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Bioemulsifiers from marine microorganisms

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This review highlights bioemulsifier-producing marine microorganisms, which are capable of producing unique metabolites having industrial applications. High molecular weight biosurfactants (bioemulsifiers) produce stable emulsions, which allow bacteria to adhere strongly to hydrophobic surfaces and then degrade large biological complexes. Bioemulsifiers are classified according to their hydrophile–lipophile balance (HLB); those having a low HLB are strong lipophiles and used as water-in-oil emulsifiers, whereas those having a high HLB are strong hydrophiles and used as oil-in-water emulsifiers. *Acinetobacter calcoaceticus* is the most promising marine microorganism used in diverse applications. In *A. calcoaceticus* RAG-1, emulsification is brought about by production of an extracellular, polymeric bioemulsifier termed emulsan. Tropical marine yeast, *Yarrowia lipolytica* NCIM 3589, produces emulsifier in the presence of alkanes or crude oil. Bioemulsifier potential is mainly dependent on its chemical nature and hence its activity can be enhanced by simple media modification or mutagenesis.

Keywords: *Acinetobacter calcoaceticus*, Bioemulsifiers, Marine microorganisms

Introduction

Marine microbes have developed unique metabolic and physiological capabilities that not only ensure survival in marine habitats but also offer potential for the production of metabolites, which would not be observed from terrestrial microorganisms[1]. Gram-negative bacteria are found in abundance (90% of sea bacterial flora) in seawater. Gram-positive forms mainly belong to *Bacillus* spp[2,3]. Bacteria specific to marine environment may or may not have obligatory requirement for sodium and seawater but have capability to perform unique biosyntheses[4]. New secondary metabolites have been reported from marine bacteria, protozoa, algae, fungi etc[4-6]. This review presents bioemulsifiers from marine microbes and compares them with those obtained from non-marine microbes.

Biosurfactants (BSs)

Microorganisms (bacteria, yeasts and fungi), having hydrophobic and hydrophilic groups, produce biosurfactants (BSs). By evolution, bacteria have adapted themselves to feeding on water-immiscible materials by producing and using a surface active product that helps bacteria in aqueous phase to adsorb, emulsify, wet and disperse or solubilize water-immiscible material[7]. Microorganisms produce low- and high-molecular weight BSs. Low-molecular weight types are glycolipids (trehalose tetraesters, dicorynomycolates, fructose lipids, sophorolipids and rhamnolipids) or lipopeptides (surfactin and viscosin). A number of reviews have focused on biosurfactants/bioemulsifiers and their various aspects like types, production and recovery, applications etc[8-12]. BSs can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors, particularly oil sector. BSs have several advantages over chemical surfactants and emulsifiers such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, higher selectivity and specific gravity at extreme temperature, pH and salinity and ability to be synthesized from renewable feedstock$[13]. Several patents have been issued on BS and bioemulsifier (BE) produced by *Acinetobacter*, *Bacillus*, *Pseudomonas* and *Candida* spp[7,14].

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Table 1 — Important types of marine microbial emulsifiers

Bioemulsifier	Producing microorganisms
BD4 emulsan[16]	*Acinetobacter calcoaceticus* BD4
Trehalose-lipid-o-dialkyl monoglycerides-protein[17]	*Pseudomonas fluorescens*
Polypeptide[18]	*Acinetobacter* sp. A3
Emulsan[19]	*A. calcoaceticus* RAG-1
	A. calcoaceticus A2
Biodispersan[19]	*A. calcoaceticus* A2
Lipid-carbohydrate-protein complex[20]	*Yarrowia lipolytica* NCIM 3589
Carbohydrate-protein complex[21]	*Rhodotorula glutinis*
Protein-polysaccharide[22]	*Streptomyces* species S1
HE39[23]	*Halomonas* TG39
HE67[23]	*Halomonas* TG67
Yansan[24]	*Yarrowia lipolytica* IMUFRJ 50682

Among potent BSs, *B. subtilis* produces surfactin while *B. licheniformis* produces lichenysin. Surfactin has an amphiphilic structure associated with high surfactant activity and extensive biological properties including affecting the growth of tumors, bacteria, fungi, viruses and mycoplasmas. Lichenysin is an effective surfactant over a wide range of temperatures, pH's and salt and calcium concentrations[15].

