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ORIGINAL PAPER

Production kinetics and tensioactive characteristics of biosurfactant from a *Pseudomonas aeruginosa* mutant grown on waste frying oils

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Abstract Various waste frying oils (WFOs) were evaluated as substrates for rhamnolipid production by *Pseudomonas aeruginosa* mutant EBN-8 in the presence or absence of rhamnolipid precursor, under single-/batch-fed conditions. Soybean WFO was the best substrate, producing 9.3 g rhamnolipid l^{-1} with the specific product yield of 2.7 g g^{-1} h, under batch-fed cultivation with the addition of rhamnolipid precursor. The surface tension of the cell-free culture broth (CFCB) was 29.1 mN m^{-1} and the interfacial tension against *n*-hexadecane was <1 mN m^{-1}. The hydrocarbon/CFCB systems showed the relative emulsion stability to be in the range of 89.7–92.3.

Keywords Biodegradation · Emulsification · Fermentation · Rhamnolipid · Surface-active · Surface tension

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Introduction

World production of edible oils, for 1999–2000, was estimated as 86.4 million tones (Mt), consisting of 85.2 Mt of vegetable oil and 1.2 Mt marine oil (Beckman 2000). Most of the edible oils and fats are used by the food industry, which generates large quantities of wastes; including residual oils, tallow, lard, soap stock, waste free fatty acids, vegetable oil refinery wastes as well as cooking oil residues (Haba et al. 2000; Benincasa et al. 2004). The costs of environmentally sound disposal of these wastes are an economic burden on industry and treatment capacity may soon outstrip the available resources. There is thus a need to better manage these wastes by recycling.

Biosurfactants are among the most versatile process chemicals. By the end of this decade, they are expected to constitute 10% of the world surfactant market, worth approximately US$ 200 million in sales (Nitschke et al. 2005). Rhamnolipids are the most common among the glycolipid biosurfactants. At low concentration rhamnolipid hydrophilic monomer head groups accumulate at the air/water interface, whereas at high concentration, the hydrophobic interactions between the amphiphiles of rhamnolipid molecules may contribute to micelle formation, which becomes significant at well-defined concentration known as the critical micelle concentration (CMC). Hydrophobic compounds are emulsified

within these micelle structures, effectively increasing their apparent aqueous solubility and availability for the uptake and degradation by bacterial cells. In this way, rhamnolipids may play an important role in accelerated bioremediation of hydrocarbon-contaminants in the environment.

Rhamnolipids perform a variety of functions in microbial cells, although there is no consensus on their actual physiological role. Their presence in the culture media may affect bacterial growth as well as bacterial surface physico-chemistry. Rhamnolipids are involved in the adhesion of microbial cells to hydrocarbons by reducing the surface tension (ST) of the phase boundary, thus making them more readily available for the microbial uptake and metabolism. This enhances the uptake of water-immiscible substrates by freeing the biodegradation process from carbon source limitation (Itoh and Suzuki 1972; Del' Arco and de Franka 2001).

Rhamnolipids can be produced using various substrates (Makker and Cameotra 2002; Raza et al. 2006). Vegetable oils and residues from the vegetable oil processing industry are among the most suitable low-cost substrates for rhamnolipid production, whereas accumulation from glucose and other hydrophilic carbon sources generates lower yields (Robert et al. 1989). Hydrocarbons, such as hexadecane, are not ideal growth substrates due to their toxicity and high cost (Desai 1987).

We have examined rhamnolipid production by a *Pseudomonas aeruginosa* mutant strain using separate canola, corn and soybean waste frying oils (WFOs) as carbon and energy sources. Single- and batch-fed cultivation setups were compared in terms of the relationship between the substrate utilized, the biomass formed and rhamnolipids produced. The effect of rhamnolipid precursor addition on the kinetics of rhamnolipid production from different WFOs, along with a spectrum of tensioactive properties of the rhamnolipid produced is reported for the first time.

Materials and methods

Microorganism and inocula

The microorganism used in this study was EBN-8, a gamma ray-induced mutant of *Pseudomonas aeruginosa* S8 (Raza et al. 2006). The culture was maintained on nutrient agar plates and was sub-cultured prior to inoculation. The inocula used were prepared in two different ways:

- Inoculum A: A single colony of the EBN-8 mutant, grown on a nutrient agar plate, was transferred to nutrient broth in an Erlenmeyer flask (250 ml) and incubated in an orbital shaking incubator (100 rpm) at 37°C for 48 h. The cells were harvested by centrifugation (7,740 *g*, 15 min), washed and resuspended in normal saline (0.89% w/v, NaCl) to an OD_{660} of 0.7.
- Inoculum B: A single colony was grown on *n*-hexadecane (1% w/v) as the carbon source in liquid salts medium (Deziel et al. 2000) at 37°C with shaking (100 rpm). When the bacterial growth reached the stationary phase (at 4 d), the rhamnolipid in the culture medium was 2.5 g l^{-1}. This culture broth as 1% (v/v) of the minimal medium was used as the inoculum.

