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Characterization of Surfactin Produced by *Bacillus subtilis* Isolate BS5

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Abstract Physical and chromatographic characterization of the surfactin biosurfactant produced by *Bacillus subtilis* isolate BS5 has been conducted to study its potentiality for industrial application. The crude extract of test surfactin appeared as off-white to buff flake-like amorphous residue with bad odor similar to sour pomegranate. Test surfactin showed solubility in aqueous solution at pH>5 with optimum solubility at pH 8–8.5. It was also soluble in organic solvents like ethanol, acetone, methanol, butanol, chloroform, and dichloromethane. Surfactin crystals appeared rectangular with blunt corners and were arranged perpendicular to each other making a plus sign. Extracted surfactin showed high surface activity, as it could lower the surface tension of water from about 70 to 36 mN/m at ~15.6 mg/l. Moreover, test surfactin exhibited excellent stabilities at high temperatures (100°C for up to 1 h at and autoclaving at 121°C for 10 min), salinities (up to 6% NaCl), and over a wide range of pH (5–13). Test surfactin in the cell-free supernatant or crude culture broth forms showed high emulsification indices against kerosene (62.5% and 59%, respectively), diesel (62.5% and 66%, respectively), and motor oil (62% and 66%, respectively). These characters can effectively make test surfactin, in its crude forms, a potential candidate for the use in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry. Chromatographic characterization of test surfactin, using high-performance liquid chromatography technique, revealed that the extracted surfactin contained numerous isoforms, of which six were found in the standard surfactin preparation (Fluka). Additional peaks appeared in the test surfactin and not in the standard one. These peaks may correspond to new surfactin isoforms that may be present in the test surfactin produced by *B. subtilis* isolate BS5.

Keywords Surfactin · *Bacillus* · Characterization · Potential application

Abbreviations

BS	biosurfactant
CFS	cell-free supernatant

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CMC	critical micelle concentration
E_{24}	emulsification index after 24 h
HPLC	high-performance liquid chromatography
RP-HPLC	reversed-phase HPLC
MSM	mineral slats medium
MMSM	molasses–MSM
ODS	octadecyl silane
SF	surfactin
ST	surface tension
TPA	total peak area
t_r	retention time
Wt	weight

Introduction

Biosurfactants are amphiphilic compounds produced by microorganisms that either adhere to cell surfaces or are excreted extracellularly in the growth medium. Many microorganisms produce biosurfactants during growth on a wide variety of substrates. Interest in biosurfactants has been generated due to their possible applications in environmental protection, crude oil drilling, and in the pharmaceutical and food-processing industries [1]. Since the earliest reports on bacterial surfactants, a variety of biosurfactants has been described. These include glycolipids, phospholipids, lipopeptides, neutral lipids, fatty acids, and lipopolysaccharides [2]. Several reports are available on the production of these biosurfactants on water-immiscible substrates, especially hydrocarbons. Reports are also available on biosurfactant production on water-soluble substrates [1]. Surfactin, one of the most effective biosurfactants, was isolated from the cell-free culture medium after growth of *Bacillus subtilis* on glucose [3].

Surfactin belongs to the lipopeptide family excreted by *B. subtilis*. Its structure is characterized by a heptapeptidic moiety linked to a β-hydroxyl fatty acid. A lactone bridge between the β-hydroxyl function of the acid and the carboxy-terminal function of the peptide confers a cyclic structure to this molecule [4]. A natural diversity occurs, giving rise to homologues, differing from each other by the length (13 to 15 atoms of carbon) and the ramification of the fatty acid chain, and to isoforms, characterized by some differences in the peptidic sequence [4].

The increasing interest for these molecules is due to their amphiphilic character, which is responsible for their excellent surface-active properties as it reduces the surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005% [5]. In addition, surfactins exhibit diverse biological activities such as antiviral and antimycoplasma [6, 7], antitumoral [8], inhibition of fibrin clot, and antibacterial properties [5]. In this study, the physical and chromatographic characters of test surfactin produced by *B. subtilis* isolate BS5 were investigated. The isolate BS5 was recovered from Egyptian soil and characterized by its high productivity of surfactin [9].

Materials and Methods

Microorganisms

B. subtilis isolate BS5, a promising surfactin producer, was obtained through an extensive screening program [9].

Culture Media

Molasses–mineral salts medium (MMSM) is a medium optimized for surfactin production by *B. subtilis* isolate BS5 that was previously developed by [10]. the composition of MMSM was as follows (per liter): molasses (160 ml); $NaNO_3$ (5 g), $ZnSO_4$ (0.16 g), $FeCl_3$ $6H_2O$ (0.27 g), $MnSO_4$ H_2O (0.017 g), $MgSO_4$ $7H_2O$ (0.4 g), NaCl (1 g), KCl (1 g), H_3PO_4 85% (10 ml), and distilled H_2O to 1,000 ml, and the pH was adjusted to 7.2 with KOH pellets.

