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Original Research

Biosurfactant Production by a Newly Isolated *Enterobacter cloacae* B14 Capable of Utilizing Spent Engine Oil

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Abstract

This work aims to investigate biosurfactant production by a bacterium capable of utilizing spent engine oil. The effects of pH and temperature on the growth of this bacterium on spent engine oil were determined. The bacterium was isolated from petroleum-contaminated soil. Based on 16S rRNA gene sequence, it was identified as *Enterobacter cloacae* with 99% identity. Growth of *Enterobacter cloacae* B14 on mineral medium containing 1% (v/v) spent engine oil was optimum when incubated at 30°C. The bacterium showed the ability to grow under a wide range of pH medium, whereas the highest specific growth rate (0.29 hr^{-1}) was obtained when grown under pH 7. Biosurfactant production by *E. cloacae* B14 was observed when grown in mineral medium containing 1% (w/v) glucose. Cell-free supernatant showed 42.2% emulsification activity (E24) against spent engine oil. Such activity was higher than some previous works and was obtained from a lower amount of biomass. Positive results from oil displacement, drop collapse and CTAB-methylene blue agar tests strongly indicated that strain B14 is an effective biosurfactant producer. Therefore, *Enterobacter cloacae* B14 has the potential for applications in bioremediation of oil-contaminated sites due to its biosurfactant production and growth on spent engine oil.

Keywords: biosurfactant; *Enterobacter cloacae*; spent engine oil; emulsification activity

Introduction

The release of spent engine oil into the environment is a common problem that seriously impacts ecological balance of ecosystems. Engine oil is comprised of many petroleum hydrocarbons, in particular polycyclic aromatic hydrocarbons (PAHs). These hydrocarbons are of great attention because of their toxicity, carcinogenicity and mutagenic nature [1]. The removal and recovery of spilled or leaked engine oil can be achieved by physical and chemical means, but these approaches are usually costly, labor intensive, and cause adverse effects to contaminated sites [2, 3]. Remediation of oil contamination by biological means or bioremediation then becomes a more effective approach. The ability of microorganisms to degrade hydrocarbons is involved in several factors, including catabolic enzyme activity, cell surface hydrophobicity and the nature of biosurfactant production [4]. Due to hydrophobicity property, bioremediation of hydrocarbons is usually

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limited by the low bioavailability of the contaminants [5]. The addition of surfactant compounds can increase the solubility of hydrophobic substrates into water, resulting in enhanced nutrient transport into microorganisms. Biosurfactants are promising active agents for removing toxic hydrocarbons from contaminated environments. Numerous studies have shown successful applications of biosurfactants for, e.g., oil removal from contaminated environments [6] and biodegradation of polycyclic aromatic hydrocarbons (PAHs) [7].

Biosurfactant is a group of diverse amphiphilic molecules including glycolipids, lipopeptides, lipoproteins, lipopolysaccharides and phospholipids [8, 9]. The advantages of biosurfactants over chemical surfactants is that biosurfactants require milder production conditions and have lower toxicity and higher biodegradability, and are more environmentally friendly [10]. Extensive studies have shown numerous groups of bacteria to be capable of synthesizing surfactants such as *Pseudomonas*, *Rhodococcus* and *Bacillus* [11]. Biosurfactants have great potential not only for bioremediation purposes, but also for applications in the food, cosmetic and pharmaceutical industries [12].

This work aims to investigate the ability of a spent engine oil-utilizing isolate to produce biosurfactants. Emulsification activity of the bacterial biosurfactant was assessed. Also, the ability of this strain to utilize spent engine oil as growth substrate was examined under varied environmental conditions such as pH and temperature.

Material and Methods

Isolation of Engine Oil-Utilizing Bacteria

Soil samples were collected near the chemical disposal site in a 10-year-old gas station in Khon Kaen, Thailand. The site has been continuously contaminated with spent engine oil and petroleum chemicals. The collection of soil samples was carried out using a sterile spatula to scoop up topsoil (depth of 0-6 cm) and put in a sterile 50 ml duran bottle until full. Three grams of soil samples were mixed with 10 ml of nitrate mineral salt (NMS) medium [13] in 100 ml-serum vials to which was added 1%(v/v) of filtered-sterile spent engine oil. Cultures were incubated at 30ºC with shaking at 150 rpm for 2 weeks. Soil suspension (1 ml) was transferred into fresh 10 ml NMS medium. The culture was incubated under similar conditions as above for another 2 weeks. Then 1 ml of culture was serially diluted and plated onto NMS agar. Plates were incubated with an open vial containing spent engine oil in order to let volatile compounds evaporate within a gas-tight jar. The jar was then incubated at 30ºC for 2-3 days or until colonies developed. Individual colonies were subcultured onto fresh plates for culture purification. Purity of single colonies was checked by Gram staining followed by observation under a microscope.

