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The Effective Emulsifying Property of Biosurfactant–Producing *Marinobacter hydrocarbonoclasticus* ST1 Obtained from Palm Oil Contaminated Sites

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Abstract—The *Marinobacter hydrocarbonoclasticus* ST1, isolated from soil contaminated with palm oil from palm oil mill factories in southern Thailand, was evaluated as a potential biosurfactant with effective emulsifying property. This strain was able to produce biosurfactant with the maximum emulsification activity of 81% and emulsification index of 60% after 60 h of cultivation under the optimized cultivation conditions. The yield of biosurfactant obtained by chloroform:methanol (2 : 1, vol/vol) extraction was 1.85 g/L. The stability of the biosurfactant was effective over a wide range of pH, temperature and salinity. The high thermostability of the biosurfactant was assessed by simultaneous thermal analyzer. In addition, a preliminary chemical characterization using FT-IR, ^{1}H-NMR and ^{13}C-NMR indicated that it is glycolipid biosurfactant. The features of this biosurfactant make it a promising product for many industrial applications involving to extreme conditions.

Keywords: biosurfactant, emulsification activity, emulsification index, *Marinobacter hydrocarbonoclasticus* ST1, palm oil

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Palm oil industry is a major agro-based enterprise in Thailand particularly in the south of Thailand. The management of the ever-increasing organic waste resulting from palm oil discharge has been one of the most worrying environmental issues in the area. Using of waste from agro-industry as a substrate for biotechnological production could not only help reducing the costs of production but also pave the way for effective waste management. In natural environments, microbes occur almost always in a mixed population composed of a multitude of different strains and species. Microbes present in oil polluted environments produce various bioactive compounds. Biosurfactants belong to the bioactive compounds that have attracted major interest and attention [1]. Hydrocarbon-contaminated sites are the most promising for the isolation of biosurfactant-producing microorganisms [2, 3]. Moreover, agro-industrial wastes are considered as the promising substrate [4, 5].

Biosurfactants and bioemulsifiers are a class of microbial origin surface active compounds (SACs). A variety of microorganisms, mainly bacteria, fungi and yeasts, can produce a wide range of SACs that are amphiphilic metabolites consisting of hydrophobic and hydrophilic moieties [6]. These compounds are mainly classified according to their molecular weight, physico-chemical properties and mode of action. They can be divided into two main groups: low-molecular-weight and high-molecular-weight SACs. The low-molecular-weight biosurfactants, such as lipopeptides, glycolipids and flavolipids, play the role in surface tension reduction whereas the high-molecular-weight bioemulsifiers are involved in formation and stabilization of emulsion [7, 8]. Generally, high-molecular-weight bioemulsifiers are polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers [9]. Bioemulsifiers are not biosurfactants (they both emulsify, but only biosurfactants have the surfactant effect of reducing surface tension) [10]. Increasing interest in SACs led to an intense research for environment friendly and

cost-efficient production of SACs. Structural diversity and functional properties of SACs make them an attractive group of compounds for potential use is wide variety of industrial and environmental biotechnology. [1]. Elucidation of the structure and chemical composition of a biomolecule is essential [11].

SACs are attracting a pronounced interest due to their potential advantages over their synthetic counterparts, and to the fact that they could replace some of the synthetics in several industrial processes such as lubrication, wetting, softening, fixing dyes, making emulsion, stabilizing dispersions, foaming, preventing foaming, as well as in food, biomedical and pharmaceutical industry, and bioremediation of organic- or inorganic-contaminated sites [12]. Many studies have been carried out with different microbes to explore the SACs production based on the above facts. Therefore, the present study focused on the isolation biosurfactant-producing bacteria from palm oil-contaminated sites in southern Thailand. The isolate producing the highest performing emulsifying property was identified and characterized.