Chemicals

All chemicals were of available high quality grades supplied by (unless otherwise indicated) El-Nasr Chemicals (Adwic), Egypt.

Production of Surfactin

Seed culture was prepared by transferring a loopful from a fresh culture grown onto nutrient agar slant into 25 ml of MSM [11] contained in 250-ml Erlenmeyer flask. The flask was incubated at 250 rpm and 30°C for 36 h.

Surfactin production was carried out in 1-l Erlenmeyer flasks containing 200-ml aliquots of MMSM. The flasks were inoculated with the seed culture at 2% *v/v* and incubated under shaking conditions (250 rpm) and at 30°C for 2.5 days.

Extraction of Surfactin

Surfactin was extracted basically according to [12] with minor modifications. Cell suspension was centrifuged (Hietech® Biofuge) 10,000×*g* for 5 min to prepare the cell-free supernatant (CFS). The CFS was acidified with 1 N HCl to pH 2 and left overnight at 4°C. The produced off-white to buff cake in the centrifuge tubes was dried in a hot-air oven at 70°C. The dried materials were transferred to 50 ml methylene chloride contained in a 250-ml conical flask and left covered overnight at room temperature with intermittent shaking. The organic extract was filtered and then the residue on the filter paper was re-extracted with another 50 ml fresh methylene chloride and refiltered again. The pooled organic phase was evaporated under vacuum (Buchi, Germany) at 40°C. The residue obtained was characterized as such or after being dissolved in 5 mM Tris–HCl buffer, pH 8.5 (the crude surfactin solution).

Analytical Methods

Measurement of Surfactin Surface Activity

This was conducted by measurement of surface tension using a du Nouy ring tensiometer (Krüss) according to [13]. Before conducting the experiment and between each pair of measurements, the sample cup was washed three times with distilled water and acetone in series and then allowed to dry. The platinum ring was similarly treated; then, it was flamed till redness and left to cool.

Measurement of Surfactin-emulsifying Activity

Measurement of the emulsification activity of surfactin-containing crude preparation (CFS) was conducted by measuring the emulsification index (E_{24}) using different oil phases [14].

Emulsions were prepared as follows: After cell removal by 20-min centrifugation at 6,000 rpm, the oil phases were added to aliquots of CFS in Wassermann tubes at an oil/CFS ratio of 3:2 *v/v*. The tubes were then vortexed at maximum speed for 3 min, and the emulsions produced were allowed to settle for 24 h at room temperature. The emulsification index (E_{24}) was calculated by determining the percentage volume occupied by the emulsion after 24 h to the total volume of the liquid phase [15].

Characterization of the Produced Surfactin

Physical Characterization Physical characterization of surfactin was mainly performed using surfactin solutions prepared in 5 mM Tris–HCl buffer (pH 8.5). In some cases, however, crude preparations (CFS or crude culture broth without cell removal) containing surfactin at 1.12 g/l were used. These crude preparations were prepared from cultures in the optimized MMSM as mentioned before under "Extraction of Surfactin."

Solubility of surfactin extract (residue remaining after extraction) was tested in water, methanol, ethanol, butanol, acetone, chloroform, methylene chloride, *n*-hexane, ethyl acetate, acetonitrile, and ethyl ether.

Crystalline appearance of extracted surfactin was determined by acidifying a surfactin solution with 1 N HCl, so that the final concentration of surfactin was 50 mg/l at pH 2±0.5. The acidified solution was overnight incubated at 4°C and then centrifuged at 10,000×*g* for 10 min. The precipitated crystalline surfactin was recovered and examined under a light microscope at a magnification of ×40, and the films were photographed [16].

Critical Micelle Concentration and Surface Tension-lowering Values A crude surfactin solution was prepared at a concentration of 175 mg/l. Then, it was appropriately serially diluted (175–0 mg/l), and the surface tension values of the prepared dilutions were measured using a du Nouy ring-type tensiometer from Krüss and as described by [13]. The lowest surface tension value reached by surfactin was recorded. The critical micelle concentration (CMC) of test surfactin was estimated from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections of a curve plotted between surfactin concentration and surface tension values [17]. In parallel, the CMC of standard surfactin was determined and used for tentative estimation of the percentage purity of test surfactin [18, 19]

The emulsification index (E_{24}) was used to measure the emulsification activity of crude surfactin preparations (CFS and cell-containing culture broth containing 1.12 g/l surfactin) against different oil phases: hexadecane, linseed oil, kerosene, diesel (Solar), and motor oil (Pennzoil oil 10W-40) and carried out as mentioned before.

