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Screening and Characterization of Biosurfactant Producing Bacteria Found in Mar Chika Lagoon

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ABSTRACT

Petroleum based products are the major source of energy for industries and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. In the present study four Bacterial strain isolated from Marchika lagoon, located in the north-west Mediterranean coast of Morocco were identified by biochemical and molecular tests. The effects of culture conditions in vitro and biosurfactant detection were studied on bacterial strains capable of degrading gasoline. Biosurfactant production was evaluated by measuring the hydrophobicity of the cell surface, the hemolytic activity, the spreading technique of Oil spreading, drop-collapse, and surface tension. Therefore a chemical identification of produced biosurfactants was investigated using Fourier Transform Infrared (IR-TR) and High-performance liquid chromatography Electrospray Ionization Mass Spectra methods. Aeromonas salmonicida seems to be the most potent biosurfactant producing showing a β-hemolysis with higher hydrophobicity index (BATH) and degree of collapse and the lowest surface tension. The optimal growth conditions including pH, temperature and the concentration of gasoline were respectively determined at 7, 25°C and 1%. HPLC and IF showed the presence of some biosurfactants and a large variety of compounds showing same properties as biosurfactants.

Key words: *Biosurfactant, Aeromonas salmonicida, Marchika lagoon, Bioremediation.*

INTRODUCTION

In the past decade, many studies have reported the effects of microbially produced surfactants on bioremediation and enhanced oil recovery. Biosurfactants are amphiphilic compounds which regroup a structurally diverse class, produced by a variety of yeast, bacteria and fungi[1].

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These biomolecules are potential alternatives of chemically synthesized surfactant in a variety of applications; because of their many advantages such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity[2-4].

These properties allow their use in many fields such as food industry as emulsifying agents, and stabilizers; pharmaceutical industry as therapeutic agents for the control of many diseases, agriculture field as biocontrol agents and biotechnology applications for the bioremediation of sites contaminated with hydrocarbon compounds[5-10].

Biosurfactants create micro-emulsions in which micelle formation occurs where hydrocarbons can be solubilized in water and vice versa[11,12]. They mainly reduce surface and interfacial tensions both in aqueous solutions and hydrocarbon mixtures[13]. These emulsification properties have been demonstrated to enhance the hydrocarbon degradation in the environment, hence making them potential tool for the oil spill pollution control[14].

Organisms used in this work were isolated from Mar Chika lagoon which is located in the north-eastern part of Morocco in the Mediterranean front between Beni Enzar and Kariate Arkmane towns. The lagoon receives domestic and industrial sewage, untreated effluents, petroleum hydrocarbons, pollution caused by fishing ships. Few studies, have evaluated the presence of natural, indigenous biosurfactant producing microbes in this lagoon and because of the nature of the waters it receives, a large variety and number of microorganisms with capacity to utilize different xenobiotics can be present in Mar Chika lagoon. In this paper, we focused on isolating and identifying the most potent biosurfactant producing strain, therefore characterization and chemical structure of produced biosurfactants were investigated.

MATERIEL AND METHODS

Microorganism strain

The bacterial strains: *Exiguobacterium aurantiacum*, *Burkholderia cepacia*, *Aeromonas salmonicida* and *Serratia Ficaria* were isolated from water and soil collected aseptically from the lagoon Marchika in Nador, located in the north-west Mediterranean coast of Morocco, facing the Alboran Sea between Beni Enzar and Kariat Arkmane. Identification of bacteria was performed through biochemical analysis using the standardized micro-method API 20E and 20NE (Biomeriaux) and the Sequence method (16S rDNA) using GenElute™ Bacterial Genomic DNA Kit and ABI 3130xl Genetic Analyzer.

Media and substrate used

1% of each bacterial strain was added to 100 ml of the minimum medium (1.36 $g.l^{-1}$ K_2HPO_4; 0.1 $g.l^{-1}$ $MgSO_4$; 0.6 $g.l^{-1}$ $SO_4(NH_4)_2$; 0.02 $g.l^{-1}$ $CaCL_2$; 0.5 $g.l^{-1}$ NaCl; 1.1 $mg.l^{-1}$ $MnSO_4$; 0.2 $mg.l^{-1}$ $ZnSO_4$; 0.2 $mg.l^{-1}$ $CuSO_4$; and 0.14 $mg.l^{-1}$ $ZnSO_4$) ,pH adjusted to 7 with 1M HCl solution. Four various carbon sources: gasoline (1%), diesel (1%), benzene (1%) and anthracene (1.3 mg/l) were tested. After 96 h of incubation at 25°C in a rotary shaker 150 rpm, absorbance was measured at 600 nm in order to evaluate the cellular growth by a spectrophotometer (Shimadzu UV-1800).

Effect of physicochemical parameters on bacterial growth

In order to determine the optimum conditions of the bacterial growth, bacterial strains were cultured at different temperatures (20, 25 and 37°C), pH (4, 7 and 10) and gasoline concentrations (0%; 0, 25%; 0, 5%; 1%; 2%; 3%; 4%).

