

Biosurfactant Production from olive oil by *Pseudomonas fluorescens*

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Biosurfactants are valuable microbial amphiphilic molecules with effective surface-active and biological properties applicable to several industries and processes. Microbes synthesize them, especially during growth on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants. In recent years natural biosurfactants have attracted attention because of their low toxicity, biodegradability, and ecological acceptability. These molecules could be widely used in cosmetic, pharmaceutical, and food processes as emulsifiers, preservatives, and detergents, and in bioremediation processes. They can be produced from various substrates, mainly renewable resources such as vegetable oils, distillery and dairy wastes. This work deals with the production of a biosurfactant by *Pseudomonas fluorescens* Migula 1895-DSMZ. Biosurfactant synthesis was followed by measuring surface tension and emulsifying index E24. The best results were obtained when using olive oil and ammonium nitrate as carbon and nitrogen sources respectively with a C:N ratio of 10. The properties of biosurfactant that was separated by acetone precipitation were investigated. The biosurfactant was a rhamnolipid-type in nature, and had a good foaming, emulsifying and antimicrobial activities. It showed stability during exposure to high temperatures (up to 120 °C for 15 minutes), high salinity (10% NaCl) and a wide range of pH.

Keywords Biosurfactant; production; factors; optimization; characterization; stability

1. Introduction

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceuticals, the oil industry, petrochemistry and the paper and pulp industry amongst others. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment. Therefore, the most significant advantage of a microbial surfactant over chemical surfactants is its ecological acceptance because it is biodegradable and nontoxic to natural environments [1-3]. Some of the advantages of biosurfactants over synthetic ones include lower toxicity, biodegradability, selectivity, specific activity at extreme temperatures, pH and salinity, the possibility of their production through fermentation, their potential applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries [4,5].

The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipid-type biosurfactants [6,7]. Several studies have been carried out to define the best ratio between carbon, nitrogen, phosphorus and iron needed to obtain high production yields. Optimising factors that affect growth in biosurfactant producing organisms with potential for commercial exploitation is of paramount importance [8].

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This work reports the effect of carbon source, nitrogen source, and carbon to nitrogen ratio on the production of biosurfactant by a commercial *Pseudomonas fluorescens* strain, and general characterization of the product.

2. Material and Methods

2.1. Organism

Pseudomonas fluorescens Migula 1895 from DSMZ was used in the present study and maintained on nutrient agar .

2.2. Media and cultivation conditions

Media and cultivation conditions Nutrient broth was used for preparation of the inoculum. The composition of the nutrient broth used was as follows: beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g in a litre of distilled water. To make nutrient agar 15.0 g of agar was added to the nutrient broth. The cultures were grown in this broth for 16–18 h at room temperature. This was used as inoculum at the 2% (v/v) level. For biosurfactant synthesis a mineral salt medium with the following composition(g/l) was utilized: Na₂HPO₄ (2.2), KH₂ PO₄ (1.4), Mg SO₄ . 7H₂O (0.6), Fe SO₄ .7H₂O (0.01), NaCl (0.05) , Ca Cl₂ (0.02), yeast extract (0.02) and 0.1 ml of trace element solution containing (g/l): 2.32 g ZnSO₄·7H₂O, 1.78 g MnSO₄·4H₂O, 0.56 g H₃BO₃, 1.0 g CuSO₄·5H₂O, 0.39 g Na₂MoO₄·2H₂O, 0.42 g CoCl₂·6H₂O, 1.0 g EDTA, 0.004 g NiCl₂·6H₂O and 0.66 g KI. pH of the medium was adjusted to 7.0 ± 0.2. Carbon and nitrogen sources were added separately. Cultivations were performed in 250 ml flasks containing 50 ml medium at room temperature, and stirred in a rotary shaker(GFL 3500) at 150 rpm for three days.