Stability at High Temperatures Thermal stability was determined according to [20] using the crude surfactin solutions. Test tubes, containing 10 ml of surfactin solution (50 mg/l), were prepared and incubated in an oven at 100°C for different time intervals (0, 5, 10, 20, 40, 60 min). After incubation, compensation of the volume was made if necessary, and then the percentage of surface tension reduction was determined from the following equation:

$$\%\ \text{ST reduction} = [(\text{ST}_{\text{before}} - \text{ST}_{\text{after}})/\text{ST}_{\text{before}}] \times 100$$

Moreover, another set of surfactin solution was autoclaved at 121°C for 10 min, and the percentage surface tension reduction values were measured for treated and untreated samples.

In addition, stability to autoclaving (121°C for 10 min) was also determined using surfactin-containing CFS by determining the emulsification index (E_{24}) before and after autoclaving [21].

Stability at Different Salinities Surfactin solutions were prepared at 50 mg/l containing different NaCl concentrations (0, 0.3, 0.9, 3, and 6 g%, *w*/*v*), and the surface tension of these solutions was measured at 30°C [22].

Stability at Different pH A set of surfactin solutions was adjusted to pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 using 1 N HCI or 1 N NaOH at a final biosurfactant concentration of 50 mg/l. The surface tension of the resultant solutions was measured using du Nouy ring tensiometer [23].

Chromatographic Characterization

HPLC of the Crude Surfactin Extract Surfactin produced by the *B. subtilis* isolate BS5 was extracted as mentioned previously from cultures in the optimized MMSM medium. The standard surfactin was from *B. subtilis*, (≥98%; Fluka, obtained from Sigma-Aldrich, Germany), and the crude surfactin solution was prepared in 5 mM Tris–HCl (pH 8.5) at 0.45 and 4.58 mg/ml, respectively. High-performance liquid chromatography (HPLC) was carried out basically as described by [12] and [24] using an Agilent 1100 HPLC system composed of vacuum degasser, autosampler, quaternary pump, column (Eclipse XDB-C18, USA), diode array detector (DAD) and an electronic integrator (HP Chemstation Software rev A.09).

An aliquot of 40 μl each of the standard surfactin and of the crude surfactin solutions were injected automatically in a reversed-phase C18 column (15.0×4.6 mm; 5 μm particle size) held at room temperature (30°C). The mobile phase was held for 10 min before each run, and then the samples were eluted using a linear gradient mobile phase composed of acetonitrile (3.8 mM):trifluoroacetic acid (80:20, *v*/*v*) at a flow rate of 1 ml/min. The eluted components were detected using DAD at 205 nm (λ_{max} of surfactin as determined experimentally). Chromatograms were analyzed using the Hewlett-Packard ChemStation software package by which peak areas were automatically calculated. Purity of test surfactin was calculated from the areas under the peaks.

Results

Physical Properties

Organoleptic Characters Crude surfactin residue obtained after the extraction appeared as off-white to buff flake-like amorphous residue with a sour pomegranate-like bad odor.

Solubility Test surfactin was found to be soluble in aqueous solution at pH>5 with optimum solubility at pH 8–8.5. It is also soluble in ethanol, acetone, methanol, butanol, chloroform, and dichloromethane but not in *n*-hexane, ethylacetate, acetonitrile, or petroleum ether.

Crystalline Appearance Test surfactin crystals appeared rectangular with blunt corners and arranged perpendicular to each other making a plus sign (Fig. 1).

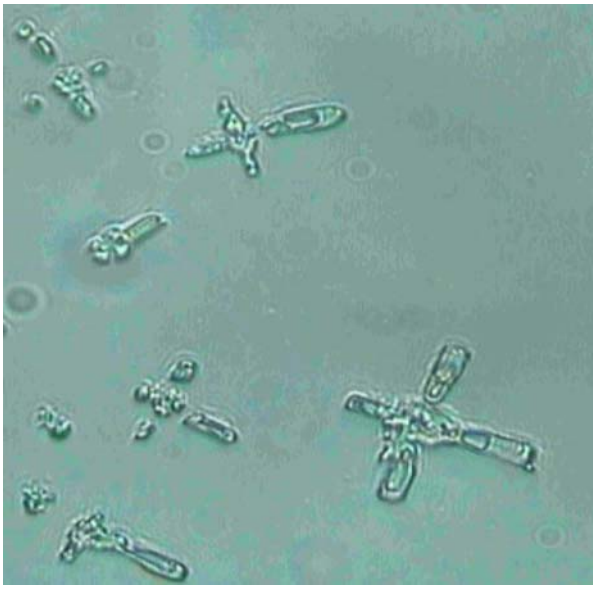

Fig. 1 Crystalline appearance of the recovered crystals of test surfactin examined under a light microscope at a magnification power of ×40

Critical Micelle Concentration and Surface Tension-lowering Values Test surfactin lowered the surface tension of water from about 70 to 36 mN/m. CMC of test surfactin was obtained from the graph, and it was found to be 15.6 mg/l (Fig. 2).

