contaminated sites using natural, less expensive, and less environmentally invasive treatments [95]. Restoration using this method can completely remove contaminants in the shortest amount of time. Oil-degrading bacteria have been identified in a variety of hydrocarbon-contaminated sites, such as oil sludge, contaminated soil, marine sediments, and other sites [50, 56]. Indigenous hydrocarbon-degrading bacteria isolated from tarball are adapted to survive at higher hydrocarbon concentrations than those extracted from other contaminated sites due to the loss of light hydrocarbon fractions during its formation [72], Payne and Phillips). Therefore, they can decompose different hydrocarbon pollutants and can be used in different contaminated sites [71].

The Taguchi method is a powerful and highly effective optimization strategy where it is possible to optimize multiple parameters with as few experiments as possible compared to other conventional optimization methods. This method provides an individual design that is economical and improves quality. The Taguchi design uses a customized model to manipulate multiple parameters at minimal cost while improving quality [83], thus reducing the margin of error and increasing the possibility of real-time application in the field [73]. The research is aimed at isolating and identifying novel hydrocarbon-degraders in tarball, then optimize their ability to degrade diesel-oil as a consortium using Taguchi experimental design. The consortium consists of different hydrocarbon decomposers extracted from the tarball, each with a different biodegradability. This consortium can be used to develop a new biological treatment strategy using the Taguchi optimization method to remove contaminated sites more quickly at a minimal cost. This is because Taguchi design can quantify the magnitude and significance of the contributions of selected environmental parameters in order to predict the maximum or minimum hydrocarbon removal over time [65]. The Taguchi method identifies the best combinations of parameters and can be used to eliminate or select important parameters that have significantly improved the bioremediation of this consortium [83, 87].

Methods

Sampling

Tarballs samples were taken in dark sterile plastic bags from the beaches of Kampung Merang, Kuala Terengganu (Latitude: N 06°32.321‘; Longitude: E 102°57.024”), Malaysia.
Samples were taken randomly when the floods were low to form a mass sample mixture of 5.0 g (grams) that was homogenized and stored at 8 °C (degrees Celsius) according to the methods described previously [71]. Terengganu is located northeast of the Malaysian Peninsula, which is bordered by the Straits of Malacca and the South China Sea. Tarballs deposited on its beaches indicate that oil pollution is taking place in the adjacent seas. Diesel-oil was chosen as a hydrocarbon substrate because it was classified as a toxic substance and consisted of hydrocarbons that are broken down by bacteria [21, 70]. The diesel-oil substrate was sterilized with a syringe filter (0.22 μm) and stored at 4 °C. All chemicals and hydrocarbon standards meet scientific standards and are sourced from Sigma-Aldrich Co., respectively. LCS and Chiron, Norway, as described previously [71].

### Bacteria isolation and identification

Bacteria capable of degrading oily hydrocarbons were isolated from the tarball (5 g) using 50.0 ml of minimal salt medium (MSM), incubated at 37 °C and 100 revolutions per minute (rpm) for 7 days. Krasowska and Sigler [55] stated that the cell wall of gram-negative bacteria is more hydrophobic (about 19–20% of lipids) than gram-positive (about 1–4% lipid content). High hydrophobicity of bacteria cells isolated from tarball will be used to form a consortium due to adhesion ability, which increases hydrocarbon degradation potential. The MSM contains 30.0 g per litre (gL−1) NaCl (sodium chloride), 2.0 gL−1 KH₂PO₄, 3.0 gL−¹ Na₂HPO₄, 1.0 gL−¹ NH₄NO₃ (Ammonium nitrate), 0.7 gL−¹ MgSO₄·7H₂O and 1.0 mL (millilitre) solution made of trace elements in 1.0 L (L) milli-Q water. The solution of trace elements contains 0.5 mg (mg) CuSO₄, 0.5 mg MnSO₄·H₂O, 10.0 mg ZnSO₄·7H₂O, 30.0 mg of FeCl₃ and 20.0 mg CaCl₂ in 1.0 L milli-Q water. The pH of the medium was adjusted to 7.5 using 0.1 M KOH solution [71]. Media were passed through an autoclave set at 121 °C and 20 min (minutes) for sterilization. Isolates were acclimated by subculturing 1.0 mL aliquot in 47.0 mL MSM amended with 4% volume per volume (v/v) diesel-oil, incubated at 37 °C and 100 rpm every 7 days for 8 weeks. After acclimation, 1.0 mL aliquot was serially diluted with sterile normal saline (0.85%) up to 10⁶ dilution factors, striped on solidified agar plates of Luria Bertani (LB) media and incubated at 37 °C for 24-h. The LB agar plates contains NaCl 10.0 gL−¹, extracted yeast 5.0 gL−¹, 10.0 gL−¹ tryptone, aga 20.0 gL−¹ and 7.5 pH, autoclaved for 20 min at 121 °C [33]. Colonies with different morphology were cultured in sterile LB broth (10.0 gL−¹ NaCl, yeast extract of 5.0 gL−¹ and 10.0 gL−¹ tryptone) at 37 °C and 120 rpm for 24 h. Pure colonies were isolated in streaks on LB agar plates [14, 33, 71]. Isolated bacteria were identified by analysing the genetic sequence of their 16S ribosomal RNA (16S rRNA) via polymerase chain reaction (PCR) and compared to a protected sequence database [112]. Pure isolate cultures were used to extract genomic DNA (deoxyribonucleic acid) for amplification using the 16S rRNA universal primer 5'-AGAGTTTGTATCCTGGCTC AG-3', which acts as the forward primer, and the 5'AGC GCTACCTTGTTACGACTT-3' is the reverse primer [71]. DNA samples were evaluated by agarose gel electrophoresis and viewed using bench-top UV transilluminator (Kodak Imaging System EDAS 290) as described in previous studies [8, 18, 36, 57]. PCR mixture had a total volume of 100 μL comprising of milli-Q water (55 μL) that is nuclease-free and ultrapure, 1 μL DNA Taq polymerase with NH₄SO₄, 10 μL MgCl₂ (25 mM), 2 μL of 20 mM deoxynucleoside triphosphates (dNTP) mix, 5 μL each of forward primer and reverse primer respectively (each 10 ppm), 1 μL DNA Taq polymerase and 2 μL solution of DNA template in accordance with earlier studies [33, 34].

