



ORIGINAL ARTICLE

Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia



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Abstract Twenty three morphologically distinct microbial colonies were isolated from soil and sea water samples, which were collected from Jeddah region, Saudi Arabia for screening of the most potent biosurfactant strains. The isolated bacteria were selected by using different methods as drop collapse test, oil displacement test, blue agar test, blood hemolysis test, emulsification activity and surface tension. The results showed that the ability of *Virgibacillus salarius* to grow and reduce surface tension under a wide range of pH, salinities and temperatures gives bacteria isolate an advantage in many applications such as pharmaceutical, cosmetics, food industries and bioremediation in marine environment. The biosurfactant production by *V. salarius* decreased surface tension and emulsifying activity (30 mN/m and 80%, respectively). In addition to reducing the production cost of biosurfactants by tested several plant-derived oils such as jatropha oil, castor oils, jojoba oil, canola oil and cottonseed oil. In this respect the feasibility to reusing old frying oil of sunflower for production rhamnolipids and sophorolipids, their use that lead to solve many ecological and industrial problems. © 2014 Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

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1. Introduction

The surface-active compounds commonly used in many industries are chemically synthesized; they are widely used in almost every sector of recent industry (Samadi et al., 2007). The expansion in environmental carefulness has led to serious consideration of biological surfactants as the most promising alternative to existing product (Henkel et al., 2012).

Biosurfactants are considered as one of the high values of microbial products, which have gained considerable interest in recent years that have become an important product of biotechnology for industrial and medical applications (Nitschke and Costa, 2007; Makkar et al., 2011). The reasons for their publicity are lower toxicity, specificity of action, simplicity of preparation and extensive applicability. Moreover, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as: organic chemicals, pharmaceuticals and cosmetics, beverages and foods, metallurgy, mining, petroleum, petrochemicals, biological control and management and many others (Banat et al., 2000; Perfumo et al., 2010; Vedaraman and Venkatesh, 2011). Over the above, biosurfactants have many advantages over synthetic ones, including bioavailability, structural diversity, specific activity at extreme salinity, temperatures and pH (Datta et al., 2011). In spite of these advantages, good attributions and a variety of potential uses of biosurfactants, efforts to commercial production have failed due to the low yield obtained and high production cost. The possibility of economical biosurfactant production to reduce pollution was caused by wastes. Increasing biosurfactant yields and decreasing production costs are essential factors affecting the efficiency of biosurfactant production process (Kosaric, 1992; Bognolo, 1999; Moussa et al., 2006). Syldatk and Hausmann (2010) found that the use of costly substrates, gave low yields and accumulation of undesirable product mixtures rather than refined biosurfactant compounds, such constraints explain why there is restricted production of biosurfactants in industry. Great varieties of agronomic, industrial by-products and material residues are recently available as nutrients for biosurfactant fermentation industry (Makkar and Cameotra, 2002; Savarino et al., 2007; Ferreira, 2008; Silva et al., 2009). Therefore hopefully tomorrow's microbial surfactants appear to depend particularly on the use of plentiful and cheap substrates for optimization of the operational cultivation conditions, which can markedly increment the yield (Mukherjee et al., 2007, 2008; Mutalik et al., 2008; Makkar et al., 2011). The world production of fats and oils is about 120 million tonnes, 81% of which are from plant sources (Brackmann and Deutschland, 2004). Most of the oils and fats are used in the food industry, which produces large amounts of waste frying oils. The disposal of frying oil waste is causing a great problem, which explains the increasing interest in the use of waste frying oils for microbial transformation (Vedaraman and Venkatesh, 2011). The beneficial effects of this field were paid attention to for isolation and characterization of biosurfactants produced by extremophiles such as halophilic and thermophilic bacteria (Mnif et al., 2009; Kumar et al., 2008; Joshi et al., 2008). This study aimed at isolation of some bacterial isolates from different sources (Oil contaminated soil, uncontaminated soil and from Red sea water at Jeddah region in kingdom of Saudi Arabia [KSA]), then screening assays for biosurfactant production from obtained isolates. The waste frying oil will be reused as a substrate for the production of cheaper biosurfactant.

2. Material and methods

2.1. Bacterial isolation

Bacterial isolates were obtained from different sources such as oil contaminated soil, uncontaminated soil and from Red sea

water from Jeddah region at KSA. Ten grams of soil was collected from soil samples, in addition to 10 ml of water sample. Then all samples were transferred to the laboratory in sterilized polyethylene containers. The direct isolation of the microorganisms was carried out using serial dilution (up to 10^{-7}) of soil samples in 0.85% sterile saline (Bordoloi and Konwar, 2008). The different samples were agitated and serial dilutions, then 1 ml from each diluted samples was plated onto the surface of Nutrient Agar medium. The plates were incubated at 30 °C for 1–5 days. Pure cultures with different morphological distinct properties were obtained by picked repetitive streaking and stored in nutrient slants at 4 °C.

