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Research Article

Optimization of Diauxienne Growth of *Pseudomonas aeruginosa* in the Bioremediation of Soils Polluted by Hydrocarbons

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Abstract

The objective assigned to this study is the optimization of azote nutriment source for the microbial bioremediation to contribute to the fight against environmental pollution through the production of biosurfactants of multidisciplinary interest. This study consists in metabolizing hydrocarbons into biosurfactants using *Pseudomonas aeruginosa ATCC 27853* strain in order to control the environmental threat in a batch of 250 ml at 37°C for 48 hours of culture. The optimization tests of nitrogen source showed that within a range of concentration [1 - 8] g/l, the optimum value was 4 g/l for KNO_3 with an emulsification index EI24 = 71.45% and an optical density DOX = 0.55 for 46h of culture. The yields obtained $Y_{x/s}$ and $Y_{p/s}$ were of 60.00% and 32.17% respectively with an amount of biosurfactants P = 580 mg, a bioconversion rate θ = 78.47% and a ratio N/C = 0.473. The comparative study between two nitrogen sources such as KNO_3 and NH_4NO_3 for a range of concentration [3 - 4] g/l demonstrated that the best source of nitrogen remains KNO_3. Indeed, the kinetic monitoring of the biomass growth, the emulsification index, the biosurfactants productivity and the substrates consumption revealed the inhibition of the strain by NH_4NO_3 after 7 hours of incubation, provoking the degeneration of the strain and slowing the removal of pollutants process. A similar study of NH_4NO_3 at a concentration of 4 g/l resulted in an emulsification index EI24 = 65.22%, a yields $Y_{x/s}$ = 18.37% and $Y_{p/s}$ = 45.17% and a bioconversion rate θ = 57.36%. The amount of the biosurfactants measured at P = 590 mg for an N/C ratio of 0.739. In fact, the results of this study demonstrated that KNO_3 promotes the growth of biomass as well as the production of biosurfactants who play a major role in the enrichment of the soil by solubilizing the toxic elements and a maximum removal of hydrocarbons following diauxic phenomena.

Keywords: Pollution; Environment; Bioremediation; Biosurfactants; Optimization

Introduction

The infiltration and the dispersal of hydrocarbons in the underground constitute a major threat for the whole ecosystem due to their pollution of the shallow ground waters [1]. According to the CETESB (1996) and the Brazilian agency of environmental control, hydrocarbons spraying covers 78% of the soil contamination which represents 90% of the crude oil in the environment and affects water sources destined for the consumption leading to a major public health problem due to the toxic properties (mutagenic and carcinogenic of the aromatic compounds) [1,2].

The bioremediation by the bacteria is based on their ability to degrade crude oil compounds through a process of assimilation as a source of carbon and energy during growth metabolism, resulting in complete mineralization of constituents [3].

The efficiency of the bioremediation process depends on several factors such as. The presence of microorganisms with catabolic capacities adapted to contaminants at the site or an inductive enzyme metabolism as *Pseudomonas aeruginosa*; presence of nutriments and physicochemical conditions favorable for the growth and for the secondary metabolism of production of xenobiotic compounds of the hydrophobic solubilization compound (biosurfactant); the composition, the concentration and the bioavailability of the contaminant. The two techniques widely used in the bioremediation are the bio-stimulation by a supply of nitrogen and phosphate the growth and the bio-increase [4,5]. Moreover, the use of biosurfactants such as rhamnolipids produced by *Pseudomonas aeruginosa* has proven to be very effective in the bioremediation of soils contaminated by hydrocarbons (David., *et al.* 2017; Eman and Andrew 2017).

Biosurfactants are surfactant amphiphilic molecules, which decrease the superficial and interfacial tension of the medium by solubilizing the crude oil compounds (Magdalena., *et al.* 2011). They are biodegradable, specific, highly selective and are active under extreme conditions of temperature, of salinity and of pH, Ellen., *et al* [1].

Their mechanism of action in very small amount is based on the formation of micelles by emulsification of the oil/water or water/oil medium. Biosurfactants participate in the mobilization, the solubilization and the emulsification of the contaminated medium by hydrocarbons (Magdalena., *et al.* 2011).

