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Effect of pH and salinity on the emulsifying capacity and naphthalene solubility of a biosurfactant produced by *Pseudomonas fluorescens*

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ABSTRACT

A biosurfactant with a low critical micelle concentration, CMC (290 mg L^{-1}), was produced by a *Pseudomonas fluorescens* strain using olive oil. Measurements of surface tension and emulsification index (E24) showed a positive effect on alkaline pH and a high level of tolerance to ionic strength of the product. Above the CMC, naphthalene solubility was affected by biosurfactant concentration (3–7 times its aqueous solubility), pH and salinity; for 0.5–1.5 g L^{-1} of biosurfactant, pH 7 and no salinity, naphthalene solubility was about 7-fold its aqueous solubility. The solubility reached a saturation value (205 mg L^{-1}) when biosurfactant concentration exceeded 1.5 g L^{-1}. For alkaline pH or high salinity (above 10%) the solubility decreased by more than 50%. The weight solubilization ratio 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 a constant value for 4.0 g L^{-1} biosurfactant irrespective of pH and salinity (in the range of 0.02–0.05 g L^{-1}). 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 contamination in extreme environments.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, and phenanthrene are hydrophobic pollutants found in contaminated soils and groundwater at many sites, particularly at coal storage, coke oven plants, and areas of coal tar spillage. They are toxic environmental pollutants that are known or suspected carcinogens or mutagens [1,2].

Bioremediation has been used as a way to remove pollutants from contaminated sites or aquifers [3,4], 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 [5].

Surfactant-enhanced remediation (SER) has been proposed as a promising technology for removing residual organics from contaminated aquifers [6,7]. This technology is based primarily on two processes: (i) micellar solubilization and (ii) mobilization of entrapped nonaqueous phase liquid (NAPL) due to reduction of interfacial tension. Below the surfactant's critical micelle concentration (CMC), surfactants exist as monomers and have only minimal effects on the aqueous solubility of organics. 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 [8].

The extent of micellar solubilization depends on many factors, including surfactant structure, aggregation number, micelle geometry, ionic strength, pH, temperature, and the size and chemistry of the solubilizate [9]. SER can be realized either by chemical surfactants such as sodium dodecyl sulfate (SDS), Triton X-100 or by using biological surfactants (biosurfactants) [10]. Biosurfactants are produced by numerous microorganisms and represent a wide diversity of chemicals and molecular structures [11]. They can be produced by a diverse group of bacteria, fungi, and yeast. By evolution, bacteria have adapted themselves to feeding on hydrophobic substrates by manufacturing and using a surface-active product that helps the bacteria to adsorb, emulsify, wet, disperse or solubilize the water-immiscible material [10,12].

Most microbial surfactants are complex molecules, comprising various structures that include lipopeptides, glycolipids, polysaccharide protein complex, fatty acids and phospholipids. These

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are potent surfactants, as they dramatically reduce surface tension (from 72 to 30 mN m^{-1}) and have low (micrograms per liter) critical micelle concentrations, which increase apparent solubilities of hydrophobic hydrocarbons by their solubilization into the hydrophobic core of micelles.

These molecules have tremendous potential for applications in the pharmaceutical, cosmetics, and food industries, as emulsifiers, surfactants and dispersants [13,14]. Indeed, they show many advantages over chemical surfactants as regards biodegradability, low toxicity and effectiveness at extreme temperatures, pH or salinity [13]. For potential application of bioemulsifier produced by hydrocarbon degrading microbes for bioremediation of a contaminated site, it is essential to understand the environmental conditions leading to the maximum efficiency.

