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REVIEW ARTICLE

Marine Surfactants: A Review

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ABSTRACT

This review highlights the biosurfactants from micro- organisms of marine origin, which are capable of producing potential surfactants of industrial and biological applications, there are so many applications of marine biosurfactants in the pharmaceutical industry as well so it can be used as renewable source in the future for obtaining the biosurfactants and bioemulsifiers from marine micro-organisms. Different class of biosurfactants helps to assess the influence of molecular weight on the properties of marine biosurfactants.

KEYWORDS: biosurfactants, glycolipids, industrial potentials, etc.

INTRODUCTION:

Biosurfactants are surface active agents having microbial origin. They have both hydrophilic and hydrophobic regions in the same molecule, due to which they partition at the interfaces between the two phases. The Biosurfactants are majorly classified into different types such as glycolipids, lipoproteins, and lippopeptides, phospholipids and fatty acids, polymeric biosurfactants and particulate biosurfactants. They have several advantages such as lesser toxicity, stability at extreme temperatures, pH, salinity, higher biodegradability, and also ability to synthesize from cheap renewable sources.

These molecules also can be classified into two major classes such as high molecular weight and low molecular weight biosurfactants, The high molecular weight biosurfactants having molecular weight higher than 1MDa. These include mostly amphipathic polysaccharide, proteins, lipopolysaccharides and lipoprotein which help to stabilize oil in water emulsion and low molecular weight biosurfactants have molecular weight 1-2 KDa which include glycolipid and lippopeptide, which effectively lowers the interfacial and surface tension. It can ba assumed that different groups of biosurfactants have different natural roles in the growth of producing micro-organisms. It is possible that two closely related organisms having same species and same genus but having different habitat can produce different biosurfactants isoforms which helps to sustain their growth in that particular habitat. The produced biosurfactants will have different physicochemical properties, so they have potential biological and industrial aspects. They also possess potential applications as anti- bacterial, antifungal, and antiviral agents. The marine environment, which occupies major part of the earth surface, has diverse type of microflora which produces biosurfactants.

MARINE BIOSURFACTANTS AND BIOEMULSIFIERS:

EXOPOLYSACCHARIDES BIOSURFACTANTS:

These are the important group of marine biosurfactants. The different genera such as *alcaligens, pseudomonas, halomonas, antarobactor* are the major type of biosurfactants producers. For example, EPS producing strain *pseudomonas putida* ML2 which is isolated from hydrocarbon polluted sediments produced emulsifiers during growth on hydrophobic substrate, naphthalene, in the stationary and exponential growth phase. The crude emulsifier obtained had molecular weight approx. 10-80KDa, and it does not contain proteins. The monosaccharide proportion was rhamnose, glucose and glucosamine in the molar ratio of 3:2:1. Similarly other biosurfactants producing marine EPS strain *planococcus maitriensis* Anita I was isolated from seawater collected from costal area. The EPS obtained from this strain contained 12.06% carbohydrates, 24.44% proteins, 11% uronic acids, and 3.03% sulphate.

High molecular weight compound biosurfactants are reported from bacterial strains like TG39, TG67 and now characterized as halomonas species, these cultures showed profused growth and emulsifier production during exponential phase, and emulsions formed were stable even after 6 months. Biopolymers from TG39 had carbohydrate content of 17.3 ±1% which considered mainly of rhamnose, glucuronic acid, and galactose, and carbohydrate content obtained from biopolymers from TG67 was 22.7%. and has the composition of glucuronic acid, glucosamine and mannose.

GLYCOLIPPOPEPTIDES AND CARBOHYDRATE LIPID PROTEIN COMPLEXES:

This class of producing biosurfactants and bioemulsifier is very important. These bioemulsifiers and

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biosurfactants are carbohydrate protein and lipid complexes or glycolippopeptides are obtained from bacteria like *corneybacterium* sp. and *halomonas* sp. As well as yeast species like *yarrowia* sp. A strain of corneybacterium kutscheri which utilized substrate like waste motor lubricant oil and peanut cake oil produces biosurfactants having the composition of carbohydrate (40%), protein (29%) and lipid (27%). The produced boisurfactant emulsified various hydrocarbons, vegetative oils and polyaromatic hydrocarbons. Yansan is another bioemulsifier being produced from a strain of *yarrowia lipolyica* which is isolated during cultivation in glucose based YPD medium. Although the emulsifier was isolated in the late stationary phase, significant emulsifying capacity was also observed in cell free supernatant from exponential phase. The protein content in this bioemulsifier was found to be higher than lipid content. Another emulsifier producer strain, *Yarrowia lippolytica* NCIM 3589 which is isolated from oil contaminated sample, it produces emulsifiers in the stationary phase of its growth. The isolated emulsifier was found to be lipid, carbohydrate and protein complex having 75% lipid, 20% carbohydrate, and 5% protein. This emulsifier stabilized oil in water emulsion with several aromatic hydrocarbons.

