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Biodegradation of Petroleum hydrocarbons and Biosurfactant production by an extremely halophilic Archaea Halovivax sp. A21

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ABSTRACT/RESUME

Abstract: *Over the recent few years, biosurfactant has played an important role in the industrial application especially in oil recovery; even in high salinity conditions. The potential of biosurfactant production by the extreme halophilic archaeon Halovivax sp. A21 in the presence of petroleum hydrocarbons (2% v/v) as sole carbon source at high salinity (25% NaCl) has been investigated. The results show the ability of Halovivax sp. A21 to grow and reduce surface tension under an optimum range of pH (7-9), salinities (15-35% NaCl) and temperature (40-45°C) for an optimized volume of 100 ml of the medium for 1000 ml capacity Erlenmeyer flasks with an optimum agitation of 120 rpm. The rates of biosurfactant production on petroleum hydrocarbons were enhanced with increasing NaCl concentration in the medium with an optimum of 25%. Biosurfactant production by Halovivax sp. A21 showed high emulsifying activity (more than 80%) and decreased surface tension (24.5 mN/m). The stability of the produced biosurfactant was determined by different physico-chemical conditions like pH, temperature and salinity. Moreover, the partial purification of the derived biosurfactant by silica gel column chromatography and Thin-layer chromatography revealed that it belongs to the lipopeptide group. Although both catechol dioxygenases participated in the degradation of petroleum hydrocarbons, more induction of catechol 1,2 dioxygenase was observed than the catechol 2,3 dioxygenase which indicated the predominance of the ortho cleavage pathways in the petroleum hydrocarbons degradation by the halophilic strain Halovivax sp. A21. The results demonstrated that strain Halovivax sp. A21 was able to increase the bioavailability of insoluble hydrocarbons, thus facilitating their uptake and their biodegradation even at high salt concentration. Likewise, the search of novel biosurfactants in extremophiles, or the use of microorganisms that present excellent*

degradation capacity together with the production of stable biosurfactant from contaminant (hydrocarbon compounds) as a carbon source seem to be particularly promising since they have particular adaptations to increase stability in adverse extreme environments.

I. Introduction

Petroleum is a complex mixture of different hydrocarbons (aliphatic and aromatic) which are certainly the most widespread on Earth because of their economic and energy interests [1,2]. Thus, hypersaline environments including natural saline lakes, salt flats, saline industrial effluents, oil fields, coastal areas, and salt marshes are often contaminated with high levels of petroleum hydrocarbons [3]. Nevertheless, contamination of these habitats constitutes a serious environmental problem mainly due to the high toxicity exhibited by aromatic hydrocarbons [4]. Bioremediation, yet, is the most effective and attractive cleaning biodegradation biotechnology to decrease the level of pollution and to recover contaminated environments [5]. The main benefit of using bioremediation is that microorganisms can destroy hazardous contaminants or turn them into less harmful forms [6]. However, in high saline environments, application of bioremediation is very challenging due to the detrimental effect of salt on microbial life; hence the necessity to use halophilic species capable of effectively degrading petroleum compounds under high salt conditions. During the last two decades, rather little but interesting and important studies have been reported about hydrocarbonoclastics microorganisms indigenous to hypersaline environments that utilize aliphatic and aromatic hydrocarbons at high salinity. Such microorganisms comprise *Bacteria*, Actinomycetes and *Archaea* [2, 7-25]. However, it has been showed that in high salinity conditions, increased biodegradation was mainly due to archaeal members, with little or no contribution from *Bacteria* [19]. Among them, some strains of the genera *Heloferax*, *Haloarcula*, *Halobacterium* and *Natrialba* seem to play an important role in the degradation of hydrocarbons, especially in extremely high salinity conditions. Although the potential use of haloarchaea in bioremediation has been extensively demonstrated, different aspects of their metabolism remain poorly known [6]. Biochemical processes of oil degradation carried out by microbes include involvement of several types of enzymatic reactions driven by (i) monooxygenases which initiate degradation of aliphatic hydrocarbons by the addition of oxygen atom(s) to the terminal or subterminal carbon, and converting them to corresponding fatty acids which are then assimilated *via* beta-oxidation, or (ii) dioxygenases by the addition of oxygen atom(s) to the alkyl moiety or aromatic ring, converting them to a few central intermediates such as catechols, protocatechuate, and gentisate through convergent pathways [26, 27, 28]. Nevertheless, few reports exist in the literature on the degradation pathways and enzymes involved in the aerobic metabolism of petroleum compounds for halophilic aerobic *Bacteria*, *Archaea*, and eukaryotes related to the non-halophilic ones [3]. On the other hand, the major limitation of microbial degradation of crude oil is its hydrophobicity [29]. Indeed, hydrocarbon-degrading microorganisms with the ability to produce biosurfactants are widely recommended for fast degradation of crude oil. These tensioactive molecules induce a better solubilization of hydrocarbons and so a better bioavailability for the oil-degraders. However, there are very few reports on biosurfactant producers in hypersaline environments [30]. The search for biosurfactants in extremophiles seems to be particularly promising because they have particular adaptations to increase stability in adverse environments [30, 31]. In this study, we have investigated the production of biosurfactant by the extremely halophilic *Archaea Halovivax sp.* A21 on petroleum hydrocarbons as sole source of carbon and energy at high salinity. Biosurfactants production was indicated by surface tension reduction and emulsification activity of cell-free supernatant. Besides, an induction of catabolic enzymes (catechol dioxygenases) was also investigated for their involvement in petroleum hydrocarbons degradation.