Carbon sources

Samples of canola, corn and soybean frying oils were purchased from a local market. Aliquots (~200 ml each) of these oils were used to fry eggs in 10 consecutive cycles. The used oils were cooled to ambient temperature and filtered twice through a vacuum filtration assembly (containing a Whattmann qualitative filter paper 42, 125 mm diameter) to remove solids. The samples were stored in separate glass jars at 4°C.

Fermentation setups and conditions

The shake flask experiments were conducted in three different fermentation setups:

- Setup I: The minimal medium (50 ml) in 250 ml Erlenmeyer flasks was supplemented with canola, corn or soybean WFOs, or unused canola, corn or soybean frying oils (2% w/v). Inoculum A (1% v/v) was added to these flasks and incubated in an orbital shaker (100 rpm, 37°C).
- Setup II: The medium, supplemented with WFO, was inoculated with inoculum B (1% v/v)

to give an initial net rhamnolipid content of 0.05 g l^{-1}.

- Setup III: The minimal medium was supplemented with separate WFOs as carbon sources (oils) in two batches. Initially carbon sources (2% w/v) and inoculum B (1% v/v) were added to the minimal media and, when the substrates were consumed (in about 4 d), the media were fed with a second batch of the respective WFOs as carbon sources (1% w/v).

To obtain high yields of rhamnolipid it is necessary to attain conditions where nitrogen and metal ions are limiting in the media. So, in each of the above fermentation setups these conditions were satisfied following Deziel et al. (2000). The chemicals used were of analytical grade. To avoid complex formation, the minimal media and carbon sources were autoclaved separately. There was also a parallel set of abiotic control flasks for each setup. Samples were taken every 24 h for 10 d during the incubation. The experiments were conducted in three independent replicates and the data reported are the mean values of three readings.

Process follow-up

Dry cell biomass (DCBM) was determined by centrifuging of the culture broth at 7,740 *g* for 15 min. The cell pellet obtained was dried overnight at 60°C and weighed. The residual WFO in the culture supernatant was determined by extracting it with two equal volumes of petroleum ether. The organic phase was evaporated under vacuum in a rotary evaporator at 70°C to obtain a constant mass of the residual oil. The rhamnolipid was extracted from the cell-free culture broth (CFCB) described by Zhang and Miller (1992). The resulting precipitate was redissolved in distilled water at neutral pH value and its rhamnose equivalents were determined following the standard orcinol assay (Chandrasekaran and Bemiller 1980). The rhamnolipid content was calculated by multiplying the rhamnose values by 3.4 (Benincasa et al. 2004).

Kinetics of fermentation process

The kinetics of fermentation experiments was studied in terms of the specific growth rate (μ, h^{-1}), product yields related to substrate consumption ($Y_{P/S}$, g g^{-1}) and to biomass ($Y_{P/X}$, g g^{-1}), biomass yield related to substrate consumption ($Y_{X/S}$, g g^{-1}), specific product yield (q_P, g g^{-1} h), and volumetric productivity (P_V, g l^{-1} h) of the culture media following the standard methods of Aiba et al. (1973).

Surface activity measurements

Equilibrium ST and interfacial tension (IFT) of the CFCB were measured by the ring method at 25°C using a Krüss K10T Tensiometer which is a modified de Noüy apparatus. The instrument was calibrated against ultrapure distilled water (ST = 72 mN m^{-1}). The IFT of the CFCB was measured against *n*-hexadecane. The CMC was determined from the break point of the ST versus dilution times curve. The dilution reduces the rhamnolipid levels below the CMC, at which point the ST of the media suddenly increases. This dilution factor is called the critical micelle dilution (CMD). Surface activity values of the CFCBs were compared by using the modified rapid drop-collapse test (Tugrul and Cansunar 2005).

