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Vol. 11(6), pp. 218-231, 14 February, 2017
DOI: 10.5897/AJMR2015.7881
Article Number: B84E98C62745
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* strain KVD-HR42 isolated from oil contaminated mangrove sediments

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Received 12 December, 2015; Accepted January 26, 2016

Pseudomonas aeruginosa **strain KVD-HR42 exhibiting growth and biosurfactant production with 1% molasses as the sole carbon source was isolated from oil contaminated mangrove sediments. Optimization of media conditions involving variations in carbon, nitrogen sources, amino acids, pH, temperature, and NaCl% were evaluated with the aim of increasing biosurfactant productivity and surface tension reduction (STR). The highest biosurfactant production of 4.83 g/L was obtained when cells were grown in mineral salts media (MSM) supplemented with 1% (w/v) molasses, $NaNO_3$, and leucine 0.1% (w/v) at 35±2°C at 150 rpm after 48 h. The results obtained from kinetics study indicated that biosurfactant production, E_{24}%, and rhamnose concentration were growth associated. However, maximum biosurfactant production occurred in the exponential growth phase and detected an increase in E_{24}% and rhamnose concentration. The Fourier transform infrared (FTIR) spectra confirmed the rhamnolipid nature of the biosurfactant. Stability studies revealed the thermostable activity of biosurfactant (110°C for 15 min) and could also withstand wide pH and NaCl ranges. Maximum oil biodegradation of 68% was achieved with 1% waste lubricant oil (WLO). The biosurfactant emulsified various hydrocarbons with varied efficiencies. However maximum E_{24}% and E_{48}% activity was exhibited with n-hexadecane (69.5 and 40%). The results reveal the potential of strain KVD-HR42 biosurfactant for the bioremediation of petroleum hydrocarbons in mangrove sediments.**

Key words: *Pseudomonas aeruginosa,* surface tension, molasses, crude oil, emulsification activity, biodegradation.

INTRODUCTION

Mangrove wetland ecosystems are important inter-tidal estuarine wetlands along the coastlines of tropical and subtropical regions exposed to anthropogenic contamination by polyaromatic hydrocarbons (PAHs) from tidal water, river water, and other land based sources. Elevated concentrations of PAHs (>10,000 ng

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g/L dry weight) have been recorded in mangrove sediments (Ke et al., 2002). Mangrove's exceptional features of high primary productivity, abundant detritus, rich organic carbon, and reduced conditions make them a preferential site for uptake and preservation of PAHs from anthropogenic inputs (Bernard et al., 1996). Such catastrophes make this preserved marine environment highly susceptible to an ecological catastrophe. Therefore, efficient strategies must be developed to monitor oil spills in such environments especially in pristine mangrove wetland ecosystems (Santos et al., 2011). Physical and chemical cleaning processes for decontamination of oil polluted areas have been inadequate in their application to synthetic counterparts. Despite decades of research, successful bioremediation of oil contaminated environment still remains a great challenge for the researchers (Perfumo et al., 2010). One of the most effective methods to treat oil-related contamination is the use of surfactants that disperse the oil and accelerate its mineralization (Makkar et al., 2011). The principle processes for their successful removal are currently believed to be microbial transformation and degradation (Gibson et al., 1975). Microorganisms are fundamental for the maintenance of primary productivity, conservation and recovery of mangroves and are directly involved in the biotransformation of nutrients, production of other metabolites, including antibiotics, proteins and enzymes and also serve as reservoirs for several molecules of biotechnological interest for example, bacteria that produce biosurfactants (Santos et al., 2011). Biosurfactants, produced by microorganisms (Nerurkar et al., 2009), are amphipathic surface active molecules containing hydrophilic and hydrophobic moieties that act by emulsifying hydrocarbons, increasing their solubilisation and subsequently rendering them available for biodegradation. They act by forming lipid micelles at the interface of immiscible liquids, such as water and oil by reducing surface and interfacial tension and blocking hydrogen bonding and hydrophilic/hydrophobic interactions (Darvishi et al., 2011). Biosurfactants can be glycolipids, lipopeptides, lipolysaccharides, polysaccharide-protein complexes, fatty acids, and lipids (Makkar et al., 2011). The rhamnolipids are class of biosurfactants that received great attention and were extensively reported to possess applications in commercial, therapeutic environmental, and biomedical (Dusane et al., 2010). Despite the fact that rhamnolipids are commercially applicable in various fields, their production at industrial scale was not yet exploited in commercial scale particularly because of their constraints, such as low yields, high production costs, expensive raw materials and inefficient product recovery methods (dos Santos et al., 2013). Therefore, these microbial derived rhamnolipids are desirable alternatives to synthetic counterparts, because of their selectivity, biodegradability, low toxicity and stability at extreme temperatures, pH levels, and salt concentrations (Nerurkar et al., 2009).

During our studies on biosurfactant producing bacteria, a *Psuedomonas* strain was isolated from mangrove sediments showing production of a biosurfactant in crude oil enriched conditions. This strain utilizes molasses as the sole source of carbon and produces a rhamnolipid biosurfactant. The current study reports the optimized process parameters using cheaper carbon sources for rhamnolipid biosurfactant production with the aim of increasing biosurfactant productivity, surface tension reduction, and its industrial applications with respect to remediation of oil from pristine mangrove sediments.

MATERIALS AND METHODS

Study area and sampling

Oil contaminated mangrove sediment samples were collected from two sampling sites: (1) Machilipatnam; (2) Avanigadda. The study was carried from June 2011 to November 2011. The mangrove sediment samples (0 to 10 cm) were collected with a soil corer during low tides and transferred to precleaned polycarbonate bottles stored at -20°C and kept frozen prior to further analysis.

Isolation and screening of biosurfactant-producing bacteria

Biosurfactant-producing bacteria were isolated using enrichment technique described by Saikia et al. (2012). Morphologically, different individual bacterial colonies were screened and streaked on Zobell marine agar (ZMA) to obtain pure cultures of the isolates. Screening experiments were carried out by adding 10 m inoculum of each isolate and grown in 500 ml flasks containing 100 ml sterilized mineral salts medium (MSM) (Saikia et al., 2012) with 1% molasses as sole carbon source. The cultivations were performed in triplicate. The pH of the medium was adjusted to 7.0±0.2 and flasks were kept in a shaking incubator at 35±2°C and 150 rpm for 6 days. Emulsification activity (E_{24}%) was determined using n-hexadecane as test hydrocarbon (Wu et al., 2008). Cetyl trimethyl ammonium bromide (CTAB) agar plate method was employed to qualitatively detect extracellular glycolipid-like biosurfactant production (Siegmund and Wagner, 1991). STR was used as a criterion for primary screening of biosurfactant producing isolates using digital tensiometer (KRUSS-Germany). *Psuedomonas aeruginosa* type strain DS10-129 (positive control strain) was used in the screening experiment.

Scanning electron microscopy

Scanning electron microscope (SEM) was used to examine morphological changes of the strain KVD-HR42 during biosurfactant production. Exponentially, grown cells in MSM supplemented with 1% (w/v) molasses were analyzed for cell morphology. Samples were prepared as per the standard method described by Naik and Dubey (2011) and subjected to SEM analysis (Shimadzu-SSX-550).