2.2. Screening assays for potential biosurfactant producing strains

The screening of the most potent surfactant strains was assayed qualitatively using different methods namely drop collapse test, oil displacement test, blue agar test, blood hemolysis test and quantitatively using emulsification activity and measurement of surface tension by the Du Nouy ring method. The strain which showed the lowest surface tension value was selected for a further study according to methods described by Bodour and Maier (1998), Youssef et al. (2004) and Sriram et al. (2011). All the experiments were done in triplicate.

2.3. Identification of biosurfactant producing bacteria

The bacterium identification was conducted by the molecular method depending on gene phylogenetic approximation. PCR amplifications of the 16S rRNA gene were performed with 16S rRNA Eu-bacterial primer (50-GAGTTTGATCCTGGCTCAG-30; 50-AGAAAGGAGGTGATCCAGCC-30) following the method described by Relman (1993). The phylogenetic tree was constructed by utilizing the neighbor-joining method and assessed with 1000 bootstrap replications. The minimum value of the nucleotide similitude percentage to define the species at the taxonomic level was 98% (Rossello-Mora and Amann, 2001). The 16S rRNA gene sequence obtained from the isolate Nrc-1 was compared with other bacterial sequences by using NCBI mega BLAST. The nucleotide sequence was aligned in CLUSTALX. The phylogenetic analyses were conducted using MEGA version 6 software (Tamura et al., 2007).

2.4. Production media and cultivation conditions

A minimal salt (MS) medium containing (g/l): KH_2PO_4 , 1.4; Na_2HPO_4 , 2.2; $(\text{NH}_4)_2\text{SO}_4$, 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6; NaCl, 0.05; yeast extract, 1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 and $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$, 0.02; was used throughout the study. The basal minimal medium was supplemented with 2 ml of trace element solution and glucose 2% (v/v) was used as the sole carbon source. The composition of trace element solution involved (g/l): ZnSO_4 , 0.29; CaCl_2 , 0.24; CuSO_4 , 0.25 and MnSO_4 , 0.17. The trace element solution was added after the production media were autoclaved, prior to inoculation by filtering it through 0.2 μm membrane filters, sterilized by filtration (Millipore Corp., Bedford, MA, USA). After incubation the cultures at

30 °C for 7 days were centrifuged, and the culture filtrates were used in the experiments.

To obtain higher productivity of the biosurfactant, a series of experiments were conducted to select the critical medium components and optimum cultivation condition in changing one variable at a time, keeping the other factors fixed at a specific set of conditions. These factors were: pH, temperature, NaCl concentration, Carbon sources, nitrogen source (N) and C/N ratio. The effects of pH, NaCl concentration and temperature on the biosurfactant production were evaluated as follows: the pH of medium was adjusted in the range between 4 and 12 and the temperature was set at 25, 30, 35, 40, 45 and 60 °C. To examine the effect of sodium chloride on biosurfactant production in an optimized medium, sodium chloride was added to the medium to achieve final concentrations of 1–10% (w/v). For evaluation the effects of several edible and non-edible oils derived from plants were studied as carbohydrate sources. The non-edible oil such as jatropha oil, castor oil, and jojoba oil and the edible oils such as canola oil, cottonseed oil and reusing the waste frying sunflower oil were supplied. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants sodium nitrate was eliminated and equimolar amounts of other nitrogen substrates were supplied. Urea, yeast extract, phenylalanine, peptone, tryptone, ammonium nitrate, ammonium sulfate, potassium nitrate, sodium nitrate, malt extract and ammonium chloride were employed at a concentration equivalent in nitrogen content to that of sodium nitrate (2.5 g/l) with the optimum carbon source. In the next series of experiments C/N ratios were varied at 10, 20, 30, 40, 50, 60 and 70 with optimized carbon and nitrogen sources by keeping a constant nitrogen source concentration of 1 g/l (Abouseoud et al., 2008a; Kiran et al., 2009). All cultivations were carried out in 250 ml flasks containing 50 ml of MS medium. The bacterial dry cell weight, surface tension, and emulsification activity were determined.

2.5. Time course of biosurfactant production kinetics in optimal medium

The kinetics of biosurfactant was followed in a modified MS medium at optimum conditions. West frying oil and urea were used as the carbon and nitrogen sources. Cultures were incubated at 50 °C, pH 9 for 7 days at 150 rpm. During culture growth, samples were collected at various time intervals. Biosurfactant production was monitored by measuring surface tension and emulsification assay of supernatant samples obtained after cell separation (Abouseoud et al., 2008b; Lotfabada et al., 2009).

2.6. Biosurfactant stability studies

The critical micelle concentration (CMC) was determined from the break point of surface tension versus its concentration, cell-free culture broth of 5 ml was collected at a regular interval of 12 h for a period of 156 h to determine the reduction in surface tension by the ring method (Bodour and Maier, 1998 and Kiran et al., 2009) using a Du Nouy Tensiometer. For the stability analysis, cell-free broth was heated to 100 °C for different time intervals, and then thermal stability was recorded after cooling to room temperature. To detect the effect of pH on activity, pH of the cell free broth was maintained at

different values using 2 N HCl. Similarly, the effect of different concentration of NaCl (0–10%, w/v) on the activity of the biosurfactant was investigated. Also emulsifying activity of culture broth and supernatant was measured by a method according to Abouseoud et al. (2008b).

