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Research Article

Optimization and Production of biosurfactant from *Bacillus cereus* KBSB1

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Abstract

Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. The unique properties of biosurfactants make them possible to replace or to be added to synthetic surfactants which are mainly used in food, cosmetics and pharmaceutical industries and in environmental applications. In this study, sixty three hydrocarbon-degrading bacteria were isolated for biosurfactant production. Among that two isolates were further used for the biosurfactant production and optimization. The isolate KBSB1 was identified as *Bacillus cereus* using 16S rRNA sequencing. In this study the isolate *Bacillus cereus* KBSB1 were grown in Mineral salt medium (MSM) with addition of 1% (v/v) crude oil. In the growth kinetic study the maximum biosurfactant production occurred at 132 h of incubation (5.67gl^{1}) and maximum biomass was observed at 132 h (5.48 g l^{-1}). In Thin layer chromatography the biosurfactant produced by *Bacillus cereus* KBSB1 was identified as lipopeptide, a class of biosurfactant. When the changes in pH, temperature and sodium chloride concentration, it does not produce any major differences in the emulsification activity and surface tension. From these results the biosurfactant was stable in the different pH levels with various temperatures and various concentration of sodium chloride. In *B. cereus* KBSB1, the highest emulsification activity with 86.23±0.56 for crude oil and lowest activity was found with heptanes (36.43±0.50). The glucose and ammonium nitrate was the best carbon and nitrogen source studied with the isolate. The environmental factors such as pH 7 and temperature 30^{o} C was found to be optimum for the biosurfactant production. Among the hydrocarbon tested the crude oil and n-hexadecane showed statistically on par results. The biosurfactant yield was higher in the presence of all the trace elements. In future study the optimization using Response Surface Methodology (RSM) will be helpful in studying the interaction between the variables.

Keywords: Biosurfactant, *Bacillus cereus*, Emulsification activity, Surface Tension, Response Surface Methodology (RSM)

Introduction

Biosurfactant have been studied recently, attracted increasing attention, due to their low toxicity, higher biodegradability and ecological acceptability compared to the highly used chemical surfactants (Shin *et al*., 2006). Biosurfactants are amphiphilic, consisting 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 (Pacwa-Plociniczak *et al*., 2011). Formation of most microbial products is a complex, highly nonlinear process. Along with other process variables, the media components play a key role in controlling yield and specific productivity (Moumita *et al*., 2009).

Even though interest in biosurfactants is increasing, these compounds do not compete economically with synthetic surfactants. To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering processes and the use of cost-free or cost credit feedstock for microorganism growth and surfactant production. The choice of inexpensive raw materials is important to overall economy of the process because they account for 50% of the final product cost and also reduce the expenses with wastes treatment. In this investigation, we tried to optimize the cultural conditions for maximum production of biosurfactant from the actinomycete *N. Amarae* (Tarek *et al*., 2006).

Several *Bacillus* species produce a lipopeptide biosurfactants; the most important one is surfactin which is produced from *Bacillus subtilis* (Nitschke and Pastore, 2006; Nitschke and Pastore, 2004). Moreover, *B. licheniformis* has the ability to produce many surface active lipopeptides (Imura *et al*., 2008; Dastgheib *et al*., 2008).

Information on biosurfactant production in *B. cereus* is limited. In earlier study, *B. cereus* produce plipastatins, a family of lipopeptides with antifungal activity was reported. *B. cereus* is a soil bacterium and widespread in nature. It is also an opportunistic human pathogen. PlcR, a pleiotropic regulator in *B. cereus*, positively regulates many genes, including those that encode extracellular virulence factors at the onset of stationary phase (Yi-Huang *et al*., 2007).

Materials and Methods

Isolation, screening and identification of the Microorganism

The mangrove soil samples were collected from various places of Cuddalore district of Tamil Nadu, India. The places are Pichavaram (boat house), South Pichavaram, Kodiyampalayam, Muzhukuthurai and Artificial Mangrove forest. From these five places a total of twenty one soil samples were collected from sediments and rhizosphere of the mangrove plant. One hundred gram of freshly collected soil samples was taken in a 250 ml Erlenmeyer flask. It was added with 10 ml of crude oil (to selectly enrich the biosurfactant producers) and thoroughly mixed and kept for incubation at room temperature (28 ± 2°C) for 30 days. The samples were moistened with water to avoid desiccation when necessary. The screening of bacterial isolates was done using hemolytic assay (Mulligan *et al*., 1984), drop collapse assay (Jain *et al*., 1991; Bodour and Miller-Maier, 1998), oil spreading test (Morikawa *et al*., 2000), and emulsification (Cooper and Goldenberg, 1987). Best biosurfactant producing strain was identified and used for the production and characterization.

Identification of bacterial isolates

The isolated bacterial strains were identified using standard biochemical and sugar fermentation test. The species level identification was done using 16S rRNA sequencing.

16S rRNA sequencing

It is important to use a pure cultivated bacterium for identification. The purification of PCR products were done by removing unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The sequencing was carried out by purifying the PCR products of approximately 1,400 bp were sequenced using 2 primers. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

The partial sequencing of the 16S rRNA gene was commercially carried out at the Yazhl Xenomics, Chennai, using universal amplification and sequencing primers and Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols provided by the manufacturer.

Phylogenetic analysis

The partial sequencing was analyzed and compared with nucleotide sequence databases in the National Center for Biotechnology Information (NCBI) website using Basic Local Alignment Search Tool (BLAST) program (http://www. ncbi.nlm.nih.gov/BLAST), in order to confer percentage sequence similarities. The evolutionary history of SOL-10 strain was inferred using the Neighbor-Joining (NJ) method. The evolutionary distances were computed using the Maximum Composite Likelihood (MCL) method. Phylogenetic analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) software (Version 4.0).

Growth and biosurfactant production

Bacteria were grown in Minimal Salt Medium (g l^{-1}) containing 1.0 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 0.05 $FeSO_4.7H_2O$, 0.1 $CaCl_2.2H_2O$, 0.001 $Na_2MoO_4.2H_2O$, 30 NaCl added with crude oil (1.0%, w/v). Flasks containing sterilized MSM were inoculated with a loopful of bacteria and flasks were maintained in an Orbital shaker for 7 days at 120 rpm, 30^oC. After 7 days of incubation, culture broth from each flask was centrifuged at 6000 rpm, 4^oC for 15 minutes and the supernatant was filtered through 0.45µm pore size filter paper (Millipore, India). This cell free culture broth was used for drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement. All the screening experiments were performed in triplicates (until otherwise mentioned) and the mean values were recorded.

Biosurfactant extraction and characterization

The MSM broth with the culture inoculums was centrifuged at 10,000 × g for 30 minutes to discard the cells and extracted with equal volume of acetone. The solvent was removed by rotary evaporation and the residue was partially purified in silica gel (60–120 mesh) column eluted with chloroform and methanol ranging from 20:1 to 2:1 (v/v) in a gradient manner. The fractions were pooled and solvents were evaporated, resulting residue was dialyzed against distilled water and lyophilized. The crude biosurfactant was expressed in g l^{-1} (Thavasi *et al*., 2011).

The critical micelle dilution (CMD) is defined as the solubility of a surfactant in an aqueous phase and is commonly used to measure the efficiency of a surfactant (Desai and Banat 1997). The extracted biosurfactant was dissolved in distilled water at concentrations ranging from 1.0 to 200 mg l^{-1} for calculation of critical micelle concentration (CMC). This is a direct measurement of surfactant concentration corresponding to the concentration of an amphiphilic component at which the formation of micelles is initiated in the solution (Abouseoud *et al*., 2008). The CMC of the produced biosurfactant was determined following standard methods (Kim *et al*., 1997; Bonilla *et al*., 2005). CMD^{-1} and CMD^{-2} were determined by measuring the surface tensions of cell free supernatant diluted 10-times and 100-times in distilled water (Kosaric 1993).