Biochemical characterization of strain KVD-HR42

Pure culture of strain KVD-HR42 was obtained by sub culturing several times onto ZMA agar and stored at -80°C in tryptic soya broth (TSB) supplemented with 30% glycerol. Morphological

characteristics were observed on ZMA and agar. Selected strain KVD-HR42 was identified by standard biochemical tests according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Molecular identification and phylogenetic analysis of strain KVD-HR42

The genomic DNA was extracted from a pure culture of selected bacterial isolate, polymerase chain reaction (PCR) amplification of 16S rDNA fragment (~1.5 kb) was carried out with universal primer set 27F (5'-GTTTGATCCTGGCTCAG-3') and 1494R (5'-CTACGGYTACCTTGTTACGAC-3'). The PCR was performed in an automated thermal cycler (Eppendorf, Germany). Purified PCR product was sequenced at 1st Base laboratories, Malaysia. The evolutionary history for aligned sequences was inferred using UPGMA method (Sneath and Sokal, 1973). The evolutionary distances were computed using Kimura 2-parameter method (Kimura, 1980) and Tajima's relative rate test (Tajima, 1993). The rate variation among sites was modeled with a gamma distribution (shape parameter=2). Evolutionary analyses were computed using MEGA 5.0 (Tamura et al., 2007). The sequences obtained from BLAST search were named after their accession numbers.

Media optimization

Medium optimization was evaluated in a series of experiments changing one variable at a time, keeping other factors fixed at a specific set of conditions by choosing optimal factors, namely, carbon sources, nitrogen sources, amino acids, temperature, pH and salinity for maximum production of biosurfactant, and STR. The carbon sources used were glucose, glycerol, molasses, soybean oil, diesel, n-hexadecane, used lubricant oil (ULO), and used vegetable oil (UVO) at a concentration of 1% (w/v). Beef extract, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, peptone, and yeast extract were employed as nitrogen sources at a final concentration of 1% (w/v). A medium without carbon and nitrogen source were treated as controls. Different amino acids, such as arginine, alanine, glutamic acid, methionine, cysteine, leucine, and valine were filter sterilized and then added to medium at a final concentration of 0.1 % (w/v). For optimization of environmental variables, the strain KVD-HR42 was grown in MSM supplemented with 1% (w/v) molasses and $NaNO_3$ incubated at different range of temperatures 25, 30, 35, 37, 42, 50, and 60°C, pH range 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 and NaCl concentration 0 to 6% (w/v), respectively. The dry cell weight was determined as per the method described by Raza et al. (2007). The data represented were the means of three replicates.

Biosurfactant production kinetics

Time-course studies were carried out in batch culture by determining the following key variables, viz. emulsification activity (E_{24}%), biosurfactant productivity (g/L), rhamnose concentration (g/L), and bacterial growth kinetics (OD_{600}). 5 ml of bacterial suspension (OD_{600}=1) was transferred to a 2000 ml flask containing 500 ml of MSM supplemented with 1% (w/v) molasses and 1% (w/v) $NaNO_3$ with optimum parameters of pH 7.5, temperature 35±2°C and NaCl 4% for 72 h at 150 rpm. Time course samples of culture medium were drawn in periodic time intervals and monitored for kinetic parameters including growth kinetics and emulsification activity, biosurfactant production, and rhamnose concentration (mg/ml). The culture broth was centrifuged at 9000 rpm at 4°C for 30 min. The supernatant was adjusted to pH 2 using 1 M sulphuric acid. The biosurfactant was extracted using equal volumes of chloroform-methanol (2:1) mixture. The organic phase was separated and the solvent was evaporated to concentrate the biosurfactant. The biosurfactant was then dried using a rotary evaporator. The dried product was washed thrice with absolute ethanol for the complete removal of residual pigments (Aparna et al., 2012) and estimated as per the method described by Li et al. (1984). Emulsification index (E_{24}%) of strain KVD-HR42 was determined by Wu et al. (2008). The concentration of rhamnose was estimated by phenol sulphuric acid method (Dubois et al., 1956).

Characterization of rhamnolipids by IR spectroscopy

Fourier Transform Infrared (FTIR) spectrometer (Perkin Elmer) was used to characterize the presence of rhamnolipids in crude extracts. Around 2 to 5 mg of the concentrated rhamnolipids was analyzed by FT-IR spectrum measurement in wave number range of 4000 to 400 cm^{-1}.

Biodegradation assay of WLO

Biodegradation experiment was conducted to study the impact of biosurfactant isolated from *P. aeruginosa* KVD-HR42 on WLO. Shake flask biodegradation experiments were carried out in 500 ml Erlenmeyer flasks with 100 ml of MSM. The experiment was conducted with 2 different sets: (1) positive control strain DS10-129 and (2) test strain KVD-HR42. The overnight grown cultures 1% (v/v, 10^3 to 10^4 CFU m/L) were inoculated into MSM supplemented with biosurfactant (0.1%, w/v) and 2.0% (w/v) of WLO. Flasks were incubated in a rotary shaker at 150 rpm in dark at 35°C for 20 days. The residual WLO concentrations were determined by mixing oil samples with equal volume of petroleum ether to extract residual WLO. Then the extracted WLO was estimated spectro-photometrically at 228 nm (Rahman et al., 2002). The percentage of degradation was estimated as the difference between initial and final concentration of the oil content in the medium. Uninoculated controls were kept to assess the natural weathering of oil and degradation.

Stability of biosurfactant

Thermal stability of the biosurfactant was determined by heating cell free culture supernatant at 30, 40, 50, 60, 70, 80, 90, 100, and 110°C for 15 min. The effect of pH and salinity on stability of biosurfactant was evaluated by altering the pH (2 to 9) and concentrations of NaCl (2 to 9%) of the cell free culture supernatant and measuring the E_{24}% and surface tension.

Emulsification activity

Emulsification activity was performed according to Wu et al. (2008). The emulsification index obtained with various hydrocarbons was expressed as the percentage of the height of emulsified layer (mm) divided by total height of the liquid column (mm)

$E_{24}\% = [(A_0 - A)] / A_0 \times 100.$

Statistical analysis

All measurements were expressed as Mean±Standard Deviation (SD) and Standard Error (SE) with each experiment conducted in triplicate. SPSS 11.0 software version was used in the statistical analyses. The criterion for significance was set at $p < 0.05$.

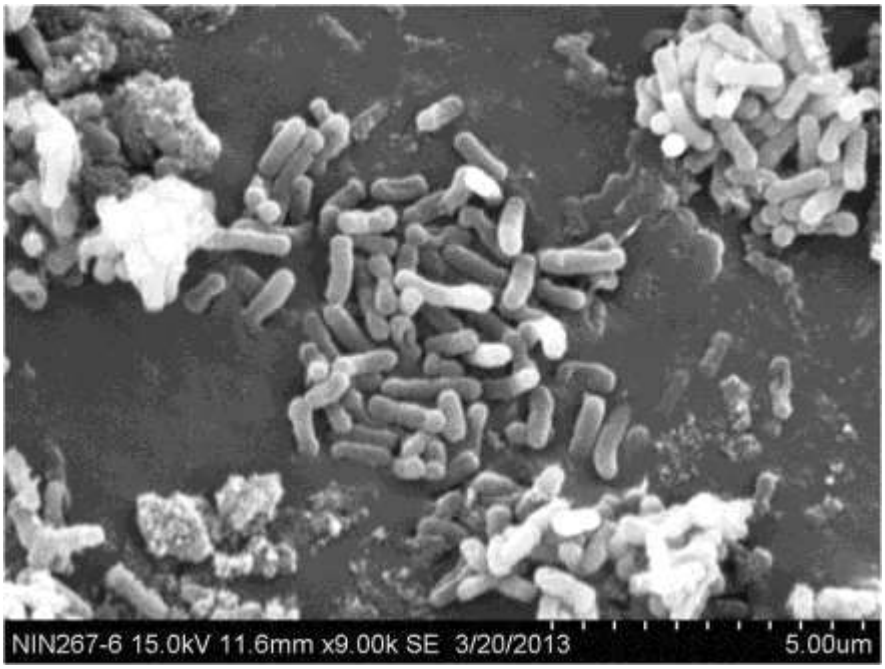

Figure 1. Photomicrograph of *Pseudomonas aeruginosa* strain KVD-HR42 with exposure to molasses using scanning electron microscopy- (SEM-magnification, 15000X).

