Purification and characterization of a surfactin-like biosurfactant produced by *Bacillus velezensis* KLP2016 and its application towards engine oil degradation

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Research

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Abstract

Engine oil used in automobiles is a threat to soil and water due to recalcitrant properties of its hydrocarbons. It pollute surrounding environment which affect both flora and fauna of earth. Microbes are able to degrade hydrocarbons containing engine oil to utilize as a substrate for their growth. Our results demonstrated that Bacillus velezensis KLP2016 (Gram +ve, endospore forming; Accession number KY214239) cell-free broth recorded an emulsification index (E 24 %) from 52.3% to 65.7% against different organic solvents, such as benzene, pentane, cyclohexane, xylene, n -hexane, toluene and engine oil. The surface tension of the cell-free broth of B. velezensis grown in Luria Bertani broth at 35°C decreased from 55 to 40 mN.m -1 at critical micelle concentration 17.2 µg/mL. The active biosurfactant molecule of cell-free broth of Bacillus velezensis KLP2016 was purified by Dietheylaminoethyl-cellulose and size exclusion chromatography, followed by HPLC (RT=1.130), UV-vis spectrophotometry (210 nm) and thin layer chromatography (R f =0.90). Purified biosurfactant molecular weight was found ~1.0 kDa, on the basis of Electron Spray Ionization-MS. A concentration of 1980×10 -2 parts per million of CO 2 was trapped in a KOH solution after 15 days incubating the bacterium in Luria Bertani broth containing engine oil (1%). Results suggests that bacterium Bacillus velezensis KLP2016 may be a promising solution to the engine oil pollution problem with achieving a bioactive biosurfactant molecule for further eco-friendly application(s).

Introduction

Environmental pollution is currently a serious problem. Engine oil used in automobiles is a very hazardous and toxic pollutant for the soil. Used engine oil that is spilled or wrongly discarded may enter storm water runoff and eventually enter into water bodies and adversely affect the environmental health of receiving water bodies and its flora and fauna [1]. Oil spills into the sea is an emerging issue, harming marine flora and fauna [2]. To save the flora and fauna of the water bodies, treatment of engine oil (main polluting agent) is very important and is the demand of the time. Different types of chemicals are used in petroleum industries for various operations, mainly in oil recovery [3]. The process releases contaminants and causes water contamination, posing health risks to living beings [4–5]. Recalcitrant hydrocarbon (present in engine oil) degrading microbes of genus Bacillus produce biosurfactants of a diverse chemical nature and molecular size, with different active role(s). These microbial biosurfactants have the capability to degrade hydrocarbons enhancing bioavailability of these hydrophobic organic compounds present in engine oil [6]. In recent years, biosurfactants have received attention due to their capacity to degrade hydrocarbons properties like biodegradability, ecological acceptability and low toxicity [7]. Biosurfactants also called green surfactants which play a key role in agriculture and these compounds (hydrophobic), which makes biosurfactants a good agent for cleaning up the environment [6]. These biosurfactants operate their work by emulsifying the non-aqueous phase liquid contaminants and increase their solubility. These features of biosurfactants facilitate contaminants export from the solid phase and allow the microorganisms adsorbed on the soil particles to access and remove the contaminant molecule [8–10].

They also have the capacity to generate a renewable source of energy from cheaper substrates [11]. Biosurfactants produced by microbial genera have been studied extensively for their role as potent surfactants, such as in the degradation of engine oil [12]. They also help in reducing the load of environment polluting agents [3]. Biosurfactants bioactive molecules are especially important due to their unique structural and biological active properties and applications [13]. Surfactin and iturin are already known to be efficient biosurfactants for degrading hydrocarbons containing engine oil [14]. The important properties that make these biosurfactants special molecules are biodegradability, lower toxicity, bioavailability, high foaming, high selectivity and specific activity at extreme temperature, pH and salinity, making them special active biomolecules [15–16].

Here, the objective of this study is to report a cost-effective solution of engine oil pollution via engine oil degradation by *B. velezensis* KLP2016 (Gram + ve, endospore forming; Accession number KY214239) bacterial strain efficiently and producing a biosurfactant in a suitable same medium. The biosurfactant was further purified and characterized, and engine degradation was investigated by GC-MS [17]. This study will be useful from both the environmental and the industrial point of view, and the outcomes of this study will be useful for cleaning environment via engine oil degradation and also will be helpful in reducing soil water contamination [18].

Materials And Methods

Production of biosurfactant by *B. velezensis* KLP2016 cells

A fresh loopful culture of *B. velezensis* KLP2016 was inoculated in 100 mL of Luria bertani broth and incubated at 200 rpm under shaking at 30ºC to get 1.0 OD of cells at 620 nm. Bacterial growth was monitored regularly and 1.0 OD cells obtained at 9 hrs incubation. For production of biosurfactants, 1000 mL of LB broth was prepared in which 4 % (v/v) of bacterial inoculum (1.0 O.D cells) was inoculated and the flasks were incubated for 72 h at 30ºC at 200 rpm. After incubation, the culture broth was centrifuged at 10,000 rpm for 10 min at 4ºC [18]. Biosurfactants containing supernatant/ cell free broth was collected for further experiments.

Measurement of emulsification index, surface tension and critical micelle concentration

Biosurfactant containing culture broth was evaluated by measuring the emulsification index (E_{24}%) using various chosen organic hydrocarbon compounds (benzene, pentane, cyclohexane, xylene, *n*-hexane, toluene and engine oil) as the substrate. In a test tube, 1.5 mL of each hydrocarbon was added to 1.5 mL *B. velezensis* cell-free broth. This combination was mixed by using a vortex for 2 min, and the content was left undisturbed for 24 h. The percentage of the emulsification index (E_{24}%) was calculated by using the following equation [12].

$$E_{24}(\%) = \frac{\text{Total height of the emulsified layer (mm)}}{\text{Total height of the liquid layer (mm)}} \times 100$$

The surface tension of cell-free broth of *B. velezensis* strain was determined by the drop weight method at 25°C and 35°C temperatures; and Luria bertani broth and Minimal Salt medium (MSM) [19]. Cell-free broths of *B. velezensis* KLP2016 grown in Luria Bertani (LB) and Minimal Salt Medium (MSM) for 72 h incubation, were used to measure the surface tension. The uninoculated LB and MSM broth (g/l) (KH_2PO_4, 1.4; Na_2HPO_4, 2.2; (NH_4) SO4, 3; $MgSO_4$, 0.6; NaCl, 0.05; yeast extract, 1; $CaCl_2$ 0.02) was taken as negative control. Critical micelle concentration (cmc) is the concentration of biosurfactant above which micelle form and further no reduction in surface tension occurs. The surface tension (γ) and critical micelle concentration (cmc) was calculated by using the following equation [19];

$$\gamma = \frac{\gamma^0 n^0 \rho}{n p^0}$$

Where γ^0 is surface tension, n^0 is number of drops and ρ^0 is density of uninoculated broths, while γ is surface tension, n is number of drops and ρ is density of cell-free fermentation broth.