Screening of the most potent biosurfactant producing strain

Bacterial Adherence to Hydrocarbon (BATH Test)

This technique was carried out using a method described by Rosenberg*et al.*[15]. The bacterial strains grown on minimum medium with gasoline as carbon source, were harvested separately and washed three times to remove any residual medium by centrifugation 8500 tr/min ,20 min. The cells were then resuspended in a buffer solution (pH = 7) containing per liter16.9g of K_2HPO_4, 7.3g of KH_2PO_4, 1.8g of urea and 0.2g of $MgSO_4.7H_2O$to give an optical density (OD) at 600 nm of 1.0 (ODc). Cell suspension and gasoline (4:1) were mixed and vortexed for 10 min, left to stand for 15min to separate the two phases. The aqueous phase was then carefully removed and the turbidity of the aqueous phase was measured (ODa). Hydrophobicity is expressed as the percentage of adherence to gasoline, which iscalculated as:

$$BATH = [1- (ODa /ODc)] \times 100 [\%].$$

Hemolytic activity

Hemolytic activity was carried out as described by Carrillo *et al.*[16]. The isolated strain was screened on blood agar plates containing and incubated at 37°C for *Exiguobacterium aurantiacum*, *Burkholderia cepacia* , *Serratia Ficaria*and 25°C for *Aeromonas salmonicida* 25°C for 24-48 h. Hemolytic activity was detected as the presence of a clear zone around a colony.

Oil spreading method

Oil spreading technique was carried out according to Morikova *et al.*[17] and Youssef *et al.*[18]. 50 ml of distilled water was added to Petri dishes followed by addition of 100 µl of gasoline to the surface of the water. Then, 10 µl of each culture was put on the gasoline surface. The diameter of the clear zone on the oil surface was measured. The control contains distilled water and gasoline without addition of the supernatant containing biosurfactants.

Drop-collapsing Test

This technique is based on destabilization of the hydrocarbon droplets by the biosurfactants. The drop-collapse technique was carried out in a 96-microwell plate as described by Jain *et al.*[19]. 100 µl of each culture supernatant was added to micro-well, and then 5 µl of gasoline was added to the surface of each culture supernatant. The droplet shape on the gasoline surface is observed after 1 min.

Measurement of surface tension:

To evaluate the production of biosurfactants, we measured the surface tension. The cell-free supernatant of each culture was then tested for its ability to reduce surface tension using a Tensiometer (Kruss K6) at 30°C, according to the ring method De Nöuy[20]. For the validity of the measurements, the calibration was carried out using distilled water and then the surface tension of the sterile minimum medium was measured before measuring of sample.

Biosurfactant extraction

The biosurfactant was extracted from culture medium after cells removal by centrifugation at 8500 rpm for 30 min. The supernatant pH was adjusted to 2 by the addition of HCl (6 M), then maintained at 4°C overnight[21]. An equal volume of ethyl acetate was added in a separation funnel, the mixture was vigorously shaken for 5 min and allowed to set until phase separation[22]. The organic phase was removed and the operation was repeated three times. The organic phase was collected and water was removed by adding anhydrous sodium sulphate then concentrated at 40°C using a rotary evaporator « Buchi Rotavapor R-200 ». The resulting green viscous product was considered as crude biosurfactants[23].

Determination of Critical Micellar Concentration (CMC)

The surface tension was determined as described above. The dried biosurfactant extract was dissolved in distilled water and serially diluted prior to surface tension measurement. The CMC was obtained from a plot of the surface tension as a function of the concentrations of biosurfactants. The concentration at which the micelles began to form was represented as the CMC. Above this concentration, there would be no reduction in surface tension.

Chemical characterization of biosurfactant

Fourier transform infrared spectroscopy

FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures[24]. The extracted biosurfactants were mixed with 100 mg of KBr and pressed with 7500 kg for 30 seconds to result a translucent KBr pellets. The IR-TR spectra were recorded on the "IR-TR-8300 spectrophotometer of Shimadzu with the spectral regionsand wave number accuracy of4000-400 cm^{-1} and 2 cm^{-1}respectively. All measurements consisted of 500 scans, and a potassium bromide pellet was used as background reference.

High-performance liquid chromatography Electrospray Ionization Mass Spectra (HPLC-ESI-MS)

HPLC-ESI-MS was used to analyze the chemical components of the biosurfactant. The sample used was prepared as follows: the pure biosurfactant was dissolved in acetonitrile (ACN) and water (1:1, v/v), centrifuged and filtered. Analysis instrument was HPLC combined with ESI mass spectrometer (Xevo G2-XS Q-TOF). Chromatographic separation was achieved with 1.7 um, 2.1*100 mm C18ACQUITY BEH. HPLC analytical conditions:

2 µl samples were injected via an autosampler with split injection at split rate of 1:4. A gradient was applied using distilled water and ACN with a flow rate of 0.35 ml/min: initially 25% CAN and 75% water, then a linear gradient over 30 min to 50% ACN and 50% water, further for 15 min to 90% ACN and 10% water and the column was washed for 0.1 min, and ultimately reequilibrated to 25% ACN and 75% water for 25 min. ESI-MS was performed in negative ion mode. The collision voltage and ionization voltage were 3 kV et -2,5 kV, respectively, using nitrogen as atomization and desolvation gas. The desolvation temperature was set at 400°C. The scan range of mass spectrum was 50–1000 m/z. The relative amount of each component was determined from the LC–MS chromatogram, using the area normalization method.

RESULTS AND DISCUSSION

Choice of substrate

Results obtained in this study show that the four isolated bacterial strains *A.salmonicida, S. ficaria, B. cepacia and E. aurantiacum* are able to grow in presence of different sources of carbon (gasoline, diesel, benzene and anthacene) tested, with Kinetics of growth that vary from one bacterium to another (Figure 1 a, b, c, d).The comparison of the various growth kinetics has made it possible to deduce that the gasoline is the most suitable substrate for their growth and the most consumed by the bacterial strains.At the level of the exponential phase the absorbances, determined fromcurves represented in figures 1 a, b, c, d, vary from *A.salmonicida* which shows the highest absorbance about 2.113, in the presence of gasoline compared to*B.cepacia, S.ficaria* and *E. aurantiacum* which respectively show 1.9; 1.7 and 0.442. A study showed that *Aeromonas* species are capable of degrading different types of crude oils with more or less rapid adaptation[25].

