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Production of Biosurfactants for Application in the Removal of Hydrophobic Contaminants Originated by the Petroleum Industry

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This work describes the application of microbial surfactants in the removal of petroleum and a derivative from marine environment. Two biosurfactants were produced by the yeasts *Candida sphaerica* and *C. lipolytica* cultivated in industrial residues as substrates during 144 h and 72 h, respectively. The surface tensions of the biomolecules were measured (25 mN/m), the production yields were calculated (8-9 g/L) and the Critical Micelle Concentrations (CMC) determined (0.03 %). Both biosurfactants were applied in samples of sea water and coral reefs artificially contaminated with petroleum and motor oil. The results showed a dispersant action of the biosurfactant from *C. sphaerica* while the biosurfactant from *C. lipolytica* showed a great oil emulsification capacity. Petroleum and motor oil percentages removal of 100 % were obtained. The possibility of application of these biosurfactants in the remediation of environments contaminated by hydrophobic hydrocarbons motivates the development of this alternative technology.

1. Introduction

Environmental contamination caused by petroleum and oil derivative spills is a contemporary concern. Storing and transport operations of crude-oil and derivatives have been recognized as critical to controlling pollution in some countries due to the number of cases reported in which leaking tanks and pipelines led to pollution (Gonzini et al., 2010). Demands from society and government and the issues of the environmental sustainability of the operations concerning transportation and storage of hydrocarbons have impelled the development of new product storage technologies and pipeline inspections. Also, other factors related to minimizing the release of chemicals into the environment are investigated, thus providing new incentives for researchers to develop clean products and processes as well as technologies for the treatment of areas contaminated by these contaminants (Mulligan, 2009). The properties of superficial and interfacial tension reduction between solids, liquids and gases make natural surfactants an important class of substances (Banat et al., 2010). From an environmental standpoint, this class of biomolecules presents a series of advantages in comparison to synthetic surfactants, such as low toxicity, biodegradability and effectiveness in a wide range of pH and temperature values (Satpute et al., 2010). Therefore, the interest in biological surface-active products has increased, in particular in those produced by microorganisms, also known as biosurfactants.
Thus, this work describes the application of microbial surfactants in the removal of petroleum and a derivative from marine environment.

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2. Materials and Methods

2.1 Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. Ground-nut oil refinery residue was obtained from ASA LTDA, Recife-PE, Brazil, and corn steep liquor from Corn Products do Brasil, Cabo de Santo Agostinho-PE, Brazil.

The ground-nut oil refinery residue was used as the main carbon source while the corn steep liquor constituted the nitrogen source. Both agroindustrial by-products also provided the other nutrients essentials for the yeast metabolism.

2.2 Microorganisms

The yeasts *Candida sphaerica* (UCP 0995) and *Candida lipolytica* (UCP 0998) were obtained from the culture collection of the Universidade Católica de Pernambuco, Brazil. The microorganisms were maintained at 5 °C on Yeast Mold Agar (YMA) slants containing (w/v): yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D-glucose (1.0 %) and agar (5.0 %). Transfers were made to fresh agar slants each month to maintain viability.

2.3 Production media

The production of the biosurfactant from *C. sphaerica* was conducted in medium containing 9 % soy bean oil refinery residue and 9 % corn steep liquor (Luna et al., 2009), while the production of the biosurfactant from *C. lipolytica* was conducted in mineral medium containing 6 % soy bean oil refinery residue and 1 % glutamic acid (Rufino et al., 2008). The media were sterilised by autoclaving at 121 °C for 20 min. The final pH of the media was 5.3 and the surface tension before inoculation was 50 mN/m.

2.4 Growth conditions

The inocula were prepared by transferring cells grown on a slant to 50 mL of Yeast Mold broth (YMB). The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm. The yeasts were cultivated in submerged culture with shaking in a New Brunswick C-24 shaker. The inocula were introduced in the amount of 10^4 and 10^8 cells/mL to each cool medium for *C. sphaerica* and *C. lipolytica*, respectively. Cultivations were carried out in Erlenmeyer flasks at 27 °C with shaking at 150 rpm for 144 and 72 h for *C. sphaerica* and *C. lipolytica*, respectively. At regular intervals, samples were withdrawn for analyses. All the assays were carried out in triplicate and did not vary more than 5 %.

