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Optimization of Culture Conditions and Biosurfactant Production from *Bacillus altitudinis* (BMSed II)

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Biosurfactants are amphiphilic compounds produced on living surfaces, mainly on surfaces of microorganisms or secreted extracellularly. Out of twenty isolates obtained from Bhitarkanika mangrove forest, five were positive for biosurfactant production. Biosurfactant production (mg/ml) and surface tension reduction (mN/m) on different days of incubation of all the five isolates when considered, only one isolate (BMSed II) was found to produce the maximum biosurfactant & highest surface tension reduction on 7 days of incubation in comparison to others. On optimization of different parameters (2% inoculum, 2% glucose, 37°C temperature and pH – 8) on potent isolate BMSed II was found to be suitable for maximum biosurfactant production of 2.14g/l. The 16S rDNA gene sequence of (BMSed II) was submitted to NCBI gene bank and found similar to *Bacillus altitudinis* (Accession number KT005170) by phylogenetic analysis. The biosurfactant produced by *Bacillus altitudinis* was characterized as a lipopeptide using Thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR) and H^1 Nuclear magnetic resonance (NMR) analysis.

Keywords: Biosurfactant, *Bacillus altitudinis*, lipopeptide, TLC, FTIR, NMR.

Biosurfactants are compounds having surface active and emulsifying activities, produced by a wide variety of bacteria, yeast, and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium. The chemical nature of the biosurfactants varies and depends upon the producing organism. *Pseudomonas aeruginosa* has been reported to produce the biosurfactant rhamnolipid [1] while *Bacillus subtilis* is known to produce surfactin [2]. Biosurfactants consists of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols[3].Biosurfactants play a number of roles including increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, binding of heavy metals, quorum sensing and biofilm formation [4]. Compared with synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, higher degradability and better environmental compatibility [5]. With their high surface activity, lower toxicity, higher biodegradability and better environmental compatibility, they are widely used in environmental applications such as enhancement of oil degradation [3], as antioxidants, as antimicrobials in the cosmetic industry [6] and anti-adhesives against several bacteria and yeasts in medical applications [4]. Moreover, biosurfactants

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can be as effective as synthetic chemical surfactants due to their higher specificity and greater biodegradability. The objectives of this study was undertaken to optimize the culture parameters to enhance the production of biosurfactants by the bacteria.

MATERIALS AND METHODS

Sample collection

Sediment samples were (approx 50gm) were collected arbitrarily from Rangani (site 1), Mahisamunda (site 2), Dangamal (site 3) and Bansurgarh (site 4) of the Bhitarkanika Wildlife Sanctuary (20°50'N latitude and 86°30' and 87° 6"E longitudes) of Odisha, India for the sample collection during the year 2012-2013 for microbiological study. Five potential isolates were selected, preserved in slants after screening for further experimental purpose.

Growth medium for biosurfactant production

Mineral salt medium (MSM) [7, 8, 9 and 13] containing the following components (g/l^{-1}) were used for the growth and production of biosurfactant producing isolates: KH_2PO_4 2.0; K_2HPO_4 5.0; 3.0; $NaNO_3$ 2.0; Nacl 0.10; $MgSO_4.7H_2O$ 0.2; $FeSO_4.7H_2O$ 0.01; $CaCl_2$ 0.01. Trace element solution having the following components (mg/l^{-1}) ZnSO4.7 H_2O 5.25; $MnSO_4.4$ H_2O 200; $CuSO_4.5$ H_2O 70.5; $NH_4MoO_4.2$ H_2O 15; $CoCl_2.6$ H_2O 200.

Effect of Days on biosurfactant production by the five potent bacterial isolates

The five potent bacterial isolates (BMSed II, BMSed IV, BRSed I, BDSed II, and BMSed V) were cultured in mineral salt medium to optimize the incubation days (1,3,5,7,9,11) days by quantification of the biosurfactant produced and reduction in surface tension.

Optimizations of culture parameters on biosurfactant production by potent bacterial isolate (BMSed II)

The optimization of different parameters was done in triplicates.[10, 11, 34, and 35]. The optimal conditions for biosurfactant production were selected on the basis of % decrease in surface tension of the medium and % increase in emulsification index (E24). Higher the % decrease in surface tension and increase in E24 value better is the biosurfactant production.

Effect of inoculum size

The optimum inoculum size was determined by using different inoculum volumes (% v/v) i.e. 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0ml, 3.5 ml, 4.0 ml respectively. To that different inoculum vol (%) of the bacterial isolate was added as above and incubated at 37°C and 150 rpm for seven days in a shaking incubator. The surface activities and emulsification index of the cell free supernatant were measured in each case.

