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Isolation and Optimization of Diesel-Oil Biodegradation using *Cellulosimicrobium cellulans* from Tarball

Bruno Martins Nkem[1], Normala Halimoon[1]*, Fatimah Md Yusoff[2] and Wan Lutfi Wan Johari[1]

[1]*Department of Environmental Sciences, Faculty of Environmental Studies, , Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Oil spill introduces hydrocarbons into the marine environment and forms oil slicks, which aggregate with other debris to form tarballs. Tarballs are composed of toxic hydrocarbons, which persist in the environment, causing economic and ecological damages. This work studied the isolation and optimization of diesel-oil biodegradation by an indigenous bacterium, identified by 16S rRNA gene sequence analysis, in tarball. An experimental methodology using a Taguchi orthogonal array was applied to optimize the effects of diesel concentration, salinity, nitrate concentration, pH, temperature, agitation speed and time. An isolated bacterium identified as *Cellulosimicrobium cellulans* removed 88.4% of diesel oil under optimized conditions, where initial diesel-oil concentration was 5% (v/v), NaCl concentration was 20 gL^{-1} and NH_4NO_3 concentration was 2 gL^{-1} in Minimal Salt Media at pH 7, 40°C and 100 revolutions per minute for 5 days. Tarballs harbor hydrocarbon-degrading *C. cellulans* that can be used under optimized conditions to design an effective oil spill bioremediation technique for mitigating oil pollution.

Keywords: Biodegradation, *Cellulosimicrobium cellulans,* diesel-oil, hydrocarbon, Taguchi

INTRODUCTION

Oil spills in marine environment emanate from various sources including natural seeps, operational discharge from ships, ruptured pipelines and accidental spills from tanker accidents and other onshore

or offshore activities (Amenaghawon et al., 2014). They are imported into the marine environment, wither into oil slicks, accumulate other debris and form tarballs.

Tarballs are made of hydrocarbon fractions that are resistant to microbial degradation via diverse weathering mechanisms such as dispersion, spreading, photooxidation, evaporation, emulsification, sinking, sedimentation, and dissolution. These hydrocarbons are lethal to marine life and the ecosystem because they contaminate the food chain. They infiltrate living cells and tissues, become immobilized within them and cause mortal deterioration (Hassanshahian et al., 2014; Perelo, 2010). They may be conveyed far away from its primary source and gradually accumulate on seaboards, causing ecological damages (Hassanshahian et al., 2014). The ability of hydrocarbons in the tarballs to resist weathering under oceanic conditions makes tarballs an excellent site for isolating hydrocarbon-degrading bacteria suited for utilizing a wider range of hydrocarbons compared to those isolated from other sources. The bacteria can be used to establish bioremediation techniques for mitigating hydrocarbon pollution.

Bioremediation techniques include cheaper and eco-friendly alternatives to mitigate hydrocarbon pollution with the prospect of large-scale implementation. They require the utilization of native microbial species in hydrocarbon-impacted sites with possible metabolic capacity to break down hydrocarbons, transforming them into simpler forms. Simpler compounds are ingested as an energy source for growth through aerobic or anaerobic pathways (Hassanshahian et al., 2014; Speight & Arjoon, 2012). The successful application of bioremediation depends on the acquisition of considerable knowledge on the type, presence and amount of indigenous hydrocarbon-degrading microbial communities in the polluted site. Other factors include the scale of hydrocarbon pollution and favorable environmental conditions such as pH, temperature, nutrient content, salinity and dissolved oxygen (Hassanshahian et al., 2014). These factors must be optimized to achieve the most competent bioremediation outcome.

This research is aimed at isolating and optimizing diesel-oil biodegradation by an indigenous bacterium isolated from Tarball. Design of experiment (D.O.E) method using Taguchi orthogonal array will be used to determine optimum conditions for achieving the most competent biodegradation. This is based on the hypothesis that diesel-oil biodegradation by hydrocarbon-degrading bacteria will be improved under optimized conditions.

