REVIEW ARTICLE

Biosurfactants from marine microorganisms

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Abstract

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Biosurfactants are the surface-active molecules synthesized by microorganisms. With the advantage of environmental compatibility, the demand for biosurfactants has been steadily increasing and may eventually replace their chemically synthesized counterparts. Marine biosurfactants produced by some marine microorganisms have been paid more attention, particularly for the bioremediation of the sea polluted by crude oil. This review describes screening of biosurfactant-producing microorganisms, the determination of biosurfactant activity as well as the recovery of marine surfactant. The uses of marine biosurfactants for bioremediation are also discussed.

Key words : biosurfactant, marine microorganisms, bioremediation, crude oil

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บทคัดย่อ

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สารลดแรงตึงผิวชีวภาพจากจุลินทรีย์ที่แยกได้จากทะเล

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สารลดแรงตึงผิวชีวภาพคือสารลดแรงตึงผิวที่ได้จากจุลินทรีย์ เนื่องจากสารลดแรงตึงผิวชีวภาพไม่มีผลเสียต่อสิ่งแวดล้อมส่งผลให้ความต้องการใช้เพิ่มมากขึ้น และอาจจะเข้ามาแทนที่สารลดแรงตึงผิวสังเคราะห์ทางเคมีได้ สารลดแรงตึงผิวชีวภาพที่ผลิตโดยจุลินทรีย์ที่แยกได้จากทะเลได้รับความสนใจมากขึ้นโดยเฉพาะสำหรับฟื้นฟูสภาวะแวดล้อมทางทะเลที่ปนเปื้อนน้ำมันดิบ บทความนี้จะกล่าวถึงการแยกจุลินทรีย์จากทะเลที่ผลิตสารลดแรงตึงผิวชีวภาพ การวัดกิจกรรมและการสกัดสารลดแรงตึงผิวชีวภาพ ตลอดจนการใช้สารลดแรงตึงผิวชีวภาพในการฟื้นฟูสภาวะแวดล้อม

ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Microbial compounds, which exhibit pronounced surface activity, are classified as biosurfactants. Biosurfactants have unique amphiphatic properties since their complex structures are composed of hydrophilic region and hydrophobic portion. As a consequence, biosurfactants can partition preferentially at the interfaces (Desai and Banat, 1997). Biosurfactants have many advantages, such as biodegradability (Zajic *et al*., 1977), low toxicity (Poremba *et al*., 1991) and environmental compatibility (Georgiou *et al*., 1990). The use of biosurfactants to protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactants during growth on hydrocarbons (Bertrand *et al*., 1993). For the sake of the environment, the use of biosurfactants is preferable to those of synthetic surfactants. However, little information on either biosurfactant produced by marine microorganisms or biosurfactants active in saline has been reported so far. This article aims to provide an overview on the production of biosurfactants by marine microorganisms and to discuss their potential use in bioremediation.

Biosurfactant classification

Biosurfactants are categorized mainly by their chemical composition and microbial origin. Generally their structures include a hydrophilic moiety consisting of amino acids or peptides; mono-, di-, or polysaccharides; and a hydrophobic moiety comprising unsaturated or saturated fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides and lipoprotein, phospholipids and fatty acids, polymeric biosurfactants, and particulate biosurfactants (Desai and Banat, 1997). In some cases, biosurfactants can be divided into low-molecular-weight molecules and high-molecular-weight polymers (Ron and Rosenberg, 2001). The low-molecular-weight biosurfactants are generally glycolipids or lipopeptide. The high-molecular-weight biosurfactants are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Rosenberg and Ron, 1999). Some biosurfactants produced from marine microorganisms are listed in Table 1.

Glycolipids: Glycolipids are carbohydrates containing long-chain aliphatic acids or hydroxyaliphatic acids. Marine bacteria such as *Alcaligenes* sp., are able to produce glucose lipid. The lipophilic component consisting of four β-hydroxydecanoic acids linked together by ester bonds is coupled glycosidically with C-1 of glucose (Figure 1). *Alcanivorax borkumensis* also produces an anionic glucose lipid with a tetrameric oxyacyl side chain. The glycolipids extracted from the cell

Table 1. Marine microorganisms and some types of biosurfactants.

