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Biosurfactant Characterization Of Bacillus Licheniformis Ns1 From Tithal Sea Beach, Gujarat, India

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Abstract

Tithal coastal site is contaminated with oil spillage due to large amount of recreational activities and industrial waste being dumped into it. Biosurfactants are complex bioactive compounds, when synthesized by halophilic bacteria have immense applications in industries, agriculture and environment. In present study, moderately halophilic bacteria NS-1, NS-7 and NS-9 were screened positive for biosurfactant among ten strains isolated from Tithal beach. The enzyme linked with the release of this surface active molecules was found to be an esterase. These three isolates were also found positive for the enzyme esterase. Further, efficient biosurfactant producer isolate NS-1 was recognized as Bacillus licheniformis through 16s rRNA characterization.The biosurfactant produced by NS-1 was characterized as lipopeptide by TLC and FTIR analysis. GCMS study revealed the fatty acid component as 3-methyl, 1, 2-diazirine with molecular weight 56 Da. Tithal coastal site is polluted by tourists frequently and crude oil spillage is also reported before. Moderately halophlic Bacillus licheniformis NS1 strain focused in this study was isolated from the same origin and can be used to remediate contaminated region by producing potential biosurfactant.

Keywords: Halophilic bacteria, Bacillus licheniformis, Surface active molecules, Lipoprotein

1. Introduction

Tithal Beach is located in the southern part of Gujarat state in Valsad district on the coast of the Arabian Sea, India. Tithal beach is one of the popular tourist attractions of Valsad district Gujarat, 20°35′53.6″N 72°53′41″E, Courtesy: (Google). This region is contaminated due to large amount of anthropogenic and industrial activities. Halotolerant or halophilic microorganisms, ready to live in saline and extreme conditions, offer a huge number impending applications in different fields of biotechnology and have a unique lipid arrangement, may have a significant task to carry out as surface-dynamic specialists. Biosurfactants are recognized as amphiphilic as they comprise both hydrophobic and hydrophilic substances. They are formed mostly on the surface of microbes or expelled extracellular. They decrease tension on surface and between fluids of different polarities and also used to increase the availability of hydrophobic substances. They have many advantages over synthetic surface active compounds as biodegradability and compatibility for environmental cleanup (Rai et al., 2011). Chemically synthesized surfactants are also harmful if applied to environment due to less degradability (Kuyukina et al., 2005). Surfactants are classified as lipopeptides, glycolipids, phospholipids, lipopolysaccharides etc. They are applicable in various fields due to its versatile properties as detergency, metal sequestering, emulsion forming and oil enhancing recovery. It seems promising to defend the marine environment. A number of marine isolates have found to produce biosurfactants when growing in the presence of immiscible substrates (Bodour et al., 2003). The archaebacterial lipid in cell wall of halophilic microscopic organisms has been appeared to have excellent surfactant properties (Kuyukina et al., 2005). The search for unique surface active molecules from halophiles seems very auspicious as they have adaptation to adverse environmental situations. A lipopeptide

surface active compound from *Bacillus licheniformis strain* BAS 50 could grow on wide range of substrates with highly saline condition [3]. In the current study, it was aimed to isolate efficient halophilic bacterial strains which produce biosurfactant and also to characterize biosurfactant produced by potential bacterial isolate for future promising applications.

2. Materials and methods

2.1 Collection of sample and halophilic strains

The water samples collection was done from Tithal beach, Gujarat, India. The medium utilized for the growth of halophiles was salt (5-15%), Tryptone (1 in gram), Glucose (2.5 in grams), Yeast extract (5 in grams) and water distilled 1 L (STGYA). The medium was supplied with the range of salt concentration from 5% NaCl to 15%NaCl for the isolation of moderate halophilic microorganisms. The bacteria grown in halophilic media were purified by single colony streaking with the same salt concentration provided.

2.2 Screening of biosurfactant producers

The isolated halophilic bacteria were transferred in BHM-Bushnell and Haas mineral salt medium containing 2% oil (crude). Screening of biosurfactant producing isolates were done by oil displacement test and hemolysis on blood agar medium. To perform displacement of oil test, 25mlwater distilled wastakeninplatesand20microliterofoilwasaddedandthen 10 microliter aliquot of supernatant was delivered tooil. Check the zone produced. Hemolysis was performed by streaking on blood agar medium to check clear zone around growing strains (Thavasi et al., 2010).