Although purity can be more accurately calculated using chromatographic methods as will be shown later, it can also be calculated from the CMC values of test and standard surfactin (10 mg/l for surfactin obtained from Sigma-Aldrich) using the following relation deduced from the finding of [19]:

$$\text{Percent of impurities} = \text{Purity}_{\text{std}} \times [(\text{CMC}_{\text{Test}}/\text{CMC}_{\text{Std}}) - 1] \times 100$$

$$\text{Percent purity} = (100 - \%\ \text{impurities})$$

Using the above equation, the purity of the test surfactin was found to be about 45%.

Emulsification Index Surfactin-containing CFS and crude culture broth showed high emulsification indices with hexadecane, kerosene, and diesel (Fig. 3). However, both of the

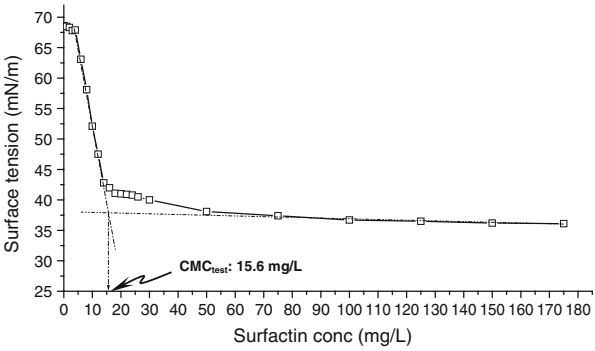

Fig. 2 A plot of surface tension as a function of concentration of test surfactin at pH 8.5 in 5 mM Tris–HCl buffer

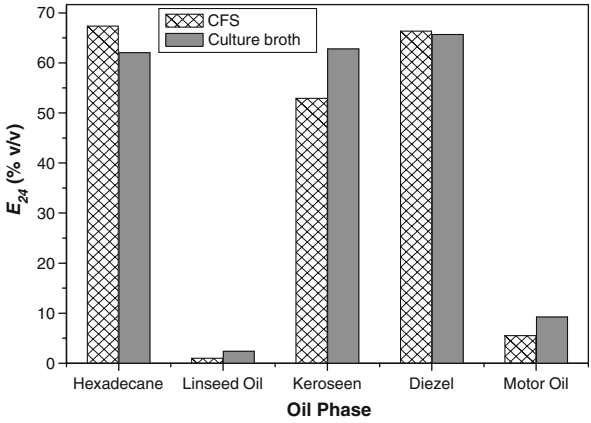

Fig. 3 E_{24} of surfactin-containing CFS against different oils

crude surfactin-containing preparations showed a very low emulsification index against motor and linseed oil.

Stability at High Temperatures Test surfactin exhibited high thermal stability because, approximately, there was no reduction in surface activity upon exposure to 100°C for up to 1 h. It is interesting to note that autoclaving at 121°C for 10 min did not reduce surface activity to any degree; it even resulted in a slight increase in surface activity. However, some white coagulation was detected at the bottom of the test tubes containing surfactin solution after autoclaving (Fig. 4).

As seen in Fig. 5, test surfactin, in its crude CFS form, tolerated well autoclaving conditions as there was no remarkable reduction in its emulsifying activity after treatment.

Stability at Different Salinities From Fig. 6, it appears that the surface activity of surfactin was nearly not affected by high sodium chloride concentrations up to 6%. Furthermore, sodium chloride alone had only a slight effect on surface tension of the used buffer (Fig. 6).

Stability at Different pHs As seen in Fig. 7, the surface activity of surfactin was greatly affected by changes in pH. It was found that the surfactin solution has nearly no surface

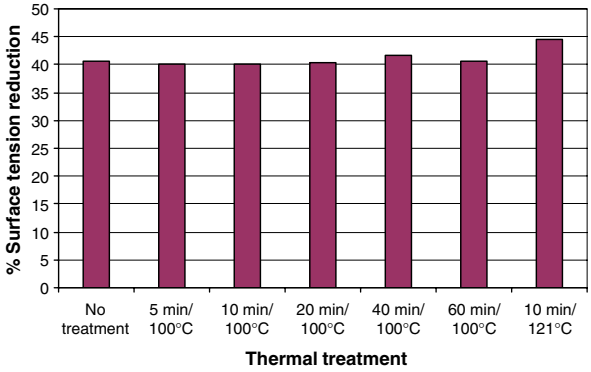

Fig. 4 Effect of temperature on surface activity of test surfactin solution (50 mg/l at a pH 8.5 in 5 mM Tris–HCl buffer). Surface activity was expressed in terms of percentage reduction in surface tension

