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Enhanced Petroleum Removal by Potent Biosurfactant Producer *Bacillus Subtilis* CC9 Strain Isolated from an Oil Field

Petrol Sahasından İzole Edilen Güçlü Biyosürfaktan Üreticisi *Bacillus Subtilis* CC9 Suşu ile Petrol Gideriminin Arttırılması

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ABSTRACT

This study aimed to enhance the removal of petroleum with potent biosurfactant producing bacterial strains (*Bacillius subtilis* CC9, *Enterobacter asburiae* WMB1, *Klebsiella oxytoca* Rizhao 536-1, and *Acinetobacter pittii* PgBE252) isolated from an oil field. The biosurfactant production was determined by different screening methods such as hemolytic activity, drop-collapse, oil-spreading, BATH, emulsification index and surface tension. While the presence of biosurfactant was detected with individual bacterial strain, *B. subtilis* CC9 strain was found to be quite effective on petroleum degradation (92%) with a high yield of biosurfactant production (0.95 ±0.05 g L^{-1}). The degradation rate constant and half-life period of *B. subtilis* CC9 strain were calculated as K = 2.533 day^{-1}, $t_{1/2}$ = 0.273. It has been clearly shown with GC/MS analysis that *B. subtilis* CC9 strain was effective on the removal of long chain *n*-alkanes (C_{31} - C_{35}) as well as short and medium chains. The FT-IR analysis also approved the presence of aliphatic and peptide moieties of biosurfactant extracted from *B. subtilis* CC9 strain. It is remarkably pointed out with this study that high yield of biosurfactant producing *B. subtilis* CC9 strain can be used in advanced petroleum bioremediation studies as an alternative to chemical surfactants.

Key Words

Bacillus subtilis, biosurfactant, petroleum, biodegradation.

ÖZ

Bu çalışmada, petrol sahasından izole edilen potent biyosürfektan üreticisi bakteriyel suşlar (*Bacillius subtilis* CC9, *Enterobacter asburiae* WMB1, *Klebsiella oxytoca* Rizhao 536-1, ve *Acinetobacter pittii* PgBE252) ile petrol gideriminin arttırılması amaçlanmıştır. Hemolitik aktivite, yayılma-daralma, petrol yayılma, BATH, emülsifikasyon indeksi ve yüzey gerilimi gibi farklı tarama yöntemleriyle biyosürfektan üretimi belirlenmiştir. Her bir bakteriyel suşta biyosürfektan varlığı tespit edilirken, *B. subtilis* CC9 suşunun biyosürfektan üretimi (0.95 ±0.05 g L^{-1}) ile beraber petrol degradasyonu (%92) üzerinde de oldukça etkili olduğu bulunmuştur. *B. subtilis* CC9 suşunun bozunma hız sabiti K = 2.533 day^{-1} ve yarılanma ömrü $t_{1/2}$ = 0.273 olarak hesaplanmıştır. GC/MS analizi ile *B. subtilis* CC9 suşunun uzun zincirli *n*-alkanların (C_{31} - C_{35}) yanı sıra kısa ve orta zincirli *n*-alkanların degradasyonunda da etkili olduğu açıkça gösterilmiştir. *B. subtilis* CC9 suşundan ekstrakte edilen biyosürfektana ait alifatik ve peptit kısımların varlığı FT-IR analizi ile doğrulanmıştır. Bu çalışma ile, yüksek verimle biyosürfektan üreten *B. subtilis* CC9 suşunun kimyasal sürfaktanlara alternatif olarak ileri petrol biyoremediasyon çalışmalarında kullanılabileceğine dikkat çekilmiştir.

Anahtar Kelimeler

Bacillus subtilis, biyosürfektan, petrol, biyoyıkım.

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INTRODUCTION

The increase in energy demand leads to more exploration, transportation, and use of petroleum, which causes accidents and oil spills in terrestrial and aquatic areas. Many technologies such as thermal evaporation, excavation and soil vapor extraction are used for cleaning petroleum-contaminated areas. However, bioremediation is the most effective treatment method which pollutants can be removed from the environment by converting them into less toxic or non-toxic compounds using indigenous microorganisms. Due to being fast and environmentally friendly, biosurfactants have been widely preferred among many treatment methods used in the bioremediation of petroleum-contaminated areas. They are surface-active compounds which are usually produced by various aerobic microorganisms like bacteria, yeasts, and filamentous fungi [1-4]. The biosurfactants are classified according to their chemical configurations such as glycolipids, lipopeptides, liposaccharides and oligosaccharides [5,6]. Due to its amphiphilic nature, biosurfactants not only increase the surface area of hydrophobic substance, but also change the property of the cell surface of microorganisms. These amphipathic compounds are important in terms of interaction of fluids with different polarity properties (oil/water), reducing surface and interfacial tension as well as ensuring the hydrophobic substrates entry into the cell. Biosurfactants act as a foaming, emulsion, and dispersing agent, which are attributed to their surface activities and have a significant impact on increasing the microbial cohesion, bioavailability, and biodegradation. They significantly support microbial growth and biodegradation by facilitating the passage of petroleum through the cell membrane [2,6]. Due to the advantages such as biodegradability, low toxicity, biocompatibility, high selectivity with specific functional groups and low sensitivity to extreme environmental conditions; biosurfactants are getting more interest day by day. Furthermore, the use of agricultural waste products as a substrate in the production process of biosurfactants causes a decrease in the production cost [6,7]. The biological surfactants have a great importance in cosmetics, pharmaceuticals, food industries, detergents, herbicide and pesticide formulations, pulp and paper, coal, textiles, ceramic processing, environmental applications, and oil industry with microbial enhanced oil recovery (MEOR) [6,8,9]. The use of biosurfactants in MEOR can be achieved by three main mechanisms (ex situ MEOR, microbial and nutrient enhancement) [10-12]. Microbial surfactants, which are effective in emulsifying and reducing the viscosity of petroleum, are considered as one of the most important MEOR mechanisms [13]. It is estimated that MEOR technology will be one of the most effective methods to be used in petroleum remediation.