Identifying a Newly Isolated Bacterium

A single colony of the isolate was grown in 1 ml of nutrient broth (NB) medium. Culture was incubated at 30°C with shaking at 150 rpm overnight. Cell pellet was collected by centrifuging at 4,000 g for 3 min. Supernatant was then completely removed. Genomic DNA of the isolate was extracted using a bacterial DNA extraction kit (Vivantis, Malaysia). PCR amplification of the 16S rRNA gene was carried out using universal primers for bacterial 16S rRNA genes, 27f: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492r:5'-TACGGYTACCTTGTTACGACTT-3' [14]. PCR amplification conditions were carried out as standard protocol. PCR products were checked on 1% (w/v) agarose gel electrophoresis and then purified using AmbiClean kit (Vivantis, Malaysia). Purified PCR product was submitted to Macrogen (Republic of Korea) for sequencing. The 16S rRNA gene sequence of the isolate was analyzed by comparison with sequences on a Genbank database on NCBI. The phylogenetic relationship between 16S rRNA gene sequences was constructed using Maximum-likelihood method with 1,000 bootstraps on MEGA 6.0 software [15].

Temperature and pH Ranges for the Growth of the Isolate on Spent Engine Oil

To investigate temperature and pH ranges for the growth of the isolate on spent engine oil, cultures were grown in 100 ml serum vials containing 10 ml NMS medium (pH 7) with 1% (v/v) spent engine oil. Incubation temperatures were varied as follows: 20, 25, 30, 37 and 45ºC. To determine pH range for growth of the isolate, pH of NMS medium was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 using 1M HCl or 1M NaOH. The experiment was carried out in triplicate. Cultures were then incubated at 30ºC with shaking at 150 rpm for 5 days. Culture samples were taken every 24 hr for colony count on nutrient agar (NA) plates. Specific growth rates, defined as a rate of an increase in biomass during a defined period of time, were calculated as an increase in CFUs over time during the exponential phase of growth. The formula for calculating specific growth rate is shown below:

$$\text{Specific growth rate} = (\log Y2 - \log Y1)/(T2 - T1)$$

… where Y1 = CFU at the start of the exponential phase (CFU/ml)
Y2 = CFU at the end of the exponential phase (CFU/ml)
T1 = Time at the start of exponential phase (hours)
T2 = Time at the end of exponential phase (hours)

Preparation of Cell-Free Supernatant for Biosurfactant Production Tests

Inoculum of a bacterium was prepared in 500 ml flasks containing 100 ml NMS with 1% (w/v) glucose

added. Flasks were incubated at 30ºC, 150 rpm, overnight. Cultures (1% (v/v)) were transferred into 500 ml flasks containing 100 ml of fresh NMS with added glucose medium. This was carried out in triplicate. Culture sample from each flask was taken every 6 hours during 5 days of incubation to test for biosurfactant production. Cultures were centrifuged to collect cell-free supernatant for use in all biosurfactant tests (oil displacement, drop collapse, CTAB-methylene blue agar, and emulsification activity). Growth of a bacterium on NMS with 1% (w/v) glucose was determined by spectrophotometry. The optical densities were correlated to a biomass concentration using an equation where OD_{600} values are linear with the values of cell dry weight (mg/L). The OD_{600}-cell mass correlation was 1.0 OD_{600} = 464.1 mg/L cell dry weight.

Oil Displacement Test

The oil displacement test was carried out according to the method by Morikawa et al., 1993 and Ohno et al., 1993 [16, 17]. Ten microliters of spent engine oil was dropped on the surface of 40 ml of distilled water in a petri dish. Then, 10 µl of cell-free supernatant was added on the layer of oil. After 1 min, a clear zone was observed.

CTAB-methylene Blue Agar Test

The ability of a bacterium to produce anionic biosurfactant was examined on CTAB-methylene blue agar plate as described by Seigmund et al., 1991 [18]. Holes on the agars were created using 4 mm cork borer. Thirty microliters of cell-free supernatant was added into the hole. Plates were then incubated at 37ºC for 48 hr. Positive anionic biosurfactant production was determined by the production of a dark-blue ring around the holes.