II. Materiel and methods

II.1.Isolation and growth conditions

The strain Halovivax sp. A21 was isolated from oil contaminated saline water collected at 1-m intervals in the ponds located close to Ain Salah, Algeria [32]. This strain was cultured in modified Mineral Salts

Basal Medium (MSB) supplemented with petroleum hydrocarbons as the sole source of carbon and energy. The modified MSB contained (in grams/liter): NaCl, 250; MgCl2, 0.5; KH2PO4, 0.45; K2HPO4, 0.9; NH4Cl, 0.3; KCl, 0.3; yeast extract, 0.01. The cultures were incubated at 40°C with shaking at 200 rpm for 22 days. The petroleum hydrocarbons were at 2 % (v/v) concentration level at the same culture parameters mentioned above. The cell growth was monitored by measuring OD600 using spectrophotometer (Perkin-Elmer, USA) [33, 34]. A solution without inoculated media was used as a control. In all cases, cultures were carried out in triplicates.

II.2.Biosurfactant activity assays

II.2.1. Emulsification activity assay (Emulsification index)

Emulsification activity was measured by a modification of the method of Cooper and Goldenberg [35]. Briefly, 2 ml of Kerosene were added to 2 ml of culture supernatant (after centrifugation at 6000 rpm for 15 min), and vortexed at high speed for 5 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification index is defined as the height of the emulsion layer divided by the total height and expressed as percentage [36].

II.2.2. Surface tension measurement

Surface tension of cell-free medium was measured at different time intervals using a KRUSS F6 tensiometer at room temperature following the Wilhelmy plate measurement technique [37].

II.2.3. Properties of emulsions

After growth on petroleum hydrocarbons, the supernatant obtained after centrifugation at 6000 rpm for 15 minutes was considered as the source of crude biosurfactant [25]. Its stability was evaluated over a range of pH (adjusted between 3 and 12 with NaOH), temperature (4, 30, 40, 50, 60,70, 80 and 100 °C for 30 min then cooled to room temperature) and NaCl concentrations (with 10, 15, 20, 25, 30 and 35% (w/v) sodium chloride in the aqueous phase). The stability of the formed emulsions (ES, %) was measured in intervals up to 48h.

II.3. Influence of environmental parameters on biosurfactant production

Effect of volume of the production medium: The medium volume with reference to the volume of the culture flasks plays a major role in the production of biosurfactant. Thereby, the strain was grown in 1000 ml of Erlenmeyer flask containing different volumes 50, 100, 150, 200, 250 and 300 ml of the medium. Followed by inoculation and incubation at 40°C for 48-72 h, the cell free medium was subjected to the measurement of emulsification activity according to the summarized procedure.

Effect of agitation: Followed by the optimization of volume, experiments were repeated again with the additional changes in the agitation at 0, 50, 100, 180 250 and 300 rpm individually, and the biosurfactant activity was measured as per the summarized procedures.

Effect of pH: In order to optimize the effective pH, the strain was grown at varied pH's 4, 5, 6, 7, 8, 9, 10 and 11. The growth and biosurfactant activity were measured at respective pH's.

Effect of temperature: To have optimum temperature for maximum production of biosurfactant, cultures were grown at different temperatures 25, 30, 35, 40, 45, 50, 55, 60°C. Meanwhile, the growth and biosurfactants activity had been measured.

