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Abstract

The purpose of this study was to elucidate the capacity of a *Pseudomonas aeruginosa* strain to metabolize hydrocarbons sludge in the production of biosurfactants to fight against environmental threats. The performance of the treatment consisted in monitoring the inductive metabolism of the strain during 48 h at a temperature of 37°C. The results showed that a removal efficiency of 96.8% and an emulsification index of 71.8% were obtained corresponding to a phosphate buffer concentration of 30 mmol/L. The main role of the biosurfactants produced was to emulsify the medium and to absorb the oils contained in the hydrocarbons sludge. This allowed to stabilize hydrocarbons oils and favored the inductive metabolism of *P. aeruginosa*. Furthermore, Physico-chemical and Fourier transform infrared spectroscopy (FTIR) analysis showed that the produced biosurfactants were of rhamnolipid type. They showed promising surfactant properties, such as a strong reduction in the surface tension of water from 72 mN/m to 40.52 mN/m, a high reactivity in the culture medium at pH7, a high osmotolerance up to 150 g/L of salt, and a critical micellar concentration of 21 mg/L. Indeed, the observed inductive metabolism constitutes an opportunity of treatment of various hydrocarbons contained in crude oil and spilled in the ecosystem to prevent pollution and damage.

Keywords: *Hydrocarbons sludge; Pollution; Refinery; Recycling;* Enzymatic induction Growth; Biosurfactants.

Introduction

Hydrocarbons sludge is an effluent provided from the crude oil refining industry resulting from various processes along the crude oil processing lines. They constitute a serious threat to the environment and need to be treated to avoid harming human health and the sustainability of the ecosystems. (Linda et al., 2016).

The study conducted by Pan et al., 2015 evaluates several essential state-of-the-art waste-to-energy (WTE) technologies including combustion, gasification and anaerobic digestion. Since strategies under appropriate policies were essential for the implementation of the WTE supply chain, a critical review on the challenging barriers and overcome strategies was performed. Several successful case studies around the world were illustrated based on their respective energy, environmental and economic benefits of the WTE supply chain for the district energy supply (DES) system. Qomarudin et al., 2010, described the problem of oil waste management as a consideration of the environmental impact of the oils contained in the waste. The production of hydrocarbons sludge is massive since oil production has increased in all producing countries as a result of its involvement in their economies, (Bhattacharyya et al., 2003); for instance, it can reach up to 2000 tons/day in Indonesia (Tse , 2003). This raise in petroleum production has increased the threat to the environment and has reached the sub-soil, (Qomarudin et al., 2010). The amount of hydrocarbons sludge continues to increase and its severity on the environment becomes worrying due to the density, and the viscosity of the oils contained. Generally, higher refining capacity is associated with a higher amount of oil sludge production (Wang et al., 2012; Guangji et al., 2013). The oil contained in the sludge crosses the earth's layer and slowly reaches the subsoil, which poses a direct risk of contamination of the subsoil and shallow groundwater.

In addition, the light hydrocarbons evaporated in the sludge are mixed with atmospheric dust, which causes air pollution during the harmattan season. This evaporation phenomenon provokes serious respiratory problems and eventually human disease and environmental damage.

Irreversible ecological disasters can be realized during releases or voluntary dumping. They present a significant toxicological risk even at low concentrations, in particular by their mutagenic and carcinogenic properties (Cravo-Laureau et Duran 2016).

In order to preserve the environment and eliminate these pollutants, it is necessary to develop reliable and effective methods of pollution control. The diagnosis of the treatment techniques used has shown major imperfections that can cause harm to human health, the ecosystem, and the environment. In this aim, Marin et al., 2005, studied the bioremediation of a refinery sludge containing hydrocarbons using agricultural techniques, to assess its ability to reduce the total content of hydrocarbons added to the soil with the sludge in a semi-arid climate and also to evaluates its effect on the microbial activity of the soil concerned.

Despite the multiple uses of physicochemical processes in the restoration of soil polluted by petroleum products, bioremediation remains the most effective, best controlled and least costly solution. Its principle is based on the complete mineralization of petroleum products which do not generate any toxic by-product. (Azubuike et al., 2016; Rajesh et al., 2017).

The addition of surfactants is a promising approach to increase the biodegradation of hydrocarbons. Nevertheless, these molecules of chemical origin present a risk to the environment due to their toxicity and their non-biodegradability (Geetha et al., 2018; Banat et al., 2000). Recently, several authors were interested in the production of biosurfactants due to the advantages presented by these biomolecules compared to their chemical counterparts, such as biodegradability, low toxicity, efficacy under extreme conditions (pH, temperature and salinity) (Makkar et al., 2011). Furthermore, as result of their emulsification and solubilization capacity, they have many potential applications covering a wide variety of fields such as medicine, the petroleum industry and the bioremediation of sites contaminated by hydrocarbons (Santos et al. ., 2017). Biosurfactants produced from *Pseudomonas aeruginosa* are constituted by the rhamnose fraction and the fatty fraction resulting from the growth on the hydrocarbon substrate, they are specific to a given carbon substrate for the growth of the producing strain.