2.5 Biosurfactants isolation

The 144-h culture from *C. lipolytica* was filtered through Whatman no. 1 filter paper and centrifuged at 5000 rpm for 20 min. The cell-free broth was concentrated (500 mL) by freeze drying and extracted two times with chloroform (1:1, by vol.) in a separatory funnel at 28 °C (Cirigliano and Carman, 1985). The culture broth free of cells of *C. sphaerica* was acidified with 6 M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4 °C, samples were centrifuged at 5,000 g for 30 min, washed twice with cold methanol and dried at 37 °C for 24-48 h.

The yield in isolated biosurfactants was expressed in g/L. Known amounts of crude precipitate were resuspended in distilled water and used for measurement of the critical micelle concentration (CMC).

2.6 Surface tension and Critical Micelle Concentration (CMC)

The surface tension was measured by the ring method using a Du Nouy Tensiometer model Sigma 70 (KSV Instruments LTD, Finland) at room temperature. The concentration at which micelles began to form was represented as the Critical Micelle Concentration (CMC). At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed. The CMC was automatically determined by measuring the surface tensions of the purified biosurfactant in distilled water up to a constant value of surface tension.

2.7 Application of biosurfactants in dispersing hydrophobic contaminant in sea water

The dispersion of lubricating motor oil was carried out in 100 mL sea water collected in the Petrochemical complex in Suape-PE, Brazil. The laboratory contamination was conducted with 5 % of the motor oil. The sample of sea water previously contaminated was treated as follows: addition of 50 mL distilled water (control) and addition of 50 mL aqueous solutions of the isolated biosurfactants from

C. sphaerica and *C. lipolytica* at 0.03 % concentration (at the CMC). The ability of dispersion, agglutination, emulsification and solubilization were visually observed.

2.8 Application of biosurfactants in removal of hydrophobic contaminants in coral reefs

The removal of lubricating motor oil and petroleum impregnated in coral reefs was carried out by using 100 mL aqueous solutions of the isolated biosurfactant from *C. sphaerica* at the following concentrations: 0.05 % (2xCMC), 0.125% (5xCMC) and 0.25 % (10xCMC). The biosurfactant from *C. lipolytica* was tested at 0.06 % (2xCMC), 0.15 % (5xCMC) and 0.3 % (10xCMC). The contaminant not removed was determined in the washed coral reef by gravimetric assay after extraction with hexane and expressed in percentage.

3. Results and Discussion

3.1 Biosurfactants yields

The production of high biosurfactant yields has been considered one of the obstacles in this area of biotechnology, since the products are isolated in small quantities and the process generates a large residual volume. In this context, the use of crude biosurfactants, ie the use of the cell free broth obtained after production has been considered a strategy for application by the oil industry, which does not require pure formulations in opposition to the food industry and pharmaceuticals (Atlas, 1991).

The yield of isolated biosurfactant from *C. sphaerica* was 9 g/L, while 8g/L was obtained for the biosurfactant from *C. lipolytica*.

Several studies have been developed in order to optimize the production yields of surfactant agents. Sarubbo et al. (2007), for example, obtained similar results to those obtained in this work for a biosurfactant produced by *C. lipolytica* using canola oil and glucose as substrates. Studies conducted by Sobrinho et al. (2008) using two industrial wastes as carbon sources, showed a yield of 4.5 g/L of the biosurfactant produced by *C. sphaerica*.

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3.2 Surface tension and Critical Micelle Concentration (CMC)

The CMC is a widely used index to evaluate surface activity. By definition, the CMC is the surfactant concentration at which an abrupt increase in surface tension is observed. Regardless of the surfactant concentration, a further decrease in the surface tension will not be observed once the CMC has been reached (Hua et al., 2003; Amaral et al., 2006).

