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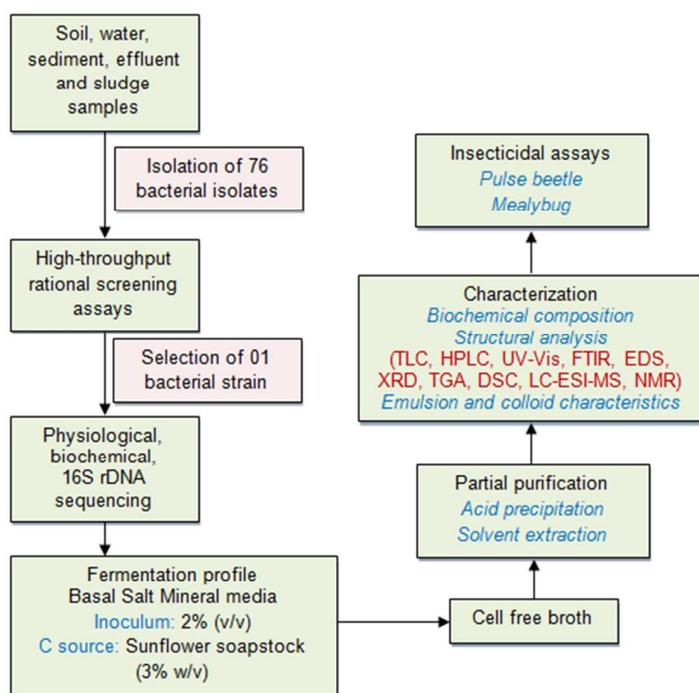
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## Graphical abstract



Production, purification and characterization of lipopeptide biosurfactant (surfactin) from *Bacillus clausii* BS02 for biocontrol activity against pulse beetle and mealybug

Lipopeptide biosurfactant from *Bacillus clausii* BS02 using sunflower oil soapstock: Evaluation of high throughput screening methods, production, purification, characterization and its insecticidal activity

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**Abstract**

A total of 76 bacterial isolates were screened for biosurfactant activity using 16 different high throughput screening methods. Based on phenotypic characteristics, 16S rDNA sequencing and FAME profiling, isolate BS02, identified as *Bacillus clausii* BS02 produced a biosurfactant ( $2.6 \text{ g l}^{-1}$ ) when grown in basal salts mineral medium with sunflower oil soapstock (3% w/v) at  $30^{\circ}\text{C}$  and 120 rpm after 42 h. The cell free broth showed 82% emulsification activity, critical micelle concentration of  $45 \text{ mg l}^{-1}$  and reduced the surface tension from  $69.07 \text{ mN m}^{-1}$  to  $30 \text{ mN m}^{-1}$ . Acid precipitated and methanol extracted crude biosurfactant had 64.3%, 28%, and 4.5% (w/w) of protein, lipid, and carbohydrate, respectively. Crude biosurfactant exhibited stability with oils, fats and non-aqueous phase liquids. Fourier transform infrared (FT-IR) spectroscopy, energy dispersive X-ray spectroscopy (EDX), and nuclear magnetic resonance spectroscopy (NMR) revealed the functional groups and bonds; X-ray diffraction (XRD) and thermogravimetry (TG) showed surface nature and thermostability of the biosurfactant, respectively. LC-ESI-MS identified the crude biosurfactant as a surfactin lipopeptide. A dose-dependent mortality against *C. chinensis* and *M. hirsutus* was observed using the crude biosurfactant, with a  $\text{LC}_{50}$  of  $50 \text{ }\mu\text{g ml}^{-1}$ , indicating first evidence of insecticidal activity against pulse beetle and mealybug.

**Keywords:** *Bacillus clausii*; Biosurfactant; Insecticidal

## 1. Introduction

Microbial biosurfactants, a heterogeneous group of amphiphilic surface-active compounds produced by bacteria, yeast and filamentous fungus, has received renewed interest in newer production trends and usage patterns due to low toxicity, high biodegradability, better environmental compatibility, high foaming capability, higher selectivity, specific activity at extreme temperature, pH, salinity, and sustainable production from renewable feedstocks.<sup>1-5</sup> These properties and ecological awareness of end-users have led to an upsurge in application of biosurfactants in dairy, food, beverage, cosmetics, detergent, textile, paint, mining, petroleum, paper/pulp, bioremediation, pharmaceuticals, biomedicine and nanotechnology.<sup>6,7</sup> However, biosurfactants have not yet been commercialized extensively owing to low production yields, high feedstock costs and purification costs and non-availability of non-pathogenic microorganisms.<sup>2,3</sup>

The vegetable oil refineries (~90) in India manufacture oil from soybean, sunflower, olive, groundnut, rapeseed, safflower, cotton, sesame, coconut, palm, mustard, rice bran, watermelon, etc.<sup>8,9</sup> and generate 2-3% (of the total oil production) alkaline (pH 10-12) greasy material (density: 1.7 mg l<sup>-1</sup>), called soapstock and acidic water (pH 2.6).<sup>10</sup> Besides, the volume of wastewater generated is up to 10 l kg<sup>-1</sup> of oil (at a rate of about 5% of the volume of refined crude oil) with chemical oxygen demand (COD) value of 2700 mg l<sup>-1</sup>.<sup>10,11</sup> Thus, the use of such low cost by-products (as low as about 10% of the price for refined oil) can (i) stabilize waste disposal by valorisation or minimization, and (ii) produce biosurfactants economically. Several previous literatures revealed that plant and vegetable oils based soapstock are better substrate for rhamnolipid production by *P. aeruginosa*, accounting for production up to 7.3 g l<sup>-1</sup>,<sup>10</sup> 11.6 g l<sup>-1</sup>,<sup>12</sup> and 15.9 g l<sup>-1</sup>.<sup>13</sup> However, efficient biosurfactant production using soapstock by *Bacillus* spp. has not been reported yet. *B. clausii* can hold a promise for production of biosurfactants since they are recognized as GRAS (Generally

Recognized as Safe) by US FDA and the German Technical Rules for Biological Materials (TRBA).

Hence, the present study attempts to explore high-throughput rational screening methods for isolating an efficient *B. clausii* isolate BS02, followed by the production of lipopeptide biosurfactants using soapstock from the sunflower oil process as the sole carbon source. Subsequently, the crude biosurfactants was partially purified and characterized. Finally, the insecticidal activity of the crude biosurfactants based on vapor toxicity was evaluated against stored-product grain pest, *Callosobruchus chinensis* and plant sap-sucking agricultural insect, *Maconellicoccus hirsutus*.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All the reagents, chemicals and media used in this study were purchased from Hi-Media, Mumbai, India until specified. Methanol and chloroform were purchased from Merck, Mumbai, India. All the reagents, chemicals, and media used in this work were of analytical grade. High quality diesel oil was supplied locally by M/s Khubchand Sagarmal (a Hindustan Petroleum franchisee). Crude/raw glycerol from biodiesel co-product stream and sunflower oil soapstock was a generous gift from Jain Irrigation Systems Ltd., Jalgaon and Rising Flora Biotech Pvt. Ltd., Jalgaon, respectively.