PCR amplification was performed using a Veriti-Applied Biosystems Thermocycler with 96 wells. Amplification conditions were determined: initial 95 °C denaturation for 5 min, 35 amplification cycles for 95 °C, denaturation for 30 s, 54 °C annealing for 30 s, 72 °C extension for 90 s, and 72 °C final extension for 10 min, based on previous literature [71, 112]. Amplified products were analysed by gel electrophoresis, purified, visualized via a UV transilluminator, and subjected to DNA sequencing using a sequencer (Applied Biosystem 3500, USA) according to the manufacturer’s instructions [8, 57, 71]. The resulting sequence was compared known database for likeness search with BLAST (Basic Local Alignment System Tool) on the NCBI (National Center for Biotechnology Information) website as similar to earlier studies [32, 71]. Extents of homologous nucleotide sequences were determined using pair-wise comparisons of matching sequences for forward and reverse primers using MEGA (Molecular Evolutionary Genetics Analysis) software 6.0 and construction of phylogenetic tree was done using neighbour-joining algorithm [26, 33]. Isolated strains were designated as strains GS, RS, WS and WR1.

### One-Factor-A-Time (OFAT) growth optimization

Optimization tests were performed using consortium of *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas baeleaica* by one-factor-at-a-time (OFAT) methodology. The parameters were examined sequentially as previously described by Agarry [4], Roslee et al. [90] and Aghamiri et al. [5], where similar parameters were manipulated to optimize bacterial growth. The sequence of parameters from first to last is as follows: initial diesel-oil concentration, agitation speed, salinity (NaCl concentration), nitrates (NH₄NO₃ concentration), pH, temperature and inoculum size. The ranges of parameters
selected in similar studies were based on the optimal range to promote bacterial growth [44, 72, 98]. Parameter levels that resulted in the highest growth of the consortium were recorded by turbidimetry at an optical density of 600 nm (OD$_{600nm}$) wavelength were established [4, 47, 90].

Pure strains of isolated bacteria were each dispatched into 25.0 mL LB broth and incubated for 24 h at 37 °C in a shaker set at 100 rpm. Isolates were harvested by centrifuging at 8,000 rpm for 10 min, mixed and suspended in normal saline (0.85% v/v) to form consortium inoculum [24, 71].

Initial diesel-oil concentration for optimal consortium growth were tested by inoculating 1.0 mL 24-h inoculum into 48.0 mL, 47.0 mL, 45.0 mL and 43.0 mL MSM supplemented with 2%, 4%, 8% and 12% (v/v) initial diesel-oil concentration [54] respectively. This was incubated for 5 days at 37 °C and 100 rpm [17, 72].

Agitation speed levels were selected by inoculating 1.0 mL 24-h consortium inoculum into 47.0 mL MSM amended with 4% (v/v) diesel-oil. Diesel-oil concentration selected produced the highest consortium growth rate after optimizing diesel-oil concentration. This was incubated in a shaker for 5 days at 37 °C with agitation speed set at 0, 50 and 150 rpm [72, 106]. Salinity (NaCl concentration) levels were selected by inoculating 1.0 mL 24-h consortium inoculum into 47.0 mL MSM containing 10.0, 20.0, 30.0, 40.0, and 50.0 g L$^{-1}$ NaCl concentrations [49, 72, 85]. Nitrate (NH$_4$NO$_3$) concentration levels were selected by inoculating 1.0 mL 24-h consortium inoculum into 47.0 mL MSM containing 1.0, 2.0, 2.5, 3.0 and 3.5 g L$^{-1}$ NH$_4$NO$_3$ concentrations [72, 98]. Levels of pH were selected by inoculating 1.0 mL 24-h consortium inoculum into 47.0 mL MSM with pH levels 6.0, 6.5, 7.0, 7.5 and 8.0 [63, 72]. Experimental flasks for salinity, nitrate concentration and pH were supplemented with 4% (v/v) diesel-oil concentration and incubated at 37 °C, 150 rpm for 5 days [47, 72]. Temperature levels were selected by inoculating 1.0 mL 24-h consortium inoculum into 47.0 mL MSM supplied with 4% (v/v) diesel-oil. They were incubated at 150 rpm with temperatures set at 32 °C, 35 °C, 37 °C, 40 °C and 42 °C for 5 days [39, 72]. Bacteria consortium was scaled-up by inoculating into 100 mL LB broth and incubated at 40 °C, 150 rpm for 24—48 h. The consortium inoculum, which consists of four isolated bacteria, was augmented to achieve an inoculum size range selected according to the optimal ranges that promote bacterial growth, as described previously in similar studies [75]. Cells were harvested, re-inoculated into 150 mL sterile LB broth and incubated under similar conditions for another 24 h. This was repeated in 200 mL LB broth using the same harvested consortium inoculum. The enlarged consortium was suspended in 2.5 mL of normal saline [17, 104]. Inoculum sizes were quantified by turbidimetry using spectrophotometer according to manufacturers’ instructions [38]. Inoculum sizes were quantified to 0.5 mL (1.1664 OD$_{600nm}$), 1.5 mL (1.1924 OD$_{600nm}$), 2.0 mL (1.138 OD$_{600nm}$) and 2.5 mL (1.248 OD$_{600nm}$), transferred into 47.5 mL, 46.5 mL, 46.0 mL and 45.5 mL MSM respectively. Medium was supplied with 4% (v/v) diesel-oil and incubated at 40 °C, 150 rpm for 5 days [39, 47]. In all experiments, control flasks for each parameter level had same constituents but without consortium inoculum [17, 72]. All experiments were performed in triplicates and the growth of the consortium was analysed by turbidimetry (OD$_{600nm}$) every day for 5 days [97, 111]. The parameter levels that caused the most growth were chosen for the Taguchi design.

### Taguchi design and biodegradation assays

The Taguchi design applies the selected parameter levels specified in the OFAT methodology in the Taguchi L$_{18}$ orthogonal array table to test the effect of different variables on the degradation of diesel-oil by consortium [5, 72, 73].

Seven parameters were investigated being agitation speed, initial diesel-oil concentration (conc.), NaCl conc. (salinity), nitrates (NH$_4$NO$_3$) conc., pH, temperature and inoculum size. According to the Taguchi L$_{18}$ design (2$^4$ X 3$^6$), six variables were investigated at three levels (-1, 0, 1) while one variable (agitation speed) was studied at two levels using eighteen (18) experimental runs [73]. Mean diesel-oil degradation (dependent variable) is outlined by the signal noise (SN) ratio [65, 87]. Nominal is best (NB) was the SN ratio of choice, calculated using the equation:

$$SN = 10 \times \log((X_D^2 - S^2/n)/S^2)$$

where,

- $n$ number of responses in the parameter level combination,
- $X_D$ mean diesel-oil degradation for each parameter level combination,
- $S$ standard deviation of the degradation responses for the given parameter level combination. Minitab 17 software for windows was used for experimental design and statistical analysis [73, 87]. Table 1 illustrates parameters and their assigned levels.