2.7. Recovery of biosurfactant

Biosurfactant was precipitated by adjusting pH of the broth cell-free culture to 2.0 using 6 N HCl and keeping it at 4 °C overnight. Pellet thus precipitated was collected by centrifugation (8000 rpm for 15 min at 20 °C) and dissolved in distilled water. Then pH adjusted to 8.0 with 1 N NaOH, and the extract was lyophilized (Abouseoud et al., 2008b).

2.8. Fourier transform infrared spectroscopy (FT-IR)

For identifying types of chemical bonds (functional groups), Fourier transform infrared spectroscopy (FT-IR) analysis can be used to elucidate some components of an unknown mixture. One milligram of partially purified biosurfactant was completely dried in a freeze dryer and then grounded with 100 mg of KBr and pressed for 30 seconds to obtain translucent pellets. Then analyzed in a FTIR (Perkin–Elmer Spectrum RX1, Shelton, Connecticut), device obtaining the spectrum in the range of 450–4000 cm^{-1} at a resolution of 4 cm^{-1} . All data were corrected for the background spectrum.

All the results of experiments were conducted in three independent replicates and the results presented were the average data.

3. Results and discussion

The isolation of a new biosurfactant producing bacterium was carried out in soil and water samples collected from Jeddah region, KSA. Twenty three morphologically distinct microbial colonies (14 Gram positive including one coccus and 13 bacilli; 9 Gram-negative bacilli) were isolated. Among the isolated bacteria, KSA-T was selected for further studies based on the primary abilities of the selected bacterial strain to hemolytic activity, oil displacement, blue agar test, drop collapse and Emulsion activity. The drop collapse test and oil displace test are indicative of surface wetting activity (Youssef et al., 2004). Emulsification index is one of the criteria to support the selection of potential biosurfactant producers as reported by Satpute et al. (2008) who found that more than one screening method should be included in the primary screening as to identify potential biosurfactant producers. Data presented in Table 1 show the screening methods for biosurfactant producing from isolated bacteria. The results of isolates of bacteria on blood agar media were similar to the work done by Mulligan et al. (1984, 1989), they have observed biosurfactant overproducer mutants with the blood agar method. Jain et al. (1991), proved the use of the drop collapse method as a sensitive and easy method to test for biosurfactant production. Dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of an anionic biosurfactant (Siegmond and Wagner, 1991). The best results in decreased surface tension (production biosurfactant) were observed in three isolates KSA-T (30 mN/m) followed by KSA-M

Table 1 Summary of the screening methods for biosurfactant producing bacteria.

Bacterial isolates	Oil spreading test	Drop collapse test	Emulsification activity	Blue agar plate test	Hemolytic activity	Surface tension (mN/m)
KSA-T	++++	+++	+++	–	+++	30
KSA-M	+++	++++	+++	–	+++	35
KSA-1	–	+	–	–	–	–
KSA-2	–	–	–	–	+	–
KSA-3	–	–	–	–	+	–
KSA-4	–	+	–	–	–	–
KSA-5	++	++	+	+	+	43
KSA-6	–	+	–	–	–	–
KSA-7	+	–	–	+	+	43
KSA-8	–	–	–	–	+	–
KSA-9	+	++	++	+	++	39
KSA-10	+	+	+	+	+	40
KSA-11	–	–	–	–	–	–
KSA-12	–	–	+	–	–	–
KSA-13	–	–	–	+	+	–
KSA-14	–	–	–	–	+	–
KSA-15	–	+	–	–	–	–
KSA-16	–	–	–	–	+	–
KSA-17	+	+	++	+	+	45
KSA-18	–	–	–	–	–	–
KSA-19	+	+	++	+	+	45
KSA-20	++	+	++	+	+	67
KSA-21	++	++	++	+	+	56

Symbol means: (–) = no result; (+) = weak result; (++) = average result, (+++) = good result, (++++) = high result.

(35 mN/m) and then KSA-9 (39 mN/m) comparing with other isolates. The isolate of KSA-T was selected for a further study as the highest biosurfactant producer for its capacity to decrease surface and interfacial tension during growth on mineral oil medium. The KSA-T strain was related to a halophilic bacterium (*Virgibacillus salarius*) at a similarity level of 88% based on 16S rRNA gene sequence analysis. Phylogenetic tree of the strain KSA-T and closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor joining tree method) is illustrated in Fig. 1.