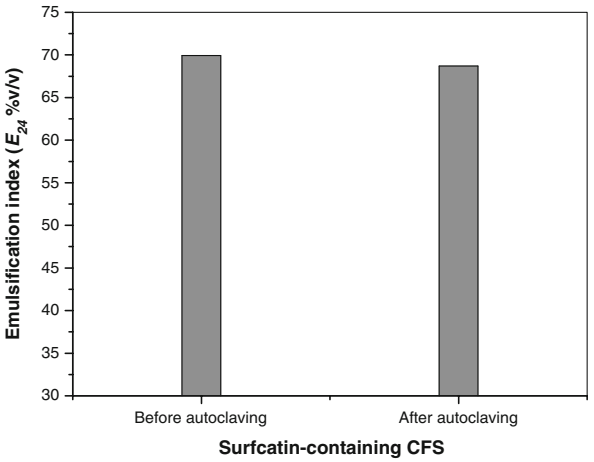

Fig. 5 Effect of autoclaving on emulsifying activity of surfactin-containing CFS (1.12 g/l) against kerosene

activity at pH values from 2 to 4, and surface activity was detected starting from pH 5 with its maximum at pH 6.

Chromatographic Characterization

HPLC Characterization The absorbance of surfactin isoforms in the standard preparation was determined at different detection wavelengths (200, 205, 210, 215, and 220 nm) with the aid of photo-DAD, and they were found to have maximum absorbance at 205 nm (data not shown).

Figure 8a shows that standard surfactin contained nine isoforms, which eluted between 6 and 20 min. Of these isoforms, six were major namely, peaks 1, 2, 4, 5, 7, and 8, and three were minor namely, peaks 3, 6, and 9. In addition, the figure shows that all surfactin isoforms were well separated except isoform numbers 7 and 8, which differed in their

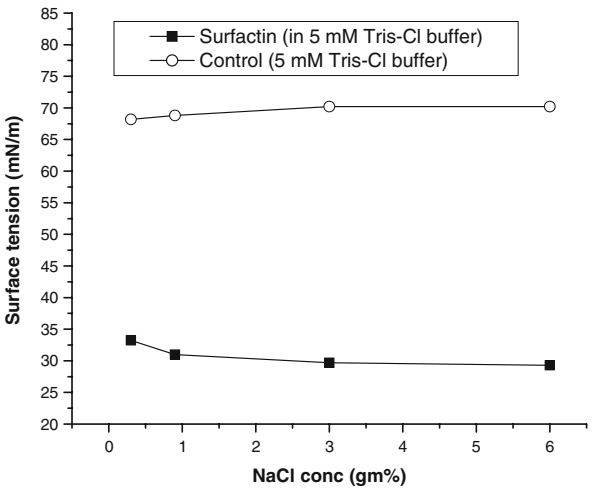

Fig. 6 Effect of salinity on surface activity (expressed in terms of surface tension values) of test surfactin. Surfactin was dissolved in 5 mM Tris–HCl buffer, pH 8.5 at 50 mg/l. Surface tension was measured using a du Nouy ring tensiometer

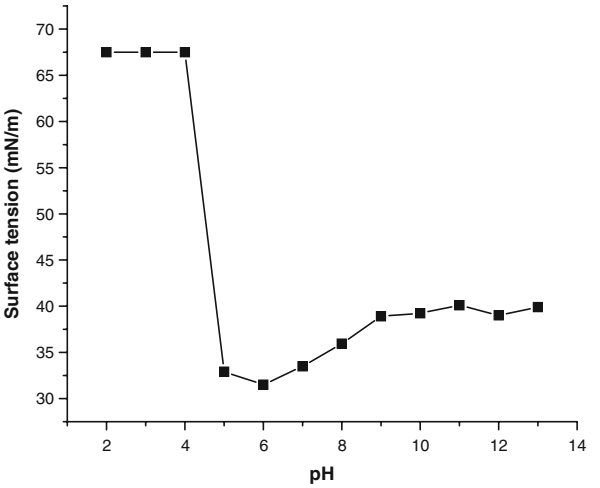

Fig. 7 Effect of pH on the surface activity of surfactin solutions. Surface activity was monitored using surface tension measurement of surfactin solutions in 5 mM Tris–HCl buffer. The surface tension value of 5 mM Tris–HCl buffer at pH 8.5 was 68.6 mN/m

retention times by less than 0.3 min, so they appeared somewhat merged. Figure 8b shows that test surfactin contained numerous isoforms, of which six were also found in the standard surfactin preparation. The peaks having very similar retention times between the standard (designated by numbers) and test surfactin (designated by letters) are arrowed in