The medium optimization was conducted in a serie of experiments changing one variable at a time, keeping the other factors fixed at a specific sets of conditions . Three factors were chosen aiming to obtain higher productivity of the biosurfactant : carbon source (C) , nitrogen source (N) and C/N ratio.The carbon sources used were n-hexadecane (2% w/v) (Merck, Darmstadt), olive oil (2% w/v) (crude commercial type) and glucose(20g/l) (Difco), with NH₄Cl as nitrogen source. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, NH₄Cl, NaNO₃ and NH₄NO₃were employed at a concentration of 1g/l with the optimum carbon source. The C/N ratio (with optimized carbon and nitrogen sources) was varied from 10 to 50 by keeping a constant nitrogen source concentration 1g/l .

2.3. Biomass and pH measurments

The dry weight technique was used to quantify microbial growth as bacterial density through the culture's absorbance at 600 nm using a UV-Vis spectrophotometer(Jenway 6305). Biomass obtained after filtration on a 0.2µ millipore was dried overnight at 105°C and weighed. The pH of the supernatant was measured with a digital pH-meter(InoLab).

2.4. Surface tension measurement

The surface tension measurement(s) of cell free supernatant 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 by centrifuging the cultures at 10000 x g for 25 min.

2.5. Emulsification index (E24)

E24 of culture samples 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 hours. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [9].

2.6. Biosurfactant production Kinetics

The kinetics of biosurfactant production was followed in batch cultures during 120 hours at optimum conditions by measuring surface tension and emulsification index E24 of supernatant samples obtained after cell separation.

2.7. Biosurfactant recovery

The culture broth was centrifuged (10000 g, 15 min) to remove the cells and thereafter sterilized with millipore membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation as described by Pruthi and Cameotra [10]. Three volumes of chilled acetone was 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 after which it was re-dissolved in sterile water.

2.8. Biosurfactant Characterization

2.8.1. Structural characterization

i. Rhamnose test

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test using the method of Dubois et al.[11]. A volume of 0.5 ml of cell supernatant was mixed with 0.5 ml of 5% phenol solution and 2.5 ml of sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm.

ii. Infrared spectra (IR)

The biosurfactant was extracted from the supernatant fluid (2 ml) with chloroform (2 ml), dried with Na₂SO₄ and evaporated on a rotary evaporator. The IR spectra were recorded on the Bruker IFS113vFTIR-spectrometer, in the 4 000 to 400 cm⁻¹ spectral region at a resolution 2 cm⁻¹, using a 0.23 mm KBr liquid cell.

2.8.2. Activity characterization

i. Foaming and emulsifying properties

The foam were produced by hand shaking a 5 g/L of crude biosurfactant solution for several minutes. The stability of the foam was monitored by observing it during 2 hours. The ability of the biosurfactant to emulsify some liquid hydrocarbons, such as dieseloil, kerosene, n-heptane and sunflower oil was determined. The sterile biosurfactant (2 ml) was added into each test tube (in a set of three) containing the substrate (2 ml). The content of the tubes were vortexed at high speed for 2 min and left undisturbed for 24 h. The emulsion index (E24) was determined as the height of the emulsion layer divided by the total height and multiplied by 100.

ii. Haemolytic and Antimicrobial activity activity

Bacterial strains were tested for haemolytic activity by plating cells onto blood agar and incubated at 37°C for 48 hours. For antimicrobial test, the concentrated culture supernatant of *Ps. fluorescens* was spotted on filter discs on top of an agar plate with freshly grown *B.subtilis*.

2.8.3. Stability characterization

To determine the thermal stability of the biosurfactant, cell-free broth was also maintained at a constant temperature range of 20–100°C for 15 minutes, and cooled at room temperature. To determine the effect of pH on activity, the pH of the biosurfactant was adjusted (2.0–11) prior to filter sterilization. The effect of addition of different concentration of NaCl on the activity of the biosurfactant was investigated. The biosurfactant was re-dissolved after purification with distilled water containing the specific concentration of NaCl (5–20%, w/v). The surface tension and E24 values of each treatment were performed as described above.

