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Chapter

Biosurfactants from Marine Microorganisms

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Abstract

The marine biosphere represents a yet underexploited natural source of bioactive compounds, mainly of microbial origin. Among them, biosurfactants (BSs) are functional molecules, which are attracting a great interest due to their biocompatibility, versatility, and applications in several biotechnological fields. BSs are surface active amphipathic compounds, containing both a hydrophilic and a hydrophobic moiety, which are grouped in low (glycolipids and lipopeptides) or high molecular weight (polymeric complexes) compounds. A number of environmental factors such as pH, salinity, temperature, and nutrient availability can affect microbial BS production. Marine microorganisms with different phylogenetic affiliations and isolated from several marine habitats (e.g., seawater, sediments, and higher organisms) worldwide (spanning from the Mediterranean Sea to Antarctica) have been reported as surfactant producers. However, most of the marine microbial world remains still unexplored. The present chapter aims at giving a general overview on the recent advances about BSs of marine origin, in order to enhance the knowledge inherent their production, chemical characterization and identification, interesting biological properties, and potential biotechnological applications.

Keywords: bacteria, surfactants, aquatic environments, biodegradability, natural tensioactive, biotechnology

1. Introduction

In aquatic ecosystems, marine microorganisms have developed unique metabolic and physiological capabilities to adapt to diverse habitats which cover a wide range of thermal, pressure, salinity, pH, and nutrient conditions [1]. In this way, the symbiotic association and the interaction of biological systems with bacteria have induced the production of a variety of bioactive compounds such as biosurfactants (BSs), antibiotics, enzymes, vitamins, [2] and more than 10,000 metabolites with a broad spectrum of biological activities that have been isolated from marine microbes. By evolution, bacteria have adapted themselves to feeding on water-immiscible materials by producing and using surface-active products that help them in aqueous phase to adsorb, emulsify, wet, and disperse or solubilize water-immiscible materials [3]. In this context, surface active metabolites as BSs have gained much attention because of their biodegradability, low toxicity, and ability to be produced from renewable and cheaper substrates, thus getting an important ecological role due to their structural, functional diversity, and the potential multidisciplinary applications in industrial and environmental fields. These features in

addition to their stability at extremes of temperatures, pH, and salinity make them commercially optimal alternatives to their chemically synthesized counterparts. For this reason, different authors have tested BSs of marine origin for environmental applications such as bioremediation processes, dispersion of oil spills, and enhancement of oil recovery. Furthermore, some BSs play an essential role for the survival of microbial producers against other competing or dangerous microorganisms by acting as biocide agents [4], and some others have shown the ability to stimulate plant and animal defense responses against microbes [5]. By considering that, these aspects have been explored especially for terrestrial bacteria, in the light of the above said characteristics, BSs from marine bacteria have been getting attention more and more as new suitable alternatives to chemical surfactants of petroleum origin in the food, cosmetic, health care industries [6], synthetic medicines and can be safe and effective therapeutic agents in medicine.

The purpose of this chapter is to provide a comprehensive overview on the recent advances about different types of marine BSs, to highlight the recent studies on the new sources of production, and to focus on the state of art of the screening methodologies for the identification, characterization, and potential biotechnological applications.

2. Biosurfactants

BSs are secondary metabolism bacterial products which exhibit surface and emulsifying activities thanks to the hydrophobic part of the molecule and the hydrophilic water soluble end. They are produced extracellularly or as a part of the cell membrane by a variety of yeasts, bacteria, and filamentous fungi from various substrates as sugars, oils, alkanes, and wastes [7]. The name *surfactant* derives from their surface chemical action, as they tend to interact at the boundary level between two phases in a heterogeneous system by forming a film which can change the properties (wettability and surface energy) of the original surface. They are mainly classified in BSs acting by reducing surface tension at the air-water interface (biosurfactants), and BSs that reduce the interfacial tensions between immiscible liquids or at the solid-liquid interface (bioemulsifiers) [8]. The investigations on BS production from microorganisms are more and more frequent and are subdivided into three main steps: (1) the potential BS producer isolation; (2) the screening for BS production; and (3) the extraction and purification step, sometimes improved with the chemical characterization of molecules. According to Ref. [6], the producer isolation and detection are crucial phases in the research for new BSs, which have to be strongly related to the aim of the investigation. While the terrestrial sources have been extensively explored, the marine environments have been focused as potential optimal source only in recent decades. It has been reported that BS-producing bacteria are widely distributed in both contaminated and undisturbed water or soil [9, 10]. Indeed, the most exploited source of microbial BS producers is represented by sediment and water samples, with different types and levels of contamination [11–13]. As a matter of fact, it is retained that microbial degraders of insoluble substrates, that is, hydrocarbons or oils, are stimulated to produce secondary metabolites that are able to enhance the cellular uptake and degradation of such compounds. In some cases, enrichment cultures have been performed with natural samples in order to favor the isolation of potential producers [14, 15]. Despite the application of this principle has gained optimal results in the discovery of new marine producers and BSs [14, 16], some authors assessed that the BS production is not strictly linked

