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Structural characterization and biological evaluation of Staphylosan (dimannooleate), a new glycolipid surfactant produced by a marine *Staphylococcus saprophyticus* SBPS-15

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ABSTRACT

Marine microbes have gained significant attention as potential biofactories for broad spectrum bioactive compounds. In the recent years, bioactive biosurfactants have warranted renewed interest from both environmental and clinical sectors as anti-biofilm agents due to their excellent properties of dispersing microbial biofilms. The present study explores a new glycolipid biosurfactant produced by a marine *Staphylococcus saprophyticus* exhibiting interesting biological activities. This glycolipid biosurfactant was purified and identified as Mannose-Mannose-Oleic acid (named as Staphylosan) based on the results of NMR, GC, GC–MS, MALDI-TOF-MS and tandem MS analysis. The surface tension and critical micelle concentration of purified Staphylosan was 30.9 mN m^{-1} and 24 mg L^{-1}. Further, it showed promising biofilm inhibition and dislodging activities against a panel of profuse biofilm forming bacteria at both single and multi-species level which were isolated from boat hull biofilm environment such as *Bacillus subtilis* BHKH-7, *Acinetobacter beijerinckii* BHKH-11, *Pseudomonas aeruginosa* BHKH-19, *Serratia liquefaciens* BHKH-23, *Marinobacter lipolyticus* BHKH-31 and *Micrococcus luteus* BHKH-39. Moreover, it exhibited anionic charge and revealed non-toxicity towards brine shrimps, suggesting its environmental safety. This is a first report on Staphylosan, a multifunctional glycolipid surfactant from a marine *Staphylococcus saprophyticus* SBPS-15, exhibiting promising anti-biofilm activities and non-toxic in nature and thus finds possible potential use in many environmental applications especially under marine conditions.

1. Introduction

From a pharmacological and biotechnological perspective, the marine environment is interesting in view of its diverse chemical and biological diversity. In the recent years, a diverse range of broad-spectrum bioactive molecules have been explored from microbes of the marine realm [1]. Among the marine explored bioactive compounds, microbial biosurfactants has gained a renewed interest due to their structural novelty, versatility and diverse properties [2,3]. Biosurfactant producers are ubiquitous in nature with reports from bacteria, fungi, yeast and actinomycetes. They are surface-active molecules which are considered as suitable green alternatives as compared to their existing chemical surfactants in use with respect to emulsification, detergency, foaming, dispersion, wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovery [4]. Besides the common surfactant properties, these surface-active molecules can interact with cell membranes of several organisms and/or with the surrounding environments and thus viewed as promising bioactive molecules with extended applications as antimicrobial, anti-biofilm, anticancer agents and so on [5].

Microorganisms can adhere to natural or artificial surfaces and form sessile and aggregated communities designated as biofilms [6]. Marine biofilms are one of the examples of unwanted biofilms that poses many technical problems and adds to financial burden, especially to the naval industry [7]. Bacteria are the most common ones responsible for biofilm formation which starts with planktonic bacterial adhesion to a surface by the use of extracellular sticky appendages (reversible adhesion). After an adaptation of its metabolic state, colonies start the production of EPS (irreversible adhesion). Living as biofilms, bacteria can resist hostile environments and antibiotics/biocides, easily access nutrient uptake and confers a more resilient colonisation [8]. Since the last decade, many researchers investigated several strategies and methods

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to control and/or kill marine biofilms which still remains a great challenge [9].

In the recent years, biological surface active molecules (so-called biosurfactants) are considered the most promising candidates for the inhibition of bacterial biofilms [10,11]. The adsorption of surface active/biosurfactant to a substratum surface alters hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes [10]. The available literature conferred that the anti-biofilm potential of biosurfactant mainly against clinical strains and not much reports exist on environmental aspects. Despite the vast diversity of microbial species in ocean and their unique metabolite biochemistry, there are only few reports available on anti-biofilm biosurfactants of marine origin [11–16]. Moreover, the available information revealed less concentrations regarding biosurfactant activity against marine biofilms and no study investigated its ecotoxicity concern. So, the need of more new research is of significant importance in examining the potentiality of less understood marine biosurfactants in the emerging trends of natural bioactive resources, hence this study was investigated since there is paucity of work using a novel structure. Further, this study undertook a systematic characterization of this novel biosurfactant isolated from a marine *Staphylococcus saprophyticus* strain SBPS-15 using various analytical techniques such as NMR, GC, GC–MS, MALDI-TOF-MS and tandem mass spectrometry. Further, this biosurfactant was studied against several potential biofilm forming bacteria isolated from a boat hull environment and its toxicity was assessed using ionic charge property and brine shrimp lethality bioassay.