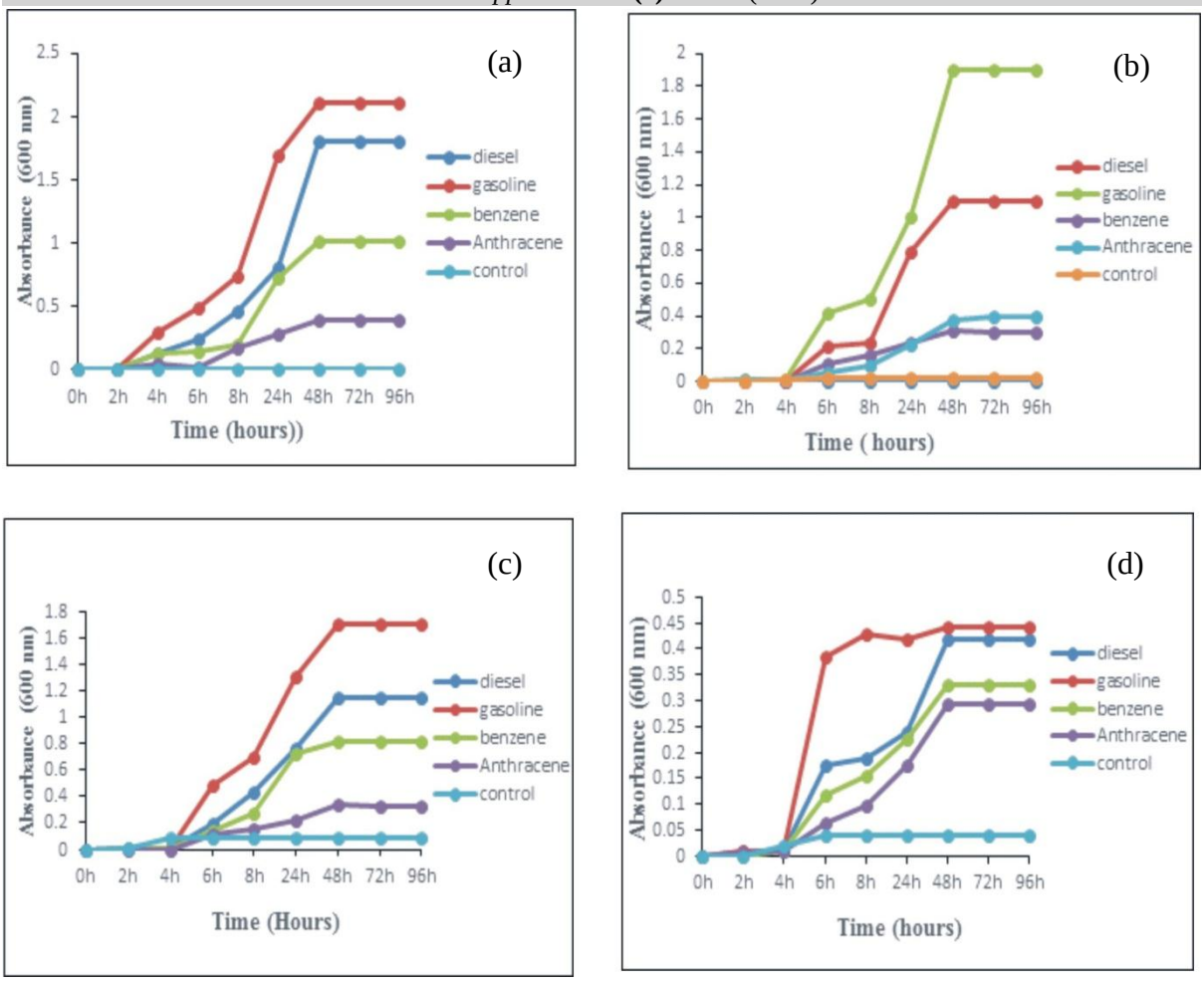

Fig. 1: Effect of Substrate on the growth of *A. salmonicida* (a) , *B. cepacia* (b), *S.ficaria* (c) *and E.aurantiacum* (d)

Effect of physicochemical parameters on bacterial growth

Effect of temperature

The effect of temperature on bacterial strains growth with gasoline as source of carbon is shown in Figure 2. The study revealed that bacterial strains are able to grow at different temperatures tested but with growth kinetics that vary from one temperature to another (Figure 2a,b,c,d).The highest amount of growth was obtained with *A .salmonicida* at 25 ° C (A= 2.3), followed by 37°C (A=2,113) after 48 hours of incubation compared to culture inoculated at 20°C (A=1,9) where the growth was slower as shown in Figure 2a. *B.cepaci, S.ficaria* and *E.aurantiacum,* reached the highest growth at 37 ° C (Figure 2 b, c, d) with absorbances of 1.9; 1.7 and 0.442 respectively.It can be concluded that the optimum temperature of *A. salmonicida* is 25°C and *B.cepaci, S.ficaria , E.aurantiacum*is 37 °C after 48 h where a stable rate of growth was observed.

