CHEMICAL ENGINEERING TRANSACTIONS, Volume 14, 2008
Edited by G. Marino, E. Bardone, A. Viglia
 ISBN 978-88-95608-02-0

Effect of the carbon and nitrogen sources on biosurfactant production by *Pseudomonas fluorescens* – Biosurfactant characterization

A.Yataghene[1], M. Abouseoud[2], R. Maachi[1], A. Amrane[3]
[1]Laboratoire Génie de la Réaction, Université Houari Boumediene, Institut de Chimie Industrielle, Alger, Algeria. E-mail: a.yataghene@yahoo.fr
[2]Centre Universitaire Yahia Fares de Médéa, Département Génie des Procédés Pharmaceutiques, Institut des Sciences de l'Ingénieur, Ain Dahab, Médéa, 26000, Algeria.
E-mail: Mahmoud103@hotmail.com
[3]UMR CNRS 6226 – Equipe CIP (Université de Rennes 1 – ENSCR)
ENSCR, 35700, Rennes, France. E-mail: abdeltif.amrane@univ-rennes1.fr

The production of biosurfactant by cells of *Pseudomonas fluorescens* Migula 1895, using the following Carbon (glucose, olive oil and hexadecane) and Nitrogen (urea, NH_4NO_3, KNO_3 and NH_4Cl) sources were examined in this work.
Olive oil and NH_4NO_3 as carbon and nitrogen sources were found to give the optimal yield of biosurfactant production (9 g L^{-1}) for an optimal C/N ratio of 10/1, and led to a decrease of the surface tension of the culture medium from an initial value of 69 mN m^{-1} to 30.5 mN m^{-1} at the end of culture. The critical micellar concentration was found to be 290 mg L^{-1}. Interfacial tension value was low with vegetable oil (2.5 mN m^{-1} with Sunflower oil) and high in case of aromatic oil (17.5 mN m^{-1} with toluene). The stability of the emulsion produced against sunflower oil was very stable during several days; leading to an emulsification index E24 of 80%. Wettability is also a useful parameter to characterize biosurfactants. The decrease of the contact angle reflects an improvement in the degree of wetting.

1. Introduction

Due to the features of high surface activity and biodegradability, biosurfactants produced by a variety of microorganismes have been studied extensively in recent years (Van Hamme et al 2006). Biosurfactants are amphiphilic compounds and are mainly classified into four categories based on the hydrophilic part: glycolipid type, fatty acid type, lipopeptide type and polymer type. Biosurfactants production is an important area of research, owing to the large number of potential applications, especially as substitutes for synthetic surfactants in oil and other industries (Banat et al 2000; Mulligan 2005).They can be used as emulsifiers, de-emulsifiers, wetting agents, foaming agents, functional food ingredients and detergents (Kosaric 1992). The major factors restricting the commercial viability of biosurfactants are the low yield and high production cost (Mukherjee et al 2006; Fiechter 1992). *Pseudomonas* are the best-known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants to enhance the uptake of such immiscible hydrophobic compounds (Al-Tahhan et al 2000; Rahman et al 2002). However, the available literature shows a lack of studies dealing with biosurfactant production by considering the use of carbon sources other than hydrocarbons.

2. Materials and Methods

2.1. Microorganisms

Pseudomonas fluorescens Migula 1895 [Al] (DSMZ; Braunschweig GERMANY) strain was used in this work.

2.2. Culture medium and fermentation condition

The *P. fluorescens* strain was pre-cultured at 30°C and 250 rpm agitation for 18h in a 250 ml Erlenmeyer flask containing 50 ml of sterilized culture medium (20 min at 120°C). The culture medium contained 1 ml of a solution (MS) of the following mineral salts (g l^{-1}): K_2HPO_4 2.7, KH_2PO_4 1.4, Mg $SO_4.7H_2O$ 0.6, $FeSO_4.7H_2O$ 0.01, NaCl 0.05 and $CaCl_2$ 0.02; 1g of the considered nitrogen source; and 2 % of the considered carbon source. The pH of the medium was adjusted to 6.8 with HCl or NaOH. Three carbon sources (Hexadecane, Glucose and Olive Oil) and four nitrogen sources (Urea, NH_4Cl, KNO_3 and NH_4NO_3) were considered.

2.3. Growth conditions

Erlenmeyer flasks containing 50 ml of sterile culture medium were inoculated with 2%(v/v) of *P. fluorescens* cells pre-culture and incubated at 30°C and 250 rpm agitation speed for 5 days.

2.4. Analytical techniques

During culture, samples were taken twice a day and the following parameters were controlled: medium acidity (pH), biomass yield (g dry matter l^{-1}), the emulsifying activity and the surface tension of the supernatant, after cells separation by centrifugation at 8000 rpm for 20 min. A decrease of the surface tension of the culture medium and an increase of the emulsifying activity allowed to characterize biosurfactant production.