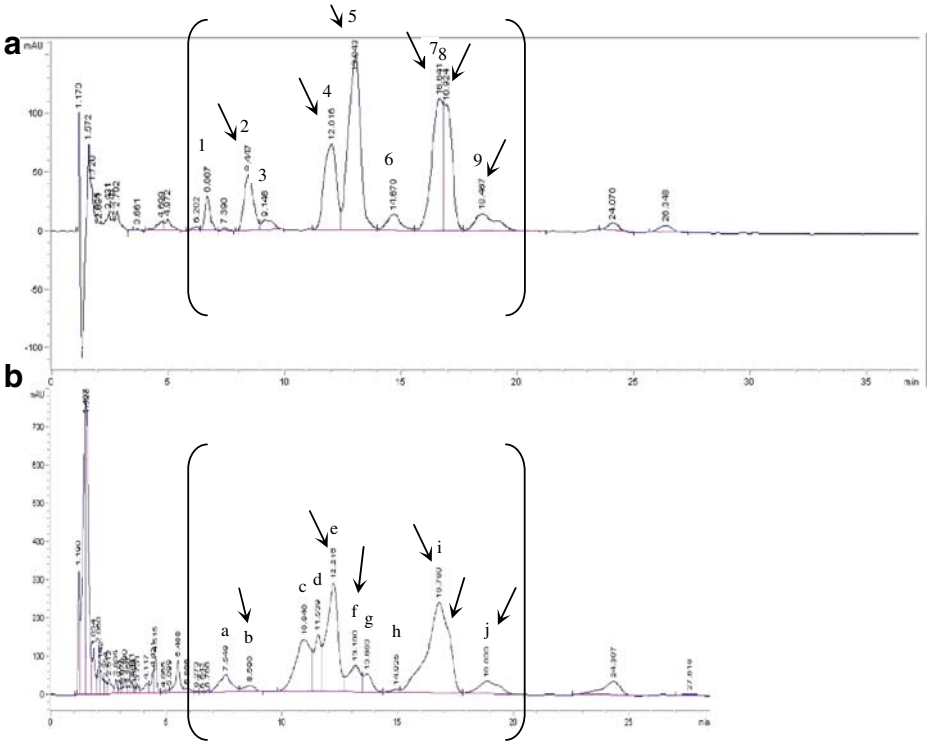

Fig. 8 Analytical reversed-phase HPLC (octadecyl silane) absorbance spectrogram of surfactin (50 mg/l in 5 mM Tris–HCl buffer) measured at 205 nm. **a** Spectrogram of standard surfactin preparation obtained from Sigma; **b** spectrogram of crude surfactin preparation obtained from *B. subtilis* isolate BS5

Table 1 Total peak areas (TPA) of standard and test surfactin[a].

	Standard surfactin	Test surfactin
TPA	18,639.04719	49,864.92002
Loaded weight (μg)	18.2	183.2

[a] TPA is the sum of the six surfactin peaks found in both test and standard samples.

both spectrograms (2, b; 4, e; 5, f; 7, i; 8, undetected; and 9, j). It appeared that the relative abundance of the different isoforms was not the same in both the test and standard. The test surfactin peak (i) that was corresponding to peak 7 in the standard spectrogram appeared as a broad peak with a right shoulder, which may refer to another surfactin isoform that is corresponding to peak 8 in the standard surfactin spectrogram.

The areas of the peaks that eluted between 6 and 20 min were summed to give the total surfactin peak area for each of the standard and the test surfactin. This calculated total peak areas were presented and compared in Table 1.

The purity of test surfactin was calculated from the equation:

$$\text{Purity}_{\text{test SF}}(\%w/w)=\left[(\text{TPA}_{\text{test SF}}\times \text{Wt loaded}_{\text{std SF}})/\text{TPA}_{\text{std SF}}\right]/\text{Wt loaded}_{\text{test SF}}\times 100$$

The purity of test surfactin was 26.6%.

Attention should be paid to the additional peaks appearing in the test surfactin spectrogram. These included five peaks, a, c, d, g, and h, that appeared at retention times of 7.549, 10.940, 11.539, 13.663, and 14.925 min, respectively.

Discussion

Characterization of surfactin biosurfactant produced by *Bacillus* isolate BS5 included organoleptic, solubility, crystalline characteristics, and chromatographic analysis using HPLC. Surfactin biosurfactants are promising environmental molecules for bioremediation purposes; therefore, the stability of test surfactin at conditions that mimic the environmental ones such as high temperature, salinities, and over a wide range of pH was also studied.

Physical Properties

Physical State and Organoleptic Characters Crude surfactin extract was found as off-white to buff flake-like amorphous residue, and this was in accordance with that published by [25]. Test surfactin had a sour pomegranate-like bad odor.