2. Materials and methods

2.1. Biosurfactant producing marine bacterium and culture conditions

The biosurfactant producing marine bacterium, *Staphylococcus saprophyticus* SBPS-15 used in this study was previously identified (GenBank accession no. KX352162) and reported to produce a promising glycolipid biosurfactant based on different screening and biochemical methods [17]. This marine bacterium was isolated from a petroleum contaminated sediment sample collected from the coastal sites of Puducherry, India, and maintained in the laboratory as an axenic culture and stocks in lyophilized form. The strain was cultured in glucose-mineral salts production medium having the following composition: Glucose (2%), NH_4NO_3 (0.3%), K_2HPO_4 (0.22%), KH_2PO_4 (0.014%), NaCl (0.001%), $MgSO_4$ (0.06%), $CaCl_2$ (0.004%), $FeSO_4$ (0.002%) and 0.5 ml L^{-1} of trace elements solution containing (per litre): 2.32 g of $ZnSO_4.7H_2O$, 1.78 g of $MnSO_4.4H_2O$, 0.56 g of H_3BO_3, 1.0 g of $CuSO_4.5H_2O$, 0.39 g of $Na_2MoO_4.2H_2O$, 0.42 g of $CoCl_2.6H_2O$, 1.0 g of EDTA, 0.004 g of $NiCl_2.6H_2O$ and 0.66 g of KI, prepared in 34 ppt sea water. The inoculum was prepared using 1% exponential phase culture in the same production medium, where the optical density ($OD_{620\ nm}$) of the inoculum was adjusted to 0.1 based on McFarland turbidity standard of 0.5, equivalent to the bacterial concentration of 1×10^8 cfu ml^{-1}. The fermentation was carried out in a 3 L laboratory fermenter (Scigenics, India) with 2.1 L working volume. The culture conditions used were: pH 8.0, temperature of 37 °C, agitation at 150 rpm and aeration at 1.0 vvm for 66 h.

2.2. Purification and structural characterization of biosurfactant

The purification of glycolipid biosurfactant from the marine bacterium *Staphylococcus saprophyticus* SBPS-15 was described earlier [17]. Briefly, the crude biosurfactant obtained from the cell free supernatant was acid precipitated followed by solvent extraction and the purification was achieved on normal phase silica gel (60–120 mesh, HiMedia Laboratories Pvt. Ltd., Mumbai, India) column chromatography with stepwise elution using methanol and chloroform solvent system. The purified glycolipid biosurfactant was eluted at 1:11 fraction and based on TLC analysis it was found to be a glycolipid which was subjected to further structural characterization studies.

The purified biosurfactant was further hydrolyzed with 6 mol L^{-1} HCl at 100 °C for 24 h and the resulting hydrolyzate was extracted with diethyl ether to obtain solvent and aqueous fractions. The solvent phase contained fatty acids whereas the aqueous phase contained sugar components which were analyzed using GC–MS and GC methods. The fatty acid components were methylated [18] and analyzed on a Thermo Trace GC Ultra coupled with Polaris Q MS and TriPlus auto-sampler using a DB-5 (0.25 mm × 30 m × 0.22 µm) column by passing helium as a carrier gas. The temperature was set between 60 °C–260 °C and ramped at a rate of 10 °C min^{-1}. The initial temperature was held for 2 min and final temperature was set at 260 °C for 10 min. The GC flow rate was 1 ml min^{-1} and the total run time was 32 min. MS was recorded at scan mode between *m/z* 50–350 with an ion trap EI^+. The temperature of the ion source was 200 °C. The mass spectrum of the fatty acid methyl ester was identified based on NIST database. Similarly, the aqueous fraction containing the sugar components were analyzed using aldononitrile acetate derivatization [19] and simultaneously 10 standard neutral sugars were also derivatized. The GC analysis was carried out with HP 6890 GC equipped with a flame ionization detector (FID) (Agilent, USA) and a DM-1 fused silica capillary column (30 µm × 0.25 µm × 0.25 µm film thickness) was used for sugar separation. Nitrogen gas was used as carrier gas at a flow rate of 1.2 ml min^{-1}. The initial column temperature was set at 175 °C for 4 min and it was increased at 4 °C min^{-1} to 225 °C held for 2 min. Thereafter, the temperature was increased again at 50 °C min^{-1} to 300 °C held for 2 min. The inlet temperature was 250 °C and the detector temperature was set at 300 °C. The results were analyzed with the help of Agilent ChemStation A.08.03.

The detailed structural characterization of the purified biosurfactant was elucidated using MALDI-TOF MS/MS analysis with a Voyager DE-Pro MALDI-ToF spectrometer (Applied Biosystems Inc., CA, USA) in reflector mode with an accelerating voltage of 20 kV. Purified sample fraction (2 µl) was mixed with equal volume of matrix solution (0.1% α-cyano-4-hydroxycinnamic acid in acetonitrile–water-TFA; 50:50:0.01, v/v/v) and the mixture was spotted onto the sample plate and dried to form matrix crystallization. Spectra were recorded in the mass ranging between 250 and 4000 Da. Further, the unhydrolyzed purified biosurfactant was dissolved in D_2O at 50 mg ml^{-1} concentration and NMR spectrum was recorded on a Bruker AV600 NMR spectrometer (Germany) at 500 MHz. Chemical shifts were expressed in parts per million (ppm) downfield from an internal standard of tetramethylsilane (TMS).

2.3. Critical micelle concentration

The critical micelle concentration (CMC) was determined by plotting biosurfactant concentration as a function of surface tension reduction of distilled water (72 mN/m) with the help of Du Nouy ring method using Digital Tensiometer (Jinhaihu, China) and the CMC value was taken at the point where the slope of the curve abruptly changed [20].

2.4. Isolation of biofilm forming bacterial strains

A battery of six different profuse biofilm forming marine bacterial strains were used in this study which were isolated from the biofilms of boat hull samples collected from Karaikal fishing harbour, Puducherry, India. Briefly, the isolation was made from randomly chosen four different biofilms formed on boat hulls in which the biofilms was scrubbed, serially diluted in pre-sterilized sea water (34 ppt) and spread plated on Zobell marine agar plates. After 48 h incubation, a total of 39 distinct morphological bacteria were isolated and pure cultured. All the bacterial strains were individually predetermined for strong adhesion forming ability. The predetermination was based on biofilm formation to the polystyrene microtitre plate as described by Stepanović and