Heterologous expression, purification, and phylogenetic analysis of oil-degrading biosurfactant biosynthesis genes from the marine sponge-associated Bacillus licheniformis *NIOT-06*

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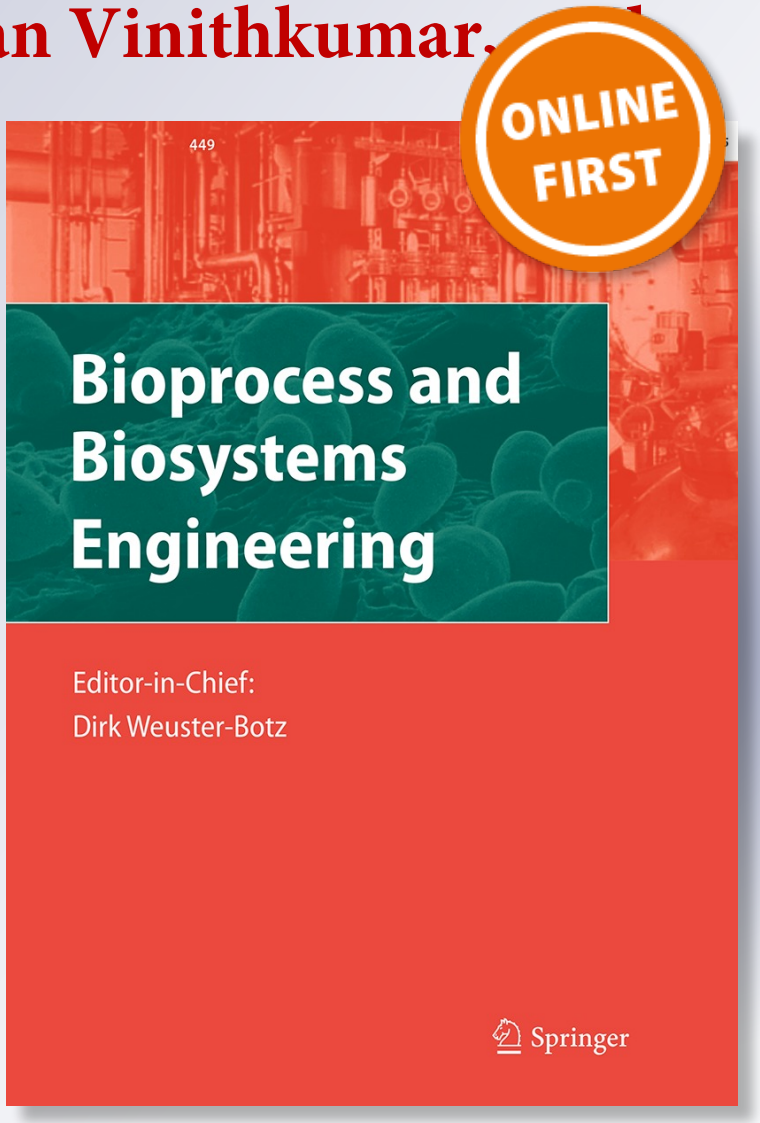

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ORIGINAL PAPER

Heterologous expression, purification, and phylogenetic analysis of oil-degrading biosurfactant biosynthesis genes from the marine sponge-associated *Bacillus licheniformis* NIOT-06

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Abstract Surfactin is a lipopeptide, composed of one β-hydroxy fatty acid, a long fatty acid moiety, and seven amino acids. In this study, the biosurfactant biosynthesis genes; 4′-pantetheinyl transferase (*sfp*), phosphopantetheinyl transferase (*sfp*O), and surfactin synthetase (*srf*A) have been characterized from the marine sponge-associated *Bacillus licheniformis* NIOT-06 from the Andaman and Nicobar Islands. The purified recombinant biosurfactant revealed excellent emulsification activity with crude oil and kerosene. Reverse-phase high-performance liquid chromatography resolved the purified recombinant biosurfactant into several fractions and one of which had significant surface tension reducing property. Fourier transform infrared spectroscopy spectrum also revealed the presence of C–N–N, alkenes, and N–H as the functional groups, and a similar overlapping pattern was observed with that of standard lipopeptide surfactin. The diversity and phylogeny of *sfp*, *sfp*O, and *srf*A gene sequences were compared with other eubacteria. The *sfp*, *sfp*O, and *srf*A gene sequences obtained from *Bacillus licheniformis* NIOT-06 were diverse and appeared to be partially conserved when compared with the GenBank reported sequences of several eubacteria.

Keywords *B. licheniformis* NIOT-06 · Marine sponge · Biosurfactant · Biosurfactant biosynthesis genes · Biodegradation

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Introduction

Biosurfactants are amphiphilic compounds, produced by bacteria, fungi, and yeast. They belong to various macromolecular classes, such as glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids, and lipopolysaccharides. The properties of biosurfactants include excellent detergency, emulsification, foaming, metal sequestration, and oil recovery. In addition, biosurfactants are promising natural surfactants that offer several advantages over the chemically synthesized surfactants, such as lower toxicity, biodegradability, and ecological acceptability [1]. Among the biosurfactants, lipopeptides are the most extensively studied and have been found in many chemoheterotrophic halophilic eubacteria [2, 3]. The biosynthesis of surfactant comprises of three genes and has been characterized in few halophiles [4]. The *sfp*, *sfp*O, and *srf*A are the genes involved in the biosynthesis of biosurfactant in eubacteria. The bacterial isolates associated with marine sponges have the ability to produce compounds that are more potential

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than other sources [5]. However, no reports are available on the characterization of biosurfactant from the marine invertebrate-associated bacteria. Marine sponges (Poriferans) are sessile filter feeders that filter bacteria from seawater by pumping in large volumes of water through their aquiferous system. They are considered as important sources for biologically active natural products, and diverse secondary metabolites have been derived from sponge-associated microbial community [6]. In the present study, *Bacillus licheniformis* from marine sponge was used for the isolation and characterization of biosurfactant.