Emulsification measurements

Emulsification index (E_{24}, %) was measured by vortexing equal volumes of CFCB and a hydrocarbon in a screw-capped graduated test tube and equilibrating at room temperature for 24 h. The E_{24} was calculated as the percent length of the emulsion layer of the total height of liquid column. The emulsifying capacity (EC) of the CFCB was determined by adding a hydrocarbon in 50 µl increments to the CFCB (2 ml) in a graduated test tube. The mixture was vortexed for 10 s after each addition and allowed standing for 2 min. This procedure was repeated until the emulsion collapsed. The volume (in ml) of hydrocarbon added before the emulsion collapsed was termed as the EC of the test CFCB. The emulsifying activity (U_{540}) of the CFCB was determined following the approach of Cirigliano and Carman (1985). Emulsion stability was analyzed on the basis of the changes in the U_{540} with time. The emulsified mixture of the CFCB with hydrocarbon was

allowed to stand for 5 min in the cuvette of spectrophotometer. Then the optical density reading was taken at 540 nm every 5 min for 50 min. The log of the absorbance was plotted versus time (results not shown). The slope of the curve was calculated and termed as decay constant (K_d), which expresses the emulsion stability of the tested CFCB. The relative emulsion volume (EV, %) and relative emulsion stability (ES, %) were measured by vortexing the CFCB (2 ml) with an equal volume of a hydrocarbon in a test tube (100 mm × 15 mm) for 2 min, allowing it to stand for 24 h, and then calculated using the following equations (Das et al. 1998):

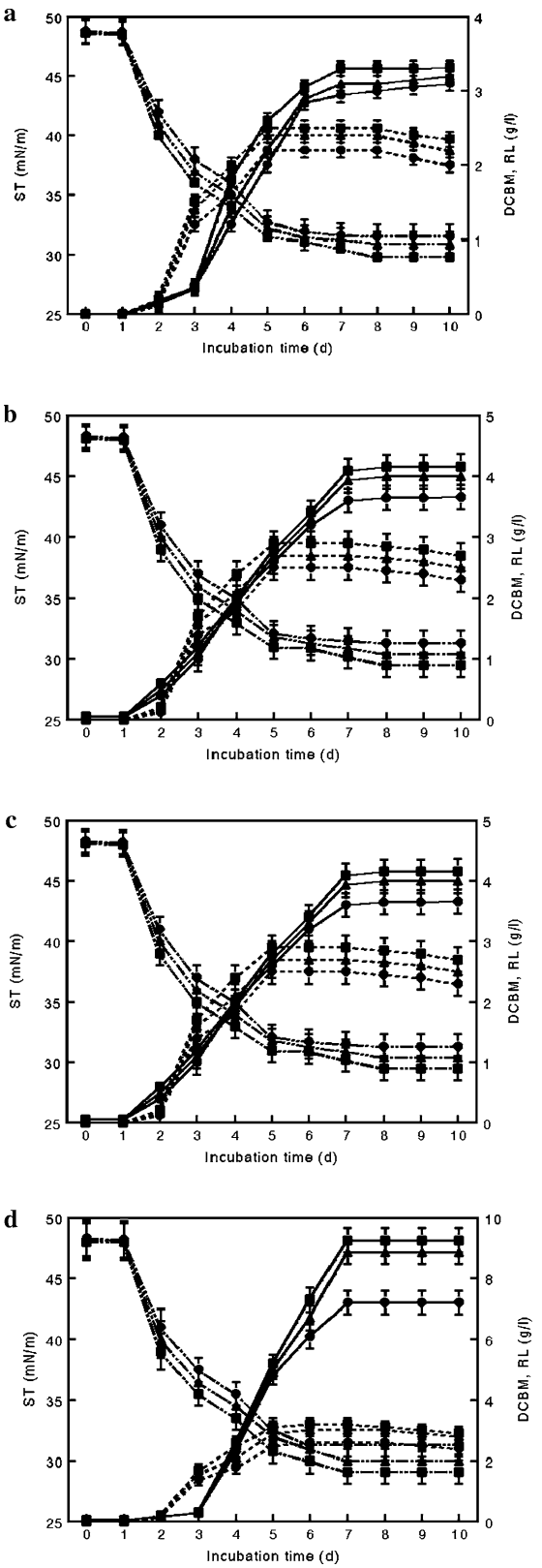
$$\mathrm{EV},\% = [\mathrm{emulsion\,height(mm)} \times \mathrm{cross-sectional\,area(mm^2)}]/[\mathrm{total\,liquid\,column}] \times 100$$

$$\mathrm{ES},\% = [\mathrm{EV}_{24},\ \%\,\mathrm{at}\,24\,\mathrm{h}]/[\mathrm{EV}_{0},\ \%\,\mathrm{at}\,0\,\mathrm{h}] \times 100.$$