In their study, Montagnolli et al., 2019 compared the effects of biosurfactants on the biodegradation of biodiesel and vegetable oils by two conceptually divergent methodologies. They found that biosurfactants caused an increase in oil absorption, increasing biodegradation performance. Also, they found that the biodiesel substrate stood out with the highest biodegradation rates. These results indicated that a rapid metabolic shift from the parent compound initially favored biodiesels in the uptake of organic carbon for a set of specialized microbial inoculum. The data has been successfully combined with mathematical models and statistical tools to describe and predict the actual environmental behavior of biodiesel and vegetable oils. The models confirmed and predicted the effectiveness of biodegradation with biosurfactants and allowed to estimate the time needed to satisfactorily remove contaminants.

The enzymes involved in the conversion of hydrocarbons in *P. aeruginosa* are of two types, namely rhamnotransferase I and II with self-inductive capacity. However, in the literature, no author was interested in the efficiency of the inductive metabolism of *P. aeruginosa* in removing the oils contained in the residual sludge of the petroleum refining industries. The bacterial genus *Pseudomonas* is characterized by a multitude type of biosurfactants and synthesizing enzymes by induction in a medium in which phosphate and nitrogen are limiting. The enzymatic induction phenomenon of *P. aeruginosa* is at the origin of the synthesis of several rhamnolipids (Hanna et al., 2011; Ellen et al., 2014). Also, the metabolic process of *P. aeruginosa* constitutes a great opportunity for the discharge sites by the presence of biosurfactants which enrich the environment with biodegradable organic substances (Abhijit et al., 2014; Tanja et al.,2017).

The challenge of this study dealing with the treatment of hydrocarbons sludge is to highlight the importance of the inductive metabolism of *P. aeruginosa* in the elimination of hydrocarbons oils in sludge. For this purpose, the physicochemical behavior of the biosurfactants produced, allowing the extraction of the oils contained in the sludge by wetting, solubilization, and emulsification was examined. In addition, the conditions for the inductive growth of the strain leading to an efficient elimination and evacuation of sludge without risk of ecosystem degradation were investigated.

Materials and methods

The strain used in this work was *P. aeruginosa* ATCC 27853. It was kindly provided by the hospital of Medea (Algeria). The hydrocarbons sludge was furnished by the Niger-SORAZ industry. It was packaged in powder form after several treatments to destroy all traces of microorganisms. The culture medium was formulated according to the medium proposed by Milena et al., 2012. It was conditioned using an oven, and then wet heated in an autoclave. Biomass growth (OD at 600 nm) and hydrocarbon consumption by COD (transmittance at 420 nm) monitoring were performed using a spectrophotometric method.

Culture medium formulation

To ensure the microbiological quality of the hydrocarbons sludge, double sterilization was undertaken. The first step consisted of humidifying the hydrocarbons sludge to reactivate all the microorganisms contained. The hydrocarbons sludge were then placed in an oven for one

hour at 180°C before being cooled to 70°C. In addition, 1 g of this suspension was dissolved in 100 mL of distilled water. This solution was then tested on a solid agar medium for a period of 48 to 72 h at 37°C. The second step consisted of sterilization with moist heat at 121°C for 15 minutes followed by the same test to evaluate the sterility (Pacheco et al., 2010).

The culture medium formulated for the production of glycolipids biosurfactantwas constituted by the following mixture: NaCl (1g/L); KNO_3 (4g/L); $MgSO_4x3H_2O$ (0.2g/L); $CaCl_2x2H_2O$ (0.05 g/L); $FeCl_3x7H_2O$ (0.01 g/L); yeast extract (6 g/L); as well as KH_2PO_4 monobasic phosphate buffer (4 g/L). 1 mL/L of the following trace elements solution was added to the culture medium: $ZnSO_4$ (50 mg); $MnSO_4$ (23 mg); $FeSO_4x5H_2O$ (10 mg); $CuSO_4$(10 mg); $CaCl_2x6H_2O$ (36 mg); H_3BO_3 (12 mg); KI (15 mg) and EDTA (80 mg). (Milena et al., 2012).