Bioemulsifiers (BEs)

Emulsifiers are classified according to their hydrophile–lipophile balance (HLB). In general, an emulsifier with a low HLB is lipophilic, whereas a high HLB confers better water solubility. Emulsifiers with low HLB are suitable for water-in-oil (W/O) emulsions and the ones with high HLB are suitable for oil-in-water (O/W) emulsions. High-molecular weight BSs, called BEs (bioemulsans), are associated with production of stable emulsions enabling bacteria to adhere to hydrophobic surfaces very strongly, with implications on biodegradation capabilities. Applications of BEs are based on HLB as follows: surface films, < 3; W/O emulsions, 3-6; wetting agents, 7-9; O/W emulsions, 10-18; detergents, 13-15; and solubilisers, 15-18. BEs are (lipo) polysaccharides, (lipo) proteins or combinations of these. Hydrophobic moiety necessary for emulsification can be protein as in BD4 emulsan from *Acinetobacter calcoaceticus* BD4, carbohydrate as in liposan from *Candida lipolytica* or lipid as in emulsan from *A. calcoaceticus* RAG-1.

Bioemulsifier Production by Marine Microbes

Marine microbes produce BEs with certain unusual properties in addition to being tolerant to pH (3-12), temperature (20-100°C) and salinity (0.5-2.0%). However, work on BEs from marine sources seems to be scanty (Table 1). *Acinetobacter* spp. is predominant group of Gram-negative bacteria in marine environment and produces a commercial BE[25]. Emulsan, a BE, produced by *A. calcoaceticus* RAG-1, has been marketed. Isolation and characterization of a mutant of *A. calcoaceticus* RAG-1, deficient in its ability to adhere to hydrocarbons, demonstrated importance of adherence in the growth of RAG-1 on hexadecane[8]. Biodispersan is an anionic heteropolysaccharide, non-dialyzable BE produced by *A. calcoaceticus* A2. *Bacillus* strains, most common Gram-positive bacteria in marine environment, have been characterized[26]. *B. licheniformis* produces lipopeptide BSs. *Halomonas eurihalina* based BE, synthesized in crude oil media, has been characterized[12]. Tropical marine yeast, *Yarrowia lipolytica*, produces BE, Yansan, which has been isolated and characterized[27]. Uses of marine BEs are reported[28].

Bioemulsifier Production by Non-marine Microbes

Reports on emulsifiers from terrestrial microbes (bacteria and fungi) are abundant. Bonilla *et al*[29] reported a BE from *Pseudomonas putida* ML2. A lysozyme sensitive BE was produced by *Bacillus* strain FE-2 during growth on organophosphorus pesticides[30]. Petroleum contaminated sites have yielded BS/BE producing bacteria[31]. Oil reservoir microorganisms have been studied for BE production[32].

Acinetobacter spp. can also be found in non-marine habitats. Alasan, an anionic alanine-containing heteropolysaccharide–protein BE, is produced by *A. radioresistens* KA-53. *A. junii* SC14 produces a proteoglycan BE. Role of esterase in release of cell-bound emulsifier and contribution of capsular polysaccharide to emulsification activity have been studied[33]. *Cyanobacterium* J1 emulsifier has been patented[34]. A BS obtained from *Nocardia* sp. L-417 is a strong emulsifying agent and emulsan stabilizing agent. Emulsanase, isolated from soil bacteria, can be used for breaking emulsions and performing structure-function studies. Cell-free esterase of *A. venetianus* RAG-I enhances emulsification activity of its emulsan[35]. An extracellular polymer, produced by continuous fermentation of *Corynebacterium hydrocarboclastus* on kerosene, exhibited good emulsifying activity. *Bacillus* spp. (*Bacillus* sp. strain IAF 343 and *B. cereus* IAF 346) produce BE[29]. *Bacillus* spp. CP912 produces extracellular polysaccharide that has emulsifying as well as flocculating activity[36]. *B. stearothermophilus* VR-8 isolate produces an extracellular BE. Rahman *et al*[37] prepared a mixed bacterial consortium of *Pseudomonas* sp. DS10-129, *Micrococcus* sp. GS2-22, *Bacillus* sp. DS6-86, *Corynebacterium* sp. GS5-66 and *Flavobacterium* sp. DS5-73, which was an efficient BS producing, oil emulsifying and degrading culture. Antarctic hydrocarbon degrading bacteria capable of producing BEs have been characterized[38].