2.3 Emulsification activity

Isolates NS1, NS7 and NS9 selected for emulsification tests as they were screened as biosurfactant producers through above tests. According to the procedure followed by Mulligan et al. 1984, for emulsification essay, 0.5 ml supernatant was mixed with 1ml lubricant oil. The mixture was vortexed with increased speed for 120 seconds. Immediately, after one day, emulsion height (cm) was found out (Mulligan et al., 1984).

2.4 Esterase Activity

Esterase activity was performed by using para-nitrophenyl acetate 100 mM as a substrate and 75 mM phosphate buffer with pH 7.0. Samples are considered as enzyme source and added to reaction. The tests with standard and control incubated at 37°C for 30 minutes. Optical density at 405nm was taken to measure esterase activity spectrophotometrically (Thavasi et al., 2010).

2.5 Identification of biosurfactant producing isolates

The isolates were characterized by morphological, gram staining and biochemical analysis. HIMVIC – biochemical characterization (KB001) kit was used for different biochemical tests. Isolate NS-1, NS-7 and NS-9 were found positive in screening and were considered for its biochemical characterization. Further, isolate NS1 has shown maximum oil displacement activity and emulsification ability, it was identified by 16srRNA analysis. Isolate NS1 was sent for 16srRNA analysis at Europhin Pvt. Ltd., Bangalore.

2.6 Extraction of biosurfactant

For extraction and partial purification of biosurfactant, the supernatant collected after centrifugation of fresh culture and was acidified as pH 2 with 1N HCL. The acidified suspension was kept overnight at4ºC and next day precipitates were collected by centrifuging for 30 minutes. Precipitates were dissolved in distilled water, 7.0 pH was set and lyophilized.

2.7 Characterization of surface active molecule

Initially, characterization was achieved by performing reaction with ninhydrin reagent and anthrone reagent, then biosurfactant produced by isolate NS-1 was characterized by TLC and FTIR. The fatty acid component was identified by Gas Chromatography Mass Spectrum analysis. The standard methods were followed according to Daxini and Mistry, 2018. The samples for FTIR and GC-MS were sent to SICART, CVM, Vallabh Vidyanagar for analysis of biosurfactant.

3. Results and Discussion

3.1 Isolation of halophilic strain

The growth of microorganisms was observed in STGYA media with different NaCl concentration. Total ten organisms were isolated in the media with 5%, 7%, 10% and 15% NaCl. These moderate halophilic isolates

were purified on the same media with its respective requirement of salt.

3.2 Screening of biosurfactant producing isolates

All the purified moderately halophilic strains were screened for the production of biosurfactant by displacement of oil test, hemolysis test and emulsification activity. Isolates NS-1 and NS-7 were able to grow in media with 7 % NaCl and NS-9 with 10% NaCl proved their moderately halophilic nature. These isolates were found as potential biosurfactant producers as they have shown oil displacement test positive as 3.5cm, 2.4cm and 2.9cm clear zones respectively. This method is based on the principle that the biosurfactant would displace oil present on the surface of water contained in a petri plate. Usually, this method of displacement of oil is known as fast and simple to carry out. Youssef et al. explained that the oil displacement is a trustworthy method to check the presence of surface active molecules (Youssef et al., 2004).

Three strains NS-1, NS-7, NS-9 have shown blood hemolysis test positive that indicated these 3 strains produced extracellular bioactive compounds. Blood agar hemolysisisanimportantscreeningtestforbiosurfactantproduction.Thecolonies of bacteriawere checked for the zone of clearance around.Hemolysisisthe breaking of RBCs by a bacteriological active compound. Variety of bacteria produce surface active molecule and possess hemolytic property. These compounds believed to act on RBCs membrane by releasing hemoglobin from it and disturbingthemembranestructure (Rosenberg and Ron, 1999).Emulsification index of NS-1 was observed as 66%, for isolate NS-1, 53% for NS-7 and 60% by isolate NS-9. Emulsification activity of NS-1 is the highest among all the three strains. Isolate NS-1 has shown maximum emulsification forming ability with oil proved its potential use in enhancing recovery of oil by bacteria.