MATERIALS AND METHODS

Sample Collection and Chemicals

Tarballs were gathered along intertidal zones of polluted shorelines located on N 05°35.161'; E 102°50.169', Rhu Sepuluh, Kuala Terengganu, Malaysia. The carbon source chosen for this research was diesel-oil because it is a blend of alkanes and PAH's which have been accounted for as ecological contaminants (Gallego et al., 2001).

Enrichment and Isolation of Hydrocarbon-degrading Bacteria

The enrichment and isolation of hydrocarbon-degrading bacteria were performed using minimal salt media (MSM). Five-gram (5g) of tarball were placed into 98 ml MSM enhanced with 2% (v/v) filter-sterilized diesel-oil. MSM was autoclaved at 121°C for 20 minutes after pH was modified to 7.5. The setup was incubated in a shaker set at 32°C and 100prm for 7 days. Freshly prepared MSM was used to subculture 1mL previously incubated culture every week for 8 weeks. This was followed by serially diluting 1 mL of 8th subculture and plated on Luria Bertani agar (LB) plates. LB agar plates were incubated at 32°C for 48 hours (Nkem et al., 2016). Colonies that were morphologically different were purified on LB agar (Deng et al., 2014).

Bacteria Identification using 16s rRNA

Purified isolated bacteria was identified by molecular characterization of its 16S rRNA using polymerase chain reaction (PCR). Genomic DNA was isolated from purified bacteria and used as a template. The 16S universal primers used for PCR include reverse primer rP2 (5'ACGGCTACCTTGTTACGACTT-3') and forward primer fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'). Taq DNA polymerase enzyme was used to catalyze the reaction (Weisburg et al., 1991). The correlation of DNA sequences was interpreted via the NCBI website using BLAST (Roy et al., 2014). A neighbor-joining methodology was used to design phylogenetic tree using MEGA 4.0 software.

Optimization of Diesel-oil Biodegradation

The experiments were carried out using pure strains of isolated bacteria and injected inside fresh LB broth. The culture was incubated in a shaker set at 100 rpm and 32°C for 24h. Bacteria were harvested after incubation and used to create an inoculum. The inoculum was used to test for optimum conditions by 'one factor at a time' method establishing parameter levels for each condition. Bacterial suspensions (0.5 optical density or 1.5 x 10^6 CFUml^{-1}) were initially screened by inoculation into 185 mL MSM amended with diesel-oil (evaporated to 5g). Initial pH was modified to 6.0, 6.5, 7.0, 7.5 and 8.0 with 1 M HCl or 1 M NaOH; incubation temperature was set at 30, 32, 35, 37 and 40°C; NaCl concentration was set at 10, 20,30, 40, and 50 gL^{-1}; concentration of NH_4NO_3 was adjusted to 0.5, 1, 1.5, 2 and 2.5gL^{-1}; incubation time was 5, 7, 10 and 14 days; initial diesel-oil concentration was set to 0.5%, 1%, 2% and 5%. Finally, agitation was controlled at 0, 50 and 100 rpm. Uninoculated controls were maintained at similar conditions for each parameter. Residual diesel-oil was extracted and diesel-oil removal was estimated (Deng et al., 2014). For each parameter, three levels with highest diesel-oil biodegradation rate were selected for application in DOE Taguchi L18 Orthogonal array (Table 1). This was applied to 18 experimental runs with nutrients and parameters amended as indicated on

Taguchi L18 Orthogonal array in Table 2 designed using Minitab 17 software (Taguchi, 1986). Samples were incubated for 5, 7 and 14 days.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Diesel-oil

Residual diesel oil was extracted from experimental run samples using dichloromethane (DCM) and evaluated using the GC-MS technique (Deng et al., 2014). All experimental runs were implemented in triplicate. Biodegradation results were calculated by comparing residual diesel-oil extracted from samples to diesel-oil extracted from controls expressed in percentage. Concentrations of n-alkanes in residual diesel-oil were estimated by analyzing external standards peak area for diesel-oil in comparison to the peak area of the diesel-oil in extracted samples and controls. The sum of n-alkane concentrations in diesel oil was defined as residual diesel-oil hydrocarbon concentration. The degradation of diesel-oil by isolated bacteria was calculated using the equation:

$$R_d = \ (D_c - D_s)/D_c \quad x \quad 100\% \tag{1}$$

where R_d represents diesel-oil biodegradation, D_c represents hydrocarbon concentration in extracted diesel-oil from control, and D_s represents hydrocarbon concentration of diesel-oil extracted from samples (Deng et al., 2014).