Among fungi, *Candida lipolytica* produces an emulsifier, liposan. A mannoprotein of *Saccharomyces cerevisiae* is an effective BE. Among molds, *Penicillium citrinum* produces an extracellular polysaccharide with emulsifying property[39]. Sarubbo *et al*[40] studied a new BE producing strain of *C. glabrata* UCP 1002.

Yield and Activity Improvement of Bioemulsifiers

BEs are composed of polysaccharides, proteins, lipopolysaccharides or complex mixtures of these polymers. However, nature of nutrient composition of growth media drastically affects the amount and chemical composition of these biopolymers. Main limiting factor of industrial and environmental applications of BSs is economics of their large-scale production; main drawbacks being: (1) Poor yields from raw substrate materials; (2) Large capital investment; (3) Reactions being carried out in dilute solution leading to poor volume efficiency for the plant; (4) Need for sterilization; (5) Problems in the control of process, like foaming; (6) Problems in product recovery and purification; and (7) Difficulties in analyzing finished products chemically due to their complex nature. Thus, use of inexpensive feedstocks is essential for competitive nutrients. Panilaitis *et al*[41] have shown that emulsan, produced by *Acinetobacter venetianus* RAG-1, can be synthesized from agro based feedstocks. Media optimization has been used effectively to increase yield of BE. Response surface methodology (RSM) has been used to optimize medium components for BE production by *Candida lipolytica*[42].

Emulsan, produced by *A. calcoaceticus* RAG-1, is a complex of anionic heteropolysaccharide and protein. Its emulsifying activity is due to the presence of fatty acids, comprising emulsan (15% dry wt) attached to polysaccharide backbone. RAG-1 emulsan does not emulsify pure aliphatic, aromatic or cyclic hydrocarbons; however, it emulsifies all mixtures that contain an appropriate mixture of an aliphatic and an aromatic (or a cyclic) alkane efficiently. *A. calcoaceticus* BD4 produces a BE, a polysaccharide-protein complex; its individual components have no emulsifying activity by themselves, but their mixing leads to a reconstitution of emulsifying activity. Protein binds to hydrocarbon initially in a reversible fashion. Polysaccharide then attaches to protein and stabilizes O/W emulsion. Alasan, produced by *A. radioresistens*, is a complex of anionic polysaccharide and protein. Emulsifying activity of alasan is due to interactions of protein and polysaccharide portions of the complex. BE activity can be improved by simple media modification or mutagenesis.

Transposon mutants of *A. calcoaceticus* strain RAG-1 have been studied to control fatty acid (FA) substitution patterns of emulsan[43]. Single insertion sites in *A. calcoaceticus* genome were confirmed for each of the selected mutants based on southern hybridizations using transposon as a probe. Site of transposon insertion was determined for each mutant by cloning genomic DNA fragment conferring kanamycin resistance and sequencing outward using primers complimentary to sites within Tn*10*PttKm cassette. These disrupted genes, involved in biosynthetic pathways of biotin, histidine, cysteine or purines, influenced level and types of FAs incorporated into emulsan. Yields of emulsan from transposon mutants were found to be lower than parent strain and depended on type of FA used to supplement growth medium. Mutants 13D (His-) and 52D (Cys-), grown on LB plus C16 and C14 respectively, exhibited enhanced emulsifying activity as compared to *A. calcoaceticus* RAG-1. Presence and composition of long chain FAs on polysaccharide backbone influenced emulsification behavior; particularly a high mole percentage of C16 (48%) and C18 (42%). These results provide an important insight into bioengineering of BE-producing microorganisms and a path towards highly tailored novel amphipathic structures.

Conclusions

Marine microorganisms present an enormous diversity of options for wider biotechnological development. Microbial emulsifiers are important secondary metabolites produced by both marine and non-marine microbes. Out of marine microorganisms, *Acinetobacter* spp. are most studied. However, emulsifiers produced by a large number of terrestrial microorganisms have been reported. Studies on bioemulsifiers from marine sources are very less as compared to terrestrial ones. Thus, marine microbes are still to be explored with regard to BEs production. Ultramicrobiota, a unique group of marine microorganisms, has not been studied at all with respect to BE production.

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