Effect of salinity: In order to optimize the effective concentration of NaCl in the medium, the strain was grown at 0, 10, 15, 20, 25, 30 and 35% NaCl. The growth and biosurfactants activity had been measured accordingly.

Effect of incubation period: In order to assess the required incubation period, the halophilic strain was grown in the optimized media for the period of 48, 72, and 96 h. Therefore, the growth and biosurfactants activity were measured.

II.4.Biochemical characterization of biosurfactant (BS)

Biosurfactant samples were initially subjected to Column chromatography and thin layer chromatography using various solvent combinations and spray reagents to identify their chemical nature.

The biosurfactant purification method was used according to Kebbouche-Gana et al. [32]. Briefly, the partially purified crud biosurfactant was dissolved in 1 ml of chloroform and subjected to column chromatography. For the purification of the produced biosurfactant, the glass column (Length – 20cm; internal diameter – 2cm) was dry packed with silica gel 75 and eluted with chloroform: methanol (2:1). After settling and equilibration, the extract dissolved in 1 ml chloroform, was gradually poured onto the column. The compounds were eluted at a flow rate of 2 ml/min. Each fraction was further inspected for surface activity to identify the fraction containing active compounds. The purity of biosurfactant was checked by thin layer

chromatography on silica gel plate as described by Kebbouche-Gana et al. [32] and the fraction showing maximum activity was lyophilized for further studies.

II.5. Protein analysis and Enzyme essays

Cells were grown in Minimal Salt Basal medium (MSB) in the presence of hydrocarbon compounds at 25% NaCl. After centrifugation at 6,000xg for 5 min, the cells were washed twice using deionized water and centrifuged after each wash under the same conditions. The cells were re-suspended in breaking buffer (50 mM Tris–HCl [pH 7.5], 5 mM ammonium sulfate, 1 M glycerol, 1 mM EDTA, 2.5 mM MgCl2, 1 mM DTT) and sonicated; the cell suspensions were centrifuged at 11,000×g for 3 min at 4°C. The supernatant obtained was used for the enzyme activity with the spectrophotometer, using specific wavelengths to each enzyme assay [43]. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity assays were performed spectrophotometrically by measuring the rate of increase in absorbance at A260nm or A375 nm due to the formation of cis, cis-muconate or 2-hydroxymuconic semi-aldehyde, respectively. Concentration of absorbing material in the sample was calculated by using the Beer-Lambert law. The coefficient was obtained by preparing a standard curve and was found to be 16,900 liters moles-1 cm-1 (for catechol-1,2-oxygenase) or 14,700 liters moles-1 cm-1 (for catechol-2,3-oxygenase). The specific enzyme activities were reported as micromoles product per minute per milligram protein. All experiments were performed with triplications.

III. Results and discussion

III.1 Growth on Petroleum Hydrocarbons and Biosurfactants Production And Characterization

According to Kebbouche-Gana et al. [32], the extremely halophilic strain Halovivax sp. A21 is gram negative, motile, catalase and oxidase positive. The partial gene sequence obtained from this halophilic strain was 400 nucleotides in length (EMBL, AM982815). It has been already shown that this strain produces stable biosurfactant and exhibited a high surface activity with a low surface tension of 28.40 mN m-1 on starch (2% w/v) as the sole carbon source at high salinity (15-30% of NaCl). The results showed that the production of biosurfactants were salt dependent. The produced biosurfactant was a glycolipopeptide.

However, as the qualitative and quantitative effect of the carbon substrate on biosurfactant production is correlated [39], we investigated in this study the production and the characterization of biosurfactant produced by the halophilic strain Halovivax sp. A21 when growing at high salinity (25% NaCl) on mineral medium with petroleum hydrocarbons as sole carbon source.

With regard to its growth, as shown in figure 1, the strain Halovivax sp. A21 seems to be able to grow rapidly and degrade petroleum hydrocarbons (2 % v/v) in a mineral medium at high salinity (25% NaCl) after 20 days at 40°C, pH 7.0. Petroleum hydrocarbons are virtually insoluble in the hypersaline growth medium. Therefore, to have access to these hydrophobic compounds, strain Halovivax sp. A21 must either secrete key enzymes to cleave them and/or produce biosurfactant to emulsify the hydrocarbons.