The hydrocarbons sludge powder 3% (w/v) was added as a source of carbon and energy to the medium (Linda et al., 2016). The solution was dissolved and magnetically stirred for 5 min for homogenization. The medium was adjusted to a pH = 6.0 ± 0.01 by the addition of sodium hydroxide solution (1N) or hydrochloric acid (1N). It was then sterilized for 15 minutes at 121ºC in an autoclave. After sterilization, the medium was cooled with tap water and the pH was then adjusted to pH = 7.0 ± 0.01 before inoculation, (Patil et al., 2014).

Culture conditions

The culture was performed in batch mode at 37°C for 48 h with an agitation speed W = 250 rpm at pH = 7.00 ± 0.01. Germination was carried out in a sterilized solid selective medium (cetrimide agar) for 24 hours containing as carbon source a mixture of n-heptane and cetane to prevent a long latency phase. The microbial cells were then seeded for 48 h of culture with a regular sampling of 10 mL every 2 h on average for kinetic monitoring (Saimmai et al., 2012).

Kinetic Monitoring

The kinetic study focused on biomass growth through the measurement of the optical density at 600 nm, which was examined versus the evolution of nitrogen, the consumption of hydrocarbons sludge and the productivity of biosurfactants. Samples of 10 mL were collected and centrifuged at W = 6000 rpm for 10, 15 and 20 min with the removal of the residual solid mass ; this latter was diluted in 10 ml of water and measured at 600 nm. Then, 2 mL of cell-free medium was taken for the preparation of the emulsifying index. The emulsion samples were left for 24 h. The emulsifying index EI_{24}% was given by the ratio between the height of

the emulsified layer on that of the whole column (Kumara et al., 2006; Aparna et al., 2011). The rest of the cell-free medium was precipitated after 24, 48, 72 and 96 h, with absolute ethanol (1:3 v/v), then centrifuged and maintained for 24 h at 4°C (Prabakaran et al., 2015). Biosurfactants were recovered and dried in a vacuum desiccator. The precipitated medium was measured by the COD-spectroscopic method at 420 nm. Samples of 2 mL were then taken, diluted and digested in a digester. The monitoring of the biomass growth was performed by optical density (OD) at 600 nm using a UV/VIS spectrophotometer (Senthil et Jayalakshmi, 2013). The nitrogen concentration in the culture medium during the treatment was determined by the iron oxidation method in acidic medium, followed by a spectrophotometric determination of ferrous ion at 510 nm.

Effect of the phosphate buffer

The effect of buffer was performed owing to its significant impact on culture; it stimulates growth, the associated production of biosurfactants and stabilizes the pH of the medium (Graziela et al., 2010). Experiments were spread on an experimental scale according to the lower and upper limits of buffer concentration and productivity. It allows to find a maximum production of biosurfactants and to determine the stimulating effect of the buffer on the growth of the strain. This assessment was done according to three key areas, namely low stimulation, optimal stimulation and repressive stimulation (Bassirou et al 2019).

Characterization of the Biosurfactants

The detection of biosurfactants in the medium during the culture was based on the emulsification index and the method of Cooper and Goldenberg (1987). In addition, a test was carried out to demonstrate the rhamnose fraction of the biosurfactants produced based on the determination of hydrocarbon groups of the biosurfactants using the method of Dubois et al (Dubois et al., 1956). A volume V = 0.5 mL of cell-free medium was collected and mixed with 0.5 mL of phenol solution (5%) and 2.5 mL of sulfuric acid. The mixture was set for 15 minutes and then the absorbance was read at 490 nm.

FTIR Characterization of crude biosurfactants

The infrared method was used to analyze the crude biosurfactants and to identify its various groups, characteristic of biomolecules (Prabakaran et al., 2015). The characterization method consisted of assaying KBr pellets of 0.23 mm containing a small amount of crude biosurfactants. The analysis was then carried out in a wavelength range from 400 cm^{-1} to 4000 cm^{-1} with a resolution of 2 cm^{-1}. The unit was controlled by software (IR/FP Solutions) which regulated the humidity and the ambient air (correction of atmospheric carbon dioxide).

Physicochemical characterization of biosurfactants.

Surface tension and CMC

Equilibrium surface tension (γ) was measured at a temperature of 25°C with a Krüss K9 digital tensiometer (Krüss, Helsinki, Finland) by the ring method of the aqueous solution at different concentrations of crude biosurfactant (Habib et al., 2013). The instrument was calibrated against ultrapure water (γ = 72 mN/m) to ensure accuracy over the entire range of surface tension. The ring was rinsed with ethanol than with water to avoid any particles or dust which may contaminate the latter (Milena et al., 2012). For the calibration of the instrument, the surface tension of distilled water was measured before each series of experiments. All assays were performed in triplicate along with distilled water as a control.