Carbohydrate moieties in the biosurfactant molecule were assayed using rhamnose (Dubois *et al*., 1956) and Molisch's test. The rhamnose test was performed by adding 0.5 mL cell supernatant to 0.5 ml 5% phenol solution and 2.5 ml sulfuric acid and incubating the sample for 15 min before measuring absorbance at 490 nm. Molisch's test was performed by adding 3 mL cell free supernatant to 1 mL 10% α-naphthol. This was followed by the addition of 1 mL concentrated sulfuric acid to the sample without disturbing it.

The crude biosurfactant extracted with chloroform: methanol was analyzed by thin layer chromatography (TLC). The TLC tank was filled

with a solvent mixture of chloroform:methanol:acetic acid:water (25:15:4:2 v/v/v/v). The chromatogram was sprayed with α-naphthol and sulfuric acid.

Activity characterization (Rashmi *et al*., 2012)

Foam was produced by hand shaking a two-day-old culture supernatant for a few minutes. The stability of the foam was monitored by observing it for 48 h. To determine the thermal stability of the biosurfactant, cell-free broth of the isolate was maintained at a constant temperature range of 20-100^{o}C for 15 min, followed by cooling at room temperature (28 ± 2^{o}C).The effect of pH and salinity on stability of the biosurfactant was evaluated by altering the pH (2–12) and the concentration of NaCl (0%–1%, 5%) of the cell free culture supernatant and measuring the surface tension and Emulsification index ($EI_{24,}$ %).

Emulsification

Cell free culture broth was used as the biosurfactant source to check the emulsification of crude oil. 1 ml of cell free culture broth was added to 5 ml of 50 mM Tris buffer (pH 8.0) in a 30 ml screw-capped test tube. Five milligram of hydrocarbon was added to the above solution and vortex-shaken for 1 minute and the emulsion mixture was allowed to stand for 20 minutes. A negative control was maintained only with buffer solution and crude oil and Triton X-100 was used as the positive control. Various hydrocarbons like xylene, crude oil, benzene, kerosene, coconut oil, heptanes, n-hexadecane, diesel and petrol were used to check the emulsification activity of the isolate.

$$E_{24}\,(\%) = \frac{\textit{The height of emulsion layer}}{\textit{The height of total solution}} \times 100$$

FTIR

The FT-IR spectra was recorded in a Thermo Niocolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 cm^{-1} using potassium bromide (KBr) pellets. The air dried biosurfactant sample was grounded with a purified potassium bromide salt to remove scattering effects from large crystals. This powdered mixture is then pressed in a mechanical press to form a translucent pellet through which the beam from spectrometer passed.

Optimization of Biosurfactant production from *Bacillus* sp., KBSB1

The selected bacterial isolate KBSB1 was studied for the biosurfactant production under the influence of certain physical and chemical factors. All the experiments were carried with five replicates. Biosurfactant production was determined by estimating the surface tension (ST) reduction and the emulsification assay (E_{24} %), dry cell biomass (DCBM).

Effect of carbon sources on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) with six different carbon sources *viz.*, glucose, glycerol, fructose, sodium citrate, mannitol and starch were prepared in a 250 ml Erlenmeyer flask and sterilized in an autoclave. It was added with1.0 ml of crude oil. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at 28 ± 2°Cfor 5 days over a shaker set at120 strokes min^{-1}. Extraction was done following the procedures of Cameotra (1995) and emulsification assay with crude oil was performed following the procedures of Banat (1995).

Effect of nitrogen sources on the production and activity of the biosurfactant

One hundred ml of MSM broth (pH 7.0) with five different nitrogen sources like yeast extract, peptone, ammonium chloride, ammonium nitrate and sodium nitrate were dispensed in 250 ml Erlenmeyer flasks and sterilized in an autoclave. A quantity of 1.0 ml of crude oil and 5.0 ml of culture inoculum were added. The flasks were incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of hydrocarbons on the production and activity of the biosurfactant

One hundred ml of MSM broth (pH 7.0) was taken in a clean 250 ml Erlenmeyer flask and sterilized. One ml of different heavy hydrocarbons *viz.*, n-hexadecane, heptane, xylene, kerosene, petrol, diesel and crude motor oil were added in different flasks. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at 28 ± 2°Cfor 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of pH on the production and activity of the biosurfactant

One hundred ml of MSM broth was prepared of varying pH 5.0, 5.5, 6.0, 6.5, 7.0 7.5 and 8.0 and sterilized. It was added with one ml of crude oil. To that 5.0 ml of bacterial inoculation of different isolates were added and incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of temperature on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) was prepared and sterilized. It was added with 1.0 ml of crude oil and different bacterial cultures were inoculated. The flasks were incubated at varying temperatures of 25, 30, 35, 40, 45 and 50°C in an incubator over shaker. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of trace elements

One hundred ml of MSM broth with 2 % glucose for *B. cereus*, glycerol for *P. aeruginosa* and pH 7.0 was prepared as follows; (a) without $MgSO_4$ (b) without $MnSO_4$, (c) without $FeSO_4$, (d) without $FeSO_4$ and $MnSO_4$, (e) without $MgSO_4$ and $FeSO_4$ (f) without $MgSO_4$, $MnSO_4$ and $FeSO_4$. One ml crude oil was added aseptically and to that 5 ml of inoculums from selected bacterial isolates was added and kept at room temperature 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Statistical Analysis of Results

All the results related to determination of emulsification activity, biosurfactants quantity and CFU counts were the average of three replicates of two separate experiments for each cultural condition. They were statistically analyzed by SPSS software (version 100) using the Duncan test performed after analysis of variance (ANOVA).