3. Results and Discussion

3.1. Optimization of cultivation medium

3.1.1. Effect of carbon source

The production of biosurfactant by the *Pseudomonas fluorescens* strain using substrates such as n-hexadecane, olive oil and glucose, is displayed in Fig.1. The use of vegetable oil as carbon sources to produce biosurfactants seems to be an interesting and low cost alternative [12].

Screening of nutrient substrates (Table 1) showed that *P. fluorescens* supported growth on all substrates although the yield was limited with glucose or hexadecane as carbon sources because of inhibition due to the decrease in pH is probably caused by the production of secondary acid metabolites such as uronic acid [13]. Biosurfactant production could occur only with hexadecane and olive oil. The strain grew on glucose but did not produce the surfactant under these conditions. Growing on hexadecane decreased the surface tension of the culture liquid without displaying a significant emulsifying activity (E24 = 10%). Olive oil was the best carbon source for surfactant synthesis: growth of the strain on this substrate decreased the surface tension to 38 dyne/cm, and the emulsifying activity was 49%. Similar results were found with *Ps. Aeruginosa* 44T1 [14]. A probable reason for this tendency that *Pseudomonas fluorescens* is lipase positive which facilitate assimilation of fatty acids contained in olive oil fractions.

Table 1 : Effect of carbon source on biomass and final pH during biosurfactant production by *Pseudomonas fluorescens*

Carbon source	Biomasse (g/l)	pH
Hexadecane	0.2	5.01
Olive oil	0.8	5.38
Glucose	0.1	3.53

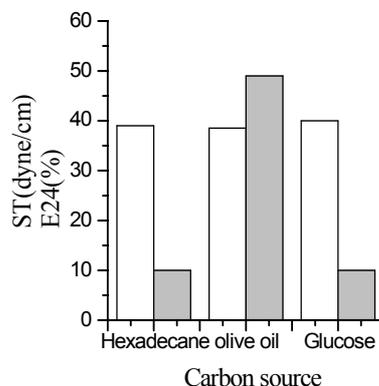


Fig. 1: Influence of carbon source on the variation of surface tension ST [] and emulsification index E24 [] during biosurfactant synthesis by *Pseudomonas fluorescens* with NH₄Cl as nitrogen source

3.1.2. Effect of nitrogen source

With olive oil as a carbon source, the choice of nitrogen source affects the biosurfactant production as depicted in Fig.2. *Pseudomonas fluorescens* is able to use nitrogen sources such as ammonia or nitrate (table 2). However, in order to obtain high concentrations of biosurfactant it is necessary to have restrained conditions of this macro-nutrient. Sodium nitrate and ammonium nitrate were the two best sources of nitrogen for growth and biosurfactant synthesis. Ammonium salts in the form of ammonium chloride was used for growth but not for biosurfactant production and caused a significant decrease in

Table 2 : Effect of nitrogen source on biomass and final pH during biosurfactant production by *Pseudomonas fluorescens*

Nitrogen source	Biomasse (g/l)	pH
NH ₄ Cl	1.0	4.03
NaNO ₃	3.3	7.33
NH ₄ NO ₃	2.2	6.98

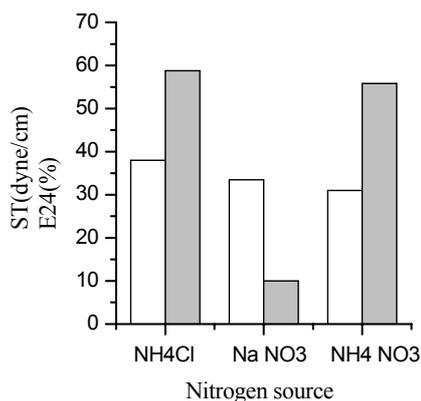


Fig.2: Influence of nitrogen source on the variation of surface tension ST (□) and emulsification index E24 (■) during biosurfactant synthesis by *Pseudomonas fluorescens* with olive oil as carbon source (2% v/v)

pH(4.03) as observed in the previous experiment (table 2). The maximum emulsifying activity (56%) and minimal surface tension (31 dyne/cm) were reached in media with NH₄NO₃. No significant change in pH was observed in this case. Similar results were reported elsewhere[13, 15, 16].