MATERIALS AND METHODS

Isolation and screening of biosurfactant-producing strains. Palm oil-contaminated soil, wastewater and decanter cake were collected from many sites of palm oil factories in Krabi, Surat Thani, Satun and Trang Province in southern Thailand. Bacteria were isolated using mineral salt medium (MSM) containing (g/L): K_2HPO_4—0.8, KH_2PO_4—0.2, $CaCl_2$—0.05, $MgCl_2$—0.5, $FeCl_2$—0.01, $(NH_4)_2SO_4$—1.0 and NaCl—5.0 [13]. The pH of the medium was adjusted to 7.0 before autoclaving. One gram/mL of sample was added aseptically to 5 mL of 0.85% NaCl and 100 μL of the suspension was spread on MSM agar supplemented with palm oil (1%, vol/vol) as a carbon source followed by incubation at 30 ± 3°C for 4–5 days. Subsequently, bacterial colonies with different morphology were picked, purified on MSM agar and kept in nutrient broth containing 20% glycerol at –20°C.

The bacterial isolates were screened for their ability to produce biosurfactant. Inocula of cultures of isolated bacteria were prepared in nutrient broth (NB, HiMedia, India) and OD_{660} was adjusted to 0.5. Then, it was inoculated in 20 mL MSM medium supplemented with 1% (vol/vol) palm oil or 1% (wt/vol) glucose as a carbon source, followed by kept on incubator shaker (200 rpm) at 30 ± 3°C for 48 h.

16S rRNA gene sequence analysis. Species identification of selected bacterial isolate was done on the basis of 16S rRNA gene sequencing. The genomic DNA was extracted and 16S rRNA sequence was amplified by using universal primer 27F and 1492R (5'-AGAGTTTGATCATGGCTCAG-3'; 5'-GGTACCTTGTTACGACTT-3') [14]. The resulting sequence was compared with sequences in the GenBank database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the nucleotide-nucleotide blast (BLASTn) network service.

Culture medium optimization and time course of biosurfactant production. MSM medium was used throughout this experiment. The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed under a specific set of conditions. Two factors were chosen aiming to obtain the higher productivity of a biosurfactant: carbon source (C) and nitrogen source (N). The carbon sources used were 1% (wt/vol) of glucose, commercial sugar, molasses, soybean oil, palm oil and rice bran oil, with 0.1% (wt/vol) $(NH_4)_2SO_4$ as a nitrogen source. A medium with no carbon source was used as the control. For evaluation of the most appropriate nitrogen sources for the production of a biosurfactant, peptone, yeast extract, urea, $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$ were employed at a concentration of 0.1% (wt/vol) with the optimum carbon source. A medium with no nitrogen source was used as control assay. Finally, optimum amount of carbon and nitrogen sources were determined. To find optimum conditions for biosurfactant production, the selected strain was grown in a 250 mL flask containing 50 mL of MSM medium using 5% (vol/vol) of inocula containing optimum amount of carbon and nitrogen sources with pH 7.0, incubated in an orbital shaker (150 rpm) at 30 ± 3°C for 72 h. Samples were taken at different time intervals to measure microbial growth by dry cell weight and its ability to emulsify palm oil by measuring emulsion activity (%EA and %EI).

Recovery of biosurfactant. For recovery of crude biosurfactant, cells were separated from the cultures broth by centrifugation at 6000 × *g* for 10 min at 4°C. The supernatant was tested for biosurfactant extraction. Five precipitation methods (acid, acetone, $(NH_4)_2SO_4$, methanol and ethanol) and solvent extraction (chloroform-methanol (2 : 1, vol/vol)) for recovery of biosurfactant were perform according to Saimmai et al. [15] with modifications. The method showing the highest emulsion activity was used to recover the crude biosurfactant from *Marinobacter hydrocarbonoclasticus* ST1. Biosurfactant production in the culture broth was detected by the method described by Cooper and Goldberg [19].