### 2.2. Enrichment, isolation and selection of biosurfactant producers

Terrestrial (soil and water) samples were collected from diverse eco-habitats (geographical coordinates: 20.99°N, 75.56°E) and detailed procedure is described in Supplementary data.

### 2.3. Screening for biosurfactant-producing bacteria using high-throughput rational screening techniques

In order to determine the ability of the isolates to produce biosurfactant, the culture supernatant from each isolate was subjected to preliminary screening methods namely tilted

glass slide test, parafilm M test, drop collapse test, hydrocarbon overlay agar assay, microplate assay, droplet assay, penetration assay, replica plate assay, bacterial adhesion to hydrocarbon (BATH) assay, blood agar hemolysis, CTAB-MB assay, oil spreading assay, emulsification index (EI<sub>24</sub>) assay, surface tension measurement, contact angle measurements, and atomized oil assay. In all the above mentioned experiments, the cell free culture supernatant of the isolates obtained from Section 2.2 were used following the protocols detailed elsewhere.<sup>14-16</sup> Determinations of contact angle are given in Supplementary data. All the experiments were done in triplicates.

#### *2.4. Identification of the selected isolate*

Phenotypic characterization, whole-cell fatty acid Methyl Ester (FAME) profiling, and 16S rDNA sequencing of the selected isolate are described in Supplementary data.

#### *2.5. Fermentative time course of growth and biosurfactant production*

A 2% (v/v) actively growing cell suspension of the selected isolate was used to inoculate in a pre-sterile BSM broth containing soapstock (3% w/v) sourced from a sunflower-oil process. Experiments were conducted, in three independent replicates, in a rotary shaker with 500 ml Erlenmeyer flasks containing 100 ml of medium and incubated at 30 °C and 120 rpm. Sample (10 ml) was drawn at appropriate time intervals and monitored for biosurfactant production and biomass growth.

#### *2.6. Isolation, partial purification and structural characterization of the biosurfactant*

Extracellular crude biosurfactant from a 72 h old culture broth of the isolate was recovered by acidification of the cell free broth and precipitation followed by solvent extraction of the acid precipitate as reported earlier.<sup>17</sup> The methanol extracts were subsequently dried and determined gravimetrically.

Compositional analysis of the crude sample was determined by ninhydrin test (proteins), anthrone assay (carbohydrate) and saponification test (lipids) according to Patowary *et al.*<sup>18</sup> Surface tension, interfacial tension, critical micelle concentration (CMC) and emulsification was determined as reported previously.<sup>19,20</sup> For determining preliminary chemical nature of the biosurfactants, crude sample was spotted on silica gel 60 thin-layer chromatography plates (F<sub>254</sub>, Merck, Darmstadt Germany). After development in chloroform/methanol/water (65:25:4, v/v), lipids were detected with iodine vapors and amino acids/peptides were detected with rhodamine B (250 mg rhodamine B in 100 ml absolute alcohol) and ninhydrin reagent (0.2% ninhydrin solution in acetone). Separation of crude extracts was done using high performance liquid chromatography (HPLC; Agilent 1100 series) equipped with a Zorbax Eclipse column [C<sub>18</sub>, 5  $\mu$ m, 4.6 (ID) $\times$ 250 mm (L)]. The column was run for 15 min with water:acetonitrile (with 0.1% TCA) (42:58) at a flow rate of 1 ml min<sup>-1</sup>. Sample injection volume was 50  $\mu$ l and detection was monitored at 210 nm. UV spectrum of serially diluted (up to 10<sup>-3</sup>) stock (1 mg ml<sup>-1</sup>) of the crude biosurfactant (in methanol) was recorded against methanol as a blank in the range of 190-400 nm. Fourier transform infrared spectroscopy (FT-IR) of the dried crude biosurfactant was done by KBr pellet method.<sup>17</sup> Energy dispersive spectroscopy (EDS) of the drop-coated grids of vacuum-dried samples (0.5% w/v) was carried out using Bruker attachment (Bruker AXS Ltd., Coventry, UK) with FESEM instrument. The results obtained were reproducible with the accuracy of  $\pm$ 5% error. Powder X-ray Diffraction (XRD) patterns were acquired on a Bruker D8 Advance instrument using Cu K $\alpha$  radiation ( $\lambda = 1.5418 \text{ \AA}$ ). A scanning rate of 0.05 $^\circ \text{ s}^{-1}$  over a wide range of the Bragg angles ( $\theta$ , 20 $^\circ \leq 2\theta \leq 80^\circ$ ) was used. For thermogravimetry (TGA-50, Shimadzu, Tokyo, Japan) and differential scanning calorimetry (DSC-60, Shimadzu, Tokyo, Japan), about 10 mg of dried crude samples were sealed in an Al<sub>2</sub>O<sub>3</sub> crucible with a lid and scanned at a rate of 10  $^\circ\text{C min}^{-1}$  under N<sub>2</sub> gas atmosphere (50 ml min<sup>-1</sup>

<sup>1</sup>) over a temperature range of 30-300°C. The LC-ESI-MS was recorded on a HCT-Ultra ETD II ion trap mass spectrometer (Bruker Daltonics, Germany) coupled to an Agilent 1100 HPLC system. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of the crude samples (dissolved in CDCl<sub>3</sub> at 50 mg ml<sup>-1</sup>) were collected on a Bruker Avance 300 MHz at room temperature with tetramethylsilane (TMS) as an internal standard.

### 2.7. Insecticidal activities of the crude biosurfactant

The insecticidal activity of the crude biosurfactant was measured by contact toxicity bioassay against adults of stored-product pest insects, the pulse beetle [*Callosobruchus chinensis* L. (Coleoptera: Bruchidae)] and the mealybug [*Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae)]. Rearing of *C. chinensis* was conducted in plastic boxes (15× 22×10 cm) containing mung beans (*Vigna radiata* L.) under a photoperiod of 15:9 (L:D) at 25±1°C and 65±5% relative humidity at Biopesticide Laboratory, School of Life Sciences.<sup>21</sup> *M. hirsutus* collected from an infested *Hibiscus rosa-sinensis* L. plant in the N.M.U. campus, Jalgaon were reared on the same host plant as per Patil *et al.*<sup>22</sup> For insecticidal activity against adults of *C. chinensis*, different concentrations of crude biosurfactant (dissolved in methanol) were sprayed on filter papers (Whatman No. 2, 5 cm diameter). For *M. Hirsutus*, test samples were applied to a small cotton ball attached to the lower end of petiole of healthy leaves of *H. rosa-sinensis* and air dried. Controls received 500 µl methanol. Each filter paper and leaf was placed in the bottom of a glass Petri plate. Ten test adult insects (1-7 days old) irrespective of sexes were placed on the filter paper or on the leaves in each dish and dish lids replaced. Mortality counts were recorded after every 12 h of exposure at 28±2 °C and relative humidity of 65-70%. Three replicates were run for each treatment and each experiment was repeated thrice on different days to avoid day-to-day variations.

### 2.8. Statistical analysis

Data are expressed as means  $\pm$  S.D from at least three independent experiments and were analyzed using Origin 6.1 statistical software (OriginLab Corporation, Northampton, MA, USA). The values of  $p < 0.05$  were considered statistically significant.