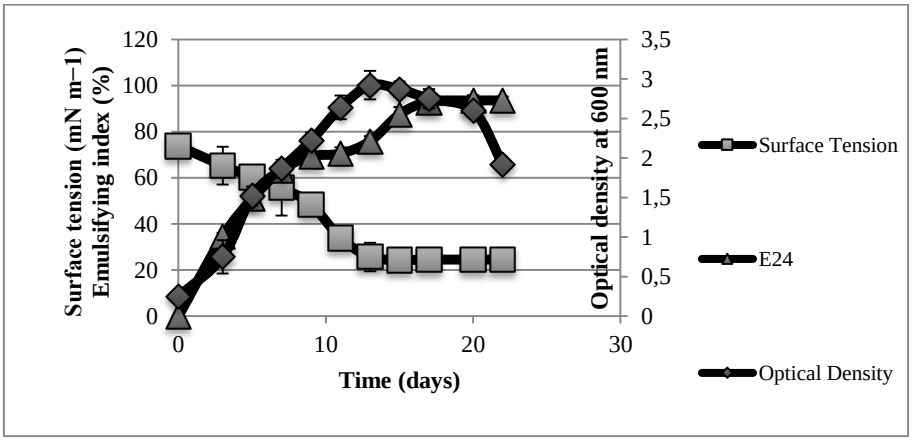

Figure 1. *Growth curve (OD), biosurfactant activity (E24) and surface tension of Halovivax sp. A21 cultured in mineral media containing petroleum hydrocarbons (2% v/v) as sole carbon source, at 48h time interval. Errors bars are ± the standard deviation of the mean.*

The potential ability to produce biosurfactant has been assessed using different assays aimed to determine the surface tension reduction and surface activity (emulsifying activity) as previously described. As shown in figure 1, the biosurfactant production is correlated with the kinetic growth of the halophilic strain Halovivax sp. A21 on petroleum hydrocarbons. This strain exhibited high emulsifying index and relevant reduction in surface tension after 22 days of growth. According to Mulligan [40] a good surfactant can lower the surface tension of water from 72 to 35 mN/m [41]. In the present study, the surface activity reduces the surface tension from 72 to 24.5 ± 1.5 mN/m with an emulsifying index of 93.5% in cell-free supernatant (figure 1). Thus, the obtained surfactant can be grouped under good/ very good extracellular biosurfactant. Note that the emulsions were stable even after 3 months. The results indicated that the produced biosurfactant was capable of effectively emulsifying petroleum hydrocarbons at high salinity. On the other hand, results on influence of environmental factors on biosurfactants production and activity on petroleum hydrocarbons showed that optimized volume of the medium for 1000ml capacity Erlenmeyer flasks was identified as 100ml with an optimum agitation of 120 rpm. Indeed, the strain Halovivax sp. A21 showed appreciable surface activity (28-30 mN/m) at pH 7-9. Similarly, the optimum temperature was identified as 40-45°C. Besides, the rates of biosurfactants production on petroleum hydrocarbons as a carbon source by the strain Halovivax sp. A21 were enhanced with increasing NaCl concentration in the medium. Optimal concentrations were 15-35% NaCl, but even with 25-35% of NaCl the hydrocarbon-biodegradation rates (represented by the growth of the halophilic strain) were higher than with 15-20% of NaCl. Moreover, the stability of the produced biosurfactant at different pH, temperature and salinity values was measured and the results are illustrated in figure 2. The surface activity was affected by lowering pH values from 3 to 5. The emulsification activity was increased by increasing the pH value from acidic to basic regions. The stability of biosurfactant in different temperatures was found to withstand even up to 100-120°C with optimum activity at 40°C. Indeed, the emulsion activity of biosurfactant did not show remarkable effect after heating at 100°C. This indicates that the produced biosurfactant seems to be thermostable. Similarly, in different salt concentration, the biosurfactant conserves its activity up to 15% of NaCl, with an optimum at 25% of salt.