Clearly, effective use of biological or synthetic surfactants to enhance the bioavailability of hydrophobic contaminants requires the optimization of surfactant/microorganisms/target environment combination(s) and better understanding of the complex interactions [12,15]. One key environmental factor impacting these interactions is the system pH, which affects both the microbes and the biosurfactant. Of course, the pH effect on microbial growth kinetics are well known, with most microbes having an optimum pH for growth in the range 5–8 (around 7 for many of them), although there are many exceptions to this trend [16]. The morphology of biosurfactants can also be significantly affected by changes in pH, which in turn affects the degree of solubility enhancement. Previously, Shin et al. [17] demonstrated that the effect of a rhamnolipid biosurfactant on the surface tension and dispersion of phenanthrene was a function of pH. Similar results were obtained with naphthalene [18].

For instance, Ishigami et al. [19] and Champion et al. [20] 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 confirmed later [21].

Similarly, the ionic strength or salinity of the medium could affect the solubility process. For anionic biosurfactants, like rhamnolipids, it has been shown that the presence of electrolytes causes a decrease in the CMC and therefore increases the solubility of hydrocarbons [22]. The formation of complex compounds between ions and surfactants could also alter micelle formation [23,24]. These studies demonstrate that the control of the pH and the salinity needs to be considered in field applications for improved performances of biosurfactant systems.

The specific objectives of this study were to examine the properties of a biosurfactant produced by a *Pseudomonas fluorescens* strain, namely, the purification process, the structural characterization, and some associated physicochemical properties, including the critical micelle concentration and the characterization of the bioemulsifier produced based on its solvent specificity and stability. A special focus is 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. Materials and methods

Naphthalene and salts were all purchased from Fisher Scientific (Illkirch, France). Commercial olive oil and gasoil were purchased from Naftal (Sonatrach, Algeria).

2.1. Biosurfactant production

A *P. fluorescens* Migula 1895 strain from DSMZ (Braunschweig, Germany) was used in the present study for biosurfactant production.

Nutrient agar media (beef extract 1.0 g L^{-1}, yeast extract 2.0 g L^{-1}, peptone 5.0 g L^{-1}, NaCl 5.0 g L^{-1}, agar 15.0 g L^{-1}—all components from Difco, BD, Le Pont de Claix, France) were used for inoculum preparation. Two loops of agar culture were used to inoculate 50 mL of nutrient broth (Difco). Seed culture was carried out for 16–18 h on a rotary shaker at room temperature. An aliquot of inoculum was used to inoculate culture medium at 2% (v/v) level. More information on the culture conditions can be found in previous papers [25,26]. The medium was also previously optimised for carbon and energy source (C), nitrogen source (N) and C/N ratio respectively, as follows [25]: 2% (v/v) olive oil and 1 g L^{-1} ammonium nitrate, leading to a C/N ratio of 10. Cultivations were performed in 250 mL flasks containing 50 mL medium at room temperature, and stirred on a rotary shaker (GFL 3500 Burgwedel, Germany) at 2.5 rev s^{-1}.

2.2. Surface tension measurement

The surface tension measurement of cell-free supernatants was determined in a K6 tensiometer (Krüss GmbH, Hamburg, Germany), using the du Nouy ring method. The values reported are the mean of three measurements. All measurements were made on cell-free broth obtained after culture centrifugation at 10,000 × *g* for 1500 s.

2.3. Emulsification index (E24)

The E24 of culture samples, which is an index of the capacity of the biosurfactant to produce stable emulsions with hydrocarbons, was determined by adding 2 mL of a hydrocarbon (gasoil) to the same amount of culture, mixing with a vortex for 2 min, and leaving to stand for 24 h. The E24 index corresponded to the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [27].

2.4. Biosurfactant recovery

The culture broth was centrifuged (10,000 × *g* for 900 s) to remove the cells and thereafter sterilized with Millipore membrane filter (Millex®-HV – 0.2 μm; Millipore SAS, Molsheim, France). The clear sterile supernatant served as a source of crude biosurfactant. The biosurfactant was recovered from the cell-free culture supernatant by cold acetone precipitation as described by Pruthi and Cameotra [28]. Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 °C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone; it was thereafter re-dissolved in sterile water.

