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Production and Properties of Biosurfactant from *Pectinatus cerevisiiphilus* CT3 Isolated from Marine Sediments

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Abstract

A marine bacterial isolate, *Pectinatus cerevisiiphilus* CT3, was able to grow and produce biosurfactant on minimal salts media using glucose and $NaNO_3$ as carbon and nitrogen sources. It was found that cellular growth and biosurfactant production in MSM were greatly affected by the medium components. After 54 h of cultivation, *P. cerevisiiphilus* CT3 was able to grow and produce surfactant, reducing the surface tension of the medium to 28.0 mN/m with a biosurfactant concentration of 3.05 g/l and a critical micelle concentration of 10 mg/l. Biosurfactant recovery by chloroform/methanol extraction showed pH and thermal stability with respect to surface tension reduction. It also showed emulsification activity and a high level of salt concentration. In addition, promising antimicrobial activity was revealed when tested against human pathogenic bacterial and fungal isolates. Based on these results, the isolated biosurfactant from the marine bacteria *P. cerevisiiphilus* CT3 revealed a broad physicochemical stability and has excellent antimicrobial properties, indicating the potential for possible use in various therapeutic and biomedical applications.

Keywords: Production, Antimicrobial activity, Biosurfactant, *Pectinatus cerevisiiphilus*, Marine bacteria

Introduction

Biosurfactants, or microbial surfactants, are a structurally diverse group of surface-active compounds produced by microorganisms. They consist of hydrophilic and hydrophobic moieties and are able to reduce surface tension and enhance the emulsification of hydrocarbons. Their hydrophilic parts are usually composed of sugars, amino acids, or polar functional groups such as carboxylic acid groups. The hydrophobic part is typically an aliphatic hydrocarbon chain of β-hydroxy fatty acids. Biosurfactants are primarily classified into glycolipids, lipopeptides, polymeric biosurfactants, fatty acids, and phospholipids [1-3] according to their molecular structures.

Biosurfactants possess a high specificity, including low toxicity, high biodegradability, and effectiveness at extreme temperatures, pH and salinity. The unique structures of biosurfactants provide specific properties that commercial surfactants usually lack [2,3]. In addition to their utilization in enhanced oil recovery, bioremediation and industrial emulsification, microbial surfactants have been found in recent years to possess several properties of therapeutic and biomedical importance, e.g., antibacterial, antifungal and antiviral properties [4-6]. They also have anti-adhesive effects against several pathogenic microorganisms [7-9]. The present study examines the production, properties and antimicrobial activity of biosurfactant obtained from marine isolates of *Pectinatus cerevisiiphilus* CT3

(AB685263), which has been preliminarily characterized as a biosurfactant-producing strain as reported in an earlier study [10].

Materials and methods

Microbial strain

Pectinatus cerevisiiphilus CT3 (accession number AB685263) was isolated from marine sediment collected from the Palian River in Trang Province in the south of Thailand [10]. The complete 16S rRNA gene sequence from *P. cerevisiiphilus* CT3 showed 100 % homology to *Pectinatus cerevisiiphilus* CECT4927T (accession number FR870446). *P. cerevisiiphilus* CT3 was maintained on nutrient agar plates and transferred monthly.

Media and cultivation conditions

Nutrient broth was used to prepare the inoculum using the following composition in distilled water: 1.0 g/l beef extract, 2.0 g/l yeast extract, 5.0 g/l peptone, and 5.0 g/l NaCl. To make nutrient agar, 15.0 g of agar was added to the nutrient broth. The strain was cultivated in the nutrient broth overnight at 30°C. This was used as inoculum at the 5 % (v/v) level. For biosurfactant production, a mineral salt medium (MSM) with the following composition was utilized: 0.8 g/l K_2HPO_4, 0.2 g/l KH_2PO_4, 0.05 g/l $CaCl_2$, 0.5 g/l $MgCl_2$, 0.01 g/l $FeCl_2$, and 5.0 g/l NaCl [10]. The pH of the medium was adjusted to 7.0. Carbon and nitrogen sources were added separately. Cultivation was performed in 250 ml flasks containing 50 ml medium at 30 °C, which were shaken in a rotary shaker at 150 rpm for 48 h.

Medium optimization

Four main factors were chosen to obtain higher productivity from the biosurfactant: carbon sources, nitrogen sources, carbon/nitrogen ratio, and inoculum concentration. The used carbon sources were glucose, glycerol, molasses, oleic acid, palm oil, soybean oil, stearic acid, and used lubricating oil at a concentration of 5 g/l. A medium with no carbon source was used as the control assay. The used nitrogen sources were beef extract, monosodium glutamate, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, peptone, and yeast extract at a concentration of 1 g/l. A medium with no nitrogen source was used as the control assay. The C:N ratio (with optimized nitrogen source) ranged 1 - 35, while the nitrogen source concentration (1 g/l) was kept constant.

Recovery of biosurfactant

Acid and ammonium sulphate precipitations and solvent systems were examined for their biosurfactant extraction properties. The four solvent systems were chloroform with methanol (2:1), cold acetone, dichloromethane, and ethanol with ethyl acetates [11-14]. The recovery method showing the highest biosurfactant activity, chloroform with methanol (2:1), was used to recover biosurfactant from *P. cerevisiiphilus* CT3.

Stability of biosurfactant

The biosurfactants were dissolved in distilled water at the critical micelle concentration (CMC), and the effects of pH, sodium chloride (NaCl) concentration and temperature on biosurfactant activity were investigated. The effects of pH were explored by adjusting biosurfactant solutions with 1.0 N HCl or NaOH to obtain a pH of 2.0 - 12.0. The effects of NaCl concentration were explored by adding NaCl to the samples to reach final concentrations of 0 - 21 % (w/v). For the thermal stability study, biosurfactant solutions were incubated at 4 - 100 ºC for 1 h and at 121 ºC for 15 min and cooled to 25 ºC. The remaining activity was then determined.

Biochemical composition of the biosurfactant

The carbohydrate content of the biosurfactant was determined by the phenol-sulfuric acid method [15] using D-glucose as a standard. Protein content was determined by the method of Lowry *et al*. [16] using bovine serum albumin as a standard, and the lipid content was estimated by following the procedure of Folch *et al.* [17].

The chemical composition of the biosurfactant was determined with thin layer chromatography (TLC). The purified biosurfactant was spotted in triplicate on readymade silica gel TLC plates (Merck, Darmstadt, Germany). After the development in $CHCl_3$:CH_3OH:H_2O (65:15:1), one of the plates was put into a jar saturated with iodine vapors to detect lipids. Another plate was sprayed with rhodamine B reagent (250 mg rhodamine B in 100 ml absolute alcohol) and visualized under UV to confirm the presence of lipids. The third plate was sprayed with ninhydrin reagent (0.2 % ninhydrin solution in acetone) and dried. It was then heated at 120 ºC for 5 min to detect peptides [18].

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is very useful for identifying types of chemical bonds (functional groups) and can therefore be used to analyze the components of an unknown mixture. The obtained biosurfactant (10 mg) was ground with 100 mg of potassium bromide and pressed with 7,500 kg for 30 s to obtain translucent pellets. Infrared absorption spectra was recorded on a Thermo Nicolet AVATAR 330 FT-IR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. Each set of measurements consisted of 500 scans, and a potassium bromide pellet was used as a background reference [19].