The bacteria of the kind *Pseudomonas* are characterized by a multitude of enzymes of synthesis of biosurfactants and their induction in a mean where the phosphate and the nitrogen are factor limitants. *Pseudomonas aeruginosa* synthesizes rhamnolipid biosurfactants in response to stimulus, which are hydrocarbons [1]. The enzymes involved in the conversion of hydrocarbons are of two types: rhamnotransferase I and II with auto-inductive capacity. Their induction results in the synthesis of several rhamnolipids (Rodrigo., *et al.* 2011).

Purpose of the Study

The main purpose of this study is to elucidate the auto-inductive capacity of *Pseudomonas aeruginosa ATCC 27853* in the bioremediation of grounds polluted by the crude oil through biostimulation combined with biosurfactants. The kinetic monitoring focused on the inductive growth kinetic (diauxic phenomena of the biomass), the emulsification of the medium, the productivity of the biosurfactant and the consumption of the carbon substrate by optimization of the quality and the quantity of the nitrogen source.

Material and Experiment

Preparation of the study site

The Niger Delta was chosen in view of the extent of the damage to the environment caused by the spills of crude oil several hectares of agricultural land and pasture and the water resources for consumption and fishing. It is a clay soil harvested from 10 localities that were heavily contaminated according to the study by Amnesty International (2015). Sampling was carried out using tubes 14.50 cm long and 1.50 cm in diameter at a depth of 8 - 10 cm from the ground. Thus, all the tubes were conditioned with 1 ml sulfuric acid at 9N *in situ* and placed under sterile conditions at –13°C before pretreatment in the laboratory, AL-Saleh and Akbar [6].

The strain *Pseudomonas aeruginosa ATCC 27853* was kindly supplied from Medea hospital (Algeria) and was stored in the laboratory under freezing temperature. It was activated on a cetrimide agar in a petri dish and then seeded on the germination medium containing a mixture of heptane and cetane as a source of carbon and energy. The culture medium was formulated according to the work of Milena., *et al* [7].

Fertilizers were successively added with 3% (P/V) of the pretreated contaminated soil in 250 ml of distilled water under agitation for 5 minutes for homogenization. The medium was adjusted to a pH of 6.00 ± 0.01 by addition of sodium hydroxide (1N) hydrochloric acid (1N) and was sterilized for 15 minutes at 121ºC in the autoclave [8,9]. The culture medium was cooled to 70°C; the pH was adjusted to 7.00 ± 0.01 before seeding. The culture was carried out in a batch with an agitation speed of 250 rpm at 37°C for 48h [9].

Kinetic monitoring

Biomass growth monitoring was carried out using optical density method at 600 nm [6,10]. Samples of 10 ml of the fermented medium were taken at regular time intervals and were centrifuged at 6000 rpm for 10, 15 and 20 minutes successively with pellet removal. The pellet was washed with distilled water to remove residues from the culture medium and then centrifuged at 6000 rpm for 20 minutes. The biomass was dried in an oven for 24h at 105°C [10-12]. The dry weight of the biomass was determined from the following relationship: (Biomass = Dry weight × 0.44) for all experiments (Palashpriya and Luyan 2013).

To determine the emulsification index EI_{24}%, 2 ml of the acellular medium obtained after centrifugation by removal of the pellet were taken and completed with 2 ml of hexadecane and were placed in a vortex for 5 minutes. The emulsified samples were then left at rest and the emulsification index was measured after 24h [10,12].

The crude biosurfactant produced by the *P. aeruginosa* strain was isolated by acid precipitation from the cell-free supernatant [10,12,13]. The medium devoid of residual fragments was precipitated by addition of absolute ethanol with a slight agitation until the precipitation of the total amount of the biosurfactant contained in the supernatant [8].

The obtained biosurfactant was washed with distilled water to eliminate as much as possible the traces of ethanol and dried in a vacuum desiccator, then was weighed and expressed in g/L of the culture medium. The amount of hydrocarbons contained in the homogenized mixture of 10 samples taken from the soil was determined by COD-Spectrophotometric at 600 nm using a UV/VIS spectrophotometer GBC 101 Scientific Equipment, driven by the "Cintral ver 2.3" software with a resolution of 0.1 [14,15].

