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Full Length Research Paper

Screening of potential biosurfactant-producing bacteria isolated from seawater biofilm

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Seawater represents a specific environment harboring complex bacterial community which is adapted to harsh conditions. Hence, biosurfactant produced by these bacteria under these conditions have interesting proprieties. The screening of biosurfactant producing strains isolated from seawater biofilm was investigated. Specific media, which have been reported to induce biosurfactant production, were used to prepare bacterial cultures and four methods; drop collapse, blue agar, blood-agar lysis and emulsification index, were applied to screen for biosurfactant production. 16 culturable, aerobic bacterial strains were isolated from biofilm in this study. Among these isolates, nine strains were Gram-positive and seven were Gram-negative. However the majority of the biosurfactant producer strains were Gram-negative belonging to different genera according to " a multitube micromethod for identification of bacteria (API system gallery)". Some of the isolated genera such as: *Bacillus, Pseudomonas, Micrococcus, Neisseria*, and *Aeromonas* are well known as biosurfactant producers, while other genera, mainly *Staphylococcus, Chrysomonas* and *Photobacterium*, were described for the first time as biosurfactant producers in this work.

Key words: Biosurfactant, seawater biofilm, bacteria.

INTRODUCTION

Surfactants are surface active agents that have a wide range of properties including lowering surface and interfacial tensions of liquids. Today, most industries, as those of paper, paint and protective coatings, textiles, agriculture, food and beverages, pharmaceutical, cosmetics use chemically synthesized surfactants (Kosaric, 2001). However, Interest in microbial surfactants has been steadily increasing in recent years since they represent a promising alternative to chemical surfactants. Biosurfactants are amphiphilic molecules of glycolipids (sophorose lipid, rhamnose lipid), hydroxylated and cross-linked fatty acids, polysaccharide-lipid complexes, lipoprotein-lipopeptides (surfactin), and phospholipids. These molecules, with specific functional groups, are often specific in their action and have proprieties of wetting and penetrating actions, spreading, hydrophylicity and hydrophobicity of surfaces, metal sequestration, microbial growth enhancement, or anti-microbial activity, Moreover; they are effective even in extreme conditions such as high temperature, pH or salinity (Kosaric, 2001). Possible applications of biosurfactants as emulsifying agents for drug transport to the infection site, supplementary agents to pulmonary surfactant and adjuvants for vaccines, have been reported by Ligia and José (2010). Some studies demonstrated that the type of biosurfactant produced by some bacterial strains is modified when growing on hydrocarbons and oils (Duvnjak and Kosaric, 1985; Robert et al., 1989; Makkar and Cameotra, 1999; Nilanjana and Preethy, 2011).

Some bacteria are able to form biofilms, forming matrixes with extracellular polymeric substances. In fact, biofilms represent a special environment where bacteria produce substances to ensure their adherence and solubilise other compounds such as unavailable substrates.

All these proprieties confer biosurfactants an environmental and health friendly status mainly due to their biodegradability, diversity, low toxicity, biocompatibility

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and digestibility (Zhaozhe et al., 2003).

Using four different methods, we screened for biosurfactant producer bacteria isolated from seawater biofilms formed in pipelines used for the refrigeration of petroleum derivatives which attain high temperatures. This approach enabled us to isolate biosurfactant producing strains that belong to bacteria genera that have not been reported to have this property.

MATERIALS AND METHODS

Sampling

Samples were collected from biofilms created in pipeline filters conducting seawater used to refrigerate oil derivatives after oil extraction process. The involved pipelines were located in the petroleum refinery of Bizerte, Tunisia. The biofilm samples were detached from the inner parts of the pipelines, placed into sterile bottles containing 50 ml phosphate-buffered saline (PBS), pH 7, and then stored at 4°C.

The PBS medium contained, per litre, 8 mg NaCl, 0.2 mg KCl, 1.15 mg Na_2HPO_4, 0.2 g KH_2PO_4, 0.1 mg $CaCl_2$ and 0.1 mg $MgCI_2.6H_2O$.

To screen bacterial strains producing biosurfactants, samples were vortexed vigorously and decanted for 4 h. The bronze colour supernatant was used to inoculate enrichment culture media.