Purification and identification of active compound extracted from culture broth of *B. velezensis* KLP2016

Ammonium sulfate (NH_4SO_4) mediated protein precipitation and dialysis

The cell-free broth was introduced with 0-20, 20-40, 40-60, 60-80 and 80-100% saturation of NH_4SO_4 at 4°C, further mixed and kept overnight at 4°C. Thereafter, the precipitates were deposited after centrifugation at 12,000 rpm for 15 min. The precipitates were reconstituted in 1 mL of 20 mM sodium phosphate buffer at pH 7.5 and checked for emulsification activity against engine oil. One unit of emulsifying activity was explicated as the quantity of emulsifier that yielded an absorbance (600 nm) of 0.1 in the assay mixture [20].

Ion exchange chromatography

The DEAE cellulose packed glass column (height 10 cm; diameter 1.5 cm) was equilibrated with 20 mM sodium phosphate buffer (pH 7.5) after activation by 0.5 M NaOH. Five mL of dialyzed biosurfactant preparation (4.0 mg protein) was loaded on the matrix in the column [21]. Column was equilibrated with 20 mM sodium phosphate buffer (pH 7.5). Unbound proteins were eluted with low ionic strength buffer (sodium phosphate buffer; pH 7.5) at a flow rate of 1 mL/min and discarded. The bound biosurfactant molecules eluted with the stepwise gradient of 0.5 M NaCl, 1 M NaCl and 1.5 M NaCl in sodium phosphate buffer (pH 7.5; 20 mM), respectively [22]. Emulsification activity and A_{280} values were evaluated against the engine oil.

Size exclusion chromatography

Sephadex G-25 packed matrix was washed off with several column volume of 20 mM sodium phosphate buffer (pH 7.5). Pooled active fraction of the DEAE was loaded on the bed surface of Sephadex G-25

column and eluted with the sodium phosphate buffer (20 mM; pH 7.5) and fractions were collected [21]. Absorbance at 280 nm and emulsification activity was evaluated against the engine oil. Active fractions were further checked with UV-vis spectrophotometer and TLC, as detailed below.

TLC and UV-VIS spectrophotometry

The fractions obtained from size exclusion chromatography, were analysed and mixed on the basis of their OD. A solvent system of chloroform: methanol: water (39:15:3; v/v) was prepared, and 5 µl sample of mixed biosurfactant fractions was applied at the point of origin of the TLC plate [23]. Lipid moiety of the molecule was detected by TLC plate sprayed with water and thereafter kept for drying. The R_f values of the biosurfactant spot on the TLC plate were evaluated using the following formula and results recorded accordingly.

$$Rf = \frac{Distance\ travelled\ by\ the\ solute\ (cm)}{Distance\ travelled\ by\ the\ solvent\ (cm)}$$

The purified biosurfactant was also analysed for ultraviolet absorbance spectrum [24] at range of 190-800 nm (UV-VIS Spectrophotometer, CARY, VARIAN).

High performance liquid chromatography analyses

The presence of biosurfactant in the purified molecule was confirmed by HPLC using an HPLC pump (Waters, USA) by a reverse phase column (Lichrosorb C18-5 µm; Merck, Germany) and 2998 photodiode assay detector [18]. The mobile phase contained acetonitrile (ACN): ammonium acetate (10 mM) in the ratio of 40: 60 (v/v) and mobile phase flow rate was adjusted at 2 mL / min. Biosurfactant sample 5 µl was injected each time and analysed at 254 nm wavelength with comparing standard biosurfactants, *i.e.*, surfactin and iturin.

ESI-MS of purified biosurfactant

A mass spectrometer (Q-TOF micro Waters 2795 UK) was used to find the molecular weight of the purified biosurfactant. The conditions for used MS were temperature source, 100^{o}C; 3000 V in positive mode; capillary voltage, cone voltage, 30 V; current source, 80.0 A and capillary voltage of 7.0 V in positive mode [21]. About 20 µl of purified biosurfactant was injected into the MS and gently ionized with CH_3OH and H_2O (80:20) using electrospray (ESI) with flow rate of 1.0 mL/min. ESI-MS results were compared with the authentic surfactin biosurfactant molecule to identify the molecular mass of the purified biosurfactant of *B. velezensis*.

Hydrocarbon degradation activity of *B. velezensis* KLP2016

Biodegradation of engine oil (K 15W-40) by *B. velezensis* KLP2016 in a biometric system

For the biodegradation of engine oil, 5% (v/v) starter inoculum of 7 h of *B. velezensis* KLP2016 culture was inoculated in the 250 mL capacity sterilized flasks each containing 100 mL MSM and LB broth. Hydrocarbon substrate (K 15W-40 Engine oil) was added at 1% (v/v) concentration in each of the sterilized flasks. One test tube containing fresh KOH (10 mL; 0.05 M) was placed in each of the flasks, and these flasks were incubated at 30°C under shaking (100 rpm) from 5 to 20 days. Inoculated and uninoculated broths were observed for their absorbance (A_{600}) and CO_2 content at 5-day intervals up to 20 days. The CO_2 gas trapped in the KOH solution was titrated by introducing 100 µl of barium chloride (w/v; saturated) and three drops of phenolphthalein with 0.05 M HCl until the appearance of the end point as the colourless solution. The difference in millilitres of HCl used to titrate KOH containing solution of control (placebo) and *B. velezensis* KLP2016 inoculated media was converted into ppm of fixed carbon dioxide as per method [17, 25]. Hydrocarbon degradation of engine oil facilitated by *B. velezensis* KLP2016 was also confirmed by Gas chromatography-mass spectrometry (GC-MS) analysis of the engine oil treated with bacterial cells.