3.1.3 Effect of Carbon/Nitrogen Ratio

Another aspect that was fundamental to improvement of biosurfactant productivity was the ratio C/N, since the best results were attained with lower values of this parameter(C/N = 10) (ST=33.5; E24= 50) (Fig.3). Higher biomass yield and pH stability were also obtained with these conditions (Table 3). These results are similar with those found using waste frying oil and sodium nitrate as carbon and nitrogen sources respectively[17]. Other studies with *Ps. aeruginosa* strain reported stimulation of rhamnolipid synthesis takes place under nitrogen-limited conditions [18].

Table 3 : Effect of carbone source on biomass and final pH during biosurfactant production by *Pseudomonas fluorescens*

C/N ratio	Biomasse (g/l)	pH
10	2.78	7.34
30	2.14	6.85
50	2.5	6.82

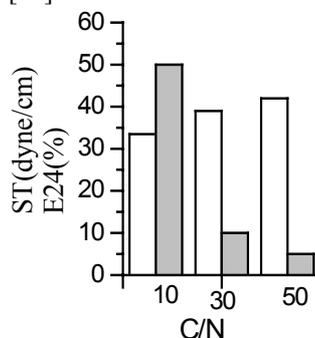


Fig.3: Influence of C/N ratio on the variation of surface tension ST (□) and emulsification index E24 (■) during biosurfactant synthesis by *Pseudomonas fluorescens* with olive oil as carbon source (2% v/v) and NH₄NO₃ as nitrogen source

3.2. Kinetics of biosurfactant production

Fig.4 shows the dependence of biosurfactant production, surface tension, emulsification index E24, biomass, and pH on time of *Pseudomonas fluorescens* cultivation in mineral medium containing olive oil as carbon and energy source (C), NH_4NO_3 as nitrogen source (N) with a C/N ratio of 10. The surface tension dropped rapidly after inoculation, reaching its lowest value (30 dyne/cm) during exponential phase after about 40 hours of growth. The E24 plot, a measure of biosurfactant concentration, showed that insufficient surfactant was initially present to form micells. At about 36 hours of growth, the surfactant concentration started to increase, reaching its maximum after about 56 hours (56%). The increase in surface tension and the decrease in E24 after 56 h of incubation shows that biosurfactant biosynthesis stopped and is probably due to the production of secondary metabolites which could interfere with emulsion formation and the adsorption of surfactant molecules at the oil-water interface [19]. These results indicate that the biosurfactant biosynthesis from olive oil occurred predominantly during the exponential growth phase, suggesting that the biosurfactant is produced as primary metabolite accompanying cellular biomass formation (growth-associated kinetics) [20]. This property suggests that biosurfactant could be effectively produced under chemostat conditions or by immobilized cells [21].

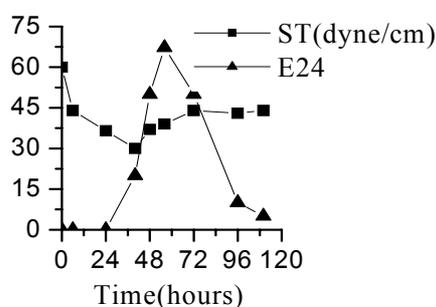


fig.4: Kinetics of biosurfactant production by *Pseudomonas fluorescens*.
with optimized media : olive oil(C) 2% ; NH_4NO_3 (N) 1g/l; C/N =10

3.3. Biosurfactant recovery and characterization

The biosurfactant was separated by an easy and reliable method without loss of its activity. A yield of approximately 2 g/l comparable to that found elsewhere [18] was obtained.

3.3.1 Rhamnose test

The rhamnose test was positive which indicates that the separated biosurfactant could be a glycolipid.