GLYCOLIPIDS:

These biosurfactants consist of a hydrophilic glycol part consisting of few sugar molecules and hydrophobic lipid portion. Different types of microbial genera like *Halomonas, Pantoea, Nocardiodes, Rhodococcus*to name a few are the ones which produce glycolipid type of biosurfactants.

ENVIRONMENTAL AND INDUSTRIAL POTENTIALS:

The marine biosurfactants have been proved as potent applicator in the environmental bioremediation. Different studies have been suggested that these biosurfactants can be used for cleaning the environment polluted with the crude oil or polyaromatic hydrocarbons. Besides their applications in the environmental cleaning, they are also useful for industrial emulsifications and stabilization processes.

The high molecular weight biosurfactants such as exopolysachharides type of biosurfactants isolated from different marine bacteria showed their efficacy in environmental cleaning and potential for industrial applications.

The marine micro-organisms producing exopolysachharides such as *Alcaligens*sp. PHY9L.86 used as 0.1% tetradecan as the sole carbon andenergy source. The culture is able to degrade 98% of the hydrocarbon substrate within 48h of its growth. The high degradation efficiency showed by this marine bacterium may be exploited in the remediation of the crude oil contaminated sites. Another bacterium producing bioemulsifier *pseudomonas putida* ML2 is also able to grow on polyaromatic hydrocarbons. EPS produced by salt tolerant strain *planacoccus maitriensis* Anita showed a positive oil spreading test even at a low concentration of 0.1% and this oil dispersing potential was retained even at acidic and alkaline pH ranges. The oil dispersing capacity of this EPS was found comparable to Tween 80 and was even better than Triton X. The EPS produced also possess good emulsification activity and could emulsify various hydrocarbons and vegetable oils. Strikingly, its emulsion with silicone and paraffin and jatropa oil showed 100% stability upto 45 days and hence strain or its biosurfactants/ bioemulsifiers can find potential applications in industrial applications as well as in enhanced oil recovery. Another emulsifier AE22 produced by *Antarctobacter* sp. was found to form stable emulsion with various food oils at neutral and acidic pH values. The results indicated that the AE22 biopolymer can be better stabilizing agent than an emulsifying agent, a characteristics of natural hydrocolloid polymers. These stabilizing and emulsification properties may find various applications in healthcare and food oil formulations. The AE22 may also be applied as a biosorbent for treatment of contaminated environments. Similarly emulsifiers from *Halomonas* sp. TG39 and TG67 showed good emulsification activity with different edible oils as well as with hexadecane and these emulsions remained stable for several months. Both the emulsifiers were also able to show stable emulsification under both neutral and acidic conditions. However, the emulsification capacity at the acidic pH was found to be lower (<45%) than neutral pH, heat treatment was also found to increase the emulsification activity of these bioemulsifiers. The emulsifying and stabilizing properties of these extracellular bioemulsifiers suggest their potential use for commercial purposes. These novel emulsifiers may substitute the presently used emulsifiers that have limited emulsifications and stabilization potential. Emulsifiers produced by *corneybacterium kutscheri* emulsified various hydrocarbons. This culture was able to degrade crude oil more efficiently with added fertilizers. The potential of this strain of corneybacterium and its biosurfactants product to emulsify and degrade hydrocarbons may prove to be potent in environmental remediation and other similar type of purposes.

Similarly emulsifiers from *Yarrowia lippolytica* called yansan showed high emulsification activity with

hydrocarbons such as toluene, xylene and styrene and perfluorohydrocarbons(PFC). The emulsification activity was retained in a wide pH range (3-9) and was fairly pH independent. This biosurfactants has potential applications in bioremediation and formulation of perfluorocarbons based emulsions. In a similar emulsifiers from *Yarrowia lipolytica* NCIM3589 were found to stabilize oil-in-water emulsions with several aromatic hydrocarbons such as benzene, xylene, toluene and 1- methyl naphthalene. However, interestingly, the emulsion was not stable with n-alkanes, though the bacterium used these as sole carbon source. The emulsifier was stable and retained its activity in wide range of pH values 2-10. It was also found to retain its activity at 80^0c for 7h andat 100^0c for 3h.