Hydrocarbon analysis by GC-MS of K 15W-40 engine oil treated with *B. velezensis* KLP2016

In order to analyse the hydrocarbon products of engine oil broken down by *B. velezensis* KLP2016, the culture broth (5, 10, 15 and 20 days) was centrifuged at 10,000 rpm, at 4°C for 10 min. From the supernatant, the upper layer was collected, filtered with syringe filter (0.22 µm) and the filtrate was analysed using GC-MS to evaluate engine oil products. The GC-MS analyses were performed using an MS5973 spectrometer with a ULBON HR-1 column (25 mm x 50 mm), with thickness of 0.25 micron, 1 mL/min flow rate of the sample injected (5 µL) with the carrier gas helium, ion source temperature 230^{o}C at 18.5 psi pressure and 20% split ratio [25]. Results were observed and recorded accordingly.

Statistical analysis: All methods are statistically analysed.

Results

Emulsification index, surface tension and critical micelle concentration of biosurfactant containing cell-free broth of *B. velezensis*

An emulsification index of ≥30% was considered as significant emulsification activity. The reported results showed that *B. velezensis* cell-free broth showed E_{24} % marked 65.7%, 59.0%, 56.1%, 61.0%, 52.3%, 65.2% and 56.2% with benzene, pentane, cyclohexane, xylene, *n*-hexane, toluene and engine oil, respectively. The surface tension of biosurfactant containing cell-free broth at 35^{o}C was reduced from 55 $mN.m^{-1}$ to 40 $mN.m^{-1}$ at 17.2 µg/mL (cmc) and surface tension at 25°C was reduced from 62 $mN.m^{-1}$ to 48 $mN.m^{-1}$ at 17.4 µg/mL of critical micelle concentration by using LB broth as a medium for cell-free broth production (Fig 1a). The surface tension at 35^{o}C was reduced from 58 $mN.m^{-1}$ to 43 $mN.m^{-1}$ at 17.6 µg/mL (cmc) and at 25^{o}C, surface tension was reduced from 65 $mN.m^{-1}$ to 50 $mN.m^{-1}$ at 18.1 µg/mL (cmc) of cell-free broth of *B. velezensis* grown in MSM broth (Fig. 1b).

Purification of biosurfactant by DEAE- cellulose and size exclusion chromatography

On the basis of emulsification activity against the engine oil, an ammonium sulphate cut in the range 20-40% showed 24.0±1.54 U/mL emulsification activity or ~60% E24%, was selected for further purification. A total of 15 fractions were collected (1.5 mL each) by elution with 0.5 M, 1 M and 1.5 M NaCl (Fig. 2a). Fractions that were eluted were checked for emulsification activity against engine oil, and the maximum activity was recorded in the case of fraction number 9 (33 U/mL). The active fractions from the DEAE column were collected and further loaded on Sephadex G-25 column for further purification. A total of 28 fractions were collected (1.5 mL each) after elution with sodium phosphate buffer. Emulsification activity against engine oil was observed in 9-17 fractions. The fractions (9-17) were checked separately then pooled for further investigations (Fig. 2b). The polled fractions of *B. velezensis* KLP2016 yielded absorbance maxima at 221 and 210 nm (Fig. 3c), which corresponded to the characteristic absorption of peptide bonds of surfactin. These results showed that the *B. velezensis* KLP2016 strain might be a producer of a biosurfactant belonging to the 'iturin or surfactin family', possessing emulsification activity against engine oil.

Identification of purified biosurfactant by TLC, HPLC and ESI-MS

A white spot was observed when the TLC plate was sprayed with water, indicating the lipophilic nature of the compound (Fig. 3d). Thus, a peptide without free amino groups (cyclic structure) might be present, as assumed after TLC results. The standard preparation of surfactin also showed a value 0.94 R_f, which was similar to the value of 0.90 R_f recorded for the biosurfactant, indicating the presence of a surfactin-like biosurfactant. The biosurfactant of *B. velezensis* KLP2016 showed retention time (RT) 1.130 min (Fig. 4c), while the authentic surfactin and Iturin A showed a RT 1.27 min and 6.066 min respectively (Fig. 4a & 4b). Thus, it appeared that the purified biosurfactant appeared to be a 'surfactin-like' biosurfactant molecule. The MS/ MS values of the peak (1058.60, 1044.62 and 1030.63 m/z) of the purified biosurfactant of *B. velezensis* KLP2016, were found similar to that present in commercial grade surfactin (Fig. 5a &b). On the basis of literature analysis [26], it was safely concluded that the purified biosurfactant produced by *B. velezensis* KLP2016 was identified as surfactin with *Mr* (~1.0 Dalton) by ESI-MS spectral analysis.

Biodegradation of Engine oil (K 15W-40) using CO2 stoichiometry analysis in a biometric system

Bacterium-inoculated MSM and LB broth gave optical density 1.762 and 2.901, respectively, after 15 days of incubation (Table 1). Engine oil degradation was confirmed by the GC-MS analysis, which indicated disappearance of prominent peaks detected in engine oil (positive control). Results showed that *B. velezensis* KLP2016 cells degrade engine oil efficiently after 15 days of incubation when grown in LB broth rather than in MSM broth (Fig. 6c). The maximum carbon dioxide content trapped in the KOH solution after 15 days of incubation in LB and MSM broth showed values of 1980×10^{-2} ppm and 825×10^{-2} ppm, respectively (Table 1). Thus, LB broth was found to be a better nutrient source for bacterial growth

in context to engine oil degradation because a higher amount of CO_2 was released then got trapped in KOH.

i. $2KOH + CO_2 = K_2CO_3 + H_2O$

ii. $K_2CO_3 + 2\ HCl = H_2O + CO_2 + 2KCl$

One molecule of K_2CO3 contains one molecule or 44 g of CO_2 . To calculate the CO_2 trapped by the KOH solution, K_2CO_3 was titrated with HCl. As per reaction, it was observed that 2 molecules of HCl are required to neutralize one molecule of K_2CO_3. CO_2 trapped after the 5^{th}, 10^{th}, 15^{th} and 20^{th} days of incubation in LB broth was observed as 880×10^{-2}, 1320×10^{-2}, 1980×10^{-2} and 1969×10^{-2} ppm, respectively.