to the hydrocarbon uptake [17, 18]. Only recently, innovative marine sources of isolation has been proposed, and researchers have focused their studies on biological matrices, that is, filter-feeding organisms as host of microbial communities specialized in the production of secondary metabolites with functional roles. As reviewed in [6], BS producers with optimal potentialities have been isolated from polychaetes [19, 20], sponges [21–25], sea pens [26], cnidarians [27, 28], and fish [29]. A common characteristic of BSs is to relax or decrease surface tension and this increases solubility so that BSs may interact with the interfaces and affect the adhesion and the detachment of bacteria [4]. All these properties confer to the BS antibacterial, antifungal, and antiviral activities [30], in addition to the pollutants removal potential. For these reasons, particular growing interest at global level is centered into new and unexplored sources of BSs as marine and deep-sea environments represent.

2.1 BS classification/types, census of BS marine microbial producers

Surfactants are generally classified according to the nature of the charge on polar moiety: anionic (negatively charged), nonionic (polymerization products), cationic (positively charged), and amphoteric (both negatively and positively charged). They can be grouped into two categories: (a) low molecular mass molecules (rhamnolipids, sophorolipids, trehalose lipids, lipopeptides phospholipids, fatty acids, and neutral lipids) [31], which lower the surface and interfacial tensions and (b) high molecular polymer mass (high molecular weight polysaccharide, polysaccharide-protein complexes, lipopolysaccharides, and lipoproteins called emulsans), which bind tightly to surfaces [32]. Various microbial species produce different BSs [33]. The most known ones are glycolipids, a class of molecules made of mono-, di-, tri- and tetra-saccharides which include glucose, mannose, galactose, glucuronic acid, rhamnose, and galactose sulfate in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among glycolipids, rhamnolipids, and trehalolipids, sophorolipids are the most studied disaccharides. Rhamnolipids are known to be produced by the *Pseudomonas* genus [31, 34], while trehalolipids are generally produced by many members of the genera *Mycobacterium*, *Nocardia* and *Corynebacterium*, *Rhodococcus erythropolis*, and *Arthrobacter* sp. [35]. Finally, sophorolipids represent a group of BSs by *Candida* spp. [31]. Another class of surfactant compounds which received considerable attention is the fatty acids such as the phospholipids derived from alkane substrates. Indeed, they represent the major components of microbial membranes which are mainly produced by hydrocarbon-degrading bacteria as *Acinetobacter* sp. *Rhodococcus erythropolis*, *Thiobacillus thiooxidans, Capnocytophaga* sp., *Penicillium spiculisporum,* and *Corynebacterium* sp., or yeasts [32]. Moreover, a number of microorganisms (e.g., *Candida lipolytica* and *Saccharomyces cerevisiae*) with different taxonomic affiliations produce exocellular polymeric surfactants called bioemulsans, composed of polysaccharides, protein, lipopolysaccharides, and lipoproteins, among which the bioemulsans produced by different species of *Acinetobacter* are the most studied [36]. Different authors described microbial BSs from marine sources (sediments, corals, sponges, sea, and hot water springs) which include several lipopeptide antibiotics with potent surface-active properties [22, 37–41]. Some authors [22] detected carbohydrate, protein, and lipid contents (20 μg/0.1 ml, 35 μg/0.1 ml, and 573 μg/0.1 ml, respectively) in the analyzed BS, suggesting a chemical structure typical of a lipopeptidic compound for the sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. Further chemical analyses [Fourier-transformed infrared spectrophotometer analysis (FT-IR), nuclear magnetic resonance (NMR), and gas chromatography

mass spectrometry (GC/MS)] were carried out by other scientists [21, 23, 28], and lipopeptidic BSs were detected for *Bacillus* spp. strains, in line with previous data on the neighboring cluster *Bacillus* as prominent lipopeptide producers. Interestingly, the lipopeptide identified in [23] was firstly demonstrated from *Brevibacterium* stain MSA13 (so-called *brevifactin*) and showed a different structure respect to that observed for other lipopeptides. Indeed, it was composed of a hydrophobic moiety of octadecanoic acid methyl ester and a peptide part contained a short sequence of four amino acids including pro-leu-gly-gly (**Figure 1**). Similarly, the possible production of novel BSs by *P. rettgeri*, *Psychrobacter* sp., and *B. anthracis* isolates was suggested by the analysis of FTIR spectra [28].