Low molecular weight compounds such as glycolipids from marine micro- organisms have the potential for industrial emulsifications and environmental remediation applications. The example of this is glycolipid producer *Halomonas* sp. ANT-3b; this may degrade n- hexadecane and use it as source of carbon to produce biosurfactants. Hence this strain can be successfully used in remediation of the oil spills especially in the cold environments. Another biosurfactants producer *pseudomonas aeroginosa* A41 produced rhamnolipid biosurfactants, that showed good stability and activity in the wide range of temperatures (40-121^0c), pH (2-12) and NaCl concentrations (0-5%). Hence this marine glycolipid producer can be used for environmental cleaning in various extreme conditions and for enhanced oil recovery purposes. The glycolipids produced by MM1 were also found to be effective emulsifiers and also nontoxic in nature. Hence these glycolipidscan be effectively used for the removal of marine oil pollution without harming the marine ecology. Another glycolipids produced by actiomycetes *Nocardiodes* sp. was able to emulsify n-parrafin and several other aromatic hydrocarbons and thus could be used for remediation of the polluted sites. The cell surface hydrophobicity is important factor that determine the microbial adhesion on surfaces including hydrophobic substrates. It is also an important step in bioremediation as this step is required for the introduction of the molecular oxygen. The cell surface of facultative anaerobe *pantoea* sp. strain A13 becomes more hydrophobic when grown in hydrocarbons than that when grown in water miscible substrate like glucose.

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. The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m. 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 or kerosene or a mixture of n-hexadecane and 2-methylnaphthalene or deodecane 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 follows:

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.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 = (HLBneeded - HLBB) ÷ (HLBA - HLBB)

When

A: % cyclohexane

HLBA: HLB value of cyclohexane (=15)

HLBB: 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.

METHODS:

- Screening for biosurfactant production
  - Microorganism and Hemolytic activity

B. megaterium, C. kutscheri and *P. aeruginosa* may be isolated from water sample using Bushnell–Haas agar with 0.1% of crude oil and identified to the species level .

Hemolytic assay is performed in blood agar plates. 50µl broth culture is spot-inoculated on to blood agar plates and incubated for 48h at 37oC. The plates are visually inspected for zone of clearance (hemolysis) around the colony. The diameter of the zone of clearance is a qualitative method used as an indicator of biosurfactant production.

Bacterial adhesion to hydrocarbons (BATH)

Cell hydrophobicity is measured by BATH assay. Bacterial cells are washed twice and suspended in a buffer

salt solution (g/l 16.9 K2HPO4, 7.3 KH2PO4) to give an optical density (OD) at 600 nm of ~ 0.5. The cell suspension (2ml) with crude oil added is vortex shaken for 3 min in test tubes (10x100mm). After shaking, crude oil and aqueous phase are allowed to separate for 1h. OD of the aqueous phase was then measured at 600nm in a spectrophotometer. Hydrophobicity is expressed as the percentage of cell adherence to crude oil calculated as follows: 100 x (1-OD of the aqueous phase/OD of the initial cell suspension). For a given sample, three independent determinations can be made and the mean value may be accounted

- Visualization of bacteria in oil droplets

A few drops of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution is added to the BATH assay culture broth and observed under the microscope. The INT turned red if it is educed inside the cells, indicating the viability and adherence of cells with crude oil droplets.

- Drop-collapse test

All three bacterial strains are cultured in mineral salts medium with 0.1% crude oil for 48 h. Screening of biosurfactant production can be performed using the qualitative drop-collapse test. Crude oil was used in this test, 2µl of oil is applied to the well regions delimited on the covers of 96-well microplates and these are left to equilibrate for 24h. 5µl of the 48h culture, after centrifugation at 12,000g for 5 min to remove cells, is transferred to the oil-coated well regions and drop size is observed 1 min later with the aid of a magnifying glass. A result is considered positive for biosurfactant production when the drop diameter was at least 1mm larger than that produced by deionized water (negative control).