Results and discussion

Kinetics of fermentation process

Time course studies of dry cell biomass (DCBM) formation, rhamnolipid production and ST reduction of the culture media of EBN-8 (as inoculum A and inoculum B) grown on different frying oils under single- and batch-fed cultivation setups (I–III) at 100 rpm and 37°C is presented in Fig. 1 EBN-8 grew and produced rhamnolipid on all of the tested growth substrates. The frying oils, being hydrophobic, exhibited prolong resistance against biodegradation due to their low water solubility, which increased their sorption to cell surface and limited their availability to degrading microorganism. This was compensated by initially providing the rhamnolipid (0.05 g l^{-1}) to the culture media to increased the apparent water solubility of the water-insoluble substrates and hence the bacterial growth. After 24 h of incubation, the biomass began to increase, reaching the highest amounts at 5 d before the stationary growth phase was established. The rhamnolipid precursor



◀ **Fig. 1** Time course study of dry cell biomass (DCBM) formation, rhamnolipid (RL) production and surface tension (ST) reduction during the inoculation of the EBN-8 mutant on different frying oils at 100 rpm and 37°C. (—) DCBM, (—) RL, (-·-·-) ST, (•) canola oil, (▲) corn oil, (■) soybean oil. (**a**) WFOs as carbon sources with inoculum A under single-batch cultivation; (**b**) unused frying oils as carbon sources with inoculum A under single-batch cultivation; (**c**) WFOs as carbon sources with inoculum B under single-batch cultivation; (**d**) WFOs as carbon sources with inoculum B under fed-batch cultivation

addition (of inoculum B) enhanced the DCBM and μ values of the EBN-8 mutant in the minimal media (Table 1). For example, in the absence of rhamnolipid (under setup I), the maximum DCBM of 2.5 g l^{-1} was observed with soybean WFO as sole carbon source (Fig. 1a). Inclusion of rhamnolipid in the medium enhanced the DCBM to 2.9 g l^{-1} (setup II; Fig. 1c), whereas the second addition of soybean WFO to the culture medium (under setup III) increased the DCBM to 3.2 g l^{-1} at 5 d (Fig. 1d) i.e., an approx. 10% increase in DCBM was observed by each up-grade of fermentation process from setup I to III. The μ values were 0.901, 0.912 and 0.924 h^{-1}, respectively. The DCBM (3.0 g l^{-1}) and μ (0.906 h^{-1}) values were followed by corn WFO as sole carbon source under the experimental setup-III at 5 d (Fig. 1d and Table 1, respectively).

The EBN-8 mutant showed the best growth yield coefficients ($Y_{X/S}$) of 0.156 and 0.17 g biomass g^{-1} substrate on soybean WFO under single-batch cultivation in the absence and initial presence of the rhamnolipid in the culture media, respectively; which was rational based on the fundamental bacterial growth theories i.e., balanced energy transfer from substrate oxidation to new cell synthesis (Chen and Zhu 2005). We observed a marked change in the substrate consumption when the minimal medium was provided with inoculum B. About 80, 85 and 90% of the substrates were utilized under setups I, II and III, respectively. The facilitated substrate degradation was attributed to the increase of cell surface hydrophobicity after extraction of lipopolysaccharides from the cellular envelope by rhamnolipids, which subsequently stimulated uptake via direct contact between the cells and hydrocarbon droplets (Al-Tahan et al. 2000). Thus, rhamnolipid addition specifically enhanced the biodegradation of hydrocarbons. Koch et al. (1991) studied the utilization of *n*-hexadecane by a *P. aeruginosa* strain in the presence of rhamnolipids. A mutant strain of *P. aeruginosa* PG201 deficient in rhamnolipid production, designated 65E12, lacked the ability to take up ^{14}C-labeled hexadecane and was incapable of growth on a range of alkanes (C_{12} to C_{19}). However, uptake of ^{14}C-labeled hexadecane and growth on alkanes

Table 1 Kinetic parameters of the rhamnolipid production by *P. aeruginosa* EBN-8 on waste frying oils (WFOs)