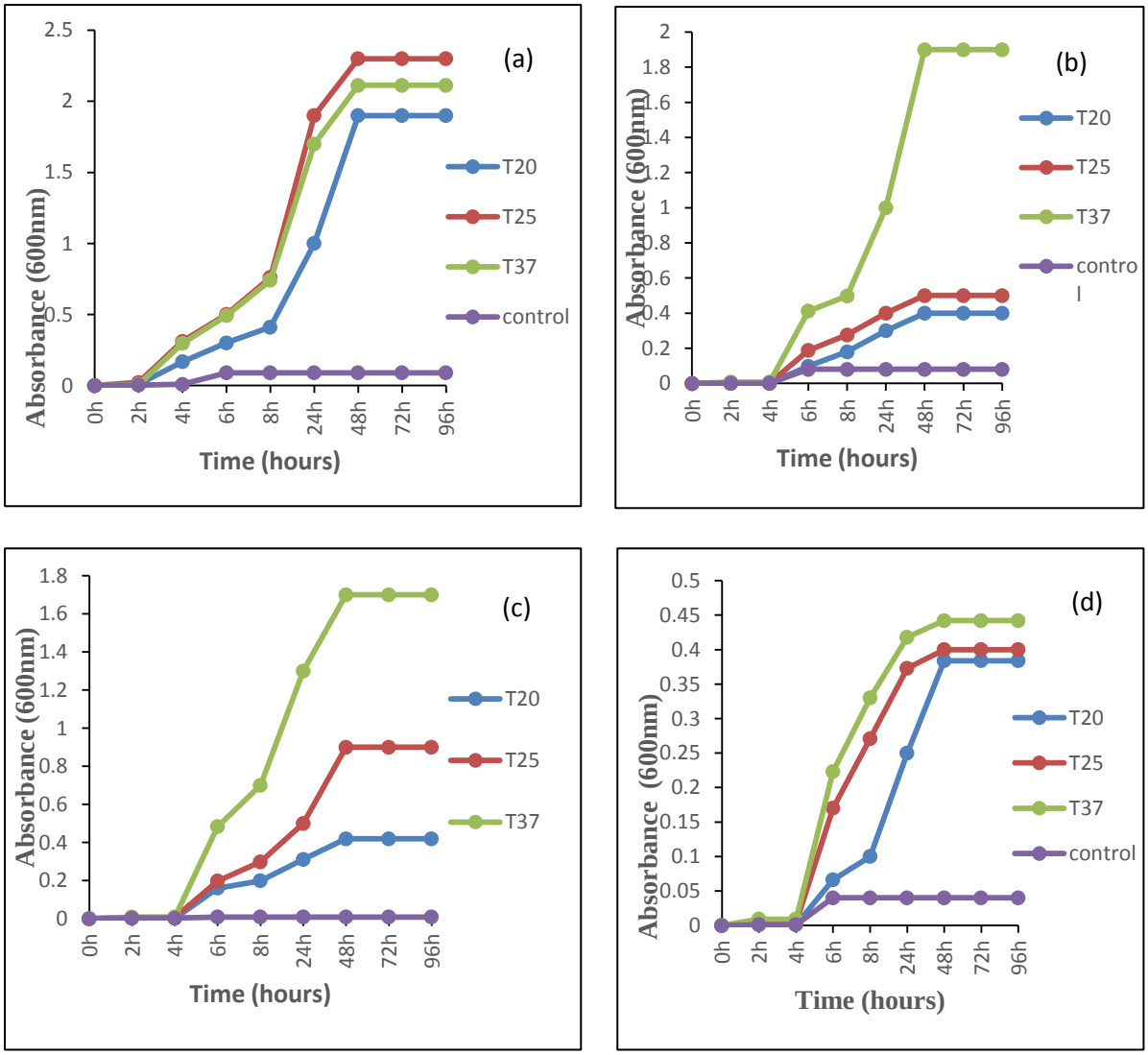

Fig. 2: Effect of temperature on *A. salmocida* (a), *B. cepacia* (b), *S. ficaria* (c) and *E. aurantiacum* (d) growth with gasoline

Effect of pH

It is observed that the bacterial strains *A.salmocida, B.cepacia, S. ficaria* and *E.aurantiacum* have maximum growth at pH = 7(A= 2.3; A= 1.9;A= 1.7 and A=0.442) (figure 3 a, b, c, d), after 48 hours the growth remains constant. While at pH 4 and 10 the growth is relatively low. This makes it possible to deduce that the growth of these different bacterial strains is negatively affected by the acidity and the basicity of the culture medium, whereas it is favorable in neutral media.

