Production and characterization of lipopeptide biosurfactants from a novel marine *Bacillus stratosphericus* strain FLU5

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ABSTRACT

This work aimed to study the potential of a newly marine bacterium, *Bacillus stratosphericus* FLU5, to produce an efficient surface active agent BS-FLU5. Biosurfactant production was examined on different carbon sources using the surface tension measurement and the oil displacement test. Strain FLU5 showed its capacity to produce biosurfactants on the most of tested substrates and in particular the residual frying oil, which is an alternative, cheap and renewable carbon source, thus minimizing the high cost of producing surfactants. MALDI-TOF MS/MS analyses confirmed the presence of lipopeptides identified as members of surfactin and pumilacidin isomers. The critical micellar concentration of the purified lipopeptides produced by strain FLU5 was 50 mg/l and, at this concentration, the surface tension of the water was reduced from 72 to 28 mN/m. Furthermore, the crude lipopeptides showed interest stability against a broad range of pH (2.1-12), temperature (10-121 °C) and salinity (0-120 g/l NaCl). The biosurfactant BS-FLU5 demonstrated negligible cytotoxic effect against mammalian cells (HEK293 human embryonic kidney cell line) at all of tested concentration (125-1000 µg/ml). The application of BS-FLU5 in oil recovery from soil contaminated by hydrocarbons (used motor oil) showed that it was more effective on the hydrocarbon-remobilization than some tested synthetic surfactants. These results highlight the applicability of the lipopeptides produced by the new marine *Bacillus stratosphericus* strain FLU5 in different fields, especially in environmental remediation processes. Production of lipopeptides is a characteristic of several *Bacillus* species, but to the best of our knowledge, this is the first report showing the potential of *Bacillus stratosphericus* for efficient production of biosurfactants or lipopeptides.

*Keywords:*Biosurfactants; Lipopeptides; *Bacillus stratosphericus;* Bioremediation

1. Intoduction

Biosurfactants, or biological surface active agents are natural amphiphilic molecules, consisting of hydrophobic and hydrophilic moieties, produced extracellulary by a wide variety of bacteria, fungi and yeasts [1]. When compared to conventional synthetic surfactants, these biocompounds have several advantages such as low toxicity, high biodegrability, digestibility, biocompatibility, low irritancy, and diversity for chemical structure and properties [2]. Moreover, biological surfactants can be produced from renewable carbon sources by biotechnological processes with low CMC, high surface activity and effectiveness even at extreme conditions of pH, temperature and salinity [3]. Due to these interesting advantages, biosurfactants have potential use in environmental applications and in petrochemical, petroleum, chemistry, food production, cosmetics and pharmaceutical industries [4]. Biosurfactants are classified, based on their molecular structure and the types of biosurfactant-producing microbial species, into mainly lipopeptides, glycolipids, phospholipids, fatty acids and polymeric surfactants [2]. Among these different groups, lipopeptides are the most popular, interesting and studied biosurfactants owing to its remarkable efficiency and commercial interest [1]. The *Bacillus* species was known as the most efficient producer of lipoeptide biosurfactants which are divided into three different families depending on their amino acids sequence: surfactins, iturins and fengycins [5]. Surfactin, a cyclic lipoheptapeptide containing a β-hydroxy-fatty-acid group as hydrophobic moiety, is the most extensively interesting and studied class of microbial surfactants due to its anti-microbiol, anti-viral and anti-tumor activities [5].

Several variants of surfactin have been described such as pumilacidin from *Bacillus pumilus* or lichenysin from *Bacillus licheniformis* [6].

Despite the advantages of microbial surfactants, production of biosurfactants is still restricted by the high cost of production. A possible strategy to reduce costs is the use of alternative, cheap and renewable substrates [7]. Two main classes of inexpensive carbon sources have been proposed for biosurfactant production: water-miscible substrates, such as molasses, starch-rich wastes and glycerol, and insoluble substrates, such as hydrocarbons, oils, and edible oily wastes [7]. The oily substrates have been proved to be good renewable carbon sources for the production of biological surfactants because as hydrophobic substrates they can possibly enhance the production of biosurfactants [8]. Among these bioresources, the utilization of waste vegetable oils as residual frying oil is becoming very important, since large quantities of cooking oil are generated in restaurants worldwide [7,9]. In addition, these waste oils can be considered as high energy sources for microorganism growth and transformation into high value products as environmental friendly surfactants [9]. Reutilization of waste oils decreases the cost of carbon source for biosurfactant production and reducing the pollution caused by these wastes, at the same time [7].

Marine environment represents a rich reservoir to explore newer compounds of commercial importance like biosurfactants, due to its diversity, nutrient availability and the exposition of marine microorganisms to extreme conditions of temperature, pressure and salinity [10]. The production of biological surfactants by marine microorganisms is yet little explored [10]. Recently, we reported the isolation and characterization of an efficient hydrocarbonoclastic marine bacterium *Bacillus stratosphericus* strain FLU5 from contaminated seawaters [11]. It has been demonstrated to be an effective degrader of a wide variety of hydrocarbons, particularly PAHs, and could be widely applied in bioremediation technology. In this present study, we report the production, purification, identification and

characterization of the biosurfactants produced by *B. stratosphericus* strain FLU5 along with its potential to remove hydrocarbons from contaminated sand by used motor oils.

2. Materials and methods

2.1. Microorganism

Bacillus stratosphericus strain FLU5, was previously isolated after enrichment culture on fluoranthene, a persistent and toxic polycyclic aromatic hydrocarbon used as the sole carbon and energy source, from contaminated seawater of the fishing harbour of Sfax, Tunisia [11]. It was selected on the basis of its remarkable capacity to grow on a wide range of aliphatic, aromatic and complex hydrocarbons. Strain FLU5 was also capable of reducing the surface tension of the cell-free medium during the growth on fluoranthene supporting the biosurfactant secretion [11]. To our knowledge, there is no data on the use of *Bacillus stratosphericus* for producing biosurfactants including lipopeptides.

