Biodegradation (2007) 18:115–121
DOI 10.1007/s10532-006-9047-9

Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes

Zulfiqar Ali Raza[1,2], Asma Rehman[2], Muhammad Saleem Khan[1] & Zafar M. Khalid[2,*]
[1]*Polymer Chemistry Laboratory, National Centre of Excellence in Physical Chemistry, University of Peshawar, 25210 Peshawar, Pakistan;* [2]*Environmental Biotechnology Division, National Institute for Biotechnology & Genetic Engineering, P.O. Box 577, Faisalabad, Pakistan (*author for correspondence: e-mail: zmkhalid@yahoo.com)*

Accepted 18 January 2006

Key words: biosurfactant, emulsion, *Pseudomonas aeruginosa*, vegetable oil wastes, yield factors

Abstract

Biosurfactant production by *Pseudomonas aeruginosa* EBN-8 mutant was studied in shake flasks on separate wastes from canola, soybean and corn oil refineries. Of the substrates tested, canola oil refinery waste (COD = 20 g l^{-1}) supplemented with sodium nitrate (at COD/N = 20) showed the best microbial growth (4.50 g l^{-1}) and rhamnolipid production (8.50 g l^{-1}), at 10 d of incubation with the specific growth rate of 0.316 h^{-1} and specific product yield of 0.597 g g^{-1} h. Its cell-free supernatant showed the critical micelle dilution (CMD) of 150 and surface tension (ST) of 28.5 mN m^{-1}.

Introduction

Biosurfactants have several benefits over chemical surfactants because of their advantages like lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, ease of production, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Desai & Banat 1997), and promising substituents for synthetic surfactants (Georgiou et al. 1992). To date, biosurfactants are unable to compete with chemical surfactants in terms of economics due to their higher production expenditures. However, one option is to produce them from some cost-free or cost-credit substrates like industrial and domestic wastes having an appropriate balance in the nutrient contents required for the microbial population. For this purpose, strong candidates have been olive oil mill effluent (OOME), agroindustrial by-products (Robert et al. 1989; Mercade et al. 1993; Mercade & Manresa 1994), distillery and whey wastes (Dubey & Juwarker 2001), used vegetable oils (Vipulanandan & Ren 2002; Haba et al. 2003), waste free fatty acids (Abalos et al. 2002) and soybean oil refinery wastes (Abalos et al. 2001). The selection of inexpensive raw material is important to the overall economy of the fermentation process in the sense that it accounts for ~50% of the final product and also reduces the wastes' treatment expenses (Makker & Cameotra 1999). *Pseudomonas* species are well known for their capability to produce rhamnolipid biosurfactants on different carbon sources (Robert et al. 1989; Mercade et al. 1993).

Vegetable oil refinery wastes (VORWs), the mixtures of water-soluble and insoluble substrates, having high carbohydrate and lipid contents, meet the criteria to be used as substrates for the biosurfactant production. In the present manuscript, these wastes have been studied as sole carbon and

energy sources by *Pseudomonas aeruginosa* EBN-8 mutant stain. The shake flask experiments were monitored by measuring the biomass and rhamnolipids accumulation in the culture media followed by determining the surface-active characteristics of the culture supernatant. The kinetics of biosurfactant production was studied in terms of yield factors, specific substrate uptake rate (q_s) and specific product yield (q_p).

Materials and methods

Growth substrates

Refinery waste samples were separately collected from the canola, soybean and corn oils processing units at a local vegetable oil refining industry. Settleable solids from the samples were removed using a separating funnel and the suspended or otherwise fibrous materials by passing the samples through a vacuum filtration assembly (containing Whatman qualitative filter paper 42, 125 mm diameter). These pre-treated samples were stored at 4 °C until needed for analyses and/or biosurfactant production.

Analyses of substrates

Total sugars in the VORWs were determined by the standard dinitrosalicylic acid (DNS) method, total carbohydrates by the standard phenol–sulfuric acid method and proteins by the Bradford microassay using bovine serum albumin as a standard protein. Residual oils in the samples were estimated by extracting 1 ml of the sample with two 5-ml volumes of petroleum ether, followed by separating the residue in a rotary evaporator (Rotavapor EL 131, BÜCHI) at 70 °C and weighed. Concentrations of Na^+ and K^+ in the samples were determined using a Flame Photometer (FP 20, SEAC) and Fe^{2+} by an Atomic Absorption Spectrometer (SpectrAA·20, Varian). The summary of results of these tests conducted in triplicates is given in Table 1. All of the chemicals used were of analytical grade.

