Environ Sci Pollut Res (2015) 22:17636–17643
DOI 10.1007/s11356-015-4937-1

RESEARCH ARTICLE

Biosurfactant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization

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Received: 18 March 2015 / Accepted: 22 June 2015 / Published online: 7 July 2015

Abstract Among 348 microbial strains isolated from petroleum hydrocarbon-contaminated soil, five were selected for their ability to produce biosurfactant based on battery of screening assay including hemolytic activity, surface tension reduction, drop collapse assay, emulsification activity, and cell surface hydrophobicity studies. Of these, bacterial isolate DSVP20 was identified as *Pseudomonas aeruginosa* (NCBI GenBank accession no. GQ865644) based on biochemical characterization and the 16S rDNA analysis, and it was found to be a potential candidate for biosurfactant production. Maximum biosurfactant production recorded by *P. aeruginosa* DSVP20 was 6.7 g/l after 72 h at 150 rpm and at a temperature of 30 °C. Chromatographic analysis and high-performance liquid chromatography-mass spectrometry (HPLC-MS) revealed that it was a glycolipid in nature which was further confirmed by nuclear magnetic resonance (NMR) spectroscopy. Bioremediation studies using purified biosurfactant showed that *P. aeruginosa* DSVP20 has the ability to degrade eicosane (97 %), pristane (75 %), and fluoranthene (47 %) when studied at different time intervals for a total of 7 days. The results of this study showed that the *P. aeruginosa* DSVP20 and/or biosurfactant produced by this isolate have the potential role in bioremediation of petroleum hydrocarbon-contaminated soil.

Keywords Petroleum hydrocarbons · Biodegradation · Biosurfactants · *Pseudomonas aeruginosa*

Responsible editor: Robert Duran

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Introduction

Environmental pollution caused by fossil fuel is an issue of the greatest importance regarding both economic growth and ecological restoration. Among fossil fuels, oil (petroleum hydrocarbon) is the principle source of energy, and it is part of major global environmental pollutants. Significant amounts of hydrocarbons from petroleum products contaminate groundwater and soil due to leaks and spills from petroleum refinery processes, oil storage tanks, and transportation (Su et al. 2011). Hydrocarbon disposal often leads to release of hydrocarbon pollutants into the environment that bring a serious ecological problems. These petroleum pollutants are both toxic to biological components of the environment and carcinogenic (Okoh 2006; Okoh and Trejo-Hernandez 2006; Obayori 2009).

Bioremediation is a process used for the degradation of hydrophobic organic compounds (HOCs) in oil-contaminated water and soil due to its environmental and economic advantages over other physicochemical remediation methods (Silva et al. 2014). So far, the main approach for enhancing the bioavailability of HOCs is the use of synthetic or natural surfactants (Walters and Aitken 2001; Mulligan 2005; Zhu and Aitken 2010), which promote the solubility of HOCs and improve the biodegradation process (Zheng et al. 2012).

Inspite of continuous research, successful bioremediation of petroleum hydrocarbon-contaminated soil is still a challange problem.

Synthetic surfactants used to release the contaminants are often toxic and representing an extra source of contamination (Banat et al. 2004). Nowadays, extensive research have been conducted on the isolation and characterization of novel surfactant-producing microbes and their use in ecological remediation (Yan et al. 2012). Biosurfactants are classified based on their chemical configuration as glycolipids, lipopeptides, lipopolysaccharides, or oligosaccharides and are produced by diverse bacterial genera (Franzetti et al. 2010). Rhamnolipid (class of glycolipid) is the most frequently used in bioremediation and oil recovery, but the major problem for commercial application of the biosurfactant has its low yield and high production cost (Wei et al. 2005). Thus, there is a need to develop a new efficient biosurfactant producer species/or strain that can produce large amount, and effective biosurfactants are desired to address the challenges (Mukherjee et al. 2006).