Optimization of the culture conditions

The nitrogen source is a determining factor for the bioremediation of soil contaminated by hydrocarbons process efficiency. Therefore, two sources of nitrogen were optimized as a limiting factor, namely: KNO_3 and NH_4NO_3 within a range [1 - 8] g/l for KNO_3 and [3 - 4] g/l for NH_4NO_3 in a medium rich of carbon for a total volume of 250 mL without renewal during 48h at 37°C with pH = 7. The limitation of concentration of NH_4NO_3 is related to the inhibi-

tory and repressive effect of NH_4 on the strain containing a high-dose in the medium [4,5,14].

Results and Discussion

The choice of *Pseudomonas aeruginosa* in bioremediation was based on its ability to grow in a poor medium of nutrient and its enzymatic induction genome characteristic. The optimization of the pollutant removal process was based on the nitrogen source and constitutes a performance process of bioremediation. This impact, not only microbial growth, but also biosurfactant productivity as a factor of mobilization, solubilization and emulsification of the polluted medium for the degradation of hydrocarbons. All experiments carried out in this study were repeated three times in order to guarantee the reproducibility of the results in a range of confidence of 95% by the statistical analysis on the correlation coefficient. The results of nitrogen optimization for NO_3 are presented in figure 1 below.

The effectiveness of the bioremediation process depends on several factors including the microorganism growth in proportion to anaerobic respiration metabolism. This growth of *Pseudomonas aeruginosa* as shown in the results of figure 1a is maximal, in proportion to the emulsification index and the amount of the biosurfactant in the medium (Figure 1b) at 4 g/l NO_3. This proportionality between these three factors characterizes a process of mobilization, solubilization and elimination of hydrocarbons contaminating the medium due to the limitation of nitrogen in the presence of a high concentration of carbon and energy sources, C_s = 9107 mg/L, in accordance with the observations of Magdalena., *et al* (2011).

Moreover, the bioconversion rate (Figure 1a) shows that the maximum elimination is obtained at 8 g/L. This inversion of proportionality with other factors is characteristic of the abundance of nitrogen in the medium reorienting the metabolism of the strain. The impact of this abundance influences the biosurfactant production in the medium as shown by the evolution of the emulsification index (Figure 1b). The results found are in accordance with the observations of Tyagi., *et al*. [4], Taccari *et al*. [5], Vanessa., *et al.* 2014; Joanna *et al*. on the production of biosurfactants and the removal of oils. On the other hand, the evolution of the N/C ratio and the yield of biomass $Y_{x/s}$ against the nitrogen amount shows that the conversion rate is a performance factor of the bioremediation process (Figure 1c).

Figure 1d characterizes the type of diauxic phenomenon of the *Pseudomonas aeruginosa* strain in the medium. This diauxienne growth implements enzymes entering within the scope of the conversion of hydrocarbons by *Pseudomonas aeruginosa* which are of two types namely rhamnotransferase I and II with auto-inductive capacity. Their induction is at the origin of the synthesis of several rhamnolipids, Rodrigo., *et al* (2011). In fact, the emulsification is a key factor of the bioremediation process and this is in adequacy with the growth, the biosurfactant productivity, the yield, the elimination rate and N/C ratio for a concentration of KNO_3 = 4 g/L as shown in figure 1a-1d.