Figure 1. Hemolysis is depicted in blood agar by NS-1 NS-7 and NS-9 isolates.

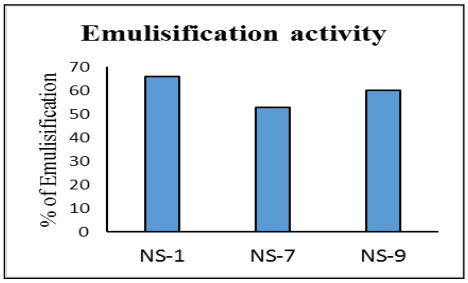

Figure-2. Emulsification activity of isolates NS-1, NS-7 and isolate NS-9

Esterase activity

The enzymatic activity of esterase enzyme produced by NS-1 was 0.183 U/ml, NS-7 0.143 U/ml and NS-9 as 0.208 U/ml (Table-1). The NS-9 strain showed maximum optical density at 405nm showed the maximum esterase activity out of all 3 strains. The significance of studying enzyme assay is for Lipolytic proteins (lipases and esterase) fundamentally catalyze both the hydrolysis and combination of ester mixes. The esterase and biosurfactant composite has been known for influential emulsification activity, which proves its application in remediation, degradation of hydrocarbon and pharmaceutical aspects.

Table-1. Esterase activity of isolates NS-1, NS-7 and isolate NS-9

	OD	Y value	U/ml
1	0.362	9986.207	0.183
2	0.282	7779.31	0.143
3	0.411	11337.93	0.208

3.4Identification of efficient biosurfactant producing strains.

Characterization of biosurfactant producing isolates was done by biochemical tests. The biochemical tests results were interpreted using Bergy's manual of systematic Bacteriology Edition-2 and shown in table-2. Probable identity of isolated strains on the basis of biochemical results were as NS-1- *Bacillus licheniformis*, NS-7 *Sporocarsina halophila* and NS-9 as *Bacillus subtilis*. Maximum biosurfactant activity was achieved by gram positive, rod shaped bacterial isolate NS-1. Moderately halophilic isolate NS1 was further characterized by 16s rRNA analysis and the strain was confirmed as *Bacillus licheniformis* (Fig. 3). The BLAST was done in NCBI and phylogenetic tree was created using the closely related species shown in table with accession number table-3. The sequence submission was done to NCBI and MN856586 was received as the accession number for *Bacillus licheniformis NS1*. This analysis is usually employed for the recognition of isolated bacterial species, and can be promising for the identification of new pathogens and microscopic organisms (Mishra, et al., 2016).

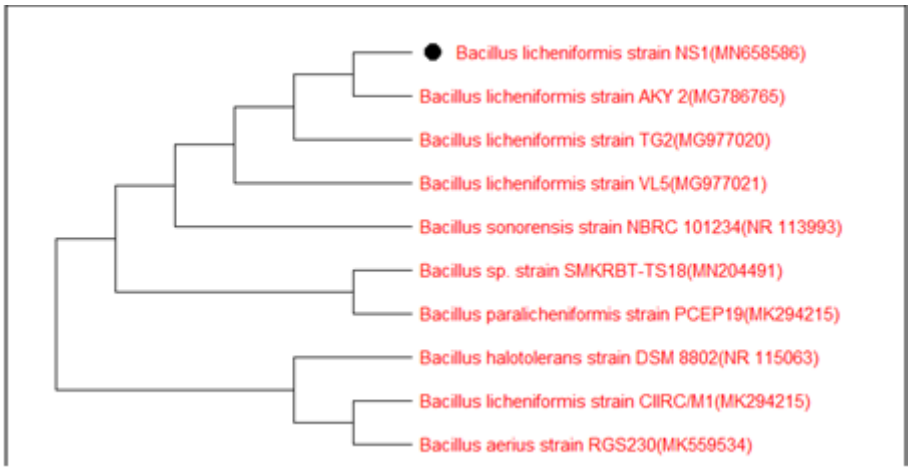

Figure-3. The phylogenetic classification and relations of NS-1 strain with other strains based on their 16s rRNA sequence.

