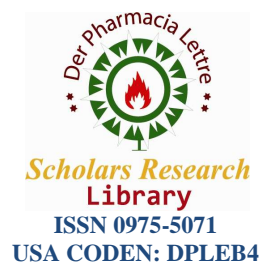




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Effect of carbon and nitrogen source on the microbial production of biosurfactants by *Pseudomonas aeruginosa*

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ABSTRACT

Rhamnolipids are biosurfactants substances produced by bacterial fermentation process of *Pseudomonas aeruginosa*, required for the remobilization of oil and used for fight against pollution. In the present study, 26 bacterial strains were isolated from soil and sewage of two different sites polluted by hydrocarbons, in which 3 selected strains producing Rhamnolipids belonging to the genus of *Pseudomonas aeruginosa* were purified, identified and used for further characterization. The secondary screening of the selected *Pseudomonas aeruginosa* revealed that one strain manifested a high performance for Rhamnolipids production. For the optimization of several parameters implicated in the biosurfactants production, five carbon sources such as olive oil, sunflower oil, glycerol, ethanol, corn germ and six nitrogen sources such as yeast extract, soybean meal, peptone, meat extract, urea, corn steep liquor has been investigated. The obtained results showed that the maximum Rhamnolipids production (2.7 g/l) was yielded by the using olive oil at a concentration of 3% as carbon source and 5 g/l of extract yeast as nitrogen source.

Key words: Rhamnolipids, *Pseudomonas aeruginosa*, polluted soils.

INTRODUCTION

Microbiological derivate surfactants or biosurfactants are heterogeneous group of surface-active molecules produced by a wide variety of bacteria, yeast, filamentous fungi, which adhere to cell surface or excrete in the supernatant of the growth culture medium [1, 2, 3].

The biosurfactants with their both hydrophobic and hydrophilic moieties, are able to reduce the surface tension and interfacial tension between two fluids at the surface and the interface respectively. Furthermore, these are also able to form micro emulsion, where hydrocarbons can solubilise [4, 5, 6].

Recently, the environmental impacts caused by chemical surfactants due to their toxicity and difficulty encountered by their degradation in the environment become great emphasis.

The increasing environmental problems, the advance in biotechnology and the emergence of more stringent laws have led the utilisation of biosurfactants as a potential alternative to the chemical surfactants available on the market [7, 8, 9].

The advantages of biosurfactants were their lower toxicity, their biodegradability, their specific activity at extreme temperatures, pH and salinity. Furthermore, their production through fermentation process, their potential applications in environmental protection and crude oil recovery, their use as antimicrobial agents in health care and food processing industries [1, 15].

The mainly produced glycolipid biosurfactants as rhamnolipids (RLs) by *Pseudomonas aeruginosa* are the most intensively studied [25]. The first process of rhamnolipids production was observed at 1946, when Bergström and co-workers (1946) has reported that only glycolipid was produced by *Pseudomonas pyocyanea*, cultivated on the culture medium containing the glucose as carbon source. The biosynthesis process of rhamnolipids production was subsequently studied *in vivo* by using various radioactive processors such as ^{14}C -acetate and ^{14}C -glycerol [8].

The biosurfactants consist one of the most virulence factors contributing to the pathogenesis of *Ps. aeruginosa* infections due to their relatively high yields after relatively short incubation periods and their easy cultivation [13].

MATERIALS AND METHODS

Bacterial strains

The used strains in this work belonging to the genus of *Ps. aeruginosa* was isolated from the most contaminated soils with diesel, in the region Remchi (Tlemcen), located in the North West of Algeria. The isolated strain was maintained on nutrient Broth agar slants, conserved at 4°C and subcultures were made every 2 weeks.

Growth conditions

The used mineral salt culture medium (MS) in this study was containing the following components (g/L): $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (1.0) ; KH_2PO_4 (0.5) ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5) ; CaCl_2 (0.01) ; yeast extract (5.0); glycerol (30.0) as sole carbon source, pH 6.8. The bacteria strains were inoculated in a 250 mL flasks containing a volume of 100 mL of culture medium MS, incubated at a temperature of 30°C by stirring in a rotary shaker at 220 rpm for seven days. The bacterial growth was monitored by measuring of the optical density at a wavelength A_{600} .

Detection of biosurfactants activity

The obtained overnight culture medium from fermentation was diluted with fresh culture medium, plated on the cetyltrimethyl-ammonium bromide (CTAB)-methylene blue agar, prepared by adding of 0.2 g CTAB, 0.005 g of methylene blue, and 15 g agar in a volume of 1L of MS culture medium. The rhamnolipids producing colonies on CTAB agar plates, were identified by the formation of dark blue halos around the colonies on a light blue-plate background [6].