Effect of pH and Temperature

The pH optimization was done inoculating the standardized inoculum of 2% in MSM at different pH (2, 4, 6, 8 10, 12) followed by incubating at 37°C in an orbital shaker at 150 rpm for 7 days.

The standardized inoculum was inoculated in MSM at optimized pH and incubated at different temperatures (25, 30, 37, 40, 45°C) in an orbital shaker at 150 rpm for 7 days.

Effect of Carbon Sources

Bacterial inoculum (2%) was inoculated in MSM at optimized pH and temperature to which was added different carbon sources comprising glycerol, glucose, sucrose, paraffin oil, hexadecane at 2% (v/v), then incubated on an orbital shaker at 150 rpm and at 37°C for 7 days. The carbon source that induced the highest biosurfactant production demonstrated by the lowest surface tension and highest emulsification index was subsequently chosen for variation in concentrations of carbon source ranging from [0.5, 1, 2, 4, 6 % (v/v)].

Effect of Nitrogen Sources

To determine the best nitrogen source for optimized production of biosurfactant, standardized bacterial inoculum was inoculated in MSM at optimized pH with added different nitrogen sources, namely, ammonium sulphate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), and ammonium chloride (NH_4Cl), then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 7 days. The nitrogen source that induced the highest level of biosurfactant production as demonstrated by the lowest surface tension activity and highest emulsification index was further considered for variation in its concentrations, ranging from 0.5-6 g/l.

Effect of salt (NaCl) concentration (%)

The effect of salt concentration (NaCl) on biosurfactant production was optimized with

different concentrations of NaCl (2%- 20% w/v). The cell free supernatant was checked for reduction in surface tension and increase in emulsification index after 7 days.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyse the effect of different culture parameters on biosurfactant production. Duncan multiple range test (DMRT) was used to test the significant (p<0.05) difference between the different level of parameters using SAS (Ver. 9.20)

Identification of biosurfactant producing bacterium

The most potent isolate was identified following a series of biochemical, physiological and morphological tests and comparing the data with ABIS, 16Sr-DNA sequencing and phylogenetic tree analysis was also carried out. The 16S rRNA gene fragment was amplified by using universal primers. Sequencing was done at Sci. Genome Laboratories Pvt. Ltd, Cochin. Genomic DNA of BMSed II bacteria was isolated and searched for 16Sr DNA gene sequence homology, using BLAST algorithm followed by the sequence alignment using ClustalW algorithm. A phylogenetic tree was built with MEGA 5.05 software [12, 13].

Production and Extraction of crude Biosurfactant

Bacteria was inoculated to mineral salt medium and incubated at 37°C with a shaking speed of 150 rpm. After 7 days of incubation the culture was centrifuged at 10,000 rpm for 30 min at 4°C. The pH of the cell-free supernatant was adjusted to 2 with the help of 6 N HCl and incubated over night at 4 °C as reported earlier [12, 13, 14, 15]. The acidified supernatant was centrifuged at 10,000 rpm for 30 min to pellet down the biosurfactants. Pellet was used as crude biosurfactant and stored at -20 °C.

Partial purification of biosurfactant by solvent extraction

Twenty millilitre of aqueous biosurfactant solution was mixed with chloroform and methanol mixture (2:1 v/v) for extraction of biosurfactant molecule into chloroform phase. After shaking vigorously for 15 min in a separating funnel, the organic phase containing biosurfactant was separated. Aqueous phase was extracted two or more times in similar manner. The pooled organic phase was concentrated to 5 ml using rotary evaporator (Buchi Model 1, Japan). The concentrated biosurfactant solution was stored at 4 °C for further analysis.

Characterization of biosurfactants

Thin layer chromatography (TLC)

Biosurfactants solution in chloroform was spotted on a TLC plate (20 X 20 cm, Silica gel-60, Merck), and eluted with chloroform: methanol: water (65:15:2) in a solvent chamber. Iodine vapour, Bromothymol blue solution (0.04% in 0.1 N NaOH), orcinol reagent (0.19 % in 53% H_2SO_4) and ninhydrin solution (0.1 % in ethyl acetate) were used as developing reagents [2, 15, 12, 13].

Fourier - transform infrared spectroscopy (FTIR)

The functional group present in the biosurfactants was determined using FTIR. The IR spectrum of biosurfactants was recorded in the 4000-400 cm^{-1} spectral region. The generated peaks were analysed using infrared absorption frequencies data base [13, 16, 17].