Bacillus licheniformis is an alkaliphilic bacterium [7] that can grow well at pH range of 7–10.5 in marine environments. It is well characterized biochemically and genetically, and it has the ability to synthesize the oil-degrading biosurfactant [8]. The objective of this study was based on heterologous expression and purification of the lipopeptide surfactant from *B. licheniformis* NIOT-06 and to determine the diversity and phylogenetic relationship of biosurfactant biosynthesis genes (*sfp*, *sfp*O, and *srf*A) with other eubacteria.

Materials and methods

Bacterial growth conditions and identification

The eubacterial strain NIOT-06 was isolated from the marine sponge *Acanthella* sp. collected from North Bay of Port Blair, South Andaman by scuba diving at a depth of 12 m. The isolate was grown aerobically in alkali bacillus medium containing 1 % peptone (w/v), 0.5 % yeast extract (w/v), 1 % glucose (w/v), 0.1 % K_2HPO_4 (w/v), 1 % Na_2CO_3 (w/v) and incubated at 37 °C. The genomic DNA was extracted following the method described by Ausubel et al. [9]. Approximately 1 mL of overnight grown NIOT-06 culture was centrifuged at 10,000*g* for 1 min in a 3K30 centrifuge (Sigma, Germany). The pellet was resuspended in 100 µL of MilliQ water and subjected to cell lysis in a boiling water bath for 5 min. After centrifugation for 5 min at 5,000*g*, 1 µL of supernatant was used for PCR amplification. The 16S rRNA was PCR amplified using the universal eubacterial primers, 16S f (5′-ACTCAAAGGAATT GACGG-3′) and 16S r (5′-TACGGCTACCTTGTTAC GACTT-3′). The amplicon was cloned in a T/A cloning vector according to the manufacturer's instructions in the InsTAclone PCR Cloning kit (MBI Fermentas, USA). The sequencing was performed on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA) using the dye termination method. The acquired 16S rRNA sequences were used in a homology search with the available sequences in GenBank using BLAST provided by NCBI (http://www.ncbi.nlm.nih.gov) for pairwise identities. Multiple sequence alignment of the sequences was performed using the CLUSTAL X version 1.81 program, and the phylogenetic tree was constructed with the MEGA version 5.0 program using neighbor-joining analysis.

PCR amplification of the surfactant biosynthesis genes

The biosurfactant genes (*sfp*, *sfp*O, and *srf*A) of NIOT-06 were PCR amplified using gene-specific primers designed using a program available at http://frodo.wi.mit.edu/primer3. PCR was performed in a 50-µL reaction mixture that contained 50 ng of genomic DNA, 0.5 µM of respective primer, 200 µM of respective dNTP (MBI Fermentas, USA), 1.25 U of *Pfu* DNA polymerase (MBI Fermentas), 1 × *Pfu* buffer, 2.5 mM $MgSO_4$, and autoclaved Millipore water. Amplification was performed in a Mastercycler (Eppendorf, Germany) under the following conditions: initial denaturation at 94 °C for 3 min followed by 30 repeated cycles of 94 °C for 30 s, 53 °C for 1 min for *sfp*; 52 °C for 1 min for *srf*A; 55 °C for 1 min for *sfp*O and 72 °C for 2 min and a final extension at 72 °C for 5 min. The PCR amplicons were analyzed on a 1.5 % agarose gel along with a DNA molecular weight marker (MBI Fermentas) and documented in a gel documentation system (UVP BioSpectrum Imaging system, USA).

Cloning and sequencing

The PCR amplicons of *sfp*, *sfp*O, and *srf*A were purified by the MinElute Gel purification Kit (Qiagen, Germany) and cloned into the pTZ57R/T vector (MBI Fermentas). The pTZ57R/T-*sfp*, *sfp*O, and *srf*A constructs were transformed into competent *Escherichia coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (rK–mk+), e14–(*mcrA*–), *supE*44, *relA*1, Δ(*lac-proAB*)/F′ (*traD*36, *proAB*+, *lac Iq*, *lacZ* ΔM15), plated on Luria–Bertani (LB) agar containing 100 µg mL^{-1} ampicillin, 50 µM isopropyl-β-D-thiogalactoside (IPTG), and 80 µg mL^{-1} X-gal, and incubated overnight at 37 °C. The white colonies were selected for PCR amplification with vector primers M13f-M13r (MBI Fermentas), and the clones with the correct insert as judged by size were sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA).