Drop Collapse Test

Drop collapse test was carried out as described by Bodour et al. 1998 [19] with slight modification. Spent engine oil was thinly coated onto a petri dish. Five microliters of cell-free supernatant was added on the oil layer. The collapse of cell-free supernatant drops was observed after 1 min.

Determining Emulsification Activity

Emulsification activity of the biosurfactant was determined as emulsification index value according to the method by Cooper et al. 1987 [20] with slight modification. One ml of cell-free supernatant was mixed with 1 ml of spent engine oil by vortexing for 2 min. The mixture was left to rest at room temperature for 24 hr. The height of emulsion layer (cm) and the total height of the mixture (cm) were measured and calculated as emulsification index. The percentage of emulsification (emulsification index; E24) was calculated according to the following equation:

$$\text{Emulsification index (E24)} = (\text{Height of emulsion layer (cm)} / \text{Total height (cm)}) \times 100$$

Furthermore, emulsification activity against crude oil was investigated. The experiment was carried out in a similar manner, but using crude oil instead of spent engine oil.

Chemicals

All chemicals used in this study were of analytical grade. Crude oil was kindly provided by PTT Global Chemical Public Company Limited, Thailand. Spent engine oil, which was provided by a local garage in Khon Kaen, was obtained from vehicles as 1-year-pooled used engine oil.

Results and Discussion

Isolating and Identifying Engine Oil-Utilizing Bacteria

In order to isolate bacteria capable of utilizing spent engine oil, we carried out an enrichment technique. After culture purification, three different colonies were obtained. To confirm the growth of these bacteria on spent engine oil, individual colonies were transferred into liquid NMS medium added with 1% (v/v) spent engine oil. The cultures were incubated for 7 days. Positive growth was determined by an increase in OD_{600} after incubation. It appeared that 2 out of 3 cultures lack the ability to regrow in liquid medium containing spent engine oil, while another could. Therefore, the isolate that could regrow on NMS agar incubated with spent engine oil was selected for use in this study. We named this isolate B14.

Isolate B14 is a Gram-negative, rod-shaped bacterium. Identification of isolate B14 was based on 16S rRNA gene sequence comparison between its gene and those of its relatives. The 16S rRNA gene sequence of isolate B14 is clustered with the genes belonging to the genus *Enterobacter*. Sequence comparison indicated that isolate B14 is closely related to *Enterobacter cloacae* with 99% identity. Isolate B14 is then identified as *Enterobacter cloacae* B14. The phylogenetic relationship between the 16S rRNA gene sequence of isolate B14 and its relatives is shown in Fig. 1.

Temperature and pH Ranges for the Growth of the Isolate on Spent Engine Oil

To investigate the effect of pH and temperature on the growth of *E. cloacae* B14 on spent engine oil, the range of pH and incubation temperatures was varied. Fig. 2 showed that *Enterobacter cloacae* B14 could

Fig. 1. The phylogenetic relationship between the partial 16S rRNA sequence (1,410 bp) of isolate B14 and the sequences of the related strains obtained from Genbank database; the tree was constructed using maximum-likelihood method with 1,000 bootstraps using MEGA 6.0[18] (bootstrap values were indicated at branch points).

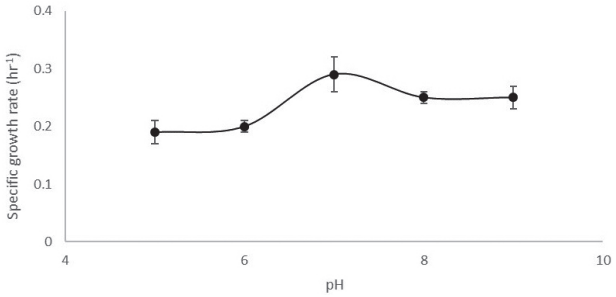

Fig. 2. Growth of *Enterobacter cloacae* B14 on NMS medium containing 1% (v/v) spent engine oil at different pH (error bars represent standard deviations of triplicate data).

grow on 1% (v/v) spent engine oil at a wide range of pH from pH 5 to pH 9. The highest specific growth rate (0.29 hr^{-1}±0.03) appeared when *E. cloacae* B14 was grown in the medium at pH 7. Moreover, *E. cloacae* B14 could grow on medium pH 7 with the least time spent during lag phase (24 hr) compared to its growth in other pH. This suggested that medium with pH 7 is optimal for growth of this strain. Therefore, any growth medium of *E. cloacae* B14 was adjusted to pH 7 for all investigations.