Nuclear magnetic resonance analysis (H^1 NMR)

Purified biosurfactants were mixed with deuterated chloroform ($CDCl_3$) and subjected to nuclear magnetic resonance (NMR) and the generated peaks were recorded. Proton NMR was performed for the biosurfactants. Peaks were assayed by comparing with the data assembled. [13, 17, 18].

RESULTS AND DISCUSSION

Identification and characterization of bacterial isolates

Sediment samples were collected arbitrarily from Rangani (site 1), Mahisamunda (site 2), Dangamal (site 3) and Bansurgarh (site 4) of the Bhitarkanika Wildlife Sanctuary of Odisha, India. Out of twenty bacterial isolates only five showed biosurfactant production.

Optimization of parameters for biosurfactant production

The cell growth and accumulation of metabolic products were strongly influenced by medium composition and other growth factors viz., temperature, pH, carbon source, nitrogen source, inoculum % etc. Thus optimization process can give high yield of metabolites. The optimization parameters for highest biosurfactant production were measured by observing the surface tension reduction and emulsification index

Effect of Days in Mineral Salt Medium by the five potent bacterial isolates

The biosurfactant screened five isolates were optimized at different incubation days [Fig 1], out of which only BMSed II showed the highest surface tension reduction of 30.66± 0.42 mN /m and maximum biosurfactant production of 0.51± 0.014 mg/ml respectively and thus, selected for further optimization of parameters.

Effects of different parameters on biosurfactant production by potent bacterial isolate (BMSed II)

Effect of pH & temperature

The microbial metabolism is largely influenced by the pH of the medium. Although BMSed II isolate grew and produced biosurfactant at a wide range of pH 6.0-10 [Fig 2.1] and pH 8.0 was selected as the best pH for biosurfactant production because it reduces the surface tension maximum (37.50 ± 0.40) mN/m and showed highest emulsification index of 65% ± 2.88 [19]. At an acidic pH (4.0) and extreme alkaline pH (12.0) there was an increase of surface tension to 43.0 ± 0.12 mN/m ,40.0 ± 0.29 mN/m and decrease in emulsification index of 40% ± 2.88, 47.5 ± 1.44 respectively . Statistical analysis showed pH at (4, 6, 12) had significant effect (p>0.05) on biosurfactant production. Any change in pH on both sides of neutral pH values caused an appreciable drop in biosurfactant production as indicated by surface tension reduction and emulsification index values.

Temperature is one of the critical parameter that greatly affected the culture growth and biosurfactant production. The optimum temperature for biosurfactant production by BMSed II isolate [Fig 2.2] was found at 37°C producing the lowest surface tension reduction at 39.96 ± 0.12 (mN/m) and highest emulsification index of 65% ± 2.88 [4] was significantly higher than the culture grown at 25°C, 30°C, 45°C . A decrease or increase in incubation temperature leads to less growth of the organism and biosurfactant production.

The activity of the biosurfactant produced by *Pseudomonas aeruginosa* at pH 4-11 was very stable values [21, 22]. *Candida lipolytica* was found to be very stable and produce biosurfactant at wide range of pH 2-12. The biosurfactants produced by different microorganisms were also found to be stable at wide range of pH, salinity and temperature [23, 24].

Effect of carbon & nitrogen source

The utilization to various carbon sources by BMSed II isolate for biosurfactant production [Fig 2.4] was tested with glucose, glycerol, sucrose, paraffin oil, hexadecane at 2% (v/v) [25-27]. Among these carbon sources tested, glucose produced the lowest surface tension and highest percentage of reduction in surface tension at 37.08± 0.2 mN/m with an emulsification index of 62% ± 1.15. Glucose was selected as the optimal carbon source since it

Table 1. TLC plate analysis of biosurfactant produced by *Bacillus altitudinis* (BMSed II).

S No	Spot developers	Spot color	Remarks
1	Iodine vapour	Brown	Organic
2	Bromothymolblue	Yellow	Lipids
3	Ninhydrin solution	Purple	Peptide
4	Orcinol reagent	No spot	Carbohydrate was absent

Table 2. FTIR result of biosurfactant produced by *Bacillus altitudinis* (BMSed II).