Table 1 shows the parameters and their respective levels, coded as -1, 0, 1, denoting levels 1, 2, and 3. Parameter levels were chosen because they yielded the highest growth for the consortium in the OFAT experiments [72, 92]. Prior to biodegradation studies, consortium inoculum was acclimated by subculturing 1.0 mL 24-h consortium culture in 47.0 mL MSM supplemented with 4% (v/v) diesel-oil. This was incubated at 40 °C, 150 rpm for 7 days. After eight (8)
subcultures, cells were harvested, washed twice with normal saline (0.85%), and grown to the appropriate volumes as indicated on Table 1 [24, 71, 72]. The experiments were performed with 1.0 mL of 24-h consortium inoculum in 99.0 mL of LB broth and incubated in a shaker set at 40 °C, 150 rpm for 24-h. Cells were harvested and inoculated into 150 mL LB broth, then incubated at 40 °C, 150 rpm for 24-h. After incubation, consortium cells were repeatedly harvested and cultured while increasing LB broth volume to 200 mL, 250 mL and 300 mL using identical incubation conditions. After the final scaling step, the cells in the consortium were harvested, washed, and re-suspended in normal saline (0.85%) to a size of 0.5 mL inoculum (0.1664 OD600nm), 1.5 mL (1.1924 OD600nm) and 2.5 mL (1.248 OD600nm) [17, 104]. Table 2 illustrates Taguchi L18 (2^1 × 3^6) orthogonal array experimental matrix for overall optimization of diesel-oil biodegradation by this consortium.

| S/number | Factors                      | Level 1(-1) | Level 2(0) | Level 3(1) |
|----------|------------------------------|-------------|------------|------------|
| 1        | Agitation (rpm)              | 50          | 150        | -          |
| 2        | Diesel conc. (% v/v)         | 4           | 8          | 12         |
| 3        | NaCl conc. (gL⁻¹)            | 20          | 30         | 40         |
| 4        | NH₄NO₃ conc. (gL⁻¹)          | 1           | 2          | 3          |
| 5        | pH                           | 6.5         | 7          | 7.5        |
| 6        | Temperature (°C)             | 37          | 40         | 42         |
| 7        | Inoculum size (mL)           | 0.5         | 1.5        | 2.5        |

Table 2 shows experimental matrix designed by Taguchi L18 (2^1 × 3^6) orthogonal array with parameter levels 1, 2 and 3. Biodegradation assays for each experimental run and control were 50 mL in total volume prepared in 250 mL Erlenmeyer flasks. The consortium inoculum was transferred to 18 triplicate flasks with MSM supplemented with diesel-oil. For each of the 18 experimental cycles, the initial diesel concentration, the size of the consortium inoculum, as well as the pH, NaCl and NH₄NO₃ concentrations in MSM were adjusted as indicated on Table 2. Incubation conditions, such as agitation rate and temperature, were also adjusted as shown in Table 2. Negative uninoculated controls in each of the 18 experiments were maintained in triplicate under similar conditions. [4, 72, 73, 92]. Diesel-oil was extracted in biodegradation studies after 30 days of incubation for all experimental cycles, triplicate, and control flasks.

### Biodegradation analysis

Diesel-oil was extracted from 50 mL of experimental growth medium for triplicate experiments and controls by mixing with 50 mL hexane/dichloromethane (DCM) in 3:1 (v/v) ratio. This was spiked with 500 µL (µL) of 10 parts per million (ppm) surrogate internal standard (SIS) for PAH's was used on the diesel-oil extract to obtain PAH's in accordance with previously described methods [31]. The alkane fraction was obtained by column chromatography. The extract was vortexed and concentrated to 1.0 mL by ultrasonication and rotary evaporation according to the methods previously described [28, 64, 115]. The
extract was passed through a glass column with an inner diameter and length of 0.9 cm and 9 cm, respectively, packed in a 1 cm layer of activated silica gel. Polar compounds and water were removed from the samples using anhydrous sodium sulfate, which was passed through the column. The extract was eluted sequentially with HPLC (high performance liquid chromatography) using hexane / DCM (3: 1) and concentrated for gas chromatographic mass spectrometry (GC–MS) analysis [33, 72]. The analysis was performed using a GC–MS instrument (Thermo Trace GC-Ultra and Thermo Focus DSQ II) supplied with a standard non-polar capillary column (DB 35-MS) measuring 30 m × 0.25 mm × 0.25 μm. The eluted extracts were injected (1 μL) onto a GC–MS capillary column with a fixed injection and detection temperature of 320 °C. Nitrogen carrier gas was fixed at a flow rate of 1.0 mL min⁻¹. The temperature was programmed to maintain at 30 °C for 2 min after heating, heating at 7 °C for 1 min; maintain at 100 °C for 5 min, heat at 15 °C for 1 min; maintain for another 10 min at 200 °C, heat for 1 min at 30 °C; hold for a further 12 min at 300 °C, then heat for 1 min at 10 °C and finally at 10 min at 320 °C [61, 71, 100]. Peak areas were used to estimate the concentration of hydrocarbons in the diesel-oil extracted from the three experimental runs and controls using this formula:

\[
\text{Sample hydrocarbon concentration nanogram per milligram (ng/mg)} = \frac{[PA/PA'] \times \text{(Final volume of hydrocarbon standard in μL)}}{\text{weight of sample being analysed (mg)}}
\]

\[
PA \text{ represents the Peak area of hydrocarbon in sample;}
PA' \text{ represents Peak area of hydrocarbon in hydrocarbon standard. Diesel-oil degradation rate } (X_D) \text{ was given as sum of individual hydrocarbon concentrations in control compared with the sum of individual hydrocarbon concentrations in sample. This was estimated with this equation:}
\]

\[
X_D = \left( \frac{D_C - D_S}{D_C} \right) \times 100\%
\]

where,

- \(X_D\) is the Percentage diesel-oil hydrocarbon degraded;
- \(D_C\) is Total concentration of extracted diesel-oil hydrocarbon in control;
- \(D_S\) is Total concentration of extracted diesel-oil hydrocarbon in experiment;
- \((D_C - D_S)/D_C\) represents Total concentration of diesel-oil hydrocarbon degraded [33, 72].

Analysis of variation (ANOVA) was used to determine the suitability of the Taguchi model and the contribution of the parameters to the mean degradation rates of diesel-oil. Regression analysis was used to estimate the direction of the influence of the parameters on the average degrees of degradation using regression coefficients [66]. Regression analysis generates an equation that shows the statistical relationship between the predicted parameters and the diesel-oil removal rate using the maximum points of the model [73]. This equation was used to calculate the predicted diesel-oil degradation rate for each experimental run [66]. The regression equation was expressed as follows:

\[
Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \epsilon_i
\]

where,

- \(Y\) is the mean diesel-oil biodegradation rates (\(X_D\));
- \(\epsilon_i\) is the statistical noise; \(\beta_0\) is the model constant;
- \(\beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7\) are regression coefficients for parameters;
- \(X_1, X_2, X_3, X_4, X_5, X_6 \text{ and } X_7\) are the parameters agitation speed, diesel-oil concentration, NaCl concentration, NH₄NO₃ concentration, pH, temperature and inoculum size respectively.