3.1. Effect of carbon source

In order to reduce the production cost of biosurfactants several plant-derived non edible oils were studied (Fig. 2A). The use of plant-derived oil as carbon sources to produce biosurfactants seems to be an interesting and low cost alternative (Abouseoud et al., 2008a). There are few reports, which utilized the vast potential of these frying oils for biosurfactant production. Fleurackers (2006) first demonstrated usage of waste frying oils for the production of biosurfactants, he has shown *Candida bombicola* ATCC 22214 can produce biosurfactants in shake flask using restaurant oil waste. It was a successful feasibility study for waste frying oils as substrate. Shah et al. (2007) studied sophorolipid production by *C. bombicola* in both batch and fed batch fermentations. They achieved a yield of 34 g/l sophorolipids on restaurant oil waste, while Zhu et al. (2007) achieved 20 g/l rhamnolipid using *Pseudomonas aeruginosa* 50 L bioreactor. These studies demonstrated the feasibility to reuse waste frying oil for production of both sophorolipids and rhamnolipids on an industrial scale. Pure paraffin oil and cottonseed oil were used as substrates for the production of surfactin using *Bacillus subtilis* (Joshi et al., 2008). In the

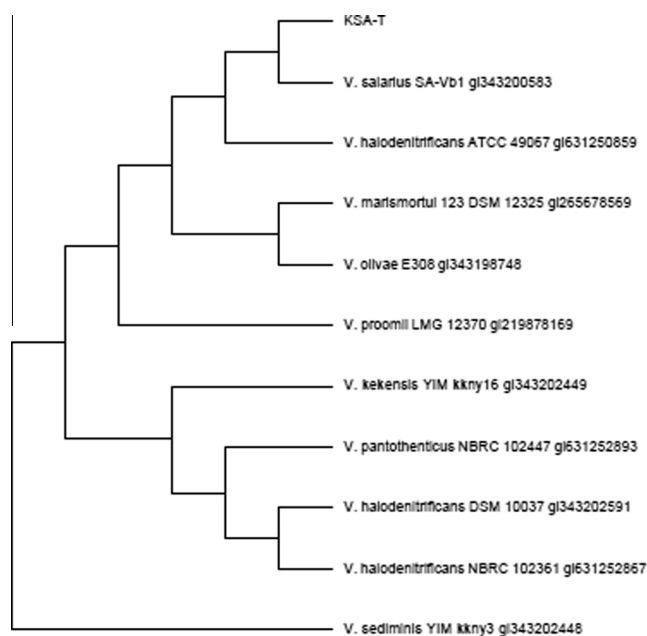


Figure 1 Phylogenetic tree of the strain KSA-T (*Virgibacillus salarius*) and closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor joining tree method).

present work, waste frying oil was the best carbon source for biosurfactant synthesis. The isolated biosurfactant decreased the surface tension to 30 mN/m and the emulsifying activity was 80%. Similar results were found with biosurfactant production from *P. aeruginosa* 44T1 (Lin et al., 1994; Morikawa