Antimicrobial activity of the biosurfactant

The extracted compound was tested for antimicrobial activity using the agar well diffusion method as described by Candan *et al*. [20]. Briefly, the extract was weighted and dissolved in distilled water at a concentration of 10 mg/ml and the filter was sterilized by using a 0.2 µm membrane filter. Each tested microorganism was suspended in sterile saline and diluted to ca. 10^6 CFU/ml for bacteria and 1×1 cm^2 for 5-day cultivated fungi (10^8 spore/ml). Microbial suspensions were overlaid onto the surface of BHI agar plates, which were dried for 20 min at room temperature. The wells (5 mm in diameter) were cut from the agar and 50 µl of extracted solution was delivered into them. After an appropriate incubation time (1 and 5 - 7 days for bacteria and fungi, respectively) the clear zone was measured [20].

Analytical methods

Biomass was determined by the dry cell weight. At different stages of fermentation, samples were mixed in pre-weighted tubes with chilled distilled water and centrifuged at 9,693 g for 30 min. The biomass obtained was dried overnight at 105 °C and weighed.

Emulsification activity was performed according to [21]. Briefly, 4 ml of oil or other hydrocarbon was added to 4 ml of aqueous solution of culture supernatant in a screw cap tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and was calculated by dividing the measured height of the emulsion layer by the mixture's total height. The result was them multiplied by 100.

The surface tension measurement of the culture supernatant was determined in a Model 20 Tensiometer (Fisher Science Instrument Co., Pittsburgh, PA, USA), using the du Nouy ring method [22]. The values reported were the mean of the three measurements. All measurements were made on cultures obtained by centrifuging the cultures at 9,000 g for 15 min at 4 °C. The CMC was determined by plotting the surface tension versus the concentration of biosurfactant in the solution [23].

All experiments were carried out in triple for the calculation of the mean value. Two well-defined synthetic surfactants, SDS and cetrimonium bromide (CTAB), were used as positive controls. Distilled water and an MSM medium were used as negative controls. All of the used chemicals were of analytical grade. Statistical analysis was performed using R software (version 2.13.1).

Results and discussion

Effect of carbon sources on growth and biosurfactant production of *Pectinatus cerevisiiphilus* CT3

The literature revealed that the type of carbon source markedly affected the biosurfactant production [21,24]. This study began with an examination of the effect of carbon sources on biosurfactant production. *P. cerevisiiphilus* CT3 was grown on all of the tested carbon sources. After 48 h cultivation, the cultures with glucose and molasses produced 2.55 and 2.41 g/l of biomass, respectively. Meanwhile, the maximum biosurfactant production was 0.51 and 0.32 mg/l for glucose and molasses, respectively. The resulting biosurfactant-containing supernatant had a surface tension reduction of 20.51 mN/m and achieved an E_{24} of 36.30 % when glucose was used as a carbon source. **Table 1** also shows a clear trend between biomass yield and biosurfactant yield, strongly depending on the carbon source used.

Table 1 Effect of carbon sources on biosurfactant production by *Pectinatus cerevisiiphilus* CT3, which was cultivated in 250 ml flasks containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h (nitrogen source: 1 % $(NH_4)_2SO_4$).

Carbon source (5 g/l)	Dry cell weight (g/l)*	Surface tension reduction (mN/m)*	Biosurfactant (g/l)*	Emulsification activity* (%)
No carbon source	$0.12\pm0.02^{f**}$	0^{e**}	0^{e**}	0^{f**}
Glucose	2.55 ± 0.21^{a}	20.51 ± 2.25^{a}	0.51 ± 0.24^{a}	36.30 ± 5.20^{a}
Glycerol	2.08 ± 0.32^{b}	13.00 ± 0.50^{c}	0.20 ± 0.08^{b}	25.25 ± 3.85^{b}
Molasses	2.41 ± 0.52^{a}	16.62 ± 0.62^{b}	0.32 ± 0.03^{b}	30.17 ± 2.51^{ab}
Oleic acid	1.80 ± 0.31^{b}	10.05 ± 2.20^{cd}	0.12 ± 0.26^{cd}	15.20 ± 3.63^{cd}
Palm oil	1.21 ± 0.15^{c}	9.25 ± 1.81^{d}	0.08 ± 0.28^{d}	10.27 ± 2.58^{e}
Soybean oil	1.41 ± 0.20^{c}	12.23 ± 3.80^{cd}	0.10 ± 0.30^{d}	12.80 ± 2.35^{de}
Stearic acid	0.94 ± 0.21^{d}	13.23 ± 2.62^{c}	0.15 ± 0.15^{bc}	17.38 ± 2.57^{c}
Used lubricating oil	0.50 ± 0.20^{e}	8.65 ± 0.25^{d}	0.10 ± 0.03^{d}	11.87 ± 2.00^{e}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Effect of nitrogen sources on growth and biosurfactant production by *Pectinatus cerevisiiphilus* CT3

With glucose as a carbon source, the choice of nitrogen source affecting the biosurfactant production is depicted in **Table 2**. After examining the most commonly used organic and inorganic nitrogen sources reported in the literature [25], it was found that $NaNO_3$ was the most efficient nitrogen source for *P. cerevisiiphilus* CT3 to produce biosurfactant, giving a high biosurfactant yield of 2.54 g/l. This yield is five times the yield obtained from using $(NH_4)_2SO_4$ as the nitrogen source. Moreover, using $NaNO_3$ as the nitrogen source did not only increase the biosurfactant yield but also resulted in an increased biomass and a higher level of surface tension reduction, at 3.25 g/l and 30.30 mN/m, respectively. $NaNO_3$ has been previously reported as a suitable nitrogen source for biosurfactant production by *Anoxybacillus* sp. [26], *Wickerhamomyces anomalus* PY189 [27], *Bacillus flexus* [28] and *Virgibacillus salariust* [29], with the final surface tension of the production yield in the range of 29.5 - 42.3 mN/m [26,27,29].

Effect of carbon to nitrogen (C:N) ratio on growth and biosurfactant production by *Pectinatus cerevisiiphilus* CT3

The C:N ratio has also been known as a vital factor influencing the performance of biosurfactant production [30]. Therefore, the effect of the C:N ratio on biosurfactant production was investigated by keeping a constant nitrogen source (1 g/l of $NaNO_3$). In terms of carbon, a lower C:N ratio resulted in lower levels of cell growth. The lowest C:N ratio used (C:N=1) resulted in 54 % lower cell growth than the highest C:N ratio used (35:1), as shown in **Table 3**. The best biosurfactant yield (2.94 g/l) was obtained at a C:N ratio of 25, whereas the productivity tended to decrease as the C:N ratio increased from 25 to 35, especially for C:N ratio >30. Some reports have stated that biosurfactant production is more efficient under nitrogen limiting conditions [31,32]. The results showed that a possible inhibitory effect on the bacterial metabolism may occur due to a likely nutrient transport deficiency. In addition, the present study also shows that an appropriate amount of nitrogen source is a prerequisite for efficient biosurfactant production with *P. cerevisiiphilus* CT3.