Effect of the salinity

The effect of salinity on aqueous solutions of crude biosurfactant was studied by adding NaCl in the range of 25 to 250 g / L with a step of 25 g / L. After addition, the samples were placed in the dark for 20 minutes. The objective was to assess the reactivity of the biosurfactant facing extreme chemical conditions. Thus, the monitoring was carried out by measurements of the surface tension after the addition of NaCl (Rajesh et al., 2014).

Effect of the pH

The stability of the biosurfactant versus pH variations was examined using a solution of 0.6 g/L of crude biosurfactant. The pH of the samples was adjusted within a range of 2 to 12 with a step of 1 for biosurfactant concentrations in the range of 1 to 600 mg/L (Habib et al., 2013). The

surface tension of the solutions was measured after adjusting the pH and after 30 minutes of contact time. This allowed to determine the optimal pH linked to the reactivity of the biosurfactant tested and also to evaluate the activity of the bioproducts regarding pH variations of the medium (Govindammal et Parthasarathi, 2013).

Displacement and oil solubilization

The oil displacement test is a method of determining the surface activity of the biosurfactant by measuring clear zone diameter. The method of displacement of the oil by solubilization was used to measure the stretching area diameter of the immiscible oil. The solubilization and displacement were provoked by the addition of an aqueous solution of crude biosurfactant at the oil-water interface (Rath et al., 2016).

The following procedure was considered to study the oil displacement, namely the expansion of the diameter of the oil droplet on a surface of distilled water: a Petri dish was filled with 50 mL of distilled water, a volume of 20 µL of oil was deposited on the surface of water, and then 15 µL of a solution of the crude biosurfactant at a concentration of 1 g/L was filled on the oil surface (Hamid et al., 2012).

Moreover, to investigate the solubility of the oil in an aqueous solution of crude biosurfactant, a Petri dish was filled with 25mL of a 1 g/L biosurfactant solution ; then, 15µL of oil were removed and deposited on the surface of the aqueous solution of crude biosurfactant. In both cases, the value of the diameter of the oil displacement was expressed in cm.

Results and discussion

The treatment efficiency characterizes the bioconversion and the efficiency of the treatment of hydrocarbon sludge to avoid damage to the environment and the ecosystem. It responds to the objective assigned to treatment, and therefore to the development of decontamination technologies in environments polluted by hydrocarbons. This allows to have a completely biodegradable and stable product, ES5D = 96.17% for an emulsification index EI_{24} = 71.80%, useful for various industries.

The results obtained include the regeneration of all the genetic material, the production of primary metabolites (enzymes, structural proteins, intra, and extracellular signaling systems, nucleic acids ...), since *Pseudomonas aeruginosa* synthesizes a complex called quorum whose maturity is essential to the growth and the production of secondary metabolites in fermentative

medium. Facing this consideration, the hydrocarbon and nitrogen substrates abruptly decreased between 0 and 3 h, leading to a treatment yield of 36.5% after 3 h. Also, a part of the substrate was used for the production of biosurfactants stimulated by the presence of hydrocarbon substrates.

The characteristic of the biosurfactants produced should highlight the importance of its biomolecules in the treatment of hydrocarbons sludge by their actions of wetting, solubilization, emulsification and extraction of the contained oils. The presence of biosurfactants in the medium promotes the contact between the microbial cells of *Pseudomonas aeruginosa* and the source of carbon and energy, namely the hydrocarbons contained in the sludge, to achieve optimal growth.

Determination of the phosphate buffer concentration

The determination of the phosphate buffer content, both as a growth stimulant and for pH stabilization was envisaged. The results regarding the evaluation of the importance of the phosphate buffer are summarized in Table 1, showing an optimum for 30 mmol/L.

Table 1. Comparative results of phosphate buffer values. Threshold and optimal concentrations (pH = 7, T = 37°C, W = 250 rpm).

In light of the results obtained and based on the tests carried out, the lowest critical value of productivity was 10mmol/L. It is important to note that the initial conductivity measurements are subject to the effect of the measured temperature and the volume of sodium hydroxide or hydrochloric acid used for pH adjustment after medium sterilization and cooling, (Cha et al., 2008; Priya et Usharani 2009). Nutrients assimilation allowed to decrease the conductivity of the medium to an extreme level, having a significant impact on the beginning of the growth and biosurfactant production. In addition, when the buffer concentration is low in the medium, the conductivity of nitrate ions dominates the overall conductivity, owing to the high conductivity of this anion.