The biosurfactant producers are commonly obtained from diverse terrestrial sources (Das and Chandran 2010). In recent decades, several novel biosurfactant producers have been discovered and was used as no-cost complete nutrient medium viz. distillery wastewater, curd whey waste, fruit processing waste for biosurfactant production (Juwarkar et al. 2008; Dubey et al. 2012a). Some microorganisms, mainly from genera *Pseudomonas* and *Mycobacterium*, were capable of oxidizing petroleum products and trace of organic acids (Zobell 1946). *Pseudomonas* sps. are known for its ability to produce extensive amounts of glycolipids (rhamolipids) and are good producers of biosurfactants (Aparna et al. 2011) and play a diverse role in bioremediation. For instance, *Pseudomonas* sp. P-1 strain, isolated from heavily petroleum hydrocarbon-contaminated soil, produced biosurfactant and used in bioremediation of hydrocarbon-contaminated soil (Pacwa-Płociniczak et al. 2014). Rhamolipid biosurfactant produced by *P. aeruginosa* BS2 strain (Juwarkar et al. 2007) used to remove cd and pb from artificially contaminated soil. Biosurfactant from *P. aeruginosa* strain PP2 showed the property to emulsify pesticides in extreme environmental conditions (Dubey et al. 2012b).

In view of this, the purpose of the present study was to isolate a bacterial strain (identified as *P. aeruginosa* DSVP20) capable of producing glycolipid biosurfactant. Further, systematic assessment on the isolated strain was to characterize the biosurfactant by nuclear magnetic resonance (NMR) and high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis and their physicochemical properties (surface tension measurement, drop collapse assay, hydrophobicity, and emulsification activity). The mineral salt medium (MSM) was used to determine the optimum medium composition for higher biosurfactant production. This work was undertaken to assess the potential application of biosurfactant in bioremediation of petroleum hydrocarbon-contaminated soil.

Materials and methods

Sample collection and isolation of microorganisms

Oil-contaminated soil samples from garage and petrol bunk were collected at 10 different locations in Roorkee, Uttarakhand, India. Microorganisms used in all experiments were isolated by the selective enrichment technique as described by Verma et al. (2006). A MSM was prepared with a composition (g/l) of NH_4Cl 2.0, KH_2PO_4 5.0, Na_2HPO_4 4.0, $MnSO_4$ 0.2, $MgSO_4$ 0.2, $FeCl_3$ 0.05, $CaCl_2$ 0.001; yeast extract, 0.01; and 0.05 ml/l of the following solution of trace elements: B (0.026 %), Cu (0.05 %), Mn (0.05 %), Mo (0.006 %), and Zn (0.07 %) with pH 7.2 according to Al-Thani et al. (2009). Briefly 1 g of the polluted soil sample was added to 100 ml sterile mineral salt medium and incubated for 7 days at 30 °C on orbital shaker at 180 rpm. Colonies of the plates were randomly subcultures to obtain pure isolates colonies, which were then used for maintenance of isolates cultures.

Screening for a biosurfactant production

Hemolytic activity was used to determine screening of isolated biosurfactant-producing strain. Surface tension measurement, drop collapse assay, and emulsification assay as described by Pemmaraju et al. (2012).

(a) Hemolytic activity

Hemolytic activity was used to determine screening of isolated biosurfactant-producing strain. The fresh single colony of cultures was streaked on blood agar plates containing 5 % (*v*/*v*) blood (HiMedia, India), respectively, and incubated at 37 °C for 48–72 h. Hemolytic activity was detected by presence of a clear zone around the bacterial colony after incubation for 48 h at 37 °C which is an indicative of biosurfactant production (Carrillo et al. 1996).

(b) Surface tension measurement

The surface tension of the culture supernatant was measured using tensiometer (Sigma 703 KSV Instruments Ltd., Finland) and using the Wilhelmy plate measurement technique as described earlier by Pallas and Pethica (1983). The Wilhelmy plate method utilizes a Platinum rectangular plate. The surface tension of MSM at 25 °C was used as a control. Cell-free broth was taken in a 50-ml glass beaker and placed onto the tensiometer platform. The surface tension of each sample was measured by

using the Wilhelmy plate. The instrument was calibrated using water to a reading of 72 mN/m, and all the measurements were taken in triplicate.

(c) Drop collapse assay

This method was performed according to Bodour and Miller-Maier (1998), in the polystyrene 96-well microtiter plate (Thermo Fisher Scientific, India). Briefly, each well of microtiter plate was coated with a thin layer of oil (2.0 μl). A 5.0 μl aliquot of the sample was delivered into the center of the well using a 25-μl glass syringe (Hamilton, USA) by holding the syringe at an angle of 45°. The coated wells were equilibrated for 24 h to ensure a uniform oil coating. The results were monitored visually after 1 h. If the drop remained beaded, the result was scored as negative were as was scored as positive if drop collapsed.