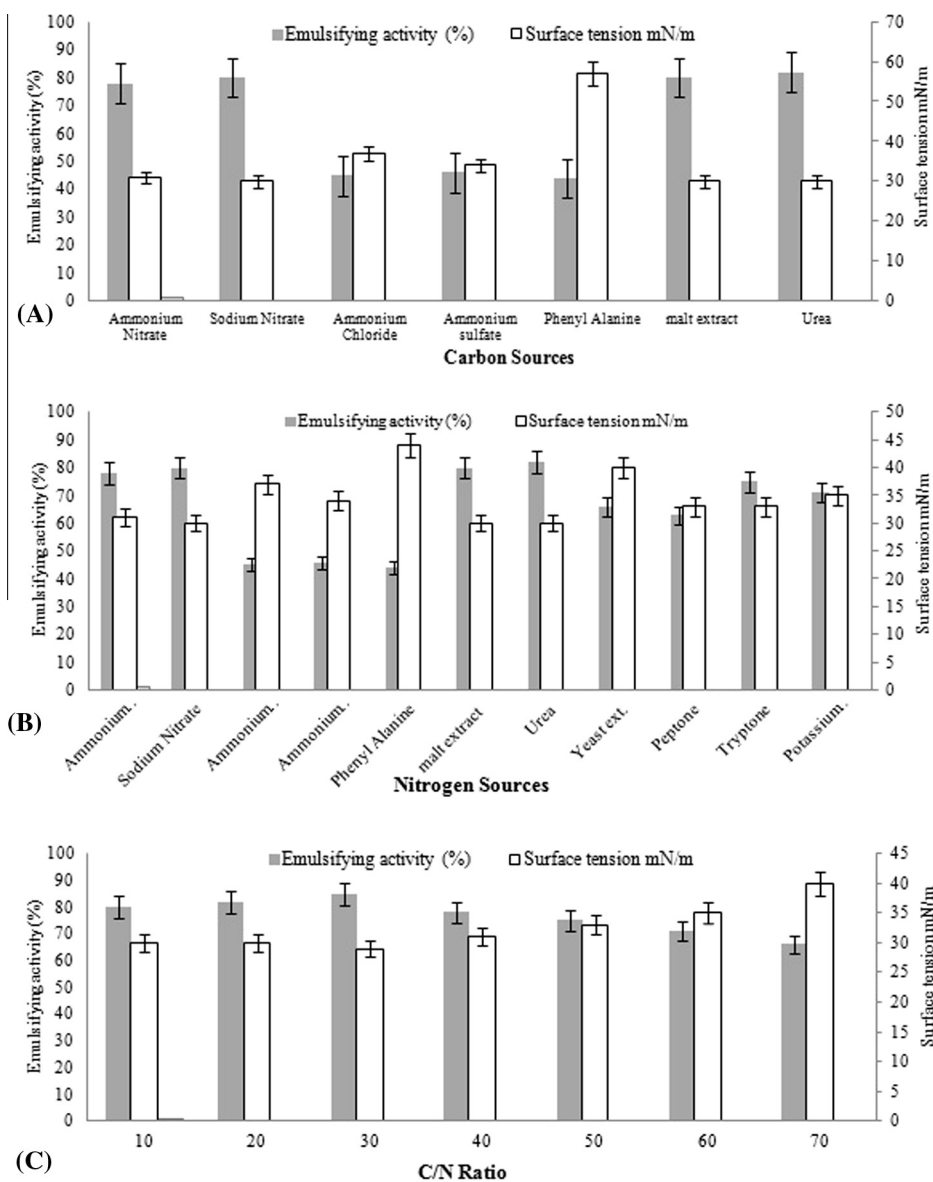


Figure 2 Effect of different carbon sources, different nitrogen sources and carbon/nitrogen ratio on biosurfactant production.

et al., 1993). Apart from various vegetable oils, oil wastes from vegetable oil refineries and the food industry were also reported as good substrates for biosurfactant production (Haba et al., 2000; Abalos et al., 2001; Bednarski et al., 2004; Nitschke and Costa, 2007).

3.2. Effect of nitrogen source

There is evidence that nitrogen sources play an essential part in the biosurfactant production process (Wu et al., 2008). The choice of how the nitrogen source influences the biosurfactant creation is illustrated in Fig. 2B. It showed that urea and NaNO_3 were the most efficient nitrogen source for almost all bacteria isolates. *V. salarius* (KSA-T) produced biosurfactants with minimal surface tension (29.5 mN/m) and maximum emulsifying activity (82%). The use of Ammonium salts in the form of ammonium nitrate and Ammonium chloride led to a

substantial increase in the growth but not for biosurfactant production and caused a noteworthy decrease in pH (4.03). These results were in agreement with Santa Anna et al. (2002), Rashedi et al. (2005) and Prieto et al. (2008). Although there is no significant difference between sodium nitrate or Urea on biosurfactant production, urea was chosen as the cheaper nitrogen source than other sodium nitrate. This result was in agreement with Abdel-Mawgoud et al. (2008) and Meyer (2011) who reported the most elevated biosurfactant production utilizing ammonium nitrate and sodium nitrate as nitrogen sources. Additionally Makkar and Cameotra (1997) and Ghribi and Ellouze-Chaabouni (2011) reported the most elevated biosurfactant production using urea. While some *Bacillus* isolates did not utilize ammonium sulfate for biosurfactant production or microbial growth, however they can utilize sodium nitrate, ammonium nitrate or potassium nitrate (Makkar and Cameotra, 1997).

Table 2 Effect of different NaCl concentrations, temperature and pH on growth and biosurfactant production by *Virgibacillus salarius* (KSA-T).

Treatments	Con./degree	Dry cell weight (g/L)	Surface tension (mN/m)	Emulsification index (%)	Biosurfactant concentration (g/l)
NaCl (%)	0.0	1.43 ± 0.4	32.0 ± 1.20	97.6 ± 4.0	0.96 ± 0.0
	2.0	2.05 ± 0.1	29.5 ± 1.20	98.6 ± 2.3	1.03 ± 0.3
	4.0	2.30 ± 1.0	28.8 ± 0.50	99.3 ± 4.4	1.6 ± 0.12
	6.0	2.3 ± 0.04	28.9 ± 0.10	94.3 ± 2.7	1.41 ± 0.4
	8.0	2.23 ± 0.0	29.0 ± 1.00	90.1 ± 1.9	1.38 ± 0.8
	10	1.3 ± 40.2	32.0 ± 1.00	74.2 ± 3.1	1.0 ± 0.40
Temperature (°C)	30	2.11 ± 0.50	32.0 ± 0.88	97.0 ± 1.0	0.90 ± 0.30
	40	2.13 ± 1.00	28.5 ± 0.20	99.5 ± 5.1	1.64 ± 0.25
	50	3.00 ± 0.04	28.5 ± 0.60	99.5 ± 3.7	1.62 ± 0.60
	60	1.00 ± 0.00	28.9 ± 1.01	99.0 ± 3.2	1.62 ± 0.10
PH	5.0	1.80 ± 0.70	31.0 ± 2.00	99.0 ± 0.2	1.0 ± 0.40
	7.0	2.00 ± 0.67	30.0 ± 1.40	99.0 ± 1.8	1.08 ± 0.6
	9.0	2.40 ± 0.18	28 ± 0.340	100 ± 5.3	1.51 ± 0.5
	11	2.10 ± 1.00	29.4 ± 0.30	96.0 ± 2.1	1.5 ± 0.20
	12	1.00 ± 0.20	31.0 ± 0.90	94.3 ± 2.7	0.85 ± 0.5

-Values are the means of three replications.