To investigate the range of temperatures in which *E. cloacae* B14 could grow, cultures containing 10 ml NMS pH 7 with 1% (v/v) spent engine oil were incubated at different temperatures for 5 days. Fig. 3 showed that the incubation temperature of 30ºC resulted in the highest specific growth rate of 0.29 hr^{-1}±0.02. It was found that strain B14 could not grow at 20ºC and 45ºC, while it could still grow at 25ºC and 37ºC. This suggested that the range of temperature allowing for growth of strain B14 is 25-37ºC, whereas 30ºC is the most desirable temperature.

The ability to grow and degrade engine oil was found as common among *Enterobacter* spp. [21, 22]. The ability of strain B14 to grow on spent engine oil suggested that it is perhaps capable of tolerating toxicity of hydrocarbon constituents in engine oil and/or utilizing those compounds for its growth. This leads to the possibility of applying this strain to bioremediate spent engine oil contamination *in situ*.

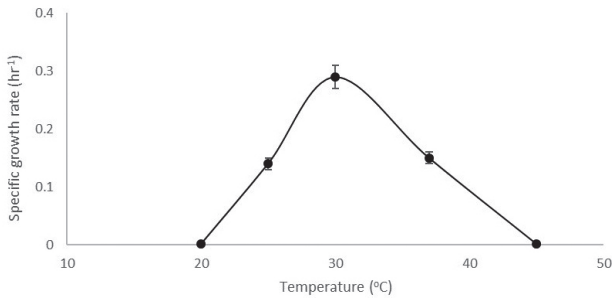

Fig. 3. Growth of *Enterobacter cloacae* B14 on NMS medium containing 1% (v/v) spent engine oil under incubation temperatures between 20-45ºC (error bars represent standard deviations of triplicate data).

Biosurfactant Production

Drop collapse, oil displacement and CTAB-methylene blue agar tests were performed every 6 hr during 5 days of incubation with cell-free supernatant samples obtained from cells grown on NMS with 1% (w/v) glucose. Culture medium without inoculated cells was used as control. The results obtained from these tests on cell-free supernatant of cells grown after 4 days, which showed the highest emulsification activity (E24 = 42.2%±4.2), are shown in Fig. 4(a-c). According to Figure 4a, a drop of cell-free supernatant collapsed on the surface of spent engine oil immediately after the addition, while a drop of negative control (NMS medium) stayed in circular shape. This suggested reduced surface tension between cell-free supernatant and oil layer. The result from oil displacement test

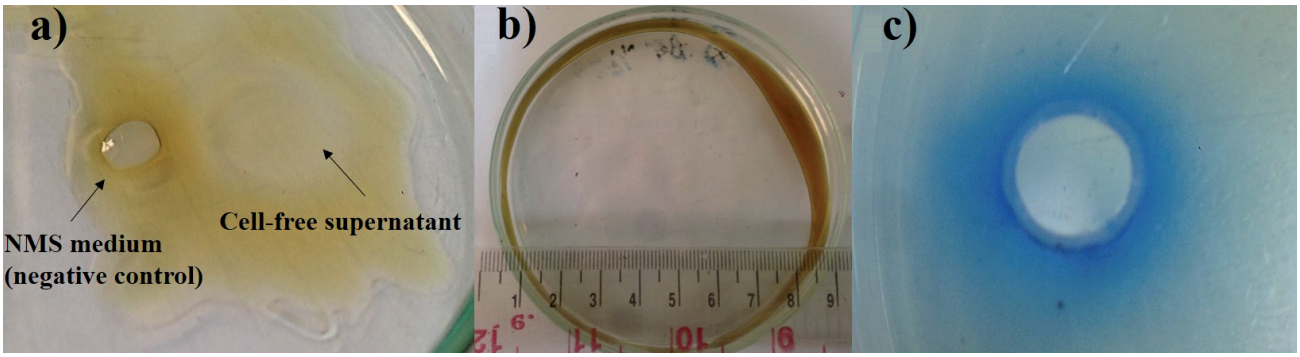

Fig. 4. Biosurfactant activity against spent engine oil of cell-free supernatant obtained from cultures grown on NMS with 1% (w/v) glucose for 4 days: a) drop collapse test of cell-free supernatant compared to NMS medium as a negative control, b) oil displacement test observed after 1 min. and c) CTAB-methylene blue agar test showing dark blue ring around the hole after 24 hr incubation.