S.no	Wave numbers (cm^{-1})	Assignment
1	2,945	–CH3
2	2,832	–CH2–
3	1,020	Ester
4	1,663	Carbonyl group of amide (C=O)
5	3855, 3734, 3260, 3255	N–H bending of Sec amides
6	1524	C=C aromatic rings
7	1,239	C-O Ether
8	1,020	C–O of ester
9	688	Amides

Table 3. Chemical shifts of Lipopeptide biosurfactant produced by *Bacillus altitudinis* (BMSed II) in H^1-NMR

S.no	H^1 chemical shift (´ in ppm)	Assignment
1	0.71–0.83	–CH3,–CH(CH3)2
2	1.22	-OH-
3	1.90	H–C–(COOR)
4	2.49-2.50	“CO–NH–
5	3.16–3.50	H–COH, –CO–H, H–COR
6	7.24	“CH=CH2
7	7.12	Water in CDCl3

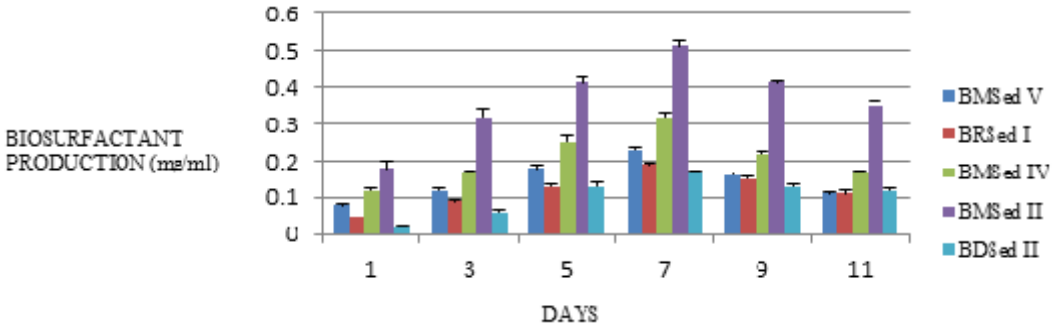

Fig. 1. Effect of days on biosurfactant production by five isolates

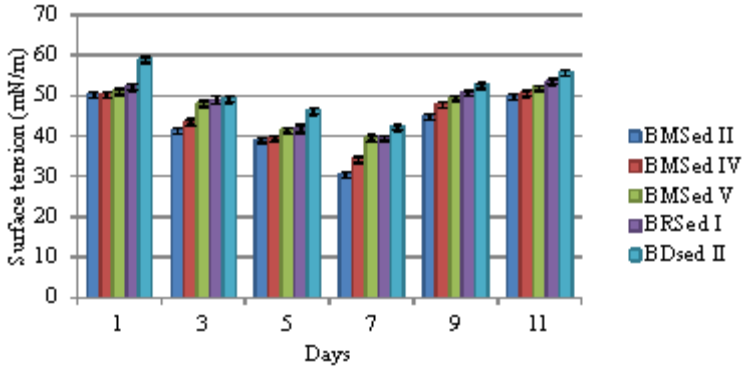

Fig. 1.1. Effect of days on surface tension reduction by five isolates

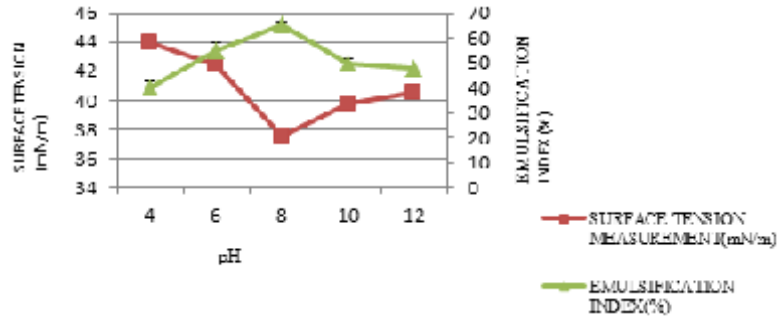

Fig. 2.1. Effect of pH on the production of biosurfactant by BMSed II

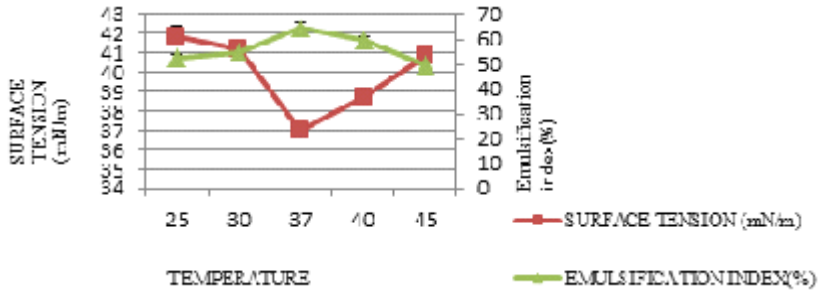

Fig. 2.2. Effect of temperature on biosurfactant production by BMSed II