Surface tension and interface tension

Surface tension and interface tension were determined by means of a K6 tensiometer (Krüss, Germany).

Determination of the emulsifying activity

To estimate the emulsifying activity, two equal volumes of supernatant and Hexadecane (2ml each) were added in a test tube and mixed at high speed for 2mn.The emulsion stability was determined after 24h. The emulsification index, E_{24} (%) was the ratio of the height of the emulsion layer by the total height of the mixture (Iqbal et al 1995).

Biomass concentration measurement

The biomass was deduced from dry cellular weight measurement. Cells were separated from culture broth by centrifugation for 20 min at 8000 rpm; they were washed twice with distilled water, dried at 105°C and weighed.

Biosurfactant Production

After centrifugation of the culture broth (20 min at 8000 rpm), the supernatant was treated with 3 volumes of acetone at 4°C. The precipitate was collected by centrifugation for 10 mn at 4000 rpm and dried under air flow.

Critical micellar concentration (CMC)

The Critical micellar concentration (CMC) corresponded to the concentration of an amphiphilic component needed to initiate the formation of micelles in the solution. The CMC is an important parameter, since no further effect is expected in the surface

activity above this concentration (Ligia 2006). The CMC was determined by measuring the surface tensions of dilutions of cell-free broth in distilled water up to a constant value of surface tension. Measurements of the surface tension of distilled water and of the mineral medium were used as controls. It was carried out by means a K6 tensiometer (Krüss, Germany).

Interfacial tension (IFT) and Foaming activity of biosurfactant

The IFT between biosurfactant solution (2 mg ml^{-1}) and different hydrocarbons (toluene, hexadecane), as well as sunflower oil was measured. For foaming activity measurement, the biosurfactant solution (1 mg ml^{-1}) was agitated manually during 1mn until foam became stable.

Biosurfactant stability

Influences of salt (0, 10, 20, 30, 40 % NaCl), pH (1.5, 6, 11 and 12) and temperature (20, 40, 60, 80 and 105°C) were examined.

Wettability Measurements

The sessile drop method was used to characterize the wettability of a layer of biosurfactant (BS) on polystyrene (PS) surface. The angles were measured using a goniometer. The energetic parameters of the BS surface free energy (γ_s) and its dispersive and no dispersive (polar) terms $(\gamma_s^d, \gamma_s^{nd})$ were calculated from the respective contact angles of water and diiomethane and the energetic parameters of water (liquid free energy, γ_l = 72.8 mJ m^{-2}, γ_l^d = 21.8 mJ m^{-2} and γ_l^{nd} = 51 mJ m^{-2}) and diiodomethane (γ_l = 50.8 mJ m^{-2}, γ_l^d = 50.8 mJ m^{-2} and γ_l^{nd} = 0 m J m^{-2}).

Fourier transforms infrared spectroscopy (FTIR)

The infrared adsorption bands identify specific molecular components and structures. For characterization, dried BS were ground with KBr and pressed to obtain pellets. Infrared absorption spectra were recorded on a FT-IR / Diffuse reflexion spectrometer, in the 4000-600 cm^{-1} range. KBr pellet was used as the background reference.

3. Results and Discussion

3.1. Optimal conditions for biosurfactant production

The carbon source is a major parameter in the production of biosurfactants (BS). The carbon sources generally used can be divided into three categories: carbohydrates, hydrocarbons and vegetable oils. In this study, the production of BS by *Pseudomonas fluorescens* was examined in the presence of glucose, hexadecane and olive oil. The following nitrogen sources were tested, NH_4Cl, KNO_3, NH_4OH and urea. In all cases, the surface tension (ST) decreased after 72 h cultivation; and this decreased was more pronounced in the case of the following couples of nitrogen and carbon sources, NH_4NO_3 and oil olive or urea and glucose (Table1). The higher value of E_{24} was recorded during *P. fluorescens* growth on urea and glucose based medium, while NH_4NO_3 and glucose based medium led to the higher biomass concentration (0.91 gL^{-1}).

Table 1. Surface tension ST and emulsification index E_{24} for the various culture media tested

	NH_4Cl		KNO_3		NH_4NO_3		Urea	
	ST	E_{24}	ST	E_{24}	ST	E_{24}	ST	E_{24}
Glucose	50	0	45	10	48	5	45	55
Oil olive	50	5	42	5	40	7	48	8
Hexadecane	52	15	43	5	50	2	50	8

Medium acidification was recorded during growth on glucose (Table 2), most likely due to the fermentative production of organic acid. However, this assumption has to be subsequently confirmed. Contrarily, no effect on pH of the use of olive oil or hexadecane was observed (Table 2). Whereas, the initial pH of culture media was 6.80.

