Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 25, No. 02, pp. 269 - 274, April - June, 2008

USE OF WEATHERED DIESEL OIL AS A LOW-COST RAW MATERIAL FOR BIOSURFACTANT PRODUCTION

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(Received: February 01, 2007 ; Accepted: February 25, 2008)

Abstract - This work aimed to investigate the capability of biosurfactant production by *Staphylococcus hominis*, *Kocuria palustris* and *Pseudomonas aeruginosa* LBI, using weathered diesel oil from a long-standing spillage as raw material. The effect of the culture media (Robert or Bushnell-Haas) and of the carbon source (spilled diesel oil or commercial diesel oil) on biosurfactant production was evaluated. Erlenmeyer flasks (250 mL) containing the cell broth were agitated (240 rpm) for 144 h at 27±2°C. Biosurfactant production was monitored according to the De Nöuy ring method using a Krüss K6 tensiometer. Considering the possibility of intracellular storage of biosurfactant in the cell wall of the cultures *S. hominis* and *K. palustris*, experiments were also done applying ultrasound as a way to rupture the cells. For the conditions studied, the cultures did not indicate production of biosurfactants. Results obtained with a hydrocarbon biodegradability test based on the redox indicator 2,6-dichlorophenol indophenol showed that only the commercial diesel was biodegraded by the cultures.
Keywords: Biosurfactant; Diesel oil; Raw material; Weathered.

INTRODUCTION

Commercial viability of biosurfactants is still limited by their high production costs, associated with inefficient recovery methods and with the use of expensive raw materials (Nitschke and Pastore, 2002). These costs can be significantly reduced with the development of cheaper processes and the use of low-cost raw materials, which account for 10-30% of the overall cost (Cameotra and Makkar, 1998). Biosurfactants can be commercially produced at levels of up to 100 g/L, as reported for rhamnolipids from *Pseudomonas* (Maier and Soberon-Chavez, 2000). This production level, combined with the use of cheap renewable substrates as organic wastes, makes the cost of biosurfactants competitive with the cost of synthetic surfactants (Makkar and Cameotra, 2002). Alternative substrates have been suggested for biosurfactant production, especially water-miscible agro-industrial wastes: molasses, whey, cassava wastewater and distillery wastes (Babu et al., 1996; Nitschke and Pastore, 2006). However, there are few examples of the use of hydrophobic wastes as cheap substrates, for instance, waste frying oils, used lubricant oils and oily sludge from petroleum refineries (Mercadé et al., 1996; Haba et al., 2000; Pirôllo, 2006). Thus, this work aimed to verify the capability of three bacterial cultures to produce biosurfactants using weathered diesel oil recovered from the groundwater at a petrol station as raw material.

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MATERIALS AND METHODS

Growth Conditions

Experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL liquid medium. Different combinations of culture media and carbon sources were evaluated. Thus, the effect of two media on biosurfactant production was tested: a) the medium described by Robert et al. (1989) and the Bushnell-Hass (BH) medium (Difco, 1984). The former medium has been employed in biosurfactant production experiments using different strains of *P. aeruginosa* (Robert et al., 1989; Benincasa et al., 2002; Silva, 2005; Pirôllo, 2006), while the BH medium is normally used in studies where the biodegradation of hydrocarbons is evaluated (Hanson et al., 1993). As carbon sources, we tested the diesel oil (W-D) recovered from the groundwater at a petrol station [whose soil was the source of the cultures *Staphylococcus hominis* and *Kocuria palustris* (Mariano et al., 2007)] and, comparatively, a commercial diesel oil (C-D) purchased from a local petrol station. The mineral media plus the carbon source [different concentrations of diesel oil (Table 1)] were sterilised together in an autoclave at 121°C and 1 atm for 15 minutes. Then, inoculum (1 mL; O.D. of 0.65 at 610 nm SHIMADZU UV-1601PC) was added to the flasks, which were kept under agitation (240 rpm) for 144 hours at 27±2°C. Experiments including the strain *Pseudomonas aeruginosa* LBI [isolated by Benincasa et al. (2002) from a hydrocarbon contaminated area] and the Robert media with the commercial diesel were not carried out because this strain had previously proved to be an efficient biosurfactant producer under these conditions (Pirôllo, 2006).

The spillage at the petrol station that released diesel oil into the groundwater occurred approximately ten years ago. Analyses of BTEX (benzene, toluene, ethylbenzene and xylenes), PAH (polyaromatic compounds) and TPH (total petroleum hydrocarbons) showed that the composition of the diesel oil had been altered probably due to both biological and physico-chemical processes (Mariano, 2006).