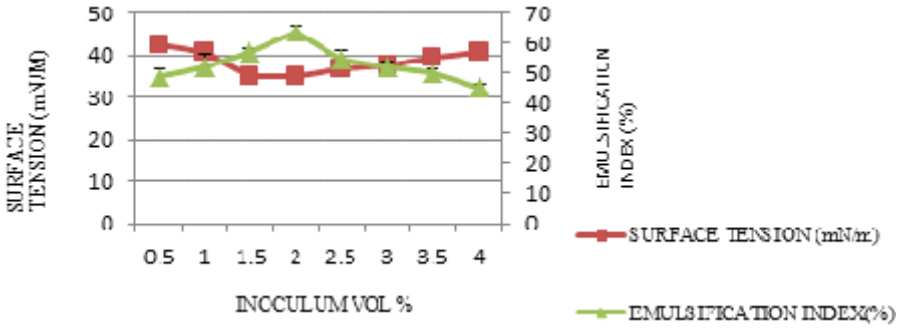

Fig. 2.3. Effect of Inoculum on the production of biosurfactant by BMSed II

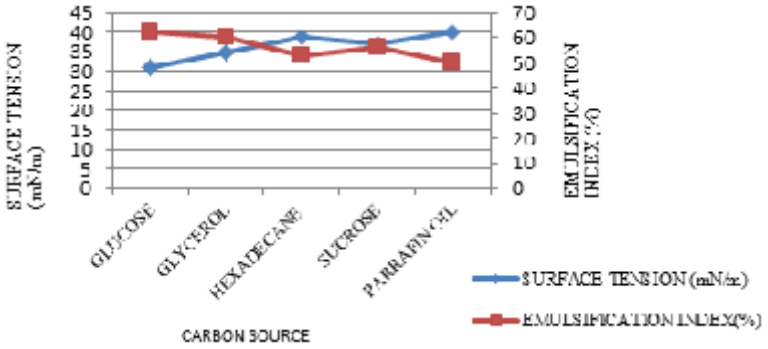

Fig. 2.4. Effect of Carbon source on the production of biosurfactant by BMSed II

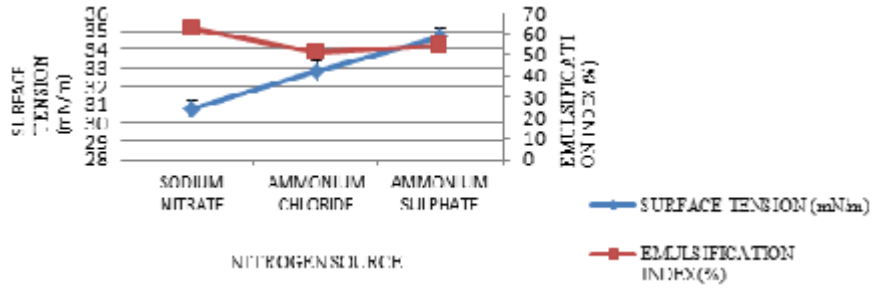

Fig. 2.5. Effect of nitrogen source on the production of biosurfactant by BMSed II

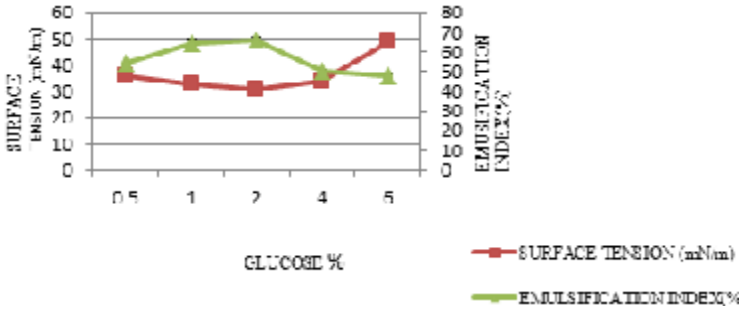

Fig. 2.6. Effect of glucose concentration (%) on the production of biosurfactant by BMSed II

resulted in the highest % reduction in surface tension. BMSed II isolate when grown at 2% v/v glucose concentration showed significant ($p>0.05$) reduction in surface tension 30.73 ± 0.58 mN/m and maximum emulsification index of about 65% ± 2.08 [14, 15]. However, no significant difference at $p>0.05$ was observed between the 0.5% (v/v), 4% (v/v) and 6% (v/v) levels of glucose on reduction in surface tension and emulsification index [Fig 2.6].