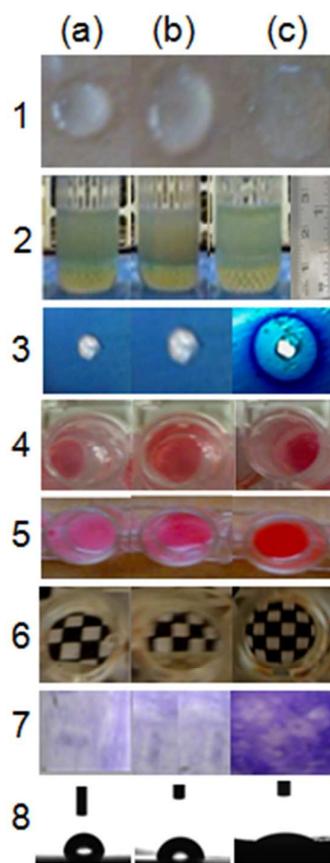
### 3. Results and discussion

#### *3.1. Enrichment, isolation and assessment of different high-throughput rational screening techniques for selecting biosurfactant producing isolates*

A total of 76 morphologically distinct bacterial isolates, which could grow in BSM supplemented with diesel and crude glycerol (2%, v/v) as sole carbon source were obtained from nine different samples of soil, water, sediment, effluent and sludge (Table S1; Supplementary data). Of these, 63% of the bacterial isolates were Gram negative. Surprisingly, no filamentous fungi or yeast were found even after three successive enrichments. It may be due to minimal nutritional ingredients [BSM plus diesel and crude glycerol (2%, v/v)] unsupportive for fungal or yeast growth. Among 76 bacterial strains, 54% isolates exhibited biosurfactant activity in the hemolytic assay (Table S2; Supplementary data). Of these, 13 bacteria showed  $\alpha$ -hemolysis while the rest were  $\beta$ -hemolytic. The cell-free culture supernatant of isolate BS01 and BS02 showed distinct and clear hemolysis halos of 2.8 and 2.4 cm, respectively, on blood-agar. Moreover, the diameter of clear zone linearly increased with the concentration of biosurfactant in culture supernatant over a concentration range for the isolate BS01 and BS02 with relatively high  $R^2$  values (Fig. S1a; Supplementary data). Of the 35 nonhemolytic strains, 23 isolates were able to produce biosurfactant both by oil spreading technique and drop collapse method, and 12 other strains showed biosurfactant activity by the oil spreading technique (Table S3; Supplementary data). On the other hand, out of the 41 positive results obtained with the blood agar method, 16 and 17 gave negative results for biosurfactant production with oil spreading technique and microplate assay, respectively. Thus, the blood agar lysis gave a high percentage of both false positives and

negatives. In drop collapse assay, 54 (71%) strains were positive (Table S2; Supplementary data). Of 22 isolates tested negative by this method (Table S3; Supplementary data), 8 isolates were also negative for biosurfactant production by oil spreading technique. The other 14 strains were positive for biosurfactant production by the oil spreading technique with diameters of clearing on the oil surface ranging from 0.5 to 0.9 cm, which suggests low levels of biosurfactant concentration (50-63 mg l<sup>-1</sup>) as per Youssef *et al.*<sup>14</sup> Consistent with this, surface tensions of seven of these strains were high ranging from 55 to 62 mN m<sup>-1</sup>. Some isolates that were negative for drop collapse activity, but they were all positive for hemolytic and BATH assays. From oil spreading assay, the following general trends could be observed: (i) isolates found with positive drop collapse results were positive for oil spreading assay also; and (ii) the maximum wide clear zone was observed on the oil-water interface (average halo diameter of 4.23 cm to 4.58 cm, respectively) for the isolate BS01 and BS02, respectively and the diameter of the clear zone linearly increased with the concentration of biosurfactant in culture supernatant over a concentration range (Fig. S1b; Supplementary data). When the degree of oil spreading of 76 isolates was compared to the surface tension of the culture, an inverse linear relationship between the diameter of the clear zone and the surface tension of the culture was obtained (Fig. S1c; Supplementary data) with a poor correlation coefficient. Wettability alteration measured by contact angle for the isolate BS01 and BS02 were 76° and 75°, respectively, indicating that biosurfactant changed the wettability toward more water-wet, which could be desirable for improving oil recovery at field scale applications. Although the contact angle data correlated well with surface tension measurements, high surface activity of a biosurfactant did not necessarily suggest high emulsifying ability. Out of 76 isolates, 60 and 35 bacteria tested negative for biosurfactant activity with CTAB-MB and blood agar assay, respectively. This integrates the screening procedure and indicates the probable production of anionic surfactant. Table S4

(Supplementary data) shows the coefficient of correlation between the best five methods (giving maximum no. of positives) that were used to detect biosurfactant production by two different statistical analyses.<sup>14</sup> Oil spreading and drop collapse method were strongly correlated with Spearman rank correlation coefficient of  $r_s = 0.91$ . However, a weak correlation was detected between drop collapse and blood agar lysis method ( $r_s = 0.541$ ) and between oil spreading and blood agar lysis method ( $r_s = 0.453$ ). The statistical test of independence (2×2 contingency table) also indicated similar conclusions about the relationships of the five methods in accordance with Youssef *et al.*<sup>14</sup>



**Fig. 1.** Preliminary screening for detecting biosurfactant activity: (a) Milli-Q water, (b) uninoculated BSM media (with crude glycerol; 2%, v/v), and (c), cell-free culture supernatant from 24 h grown culture of BS02. 1, Parafilm M test; 2, EI<sub>24</sub> assay against kerosene; 3,

CTAB-MB assay; 4, droplet assay; 5, penetration assay; 6, microplate assay; 7, replica plate assay; 8, water contact angle measurement. All experiments were performed in triplicates.

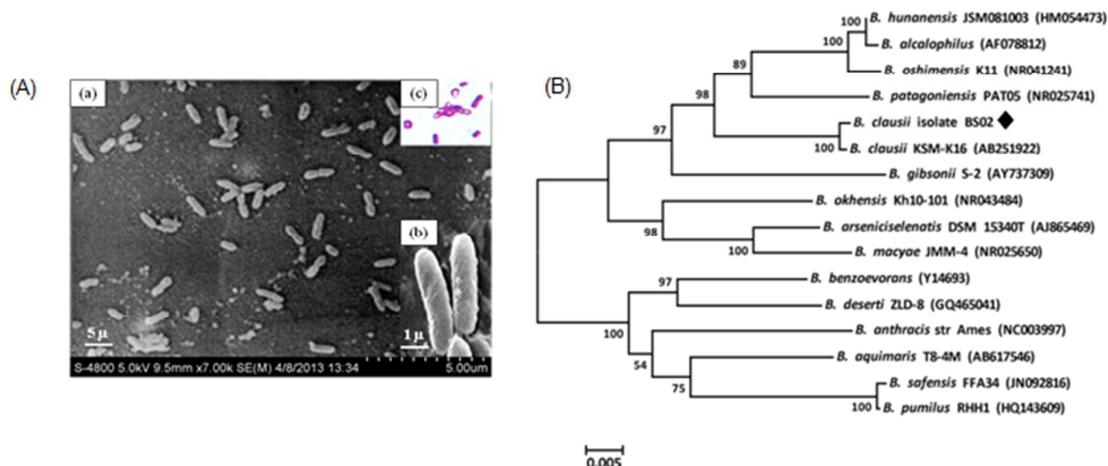
From the data of Table S2, S3, S4 and Fig. 1, isolate BS02 was selected for subsequent experiments as it showed (i) distinct and clear hemolysis halos (2.4 cm) on blood-agar; (ii) a wide clear zone (average halo diameter of 4.58 cm) on the oil-water interface in OSA; (iii) coalescence over oil covered glass slide in drop collapse test; (iv) highest reduction of surface tension (from 71.14 to 30.06 mN m<sup>-1</sup>) as compared to other isolates; and (iv) lowest water contact angle (75°).