Surface Tension Measurement

After the incubation period, the surface tension of the cell-free culture was measured employing the De Nöuy ring method with a Krüss K6 tensiometer.

Verification of Bioemulsifiers

For experiments 1, 4, 5, 11, 14 and 15 (Table 1), the emulsion index (E24) was determined in accordance with Iqbal et al. (1995) for the weathered diesel oil (W-D) or kerosene.

Application of Ultrasound

Considering the possibility of intracellular storage of biosurfactant in the cell wall (Gomes et al., 2004), in some experiments (Table 1) ultrasound was also applied as a way to rupture the cells to obtain the release of biosurfactant into the broth. The ultrasound was irradiated in the culture broth (with the cells) before measuring the superficial tension. A cells disrupter (UNIQUE) was employed (ultrasonic frequency of 20 KHz, 306 W). For experiments 10 and 20, the ultrasound was irradiated for 20 minutes and for experiments 6, 8, 16 and 18, for 10 minutes. The ultrasound application was only employed in experiments with the cultures *S. hominis* and *K. palustris* because the strain *P. aeruginosa* LBI is an extracellular biosurfactant producer (Benincasa et al., 2002).

Diesel oil Biodegradability Test

The diesel oil biodegradability test was done using the redox indicator 2,6-dichlorophenol indophenol (DCPIP) (Hanson et al., 1993). During the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors. By incorporating an electron acceptor such as DCPIP into the culture medium, it is possible to ascertain the ability of the microorganism to utilise hydrocarbon substrate by observing the colour change of DCPIP from blue (oxidised) to colourless (reduced). The capability of the cultures *S. hominis*, *K. palustris* and *P. aeruginosa* LBI to degrade both diesel oils (W-D and C-D) was verified. Inoculum was added (125 μL, O.D. = 0.55) to test tubes (duplicates) that contained sterile BH medium (7.5 mL) and 50 μL of diesel oil. The concentration of DCPIP was 27 mg/mL. Test tubes were kept under agitation (240 rpm) at 27±2°C.

RESULTS AND DISCUSSION

Table 1 shows the results of superficial tension measures of the culture broth without inoculum

(initial superficial tension) and after incubation time (final superficial tension) in accordance with the experimental conditions. Results demonstrate that no significant decrease in superficial tension was verified in any of the experiments. According to Haba et al. (2000), good biosurfactant producers were considered to be those that decrease the surface tension to 40 mN/m or less. Thus no biosurfactant production was obtained in the experiments. In other works, the strain *P.aeruginosa* LBI was capable of producing the rhamnolipid biosurfactant using soapstock (Moraes et al., 2002), mannitol and glycerol (Silva, 2005) and kerosene, diesel oil, crude oil and oily sludge (Pirôllo, 2006).

Emulsification tests in experiments 1, 4, 5, 11, 14 and 15 demonstrated that no bioemulsifier was produced by the cultures *S. hominis* and *K. palustris*, since the E24 for both diesel oil and kerosene was zero. Carrying out this test is important because not all biosurfactants show the property of reducing the surface tension. However, some biosurfactants, such as emulsan, are good emulsifier agents of hydrocarbons (Kim et al., 1997). According to Ron and Rosemberg (2001), biosurfactants with low-molecular-weight molecules lower surface and interfacial tensions efficiently, while high-molecular-weight polymers are good emulsifiers. The rhamnolipid biosurfactant produced by the strain *P. aeruginosa* LBI from commercial diesel (Pirôllo, 2006), was capable of emulsifying different compounds, such as benzene, kerosene, diesel oil and crude oil.

Table 1: Summary of the experimental conditions and results of the superficial tension measurement