Recently, the isolation of biosurfactant producing microorganisms, characterization, and the usage of biosurfactants in different remediation studies have been of great importance [9,14]. In this context, this study focused on increasing the petroleum degradation efficiency of microorganisms capable of producing biosurfactant isolated from an oil field.

MATERIALS and METHODS

Sample collection

The collected soil sample was transported from an oil field (Manisa, Turkey) through cold chain transport to Hacettepe University Environmental Biotechnology Laboratory, Ankara, Turkey within 24h.

Enrichment of soil samples

Bushnell Haas (BH) (Sigma-Aldrich, USA) medium containing 1% (v/v) of petroleum and 0.1% (w/v) of glucose (Merck, USA) were used for the enrichment of sample. 1 g of dry soil sample was dissolved in sterile 0.9% NaCl (pH 7.0) and left for 24 h. Enrichment process was carried out at 30 °C for 3 days in rotatory shaker (Miprolab, Turkey) [15].

Isolation and identification of bacterial strains

0.1 mL of enriched sample was inoculated on Nutrient Agar (NA) (Merck, USA) and incubation was carried out at 30 °C for 24 h. The isolated bacterial strains identified with 16S rRNA gene analysis.

Preparation of inoculum

The preparation of bacterial inoculum was carried out as described by Bilen Ozyurek and Seyis Bilkay before [16].

Biosurfactant production

2% (v/v) of individual bacterial culture was inoculated into BH medium supplemented with 1% (v/v) petroleum and 1% (w/v) yeast extract (Merck, USA) and incubated at 30 °C for a week in a rotatory shaker [17,18].

Screening assays for biosurfactant production

Following the incubation period, the bacterial growths were measured at $OD_{600\ nm}$ by UV spectrophotometer. Following this, the whole broth was centrifuged (4650 × *g*, Eppendorf 5810R, Sigma-Aldrich, USA) (10752 × *g*, Eppendorf 5417C) for 10 min. The supernatant was filtered through 0.45µm CA syringe filter (Millipore, Sartorius, USA), and were transferred to a clean test tube. Deionized water and Triton X:100 was used as control groups [19].

Hemolytic activity

Hemolytic activity was performed as described before by Banat [20] and Carrillo et al. [21]. A pure culture of each bacterial strain was inoculated on blood agar (Merck, USA) and incubation was carried out at 37 °C for 24 h. All plates were evaluated according to the hemolytic activities of bacterial strains.

Drop-collapse

The method was carried out as described before by Bodour et al. [22]. The change in drop size was detected within 1 min. [19].

Oil spreading

The method was performed as described before by Hassanshahian [23]. The diameter of the transparent zone was calculated after 30 s.

Bacterial adhesion to hydrocarbons (BATH)

The BATH assay was carried out as previously described by Rosenberg et al. [24]. The percentage of cell adherence to hydrocarbon was calculated according to Van der Vegt et al. [25].

Emulsification index (E_{24})

The emulsification index was measured as described previously by Plaza et al. [26]. The percentage of the emulsification index was calculated according to Shahaliyan et al. [27].

Surface tension

The surface tension measurement was carried out with Optical Tensiometer (Biolin Scientific, Attension Theta).

Petroleum degradation

The petroleum degradation of each bacterial strains was investigated according to Rahman et al. [28]. Precultured of all bacterial strains were inoculated into 50 mL BH medium supplemented with 1% (v/v) petroleum and 1% (v/v) Triton X:100 (Merck, USA) and 0.1% (w/v) yeast extract. The incubation period was carried out at 30 °C for a week in a rotatory shaker (Miprolab, Turkey). The experiments were carried out in triplicates.

Gravimetric analysis

The degradation of petroleum was calculated according to Bilen Ozyurek and Seyis Bilkay [29].

The kinetics of petroleum degradation

The petroleum degradation conforms the first-order reaction kinetics and was calculated according to Al-Hawash et al. [30].

Gas Chromatography/Mass Spectrophotometry (GC/MS) analysis

The GC/MS analysis was carried out according to Bilen Ozyurek and Seyis Bilkay [29] with using TRB-1 GCMS-QP-2020 (Shimadzu, Tokyo, Japan).