Biosurfactants productivity showed a steady evolution until 40 hours, while there was an increase of almost 10% at 48 h (EI_{24} = 57.2%) (Fig. 1); this result is in agreement with previous reports (Senthil et Jayalakshmi, 2013). This account for the inefficiency of the buffer, since the conductivity at the beginning of biosurfactants production (G = 11.20 mS/cm after 3 h of

culture) had not reached the threshold value of productivity observed with 30 mmol/L after 48 h, G = 15.10 mS/cm. This conductivity value was however observed after 48 h of culture, (Ould boudia, 2013). The increased production of biosurfactants obtained at 40 h can be related to a deficiency in nutrients and especially hydrocarbons substrate. Indeed, such nutrients limitation induced an increase in biosurfactants production to solubilize the remaining hydrocarbons substrate (20.12 mg/L for 10 mmol/L of phosphate buffer).

It can also be noted that the consumption of nitrogen (nitrate) with respect to hydrocarbons was 0.20 g/g of carbon source. Nitrates consumption for 10 mmol/L buffer was nearly identical to the value observed for 30 mmol/L. The stimulating effect of the buffer was therefore observable in these conditions since this latter did not induce a significant difference regarding the lowering of the conductivity of the medium allowing maximal growth of the strain. Therefore, as shown through the emulsification index (Table 1), an increase in the production of biosurfactants was observed from 40 h of culture due to nutrients starvation (Wei et al., 2005; Whang et al., 2008). Productivity was significantly lower both in terms of biomass and product level, confirming the importance of the buffer for the growth of the strain.

Fig. 1. Evolution of the emulsification index versus time (pH = 7, T = 37°C, W = 250 rpm, KH_2PO_4 = 10 mmol/L, $p \leq 0.05$).

Whereas, the value of 50 mmol/L buffer showed to be critical in terms of repression of the strain by the salinity of the medium (Ilori et al., 2005), the emulsification index at this level showed a maximum at 5 h before declining abruptly up to 48 h. Medium conductivity did not decrease to a threshold limit allowing growth and biosurfactant production, owing to the lack of salt tolerance of the used strain; conductivity remained almost constant throughout culture (Table 1). From this, it can be assumed that nitrate respiration most likely occurred throughout the culture.

As shown in Fig 2a and b, the best emulsification was obtained between 3 h and 7 h. Indeed, to produce biosurfactants without variations of the other medium components, the buffer concentration must satisfy the optimal physiological needs of the strain to allow an adequate response to the stimulus. Therefore, the concentration of 50 mmol/L exceeded the optimal needs of *Pseudomonas aeruginosa* ATCC 27853 in the considered medium and hence caused a repressive stimulation of the strain (Gorna et al., 2011; Al-Saleh et Akbar 2015).

In agreement with the work of Anna et Partchasarathi (2014), owing to the excess of salinity due to the phosphate buffer, cell lysis was observed since the considered *Pseudomonas* strain

was not osmotolerant. As shown in Fig 2a and b, this effect can be compared to that of NaCl (Rikalović et al., 2012; Anna et Partchasarathi, 2014).

The N/C ratio showed that 0.67 g of nitrate was needed to consume 1 g of carbon, namely three times the normal respiration observed for the value of 30 mmol/L. This shows that the environment was not optimal for high biosurfactants production, since the components of the environment were not well balanced (Dos-Santos et al., 2010; Senthil et Jayalakshmi 2013). In addition, an approximate lethality and inhibition of the emulsification index were estimated to be 27.3% and 19.9%, respectively. Lethality and inhibition were deduced from the ratios of the emulsification index values at 24 and 48 h for the former and 7 and 22 h for the latter (Bassirou et al., 2019), according to the following equations:

$$Lethality = \frac{EI_{24\ from\ 24h\ to\ 48h}}{EI_{24\max obtained}}\ x100\% - 100\% \quad (1)$$

$$Inhibition = \frac{EI_{24\ from\ 7\ h\ to\ 48h}}{EI_{24\max obtained}}\ x100\% - 100\% \quad (2)$$

Fig. 2. Biomass (a) and Emulsification index versus time (b) (pH = 7, T = 37°C, W=250 rpm, KH_2PO_4 = 50 mmol/L, $p \leq 0.05$).

Culture conditions

Fig 3 a, b, c and d shows growth in two-steps, illustrating enzymatic induction growth namely diauxic phenomena. Regarding the results represented in Fig. 3, the first growth phase, until 20 h, corresponded to the consumption of the most readily treatment hydrocarbons contained in the sludge and then the less biodegradable hydrocarbons contained was assimilated until 48 h of culture (Bassirou et al., 2017).

The treatment efficiency can be estimated to be θ = 96.8% with a final hydrocarbons source concentration C = 250.1 mg/L and a nitrogen to carbon ratio N/C = 20.85%; consequently, the biomass on substrate yield was $Y_{X/S}$ = 52.7% and the product on substrate yield was $Y_{P/S}$ = 20.3% over 48 h of culture. The quantitative productivity showed two maxima (Fig 3d), P_1 =

64.1 mg at 7 h and P_2 = 75.6 mg at 28 h corresponding to each exponential phase of growth (Fig. 3a).