In the marine context, the group of glycolipids has been widely studied because they are produced by a broad spectrum of bacteria isolated from various marine matrices, both animals (Annelida, sea pen *Pteroeides*, and fish gut) and contaminated soils (Arctic and Antarctic sediments) [14, 19, 20, 24, 26, 29, 42]. A glycolipopeptide BS produced by the coral-associated *Bacillus* sp. E34 was identified by means of FT-IR analysis [27].

A list of various studied BS types with the respective microbial producers and the source of isolation is shown in **Table 1**.

Finally, other studies drew their attention on a group of heterogeneous high molecular weight bioactive compounds not strictly defined as biosurfactants but endowed with interfacial properties, called exopolysaccharides (EPSs), often originated from marine both prokaryotes and eukaryotes (cyanobacteria and microalgae) and extreme environments [43].

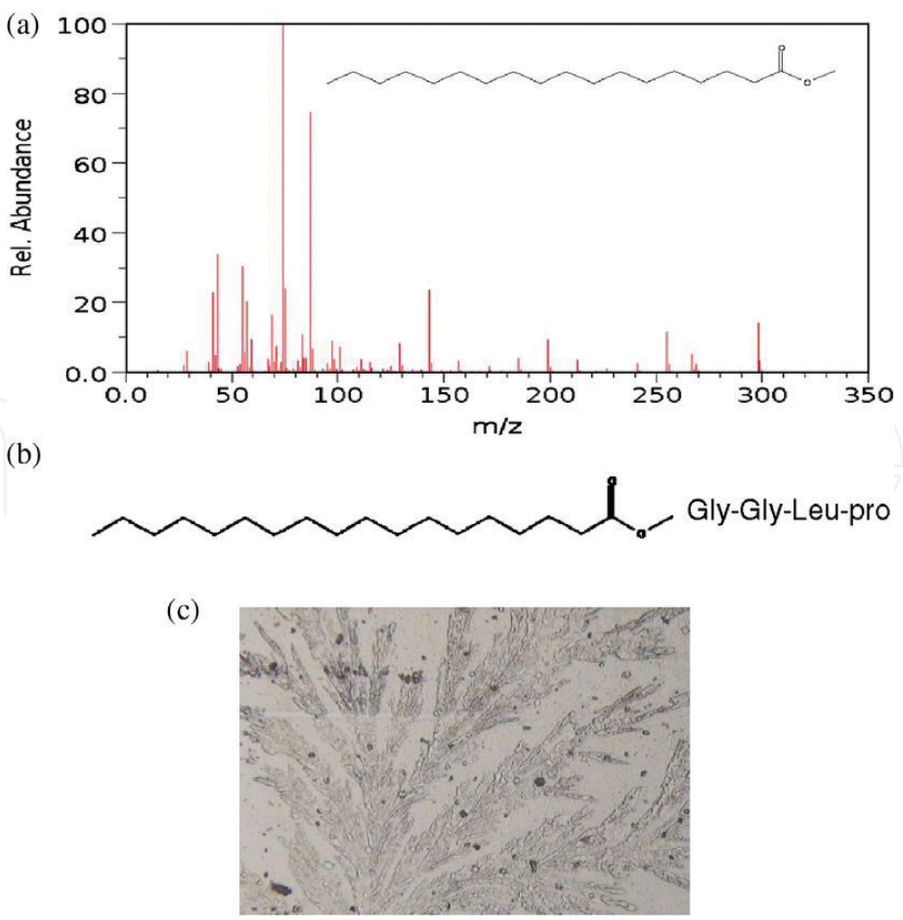

Figure 1.
MS spectra of octadecanoic acid methyl ester (a), probable structure of octadecanoic acid methyl ester with peptide moiety (b), crystalline appearance of the recovered crystals of lipopeptide (MSA13) examined under a light microscope at 40× [23].