(Fig. 4b) also suggested that cell-free supernatant could spread the oil drop on water. This phenomenon is a preliminary determination of the presence of surface active compounds and correlated to its concentration [23]. Likewise, the result from CTAB-methylene blue agar test showed strain B14 to be capable of producing anionic surfactant. This resulted in the formation of complex mixture between anionic surfactant and cationic methylene blue, which showed as a dark blue ring around the hole (Fig. 4c). Anionic biosurfactants have great advantages for enhanced metal desorption from soils by forming complexes with metals [24, 25]. Altogether, the presence of surface active compounds in *E. cloacae* B14 provide strong evidence supporting this strain having the ability to produce and release active biosurfactant into the culture medium.

Emulsification Activity Against Spent Engine Oil and Crude Oil

In order to investigate emulsification activity of cell-free supernatant against spent engine oil and crude oil at different growth phases, samples were collected from cultures grown in NMS with 1% (w/v) glucose for 5 days. Emulsification activity against spent engine oil and crude oil of cell-free supernatant collected from cells grown at different times is shown in Fig. 5.

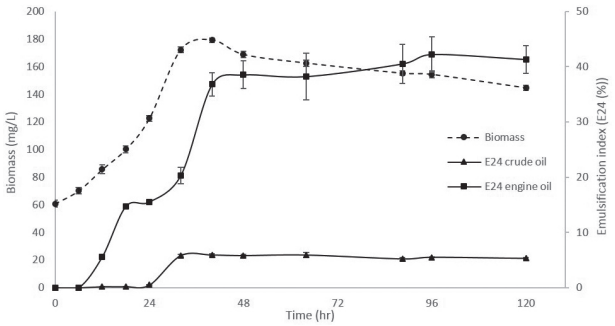

Fig. 5. Emulsification activity (E24) against spent engine oil and crude oil of cell-free supernatant obtained from cells grown on NMS with 1% (w/v) glucose (error bars represent standard deviations of triplicate data).

Enterobacter cloacae B14 could grow well (specific growth rate of 3.82 hr^{-1}±0.24) on NMS with 1% (w/v) glucose within 2 days of incubation. Emulsification activity of cell-free supernatant against crude oil was stably low throughout the growth of this strain. This is probably due to higher viscosity of crude oil, which obstructed the accessibility of biosurfactant into it. In contrast, emulsification activity (E24) against spent engine oil indicated that biosurfactant of strain B14 was greatly active during the stationary phase. The highest emulsification activity of 42.2%±4.2 was detected in cell-free supernatant of cells grown after 4 days of incubation. This suggested that the produced biosurfactant from *Enterobacter cloacae* B14 could effectively emulsify spent engine oil with a hydrophilic environment. Furthermore, we found that either the room temperature or 30ºC resulted in the same activity, so we selected room temperature to measure E24, as was generally used [20]. The ability to form stable emulsions is not very common [26, 27]. Such a feature in addition to the ability to reduce surface tension is very useful for bioremediation of petroleum-polluted environments and enhanced oil recovery [28, 29]. This is because a biosurfactant with such efficiency capable of increasing bioavailability of hydrophobic compounds to microorganisms possessing the ability to degrade petroleum. Using biosurfactant instead of chemical dispersant is advantageous because it is biodegradable and thus results in decreased toxicity after remediation.

Although a number of studies have reported various genera of bacteria possessing the ability to produce biosurfactants, reports indicating bacteria in the genus *Enterobacter* as biosurfactant producers are not very widespread. The results from this work become more evidence suggesting the potential of *E. cloacae* members to produce biosurfactant. The study of biosurfactant production from bacterial strains of *Enterobacter cloacae* has been an active area of research. For example, *Enterobacter* spp. produced biosurfactants by using a variety of growth substrates such as cane sugar, soybean

Table 1. Comparative data of the maximum emulsification activity (%E24), the amount of biomass (mg/l) at the time of maximum %E24 was yielded of *E. cloacae* B14 and those of other bacterial strains.