3.3.2. Infrared spectroscopy analysis

Infrared spectra analyses of the extracts from noninoculated control media and from media inoculated with *Ps. Fluorescens* were performed on the 5 day of cultivation (data not shown). New characteristic bands were found in the IR spectrum of the inoculated culture fluid. In the region $3000\text{-}2700\text{ cm}^{-1}$ were observed several C-H stretching bands of CH_2 and CH_3 groups. The deformation vibrations at 1467 and 1379 cm^{-1} also confirm the presence of alkyl groups. Carbonyl stretching band was found at 1745 cm^{-1} which is characteristic for ester compounds. The ester carbonyl group was also proved from the band at 1250 cm^{-1} which corresponds to C-O deformation vibrations.

3.3.3. Activity characterization

i. Foaming and emulsifying activity

Aqueous solutions of biosurfactant showed good foaming stability. Total disappearance of the foam was detected after 2 hours. In addition to surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator [9]. As shown in Table 4, all the hydrocarbons tested served as substrates for emulsification by the biosurfactant. Diesel oil and kerosene were the best substrates(55%). Sunflower oil were less good substrates for emulsification (45%).

Table 4: Emulsification activity (E24) of biosurfactant against different hydrocarbons

Hydrocarbon	E24(%)
Diesel oil	55
Kerosene	54
Heptane	50
Sunflower oil	45

ii. Haemolytic and antimicrobial activity

The culture supernatant contained abundant amounts of hemolysin as the diameter of the hemolytic zone was 11 mm. The same overall pattern was seen in the *B. subtilis* inhibition test. A clear growth inhibition zone with a diameter of 25 mm was quantified when using the concentrated culture supernatant of *Ps.fluorescens* .

3.4. Stability tests

3.4.1 Temperature stability

The stability of biosurfactant was tested over a wide range of temperature. The biosurfactant produced by *Pseudomonas fluorescens* was shown to be thermostable (Fig.5[A]). Heating of the supernatant to 100 °C (or its autoclaving at 120 °C) caused no significant effect on the biosurfactant performance . The surface tension reduction and emulsification activity were quite stable at the temperatures used (ST = 36% ; E24 = 40-48%) in comparison with synthetic surfactants such as SDS which exhibits a significant loss of emulsification activity beginning at 70 °C [22].

3.4.2. pH effect

Fig. 5 [B] shows the effect of pH on the biosurfactant properties. These results indicate that pH increase has a positive effect on surface tension ST(decrease from 34 to 30 dyne/cm) and on emulsion stability (15% increase of E24) . This could be caused by a better stability of fatty acids-surfactant micells in the presence of NaOH and the precipitation of secondary metabolites at higher pH values. The effect of pH on surface activity has been reported for biosurfactants for different microorganisms [21]

3.4.3. Effect of Salinity

Fig.5[C] shows the effect of sodium chloride addition on surface tension(ST) and emulsification index E24 of *Pseudomonas fluorescens* biosurfactant. Little changes were observed in either parameters with addition of up to 20% w/v sodium chloride.

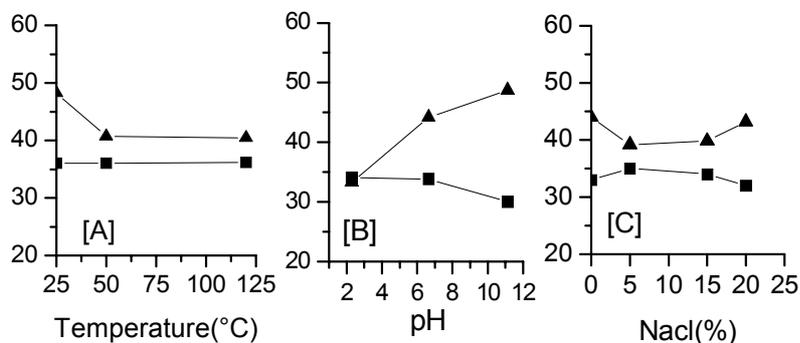


fig.5: Effect of temperature[A], pH[B] and salinity(NaCl=[C]) on biosurfactant activity.

—■— Surface tension ST(dyne/cm)
—▲— Emulsification index E24(%)

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