Origin	BS	Bacterial Affiliations	Reference
Natural samples			
Seawater, sediment enrichments		*Alcanivorax borkumensis, Paracoccus marcusii*	[65]
Sand/soil samples and seawater	ND	*Catenovulum* sp., *Cobetia* sp., *Glaciecola* sp., *Serratia* sp., *Marinomonas* sp., *Pseudomonas* sp., *Pseudoalteromonas* sp., *Psychromonas* sp., *Rhodococcus* sp.,	[11]
Oil-spilled seawater samples	ND	*Acinetobacter* sp., *Arthrobacter* sp., *Bacillus* sp, *Gluconobacter* sp., *Pseudomonas* sp.	[12]
Seawater	Glycoprotein	*Pseudoalteromonas* sp.	[42]
Black sea, Red sea, hot water springs	ND	*Bacillus* spp.	[40]
Shoreline marine sediment enrichments	Glycolipid	*Pseudomonas* spp., *Pseudoalteromonas* spp., *Idiomarina sp., Rhodococcus* spp.	[14]
Seawater sediment, shells samples	ND	*Acinetobacter* spp., *Bacillus* spp, *Pseudomonas* sp.	[30]
Seawater samples	ND	*Pseudomonas aeruginosa, Aeromonas hydrophila*	[13]
Seawater samples	Lipopeptide	*Halobacterium salinarum*	[50]
Seawater enrichments	ND	*Bacillus cereus, B. megaterium, B. subtilis, Branhamella catarrhalis, Citrobacter intermedius, Corynebacterium kutscheri, C. xerosis, Enterobacter aerogenes, Escherichia coli, Klebsiella ozaenae, Lactobacillus casei, L. delbrueckii, Proteus inconstans, Pseudomonas aeruginosa, P. fluorescens, P. diminuta, P. mallei, Staphylococcus aureus*	[15]
Biological Matrices			
Callyspongia diffusa	Lipopeptide	*Alcaligenes* sp., *Bacillus* sp., *Halomonas* sp.	[21]
Sparus aurata	Glycolipid/Rhamnolipid	*Pseudomonas* spp., *Acinetobacter* sp., *Sphingomonas* spp., *Aeromonas* sp.	[29]
Pteroeides spinosum	Glycolipid	*Brevibacterium* sp., *Vibrio* sp.	[26]
Callyspongia diffusa	Lipopeptide	*Nocardiopsis alba*	[22]
Dendrilla nigra	New Lipopetide (Brevifactin)	*Brevibacterium aureum*	[23]
Dendrilla nigra	Glycolipid	*Brachybacterium paraconglomeratum*	[24]
Sarchophyton glaucum	Glycolipopeptide	*Bacillus* sp. E34	[27]
Acropora digitifera	Lipopeptide/New BS	*Providencia rettgeri, Psychrobacter* sp., *Bacillus flexus, Bacillus anthracis, Psychrobacter* sp., *Bacillus pumilus*	[28]
Megalomma claparedei, Sabella spallanzanii Branchiomma luctuosum enrichment cultures	ND	*Joostella* spp., *Cellulophaga* spp., *Thalassospira* spp., *Pseudovibrio* spp., *Pseudomonas* spp., *Alcanivorax* spp., *Cellulophaga* spp., *Cobetia* sp., *Cohaesibacter* spp., *Idiomarina* spp., *Marinobacter* sp.	[19]
Megalomma claparedei, Sabella spallanzanii Branchiomma luctuosum		*Psychrobacter sp., Pseudoalteromonas spp., Vibrio sp., Maribacter sp., Cellulophaga spp., Tenacibaculum spp., Citricoccus spp., Staphylococcus sp.*	[20]

ND: not determined.

Table 1.
Overview of various studied BS types with the respective marine producers and the source of isolation.

3. Natural roles and biotechnological applications

Biosurfactants have various functions which are often unique for the physiology and ecology of their microbial producers. As stated above, one of the most interesting roles from the environmental point of view is represented by the different strategies adopted by microorganisms to enhance the bioavailability and the access to hydrophobic compounds as carbon source [44]. A mechanism proposed by which hydrocarbons became incorporated within the hydrophobic core of the BS micelles is shown in **Figure 2**. This process studied with alkanes as model substrates and referred to as "micelle solubilization" [45], favors their dispersion into the aqueous phase and their bioavailability for uptake by cells.

The potential application of BSs in hydrocarbon bioremediation has been investigated for marine microorganisms from different origins and allowed to obtain interesting results. In this case, the use of contaminated samples for isolation of potential BS producers is extremely encouraged. Strains affiliated to *Rhodococcus* spp. were reported as capable of reduce surface tension in the presence of oily substrates, and the extracted BSs have been proved optimal enhancer

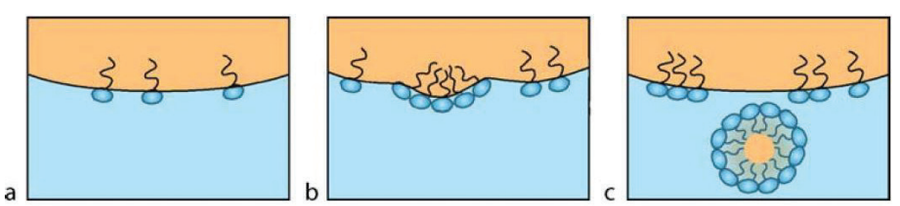

Figure 2.
Mechanism of hydrocarbon solubilization so called "micelle solubilization" within biosurfactant micelles: biosurfactants at the interface between the aqueous and hydrocarbon phases [45].