Table 2. Biochemical results of efficient biosurfactant producing strains

No.	Tests	NS-9	NS-1	NS-7
1	Indole test	Negative	Negative	Negative
2	Methyl red test	Negative	Negative	Positive
3	Vogue's Proskaeur	Negative	Negative	Negative
4	Citrate utilization test	Positive	Positive	Positive
5	Glucose	Negative	Positive	Negative
6	Adonitol	Negative	Negative	Negative
7	Arabinose	Variable	Variable	Variable
8	Lactose	Negative	Negative	Negative
9	Sorbitol	Variable	Positive	Variable
10	Mannitol	Variable	Variable	Variable
11	Rhamnose	Negative	Negative	Negative
12	Sucrose	Variable	Positive	Positive

3.5Extraction of Biosurfactant

Biosurfactant production was followed by extraction and partial purification. Biosurfactant produced by isolate NS-1 was again checked for oil displacement activity to confirm presence of surface active molecule. Positive oil displacement test has shown the presence and effectiveness of biosurfactant produced. It has shown same activity as before and further used for characterization.

3.6Characterization of biosurfactant from efficient bacterial isolate

Characterization studies gave idea about the nature and combination of molecules in the isolated biosurfactant compound. The biosurfactant was partially purified from NS-1 strain and characterized preliminary by biochemical reaction with ninhydrin and anthrone. The positive results with ninhydrin and negative with anthrone has analysed the presence of peptides and the compound devoid of carbohydrate molecule. This nature of molecule was confirmed by performing Thin Later Chromatography. The Rf value of extracted biosurfactant was found to be 0.46. which is characteristic of protein present in extracted biosurfactant. Thin layer chromatography is the best way to partially characterize the nature of biosurfactant. According to the results of TLC the nature of biosurfactant is peptide or lipopeptide (Santhini and Parthasarathi, 2014). Further characterization studies were focused on Fourier Transfom Infrared (FTIR) and GCMS analysis.