### Results and discussion

#### Isolation and identification

Isolation of hydrocarbon-degrading bacteria is essential to preserve terrestrial and marine ecosystems that are constantly affected by oil pollution [17, 56, 95]. The study found that oil-contaminated tarballs taken from the beaches of Terengganu, Malaysia consisted of alkanes and polycyclic aromatic hydrocarbons (PAHs) (data not shown). The tarballs were greasy, dark in colour and basic in nature (pH 8.5). Oil-degrading bacteria were isolated from the tarballs by enrichment and purified on LB agar incubated at 37 °C for 24 h. Morphological individual colonies, initially labelled with RS, GS, WR1, and WR2 strains, were identified by analysis of their 16S rRNA gene sequence. Results revealed that linear stretch of 1,477 base pairs (bp) for strains RS, GS, WR1 and WR2 were 99.9% identical to strains *Pseudomonas stutzeri* DSM 5190 (NR_114751.1), *Cellulosimicrobium*...
cellulans ATCC 12,830 (NR_115251.1), Acinetobacter baumannii CIP 70.34 (NR_116845.1) and Pseudomonas balearica SP1402 (NR_025972.1) respectively as illustrated on Figs. 1, 2, 3 and 4 respectively.

Figures 1, 2, 3, and 4 show phylogenetic trees reflecting the evolutionary relationships of bacteria isolated from oil-contaminated Tarball and identified by their 16S rRNA gene sequence as Pseudomonas stutzeri, Cellulosimicrobium cellulans, Acinetobacter baumannii and Pseudomonas balearica. This means that the Tarball bacterial communities taken from Terengganu Beach, Malaysia, consisted of Gammaproteobacteria (P. stutzeri, A. baumannii and P. balearica) and Actinobacteria (C. cellulans) with Gammaproteobacteria being the dominant group. This coincides with several reports stating that Proteobacteria and Actinobacteria appeared in oil mousse (formerly the tarball form), with Gammaproteobacteria being the most abundant group [43, 59]. Several reports indicate that hydrocarbon-degrading bacteria can be isolated from sites contaminated with different hydrocarbons. It has been reported that most of them can decompose n-alkanes and PAHs from contaminated crude oil, petroleum or petroleum products. [30, 99]. Pseudomonas stutzeri is a class of gram-negative Gammaproteobacteria that are reported to thrive in a variety of ecological niches due to their ability to adapt to a wide temperature range (4—40 °C) and high genetic versatility [37, 44]. Studies have shown that P. stutzeri, isolated from oil sludge and marine sediments, was able to selectively use an organic carbon source in chemically specified media for growth and energy [37, 52, 106]. The Pseudomonas stutzeri isolated in this study were able to thrive on tarball due to their ability to utilize hydrocarbon substrates [44, 52]. Cellulosimicrobium cellulans is a class of gram-positive Actinobacteria class that thrives at high temperatures (about 42 °C).
and under very saline conditions. [35, 42]. They are hemi-
cellulolytic and cellulolytic microbes capable of producing
diverse degradative enzymes such as protease, glucosidase,
chitinase and glycoside hydrolase. *Cellulosimicrobium cel-
lulans* was formerly isolated from soils in Libyan desert pol-
luted with oil and was reported to utilize alkanes and PAH’s
in crude oil due for growth [94].

*Acinetobacter baumannii* are gram-negative *Gammapro-
teobacteria* that are ubiquitous due to their different meta-
biological capacities and have been used for a variety of biotechno-
logical purposes [58, 102]. Previously, they were isolated
from refinery sludge and could grow in contaminated areas
where the only source of carbon was crude or diesel oil [49,
68]. *Pseudomonas balearica* is another class of gram-neg-
ative *Gammaproteobacteria* that is genotypically and mor-
phologically closely related to *Pseudomonas stutzeri* [37].
However, it retains different phenotypical attributes and
16S rRNA sequence when compared to another *P. stutzeri*
genomovars. They can thrive at 46 °C and retain the capacity
to mineralize xylose as only carbon source [16]. Previous
reports stated that *P. balearica* was part of a consortium
used for degrading total petroleum hydrocarbons (TPH) in
crude oil [114]. *Pseudomonas balearica* strain UKMS3P3
isolated from oil-polluted Mathura Refinery soil metabo-
lized n-alkanes and PAH’s in diesel-oil [76].

**One-Factor-A-Time (OFAT) growth optimization**

The growth of a consortium of isolated bacteria in diesel-oil
was initially optimized by the OFAT method to determine
the parameter levels of the Taguchi method. The effects
of agitation speed, initial diesel-oil concentration, NaCl
concentration, NH₃NO₄ concentrations, pH, temperature
and inoculum size on consortium growth were investi-
gated. Results were presented on Fig. 5a-g and Appendix
1 (Table 1) showing consortium growth (absorbance at
OD₆₀⁰nm) recorded by turbidity every day for 5 days. Day 6
on Fig. 5a-g represents average growth after 5 days incuba-
tion [97, 111].

Figure 5a shows agitation speeds 50 and 150 rpm
recorded the highest consortium growth (0.902 and 1.319
OD₆₀⁰nm respectively) on Day 6. This means that the high
mixing rate has led to significant growth in the consortium,
similar to previous reports that high aeration is required for
bacterial growth because it increases the amount of dis-
solved oxygen [17]. The result also shows that all the bacte-
ria in the consortium favoured the aerobic use of diesel-oil
Figure 5 shows that the growth of the consortium was highest at the initial diesel-oil concentration of 4%, 8% and 12% (v/v), indicating that the growth of the consortium was more favourable at the higher initial diesel-oil concentration. This is almost the same as in previous studies, which suggest that the population of hydrocarbon decomposers can increase abruptly from 1 to 100% when petroleum-based hydrocarbons are added to an unpolluted environment due to increased bioavailability [91].

Earlier studies have confirmed that marine bacteria grow better in a hypersaline medium high in NaCl because of improved nutrient transport and assimilation under such conditions [77]. Consortium growth increased because they were of marine origin [22].

