Microbiological Research 194 (2017) 1–9

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Aneurinifactin, a new lipopeptide biosurfactant produced by a marine *Aneurinibacillus aneurinilyticus* SBP-11 isolated from Gulf of Mannar: Purification, characterization and its biological evaluation

Shanmugasundaram Senthil Balan[a,c], C. Ganesh Kumar[b,*], Singaram Jayalakshmi[a]

[a] CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Porto Novo, Tamil Nadu 608502, India
[b] Medicinal Chemistry and Pharmacology Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India
[c] Present address: Department of Medicinal Plant Biotechnology, Sharmila Institute of Medicinal Products Research Academy, Thanjavur, Tamil Nadu 613007, India

ARTICLE INFO

Article history:
Received 2 May 2016
Received in revised form 13 October 2016
Accepted 22 October 2016
Available online 26 October 2016

Keywords:
Aneurinibacillus aneurinilyticus
Lipopeptide
Biosurfactant
Emulsification
Antimicrobial
Oil recovery

ABSTRACT

Biosurfactants are microbial-derived amphiphilic molecules having hydrophobic and hydrophilic moieties produced by bacteria, fungi, yeasts and algae and are extracellular or cell wall-associated compounds. In an ongoing survey for bioactive microbial metabolites from microbes isolated from diverse ecological niches, a new lipopeptide biosurfactant was identified from a marine bacterium; Aneurinibacillus aneurinilyticus strain SBP-11, which was isolated from a marine diversity hotspot, Gulf of Mannar, India. A new lipopeptide biosurfactant was purified and characterized based on TLC, FT-IR, NMR, GC–MS, HPLC, MALDI-TOF-MS and tandem MS analysis as Stearic acid-Thr-Tyr-Val-Ser-Tyr-Thr (named as Aneurinifactin). The critical micelle concentration of Aneurinifactin was 26 mg L^{-1} at a surface tension of 26 mN m^{-1}. Further, the biosurfactant showed stable emulsification at a wide range of pH (2–9) and temperature up to 80 °C. Aneurinifactin showed promising antimicrobial activity and concentration dependent efficient oil recovery. This is the first report on Aneurinifactin, a lipopeptide biosurfactant produced by a marine A. aneurinilyticus SBP-11, which could be explored as a promising candidate for use in various biomedical and industrial applications.

1. Introduction

Microbial compounds exhibiting pronounced surface and emulsifying activities are classified as biosurfactants which include glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Marchant and Banat, 2012; Gudina et al., 2016). They have both hydrophilic and hydrophobic moieties that tend to interact with phase boundaries in a heterogeneous system to solubilize the organic molecules (Banat et al., 2010). In view of the diverse chemical structures, biodegradability, lower toxicity and effectiveness to function at extreme temperature, pH and salinities qualifies these biosurfactants as suitable green alternatives as compared to their synthetic counterparts, which are amenable for application in diverse niche areas such as agriculture, pharmaceutical, cosmetics, food industries, oil recovery and environmental remediation (Nitschke and Costa, 2007; Pacwa-Płociniczak et al., 2011; Sachdev and Cameotra, 2013; Gudina et al., 2013; Shekhar et al., 2015). Moreover, several biosurfactants such as surfactin (Cao et al., 2010), iturin (Arrebola et al., 2010), rhamnolipids (Cortes-Sanchez et al., 2013), sophorolipids (Hirata et al., 2009), mannosylerythritol lipids (Arutchelvi et al., 2008) and trehalose lipid (Christova et al., 2015) exhibited antibacterial, antifungal or antitumor activities making them potential alternatives to conventional therapeutic agents for use in many biomedical applications (Cortes-Sanchez et al., 2013; Gudina et al., 2013).