Table 2 Effect of nitrogen source on biosurfactant production by *Pectinatus cerevisiiphilus* CT3, which were cultivated in 250 ml flask containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h (carbon source: 5 g/l glucose).

Nitrogen source (1 g/l)*	Dry cell weight (g/l)*	Surface tension reduction (mN/m)*	Biosurfactant (g/l)*	Emulsification activity (%)*
No nitrogen source	$0.52 \pm 0.10^{d**}$	0^{d**}	0^{c**}	0^{d**}
$(NH_4)_2SO_4$	2.55 ± 0.21^{c}	20.51 ± 2.25^{c}	0.51 ± 0.20^{d}	36.30 ± 4.20^{b}
$NaNO_3$	3.25 ± 0.81^{b}	30.30 ± 2.00^{a}	2.54 ± 0.40^{a}	50.10 ± 5.12^{a}
NH_4Cl	2.18 ± 0.72^{c}	25.70 ± 2.21^{b}	1.24 ± 0.63^{b}	39.12 ± 3.05^{b}
NH_4NO_3	2.52 ± 0.88^{c}	23.10 ± 3.71^{bc}	1.14 ± 0.24^{b}	36.25 ± 3.20^{b}
Beef extract	3.92 ± 0.60^{a}	15.50 ± 2.57^{d}	0.89 ± 0.18^{c}	24.16 ± 4.55^{c}
Momosodium glutamate	4.18 ± 1.53^{a}	20.19 ± 2.20^{c}	0.62 ± 0.09^{d}	20.50 ± 4.61^{c}
Peptone	4.09 ± 0.85^{a}	26.34 ± 3.51^{b}	1.30 ± 0.84^{b}	38.28 ± 4.32^{b}
Yeast extract	4.14 ± 0.68^{a}	22.00 ± 4.25^{bc}	0.65 ± 0.15^{d}	22.60 ± 2.30^{c}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Table 3 Effect of carbon to nitrogen (C:N) ratio on biosurfactant production by *Pectinatus cerevisiiphilus* CT3, which were cultivated in 250 ml flask containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h.

C:N	Dry cell weight (g/l)*	Surface tension reduction (mN/m)*	Biosurfactant (g/l)*	Emulsification activity* (%)
1	$1.81\pm0.09^{e**}$	$12.50\pm2.18^{e**}$	$0.93\pm0.21^{d**}$	$30.21\pm2.84^{d**}$
5	3.25 ± 0.81^{c}	30.30 ± 2.00^{d}	2.54 ± 0.40^{c}	50.10 ± 5.12^{c}
10	3.52 ± 1.03^{cd}	33.91 ± 3.80^{cd}	2.62 ± 0.29^{c}	55.42 ± 4.36^{b}
15	3.67 ± 1.02^{bc}	35.02 ± 4.12^{bc}	2.71 ± 0.47^{bc}	56.11 ± 5.21^{b}
20	3.89 ± 1.01^{ab}	39.30 ± 5.28^{a}	2.85 ± 0.11^{ab}	57.65 ± 4.64^{ab}
25	4.01 ± 0.74^{a}	42.15 ± 4.61^{a}	2.94 ± 0.20^{a}	59.20 ± 3.52^{a}
30	4.00 ± 0.13^{a}	42.14 ± 3.20^{a}	2.91 ± 0.13^{a}	58.50 ± 6.51^{a}
35	3.74 ± 1.25^{bc}	35.32 ± 4.15^{bc}	2.58 ± 1.11^{c}	55.81 ± 5.20^{b}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Effect of inoculum concentration on growth and biosurfactant production by *Pectinatus cerevisiiphilus* CT3

The effect of the inoculum concentrations on the growth and biosurfactant production of *P. cerevisiiphilus* CT3 cultivated in optimal medium (25 g/l glucose and 1 g/l $NaNO_3$) is illustrated in **Table 4**. Cell growth of *P. cerevisiiphilus* CT3 increased when the inoculation concentration increased. Increasing the inoculum concentration from 2.0 to 5.0 % (v/v) resulted in a higher level of surface tension reduction (19.5 - 42.15 mN/m) and emulsification activity (35.12 - 42.15 %). However, further increase in inoculum concentration did not affect surface tension reduction and emulsification activity. These results were in accordance with [13], who reported that increasing the inoculum's size from 1.0 to 5.0 (v/v) resulted in an increase of biomass and biosurfactant yield from *Selenomonas ruminantium* CT2. However, an inoculum concentration of more than 5.0 (v/v) did not have a significant impact in enhancing the biosurfactant production.

Table 4 Effect of inoculum concentrations on biosurfactant production by *Pectinatus cerevisiiphilus* CT3, which were cultivated in 250 ml flask containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h.

Inoculum concentration (%, v/v)	Dry cell weight (g/l)*	Surface tension reduction (mN/m)*	Biosurfactant (g/l)*	Emulsification activity* (%)
2	2.18±0.95^{d**}	19.50±3.81^{d**}	1.95±0.18^{c**}	32.12±5.40^{d**}
3	3.05±1.58^{c}	23.63±2.57^{c}	2.14±0.54^{c}	45.10±3.21^{c}
4	3.51±1.03^{b}	30.11±3.09^{b}	2.52±0.98^{b}	50.21±4.36^{b}
5	4.01±0.74^{a}	42.15±4.61^{a}	2.94±0.20^{a}	59.20±3.52^{a}
6	4.09±1.84^{a}	42.30±4.81^{a}	2.85±0.63^{a}	59.51±5.41^{a}
7	4.11±1.41^{a}	42.50±4.06^{a}	2.94±0.21^{a}	59.01±3.22^{a}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Time course of growth and biosurfactant production by *Pectinatus cerevisiiphilus* CT3

Time course studies were conducted on growth and biosurfactant production by *P. cerevisiiphilus* CT3 in MSM (pH 7.0) supplemented with 25 g/l glucose and 1 g/l $NaNO_3$ as carbon and nitrogen sources, respectively. A 5 % (v/v) overnight inoculum concentration was incubated at 30 °C in a rotary shaker at 150 rpm for 72 h (**Figure 1**). It was observed that this strain started to excrete biosurfactant after the lag phase, which was after 6 h. of cultivation, as indicated by a decrease in the surface tension of the culture media. Under the studied conditions, the log phase occurred and ranged 9 - 48 h. The surface tension of the culture medium was markedly reduced and it reached a minimum (28.0 mN/m) after 51 h of cultivation. However, the highest level of biosurfactant yield (3.05 g/l) and emulsification activity (60 %) was obtained as the cultivation time approached 54 h, which corresponded to the stationary phase of the microbial growth. Therefore, it can be concluded that the biosurfactant produced by *P. cerevisiiphilus* CT3 is a growth-associated production, or primary metabolite. In this case, there is a parallel relationship between the substrate utilization, growth, and biosurfactant production [33]. Growth-associated production of biosurfactant has been reported for *Pseudomonas fluorescens* Migula 1895-DSMZ [34], *Selenomonas ruminantium* CT2 [13], *Deinococcus caeni* PO5 and *Halobacteriaceae archaeon* AS65 [35]. From the results obtained, it can be seen that a cultivation time of 54 h can give the highest biosurfactant activity.