Figure 5d indicates that NH₄NO₃ concentrations 1.0, 2.0 and 3.0 gL⁻¹ had best consortium growth. Ammonium and nitrate ions are important building blocks for amino acids and nucleotides, which means that high concentrations will directly increase bacterial growth [20, 107]. Figure 5e demonstrates that media pH 6.5, 7.0 and 7.5 yielded highest consortium growth. The growth of the consortium increases at neutral or near-neutral pH. Studies have shown that most bacteria grow best at a neutral pH of 6.5 to 7.5. This is because enzyme activity and protein synthesis are maximized around these pH levels [75, 113]. Figure 5f illustrates that at temperatures 37, 40 and 42 °C consortium grew the largest. This may be associated with increased catalytic
activity of metabolic enzymes and high-temperature solubility of diesel fuel [40]. Studies have shown that at high temperatures, diesel becomes more soluble and less viscous, making it more bioavailable to bacteria [46]. Figure 5g shows that the 0.5 mL, 1.5 mL, and 2.5 mL inoculum sizes of the initial consortium had the highest mean growth. This indicates that the growth of the consortium increased at all levels of the initial inoculum size studied. When the inoculum size is small (0.5 mL), competition between bacterial species is minimal. Bacterial cells make efficient use of the diesel carbon source and the population is growing exponentially [90]. When the inoculum is large (1.5 mL or 2.5 mL), the use of diesel-oil also increases, resulting in a decrease in sensitivity to hydrocarbon toxicity and an increase in population [1]. In all experiments, the parameter ranges of the Taguchi method were chosen as the best parameter levels because the higher bacterial biomass indicates increased biodegradation of diesel-oil [75, 90, 97].

Taguchi experimental design and biodegradation

The Taguchi method was used to optimize the biodegradation of diesel-oil by a consortium of isolated bacteria and to determine the contribution value of the predicted parameters [66, 83, 87]. Parameters and respective levels were applied to eighteen (18) experimental runs (Table 2). Mean diesel-oil degradation ($X_{D}$) were calculated by comparing peak areas after GC–MS analysis. Table 3 shows Observed and Predicted diesel-oil degradation rates ($X_{D}$) by consortium of isolated bacteria using Taguchi $L_{18}$ Design of Experiments (DOE).
Table 3 shows experimental run 18 had a maximum mean diesel removal of 93.6%, while runs 15, 16 and 17 also had a mean diesel removal of more than 85% (88.1%, 89.9% and 88.1%, respectively). Run 18 had an inoculum size of 2.5 mL, an initial diesel-oil concentration of 12% (v/v), NaCl concentration of 40.0 gL⁻¹ and NH₄NO₃ concentration of 2.0 gL⁻¹ in MSM with pH of 6.5. Incubation conditions were 40 °C and 150 rpm for 30 days (Table 2). At least 41.5% of the diesel-oil was removed in Experiment 6. Therefore, the optimization of the degradation response will be directed to the maximum and minimum degradation of 93.6% and 41.5%, respectively. The highest rate of decomposition recorded in Run 18 was attributed to several parameters whose levels supported the growth of the consortium. Diesel-oil uptake can be explained by the growth of a bacterial consortium that survived the initial lag phase and did not increase the number of living cells in the consortium during the first few days. Diesel oil removal was minimal as the cells in the consortium adapted to the medium conditions by synthesizing RNA, enzymes, and possibly biosurfactants [48, 110]. Exponential growth continues for up to 30 days with increased diesel removal due to larger consortium cell population [2]. Due to the high stirring speed (150 rpm), the medium became more aerated and increased the amount of dissolved oxygen, resulting in the consortium growing at a high rate [17]. High growth in the consortium requires a sufficient concentration of diesel-oil as carbon source (12% v / v) to sustain growth [95]. High temperatures of 40 °C enhanced enzyme activities while making the carbon source more soluble and less viscous making them more accessible to consortium [46]. High nitrate concentration (2.0 gL⁻¹) also increased bacteria growth and diesel-oil utilization due to the availability of building blocks for cell formation [107]. High Salinity (40.0 gL⁻¹) and near-neutral pH were optimal for expression of degrading enzymes in consortium bacterial cells because they were all isolated from marine Tarball samples where similar conditions were natural for their survival [22, 75]. The number of active bacterial cells also played an important role in ensuring the highest rate of degradation in Experiment 18, as the cells were less sensitive to hydrocarbon toxicity as their population grew to increase the rate of diesel utilization [1, 90]. GC–MS analysis revealed that both long and short-chain n-alkanes in the diesel-oil were degraded by the consortium in Experiments 6 and 18 after 30 days. Table 4 shows the average amount of n-alkanes in diesel removed by the consortium of isolated bacteria after 30 days of incubation for experimental runs 6 and 18 at an initial diesel concentration of 12% (v/v) in 50 mL MSM.

Table 3 Mean Observed and Predicted diesel-oil degradation rates (X₀) by consortium of isolated bacteria after 30 days

| Experimental runs | Observed Degradation (%) | Predicted Degradation (%) | SD* |
|-------------------|--------------------------|----------------------------|-----|
| 1                 | 42.5                     | 42.17                      | 5.68|
| 2                 | 62                       | 46.11                      | 3.19|
| 3                 | 50.7                     | 48.9                       | 5.58|
| 4                 | 46.6                     | 51.8                       | 4.34|
| 5                 | 47.6                     | 51.14                      | 4.26|
| 6                 | 41.5                     | 47.26                      | 4.86|
| 7                 | 53.3                     | 54.6                       | 4.74|
| 8                 | 50.2                     | 49.39                      | 4.86|
| 9                 | 56.2                     | 59.23                      | 5.24|
| 10                | 63.1                     | 71.68                      | 4.81|
| 11                | 84.5                     | 85.14                      | 5.18|
| 12                | 77.9                     | 76.49                      | 4.86|
| 13                | 77.4                     | 76.89                      | 4.86|
| 14                | 74.9                     | 78.67                      | 4.34|
| 15                | 88.1                     | 90.81                      | 4.26|
| 16                | 89.9                     | 81.94                      | 5.18|
| 17                | 89.8                     | 86.08                      | 4.86|
| 18                | 93.6                     | 91.34                      | 4.81|

SD* Standard deviation
Bacteria in consortium such as *Cellulosimicrobioum cellulans* and *Acinetobacter baumanniii* have been reported to retain genes coding for n-alkane dioxygenase and hydroxylases enzymes which grants them preference for utilization of n-alkanes from Decane (n-C10) to Triacontane (n-C30). *Pseudomonas stutzeri* and *Pseudomonas balearica* are also known to retain similar genes in addition to their ability to produce biosurfactants which aid degradation of complex hydrocarbons [51, 78]. Therefore, this consortium can be used to develop a new bioremediation strategy complemented by Taguchi optimization that can achieve faster decontamination of contaminated sites at minimal costs.