The relationship between surface tension and concentration of the isolated biosurfactant solutions was determined in an automatic tensiometer. The biosurfactant produced exhibited excellent surface tension reducing activity. The surface tension of water of 70 mN/m decreased to 25 mN/m by increasing the solution concentration up to 0.025 % and 0.03 % for the biosurfactants produced by *C. sphaerica* and *C. lipolytica,* respectively (Figure 1A, B). Further increase in the concentration of the biosurfactant solution did not reduce the surface tension of water, indicating that the CMC was reached at this concentration.

The biosurfactants produced showed a lower minimum surface tension than that of the biosurfactant from *C. lipolytica* (32 mN/m) (Rufino et al., 2007), from *C. glabrata* (31 mN/m) (Sarubbo et al., 2006), from *C. antarctica* (35 mN/m) (Adamczak and Bednarski, 2000) and from *Yarrowia lipolytica* (50 mN/m) (Gallert and Winter, 2002). The biosurfactants produced showed also a smaller CMC value than those of other biosurfactants from yeasts described in the literature, as values of 2.5 % found for biosurfactants from *C. lipolytica* (Sarubbo et al., 2007) and *C. glabrata* (Sarubbo et al., 2006), and of 0.6 % for the biosurfactant from *C. antarctica* (Adamczak and Bednarski, 2000).

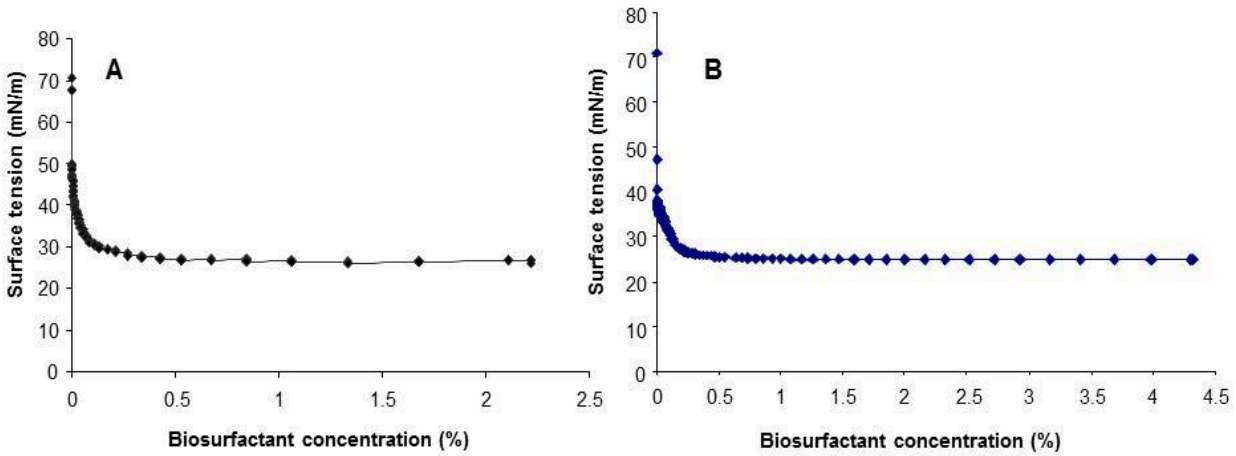

Figure 1: Surface tension versus concentration of the isolated biosurfactant produced by Candida sphaerica grown in distilled water supplemented with 9.0 % ground-nut oil refinery residue plus 9 % corn steep liquor (A); Surface tension versus concentration of the isolated biosurfactant produced by Candida lipolytica grown in mineral medium supplemented with 6.0 % ground-nut oil refinery residue plus 1.0 % glutamic acid (B)

3.3 Application of biosurfactants in dispersing hydrophobic contaminant in sea water

Apart from the industrial applications of biosurfactants envisage, their application in the oil industry is one of the potential uses which requires lower purity specifications so that whole cell broth could be used, eliminating the purification steps that represent almost 60 % of the total production costs (Desai and Banat, 1997; Dubey and Juwarkar, 2001).

Addition of surfactants can be expected to enhance hydrophobic pollutants removal through the processes of solubilization and mobilization, as described by Bai et al., (1997).