Expression of biosurfactant genes

The recombinant plasmid pTZ57R/T-*sfp*, *sfp*O, and *srf*A constructs were double digested with *Sac*I and *Sma*I (MBI Fermentas) and purified by the MinElute gel purification kit. The purified *sfp*, *sfp*O, *srf*A genes were religated into pQE30 (Qiagen, Germany), which had previously been digested and purified. The resulting recombinant expression vector pQE30-*sfp*, *sfp*O, and *srf*A cassettes were transformed into competent *E. coli* M15. The

overexpression of biosurfactant genes in the host cell was performed by following the methodology defined in the QIAexpress Type IV Kit (Qiagen). A single colony of the recombinant clones were inoculated into 10 mL of LB broth containing 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} kanamycin and incubated overnight at 37 °C. Approximately 5.0 mL of the culture was transferred into 100 mL LB broth containing 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} kanamycin and incubated at 37 °C until the OD_{600} value reached 0.6–0.7. IPTG was added to the culture broth at the final concentration of 1 mM and was continuously incubated at 37 °C for 4 h.

Purification of the recombinant surfactant

The induced recombinant clones were harvested by centrifugation at 10,000*g* for 20 min at 4 °C. Cell pellets were resuspended in 2 mL lysis buffer containing 10 mM imidazole, 50 mM NaH_2PO_4 pH 8.0, and 300 mM NaCl and lysed by vigorous vortexing. The suspension was further centrifuged at 13,000*g* for 10 min and the supernatant was loaded into a Ni–NTA column (Qiagen) with 1 mL of resin preequilibrated in lysis buffer. The column was washed 8 times with 1 mL wash buffer containing 20 mM imidazole, 50 mM NaH_2PO_4 pH 8.0, and 300 mM NaCl, and 4 times with 500 μL elution buffer containing 200 mM imidazole, 50 mM NaH_2PO_4 pH 8.0, and 300 mM NaCl. The eluted fractions were resuspended in 1 × SDS-PAGE sample buffer and lysed in boiling water bath for 3 min. The cells were centrifuged at 4,000*g* for 10 min and the supernatant was checked for expression of soluble proteins. The expression of target proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [10] along with protein ladder (MBI Fermentas) for the estimation of molecular mass.

Preparation of lipopeptide extract

Extraction of the lipopeptide was performed as described previously [11]. Briefly, the cells obtained from a 100 mL culture were resuspended with 1 mL of 50 mM Tris-HCl buffer (pH 8.0) and treated with 20 μg of lysozyme at 37 °C for 5 min. The mixture was then incubated with 0.5 mg of DNase I and 2.0 mg of RNase A at 37 °C for 10 min. The cell lysate was then treated with 1 mM phenylmethylsulfonyl fluoride [PMSF] (Sigma, USA), 0.5 mM EDTA, 0.1 M NaCl and centrifuged at 10,000*g* for 20 min. The protein concentration was determined according to the method of Bradford [12] using bovine serum albumin as the standard.

Protein assays

Determination of recombinant biosurfactant activity

The emulsifying activity of biosurfactant with different hydrophobic substrates was performed according to the methods described by Cirigliano and Carman [13]. For determination, 2 mL of the recombinant biosurfactant was combined with an equal volume of sodium acetate buffer (0.1 M) and 1 mL of hydrophobic sources (olive oil, vegetable oil, kerosene, crude oil, and waste engine oil) and SDS as the positive control. The reaction mixture was vigorously vortexed for 2 min and the absorbance of the aqueous phase obtained after 10 min was measured in a Lambda 25 UV–Vis spectrophotometer (PerkinElmer) at 540 nm. One unit of emulsifying activity was defined as the amount of surfactant that changed the absorbance of the emulsion by 1.0.

RP-HPLC analysis of recombinant biosurfactant

Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed with a reverse phase column (XTerra MS C_{18} 250 × 4.6 mm, 5μm) on a Waters 4,707 series HPLC system equipped with diode array detector (DAD). The elution was done with 80 % of gradient acetonitrile (0.1 % triflouroacetic acid) at 27 °C with the flow rate of 1 mL min^{-1}. Purified biosurfactant/peaks eluted from the column was detected by UV detector at 205 nm. Identification and quantification of biosurfactant were carried out using surfactin (Sigma, USA) as the standard.

FT-IR analysis of recombinant biosurfactant

Fourier transform infrared spectroscopy (FT-IR) was performed to determine the functional groups and the chemical bonds present in the recombinant biosurfactant. The samples were processed by dispersing in potassium bromide and analyzed in a FT-IR spectrometer (IR Affinity-1, Shimadzu, Japan). The IR spectra were obtained in the range of 400–4,000 wave numbers (cm^{-1}).

In silico sequence analysis

The nucleotide sequences obtained were compared against database sequences using BLAST provided by NCBI (http://www.ncbi.nlm.nih.gov). The sequences that are closely related were obtained for multiple sequence alignment and phylogenetic analysis using MEGA version 6.0 program [14]. The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (http://www.expasy.org/tools/protparam.html).

Results and discussion

Molecular identification of the potent strain (NIOT-06)

The 16S rRNA sequences (1,495 bp) generated in this study were deposited in GenBank under accession number KC899772. Upon analysis with the BLAST program and phylogenetic analysis, it was established that the deduced nucleotide sequences of NIOT-06 were highly homologous (99 %) with the reported 16S rRNA sequences of *Bacillus licheniformis* (GenBank accession no. DQ071568) (Fig. 1a). Based on the morphological and biochemical characteristics (Table 1) and the phylogenetic analysis, the isolate NIOT-06 was identified as *B. licheniformis* NIOT-06.