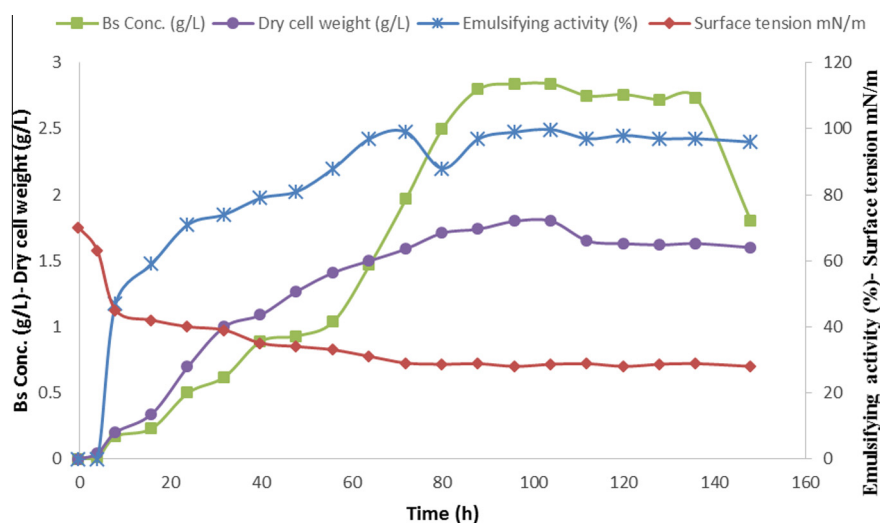


Figure 3 The growth, biosurfactant production and surface tension profiles of *Virgibacillus salarius* (KSA-T) isolate grown in new optimized MS medium at 40 °C, pH 9 and 150 rpm.

3.3. Effect of carbon/nitrogen ratio

In many biological processes, the *C/N* ratio is a critical factor, thus affecting product accumulation (Silva et al., 2009). The influence of *C/N* ratio was investigated using a wide range of *C/N* ratio (10:1–70:1) with frying oil and urea as carbon and nitrogen source, respectively. As shown in Fig. 2C, the maximum emulsifying activity (85%), and minimal surface tension (29 mN/m), were attained at a *C/N* ratio of 30:1, while any excess in the *C/N* ratio has no obvious effect on biosurfactant production or biomass of *V. salarius* (KSA-T). These results were in agreement with Santa Anna et al. (2002), Rashedi et al. (2005) and Chen et al. (2005) who found that a low *C/N* ratio is more effective in increasing biosurfactant production using waste frying oil and sodium nitrate.

3.4. Effect of sodium chloride, temperature and pH on biosurfactant production

Recent progresses in the field of extreme biosurfactant have prompted the acceleration of utilization of this molecule in different industrial applications. Specifically, biosurfactants from thermophilic microorganisms have found the most useful commercial utilization to date due to their general characteristic soundness (Demirjian et al., 2001) As well, since the majority of the modern industrial applications work at temperatures above 40 °C, the significance of thermostable biosurfactants is of extraordinary importance. The effects of salinity, temperature and pH on the growth and biosurfactant production of *V. salarius* (KSA-T) are shown in Table 2.