2.5. Critical micelle concentration

The critical micellar concentration corresponded to the concentration of an amphiphilic component at which the formation of micelles is initiated in the solution. Purified biosurfactant from *P. fluorescens* was dissolved in aqueous solutions at concentrations ranging from 0 to 6 g L^{-1}. The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration [29]. For each concentration, surface tension measurement was carried out in a K6 tensiometer (Krüss GmbH) using the du Nouy ring method until a constant value was reached.

2.6. Stability tests

It was based on the determination of pH and salinity (NaCl) effects on the activity of the biosurfactant. To determine the effect of pH on the activity, the pH of the biosurfactant was adjusted at a value in the range of 2–11 prior to filter sterilization. The effect of NaCl on the activity of the biosurfactant was investigated by adding

NaCl in the range of 5–20% (w/v). The biosurfactant was then re-dissolved after purification with distilled water containing NaCl. In each case, surface tension and E24 values were performed as described above.

2.7. Naphthalene solubilization assays

Naphthalene was solubilized by adding solid naphthalene (2 g L^{-1}) into surfactant solutions at room temperature. The amount of naphthalene in the system was higher than the amount of naphthalene that could be solubilized. Triplicate tests were conducted for each surfactant concentration ranging from 0.1 to 4 g L^{-1}. Solutions were adjusted to initial pH values of 4, 7 and 10 by the addition of 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH solution, as needed at 0% salinity. Similar tests were performed in order to study the effect of salinity on naphthalene solubility, by keeping neutral pH value while adjusting the concentration of added NaCl to 5, 10 and 15 g L^{-1}. Experiments were conducted in erlenmeyers (50-mL working volume), sealed with Teflon-lined screw caps and continuously stirred on a rotary Shaker (New Brunswick Scientific, NJ) at 3.33 rev s^{-1} and under ambient temperature (25 ± 2 °C).

Measurements of naphthalene concentrations at regular intervals showed that the solubility limit was reached after 48 h. Hence, after 48 h samples were transferred into 10-mL centrifuge tubes in order to settle the non-solubilized solid naphthalene (4000 rpm at 2700 × *g* for 600 s). The supernatant was analyzed for naphthalene concentration after direct sampling by measuring the absorbance at 274 nm wavelength with quartz cuvettes of 10 mm path length using an UV–vis spectrophotometer (Jenway 6305, Dunmow, Essex, England). It should be noted that subsequent dilutions were made whenever needed and surfactant concentration was kept the same in both the reference and the measurement cells to minimize the effect of surfactant on UV absorbance. All experiments were performed at room temperature, 22–27 °C. Experiments were done in triplicate and the average values are reported. The limit of detection (LOD) of naphthalene was 1 mg L^{-1}.

3. Results and discussion

3.1. Biosurfactant production, separation and characterization

Free and immobilized cell cultures were carried out in duplicate. *P. fluorescens* growth in mineral medium containing olive oil as carbon and energy source (C) and NH_4NO_3 as nitrogen source (N), corresponding to a C/N ratio of 10, led to a decrease of the surface tension, until reaching minimum values of 30 and 35 mN m^{-1} for free and immobilized cell cultures (not shown), in agreement with earlier studies [25,26], where more details on biosurfactant production by free and immobilized cells can be found.

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 [30]. The critical micelle 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 mN m^{-1} [26]. Biosurfactant concentrations above the CMC produced only a weak decrease of the surface tension, indicating that biosurfactant molecules began to aggregate [31]. Compared to the available literature, similar CMC values were found for biosurfactants produced by *Flavobacterium* sp. on glucose as the carbon source (CMC = 300 mg L^{-1}) [32] and by a *Rhodococcus* strain using residual sunflower frying oil as substrate (CMC = 287 mg L^{-1}) [33].

