Production of a biosurfactant by *Pseudomonas fluorescens* – Solubilizing and wetting capacity

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Production of biosurfactant by free and alginate entrapped cells of *Pseudomonas fluorescens* Migula 1895-DSMZ was investigated using olive oil as the sole carbon and energy source. Diffusional limitations in alginate beads affected the kinetic of biosurfactant production when compared to that obtained with free cells culture. Nevertheless, the emulsion stability was improved and fewer by-products interfered with the biosurfactant activity. After separation by acetone precipitation, the biosurfactant showed a rhamnolipid-type in nature, and had a good foaming and emulsifying activities. The critical micellar concentration (CMC) was found to be 290 mg l^{-1}. The product exhibited a positive effect to alkaline pH and demonstrated a high level of tolerance to ionic strength. Above the CMC, naphthalene solubility was deeply affected by biosurfactant concentration, pH and salinity. A quantitative estimation of the effectiveness of the solubility process was obtained by calculating the weight solubilization ratio (WSR). The WSR decreased from 0.63 to 0.015 for increasing biosurfactant concentration up to 1.5 g L^{-1}, alkaline pH or high salinity; and reached an almost constant value for 4.0 g L^{-1} of biosurfactant irrespective of the pH and the salinity. In all cases the solubility of naphthalene in water was enhanced by the biosurfactant addition, showing its potential for application in bioremediation of polycyclic aromatic hydrocarbons (PAH) contamination in extreme environments.

Keywords: Biosurfactant; Production; Characterization; Naphthalene; Solubility; Wetting capacity.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, and phenanthrene are hydrophobic pollutants found in contaminated soils and groundwater. They are toxic environmental pollutants that are known or suspected carcinogens or

mutagens (Jacob, 1996). Bioremediation has been used as a general way to remove them from contaminated sites or aquifers (Johnsen et al., 2005), but their biodegradation is rather limited due to their low bioavailability because of their sparingly soluble nature. Surfactant-mediated biodegradation is a promising alternative. The presence of surfactants can increase the solubility of PAHs and hence potentially increase their bioavailability (Edwards et al., 1991). Most microbial surfactants are complex molecules (Desai and Banat, 1997). These are potent surfactants, as they dramatically reduce surface tension (from 72 to 30 dynes cm^{-1}) and have low critical micelle concentrations (CMC), which increase apparent solubilities of hydrophobic hydrocarbons by their solubilization into the hydrophobic core of micelles.

They can be produced by various bacteria, fungi, and yeast. These molecules have tremendous potential for various applications. Indeed, they show many advantages over chemical surfactants as regards biodegradability, low toxicity and effectiveness at extreme temperatures, pH or salinity (Banat et al., 2000). The most significant advantage of a microbial surfactant over chemical surfactants is its ecological acceptance because it is biodegradable and non-toxic to natural environments (Abu-Ruwaida et al., 1991; Desai and Banat, 1997).

The genus *Pseudomonas* has the availability to use various substrates to produce rhamnolipid-type biosurfactants (Desai and Banat, 1997). Optimizing factors that affect growth in biosurfactant producing organisms with potential for commercial exploitation is of paramount importance (Soumen et al., 2006). The specific properties of a biosurfactant produced by a *P. fluorescens* strain, namely, the purification process, the structural characterization, and some associated physicochemical properties, including the critical micelle concentration (CMC) and the characterization of the bioemulsifier produced based on its solvent specificity and stability was therefore examined in this work.

Surfactant-enhanced remediation (SER) has been proposed as a promising technology to remove residual organics from contaminated aquifers (Noordman et al., 2002). This technology is primarily based on two processes: (i) micellar solubilization and (ii) mobilization of entrapped non-aqueous phase liquid (NAPL) due to the reduction of interfacial tension. Micellar solubilization occurs when the surfactant concentration exceeds the CMC, where the aqueous solubility of organics is enhanced by the incorporation of hydrophobic molecules into surfactant micelles (Li and Chen, 2009). SER can be performed either by chemical surfactants such as sodium dodecyl sulfate (SDS), Triton X-100 or by using biological surfactants (Biosurfactants) (Kosaric, 2001). One key environmental factor impacting surfactant/microorganisms/target environment combination(s) is the system pH (Paria, 2008), which affects both the microbes and the biosurfactant. The morphology of biosurfactants can be significantly affected by changes in pH, which in turn affects the degree of solubility enhancement. The effect of a rhamnolipid biosurfactant on the surface tension and dispersion of phenanthrene and naphthalene was shown to be a function of the pH (Vipulanandan and Ren, 2000; Shin et al., 2004). For instance, Ishigami et al. (1987) and Champion et al. 1995) have shown that the morphology of rhamnolipid biosurfactants is a function of pH, changing from lamellar, to vesicular and ultimately micellar as the pH is increased, which was subsequently confirmed (Shin et al., 2008). Similarly, the ionic strength or salinity of the medium could affect the solubility process. A special focus was therefore made on

the ability to solubilize a model organic compound, naphthalene. The effects of pH and salinity on naphthalene solubilization using a biosurfactant solution are also reported.