2.2. Chemicals and culture media

Nutrient broth medium (NB) contained (g/l): 15 peptone, 3 yeast extract, 1 glucose and 6 NaCl. The basal medium (BM) consisted of 0.3 g KH_2PO_4, 0.4 g NH_4Cl, 0.33 g $MgCl_2.6H_2O$, 0.05 $CaCl_2.2H_2O$ g, 30 g NaCl and 1 ml trace-element solution [12] per liter of distilled water. The culture media were sterilized by autoclaving at 121 °C for 20 min. Complex hydrocarbons including diesel fuel and motor oil were obtained from Shell Company (Sfax, Tunisia) and sterilized by filtration (pore size 0.45 µm; Millipore), while crude oil was collected from "Thyna Petroleum Services" (Sfax, Tunisia) and sterilized by autoclaving. Olive oil and corn oil were obtained from commercial sources. Residual frying oil was collected from a restaurant located in Sfax, Tunisia. These vegetable oils were sterilized by filtration (pore size 0.45 µm; Millipore). The solutions of glycerol and chemical

surfactants including Tween 20, Tween 80, Titon X-100 and sodium dodecyl sulfonate (SDS) were purchased from Sigma Aldrich Company (98-99 % purity). Chemical surfactants were dissolved in water at concentrations corresponded at their CMCs and sterilized by filtration (pore size 0.45 µm; Millipore).

2.3. Production of biosurfactants from Bacillus stratosphericus FLU5 using various carbon sources

The ability of strain FLU5 to produce biosurfactants from different carbon sources was studied by adding different substrates (Crude oil, diesel fuel, motor oil, used motor oil, corn oil, olive oil, residual frying oil and glycerol) at concentration of 1% (v/v) into flasks containing BM under agitation of 180 rpm at 37 °C. The production of biosurfactants on a rich medium (NB) by strain FLU5, was also evaluated. Experiments were carried out in triplicate with an inoculums size of 5% (v/v). The cell growth was confirmed by OD at 600_{nm} measurement. To evaluate the ability of strain FLU5 to produce biosurfactants, the determination of surface tension and the oil displacement test, were assessed, as described below.

2.4. Biosurfactant extraction and purification

In order to extract biosurfactant, the culture broth (1 L) of strain FLU5 on every carbon sources was centrifuged at 7500 rpm for 20 min to remove the cells. The supernatant was acidified with 2 N HCl to pH 2.0 and incubated at 4 °C overnight, following which the biosurfactant was extracted two times using an equal volume of ethyl acetate. The organic phase was separated and concentrated in a rotary evaporator yielding a viscous yellowish biosurfactant product. The crude biosurfactant was subsequently dried and determined gravimetrically. The extraction of biosurfactant was realized after 2 days for nutrient broth, olive oil and corn oil, after 3 days for residual frying oil and glycerol and after 4 days for crude oil, diesel fuel, motor oil and used motor oil.

For purification, crude biosurfactant produced on Nutrient Broth were chromatographed on a silica gel column (60 Mesh) (Merck, Darmstadt, Germany) eluted by a mixture of chloroform/methanol/water in the ratio of 65:25:4 (v/v/v). Fractions of 1 ml were analyzed by thin layer chromatography (TLC) on silica gel plates 60 G (Machery-Nagel, düren, Germany) with the same mobile phase. The resulting spots on the TLC were detected by spraying with a solution of ninhydrin specific for free amino groups and phosphomolybdic acid specific for fatty acid groups. Fractions showing the presence of both amino acid and fatty acid parts were analyzed by tandem mass spectrometry (4800 Plus MALDI TOF/TOF, AB SCIEX, CA, USA) as described by Coronel et al. [13].

2.5. Surface tension and oil displacement test measurements

The surface tension was determined in triplicate by a GibertiniTensiometr (Milan, Italy) [14]. The oil displacement test was determined by adding 20 ml of distilled water to a Petri dish (diameter 90 mm). Subsequently, 100 µl of crude oil was layered onto the surface of the water forming a thin oil layer, and then 200 µl of cell free supernatant was placed onto the center of the oil slick. The diameter of the clear zone on the oil surface was measured [15,16].

2.6. Determination of critical micelle concentration (CMC)

The critical micelle concentration (CMC) of the crude and the purified biosurfactant was determined by plotting the surface tension as a function of the biosurfactant concentration. Biosurfactants were dissolved in Milli-Q water at concentration ranging from 0 to 500 mg/l (crude biosurfactant) or 0 to 100 mg/l (purified biosurfacatnt). For each concentration, surface tension measurement was measured until a constant value was reached. Results are expressed as the mean of three independent tests ± standard deviation [14].

2.7. Effect of pH, temperature and salinity on biosurfactant stability

The cell-free supernatants of strain FLU5 were exposed at different pH (pH 2.1 to pH 12); at various concentrations of NaCl (from 0 to 300 g/l) and at different temperatures (from 4 to 121 °C.). Subsequently, the surface tension was measured immediately for pH and salinity tests and after overnight incubations for temperatures ranging from 4 to 70 °C, one hour for temperature 100 °C and after autoclaving (20 min) for a temperature of 121 ° C [17].

2.8. Cytotoxicity assay

Cytotoxicity level of the biosurfactant BS-FLU5 was determined against HEK293 human embryonic kidney cell line using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay [18]. Briefly, the cells were seeded in 96 well plates at a density of 3. 10^4 and treated with BS-FLU5 at different concentrations (125 -1000 µg/ml), for 24 h and 48 h. Then, 10 µl of 5 mg/ml MTT solution were added to each well and incubated for 6 h at 37 °C and 5% CO_2. Finally, the formazan crystal formed was dissolved in 10% SDS and the absorbance was detected at 570 nm using microplate reader (Thermo Scientific Varioskan Flash). Cell viability of the different treatments was calculated as a percentage of the viable cells compared to control cells (cells treated with medium only). Three replicate wells were set for each treatment.

2.9. Application of BS-FLU5 in the removal of hydrocarbons from contaminated soil

Biosurfactant suitability for enhanced oil recovery was carried out using contaminated soil collected from a garden located in Sfax (Tunisia), with (20%, v/w) of used motor oil. Samples of 10 g of contaminated soil were transferred to 200-ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 20 ml water (control) or 20 ml of the cell-free broth of strain FLU5 using residual frying oil as carbon source or 20 ml of a solution of the isolated biosurfactant at the CMC (0.025%, m/v) and 20 ml of solutions of chemical surfactants: Tween 20 (0.05%, m/v), Tween 80 (0.0016%, m/v), Triton X-100 (0.0155%, m/v)

and sodium dodecyl sulfate SDS (0.2304%, v/v), at their CMC. The samples were incubated on a rotary shaker (180 rpm) for 24 h at 30 °C and then were centrifuged at 6000 rpm for 20 min for separation of the laundering solution and the soil. The supernatant phase was extracted two times (v/v) using hexane. The amount of oil residing in the soil after the impact of surfactants was gravimetrically determined [17].