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 mN m^{-1}

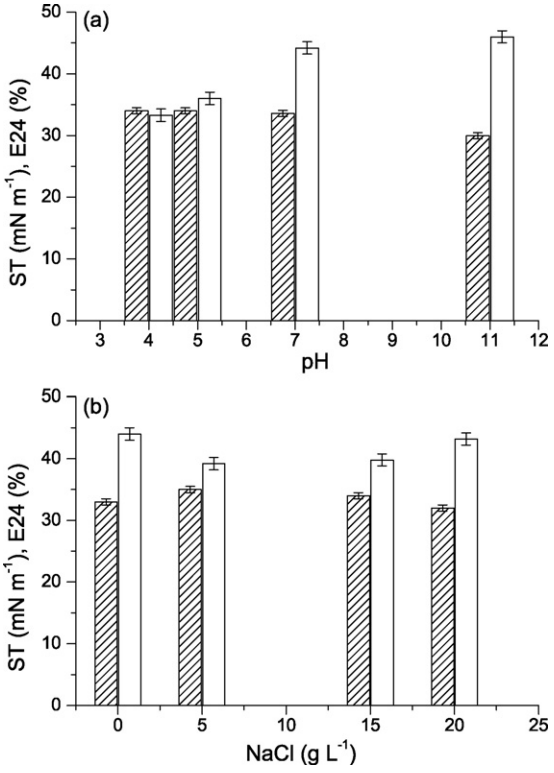

Fig. 1. Effect of pH (a) and salinity (b) on surface activity ST (medium grid bars) and emulsification index E24 (open bars) of the biosurfactant produced by *Pseudomonas fluorescens*.

while the latter increased by 38% for a pH increase from 4 to 11 (Fig. 1), in agreement with the available literature [34]. 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. In contrast, NaCl addition in the range 0–20% had only a weak effect on surface tension and emulsification index of *P. fluorescens* biosurfactant (Fig. 1).

3.2. Naphthalene solubility

The solubilization of naphthalene in biosurfactant aqueous solutions at different pH values and salinities is represented on Figs. 2 and 3. It could be seen that at biosurfactant concentration

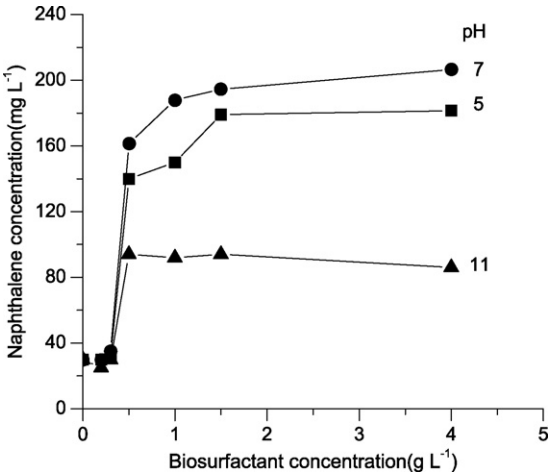

Fig. 2. Variation of naphthalene solubility with biosurfactant concentrations at different pH values in absence of NaCl addition.

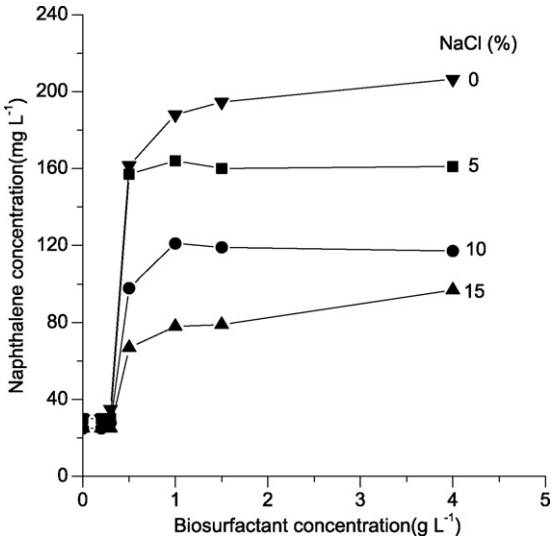

Fig. 3. Variation of naphthalene solubility with biosurfactant concentrations at pH 7 and various salinities.