RESULTS AND DISCUSSION

Isolation, screening, and identification of strain KVD-HR42

The mangroves receive large inputs of crude oil from anthropogenic sources and potentially sequester a huge fraction in its sediments. Despite the fact that the mangrove ecosystems of Krishna estuary is away from the major ports, still the oil spill risk is moderate due to ship breakers, offshore oil wells, spillage from oil tankers and accidents (Ramasubramanian and Ravishankar, 2004). Crude oil is also spilled during loading and unloading operations; further influencing the physico-chemical conditions in different channels of the mangroves. Moreover, these selected sampling sites are land locked with a narrow outlet of the Krishna estuary. As a result, there is lack of water movement which may account for concentration of crude oil at these sites.

A total of 72 bacterial isolates were obtained from the mangrove sediment samples of Krishna estuary, out of which, fourteen isolates showed emulsification activity and STR potential. The strain KVD-HR42 produced excessive amounts of foam when grown on crude oil containing MSM and therefore STR indicates the ability of a biosurfactant to remove oil from mangrove sediments by reducing the capillary force responsible for holding crude oil and soil together (Calvo et al., 2009). Microscopic examination revealed that the strain was gram negative rod shaped with motility, oxidase, catalase, nitrate reduction, and arginine decarboxylase reaction positive. The enrichment culture technique using crude oil showed lowest percentages of positive isolates. Approximately 19 to 20% of isolates from the crude oil contaminated sediments were biosurfactant producers. These results may be attributed to the fact that high concentrations of crude oil did not select for bacteria able to produce biosurfactants. This is consistent with the reported dominance of used lubricant oil resistant bacteria in mangrove sediments (Saimmai et al., 2012). As depicted in SEM image (Figure 1), cell aggregation was observed in *P. aeruginosa* strain KVD-HR42, due to unique cell adaptation to molasses, suggesting the production of complex polymeric biosurfactants surrounding the cells that facilitate in cell to cell communication, thus promoting microbial interactions, quorum sensing, biofilm formation, and swarming motility (Franzetti et al., 2012).

In the screening experiment, cell free supernatant of strain KVD-HR42, showed the highest surface tension reduction from 61.46±0.12 to 30.73±0.17 mN/m and emulsification activity (E_{24}%) of 66.5% as compared to other isolates, respectively. The biosurfactant production was qualitatively determined by CTAB method evidenced from the formation of dark blue halos around the colonies, assuming that the surfactants secreted by selected isolates might be rhamnolipid-like biosurfactants (Table 1). Subsequently, based on the maximum STR, the 16S rRNA gene identification was done for the selected isolate KVD-HR42. The partial sequence of 16S

Table 1. Screening of selected biosurfactant producing bacterial isolates for emulsification activity (E24%), Rhamnolipid Production & STR.

Isolate name	Emulsification activity (E_{24}%)	Rhamnolipid production	Incubation period (No. of days) for surface tension reduction (STR)						
			0	1	2	3	4	5	6
Control[a]	0	0	74.2±0.8	72.2±0.20	71.3±0.08	71.23±0.14	71.15±0.12	71.03±0.08	71.5±0.26
*DS10-129[b]	68	+++	62.46±0.12	42.43±0.14	31.73±0.17	32.30±0.15	33.74±0.11	34.92±0.46	35.32±0.23
KVD-HS 42	-	-	63.54±0.25	58.16±0.23	58.11±0.17	58.08±0.30	57.17±0.12	57.06±0.14	58.16±0.14
KVD-HS 41	50.06	-	61.2±0.15	58.8±0.45	47.3±0.11	49.76±0.38	53.43±0.14	53.53±0.17	55.06±0.12
KVD-HS 45	50.82	++	61.4±0.11	56.86±0.24	44.16±0.12	48.4±0.11	52.26±0.17	53.2±0.11	56.16±0.12
KVD-HL 33	46.34	-	63.33±0.38	57.23±0.27	47.16±0.12	49.56±0.26	53.36±0.14	55.23±0.18	58.43±0.24
KVD-HL 32	45.3	-	63.63±0.18	59.01±0.11	48.3±0.15	54.26±0.14	57.43±0.12	58.06±0.14	58.29±0.05
KVD-HL 31	51.02	-	61.83±0.27	55.21±0.40	46.63±0.08	48.81±0.10	49.4±0.34	53.53±0.35	54.14±0.44
KVD-HL 42	47.58	-	61.6±0.23	53.96±0.54	46.23±0.14	47.26±0.08	47.58±0.21	48.52±0.28	49.16±0.38
KVD-HL 41	48.63	-	63.73±0.26	55.26±0.17	45.26±0.14	46.13±0.08	48.34±0.28	49.16±0.08	52.1±0.05
KVD-HV 32	53.32	-	63.2±0.15	57.46±0.20	46.03±0.14	48.51±0.28	49.7±0.15	52.03±0.31	54.2±0.11
KVD-HM 52	62.5	++	62.7±0.05	46.53±0.31	34.73±0.37	34.51±0.25	35.77±0.14	35.85±0.34	36.36±0.12
*KVD-HR 42[c]	66.5	+++	61.46±0.12	42.43±0.21	30.73±0.17	31.30±0.17	32.74±0.212	33.32±0.46	33.52±0.23
KVD-HM 54	47.3	-	63.46±0.12	51.3±0.26	42.56±0.47	43.46±0.08	47.1±0.05	47.13±0.08	47.67±0.27
KVD-DL 31	52.78	++	62.4±0.25	43.03±0.53	38.73±0.37	41.3±0.11	43.33±0.08	44.76±0.39	45.3±0.15
KVD-DM 52	54.46	-	63.06±0.27	43.46±0.73	41.03±0.20	42.3±0.17	43.93±0.37	44.13±0.14	44.8±0.15

[a]Media control. [b]Positive control (*Pseudomonas aeruginosa* strain DS10-129). [c]Test (*Pseudomonas aeruginosa* strain KVD-HR42) present study.

rRNA gene is available on GenBank with an accession number KJ872835. Figure 2 shows the UPGMA phylogenetic tree based on 16S rDNA sequences of the best biosurfactant producer strain KVD-HR42 isolated in this study and few other microorganisms previously reported in the literature as biosurfactant producers. The UPGMA algorithm showed that this strain formed a coherent cluster with the clade that comprised *Pseudomonas* species as supported by bootstrap confidence level of 99% and a phylogenetic tree was presented in Figure 2. Tajima's relative rate test was performed and its χ^2 test statistic was 17.64 (P =0.00003) with 1 degree of freedom. P-value less than 0.05 was often used to reject the null hypothesis of equal rates between lineages. The UGPMA tree showed the wide phylogenetic diversity of the isolates which are distributed between the divisions of Actinobacteria, Firmicutes, Proteobacteria, and Archeabacteria. The strain KVD-HR42 has the highest similarity with gamma-subclass of Proteobacteria, in particular to the genus *Pseudomonas* spp. Of the Proteobacteria, most of the isolates reported from mangrove sediments falls in the cluster of γ-Proteobacteria, as found in a previous study (De Sousa and Bhosle, 2012). Several strains of *Pseudomonas* spp. were previously isolated from various petroleum hydrocarbons and oil spillages (Saimmai et al., 2012; De Sousa and Bhosle, 2012) and have been associated with a number of biotransformations of chemically-synthesized molecules (Nishino and Spain, 2006). In spite of its ubiquity and an affluence of fascinating metabolic capacities, relatively few reports have been published from the mangrove wetland ecosystems. The surfactant molecules produced by this genus can be different from one species to another. It is thus plausible to hypothesize that unique properties of this molecule may be related with the production of industrially important novel surface active compounds. Such biosurfactant moieties could be vital for containment of environmental pollution; due to the affinity of biosurfactants used in degradation of crude oil environmental polluters (Ozturk et al., 2012).