Purification and functional characterization of recombinant biosurfactant

The biosurfactant biosynthesis gene cluster from *Bacillus licheniformis* NIOT-06 was successfully cloned, sequenced, and transferred to the bacterial expression vector pQE30. In order to analyze the expression of recombinant biosurfactant, a colony of *E. coli* M15 (pREP4) cells harboring pQE30-*sfp*, *sfp*O, *srf*A genes were grown to mid-log

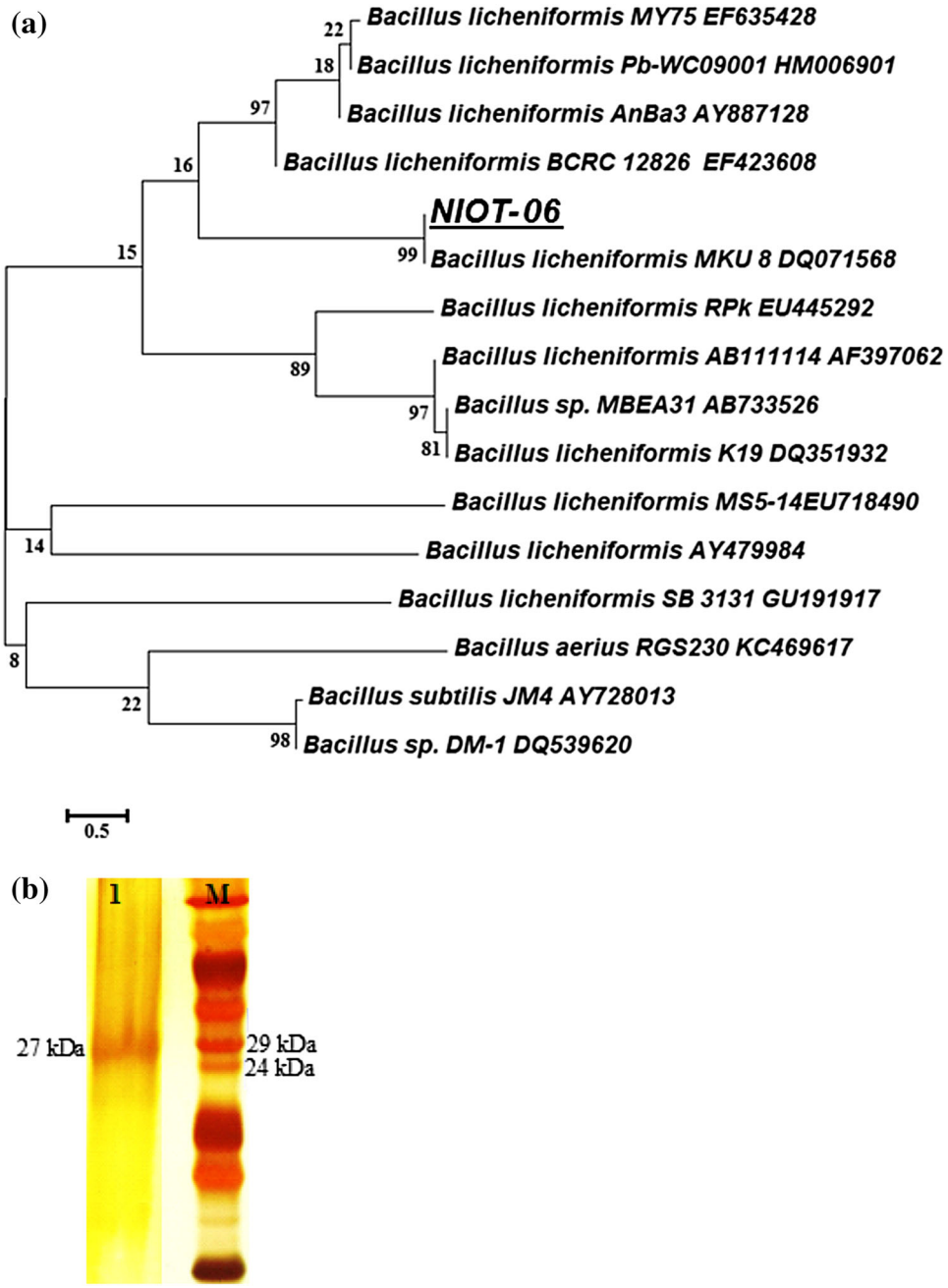


Fig. 1 **a** Phylogenetic tree based on 16S rRNA sequences using neighbor-joining method for the isolate, NIOT-06 and the GenBank sequences. Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position. **b** Silver stained SDS-PAGE spectrum of the purified recombinant biosurfactant. *Lane 1*, Ni–NTA purified biosurfactant; *Lane M*, protein molecular mass marker

and induced with IPTG for 4 h. After induction, the cleared cell lysate was chromatographed in a Ni–NTA resin column in order to purify recombinant biosurfactant in a single step. After elution with 200 mM imidazole, a predominant band with an apparent molecular mass of 27 kDa was observed in SDS-PAGE (Fig. 1b). Molecular weight of the purified recombinant biosurfactant was similar to the biosurfactant reported from *Klebsiella* sp. [15]. Hence it was concluded that the recombinant biosurfactant is a lipopeptide.