Results

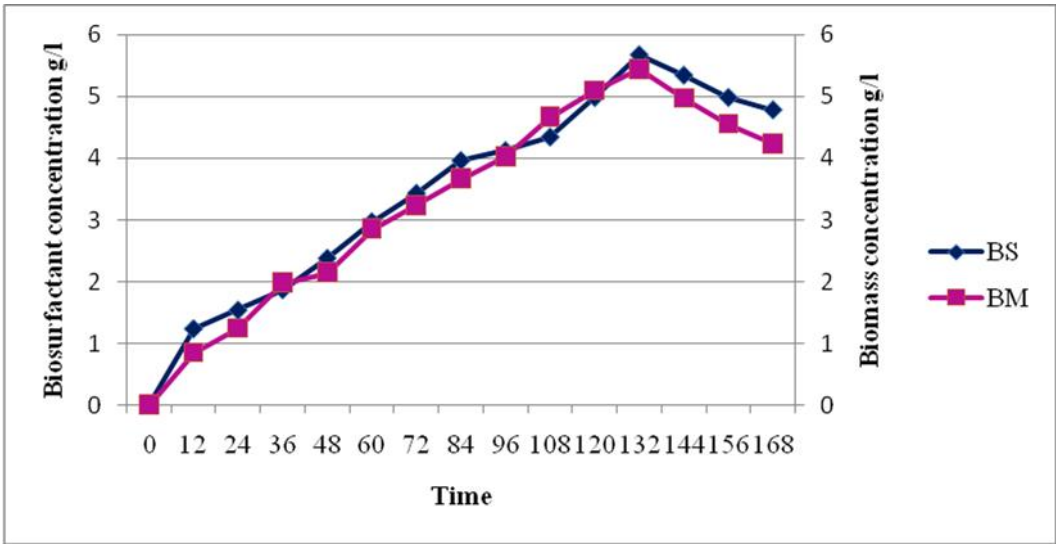
Growth and biosurfactant production

The isolate KBSB1 was identified as *Bacillus cereus* and the sequence data was submitted to Genbank and got the accession no as KJ920195. Bacteria were grown in Minimal salt medium added with crude oil (1.0%, w/v). After 7 days at 120 rpm and 30^{o} C incubation the culture broth was centrifuged and the supernatant was collected and by using equal volume of acetone the filtrate was extracted. This method of extraction was used to extract the biosurfactant from the *B. cereus*. The precipitate was observed after incubation, collected by centrifugation and pellet was resuspended with the solvent and stored at 4 ^{o} C. The stored crude biosurfactant was further characterized. The emulsification activity with various hydrocarbons and stability characterization was studied by altering the pH, temperature and NaCl concentration. The biosurfactant production was studied using 2.0% Crude oil supplemented with 1% glucose in the MSM medium. Fig. 1 shows the time course of biosurfactant production of *B. cereus*. Maximum biosurfactant concentration of biosurfactant occurred at 132 h of incubation (5.67 g l^{-1}) and maximum biomass was observed at 132 h (5.48 g l^{-1}).

Chemical characterization of the biosurfactant
Preparative column chromatography

After solvent extraction, crude biosurfactant was subjected to preparative column chromatography.

Fig. 1: Growth and biosurfactant production by *Bacillus cereus* KBSB1 using crude oil



The sample-loaded column was first washed with n-hexane (~ 200ml), followed by chloroform (~ 300ml) finally Chloroform: Methanol mobile phases were then applied in a sequence: 1:1 v/v (200ml) and 1:2 v/v (200ml) at a flow rate of 1 ml/min. The biosurfactant fractions were collected separately, freeze-dried, weighed and characterized. The eluted fraction was subjected to TLC.

Thin layer chromatography

Each of the Column chromatography purified fraction was applied on preparative silica gel plates. Chromatograms were developed with the following saturation buffer and spraying agents. The R_f value of each macromolecule was noted using the formula:

R_f = Distance travelled by the substance/
Distance travelled by the solvent

Ten microliter of biosurfactant in methanol was spotted on a silica gel thin layer chromatography (TLC) plate (Silica gel 60; Merck, Darmstadt, Germany). The compounds were separated using a mobile phase of chloroform/methanol/water (65:25:4,*v/v/v*). For the detection of peptides, the dry plates were sprayed with 0.25% ninhydrin in acetone and kept at 105^{o}C for 5 min. For the detection of lipids, the plates were exposed to iodine. For further analysis, TLC purified lipopeptide biosurfactant was used. *Bacillus cereus* KBSB1 was identified as lipopeptide class of biosurfactant using TLC analysis. Detection of TLC plate with iodine showed spot having R*f* value of 0.8.

Activity characterization

Aqueous solutions of biosurfactant produced from the selected bacterial isolates showed good foaming stability. Total disappearance of the foam was detected after 2 h for all the isolates. The isolate KBSB1 showed emulsification activity of about 69.54 per cent. The CMC value of the isolate KBSB1 was found to be 78.56 mg/l.

Stability characterization

To determine the thermal stability of the biosurfactant, cell-free broth of *Bacillus cereus* KBSB1 were maintained at a constant temperature range of 20-100^{o}C for 15 min, followed by cooling at room temperature (28 ± 2^{o}C). To determine the effect of pH on activity, the pH of the biosurfactant was adjusted (2.0-12) prior to filter sterilization (Whatman qualitative filter paper 42, 125 mm diameter). To study the effect of addition of different concentration of NaCl on the activity of the biosurfactant, the biosurfactant was re-dissolved after purification with distilled water containing the specific concentration of sodium chloride (0.2 to 5 per cent, W/V).

The Fig. 2, 3, 4 shows the stability of the isolates towards the changes in the pH, Temperature, NaCl concentration. In the case of stability of the biosurfactant towards the variations in the pH may alter the emulsification activity of the surfactant. pH range between 6-8 the highest emulsification activity was found. In the pH level of 2 and 12 the emulsification activity was greatly reduced and moderate activity was observed with the pH 4 and 10. The temperature may also have a significant role in the activity of emulsification of biosurfactant. In the lowest and highest temperature the activity was greatly reduced but good activity was observed with 20^{o}C and 40^{o}C.

When compared with pH and temperature the sodium chloride concentration does not produce any major differences in the emulsification activity. 5% concentration showed lowest emulsification activity. From these results the biosurfactant was stable in the different pH levels with various temperatures and various concentration of sodium chloride.

Emulsification activity

Biosurfactant isolated from *Pseudomonas aeruginosa* PBSC1 showed maximum emulsification activity against crude oil. Similar kind of results was observed with the *B. cereus* KBSB1 biosurfactant. Emulsification activities of the biosurfactant with different hydrocarbons were illustrated in (Fig. 5).The emulsion formed by the biosurfactant against each hydrocarbon was stable for 1 month. The crude oil is used as a substrate for the further production and characterization of biosurfactant. In *B. cereus* KBSB1, the highest emulsification activity with 86.23±0.56 for crude oil and lowest activity was found with heptanes (36.43±0.50).

FTIR analysis of Biosurfactant from *B. cereus* KBSB1

In case of *B. cereus* KBSB1 a lipopeptide biosurfactant was synthesized and confirmed by the FT-IR analysis. The FTIR spectrum (Fig. 6) of the sample showed strong absorption bands of peptides at 3542.29 cm^{-1}, 1648.09 cm^{-1}, and 1431.32 cm^{-1}. These bands resulted from the stretching mode of N-H, stretching mode of the C=O bond, and the deformation mode (combined C-N stretching mode) of the NH bond, respectively. The spectrum showed a broad absorbance peak centered around 3485.55 cm^{-1}, ranging from 3100 cm^{-1} to 3600 cm^{-1}. This is a typical feature of compounds containing carbon and amino groups and is caused due to stretching vibrations of C-H and N-H bonds, present in the compound. The absorbance peak at 1648.09 cm^{-1} implies that peptide groups are present in the sample. A weak absorbance signal at 1431.32 cm^{-1} was due to bending vibrations of C-H bonds associated with alkyl chains. Another absorption peak at 1133.52 cm^{-1} was due to C – O stretching vibrations related to esters.