Biosurfactant	Organisms	Reference (s)
Glycolipids		
Glucose lipids	*Alcanivorax borkumensis*	Abraham *et al* (1998)
	Alcaligenes sp.	Poremba *et al* (1991)
Trehalose lipids	*Arthrobacter* sp.	Schulz *et al* (1991)
Lipoproteins		
Ornithine lipids	*Myroides* sp. SM1	Maneerat et al (2005a)
Phospholipids and fatty acids		
Bile acids	*Myroides* sp. SM1	Maneerat *et al* (2005b)
Polymeric biosurfactants		
Lipid-carbohydrate-protein	*Yarrowia lipolytica*	Zinjarde and Pant (2002)
	Pseudomonas nautica	Husain *et al* (1997)
Particulate biosurfactants		
Whole cells	Variety of bacteria	Denger and Schink (1995); Gilewicz *et al* (1997); Zinjarde and Pant (2002)

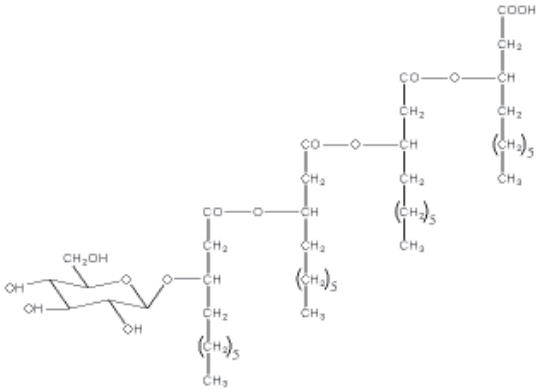

Figure 1. Structure of glucolipid from *Alcaligenes* sp. (Poremba *et al.*, 1991).

wall consist of this biosurfactant N-terminally esterified with glycine. Ten different derivatives of lipid type have been identified. The varying chain length of one or two of the four β-hydroxy fatty acids (C_6, C_8 and C_{10}) was reported (Figure 2). Carbon source has been known to influence the structure of glycolipid. For example, *Arthrobacter* sp. SI 1 produced disaccharide trehalose (trehalose tetraester) (Figure 3a) which is acylated by three fatty acids (C_8-C_{14}) and one succinyl group when the carbon source is mihagol-S. However, trehalose dicorynomycolates (trehalose diester) and produced when ethanol is used (Figure 3b).

Phospholipids and fatty acids: *Myroides* sp. SM1 was found to produce bile acids; cholic acid, deoxycholic acid and their glycine conjugate (Figure 4) when cultivated in Marine Broth (Maneerat *et al*., 2004).

Polymeric biosurfactants: The production of polymeric biosurfactant by *Pseudomonas nautica* was achieved (Husain *et al*., 1997). The major constituents were proteins, carbohydrates and lipid at the ratio of 35:63:2, respectively. Zinjarde and Pant (2002) also reported that *Yarrowia lipolytica*, a tropical marine strain produced the emulsifier in the presence of alkanes or

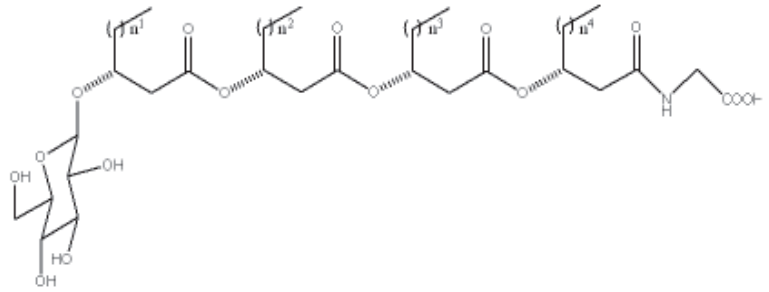

Figure 2. Glucolipids from *Alacnivorax borkumensis* (Abraham *et al.*, 1998).

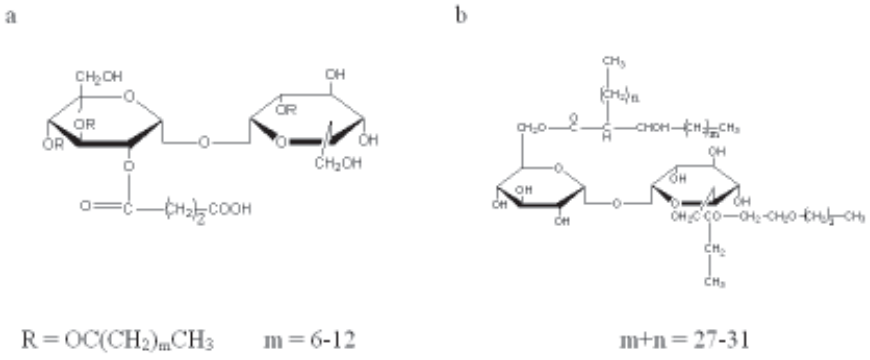

Figure 3. Trehalose tetraester (a) and trehalose diester (b) from *Arthrobacter* sp. SI 1 (Schulz *et al.*, 1991).