Solubility It was observed that surfactin has its optimum aqueous solubility at alkaline pHs (pH 8–8.5). This phenomenon is attributed to the acidic nature of surfactin. Surfactin contains two carboxylic groups, one corresponding to a glutamic acid residue and the other corresponding to the an aspartic acid residue of the peptide moiety of the lipopeptide surfactin; these groups confer the anionic nature to surfactin. In a study conducted by [26], it was found that surfactin started to precipitate out of the production medium at pH≤5 and was dissolved completely when the pH returned to 6.1 [26]. Test surfactin was also soluble in ethanol, acetone, methanol, butanol, chloroform, and dichloromethane but not in *n*-hexane, ethylacetate, acetonitrile, or petroleum ether. This pattern of solubility was in accordance with that published by [25].

Crystalline Appearance Acidification of the aqueous preparation of surfactin, which is an acidic molecule, resulted in the precipitation of its crystals, appearing, under the microscope, rectangular with blunt corners and arranged perpendicular to each other making a plus sign (Fig. 1).

Critical Micelle Concentration and Surface Tension-lowering Value One of the main characteristics of surfactants is their tendency to adsorb at interfaces in an oriented fashion as a consequence of their amphipathic structure. As surfactant concentration increases, the surface tension of the surfactant solution decreases up to a certain value and then becomes almost constant due to the interface saturation with the surfactant molecules. The concentration at which this phenomenon occurs is known as the CMC and is determined from the break point of the surface tension versus concentration curve [27]. For practical purposes, it is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be determined from the CMC of the biosurfactant [28].

In this study, test surfactin showed to be a highly effective biosurfactant since its crude solution could lower the surface tension of water from about 70 to 36 mN/m. However, it was documented that surfactin can lower the surface tension to even lower values, reaching to 27 mN/m [29]. This contradiction may be attributed to the low purity of the test surfactin preparation (25%). The test surfactin showed to be highly efficient as well, if compared with synthetic surfactants that have minimum CMCs of 590 to 2,000 mg/l [30], since its CMC value was found to be 15.6 mg/l. In the literature, surfactin was documented to be even more efficient since its CMC could reach 11 mg/l [30]. However, some variations in CMC values (13, 22, and 17 mg/l) for surfactin have been stated by other authors [19, 31, 32]. Variations in CMC values were shown to be dependent on the nature of the solvent surfactin is dissolved in as well as the purity of surfactin preparation [31, 18]. The higher surface tension reduction and CMC values (36 mN/m and 15.3 mg/l, respectively) of test surfactin produced by *B. subtilis* isolate BS5 were expected. This is because these values were determined using a crude surfactin extract (purity 26.6%).

In the present study, it was found that the purity of surfactin produced in MMSM as calculated from CMC values was 45%, although its purity as calculated chromatographically was 26.6%. The former method is dependent on biosurfactant activity; however, the latter method is dependent on biosurfactant concentration. This discrepancy, may explain the lack of coherency between surfactin activity and surfactin concentration that was noted during surfactin production experiments [10].

Emulsification Index The emulsifying power is another important character of any surfactant. Therefore, it was assessed for the test surfactin in the crude CFS or crude culture broth forms using the emulsification index (E_{24}) against different oil phases.

Surfactin-containing CFS and culture broth showed high emulsification indices with hexadecane, kerosene, and diesel. However, neither of the crude surfactin-containing preparations could emulsify motor or linseed oil to a considerable level.

It should be realized that the emulsifying activity was measured using an oil/water ratio of 3:2, which means that the oil phase constitute 60% of the total volume. This means that E_{24} values greater than or equal to 60 entails a complete emulsification of the oil phase. This condition occurred with surfactin-containing culture broth against kerosene and with both crude forms against hexadecane and diesel.

The ability of surfactin to emulsify crude oil products (hexadecane, kerosene, and diesel), which may facilitate their microbial assimilation, could be useful for pollution treatment of oil-contaminated environments [15, 33].

Stability of surfactin to drastic conditions (high temperature and salinities and over a wide pH range) deserved to be studied to assess their applicability in bioremediation where such conditions commonly prevail.

Stability at High Temperatures The stability of surfactin at different temperatures is an important issue that deserved to be investigated. Test surfactin was prepared at an appropriate discriminative concentration (lowest concentration that can produce maximum reduction in surface tension value of water), which was determined from Fig. 2. This concentration was selected to be at the linear declining part of the curve just preceding the plateau where minor changes in surfactin activity (that may result from exposure to the tested conditions) will be accompanied with measurable changes in surface tension. This concentration was 50 mg/l of surfactin.