**Effects of parameters on diesel-oil biodegradation by consortium**

Mean diesel-oil degradation rates ($X_D$) from experimental runs (Table 3) were analysed to determine combined and individual contributions of each parameter on diesel-oil degradation. ANOVA was executed to test significance of the model and its fitness.

Table 5 shows model fitness was significant since determination coefficient ($R^2 = 90.89\%$) is large, implying change in mean diesel-oil degradation were caused by investigated parameters. It means there is acceptable correlation between observed and predicted diesel-oil degradation response. This means combination of all parameter significantly improved diesel-oil removal by 90.89% when this consortium were utilized across experimental runs [66, 73]. Probability of different parameter levels having statistically different effects on diesel-oil degradation was expressed by the model F-value of 14.25 and "$P > F$” was $< 0.0001$ (Table 5). Therefore, model fitted experimental datasets significantly with 0.01% likelihood large F value would happen at the time of optimization [66, 73].

This means that the combination of mixing rate (agitation speed), initial diesel concentration, salinity, nitrate concentration, temperature, pH, and inoculum size simultaneously affected the nutrient flow in the biodegradation system, thus increasing the diesel metabolism by the consortium. This is similar to Umar et al. [106] stating that the effects and interactions of mixing rate, pH, temperature, salinity, and inoculum volume significantly improved PAH degradation by *Enterobacter sp. MM087*. Simultaneous exposure to the parameters increased the expression of enzymes between the isolates and thus increased the rate of diesel-oil removal.

### Table 4 Mean diesel-oil n-alkanes removed by consortium of isolated bacteria after 30 days incubation for Runs 6 and 18

| Carbon number | Hydrocarbon | Run 6 (%) | Run 18 (%) |
|--------------|-------------|-----------|------------|
| n-C10        | Decane      | 81.2      | 87.6       |
| n-C11        | Undecane    | 51.5      | 93         |
| n-C12        | Dodecane    | 37.7      | 89         |
| n-C13        | Tridecane   | 35        | 93.3       |
| n-C14        | Tetradecane | 33        | 90.6       |
| n-C15        | Pentadecane | 41.2      | 92.2       |
| n-C16        | Hexadecane  | 30.6      | 92.2       |
| n-C17        | Heptadecane | 54.9      | 93.5       |
| n-C18        | Octadecane  | 16        | 93.7       |
| n-C19        | Nonadecane  | 6.9       | 95.1       |
| n-C20        | Eicosane    | 7.1       | 96.2       |
| n-C21        | Heneicosane | 55.8      | 97.5       |
| n-C22        | Docosane    | 36.6      | 95.4       |
| n-C23        | Tricosane   | 47.6      | 96.9       |
| n-C24        | Tetracosane | 54.8      | 97.6       |
| n-C25        | Pentacosane | 50.7      | 93.4       |
| n-C26        | Hexacosane  | 59.1      | 91.96      |
| n-C27        | Heptacosane | 91        | 94.6       |
| n-C28        | Octacosane  | 85.6      | 94         |

### Table 5 Analysis of variance for Taguchi quadratic model terms

| Parameters      | Df | Adj. SS** | Adj. MS*** | F-value | P-value | Contribution (%) |
|-----------------|----|-----------|------------|---------|---------|------------------|
| Model           | 7  | 5183.08   | 740.44     | 14.25   | 0.000   | 90.89            |
| Agitation       | 1  | 4620.81   | 4620.81    | 88.94   | 0.000   | 81.03            |
| Diesel conc     | 1  | 226.20    | 226.20     | 4.35    | 0.063   | 3.97             |
| NaCl conc       | 1  | 102.08    | 102.08     | 1.96    | 0.191   | 1.79             |
| NH4NO3 conc     | 1  | 116.56    | 116.56     | 2.24    | 0.165   | 2.04             |
| pH              | 1  | 2.34      | 2.34       | 0.05    | 0.836   | 0.04             |
| Temperature     | 1  | 99.21     | 99.21      | 1.91    | 0.197   | 1.74             |
| Ino. size       | 1  | 15.87     | 15.87      | 0.31    | 0.593   | 0.28             |
| Other Errors    | 10 | 519.52    | 51.95      |         |         | 9.11             |

$Df$ Degree of freedom

$Adj. SS$ Adjusted sum of squares

$Adj. MS$ Adjusted mean squares
Table 5 also shows agitation speed (mixing rate) had the largest statistically significant individual contribution of 81.03% ($P < 0.05$; 0.000). This implies agitation speed individually caused 81.03% change in mean diesel-oil biodegradation similar to previous studies stating that microbial respiration and biomass significantly influenced removal of total petroleum hydrocarbons in an oil-impacted environment [5]. The rate of mixing directly affects dissolved oxygen, which is a major component of aerobic biodegradation. Oxygen acts as an electron acceptor and must be infused into the methyl terminal groups to initiate aerobic biodegradation [23]. Initial diesel-oil concentration, nitrate concentration, salinity, temperature, inoculum size and pH had no statistically significant contributions ($P > 0.05$) to diesel-oil removal (Table 5). However, ‘missing’ parameters had low contribution (9.11%), implying the model adequately explained how predictor parameters significantly improved diesel-oil degradation by this consortium [87].

The size and direction of changes in diesel-oil degradation rate caused by each parameter were given as regression coefficients (Table 6) illustrated in the regression equation, where diesel-oil degradation rate ($Y$) is a function of investigated parameters as given:

$$Y = -18 + 0.3204 \text{Agitation speed} + 2.17 \text{Diesel conc.} + 0.292 \text{NaCl conc.} - 3.12 \text{NH}_4 \text{NO}_3 \text{conc.} - 0.88 \text{pH} + 1.143 \text{Temperature} + 1.15 \text{Inoculum size}$$

Table 6 demonstrates agitation speed had largest statistically significant ($t$-value = 9.43; $P < 0.05$, 0.000) positive effect on diesel-oil degradation by consortium in agreement with results on Table 5. Mean change in diesel-oil degradation increased significantly by 0.3204 when agitation speed increased by one unit (Table 6). This is attributed to increase in bacteria growth and diesel utilization due to high dissolved oxygen and nutrient absorption. Consortium preferred aerobic conditions since all bacteria strains thrived in Tarball under aerobic conditions. Therefore, oxygen is essential to achieve optimal bioremediation using this consortium [9]. Table 6 also confirms that initial diesel-oil concentration, salinity (NaCl concentration), nitrate ($\text{NH}_4 \text{NO}_3$) concentration, pH, temperature and inoculum size individually had less effect on the decomposition of diesel-oil by the consortium, which is not statistically significant ($P > 0.05$). Agitation speed, initial diesel-oil concentration, salinity (NaCl concentration), temperature and inoculum size had positive (synergistic) effects, while $\text{NH}_4 \text{NO}_3$ and pH had negative (antagonistic) effects on diesel-oil removal by consortium of isolated bacteria (Table 6). This means that high values of these parameters will increase the average degradation of diesel-oil by the consortium when the parameters have a positive effect and the average degradation will decrease when the parameters have a negative effect [66, 73].