2. Culture medium optimization

The available literature shows a lack of studies dealing with biosurfactant production by considering the use of carbon sources other than hydrocarbons. In this aim, the use of vegetable oil as carbon sources to produce biosurfactants seems to be an interesting and low cost alternative (Abouseoud et al., 2008a). Screening of some nutrient substrates showed that olive oil was the best carbon source for surfactant synthesis by a commercial strain of *P. fluorescens*: growth of the strain on this substrate decreased the surface tension to 38 dyne/cm, and the emulsifying activity was 49%; this can be most likely related to the fact that *P. fluorescens* is lipase positive which facilitate assimilation of fatty acids contained in olive oil fractions. Similar results were found with *P. aeroginusa* 44T1 (Robert et al., 1989). Nitrogen source examination showed that maximum emulsifying activity (56%) and minimal surface tension (31 dyne/cm) were achieved with NH_4NO_3 and no significant change in pH was observed in agreement with the literature (Ochsner et al., 1995). The optimum C/N ratio was 10 (ST = 33.5 dyne/cm; E24 = 50 %). These results were similar with those found using waste frying oil and sodium nitrate as carbon and nitrogen sources (Santos et al., 2002).

3. Kinetics of biosurfactant production

Due to the low produced amounts, biosurfactant recovery from the culture medium account significantly in the final cost production. Moreover, the amphiphilic characteristic of these molecules increased the difficulty of recovering (Banat et al., 2000). In this aim, immobilization of living cells in porous support offers enormous advantages in continuous production of biosurfactant. It is an efficient way to reduce the cost of product recovery, as the growth and the product formation phases can be separated and substrate inhibition could be avoided (Siemann and Wagner, 1993). Entrapment in insoluble calcium alginate is recognized as a rapid, non-toxic, inexpensive, versatile method which was successfully used for biosurfactant production (Siemann and Wagner, 1993).

Free and immobilized cell cultures of *P. fluorescens* were therefore compared. During free cells culture of *P. fluorescens* growth in mineral medium containing olive oil as carbon and energy source and NH_4NO_3 as nitrogen source, corresponding to a C/N ratio of 10, the surface tension dropped rapidly after inoculation, reaching its lowest value (30 dyne cm^{-1}) during exponential growth phase, while the emulsification index reached a maximum value of 67 %. The following increase of surface tension and decrease of the emulsification index characterized cessation of biosurfactant biosynthesis, most likely due to the production of secondary metabolites which could interfere with emulsion formation (Bonilla et al., 2005). Biosurfactant biosynthesis during the exponential growth phase suggested that the biosurfactant was produced as a primary metabolite (Persson et al., 1988), suggesting a more efficient biosurfactant production under chemostat conditions or by immobilized cells (Klein and Wagner, 1987).

Although the minimum surface tension value recorded (35 dyne cm^{-1}) during immobilized cell culture was slightly greater than that obtained with free cells, the

formed emulsion was more stable (E24 = 62 %), most likely due to a low content of secondary metabolites, which could interfere as in the case of free cells (Bonilla et al., 2005). The lower rates recorded during immobilized cell culture, if compared to free cell culture, was caused by diffusional limitations of nutrients from the bulk of the culture through the gel bead on one hand, and product (biosurfactant) diffusion from the immobilized cells to the surrounding medium on the other hand. It should be noted that after reaching its minimum value surface tension remained then constant throughout culture, contrarily to the abrupt decrease recorded during free cell culture. In addition, the decrease consecutive to the E24 maximum value was clearly less pronounced with immobilized cells. This showed that immobilization led to more physiological stability of cells.

4. Biosurfactant separation and characterization

The cold acetone precipitation method used for biosurfactant recovery led to an approximate yield of 2 ± 0.1 g L^{-1}, which was similar to those reported in the available literature (Haba et al., 2000). The critical micellar concentration, namely a sudden change in the surface tension, was 290 ± 0.2 mg L^{-1} for the isolated biosurfactant and the corresponding surface tension was 32 dyne cm^{-1} (Abouseoud et al., 2008b). Biosurfactant concentrations above the CMC produced only weak decrease of the surface tension, indicating that biosurfactant molecules began to aggregate (Sanchez et al;, 2007).