Table.2. pH for different culture media

	glucose	olive oil	hexadecane
	pH	pH	pH
NH_4Cl	4.48	7.5	7.43
KNO_3	3.7	7.3	7.2
NH_4NO_3	3.5	6.3	7.3
Urea	7.9	7.51	7.6

The following carbon on nitrogen ratio 10, 20, 30, 50 were then considered for glucose + urea and olive oil + NH_4NO_3 based media. ST and E_{24} varied weakly in the range of concentrations tested, with optimal values of 28.5 mN m^{-1} and 40% during growth on olive oil and NH_4NO_3, when the lowest C/N ratio was considered (10). The minimum ST value (28.5 mN m^{-1}) was recorded at 110h. Surface tension decrease and emulsification index increase characterize biosurfactant production. After acetone precipitation and drying of the biosurfactant contained in the supernatant, between 6 and 9 g l^{-1} of biosurfactant were produced after 6 days of culture.

The emulsification index of the produced biosurfactant, determined at 24h (E_{24}), was found to be the highest when sunflower oil was considered (80%).

The critical micellar concentration (CMC) of the biosurfactant produced by *P. fluorescens* was 290 mg L^{-1} and occurred for a surface tension of 37 mN m^{-1}, as it can be deduced from Fig.1.

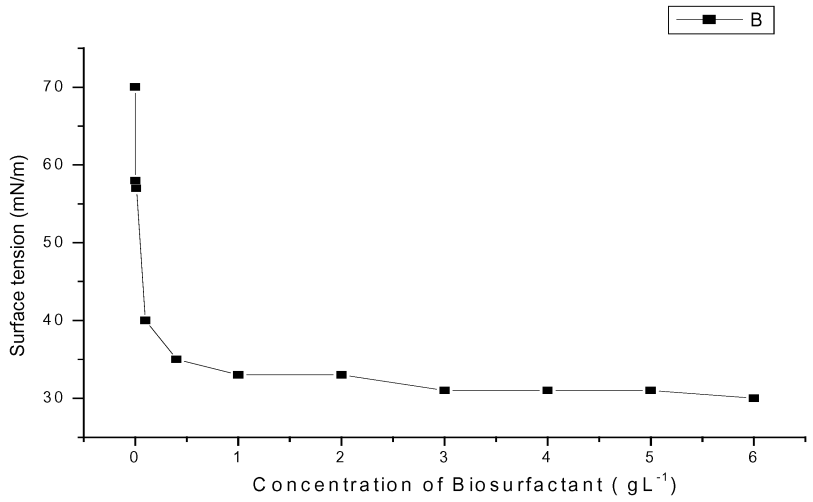


Fig.1.Determination of the critical micellar concentration (CMC) of the biosurfactant produced by *P. fluorescens*.

Table 3. Interfacial tension (IFT) of toluene, hexadecane and sunflower oil.

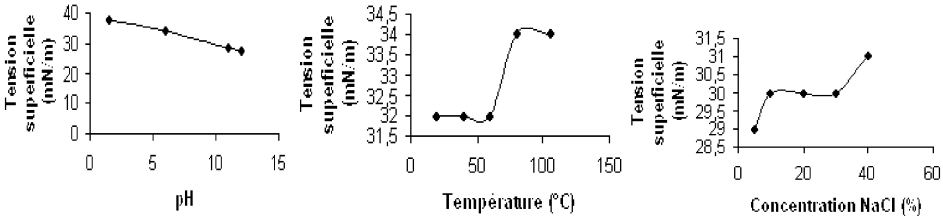
	Toluene	Hexadecane	Sunflower
Interfacial tension (mN m^{-1})	17.5	12	2.5

As expected, interfacial tension of the biosurfactant produced was low with vegetable oil and high with aromatic compound.

During growth on sunflower oil, a nearly constant value of the emulsification index was recorded for several days (E_{24} = 80%).
After manual agitation of the biosurfactant solution (1 mg l^{-1}) for 1 min, foam stability was shown for at least 1h.

3.2. Stability of the biosurfactant

In Fig.2a, the augmentation of the pH decreased the surface tension. In the range 20 to 60°C (Fig.2b) and 10 to 30 % NaCl (Fig.2c), the surface tension (ST) remained nearly constant.



(a) (b) (c)

Fig.2.Effect of pH (a), temperature (b) and salinity (c) on the biosurfactant produced during *P. fluorescens* growth.