Hydrocarbon analysis of engine oil (K 15W-40) by GC-MS

The uninoculated LB-broth containing engine oil exhibited more peaks than *B. velezensis* KLP2016 inoculated/ treated engine oil, after 5 and 15 days treatment (Fig. 6b& 6c). Engine oil was broken down into methylsulfonyl, borane, pyridine, piperazine, octanamide, ethylene, diethyl propyl and benzenenamine, as can be seen in Fig. 6, on the basis of variation in the peaks generated by the GC-MS.

Discussion

Due to the hazardous effects of engine oil and associated hydrocarbons, it is urgent need to find methods of controlling and biodegrading them to safeguard the environment and human welfare. Biosurfactants have been successfully used in cleaning up polluted areas at low cost and high efficiency [27]. Biosurfactant-mediated remediation of hydrocarbons containing engine oil is an eco-friendly approach, which is able to transform toxic substances into nontoxic compounds, and this technique is an effective technology for the treatment of soil and water contamination [28]. In earlier reports, many methods for screening biosurfactants have been discussed, such as the haemolytic assay, BATH assay, oil spreading, drop collapse and surface tension measurement [29]. In earlier reports, these methods have been noted as screening methods, excluding surface tension measurement which is the key parameter for detecting surfactant activity [30]. Oil spreading is a widely used and effective biosurfactant screening method to detect the potential biosurfactant-producing microbes in the mixtures [31]. This method is a rapid detection method, which can be applied when the activity/quantity of biosurfactant is low in the respective fermentation medium [32].

In our study, the bacterium *B. velezensis* KLP2016 cell-free broth showed excellent biosurfactant properties, as was evident on the basis of data of emulsification activity, surface tension measurement and critical micelle concentration. All these methods strongly detected the biosurfactant nature of *B. velezensis* KLP2016, as it reduced surface tension up to 40 $mN.m^{-1}$ in an *in vitro* assay at 35°C after using cell-free broth of *B. velezensis* grown in LB broth. The critical micelle concentrations (cmc) of cell-free broth of *B. velezensis* grown in LB at 35°C and 25°C were 17.2 µg/mL and 17.4 µg/mL, respectively

while in MSM broth at 35^{o}C and 25 ^{o}C were 17.6 µg/mL and 18.1 µg/mL respectively. E_{24} % of the cell-free broth of *B. velezensis* was observed as 65.7%, 59.0%, 56.1%, 61.0%, 52.3%, 65.2% and 56.2% against benzene, pentane, cyclohexane, xylene, *n*-hexane, toluene and engine oil, respectively.

Bacterial biosurfactants are generally peptides containing a small lipidic moiety and gel permeation, hydrophobic interaction and ion exchange methods are generally employed for the purification from cell-free fermentation broth of *B. velezensis* KLP2016. In previous studies, ion exchange chromatography has been reported for the purification of biosurfactants [33]. Another lipopeptide-like biosurfactant was purified by using DEAE anion exchanger chromatography, followed by an HPLC [34] or a HiTrap Q system [35]. In the earlier reports, molecular sieve chromatography was also used to resolve the low molecular mass biosurfactant by using Sephadex as the matrices [35]. Ion exchange chromatography is also very effective in eliminating coloured contaminating molecules from the biosurfactant fraction, and this technique resolved the antibiotic biosurfactant peak from other chromatographic peaks [24].

UV-Visible spectrophotometry (210 nm) and thin layer chromatography (R_f 0.90) confirmed the purity of a biosurfactant molecule. A dense white spot in the TLC of the purified biosurfactant molecule at R_f 0.90 confirmed the presence of a lipid moiety in the purified molecule. In our study, the purity check and confirmation of the biosurfactant molecule was further detected by HPLC. Detection of a prominent single peak during HPLC indicated the purity of the surfactin type biosurfactant produced by *B. velezensis* KLP2016. Furthermore, the ESI-MS data confirm the *Mr* ~ 1.0 kDa of the purified surfactin-type biosurfactant. The purified surfactin biosurfactant molecule from this strain (*B. velezensis* KLP2016) was found to be a strong degrader of engine oil, as compared to previous reports [17].

The adaptation of microbial communities to hydrocarbons increases their hydrocarbon degradation rates [36]. In the present study, *B. velezensis* KLP2016 growing cells were observed to be a potent degrader of engine oil. In this approach, engine oil degradation occur by the *B. velezensis* KLP2016 bacterium strain in which engine oil was used by the bacterium as a substrate for its growth on the basis of previous studies and a surfactin biosurfactant was released by the *B. velezensis* KLP2016 bacterium in the production medium [37]. The highest value of CO_2 was recorded to be 1980×10^{-2} ppm, which was trapped in the KOH solution after 15 days of incubation of *B. velezensis* grown in LB broth containing engine oil (1%). Engine oil degradation in our study was found to be ~ 1000 times higher than the previously reported value of 656 µmol [17], which shows the efficiency of the *B. velezensis* strain.

The high efficiency of the *B. velezensis* strain for engine oil degradation may be due to the high production of the biosurfactant molecule, which degrades by binding hydrophobically to the engine oil. LB broth appeared to be the best nutrient source to sustain bacterial growth as well as providing an efficient adjustment of engine oil for the degradation, which was qualitatively and quantitatively analysed by GC-MS on the 5th and 15th days. Engine oil degradation by *B. velezensis* KLP2016 was very efficient, and it indicated a potential for using *B. velezensis* KLP2016 in the treatment of oil-spills. This approach to hydrocarbon degradation, which achieves a valuable compound, surfactin, as a by-product, can be used to solve problems such as oil spills and soil water contamination.

This bacterium is, therefore, reported as an engine oil degrader which is highly efficient in LB medium. ~75% more engine oil was degraded by *B. velezensis* KLP2016 cells using LB medium than MSM medium. The released bioactive biosurfactant is considered to be a surfactin-like molecule after the purification and characterization studies. Thus, *B. velezensis* KLP2016 has been proved to be an efficient engine oil degrader which also generates valuable compounds as a by-product, such as surfactin. Therefore, use of *B. velezensis* strain can be a better solution organism for engine oil degradation than the conventional one.