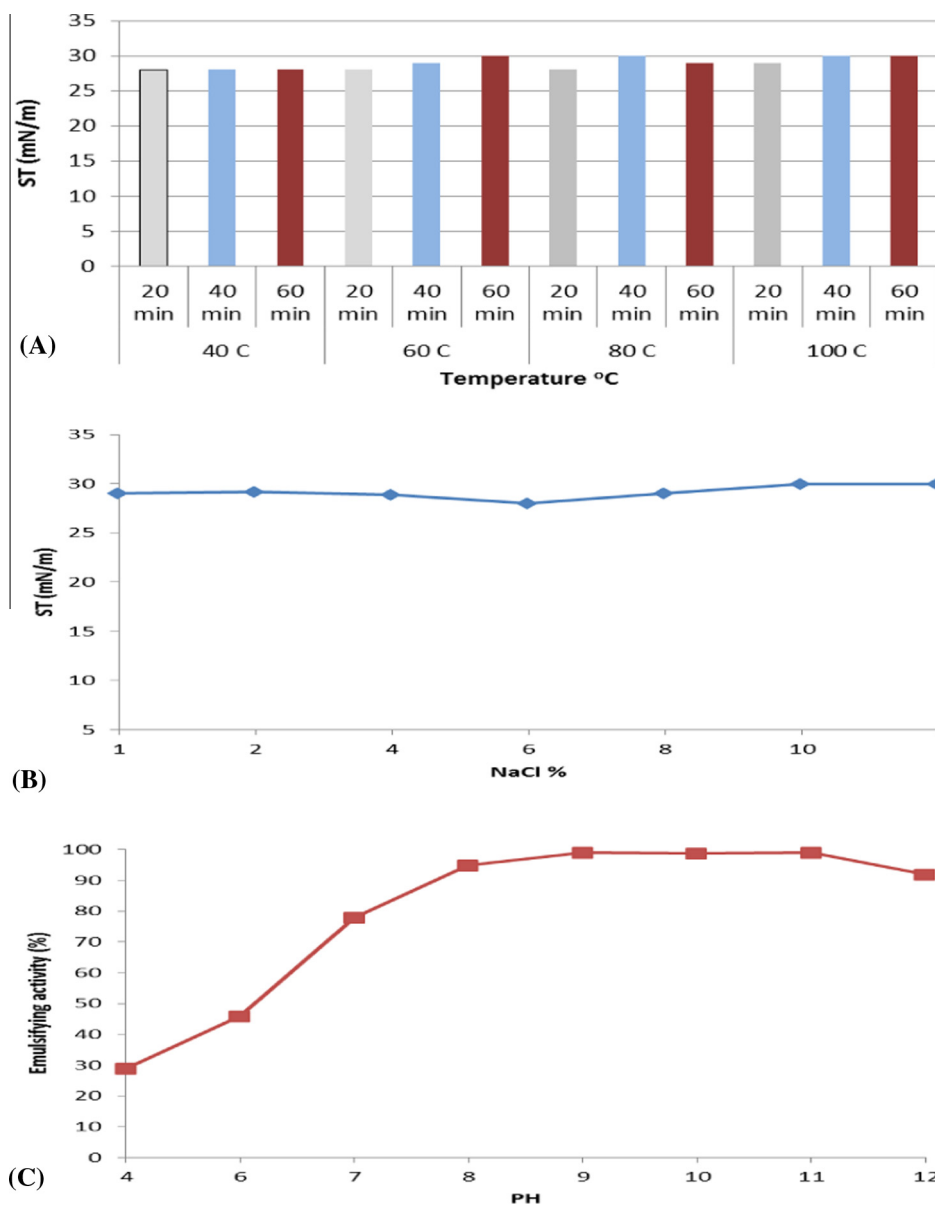


Figure 4 Effect of temperature (A), pH (B) and salinity (C) on biosurfactant stability.

The strain *V. salarius* (KSA-T) was found to be halophilic as highest biosurfactant synthesis was obtained in the presence of 4% (w/v) of NaCl, however, in the presence of 10% (w/v) of NaCl it lost 25% of its activity. In addition, the results in Table 2 show the strain *V. salarius* (KSA-T) was found to be moderately thermophilic and attained the ideal growth and maximum biosurfactant production in the temperature range of 45–50 °C.

On the other hand, the research concentrated on the choice of alkaline biosurfactant from isolated bacteria, due to the enormous utilization of biosurfactant molecules in detergent manufacture. The pH of the medium was increased, the biosurfactant generation increased as well, since the pH11 after that the biosurfactant production started to decrease. The strain *V. salarius* (KSA-T) was shown maximum biosurfactant production when pH increases to 9. The pervious results were in agreement with (Hamzah et al., 2013).

A typical time course profile of the growth Kinetics and biosurfactants production by *V. salarius* (KSA-T) isolate was performed at pH 9.0, 40 °C and 4% (w/v) salinity Fig. 3. Biosurfactant yield reached its maximum (2.8 g/l) at the end of the exponential growth phase. A significant reduction in the surface tension of *V. salarius* supernatant (30 mN/m) was determined after 8 h of incubation, then reaching its minimal value (29 mN/m) after about 3 days of growth by the end of the exponential phase. Thereafter, a slight reduction in the surface tension was up to the end of cultivation. That may be attributed to the critical micelle concentration (CMC) value, in which the surface tension stayed stable (30 mN/m). The increase in extra-cellular biosurfactant concentration might be the result of the cell-bound biosurfactant molecules released into the broth medium (Deziel et al., 1999). The results revealed that the production of biosurfactants from frying oil occurred predominantly throughout the exponential phase