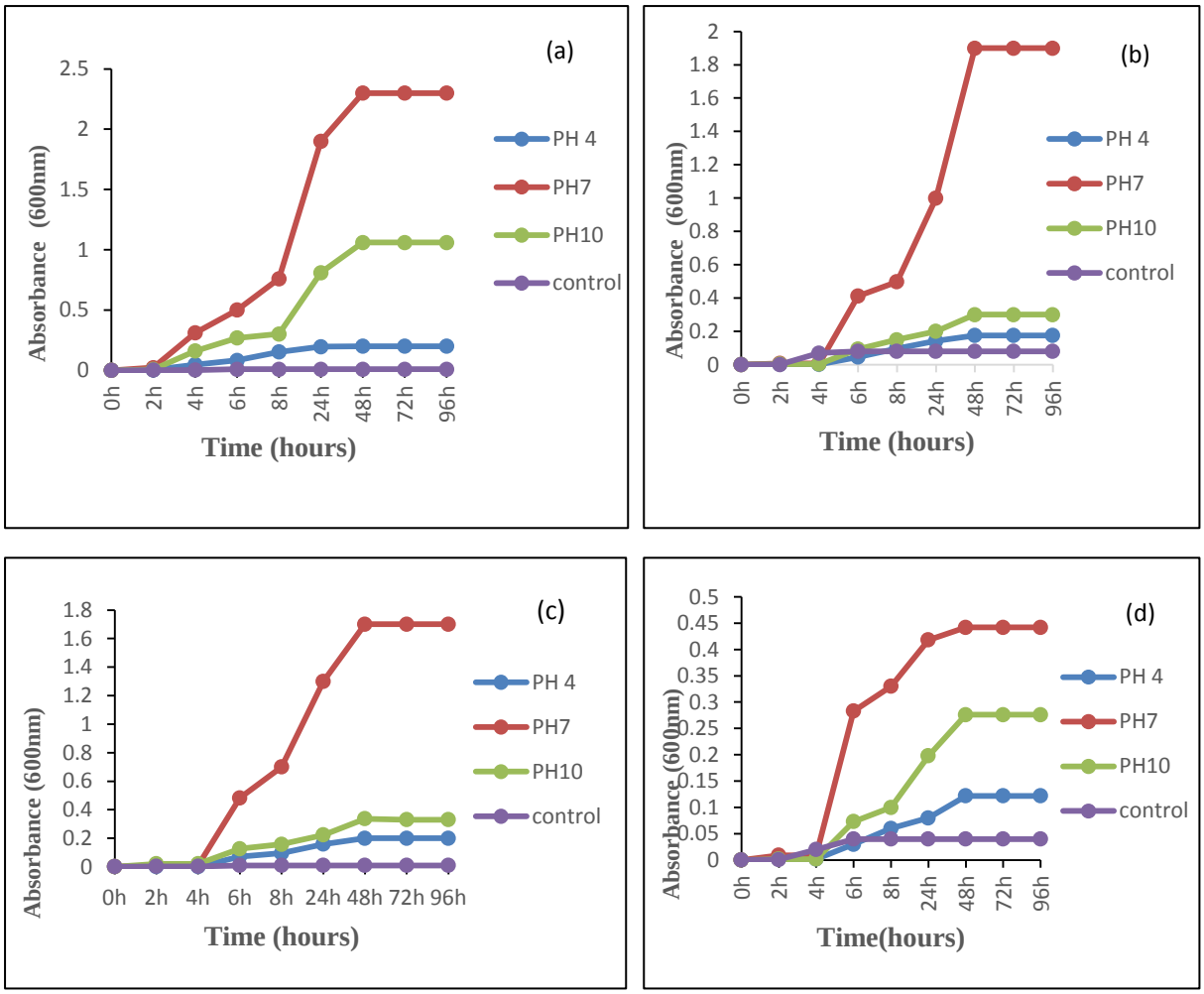

Fig. 3: Effect of pH on growth of *A.salmonicida* (a), *B. cepacia* (b), *S. ficaria* (c) and *E.aurantiacum* (d) with gasoline

Effect of gasoline concentration

The effect of gasoline concentration on cell growth was revealed on figure 4.The appearance of the curves shows that the bacterial growth increases progressively with the increase of the concentration of gasoline. The results for the four strains *A.salmonicida, B.cepacia, S. ficaria* and *E. aurantiacum* showed high absorbances at a concentration of 1% of gasoline respectively 2.3; 1.9; 1.7; 0.442, but no growth was detected above 2%. The reason for decreased consumption of gasoline oil at high concentration may be attributed due to stress of hydrocarbons on bacterial species.

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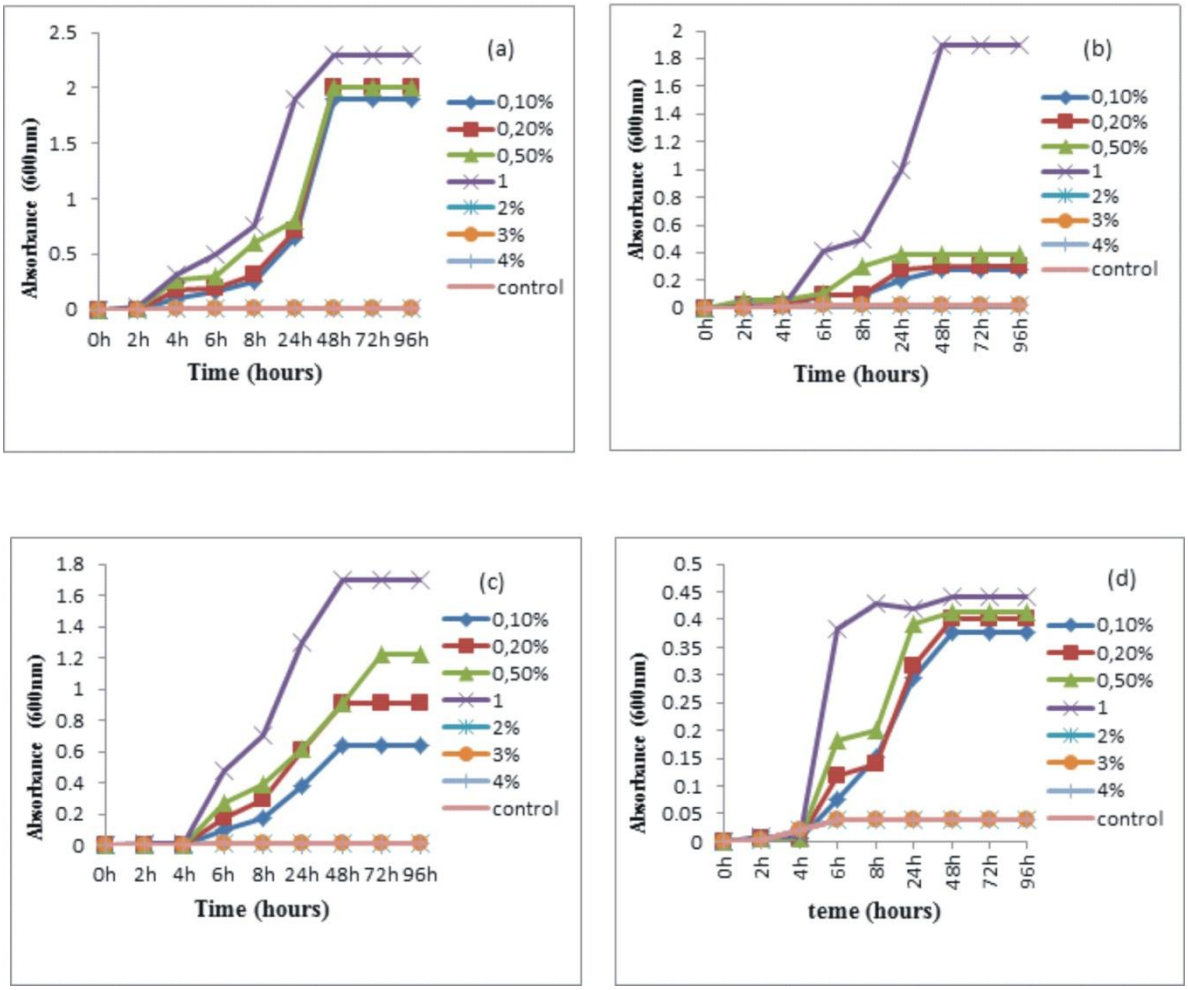