RESULTS AND DISCUSSIONS

Isolation and Identification of Hydrocarbon-degrading Bacteria in Tarball

Colonies of purified strains were pale-yellow, tiny and circular with moist pigmentation. The rod-shaped, gram-positive bacilli had raised elevation and entire margin. These morphological characteristics are like those of *Cellulosimicrobium cellulans* (Antony et al., 2009; Schumann et al., 2001). Gene sequence analysis of 16S rRNA identified a linear stretch of 1,477 bp with 99% similarity to those of strains *C. cellulans* (GenBank accession number NR_119095), as shown in Figure 1. The phylogenetic tree was designed with
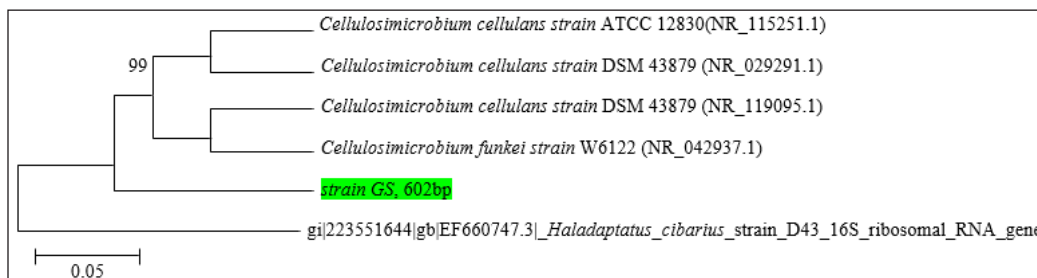

Figure 1. Phylogram showing phylogenetic relationships of isolated bacteria with a linear stretch of 1,477 bp. Selected outgroup based on 16S rRNA sequences is *Haladaptatus cibarius*. Trees were calculated by a neighbor-joining algorithm with 50% conservation filter. Bootstrap values (1000 replicates) are indicated by the numbers at the nodes

identical sequences by neighbor-joining methodology. These results indicate that the closest relative of isolated bacteria was *Cellulosimicrobium cellulans* DSM 43879 (NR_119095.1).

Cellulosimicrobium cellulans are universally distributed, with some strains formerly isolated as native hydrocarbon degraders from sites impacted by crude-oil with capabilities for utilizing hydrocarbons as a carbon and energy source (Aliakbari et al., 2014; Shaieb et al., 2015). Previous reports have also shown they are capable of thriving in a medium with crude-oil concentrations of as low as 1%. This makes them suitable for use in oil bioremediation (Yakimov et al., 2004).

Optimization of Diesel-oil Biodegradation

The Taguchi approach was used to design and analyze the effect of environmental parameters thereby optimizing diesel-oil biodegradation by *C. cellulans* (Taguchi, 1986). The parameters studied included agitation, diesel-oil concentration, NaCl concentration, NH_4NO_3 concentration, pH, temperature and incubation time. Parameter levels in Table 1 were selected by choosing the three best levels that yielded the most diesel-oil degradation after initially implementing the 'one factor at a time' optimization method. These levels were applied in an L18 orthogonal array to determine optimum conditions, which were then evaluated by calculating the degradation rate of extracted diesel-oil after incubation and GC-MS analysis.