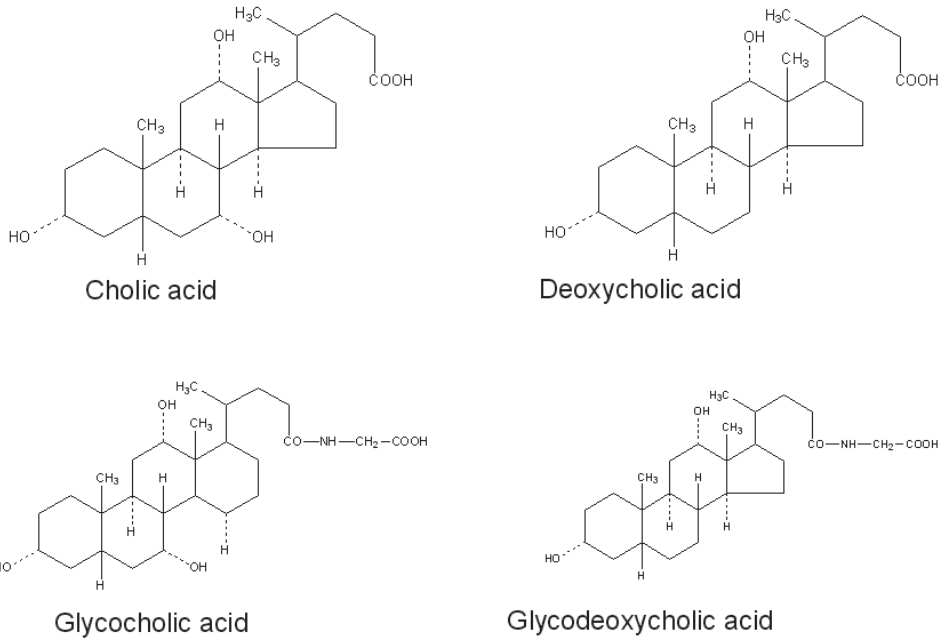

Figure 4. Bile acids from *Myroides* sp. SM1 (Maneerat *et al.*, 2005b).

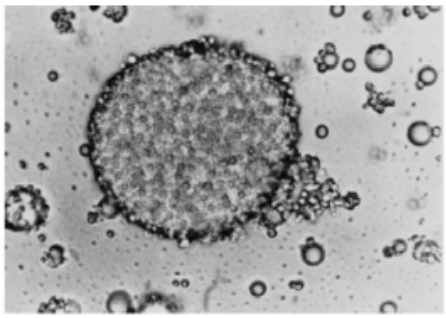

Figure 5. Microscopic observation of *Yarrowia lipolytica* NCIM 3589 grown on n-hexadecane after incubation for 48 h (Zinjarde and Pant, 2002).

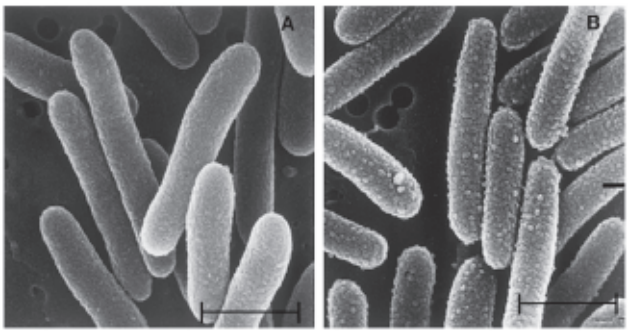

Figure 6. Electron micrographs of *Sphingomonas* sp. A) Cells grown on sodium acetate; B) Cells grown on 2-methylphenanthrene (Gilewicz *et al.*, 1997).

crude oil. An emulsifier (lipid-carbohydrate-lipid) complex was associated with the cell wall in the earlier stages of growth but displayed the extracellular emulsifier activity in the stationary phase.