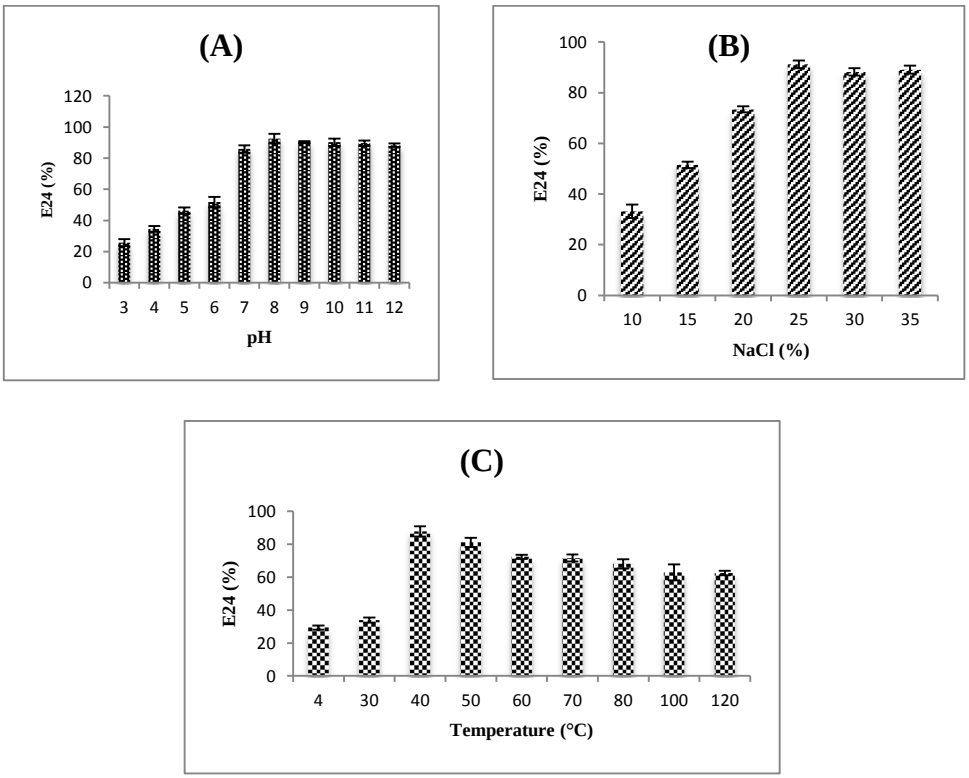

Figure 2. *Effect of pH (A), salinity (B) and temperature (C) on biosurfactant stability. Errors bars are ± the standard deviation of the mean.*

Since the applicability of biosurfactants in several fields depends on their stability at different environmental conditions such as NaCl, temperatures and pH values, these results showed the stability of the produced halophilic biosurfactant and its potential use for the bioremediation of hypersaline and hyperalkaline environments contaminated by petroleum hydrocarbons. Thus, the success of bioremediation of oil spill not only depends on the ability of the strains, but also on physical, chemical and biological conditions in the contaminated environment [42]. The same stable proprieties were also observed with the biosurfactants produced by the strain Natrialba sp. C21 when it grew on phenol as sole carbon source at 25% of NaCl [25]. Furthermore, purified biosurfactant was eluted by normal phase column chromatography which was carried out for the crude extract produced by the strain Halovivax sp. A21 after growth on petroleum hydrocarbon as carbon source. Then, the obtained information from TLC confirmed the presence of lipopeptide with protein and lipid fractions.

There are still few studies on the mechanisms of the hydrocarbon uptake and biosurfactants production by halophilic or extremely halophilic strains. Halomonas eurihalina strain H-28, a moderately halophilic bacterium, was reported to produce an extracellular polysaccharide not only in media with glucose but also in media supplemented with hydrocarbons such as n-tetradecane, n-hexadecane, n-octane, xylene, mineral light oil, mineral heavy oil, petrol, or crude oil [43]. Sarafin et al. [30] have characterized a biosurfactant belonging to the lipopeptide groups from a halophilic bacteria Kocuria marina BS-15 isolated from solar salt works in India, after growth on crude oil at 10% NaCl. As well, Elazzazy et al. [44] have also isolated an alkali-halo-thermophilic bacteria Virgibacillus salarius producing Rhamnolipids under extreme environmental conditions. This biosurfactant keeps its activity stable over a wide range of temperatures between 30 and 100 °C, alkaline pH and hyper salinity over 10%. Among haloarchaea, apart from the fact that the ether-linked phytanyl membrane of Halobacteriaceae showed emulsification properties, which was effective in enhancing the efficiency of oil recovery [45], only some strains belonging to the genera Haloferax, Natrialba, Halovivax and Haloarcula were described for their capacities to produce peptidoglycolipids or exopolysaccharides to solve the hydrocarbons bioavailability issue in highly saline environments [4, 25, 32, 46, 47]. These results suggest the potential role of strains belonging to the Halobacteriaceae family which can degrade aromatic compounds in oil-polluted hypersaline environments.