Characterization of biosurfactant. *Stability of biosurfactant.* The crude biosurfactant (1 mg/L) was dissolved in distilled water. To investigate the effects of pH, sodium chloride, magnesium chloride, calcium chloride and temperature on emulsion activity, the biosurfactant solution was adjusted with 1 N HCl or 1 N NaOH to obtain a pHs from 2.0 to 12.0. NaCl was added to the sample to obtain the final concentrations of 0–12% (wt/vol), $MgCl_2$ and $CaCl_2$ used in the range of 0–0.1% (wt/vol). For the thermal stability study, biosurfactant solution was incubated at 25–100°C for

1 h and at 110 and 121°C for 15 min and cooled to 30°C. The remaining activity was then determined.

FT-IR. Structural characteristics of biosurfactant were determined using FT-IR spectrophotometer (Bruker, Germany). The partially purified biosurfactant was ground, mixed with KBr powder and then pressed to form a pellet for measurement in a frequency range of 4000–400 cm^{-1}.

^1H and ^{13}C NMR. NMR data was obtained to elucidate chemical structure of the partially purified biosurfactant. The sample was tested as solution in chloroform. ^1H and ^{13}C NMR spectra were recorded on a Model Varian Unity@ Inova 500 spectrometer (Germany) (operation frequency of 499.573 MHz for ^1H and 125.632 MHz for ^{13}C). The chemical shifts were expressed in ppm relative to a resonance of Me_4Si used as an internal standard.

Thermogravimetric analysis. Thermal behavior of the biosurfactant was studied by thermogravimetric analysis and differential scanning calorimetry (DSC) using simultaneous thermal analyzer (STA 8000, Perkin Elmer, USA). The sample was subjected to a temperature in the range of 30–480°C under nitrogen atmosphere at a rate of 10°C/min and the corresponding weight loss was determined.

Analytical methods. Biomass determination was done in terms of dry cell weight. At different times of fermentation, samples were mixed in pre-weighted tube with chilled distilled water and centrifuged at 6000 × *g* for 30 min. The biomass obtained was dried overnight at 105°C and weighed.

Tests for determination of the emulsification activity (%EA) and emulsification index (%EI) were performed to evaluate the emulsifying ability of culture supernatant following the method described by Cooper and Goldberg [16]. The activity was determined by mixing 1 mL palm oil with 1 mL culture supernatant in a test tube, vortexing at high speed for 2 min and the mixture was allowed to stand for 1 h (%EA) and 24 h (%EI). Upon standing, a creamy emulsion was formed when an emulsifier was present. Then the total height of the suspension and the height of the emulsified layer were determined by a measuring scale. Emulsion activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

All experiments were carried out in triplicate for the calculation of the mean value. All chemicals used were of analytical grade. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, USA).

RESULTS AND DISCUSSION

Isolation and screening of biosurfactant-producing strains. MSM with 1% (wt/vol) palm oil as sole carbon source was used to isolate biosurfactant-producing bacteria from soil, wastewater and decanter cake collected from many sites of palm oil factories in Krabi, Surat Thani, Satun and Trang Province in southern Thailand. A total of 421 bacterial isolates were obtained based on distinctly different colony morphology and all samples were screened for biosurfactant production by using 1% (wt/vol) palm oil or glucose as sole carbon source. Among them, 20 bacterial isolates were identified as biosurfactant-producing bacteria using emulsification activity test (%EA). Eight and twelve isolates showed emulsification activity by using glucose and palm oil as sole carbon source, respectively. The most of isolated bacteria (75%) were Gram-negative (Table 1). It has previously been reported that most bacterial isolates from site with a history of contamination by oil are Gram-negative and this may be a characteristic that contributes to survival of these populations in such harsh environments [4, 15, 17].

The most of bacterial isolates demonstrated emulsification activity with palm oil between 25–45%. Potential biosurfactant should have emulsification activity greater than 50% [18]. Only 4 isolates (KB2, ST1, ST2 and TR31) showed %EA more than 50%. All bacterial isolates had different emulsion activity depending primarily on the microbial strain, source of carbon and nitrogen, pH and temperature and addition of water-immiscible substrates to media [19]. The isolate that showed the highest %EA was isolate ST1 (55.01%).