(d) Emulsification test

The bacterial broth was centrifuged and was studied for its emulsifying ability by a modified method of Cameron et al. (1988). Cell-free broth (2 ml) was pippeted into the screw cap test tube, and 3 ml of kerosene was then added. The mixture was vortexed at high speed for 2 min and left at room temperature. The result was observed after 24 h for the stability of emulsion. The total volume of the mixture, volume of emulsified, and volume of non-emulsified phase was observed. The emulsification index was calculated by the following equation:

$$E24 = \frac{\text{Height of emulsion layer} \times 100}{\text{Total height}}$$

Cell surface hydrophohicity technique

Bacterial strains were selected on the basis of the above screening method, and they were subjected to a cell surface hydrophobicity technique for further assessment of biosurfactant production. In this technique, hydrophobic interaction chromatography (HIC), salt aggregation test (SAT), bacterial adherence to hydrocarbons (BATH), and replica plate test (RP) were performed as described by Pruthi and Cameotra (1997).

(a) HIC

In this method, phenyl Sepharose CL-4B, bed volume approximately 0.6 ml was used as the column packing matrix. The column was equilibrated with a solution of 4.0 M NaCI in 0.5 M citrate buffer. The cell suspension was prepared in the same solution which served for equilibrating the gel. The cell suspension (0.1 ml) was introduced on the gel followed by 3 ml of the equilibrating solution. The elute (non-retained bacteria) was compared with the original bacterial suspension by measuring the absorbance at 540 nm and the results recorded as a percentage of retained bacteria (hydrophobic index (HI)).

(b) SAT

Sodium phosphate (0.002 M, pH 6.8) was used to dilute a solution of 4 M $(NH_4)_2SO_4$ in 0.002 M sodium phosphate pH 6.8. Serial dilutions were made giving $(NH_4)_2SO_4$ concentration ranging from 4.0 to 0.2 M differing by 0.2 M per dilution. A bacterial suspension of 25 μl (approx. 10^{10} cfu/ml) in 0.002 M sodium phosphate buffer pH 6.8 was mixed with an equal volume of salt solution into 24-well tissue culture tray. The bacterial/salt mixture was gently rocked for 2 min at 25 °C, and visual reading was performed against a black background. The results were expressed as the lowest molarity of ammonium sulphate causing bacterial aggregation.

(c) BATH

Bacterial suspensions were prepared at intervals in buffer (pH 7.1) containing K_2HPO_4, 22.2 g/l; KH_2PO_4, 7.26 g/l; urea, 1.8 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; and distilled water to 1000 ml. The suspension were dispensed as 1.2 ml into 10-mm diameter test tubes. The 0.2 ml of hydrocarbons (dodecane, hexadecane, pristane) were added. Following preincubation at 25 °C for 10 min, the mixtures were agitated for 2 min, stood at room temperature for 15 min. This ascertain hydrocarbon separation. The turbidity of the aqueous phase was measured before and after treatment. Results were recorded as the percentage absorbance of the aqueous phase after treatment relative to the initial absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension.

(d) Adherence to polystyrene: RP

In this technique, 25 mm diameter discs cut from sterile disposable polystyrene petri dishes were used. They were pressed firmly on to confluent agar surface growth, and the replica colonies obtained on the polystyrene surface were washed in running water for 2 min. To facilitate visualization and comparison with the original colonies, the replica was fixed by dipping in methanol and staining with crystal violet. Positive was scored when greater than 50 % coverage of the disc by adherence cells was obtained. This procedure was repeated at different time intervals in order to check the age-dependent hydrophobicity of the bacterial colonies.

Identification of the isolate

Bacterial isolates that displayed a highest biosurfactant production ability using cell surface hydrophobicity studies was

Table 1 Surface-active properties of biosurfactant produced by *Pseudomonas aeruginosa* DSVP20 and concentration of glycolipid production

Drop collapse	Hemolysis	Emulsification index [E24] (%)							Glycolipid yield (g/l)
		Carbon source							
		Hexane	Toluene	Dodecane	Kerosene	Tetradecane	Hexadecane	Motor oil	
+	+	64.3±2.9	72.8±3.2	68.4±1.7	78.1±2.8	64.3±2.1	72.4±3.4	70.5±1.8	6.7±0.2

± Standard deviation of three independent experiments

selected and identified by partial 16S ribosomal RNA (rRNA) using forward primers (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-AAGGAGGTGATCCAGCCGCA-3′) (GenScript, India) and conventional biochemical tests in accordance with Bergey's manual of systematic bacteriology performed as earlier reported by Pemmaraju et al. (2012).