The stability of the biosurfactant tested over a wide range of pH showed that pH increase had a positive effect on surface tension and emulsion stability; the former decreased from 34 to 30 dyne cm^{-1} while the latter increased by 38 % for a pH increase from 4 to 11, in agreement with the available literature (Abu-Ruwaida et al., 1991). This could be caused by a better stability of fatty acids-surfactant micelles in presence of NaOH and the precipitation of secondary metabolites at higher pH values. Contrarily, NaCl addition in the range 0-20 % had only a weak effect on surface tension and emulsification index of *P. fluorescens* biosurfactant.

5. Naphthalene solubility

It has been shown that at biosurfactant concentration below CMC (290 mg L^{-1}), no significant change in naphthalene solubility occurred after addition of biosurfactant in comparison to its solubility in water at the same temperature (25 – 30 mg L^{-1}). Above the CMC, the biosurfactant was effective in enhancing naphthalene solubility and a relationship between naphthalene solubility and biosurfactant concentration up to about (0.5 g L^{-1}) can be observed. A linear relationship between hydrophobic compound solubility and surfactant concentration beyond CMC has been well established for commercial surfactants and biosurfactants (Edwards et al., 1991). In our case such linearity correlation could not be checked but a sigmoidal curve fitted rather accurately experimental data ($R^2 = 0.995$). Below the CMC, The biosurfactant mainly existed as monomers and did not contribute to the solubility of naphthalene, while above the CMC the added surfactants formed micelles (a transient aggregate of surfactant molecules) and hence enhanced the solubility. Thus the increase in apparent solubility above the biosurfactant CMC might be due to micelle formation since the concentration of the

surfactant monomers remained relatively constant when the surfactant concentration was above its CMC (Edwards et al., 1991).

For a given biosurfactant concentration, pH and salinity have tremendous impact on the solubility of naphthalene. Saturation value or maximum solubility depends strongly on these factors. For instance, for a biosurfactant solution of 0.5 g L^{-1} at pH 7, naphthalene solubility was 160 mg L^{-1} which represents more than 5 folds its solubility in water. At more acidic pH (5) or alkaline pH (11), maximum solubility decreased, in agreement with available study dealing with biosurfactant production by *Pseudomonas sp.* (Vipulanandan and Ren, 2000). At a given biosurfactant concentration above the CMC, the naphthalene solubility decreased for increasing salinity from 0 to 15% w/v (at pH 7). Variations of the apparent solubility with the pH are possibly related to the biosurfactant nature, forming different pH-dependent structures of aggregates (Shin et al., 2004). These aggregate structures lead to the formation of micelles of smaller volumes resulting in less solubilizing capacity. The pH and salt sensitivity of the biosurfactant will therefore vary according to the specific structure of the materials (Mohanty and Caneba, 2006).

The final structure of the isolated biosurfactant is under investigation to improve the knowledge concerning the relation between the micelle structure and the pH or the salinity and their effect on naphthalene solubility.

6. Estimation of Solubility Effectiveness

A measure of the effectiveness of a surfactant in solubilizing a given compound is the molar solubilization ratio (MSR) or weight solubilization ratio (WSR) (edwards et al., 1991). The WSR value is defined as the amount of solubilized hydrocarbon per amount of surfactant, and hence corresponds to an increase in solubilizate concentration per unit increase in micellar surfactant concentration. In the presence of an excess of hydrophobic organic compound, the WSR is (Myers, 2006):

$$\text{WSR} = ([St] - [S_{CMC}]) / (Ct - CMC) \tag{1}$$

Where [St] is the total apparent solubility of polycyclic aromatic hydrocarbons PAH in biosurfactant solutions at a biosurfactant concentration Ct above the CMC; [S$_{CMC}$] is the apparent solubility of PAH at CMC taken equal to their water solubility (Sw).

The highest solubility effectiveness was obtained for 0.5 g L^{-1} biosurfactant, and then decreased for increasing biosurfactant concentration till reaching a minimum value for 4.0 g L^{-1} biosurfactant. A slight pH increase (5 to 7) or salinity (0 to 5%) had only a low effect on solubility effectiveness, while alkaline pH values and high salinity (above 10%) induced more than 50% decrease of the WSR at biosurfactant concentration of 0.5-1 g L^{-1}. The WSR values could be compared to those obtained with synthetic surfactants such as SDS (0.035), Triton X-100 (0.073) at pH 7 or with a biosurfactant produced by *Pseudomonas sp.* (0.17 at pH 7 and 0.063 at pH 10.5) (Vipulanandan and Ren, 2000).

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