Recovery of biosurfactant

Crude extract of biosurfactant was recovered from the culture supernatant of *P. cerevisiiphilus* CT3 by precipitation and extraction with several organic solvents. The highest yield (5.45 g/ml) was obtained from acid precipitation; however, the CMC and surface tension at CMC of this method was high (40 mg/l and 60 mN/m, respectively) when compared with other recovery methods (**Table 5**). Normally, biosurfactants recovered by acid precipitation are high molecular-weight biosurfactants which tend to exhibit emulsification activity rather than the reduction of surface tension [13,14]. Among the six methods, chloroform:methanol was the most efficient in biosurfactant recovery with 3.05 g/l, 10 mg/l and 28.0 mN/m for yield, CMC and surface tension at CMC, respectively. It has also been previously reported that the extraction of biosurfactants consisting of hydrophilic and hydrophobic parts by a mixture of chloroform and methanol is highly efficient [11,36]. The efficiency of chloroform:methanol extraction may be a result of this solvent system containing both non-polar (chloroform, Log P = 1.97) and highly polar (methanol, Log P = -0.74) solvents. Thus, it is more effective than the single solvent (ethyl acetate, Log P = 0.73) used. Chloroform:methanol extraction has been reported for biosurfactant recovery from *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9 [37], *Leucobacter komagatae* 183 [11] and *Oleomonas sagaranensis* AT18 [12].

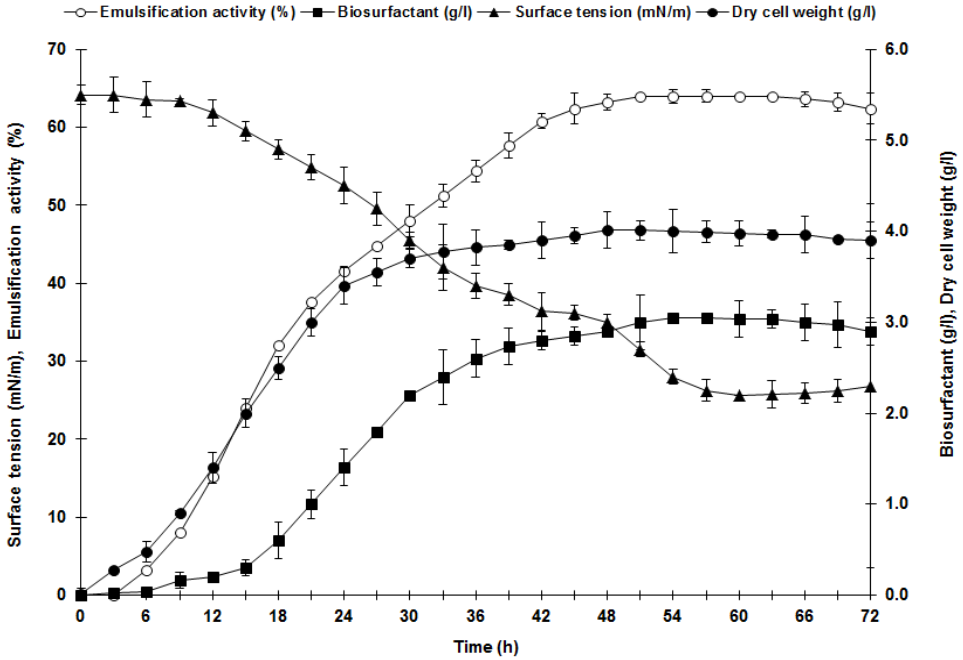

Figure 1 Time course of growth and biosurfactant production by *Pectinatus cerevisiiphilus* CT3 in optimal medium (25 g/l glucose and 1 g/l $NaNO_3$) at 150 rpm and 30 °C). Bars indicate standard deviations from triplicate determinations.

Table 5 Effect of extraction methods on yield and critical micelle concentration (CMC) of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3.

Recovery method	Yield (g/l)*	Critical micelle concentration (mg/l)*	Surface tension (mN/m)*
Acetone precipitation	5.15±1.12^{b**}	35.00±0.00^{b**}	55.0±2.50^{b**}
Acid precipitation	5.45±1.87^{a}	40.00±0.00^{a}	60.0±5.00^{a}
$(NH_4)_2SO_4$ precipitation	5.02±0.54^{b}	20.00±0.00^{c}	42.5±4.50^{c}
CH_3Cl:MeOH extraction	3.05±0.42^{d}	10.00±0.00^{d}	28.0±3.50^{f}
Ethanol precipitation	3.71±0.35^{c}	20.00±0.00^{c}	30.5±4.50^{e}
Ethyl acetate extraction	3.25±0.47^{d}	20.00±0.00^{c}	35.5±3.50^{d}

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Surface tension and Critical Micelle Concentration (CMC) of the biosurfactant

The surface tension of biosurfactant solution from *P. cerevisiiphilus* CT3 was measured as a function of concentration in order to compare the surface activities of excreted biosurfactant. A comparison of surface tension with biosurfactant concentration indicated that the surface tension of pure water rapidly decreased as the concentration of biosurfactant increased, and a minimum surface tension (28.0 mN/m) was observed (**Figure 2**). From the break point of the plotting of surface tension against biosurfactant concentration, the critical micelle concentration (CMC) was 10 mg/l. The CMC is a key characteristic of a surfactant [2,3]. Before the CMC is reached, according to the concentration of surfactant, the surface tension significantly varies; however, the surface tension remains relatively unchanged after reaching the CMC [1,4]. Temperature, pressure, and the presence or concentration of other surface-active substances and electrolytes play important roles as controllers of the CMC value for a given dispersant in a given medium [8]. Micelles only show above critical micelle temperature [6,7]. Biosurfactant production from *P. cerevisiiphilus* CT3 showed a lower minimum surface tension and CMC value than that of biosurfactant from *Bacillus subtilis* (29.0 mN/m, 40 mg/l) [38], *Rhodococcus* sp. strain PML026 (29.0 mN/m, 250 mg/l) [39], *Bacillus subtilis* LB5a (26.6 mN/m, 33.0 mg/l) [40], *Bacillus subtilis* PT2 (26.4 mN/m, 25.0 mg/l) and *Pseudomonas aeruginosa* SP4 (28.3 mN/m, 120 mg/l) [41], *Pseudomonas aeruginosa* S6 (33.9 mN/m, 50 mg/l) [42], *Pseudomonas aeruginosa* Bs20 (29.5 mN/m, 13.4 mg/l) [43], and *Lactobacillus paracasei* (41.8 mN/m, 250 mg/l) [44].