Conclusion

The bacterium *Bacillus velezensis* KLP2016 (Accession number KY214239) showed excellent biosurfactant properties checked by surface tension and critical micelle concentration measurement. Surfactin type biosurfactant was found after the characterization by ESI-MS and HPLC. In this study, hydrocarbon(s) containing engine oil was degraded by the *Bacillus velezensis* KLP2016 (Accession number KY214239) strain where hydrocarbons probably might be used as a carbon source for bacterial growth and additionally, a biosurfactant released by the bacterium may be used further for its wide applications. This biosurfactant-based approach to engine oil degradation is highly promising and may play a key role in the reduction of soil and water pollution in the near future.

Declarations

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Author contribution

SSK, KRM, RD, AS designed the experiments while KRM, RD, KS and SK performed the experiments in the laboratory. KRM wrote paper and SD, AKM and OLF helped in writing and reviewing the research paper and provided valuable suggestions.

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Research involving animal and human rights

There are no human participants and/or animals involved in this study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this present study are included herewith this article.

Conflict of interest

The authors declare that they have no competing interests.

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Tables

Table 1: Growth of *B. velezensis* KLP2016 in MSM and LB broth containing engine oil at 30°C in a shake flask culture

Incubation time (Days)	(MSM broth+ Engine oil+ *B. velezensis*)		(LB broth+ Engine oil+ *B. velezensis*)	
	OD_{600} nm	Fixed carbon dioxide (ppm)	OD_{600} nm	Fixed carbon dioxide (ppm)
0	0.665	-	0.742	-
5	1.312	550×10^{-2}	2.085	880×10^{-2}
10	1.421	770×10^{-2}	2.402	1320×10^{-2}
15	**1.762**	825×10^{-2}	**2.901**	1980×10^{-2}
20	1.667	814×10^{-2}	1.720	1969×10^{-2}

Figures

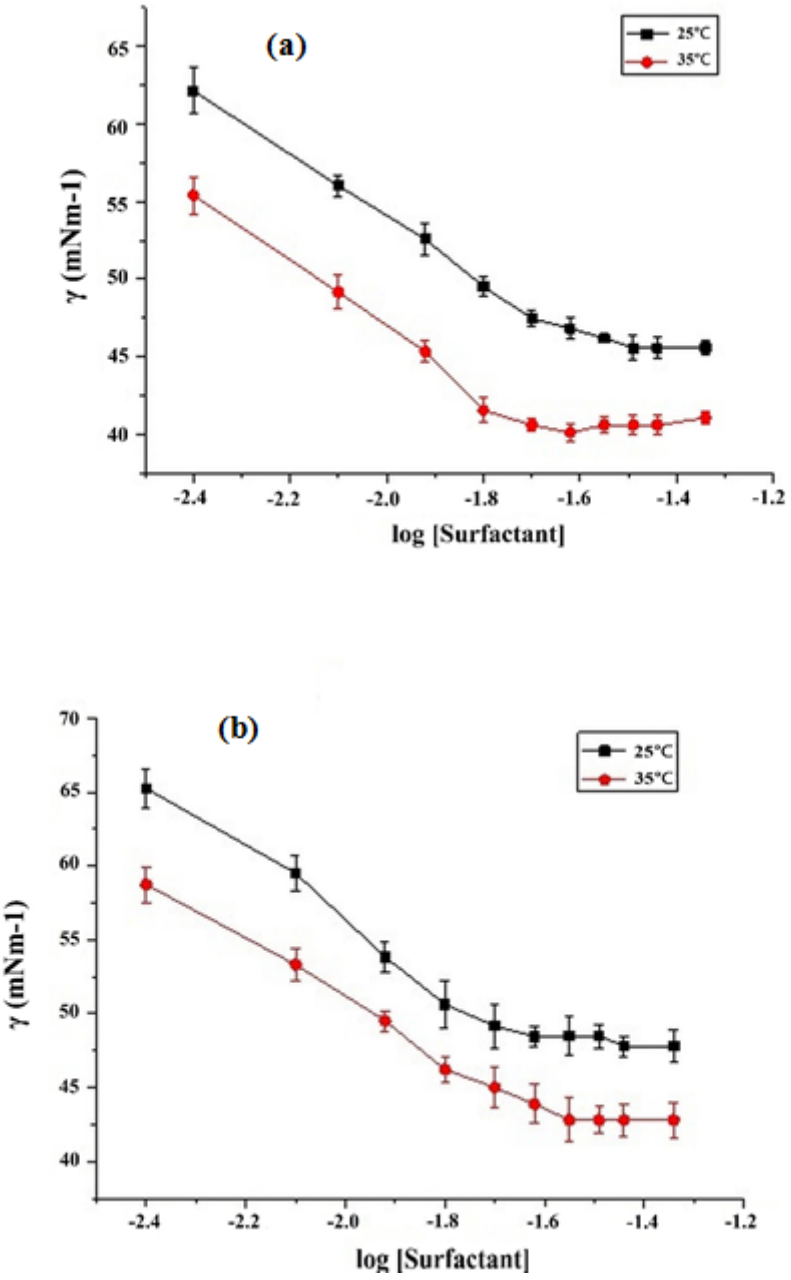
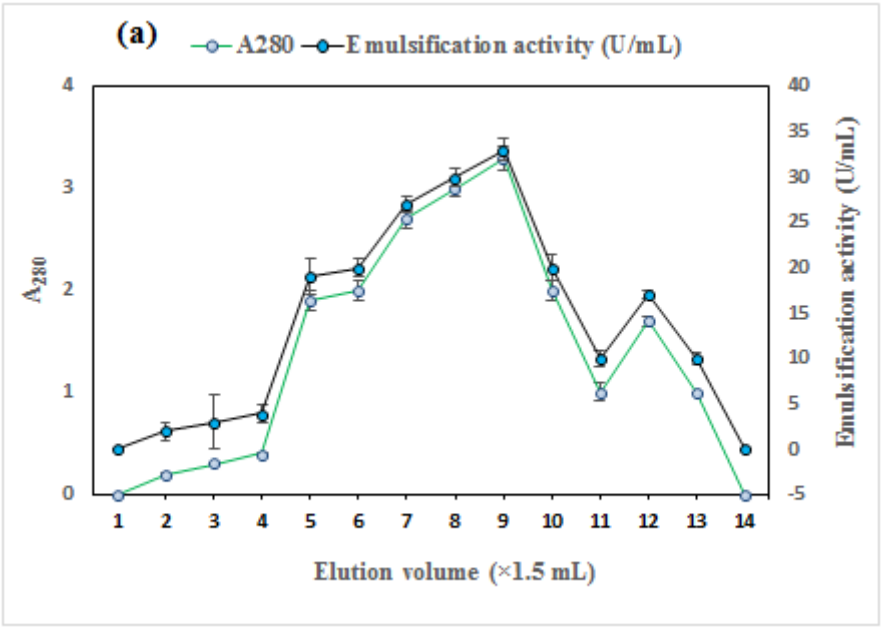


Figure 1

Surface tension and cmc measured against the logarithm concentration of biosurfactant containing cell-free broth. (a) surface tension and cmc of cell-free broth from B. velezensis inoculated in LB broth; (b) surface tension and cmc of cell-free broth from B. velezensis inoculated in MSM broth. Surface tension at 35oC was reduced from 55 to 40 mN.m-1 by cell-free broth of B. velezensis grown in Luria Bertani broth.