for n-hexadecane biodegradation at 13°C and of tetradecane [11, 14]. The studies described in [26] evaluated the BS production in the presence of hydrocarbonic substrates to test the potential of *Brevibacterium* and *Vibrio* spp. strains in the field of bioremediation and reported the diesel oil as better utilized carbon source. The hydrocarbon remediation aspect was also deeply studied by investigating *Joostella* sp. A8, *Alcanivorax* sp. A53, and *Pseudomonas* sp. A6 for BS production and diesel oil degradation in pure culture and co-culture conditions [46]. Interestingly, the biodegradation rates and the efficiency increased in co-culture of 99.4 and 99.2%, respectively. Furthermore, in Ref. [27], a glycolipidic biosurfactant produced by the coral-associated *Bacillus* with a removing capacity of about 45% was reported as optimal candidate for oil removal. The bioremediation potential of bacterial BSs has been investigated also in terms of chelating activity toward heavy metals, despite in this context, the literature is scarce. In fact, several bacteria have been reported as able to produce BSs in the presence of heavy metals such as Cd, Cu, and Zn, as *Joostella* sp. A8 and *Alcanivorax* sp. A53 [47, 48]. Despite, the heavy metal removal is an interesting topic, it remains still largely unexplored [47–49] for marine bacteria and need to be improved especially for metal chelation in aqueous systems. BS-producing microorganisms have developed other important physiological functions as response to needs and life style, such as antimicrobial activity (mainly lipopeptide and glycolipid surfactants), biofilm formation or processes of motility and colonization of surfaces. Immunomodulation and enzyme inhibition, have been detected for several BSs from marine environments or not. The antimicrobial activity of BS has been studied *in vivo* and *in vitro* and a broad spectrum of this activity against Gram-positive, Gram-negative, fungi, viruses, algae, etc., so as different modes of action were detected [5]. Both lipopeptides and glycolipids of marine origin have been proved as active against several bacterial pathogens. Indeed, the lipopeptide produced by *N. alba* [22] exhibited antibacterial activity against *E. faecalis*, *B. subtilis*, and the pathogenic yeast *C. albicans*. Similarly, the sponge-associated *Brevibacterium aureum* MSA13 and *Brachybacterium paraconglomeratum* MSA21 were proved as BS producers with a wide antibacterial activity toward several pathogens such as *B. subtilis*, *C. albicans E. coli*, *E. faecalis*, *K. pneumonia, M. luteus*, *P. aeruginosa*, *P. mirabilis*, *Streptococcus* sp., *S. aureus,* and *S. epidermidis* [23, 24]. The antibacterial and antifungal activities were also found in the BSs produced by *Halobacterium salinarum* [50]. It is quite fascinating the mechanism by which BS producers act. Various studies observed that the formation of a film on an interface after the excretion of a BS determines the attachment of certain microorganisms to the interface while inhibiting others. Therefore, it can be stated that some microorganisms can use their BS to regulate the cell surface properties in order to attach or to detach from surfaces according to need. In this respect, some authors [51] reported about studies on the mechanism by which bioemulsifier producing microorganisms regulate biofilm formation. Interestingly, the biofilm formation inhibition of *P. aeruginosa* ATCC10145 is highlighted and this seems to be determined to the BSs produced by a coral mucus associated strains [28]. According to [49], the detection of microorganisms able to produce BSs with such activities is fundamental for a reduced utilization of synthetic surfactants, and it favors the increase of biodegradable compounds. Besides, the existence of a horizontal transfer of high molecular weight emulsifiers from the producing microorganisms to heterologous bacteria was highlighted. In this case, the first step of this process is to bind to the surface of a group of bacteria by changing their surface properties in order to transport the emulsifier into the recipient cells. This has significant ecological implications for building a network of microbial BS producing strains in natural microbial communities. Last but not least, a new role for rhamnolipids in stimulation of plant and animal defense responses has emerged [5].

In particular, rhamnolipids have been demonstrated to have a direct biocide action on bacteria and fungi and to increase the susceptibility of certain Gram-positive and Gram-negative bacteria to specific antibiotics. Indeed, these biomolecules have been known as exotoxins produced by pathogens and are described as a new class of microbe-associated molecular patterns (MAMPs) that is, molecular signals which activate a large battery of defense-related genes of plants and animals by which a more specific immune response is determined [5, 52].