On others hand, the indirect way for the exploration of rhamnolipids producing colonies is based on their hemolytic properties, which the rhamnolipids producing strains was inoculated on blood agar plates. The Formation of a halo clear around the colony indicated the presence of RLs [8].

Furthermore, the drop collapsing test is a more sensitive method for the rapid screening of rhamnolipids producing colonies by various isolates [12]. This assay consists to apply a drop of bacterial culture supernatant over a plate covered with oil. The droplet will spread over the oil only if the culture supernatant sample contains RLs.

Quantification of rhamnolipids

Rhamnolipids were purified by separating of the cells biomass from supernatant of the inoculated culture medium by centrifugation (12500 g) for 10 min at a temperature of 4°C. The recuperated supernatant of the culture medium was then acidified by the using 4 N hydrochloric acid adjusted to pH-value of 3.0, the precipitated rhamnolipids was collected by centrifugation (12000 g). After that, the RLs were extracted for three times with a chloroform-Methanol (1:1) mixture, which was then evaporated and leaving behind relatively pure Rhamnolipids having an oil-like appearance [26].

The extract fractions were further separated on thin-layer chromatography (TLC) by the using of aluminium silica gel 60 F₂₅₄ plates and a chloroform: methanol: distilled: water (65:25:4) solvent system. The RLs was visualized by the using the orcinol test [14]. The quantification of RLs has achieved by orcinol assay [5].

This test was used for direct assessment of the amount of glycolipid in the culture supernatant. For this purpose, a volume of 400 µl of solution containing 1,5 g of orcinol diluted in 100 mL of H_2SO_4 (30%) was added to 200 µl of each sample with suitable dilution. After heating for 30 min at temperature of 80°C, the samples were cooled to room temperature and the DO_{420} was measured. The RLs concentration was calculated from a standard curve prepared with L-rhamnose monohydrate and expressed as rhamnose monohydrate equivalents (RME) (mg/mL).

Optimization of carbon and nitrogen source for biosurfactants production

In order to explore the effect of carbon and nitrogen sources on the Rhamnolipids production, the investigated carbon source in MS culture medium was replaced by using ethanol (30 g/L), corn germ (30 g/L), sunflower oil (30

g/L) and olive oil (30 g/L) and the used nitrogen source was also replaced by meat extract (5g/l), corn steep liquor (5g/l), urea (5g/l), soybean flour (5g/L) and peptone (5g/L) .

Study of bacterial cell growth and rhamnolipids production

The Study of bacterial cell growth and rhamnolipids production were carried out at optimized culture conditions between inoculum of fresh overnight culture and 7 days old culture. In this experiment, variations in biomass and RLs production were recorded at different time intervals over a period of 7 days. Biomass was expressed as optical density (OD) of culture broth measured at 600 nm.

RESULTS AND DISCUSSION

Detection and quantification of the surface-active compounds

The haemolysis of blood agar was first used for the screening of the biosurfactant-producing bacteria [3] and the method of Moran and co-workers (2002) was applied for the quantification of the amount of the produced surfactin by *B. Subtilis*, where was demonstrated the presence of the biosurfactants caused the lyses of the red blood cells on the agar. The inoculated *P. aeruginosa* on blood agar shows remarkable hemolysis zones, However, the obtained hemolytic zone formation on blood agar plates is not only dependant on the produced RLs and may be affected by divalent ions and other hemolysins produced by the bacteria under investigation. The accumulation of the insoluble ion pair precipitate in the agar plate containing methylene blue exhibited dark blue color against the light blue background.

The obtained diameter of the dark blue region has been shown to be semi quantitatively proportional to the concentration of the Rhamnolipids [22, 24].

The colony manifested the largest dark blue halo was identified as *P. aeruginosa* and selected for further investigation. Jain and co-workers (1991) has suggested the drop collapse method as a sensitive and easy for the investigation of the biosurfactant production. However, the drop collapse technique was only applied as a qualitative method for detection of biosurfactant production [4].

The obtained results showed that the culture supernatants *P. aeruginosa* manifested permanent movement, explained by the presence of produced biosurfactants.

The excreted biosurfactants in the supernatant culture of *P. aeruginosa* are most probably a mixture of Rhamnolipids, the amphiphilic surface-active glycolipids [10, 23].

From 26 isolated strains belonging to the family of Pseudomonaceae, the highest displayed producing biosurfactant strain was selected and identified as *P. aeruginosa*.