Based on the related literature, the comparison of the emulsification index obtained in 30 mmol/L buffer and the concentration of biosurfactants produced (C = 1560 mg/L) suggests that the diauxic growth in the present work occurred on a substrate approaching a lighter substrate as kerosene for the first phase and diesel for the second phase (Wei et Chu , 2002). Furthermore, the results of Fig. 3 demonstrated the presence of two hydrocarbons sources in the medium and growth by enzymatic induction or diauxic growth, which characterizes the metabolic pathway of the strain. The two peaks reflected the presence of two rhamnolipids, because of the specificity of biosurfactants to the carbon substrate and the productivity associated with growth.

Fig. 3. Evolution of Growth (a); hydrocarbons substrate consumption (b); nitrogen uptake (c) and biosurfactants product (d) versus time (pH=7, T=37°C, W=250 rpm, KH_2PO_4 =30 mmol/L, $p \leq 0.05$).

Following the Cooper and Goldenberg method, the emulsification kinetics showed the existence of two peaks, corroborating the growth of the strain and the evolution of substrates assimilation. As shown in Fig. 4, the values of the emulsification indexes EI_{24} found are of 46.70% at 7 h (1) and 71.80% at 46 h (2). These two emulsification peaks correspond perfectly to inductive or diauxic growth

Fig.4. Emulsification index versus time (pH=7, T=37°C, W=250 rpm, KH_2PO_4 =30 mmol/L, $p \leq 0.05$).

The stability of the emulsion over five days allowed to test the biosurfactants according to the time and the environmental factors. According to Figure 5, the emulsions remained stable after five days. They showed ES5D stability of 81.63% for the first growth phase with an EI_{24} of 46.70% and ES5D stability of 96.17% for the second growth phase with an EI_{24} of 71.80%. This stability of the formulated foaming is important for the treatment on-site of the sludge discharges without risk of degradation of the ecosystem due to the capacity of the biosurfactants to extract the oils contained in the sludge (Yan et al., 2012). The treatment yield was intrinsically dependent on the stability of the formulated foaming.

Fig. 5. Stability of the emulsions against the time after five days

(pH=7, T=37°C, W=250 rpm, KH_2PO_4 = 30 mmol/L, $p \leq 0.05$).

Characterization of the biosurfactants

Biosurfactants were characterized by highlighting their presence in the cell-free medium. For this purpose, emulsions were prepared under Cooper and Goldenberg conditions. Consistency and stability can be related to the emulsification index after 24 h. Indeed, no disintegration of the emulsion was observed during the five days of monitoring at ambient temperature. The quality of the foam proves the importance of the molecular weight of the biosurfactants produced. The formation of flocs was very fast because of their high molecular weight. The rhamnose test, at 490 nm absorbance, was positive, showing their glycolipids structure and rhamnolipid type.

Infrared Characterization of the crude biosurfactants

The obtained spectrum showed two regions with two peaks of maximum absorbance characteristics of the functional groups. The first of high intensity included two amplitudes. The first at 3420 cm^{-1} (OH^- band alcohol, typical of polysaccharides) and the second at 3277.17 cm^{-1} (OH^- band characteristic of carboxylic acid). This band constitutes a stretching vibration of the O-H groups, carboxylic acid dimmers, with large absorption of the band O-H between 3300 and 2500 cm^{-1}; while the second region had a peak of high intensity of narrowband which absorbed at 1105 cm^{-1}. The characteristic band of carboxylic acids was easily distinguishable from that of alcohol by its lower and extended absorbance, covering the C-H bands, in contrast to that of alcohol between 3420 cm^{-1} and 1105 cm^{-1}. Besides these bands, the spectrum showed two bands of 2862.46 cm^{-1} and 2926.11 cm^{-1} (C-H bands characteristic of a CH_2-CH_3 hydrocarbon chain).

Other bands can also be observed, at 1647.26 cm^{-1} (characteristics of C=O ester groups) and at 1454.38 cm^{-1} (characteristics of C–H, asymmetric of CH_3) (Amani et al., 2010; Pereira et al., 2013). Thus, the extended C-O band, which absorbs from 1105.25 to 1384.94 cm^{-1}, characterized the presence of a bond between the carbon atoms and the hydroxyl groups of the chemical structure of rhamnose. According to Govindammal et Partchasarathi (2013); Anna et Partchasarathi (2014), the maximum absorption observed at 3466.24 cm^{-1} and 3424.60 cm^{-1} can be attributed to OH^- groups and those at 881.50 cm^{-1} and 698.25 cm^{-1} are characteristic of CH_2

groups ; all characteristics of the rhamnose cycle, showing that the obtained infrared spectrum was consistent with the glycolipids structure and the rhamnolipid type of the obtained biosurfactants (Fig. 6).