- Emulsification assay

Partially purified biosurfactant (5mg) is dissolved in 5ml of Tris buffer (pH 8.0) in 30ml test tubes. Hydrocarbons like waste motor lubricant oil, crude oil, peanut oil, diesel, kerosene, naphthalene, anthracene and xylene are tested for emulsification activity. 5mg of hydrocarbon is added to the above solution and shaken well for 20 min the mixture is allowed to stand for 20 min. The optical density of the emulsified mixture is measured at 610nm and the results are expressed as D610. Emulsification activity of the biosurfactant is compared with Triton X-100 (1mg/ml), concentration and conditions for emulsification study are maintained similar to that of biosurfactant.

- Cell growth and Biosurfactant production

➢ Biosurfactant production in fermentor

Laboratory scale biosurfactant production may be performed in a 3L fermenter working volume. Optimization of culture conditions is carried out and reported elsewhere. The culture conditions are as follows - pH 8.0, temperature 38oC, salinity 30‰ (w/v) and 2.0% substrate concentration and 8.0 mg/l of dissolved oxygen (DO). Substrates used may be crude oil, peanut oil cake and waste motor lubricant oil

➢ Estimation of growth

Two milliliters of culture broth is collected at 12h intervals for a period of 168h and the biomass is estimated gravimetrically. For gravimetric estimation of biomass 1ml of broth culture is taken and allowed to stand for some time. When the oil phase separated, the bottom phase with cells is siphoned out and filtered through a 0.45µm sized Millipore filter paper. The filter paper with cells is dried at 80oC in a hot air oven for a period of 24h and weighed; a control is maintained to exclude the weight of crude oil adhered to the filter. Biomass is quoted in terms of mg/ml (dry weight).

➢ Purification of biosurfactant

Culture broth has to be centrifuged at 12,000g for 20min and extracted twice with chloroform and methanol (2:1v/v). The solvents are removed by rotary evaporation and the residue is purified in a silica gel (60–120 mesh) column and the elution are made with chloroform and methanol ranging from 20:1 to 2:1v/v in a gradient manner and fractions are obtained. The fractions are pooled and the solvents are evaporated, the resulting residue is dialysed against distilled water and lyophilized. Weight of the biosurfactant can be expressed in terms of mg/ml (dry weight).

- Characterization of biosurfactant

➢ Biochemical composition of biosurfactant

Chemical composition of the biosurfactant can be analyzed following standard methods. Carbohydrate content of the,biosurfactant can be determined by the phenol–sulfuric acid method using D-glucose as a standard. Protein content can be determined by the Lowry et al. method using bovine serum albumin as a standard and lipid content can be estimated

➢ Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. One milligram of freeze-dried partially purified biosurfactant iss grinded with 100mg of KBr and pressed with 7500kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra are recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01cm-1, respectively. All measurements consisted of 500 scans, and a KBr pellet is used as background reference.

➢ Mass spectrometric analysis of biosurfactant

Biosurfactant can be dissolved in methanol and mixed thoroughly. The mass spectrometric analysis of the biosurfactant can be carried out in LCQTM quadrupole iontrap mass spectrometer utilizing electrospray ionization (ESI). Standard solutions and samples under investigation are infused into the mass spectrometer at a flow rate of 10µl/min. In the ESI, nitrogen and auxiliary gas flows are maintained at 50 and 5ml/min respectively and refer to arbitrary values set by the software. The heated capillary temperature was 250oC and the spray voltage is set to 5kV. Negative ion mode is used and scanning can be done at 50–2,000 *m/z* range.

BIOLOGICAL ACTION OF MARINE BIOSURFACTANTS:

Biosurfactants may have several therapeutic and biological applications, yet they are not assessed extensively for the biological activities. For example, glycolipid obtained from *nocardioides* sp. Caused hemolysis and inhibited *bacillus subtilis* cells. And also it is able to modify the cell surface hydrophobicity of other bacterial strains which indicated their role in attachment and detachment of bacteria on certain surfaces. Another biosurfactant of lipopeptide class, *bacillus pumilus* KMM 150 caused anomalies in the development process of echinus ova. Its ovicidal and cytotoxic effect against cells in the early stages of the development may be potentially used as contraceptive agent or agent for safe termination of unwanted pregnancy.

CONCLUSION:

Marine micro- organisms possess enormous diversity of action for vast technological development. Studies on marine biosurfactants are very less than terreserial ones. So by properly assessing the biological and industrial properties, marine biosurfactans can be evaluated. Marine biosurfactant have been proven as the promising agents for bioremediation of hydrocarbons, particularly oil polluted in marine environment. Marine biosurfactant can be used for therapeutic purposes so they should be assessed extensively for the biological activities.

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