Carbon source	μ (h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{P/X}$ (g g^{-1})	q_p (g g^{-1} h)	P_V (g l^{-1} h)
Setup I						
Canola WFO	0.786 ± 0.061	0.138 ± 0.009	0.186 ± 0.012	1.364 ± 0.095	1.072 ± 0.081	0.018 ± 0.001
Corn WFO	0.862 ± 0.064	0.15 ± 0.011	0.194 ± 0.013	1.292 ± 0.091	1.114 ± 0.085	0.018 ± 0.001
Soybean WFO	0.901 ± 0.07	0.156 ± 0.011	0.206 ± 0.015	1.320 ± 0.094	1.189 ± 0.087	0.020 ± 0.001
Setup II						
Canola WFO	0.822 ± 0.066	0.147 ± 0.011	0.215 ± 0.011	1.460 ± 0.095	1.200 ± 0.090	0.022 ± 0.001
Corn WFO	0.875 ± 0.07	0.159 ± 0.012	0.231 ± 0.012	1.456 ± 0.095	1.274 ± 0.095	0.023 ± 0.001
Soybean WFO	0.912 ± 0.077	0.17 ± 0.013	0.241 ± 0.015	1.414 ± 0.091	1.289 ± 0.097	0.024 ± 0.001
Setup III						
Canola WFO	0.852 ± 0.065	0.096 ± 0.007	0.267 ± 0.017	2.769 ± 0.167	2.359 ± 0.195	0.043 ± 0.002
Corn WFO	0.906 ± 0.068	0.111 ± 0.008	0.328 ± 0.02	2.953 ± 0.150	2.675 ± 0.197	0.053 ± 0.002
Soybean WFO	0.924 ± 0.081	0.118 ± 0.008	0.344 ± 0.022	2.906 ± 0.145	2.685 ± 0.205	0.055 ± 0.002

The minimal media (Deziel et al. 2000) were supplemented with separate WFOs as carbon sources in two batches at 100 rpm and 37°C. In the first, carbon sources (2% w/v) along with inoculum B (1% v/v) were added to the minimal media and when the substrates were consumed (in about 4 d) the media were fed with the second batch of respective carbon sources (1% w/v). The values given were obtained from three parallel studies ($n = 3$). μ = specific growth rate, $Y_{X/S}$ = biomass related to substrate, $Y_{P/S}$ = product yield related to substrate, $Y_{P/X}$ = product yield related to biomass, q_p = specific product yield, P_V = volumetric productivity

was restored by the addition of rhamnolipids (Beal and Betts 2000).

Rhamnolipid production was below detection limit until 24 h under setup I. Firstly, the rhamnolipid production appeared to be growth-associated as the rhamnolipid contents of the culture media increased and the ST decreased with the increase in biomass over the first 5 d, and then it switched to growth-limiting production. Most of the rhamnolipid was produced after the growth had ceased, and increased throughout the stationary phase, reaching the maximum production after 7 days under either setup. The growth-limited production is characterized by a sharp increase in rhamnolipid production caused by limitation of a component required for growth.

Generally, it is believed that rhamnolipids are produced under nitrogen limiting conditions. Actually, such conditions do not favor rhamnolipid production, as the production starts with the exhaustion of nitrogen in the medium (Robert et al. 1989). The use of inoculum B (containing cells plus 0.05 g rhamnolipid ml^{-1}) as compared to the inoculum A (containing only cells) increased the rhamnolipid production from 3–3.3 g l^{-1} to 3.6–4.1 g l^{-1} using different WFOs under the setup shift from I to II, respectively. The second feeding of respective substrates (under setup III), enhanced the product formation (7.2–9.3 g rhamnolipid l^{-1}) 2–2.3 times the yields obtained under single-batch cultivation (set up II); accompanying the inoculum B in both cases. As a whole, the rhamnolipid precursor addition (from inoculum B) increased the growth and product yields. This might be attributed to an increase the hydrophobicity of the cells surface, thereby increasing direct physical contact with slightly soluble substrates, or it may increase the bioavailability of the hydrophobic substrates by increasing their solubilities (Shreve et al. 1995). The best rhamnolipid production of 9.3 g l^{-1} with the $Y_{P/X}$ of 2.9 g g^{-1} was observed with soybean WFO plus inoculum B under the batch-fed fermentation. The net rhamnolipid production was obtained in two stages. During the first (0–120 h), 2.6 g rhamnolipid l^{-1} was produced with the P_V of 0.022 g rhamnolipid l^{-1} h. During the second (120–168 h), the concentration further increased by 6.3 g rhamnolipid l^{-1} and most of the product (9.3 g rhamnolipid l^{-1}) with the q_P of 2.685 g g^{-1} h and the P_V of 0.055 g rhamnolipid l^{-1} h was secreted into the culture medium. The rhamnolipid yields reported for setups II and III are excluding the initial rhamnolipid supplement (0.05 g l^{-1}) to the media.