4. Methods and screening procedure for testing BS production

The screening procedure is constantly based on the performance of a selection of standard tests, differently chosen by authors with the attempt to carry out a fast and economic selective procedure. The BS chemical diversity is very wide and also different in their properties; thus, the screening procedure has to probe all the multifaceted activities, from the interfacial to the emulsifying, from the chelating to the foaming stabilization functions, and so on. The interfacial actions are generally explored by direct measurements of surface tension, through the evaluation of the force required to detach a ring or loop of wire (Du-Noüy method), or a platinum plate (Wilhelmy plate method) from an interface or surface [53, 54]. These methods ensure the advantages of accuracy and easiness, despite they require specialized equipment, and the impossibility to perform the measurements on different samples simultaneously. Other direct surface tension measurements have been reported, but they are actually considered not recommendable for an efficient screening procedure [55, 56]. The surface tension evaluation by direct measurement is one of the most commonly test reported for marine isolates [11, 15, 19, 20, 25, 26, 28, 47]. Many screening methods are based on indirect measure of surface/interfacial tension, such as drop collapse method, titled glass slide test, oil spreading assay, penetration assay, and microplate assay. The main advantages are represented by the possibility to screen more samples in a quick way, although a low sensitivity due to the strong dependence on BS concentration was highlighted. The methods are indeed based on distortion visual effects caused by the BS presence and are generally performed on supernatants and some authors suggested to color them in order to evidence the visual effects [57–59]. Among them, the oil spreading and the drop collapse assays are the most reported for marine bacteria [11–13, 21–24, 26, 28, 30, 50] for its rapidity and easiness, in addition to the requirement of small volume of sample, and simple and easily available equipment [56].

A second group of screening tests is differently based on the evaluation of emulsifying activity and is generally carried out with some modifications, with regards to volume of culture/supernatant, to the hydrocarbon used as test, and to vortexing time. The most used tests are the emulsifying activity test, based on a quick observation of emulsion occurrence [60] and the E_{24} index detection, based on the occurrence of emulsions stable over the time. It was applied in many screenings for marine BS producers [11–13, 19–25, 28, 30, 46–48, 50], and most authors reported some modifications: it was tested with cell broth instead of cell-free supernatant, whereas kerosene has been replaced in some cases by other hydrophobic compounds, for example, hexadecane, crude oil, vegetable oil, and diesel oil [13]. Surface activity and emulsification capacity do not always correlate [56]. Indeed, different studies [7, 14, 19] observed and explained this aspect considering that some BSs might stabilize (emulsifiers) or destabilize (de-emulsifiers) the emulsion so that the emulsification test alone fails to identify compounds with surfactant activity which destabilizes the emulsions. However, while the surface activity assay could give just an indication of BS production, the detection of stable emulsion

index correlates to the surfactant concentration. According to the Refs. [20, 25], the surface tension measurement and the emulsification activity assays could be complementary and represent the basic tests to include in a screening procedure, allowing to detect both low molecular mass BSs with efficiency in surface and interfacial tension reduction and high molecular mass BSs more effective as emulsion stabilizers. In addition to the above standard screening tests, several authors reported the use of specific assays, the cetyltrimethylammonium bromide (C-TAB) agar plate assay, and the blood agar assay, useful to detect anionic BSs, but not enough sensitive to detect BS producers. The first one is based on the interaction between anionic surfactants eventually present and the cationic surfactant cetyltrimethylammonium bromide contained in a methylene blue stained mineral salts agar plate; the consequent creation of a blue dark halo around the colonies detects the presence of the BS producers. The blood agar assay is differently based on the hemolytic actions of biosurfactants—α, β, and γ hemolysis—on solid medium containing defibrinated blood as greenish or clarification halos around the bacterial colonies. The use of these specific assays have been reported in most of the above mentioned references, but it has been reduced over the years, because some authors signaled the possible harmfulness toward bacterial growth, the low specificity, and the possible occurrence of false positive/negative. In [19, 20], the two assays are performed on 69 and 96 isolates of different marine origin, respectively, suggesting the use of such tests as integration of a more deep screening procedure. To the list of screening test, it is necessary to mention some other tests with important implications in the screening quality and for considerations about bioremediation purposes. In [46], the authors reported the BS-mediated hydrocarbon degradation by *Joostella* sp. A8 grown in pure culture and consortia, as result of a BS production monitoring in which the bacterial adhesion to hydrocarbons assay (BATH assay) was included. This is a simple photometrical assay described for the first time in Ref. [61] for indirect evaluation of BS production by measuring the hydrophobicity of bacteria. The use of such test in relation to BS production is still a debating issue in this field, because the authors are divided between those who believes that a correlation between cell hydrophobicity, biodegradation efficiency, and BS production exists [62, 63] and who instead claims that the changes in cellular surface properties could be affected by several parameters and may not be necessarily associated to biodegradation ability [64]. Finally, some other minor tests have been reported by several authors for BS production by marine bacteria, but their efficiency have to be improved: this is the case of penetration assay [26], the hydrocarbon overlay agar method [13, 30], tilted glass slide test (TGS test) [30], and lipase activity [21–24, 28]. A number of additional screening tests are well reviewed in [59].