### 3.2. Taxonomic affiliation of the biosurfactant producing bacterial strain BS02

Colonies of isolate BS02 were non-pigmented, cream-white, circular, irregular, rough, glistening, erose to lobate, and either slightly raised or low convex. The cells appeared to have translucent, spreading edges with age. The growing cells (on BSM agar supplemented with diesel and/or crude glycerol (2%, v/v) at 30°C for 48 h) were Gram positive, facultatively aerobic, motile, sporulating, and rod-shaped (0.8-1.0 µm × 2.0-2.5 µm) (Fig. 2A) that grew at pH 6.5-10.0 with an optimal pH of 7.0-8.0, and at 10-41°C with an optimal temperature of 30°C. The isolate showed salt tolerance up to 0.2 M NaCl (10% w/v) at pH 9, indicating obligate alkalophile nature. The phenotypic (Table S5; Supplementary data) and FAME profile (Table S6; Supplementary data) of isolate BS02 were similar with those of closely related type strains described in the literature, suggesting affiliation to the *Bacillus* genus.<sup>23-25</sup>

For phylogenetic analysis, the almost complete 1,422-bp-long 16S rRNA gene was PCR-amplified by using bacterial universal primers, sequenced, and deposited in Gen-Bank under accession number JQ229779. Pairwise sequence analysis and the phylogenetic tree constructed using the neighbour-joining method suggested that strain BS02 is a member of

the genus *Bacillus* and formed a clade with *Bacillus clausii* KSM K-16 (AB251922) at 100% bootstrap value (Fig. 2B).

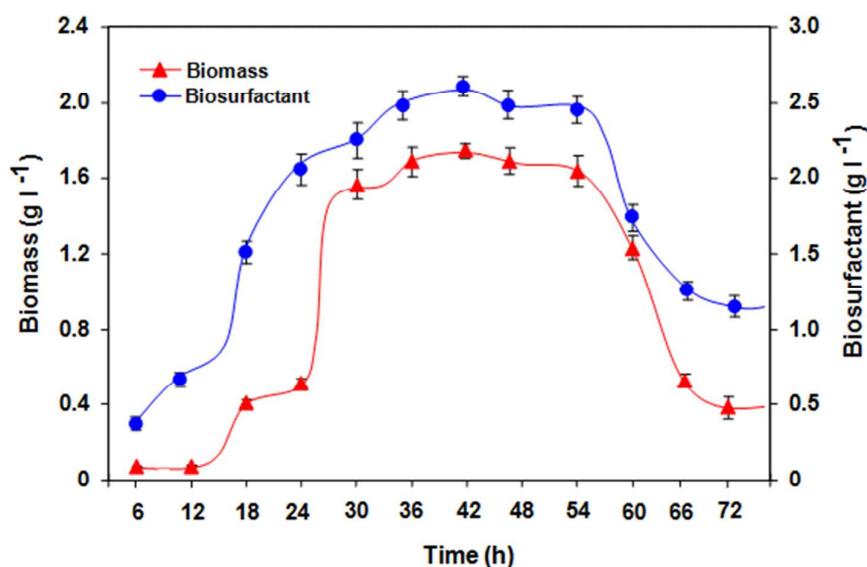


**Fig. 2.** (A) FESEM photomicrographs of strain BS02 at (a) 7.0k x magnification and (b) 20.0k x. Snap (c) represent Gram staining of BS02 at 100 x. (B) Evolutionary relationships of strain BS02 with its relatives inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Figures next to the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances are in the units of the number of base substitutions per site. Text in the parenthesis is GenBank accession numbers.

### 3.3. Growth and biosurfactant production profile

The fermentative time-course profile of biosurfactant production by isolate BS02 as a function of time (Fig. 3) depicted that it was detected in the early logarithmic phase of bacterial growth at 24 h. The microbial growth was characterized by a transient lag phase for 18 h followed by an exponential growth phase, which was complete after 54 h of incubation. The biosurfactant production reached to its maximum of 2.6 g l<sup>-1</sup> after 42 h of incubation with the  $Y_{P/X}$  (product yield related to biomass formed) of 1.625 g g<sup>-1</sup> and the  $P_V$  (volumetric productivity) of 0.036 g l<sup>-1</sup> h<sup>-1</sup>. A noticeable decrease in surface tension of the broth culture

(from  $69.07 \text{ mN m}^{-1}$  to  $30 \text{ mN m}^{-1}$ ) during the exponential growth phase indicated a rationally parallel relationship between biosurfactant production and surface tension reduction (Table S7; Supplementary data). The reduction in surface tension remained constant until 72 h, when the culture attained the stationary growth phase. Interestingly, a decrease in biosurfactant production was observed after 54 h. It has been reported earlier that in batch fermentation, biosurfactant could be utilized as substrate by the bacteria when the original source of substrate is limited.<sup>26</sup> This would explain the sudden drop in the productivity as the isolate approached stationary phase. These findings indicate two aspects: firstly, the biosurfactant biosynthesis occurred predominantly during exponential growth phase, suggesting that (i) the biosurfactant is produced as primary metabolite accompanying the formation of cellular biomass and (ii) the production profile exhibited growth-associated kinetics.<sup>26,27</sup>; secondly, biosurfactant production in the presence of soapstock as a sole carbon source may alleviate the growing environmental burden associated with by-product of the vegetable oil processing industry.



**Fig. 3.** Time course profile of growth of the strain BS02 on BSM medium supplied with sunflower soapstock (3%, w/v) as the sole carbon source accompanied by biosurfactant

production. All values are mean±S.D of triplicates. Small (non-visible) standard deviations are within the symbols. Other process parameters: pH, 8.5; temperature, 30°C; agitation speed, 120 rpm.