Furthermore, the use of the thin-Layer Chromatography, has revealed the presence of two typical glycolipid spots after the orcinolsulfuric staining at R_f 0.77 and 0.62 corresponding to the reference mono-rhamnolipid and di-rhamnolipid from *P. aeruginosa* (Figure 1).

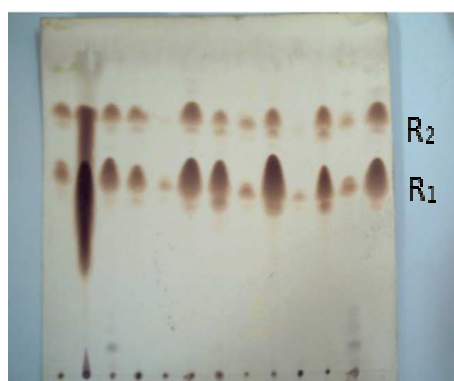


Figure 1: Illustration of the thin layer chromatography of different broth in the presence of several carbon and nitrogen source, R1: di-rhamnolipid; R2: mono-rhamnolipid

Effect of carbon source on the RL production

The obtained results of the effect of several carbon source such as sunflower oil, olive oil, glycerol, ethanol and corn germ on the production of RLs by *P. aeruginosa*, inoculated in the MS culture medium is presented in Figure 2.

The histogram shows that the yield of RLs and bacterial dry weight are the highest by the using of olive oil as source of carbon (2.7 g/L and 3.93 g/L respectively) and glycerol (1.9 g/L and 3.45 g/L). Santa Anna *et al.* (2002) has reported that the use of glycerol yielded more Rhamnolipids than others used different oils. The use of glycerol as carbon sources for RLs production seems to be an interesting and low cost alternative [10, 11]. An important RLs production was achieved by the using of the sunflower oil (1.6 g/L) and ethanol (1.2 g/L), accompanied with a cell dry weight of 3.45 g/L and 0.63 g/L respectively.

Some reports showed that the used vegetable oils were more efficient substrates for Rhamnolipids production by *P. aeruginosa*, when compared with glucose, glycerol, and hydrocarbons [16,18]. Low Rhamnolipids production was obtained by the using of corn germ (0.5 g/L). Therefore, the glycerol was selected as the optimal carbon source. The use of vegetable oil and glycerol as carbon sources for Rhamnolipids production seems to be an interesting and low cost alternative [21].

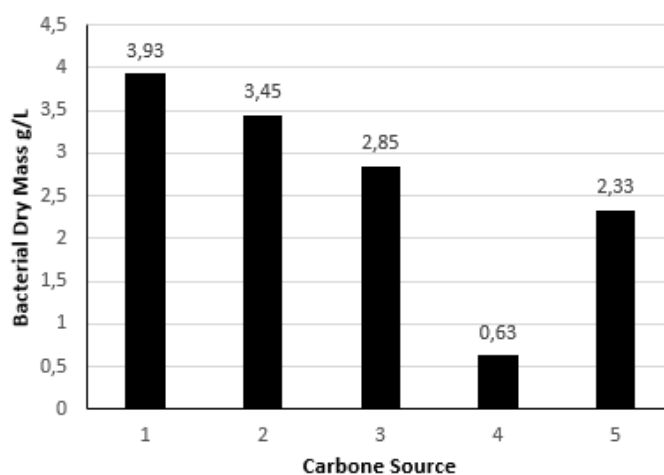


Figure 2: Effect of carbon source such as 1olive oil (1), sunflower oil (2), glycerol (3), ethanol (4), corn germ (5) on the production of Rhamnolipids by *P. aeruginosa*

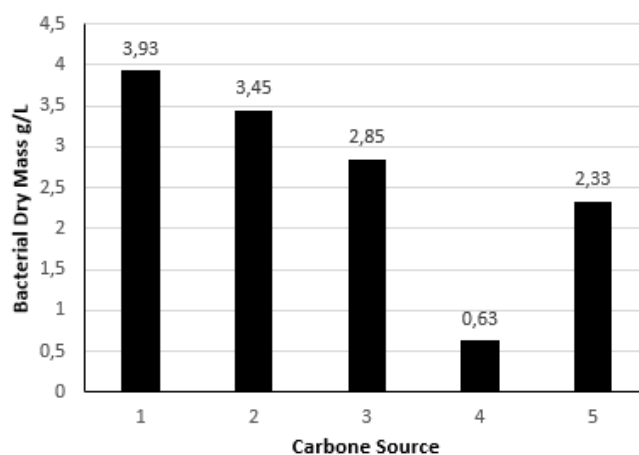


Figure 3: Effect of carbon source such as olive oil (1), sunflower oil (2), glycerol (3), 4-ethanol (4), corn germ (5), on the bacterial dry weight of *P. aeruginosa*

Effect of nitrogen source on the production of RLs

The use of MS culture medium supplemented with glycerol as a source of carbon and six several nitrogen sources such as meat extract, yeast extract, soybean flour, peptone and corn steep liquor, their effect on the RLs production by *P. aeruginosa* has been investigated.