Bacteria

The present work is in continuity to investigate the growth behaviour of hydrocarbon utilizing gamma ray-induced mutant strain *P. aeruginosa* EBN-8 (Iqbal et al. 1995) on distant carbon substrates like VORWs. *P. aeruginosa* EBN-8 mutant was obtained by subjecting the cell suspension of *P. aeruginosa* S8 parent strain to the best gamma radiation dose of 400 Gy with 3 log kill, then plating on nutrient agar and incubating at 37 °C (Iqbal et al. 1995). The EBN-8 mutant strain was enriched in the VORW media (of the composition mentioned below) in 250 ml Erlenmeyer flasks in an orbital shaker at 37 ± 1 °C and 100 rpm. The cells were collected by centrifugation (at 7740 *g* for 15 min), washed alternatively with filter-sterilized *n*-hexane and saline (0.89% w/v, NaCl), and resuspended in saline to adjust an optical density of 0.7 at 660 nm, to be used as inoculum.

Table 1. Composition of different vegetable oil refinery wastes, diluted to COD value of 20.0 ± 0.1 g l^{-1} and at pH 7.0 ± 0.1

Parameter	Canola ORW	Soybean ORW	Corn ORW
Total solids (105 °C)	11.53 ± 0.02	10.93 ± 0.01	11.42 ± 0.02
Total volatile matter (%)	98.90 ± 0.05	98.20 ± 0.06	98.80 ± 0.05
Minerals (550 °C)	3.26 ± 0.01	3.18 ± 0.01	3.05 ± 0.01
Residual oils	9.68 ± 0.03	9.55 ± 0.03	9.63 ± 0.02
Total sugars	0.45 ± 0.01	0.37 ± 0.01	0.40 ± 0.02
Total carbohydrates	0.79 ± 0.03	0.71 ± 0.02	0.73 ± 0.02
Phenols	0.042 ± 0.001	0.047 ± 0.001	0.057 ± 0.001
Proteins	0.77 ± 0.03	0.79 ± 0.02	0.75 ± 0.01
COD/N ratio	185.00 ± 6.30	245.50 ± 8.20	205.00 ± 7.00
Na^+ (mg l^{-1})	838.59 ± 5.00	798.95 ± 7.10	858.33 ± 9.25
K^+ (mg l^{-1})	40.69 ± 0.75	38.95 ± 0.70	42.25 ± 0.84

The values are given in g l^{-1} or specified, otherwise. The results are averages of three concordant readings.
ORW = oil refinery waste.

Shake flask experiments

Microbial growth and biosurfactant production experiments were conducted in 250 ml Erlenmeyer flasks each containing 50 ml of the culture medium with VORW, diluted to COD of 20.0 g l^{-1}. KH_2PO_4, K_2HPO_4, $CaCl_2{\cdot}2HO_2$, $MgSO_4{\cdot}7H_2O$ and $FeCl_3{\cdot}6H_2O$ were added to the media to attain the concentrations of P, Ca, Mg and Fe, as proposed by Bushnell & Hass (1941). Separate synthetic nitrogen sources like sodium nitrate, ammonium nitrate and ammonium sulfate were also supplied to adjust the COD/N ratios of the media to 10, 20 or 30. The pH value was set at 7.0 by K_2HPO_4, following the sterilization. The flasks were provided with 2% (v/v) inocula and incubated at 37 ± 1 °C and 100 rpm in an orbital shaker. A parallel set of abiotic control-flasks was also incubated provided with the same nutritional conditions, but without bacteria.

Measurement of metabolites produced

Bacterial growth was measured by removing the cells from the culture broth by centrifugation at 7740 *g* for 15 min. The cell-free culture broth (CFCB) was used for analytical measurement and biosurfactant recovery; and the cell pellet was washed with sterile *n*-hexane and saline, and desiccated in an electric oven at 60 °C to determine the dry cell biomass (DCBM). The whole cell proteins were also measured to confirm the results of DCBM following the standard Bradford microassy.