The pH of the culture medium decreased from an initial value of 11.1±0.1 to a maximum of 9.3±0.15 after 48 h of growth, and remained constant thereafter during the subsequent fermentation period. The ability of the isolate to tolerate such alkaline pH during biosurfactant production using soapstock is noteworthy. Earlier literature reports showed that the pH increased from (i) 6.8 to 7.7 for *B. subtilis* in mineral medium containing purified glycerol,<sup>28</sup> (ii) 5.4 to 7.6 for *B. subtilis* LB5a grown on mineral medium containing cassava wastewater,<sup>29</sup> and (iii) 6.9 to 8.0 for *B. subtilis* LSFM-05 cultivated on minimal medium containing raw glycerol.<sup>30</sup>

#### 3.4. Partial recovery of the crude biosurfactant

The literature shows different methods for biosurfactant purification, which are dependent on various properties such as acid precipitation, cold acetone extraction, electro-coagulation/flotation, solid phase extraction etc. About 2.5 g l<sup>-1</sup> of crude biosurfactants as white solid was isolated from acid precipitated and ethyl acetate extracted 1 l culture of *B. clausii* BS02.

Several strategies have been developed to optimize the production and recovery of purified surfactin using diverse carbon sources, media ingredients, bioreactor design and downstream processes.<sup>16</sup> The objective of this work was not to optimize the biosurfactant production but to use the partially purified lipopeptidic compounds as a source of biocontrol agent. Nevertheless, the production yield obtained here is in agreement with the ranges previously reported for surfactin in suboptimal conditions (Table S8 in Supplementary data). The literature data suggests that *B. subtilis* ATCC 21332 is a reference strain and it is usually known as a standard surfactin producer in the range of 109.5 to 800.0 mg l<sup>-1</sup> depending on the

culture medium.<sup>31-33</sup> Based on the information summarized in Table S8, the production of surfactin reported here is lower than previous studies using chemically defined optimized media or using solid-state fermentation. However, it is comparably higher than the earlier literature results obtained with low-cost wastes such as crude oil, biodiesel glycerol, squid pen powder, date molasses, okara and sugarcane bagasse, cashew apple juice, beer wastewater etc. Since one of the motivations of this work was to reduce the costs of producing lipopeptides, the provision of mechanically complex bioreactor designs and labor intensive substrate handling was not considered like other researchers.<sup>34</sup> Notably, this is the first report on the use of sunflower oil soapstock as the sole carbon source for surfactin production by *B. clausii*. This may pave a way for commercial production and bulk applications of surfactin for phytopharmaceutical products since low-cost substrates accounts for 50% of the overall production costs.<sup>35</sup>

### 3.5. Characterization of the crude biosurfactant

#### 3.5.1. Physicochemical characterization

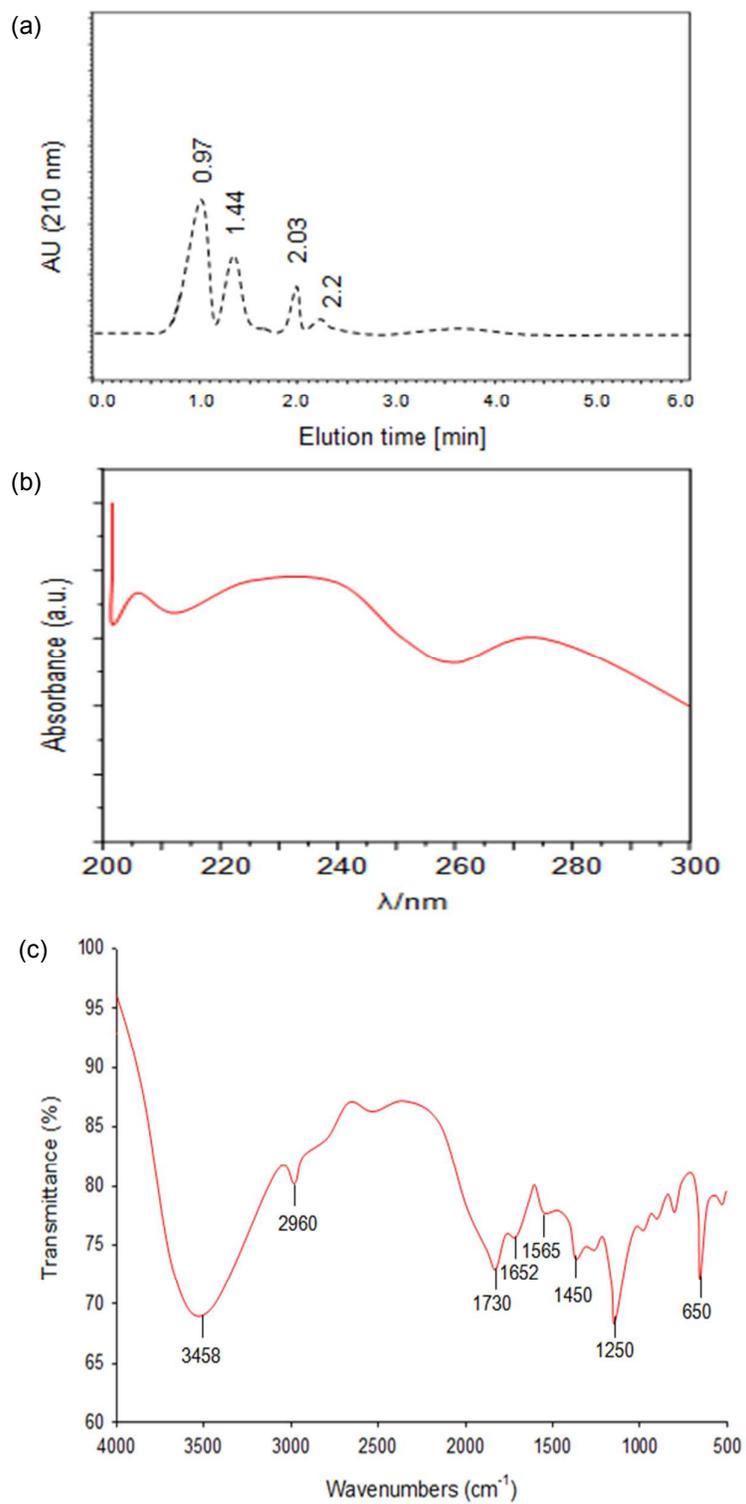
Unless otherwise stated, all the experiments were performed with the crude biosurfactant displaying maximum surface-active properties. Compositional analyses revealed that the biosurfactant produced by *B. clausii* BS02 might be a lipoprotein primarily consisting of protein with a relative percent of 64.3% (w/w) and 28% (w/w) of lipid. A minor fraction of carbohydrate (4.5%, w/w) might have arisen from the existence of the residual cell debris in broth co-precipitated with biosurfactant during the extraction process. The physicochemical and biochemical properties of the isolated biosurfactant are shown in Table S9 (Supplementary data). The biosurfactant formed stable emulsions with different non-aqueous phase liquids used, confirming its usefulness in applications such as soils remediation and household cleaning systems, among others. The efficiency of this biosurfactant in reducing the surface and interfacial tension between water and various hydrophobic substances makes

it a potential candidate in microbial enhanced oil recovery (MEOR). Similarly, it is anticipated that the biosurfactant from this strain could be used for solubilizing high fat and oil prevalent in dairy industry wastewater.