The obtained results (Figure 4) in the presence steep liquor and meat extract as unique nitrogen sources indicated that these sources were not suitable for an optimal RLs production with a rate of 0.12 g/L and 0.6 g/L respectively.

The Figure 4 showed that the highest RLs production and cell dry weight were achieved by the using of yeast extract (1.9 g/L and 2.87 g/L) respectively. However, the use of soy flour yielded a rate of biosurfactants of 1.6 g/L, and 0.45 g/L of cell dry weight. The produced RLs in the supplemented MS culture medium on peptone and urea was low. Whereas, the yeast extract was the best nitrogen source for growth and RLs Production. Therefore, the yeast extract was selected as the optimal nitrogen source.

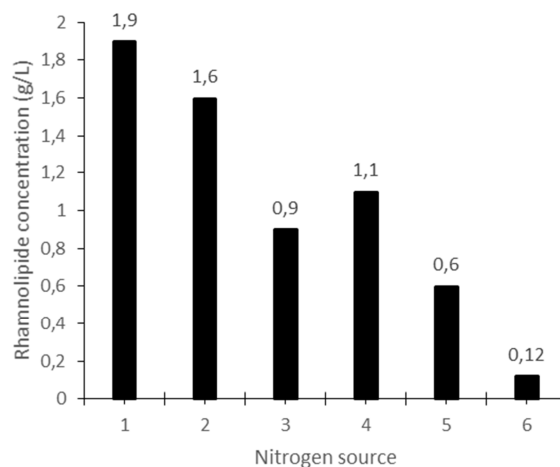


Figure 4: Effect of nitrogen source such as yeast extract (1), soy flour (2), 3-peptone (3), urea (4), 5-meat extract (5), corn steep liquor (6) on the production of Rhamnolipids by *P. aeruginosa*

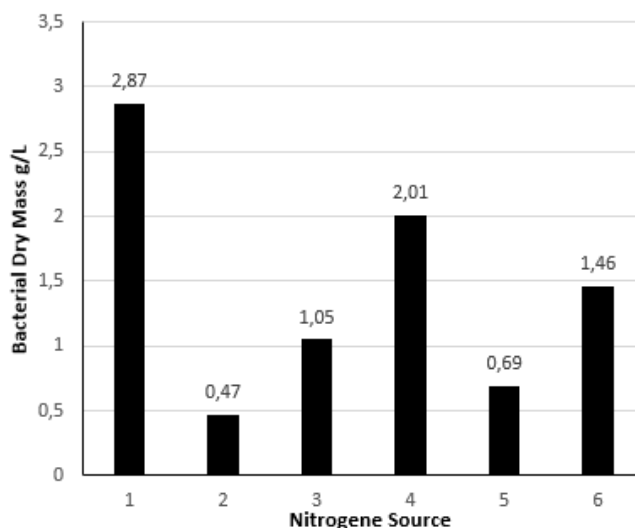


Figure 5: Effect of nitrogen source such as yeast extract (1), soy flour (2), 3-peptone (3), urea (4), 5-meat extract (5), corn steep liquor (6) on the bacterial dry weight of *P. aeruginosa*

Bacterial cell growth and Rhamnolipids production

The RLs Production was high yielded by some microorganisms, especially *Pseudomonas spp* [17]. The obtained results indicated the profile of bacterial growth and RLs production in the fermentation medium containing glycerol as carbon source and yeast extract as nitrogen source (Figure 6). The exponential phase extended up to 10 hours, where RLs production and bacterial cell growth was found to be normal. This observation suggests that the produced biosurfactants in the culture broth is a secondary metabolite [7]. During the stationary phase of cell growth, the RLs production increased at a higher rate and maximum product accumulation occurred after 40 h of cultivation (1.9 g/L). After 40h incubation, the concentration of RLs was dropped, may be due to the degradation of these molecules.

Rhamnolipids yield was then calculated by a coefficient of 3.4 obtained from the correlation of pure rhamnolipids/rhamnolipose (1.0 mg of rhamnolipose corresponds approximately to 3.4 mg of RLs) [20]. The RLs production is typical secondary metabolite and increased considerably in the stationary phase.