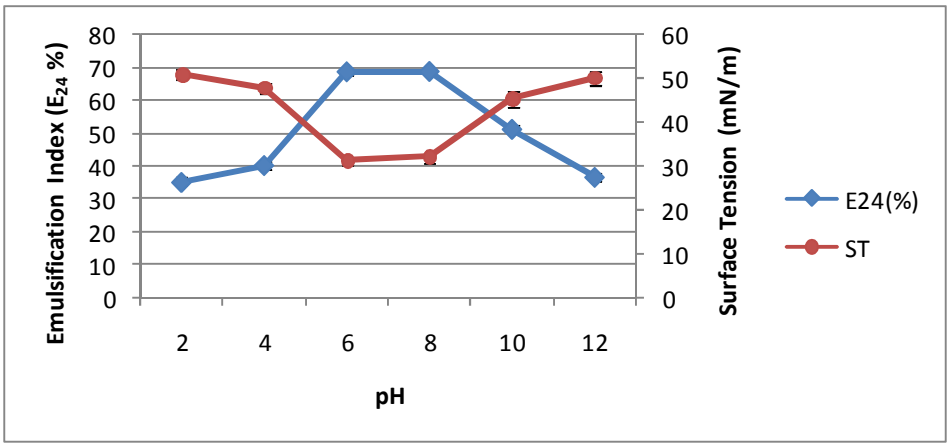
Effect of different Carbon sources

Among the different carbon sources tested the isolate *B. cereus* KBSB1 produced maximum biosurfactant using glucose as a sole carbon source (5.23 g/l), followed by glycerol with 3.96 g/l (Table 1, Fig. 7). The maximum surface tension was recorded for the isolate was 31.32 mN/m when glucose used as a carbon source. The dry cell biomass was measured for about 4.54 g/l followed by glycerol (4.23 g/l). The lowest value was observed with sodium citrate (0.98 g/l). The isolate *B. cereus* KBSB1 was able to utilize almost all the carbon sources but glucose produced best results on the production of biosurfactantas well as produced highest amount of dry cell biomass. The isolate was able to reduce the interfacial tension in the cell free supernatant when glucose was used. The highest emulsification activity was recorded for about 78.12 per cent for glucose and followed by 72.56 per cent for glycerol by the isolate. When sodium citrate used as a carbon source it produced lower amount of biosurfactant and dry cell biomass. From the above study the best carbon source studied was glucose.

Effect of nitrogen source

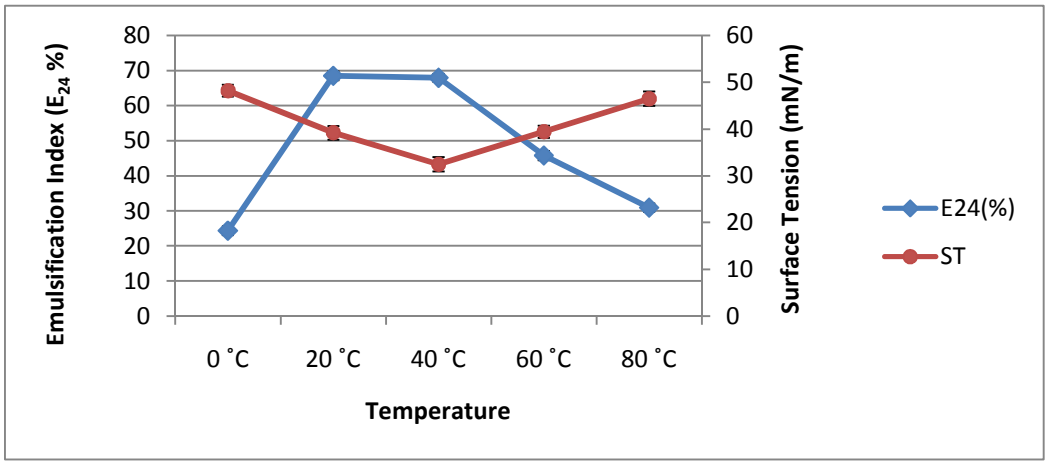
Regarding the nitrogen sources, the highest biomass production was obtained using ammonium nitrate as the sole nitrogen source for isolates *B. cereus* KBSB1. The table 2, Fig 8 shows the effect of

Fig. 2: Stability of biosurfactant toward the changes in pH



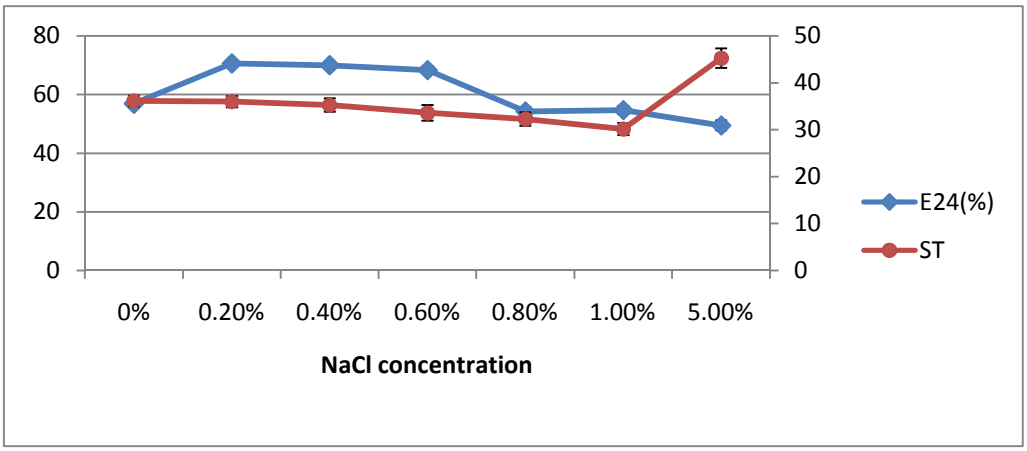
Value is a mean of five replicates ± SD

Fig. 3: Stability of biosurfactant toward the changes in temperature



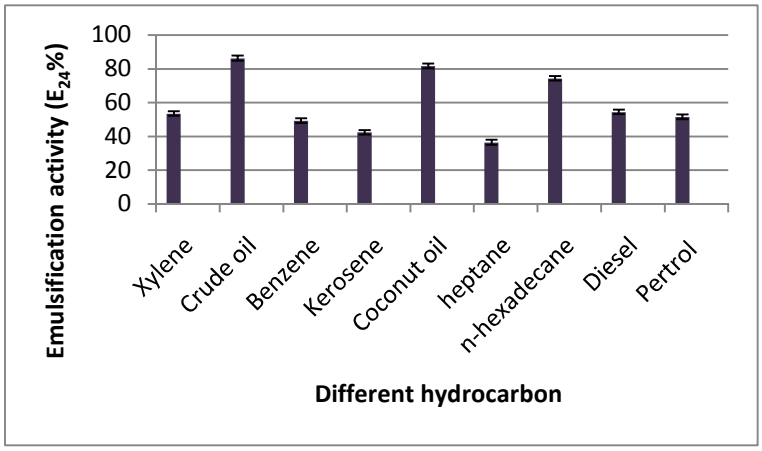
Value is a mean of five replicates ± SD

Fig. 4: Stability of biosurfactant toward the changes in NaCl concentration



Value is a mean of five replicates ± SD

Fig. 5:Emulsification activity of *B. cereus* KBSB1 with various hydrocarbon



Value is a mean of five replicates ± SD

Fig. 6: FTIR spectrum of Biosurfactant from *B. cereus* KBSB1

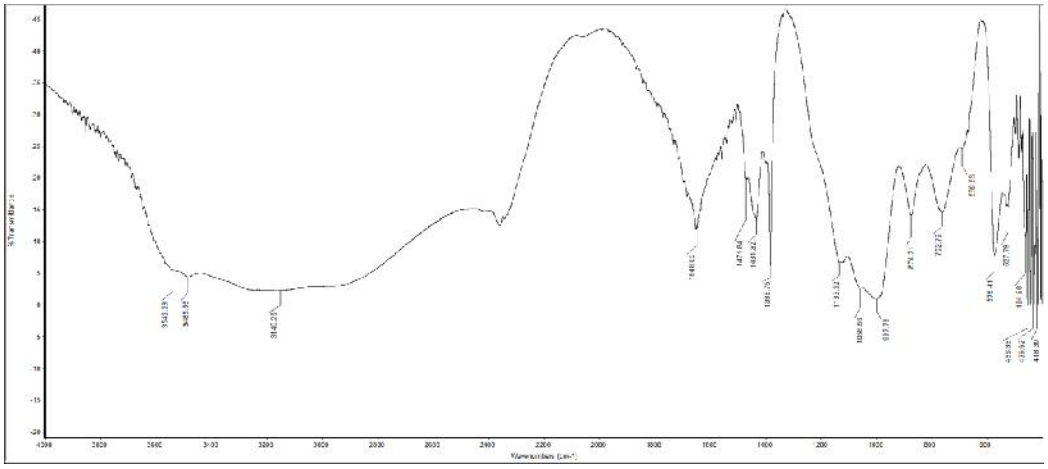


Table 1: Effect of different carbon * sources on the isolated organism's biomass, surface tension and emulsification activity