Isolation and partial purification of biosurfactant

The isolation scheme employing acetone precipitation from the culture broth was used for biosurfactant extraction (Pruthi and Cameotra 1995). The precipitate was collected by centrifugation at 5000 rpm, for 30 min and dried. The precipitate was dissolved in 0.4 M HCL and extracted twice with chloroform-methanol (2:1). Crude biosurfactant obtained was further purified using (column size 2 cm×30 cm) silica column containing activated Silica gel 60 (Merck Germany), particle size 0.063–2 mm. Polar lipid fractions were eluted with $CHCl_3$ and then by chloroform–methanol (9:1 *v*/*v*).

Thin-layer chromatography

Thin-layer chromatography was used to examine polar lipids (Monteiro et al. 2007). Briefly, the samples was spotted on Silica gel 60 F_{254} plates (20 cm×20 cm, Merck) and were developed using the solvent system chloroform-methanol-acetic acid (65:15:2 *v*/*v*/*v*). Plates were sprayed with orcinol-H_2SO_4 and incubated at 100 °C for 5 min.

Structural characterization

Determination of the chemical structures of components present in crude biosurfactant was performed with the use of mass spectroscopy and NMR.

HPLC/MS

Glycolipid preparations was separated and identified by HPLC/MS using a Waters 2690 separation module (Waters Co., Midford, USA). Samples (20 μl) was dissolved in methanol (Sigma-Aldrich, India) and analyzed 15 cm×2.1 mm Symmetry C_{18} 3.5-μm columns (Astec, Sigma-Aldrich, USA). MS was performed using a Micromass ZMD mass spectrometer containing atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) probes (Waters Co. USA) was next used for structure interpretation of the chromatograph-separated products.

NMR spectroscopy

^{1}H NMR was used to structural characterization of the biosurfactant molecule. One-dimensional ^{1}H nuclear magnetic resonance (NMR) spectrum spectra was recorded on 298K (the number of data points per parts per million of the plot) on a 500 MHz NMR spectrometer (Bruker, Rheinstetten, Germany). The samples were prepared as solutions in 100 % $CDCl_3$ (Sigma-Aldrich, India), using approximately 1–3 mg of the biosurfactant (Pereira et al. 2012), and tetramethyl

Table 2 Cell surface hydrophobicity assay of some selected isolates

Strains	Assay					
	Hydrophobic interaction chromatography (HIC)	Salt aggregation test (SAT)	Replica plate test (RP)	Bacterial adherence to hydrocarbons (BATH)		
				Dodecane	Hexadecane	Pristane
DSVP2	91	1.3	−	14	18	19
DSVP9	95	>2.0	+	80	76	79
DSVP11	28	1.2	−	16	15	18
DSVP18	22	>3.0	−	90	87	75
DSVP20	98	1.0	+	14	12	14

Table 3 Biochemical characterization of biosurfactant-producer strain DSVP20

Biochemical characterization of DSVP20	
Gram stain	-ve
Shape	Rod
Motility	+ve
Spore formation	-ve
VP test	-ve
Indole production	-ve
Urease test	+ve
Starch hydrolysis	-ve
Catalase test	+ve
Oxidase test	+ve
Utilization of glucose	-ve
Utilization of glactose	-ve

silane (TMS) (Sigma-Aldrich, India) was used as the internal standard. The results obtained by the application of one-dimensional NMR spectral methods were utilized to characterize the biosurfactant molecule.

Bioremediation using pure hydrocarbon sources

Batch studies under shake flask conditions were carried out to study the degradation of pure hydrocarbons namely tetra methyl pentadecane or pristane ($C_{19}H_{40,}$ a branched alkaline compound), eicosane ($C_{20}H_{42,}$ a C-20 n-alkane), and fluoranthene ($C_{16}H_{10}$, a polycyclic aromatic hydrocarbon) under optimal growth conditions. Only 0.1 % of these substrates was added in different experimental flasks. Bacterial growth was estimated by determination of cell dry biomass. Degradation was estimated by extracting the undegraded hydrocarbons from different experimental flasks with after 7 days (aliphatic fraction was dissolved in 10 ml hexane and the aromatic fraction in 5 ml acetone) and analyzed with gas chromatography (Hewlett Packard 5890 series II) (Verma et al. 2006). The cell-free supernatant was also analyzed for change in pH and reduction in surface tension. Biosurfactant was also recovered using acid precipitation.