A parallel set of experiments was also conducted with unused canola, corn or soybean frying oils as carbon sources. The unused soybean frying oil was the best substrates, of this category, producing 3.1 g rhamnolipid l^{-1} with the $Y_{P/X}$ of 1.348 g g^{-1} at 7 d of incubation under fermentation setup I (Fig. 1b). These results were comparable to those obtained with the WFOs as carbon sources under same experimental setup. Clearly WFOs could replace the native vegetable oils as economical carbon sources for biosurfactant production. *P. aeruginosa* 44T1 when grown on olive oil produced 9 g rhamnolipid l^{-1} with the $Y_{P/S}$ of 0.45 g g^{-1} (Parra et al. 1990), *P. aeruginosa* 47T2 grown on frying oils (sunflower/olive) accumulated 2.7 g rhamnolipid l^{-1} in the culture media with the $Y_{P/X}$ of 0.34 g g^{-1} (Haba et al. 2000) and *P. aeruginosa* DS10-129 produced 4.3 and 2.9 g rhamnolipid l^{-1} using soybean and sunflower oils, respectively, as carbon sources (Rahman et al. 2002).

Surface-active properties

The tensioactive properties of the rhamnolipid-containing CFCB of the EBN-8 mutant grown on different WFOs as carbon sources under batch-fed cultivation (setup III) are given in Table 2. The efficiency of rhamnolipid was measured by the concentration it required to significantly reduce the ST of water, whereas the minimum value to which the ST can be reduced is a measure of its effectiveness. The STs of the CFCBs of EBN-8 mutant on each of the used and unused frying oils as carbon sources in the minimal media reduced to 30 ± 1 mN m^{-1} from ~48 mN m^{-1} after 7 d of incubation (Fig. 1a–d). The lowest ST value of 29.1 mN m^{-1} was obtained with soybean WFO as sole carbon source with inoculum B. The results of ST measurements and those of quantitative analysis of the CFCB for rhamnolipid following the orcinol method were consistent.

Table 2 Comparison of tensioactive characteristics of the cell-free culture broths (CFCBs) of *P. aeruginosa* EBN-8 grown on waste frying oils (WFOs)

Parameter	Canola WFO	Corn WFO	Soybean WFO
Critical micelle dilution (times)	180 ± 9	200 ± 10	220 ± 12
Surface area of 10 μl drop (cm^2) on			
n-Hexadecane-coating	3.1 ± 0.2	4.2 ± 0.3	9.3 ± 0.5
Paraffin oil-coating	2.7 ± 0.2	4.0 ± 0.3	8.2 ± 0.4
Kerosene oil-coating	2.6 ± 0.2	4.1 ± 0.3	7.5 ± 0.4
Emulsification index, E_{24} (%) versus			
n-Hexadecane	65.3 ± 4.3	64.6 ± 4.4	73.6 ± 4.8
Paraffin oil	60.0 ± 4.1	61.5 ± 4.2	69.2 ± 4.7
Kerosene oil	62.1 ± 4.2	62.3 ± 4.3	67.8 ± 4.6
Emulsifying capacity, EC (ml hydrocarbon ml^{-1} CFCB) versus			
n-Hexadecane	22.2 ± 1.8	23.3 ± 1.9	25.0 ± 2.1
Paraffin oil	20.5 ± 1.7	21.2 ± 1.7	22.4 ± 1.8
Kerosene oil	22.9 ± 1.8	25.3 ± 2.0	28.2 ± 2.2
Emulsion activity (U_{540}) versus			
n-Hexadecane	0.21 ± 0.01	0.24 ± 0.02	0.30 ± 0.02
Paraffin oil	0.17 ± 0.01	0.19 ± 0.01	0.24 ± 0.02
Kerosene oil	0.15 ± 0.01	0.17 ± 0.01	0.20 ± 0.01
Decay constant, K_d (Δ log U_{540} min^{-1}) versus			
n-Hexadecane	–0.46 ± 0.02	–0.39 ± 0.02	–0.05 ± 0.002
Paraffin oil	–0.47 ± 0.02	–0.40 ± 0.02	–0.05 ± 0.002
Kerosene oil	–0.48 ± 0.02	–0.40 ± 0.02	–0.06 ± 0.002
Relative emulsion volume (at 0 h), EV_0 (%) versus			
n-Hexadecane	76.7 ± 4.9	76.7 ± 5.0	83.3 ± 5.2
Paraffin oil	71.7 ± 4.6	73.3 ± 4.6	81.7 ± 5.1
Kerosene oil	75.0 ± 4.7	75.0 ± 4.8	80.0 ± 5.1
Relative emulsion volume (at 24 h), EV_{24} (%) versus			
n-Hexadecane	70.7 ± 5.1	70.0 ± 5.2	79.7 ± 5.4
Paraffin oil	65.0 ± 4.5	66.6 ± 4.6	75.0 ± 5.3
Kerosene oil	67.3 ± 4.7	67.5 ± 4.7	73.4 ± 5.2
Relative emulsion stability, ES (%) versus			
n-Hexadecane	92.3 ± 6.7	91.3 ± 6.5	90.3 ± 6.5
Paraffin oil	90.7 ± 6.5	90.8 ± 6.4	91.8 ± 6.6
Kerosene oil	89.7 ± 6.4	90.0 ± 6.4	91.8 ± 6.5