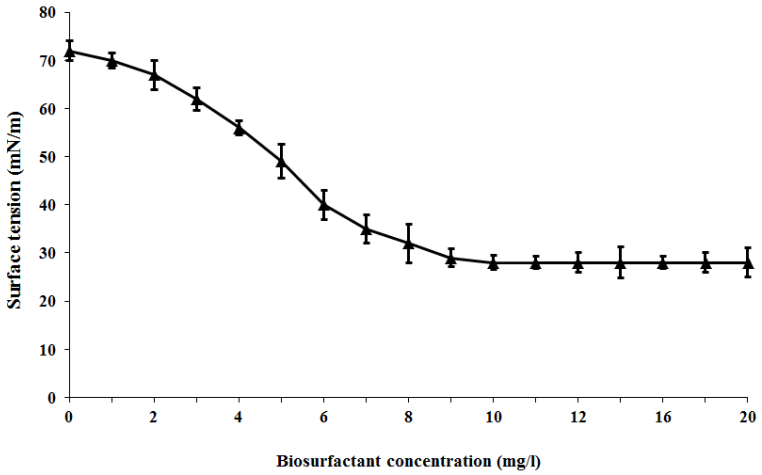

Figure 2 Surface tension vs biosurfactant concentration produced by *Pectinatus cerevisiiphilus* CT3.

Effect of temperature, pH and salinity on biosurfactant stability

The results obtained from thermal stability analysis of biosurfactant over a wide range of temperatures (4 - 121 ºC) revealed that the biosurfactant from *P. cerevisiiphilus* CT3 was thermostable (**Figure 3**). Heating of the biosurfactant solution up to 100 ºC (or its autoclaving at 121 ºC) caused little effect on emulsification capacity and surface tension activity. An approximately 15 % reduction in emulsification activity (E_{24}) and surface tension was observed after autoclaving at 121 ºC for 20 min. The surface tension reduction and E_{24} were relatively stable at such temperatures (ST ≈ 29 mN/m, E_{24} ≈ 57).

Figure 4 shows the effect of pH on the biosurfactant stability. The emulsification ability and surface activity of the obtained biosurfactant remained relatively stable for pH changes between pH 6 and 11, showing higher stability for alkaline conditions than acidic conditions. At pH 12, the E_{24} and surface tension values were 57 % and 32 mN/m, respectively. However, at pH 5, E_{24} decreased to 40 % and surface tension increased to 45 mN/m. In addition, for pH values lower than 4, the samples become turbid, due to partial precipitation of the biosurfactant. The optimum pH for both parameters, namely biosurfactant activity (ST = 28 mN/m) and emulsification capacity (E_{24} = 60), was determined to be 6 - 7.

Figure 5 demonstrates the effect of NaCl on the surface tension and E_{24} of obtained biosurfactant. As shown in Figure 5, negligible changes were observed in the surface activity for an increase in the NaCl concentration up to 12 %. Likewise, an increase in NaCl concentration up to 15 % did not significantly affect E_{24}. However, both E_{24} and surface activity were affected at NaCl concentrations higher than 15 %, with E_{24} decreasing to less than 50 % and surface activity increasing to more than 35 mN/m.

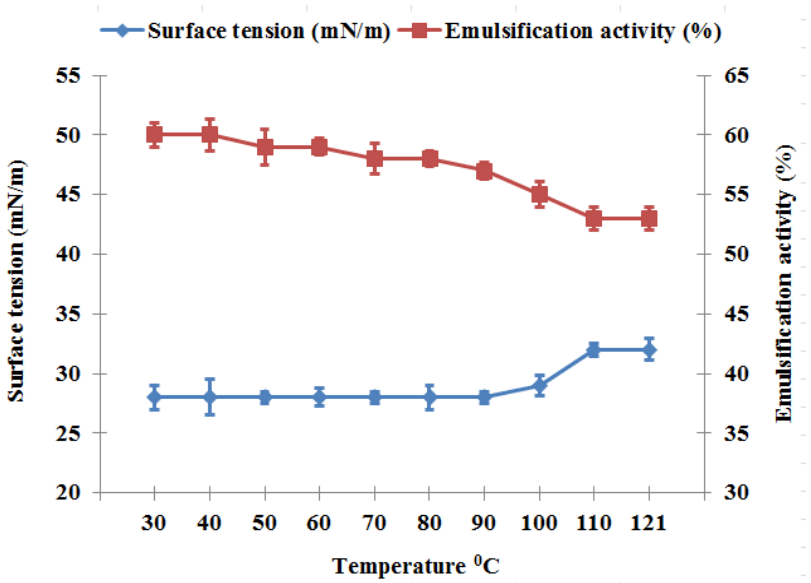

Figure 3 Effect of temperature on activity of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3. Bars represent the standard deviation from three determinations.

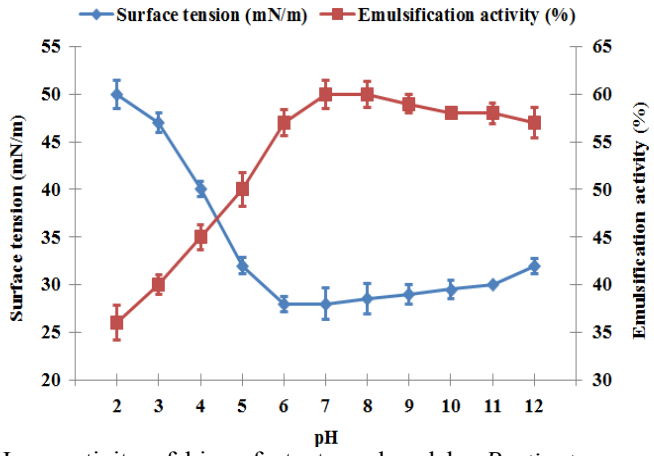

Figure 4 Effect of pH on activity of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3. Bars represent the standard deviation from three determinations.

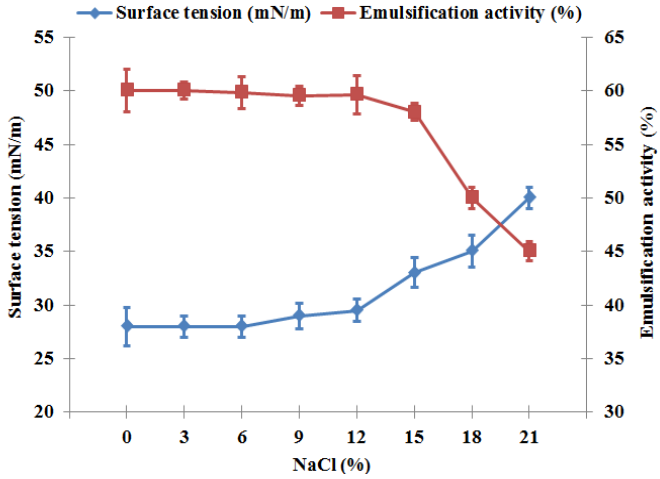

Figure 5 Effect of NaCl concentration on activity of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3. Bars represent the standard deviation from three determinations.