Result and discussion

In the present study, a total of 348 bacterial isolates were isolated from oil-contaminated soil samples by plate and dilution techniques. They were further screened for biosurfactant activities by surface tension reduction assay, hemolytic test, drop collapsing, and emulsification index (Table 1). It was reported that to identify potential biosurfactant producers, more than one screening method should be involved in the primary screening (Satpute et al. 2008). Only five isolates showed positive results for all four screening methods viz., surface tension reduction assay, hemolytic test, drop collapsing, and emulsification index. These five isolates were further tested for maximum biosurfactant production by cell surface hydrophobicity studies. Among the selected isolates, DSVP20 showed maximum biosurfactant-producing ability (Table 2). The best isolate DSVP20 was identified using biochemical analysis as per Bergey's manual of determinative bacteriology (Table 3) and 16S rRNA analysis. Results revealed that the PCR amplified product of 1.5 kb was produced, which corresponds to 16S rRNA gene. The phylogenetic analysis showed that the 16S rRNA gene sequences of bacterial isolate DSVP20 (NCBI GenBank accession no. GQ865644) had 99 % sequence similarity with *P. aeruginosa* (Fig. 1).

Members of the genus *Pseudomonas* have a complex enzymatic system and show a wide variety of physiological and metabolic properties. Similarly, they are the most predominant group of microbes that degrade xenobiotic compounds. It is well documented that various bacteria from genus *Pseudomonas*, including *P. aeruginosa* strains, inhabit hydrocarbon-contaminated soils and can break a wide range of different organic compounds (Glick et al. 1994; Hong et al. 2005; Saikia et al. 2012; Pacwa-Płociniczak et al. 2014). They are the well-known bacteria that have the capability of utilizing a number of aliphatic and aromatic hydrocarbon compounds as a carbon and energy sources (Das and Chandran 2010; Kadali et al. 2012; Puškárová et al. 2013). Monitoring of maximum biosurfactant production and reduction in surface tension during growth of *P. aeruginosa* DSVP20 on pristane, eicosane, and fluoranthene at intervals of 24 h revealed that with increase in dry biomass, there was a reduction in

Fig. 1 Neighbour-joining phylogenetic tree of the strain DSVP20 based on 16S rRNA gene sequence comparisons and closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor-joining tree method). The *scale bar* indicates 0.0001 nucleotide substitutions per nucleotide position. The *numbers at node* show the bootstrap values obtained with 1000 resampling analyses

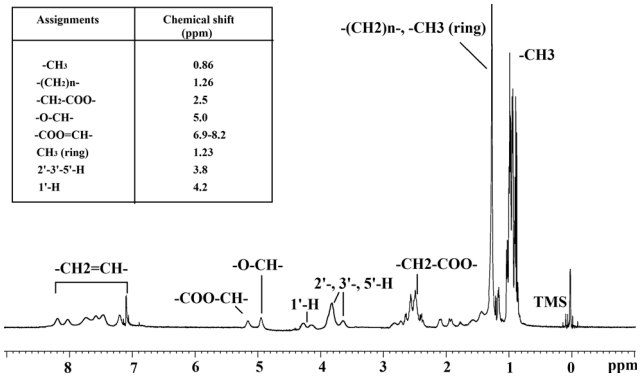

Fig. 2 ^{1}H nuclear magnetic resonance (NMR) spectrum of purified biosurfactant produced by *Pseudomonas aeruginosa* DSVP20

surface tension values of 27.6 and 32.4 mN/m after 3 days for pristane, eicosane, respectively, and 42.43 mN/m after 5 days for fluoranthene. This coincides with the maximum biosurfactant production (g/l) 6.315, 6.813, and 3.932 on pristane, eicosane, and fluoranthene, respectively (Fig. 3a, b, c). These results are in accordance with previous reports which revealed a direct relationship between the production of surface-active compounds with a reduction in the surface tension values (Verma et al. 2006). It was reported that during evaluation of paraffin biodegradation and biosurfactant production by *P. aeruginosa* in the presence of crude oil, the surface tension reduction was achieved during biosurfactant production (Queiroga et al. 2003). The simultaneous pH drop with an increase biomass indicate that *P. aeruginosa* is capable of utilizing hydrocarbons as carbon source (Fig. 3a, b, c).