The minimal media (Deziel et al. 2000) were supplemented with separate WFOs as carbon sources in two batches at 100 rpm and 37°C. In the first, carbon sources (2% w/v) along with inoculum B (1% v/v) were added to the minimal media and when the substrates were consumed (in about 4 d) the media were fed with the second batch of respective carbon sources (1% w/v). The CFCBs were obtained by centrifuging the 10 d culture samples at 7,740 *g* for 15 min. The values given were obtained from three parallel studies ($n = 3$)

Below the CMC, the surface activity of the medium depends only on the concentration of monomeric surface-active molecules; hence the ST of a diluted sample increases. The break points of the experimental curves (not shown) yielded CMCs of 42, 44 and 45 mg l^{-1}, respectively, with soybean, corn and canola WFOs, under batch-fed fermentation cultivated with the inoculum B. Zhang and Miller (1995) reported the CMC of rhamnolipid produced from alkanes as 50 mg l^{-1}. The CMD values of different CFCBs were in the range of 180–220 (Table 2). These results indicate that the rhamnolipid biosurfactant produced by EBN-8 is both efficient and effective.

The reduction of tension at an interface by a biosurfactant in an aqueous medium, when the second phase is liquid, is known as liquid–liquid IFT. The biosurfactant tends to absorb at the

interface in an oriented fashion as a consequence of its amphiphatic structure, which is responsible for lowering the IFT and increasing the miscibility of the liquids. The IFT values of the CFCBs obtained from WFOs, as measured against *n*-hexadecane were below 1 mN m^{-1} and that of control was 24 ± 1 mN m^{-1}. Such dramatically low liquid–liquid IFT values exhibited by a biosurfactant is effective in promoting hydrocarbon emulsification and enhanced oil recovery, and, in particular, in mobilizing bound hydrophobic substrates, making them available for biodegradation (Rosenberg and Ron 1999) This property is useful for the bioremediation of oil-contamination.

The surface areas of 10 µl drops of the control medium on the hydrocarbon (*n*-hexadecane, paraffin oil or kerosene oil)-coated surfaces were 0.3 ± 0.1 cm^2 and those of the CFCBs were in the range 2.6–9.3 cm^2 (Table 2). The collapsing of CFCB-drop on the oil-coated surface showed its possession of some biosurfactant, which lowered the ST and IFT between the oily surface and the liquid medium, whereas non-biosurfactant-containing drop of the control medium remained stable.

Emulsification properties

The E_{24} values of the CFCBs of EBN-8 mutant grown on different WFOs, measured against various hydrocarbons, were between 60% and 73.6%, as given in Table 2. The best E_{24} of 73.6% vs. *n*-hexadecane was exhibited by the CFCB obtained from soybean WFO as the source of carbon and energy. The rhamnolipid showed the ability to emulsify hydrocarbons depending on its concentration of the CFCB. The emulsions lost ~10% of their stability after 24 h of ageing.

For various CFCBs of EBN-8 mutant, grown on different WFOs, the emulsifying capacities ranged from 20.5% to 28.2% (Table 2). The CFCB from soybean WFO showed the most efficient EC of 28.2% vs. kerosene oil (i.e., 1 ml of the CFCB could convert 28.2 ml of kerosene oil into an emulsion).

Emulsification activities of various CFCBs obtained from different WFOs were measured versus different hydrocarbons (Table 2). The results revealed that the CFCB from soybean WFO showed the highest U_{540} of 0.3 against *n*-hexadecane. The emulsion stabilization ability of the CFCB was expressed by the decay constant, K_d (the slope of emulsion decay plot). Emulsion decay plots were drawn for different hydrocarbons in the presence of the CFCBs (Figure not shown), and the respective K_d values were calculated (Table 2). The stability of the emulsion was particularly good with the CFCB from soybean WFO against *n*-hexadecane or paraffin oil with the highest K_d of –0.05, indicating a high emulsion stability against hydrocarbons. These U_{540} values of the biosurfactant produced show that it could be used as a bioemulsifier for hydrocarbons and oils, giving stable emulsions.

The EV values of various CFCBs from soybean, corn and canola WFOs against different hydrocarbons at 0 h and 24 h are presented in Table 2. All of them showed good emulsion forming capacity versus different hydrocarbons e.g., 83.3% EV was exhibited by the CFCB from soybean WFO, as measured against *n*-hexadecane. After 24 h of ageing, the average drop in the EV values was only 8–10%. Sometimes, a CFCB might form an emulsion of a high EV but it might lack stability (its K_d value might be of lower order). So, the ES actually measured the emulsion stability capacity of the CFCB against various hydrocarbons (Table 2). The highest ES of 92.3% was observed with the CFCB from canola WFO as measured against *n*-hexadecane.