Screening for culturable biosurfactant-producing isolates

Two media (media A and B) allowing adequate production of bacterial biosurfactant were prepared. Medium A contained, per litre, 4 g Na_2HPO_4, 1.5 g KH_2PO_4, 1 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.005 g of iron ammonium citrate, 0.01 g $CaCl_2$ and 2% glycerol (Bodour and Miller-Maier, 1998); the pH was adjusted to 7.2. The medium B contained, per litre, 1 g meat extract (Biorad), 1 g yeast extract (Biorad), 2 g N-Z amine (Sigma), 0.02 g $CaCl_2$ and 10% (w/v) mineral oil (Sigma) (Cheeptham, 1999); the pH of the medium B was adjusted to 7.2. Fifty millilitre of each media A and B were mixed in a 250-ml flask and the mixture was inoculated with 1 ml of supernatant. Flasks were incubated at 30°C for seven days, with agitation at 200 rpm. After incubation, 100 µl of enriched cultures were spread on agar plates containing 2% glucose as the sole source of carbon and energy, mineral salt medium (MSM), supplemented with and 18 g L^{-1} of agar.

In this set of experiment, we purified the different cultured isolates by repeated streaking on the same medium (MSM + 2% glucose + 18 g L^{-1} agar) and identified them using morphological and biochemical characteristics along with an API system gallery, (API 20E, API 50CH, API Staph, and APILAB 3.3.3 software, Biomérieux). Each bacterial isolate was checked for production of biosurfactant, using the following methods cited below. Sodium dodecyl sulphate (SDS) solution (1 g L^{-1}) (Sigma) and sterile distilled water were used as positive and negative controls respectively.

Blue agar plate method (BAPM)

Pure culture of each isolate was streaked in mineral salt agar medium containing, per liter, 20 g glycerol, 0.7 g KH_2PO_4, 0.9 g Na_2HPO_4, 2 g $NaNO_3$, 0.4 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.2 g cethyl trimethyl ammonium bromide (CTAB), 0.005 g methylene blue, 15 g agar, (Siegmund and Wagner, 1991) and 2 ml oligoelements (Van Hamme and Ward, 2001) solution (acidified with HCl 37%). The oligoelements medium contain, per litre, 1.5 g nitrilotriacetic acid, 5.0 g $MnSO_4·2H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2·2H_2O$, 0.1 g $ZnSO_4·7H_2O$, 0.01 g $CuSO_4·5H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3·16H_2O$, 0.01 g H_3BO_4 and 0.01 g $Na_2MoO_4·2H_2O$.

After incubation for period of 48 h at 30°C, a dark blue halo around the culture was formed indicating biosurfactant production. The halo intensity is proportional to quantity of biosurfactant produced.

Blood agar lysis method (BALM)

Hemolytic activity was detected after inoculation of the isolates in MSM, supplemented with 2% glycerol as the source of carbon and energy, and 5% fresh sheep blood. Hemolytic zones formed around colonies indicated biosurfactant production. Plates were incubated at 30°C for 48 h (Youssef et al., 2004).

Modified drop collapse test (MDC)

A 96-well microtiter plate was rinsed subsequently with hot water (three times), ethanol (75%), distilled water, then dried with air (Bodour and Miller-Maier, 1998). After preparation, well surfaces were coated with a thin layer of crude oil and equilibrated for 24 h to ensure uniform oil coating. A drop of each bacterial culture was then transferred on the prepared oil layers. The result is scored positive if the drop collapse and negative if the drop remained having round shape.

Determination of emulsification index (EI)

To determine the emulsification index, the isolates were cultured in MSM supplemented with 2% glycerol and 10% mineral oil. After incubation at 30°C for five days, residual mineral oil was eliminated after decantation. For each isolate, 2 ml of cell suspension was mixed with 3 ml of mineral oil and vortexed for 2 min. The test tubes were maintained at 30°C and the height of the emulsion layer was measured after 24 h to determine the emulsification index (Viramontes-Ramos et al., 2010). The emulsion test was run for both the mineral and the crude oil. Negative control test was run with uninoculated assay. To determine the emulsification index (EI) the following equation was used.

$$EI = \frac{\text{Height of emulsion}}{\text{Height of total solution}} \times 100\%$$

RESULTS AND DISCUSSION

Biofilms, created in pipelines conducting seawater represents a specific environment harboring complex bacterial community. At the outlet of these pipelines, the seawater temperature was 50°C and the salinity was 35 g L^{-1}.