Biosurfactant Characteristics

The chemical nature of biosurfactant from *P. cerevisiiphilus* CT3 was observed as a single spot on TLC. This fraction showed a positive reaction with ninhydrin reagent and iodine vapor, indicating the presence of peptide and lipid moieties in the molecule (data not shown). The biochemical composition of the biosurfactant revealed that it is a mixture of proteins and lipids in a combination of 69 and 25 %, respectively. These results indicated the existence of lipopeptide biosurfactant. The lipopeptide nature of the biosurfactant was further confirmed by FT-IR spectra of the compound (**Figure 6**). The IR spectrum in KBr showed bands characteristic of peptides at 3,432 - 3,306 cm^{-1} (NH stretching mode). At 1,654 cm^{-1}, the stretching mode of a CO-N bond was observed, and at 1,552 cm^{-1}, a deformation mode of the NH bond combined with an NH stretching mode occurred. The presence of an aliphatic chain was indicated by the CH stretching modes at 2,958 - 2,854 and 1,464 - 1,022 cm^{-1}. These results strongly indicate that the biosurfactant contains aliphatic and peptide-like moieties. The biosurfactant composition contains more aliphatic than peptide-like moieties, making it preferable for use as a hydrophobic solvent rather than in a hydrophilic solution [45,46].

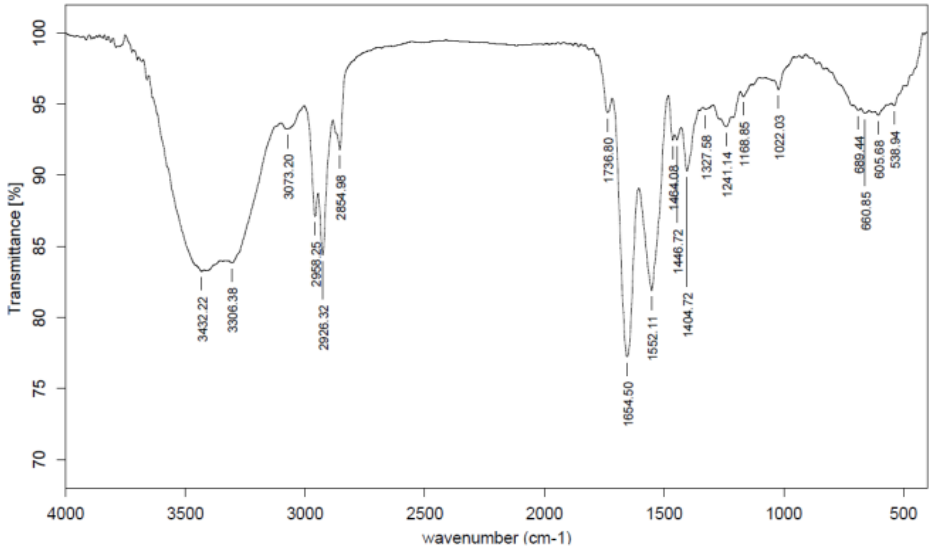

Figure 6 Fourier transform infrared spectrum of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3.

Antibacterial activity of biosurfactant

The antibacterial activity of biosurfactant from *P. cerevisiiphilus* CT3 is shown in **Tables 6** and **Figure 7**. Biosurfactant from *P. cerevisiiphilus* CT3 was found to have antimicrobial activity against several strains of the tested bacteria. The biosurfactant showed inhibitory activity against Gram-positive bacteria *B. cereus* and *S. aureus* and Gram-negative bacteria *E. coli*, *S. Thyphi*, *V. vulnifus* and *V. choleare*. The highest concentration tested (200 mg/ml), biosurfactant from *P. cerevisiiphilus* CT3 was active against all tested bacterial strains. The antimicrobial activity against Gram-positive bacteria was lower than the activity against Gram-negative bacteria. The biosurfactant was able to inhibit *B. cereus* and *S. aureus* in concentrations as low as 25 mg/ml. However, the MIC values for most of the tested Gram-negative bacteria (*E. coli*, *S. thyphi*, *V. vulnifus* and *V. choleare*) were 50 mg/ml. High MIC-values correlating with higher resistance were often seen when Gram-negative microorganisms were tested [47]. Gram-negative bacteria have been reported to be more resistant to external substances [48]. This higher level of resistance has been attributed to the presence of lipopolysaccharides in their outer membranes, making them naturally resistant to certain antibacterial agents [49]. On the other hand, Gram-positive organisms that were tested showed higher sensitivity towards the biosurfactant than the Gram-negative bacteria. The reason could be attributed to the differences between their cell wall compositions. Gram-positive bacteria contain an outer peptidoglycan layer, which is an infective permeability barrier [50].

Table 6 Antimicrobial activity of biosurfactant produced by *Pectinatus cerevisiiphilus* CT3 by agar well diffusion method against bacteria and fungi.

Microorganisms	Antimicrobial zone diameter (mm) at concentration (mg/ml)*				
	200	100	50	25	12.5
Gram-positive bacteria					
Bacillus cereus	20.23±3.21^{ab**}	18.53±3.06^{ab**}	11.17±4.15^{b**}	7.94±2.01^{b**}	0
Staphylococcus aureus	18.27±4.25^{bc}	16.93±6.15^{bc}	10.57±3.12^{bc}	6.06±2.15^{b}	0
Gram-negative bacteria					
Escherichia coli	12.11±2.10^{d}	8.53±1.06^{ef}	7.57±2.11^{de}	0	0
Salmonella Thyphi	10.14±2.15^{d}	7.27±2.15^{ef}	6.23±1.05^{de}	0	0
Vibrio vulnificus	20.48±4.10^{ab}	18.13±2.06^{ab}	10.97±3.12^{bc}	0	0
Vibrio cholerae	12.37±1.15^{d}	8.27±1.06^{ef}	7.03±2.15^{de}	0	0
Fungi					
Aspergillus niger	18.01±5.54^{bc}	15.52±7.21^{bc}	12.23±3.25^{b}	6.03±2.12^{b}	0
Aspergillus oryzae	16.86±5.29^{c}	12.97±4.54^{d}	10.4±4.87^{bc}	8.13±2.42^{ab}	0
Candida albicans	12.64±2.02^{d}	10.52±2.03^{de}	8.01±2.53^{cd}	6.59±1.02^{b}	0
Mucor sp.	22.43±4.12^{a}	19.43±1.37^{a}	15.05±2.84^{a}	9.59±1.02^{a}	0
Penicillium sp.	9.04±2.17^{d}	6.73±4.32^{f}	0	0	0
Phytophthora palmivora	20.38±3.62^{ab}	17.51±2.80^{abc}	12.66±1.09^{b}	8.03±2.51^{ab}	0
Rhizopus oryzae	10.81±2.21^{d}	8.80±3.60^{ef}	5.81±2.20^{e}	4.87±1.21^{c}	0

* Values are given as means ± SD from triplicate determinations.
** Different superscript letters in the same column indicate significant differences ($p < 0.05$).

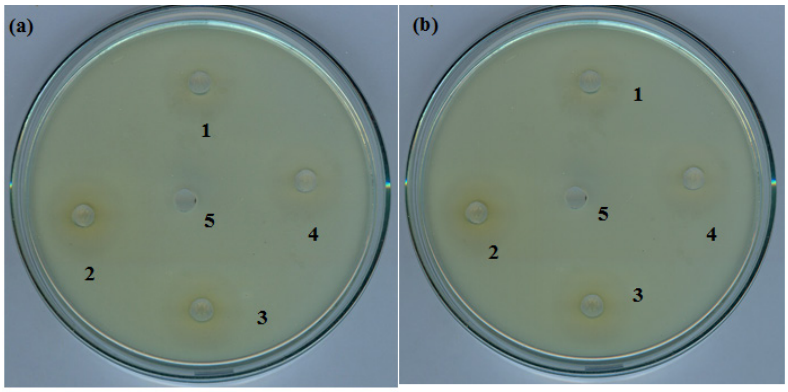

Figure 7 Antibacterial activity of biosurfactant from *Pectinatus cerevisiiphilus* CT3 against *Bacillus cereus* (a) and *Staphylococcus aureus* (b). Numbers 1 - 4 denote 25 mg/ml of biosurfactant dissolved in distilled water. Distilled water (5) was used as a control.