Biosurfactant extraction

The cell-free culture supernatant of *B. subtilis* CC9 strain (A1) was adjusted to pH 2.0 with 6N HCl (hydrochloric acid) and stored at 4°C for 24 h. Following the centrifugation, the supernatant extracted with an equal volume of chloroform: methanol (2:1) (v/v) (Merck, USA), was shaken and left for phase separation. Following the removal of an organic phase, the solvents were evaporated in fume hood for an hour (Hedlab, Turkey). It was suspended in double-deionized water (pH 7.0) and evaporated at 60°C (Memmert, Germany). The crude biosurfactant was quantified as gravimetrically (g/L). The biosurfactant was lyophilized and stored in the freezer at -20 °C [31,32].

Characterization of biosurfactant

The functional groups of the biosurfactant were characterized by Fourier Transform-Infrared Spectrum (FT-IR) (Thermo Fisher, Nicolet is50, USA).

RESULTS and DISCUSSION

Bacterial strains

The bacterial strains were identified as *Bacillius subtilis* CC9, *Enterobacter asburiae* WMB1, *Klebsiella oxytoca* Rizhao 536-1, and *Acinetobacter pittii* PgBE252 (Table 1).

Table 1. The identification of bacterial strains isolated from an oil field soil.

# of bacterial strains	Microorganisms	Reference strains	Similarity (%)
A1	*Bacillius subtilis*	MK720678.1 *Bacillius subtilis* CC9	99
A2	*Enterobacter asburiae*	KF901493.1 *Enterobacter asburiae* WMB1	99
A3	*Klebsiella oxytoca*	MN249579.1 *Klebsiella oxytoca* strain Rizhao 536-1	99
A4	*Acinetobacter pittii*	MH144322.1 *Acinetobacter pittii* PgBE252	99

A1: *Bacillus subtilis*, A2: *Enterobacter asburiae*, A3: *Klebsiella oxytoca*, A4: *Acinetobacter pittii.*

While *B. subtilis* is included in a group of Gram-positive microorganisms, the other bacterial strains are also Gram-negative. In the literature, *Pseudomonas sp., Bacillus sp., Acinetobacter sp., Klebsiella sp., Enterobacter sp, Salmonella sp*, *Ochrobactrum sp.* and *Mycobacterium sp.* are the most common bacterial strains isolated from hydrocarbon-contaminated areas [33,34]. Borah et al. [35] reported that *Serratia sp.,* was isolated from a petroleum-contaminated site. In particularly, Gram-negative bacterial strains such as *Pseudomonas sp.* and Gram-positive bacteria such as *Bacillus sp., Rhodococcus sp., Staphylococcus sp., and Exiguobacterium sp.* have also been found to be hydrocarbon tolerant [36]. Although Gram-negative bacteria are also known to be more tolerant to hydrocarbons than Gram-positive bacteria due to their highly impermeable outer membrane, Gram-positive bacteria also show high resistance to toxic effects of petroleum hydrocarbons. In this context, it is difficult to compare the hydrocarbon tolerance between Gram-positive and Gram-negative bacterial strains [37].

Biosurfactant production

Biosurfactants enhance the emulsification of hydrocarbons by converting them into smaller, soluble, and less toxic forms [35]. The production of biosurfactant by various microbial strains was also investigated by different screening methods [38,39]. Accordingly, the screening tests for biosurfactant production of four bacterial isolates (A1, A2, A3, A4) were shown (Table 2).

For the primary screening of biosurfactant presence, the association between the hemolytic activity and biosurfactant production was evaluated. Although, the lysis of blood agar was observed with all bacterial strains (A1, A2, A3, A4), hemolytic activity of A1 strain was more noticeable (Table 2). Varadavenkatesan and Ramachandra Murty [40] demonstrated that three of the microbial strains (RT2, RT7 and RT10:*Bacillus siamensis*) had an excellent hemolytic activity. However, different compounds, without surfactant molecules, produced by microorganisms can also cause hemolysis on blood agar. Therefore, the presence of biosurfactants should be supported by further screening methods. So, the surface-active properties of bacterial strains were also analyzed by drop-collapse and oil-spreading methods. These methods have advantages of being fast, easy, and not requiring special equipment [26]. In drop-collapse method, A1 strain collapsed the drop of petroleum within 1 min. However, the cell-free culture su-

Table 2. The screening tests for biosurfactant production.

# of bacterial strains	Hemolytic activity	[a]Drop-collapse	[b]Oil-spreading (mm)
A1	++	+++	++++
A2	+	++	++
A3	+	++	++
A4	+	++	+++

[a]Change in drop within one minute (+++) and after one minute (++).

[b]The diameter of transparent zone of ~5.0 mm (++++), ~4.0 mm (+++), ~2.0 mm (++).