BMSed II isolate was able to utilize all types of nitrogen sources tested, including ammonium chloride, ammonium sulphate & sodium nitrate but among them sodium nitrate produced the highest surface tension reduction at 30.80 ± 0.43 mN/m [25, 10] with an emulsification index of 62.4

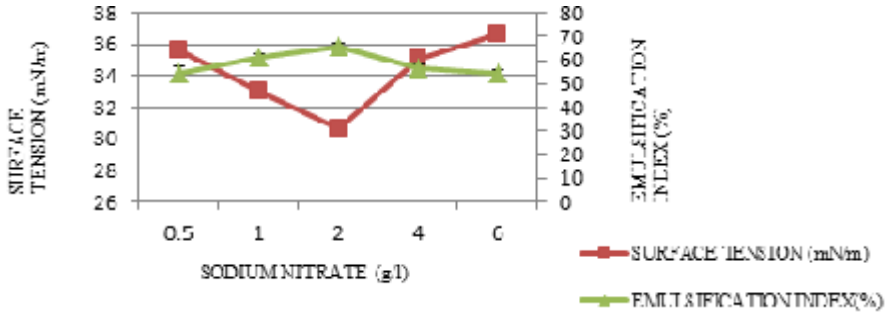

Fig. 2.7. Effect of sodium nitrate concentration (g/l) on the production of biosurfactant by BMSed II

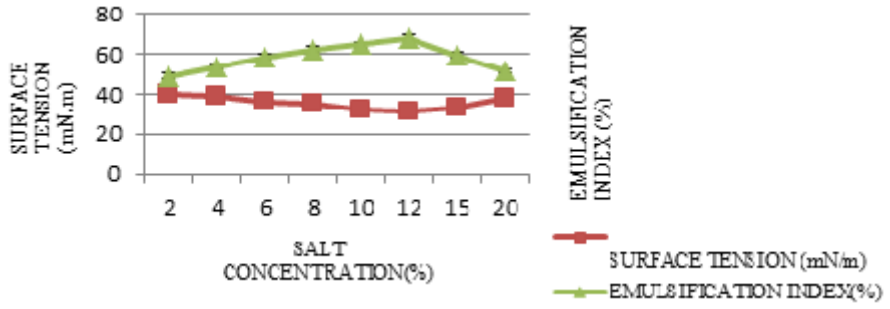

Fig. 2.8. Effect of salt concentration (%) on the production of biosurfactant by BMSed II

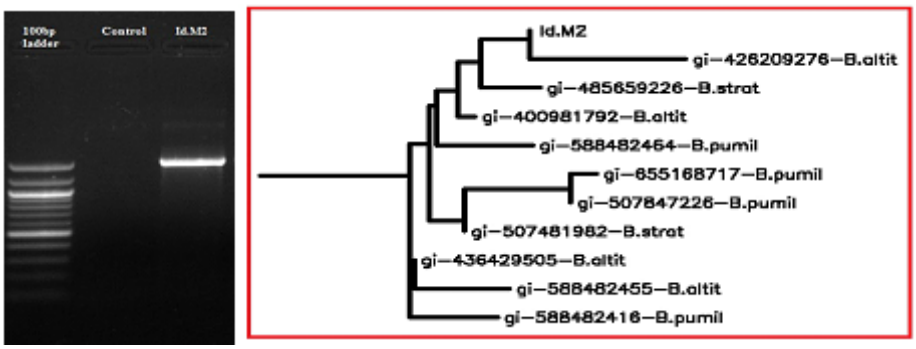

Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA sequences showing relationships between *B.altitudinis* (KT005170)

± 1.58 %. These results are in agreement with [28, 29] who mentioned sodium nitrate as the best nitrogen source for biosurfactant production. No significant difference between ammonium chloride, ammonium sulphate, except for $NaNO_3$ for biosurfactant production was observed [Fig 2.5]. Addition of sodium nitrate 2g/l in MSM medium [Fig 2.7] resulted highest percentage of reduction in surface tension of 30.66 ± 0.29 mN/m and highest emulsification index of 65.8 % ± 1.29. Enzymes needed for biosurfactant production were produced during the organism's stationary growth phase. Any change in one or more environmental parameters such as nutrient, temperature and pH, growth is inhibited. Since carbon, hydrogen and oxygen are the only important elements for the development of molecular structure of glycolipids, biosurfactant production does not need any additional nitrogen-containing salts and the production of biosurfactant continues as long as the carbon source and oxygen are available [30].