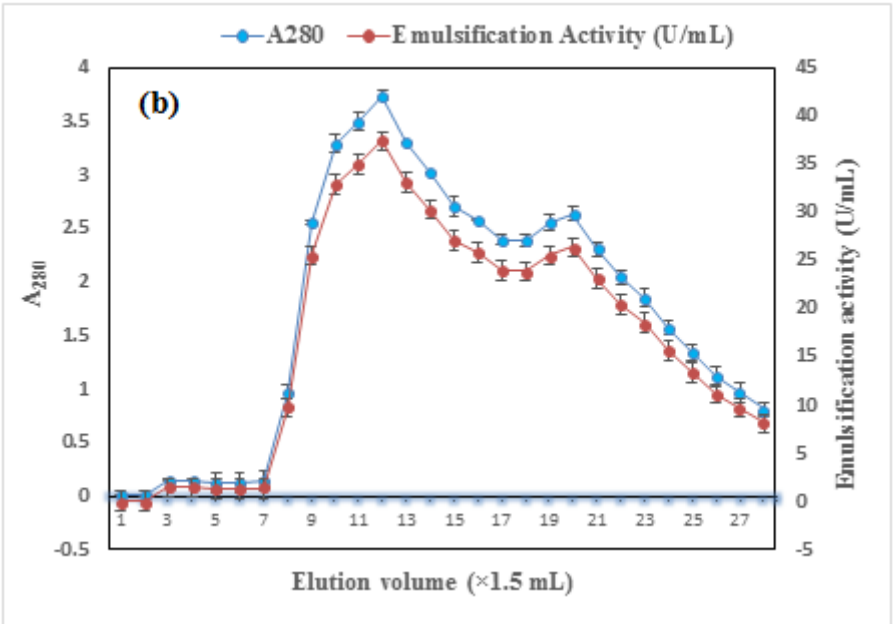


Figure 2

Purification of a biosurfactant and its emulsification activity against engine oil. (a) DEAE column fractions emulsification activity against engine oil and A280; (b) Sephadex G-25 column eluted fractions and emulsification against engine oil.

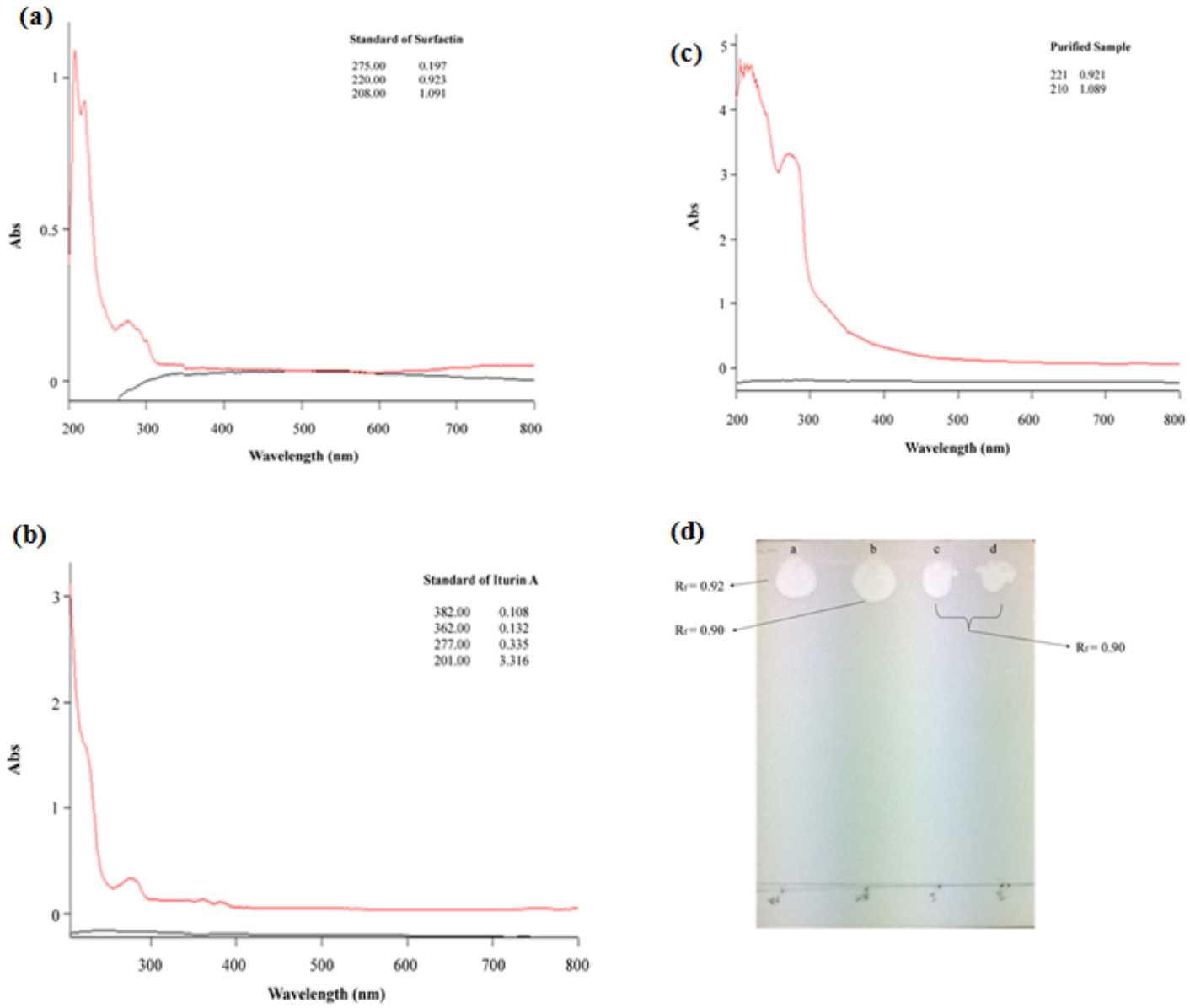


Figure 3

(a) Standard of Iturin A; (b) standard of surfactin; (c) UV visible spectra showing absorbance maxima of biosurfactant containing purified fractions of B. velezensis KLP2016 at 221 and 210 nm; (d) Spot(s) of the cell-free broth sample and standard on TLC plate, (a) iturin, (b) surfactin, (c&d) Purified biosurfactant from Bacillus velezensis KLP2016.