Extraction and quantification of biosurfactants

Crude biosurfactants were extracted from the CFCB by acid precipitation followed by liquid–liquid extraction, according to Zhang & Miller (1995). The extracts were dried, weighed and re-dissolved in distilled water to determine rhamnolipids (in terms of rhamnose equivalents) by the standard orcinol assay. Rhamnolipids were calculated by multiplying rhamnose amount by a factor of 3.4 (Banincasa et al. 2004). The kinetics of fermentation process was studied following the standard methods of Aiba et al. (1973).

Analytical procedures

Surface tension (ST) and interfacial tension (IFT) of the CFCB against *n*-hexadecane were measured with a Krüss K10T Tensiometer working according to the standard de Noüy method. The critical micelle dilution (CMD) was determined by measuring the ST of serial dilutions of CFCB in distilled water at pH 7.0 and the emulsification strength (E_{24}) by vortexing the equal volumes of the CFCB and different hydrocarbons or crude canola oil at high speed for 2 min and then determining the percentage volume occupied by the emulsion after 24 h of equilibration at 25 °C.

Results and discussion

Biosurfactant production

Effect of separate nitrogen sources like sodium nitrate, ammonium nitrate or ammonium sulfate at different COD/N ratios on the production of rhamnolipid biosurfactant by EBN-8 from separate oil refinery wastes like of canola, soybean or corn, after 10 d of growth is shown in Figure 1. The results indicated that canola oil refinery waste medium amended with sodium nitrate to the COD/N ratio of 20 gave the highest amount of rhamnolipids (8.50 g l^{-1}). The strain was able to utilize all the selected nitrogen sources at all specified COD/N ratios to different extent, but the preferred nitrogen source was observed as sodium nitrate. Although refinery wastes were rich in other mineral contents, yet were deficient in nitrogen. Therefore, some separate inorganic nitrogen sources were added to the media to maximize the rhamnolipid yield. Table 2 shows that with canola oil refinery waste (at COD/N ratio of 20), sodium nitrate (product yield, $Y_{P/X} = 1.889$ g g^{-1}) was observed to be more effective nitrogen source than ammonium nitrate ($Y_{P/X} = 1.853$ g g^{-1}) or ammonium sulfate ($Y_{P/X} = 1.864$ g g^{-1}). Similar trends were observed with separate soybean and corn oil refinery wastes as growth substrates by EBN-8 (Figure 1). The higher nitrogen concentrations enhanced the bacterial growth but suppressed the biosurfactant formation; indicating a nitrogen-limited biosurfactant production.

After optimizing the nitrogen source, on the basis of product yield, the microbial growth, rhamnolipid production and ST changes with time were studied (Figure 2). After 36 h of lag phase, the microbes entered the exponential growth phase (36–144 h); at the end of this phase, maximum