Fig. 4: Effect of gasoline concentration on growth of *A. salmonicida* (a), *B. cepacia* (b), *S. ficaria* (c) and *E. aurantiacum* (d)

Screening for biosurfactant production

In this study, a variety of tests were adopted for the screening of the biosurfactant production by *A.salmonicida, B.cepacia, S.ficaria* and *E.aurantiacum.* The responses of the strain to the screening methods are shown in Table1.

Table 1: Screening of biosurfactant-producing bacteria using four methods

Bacterial strains	Hydrophobicity index BATH (%)	Hemolytic activity	Drop Collapse (mm)	Oil spreading (cm)
AS	82,5	++++	3,7	3,4
BC	38,3	+++	2,2	1,5
SF	27	+++	1 ,9	2,1
EA	8	-	0,3	-
Control	0	-	-	-

According to results in table 1*A.salmonicida* showed a high capacity to adhere to the droplets of gasoline with a higher hydrophobicity index (BATH) of 82.5%.Therefore, the increase in the percentage of hydrophobicity index indicates a higher affinity of the cells to the hydrocarbon[26]. This suggests that *A. salmonicida* has a high affinity for gasoline.

Those with lower hydrophobicity cellular: *B.cepacia*, *S.ficaria* and *E.aurantiacum* with a percentage of 38.3; 27 and 8% can also be capable of producing biosurfactants and degrading hydrocarbons but with a longer adaptation period. It has been suggested that cells with a greater hydrophobicity are more likely to adhere to hydrophobic compounds than those with a low hydrophobicity, and are better able to absorb the hydrocarbons[27]. This observation suggests that cellular hydrophobicity is also an indication of the production of biosurfactants[28].

The results obtained in table 1report that *A.salmonicida, S.ficaria* and *B.cepacias*howed a ß-hemolysis corresponding to a clear area (hemolysis) on plates of blood Agar but the largest diameter was observed with *A.salmonicida* representing a diameter of 1.2 cm, thus indicating the presence of a high concentration of biosurfactants.

As shown in table 1 drop collapse method has a most positive response andit was found that the *A.salmonicida* showed a high value: 3.7 mm suggesting a high degree of collapse. In the Drop Collapse test, in the presence of biosurfactants, the interfacial tension between the water drop and the hydrophobic surface is reduced, which results in its propagation on the hydrophobic surface[29].

Morikawa *et al.*[30] showed that the area of displacement by a surfactant-containing solution is directly proportional to the concentration of the two biosurfactants tested. The results obtained are noted in Table 1.Three strains tested showed a positive result for oil spreading but with different diameters. A clear zone was observed for *B. cepacia* and *S. ficaria* with values of 1.5, respectively; 2.1 cm, but the largest diameter is observed in *A.salmonicida* with a diameter of 3.4 cm indicating the presence of a high concentration of biosurfactants These results show that *A.salmonicida* produces higher concentrations of biosurfactants.

Measurement of surface tension has been used by many researchers to measure the surface properties of biosurfactants[31,32]. Figure 5 shows the values of the surface tension measured for the bacterial strains after 96

hours with gasoline.In this study, the lowest recorded surface tension was 34.5 mN.m^{-1} obtained with *A.salmonicida* whereas *B.cepacia*, *S.ficaria* and *E.aurantiacum* showed a small decrease in surface tension with values close to: 53; 55; 59 mN.m-1 respectivelyafter 48 hours. The reduction of the latter confirms that there is production of specific biomolecules having surface-active properties, such as biosurfactants, known to cause the lowering of the surface tension[33]. Similar results were obtained by Somayeh *et al.*[34] who were able to isolate *P. aeruginosa* with the ability to produce biosurfactants and reduce the surface tension of the culture medium to 36 mN.m^{-1}.