4.1 BS production conditions

There are two primary pathways for bacterial BS biosynthesis: the way of hydrocarbons and the way of carbohydrates [9, 14], and their production is influenced by the availability, types of carbon sources, and the balance between carbon-nitrogen and other limiting nutrients [10]. The effects of several parameters on marine bacterial BS production have been explored by several authors, with particular focus on carbon source, temperature, pH, and NaCl concentration [20, 22–27]. The bacterial BS production has been generally observed at early stationary phase of growth [21, 22, 44, 65], or at exponential phase [25, 27]. In Ref. [22], the authors hypnotized that *N. alba* releases a cell bound biosurfactant into the culture broth which leads to an increase in extra cellular BSs. Moreover, the same authors evidenced a good BS production by the strains at all the tested conditions, even if they detected for *Nocardiopsis alba* the optimum conditions for BS production at pH 7, temperature

30°C, and 1% salinity with glucose and peptone supplementation as carbon and nitrogen sources, respectively. In [21], the authors confirmed that carbon source and its concentration are affecting parameters for BS production, and established that glycerol, peptone, ferrous sulfate, and incubation time exhibited significant effect, with optimum levels as pH 7, temperature at 37°C, and salt concentration 2% for *B. amyloliquefaciens.* In particular, the authors reported that glycerol used as a carbon source showed the highest BS production (up to 6.76 g/l). According to Ref. [24], the studies performed a more deep optimization analysis to investigate the BS production by the sponge-associated *Brachybacterium paraconglomeratum* and indicated a yeast extract nitrogen source as factor enhancing up to 60% biosynthesis activity. Positive effects were also exhibited by the supplementation with 2% of NaCl, a pH level of 7.0, and a 30°C temperature. Moreover, asparagine resulted highly effective for BS production followed by glycine, leucine, and valine, and a requirement of $CuSO_4$ as a metal supplement was requested by the strain for optimum production of BS. In [20], the authors investigated the influence of salinity and temperature on the BS production by polychaete-associated isolates and showed that the NaCl concentration strongly influenced the surface tension reducing activity and emulsification rate in major level rather than temperature. Nevertheless, the authors reported that the strain *Marinobacter* sp. A1 exhibited the best performances at 15°C and in the absence of NaCl, by suggesting that limited conditions could act as stimulating factors. Interestingly, several researchers [19, 20, 25] also reported the BS production in the presence of hydrocarbons. The BS synthesis under solid state cultivation (SSC) was investigated for *Brevibacterium aureum* MSA13, which increased its production with pre-treated molasses, glucose, and acrylamide [23] as substrates. The report is interesting because it represents the first attempt in which acrylamide was used as nitrogen source, and the SSC conditions have been proven to be a preferred bioprocess for the BS production and optimization. In [27], the authors suggested a parallel relationship between bacterial growth and productivity and tested several carbon sources (sugar cane molasses, olive oil, corn oil, motor oil, and kerosene) among which molasses resulted the better one, as previously reported for non-marine BS producers [40]. This finding evidenced the possible importance of low cost substrate employment, which could solve the problems of high costs for BS production. As suggested by other researchers who revealed yeast extract and tryptone as significantly positive factors for BS production, nitrogen is an aspect to be carefully treated, as important constituent of the peptide part of lipopeptidic BSs [27]. Similarly, phosphate source has to be regulated to ensure a good bacterial growth, with positive influence on BS production. Finally, calcium source has also been reported as important positive factor and enhancer of emulsification activity. Due to this deep optimization approach, the authors achieved an increase of BS production with emulsification indexes from 60 to 77%.