A1: *Bacillus subtilis*, A2: *Enterobacter asburiae*, A3: *Klebsiella oxytoca*, A4: *Acinetobacter pittii.*

pernatants of A2, A3, A4 strains could collapsed the petroleum after 1 min (Table 2). Zhou et al. [9] showed that the culture supernatant of *Bacillus sp.* led to a collapse of droplet in microwell-plate. But this method is not as sensitive as oil-spreading on determining the low amount of biosurfactant presence [41,42]. While high level of biosurfactant presence was determined in A1 (~5.0 mm) and A4 (~4.0 mm), ~2.0 mm of zone formation was observed in A2 and A3 strains by oil-spreading method (Table 2). Nayariserri et al. [32] observed that *Staphylococcus sp.* and *Bacillus sp.* strains had positive results with a transparent zone of 1.8 mm and 2.5 mm, respectively. The transparent zone on petroleum surface was associated with surface activity of microorganisms [39]. In this context, it is obvious that there is a direct relationship between the diameter of the spread of oil and the biosurfactant concentration [43].

The emulsification abilities of bacterial strains (A1, A2, A3, A4) were investigated with different hydrocarbons such as *n*-decane, *n*-hexadecane and petroleum. As it was shown in Table 3, the highest emulsification index values of *n*-decane, *n*-hexadecane and petroleum were detected with *Bacillus subtilis* CC9 strain (A1). The highest and lowest of E_{24} values were obtained with *n*-decane and petroleum, respectively (Table 3). Because of the solubilization or emulsifying properties of biosurfactants differ according to hydrocarbons, the E_{24} values also vary for each hydrocarbon [4,44]. While *Bacillus sp.* and *Acinetobacter sp.* formed stable emulsions with *n*-decane, emulsion formed by *Acinetobacter* sp. was also small [45]. Cai et al. [46] observed that *Acinetobacter sp.* isolates emulsified 62.5% of hydrocarbons, whereas *B. subtilis* was emulsified only 40%. E_{24} value changes according to growth phase of bacteria, bacterial interactions, and the structure of hydrophobic compounds [47]. Furthermore, it also varies depending on the viscosity, droplet size, volume, temperature, and pH [48].

As it was determined that A4 strain showed more adherence to *n*-decan, *n*-hexadecane, and petroleum than other bacterial strains (A1, A2, A3) (Table 4). A1 strain showed maximum adherence to petroleum. While A2 and A3 strains showed maximum adherence to *n*-hexadecane, minimum adherence was seen to petroleum because of being a complex organic compound**.** Shoeb et al. [8] determined that bacterial strains showed maximum adhesion to *n*-hexane. Chettri et al. [49] was observed that bacterial cell surface hydrophobicity of *A. pittii* strain ABC showed high cell surface hydrophobicity (BATH 60.88 ± 3.5%) to crude oil. Nayariserri et al. [32] found that the highest cell adherence was observed with *Bacillus sp.* and *Staphylococcus sp.* and the least cell adherence was observed with *E. coli*.

Table 3. The E_{24} results obtained with *n*-decan, *n*-hexadecane, and petroleum.

	Emulsification index (%)		
# of bacterial strains	n-decane	n-hexadecane	petroleum
A1	71.42	67.86	53.5
A2	67.86	64.28	51.7
A3	69.00	66.67	59.2
A4	66.67	62.96	53.5

A1: *Bacillus subtilis*, A2: *Enterobacter asburiae*, A3: *Klebsiella oxytoca*, A4: *Acinetobacter pittii.*

Table 4. The BATH results obtained with *n*-decane, *n*-hexadecane and petroleum.

	BATH (%)		
# of bacterial strains	n-decane	n-hexadecane	petroleum
A1	52.2	55.1	59.2
A2	49.0	55.3	38.3
A3	51.5	57.0	50.7
A4	53.4	59.4	59.4

A1: *Bacillus subtilis*, A2: *Enterobacter asburiae*, A3: *Klebsiella oxytoca*, A4: *Acinetobacter pittii.*

Bio-removal of petroleum

Recently, increasing on the removal of petroleum hydrocarbons with bacteria and their microbial products has emerged as a promising technology to reduce the negative effects of pollutants [50]. Xu et al. [51] reported that 79 genera of bacteria have petroleum degradation capacity. Bacteria have co-evolved with toxic environments by developing various countermeasures against pollutants, such as increasing the degree of contact with hydrocarbon compounds through surfactants [52,53]. Especially, different indigenous bacterial strains can degrade the *n*-alkane fractions of petroleum hydrocarbons [51]. Accordingly, the petroleum degradation capacities and bacterial growths of biosurfactant producing strains (A1, A2, A3, A4) were detected (Figure 1). As it was clearly showed that maximum petroleum degradation (92%) was obtained with *B. subtilis* CC9 (A1) strain with its high capacity of hemolytic activity, drop-collapsing, oil spreading, emulsification index (E_{24} 53.5) and hydrophobicity (BATH 59.2) (Table 2, Table 3, Table 4).