3. Results and discussion

3.1. Assessment of biosurfactants production by strain FLU5 on different carbon sources

To determine the capacity of strain FLU5 to product biosurfactants, the use of nutrient broth (NB) and various carbon sources including crude oil, diesel oil, motor oil, used motor oil, corn oil, olive oil, residual frying oil and glycerol in the presence of basal medium was tested. As shown in table 1, strain FLU5 was able to utilize all the substrates tested as sole carbon and energy sources, in the presence of 30 g/l NaCl and at 37 °C. In addition, the growth was accompanied with biosurfactant production as shown by the reduction of surface tension and the oil displacement test (Table 1). Strain FLU5 showed a better growth on nutrient broth and vegetable oils (olive oil; residual frying oil and corn oil) than hydrocarbons (crude oil, used motor oil, motor oil and diesel fuel). During the growth of strain FLU5 on the different used substrates, the surface tension decreased, especially for nutrient broth and vegetable oils (RST between 28 and 33 mN/m) (Table 1). The criterion used for classification as a biosurfactant-producer is the ability to reduce the surface tension of a solution to 40 mN/m [16]. Moreover, the determination of the diameter of the clear zone, by using the oil displacement test during the growth of strain FLU5 on different substrates showed the formation of large and distinct halo zones of diameters between 3.5 and 8.7 cm, indicating adequate biosurfactant production. The highest values of diameter of the clear zone were obtained in the presence of nutrient broth (ODT = 7.2 cm) and vegetable oils (olive oil, corn oil and residual frying oil) (ODT = 8.7; 8.7 and 8.5 cm, respectively), as substrates (Table 1).

The biosurfactant yield was affected by the type of carbon substrate used. Maximum biosurfactant production (2.38 g/l) was observed when nutrient broth was used as the carbon source. The highest production in nutrient broth may be due to its high total proteins contents. The amounts of biosurfactants produced were found to be 2.25 g/l, 1.92 g/l and 1.88g/l when grown on olive oil, corn oil and residual frying oil, respectively. Least biosurfactant production (0.12-0.27 g/l) was observed on following carbon source glycerol, crude oil, diesel fuel, motor oil and used motor oil (Table 1). It was mentioned in the literature that hydrophobic substrates, including hydrocarbons and vegetable oils could induce the production of biological surfactants [8]. Nevertheless, the low synthesis of biosurfactants in the presence of hydrocarbons can be explained by the fact that these compounds are less biodegradable, due to their complex structures, leading to weak bacterial growth and thereafter low production of surfactants [19].

3.2. Production of biosurfactants by strain FLU5 using residual frying oil

From an economic perspective, the use of cheap alternative substrates is an important factor for successfully developing environmental friendly surfactants production. Alternative low cost substrates have been suggested for economical biosurfactant production, among which we can cite: molasses, cheese whey, dairy wastes, sludge palm oil, potato process effluents, vegetable oil refinery wastes, and more [20]. Water-immiscible substrates such as vegetable oils or hydrocarbon compounds are widely used for biosurfactant production [21]. Among the tested hydrophobic carbon sources, residual frying oil, due to its composition, its easy availability and its economical advantages, was found to be a prominent substrate for biosurfactant production by strain FLU5. In fact, large quantities of residual frying oil, which is a major source of nutrient rich low cost fermentative waste, are produced by the food industry and at the domestic scale [9]. After being used, cooking oil changes its composition and contains more than 30% of polar compounds depending on the type of frying, the variety

of food and the number of times it has been used [9]. Strain FLU5 showed an important capacity to use residual frying oil as carbon source (OD_{max} = 1.87) (Table 1, Fig. 1). In addition, during the growth of strain FLU5 on residual frying oil, the surface tension decreased from 64 to 36 mN/m, after 3 days of incubation at 37 °C and remained stable during the stationary phase (Fig. 1). The oil displacement test showed also the formation of a halo with an important diameter (8.5 cm), after the addition of culture supernatant of strain FLU5 growing on residual frying oil (Fig.1). These results support the potentiality of strain FLU5 to produce surface active agents using residual frying oil as carbon source. Consequently, strain FLU5 of *Bacillus stratosphericus* could be used to reduce wastes generated by food industries, and to convert residual frying oil, an alternate low-cost carbon source into higher value products. These biosurfactants can be utilized with minimum purity specification and hence applied for enhanced oil recovery.

There are few reports studying the production of biosurfactants using residual frying oils [7]. In this respect, *Pseudomonas aeruginosa* 47T2 NCIB 40044 used waste frying oil, an economic renewable substrate, to produce extracellular rhamnolipids [9]. Moreover *Rhodococcus erythroplis* 16 LM.USTHB can produce glycolipids using residual sunflower frying oil [22]. Furthermore, the efficiency of rhamnolipid biosurfactant production by the *Pseudomonas aeruginosa* D from waste frying coconut oil was investigated [23]. Recently, biosurfactant production by *Bacillus pumilis* CCT 2487 using waste frying oil was studied [24].

3.3. Characterization of biosurfactant produced by strain FLU5 (BS-FLU5)

The crude biosurfactant BS-FLU5 produced on nutrient broth and extracted from the acid precipitate was initially characterized by TLC which revealed a pink spot when sprayed with ninhydrin reagent which affirmed the presence of amino acids. The plate, when sprayed with phosphomolybdic acid reagent, produced a blue violet spot indicating the presence of

fatty acid groups (data not shown). The obtained results confirmed the presence of lipopeptide biosurfactant [1]. Further characterization was carried out by fractionation of lipopeptides using silica gel column. TLC was used to compare the level of each fraction migration in order to collect the similar ones, according to their molecular weight. Many fractions were collected but a single fraction showing the presence of both amino and fatty acids and the capacity to reduce the surface tension was analyzed by MALDI-TOF.

Mass spectra analysis of purified lipopeptides BS-FLU5 showed the presence of one well resolved cluster of peaks, at *m/z* values between 1044 and 1100 Da (Fig. 2). By comparing the mass with the mass numbers reported for the lipopeptide complexes from other *Bacillus* strains, the group of peaks could be assigned to surfactin isomers [6,25–31]. The mass spectra reported in Fig. 1 revealed the presence of five major $[M+Na]^+$ peaks at m/z 1044.7; 1058.8; 1072.8; 1086.8 and 1100.8 Da which differ each other by *m/z* 14. The peak with a *m/z* 1044.7 corresponded to the mass of $[M+Na]^+$ ion of surfactin with a fatty acid (FA) chain length of 14 or 15 carbon atoms, or pumilacidin C14. The peak at m/z 1058.8 could be assigned as sodium adducted of surfactin C15 or pumilacidin C14 or C15. The peaks at *m/z* 1072.8 Da and 1086.8 Da could be assigned as sodium adduct of pumilacidin C15 or C16 and pumilacidin C16 or C17, respectively. The last peak at *m/z* 1100.8 Da corresponded to the mass of $[M+Na]^+$ ion of pumilacidin with a fatty acid chain length of 17 (Fig. 2).