Strains that belong to genus *Pseudomonas* are the greatest producers of biosurfactant. Since biological surface-active compounds are considered to be very valuable in enhancing the bioavailability of petroleum hydrocarbon pollutants in soil, the capability of biosurfactant production along with the capacity to degrade hydrocarbons make *pseudomonads* one of the most useful microorganism in bioremediation of petroleum hydrocarbon-contaminated soils. In order to check if DSVP20 yields biosurfactants, the surface-active properties of cell-free supernatant of DSVP20 strain were determined (Table 1). The surface tension of the supernatant was reduced, and the flat shape of the oil drop added to the surface of the culture supernatant, indicating a positive result in the drop collapse method, was observed. Additionally, the high emulsification activities of the strain DSVP20 were observed when different carbon source were used. The concentration of the produced glycolipid was high and reached the value of 6.7 g/l

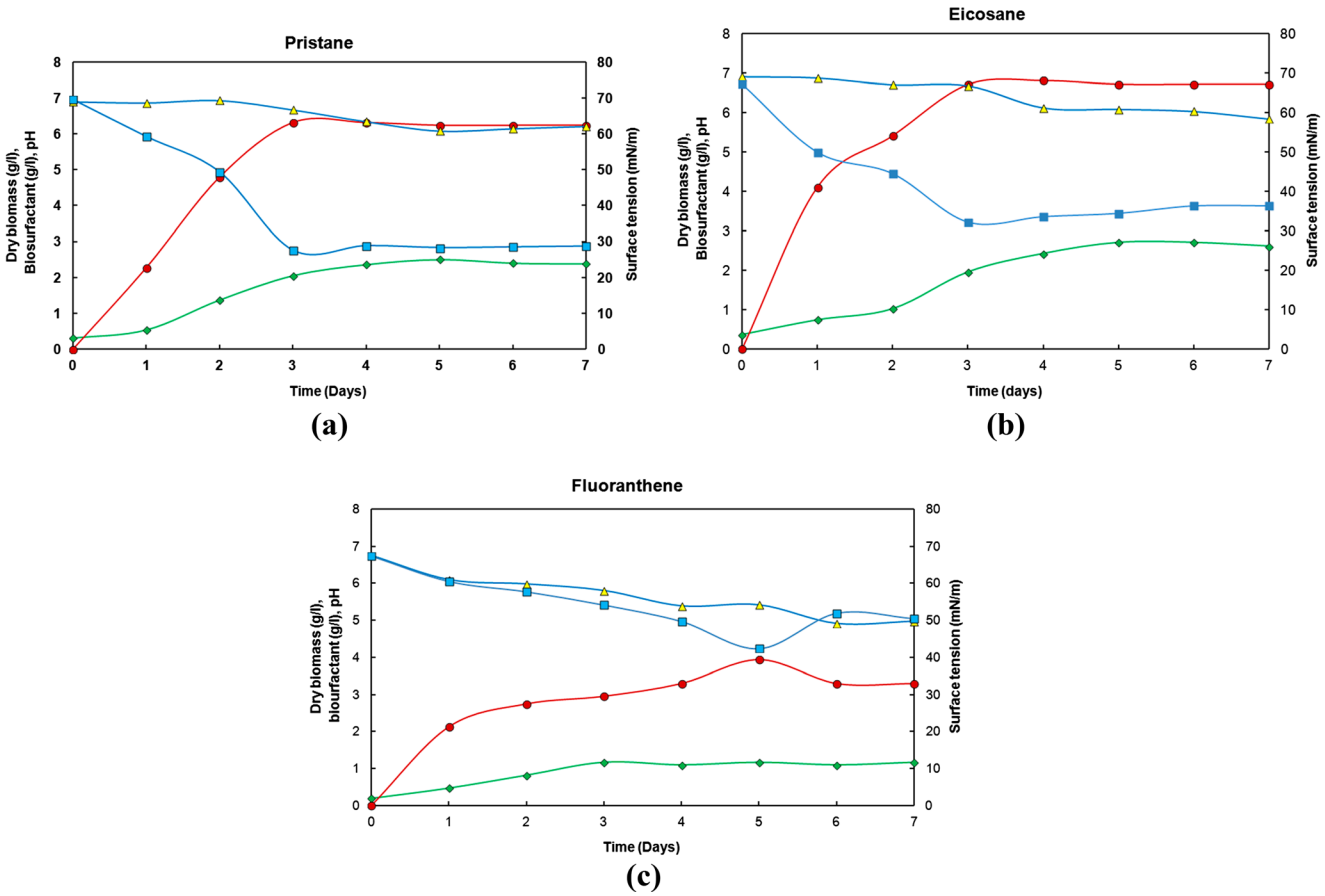

Fig. 3 Growth of *P. aeruginosa* DSVP20 on **a** pristane, **b** eicosane, and **c** fluoranthene at intervals of 24 h: dry biomass (*green diamonds*), biosurfactant production (*red circles*); surface tension value (*blue squares*); pH (*yellow triangles*)

(Table 1). The ability of DSVP20 to synthesize biosurfactants was tested on a blood agar plate, and a clear zone appeared in the hemolytic activity on blood agar which showed the positive result for the hemolytic activity of DSVP20. The blue agar plate method, mainly developed for the detection of glycolipid production such as rhamnolipids by *Pseudomonas* sps., was suggested by Siegmund and Wagner (1991).

Crude biosurfactant was isolated using acetone precipitation, organic extraction, and column chromatography. *Pseudomonas s*ps. are known to produce different type of biosurfactants. For example, *P. aeruginosa* produces a mixture of homologues RL1, RL2, RL3, and RL4 (Syldatk and Wagner 1987). A simple method using surface tension reduction value can be used to screen rhamnolipids produced by a wide range of *Pseudomonas* species. Chemical composition and structural characterization of the biosurfactant produced by *P. aruginosa* DSVP20 were evaluated by thin-layer chromatography (TLC), HPLC/MS, and NMR. TLC results suggested that the isolated surface-active product from *P. aruginosa* DSVP20 was composed of