Since anaerobic strains are much more difficult to isolate and manipulate, only aerobic strains have been isolated. Thus, under aerobic conditions, 16 culturable bacterial strains were isolated from the collected biofilm. Using API system gallery, bacterial identification revealed that the isolated strains belong to different genera (*Bacillus, Staphylococcus, Micrococcus, Aeromonas, Pseudomonas, Photobacter, Chrysomonas* and *Neisseria*).

Table 1. Characterization and identification of the isolated strains.

Isolates reference	Gram stain	Microbial group affiliation	MDC	BALM	BAPM	Emulsion index (EI) (%)	
						Crude oil	Mineral oil
A1	G+	*Bacillus coagulans*	-	-	-	2.15 ± 0.58	3.26 ± 0.62
S1	G+	*Bacillus circulans*	-	+	-	3.65 ± 0.45	3.03 ± 0.94
13B	G+	*Micrococcus* spp	+	+	+	8.36 ± 1.57	18.16 ± 2.75
AP5	G+	*Staphylococcus aureus*	+	+	+	7.96 ± 1.47	12.39 ± 2.64
A5	G+	*Staphylococcus capitis*	-	+	-	3.75 ± 1.07	3.36 ± 0.88
AP	G+	*Staphylococcus hominis*	-	+	-	3.65 ± 1.4	4.22 ± 1.09
S5	G+	*Staphylococcus saprophiticus*	+	-	+	10.85 ± 1.33	17.2 ± 0.87
12H	G+	*Staphylococcus epidermidis*	+	-	+	9.04 ± 0.56	13.76 ± 0.96
G	G+	*Staphylococcus* spp	-	+	-	3.62 ± 0.48	4.94 ± 1.89
F1	G-	*Aeromonas* spp	+	+	+	6.03 ± 0.92	9.47 ± 0.98
AP6	G-	*Pseudomonas alcaligenes*	+	+	+	13.34± 0.99	20.29 ± 2.1
14G	G-	*Pseudomonas* spp	+	+	+	15.63± 0.67	17.85 ± 3.88
E2	G-	*Pseudomonas aeruginosa*	+	+	+	34.34± 0.99	42.02 ± 0.8
S4	G-	*Photobacterium damsel*	+	+	+	7.53 ± 1.19	9.43 ± 0.47
A7	G-	*Chrysomonas luteola*	+	+	+	7.93 ± 0.56	13.30 ± 0.93
13C	G-	*Neisseria* spp	-	-	-	4.74 ± 0.58	4.48 ± 2.7
Positive control (SDS 1 g L^{-1})			+	+	+	57.33 ± 0.15	64.56 ± 0.97
Negative control (SDW)						1.49 ± 0.23	1.66 ± 0.16

G+, Gram positive; G-,Gram negative; MDC, modified drop collapse, BALM, blood agar lysis method; BAPM, blue agar plate method; SDW, sterile distilled water.

Among these isolates, nine strains were Gram-positive and seven Gram-negative (Table 1).

Among the Gram-positive strains, five isolates (*Bacillus coagulans*, *Bacillus circulans*, *Staphylococcus capitis*, *Staphylococcus hominis* and *Saphylococcus* spp.) were not considered to be biosurfactant producers based on the MDC and BAPM results. In addition, the emulsion index of these strains was usually lower than 4.

Although *S. capitis*, *S. hominis* and *Saphylococcus* spp. were known to be unable to produce biosurfactants, it was found that they had the ability to lyse blood cells. Except for *B. coagulans*, *Staphylococcus saprophiticus* and *Staphylococcus epidermidis*, all Gram-positive strains were hemolytic as demonstrated by the BALM results.

Apart from the strain identified as *Neisseria* spp., all other Gram-negative strains were biosurfactant producers as revealed by the methods used (Table 1). Within these bacterial populations, the highest emulsion index for mineral and crude oil, 42.02 and 34.34%, respectively was registered for *P. aeruginosa*.