5. Genetic regulation

The genetics of microbial surfactant synthesis is important because it represents a primary factor determining their productivity. It has been studied by the use of mutants naturally occurring or induced by transposition. However, the screening for such mutants is difficult because of the loss of ability to produce the surfactant that does not result in an easily selectable phenotype or may be lethal. The regulation at the molecular level of BS production has been mainly investigated for the rhamnolipid produced by *Pseudomonas aeruginosa* and for a few lipopeptides of Bacilli. The BS production has proved to be controlled by *quorum sensing*

mechanism, a cell density dependent gene regulation process allowing bacterial cells to express certain specific genes on attaining high cell density [66]. With this mechanism, at the base of the bacterial production of secondary metabolites, bacteria produce a small diffusible signal molecule which accumulates in the growth medium and determine the gene activation when the microorganisms are in high densities. According to [49], the genes responsible for lipopeptide BS biosynthesis from *Bacillus* and *Pseudomonas* species display a high degree of structural similarity among themselves. The lipopeptide surfactin is produced as a result of nonribosomal biosynthesis. The mechanism is quite complex, as peptide synthetase for the amino acid moiety of surfactin is encoded by four open reading frames (ORFs) in the *Srf*A operon. The gene controlling the peptide synthetase consists of *Srf*AA (SrfORF1), *Srf*AB (SrfORF2), and *Srf*AD which activate each other. As a matter of fact, a more complex mechanism drives the *srf*A operon expression by controlling the level of various molecular signals [67]. On the other hand, structural genes required for the synthesis of the lipopeptidic BS lichenysin have been isolated, and a high sequence homology with *srf*A of surfactin synthetase was observed. Indeed, the lichenysin biosynthesis operon (called *lic operon*) was cloned and sequenced by revealing a composition of three peptide synthetase genes (lic A, lic B, and lic C) [68]. With respect to the genes which regulate the synthesis of glycolipids as rhamnolipids, they had been isolated, characterized, and their introduction into other species allowed the production of rhamnolipids in heterologous hosts [66]. The genes involved in the rhamnolipid biosynthesis are plasmid encoded and act during the late exponential early stationary phase as a consequence of the higher cell density. The synthesis of rhamnolipids in *P. aeruginosa* is carried out by the *rhl*AB operon, and a few additional genes are required [51, 45]. In particular, the biosynthetic pathway involved the genes *rhl*A, B, C, R, and I. *rhl*AB operon and *rhl*C gene encode the two rhamnosyltransferases (proteins that resides in the periplasm) responsible for the synthesis, transport or the stabilization of the rhamnosyltransferase within and in the cytoplasmatic membrane. *Rhl*A, B genes are organized in one operon and are coexpressed from the same promoter [67]. *Rhl*R and *rhl*I genes act as regulators of the *rhl*A, B gene expressions, and in turn are regulated by other genes (*las*R and *las*I) of a second quorum sensing system found in a different region of the *Pseudomonas aeruginosa* chromosome [67, 69]. As a matter of fact, the circuit of rhamnolipid production is promoted by other regulatory factors triggered by environmental conditions such as C/N ratio and inhibited by higher iron concentration [70], while the transcription of *rhl*AB genes is overexpressed under nitrogen-limited medium. As regards to the genetic regulation of high molecular weight heteropolysaccharide bioemulsifiers, they are more complex than the low molecular weight lipopeptides or rhamnolipids because they require a larger number of genes, and the genetic organization is even more complicated for polysaccharides-protein complexes [49]. However, although several structural and regulatory genes have been identified for the BS production, this aspect has been mainly improved for bacteria of terrestrial origin, while it has been scarcely explored for marine microorganisms. To the best of our knowledge, the only reports in this regard are in Refs. [23, 71]. In particular, these studies were addressed to the polyketide synthases (PKSs), the nonribosomal peptide synthases (NRPS), and large multifunctional proteins, modular proteins involved in the production of bioactive molecules. The research group investigated the possible correlation between the PKS gene and the BS biosynthesis in sponge-associated BS actinobacterial producers. In Ref. [23], the authors detected the presence of *pks* II gene in *B. aureum* MSA13, by suggesting the possible biosynthesis of secondary metabolites with antibiotic and surfactant properties. Furthermore, the studies described in [71] provided interesting insights into the KS genes of *Brevibacterium* and *Brachybacterium*, by confirming through

molecular approaches that marine resources should be better explored for biodiscovery purposes. The molecular genetics of BS production is still in progress, and important genetic tools (plasmids, transposons, and gene libraries) are still to be developed, as well as further studies could allow to develop a biosynthetic engineering approach to design novel biomolecules.

6. Conclusions

The research on BS is still undergoing evolution and requires many improvements in several aspects. The results obtained in the last decades from the marine resources are very encouraging, both in terms of BS chemical diversity and in terms of the BS effectiveness and microbial production capacity. Despite this, new tools and improvements are necessary for a better comprehension of the genetic regulation, in order to exploit these mechanisms for a potential large-scale production. The discovery of new BSs must represent the main goal, and should be accompanied by appropriate chemical analysis, and identification of the most active fractions. The screening methods must be standardized and refined, and above all the study should be planned on the basis of the application of interest. Marine bioprospecting and the blue biotechnology are research areas that deserve to be explored, which are worth focusing on, and which could allow significant scientific contributions and useful applications for humans.

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