Carbon sources	*Bacillus cereus* KBSB1	
	DCBM (g/l)	Emulsification Index (E_{24}) (%)
Glucose	4.54±0.18[a]	78.12±0.02[a]
Glycerol	4.23±0.04[b]	72.56±0.08[b]
Fructose	1.45±0.16[e]	59.45±0.24[e]
Sodium citrate	0.98±0.12[f]	53.17±0.10[f]
Mannitol	3.78±0.22[c]	68.34±0.18[c]
Starch	2.56±0.08[d]	66.14±0.22[d]

*- 2 per cent concentration

DCBM – Dry cell biomass; E_{24} – Emulsification index

Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table 2: Effect of different nitrogen sources on the isolated organism's biomass, surface tension and emulsification activity

Nitrogen source	*Bacillus cereus* KBSB1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
Yeast extract	2.92 ± 0.22^{d}	65.18 ± 0.16^{d}
Peptone	3.53 ± 0.12^{c}	69.68 ± 0.07^{c}
Ammonium chloride	4.24 ± 0.06^{b}	76.35 ± 0.22^{b}
Ammonium nitrate	4.65 ± 0.22^{a}	79.32 ± 0.15^{a}
Sodium nitrate	1.38 ± 0.08^{e}	54.29 ± 0.10^{e}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table 3: Effect of different pH on the isolated organism's biomass, surface tension and emulsification activity

pH	*Bacillus cereus* KBSB1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
5.0	2.92 ± 0.08^{e}	65.13 ± 0.20^{d}
5.5	3.24 ± 0.12^{d}	69.34 ± 0.32^{c}
6.0	3.85 ± 0.22^{c}	71.45 ± 0.22^{b}
6.5	4.45 ± 0.10^{b}	76.12 ± 0.21^{a}
7.0	4.87 ± 0.18^{a}	78.34 ± 0.08^{a}
7.5	4.35 ± 0.22^{b}	69.16 ± 0.16^{c}
8	3.48 ± 0.04^{d}	62.56 ± 0.08^{e}
8.5	1.84 ± 0.02^{f}	56.13 ± 0.12^{f}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table 4: Effect of different Temperature on the isolated organism's biomass, surface tension and emulsification activity

Temperature (°C)	*Bacillus cereus* KBSB1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
25	4.12 ± 0.10^{b}	74.45 ± 0.12^{b}
30	4.56 ± 0.12^{a}	78.98 ± 0.14^{a}
35	3.97 ± 0.14^{b}	69.32 ± 0.06^{c}
40	2.68 ± 0.22^{c}	51.34 ± 0.22^{d}
45	1.45 ± 0.08^{d}	49.67 ± 0.34^{e}
50	0.84 ± 0.16^{e}	48.65 ± 0.08^{f}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table 5: Effect of different hydrocarbons on the isolated organism's biomass, surface tension and emulsification activity

Hydrocarbons (1%)	*Bacillus cereus* KBSB1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
n-hexadecane	4.35 ± 0.12^{b}	72.42 ± 0.43^{b}
Heptanes	3.96 ± 0.20^{c}	69.74 ± 0.54^{c}
Xylene	1.75 ± 0.46^{g}	55.38 ± 0.32^{f}
Kerosene	3.14 ± 0.32^{d}	65.35 ± 0.36^{d}
Petrol	2.17 ± 0.26^{f}	59.84 ± 0.41^{e}
Diesel	2.85 ± 0.10^{e}	58.22 ± 0.38^{e}
Crude motor oil	4.98 ± 0.32^{a}	75.86 ± 0.26^{a}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table 6: Effect of different trace elements on the isolated organism's biomass, surface tension and emulsification activity

Trace elements	*Bacillus cereus* KBSB1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
Control	4.98±0.31[a]	70.05±0.12[a]
Mg_2SO_4(0.5 g/l) free	4.12±0.09[b]	64.53±0.08[b]
$MnSO_4$(trace) free	4.04±0.12[c]	63.98±0.21[b]
FeSO4 (0.01g/l) free	3.98±0.22[c]	63.42±0.32[b]
Mg2SO4 + FeSO4 free	3.78±0.10[d]	59.85±0.16[c]
MnSO4 + FeSO4 free	2.85±0.04[e]	59.12±0.13[c]
Mg2SO4 + MnSO4 + FeSO4 free	1.76±0.15[f]	54.32±0.06[d]

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Fig 7: Effect of different carbon sources on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1

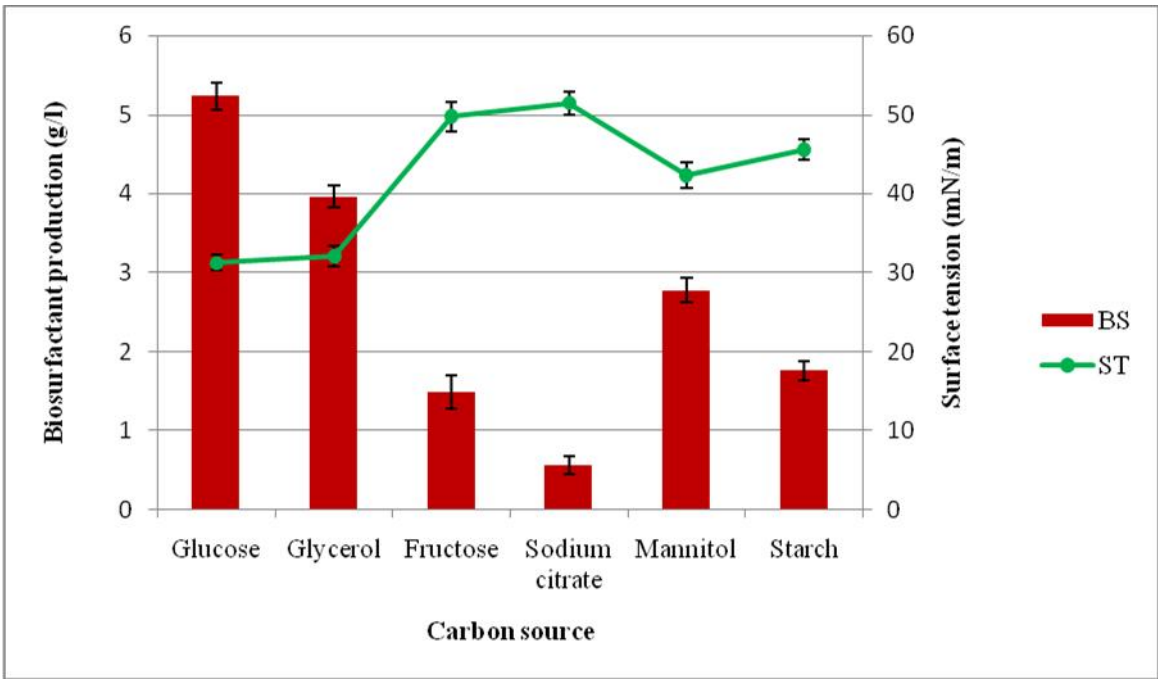
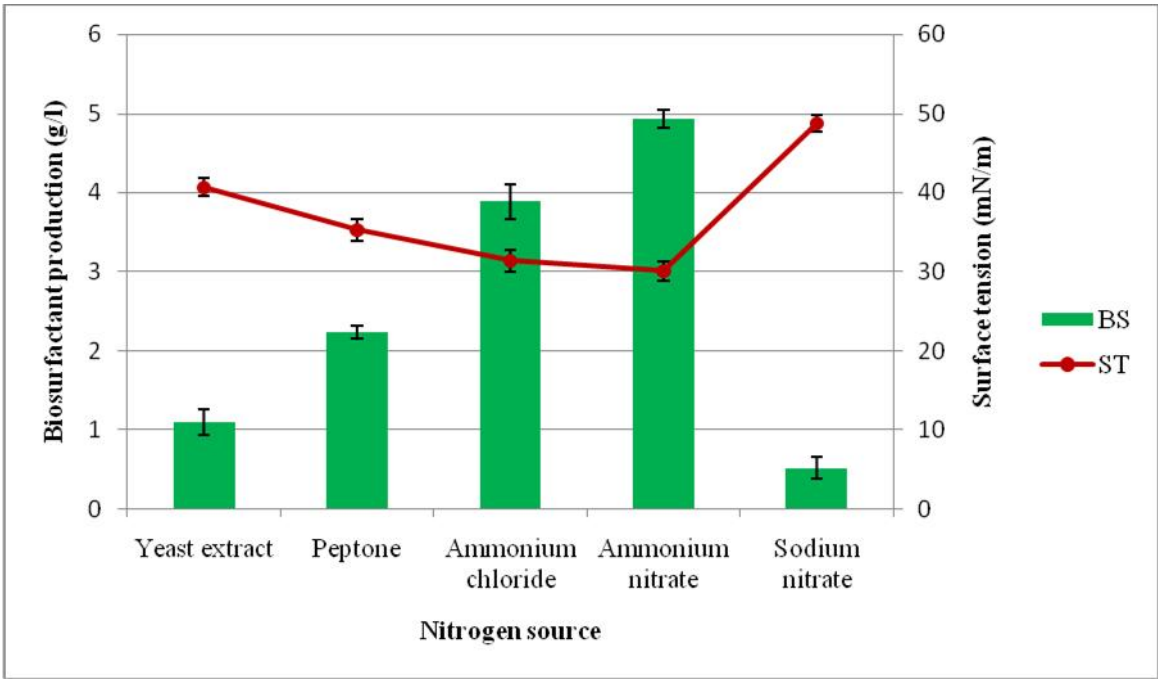