Considering all positive results, we noted that emulsion index for mineral oil is usually more important than that of crude oil. This can be explicated by a variability of biosurfactants production following available carbon source. In fact, Jitendra and Ibrahim (1997) reported that the production of rhamnolipid by *Pseudomonas* spp. is related to the used carbon source and its water solubility. Biofilms are a special environment that includes a variety of microorganisms. To ensure their adherence, many bacterial strains produce polymers. In addition to their role as adhesive substances, many polymers have antibiotic and biosurfactant effects (Korenblum et al., 2005). These molecules can be tailor-made to suit different applications by changing growth substrates or growth conditions, which confer original proprieties to biosurfactants (Rodrigues et al. 2006). In this study, we screened biosurfactant producing strains from biofilms formed in marine and hot environment.

The reduced number (16 strains) of isolates producing biosurfactant found in our study may be due to the inner conditions existing in prevalent in pipelines (temperature and salinity) or to some exopolysaccharides produced by some bacterial strains. In fact, Inmaculada et al. (2006) reported that halophillic strains such as *Halomonas maura* are capable of producing biofilms and that exopolysaccharides forms an integral part of the structural organisation of these biofilms. The production of exopolysaccharides is regulated by a cell-to-cell communication mechanism. Furthermore, this mechanism involves changes in the concentration of diffusible autoinducer to provide a regulatory signal in response to bacterial population density.

The preliminary bacterial identification reveals that isolated strains belong to different genera (*Bacillus*, *Staphylococcus*, *Micrococcus*, *Aeromonas*, *Pseudomonas*, *Photobacter*, *Chrysomonas* and *Neisseria*). While studied biofilms is formed in marine environment, some genera were known essentially in hospital fields such as *Aromonas*, *Chrysomonas*, *Neisseria* and *Pseudomonas*. The isolation of these strains from saline and hot water revealed their resistance to harsh conditions of salinity and temperature.

Since some strains showed positive biosurfactant production following one method and negative following other methods, it seems difficult to confirm the biosurfactant production using only one method. In view of this, it appeared that several screening methods have to be combined in order to understand the ability of bacteria to produce biosurfactant. Hence, for efficient detection of potential biosurfactant producers, combination of various screening methods is required.

Blood agar lysis method has been recommended as a simple and easy method to test biosurfactant activity (Banat, 1993; Yonebayashi et al., 2000) and has been used to quantify surfactin and rhamnolipids (Johnson and Boese-Marrazzo, 1980; Moran et al., 2002), as well as to screen biosurfactant production by new bacterial isolates (Mulligan et al., 1984; Banat, 1993; Yonebayashi et al., 2000). Carrillo et al. (1996) found an association between hemolytic activity and surfactant production, and recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity. In our study, using this method, two strains identified as *S. epidermidis* and *S. saprophyticus* did not show any hemolytic activity. On the contrary, these strains were shown to be biosurfactant producers using MDC, BAPM and EI. Moreover, the three strains identified as *S. capitis, S. hominis* and *Staphylococcus* spp. were found to have hemolytic activity but they were not biosurfactant producers when tested by MDC, BAPM and EI methods (Table 1). Results obtained with BALM were contradictory compared to other used methods; informing that this cannot be used as a single and exclusive evaluation of biosurfactant production. These results corroborate the findings of Youssef et al. (2004) and confirm that not all biosurfactants have a hemolytic activity and other compounds different from biosurfactants cause hemolysis. In his study, it was found out that, of the 81 strains that did not lyse blood agar, 31 were positive for biosurfactant production both by oil spreading technique and drop collapse method, and nine other strains were positive for biosurfactant production by the oil spreading technique.

Drop collapse was suggested to be used as a sensitive and easy method to test production of biosurfactant (Jain et al., 1991) and could be quantitative (Bodour and Miller-Maier, 1998). However, in this study, it was only applied as a qualitative method to detect biosurfactant production.

Using the BAPM, the compound CTAB revealed the presence of only glycolipidic and anionic biosurfactant (Youssef et al., 2004; Anand et al., 2009). Thus, the bacterial potential to produce other types of biosurfactant could be detected with both MDC and EI.