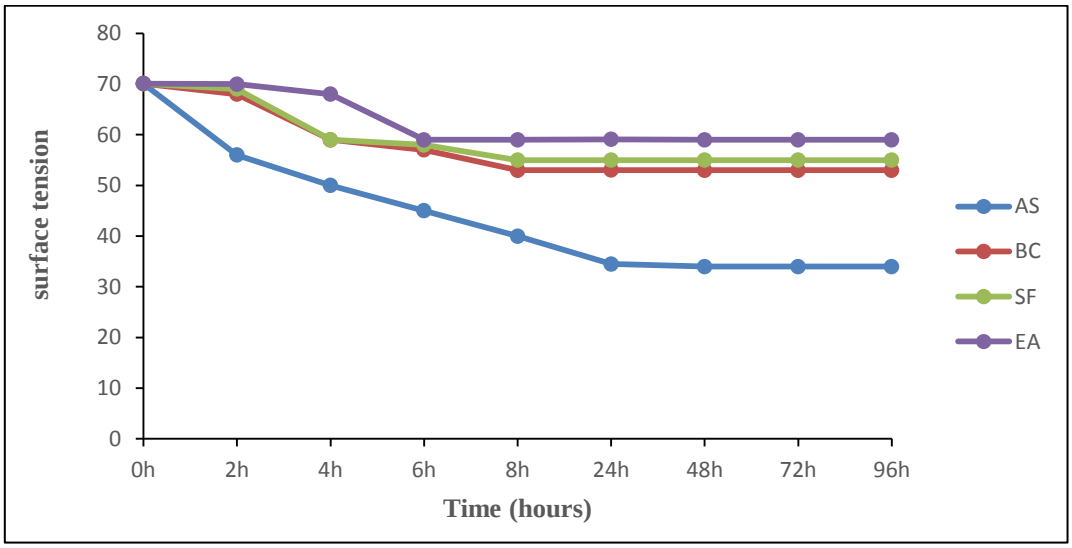

Fig. 5: Measurement of the surface tension in the culture medium of *A. salmonicida*, *B. cepacia*, *S. ficaria* and *E. aurantiacum* with gasoline

This result was in agreement with those obtained from the haemolysis and cell hydrophobicity test as well as the drop collapse, confirming that the *A.salmonicida* strain was the most potential in the production of biosurfacatnts.

Characterization of biosurfactants produced by *A.salmonicida*

Fourier Transform Infrared (IR-TR)

The functional groups of the biosurfactants produced were analyzed by IR-TR spectroscopy to confirm further that the biosurfactants extracted were biosurfactants of lipopepetidic nature.

Figure 6 shows the infrared spectrum of biosurfactants produced by *A.salmonicida* in a medium containing gasoline as the sole source of carbon.

According to the obtained profile, the presence of the groups: alkenes = C-H represented by a stretch peak of approximately 3012 cm^{-1}, 2927 cm^{-1}and 2854 cm^{-1} are attributed to asymmetric stretching) and symmetrical (CH_2) of the methylene group, and also corresponds to the aliphatic chain of the CH stretch , in addition, a symmetrical stretch peak of about 1754 cm^{-1} indicates the presence of carbonyl ester groups (C = O in COOH), and the peak 1458 cm^{-1} corresponds to the COH groups of the carboxylic acid (COOH) And also CH = O of the aliphatic aldehyde[35]. 1377 cm^{-1} corresponds to the C-H groups and to the CH_3 vibrations, the aliphatic amine group C-N has been proved from the band at 1240 cm $^{-1}$. The 1165 cm $^{-1}$ peak corresponds to the C-O groups of the tertiary alcohol and the 723 cm $^{-1}$ peak corresponds to the aromatic C-H groups. In fact, a lipid is composed of hydrocarbon chain, nitrogen group, phosphate and alcohol functions. This suggests that their presence is a characteristic that the biosurfactants produced by *A.salmonicida* are of lipid nature[36].

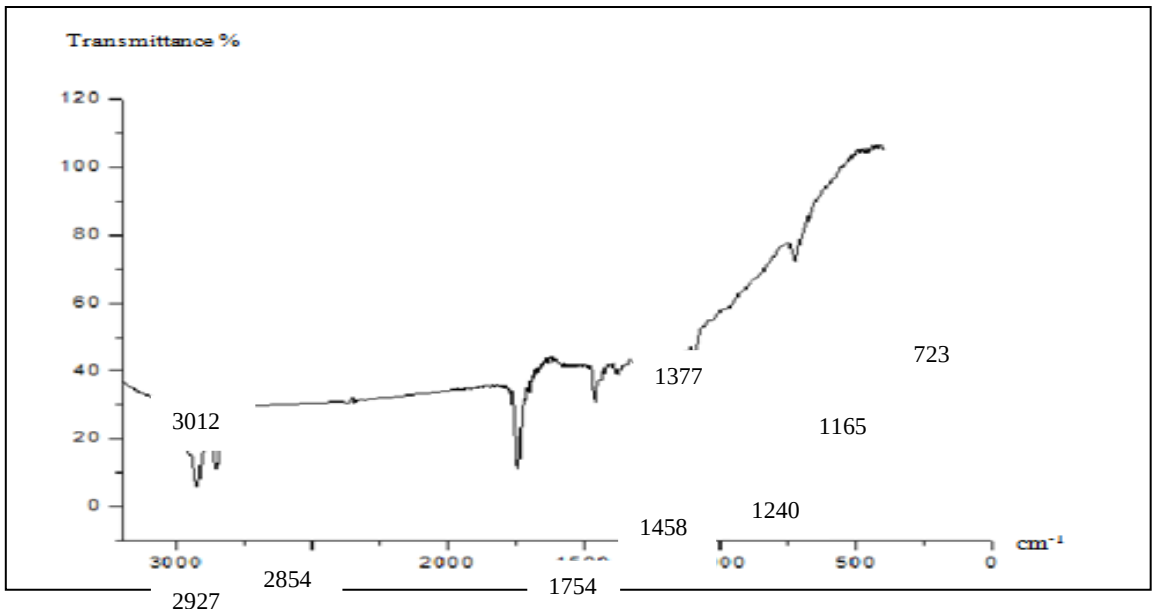

Fig. 6: Infrared spectrum of biosurfactants produced by *A. salmonicida* grown with gasoline as the sole carbon source