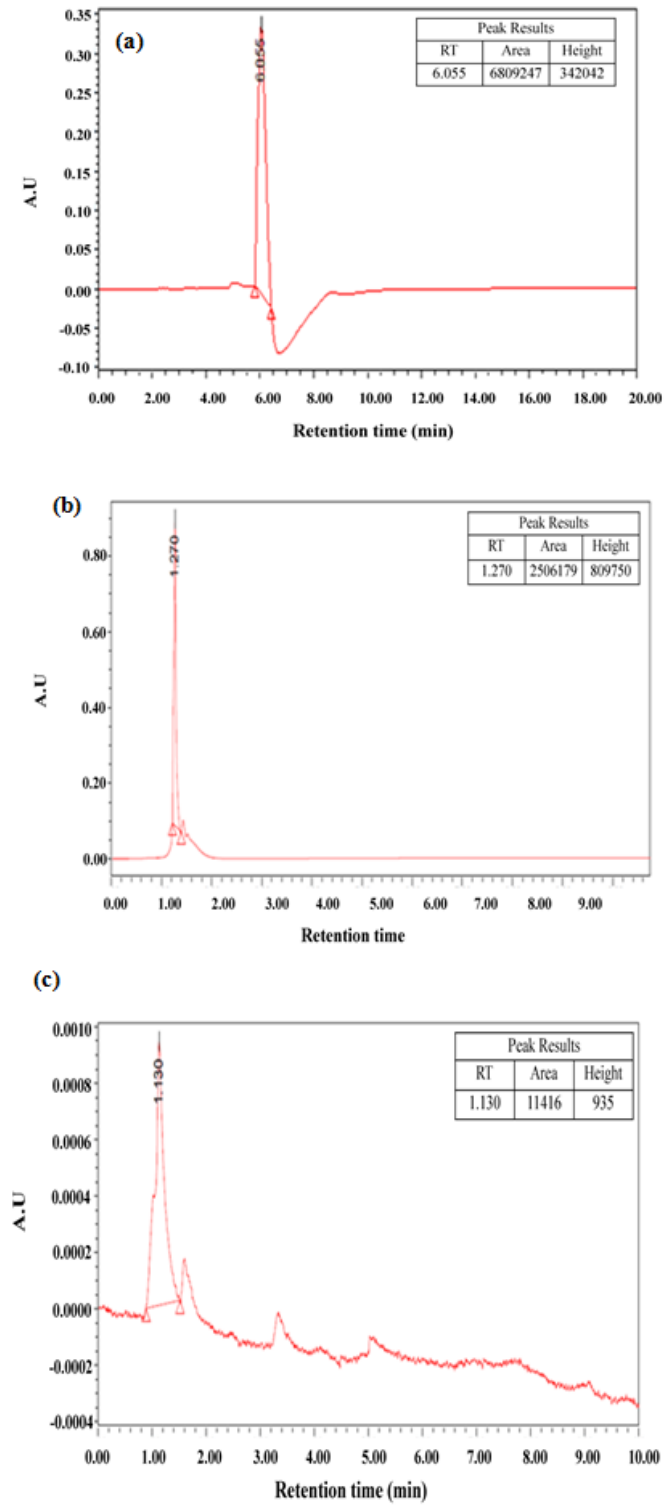


Figure 4

HPLC chromatogram of purified biosurfactant and biosurfactant standards. (a) standard of iturin A; (b) standard of surfactin; (c) chromatogram of active Sephadex G-25 fraction. The purified biosurfactant might be a surfactin showing ~ similar retention time to that of surfactin.