Fig. 6. Spectral IR characterization of biosurfactants produced by *Pseudomonas aeruginosa* ATTC 27853.

Glycolipids are composed of carbohydrates and long-chain aliphatic acids or hydroxyl aliphatic acids. An example of the most widely studied glycolipids is rhamnolipids produced by *P. aeruginosa* strains. Although there are several types of rhamnolipid species, all of them possess similar chemical structures. In general, the two major types of rhamnolipid species are l-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or mono-rhamnolipid (Rha–C_{10}–C_{10}) and l-rhamnosyl l-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or di-rhamnolipid (Rha–Rha–C_{10}- C_{10}) (Prabakaran et al., 2015).

In comparison to the results obtained by Prabakaran et al., (2015) by FTIR analysis of biosurfactants products and Habib et al. (2013) related to the physicochemical characterization of mono-rhamnolipid, the biosurfactants product in the present study according to the FTIR result analysis are di and mono-rhamnolipids. According to the enzymatic induction phenomenon (diauxic growth) displayed in the fig. 3, the difference in the composition of rhamnolipid species might be due to the age of the culture, the strain of *P. aeruginosa*, substrate composition and the specific culture conditions (Habib et al., 2013). Although the bacterium produced predominantly a di-rhamnolipid, it also produced a mono-rhamnolipid in minor proportion.

Physicochemical characterization of the biosurfactants

Surface tension and CMC

The CMC is an index used to evaluate the surface-active activity that is the minimum biosurfactants concentration needed to achieve the lowest values of surface tension, from which the formation of micellar aggregates begins (Juliana et Crispin, 2013). Fig.7 exhibits the variation of the surface tension according to the concentration of biosurfactants in aqueous solutions. Indeed, the crude biosurfactants showed a lowering capacity of the surface tension of water from γ = 71.80 mN/m at 25°C to γ = 40.52 mN/m with a crude biosurfactants

concentration C = 600 mg/L. The critical micellar concentration of the biosurfactant was CMC = (21.0 ± 0.1) mg/L for a series of samples between C = [0 – 150] mg/L as shown in Fig 7. It is significantly above sodium dodecyl sulfate (SDS), a synthetic surfactant commonly used in many industrial sectors, which has a CMC of 21.0 mg/L in the same conditions test (Juliana et Crispin, 2013).

Fig. 7. Evolution of the surface tension versus the biosurfactants concentrationconcentration (pH = 7, T = 37°C, W=250 rpm, KH_2PO_4 = 30 mmol/L, $p \leq 0.05$).

Effect of the salinity

The results of the stability effect related to the action of NaCl on the biosurfactants showed a strong reactivity in the medium below the CMC in terms of lowering the surface tension, even exceeding the behavior observed in the absence of salt. It was found that the addition of NaCl from 25 to 100 g/L, for biosurfactant concentrations ranging from 0 to 20 mg/L increased the efficiency of the biosurfactants to reduce the surface tension of the medium owing to the competition induced between the electrolytes.

However, beyond the CMC a slight increase in the surface tension was observed despite a very good reactivity of the crude biosurfactants. This increase should be most likely related not only to a competition effect, but even more to medium saturation by the electrolytes.The saturation concentration of NaCl was C =150 g/L for a concentration C = 37mg/L of biosurfactants. The maximum reduction of the surface tension was γ = 32.36 mN/m. Several authors described similar behavior for biosurfactants produced by other microorganisms, *P. putida*, *Bacillus subtilis*, *P. aeruginosa*, *B. cereus* and *Rhodococcus erythropolis* (Morikawa et al., 2000; Xia et al., 2011).

Effect of the pH

The stability's tests related to the pH indicated the ability of biosurfactants to react facing an increase of the ionic strength above and below the CMC, in terms of lowering the surface tension after pH adjustment. The results showed that pH = 7 was optimal for biosurfactant reactivity allowing a sufficient lowering of the surface tension (Fig.8).

The pH is an important factor, it can limit or enhance the effect of biosurfactants in the medium. The effect of the pH is characterized by a modification of the surface charges of the

biosurfactants contained in the crude extract depending on the critical micellar concentration. It is at the origin of the solubilizing, wetting, foaming and emulsifying capacity of biosurfactants during culture. In an acidic medium, the surface tension decreased gradually until a value C = 18.75 mg/L . The electrical competitiveness of the biosurfactants resulted from negative charges which allow the stability of molecules, particularly di-rhamnolipids, in agreement with the related literature (Amani et al., 2010; Xia et al., 2011)

From pH 4, the acidity of the medium caused a considerable decrease in surface tension by modifying the specific CMC due to mono-rhamnolipids. The CMC previously determined depended on the variation of the pH of the medium and decreased with acidic pH. In an alkaline medium, the decrease in surface tension was due to the presence of highly reactive mono-rhamnolipids in this pH range. This reactivity can be related to their negative repulsive charges which stabilize the emulsification of the medium in terms of the heterogeneous nature of the fatty acids of the mono-rhamnolipids produced by *P. aeruginosa* (Özdemir et al., 2004 : Zhong et al., 2008).