Antifungal activity of biosurfactant

The results of antifungal activity testing of biosurfactant from marine isolated *P. cerevisiiphilus* CT3 are given in **Table 6**. At the highest tested concentration, the obtained biosurfactant was active against all seven tested fungal strains. The highest inhibition was found for *Mucor* sp. followed by *Phytophthora palmivora, Aspergillus niger Aspergillus oryzae, Candida albicans, Rhizopus oryzae* and *Penicillium* sp., respectively. **Figure 8** shows the antifungal activity of the biosurfactant from isolated *P.*

cerevisiiphilus CT3 against *Mucor* sp. and *P. palmivora*. Several biosurfactants are known to contain peptides with antibiotic properties against bacteria, fungi and yeasts [3]. However, this is the first report describing the antimicrobial activity of biosurfactant obtained from genus *Pectinatus*.

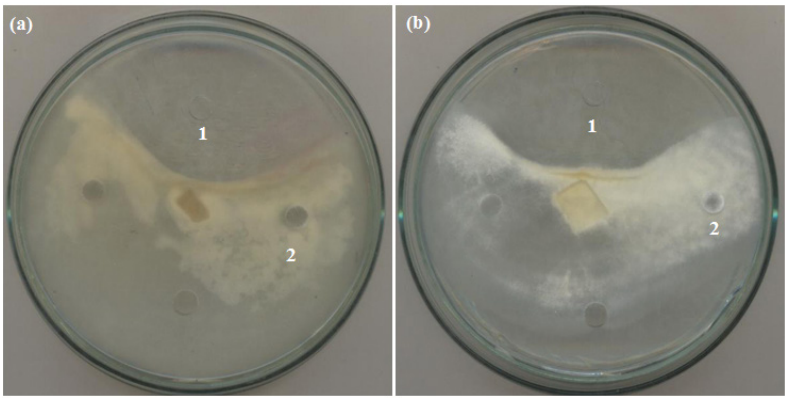

Figure 8 Antifungal activity of biosurfactant from *Pectinatus cerevisiiphilus* CT3 against *Mucor* sp. (a) and *Phytophthora palmivora* (b) after incubation in Sabouraud Dextrose Agar for 7 days. Number 1 denotes 25 mg/ml of biosurfactant dissolved in distilled water. Distilled water (2) was used as a control.

Conclusions

This study examined the optimization for biosurfactant production by *P. cerevisiiphilus* CT3. The growth characteristics were obtained, and the properties of the biosurfactant were studied for indications of its possible industrial application. The potential of this biosurfactant for industrial uses was examined by studying its physical properties, i.e., the surface tension, CMC and emulsification activity. The chemical characterization of the biosurfactant obtained from FT-IR spectroscopy revealed the presence of lipopeptide in the molecules. The properties of the biosurfactant that were obtained indicate its suitability as an alternative to synthetic medicines and antimicrobial agents, indicating that it may be used as a safe and effective therapeutic agent.

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Reference

[1] JD Desai and IM Banat. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev*. 1997; **61**, 47-64.
[2] AM Abdel-Mawgoud, F Lepine and E Deziel. Rhamnolipids: Diversity of structures, microbial origins and roles. *Appl. Microbiol. Biotechnol*. 2010; **86**, 1323-36.
[3] IM Banat, A Franzetti, I Gandolfi, G Bestetti, MG Martinotti, L Fracchia, TJ Smyth and R Marchant. Microbial biosurfactants production, applications and future potential. *Appl. Microbiol. Biotechnol*. 2010; **87**, 427-44.
[4] LR Rodrigues and JA Teixeira. Biomedical and therapeutic applications of biosurfactants. *Adv. Exp. Med. Biol.* 2010; **672**, 75-87.

[5] G Seydlová and J Svobodová. Review of surfactin chemical properties and the potential biomedical applications. *Cent. Eur. J. Med.* 2008; **3**, 123-33.
[6] I Mnif and D Ghribi. Review lipopeptides biosurfactants: Mean classes and new insights for industrial, biomedical, and environmental applications. *Biopolymers* 2015; **104**, 129-47.
[7] SK Satpute, GR Kulkarni, AG Banpurkar, IM Banat, NS Mone, RH Patil and SS Cameotra. Biosurfactant/s from Lactobacilli species: Properties, challenges and potential biomedical applications. *J. Basic. Microb.* 2016; **56**, 1140-58.
[8] M Nitschke and SS Silva. Recent food applications of microbial surfactants. *Crit. Rev. Food. Sci*. 2018; **58**, 631-8.
[9] EJ Gudiña, JA Teixeira and LR Rodrigues. Biosurfactants produced by marine microorganisms with therapeutic applications. *Mar. Drugs* 2016; **14**, 38.
[10] A Saimmai, O Rukadee, T Onlamool, V Sobhon and S Maneerat. Characterization and phylogenetic analysis of microbial surface active compounds producing bacteria. *Appl. Biochem. Biotechnol*. 2012; **168**, 1003-18.
[11] A Saimmai, V Sobhon and S Maneerat. Production of biosurfactant from a new and promising strain of *Leucobacter komagatae* 183. *Ann. Microbiol*. 2012; **62**, 391-402.
[12] A Saimmai, O Rukadee, T Onlamool, V Sobhon and S Maneerat. Isolation and functional characterization of a biosurfactant produced by a new and promising strain of *Oleomonassagaranensis* AT18. *World J. Microbiol. Biotechnol.* 2012; **28**, 2973-86.
[13] A Saimmai, T Onlamool, V Sobhon and S Maneerat. An efficient biosurfactant-producing bacterium *Selenomonas ruminantium* CT2, isolated from mangrove sediment in south of Thailand. *World J. Microbiol. Biotechnol*. 2013; **29**, 87-102.
[14] A Saimmai, S Udomsilp and S Maneerat. Production and characterization of biosurfactant from marine bacterium of *Inquilinus limosus* KB3 grown on low-cost raw materials. *Ann. Microbiol*. **63**, 1327-39.
[15] M Dubois, KA Gilles, JK Hamilton, PA Rebers and F Smith. Colorimetric method for determination of sugars and related substances. *Anal. Chem*. 1956; **28**, 350-6.
[16] OH Lowry, NJ Rosebrough, AL Farr and RJ Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 1951; **193**, 265-75.
[17] JM Folch, M Lees and HS Stanly. A simple method for the isolation and quantification of total lipids from animal tissues. *J. Biol. Chem*. 1956; **226**, 497-509.
[18] P Das, S Mukherjee and R Sen. Substrate dependent production of extracellular biosurfactant by a marine bacterium. *Bioresource Technol*. 2009; **100**, 1015-19.
[19] A Saimmai, V Sobhon and S Maneerat. Molasses a whole medium for biosurfactants production by Bacillus strains and their application. *Appl. Biochem. Biotechnol*. 2011; **165**, 315-35.
[20] F Candan, M Unlu, B Tepe, D Daferera, M Polissiou, A Sokmen and HA Akpulat. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *Millefolium* Afan. (Asteraceae). *J. Ethnopharmacol*. 2003; **87**, 215-20.
[21] JY Wu, KL Yeh, WB Lu, CL Lin and JS Chang. Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. *Bioresource Technol*. 2008; **99**, 1157-64.
[22] SH Kim, EJ Lim, SO Lee, JD Lee and TH Lee. Purification and characterization of biosurfactants from *Nocardia* sp. L-417. *Biotechnol. Appl. Biochem*. 2000; **31**, 249-53.
[23] AS Monteiroa, TT Mirandaa, I Lulab, AML Denadaic, RD Sinisterra, MM Santoroe and VL Santosa. Inhibition of *Candida albicans* CC biofilms formation in polystyrene plate surfaces by biosurfactant produced by *Trichosporon montevideense* CLOA72. *Colloid Surface B* 2011; **84**, 467-76.
[24] HS Jeong, DJ Lim, SH Hwang, SD Ha and JY Kong. Rhamnolipid production by *Pseudomonas aeruginosa* immobilized in polyvinyl alcohol beads. *Biotechnol. Lett*. 2004; **26**, 35-9.