Liquid chromatography coupled with mass spectrometry

The partially purified biosurfactants produced by the bacterial strain *A.salmonicida* with gasoline as a carbon source were analyzed by liquid chromatography coupled with LC-MS mass spectrometry. The chromatogram showed twelve fractions of the resulting major biosurfactants identified as surfactant compounds with different retention times (Figure 7).

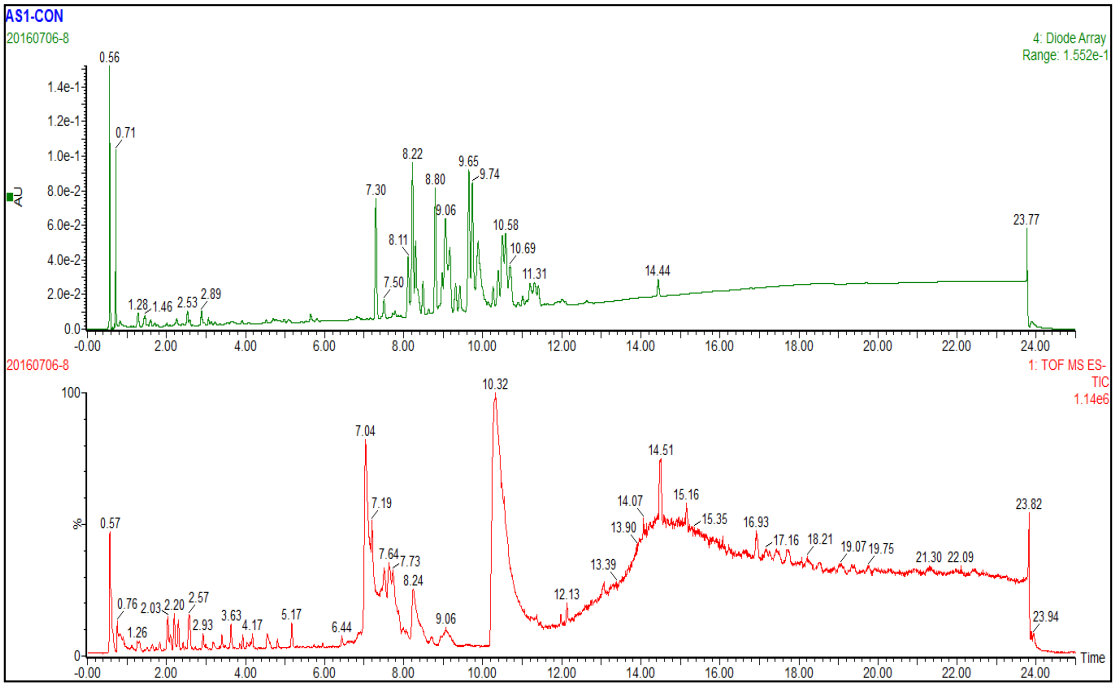

Fig. 7: LC-ESI-MS chromatographic profile of *A.salmonicida* biosurfactants with gasoline as a carbon source

Analysis of the LC-MS results indicates the presence of a wide variety of compounds in the partially purified extract. Among the compounds, some have been identified having a surfactant power, the fractions have been analyzed and listed in Table 2. Among these compounds, the following are distinguished:

Table 2: Relative position of the LC-MS peaks for the surfactant compounds of the extract

Name of Compound	Retention time (RT)	Mass(Da)
Betaïne	0,57	117,07898
Phénylpropanoïc acid	1,26	165,07898
Gentianine	2,57	175,06333
6*α*-Methylprednisolone	5,17	374,20932
Glycocholic acid	6,44	465,30904
Laurylsulfate	7,04	203,13101
Di (Oxyethylene) lauryl- sulfate	7,19	353.2000
Tri-(Oxyethlene) lauryl sulfate	7,64	397.2260
1-Monopalmitin	7,73	330,27701
Crotamiton	8,24	203,13101
Linoleic acid	10,32	278,22458
Ricinoleic acid	14,51	298,25079
Dodemorph	15,16	281,27186

Laurylsulfate, Di (Oxyethylene) lauryl-sulfate, Tri-(Oxyethlene) lauryl sulfate, are an anionic surfactants used in many cleaning and hygiene products. Glycocholic acid: substance possessing a hydrophilic pole and a hydrophobic pole involved in the emulsification of various fats, it is suggested that it belongs to anionic surfactants[37-38]. Ricinoleic acid: major fatty acid is a hydroxylated unsaturated fatty acid. Polyglycerol polyricinoleate, consisting of oligo-ricinoleic acid chains transcribed on polyglycerol via the formation of an ester bond, is a biocompatible and biologically compatible emulsifier well known as a food additive and also used in pharmaceutical products[39- 40].

In conclusion, the present study focused on the degradation of gasoline oil by a bacterial species isolated from Marchika lagoon. It also investigated the evaluation of optimum conditions for producing biosurfactants. The results indicate that *Aeromonas salmonicida* have great potential for remediation and can be used for effective degradation of gasoline oil from industrial effluents contaminated with gasoline oil.

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