Table 1 Characteristic features of *Bacillus licheniformis* NIOT-06

Morphological and biochemical characteristics	Observations
Grams staining	Positive
Motility test	Positive
Indole production	Positive
Methyl red test	Negative
Carbohydrate fermentations with lactose	Acid with gas
Sucrose	Acid with gas
Dextrose	Acid with gas
Nitrate reduction	Positive
Citrate utilization	Positive
Catalase activity	Positive
Starch hydrolysis	Positive
Caesin hydrolysis	Positive
Urease activity	Positive

Table 2 Emulsification index (E_{24}) of recombinant biosurfactant at different time intervals

Stage of cells	Emulsification index E_{24} (%)		
	sfp	*sfp*O	*srf*A
Just before induction	16.10	21.50	25.41
After induction			
1 h	20.31	30.50	34.72
3 h	45.10	52.34	59.77
4 h	68.91	71.54	74.70

The expression and functional activity of the recombinant biosurfactant was confirmed by determining the emulsifying activity and emulsification index. The emulsification index of the expressed cells was three times more than that of uninduced recombinant clones (Table 2). The biosurfactant biosynthesis genes have also been functionally characterized in *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus tequilensis* [16–18].

Emulsifying activity of recombinant biosurfactant

The emulsifying capacity and stability of recombinant biosurfactant was found elevated for kerosene and crude oil (4.9 and 4.3 units, respectively) (Fig. 2). The emulsifying capacity and the stability of the recombinant biosurfactant were recorded higher against the tested hydrocarbons than oils. These results are comparable to a previous report on the emulsification activity of surfactant from *Brevibacterium aureum* MSA13 [19]. A comparison of the previous report by Sausa and Bhosle [20] with our results revealed that the emulsification activity of our compound was more stable and also able to stabilize the emulsion formed with all of the hydrophobic compounds evaluated.

HPLC analysis of recombinant biosurfactant

Quantification of recombinant biosurfactant was performed in RP-HPLC. The chromatograms of recombinant biosurfactant revealed three major peaks/fractions at retention times of 6.86, 9.15, and 10.74 min (Fig. 3a). All these fractions had excellent surfactant property as they

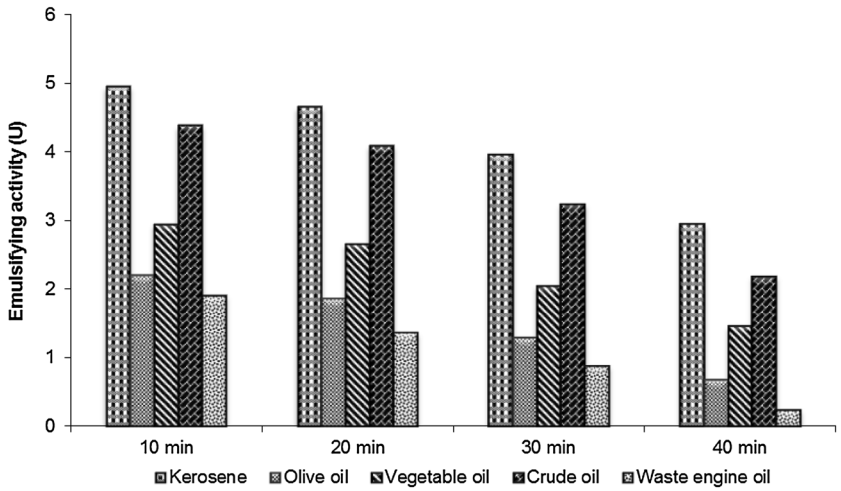


Fig. 2 Emulsifying activity of the purified recombinant biosurfactant on hydrophobic substrates