Particulate biosurfactants: Extracellular membrane vesicles partitioned hydrocarbons to form a microemulsion, which plays an important role in alkane uptake by microbial cells (Figure 5). The cellular lipid content of *Pseudomonas nautica* increased in eicosane-grown cells up to 3.2 fold, compared with acetate-grown cells. Phospholipids, mainly phosphatidylethanolamines and phosphatidylglycerides, were accumulated in eicosanes-grown cells (Husain *et al*., 1997). For *Sphingomonas* sp., the cell surface of this strain was covered with extracellular vesicles when grown on polyaromatic hydrocarbons. Nevertheless, the surfaces were smooth when cells were grown on a hydrophilic substrate such as acetate (Figure 6).

Screening of biosurfactant-producing microorganisms

Biosurfactant-producing microorganisms can be screened using different assays. Yuste *et al*. (2000) assayed the production of rhamnolipids by colorimetric measuring of the amount of methyl pentoses in culture supernatants and by the ability to form clear halos in methylene blue/cetyltrimethylammonium bromide (CTAB) plate or N-cethylpyridinium chloride-methylene blue agar plate (Lin *et al*., 1998). Hemolysis of red blood cell was also used for screening biosurfactant-producing microorganisms (Carrillo *et al*., 1996; Fiebig *et al*., 1997). Schulz *et al*. (1991) used a direct thin-layer chromatography technique for a rapid characterization of biosurfactant-producing bacterial colonies. Colony surrounded by an emulsified halo on L-agar plate coated with oil was determined as biosurfactant producer as described

by Morikawa *et al*. (1992). Emulsification power (E-24) was measured by thoroughly mixing an equal volume of the culture supernatant with kerosene for 1 min. The percentage of volume occupied by the emulsion after 24 h was recorded according to the method of Haba *et al*. (2000). A sensitive rapid method, a drop-collapsing test was advised for screening bacterial colonies that produce surfactants. Drops of cell suspensions of surfactant-producing colonies collapsed an oil-coated surface. (Jain *et al*., 1991).

Measurement of biosurfactant activity

Surface and interfacial tensions, stabilization of emulsions, and hydrophilic-lipophilic balance (HLB) are used as the indices for biosurfactant activity. Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer (Denger and Schink, 1995). The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m (Cooper, 1986).

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid phase. The emulsion activity is assayed by the ability of the biosurfactant to generate the suspension of hydrocarbon such as n-hexadecane (Kim *et al*., 2000) or kerosene (Haba *et al*., 2000) or a mixture of n-hexadecane and 2-methylnaphthalene (Navon-Venezia *et al*., 1995) or dodecane (Burd and Ward, 1996), etc., in an aqueous assay system.

The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification. The HLB value conception is an often used tool for the emulator choice. The HLB is calculated as follow (Oberbremer *et al*., 1990):

HLB value = 20 × (molecular weight of the hydrophilic part/molecular weight of the whole molecule)

In the case of ionic surfactants or if hydrophilic and hydrophobic moieties are not arranged equally within the molecule, the formula gives no proper result. Therefore, the HLB value will be measured experimentally. Vollbrecht *et al*. (1999) determined the HLB value of glycolipid in the emulsion system containing water and hydrophobic phase. The mixture of cyclohexane and soybean oil was used as hydrophobic phase. The HLB value needed for obtaining the emulsion of the hydrophobic phase was calculated as follows:

$$A = (HLB_{needed} - HLBB) \div (HLB_A - HLB_B)$$

When
A : % cyclohexane
HLB_A : HLB value of cyclohexane (=15)
HLB_B : HLB value of soybean oil (= 6)

Also, the amount of hydrophobic mixture, which leads to the most stable emulsion, can be calculated based on the HLB-value of the used surfactant.

Recovery of marine biosurfactants

The glucose lipid was extracted from cell wall of *Alcanivorax borkumensis*. Wet cells were suspended in methanol/dichloromethane/phosphate buffer and treated with an ultrasonic probe. The sample was centrifuged and the dichloromethane phase was filtered through dry Na_2SO_4 and a hydrophobic filter. The lipids were fractionated using column chromatography by sequential elution with dichloromethane, acetone and methanol which resulted in three fractions of different polarity: neutral, glyco- (glycolipid) and phospholipids (Abraham *et al*., 1998). Glucose lipid from *Alcaligenes* sp. and trehalose lipid from *Arthrobacter* sp. have been recovered by acidification, followed by extraction in ethyl acetate. Further purification was carried out by column and thick layer chromatography (Schulz *et al*., 1991; Passeri *et al*., 1992).