Test surfactin solutions showed very good thermal stability. There was no reduction in surface activity to any degree upon exposure to 100°C for up to 1 h. It is interesting to note that autoclaving at 121°C for 10 min did not also reduce surface activity to any degree; it even resulted in a slight increase in surface activity. This increase in activity may be attributed to the heat-dependent coagulation and precipitation of substances such as proteins contaminating the surfactin solution; such substances might be coextracted with surfactin during extraction steps. This coagulation was demonstrated by the presence of white coagulum at the bottom of the test tubes containing the surfactin solution after autoclaving.

Purification accounts for up to 60% of the total production cost of biosurfactants [34]. Because of economic considerations in the oil industry, most biosurfactants would be required either as whole-cell culture broths or crude preparations [34]. Therefore, the applicability of test surfactin at high temperatures in their crude form (CFS) without prior costly extraction steps was additionally investigated. Upon autoclaving of test surfactin-containing CFS at 121°C for 10 min, it was found that it tolerated well autoclaving conditions as such treatment resulted in no remarkable reduction in its emulsifying activity.

The thermal stability exhibited by the test surfactin either in the crude CFS or the extracted form widens the scope of its applicability at conditions where high temperatures prevail as in microbially enhanced oil recovery (MEOR).

Stability at Different Salinities It was necessary to study the effect of salinities on the activity of the test surfactin to investigate its applicability in bioremediation of contaminated marines. It was found that the surface activity of test surfactin was nearly not affected by high salinities (Fig. 6), even at salinities reaching double the highest sea salinity in the world (3%, as stated in http://en.wikipedia.org/wiki/Seawater). Therefore, it can be concluded that surfactin is an excellent candidate, if compared with chemical surfactants, to be used in bioremediation of contaminated marines where high salinities prevail. Chemical surfactants, however, are deactivated by 2–3% salt concentrations and cause environmental toxicity [30].

Stability at Different pHs The stability of test surfactin at different pH values is also an important issue that can affect its application spectrum. Therefore, this was investigated in this study.

The effect of pH on surface activity of surfactin was significant. It was found that surfactin solution has nearly no surface activity at pH values from 2 to 4, and surface activity was detected starting from pH 5 with its maximum at pH 6. From the above

findings, it can be concluded that surfactin biosurfactant is generally more active at pHs around neutrality; however, highly acidic pH conditions cause more reduction in surface activity than highly alkaline conditions. These findings are in accordance with that obtained by [26]. They reported that surfactin started to precipitate out of the production medium at pH≤5 and was dissolved completely when the pH returned to 6.1 [26].

As a result, physical characterization revealed that test surfactin has excellent surface and emulsifying activities as well as it showed high stabilities at elevated temperatures, high salinities, and over a wide pH range (5–13). These properties make surfactin biosurfactants potential candidates to be used in bioremediation of contaminated sites and in the petroleum industry (MEOR) where drastic conditions commonly prevail. Furthermore, crude surfactin-containing preparations such as CFS and culture broth (without cell removal) gave high emulsifying activities against different oil phases. This complies with the present need for the use of biosurfactants in their crude form for the purpose of cost reduction. The crude culture broth form is particularly applicable in the field of oil industry where higher purity specifications are not required [2]. Therefore, crude culture broth-containing surfactin is a candidate for direct use in the field of oil industry.

Chromatographic Characterization

Some authors demonstrated that surfactin has six isoforms [24]; others stated that so far at least nine different surfactin structures have been identified [35]. These isoforms differ in the chain length β-hydroxy fatty acid, which is most commonly C13 to C15 [36].

In a previous study, it appeared that these isoforms were inseparable in thin-layer chromatography (TLC) experiments as they appeared as a single separated spot [9]. Surfactin isoforms were also inseparable in TLC experiments conducted by many authors [37, 38]. Therefore, HPLC analysis was performed in this study on a crude surfactin solution for determining the different surfactin isoforms that may be present and for studying their distribution in comparison with that in a standard surfactin solution (Fluka).

Standard surfactin (Fig. 8a) shows nine isoforms; however, test surfactin (Fig. 8b) shows numerous isoforms, of which six were also found in the standard surfactin preparation. The difference in the relative abundance of the different isoforms in the test and standard may be related to strain variations. The areas of all peaks eluting between 6 and 20 min of the test and standard surfactin were used to calculate the purity of test surfactin, which was found to be 26.6%.

The peaks that are present in the test surfactin spectrogram but not in the standard one are suggested to be of the new surfactin isoforms present in the test surfactin. These isoforms may differ in the lipid and/or peptide moiety of surfactin. [39] stated that surfactin consists of a family of lipopetides with similar chemical structures, i.e., isoforms, which slightly differ in their physicochemical properties due to (1) variations in the chain length and branching of its hydroxy fatty acid component as well as (2) substitutions of the amino acid components of the peptide ring. These variations depend on the used *B. subtilis* strain and the nutritional and environmental conditions [39].

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