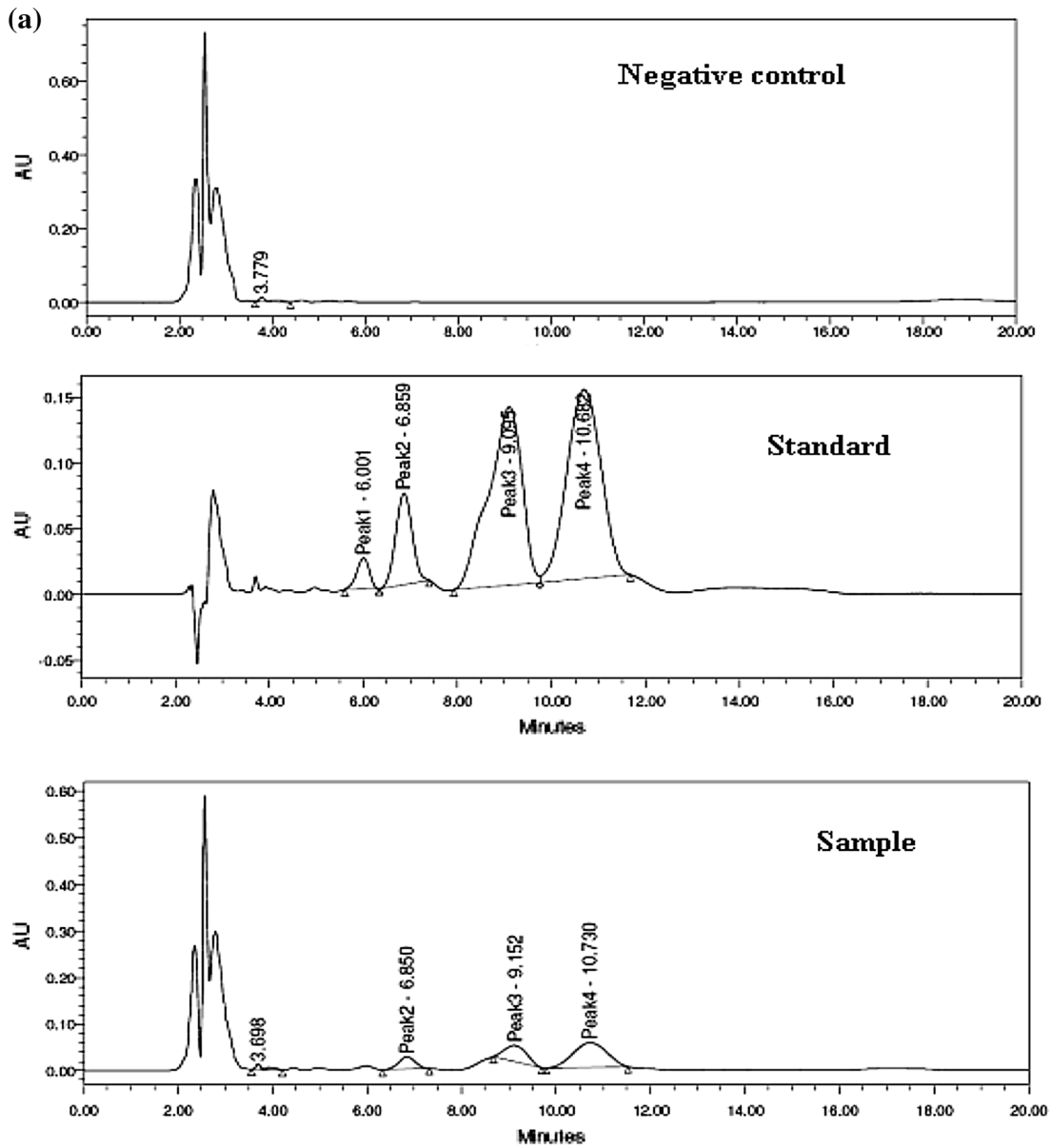


Fig. 3 **a** HPLC chromatogram of recombinant biosurfactant. **b** FT-IR spectrum of the recombinant biosurfactant

reduced the surface tension of water to a greater extent. Among these fractions, fraction 3 (retention time: 10.74 min) showed the highest surface tension reduction activity. Similar surface tension activity was previously reported for the purified HPLC fractions of the biosurfactant obtained from *Bacillus subtilis* 20B [21]. The HPLC analysis of purified biosurfactant also revealed the appearance of additional peaks in the test samples, which was not observed in control samples. Concentration of the recombinant biosurfactant was 0.71 g L^{-1} of expressed cells. The retention time of authentic surfactin standard correlated well with the additional peak obtained for test samples.

Biochemical analysis of recombinant biosurfactant by FT-IR

Fourier transform infrared spectroscopy spectrum of the recombinant biosurfactant contained a peptide component at 1,655 cm^{-1} and the N–H bond combined with C–N mode at 1,593.27 cm^{-1}. The presence of strong aliphatic chains (C–H) were also observed at 1,458.25 cm^{-1} and 2,961.82 cm^{-1} (Fig. 3b). These results strongly authenticated that the recombinant biosurfactant contained the aliphatic and peptide like moieties. Moreover, the presence of these functional groups also confirmed the recombinant biosurfactant as a lipopetide. A lipopeptide biosurfactant

Fig. 3 continued

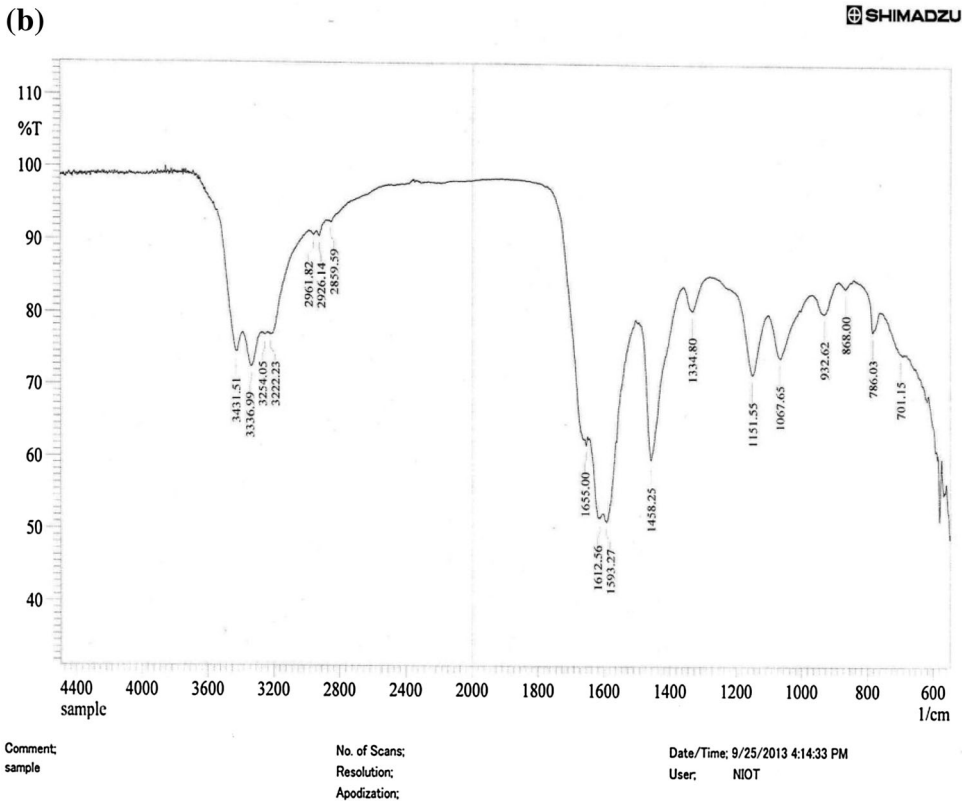


Table 3 Sequence identity shared by *sfp*, *sfp*O, and *srf*A gene sequences of *B. licheniformis* NIOT-06 with other closely related species