Ornithine lipids were extracted from culture supernatant of *Myroides* sp. SM1 twice with an equal volume of chloroform/methanol (1:1) at pH 7 and purified by normal and reverse phase silica

gel column chromatographies (Maneerat *et al*., 2004a). n-Hexadecane-emulsifier, bile acids, were also obtained from culture supernatant of *Myroides* sp. SM1. Culture supernatant was acidified with 6 N HCl to pH 2.0 and extracted two times with an equal volume of ethyl acetate. The extract was subjected to column chromatography on reverse-phase silica gel with stepwise elution with methanol or acetonitrile (Maneerat *et al*., 2004b).

Lipid-carbohydrate-protein complex from tropical marine yeast *Yarrowia lipolytica* was extracted from cell wall. The cells were suspended in buffer and the compounds were extracted with diethyl ether (Zinjarde and Pant, 2002).

Marine biosurfactants in bioremediation

Pollution of sea by crude oil, mostly caused by stranding of tankers, is one of urgent and serious environmental issues over the world. Ship operations also produce wastes that are collected in the lowest part of the hull, called the bilge area. This oil-containing bilge waste must be managed properly to avoid environmental pollution (Olivera *et al*., 2003). With those concerns, there is a large body of research on oil-spill bioremediation. Biodegradation of a given hydrocarbon depends on its dispersion state. The biodegradation is maximized when the water-insoluble substrate is dissolved, solubilized, or emulsified (Mattei *et al*., 1986). Synthetic detergents used to clean up these spillages have often led to more destruction of the environment. From an environmental viewpoint it is important that all substances released into the environment are degradable, firstly to assess their potential for causing environmental damage and secondly to safeguard against the possibility of future harm due to build-up in the environment. Biosurfactants are either extracellular compounds or localized on the cell surface. For the latter case, the microbial cell itself is a biosurfactant and adheres to hydrocarbon. In these ways, biosurfactants are capable of increasing the bioavailability of poorly soluble polycyclic aromatic hydrocarbons such as phenanthrene (Gilewicz *et al*., 1997; Olivera *et al*., 2003) and resins (Venkateswaran *et al*., 1995). Biodegradation of hydrocarbons by native microbial populations is the primary mechanism by which hydrocarbon contaminants are removed from the environment (Venkateswaran *et al*., 1991; Delille, 2000). *Acinetobacter* sp. T4 degraded components in both saturated and aromatic fractions from Arabian light crude oil, and produced metabolites that could support the growth of *Pseudomonas putida* PB4. The latter strain grew on these metabolites and degraded aromatic compounds present in the crude oil (Komukai-Nakamura *et al*., 1996). The addition of microbial inocula prepared from nonindigenous populations (bioaugmentation) increased about 30% degradation of hydrocarbon compared with the treatment without bioaugmentation (Dave *et al*., 1994). In some cases, the effectiveness of bioremediation was increased by adding fertilizers (Bragg *et al*., 1994). Recently Maki *et al*. (2003) concluded that excessive amounts of macronutrients are required to accelerate oil biodegradation and that fertilization is only effective in the early stages.

Although many studies have focused on the enhancement of biodegradation as a procedure for cleaning the environment of oil, few studies on the production of toxic or teratogenic metabolites from the enhanced biodegradation of weathered crude oil have been conducted. The effect of water soluble or neutral fraction material (WSF) after degradation of weathered crude oil on embryonic and larval of Pacific herring, *Clupea pallasi*, was investigated. High mortality was noted in larvae exposed to the 10% WSF beginning at 4 and 48 h postfertilization. Most of these larvae died 5 to 8 days after hatching when they elicited vertebral displacements at a time concurrent with the onset of feeding behavior (Middaugh *et al*., 1998). Exposure of grass shrimp (*Palaemonetes pugio*) embryos to neutral WSF resulted in high embryo mortalities and toxicity of neutral WSF also demonstrated on larvae of mysids (*Mysidopsis bahia*) (Shelton et al., 1999). These results imply that unique compounds were accumulated during degradation and may have been responsible for increased toxicity. However, from these results, it is not clear whether neutral WSF and its associated toxicity is transient or long-lived. Nor is it clear whether it could reach

toxic concentrations in open aquatic environments before being dissipated by dilution. In less open system, such as salt marshes and estuarine pools, however, bioremediation to accelerate rates of oil biodegradation may have significant adverse effects.

Conclusion

Biosurfactants have been proven as the promising agents for bioremediation of hydrocarbons, particularly oil polluted in marine environment. Nevertheless, metabolites from bioremediation process show the toxicity to some marine organisms. Therefore, novel microorganisms should be intensively screened for bioremediation without causing adverse effects.

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