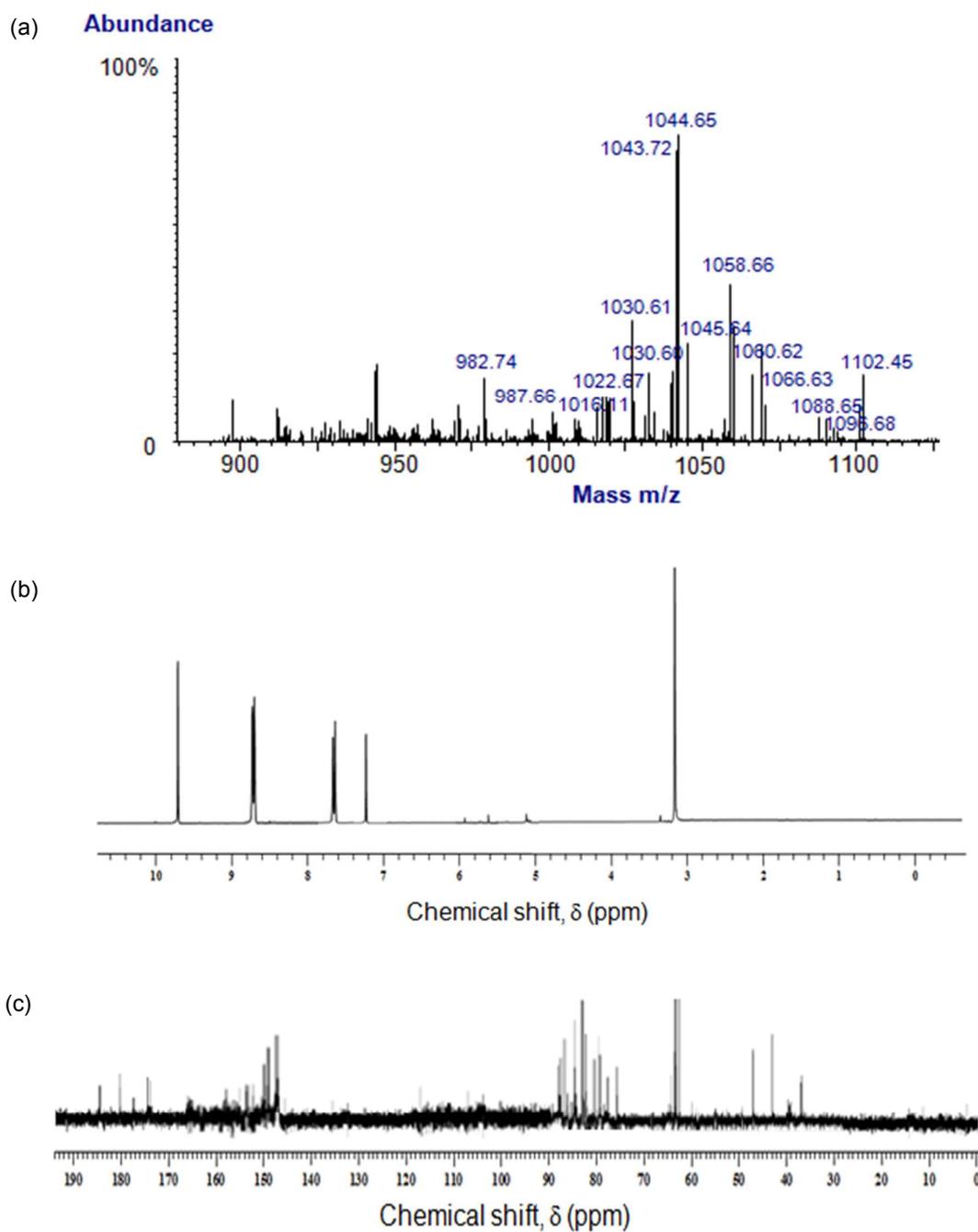
### 3.5.2. Structural characterization

The biosurfactant purified from the culture supernatant revealed one major yellow spot in TLC after spraying with saturated iodine vapors denoting the presence of lipids. Upon rhodamine-B solution treatment, the same spots showed fluorescence under UV confirming the presence of lipids. Violet spots were developed at the same locations after treatment with 0.2% ninhydrin solution and subsequent heating at 120°C showing presumptive presence of amino acids. These results thereby indicated that the surface-active compounds were lipopeptide in nature. Fractionation of crude biosurfactants from strain BS02 by HPLC on a C<sub>18</sub> column resolved them into four biosurfactant fractions eluting approximately at retention times of 0.97, 1.44, 2.03 and 2.2 min (Fig. 4a). These results are similar to those of previous reports.<sup>36,37</sup> The UV spectrum of the crude biosurfactant showed that the maximum wavelength area of absorption spectra was 200-240 nm due to n-σ\* and/or π-π\* transitions, which characterizes functional groups like amine, carboxyl, carbonyl and ester<sup>38</sup> (Fig. 4b). The broadness of this peak might be due to overlap with another maximum at 237 nm characteristic of lipopeptide biosurfactants.<sup>39</sup> Moreover, a shoulder peak was observed around 260-280 nm which is commonly attributed to π-π\* electron transitions in aromatic and poly-aromatic compounds found in most conjugated molecules like proteins.<sup>38</sup> The FTIR spectrum (Fig. 4c) showed a broad stretching peak at 3458 cm<sup>-1</sup> resulting from the -N(CH<sub>3</sub>)<sub>2</sub> group. The presence of an aliphatic chain was represented by the C-H stretching modes at 2854-2960 cm<sup>-1</sup> and 1450-1380 cm<sup>-1</sup>, whilst ν = 1730 cm<sup>-1</sup> reflected lactone carbonyl absorption. At 1652 cm<sup>-1</sup>, the stretching mode of a CO-N bond was observed and 1559-1565 cm<sup>-1</sup> were attributed to the deformation mode of the NH bond combined with C-N stretching mode.

These results infer that biosurfactant produced contains aliphatic hydrocarbons as well as a peptide like moiety.<sup>37</sup> The adsorption peaks at 1250 and 650  $\text{cm}^{-1}$  could be attributed to the presence of sulphate group as S-O and C-O-S.<sup>26</sup> Presence of sulphur was further confirmed by the elemental analysis of biosurfactant using EDX. The qualitative elemental analysis performed by SEM-EDX (Fig. S2; Supplementary data) revealed the weight and atomic percentage of different elements present (C, N, O, Na, S, and Ca) in the partially purified biosurfactant. The presence of sulphate and carboxyl functional groups in the biosurfactant indicated its anionic character. In addition, the distribution of cations such as Na, and Ca in the biosurfactant suggested their binding to the negative charges of the sulfate groups resulted in the greater availability of these elements to microbes essential for their survival and growth.<sup>38</sup> The XRD profile exhibited maximum diffraction peaks at *ca.* 28°, 30°, 32° and 49.5° (Fig. S3; Supplementary data). The results obtained are in agreement with previous studies.<sup>40</sup> Powder XRD pattern revealed that the biosurfactant is amorphous with crystallinity index ( $CI_{\text{xrd}}$ ) = 0.417. Dynamic thermogravimetry of the lipoprotein (Fig. S4a; Supplementary data) revealed an initial weight loss (7.64%) between 30 and 140°C was attributed to loss of trapped moisture within the molecules. This is followed by a drastic weight loss of 64.83% in the second phase of degradation at around 250-300°C, which may be attributed to the decomposition of the labile component in the biosurfactant. Finally, non-labile component was decomposed and the complete loss of the biosurfactant was detected at 400°C. The DSC thermogram (Fig. S4b; Supplementary data) over the temperature range of 30-300°C showed crystallization temperature ( $T_c$ ) of 100.5°C (onset temperature 96.77°C). The melting peaks were found at 225°C (onset temperature 217.43°C) for  $T_{m1}$  and 275°C (onset temperature 269.45°C) for  $T_{m2}$ . In consistence with XRD, approximately equal crystalline index ( $CI_{\text{DSC}} = 0.41$ ) was observed for the lipoprotein biosurfactant from DSC



**Fig. 4.** (a) RP-HPLC of isolated crude biosurfactant from *B. clausii* BS02. Fractionation was done as described in text. (b) UV-visible and (c) FTIR spectrum of the crude biosurfactant.