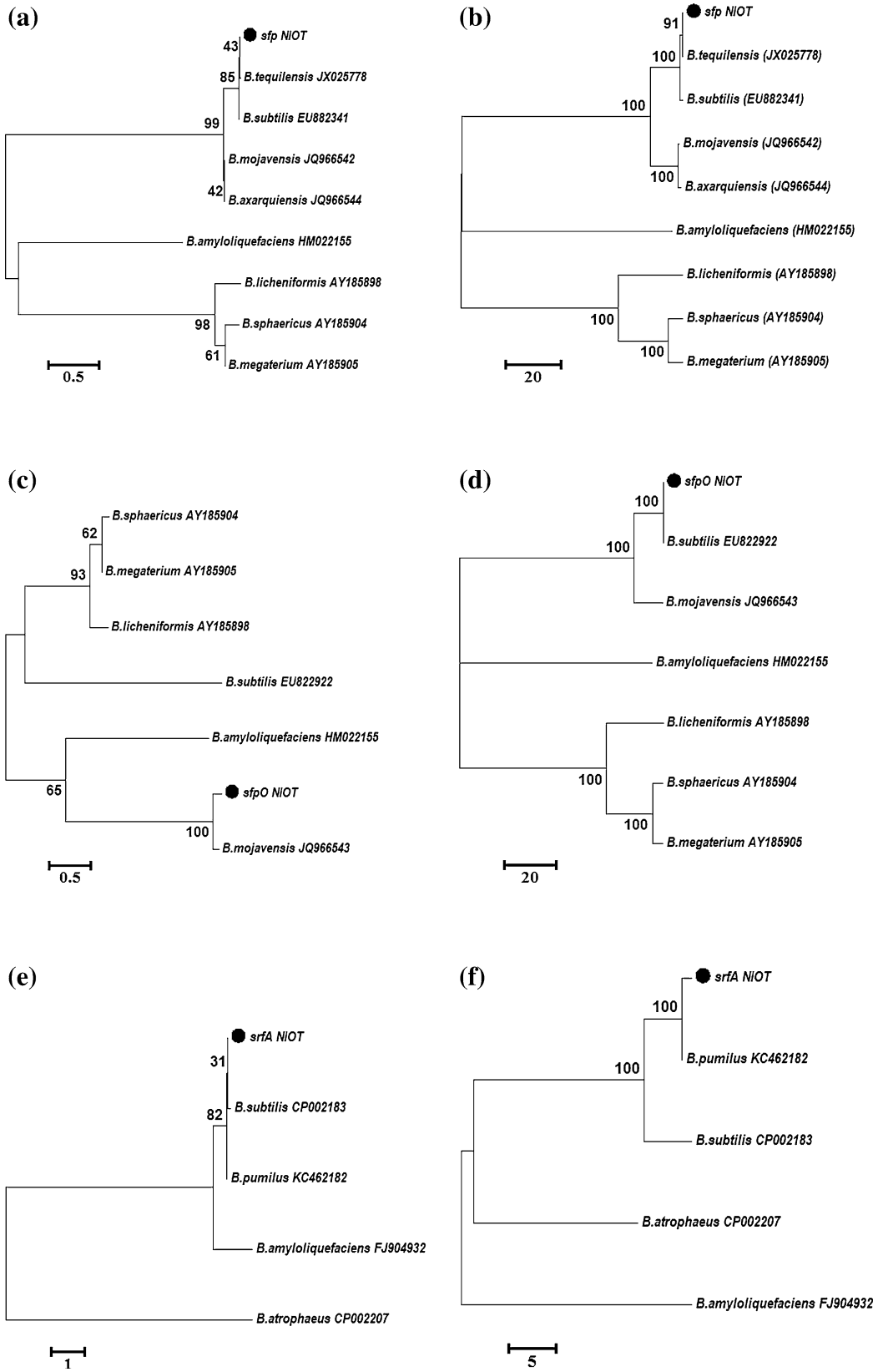
Gene	Organism	Percentage identity	No. of overlapping amino acids
sfp	*B. tequilensis*	92	206
	B. subtilis	94	199
	B. amyloliquefaciens	94	192
	B. sphaericus	95	169
	B. mojavensis	86	193
	B. axarquiensis	86	193
	B. megaterium	72	162
	B. licheniformis	73	156
*sfp*O	*B. subtilis*	96	203
	B. amyloliquefaciens	93	180
	B. sphaericus	97	168
	B. mojavensis	85	180
	B. megaterium	85	180
	B. licheniformis	80	165
*srf*A	*B. subtilis*	96	231
	B. amyloliquefaciens	76	133
	B. atrophaeus	83	195
	B. pumilus	51	121

from *Bacillus* sp. BS-3 with the similar IR spectrum was previously reported by Donio et al. [3].