Optimization of the production media

Among seven carbon sources tested, highest

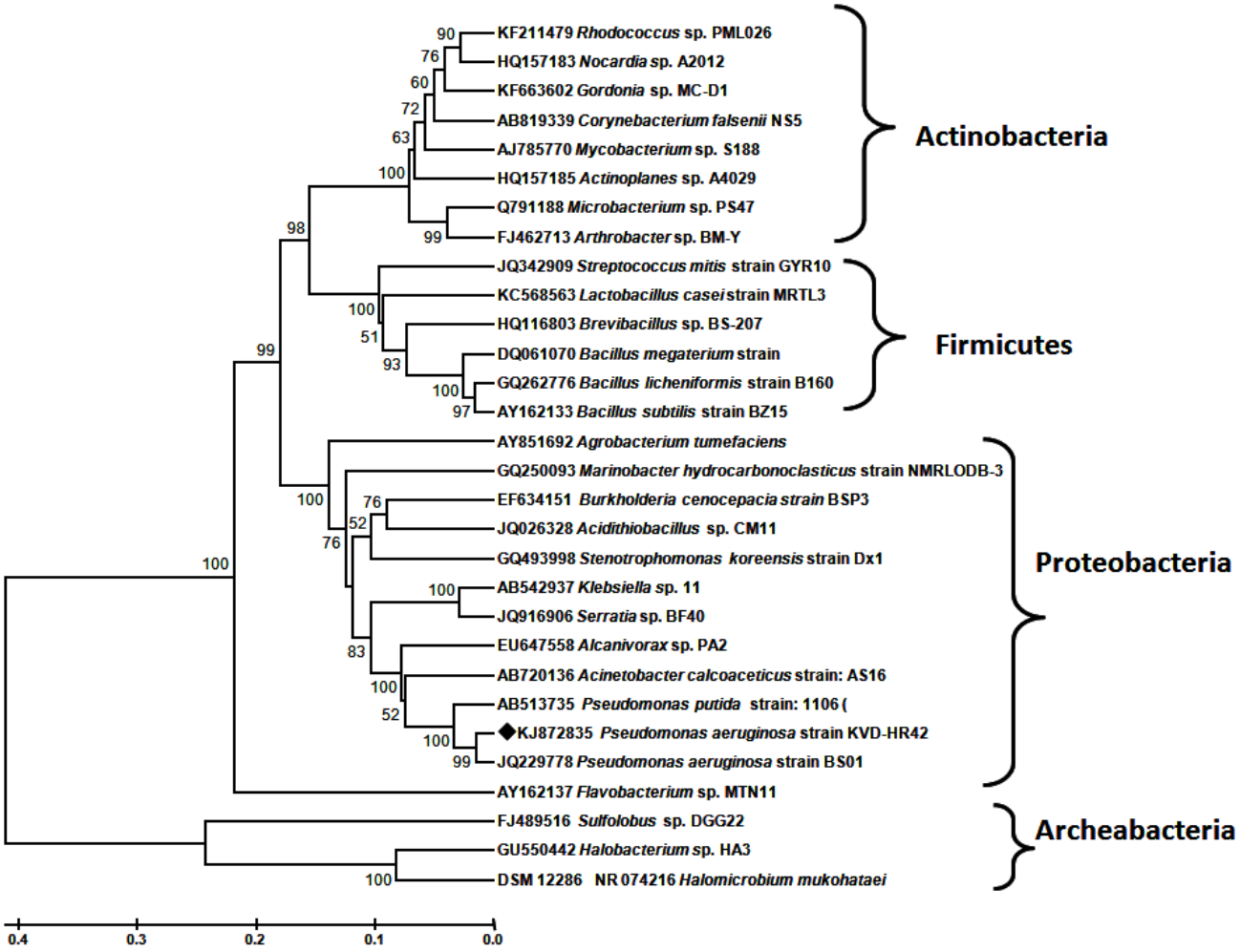

Figure 2. Unrooted UPGMA phylogenetic tree based on 16S rRNA gene comparison of the bacterial strain featured in this study and microorganisms previously described in literature for biosurfactant production. Bootstrap probability values of <50% were omitted from the figure. Scale bar indicates substitutions per nucleotide position. GenBank accession numbers are given in parenthesis.

biosurfactant production of up to 4.83 g/L and dry cell weight of 4.04 g/L, were achieved using 1% v/v of molasses as sole carbon source (Figure 3). However, only glucose, had comparable influence (4.21 g/L and dry cell weight of 4.1 g/L) on the strain KVD-HR42 biosurfactant production than other carbon sources tested. Addition of $NaNO_3$ as sole source of nitrogen with a concentration of 1% (w/v) in molasses containing MSM resulted in the highest biosurfactant production (4.63 g/L) (Figure 3). Organic nitrogen sources including yeast extract, beef extract, and peptone had moderately comparable influence on biosurfactant production. The effect of various amino acids was also examined on strain KVD-HR42 to optimize biosurfactant production, dry cell weight, and STR. Interestingly, our data evidenced that addition of 0.1% (w/v) leucine as an auxiliary nitrogen source to MSM caused a significant increase in biosurfactant production yield more than two fold (4.67 g/L) compared to control (Figure 3). The biosurfactant production was also affected by pH of culture medium. When initial pH was set to 8.0 increases in surface tension, 35.4 mNm^{-1} was observed and biosurfactant production evidenced 3.18 g/L in culture medium. At pH <6.0, the biosurfactant production was decreased followed with an increase in STR (Figure 4). The optimum pH for growth and biosurfactant production was determined to be 7.5. Likewise, an increase in temperature up to 42°C did not cause significant effect on STR. However, at the highest temperature 60°C, STR was sternly decreased to 38.2 mNm^{-1}. As shown in Figure 4, there is an inverse relation between NaCl concentration and biosurfactant production yield. Gradual addition of 4 to 6% NaCl to molasses containing MSM led to a rigorous decrease in biosurfactant production 4.17 to 2.82 g/L. At lowest level of NaCl (1% w/v), the production yield reached to 2.95 and 3.64 g/L dry cell weight was