MALDI-TOF MS/MS analysis was also used in order to obtain more precise information on the chemical structure of lipopeptides. The fragment ion patterns of the parent ions at *m/z* 1044.7, 1058.8, 1072.8 and 1086.8 Da, reported in Fig. 3, shows fragments that can correspond to differences among some amino acids in the peptide moiety.

The fragmentation pattern of the pic *m/z* 1044.7 resulted in the appearance of two product ions series deriving from the initial opening of the lactone ring (Fig. 3A). The first

series included the fatty acid chain and the N-terminal product ions at *m/z* 931.6 and 800.5 corresponded respectively, to the losses of Leu / Ile (-113 Da) and Leu / Ile-Leu- H_2O (-244 Da), from the parent ion *m/z* 1044.7. Therefore, the amino acid residue at position 7 is a Leu or Ile. The second series enclosed the peptidic moiety inside the C-terminal product ions at *m/z* 707.4; 594.4 and 481.3, corresponding respectively, to the loss of C14 *β*-hydroxyl fatty acid chain-Glu (-337 Da), C14 *β*-hydroxy fatty acid side chain-Glu-Leu (-450 Da) and C14 *β*-hydroxy fatty acid chain-Glu-Leu-Leu (-563 Da) from the precursor ion *m/z* 1044.7 (Fig. 3A). The obtained results indicated that the peak at m/z 1044.7 is unambiguously a surfactin, with a fatty acid chain of 14 carbons, and Leu or Ile residue at position 7.

The same fragmentation model was observed with the sodiated molecule $[M+Na]^+$ at m/z 1058.8 (Fig. 3B). The fragmentation resulted in the appearance of product ions at m/z 945.6; 832.6 and 814.5, corresponding respectively of the consecutive losses of Leu / Ile (-113 Da), Leu / Ile-Leu (-226 Da) and Leu / Ile-Leu-H_2O (-244 Da). Therefore, the amino acid residue at position 7 is a Leu or Ile. Other peaks were observed at m/z 707.5; 594.4 and 463.3, corresponding respectively, to the losses of loss of C15 *β*-hydroxy fatty acid chain-Glu (-351 Da), C15 *β*-hydroxy fatty acid side chain-Glu-Leu (-464 Da) and C15 *β*-hydroxy fatty acid chain-Glu-Leu-Leu-H_2O (-595 Da) from the precursor ion *m/z* 1058.8 (Fig. 3B). The obtained results indicated that the peak at m/z 1058.8 is unambiguously a surfactin, with a fatty acid chain of 15 carbons, and Leu or Ile residue at position 7. Our results of fragmentation of the parent ions *m/z* 1044.8 and 1058.8 are in accordanace with those of and Pecci et al. and Jemil et al. [27,32].

In a similar manner, the mass peaks at *m/z* = 1072.8 (Fig. 3C) and 1086.8 (Fig. 3D) found in the mass spectrum of purified BS-FLU5 were identified as pumilacidins containing a *β*-hydroxyl fatty acid with a chain length of 15 and 16 carbon atoms, respectively. These

obtained results of fragmentation of the parent ions *m/z* 1072.8 and 1086.8 are in accordance with those of Branquinho [33]. .

Lipopeptides are among of the most commonly and interesting class of biosurfactants and they present a wide range of useful properties to be explored in several fields. Among the produced lipopeptides, surfactin known as a powerful biosurfactant, is the most recognized family [1]. Many studies have been reported on the involvement of bacteria belonging to *Bacillus* genus in production of biosurfactants including lipopeptides, but there are no reports that specifically mention the production of biosurfactants or lipopeptides by *B. stratosphericus* strains. Recently, Bezza et al. [34] reported the ability of a consortium culture of different species including *Bacillus stratosphericus* to degrade PAHs and to produce biosurfactants. Among *Bacillus* species capable of producing lipopeptides, we can cite: *B. subtilis* [30, 35–39], *B. pumilis* [40,41], *B. licheniformis* [36, 42–44], *B. amyloliquefaciens* [45–47], *B. mojavensis* [6,48,49], *B. tequilensis* [50] and *B. methylotrophicus* [32].

The ability of a biosurfactant to decrease the surface tension of a solution to less than 40 mN/m was considered to be a good characteristic of a potent tensioactive [51]. The crude biosurfactant of strain FLU5 was capable of reducing the surface tension of water from 72 to 34 mN/m. Moreover, the purified lipopeptides produced by strain FLU5 were able to bring down the surface tension of water to 28 mN/m, confirming the property of an efficiency surface active agent. Critical micelle concentration (CMC), which is defined as the concentration of surfactant above which micelles form and no further effect can be observed on the surface activity, is an important parameter during the evaluation of activity of a surfactant activity. Fig. 4A shows the plot of surface tension versus crude biosurfactant concentration that allowed calculating the CMC as being 250 mg/l and the corresponding surface tension was 34 mN/m. This concentration (CMC) decreases in the case of purified

lipopeptides, with a value of 50 mg/l, and, at this concentration, it was able to reduce the surface tension of the water from 72 to 28 mN/m Fig. 4B. The CMC value of any tensioactive agent is an indicator of its surfactant potentiality [52]. Thus, a powerful surfactant has a low CMC value. Moreover, the CMC value indicates the degree of purity of surfactant and thus, the CMC value decreases as the degree of purification increases [17,52]. This can explain the difference in CMC values between the crude and the purified biosurfactants BS-FLU5. The obtained result indicated that the lipopeptides produced by strain FLU5 was comparatively more effective than some other biosurfactants reported in literature because of its low CMC value (50 mg/l) [1]. *Bacillus* genus showed smaller CMC and surface tension values than other genera, such as *Pseudomonas* and *Candida* [53]. In this context, surfactin produced by *Bacillus subtilis* ATCC 21332 was able to reduce surface tension to less than 30 mN/m with critical CMC value of 45 mg/l [54]. The lipopeptides produced by a *Bacillus* sp. ZG0427 exhibited significant reduction of surface tension of water to 24.6 mN/m, with a CMC 50 mg/l [16]. Other study showed a notable ability of surfactin produced by *Bacillus cereus* NK1 to decrease tension surface to 36 mN/m at CMC of 45 mg/l [1].