Bacillus sp., isolated from different contaminated areas, has a great degradation capacity in the bioremediation process [54]. Zhuang et al. [55] and Calvo et al. [56] reported that *Bacillus naphthovorans* and *Bacillus pumilis* were effective on the degradation of naphthalene. It is known that hydrocarbon-degrading bacteria can increase the uptake of hydrophobic substrate by producing biosurfactants. Especially *Bacillus* genus has a capacity to produce the most effective surface-active compound in the lipopeptide structure. In a recent study, it was found that biosurfactant producing *B. cereus* BN66 strain was also effective on petroleum degradation [57]. The simultaneous occurrence of biosurfactant production and petroleum degradation clearly indicates that biosurfactant-producing strains use various petroleum components as carbon sources, enhancing the overall degradation process of petroleum [17,58]. Since the solubility of petroleum is the limiting factor for the degradation process, to facilitate oil uptake, most microorganisms release their extracellular biosurfactants, increasing the surface area of the substrates and emulsifying them. They form their own micro-environments and enhance emulsification by the release of certain compounds via quorum sensing [2].

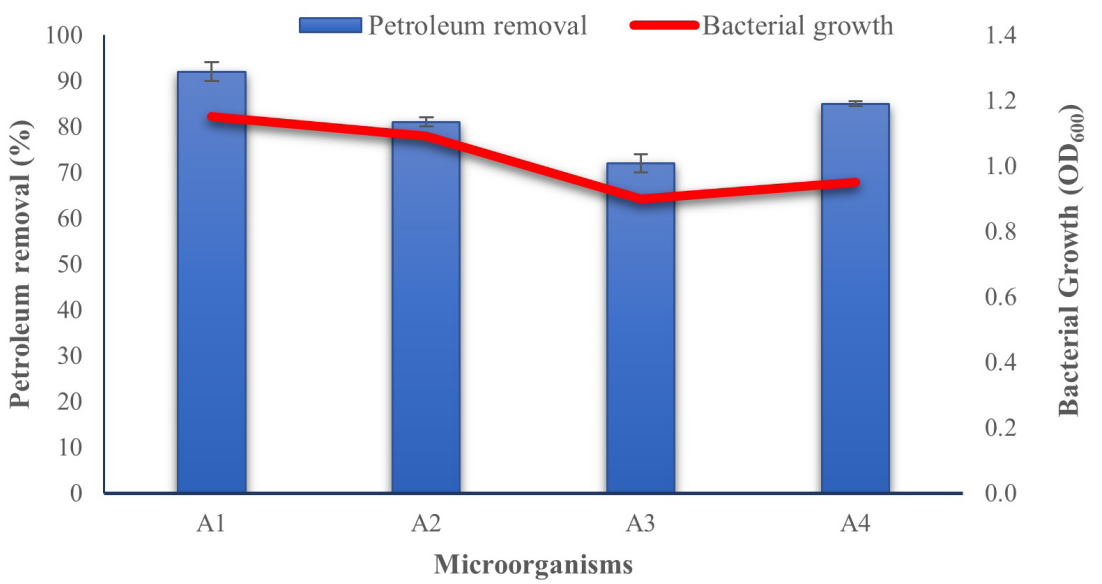

Figure 1. The removal capacities and bacterial growths of biosurfactant producing strains.

*Incubation period was carried out at 30 °C and 150 rpm for 7 days. Results are the average of three measurements. Standard deviations are shown on the graph.

A1: *Bacillus subtilis*, A2: *Enterobacter asburiae*, A3: *Klebsiella oxytoca*, A4: *Acinetobacter pittii.*

A. pittii strain (A4) achieved 85% of petroleum degradation with its high degrees of hydrophobicity (BATH 59.4) and emulsification index (E_{24} 53.5) (Table 3, Table 4, Figure 1). Chettri et al. [49] showed that the potent biosurfactant producer *Acinetobacter pittii* strain ABC could degrade 88% and 99.8% of the *n*-hexane within 5 and 10 days of incubation periods, respectively. While *Gordonia amaroe* produced biosurfactant within hexadecane-containing medium, the highest biosurfactant production was observed with starch-containing medium by *Klebsiella sp.* RJ-03 isolated from petroleum-contaminated area [38,59]. Even though the results of the emulsification index (E_{24} 51.7) and bacterial cell adherence to petroleum (BATH 38.3) of A2 strain were low, 81% of petroleum degradation was also detected. However, emulsification index (E_{24} 51.7) and bacterial cell adherence to the petroleum (BATH 50.7) were better with A3 strain than A2, the removal efficiency of petroleum was only 72% (Table 3, Table 4, Figure 1). This can be explained by the fact that petroleum contains a complex mixture of various hydrocarbons and the degradation tendencies of these microorganisms towards these components are also different [60]. Furthermore, the degradation efficiencies of microbial strains vary according to the metabolic capacities of microorganisms and their tolerance to the target pollutant [61]. Considering the degradation efficiencies of bacterial strains, the highest rate constant was shown with A1 strain (2.533 day^{-1}, $t_{1/2}$ = 0.273) followed by A2 (1.648 day^{-1}, $t_{1/2}$ = 0.420), A3 (1.272 day^{-1}, $t_{1/2}$ = 0.545) A4 (1.887 day^{-1}, $t_{1/2}$ = 0.367) in a decreasing order. The results are also consistent with studies in the literature [62,63].