However, for pH values greater than 7, most of the mono-rhamnolipid molecules are negatively charged, while they are neutral at pH 4. Thus, the determined CMC was characteristic of a mixture of mono and di-rhamnolipids produced simultaneously by the strain depending on the nature of the carbon source (Habib et al., 2013). For low ionic strengths (NaCl= 0-50 mM), the neutral mono-rhamnolipid had a CMC value less than the ionized form. This difference could be explained by the presence of electrostatic repulsions between the neutral and negatively charged mono-rhamnolipid molecules, which made their association in the micellar aggregates more difficult, compared to the neutral molecules present at pH 4.0.

Similar results were found using *P. aeruginosa*, *B. subtilis*, *R. erthropolis*, *P. putida* and *B. cereus* (Morikawa et al., 2000; Chen et al., 2010; Ikhwani et al., 2017).

Fig. 8. Evolution of the surface tension of the crude biosurfactant versus pH **(a)** and NaCl amount **(b)**
(T = 37°C, W=250 rpm, KH_2PO_4 = 30 mmol/L, $p \leq 0.05$).

Displacement and solubilization of oil

The purpose of the displacement and the solubilization of oil is to highlight the ability of the biosurfactants to solubilize and expand the oil droplet deposited on a hydrophilic surface. A high capacity of the biosurfactants to solubilize and to move the oil after the contact of 20 µL

of a1 g/L biosurfactants solution with 15 µL of oil deposited on 50 mL of distilled water was shown. This confirmed the high capacity of the biosurfactants in the solubilization of immiscible liquids, by lowering the interfacial tension between oil and water and by an enlargement of the oil diameter from 0 to 2.5 cm. This displacement occurs due to the decrease of the oil viscosity.

Hamid et al. (2012) and Rath et al. (2016) described qualitatively the activity of the biosurfactants by crossing the barrier of the oil droplet deposited on the surface of the water by the solution of biosurfactant which gradually expands the oil diameter. It is in agreement with the observed action of the biosurfactants produced by *Pseudomonas aeruginosa* ATCC 27853 applied to the oil droplet deposited on the surface of the water.

Furthermore, the displacement of the oil on 25 mL of biosurfactants at a concentration of 1 g/L reached up to 5 - 8 cm ; it is in agreement with the related literature (Rath et al., 2016). Indeed, the oil was dissolved in the solution of biosurfactants by expanding its diameter. This result highlighted the solubilizing capacity of the produced biosurfactants and can be the subject of a more widespread application for the environmental safeguarding and protecting against the pollution of hydrocarbons sludge of refinery.

Conclusion

The results obtained showed the performance and the efficiency of the treatment of hydrocarbons sludge of *Pseudomonas aeruginosa* strain using the combined effect of biosurfactants and inductive metabolism. This observed phenomenon showed the ability of *Pseudomonas aeruginosa* to grow on complex hydrocarbons. The presence of biosurfactants had a positive effect on the mobility of hydrophobic components of soil, facilitating the leaching of hydrocarbons contained in the sludge. The mechanism of wetting, solubilization, emulsification and extraction by biosurfactants of *Pseudomonas aeruginosa* is an useful way to remove hydrocarbons oils. Ultimately, the application of the crude biosurfactants in the solubilization and the displacement of oil confirmed the promising properties of the rhamnolipids produced and hence, to prevent pollution and damage, it constitutes an opportunity of treatment of various hydrocarbons contained in crude oil and spilled into the ecosystem.

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Conflict of interest

The authors declare that there is no conflict of interests.

Author Disclosure Statement

No competing financial interests exist.

Abbreviation

C: concentration (mg/L) ;
CMC: critical micellar concentration (mg/L);
COD: chemical oxygen demand
DES: district energy supply;
EI_{24}: emulsification index (%);
FTIR: Fourier Transformer Infrared;
G: conductivity (mS/cm);
OD: optical density;
Rp: speed of the biosurfactants production (mg/h.L);
V: volume (mL);
W: speed of rotation (rpm);
WTE: waste-to-energy;
X: normalized biomass (g);
$Y_{X/S}$: yield of biomass/substrate (%);
$Y_{P/S}$: yield of product/substrate (%)
γ: surface tension (mN/m);

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