Table 1
Selected factors and assigned levels

Serial number	Factors	Level 1(-1)	Level 2(0)	Level 3(1)
1	Agitation (rpm)	50	100	-
2	Diesel Conc. (% v/v)	1	2	5
3	NaCl Conc. (g/L)	20	30	40
4	NH_4NO_3 Conc. (g/L)	0.5	1	2
5	pH	6.5	7	8
6	Temperature (^{0}C)	32	37	40
7	Time (Days)	5	7	14

Factor level codes: -1, 0, 1 for levels 1, 2, 3

The selected experimental design was $2^1 \times 3^6$ Taguchi L18 orthogonal array. Agitation had 2 levels while other six parameters were monitored at 3 levels, totaling 18 experimental runs (Table 2) performed in triplicate. Another 18 runs operated at similar conditions were uninoculated controls. Diesel-oil biodegradation yield by *C. cellulans* for all 18 experimental runs were calculated by comparing with control runs after extracting residual diesel-oil extraction and analyzing removal rate by GC-MS. This was designated as 'R_d%' in Table 2. Experimental runs 16 and 11 recorded the highest diesel-oil biodegradation at

88.4%, and 79.8%, respectively. Run 16 had 5% (v/v) initial diesel-oil concentration, 2.5 mL (0.5 optical density) inoculum size, 20 gL^{-1} NaCl concentration and 2 gL^{-1} NH$_4$NO$_3$ concentration in MSM at pH 7, incubated at 40^{0}C with 100 revolutions per minute (rpm) for 5 days. Run 11 had initial diesel-oil concentration of 1% (v/v), inoculum size of 2.5 mL (0.5 O.D), 30 gL^{-1} NaCl concentration and 0.5 gL^{-1} NH$_4$NO$_3$ concentration in MSM at pH 6.5, incubated at 40^{0}C, 100 rpm for 14 days.

Table 2
L18 (2^1 x3^6) orthogonal array of the designed experiments and their average Biodegradation response in terms of R$_d$

E. Runs	Agitation	Diesel Conc.	NaCl Conc.	NH$_4$NO$_3$ Conc	pH	Temperature	Incubation. Time	R$_d$ (%)
1	-1	-1	-1	-1	-1	-1	-1	27.1
2	-1	-1	0	0	0	0	0	58.25
3	-1	-1	1	1	1	1	1	41.42
4	-1	0	-1	-1	0	0	1	13.31
5	-1	0	0	0	1	1	-1	36.46
6	-1	0	1	1	-1	-1	0	8.14
7	-1	1	-1	0	-1	1	0	32.31
8	-1	1	0	1	0	-1	1	26.65
9	-1	1	1	-1	1	0	-1	3.71
10	0	-1	-1	1	1	0	0	45.2
11	0	-1	0	-1	-1	1	1	79.9
12	0	-1	1	0	0	-1	-1	45.5
13	0	0	-1	0	1	-1	1	56.1
14	0	0	0	1	-1	0	-1	43.3
15	0	0	1	-1	0	1	0	30.99
16	0	1	-1	1	0	1	-1	88.43
17	0	1	0	-1	1	-1	1	32.73
18	0	1	1	0	-1	0	0	30.14

Factor level codes: -1, 0 and 1 for levels 1, 2 and 3

The results also showed that run 9 had the least diesel-oil removal (3.7%) with 5% (v/v) initial diesel-oil concentration, 40 gL^{-1} NaCl concentration and 0.5 gL^{-1} NH$_4$NO$_3$ concentration in MSM at pH 8, incubated at 37°C with 50 rpm for 5 days. Runs 11 and 16 both had highest degradation yield at agitation 100 rpm (coded 0) while run 9 had the least biodegradation yield at agitation 50 rpm (coded -1). This suggests that agitation influences biodegradation. Previous reports agree with this by indicating aeration influences the amount of dissolved oxygen in a marine system. As such, higher agitation means higher dissolved oxygen which increases the rate of aerobic biodegradation (Baker & Moore, 2000). This implies that the diesel-oil biodegradation of *C. cellulans* follows an aerobic biodegradation pathway.