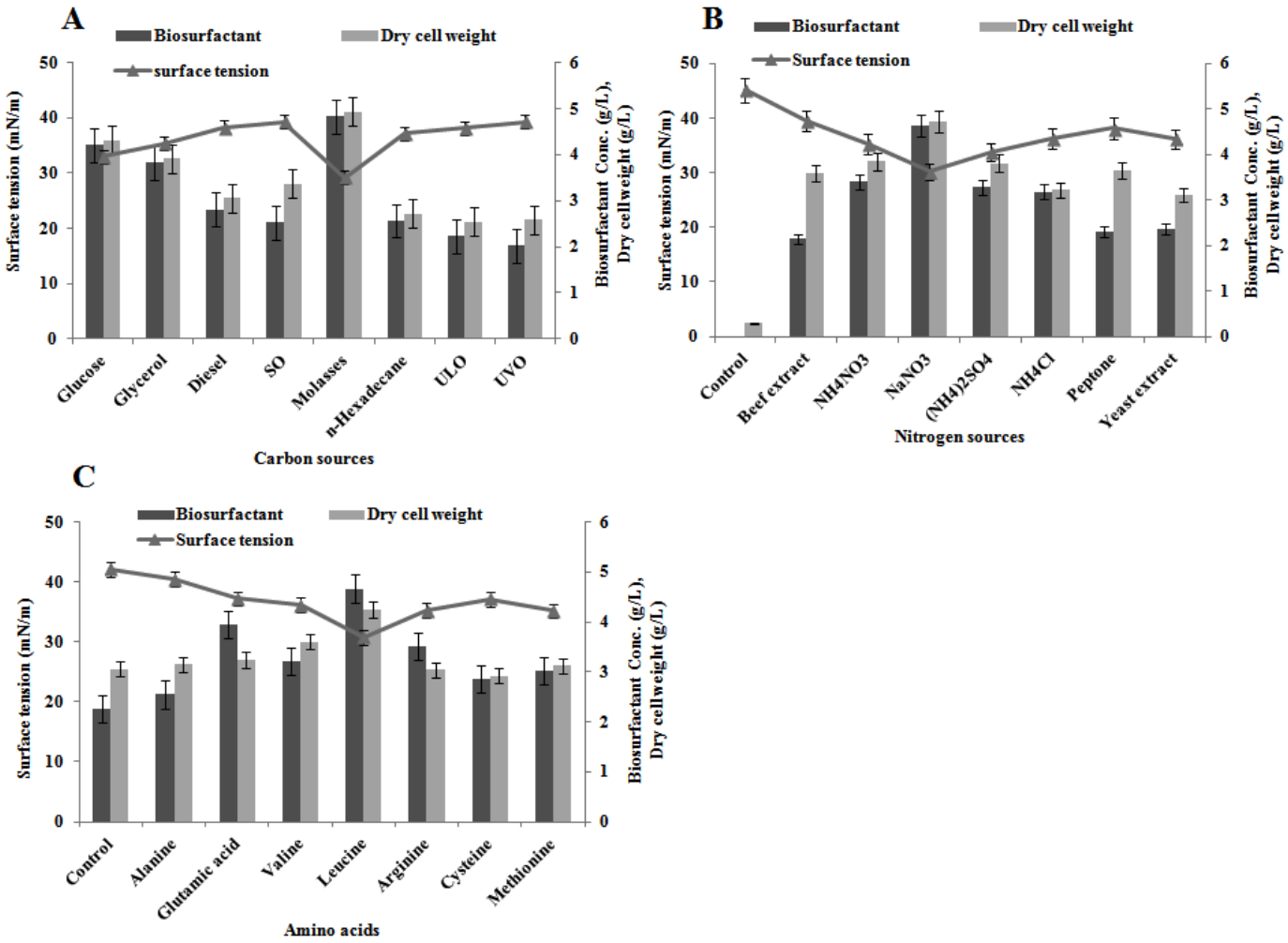

Figure 3. Optimization of Carbon (1%), Nitrogen (1%) and Amino acid (0.1%) for maximum biosurfactant production. The error bars represent mean ± standard deviation (n = 3).

obtained under similar conditions.

Our results also corroborate with several previous reports, emphasizing the utilization of industrial by-products and vegetable oils such as cheese whey, molasses, peanut cake, olive oil mill effluents as cost-effective alternative substrates for microbial growth and biosurfactant production (Onbasli and Aslim, 2009; Haba et al., 2003). The biosurfactant production kinetics of strain KVD-HR42 was strongly dependent on media composition, affecting its production yield. In shake-flask experiments, the change of carbon source employed, affected on dry cell weight and biosurfactant secretion. The preferred carbon source, molasses produced the highest biosurfactant yield. The highest production in MSM containing molasses may be due to its high hydrophobic rhamnolipid contents. Similar results have been obtained when *Pseudomonas* spp. grown on glycerol as sole source of carbon (Saikia et al., 2012). The ability of KVD-HR42 strain to utilize molasses as carbon source is a significant advantage as it provides an alternative use for glucose, provided that its biosurfactant production can be enhanced to economically viable values. Biosurfactant production with 1% carbon source reported in this study (4.83 g/L) was comparatively higher than previous reports on use of olive oil (2%), palm oil (2%), coconut oil (2%), soybean oil, safflower oil, and glycerol as carbon sources for biosurfactant production (Rahman et al., 2002). Preferences for complex compounds over simpler counterparts have been previously reported in *P. putida* strains (Basu et al., 2006). The genus *Pseudomonas* is capable of using different substrates such as n-paraffins, molasses, and vegetable oils which are known as good carbon and energy sources to produce rhamnolipid type biosurfactants (Syldatk et al., 1984; Manresa et al., 1991). The growth of microorganisms on hydrocarbons is frequently associated with the production of biosurfactants that can assist in emulsification of these

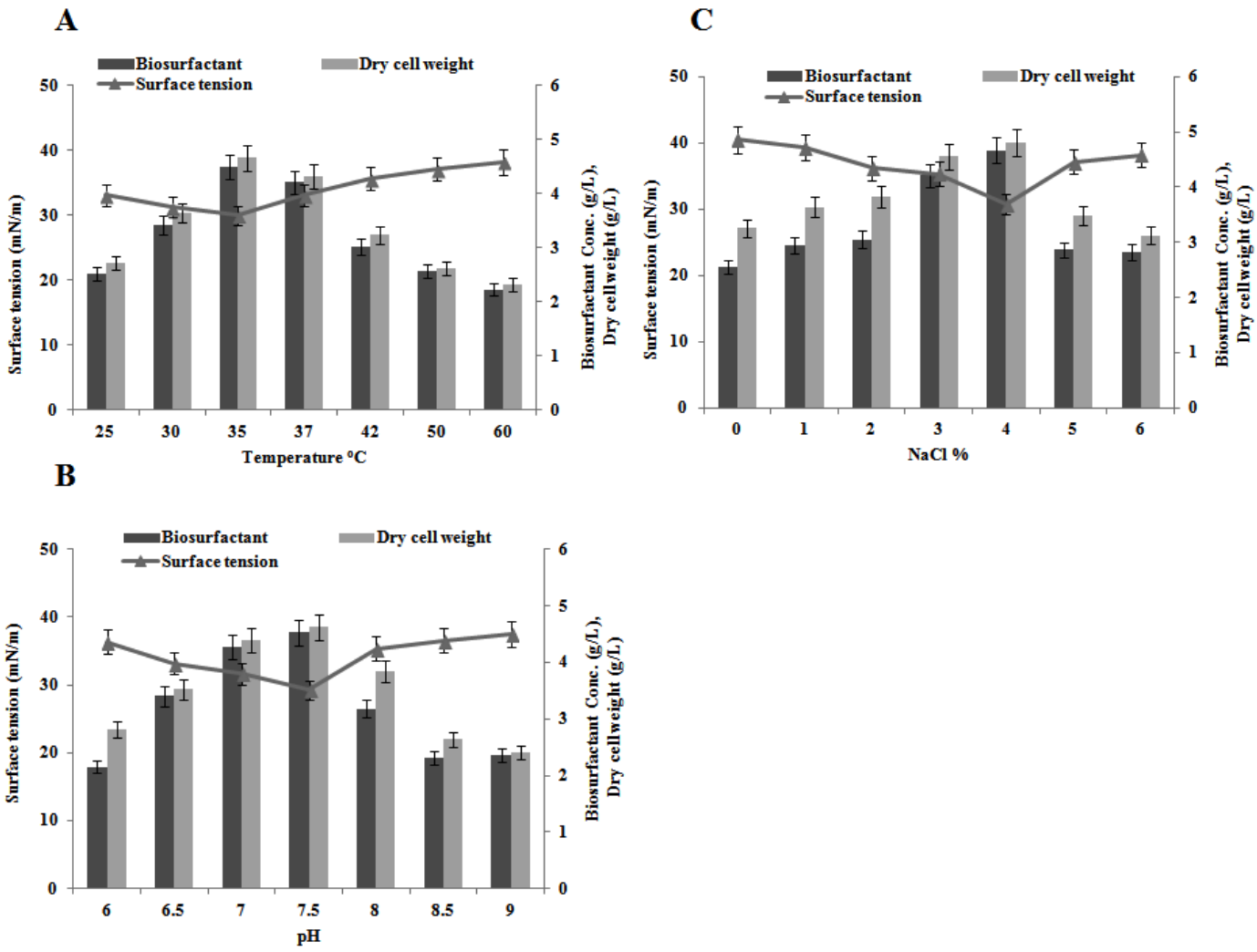

Figure 4. Optimization of pH, temperature, and NaCl% for maximum biosurfactant production. The error bars represent mean ± standard deviation (n = 3).