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 [5,24,35]. In our case such linearity correlation could not be checked but a sigmoidal curve fitted rather accurately experimental data (mean correlation coefficient R^2 = 0.995—fitted curves not shown).

Indeed, the solubility phenomenon is related to the number of strong interaction forces (bonds) with the solute. Above a certain biosurfactant concentration, interactions between biosurfactant–solid (naphthalene) molecules become weaker than biosurfactant–biosurfactant interactions. This could destabilize the formation of micelles and hence biosurfactant molecules tend to form a sort of complex structures or aggregates, which limits the solubilization of further naphthalene molecules. Since the produced biosurfactant is a mixture of compounds (glycolipids and carboxylic acid), an increase of the concentration resulted most likely in the creation of strong interactions between these two polar compounds, which weakens interactions with naphthalene. Above 0.5 g L^{-1}, the solubility of naphthalene continued to increase until reaching a saturation value. 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 because the concentration of the surfactant monomers remained relatively constant when the surfactant concentration was above its CMC [5,36].

The addition of sub-CMC amounts of biosurfactant increased the apparent solubility of naphthalene by several orders of magnitude, and it is clear that pH and salinity have tremendous impact on the solubility of naphthalene for a given biosurfactant concentration (Figs. 2 and 3). It was also observed that saturation value or maximum solubility depends strongly on these factors. For example, 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. It reached a maximum saturation concentration value of 205 mg L^{-1} for a biosurfactant concentration of 2 g L^{-1}. At more acidic pH (5) or alkaline pH (11), maximum solubility decreased to 180 and 92 mg L^{-1} (Fig. 2), in agreement with the study dealing with biosurfactant production by *Pseudomonas sp*. [18].

At a given biosurfactant concentration above the CMC, the naphthalene solubility decreased for increasing salinity from 0 to 15% (w/v) (at pH 7) (Fig. 3). Saturation values between 200 and 100 mg L^{-1} for 0 and 15% salinity respectively were recorded for a biosurfactant concentration of 0.5–1 g L^{-1}.

Variations of the apparent solubility with the pH are possibly related to the biosurfactant nature, forming different pH-dependent structures of aggregates [17]. 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 [37].

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.

These results are of great interest in using the produced biosurfactant in bioremediation process, since its efficiency was maximum, namely maximum solubility and therefore bioavailability of a model contaminant (naphthalene) was obtained at neutral pH and low salinity *viz*. the natural growth conditions of most hydrocarbon degrading microorganisms. Quantitative estimation of this efficiency can be obtained by calculating the solubilization ratio.

3.3. 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) [5]. The WSR value is defined as the amount of solubilized hydrocarbon per amount of surfactant. Hence, the WSR corresponds to an increase in solubilizate concentration per unit increase in micelle surfactant concentration. In the presence of an excess of hydrophobic organic compound, the WSR is given by the following equation [9]:

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

where [St] is the total apparent solubility of PAH (mass concentration) in biosurfactant solutions at a particular total biosurfactant concentration Ct, above the CMC; [S_{CMC}] is the apparent solubility of PAH at CMC taken equal to their water solubility (Sw), since it varied very slightly up to the CMC of the surfactant. The commonly used units to present solubilization curves were considered in this study, namely milligrams per liter for the solubilized compounds and grams per liter for the surfactant. The WSR values at different pH values and NaCl concentrations are summarized in Figs. 4 and 5.

The highest solubility effectiveness was obtained for a biosurfactant concentration of 0.5 g L^{-1}, and then decreased with increasing biosurfactant concentration till reaching a minimum value for a biosurfactant concentration of 4.0 g L^{-1}. A slight increase in pH (5–7) or salinity (0–5%) 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) [18].