As many factors influence the effectiveness of biosurfactant activities, the stability of BS FLU5 against challenging environmental conditions (pH, temperature and salinity) was evaluated to confirm the application of BS-FLU5 during remediation. The BS-FLU5 stability against various pH was tested at pH ranging from 2.1 to 12. It was observed that the surface tension of the biosurfactant solution remained steady at about 34 mN/m under different pH (2.1-12) (Fig. 5A). Similar results were found for lipopeptides produced by *Bacillus subtilis* ICA56 and showed that for all evaluated pHs (2-12), the surface tension remained almost constant and under 40 mN/m [5]. Meanwhile, other studies reported that the activity of lipopeptides was affected at alkaline conditions (pH 2-4) [34]. It was mentioned in previous

research that surfactin biosurfactant is generally more active at pHs around neutrality; however, highly acidic pH conditions cause more reduction in surface activity than highly alkaline conditions [34]. The thermal stability of BS-FLU5 showed that the product retained almost the same surface tension for a range of temperature from 4 to 121 °C (ST is between 34 and 37 mN/m) (Fig. 5B). This finding was in agreement with previous studies which demonstrated the great stability of lipopeptides at a wide range of temperatures [5, 16]. The stability of BS-FLU5 at high temperature (121 °C) suggested its usefulness in industrial processes and in microbial enhanced oil recovery operations [2,17]. The BS-FLU5 was stable during the increase of NaCl concentration from 0 to 120 g/l (ST is about 34 mN/m) (Fig. 5C). However, this stability was affected above 150 g/l NaCl (ST is about 38 mN/m at 150 g/l NaCl and 43 mN/m at 250 g/l NaCl). This behavior can be explained that the high salt concentrations can considerably reduce the size and shape of the micelle, then affecting the functional properties of a tensioactif [5]. The stability of the product at high concentrations of salt supports its application in the bioremediation of contaminated marine environments. In fact, once the microorganism was isolated from marine environment, the stability at high concentrations of salt was expected due to the adaptation of the bacterium and its metabolites to these conditions, which is the case of strain FLU5 [10]. These results highlight the potential applicability of the biosurfactant BS-FLU5 even at extreme conditions of pH, temperature and salinity.

3.4. Cytotoxicity of BS-FLU5

In order to investigate the possibility of the biosurfactant produced by *B. stratosphericus* FLU5 for industrial use, the cytotoxicity level of this product was evaluated. The application of BS-FLU5 showed a negligible cytotoxic effect on the HEK293 human embryonic kidney cell line, suggesting its utility as a biological material. At 24 h of the treatment, the cell viability was 96% in the case of 1000 µg/ml of BS-FLU5 (maximum

concentration), whereas 100% cell viability was achieved in the case of control. As the time of incubation increases, the cell viability is slightly reduced and after 48 h of incubation it was observed that viability of cells in the control is 99%, whereas in the case of 1000 µg/ml (maximum concentration), the viability was reduced to 92% (Fig. 6). In pursuant to ISO 10993-5, 2009, cell viability above 80% can be considered as non-toxic in nature [55]. Results obtained showed that the viability range of HEK293 human embryonic kidney cells was of 99 to 92 % on being treated with 250 to 1000 µg/ml of the crude BS-FLU5 (Fig. 6). Interestingly, no cytotoxicity was observed with HEK293 cells even when high concentrations were used (up to 1000 mg/l). This confirms the possible applicability of this tensioactif BS-FLU5 in biological uses. At present, there are little publications strictly devoted to toxicity of biosurfactants and they are commonly considered as low- or non-toxic. In this respect, the lipopeptides PE1 and PE2 produced by strain *Paenibacillus ehimensis* B7 showed a negligible cytotoxicity (Cell viability > 95%), against HEK293 cells at all of concentrations that were tested (1 µg/ml to 128 µg/ml) [56]. Moreover, the rhamnolipids produced by *Pseudomonas aeruginosa* PG1 did not exhibit any cytotoxic effect to mouse L292 fibroblastic cell line at concentration of 250 mg/l [57]. Furthermore, sophorolipids SLs produced by a non-pathogenic yeast *Candida bombicola*, exhibited a low cytotoxicity on human keratinocytes as the same as surfactin, which has already been commercialized as cosmetic material [58].

3.5. Application of BS-FLU5 in hydrophobic contaminants removal

In order to investigate the application of the biosurfactant produced by *Bacillus stratosphericus* FLU5 in hydrocarbon removal, a preliminary experiment using the crude biosurfactant BS-FLU5, the cell-free supernatant of culture FLU5 on residual frying oil and synthetic surfactants (Triton X-100, Tween 20, Tween 80 and SDS) was performed to verify the removal of the used motor oil pollutant from soil samples (Fig.7). The biosurfactant BS-FLU5 was more effective on the hydrocarbon remobilisation than the chemical surfactants.

The effect of solubilization of hydrocarbons was again more marked using cell-free broth containing the biosurfactant. In fact, a solubilization of used motor oil adsorbed in the soil of 3 times and 5 times was made, with BS-FLU5 and with cell-free broth, respectively, compared to the water control (Fig.7). However, the chemical surfactants showed a solubility which varies between 1.55 and 2.8 times compared to the water control.

In this respect, Chebbi et al. [59] reported that the crude biosurfactant BSW10 produced by *Pseudomonas aeruginosa* strain W10 revealed a great hydrocarbon- remobilization ability compared with the synthetic surfactant SDS, with around 2.4 fold solubility [59]. Previous studies mentioned the capacity of cell-free fermented broth contained a biosurfactant produced by *Bacillus subtilis* ICA56 to remove residual motor oil from sand [5]. Similarly, the crude rhamnolipid-containing preparations (cell-free broth) produced by strains *Pseudomonas aeruginosa* UCP0992 and *Pseudomonas* sp. 2B gave high capacity to remove hydrophobic contaminants of residual oil adsorbed in the soil [2,17]. Hence it was possible to use directly the whole-cell broth containing biosurfactants, without purification steps that accounts for up to 60% of the total production costs [17]. It may be suggested that the isolated *Bacillus stratosphericus* FLU5 is suitable candidate for oil industries applications and enhanced oil recovery, which require lower purity specifications, and consequently, lower costs for production.