hydrophobic substrates in growth medium (Calvo et al., 2009). Syldatk et al. (1984) found an overproduction of rhamnolipids by *Pseudomonas* spp. which was caused by nitrogen limitation in the medium. Our results were in tune with a previous report, in which the highest rhamnolipid production was attained by *Pseudomonas* 44Ti when grown on olive oil with sodium nitrate as sole nitrogen source (Manresa et al., 1991). These investigators also demonstrated that nitrogen restriction improved production of some biosurfactant, but changed the composition of biosurfactant as well. In this context, a similar study was conducted to investigate the effect of $NaNO_3$ as nitrogen source on biosurfactant production kinetics (de Santana-Filho et al., 2015). These observations suggest that, the improved biosurfactant production by *P. aeruginosa* strain KVD-HR42 may be occurred as a result of uptake of leucine as substrate for surfactant biosynthesis, thus enhancing the biosurfactant yield. However, previous studies revealed that, some amino acids are exceptional substrates as nitrogen sources for the production of surfactin by *Bacillus subtilis* (Sandrin et al., 1990). The effect of environmental variables, namely, temperature, pH, and NaCl%, on *P. aeruginosa* strain KVD-HR42, had a profound influence on surface tension, biosurfactant production, and dry cell weight and results are presented in Figure 4. Basic values >pH 8.0 were found to have exercised a more unfavourable effect on surface tension, biosurfactant production, and dry cell weight comparative to acidic pH values. However, in a recent report more prominent STR potential was observed at alkaline pH values in *P. aeruginosa* MA01 which could be attributed to formation of lipid vesicles (Khoshdast et al., 2011). The tolerance of biosurfactants to NaCl has been earlier reported as being restricted to NaCl 5% (Illori et al., 2005) and 15% (Darvishi et al., 2011).

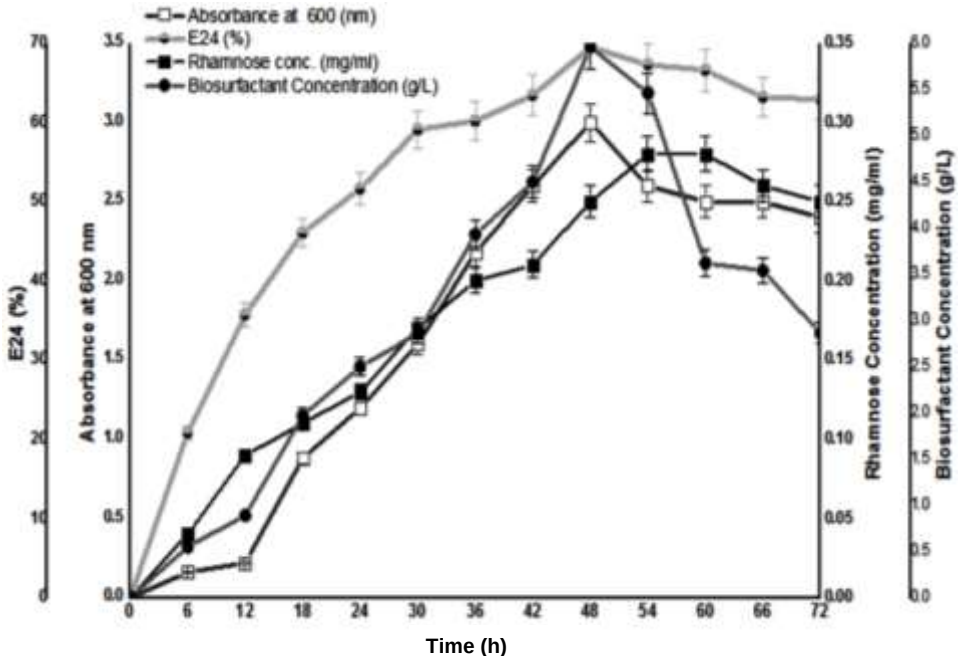

Figure 5. Time course study of absorbance at 600 nm, biosurfactant production, rhamnose concentration and E24% by *Pseudomonas aeruginosa* strain KVD-HR42 in 1% molasses containing MSM.

Biosurfactant production kinetics

The optimized culture parameters were used for the time course study. Kinetic study revealed the dependence of each biochemical variable, namely, biosurfactant production (g/L), rhamnose concentration (mg/mL), growth ($O.D_{600}$ nm), and emulsification index (E_{24}%) on time of strain KVD-HR42 when supplemented with molasses and $NaNO_3$ as carbon and nitrogen sources, respectively (Figure 5). The E_{24}% plot, a measure of biosurfactant concentration, showed that insufficient surfactant was initially present after 12 h to form micelles in culture broth. According to the data obtained from time course study, it revealed that the biosurfactant production was growth associated and started secreting in early exponential phase after 18 h, detecting an increase in emulsification activity (E_{24}%) and rhamnose concentration (mg/ml). Production yield after 72 h of incubation was only 2.46 g/L. The total amount of rhamnolipid accumulated was 0.35 mg/ml, respectively. However, maximum biosurfactant production occurred in the late exponential growth phase (48 h). Bacterial cell growth in aforementioned experiments conducted with molasses as sole carbon source revealed that cell growth was in its maximum at 48 h of incubation (Figure 5), which is comparable to the observation made in a study by Lotfabad et al. (2009). The decrease in E_{24}% after 48 h of incubation shows that biosurfactant biosynthesis stopped and is probably due to the production of secondary metabolites which could impede with emulsion formation and the adsorption of surfactant molecules at the oil-water interface (Bonilla et al., 2005). These results indicate that the biosurfactant biosynthesis using molasses occurred predominantly during the exponential growth phase, suggesting that the biosurfactant is produced as primary metabolite accompanying dry cell weight as evidenced in the growth-associated kinetics (Persson et al., 1988). Previous studies reported that rhamnolipid biosurfactant was produced during the logarithmic and stationary phases of bacterial growth and amount of production increased after then (Zhang and Miller, 1995). Onbasli and Aslim (2009) stated that *Pseudomonas luteola* and *Pseudomonas putida* produced rhamnolipid of 0.23 and 0.24 g/L at the 48th h; 0.38 and 0.36 g/L at the 72 h, respectively. It is seen that the results in our study are similar to those of the mentioned studies.

Identification of rhamnolipids by IR spectroscopy

Based on the observations made from the resultant FTIR spectroscopy data (Figure 6), the broad band at regions 3397 to 3534 cm^{-1} indicate the presence of hydroxyl groups (-OH) free stretch due to hydrogen bonding and N-H str modes. The broad observed in the regions (2958 to 2854 cm^{-1}) infers the presence of aliphatic, symmetrical, and asymmetrical CH_3, CH_2, and -C-H- vibrations in lipids. Further bands observed at 1736 and 1638 cm^{-1} points to the occurrence of carbonyl (C=O) stretching and