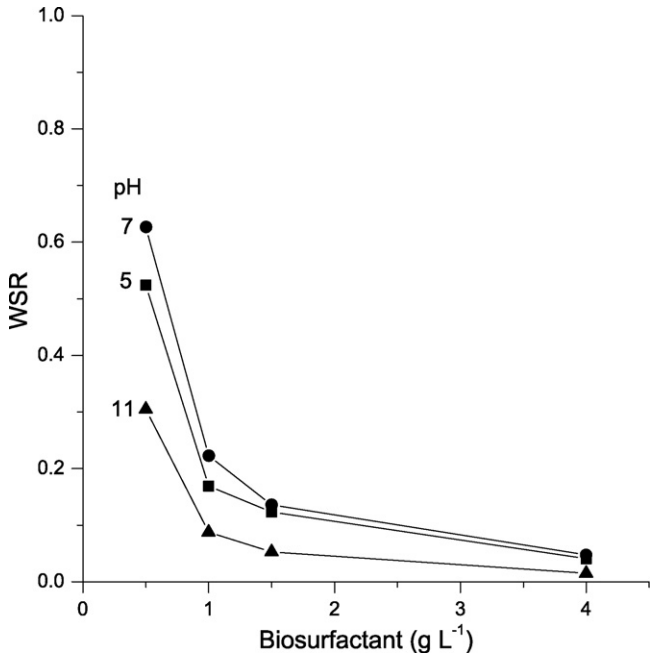

Fig. 4. Effect of pH on the solubilization ratio of naphthalene in biosurfactant solutions (WSR) in absence of salinity.

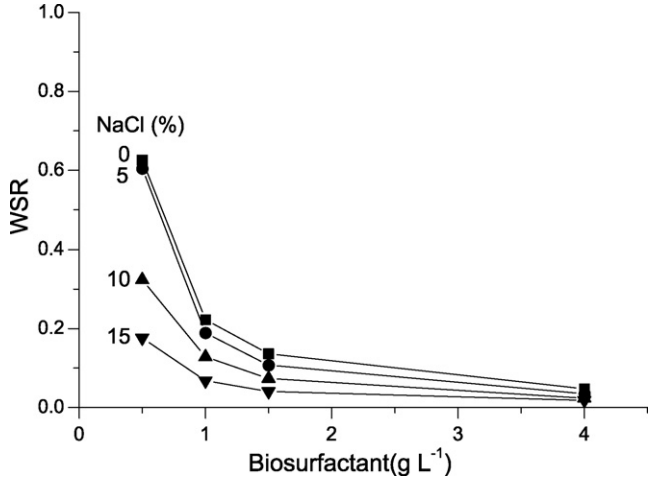

Fig. 5. Effect of NaCl concentrations on the solubilization ratio of naphthalene in biosurfactant solutions (WSR) at neutral pH (7).

4. Conclusions

A biosurfactant produced by a *P. fluorescens* strain was separated and purified by acetone precipitation. Its CMC value was determined by the dilution method and was found to be similar to those found in the available literature [32,33].

The ability of the biosurfactant to emulsify and solubilize hydrocarbons was examined in this work. The influence of pH and salinity on the emulsifying and solubilization capacities was studied. The stability and activity of the emulsion formed with various organic solvents showed that alkaline pH favoured surface activity, while salinity had insignificant effect. The solubility of naphthalene in water was enhanced by a factor of approximately 6.5 by the addition of biosurfactant at concentrations above CMC reaching a saturation value at concentrations in the range 0.5–1.5 g L^{-1}. Maximum solubility was attained for a pH range between 5 and 7 in salt-free water or for weak salinity (less than 10%, w/v) at neutral pH. The structure of the biosurfactant molecule, which has not been identified yet, and its interaction with physicochemical factors such as pH and salinity seem to affect micelles formation and shape and therefore affect the solubility of hydrocarbons. It was quantitatively confirmed with the estimation of the efficiency of solubilization process by calculating the solubility ratio.

In conclusion, the biosurfactant produced in this study can be a valuable resource for enhancing bioavailability and subsequent mineralization of PAHs in a contaminated system, since maximum efficiency was achieved for physicochemical conditions favourable for growth of most hydrocarbon degrading bacteria.

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