Effect of inoculum volume (%)

The production of biosurfactant from BMSed II isolate was affected by change in inoculum volume. Maximum reduction of surface tension of 34.90 ± 0.43 (mN/m) and highest emulsification index of about 64.23% ± 1.3 was obtained with 2 vol % inoculum [Fig 2.3]. Any change to both lower and higher inoculum volume gives poor result. Similar results are in agreement with [31] which show 2% inoculum is suitable for optimum biosurfactant production. Statistical analysis showed no significant difference between 1.5, 2, and 2.5,3 inoculum volume (%).

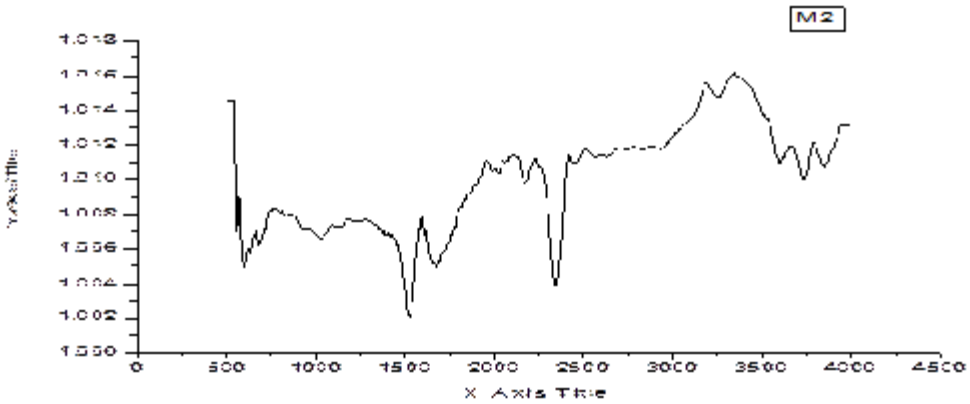

Fig. 4. FTIR spectra of the biosurfactant produced by BMSed II (*Bacillus altitudinis* KT005170)

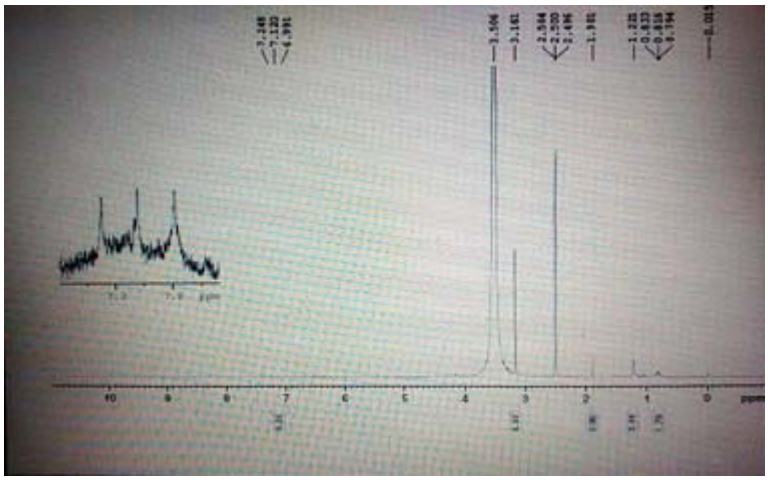

Fig. 5. H^1 -NMR spectra of biosurfactant produced by BMSed II (Bacillus altitudinis KT005170)

Effect of salt concentration (%)

Effect of salinity variations on biosurfactant production was studied with NaCl concentration ranging from 2-20% [Fig 2.8]. 12% salt concentration resulted significantly ($p>0.05$) higher reduction in the surface tension of 30.93 ± 0.29 (mN/m) and increase in emulsification index of 67.36 % ± 1.97 than the other salt concentration. Different level of salt concentration of 6, 8, 10, 12, and 15 % had significant ($p>0.05$) differences in the reduction of surface tension. However no significant difference between salt concentration of 2, 4 and 20% on reduction of surface tension was observed.

Salt concentration also affected biosurfactant production depending on its effect of cellular activity which is very near to the results obtained by [32]. He isolated Bacillus licheniformis BAS50 which grew and produced a lipopeptide surfactant when cultured on a variety of substrate at salinities of 13% NaCl. Depicted results showed that the biosurfactant production was optimal at 5% NaCl.

Highest biosurfactant production (2.14g/l) was observed in BMSed II under optimized condition such as temperature 37°C , pH 8, 2% inoculum, and 2% glucose.