Synergistic effect of agitation speed on diesel-oil removal was demonstrated by the fact that highest mean diesel-oil removal of 89.9% (Run 16), 89.8% (Run 17) and 93.6% (Run 18) were recorded at 150 rpm (Tables 2 and 3). Mean degradation reduced to 53.3% (Run 7), 50.2% (Run 8) and 56.2% (Run 9) when incubated at 50 rpm with similar initial diesel-oil concentration (12% v/v) as shown on Tables 2 and 3. Lowest degradation of 41.5% (Run 6) was also recorded at 50 rpm (Tables 2 and 3). This means that the high mixing speed (150 rpm) caused a high decomposition of diesel-oil by the bacterial consortium. This is in line with previous reports that diesel decomposition was faster in highly aerated media due to increased dissolved oxygen [2, 17, 49, 69]. Nutrient uptake is improved when the mixing rate is high due to proper mixing of the bacterial consortium and diesel. This improves the mass transfer of oxygen, substrates, by-products, and other products and thus increases the rate of biodegradation [45]. The strains used in this consortium require a highly aerated biodegradation system because they have been accustomed to thriving in a marine environment that is well ventilated [2].

### Table 6 Regression coefficients for parameters and their effects on diesel-oil biodegradation by consortium of isolated bacteria after 30 days

| Sources             | Coefficients | SE Coefficients* | t-value | P-value | 95% CI** |
|---------------------|--------------|------------------|---------|---------|----------|
| Constant            | -18.0        | 45.0             | -0.40   | 0.697   | -118.2   |
| Agitation           | 0.3204       | 0.034            | 9.43    | 0.000   | 0.245    |
| Diesel conc.        | 2.17         | 1.04             | 2.09    | 0.063   | -0.15    |
| NaCl conc.          | 0.292        | 0.208            | 1.40    | 0.191   | -0.172   |
| $\text{NH}_4 \text{NO}_3$ conc. | -3.12 | 2.08           | -1.50   | 0.165   | -7.75    |
| pH                  | -0.88        | 4.16             | -0.21   | 0.836   | -10.16   |
| Temperature         | 1.143        | 0.827            | 1.38    | 0.197   | -0.7     |
| Inoculum size       | 1.15         | 2.08             | 0.55    | 0.593   | -3.49    |

*SE Coefficients Standard error coefficients

**CI Confidence interval

\(\text{CI}^{**}\)
Initial diesel-oil concentration also had synergistic effect on diesel-oil degradation by consortium. Diesel-oil was removed by 77.4% (Runs 13), 74.9% (Runs 14) and 88.1% (Runs 15) when initial diesel-oil concentration was 8% (v/v) at 150 rpm agitation speed (Tables 2 and 3). Diesel-oil removal increased slightly when initial concentrations were raised to 12% (v/v) with removal rate of 89.9%, 89.8% and 93.6% recorded by Runs 16, 17 and 18 respectively at similar agitation speed (Tables 2 and 3). This agrees with reports by Imron and Titah [49] stating that Acinetobacter baumannii achieved best degradation when initial diesel-oil concentration was highest due to increased bioavailability of carbon source while lowest growth was observed at 1% (v/v) initial concentration. The high concentration of diesel-oil promotes the growth of the consortium, which in turn increases the rate of degradation, as the bacterial strains have been adapted to survive in the heavy hydrocarbon fractions found in tarballs [46].

Synergistic effect of Salinity (NaCl concentration) was evident on Table 3 showing lowest mean diesel-oil removal of 63.1% (Run 10) recorded at 20.0 g L\(^{-1}\) NaCl concentration and 150-rpm (Table 2). At the same agitation speed, diesel-oil removal increased to 74.9% (Run 14) and 77.9% (Run 12) when NaCl concentration was raised to 30.0 g L\(^{-1}\) and 40.0 g L\(^{-1}\) respectively (Tables 2 and 3). Maximum diesel-oil removal of 93.6% (Run 18) was attained when NaCl concentration increased to 40.0 g L\(^{-1}\) under similar conditions (Tables 2 and 3). The consortium favoured the use of diesel-oil under saline conditions, as they were isolated from the tarballs formed in the marine environment [105]. This agrees with reports by Imron and Titah [49] stating that Vibrio alginolyticus isolated from marine environment, degraded diesel-oil under highly saline conditions. This is because saline conditions increased enzyme expression and balanced osmotic potential by marine-originated hydrocarbon-degrading bacteria [77].

Temperature also had synergistic effects on diesel-oil removal as shown on Table 3 where mean diesel-oil removal increased from 89.8% (Run 17) to 93.6% (Run 18) when temperature was raised from 37 °C to 40 °C at 12% (v/v) initial diesel-oil concentration and 150 rpm agitation speed. At similar initial diesel-oil concentration and 50 rpm, degradation also increased from 50.2% (Run 8) to 53.3% (Run 7) when temperatures increased from 37 °C to 42 °C (Tables 2 and 3). This is similar to reports by Umar et al. [106] stating that high temperatures favoured PAH degradation by Enterobacter sp. MM087 due to increased hydrocarbon assimilation by bacteria and heightened enzymes activities. High temperatures made hydrocarbons more soluble, less dense, less viscous and more bioavailable [103]. The degree of biodegradation of hydrocarbons usually decreases at lower temperatures due to reduced volatility and high viscosity [46].

Inoculum size also affected diesel-oil biodegradation by consortium positively. Diesel-oil was removed by 89.9% (Run 16) at 0.5 mL consortium inoculum size with 12% (v/v) initial diesel-oil concentration and 150 rpm (Tables 2 and 3). Diesel-oil removal increased to 93.6% (Run 18) when consortium inoculum size was raised to 2.5 mL under similar initial diesel-oil concentration and agitation speed (Tables 2 and 3). Similar results were observed when inoculum size was increased from 0.5 to 1.5 mL at an initial diesel-oil conc. of 8% (v/v) and 150 rpm with diesel-oil removal increasing from 74.9% (Run 14) to 88.1% (Run 15). The large size of the inoculum means a higher number of metabolically active bacteria that interact with diesel at any time, which increases the rate of biodegradation [29]. This agrees with reports by Ibrahim et al. [47] affirming high concentration of microbes raised PAH degradation because more microbes act on the hydrocarbon substrate.