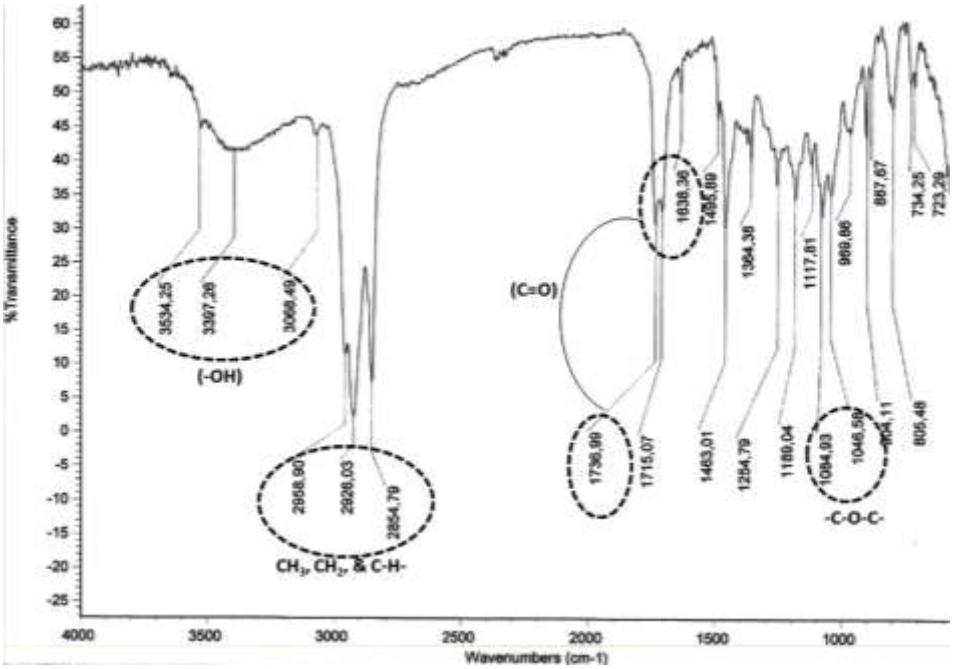

Figure 6. Fourier transform infrared spectrum of biosurfactant produced by *Pseudomonas aeruginosa* strain KVD-HR42.

amide-I group. The other two peaks were observed between 1117 and 1046 cm^{-1} and indicates the presence of C-O-C group vibrations in the cyclic structures of carbohydrates. Moreover, the bands observed in regions 805 to 904 cm^{-1} correspond to the glycosidic linkage type. In a study by Mehdi et al. (2011), similar results were found with the rhamnolipid biosurfactant.

Stability characterization

The results obtained from thermal stability analysis of biosurfactant over a wide range of temperature (30 to 110°C) revealed that the biosurfactant from *P. aeruginosa* strain KVD-HR42 was shown to be thermostable (Figure 7A). The biosurfactant was thermostable (up to 110°C for 15 min) and caused a diminutive effect on the surface tension and emulsification activity. It was found that only 20% of E_{24} becomes lost, when the biosurfactant was incubated at higher temperature. The surface tension reduction and E_{24}% were relatively stable at the temperatures used (ST=32 mNm^{-1}, E_{24}%=44%, respectively). It is very interesting to note that the biosurfactant isolated may be used in microbial enhanced oil removal processes where high temperatures are prevailed. As can be seen in Figure 7B, the optimum pH for biosurfactant activity (ST=27.5 mNm^{-1}) and emulsification capacity (E_{24}%=49.32) was determined to be 7.0. However, the emulsification activity was significantly reduced between pH 8.0 and 9.0. Similar result had been reported for biosurfactants produced by *B. subtilis* TD4 and *P. aeruginosa* SU7 was also stable in a wide range of pH (Saimmai et al., 2012). Figure 7C demonstrated an increase in NaCl concentration; up to 18% did not have a significant effect on E_{24}. However, at the highest level of NaCl (24%), E_{24}% dropped severely to 43.6% and surface activity was increased as well (48 mNm^{-1}). NaCl activated biosurfactant activity of many strains isolated from several environmental sources including mangrove sediments (Saimmai et al., 2013). More recently, biosurfactant produced by *P. aeruginosa* MA01 has shown good stability of surface tension and emulsion in the presence of NaCl up to 300 g/L (Abbasi et al., 2012). Our findings indicate that the biosurfactant has potential application over a wide range of temperature, pH values and saline environment.

Biodegradation of WLO

The strain KVD-HR42 was tested for its ability to grow in MSM with WLO as carbon source. The biodegradation of WLO in the laboratory scale experiment inferred that, maximum biodegradation was found with strain KVD-HR42 biosurfactant (68%), whereas the control strain depicted the maximum biodegradation of 65%, respectively (Figure 8). The supplementation of biosurfactant to the medium improved the biodegradation of WLO compared to the uninoculated control. Growth of microorganisms on hydrocarbons is often associated with

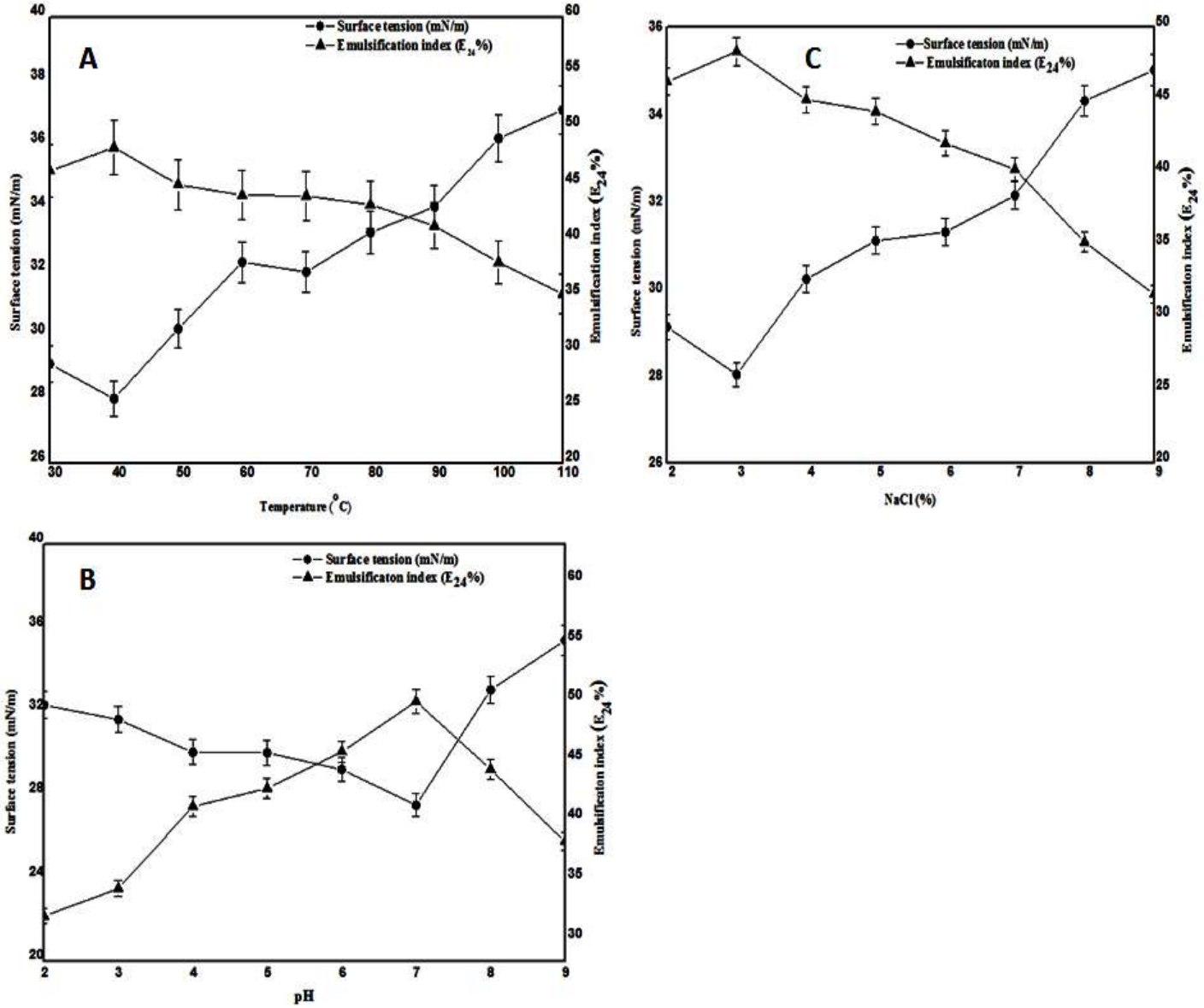

Figure 7. Effect of temperature (A), pH (B) and NaCl (C) on activity of crude biosurfactant produced by *Pseudomonas aeruginosa* strain KVD-HR42.

the production of biosurfactants that can support in the emulsification of hydrophobic substances in the growth medium (Calvo et al., 2002). It is interesting to note that biodegradation capabilities involving biosurfactants are used for hydrocarbon remediation (Ozturk et al., 2012).