Sequence and phylogenetic analysis of biosurfactant biosynthesis genes

The Open Reading Frame (ORF) of biosurfactant biosynthesis genes in *Bacillus licheniformis* NIOT-06 comprises of three genes: *sfp* (GenBank accession no. KC846090), *sfp*O (KC846091), and *srf*A (KC846092) which encode proteins of 224, 212, and 242 amino acids with the calculated molecular masses of 26,167, 24,758, and 27,646 Daltons, respectively. BLAST analysis revealed that, the deduced amino acid sequence of *sfp, sfp*O, and *srf*A was partially homologous to the biosurfactant biosynthesis genes of several eubacteria.

The *sfp, sfp*O, and *srf*A sequences from *Bacillus licheniformis* NIOT-06 were analyzed with nucleotide and amino acid sequences of other eubacteria viz., *B. tequilensis* (GenBank accession no. JX025778), *B. subtilis* (EU882341), *B. amyloliquefaciens* (HM022155), *B. sphaericus* (AY185904), *B. mojavensis* (JQ966542), *B. axarquiensis* (JQ966544), *B. megaterium* (AY185905), *B. licheniformis* (AY185898), *B. pumilus* (KC462182) using Clustal X software. Amino acids



◀**Fig. 4** The phylogenetic tree analysis by MEGA6 program using neighbor-joining algorithm with 1,000 bootstrap replicates. *Scale bar* represents the differences in conserved region of the gene between organisms. **a** Nucleotide sequences of *sfp* gene. **b** Amino acid sequences of *sfp* gene. **c** Nucleotide sequences of *sfp*O gene. **d** Amino acid sequences of *sfp*O gene. **e** Nucleotide sequences of *srf*A gene. **f** Amino acid sequences of *srf*A gene

analysis revealed that the *sfp* gene encoded protein belongs to 4′-pantetheinyl transferase ACPS super family and had partial homology with pantetheinyl transferase of other eubacteria. *Bacillus tequilensis* revealed 92 % identity with 206 aa overlap; *B. subtilis*, 94 % identity with 199 aa overlap; *B. amyloliquefaciens*, 94 % identity with 192 aa overlap; *B. sphaericus*, 95 % identity with 169 aa overlap; *B. mojavensis*, 86 % identity with 193 aa overlap; *B. axarquiensis*, 86 % identity with 193 aa overlap; *B. megaterium*, 72 % identity with 162 aa overlap, and *B. licheniformis,* 73 % identity with 156 aa overlap. Amino acid analysis of *sfp*O gene suggests that, the encoded protein belongs to ACPS super family and the *sfp*O of *B. licheniformis* NIOT-06 had highly conserved regions with phosphopantetheinyl transferase similar to other bacteria. *Bacillus subtilis* accomplished 96 % identity with 203 aa overlap; *B. amyloliquefaciens*, 93 % identity with 180 aa overlap; *B. sphaericus,* 97 % identity with 168 aa overlap; *B. mojavensis,* 85 % identity with 180 aa overlap; *B. megaterium,* 85 % identity with 180 aa overlap, and *B. licheniformis* has 80 % identity with 165 aa overlap. Amino acid analysis of *srf*A gene proposed that, the encoded protein belongs to surfactin synthetase family and the sequences of *B. licheniformis* NIOT-06 strain had partial homology with surfactin synthase of previous database reports. The identity shared by *sfp*, *sfp*O, and *srf*A gene sequences of *B. licheniformis* NIOT-06 with other closely related species are tabulated in Table 3.

Phylogenetic tree based on evolutionary distances was constructed with nucleotide and amino acid sequences of *sfp, sfp*O, and *srf*A using neighbor-joining method with 1,000 bootstrap replicates. Tree at nucleotide and amino acid level of *sfp* (Fig. 4a, b), *sfp*O (Fig. 4c, d), and *srf*A (Fig. 4e, f) reveals the phylogenetic similarity of biosurfactant biosynthesis genes from *B. licheniformis* NIOT-06 with other eubacteria. Nucleotide sequence analysis of *sfp* gene revealed a significant homology with *B. tequilnesis* and *sfp*O gene shared the similar homology with *B. sphaericus*. Amino acid sequences of *sfp* and *sfp*O genes displayed a significant homology with *B. sphaericus*. The *srf*A gene revealed a significant homology with *B. pumilus* at both nucleotide and amino acid levels. The phylogenetic analysis construes that, even though the biosurfactant biosynthesis pathway is evolutionarily well conserved with respect to the genes and enzymes involved, few differences in their organization and regulation could occur in various eubacteria.

Conclusion

In this study, we report the heterologous expression of biosurfactant biosynthetic genes from the marine sponge-associated *Bacillus licheniformis* NIOT-06 in *E. coli*. The engineered *E. coli* strain has potential industrial application since it produces biosurfactant at high rates and can avoid the complex down-streaming process associated with the conventional bioprocess. On phylogenetic analysis, the *sfp, sfp*O, and *srf*A genes of *B. licheniformis* NIOT-06 was found to be highly conserved among the bacterial species. The *sfp* gene has highest similarity between bacterial species compared to the *sfp*O and *srf*A genes. Based on the phylogenetic analysis, *B. licheniformis* NIOT-06 and *B. subtilis* were clustered together for all the genes except for the *sfp*O nucleotide sequence, which formed a separate cluster. The genes involved in the biosynthesis of biosurfactant in *B. licheniformis* NIOT-06 and *B. subtilis* are comparatively well conserved compared to other bacteria both at nucleotide and amino acid level.

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