**Fig. 5.** (a) LC-ESI-MS, (b)  $^1\text{H}$  NMR spectrum and (c)  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$  at  $25\text{ }^\circ\text{C}$ .) of crude biosurfactant from *B. clausii* BS02.

thermogram and little variation may be due to uncertainties in placing base line for area integration.

The ESI mass spectra of the purified biosurfactant showed that there were well-resolved group of peaks at  $m/z$  values between 1000 and 1100 (Fig. 5a) which may be attributed to the different variants of surfactins, as previously described.<sup>30,41</sup> Each surfactin homolog or isoforms can be attributed to the protonated forms, with sodium adducts. The ions of greatest abundances in the sample were those of  $[M+Na]^+$  peaks at  $m/z = 1022, 1030, 1044, 1058$  and it correspond to the heptapeptide moiety (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) characteristic of surfactins, linked to a  $C_{13}, C_{14}$  or  $C_{15}$  hydroxy fatty acid chain, respectively.<sup>41</sup> The  $^1H$  NMR ( $CDCl_3$ , 300 MHz) spectrum revealed the presence of aldehyde proton at  $\delta = 9.6$  ppm; aromatic protons at  $\delta = 7.22-7.68$  ppm; a peptide backbone (N-H at 8.0-7.2 ppm) and *N*-methyl protons at  $\delta = 3.2$  ppm (Fig. 5b). The chemical shift at 5.2 ppm detected an ester carbonyl group indicating the presence of a lactone ring in the structure of the biosurfactant. From the  $^{13}C$  NMR ( $CDCl_3$ , 75.5 MHz) spectrum (Fig. 5c), *N*-methyl carbon signals was detected at  $\delta = 39.98$  ppm, aromatic ring carbon (attached with the carbonyl group) at  $\delta = 154$  ppm, and carbonyl signals of the aldehyde group at  $\delta = 185$  ppm. A chemical shift at 49.15 ppm indicated a methoxy group attached to a Glu or Asp residue, which is similar with that of the antiadhesin ester and C15-surfactin-O-methyl ester.<sup>42</sup> These results showed close resemblance with previous reports of lipopeptide biosurfactant.<sup>30</sup>

### 3.6. Insecticidal activities of lipoprotein biosurfactant in insect pests

As shown in Table S10 (Supplementary data), the treatment of partially recovered biosurfactant led to a dose-dependent mortality against the adults of *C. chinensis* (L.), reared on mung beans (*Vigna radiata* L.), producing >50% mortality at  $50 \mu g ml^{-1}$  within 48 h and 100% mortality at  $100 \mu g ml^{-1}$  within 36 h. Similarly, in case of *M. Hirsutus*, an exposure period of 36 h was required for 100% mortality at  $100 \mu g ml^{-1}$  with  $LC_{50}$  of  $50 \mu g ml^{-1}$ . The

literature survey on microbial bioinsecticides from Gram positive, spore-forming bacterium *B. subtilis* suggested their (i) mosquito larvicidal and pupicidal activities of cyclic lipopeptides,<sup>43</sup> particularly against *Anopheles culicifacies*, the primary vector of malaria in India; and (ii) larvicidal activities against Mediterranean flour moth (*Ephesia kuehniella*) (Lepidoptera: Pyralidae),<sup>44</sup> *Spodoptera littoralis* (Lepidoptera: Noctuidae),<sup>45</sup> carob moth (*Ectomyelois ceratoniae*)<sup>46</sup> and olive moth (*Prays oleae*).<sup>47</sup> To the best of our knowledge, this is the first report of insecticidal activity of the biosurfactant from *B. clausii* against *C. chinensis* (L.) and *M. hirsutus* adults. Surfactin and its isoforms are cyclic heptalipopeptide class of biosurfactants constituting of both lipophilic and hydrophilic moieties. It is therefore hypothesized that as the insect crawls over the contact surfaces, uptake of biosurfactants may take place through two routes: epicuticular waxes present in the host cuticle, and/or through the intersegmental membranes. This led to leakage or dehydration of the insect cellular components. Nevertheless, the exact mode of action and a field assessment of the insecticidal activity are necessary for the development of formulation of a novel agricultural biopesticide.

#### 4. Conclusions

An efficient biosurfactant-producing and crude-oil emulsifying *B. clausii* BS02, isolated from dairy wastewater is reported using sunflower oil soapstock as the sole carbon and energy source. Putative structural characterization of the crude extract showed it to be mixture of surfactin and its isoforms. The properties of the crude lipopeptide in terms of its CMC, minimum surface tension, interfacial tension, emulsification activity, oil and fat solubilization activity revealed potential applications of the biosurfactant in biotechnological, biomedical and environmental sectors. Being insecticidal to stored-product lepidopteran and plant sap-sucking insect pests, the lipopeptide biosurfactant may be utilized in insect pest management. Further, *B. clausii*, in contrast to other pathogenic biosurfactant-producers, are classified as

GRAS (Generally Recognized as Safe) by US FDA and, therefore, hold potential advantages for their use in industrial scale production and medical applications.

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### References

- 1 E.J. Gudiña, V. Rangarajan, R. Sen, L.R. Rodrigues, *Trends Pharma. Sci.* 2013, **34**, 667-675.
- 2 P. Bharali, S.P. Singh, S. Gogoi, N. Dutta, B.K. Konwar, *RSC Adv.*, 2014, **4**, 38698-38706.
- 3 P. Saranya, S. Swarnalatha, G. Sekaran, *RSC Adv.*, 2014, **4**, 34144-34155.
- 4 A. Khopade, B. Ren, X.-Y. Liu, K. Mahadik, L. Zhang, C. Kokare, *J. Colloid Interface Sci.* 2012, **367**, 311-318.
- 5 M. Jadhav, S. Kalme, D. Tamboli, S. Govindwar, *J. Basic Microbiol.* 2011, **51**, 385-396.
- 6 S. Shekhar, A. Sundaramanickam, T. Balasubramanian, *Crit. Rev. Environ. Sci. Technol.* 2014, doi: 10.1080/10643389.2014.955631.