The obtained results of the inoculation of *Pseudomonas aeruginosa* in the fermentation medium in the absence of a carbon source, showed a feeble bacterial cell growth, accompanied with the low yield fall of RLs production during the first 24 hours (Figure 7).

The addition of glycerol at a concentration of 3% in the fermentation medium after 24 hours incubation times indicated that the bacterial cell growth accelerated and the RLs production rate was triggered and reached a high rate of 1.79 g/L at the 60th hours. From the 3rd day, the bacterial cell growth reached the stationary phase and the RLs production decreased to a level of 0.7 g/L in the 96th hours.

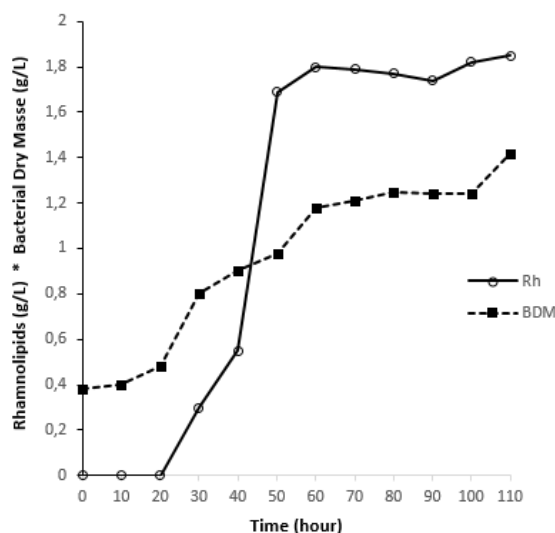


Figure 6 : Bacterial growth of *Ps. aeruginosa* and Rhamnolipids production in the presence of 3% of glycerol, incubated at a temperature of 30°C by stirring in a rotary shaker at 220 rpm for 110 hours

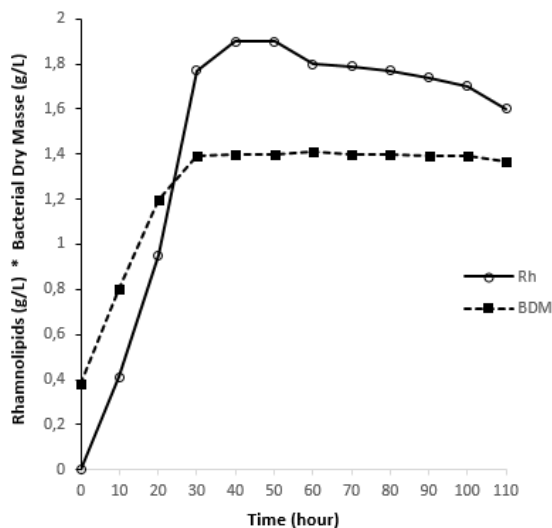


Figure 7: Bacterial growth of *Ps. aeruginosa* and Rhamnolipids production in the presence of 3% of glycerol for 24th and 1% for 100th hours

CONCLUSION

The isolated strain from contaminated soil was identified as *Pseudomonas aeruginosa*, it is capable of effectively producing Rhamnolipids by the using several carbon and nitrogen sources. Furthermore, the identified strain has manifested a important potential for the biosurfactants production and the application in bioremediation of oil pollutants.

Among the five investigated carbon and six nitrogen sources, the used olive oil and glycerol was presented as the most efficient carbon sources, whereas the yeast extract as the best nitrogen source for Rhamnolipids production. The optimization of several parameters implicated in the RLs production in batch cultures indicated that optimum of temperature, pH-value and agitation rate were 30°C, pH 6,8 and 220 rpm, respectively.

Furthermore, the TLC analysis of the RLs showed that the purified product contained mono-rhamnolipids and di-rhamnolipid.

In conclusion, the biosurfactant produced by *P. aeruginosa* could be used for potential application in bioremediation of crude oil contamination. Whereas, Rhamnolipids produced by *Ps. aeruginosa* presented a great potential for biotechnological and biopharmaceutical applications, due their biological properties. Further studies should be done for the characterization of the chemical structure and cellular toxicity of these compounds.

Acknowledgement

We would like to express my sincere thank to the Ministry of Higher Education and Scientific Research of Algeria for the financial support to Mr. Harrachi Abdelkader for providing us with the bacterial strains and to Prof. Dr. Mohamed Benali, Director of Laboratory for Biototoxicology, for your encouragement, your support and your useful discussion. Without the kind cooperation of the above mentioned people the work could not have been possible.

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