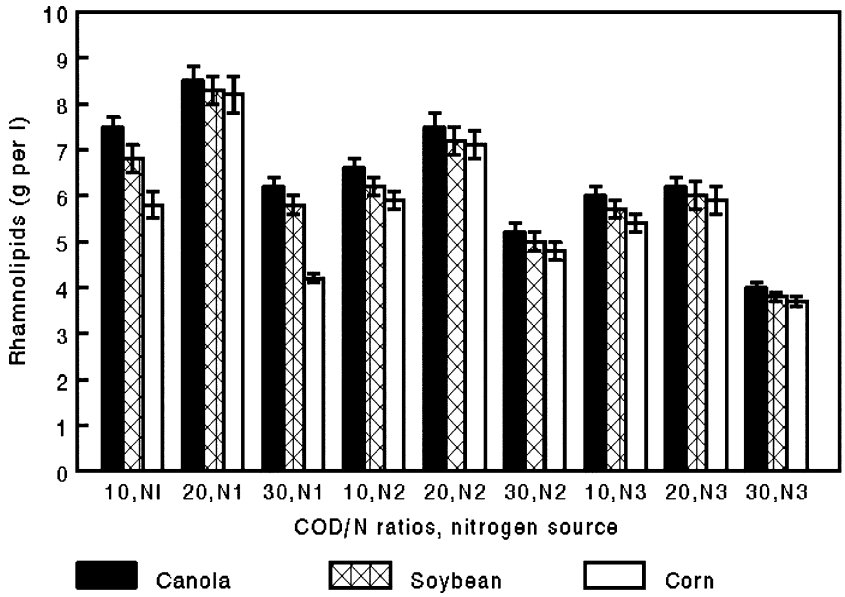

Figure 1. Effect of different nitrogen sources on the production of rhamnolipids by the EBN-8 on different vegetable oil refinery wastes at 20.0 mg COD l^{-1} and the COD/N ratio of 20 in 250 ml Erlenmeyer flasks in an orbital shaker at pH 7.0 ± 0.1, 37 ± 1 °C and 100 rpm in an orbital shaker, at 10 d of fermentation. The results are averages of three concordant readings. N1, sodium nitrate; N2, ammonium nitrate; N3, ammonium sulfate.

Table 2. Comparison of kinetics of growth specific rate (μ), product formation related to substrate consumption ($Y_{P/S}$) and dry cell biomass ($Y_{P/X}$), bacterial growth related to substrate consumption ($Y_{X/S}$), specific substrate utilization rate (q_s) and specific product yield (q_p) during growth of the EBN-8 on different vegetable oil refinery wastes (COD = 20.0 ± 0.1 g l^{-1}) amended with different nitrogen sources at the COD/N ratio of 20 in 250 ml Erlenmeyer flasks in an orbital shaker at 37 ± 1 °C, pH 7.0 ± 0.1 and 100 rpm

C & N sources	μ (h^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{P/X}$ (g g^{-1})	$Y_{X/S}$ (g g^{-1})	q_s (g g^{-1} h)	q_p (g g^{-1} h)
Canola ORW						
$NaNO_3$	0.32 ± 0.01	0.47 ± 0.02	1.89 ± 0.15	0.25 ± 0.01	1.26 ± 0.10	0.60 ± 0.04
NH_4NO_3	0.31 ± 0.01	0.46 ± 0.02	1.85 ± 0.14	0.25 ± 0.01	1.25 ± 0.09	0.57 ± 0.03
$(NH_4)_2SO_4$	0.30 ± 0.01	0.46 ± 0.02	1.86 ± 0.14	0.24 ± 0.01	1.25 ± 0.09	0.57 ± 0.03
Soybean ORW						
$NaNO_3$	0.34 ± 0.02	0.43 ± 0.02	1.56 ± 0.12	0.27 ± 0.01	1.26 ± 0.09	0.54 ± 0.02
NH_4NO_3	0.34 ± 0.01	0.41 ± 0.02	1.50 ± 0.12	0.27 ± 0.01	1.24 ± 0.09	0.51 ± 0.02
$(NH_4)_2SO_4$	0.33 ± 0.01	0.40 ± 0.01	1.51 ± 0.11	0.27 ± 0.01	1.24 ± 0.08	0.50 ± 0.02
Corn ORW						
$NaNO_3$	0.28 ± 0.01	0.38 ± 0.01	1.64 ± 0.12	0.23 ± 0.01	1.22 ± 0.08	0.46 ± 0.02
NH_4NO_3	0.27 ± 0.01	0.35 ± 0.01	1.58 ± 0.12	0.22 ± 0.01	1.22 ± 0.08	0.43 ± 0.02
$(NH_4)_2SO_4$	0.27 ± 0.01	0.35 ± 0.01	1.55 ± 0.11	0.22 ± 0.01	1.20 ± 0.07	0.42 ± 0.02

The results are averages of three concordant readings.
ORW = oil refinery waste.

growth was observed as 4.80 g DCBM l^{-1} with soybean oil refinery waste as carbon source and sodium nitrate as nitrogen source at the COD/N ratio of 20. Rhamnolipid production started at 48 h of incubation and completed in two stages. During the first stage (48–144 h), the production was growth associated and lasted for 4.50 g DCBM and 4.30 g rhamnolipid l^{-1} with $Y_{P/X}$ of 0.977 g g^{-1} with canola oil refinery waste. After this, nitrogen in the medium was depleted and the strain reached the stationary growth phase. In this phase, biosurfactant concentration gradually