Experiment	Culture[1]	Medium[2]	Carbon source[3]	Diesel oil conc. (% v/v)	Ultrasound application	Superficial tension (mN/m)	
						initial	final
1	1	1	W-D	1	no	53.0	62.0
2	1	1	W-D	5	no	52.4	51.2
3	1	1	W-D	10	no	52.4	53.3
4	1	1	W-D	20	no	54.0	53.0
5	1	1	W-D	30	no	52.0	51.5
6	1	1	W-D	10	yes	50.0	55.5
7	1	1	C-D	10	no	51.5	56.0
8	1	1	C-D	10	yes	51.5	57.0
9	1	2	W-D	10	no	50.0	55.5
10	1	2	W-D	10	yes	50.0	59.9
11	2	1	W-D	1	no	53.0	52.5
12	2	1	W-D	5	no	52.4	49.1
13	2	1	W-D	10	no	52.4	51.2
14	2	1	W-D	20	no	54.0	53.0
15	2	1	W-D	30	no	52.0	51.0
16	2	1	W-D	10	yes	50.0	57.0
17	2	1	C-D	10	no	51.5	53.5
18	2	1	C-D	10	yes	51.5	57.5
19	2	2	W-D	10	no	50.0	49.0
20	2	2	W-D	10	yes	50.0	57.0
21	3	1	W-D	30	no	52.0	48.5
22	3	2	W-D	10	no	61.0	58.0
23	3	2	C-D	10	no	52.5	45.0

[1]Culture: 1 - *K. palustris;* 2 - *S. hominis*; 3 - *P. aeruginosa* LBI;
[2]Medium: 1 - Robert et al. (1989); 2 - BH (Bushnell and Haas)
[3]Carbon source: W-D (weathered diesel oil recovered from the groundwater at a petrol station); C-D (commercial diesel oil)

The results obtained with the biodegradability test are presented in Table 2. Examining the results for *P. aeruginosa* LBI, it can be seen that they agree with those obtained in the biosurfactant production, i.e., this culture was only capable of producing biosurfactant using a carbon source (C-D) that could be biodegraded (Pirôllo, 2006).

Both cultures *S. hominis* and *K. palustris* demonstrated the capability to degrade the commercial diesel oil. However, it is known that not all hydrocarbonoclastic microorganisms are able to produce biosurfactants. Some microbial cells show high superficial hydrophobicity and are considered a biosurfactant, such as hydrocarbonoclastic bacteria, some species of Cyanobacteria and some pathogens such as *S. aureus* and *Serratia* sp. (Nitschke and Pastore, 2002). No study relating the cultures *S. hominis* and *K. palustris* to hydrocarbon biodegradation and biosurfactant production was found. Nevertheless, Gomes et al. (2004) describe the biosurfactant storage in the cell wall of a strain of *S. aureus*.

Analyzing the effect of culture medium on biosurfactant production, in Pirôllo (2006) the culture *P. aeruginosa* LBI was capable of producing biosurfactant in Robert medium and using commercial diesel oil as carbon source. This suggests that the BH medium (as employed in experiment 23) can not be suitable for biosurfactant production, especially in cases where hydrocarbons are used as raw material. This medium does not have any easily biodegradable carbon source, such as yeast extract, which can be used as a start-up for the microorganisms. Moreover, BH medium has a high iron concentration. Ramana and Karanth (1989) observed that the presence of iron in the medium inhibited the production of glycolipids by *P. aeruginosa* CFTR-6, mainly in concentrations of $FeSO_4.7H_2O$ higher than 0.01 g/L. This fact was also verified by Silva (2005) with the strain *P. aeruginosa* LBI. Further discussions about the effect of culture medium on biosurfactant production can be found in Guerra-Santos et al. (1984) and Kim et al. (2003).

Another question to be considered is that even cultures with proved capability to produce biosurfactant, for instance *P. aeruginosa* LBI, can not show an immediate positive response to raw materials whose characteristics were altered by physico-chemical and biological mechanisms due to a long period these compounds were under environmental conditions, as the weathered diesel oil used in this work, in which there are more recalcitrant fractions (Mariano, 2006). This fact may in some cases lower the in-situ production of biosurfactants in bioremediation processes of long-standing contaminated areas based on the addition of these microorganisms.

Attempts to use hydrophobic wastes as cheap substrates must not be disregarded, since other studies have proved that it is possible. Moreover, the use of low-cost raw material in production of biosurfactants may not only minimise industrial pollutants, but also simultaneously generate extremely useful and value-added products.

Table 2: Biodegradability test using DCPIP

Culture	Carbon source	Decolourization
S. hominis	W-D	no
	C-D	yes (after 4 days)
K. palustris	W-D	no
	C-D	yes (after 3 days)
P. aeruginosa LBI	W-D	no
	C-D	yes (after 3 days)

W-D - weathered diesel oil; C-D - commercial diesel oil
Obs: during the 18-day test, no decolourization of the substrate control (without inoculum) or of the inoculum control (without diesel oil) was observed.

ACKNOWLEDGMENTS

The authors acknowledge the support received from the Agência Nacional do Petróleo, Gás Natural e Biocombustíveis (ANP) (PRH-05) and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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