The solubilization power of any surfactant is dependent on the ability of the surfactant to increase the apparent aqueous-phase solubility oh hydrophobic constituents. A dramatic enhancement in hydrocarbons constituent solubility is commonly observed above the critical micelle concentration (CMC), which is attributed to partitioning into the hydrophobic core of surfactant micelles. In this process, high surfactant concentrations are generally required because the solubility of hydrophobic constituents in surfactant solution depends entirely on the surfactant concentration (Bai et al., 1997).

Mobilization can be divided into displacement and dispersion. Displacement is the release of hydrocarbon droplets from porous media owing to a reduction in interfacial tension. From a theoretical perspective, entrapped hydrocarbons will undergo displacement if the interfacial tension between the aqueous and hydrophobic phase is reduced sufficiently to overcome the capillary forces that cause the formation of residual saturation (Bai et al., 1997).

Dispersion is the process in which the hydrocarbon is dispersed into the aqueous phase as very small emulsions. Emulsions are generally not thermodynamically stable. However, owing to kinetic constraints, they may remain stable for significant time periods. Dispersion is related to both the interfacial tension and the surfactant concentration, and is different from displacement in that the displacement process is only related to the interfacial tension between aqueous and hydrophobic phases and no emulsion form (Bai et al., 1997).

In order to investigate the efficiency of the biosurfactants produced, a preliminary experiment using the cell-free broth containing the surfactant were performed to verify the removal of hydrocarbons from sea water.

The biosurfactant produced by *C. sphaerica* showed high dispersant activity for the lubricating oil of car engine, which could facilitate the targeting of oily spots in the ocean, while the biosurfactant produced by *C. lipolytica* showed emulsifying activity on oil, suggesting the solubilization of oil with formation of small droplets that would facilitate the access of hydrocarbon degrading microorganisms and the consequent acceleration of bioremediation. The results were observed when low amounts of

biosurfactants were used, thus demonstrating the potential of these compounds in transporting and solubilizing oil spots on marine aquatic environment.

3.4 Application of biosurfactants in removal of hydrophobic contaminants in coral reefs

Tables 1 and 2 show the results of removal of hydrophobic contaminant adsorbed in coral reefs by the biosurfactants produced.

The results obtained showed that the biosurfactant from *C. sphaerica* was able to remove all the petroleum and lubricating oil of car engine when used in the highest concentration. The solution at 0.125 % also showed to be effective. The biosurfactant from *C. lipolytica* removed high percentages of the contaminants at the highest concentration of 0.3 %.

Table 1: Removal of motor oil from coral reefs by the biosurfactant produced by Candida sphaerica UCP0995

Treatments (aqueous solutions)	Motor oil (%)	Petroleum (%)
Biosurfactant at 0.05 %	46	43
Biosurfactant at 0.125 %	64	100
Biosurfactant at 0.25 %	100	100

Table 2: Removal of motor oil from coral reefs by the biosurfactant produced by Candida lipolytica UCP0998

Treatments (aqueous solutions)	Motor oil (%)	Petroleum (%)
Biosurfactante at 0.06 %	51	13
Biosurfactante at 0.15 %	77	26
Biosurfactante at 0.3 %	100	70

Although the literature is sparse in describing methods of removing oils in solid surfaces, the application of biosurfactants in removing adsorbed hydrocarbons in soils has been reported frequently, as will be described below.

Sobrinho et al. (2008) observed for the biosurfactant isolated from *C. sphaerica,* removal of 65 % motor oil adsorbed in a sand sample. The biosurfactant from *C. antarctica* removed 50 % motor oil adsorbed in sand (Adamczak and Bednarski, 2000) while the biosurfactant from *C. glabrata* at 2.5 % removed 84 % motor oil (Luna et al., 2009). Another research with *Rhodococcus* cultivated in n-hexadecane revealed a removal of 82% of crude oil contained in a column (Kuyukina et al., 2005).

4. Conclusions

The results obtained demonstrate indicate that the biosurfactants are suitable for use in the petroleum industry and in environmental applications such as enhanced oil recovery, cleaning of oil reservoirs, reducing oil viscosity for crude oil transportation, and decomposition of spilled oils in soil or marine environments.

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