3.3. Fourier transforms infrared spectroscopy

Characteristic bands were found in the IR spectrum of biosurfactant, in the range 3000-2700 cm^{-1}, which are characteristics of several C-H stretching bands of CH_2 and CH_3 groups. The deformation vibrations at 1479 and 1413 cm^{-1} also confirmed the presence of alkyl groups. Carbonyl stretching band was found at 1732 cm^{-1} which is characteristic of ester compounds. The ester carbonyl group was also proved from the band at 1250 cm^{-1}, which corresponded to C-O deformation vibration.

3.4. Wettability and Surface Energy

The polystyrene (PS) surface with a biosurfactant layer was used for contact angle measurements of water and diiodomethane. The decrease of the contact angle reflects an improvement in the degree of wetting (Audic et al 2000). Indeed, contact angle measurements can be used to approch the wetting degree.

The surface energy γ_s was the sum of its dispersive and non dispersive components, $\gamma_s^d, \gamma_s^{nd}$:

$$\gamma_s = \gamma_s^d + \gamma_s^{nd} \quad (1)$$

The effects of the dispersive and non dispersive forces corresponded to long-range Lifschitz – van der Waals interactions and to short-range polar interactions, respectively (Poncin-Epaillard 1997); although the non dispersive energy could be expressed by a donor and an acceptor part (Hollander et al 1994). In our analysis, we considered the following model for the γ_s^d and γ_s^{nd} terms of the dispersive energy γ_s :

$$\gamma_l(1+\cos\theta) = 2\sqrt{\gamma_l^d \quad \gamma_s^d} + 2\sqrt{\gamma_l^{nd} \quad \gamma_s^{nd}} \quad (2)$$

Contact angles (degrees) were measured at 25° on PS surface with and without a layer of biosurfactant. Water and diiodomethane were employed to calculate γ_s^d, γ_s^{nd} and γ_s. The contact angles obtained for PS with BS were: θ_{water} = 43° and $\theta_{diiodomethane}$ = 58°. The obtained values are given in Table 4.

Table 4. Surface free energy (γ_s^d , γ_s^{nd} , γ_s) of polystyrene surface with a layer of biosurfactant.

γ_s (mJ m^{-2})	γ_s^{nd} (mJ m^{-2})	γ_s^d (mJ m^{-2})
57.2	27.5	29.7

The water contact angle decreased from 57° without biosurfactant to 43° in presence of biosurfactant. The surface free-energy was positive; the dispersive and non dispersive components were of the same order of magnitude. The decrease of the contact angle in presence of biosurfactant characterized a change of surface wettability.

4. References

Al-Tahhan R.A., T.R. Sandrin, A.A. Bodour and R.M. Maier, 2000, Rhamnolipid-induced removal of lipopolysaccharide from Pseudonomas aeruginosa : effect on cell surface properties and interaction with hydrophobic substrates. Appl. Environ. Microbiol. 66, 3262.

Audic J.-L., F. Poncin-Epaillard, D. Reyx and J.-C. Brosse, 2000, J. Appl. Polymer. Sci. 72, 1384.

Banat I.M., R.S. Makkar and S.S. Cameotra, 2000, Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53, 498.

Beal R. and W.B. Betts, 2000, Role of rhamnolipid biosurfactant in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J. Appl. Microbiol. 89, 158.

Desai J.D. and I.M. Banat,1997, Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61, 47.

Fiechter A., 1992, Biosurfactants: moving towards industrial application. Trends Biotechnol. 10, 208.

Hollander A., J. Behnisch and H.J. Zimmermann, 1994, Chimical Analysis of Polymer Surfaces on a Molecular Scale. Polym. Sci. Part A: Polym. Chem. 32, 699.

Iqbal S., Z.M. Khalid and K.A. Malik, 1995, Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of *Pseudomonas aeroginosa*. Lett. Appl. Microbiol. 21, 176.

Kosaric N., 1992, Biosurfactant in industry. Pure Appl. Chem. 64, 1731.

Mukherjee S, P . Das and R. Sen, 2006, Towards commercial production of microbial surfactants. Trends Biotechnol. 24, 509.

Mulligan C.N., 2005, Environmental applications for biosurfactants. Environ. Pollut. 133, 183.

Nitschke M. and G.M. Pastore, 2006, Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresource Technol. 97, 336.

Poncin-Epaillard F. and S. Vallon S, 1997, Illustration of surface crosslinking of different polymers treated in argonplasma. Macromol. Chem. Phys. 198, 2439.

Rahman K.S.M., T.J. Rahman, S. McClean, R. Marchant and I.M. Banat, 2002, Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. Biotechnol. Prog. 18, 1277.

Rodrigues L.R., J.A.Teixeira and C. Henry, 2006, Isolation and partial characterization of a biosurfactant produced by *Streptococcus thermophilus* A. Colloides Surf. B: Biointerfaces 53, 105.

Van Hamme J.D., A. Singh and O.P. Ward, 2006, Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol. Adv. 24, 604.