Fig. 8: Effect of different nitrogen sources on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1



nitrogen sources on the production of biosurfactant by the isolates. The lowest surface tension values, which corresponded to the highest biosurfactant productions, were obtained with ammonium nitrate for isolate (30.08 mN/m). The highest biosurfactant production was 4.93 g/l followed by 3.89 g/l. The highest emulsification activity was found to be 79.32 per cent in case of ammonium nitrate used as a sole carbon source.

Taking into account the amounts of biosurfactant produced with the different carbon and nitrogen sources, and in order to standardize the medium for all isolates, glucose and ammonium nitrate were selected for the production of biosurfactants in case of *B. cereus* KBSB1 to be further used in the chemical characterization experiments.

Effect of different pH

The effect of pH, on the activity and biosurfactant production of the selected isolate were presented in Table 3, Fig. 9. The pH of 7.0 has showed a statistically significant result on cell biomass, production and biosurfactant activity by the isolate *B. cereus* KBSB1. The maximum cell dry biomass was achieved at a pH of 7, 4.87 g/l by *B. cereus* KBSB1. The highest surface tension reduction (29.45 mN/m) and emulsification activity (78.34 per cent) was recorded at a pH of 7.0 for *B. cereus* KBSB1. The highest biosurfactant production was observed by the isolate *B. cereus* KBSB1 was 5.32 g/l at pH 7.0. Any change to both lower or higher pH values caused an appreciable drop in biosurfactant production indicated by surface tension reduction and emulsification index values.

Effect of different temperature

The effect of various temperatures like 25°C, 30 °C, 35°C, 40°C, 45°C and 50 °C on the growth, biosurfactant production, surface tension and emulsification activity was studied. The optimum condition of temperature observed was 30 °C and followed by 25 °C. The highest biosurfactant production was recorded as 4.98 g/l for the isolate *B. cereus* KBSB1 at the temperature of 30°C (Table 4, Fig. 10). The temperature 25°C and 35°C produced statistically on par results for the biosurfactant production, dry cell biomass weight, surface tension reduction and emulsification activity.

Fig. 9: Effect of different pH on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1

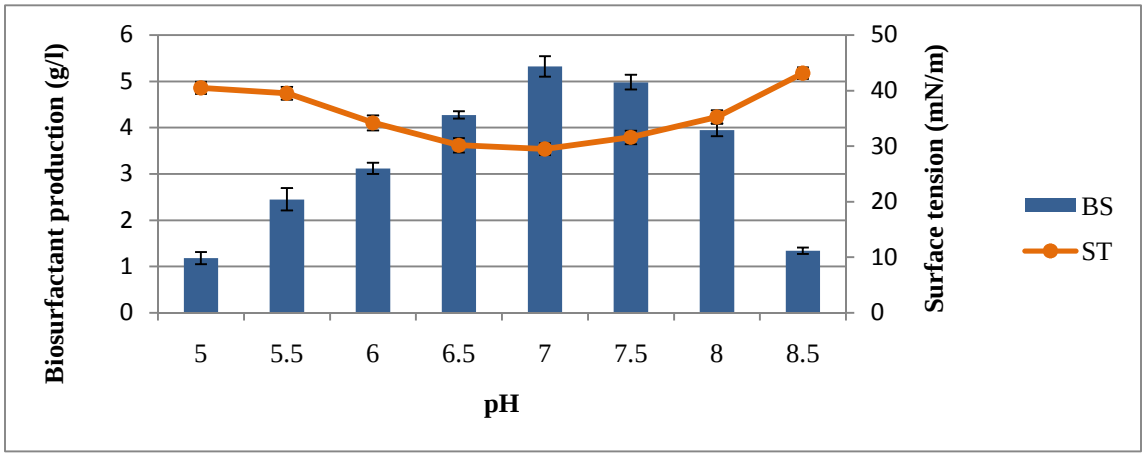


Fig. 10: Effect of different temperature on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1