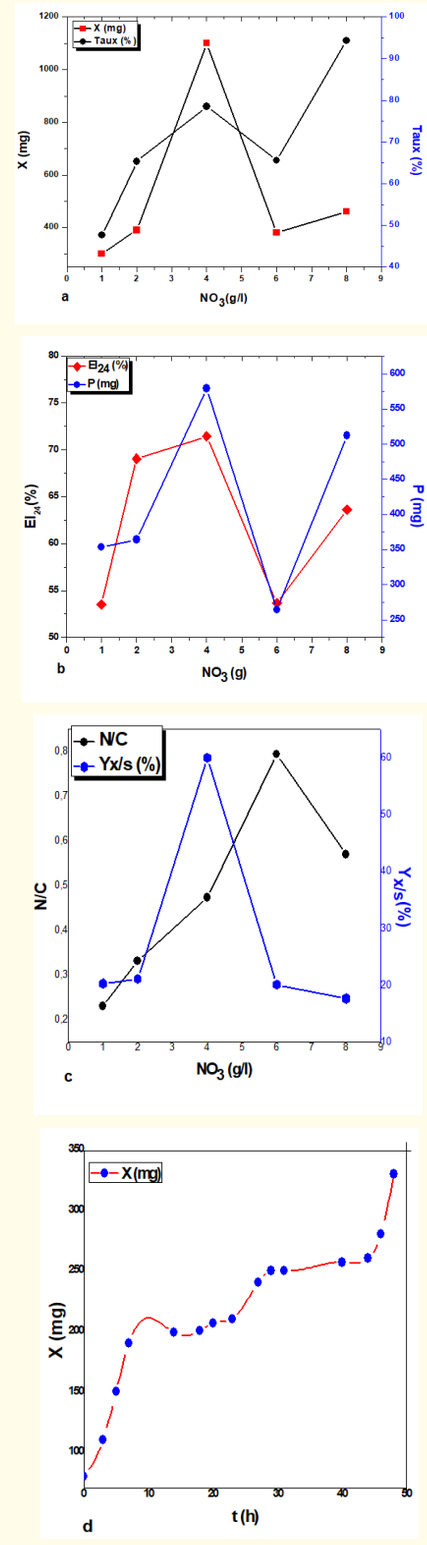

Figure 1: Maximum growth of Pseudomonas aeruginosa and biodegradation rate of hydrocarbons, (b) Emulsification index of the contaminated medium and the amount of the biosurfactant produced against the concentration of NO_3.

However, the change of the nitrogen source showed that the quality of nitrogen affects considerably the efficiency of the bioremediation process. The ammonium ion has an inhibitory effect on the growth of *Pseudomonas aeruginosa* (Table 1). This inhibition is at the origin of the blocking of the quorum sensing synthesis whose maturity is essential for the growth and the production of secondary metabolites in the fermentative medium. The results are depicted in table 1. El-Sheshtawy and Doheim [14] in their work, observed the same behavior of *Pseudomonas aeruginosa* in the presence of the ammonium ion.

Parameters								
	P (mg)	**θ %**	**$Y_{x/s}$ %**	**$Y_{p/s}$ %**	**EI_{24} %**	**N/C**	**X_0**	**X_{max}**
3 g/l	341,10	54,29	30,74	27,59	61,90	0,4362	0,08	0,42
4 g/l	590,00	57,36	18,37	45,17	65,22	0,7309	0,08	0,33

Table 1: Results of the evaluation of NH_4NO_3 on the performance of the bioremediation process (X_0 and X_{max} in mg).

In comparison of the results with the KNO_3, growth is three times less important as a first factor of the efficiency of bioremediation processes that characterizes a biosurfactant productivity yield of 2/3 compared with KNO_3 = 4 g/l. Despite emulsification EI_{24}= 65, 22%, the conversion rate does not exceed less than 60% of the total amount of hydrocarbons in the medium. The effect of the ammonium ion also affects the process of mobilization, solubilization and emulsification by affecting the quality of the biosurfactants in spite of a maximum amount produced P = 590 mg for NH_4NO_3 = 4 g/l, according to the conversion rate [16-27].

Conclusion

The synthesized biosurfactants have a great potential to solubilize the oils contained in the polluted soils, to reduce the surface tension and the interfacial tension of the immiscible liquids and to modify the diameter of the oil in the water. The objective was to produce biosurfactant microbially using hydrocarbons contained in sludge as a source of carbon and energy. The experiments carried out in this study showed that potassium nitrate is the best source of nitrogen for the *Pseudomonas aeruginosa* strain in the process of bioremediation of contaminated soils by hydrocarbons. Moreover, the results demonstrated that *Pseudomonas aeruginosa* is able to grow on several sources of carbon and energy, which characterize the complex medium formed by petroleum hydrocarbons polluting the soil. The optimization of the diauxienne growth capacity allows a more efficient bioremediation of soils polluted by hydrocarbons.

Indeed, the obtained results showed that ammonium nitrate increases the kinetic time of the pollutants removal process because of the inhibitive effect of the ammonium ion on the strain. Which not only reduces the possibility of enriching the soil with biosurfactant of a good quality of mobilization, solubilization and emulsification of the medium, but also increases the consumption of nitrogen for low conversion rates and decrease the speed of the removal of the pollutants.

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