Emulsification activity

The biosurfactant produced by strain KVD-HR42 demonstrated emulsification with a number of hydrocarbons (Figure 9). Among the aliphatics used, n-hexadecane served as the best substrate for emulsification and xylene served the best substrate among the aromatic compounds. The highest E_{24}% and E_{48}% were obtained with n-hexadecane (69.5 and 40%), whereas the lowest values were obtained with toluene (52 and 33%). This results may be attributed to the fact that rhamnolipid enables to function as an emulsifier for immiscible substances. Moreover, a variety of different hydrophobic substrates were efficiently emulsified by *P. aeruginosa* strain KVD-HR42 supernatant. It was reported in a study that n-hexadecane was attracted to the cell surface of the 21 BN strain of *P. putida* at a rate of 72% (Tuleva et al., 2002). In a different study by Noordman and Janssen (2002), it was found that n-hexadecane was attracted to the cell surfaces of the strains of biosurfactant producer *P. aeruginosa* 18 UG2,

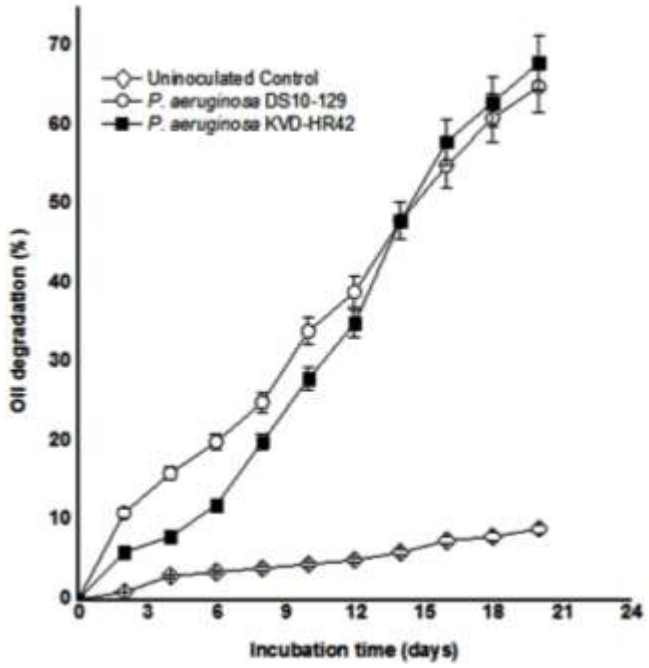

Figure 8. Percentage degradation of Waste lubricant oil (WLO).

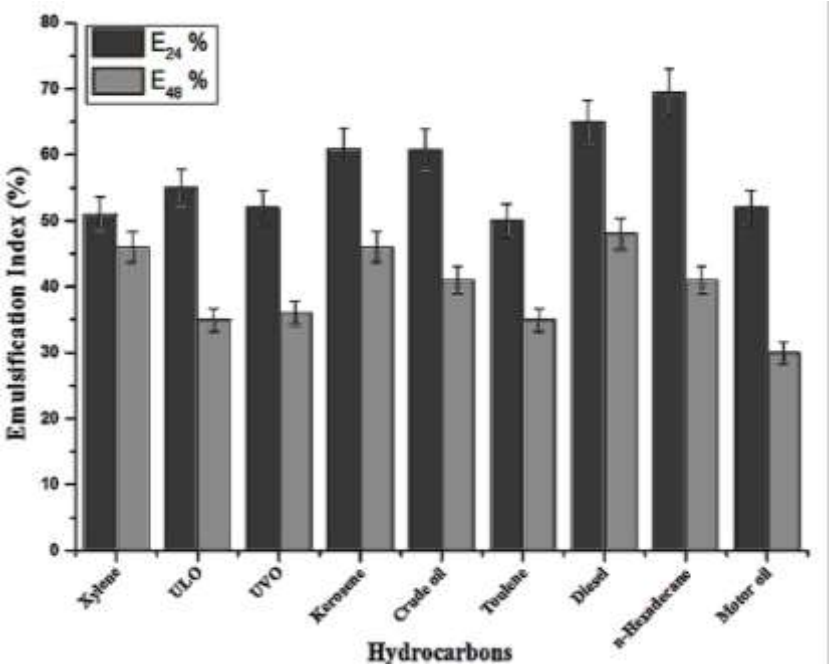

Figure 9. Comparative emulsification activity of the strain KVD-HR42 biosurfactant with various hydrocarbons. The error bars represent mean ± standard deviation (n = 3).

Acinetobacter calcoaceticus RAG1, *Rhodococcus erythropolis* DSM 43066, and *Rhodococcus erythropolis* ATCC 19558 at rates of 42, 81, 30, and 12%, respectively. The results indicated that the strain KVD-HR42 biosurfactant was capable of effectively emulsifying both aromatic and aliphatic hydrocarbons, suggesting that it could be used for hydrocarbon remediation and oil recovery (Ilori et al., 2005). Extensive work has been done on the role of biosurfactants as effective factors for emulsification of hydrocarbons (Calvo et al., 2009).

Currently, there is an escalating interest in the identification of novel biosurfactants for environmental cleanup and bioremediation (Muthusamy et al., 2008). The frequency and occurrence of biosurfactant producers in PAH's and hydrocarbon contaminated mangrove sediments has not been well researched, however, strain KVD-HR42 could play a pivotal role in biodegradation of PAH's. Therefore, it is all the more necessary to study the structure–function relationships of the strain KVD-HR42 biosurfactant and the factors that regulate its biosynthesis. The biosurfactant obtained from *P. aeruginosa* strain KVD-HR42 employing molasses as substrate having rhamnolipid content and STR potential may provide a promising focus for further investigations on its application as a compound with efficient biological activity for enhanced oil recovery management in mangrove sediments. The examination of the kinetic parameters, such as product yield formation should be taken into account to develop a high efficient production process. A precise chemical and structural analysis of strain KVD-HR42 biosurfactant is currently in progress.

Conclusion

Considering the importance of mangrove wetland ecosystem and susceptibility of such areas to oil spills, the present study was taken up. In this study, the screening of biosurfactant producing bacteria, molecular strain characterization, media optimization, kinetics, stability studies, biodegradation potential, and emulsification activity of biosurfactant produced by mangrove sediment bacterium *P. aeruginosa* strain KVD-HR42 and its importance with respect to remediation of oil from pristine mangrove sediments were demonstrated. Optimizing factors that affected growth in strain KVD-HR42 with a potential for commercialization is of paramount importance. The results on biodegradation and emulsification activity revealed the potential use of this biosurfactant in bioremediation of hydrocarbon pollutants, which emphasized that biosurfactants could be synthesized from a cheaper carbon source like molasses. This approach will make the bioremediation process an economically and environmentally feasible technology. Strain KVD-HR42 therefore holds good potential for effective bioremediation of hydrocarbons in the mangrove wetland ecosystem and oil spill management.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

ACKNOWLEDGEMENT

The financial assistance provided by University Grants Commission, New Delhi - Rajiv Gandhi National Fellowship (RGNF), as Junior Research Fellowship (JRF) to Ms. K.V. Deepika under the Grant No:F.16-1723(SC)/2010(SA-III), is greatly acknowledged.

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