- 7 Fakruddin Md, *J. Pet. Environ. Biotechnol.* 2012, **3**, 124. doi:10.4172/2157-7463.1000124.
- 8 A. Daverey, K. Pakshirajan, *Food Res. Int.* 2009, **42**, 499-504.
- 9 R.A. Pandey, P.B. Sanyal, N. Chattopadhyay, S.N. Kaul, *Resour. Conserv. Recy.* 2003, **37**, 101-117.
- 10 M. Benincasa, F.R. Accorsini, *Bioresour. Technol.* 2008, **99**, 3843-3849.
- 11 I.O. Moraes, M. Benincasa, R. Monte Alegre, *Braz. J. Food Technol.* 2002, **5**, 145-149.
- 12 M. Benincasa, A. Abalos, I.O. Moraes, A. Manresa, *Anton. van Leeuw.* 2004, **85**, 1-8.
- 13 M. Benincasa, J. Contiero, A. Manresa, I.O. Moraes, *J. Food Eng.* 2002, **54**, 283-288.
- 14 N.H. Youssef, K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp, M.J. McInerney, *J. Microbiol. Methods* 2004, **56**, 339-347.
- 15 C.-Y. Chen, S.C. Baker, R.C. Darton, *J. Microbiol. Methods* 2007, **70**, 503-510.
- 16 S.K. Satpute, A.G. Banpurkar, P.K. Dhakephalkar, I.M. Banat, B.A. Chopade, *Crit. Rev. Biotechnol.* 2010, **30**, 127-144.
- 17 P. Das, S. Mukherjee, R. Sen, *J. Appl. Microbiol.* 2008, **104**, 1675-1684.
- 18 K. Patowary, R.R. Saikia, M.C. Kalita, S. Deha, *Ann. Microbiol.* 2014, doi:10.1007/s13213-014-0854-7.
- 19 D. Kundu, C. Hazra, N. Dandi, A. Chaudhari, *Biodegradation* 2013, **24**, 775-793.
- 20 C. Hazra, D. Kundu, P. Ghosh, S. Joshi, N. Dandi, A. Chaudhari, *J. Chem. Technol. Biotechnol.* 2011, **86**, 185-198.
- 21 B.K. Salunke, H.M. Kotkar, P.S. Mendki, S.M. Upasani, V.L. Maheshwari, *Crop Prot.* 2005, **24**, 888-893.
- 22 S.V. Patil, C.D. Patil, R.B. Salunkhe, V.L. Maheshwari, B.K. Salunke, *Crop Prot.* 2011, **30**, 1553-1556.

- 23 Y. Nogi, H. Takami, K. Horikoshi, *Int. J. Syst. Evol. Microbiol.* 2005, **55**, 2309-2315.
- 24 N.K. Asha Devi, K. Balakrishnan, R. Gopal, S. Padmavathy, *Curr. Sci.* 2008, **95**, 627-636.
- 25 G. Mouloud, H. Daoud, J. Bassem, I.L. Atef, B. Hani, *Appl. Biochem. Biotechnol.* 2013, **171**, 2186-2200.
- 26 R. Chandankere, J. Yao, M.M.F. Choi, K. Masakorala, Y. Chan, *Biochem. Eng. J.* 2013, **74**, 46-53.
- 27 C.D. Cunha, M. Rosario, A.S. Rosado, S.G.F. Leite, *Process Biochem.* 2004, **39**, 2277-2282.
- 28 F.A.S.L. Reis, E.F.C. Servulo, F.P. de Franca, *Appl. Biochem. Biotechnol.* 2004, **115**, 899-912.
- 29 F.F.C. Barros, A.N. Ponezi, G.M. Pastore, *J. Ind. Microbiol. Biotechnol.* 2008, **35**, 1071-1078.
- 30 A.F. de Faria, D.S. Teodoro-Martinez, G.N.O. Barbosa, B.G.Vaz, I.S. Silva, J.S. Garcia, M.R. Totola, M.N. Eberlin, M. Grossman, O.L. Alves, L.R. Durrant, *Process Biochem.* 2011, **46**, 1951-1957.
- 31 D.G. Cooper, C.R. Macdonald, S.J.B. Duff, N. Kosaric, *Appl. Environ. Microbiol.* 1981, **42**, 408-412.
- 32 F.-C. Hsieh, M.-C. Li, T.-C. Lin, S.-S. Kao, *Curr. Microbiol.* 2004, **49**, 186-191.
- 33 M. Sousa, V. M. M. Melo, S. Rodrigues, H. B. Sant'ana, L. R. B. Goncalves, *Bioprocess Biosyst. Eng.* 2012, **35**, 897-906.
- 34 C.T. Slivinski, E. Mallmann, J. Magali de Araújo, D.A. Mitchell, N. Krieger, *Process Biochem.* 2012, **47**, 1848-1855.
- 35 C. Mulligan, B. Gibbs, In: Kosaric N (Ed.), *Biosurfactants: production, properties, applications.* Marcel Dekker, NY, 1993, pp. 329-371.

- 36 K. Das, A.K. Mukherjee, *Appl. Microbiol. Biotechnol.* 2005, **69**, 192-195.
- 37 S. Joshi, C. Bharucha, A.J. Desai, *Bioresour. Technol.* 2008, **99**, 4603-4608.
- 38 R.P. Singh, M.K. Shukla, A. Mishra, P. Kumari, C.R.K. Reddy, B. Jha, *Carbohydrate Polym.* 2011, **84**, 1019-1026.
- 39 W. Ismail, I. S. Al-Rowaihi, A. A. Al-Humam, R.Y. Hamza, A.M. El Nayal, M. Bououdina, *Int. Biodet. Biodegrad.* 2013, **84**, 168-178.
- 40 R. Chandankere, J. Yao, M. Cai, K. Masakorala, A.K. Jain, M.M.F. Choi, *Fuel* 2014, **122**, 140-148.
- 41 J.F.B. Pereira, E. J. Gudiña, R. Costa, R. Vitorino c, J.A. Teixeira, J.A.P. Coutinho, L.R. Rodrigues, *Fuel* 2013, **111**, 259-268.
- 42 X.-Y. Liu, S.-Z. Yang, B.-Z. Mu, *Process Biochem.* 2009, **44**, 1144-1151.
- 43 I. Geetha, A.M. Manonmani, *Lett. Appl. Microbiol.* 2010, **51**, 406-412.
- 44 D. Ghribi, M. Elleuch, L.M. Abdelkefi, S. Ellouze-Chaabouni, *J. Stored Prod. Res.* 2012, **48**, 68-72.
- 45 D. Ghribi, L. Abdelkefi, H. Boukadi, M. Elleuch, S. Ellouze-Chaabouni, S. Tounsi, *J. Invertebr. Pathol.* 2012, **109**, 183-186.
- 46 I. Mnif, M. Elleuch, S.E. Chaabouni, D. Ghribi, *Crop Prot.* 2013, **50**, 66-72.
- 47 D. Ghribi, I. Mnif, H. Boukadi, K. Radhouan, S. Chaabouni-Ellouze, *Afr. J. Microbiol. Res.* 2011, **5**, 4927-4936.