Runs 11 and 16 had similar agitation speed and temperature, but initial diesel-concentration increased from 1% to 5% (v/v), respectively. High biodegradation yield (79.8%) was achieved with nitrate being 0.5 gL^{-1} at an initial diesel-oil concentration of 1%. Further increase in nitrate concentration by 2.0 gL^{-1} with higher initial diesel-oil concentration (5%) produced higher removal (88.4%). However, equal initial diesel-oil concentration (5%) with reduced nitrate concentration (0.5 gL^{-1}) removed the least diesel-oil (3.7%). This suggests that higher diesel-oil concentrations must be accompanied by a corresponding increase in nitrate concentration to achieve higher biodegradation yield. Additionally, equal nitrate concentration (0.5 gL^{-1}) with high initial diesel-oil (5%) led to the lowest biodegradation yield in run 9; and low initial diesel-oil (1%) gave rise to higher biodegradation yield in run 11. This could be caused by increased bioavailability of diesel-oil when concentrations are reduced or rise in toxicity when concentrations are high (Mohajeri et al., 2010). Previous studies have reported that the influence of oil concentration is higher than nitrates as the individual coefficient of nitrogen is positive while oil is negative. However, the interaction effect between them is negative (Mohajeri et al., 2010).

Past research utilizing *Cellulosimicrobium cellulans* for diesel-oil biodegradation under the un-optimized condition when an initial diesel-oil concentration of 2% in MSM at 32ºC for 10 days at 100 rpm yielded 64.4% degradation of diesel-oil (Nkem et al., 2016). The optimized conditions from runs 11 and 16 (initial diesel-oil concentration being 2% and 5% respectively) improved biodegradation by 15.4% and 24% respectively. This suggests the optimization of diesel-oil biodegradation by *C. cellulans* using Taguchi's improved biodegradation.

Several reports have indicated that *Cellulosimicrobium species* either harbor hydrocarbon-degrading genes or produce enzymes that enhance their ability to break down hydrocarbon. Reports have also illustrated that certain strains harbor dioxygenases and hydroxylases that preferentially degrade alkanes C_{10} to C_{30} (Aliakbari et al., 2014; Shaieb et al., 2015). This justifies their ability to be used in bioremediation experiments. Studies have also shown *C. cellulans* is capable of producing cellobiose dehydrogenase (E.C. 1.1.99.18) and cellobiohydrolase enzymes which aid degradation of polycyclic aromatic hydrocarbons (PAH's) and other petroleum hydrocarbons to glucose (Juhasz & Naidu, 2000). A novel strain of *C. cellulans* was reportedly capable of breaking down a 5-ring benzo (a) pyrene into less complex 1 to 4 ringed compounds, at the same time utilizing them as its sole carbon and energy source (Juhasz & Naidu, 2000; Qin et al., 2017). Reports have also illustrated that another *C. cellulans* strain produced a posteriori extracellular enzyme capable of fermenting organic compounds to produce carbon and energy (Vogt et al., 2005).

These reports establish the fact that *C. cellulans* possess metabolic characteristics for breaking down different hydrocarbon pollutants. This potentially defines its importance in

the development of bioremediation technique for mitigating environmental oil pollution. Efficient bioremediation can be achieved by successfully optimizing the environmental parameters which influence the biodegradation system (Palanisamy et al., 2014).

CONCLUSIONS

Biodegradation offers an ecologically friendly and cost-effective technique for mitigating oil pollution. In this investigation, indigenous hydrocarbon-degrading bacteria identified as *Cellulosimicrobium cellulans* were isolated from a tarball. When exposed to diesel oil, this bacterium removed 88.4% of diesel-oil hydrocarbons under optimized conditions with an initial diesel-oil concentration of 5% (v/v), NaCl concentration of 20 gL^{-1}, NH_4NO_3 concentration of 2 gL^{-1} in Minimal Salt Media at pH 7 after incubating in a shaker at 40^0C and 100 rpm for 5 days. Previous studies have indicated that these bacteria possess metabolic characteristics such as hydrocarbon-degrading genes or enzymes which accord them the ability to metabolize hydrocarbons as carbon and energy source. Tarballs contain high molecular weight hydrocarbons; hence, bacteria trapped within like *C. cellulans* are potentially hydrocarbon-degraders. They can be used to design a bioremediation technique with improved efficiency under optimized conditions. This method can then be used to clean up oil spills in both marine and freshwater environments.

ACKNOWLEDGMENTS

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