4. Conclusion

In this present study, *Bacillus stratosphericus* FLU5, a novel biosurfactant-producing bacterium was isolated from contaminated seawater by hydrocarbons. Biosurfactant production was shown in the presence of a large variety of carbon sources. The use of residual frying oil as substrate is a promising and cheap alternative for the production of biosurfactants due to its easy availability and its rich composition. After extraction and purification, BS-

FLU5 biosurfactant, was found to be constituted of surfactin and pumilacidin isomers, using MALDI-TOF MS/MS analyses. The lipopeptides produced by *Bacillus stratosphericus* FLU5 show a stable surface tension reduction capacity under high temperatures (up to 121 °C), different pH (2.1-12) and saline conditions (0-120 g/l). This high stability of BS-FLU5 at extreme conditions supports its application in the bioremediation in hot, alkaline and hypersaline environments such as for bioremediation of oil spills at sea. The biosurfactant BS-FLU5 demonstrated negligible cytotoxic effect against HEK293 human embryonic kidney cell line, even at high concentration (up to 1000 µg/ml). The application of the lipopeptides from strain FLU5 in oil recovery from soil contaminated by used motor oil showed that BS-FLUS could remobilize hydrocarbons more effectively than the four suynthetic surfactants: Tween 20, Tween 80, Triton X-100 and SDS. These results highlight the interest for potential use of the lipopeptides produced by the new marine *Bacillus stratosphericus* strain FLU5 in a wide variety of industrial, environmental and biotechnological applications.

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References

[1] M.I. Sriram, K. Kalishwaralal, V. Deepak, R. Gracerosepat, K. Srisakthi, S. Gurunathan, Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus cereus* NK1, Colloids Surfaces B: Biointerfaces. 85 (2011) 174-181. doi:10.1016/j.colsurfb.2011.02.026.

[2] A. Aparna, G. Srinikethan, H. Smitha, Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B, Colloids and Surfaces B: Biointerfaces. 95 (2012) 23-29. doi:10.1016/j.colsurfb.2012.01.043.

[3] F. Anjum, G. Gautam, G. Edgard, S. Negi, Biosurfactant production through *Bacillus* sp. MTCC 5877 and its multifarious applications in food industry, Bioresource Technology. 213 (2016) 262-269. doi:10.1016/j.biortech.2016.02.091.

[4] O. Pornsunthorntawee, N. Arttaweeporn, S. Paisanjit, P. Somboonthanate, M. Abe, R. Rujiravanit, S. Chavadej, Isolation and comparison of biosurfactants produced by *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery, Biochemical Engineering Journal. 42 (2008) 172-179. doi:10.1016/j.bej.2008.06.016.

[5] Í.W.L. de França, A.P. Lima, J.A.M. Lemos, C.G.F. Lemos, V.M.M. Melo, H.B. de Sant'ana, L.R.B. Gonçalves, Production of a biosurfactant by *Bacillus subtilis* ICA56 aiming bioremediation of impacted soils, Catalysis Today. 255 (2015) 10-15. doi:10.1016/j.cattod.2015.01.046.

[6] H. Ayed, N. Hmidet, M. Béchet, M. Chollet, Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21, Process Biochemistry. 49 (2014) 1699-1707. doi: 10.1016/j.procbio.2014.07.001.

[7] R. Makkar, S. Cameotra, I. Banat, Advances in utilization of renewable substrates for biosurfactant production, AMB Express. 1 (2011) 5. doi:10.1186/2191-0855-1-5.

[8] B. Vijaya, N.R. Jayalakshmi, K. Manjunath, Enumeration of biosurfactant producing microorganisms from oil contaminated soil in and around Bangalore (India). International Journal of Current Science. 5 (2013) 86-94.

[9] E. Haba, M. Espuny, M. Busquets, Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils, Journal of Applied Microbiology. 88 (2000) 379-387. doi: 10.1046/j.1365-2672.2000.00961.

[10] W. Vilela, S. Fonseca, Production and properties of a surface-active lipopeptide produced by a new marine *Brevibacterium luteolum* strain, Applied Biochemestry and Biotechnology. 174 (2014) 2245-2256. doi: 10.1007/s12010-014-1208-4.

[11] D. Hentati, A. Chebbi, S. Loukil, S. Kchaou, J.-J. Godon, S. Sayadi, M. Chamkha, Biodegradation of fluoranthene by a newly isolated strain of *Bacillus stratosphericus* from Mediterranean seawater of the Sfax fishing harbour, Tunisia, Environmental Sciences and Pollution Research. 23 (2016) 15088-15100. doi:10.1007/s11356-016-6648-7.

[12] F. Widdel, N. Pfennig, Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids, Archives of Microbiology. 129 (1981) 395-400. doi:10.1007/BF00406470.

[13] J. Coronel-León, A. Marqués, Optimizing the production of the biosurfactant lichenysin and its application in biofilm control, Journal of Applied Microbiology. 120 (2016) 99-111. doi: 10.1111/jam.12992.

524 [14] S. Mnif, M. Chamkha, M. Labat, S. Sayadi, Simultaneous hydrocarbon biodegradation
525 and biosurfactant production by oilfield-selected bacteria., Journal of Applied
526 Microbiology. 111 (2011) 525-36. doi:10.1111/j.1365-2672.2011.05071.x.
527 [15] M. Hassanshahian, Isolation and characterization of biosurfactant producing bacteria
528 from Persian Gulf (Bushehr provenance), Marine Pollution Bulletein. 86 (2014) 361-
529 366. doi:10.1016/j.marpolbul.2014.06.043.
530 [16] H. Zhou, J. Chen, Z. Yang, B. Qin, Y. Li, X. Kong, Biosurfactant production and
531 characterization of *Bacillus* sp. ZG0427 isolated from oil-contaminated soil, Annals of
532 Microbiology. 65 (2015) 2255-2264. doi: 10.1007/s13213-015-1066-5.
533 [17] S.N.R.L. Silva, C.B.B. Farias, R.D. Rufino, J.M. Luna, L.A. Sarubbo, Glycerol as
534 substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992,
535 Colloids and Surfaces B: Biointerfaces. 79 (2010) 174-183.
536 doi:10.1016/j.colsurfb.2010.03.050.
537 [18] F. Hadrich, Z. Bouallagui, H. Junkyu, H. Isoda, S. Sayadi, The α-Glucosidase and α-
538 Amylase Enzyme Inhibitory of Hydroxytyrosol and Oleuropein, Journal of Oleo
539 Science. 8 (2015) 835-843.
540 [19] A. Chebbi, D. Hentati, H. Zaghden, N. Baccar, F. Rezgui, M. Chalbi, S. Sayadi, M.
541 Chamkha, Polycyclic aromatic hydrocarbon degradation and biosurfactant production
542 by a newly isolated *Pseudomonas* sp. strain from used motor oil-contaminated soil,
543 International Biodeterioration & Biodegradation. 122 (2017) 128-140. doi:
544 10.1016/j.ibiod.2017.05.006.
545 [20] R. Makkar, S. Cameotra, An update on the use of unconventional substrates for
546 biosurfactant production and their new applications, Applied Microbiology and
547 Biotechnology. 58 (2002) 428-434. doi:10.1007/s00253-001-0924-1.
548 [21] I.M. Banat, S.K. Satpute, S.S. Cameotra, R. Patil, N. V Nyayanit, G. Spano, Cost