glycolipid. TLC of curde biosurfactant revealed two positive spots: one major spot (R_f=0.5) and second spot (R_f=0.36) representing di-rhamnolipids and mono-rhamnolipids, respectively (Monteiro et al. 2007). The product from *P. aruginosa* DSVP20 was next submitted to HPLC/MS analysis to confirm the presence of glycolipid. Glycolipid structural information was obtained through the use of mass detector equipment with an ESI probe. Results showed a peak at a retention time of 4.8 min for active fractions exhibiting lowest surface tension value. Molecular ion peaks were obtained at 479.1, 339.2, and 333.1 indicated the production of a rhamnolipid type of biosurfactant. Successful use of HPLC for effective separation of rhamnolipids have been demonstrated by Li et al. (2011).

The chemical configuration of the glycolipid present in the analyzed mixture of biosurfactants was confirmed by ^{1}H NMR spectroscopy. The obtained ^{1}H NMR spectra strongly specified that the oily substance produced by DSVP20 was a mixture of glycolipids (two forms of rhamnolipids) not another group of biosurfactant. The chemical shifts at 0.86 ppm showed the presence of CH3 and similarly characteristic chemical shifts at 1.26 ppm for –(CH2)n–, 2.5 ppm for –CH2COO–, 5.0 ppm for –OCH–, and 5.2 for –COO-CH– (Fig. 2). The spectrum is in agreement with results obtained by other researchers, which indicates the same characteristics of rhamnolipid biosurfactant (Price et al. 2009; Raza et al. 2009; Monteiro et al. 2007).

In conclusion, *P. aeruginosa* DSVP20 strain is unique as it degrades both aliphatic and aromatic hydrocarbons besides being a biosurfactant producer. Growth-dependent studies for biosurfactant production suggest that interaction between these components is necessary for efficient biosurfactant assimilation. Microbial diversity can be explored to raise microbes capable of biosurfactant production and hydrocarbon uptake, *P. aeruginosa* encompasses both these features.

Acknowledgments The authors are grateful to Deanship of Scientific Research and College of Food and Agriculture Science Research, King Saud University Riyadh for providing research support.

Conflict of interest We comfirm that there is no conflict of interest upon publication of this paper.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was not necessary because the study did not involve human participants.

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