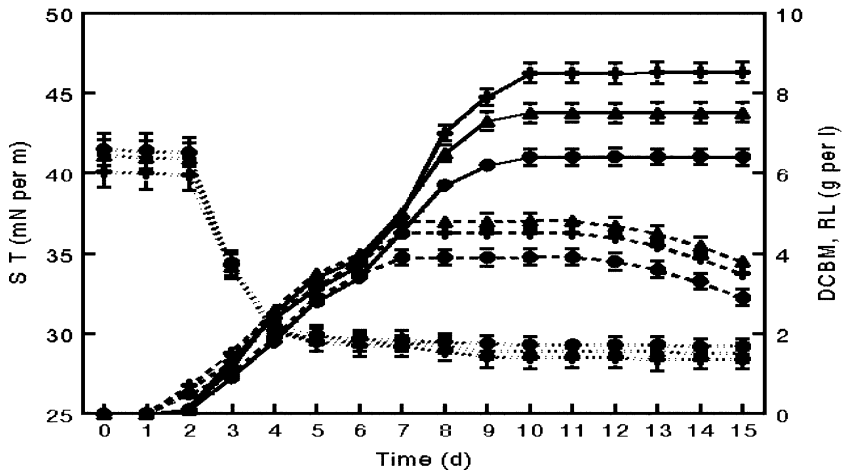

Figure 2. Changes in surface tension (ST) dry cell biomass (DCBM) and rhamnolipids (RL) accumulation with time by EBN-8 on different vegetable oil refinery wastes at 20.0 g COD l^{-1} and the COD/N ratio of 20 in 250 ml Erlenmeyer flasks in an orbital shaker at pH 7.0 ± 0.1, 37 ± 1 °C and 100 rpm. The results are averages of three concordant readings. (+), canola oil refinery waste; (▲), soybean oil refinery waste; (●), corn oil refinery waste; (· · · ·), surface tension; (- - -), DCBM; (—), RL.

increased to the final production (8.50 g rhamnolipid l^{-1}) with $Y_{P/X}$ of 1.889 g g^{-1} at 240 h of incubation and most of the product was liberated into the medium. This exhibited that biosurfactants were produced as secondary metabolites. After 12 d, the death phase started and no subsequent increase in rhamnolipid production was observed (Figure 2). The whole cell proteins produced by EBN-8 on canola, soybean and corn oil refinery wastes amended with sodium nitrate at 10 d of incubation were 1.9, 2.1 and 1.6 g l^{-1}, respectively.

Mercade et al. (1993) were able to produce 6.4 g rhamnolipids l^{-1} from OOME by *Pseudomonas* sp. JAMM. Haba et al. (2000) observed 2.7 g rhamnolipid l^{-1} from waste frying oil by *P. aeruginosa* 47T2 NCIB 40044. Rahman et al. (2002) observed 4.31 g rhamnolipid l^{-1} by *P. aeruginosa* DS10-129 with soybean oil as growth substrate at 288 h of incubation. In our study, the product yield ($Y_{P/S}$) of 0.472 g rhamnolipids g^{-1} canola oil refinery waste by *P. aeruginosa* EBN-8 was higher than 0.382 g rhamnolipid g^{-1} olive oil by *P. aeruginosa* 44T1 (Robert et al. 1989), 0.058 g rhamnolipid g^{-1} OOME by *Pseudomonas* sp. JAMM (Mercade et al. 1993), 0.405 g rhamnolipid g^{-1} canola oil by *P. aeruginosa* UW-1 (Sim et al. 1997), and 0.089 and 0.093 g rhamnolipid g^{-1} whey and distillery wastes, respectively, by *P. aeruginosa* BS2 (Babu et al. 1996). The results of specific growth rates, yield factors, specific substrate uptake rate and specific product yield with different VORWs amended with different N-sources at the COD/N ratio of 20 are given in Table 2. The highest specific growth rate of 0.344 h^{-1} was observed with soybean oil refinery waste and sodium nitrate as C- and N-sources, respectively. Specific product yield (0.597 g g^{-1} h) was the highest with canola oil refinery waste amended with sodium nitrate followed by (0.574 g g^{-1} h) with the same substrate but amended with ammonium nitrate to the COD/N ratio of 20.