According to biosurfactant screening assays (hemolytic activity, drop-collapse, oil spreading, E_{24} and BATH) and petroleum removal efficiencies of bacterial strains (A1, A2, A3, A4), *Bacillus subtilis* CC9 (A1) was found as the most effective strain on biosurfactant production. A decrease in surface tension and an increase in emulsifying activity were observed during the biosurfactant production process. Accordingly, it was detected that the secretion of biosurfactant started with the late-exponential growth phase reaching values of 0.95 ±0.05 g L^{-1} at the stationary growth phase (Figure 2).

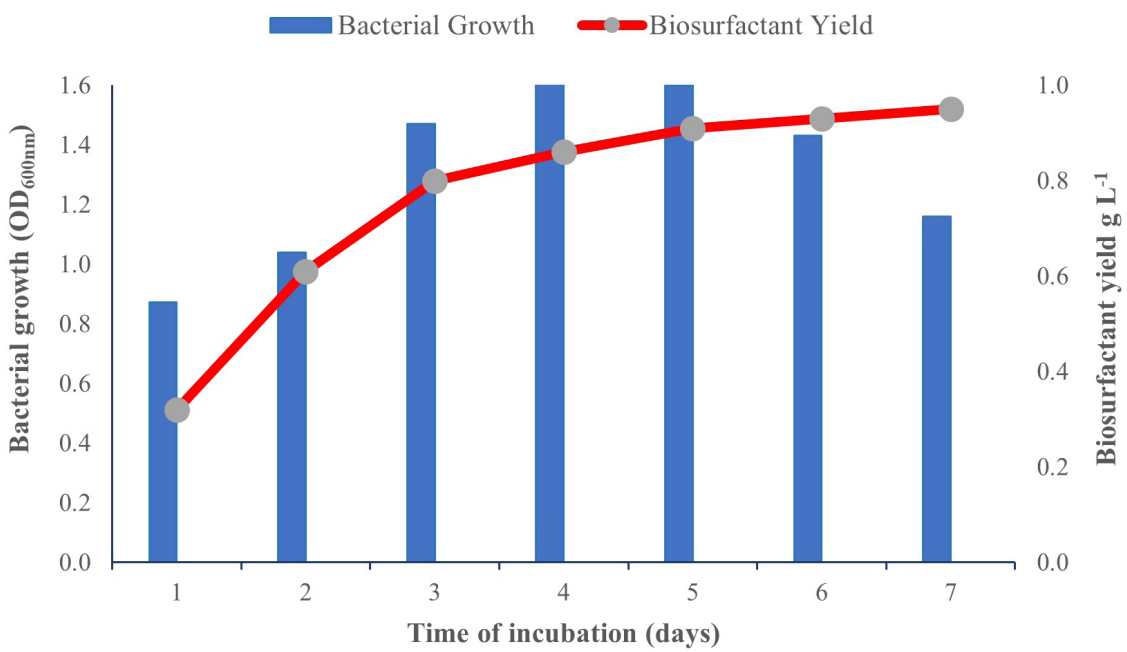

Figure 2. Time-course of biosurfactant production by *B. subtilis* CC9 strain.

*Incubation period was carried out at 30 °C and 150 rpm for 7 days.

Since biosurfactants are secondary metabolites, the maximum production occurs at the stationary growth phase [64]. The surface tension of culture was reduced to 29.5 ±0.3 mN m^{-1} which signifies the production of biosurfactant. Pereira et al. [65] showed that low surface tension values (<35 mN m^{-1}) are directly associated with high yield of biosurfactant production. The yield of partially purified biosurfactant of A1 strain was 0.95 ±0.05 g L^{-1} at the end of 7 days of incubation period. Faria et al. [66] obtained that *B. subtilis* LSFM-05 strain was produced 0.93 ± 0.4 g L^{-1} of biosurfactant with 5% of raw glycerol as a carbon source. Barros et al. [67] and Davis et al. [68] reported the high yield of biosurfactant production (1.67 g/L and 2.4 g/L) with the use of easily metabolized glucose and potato as carbon sources.

GC/MS analysis

The GC/MS analysis indicated the *n*-alkane constituting the content of petroleum (Table 5). According to the results of GC/MS analysis, the removal of *n*-alkane fractions of petroleum by *B. subtilis* CC9 (A1) strain was shown (Figure 3). Accordingly, it was detected that the *n*-alkanes in the range of C_{10} – C_{14} were removed above 90%; C_{15} - C_{21} and C_{31} - C_{35} were removed above 80%; C_{22}, C_{27} - C_{30}, C_{36} were removed above 70%; C_{23} - C_{26} were removed above 60%, respectively. It is clearly stated that the removal of short chain *n*-alkane fractions by potent strain was about 90%. The results were similar to Christova et al. [57] and Tanzadeh et al. [18]. Al-Dbahi et al. [69] showed that *B. subtilis* strain was effective on the removal of *n*-alkanes fractions in the range of C_9 - C_{14} and C_{15} - C_{20}. Moreover, *B. subtilis* CC9 strain was also effective on the degradation of long chain *n*-alkanes (C_{31} - C_{35}) as well as short and medium chains (Figure 3). However, *B. cereus* J3 strain was more effective on removal of short chain (C_9-C_{13}) and medium chain (C_{13}–C_{25}) *n*-alkanes than that long chains' (C_{25} - C_{36}) [18]. Additionally, Dwivedi et al. [70] indicated with GC/MS analysis that *Bacillus methylotrophicus* ALK-16 degraded 8.11% of petroleum. When C_{20}, C_{23}, C_{24}, C_{26}, and C_{29} *n*-alkanes were degraded as 4.44%, 19.24%, 20%, 3.92%, 4.86, C_{30} and C_{34} *n*-alkanes were only degraded as 11.84% and