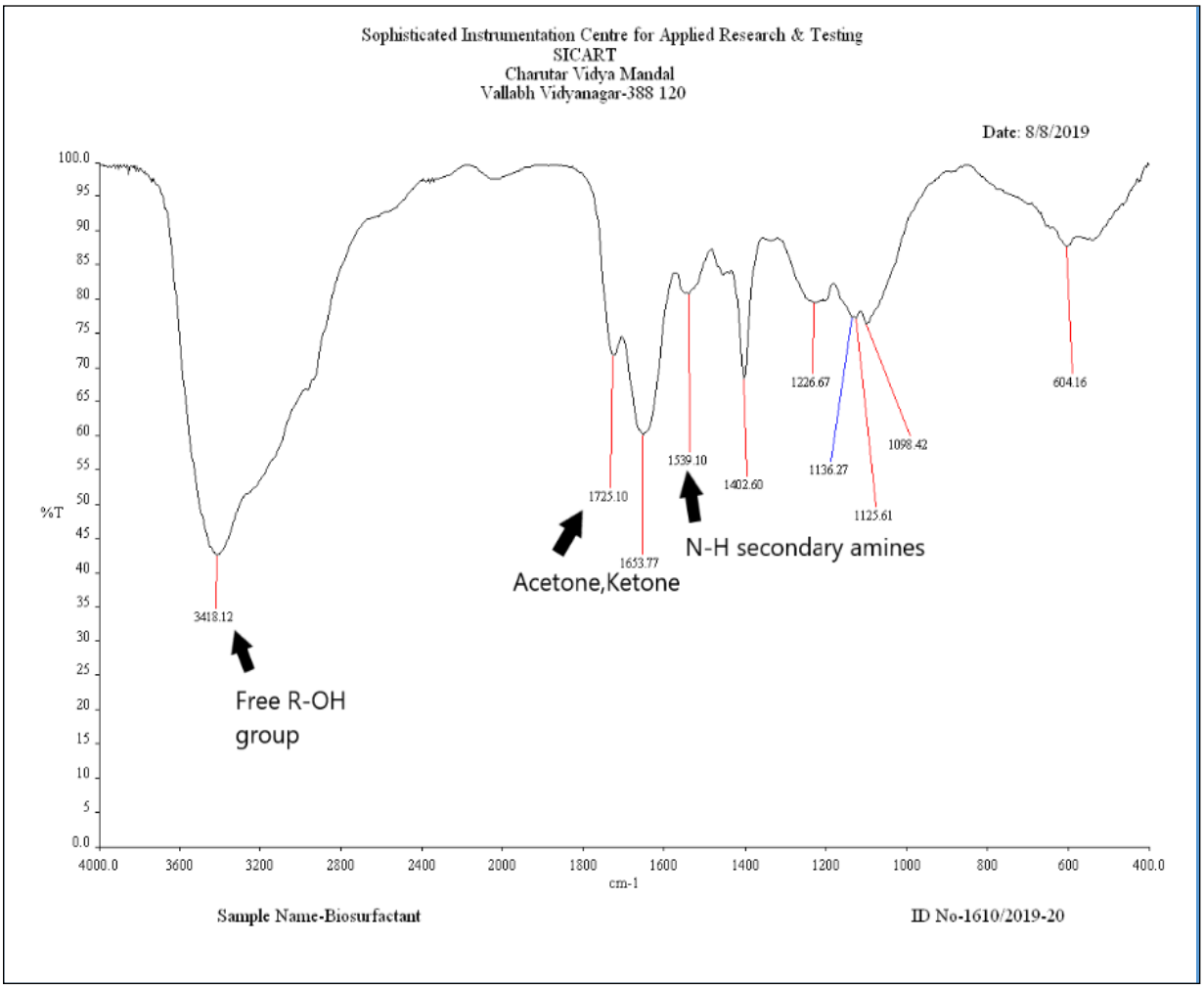

Figure 4. FTIR of biosurfactant from isolate NS-1

The FTIR Peak of the Biosurfactant depicts the nature of surfactant as lipopeptide. Based on the stretching of the peaks from FTIR, the results were interpreted as 1725-acetone, ketone, 3418.12-Free R-OH group, 1653-alpha, beta unsaturated ketones C=O, 1539-N-H Secondary Amines,1125-tertiary alcohol, 604.16-C-cl, Chlorine, Halides. The functional groups found in the compound as C-C, C=O, C-H, N-N, N-H, and C-O. The FTIR analysis reflected the synthesis of a biosurfactant which is lipopeptide in nature. As studies conducted on biosurfactants, this is the standard method to find the nature. The band 1725 cm-1 confirms ester group. Hence, the nearness of amide bond at 1653 cm-1 was because of C = O stretch. The closeness of these groups also shown that they have a place in lipopeptide family (Dehghan-Noudeh et al., 2005).