Surface-active properties of biosurfactants

The surface-active properties of a biosurfactant mainly depend on its ability to reduce surface and IFTs, CMD value and formation of stable emulsions with different water-immiscible substrates. The initial lower ST (41 ± 1 mN/m) of the VORWs media might be due to colloidal aggregation of proteins, residual oil and particulate material (Martinez-Moreno 1972). The mutant strain lowered the ST of the culture medium from 41.1 ± 0.3 to 29.0 ± 0.4 mN m^{-1} on all the three carbon sources used at all the specified COD/N ratios and that of control media remained 40.0 ± 0.8 mN m^{-1} throughout the incubation period. This indicated the production of a surface-active compound such as rhamnolipid in the treated media. The IFT of the CFCB of EBN-8 against *n*-hexadecane dropped to < 1 from 21 mN m^{-1} at 10 d of growth (Table 3). Stable emulsions of different hydrocarbons or

Table 3. Comparison of surface-active properties of the cell-free culture broth (CFCB) of EBN-8 on different vegetable oil refinery wastes (COD = 20.0 g l^{-1}) amended with sodium nitrate to the COD/N ratio of 20, at 10 d of growth

Parameter	Canola ORW		Soybean ORW		Corn ORW	
	CFCB	Control	CFCB	Control	CFCB	Control
IFT (Mn m^{-1})	21.1 ± 0.5	0.70 ± 0.03	22.2 ± 0.6	0.90 ± 0.04	22.4 ± 0.5	0.95 ± 0.04
CMD	6.2 ± 0.2	150.0 ± 4.5	5.6 ± 0.2	100.0 ± 2.5	1.5 ± 0.1	80.0 ± 2.0
Emulsification (%)						
(*n*-hexadecane)	11.0 ± 0.2	71.0 ± 0.4	10.0 ± 0.2	70.0 ± 0.5	5.0 ± 0.5	65.0 ± 0.4
(*n*-heptadecane)	9.0 ± 0.1	67.1 ± 0.5	8.0 ± 0.1	68.7 ± 0.4	4.0 ± 0.1	63.7 ± 0.4
(paraffin oil)	10.0 ± 0.2	69.2 ± 0.5	9.0 ± 0.1	70.2 ± 0.5	5.0 ± 0.1	60.0 ± 0.5
(kerosene oil)	8.0 ± 0.1	68.3 ± 0.4	7.0 ± 0.1	65.0 ± 0.4	4.0 ± 0.1	64.5 ± 0.4
(crude canola oil)	12.0 ± 0.2	74.0 ± 0.3	11.0 ± 0.2	73.7 ± 0.6	6.0 ± 0.2	72.3 ± 0.5

The results are averages of three concordant readings.
ORW = oil refinery waste; IFT = interfacial tension; CMD = critical micelle dilution.

crude canola oil with the CFCB of EBN-8 from different VORWs were observed even after 24 h of equilibration at room temperature. Emulsification values lied between 60% and 74% (Table 3). The best emulsification (74%) was observed with the crude canola oil.

Conclusions

The experiments conducted using variuos VORWs as carbon sources showed that nitrogen depletion enhanced the rhamnolipid production, and with a particular substrate, the specific product formation rate was directly proportional to the specific growth rate. However, the best specific product yield (0.597 g g^{-1} h) was obtained with the canola oil refinery waste and sodium nitrate as carbon and nitrogen sources, respectively, at the C/N ratio of 20. The optimized yield factors can be incorporated to design a culture media of *P. aeruginosa* EBN-8 on different VORWs to get an optimal microbial growth resulting in a significant and efficient rhamnolipid production.

Acknowledgements

The authors acknowledge the Higher Education Commission, Islamabad for funding for this research, Ch. Muhammad Akram, General Manager, Madni Ghee Mills, Faisalabad for providing the VORWs samples and Mr. Muhammad Afzal, Senior Scientist, NIBGE for his lab's facilities.

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