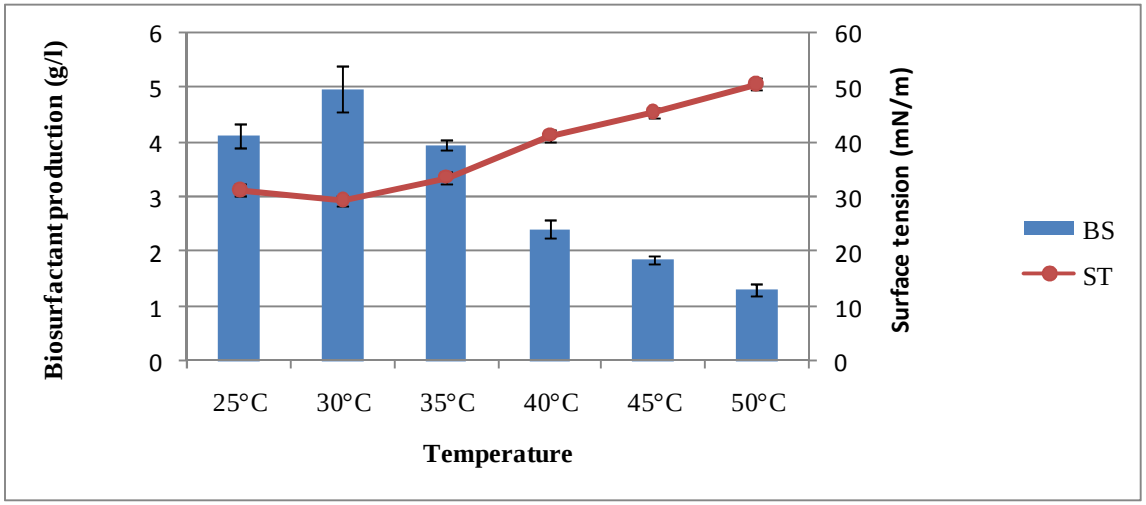
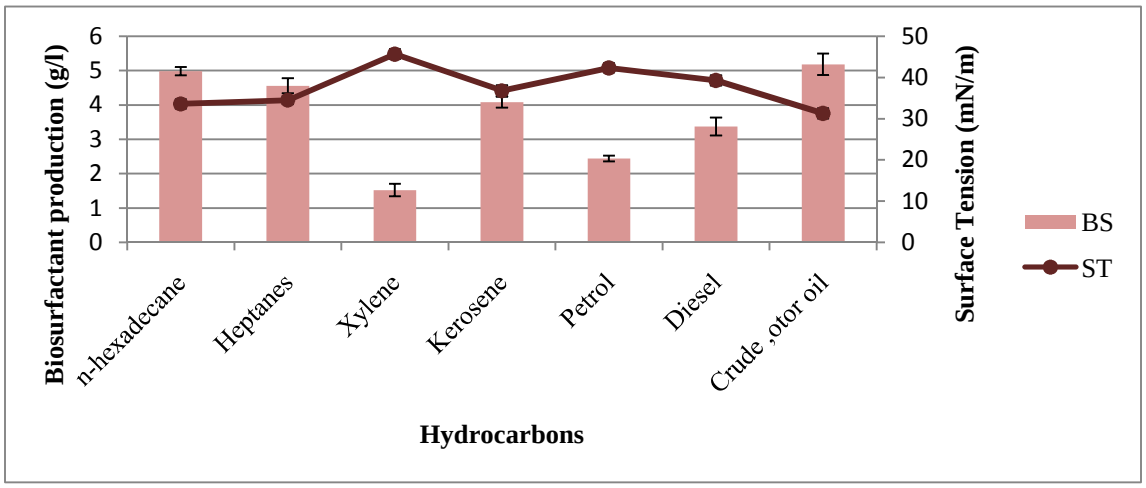


Fig. 11: Effect of different hydrocarbons on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1



The highest emulsification activity was measured as 78.98 per cent, for the isolate *B. cereus* KBSB1. Temperature is one of the critical parameter that greatly affected the culture growth and the biosurfactant production.

Effect of hydrocarbons

In the different hydrocarbons tested the crude motor oil significantly influenced dry cell biomass 5.18 g/l, surface reduction ability 31.25 mN/m of the isolate *B. cereus* KBSB1 (Table 5, Fig. 11). Petrol and Xylene have not enhanced the dry cell biomass. To improve biosurfactants production yield, different oils and hydrocarbons were added and these have a significant role in the production of biosurfactant and increase the yield parameters. The highest emulsification activity was found to be 75.86 per cent followed by 72.42 per cent for the crude oil and n-hexadecane respectively.

Effect of trace elements

The trace elements in the medium also influence the biosurfactant production level. The maximum biosurfactant production was observed in the control that means in the presence of all three trace elements. The medium without each trace elements produce on par results statistically (Table 6, Fig. 12). The highest biosurfactant production recorded for the isolate *B. cereus* KBSB1 was 5.42 g/l, the surface tension was 30.13 mN/m. Due to the ability of biosurfactants to degrade aromatic compounds, addition of hydrocarbons into the culture medium-enhanced biosurfactants production for the isolates.

Discussion

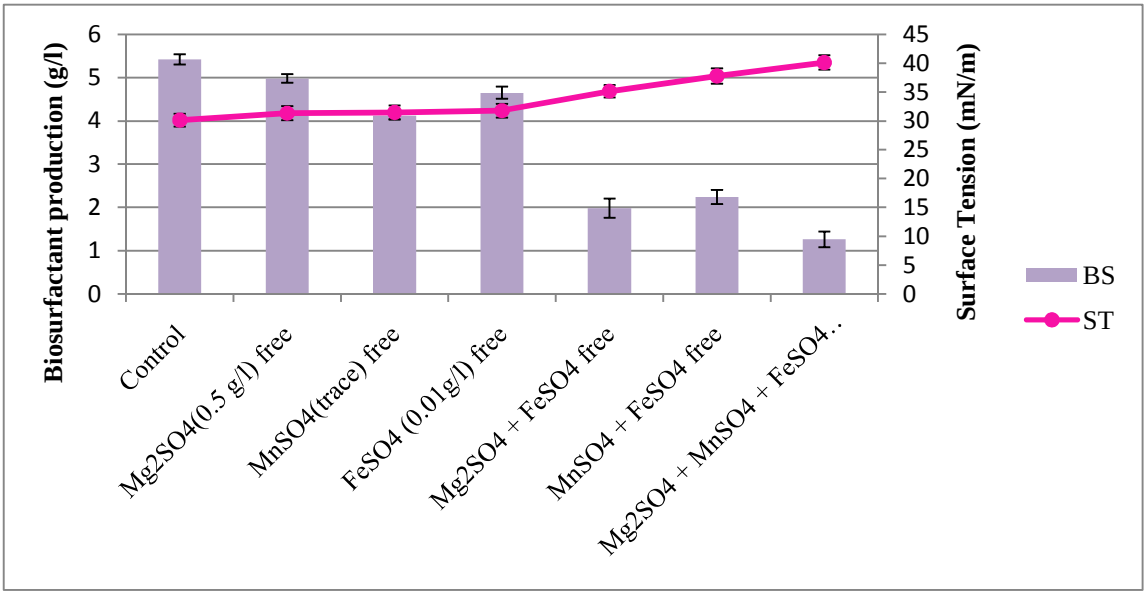
Ainon *et al*. (2013) isolated 20 isolates and screened them for the biosurfactant production. Among that, only *Pseudomonas aeruginosa* uKMP14t showed a positive result in the drop-collapse test. The other five isolates showed positive result in the microplate analysis while nine isolates were detected positive by the oil-spreading technique. These results suggested that the oil-spreading technique is more sensitive than the other methods for biosurfactant detection in the supernatant from a culture medium (Ainon *et al*., 2013). According to Youssef *et al*., (2004), the drop-collapse method is not as sensitive as the oil-spreading technique in detecting low levels of biosurfactant production. Similarly, microplate analysis was unable to detect the presence of surfactant at low levels. Bodour and Miller-Maier (1998) reported that the quantitative analysis including emulsification index (% E_{24}) and surface tension measurement was found to be a more reliable method for quantification of the soluble biosurfactant. The isolate selected was able ro reduce the surface tension below 40 mN/m and maintained at least 50% of the original emulsion volume 24 h after formation of emulsification (Willumsen and Karlson 1997).

Satpute *et al.* (2008) suggested that single screening method is not suitable to identify all types of biosurfactants and recommended more than one screening methods should be included in the primary screening as to identify potential biosurfactant producers. Therefore, in this study, drop collapsing test, oil displacement test and emulsification activity were used to screen the biosurfactant producer.