The marine environment comprises of >70% of the Earth's surface harboring a rich biological and chemical diversity (Proksch et al., 2003; Bhatnagar and Kim, 2010; Blunt et al., 2012, 2015). Earlier studies substantiate the isolation of new biosurfactant producing microorganisms in view of the biosurfactant heterogeneity with species diversity (Bodour et al., 2003; Kennedy et al., 2011). Considerable advances were made in the recent past on the isolation and screening for biosurfactant producing strains from marine habitats having unique metabolic and physiological capabilities to thrive in extreme habitats and the ability to produce novel metabo-

* Corresponding author.
E-mail addresses: cgkumar@iict.res.in, cgkumar5@gmail.com (C.G. Kumar).

http://dx.doi.org/10.1016/j.micres.2016.10.005
0944-5013/

lites (Satpute et al., 2010; Das et al., 2010; Imhoff et al., 2011); however, the biosurfactants produced by marine microorganisms have been less explored as compared to the microorganisms isolated from terrestrial samples or hydrocarbon-polluted environments (Jackson et al., 2015; Gudina et al., 2016). The predominant bacterial genera from marine environments reported to produce biosurfactants include *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Halomonas*, *Bacillus*, *Myroides*, *Corynebacterium* and *Alteromonas* (Satpute et al., 2010; Gudina et al., 2016).

In the present study, we report for the first time a promising biosurfactant producing marine *Aneurinibacillus aneurinilyticus* strain SBP-11 isolated from the Gulf of Mannar, South India, and an important coral inhabiting marine biodiversity hotspot of Asia. A new lipopeptide biosurfactant was characterized from strain SBP-11 using different analytical techniques such as TLC, FT-IR, NMR, GC–MS, HPLC, MALDI-TOF-MS and tandem mass spectroscopy. The biosurfactant was also evaluated for the antimicrobial activity and for application in crude oil recovery.

2. Materials and methods

2.1. Isolation, screening and identification of biosurfactant producing marine bacteria

Six sea sediment samples were collected from the coastal sites of Gulf of Mannar, Pamban, Tamilnadu, India, from different sites at 100 m equal distance using a Petersen grab sampler. These samples were transported to the laboratory in 250 ml pre-sterilized bottles and stored at 4 °C until further processing. The central portions of the samples were serially diluted using pre-sterilized sea water and spread plated on Bushnell Haas agar prepared with sea water and supplemented with 1% (v/v) crude oil. After 4 days incubation, morphological distinct colonies were isolated, and subcultured 4–5 times on Zobell marine agar plates to obtain axenic cultures and were preserved as lyophilized stocks stored at 4 °C for further studies. The isolated bacterial strains were subjected to different screening tests such as oil displacement test (Morikawa et al., 1993), drop collapse method (Youssef et al., 2004) lipase activity (Kiran et al., 2010) and emulsification index (EI_{24}) (Cooper and Goldenberg, 1987) for identifying the most promising strains. The promising strain was identified based on morphological, physiological and biochemical identification methods as well as 16S rRNA sequencing using the bacterial universal primer set of Eubac 27F (5′- AGAG TTTG ATCM TGGC TCAG -3′) and 1492R (5′- GGTT ACCT TGTT ACGA CTT -3′). The PCR product was purified using the QIAGEN PCR purification kit for sequencing and later sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). Sequence homologies were examined using BLAST version 2.5.0 of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary distance was computed using the maximum-likelihood method (Tamura et al., 2004) and the evolutionary analysis was performed using MEGA 7 (Kumar et al., 2016). The morphological, physiological and biochemical characteristics of the promising strain were determined as per the systematic procedures of *Bergey's Manual of Systematic Bacteriology* (Vos et al., 2009). The morphological and biochemical characteristics were performed as described by *Laboratory Exercises in Microbiology* (Harley, 2013), further detailed carbon source utilization tests were performed using Biotype 100 gallery (API System, bioMerieux, Paris, France) as per the manufacturer instructions.