549 effective technologies and renewable substrates for biosurfactants' production,
550 Frontiers in Micobiology. 5 (2014) 697. doi:10.3389/fmicb.2014.00697.
551 [22] Z. Sadouk, H. Hacene, Biosurfactants production from low cost substrate and
552 degradation of diesel oil by a *Rhodococcus* strain, Oil ans Gas Science and
553 Technology. 63 (2008) 747-753.doi: 10.2516/ogst:2008037.
554 [23] S. George, K. Jayachandran, Production and characterization of rhamnolipid
555 biosurfactant from waste frying coconut oil using a novel *Pseudomonas aeruginosa* D,
556 Journal of Applied Microbiology. 114 (2013) 373-383. doi: 10.1111/jam.12069/full.
557 [24] J. Oliveira, C. Garcia-Cruz, Properties of a biosurfactant produced by *Bacillus pumilus*
558 using vinasse and waste frying oil as alternative carbon sources, Brazilian Archives of
559 Biology and Technlogy. 56 (2013) 155-160. doi: 10.1590/S1516-89132013000100020.
560 [25] H. Chen, L. Wang, C. Su, G. Gong, Isolation and characterization of lipopeptide
561 antibiotics produced by *Bacillus subtilis*, Letters in Applied Microbiology. 47 (2008)
562 180-186. doi: 10.1111/j.1472-765X.2008.02412.
563 [26] P. Kim, J. Ryu, Y. Kim, Y. Chi, Production of biosurfactant lipopeptides Iturin A,
564 fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum*
565 *gloeosporioides*, Journal of Microbiology and Biotechnology. 20 (2010) 138-145. doi:
566 10.4014/jmb.0905.05007.
567 [27] Y. Pecci, F. Rivardo, M. Martinotti, LC/ESI-MS/MS characterisation of lipopeptide
568 biosurfactants produced by the *Bacillus licheniformis* V9T14 strain, Journal of Mass
569 Spectrometry. 45 (2010) 772-778. doi: 10.1002/jms.1767.
570 [28] C. Luo, X. Liu, H. Zhou, X. Wang, Identification of four NRPS gene clusters in
571 *Bacillus subtilis* 916 for four families of lipopeptides biosynthesis and evaluation of
572 their intricate functions to the typical, Applied and Environmental Microbiology.
573 (2014). doi: 10.1128/AEM.02921-14.

574 [29] C. Luo, H. Zhou, J. Zou, X. Wang, R. Zhang, Bacillomycin L and surfactin contribute
575 synergistically to the phenotypic features of *Bacillus subtilis* 916 and the biocontrol of
576 rice sheath blight induced by *Rhizoctonia solani*, Applied Microbiology and
577 Biotechnology. 99 (2015) 1897-1910. doi:10.1007/s00253-014-6195-4.
578 [30] I. Mnif, A. Grau-Campistany, J. Coronel-León, Purification and identification of
579 *Bacillus subtilis* SPB1 lipopeptide biosurfactant exhibiting antifungal activity against
580 *Rhizoctonia bataticola* and *Rhizoctonia solani*, Environmental Sciences and Pollution
581 Research. 23 (2016) 6690-6699. doi:10.1007/s11356-015-5826-3.
582 [31] N. Price, A. Rooney, J. Swezey, Mass spectrometric analysis of lipopeptides from
583 *Bacillus* strains isolated from diverse geographical locations, FEMS Microbiology
584 Letters. 271 (2007) 83-89. doi: 10.1111/j.1574-6968.2007.00702.
585 [32] N. Jemil, H. Ben Ayed, N. Hmidet, M. Nasri, Characterization and properties of
586 biosurfactants produced by a newly isolated strain *Bacillus methylotrophicus* DCS1
587 and their applications in enhancing solubility of hydrocarbon, World Journal of
588 Microbiology and Biotechnology (2016) 32:175. doi: 10.1007/s11274-016-2132-2.
589 [33] FRGPN Branquinho, Unveiling population diversity, biosurfactant and antibacterial
590 agents production of *Bacillus pumilis* group species. Thesis, Chapter 4: Species-
591 specific surfactin-like combinations of biosurfactants within *Bacillus pumilus* group,
592 Faculty of Pharmacy of the University of Porto (2014) 351-389.
593 species: diversity and anti-biofilm activity of a surfactin-like biosurfactant (2014) thesis
594 [34] F. Bezza, E. Chirwa, Biosurfactant-enhanced bioremediation of aged polycyclic
595 aromatic hydrocarbons (PAHs) in creosote contaminated soil, Chemosphere. 144
596 (2016) 635-644. doi: 10.1016/j.chemosphere.2015.08.027.
597 [35] A. Abdel-Mawgoud, M. Aboulwafa, Characterization of surfactin produced by Bacillus
598 subtilis isolate BS5, Applied Biochemistry and Biotechnology. 150 (2008) 289-303.