III.2 Dioxygenase activity

As evident from Figure 3, activities of the degradative enzymes catechol 1,2 dioxygenase (C12O) and catechol 2,3 dioxygenase (C23O) were induced during the incubation period of the halophilic strain Halovivax sp. A21 on petroleum hydrocarbons (2% v/v) as carbon source at high salinity (25% NaCl).

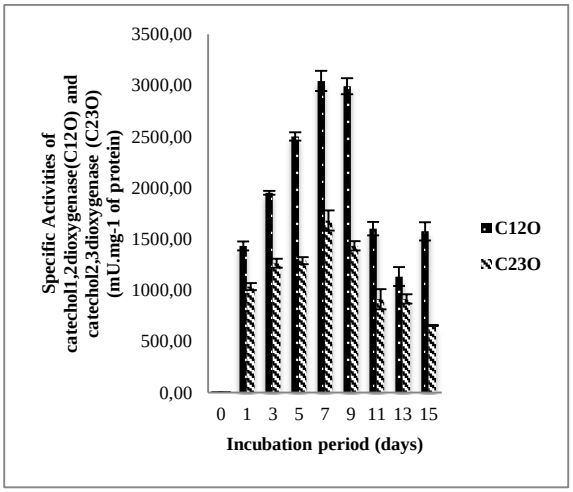

Figure 3. *Activities of catechol 1,2 dioxygenase and catechol 2,3 dioxygenase during the petroleum hydrocarbons degradation by the halophilic strain Halovivax sp. A21 at 40°C, pH7.0, 120 rpm and 25% NaCl.*

The specific activities of catechol 1,2 dioxygenase and catechol 2,3 dioxygenase gradually increased and reached to the peak (3045,03±98,7 mU mg-1 of protein and 1680,84 ± 99,3 mU mg-1 of protein) after 7 days of incubation and then the enzyme activities declined. Thus, the halophilic strain Halovivax sp. A21 showed the degradation of aromatic compounds of the petroleum hydrocarbons through ortho and meta cleavage. However, ortho cleavage pathway was predominant as compared to meta cleavage as reflected by the higher activity of catechol 1,2 dioxygenase than catechol 2,3 dioxygenase. Little is known about the degradation of petroleum hydrocarbons by extremely halophilic Archaea. Only two extreme halophilic Haloferax strains and one strain of each Halobacterium and Halococcus (isolated from a hypersaline coastal area of the Arabian Gulf) were able to grow on a mineral salt medium with crude oil vapor as a sole source of carbon and energy in the presence of up to 4.5 M NaCl or higher [16]. However, no information about their enzyme activities was mentioned. Nevertheless, Erdoğmus et al. [24] and Khemili-Talbi et al. [25] reported the ability of many archaeal strains belonging to Halobacterium, Halorubrum, Halobacterium Haloarcula, Haloferax sp. and Natrialba groups to degrade some aromatic hydrocarbons (such as phenol, naphthalene, pyrene,

p-hydroxybenzoic acid and/or phenanthrene) at over to 20 % (w/v) NaCl by ortho cleavage pathway. The presence of catechol dioxygenases in the family Halobacteriaceae has been shown in those works (including the present study), but a further work is needed to elucidate the mechanisms of action of these halophilic enzymes (and interestingly the early enzymes in the pathway), which maintain their catalytic properties in saline environments.

IV. Conclusion

According to our knowledge, Halovivax sp. C21 is the first archaeal belonging strain to this genus having the ability to degrade petroleum hydrocarbons (2% v/v) at high salinity (25% of NaCl). This strain shows its ability to produce lipopetides which keep their activities stable over a wide range of temperatures between 40 and 120°C, alkaline pH and hyper salinity over 20%. At the present day, the properties of the obtained biosurfactant were highly encouraging the eco-sustainable bioremediation that can be achieved in saline environments.

Likewise, the strong character of the produced biosurfactants makes it useful for industrial applications under extreme conditions of salinity, temperature and pH; such as food, cosmetics and pharmaceutical industries. Moreover, the catechol 1,2 dioxygenase was more induced than the catechol 2,3 dioxygenase, which clearly indicates the predominance of the ortho cleavage pathways in the petroleum hydrocarbons degradation by the halophilic strain Halovivax sp. A21. This haloarchaeal strain may be effectively used for the bioremediation of oil-contaminated environments.

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