Nitrates concentration had antagonistic effects on diesel-oil biodegradation by this consortium when 1.0 g L\(^{-1}\), 2.0 g L\(^{-1}\) and 3.0 g L\(^{-1}\) NH\(_4\)NO\(_3\) concentration were used in MSM. Diesel-oil removal decreased from 93.4% to 89.9% when NH\(_4\)NO\(_3\) concentration increased from 2.0 g L\(^{-1}\) (Run 18) to 3.0 g L\(^{-1}\) (Run 16) at 150 rpm and 12% (v/v) diesel-oil concentration (Table 2 and 3). This contradicts reports such as Imron and Titah [49] that nitrogen addition had a positive effect on bacterial growth. This means that high nitrate concentrations would lead to excessive bacterial growth and depletion of dissolved oxygen. This leads to overpopulation and increased competition between bacterial cells [2]. This causes the death of some bacteria cells and decreases the population of active hydrocarbon-degraders. This correspondingly causes a negative biodegradation response [67].

The effects of pH on diesel-oil biodegradation were also antagonistic. Diesel oil removal increased from 89.8% (Run 17) to 93.6% (Run 18) when pH levels dropped from 7.5 to 6.5 respectively when initial diesel-oil concentration and agitation speed are 12% (v/v) and 150 rpm respectively (Tables 2 and 3). This means consortium favourably utilized diesel-oil at neutral or slightly acidic (6.5 to 7.0) pH similar to earlier reports [10, 84]. Neutral or near-neutral pH facilitates the action of several hydrolysing enzymes and thus increases the degradation yield of diesel-oil [80, 86].

**Validation of optimization**

Analytical optimization was performed according to the desired functions. Experiments were performed to evaluate the optimal degradation of diesel-oil when process parameters were determined at low, optimal, and high levels using the Taguchi method [65]. The biodegradation response goal was set to maximum with observed low diesel-oil removal of 41.5% and high diesel-oil removal of 93.6% (Table 7).
For this experiment, desirability was 1.000. Results showed maximum diesel-oil removal of 99.74% was predicted with the current optimization parameters. Errors (%) between observed and predicted degradation was used to validate experiments [73]. This was calculated using the equation:

$$\text{Error} = \frac{\text{Observed} - \text{Predicted}}{\text{Observed}} \times 100$$

Table 7 shows the three levels of parameter settings (low, optimal, and high) established in the Taguchi experimental project to optimize diesel-oil decomposition using a consortium of isolated bacteria. Errors (%) for all parameter settings dropped below 5%, suggesting that process parameter optimization by Taguchi method was competent and reliable for optimizing diesel-oil biodegradation using consortium of isolated bacteria. Optimal parameter settings for achieving highest predicted diesel-oil degradation of 99.74% includes:

- an inoculum size of 2.5 mL, an initial diesel-oil concentration of 12% (v/v), NaCl concentration of 30.0 g L⁻¹ and NH₄NO₃ concentration of 2.0 g L⁻¹ in MSM with pH of 7.0.
- Incubation conditions were 42 °C and 150 rpm for 50 mL bioremediation culture (Table 7).

### Conclusion

Taguchi L₁₈ design was successfully used to optimize the biodegradability of diesel-oil using a consortium of newly isolated hydrocarbon-degrading bacteria in tarball identified as _Pseudomonas stutzeri, Cellulosimicrobium cellulans, Acinetobacter baumannii_ and _Pseudomonas balearica_. This indicates that oil-degrading bacteria are found in tarballs, which can use diesel-oil as their sole carbon source because they are adaptable and metabolically diverse. Taguchi design established optimal parameter settings for agitation speed, initial diesel-oil concentration, salinity (NaCl concentration), nitrate (NH₄NO₃) concentration, pH, temperature and initial inoculum size. These optimal settings can be used to improve the quality of hydrocarbon biodegradation using this consortium, as these parameters have significantly improved diesel-oil removal as found in this study. Optimal settings of agitation speed (aeration) individually improved diesel-oil removal significantly in a positive direction while individual effects of other parameters were not significant. The knowledge gained in this study can be used to develop a new bioremediation strategy that uses a consortium of native bacteria in tarballs with the Taguchi optimization method. This strategy can decontaminate oil-contaminated sites with minimal time and cost compared to other existing methods because Taguchi’s design is optimized with minimal experimentation. Additionally, Taguchi method can be used to determine fitness of bioremediation exercise and eliminate parameters capable of causing antagonistic effects during the bioremediation process. Priority can be given to significant parameters with synergistic contributions thereby saving time and cost. This is important when remediation is subject to monitoring and pressure from environmental regulators. Further work must be done to test the bioremediation ability of this consortium on large scale under optimized conditions illustrated in this study. Future studies can also elaborate on the synergistic relationship between two or more bacteria in a consortium during biodegradation.

### Abbreviations

- µL: Microliter; 16S rRNA: 16S ribosomal RNA; A. baumannii: Acinetobacter baumannii; Adj. SS: Adjacent sum of squares; Adj. MS: Adjusted mean squares; ANOVA: Analysis of Variance; BLAST: Basic Local Alignment System Tool; °C: Degree Celsius; C. cellulans: Cellulosimicrobium cellulans; CI: Confidence interval; conc.: Concentration; DCM: Dichloromethane; df: Degree of freedom; DNA: Deoxyribonucleic Acid; dNTP: Deoxy nucleoside triphosphates; g: Gram; GC-MS: Gas Chromatography Mass Spectrometry; gL⁻¹: Gram per litre; h: Hours; HPLC: High performance liquid chromatography; L: Liter; LB: Luria Bertani; MEGA: Molecular Evolutionary Genetics Analysis; mg: Milligram; mL: Milliliter; MSM: Minimal salt media; NaCl: Sodium chloride; NCBI: National Center for Biotechnology Information; ng/mg: Nanogram per milligram; NH₄NO₃: Ammonium nitrate; OD₆₀₀nm: Optical density at 600 nm; OFAT: One-factor-a-time; P. balearica: Pseudomonas balearica; PCR: Polymerase chain reaction; ppm: Parts per million; P. stutzeri: Pseudomonas stutzeri; rpm: Revolutions per minute; SE Coefficients: Standard error coefficients; secs: Seconds; SIS: Surrogate internal standard; SN: Signal noise; v/v: Volume per volume.

### Supplementary Information

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Data availability We further confirm that datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication We the authors declare our consent for publication of original manuscript that has not been published before and is not currently being considered for publication elsewhere.

Competing interests We wish to confirm that there are no known competing interests associated with this publication. We would like to draw the attention of the Editor to the following publication of one or more of us that refer to aspects of the manuscript presently being submitted: Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of Cellulosimicrobium cellulans and Acinetobacter baumannii from tarball at Terengganu beach, Malaysia: https://doi.org/10.1016/j.marpolbul.2016.03.060

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