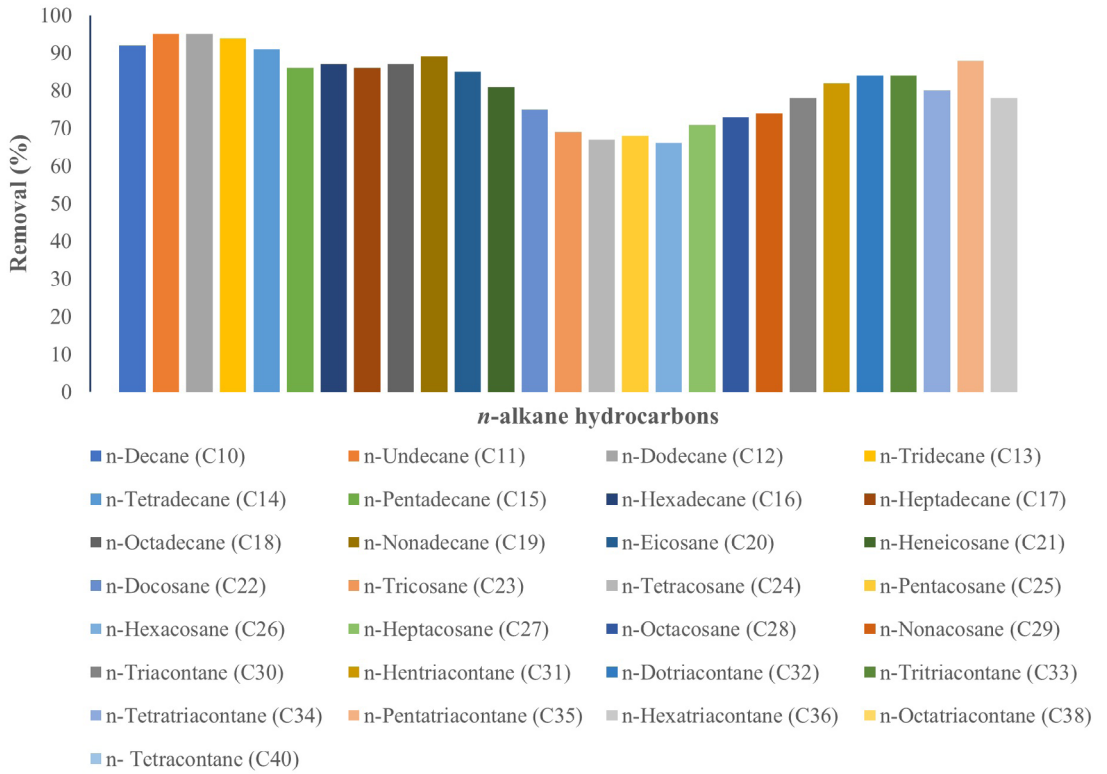

Figure 3. The removal of *n*-alkane fractions of petroleum by *B. subtilis* CC9 strain.

*Incubation period was carried out at 30 °C and 150 rpm for 7 days.

7.60%. Considering the similar studies in literature, it can be stated that potent biosurfactant producer *Bacillus subtilis* CC9 strain is effective on the removal of short, medium, and long chain *n*-alkanes that constitute the content of petroleum.

Characterization of biosurfactant

The functional groups and bond types of partially purified biosurfactant produced by potent *Bacillus subtilis* CC9 strain (A1) were analyzed by FT-IR (Figure 4). The FT-IR spectrum of biosurfactant were evaluated according to the similar studies in the literature [65,66,71,72]. The peaks at 3296 to 3059 cm^{-1} were due to the N-H stretching mode. The presence of an aliphatic chain ($-CH_3$, $-CH_2$) was clearly showed by absorbing peaks from 2926 to 2867 cm^{-1} and 1454–1205 cm^{-1}. Because of the stretching mode of the CO–N bond and the deformation mode of the N–H bond combined with the C–N stretching mode, peptide components were absorbed at 1648 cm^{-1} and 1535 cm^{-1}. The absorbing bands in 1725 and 1064 cm^{-1} are due to the carbonyl group and C-H bonding, respectively. The structure of biosurfactant was characterized as lipopeptide due to the presence of the aliphatic and peptide moieties.