Conclusions

The fermentation study shows that WFOs have an excellent potential to be used as growth substrates for rhamnolipid production by *P. aeruginosa* EBN-8 and hence to remediate pollution and manage waste. The use of such waste substrates for rhamnolipid production is an ecologically acceptable option, as it would reduce the waste treatment costs and add economic value to industrial residues. The rhamnolipid produced exhibited significant surface-active and emulsification properties and a high level of emulsion stability. These properties have potential commercial and environmental applications. For this purpose, we have evaluated different

fermentation setups to optimally produce the rhamnolipid biosurfactant from the waste substrates.

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References

Aiba S, Humphrey AE, Millis NF (1973) Biochemical engineering. Academic Press, New York, pp 75–106

Al-Tahan RA, Sandrin TR, Bodour AA, Maier RM (2000) Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiol 66:3262–3268

Beal R, Betts WB (2000) Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J Appl Microbiol 89:158–168

Beckman C (2000) Vegetable oil: situation and outlook. Bi-weekly Bull 13:1–6

Benincasa M, Abalos A, Oliveira I, Manresa A (2004) Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. Anton van Leeuwen 85:1–8

Chandrasekaran EV, Bemiller JN (1980) Constituent analyses of glycosaminoglycans. In: Whistler RL, Wolform ML (eds) Methods in carbohydrate chemistry. Academic Press, New York, pp 89–96

Chen G, Zhu H (2005) *lux*-Marked *Pseudomonas aeruginosa* lipopolysccharide production in the presence of rhamnolipid. Coll Surf 41b:43–48

Cirigliano MC, Carman GM (1985) Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*. Appl Environ Microbiol 50:846–850

Das M, Das SK, Mukherjee RK (1998) Surface active properties of the culture filtrates of a *Micrococcus* species grown on *n*-alkanes and sugars. Biores Technol 63:231–235

Del' Arco JP, de Franka FP (2001) Influence of oil contamination levels in hydrocarbon biodegradation in sandy sediment. Environ Pollut 110:515–519

Desai JD (1987) Microbial surfactants: evaluation, types, production and future applications. J Sci Indus Res 46:440–449

Deziel E, Lepine F, Milot S, Villemur R (2000) Mass spectrometry monitoring of rhamnolipids from a growing culture of *Pseudomonas aeruginosa* strain 57RP. Biochim Biophys Acta 1485:145–152

Haba E, Espuny MJ, Busquets M, Manresa A (2000) Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J Appl Microbiol 88:379–387

Itoh S, Suzuki T (1972) Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in *n*-paraffin-utilizing ability. Agric Biol Chem 36:2233–2235

Koch AK, Kappeli O, Fiechter A, Reiser J (1991) Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J Bacteriol 173:4212–4219

Makker RS, Cameotra SS (2002) An update on the use of unconventional substrates for biosurfactant production and their new applications. Appl Microbiol Biotechnol 58:428–434

Nitschke M, Costa SG, Contiero J (2005) Rhamnolipid surfactants: an update on the general aspects of these remarkable biomolecules. Biotechnol Prog 21:1593–1600

Parra JL, Pastor J, Comelles F, Manresa MA, Bosch MP (1990) Studies of biosurfactants obtained from olive oil. Tenside Surf Det 27:302–306

Rahman KSM, Rahman TJ, McClean S, Marchant R, Banat IM (2002) Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost materials. Biotechnol Prog 18:1277–1281

Raza ZA, Khan MS, Khalid ZM, Rehman A (2006) Production of biosurfactant using different hydrocarbons by *Pseudomonas aeruginosa* EBN-8 mutant. Z Naturforsch 61c:87–94

Robert M, Mercade ME, Bosch MP, Parra JL, Espuny MJ, Manresa MA, Guinea J (1989) Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol Lett 11:871–874

Rosenberg E, Ron EZ (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162

Shreve GS, Inguva S, Gunnam S (1995) Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*. Mol Mar Biol Biotechnol 4:331–337

Tugrul T, Cansunar E (2005) Detecting surfactant-producing microorganisms by the drop-collapse test. World J Microbiol Biotechnol 21:851–853

Zhang Y, Miller RM (1995) Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of *n*-alkanes. Appl Environ Microbiol 61:2247–2251

Zhang Y, Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfacatnt). Appl Environ Microbiol 58:3276–3282