Microorganisms	Biomass (mg/L)	Emulsification activity (%E24)	References
Enterobacter cloacae B14	154.3	42.2	This work
Enterobacter sp. MS16	1,500	70.5	Jadhav et al., 2011 [30]
Enterobacter sp. LS1	1,310	44	Wong-Villarreal et al., 2016 [34]
Enterobacter sp. LS8	1,314	50	
Enterobacter sp. CG101	1,060	15	
Rhodococcus erythropolis	240	36	Pacheco et al., 2010 [35]
Rhodococcus rhodochrous	560	43	Suryani et al., 2016 [36]
Pseudomonas sp. MRBSIT1	NA	64.22	Jay et al., 2017 [37]
Achromobacter sp. PS1	NA	69.9	
Pseudomonas putida	366	52	Suryani et al., 2017 [38]
Bacillus atrophaeus 5-2a	330	58.34	Zhang et al., 2016 [39]
Bacillus sp. BK34(7)	NA	44-97	Gagelidze et al., 2016 [40]
Bacillus sp. BK10(4)	NA	42-97	
Bacillus sp. RT10	3,200	70	Varadavenkatesan et al., 2013 [41]
Bacillus subtilis ATCC21332	3,800	27	Pérez-Armendáriz et al., 2013 [42]
Klebsiella sp. UPAEP9	3,500	58	
Serratia marcescens	3,800	70	
Candida inconspicua	5,100	40	
Acinetobacter venetianus ATCC31012	7,000	30	
Yarrowia lipolytica	900	38.9	Fontes et al., 2010 [43]

NA: Not available

oil, molasses and sunflower oil cake [30]. Sarafzadeh and his colleagues showed successful techniques for enhanced oil recovery by using biosurfactant-producing *Enterobacter cloacae* [31-33]. In order to reduce biosurfactant production cost for industrial applications, some studies tried to use low-cost substrates such as agro-industrial wastes to enhance biosurfactant yield. *Enterobacter* spp. isolated from rhizospheric environment in Mexico was capable of utilizing glycerol and lactoserum for their growth and production of biosurfactants [34]. However, emulsification activity of the produced biosurfactant by *Enterobacter* sp. NCCP-291 was lower than 20%. The other two strains of *Enterobacter* spp. in that study performed emulsification activity of about 40-50%, which is approximately in a similar range to the activity obtained from *E. cloacae* B14 in this work. Emulsification activity (42.2%±4.2) of cell-free supernatant from strain B14 was higher than the activities obtaining from some previous works (see Table 1). Such activity was even higher than that of *Acinetobacter venetianus* ATCC 31012 (30%) and *Bacillus subtilis* ATCC 21332 (27%), which are the two well characterized oil-degrading and biosurfactant-producing strains [42]. More than 50% of the studied *Rhodococcus erythropolis* strains, reported in the other work, produced biosurfactants with activities less than 29% [44], which suggests a promising application of biosurfactant from *E. cloacae* B14 as an oil removal enhancer.

The biomass required to produce biosurfactant with emulsification activity of 42.2% by *E. cloacae* B14 is approximately 154.3 mg/L, which is quite a small amount compared to other works as shown in Table 1. The emulsification activity by *E. cloacae* B14 should significantly increase if the biomass further increases because biosurfactant concentration in a cell-free supernatant depends on the amount of biomass producing it. Therefore, it is worth further optimizing growth conditions for maximum production of biosurfactant by this strain. This can be achieved by, for example, optimizing the carbon-to-nitrogen ratio (C:N) in growth medium and changing carbon and/or nitrogen sources, since biosurfactant yield is significantly affected by the nature of carbon and nitrogen sources [45] – all of which are planned for the future.

Conclusions

Enterobacter cloacae B14 was isolated from soil contaminated with spent engine oil and petroleum chemicals collected near a gas station. The strain could grow on spent engine oil in a wide range of pH medium (pH 5-9) under moderate temperatures. Moreover, this strain could produce effective biosurfactant by using cheap substrate (glucose). Biosurfactant production and the ability of *E. cloacae* B14 to grow on spent engine oil suggests its promising application in bioremediation of a variety of environments where leakage of spent engine oil occurred, such as in marine environments, soil and underground water. This strain could potentially be used as a biodegradable surfactant agent, which would promote dispersal of spent engine oil in aqueous phase and increase bioavailability of hydrophobic substrate for other microorganisms to degrade oil. Optimization for higher biosurfactant production by *E. cloacae* B14 has been further studied by our group.

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Conflict of Interest

The authors declare no conflict of interest.

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