Table 5. GC-MS analysis of *n*-alkane fractions of petroleum.

n-Alkane fractions	Initial day	7th day
n-Decan (C10)	151.3	12.2
n-Undecane (C11)	219.4	11.4
n-Dodecane (C12)	239.0	12.5
n-Tridecane (C13)	246.7	14.5
n-Tetradecane (C14)	227.3	20.9
n-Pentadecane (C15)	258.7	37.2
n-Hexadecane (C16)	225.1	29.4
n-Heptadecane (C17)	249.6	33.7
n-Octadecane (C18)	195.6	24.9
n-Nonadecane (C19)	213.1	22.8
n-Eicosane (C20)	187.0	28.5
n-Heneicosane (C21)	193.6	36.8
n-Docosane (C22)	198.4	50.0
n-Tricosane (C23)	222.5	69.1
n-Tetracosane (C24)	235.8	78.5
n-Pentacosane (C25)	239.2	77.5
n-Hexacosane (C26)	181.7	61.2
n-Heptacosane (C27)	149.6	43.7
n-Octacosane (C28)	111.4	30.1
n-Nonacosane (C29)	93.8	22.3
n-Triacontane (C30)	70.1	15.1
n-Hentriacontane (C31)	67.8	12.3
n-Dotriacontane (C32)	56.7	9.28
n-Tritriacontane (C33)	49.2	7.70
n-Tetratriacontane (C34)	40.8	8.95
n-Pentatriacontane (C35)	30.7	<3.78
n-Hexatriacontane (C36)	12.3	<2.81
n-Octatriacontane (C38)	<5.73	<5.73
n-Tetracontane (C40)	<6.93	<6.93

Note: The values are given as ppm (parts-per million). The results are shown the remaining *n*-alkanes at the end of 7th days of incubation period.

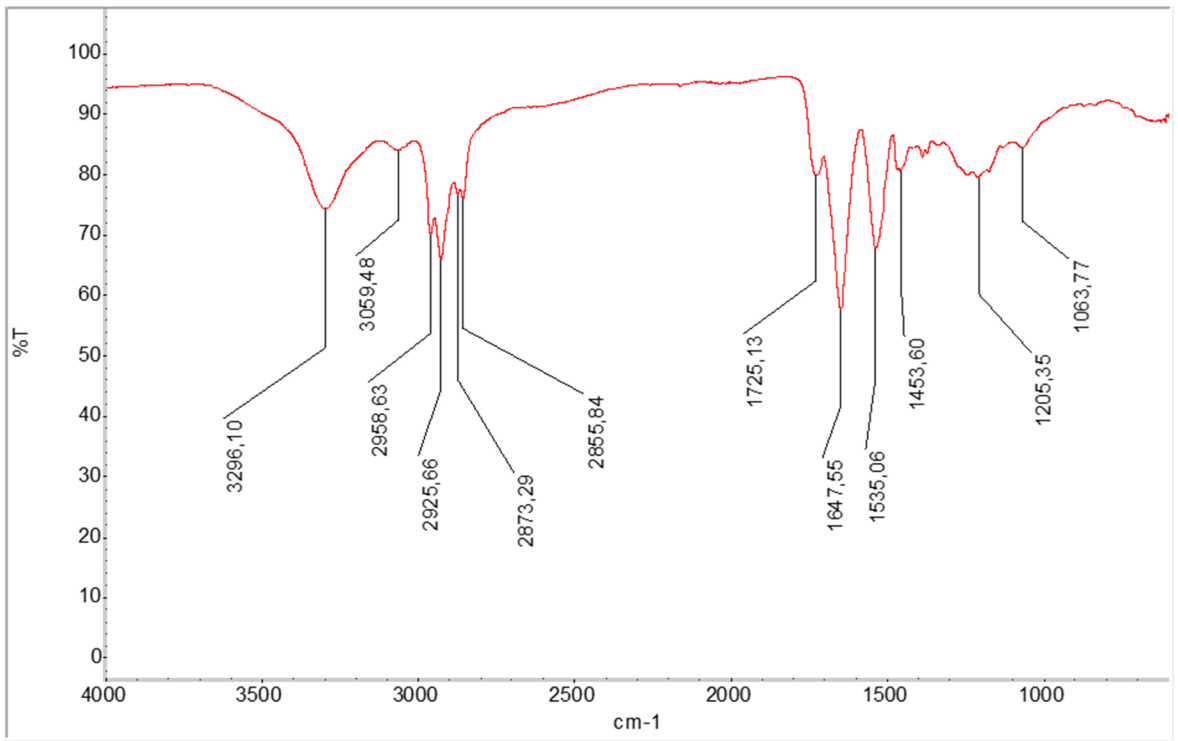

Figure 4. The FT-IR spectrum of biosurfactant extracted from *B. subtilis* CC9 strain.

CONCLUSION

B. subtilis CC9 (A1) strain was quite effective on petroleum degradation (92%) with a high yield of biosurfactant (0.95 ±0.05 g^{-1}) production. Moreover, the strain was also effective on the removal of long chain *n*-alkanes (C_{31} - C_{35}) as well as short and medium chains. The presence of aliphatic and peptide moieties of biosurfactant was also approved. It is clearly emphasized with this study that *Bacillus subtilis* CC9 strain has a great capacity to produce high yield of biosurfactant, which can be increased with further optimization studies, can replace the chemical analogues, and offers ecological advantages on petroleum bioremediation.

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