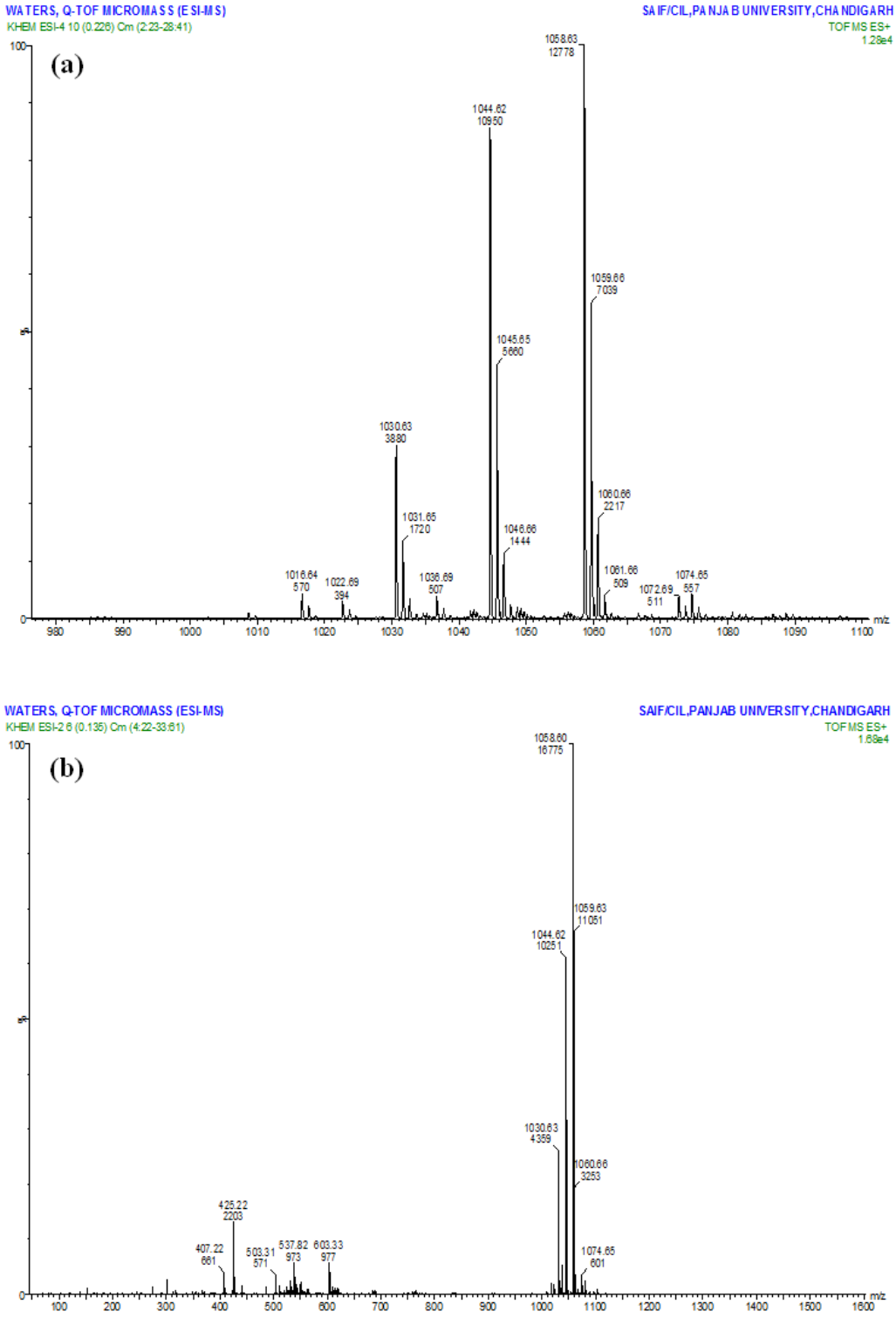


Figure 5

ESI-MS spectra. (a) standard surfactin (Sigma Aldrich, USA); and (b) purified biosurfactant of B. velezensis KLP2016. The peaks of both molecules showed a pattern with adduct of Mr (14) of CH2 group that indicated the presence of homologues with different carbon length(s).

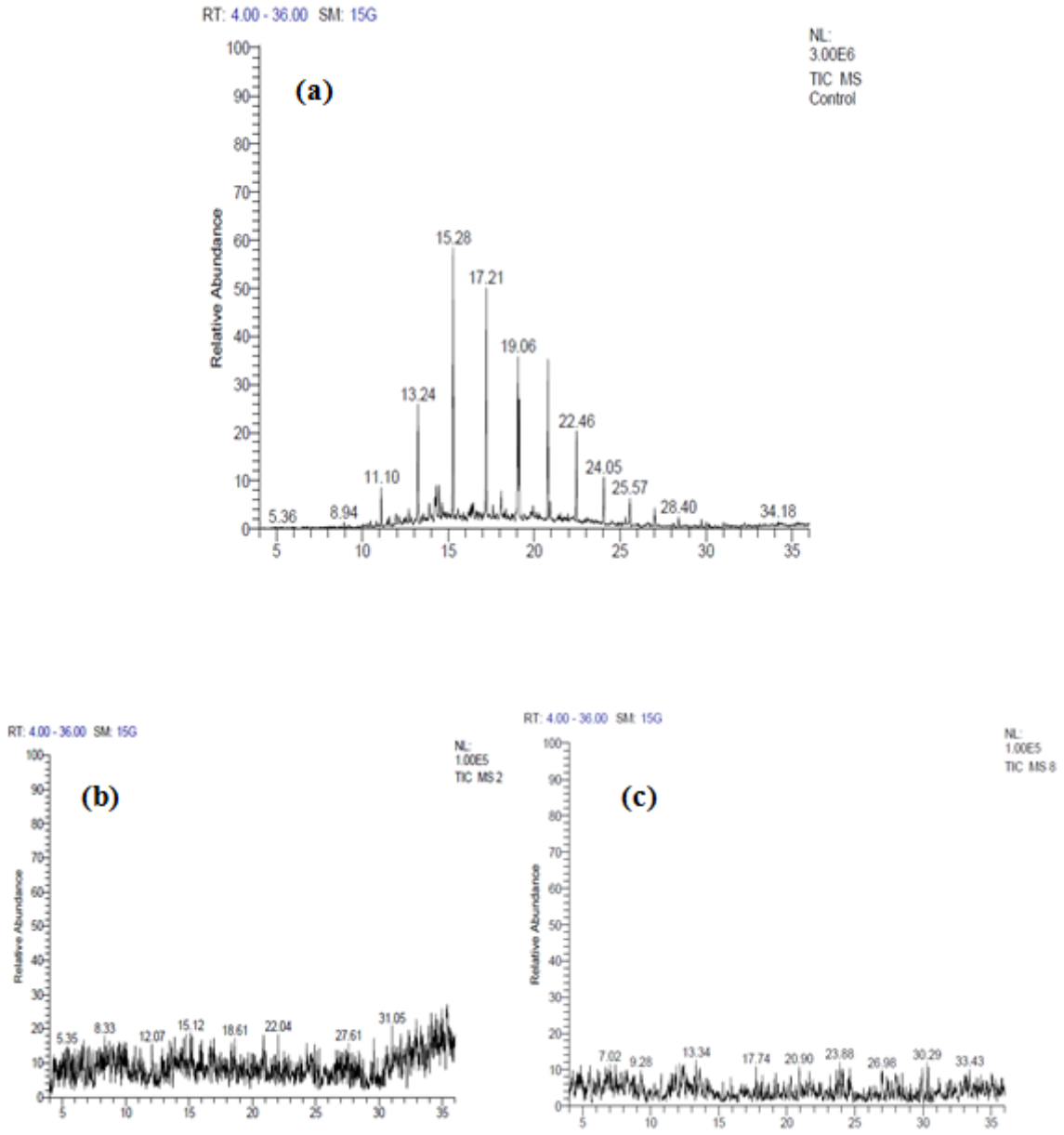


Figure 6

GC–MS analysis of Engine oil (supplemented in LB and MSM broth) without treatment and at 5 and 20 days of treatment with B. velezensis KLP2016. (a) GC-MS spectra of Engine oil (without treatment); (b) 5 days treatment of engine oil with B. velezensis KLP2016 in LB; and (c) 15 days treatment of engine oil with B. velezensis KLP2016 in LB medium.