[25] YH Wei, CL Chou and JS Chang. Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. *J. Biochem. Eng*. 2005; **27**, 146-54.
[26] NF Khairuddin, TETZ Mulok, KA Khalil, WSAW Omar and SH Saleh. *Screening of Medium with Different range of Waste Frying Oil (WFO), Sodium Nitrate (NaNO3) and Sodium Chloride (NaCl) for Biosurfactant Production by Thermophilic Anoxybacillus sp. using Fractional Factorial Design (FFD). In*: M Yusoff, N Hamid, M Arshad, A Arshad, A Ridzuan and H Awang. (Eds.). Springer, Singapore, 2015.
[27] C Dejwatthanakomol, J Anuntagool, M Morikawa and J Thaniyavarn. Production of biosurfactant by *Wickerhamomyces anomalus* PY189 and its application in lemongrass oil encapsulation. *Sci. Asia* 2016; **42**, 252-8.
[28] AK Yadav, S Manna, K Pandiyan, A Singh, M Kumar, H Chakdar, PL Kashyap and AK Srivastava. Isolation and characterization of biosurfactant producing *Bacillus* sp. from diesel fuel-contaminated site. *Microbiology* 2016; **85**, 56-62.
[29] AM Elazzazy, TS Abdelmoneim and OA Almaghrabi. Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. *Saudi. J. Biol. Sci.* 2015; **22**, 466-75.
[30] AS Santos, AP Sampaio, GS Vasquez, LMS Anna, N Pereira and DM Freire. Evaluation of different carbon and nitrogen sources in the production of rhamnolipids by a strain of *Pseudomonas*. *Appl. Biochem. Biotechnol*. 2002; **98**, 1025-35.
[31] M Benincasa, J Contiero, MA Manresa and IO Moraes. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. *J. Food. Eng*. 200; **254**, 283-8.
[32] HS Kim, JW Jeon, BH Kim, CY Ahn, HM Oh and BD Yoon. Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, by *Candida* sp. SY16 using fed-batch fermentation. *Appl. Microbiol. Biotechnol*. 2006; **70**, 391-6.
[33] L Rodrigues, J Teixeira, R Oliveira and HC der Mei. Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Process. Biochem*. 2006; **41**, 1-10.
[34] M Abouseoud, R Maachi, A Amrane, S Boudergua and A Nabi. Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. *Desalination* 2008; **223**, 143-51.
[35] CC Chooklin, S Maneerat and A Saimmai. Utilization of banana peel as a novel substrate for biosurfactant production by *Halobacteriaceaearchaeon* AS65. *Appl. Biochem. Biotechnol*. 2014; **173**, 624-45.
[36] MS Kuyukina, IB Ivshina, SO Makarov, LV Litvinenko, CJ Cunningham and JC Philip. Effect of biosurfactants on crude oil desorption and mobilization in a soil system. *Environ. Int*. 2005; **31**, 155-61.
[37] A Saimmai, V Sobhon and S Maneerat. Molasses as a whole medium for biosurfactants production by Bacillus strains and their application. *Appl. Biochem. Biotechnol*. 2011; **165**, 315-35.
[38] DA Vaz, EJ Gudina, EJ Alameda, JA Teixeira and LR Rodrigues. Performance of a biosurfactant produced by a *Bacillus subtilis* strain isolated from crude oil samples as compared to commercial chemical surfactants. *Colloid Surf. B* 2012; **89**, 167-74.
[39] DA White, LC Hirda and ST Ali. Production and characterization of a trehalolipid biosurfactant produced by the novel marine bacterium *Rhodococcus* sp., strain PML026. *J. Appl. Microbiol*. 2013; **115**, 744-55.
[40] M Nitschke and G Pastore. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. *Bioresource. Technol*. 2006; **97**, 336-41.
[41] O Pornsunthorntawee, P Wongpanit, S Chavadej, M Abe and R Rujiravanit. Structural and physicochemical characterization of crude biosurfactant produced by *Pseudomonas aeruginosa* SP4 isolated from petroleum-contaminated soil. *Bioresource. Technol*. 2008; **99**, 1589-95.

[42] H Yin, J Qiang, Y Jia, J Ye, H Peng, H Qin, N Zhang and B He. Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. *Process Biochem*. 2009; **44**, 302-8.
[43] AM Abdel-Mawgoud, MM Aboulwafa and NAH Hassouna. Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Appl. Biochem. Biotechnol*. 2009; **157**, 329-45.
[44] EJ Gudina, JA Teixeira and LR Rodrigues. Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei. Colloid. Surf. B* 2011; **76**, 298-304.
[45] JF Liu, SM Mbadinga, SZ Yang, JD Gu and BZ Mu. Chemical Structure, property and potential applications of biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation. *Int. J. Mol. Sci.* 2015; **16**, 4814-37.
[46] K Patowary, R Patowary, MC Kalita and S Deka. Characterization of biosurfactant produced during degradation of hydrocarbons using crude oil as sole source of carbon. *Front. Microbiol.* 2017; **8**, 279.
[47] R Tepsorn. 2009, Antimicrobial Activity of Thai Traditional Medicinal Plants Extract Incorporated Alginate-tapioca Starch based Edible Films against Food Related Bacteria Including Foodborne Pathogens. Ph. D. Dissertation, University of Hohenheim, Germany.
[48] PS Negi, AS Chauhan, GA Sadia, YS Rohinishree and RS Ramteke. Antioxidant and antimicrobial activities of various seabuckthorn (*Hippophaer hamnoides* L.) seed extracts. *Food. Chem*. 2005; **92**, 119-24.
[49] H Nikaido and M Vaara. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* 1985; **49**, 1-32.
[50] R Scherrer and P Gerhardt. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. *J. Bacteriol*. 1971; **107**, 718-35.