599 doi:10.1007/s12010-008-8153-z.
600 [36] Q. Liu, J. Lin, W. Wang, H. Huang, S. Li, Production of surfactin isoforms by *Bacillus*
601 *subtilis* BS-37 and its applicability to enhanced oil recovery under laboratory
602 conditions, Biochemical Engineering Journal. 93 (2015) 31-37. doi:
603 10.1016/j.bej.2014.08.023.
604 [37] F. Rivardo, R. Turner, G. Allegrone, H. Ceri, Anti-adhesion activity of two
605 biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial
606 pathogens, Applied Microbiology and Biotechnology. 83 (2009) 541-553. doi:
607 10.1007/s00253-009-1987-7.
608 [38] H. Zeriouh, A. Vicente, A. Pérez- García, Surfactin triggers biofilm formation of
609 *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity,
610 Environmental Microbiology. 16 (2014) 2196-2211. doi: 10.1111/1462-2920.12271.
611 [39] S. Yoon, J. Kim, Y. Lim, S. Hong, J. Song, Isolation and characterization of three kinds
612 of lipopeptides produced by *Bacillus subtilis* JKK238 from Jeot-Kal of Korean
613 traditional fermented fishes, Korean Society for Microbiology and Biotechnology. 33
614 (2005) 295-301.
615 [40] C.T. Slivinski, E. Mallmann, J. Magali De Araújo, D.A. Mitchell, N. Krieger,
616 Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation
617 using a medium based on okara with sugarcane bagasse as a bulking agent, Process
618 Biochemistry. 47 (2012) 1848–1855. doi:10.1016/j.procbio.2012.06.014.
619 [41] T. Fooladi, N. Moazami, P. Abdeshahian, A. Kadier, Characterization, production and
620 optimization of lipopeptide biosurfactant by new strain *Bacillus pumilus* 2IR isolated
621 from an Iranian oil field, Journal of Petroleum Science and Engineering. 145 (2016)
622 510-519. doi: 10.1016/j.petrol.2016.06.015.

[42] Y. Li, N. Haddad, S. Yang, B. Mu, Variants of lipopeptides produced by *Bacillus licheniformis* HSN221 in different medium components evaluated by a rapid method ESI-MS, International Journal of Peptide Research and Therapeutics. 14 (2008) 229-235. doi:10.1007/s10989-008-9137-0.

[43] S. Tendulkar, Y. Saikumari, V. Patel, Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen Magnaporthe, Journal of Applied Microbiology. 6 (2007) 2331-2339. doi: 10.1111/j.1365-2672.2007.03501.

[44] J. Coronel-León, G. de Grau, A. Grau-Campistany, Biosurfactant production by AL 1.1, a *Bacillus licheniformis* strain isolated from Antarctica: production, chemical characterization and properties, Annals of Microbiology. 65 (2015) 2065-2078. doi:10.1007/s13213-015-1045-x.

[45] N. Buensanteai, G. Yuen, S. Prathuangwong, The biocontrol bacterium *Bacillus amyloliquefaciens* KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant, Thai Journal of Agricultural Science. 41 (2008) 101-116.

[46] L. Sun, Z. Lu, X. Bie, F. Lu, S. Yang, Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from *Scutellaria baicalensis* Georgi, World Journal of Microbiology and Biotechnology. 22 (2006) 1259-12-66. doi: 10.1007/s11274-006-9170-0.

[47] V. Alvarez, D. Jurelevicius, J. Marques, *Bacillus amyloliquefaciens* TSBSO 3.8, a biosurfactant-producing strain with biotechnological potential for microbial enhanced oil recovery, Colloids and Surfaces B: Biointerface B. 136 (2015) 14-21. doi: 10.1016/j.colsurfb.2015.08.046.

[48] M. Snook, T. Mitchell, D. Hinton, Isolation and characterization of Leu7-surfactin

from the endophytic bacterium *Bacillus mojavensis* RRC 101, a biocontrol agent for *Fusarium verticillioides*, Journal of Agricultural and food Chemistry. (2009). doi: 10.1021/jf900164h.

[49] Z. Ma, N. Wang, J. Hu, S. Wang, Isolation and characterization of a new iturinic lipopeptide, mojavensin A produced by a marine-derived bacterium *Bacillus mojavensis* B0621A, The Journal of Antibiotics. 65 (2012) 317–322. doi:10.1038/ja.2012.19.

[50] A.K. Pradhan, N. Pradhan, G. Mall, H.T. Panda, L.B. Sukla, P.K. Panda, B.K. Mishra, Application of Lipopeptide Biosurfactant Isolated from a Halophile: *Bacillus tequilensis* CH for Inhibition of Biofilm, Applied Biochemistry and Biotechnology. 171 (2013) 1362–1375. doi:10.1007/s12010-013-0428-3.

[51] M. Pacwa-Płociniczak, G. Płaza, Environmental applications of biosurfactants: recent advances, International Journal of Molecular Sciences. 12 (2011) 633-654. doi:10.3390/ijms12010633.

[52] S. Mukherjee, P. Das, C. Sivapathasekaran, R. Sen, Antimicrobial biosurfactants from marine *Bacillus circulans*: extracellular synthesis and purification, Letters in Applied Microbiology. 48 (2009) 281–288. doi: 10.1111/j.1472-765X.2008.02485.

[53] A. Dadrasnia, S. Ismail, Biosurfactant production by *Bacillus salmalaya* for lubricating oil solubilization and biodegradation, International Journal of Environmental Research and Public Health. 12 (2015) 9848-9863. doi: 10.3390/ijerph120809848.

[54] L.-M. Whang, P.-W.G. Liu, C.-C. Ma, S.-S. Cheng, Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil, Journal of Hazardous Materials. 151 (2008) 155–163. doi:10.1016/j.jhazmat.2007.05.063.

673 [55] ISO Report, Biological Evaluation of Medical Devices, Part 5: Tests for In Vitro
674 Cytotoxicity. International Organization for Standardization, Geneva.10993 (2009)-5.
675 [56] Z. Huang, Y. Hu, L Shou, M Song, Isolation and partial characterization of cyclic
676 lipopeptide antibiotics produced by *Paenibacillus ehimensis* B7, BMC Microbiology.
677 13 (2013) 87.
678 [57] K. Patowary, R. Patowary, MC. Kalita, S. Deka, Characterization of Biosurfactant
679 Produced during Degradation of Hydrocarbons Using Crude Oil As Sole Source of
680 Carbon, Frontiers in Microbiology. 8 (2017) 279. doi: 10.3389/fmicb.2017.00279.
681 [58] Y. Hirata, M. Ryu, Y. Oda, K. Igarashi, A. Nagatsuka, T. Furuta, M. Sugiura, Novel
682 characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-
683 foaming surfactants, Journal of Bioscience and Bioengineering. 108 (2009) 142–146.
684 [59] A. Chebbi, D. Hentati, H. Zaghden, N. Baccar, F. Rezgui, M. Chalbi, S. Sayadi, M.
685 Chamkha, Polycyclic aromatic hydrocarbon degradation and biosurfactant production
686 by a newly isolated *Pseudomonas* sp. strain from used motor oil-contaminated soil,
687 International Biodeterioration and Biodegradation. 122 (2017) 128-140. doi:
688 10.1016/j.ibiod.2017.05.006
689