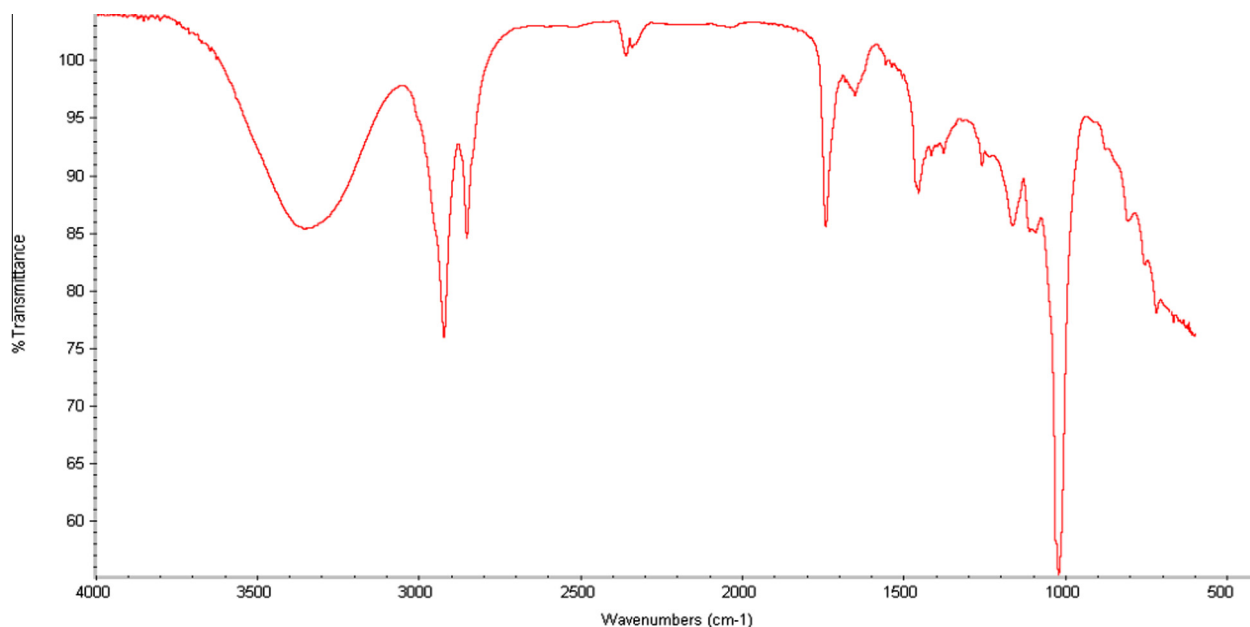


Figure 5 Fourier transform infrared spectra (FT-IR) of the biosurfactant produced by *Virgibacillus salarius* (KSA-T).

indicating that the biosurfactant is a primary metabolite and produced accompanying cellular biomass formation (Khopade et al., 2012), and the new optimized parameters are in favor of the increase of the generation time of *V. salarius* (KSA-T) isolate allowing more time to produce biosurfactants which was shown to be a growth-associated metabolite.

3.5. Stability study of the produced biosurfactant

The stability of biosurfactants at different salinity, pH and temperature values was measured and the results are illustrated in Fig. 4. The surface tension and emulsion activity of biosurfactant did not show any remarkable effect at a high temperature of 100 °C and remained without any significant increase on the surface tension capacity of the biosurfactant solution over different time intervals, therefore it was found that the biosurfactant mixture is thermally stable. In addition, the salinity and pH stability analysis were carried out, revealing that, the biosurfactant conserves its ability to decrease surface tension up to 12% NaCl. On the other hand, the activity was strongly affected by lowering pH values from 2 to 5. The emulsification activity was decreased by decreasing the pH value from basic to an acidic region; this may be due to partial precipitation of the biosurfactant (Abouseoud et al., 2008a; Khopade et al., 2012).

3.6. Fourier transform infrared spectroscopy (FT-IR)

The FTIR is the most useful analysis for identifying types of chemical bonds (functional groups) present in the biologically active fraction of an unknown biosurfactant and thus determines its chemical nature. The FT-IR spectrum is illustrated in Fig. 5, which shows extending vibration at 3300–3500 cm^{-1} which is characteristic of N–H stretching vibrations, indicating strong hydrogen bonding. The strong band peak at 3000–2900 cm^{-1} , was characteristic of aliphatic chains' ($-\text{CH}_3$, $-\text{CH}_2$) stretching vibrations. The appearance of a weak

absorbance signal at 2300–2400 cm^{-1} may be due to C=N stretch, The bands observed at 1650 cm^{-1} was a definite indicator of linkages between the amides I and II. The absorbance in this region was significant in the presence of the peptide group in the molecule. High intensity peak in the region of 1000–1100 cm^{-1} was assigned to O–C–O extend vibrations of carboxylic acids, aldehydes and ketones. It is remarkable, indicating the oxidation of the hydroxyl groups of hydrolysates (originated from the medium peptides). The following vibrations observed at 800–500 cm^{-1} may be indicative of methylene scissoring vibrations from the proteins in the bacterial filtrate. This FT-IR profile of the biosurfactant showed similarity to surfactin, a lipopeptide biosurfactant and other lipopeptide biosurfactants like arthrfactin (Morikawa et al., 1993) and lichenysin (Lin et al., 1994) confirming the lipopeptide nature of a biosurfactant.

4. Conclusion

The above-mentioned studies emphasize the potential uses of plant oils and related substrates for the biosurfactant synthesis. In addition, it might be deduced that this biosurfactant keep its activity stable over a wide range of temperatures between 30 and 100 °C, alkaline pH and hyper salinity over 10%. These results refer to the strong character of the biosurfactant its usefulness for industrial applications under extreme conditions of salinity, temperature and pH, such as, pharmaceutical, cosmetics and food industries and for bioremediation in marine environment.

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