Besides, it seems that the emulsification index depends on the substrate used for emulsification. In fact, although the positive results registered with blue agar and drop collapse method, the strains identified as *S. aureus*, *Micrococcus* spp., *Pseudomonas* spp., *P. aeruginosa*, *Photobacter damsela* and *Chrysomonnas luteola* seemed to be more efficient to emulsify mineral oil over crude oil. The higher emulsification index for mineral oil could be explained by either the use of mineral oil as carbon source in the culture preparation and isolation, or by the specificity of the produced biosurfactant. Indeed, some studies have demonstrated that carbon source used for microbial growing, influenced the production of different biosurfactant types (Duvnjak and Kosaric, 1985; Robert et al., 1989; Makkar and Cameotra, 1999).

In this study, considering the MDC, BAPM and EI, it is concluded that the number of Gram-negative biosurfactant producing strains (six strains) was higher than Gram-positive one (four strains). Our results corroborate with that of Satpute et al. (2008) who found, using eight different methods, that the number of Gram-negative biosurfactant producer strains in seawater and sea sediment is more pronounced than that of Gram-positive strains. Nevertheless, in soil samples, Bodour et al. (2003) found that out of 45 putative biosurfactant producers, 47% bacteria were Gram-negative and 53% were Gram-positive. Based on the above discussion, it can be concluded that the biosurfactant production is related to the environmental conditions and the substrates used.

The genus *Bacillus* spp. has been reported to produce a variety of lipoprotein surfactants with many isoforms such as surfactin, iturin, fengycin, lichenysin (Bodour et al., 2003). In our study, we have isolated only two *Bacillus* species (*B. coagulans* and *B. circulans)*.

Furthermore, in the current study, the emulsification index obtained with all *Pseudomonas* species was more pronounced when using mineral oil than crude oil. Indeed,

Sean Norman et al. (2004) reported that *P. aeruginosa*, as well as other species of the genus *Pseudomonas* were frequently isolated from petroleum-contaminated sites and were capable of producing metabolites (that is, alginate, rhamnolipid and pyocyanin) that enhance its competitiveness and survival. Moreover, water-soluble carbon sources such as glycerol, glucose, mannitol and ethanol, have all been used for rhamnolipid production by *Pseudomonas* spp. However, the biosurfactant product was inferior to that obtained with water-immiscible substrates, such as n-alkanes and olive oil (Jitendra and Banat, 1997).

Although few interests were given to *Staphylococcus* as a biosurfactant producer, we found that three species of this genus demonstrate positive results using four different methods. In fact, Jitendra and Banat (1997) reported that surface activity in pathogenic bacteria is attributed to several cell surface components, such as protein A for *Staphylococcus aureus*. According to our knowledge, *Chrysomonas luteola* and *Phtobacterium damsela* isolated in this study have not been described as biosurfactant producers elsewhere although they have been frequently isolated from seawater and biofilms (Fouz et al., 2000; Laramée et al., 2000).

The salinity tolerance, biofilm adherence and biosurfactant production found in *S. aureus, S. saprophyticus, S. epidermidis, C. luteola* and *P. damsela,* seem to be interesting for both environmental and medical applications and need further investigation to know the molecular biosurfactant structure. In addition, biosurfactants role as anti-adhesive agents against several pathogens may indicate their utility as suitable anti-adhesive coating agents for medical insertional materials, leading to a reduction in hospital infections without the use of synthetic drugs and chemicals (Rodrigues et al., 2006; Ligia, 2011).

Further detailed biochemical and molecular studies of these compounds are required in order to unravel their structure and identification. Also, chromatographic and spectrometric analyses will have to be performed to elucidate their primary molecular structure. The preliminary results obtained in the current study constitute a stimulus for future research aiming at the evaluation of the potential antibacterial function of these compounds as well as their competences to enhance the availability of various hydrocarbonic compounds such as polyaromatic hydrocarbon and pesticides for microbial degradation.

Conclusion

In the present study, using four qualitative methods, we demonstrated that many bacteria, isolated from seawater biofilm, were able to produce biosurfactants. Among isolated bacteria, some genera such as *Pseudomonas* were well known as biosurfactant producers, others were revealed, for the first time, to be able to produce biosurfactants. Thus, more interest must be given to others genus such as *Staphylococcus*, *Photobacter* and *Chrysomonas* in order to characterize the molecular structure of these biosurfactants which can potentially be used in the environmental and medical fields.

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