Biosurfactants are products of microbial metabolism and there-fore it is possible to produce a commercially viable biosurfactant using low-cost raw materials or high-pollutant wastes. Biosurfactant production using molasses, or whey as a carbon source under shaking mode has already been reported (Dubey and Juwarkar, 2001).

Acrylamide 3% (w/w) as nitrogen source increased the production of biosurfactant significantly followed by beef extract as alternate nitrogen source. This report would be the first on the use of acrylamide as nitrogen source. Acrylamide, a neurotoxic monomer with extensive industrial applications was found to be degraded by the microorganisms present in a tropical garden soil. A bacterium capable of degrading acrylamide was isolated from this soil by enrichment (Shanker *et al*., 1990). Acrylamide was used a nitrogen source to stimulate methanogenesis (Haveroen *et al*., 2005) and as organic nitrogen source for soil microorganisms (Kay-Shoemaker *et al*., 1998).

Fig. 12: Effect of different trace elements on the production of biosurfactant (g/l) and surface tension of *Bacillus cereus* KBSB1



The strain *B. aureum* MSA13 utilized up to 20% of FeSO4 and fur-ther increase in the metal concentration decrease the activity. Such high concentration of metal has never been reported for the production of biosurfactant. Wei and Chu (1998) recommended raising iron concentrations from the micromolar to the millimolar level to greatly enhance the surfactin production from *B. subtilis* ATCC 21332. Increasing the magnesium concentration to 20% inhibits the biosurfactant activity but trace amount of magnesium is required for biosurfactant production.

A large number of cyclic lipopeptide including decapeptide antibiotics (gramicidine) and lipopeptide antibiotics (polymyxins) produced by *Bacillus brevis* (Marahiel *et al*., 1977) possess remarkable surface active properties. The cyclic lipopeptide surfactin produced by *B. subtilis* is one of the most powerful biosurfactant known today. It is composed of a seven amino acids ring structure coupled to one molecule of 3-hydroxy-13, methyl-tetradecanoic acid. The lipopeptide biosurfactant are most prominently known as surfactin produced mostly by the *Bacillus* sp. Despite similar global structures, surfactins, iturins and fengycins differ in some aspects regarding their biological activities.

For instance, iturins and fengycins display a strong antifungal activity, while surfactins are not fungitoxic by themselves. Surfactins show weak antibacterial properties but iturins may be strongly inhibitory to the growth of some gram-positive bacteria. The lipopeptide biosurfactant produced by *B. aureum* MSA13 was a broad-spectrum antimicrobial compound. Surfactins, but not iturins, are involved in biofilm formation by *B. subtilis* cells, suggesting specific surface and membrane-active properties for these lipopeptides (Hofemeister *et al*., 2004). The biofilm producer primarily initiates biofilm at the gap between air and liquid interfaces. Considering the action of biosurfactants in air–liquid interfaces, the lipopeptide biosurfactant produced by the strain *B. aureum* MSA13 might have a functional role on its biofilm formation and stability. Biosurfactant production by *Brevibacterium* sp. isolated from oil contaminated soil and its effect on various carbon sources are reported previously (Samadi *et al*., 2007).

Biosurfactants are promising environmental molecules for bioremediation purposes; therefore, the stability of biosurfactants at high temperature, salinities, and over a wide range of pH was studied.

One of the significant findings of this study was the thermo stability of biosurfactant produced by *B. aureum* MSA13. The biosurfactant produced by *B. aureum* MSA13 was stable even at autoclaving. Such extreme stability was reported by Abdel-Mawgoud *et al*. (2008) for the *P. aeruginosa* strain.

It was found that the biosurfactant produced by the marine actinobacterium was stable at high NaCl (up to 5% NaCl). Chemical surfactants, however, are deactivated by 2–3% salt concentration (Bognolo, 1999). In our study also the biosurfactant was able to be stable at various pH, temperature and NaCl concentration. There was a slight change in the biosurfactant activity with the higher NaCl concentration (5%).

Development of yellow colour positive bands confirmed the presence of lipids in lipid chromatography, but Rf values of bands varied with each sample due to the linkage of peptides with varying length of fatty acids. Presence of amino acids valine, isoleucine, leucine and asparagine were confirmed using ninhydrin in TLC plates and further by development of colour in Lowry et al. assay. Plipastatin is a lipopeptides biosurfactant produced by *B. cereus* (Nishikiori *et al.,* 1986).

The carbon source was found to affect the cell mass to a great extent. As the biosurfactant is cell-wall associated, high cell density is desirable (Bicca *et al*., 1999). Hydrocarbons added to the fermentation medium are known to induce the production of biosurfactant (Bento and Gaylarde, 1996).

Several medium components influenced the formation of biosurfactant by the cells. One of the goals of this investigation was use the cheap materials for production, so we studied different commercial oils as a carbon source instead of n-hexadecan, olive oil was considered as the best carbon source based on surface tension not on weight; also different concentrations of olive oil were studied and found that 30 ml/l of olive oil was designed as the best concentration for biosurfactant production. Biosurfactant production has been demonstrated in the presence of water-soluble substrates, hydrocarbons and oils. The type of surfactant formed when growing on these carbon sources can be influenced (Makkar and Cameotra, 1999; Duvnjak, and Kosaric, 1985; Robert *et al*., 1989). Rhamnolipid concentration of 4.9, 5.4 and 4.8 g/l when sunflower, olive and soybean oils, respectively were used as carbon sources by *Pseudomonas aeruginosa* LB1 (Benincasa *et al*., 2002). A concentration of 11 g/l of rhamnolipids was found when *P. aeruginosa* UW-1 grew in Canola oil (Sim *et al*., 1997) and isolate of *P. aeruginosa* DS10-129 produced 4.3 and 2.9 g/l of rhamnolipids using soybean and safflower oil, respectively (Rahman *et al*., 2002). The carbon source, particularly the carbohydrate, has a major effect on the type of glycolipids formed. Glucose, fructose and sucrose lipids are formed by *Arthrobacter paraffineus* and several species of *Corynebacterium*, *Nocardia* and *Brevibacterium* during growth on the corresponding sugar (Suzuki *et al*., 1974).

NH_4NO_3 in presence of yeast extract was the best nitrogen source and the concentration 0.46 g/l NH4NO3 and 0.2 g/l yeast extract were the best concentration for biosurfactant production. The type of nitrogen present (Whether NH4 +, NO3-, urea or amino acid) influences the biosurfactant produced (Robert *et al*., 1989; Duvnjak *et al*., 1983; Haba *et al*., 2000). Interesting observations relate to the effect of nitrogen limitation that appears to stimulate biosurfactant production and overproduction by some microorganisms (Guerra-Santos *et al*., 1984; Suzuki *et al*., 1974). The nitrogen source in the medium influences the production of biosurfactant (Desai *et al*., 1994). *Arthrobacter paraffineus* showed a preference of ammonium salts and urea as the nitrogen source (Duvnjak *et al*., 1983). Robert *et al*. (1989) while investigating rhamnolipid production by *Pseudomonas* 44Ti on olive oil reported that sodium nitrate was the best nitrogen source. Similar results have been noted for *Pseudomonas aeruginosa* (Ramana *et al*., 1989) and *Candida tropicalis* IIP-4 (Singh *et al*., 1990). Maximum biosurfactant production by *N. amarae* was found after 14 days incubation time.

Conclusion

The isolate *Bacillus cereus* KBSB1 was optimized to produce the biosurfactant in an effective manner. Further the optimization using Response Surface Methodology (RSM) will give better yield of biosurfactant by the isolate.

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