Identification of biosurfactant – producing organism

The 16Sr DNA gene sequencing and the phylogenetic tree confirmed the isolate as *B. altitudinis* with an Accession number (KT005170) in [Fig 3]. Many microbes from different sources have been reported to produce biosurfactants of different types. The biosurfactant producing bacteria produced by *B. altitudinis (*BMSed II) used in the present study produced a lipopeptide type of biosurfactant. Reports are there pertaining to the relationship between the genus and species of microorganism and the type of biosurfactant it produced. It depends more on species rather than genus level diversity [25]. Bacillus mostly produces lipopeptide type of biosurfactants, such as subtilin and surfactin which has been studied in detail [33, 6, 15, 18]. Some researchers have reported another bacterium *Bacillus megaterium* belonging to same genus capable of producing a glycolipid type of biosurfactant [34].

Characterization of Biosurfactants

The characteristic color development after immerging the completely run TLC plate in various developing reagents provides information about the chemical nature of the crude biosurfactant. A brown spot on exposure to iodine vapour indicated the crude biosurfactant to be an organic compound. A yellow spot due to Bromothymol blue featured the lipid nature of organic crude biosurfactant. Purple spot of ninhydrin solution attributed the presence of peptide group in organic lipid compound. Absence of a spot in orcinol reagent confirmed the absence of carbohydrate moiety in the partially crude biosurfactant presented in [table 1]. From the above observations, the crude biosurfactant was confirmed to be a lipopeptide type of the biosurfactant giving different color in developing reagent [2, 13].

Fourier transforms infrared analysis

FTIR Spectral analysis of the biosurfactant in [Fig.4 and table.2] inferred that, wave number 3855,3734,3260,3255 cm^{-1} indicated the presence of N-H bonds of secondary amines [16-18]. Peaks at 2945and 2832 cm^{-1} wave numbers confirmed the –C-H stretching ($-CH_3$, $-CH_2$) of the long hydrophobic R chain of lipid [16, 18, 13]. The absorbance peak at 1663 cm^{-1} indicated the presence of carbonyl group (C=O) of amide which confirms the peptide fraction of biosurfactant. Presence of C-O bond was found at 1020 cm^{-1} that indicated the presence of stretching vibration related to ester [12, 13]. Peak at wave number 688 cm^{-1} also indicated the presence of amide [13, 18]. Dissolved atmospheric CO_2 was denoted as peak number 2336 cm^{-1}.

From the above interpretation, it can be concluded that the biosurfactant produced by *Bacillus altitudinis* (BMSed II) posses both lipid moiety and peptide moiety. So the biosurfactant produced by *Bacillus altitudinis* may be a lipopeptide.

Nuclear magnetic resonance analysis

^{1}H-NMR data analysis (Fig5. and table 3) of the biosurfactant demonstrated the peaks at ´=0.71–0.83 ppm for $–CH_3$ and $–CH(CH_3)_2$ of long fatty acid chain. Peaks at ´=1.22 ppm confirmed the presence of the hydroxyl proton (–OH). The proton of H–C–(COOR) was demonstrated by the peaks at ´= 1.90 ppm. The peaks at ´=7.24 indicated the presence of a double bond (–CH=CH2) in the fatty acid chain. These observations confirm the

existence of a lipid group with a double bond in its R group. The proton of the amide bond/peptide ("CO–NH–) was defined by the peaks at ´=2.49–2.50 ppm. Alcoholic and ethereal protons (H–COH, H–COR) were confirmed by peaks at ´=3.16–3.50 ppm. Water in CDCl3 solvent was demonstrated by the peaks at ´=7.12ppm. [14, 18, 13]

CONCLUSION

A biosurfactant producing microorganism *Bacillus altitudinis* (BMSed II) having Accession number KT005170, was isolated from Bhitarkanika mangrove forest sediment. Optimum biosurfactant were produced by *Bacillus altitudinis* (BMSed II) when grown in MSM medium containing 2% (v/v) of glycerol and 2g/l sodium nitrate at pH8.0, when incubated at 37°C and shaken at 150 rpm for 7 days. The biosurfactant produced reduced the surface tension to 34.71mN/m and was partially characterised by TLC and FTIR analysis, and was chemically confirmed to be a lipopeptide in nature.

Large scale production of biosurfactants by the present isolate in a suitably designed bioreactor system promises its efficient applicability at an industrial scale. This can bring about a change in present scenario by replacing traditional chemical surfactants by natural biosurfactants. Hence in the near future, the biodegradable and eco-friendly nature of biosurfactants will mark them as indispensible green molecules assuring applications in the fields of mineralogy, bioremediation, pharmaceutics, and biomedicine.

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