2.2. Medium and fermentation conditions

Strain SBP-11 was cultured in a 3 L fermenter (Scigenics, India) with 2.1 L working volume for biosurfactant production in glucose-yeast extract-mineral salts (GYMS) medium prepared in natural sea water (34 ppt) having the following composition (per litre): glucose (1.5 g), K_2HPO_4 (5.2 g), KH_2PO_4 (3.18 g), NH_4Cl (0.54 g), yeast extract (0.5 g), $MgSO_4$ (0.12 g) and 5 mL L^{-1} of trace elements solution containing (per litre): 0.287 g $ZnSO_4{\cdot}7H_20$; 0.249 g $CuSO_4{\cdot}5H_20$; 0.223 g $MnSO_4{\cdot}4H_20$; 0.124 g $Na_2MoO_4{\cdot}2H_20$; 0.118 g $CaCl_2{\cdot}6H_2O$; 0.083 g KI and 0.03 g H_3BO_3. An overnight grown culture in Zobell Marine 2216 broth was used as a source of inoculum. The fermentation conditions used were: pH 8.0, temperature of 37 °C, agitation at 150 rpm and aeration at 1.0 vvm for 90 h. At regular intervals, samples were withdrawn and centrifuged at 2700 × *g* for 15 min and the cell pellets were used for estimation of cell biomass on dry weight basis and the cell free supernatants were used for the determination of production peak time on the basis of 24 h emulsification index (Khopade et al., 2012). The growth kinetics profile as a function of time was plotted with reference to biosurfactant production and bacterial growth.

2.3. Purification and biochemical characterization of the biosurfactant

The fermented medium was centrifuged to remove the cell biomass and to obtain a cell free supernatant which was subjected to acid precipitation at pH 2 using 6 N HCl. At pH 2, the biosurfactant converts to a protonated form, making it less soluble in water and favors easy recovery. The treated broth was stored overnight at 4 °C for biosurfactant precipitation, which was collected by centrifugation at 8000 × *g* for 20 min and the acidified biosurfactant was neutralized using phosphate buffer (pH 7) solution. The resultant biosurfactant was extracted with an equal volume of ethyl acetate and the organic phase was pooled, concentrated and dried on a rotary vacuum evaporator (Lablinks PBU-6, India). Further, the isolated biosurfactant was purified by C_{18} silica gel (230–400 mesh) column chromatography using step-wise elution with methanol and water ranging from 0:100 to 100:0 (v/v) at a flow rate of 0.5 mL min^{-1} at room temperature. A total of 41 fractions were collected and each fraction was examined for maximum biosurfactant activity and the purity was checked by thin layer chromatography. The fractions exhibiting maximum activity were pooled, concentrated by rotary vacuum evaporation and lyophilized. The concentrated biosurfactant was analyzed on silica gel 60 TLC plates (F_{254}, Merck) and developed in different solvent mixtures of 96% ethanol:water (7:3) for peptide, chloroform:acetic acid:water (60:30:10) for sugars and chloroform:methanol:water (65:25:4) for lipids. The spots on the developed TLC plates were visualized using ninhydrin reagent to detect peptide, anthrone reagent for sugars and lipid portion with rhodamine B reagent.

Based on the preliminary TLC examination, the biosurfactant was hydrolyzed with 6 mol/L HCl at 100 °C for 24 h and the resulting hydrolyzate was extracted with diethyl ether to obtain solvent and aqueous fractions. The solvent and aqueous phases containing fatty acid and amino acid components were analyzed by GC–MS and HPLC methods. The fatty acid components were methylated (Jenkins et al., 2001) and analyzed using a DB-5 (0.25 mm × 30 m × 0.22 μm) column on a Thermo Trace GC Ultra interfaced with Polaris Q MS and TriPlus auto-sampler by passing helium as a carrier gas. The temperature was set between 60 °C to 260 °C and ramped at a rate of 10 °C min^{-1}. The initial temperature was held for 2 min and final temperature of 260 °C for 10 min. The flow rate was 1 mL min^{-1} and the total run time was 32 min. MS was performed at scan mode between *m*/*z* 50–300 with an ion trap EI^+. The temperature of the ion source was 200 °C. The mass