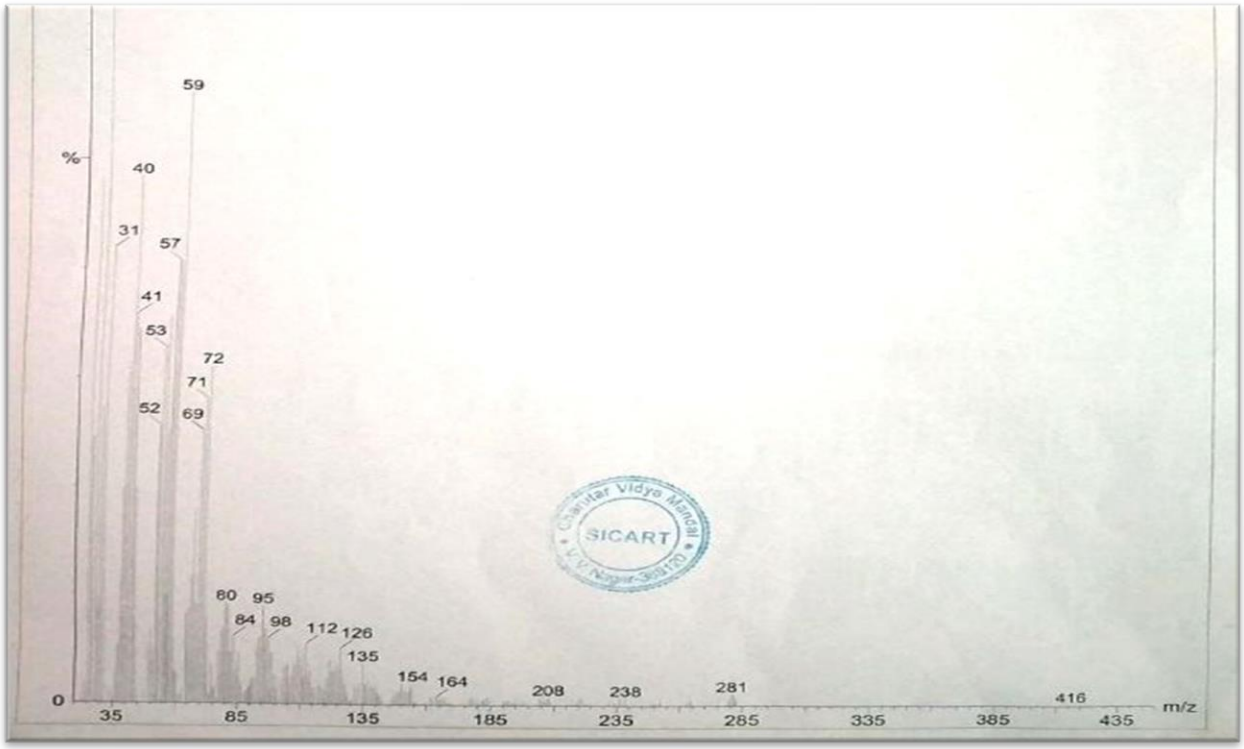

Figure 5. GCMS analysis of biosurfactant from NS-1

The surface reduction capacity is essentially depending on the components present in surfactant. The fatty acid molecule imparts key role in providing surface tension property to the surfactant produced by microorganisms. Wiącek (2015) also reported the effects of phospholipid biosurfactant and different parameters on the emulsifying ability (Wiącek and Adryańczyk, 2015). Gas chromatography peaks added information about the fatty acid component and it was also compared the functional groups finalized in FTIR. The probable peak from chromatography results was detailed characterized by mass spectral analysis for determination of molecular weight and identification of fatty acid. GCMS data revealed the fatty acid component as 3-methyl 1, 2-diazirine with molecular weight 56 Da.

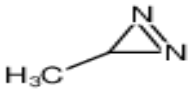

Figure 6. Structure of 3-methyl, 1-2 Diazirine

The Structure of 3-methyl, 1-2 Diazirine is shown in figure-6. It is classified as natural particles comprising of a carbon bound to two nitrogen molecules, which are two fold attached to one another, shaping a cyclopropene-like ring. This component was also found as a part in detergent with surface properties. Diazirines are regularly utilized as photoreactive crosslinking reagents (Chen et al., 2017).The molecule containing either an aliphatic or trifluoromethyl phenyl diazirine, can also be used as a promising therapeutic molecule. Diazirine combination (Wu and Zhan, 2019).

4. Conclusion

Moderately halophilic isolate NS-1 was selected for biosurfactant production among all other screened bacteria based on its maximum emulsification and oil displacement activity. NS-1 strain was identified as *Bacillus licheniformis* by 16srRNA analysis. Biosurfactant from *Bacillus licheniformis* NS-1 was characterized as lipopeptide in nature. Positive esterase activity has also proved its significant role in enhancing emulsification capacity of surface active molecule Biosurfactant produced by *Bacillus licheniformis* was characterized as lipopeptides by TLC and FTIR analysis. Mass spectra analysis identified the fatty acid component as 3-methyl 1,2 di-azirine with low molecular weight 56 Da. Tithal beach is usually contaminated by anthropogenic activities and *Bacillus licheniformis* isolated from this region has shown tremendous biosurfactant activity along with the presence of esterase. The complex of esterase and biosurfactant has been proven to have potent emulsification activity, which signifies the application of this isolate in hydrocarbon biodegradation and

microbial enhanced oil recovery.

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