Identification of selected strain. The isolate ST1 was identified by combining the alignment results of the 16S rRNA sequence analysis with biochemical and physiological characteristics. Based on the 16S rRNA gene sequence and using the GenBank BLAST tool, this isolate ST1 was found to be closely related to *Marinobacter hydrocarbonoclasticus* ss40 with a percentage of similarity of 100%. The 16S rRNA sequence of the strain ST1 was deposited in the GenBank database under accession number JN160781. The genus *Marinobacter* belongs to the family Alteromonadaceae, in Proteobacteria [20]. It is a Gram-negative, rod-shaped (coccobacilli, straight/curved), non-spore forming bacterium which shows motility by means of single unsheathed polar flagellum. *Marinobacter* (previously named as *Alteromonas*) produces large amounts of a non-dialyzable bioemulsifier [21]. Uad et al. [22] characterized *Marinobacter* spp. isolated from seawater able to produce bioemulsifier and to show hydrocarbon-degrading ability. Moreover, Mohanram et al. [17] screened and characterized surface-active agent-producing, oil-degrading marine bacteria of Mumbai Harbor (India). *M. hydrocarbonoclasticus* strains MR2, RMR3, and RMR38 showed high stable emulsion index.

Culture medium optimization and time course of biosurfactant production. The cell growth and biosurfactant production can be influenced by the composition of the medium. Appropriately, the effect of various carbon and nitrogen sources on the growth of *M. hydrocarbonoclasticus* ST1 and biosurfactant production was

Table 1. Gram's staining and emulsification activity (%EA) of isolated strains

Isolate	Firm	Carbon source	Gram's stain	EA, %
KB1	Krabi Oil Co., Ltd	Glucose	Negative	41.50 ± 3.80*
KB2	Krabi Oil Co., Ltd	Palm oil	Positive	52.51 ± 6.30
KB3	Nam Hong Palm oil Co., Ltd	Glucose	Negative	31.87 ± 4.41
KB4	Nam Hong Palm oil Co., Ltd	Palm oil	Negative	40.42 ± 5.17
KB5	Palmmorich Co., Ltd	Palm oil	Negative	35.54 ± 6.22
KB6	Palmmorich Co., Ltd	Glucose	Negative	28.93 ± 3.18
SR1	Southern Palm Co., Ltd	Palm oil	Positive	35.58 ± 5.01
SR2	Thai Palm Development Co., Ltd	Palm oil	Negative	30.45 ± 3.22
SR3	Thai Palm Development Co., Ltd	Glucose	Negative	34.53 ± 2.87
SR4	Larpthavee Palm Oil Co., Ltd	Palm oil	Negative	30.50 ± 3.57
SR5	Larpthavee Palm Oil Co., Ltd	Glucose	Negative	32.17 ± 7.52
SR6	Saharungsab Palm Oil Co., Ltd	Palm oil	Positive	44.82 ± 8.18
ST1	Thai Palm Development Co., Ltd	Palm oil	Negative	55.01 ± 5.27
ST2	Thai Palm Development Co., Ltd	Glucose	Negative	51.64 ± 4.85
ST3	Saharungsab Palm Oil Co., Ltd	Palm oil	Negative	42.57 ± 7.85
ST4	Saharungsab Palm Oil Co., Ltd	Glucose	Negative	37.82 ± 5.07
ST5	Saharungsab Palm Oil Co., Ltd	Palm oil	Positive	25.10 ± 4.88
TR1	Trang Palm Oil Co., Ltd	Palm oil	Positive	30.09 ± 5.42
TR2	Trang Palm Oil Co., Ltd	Glucose	Negative	38.27 ± 6.08
TR3	Trang Palm Oil Co., Ltd	Palm oil	Negative	51.38 ± 4.89

* Results represented mean ± standard deviation from 3 determinations.

Table 2. Effect of carbon source on biosurfactant production by *M. hydrocarbonoclasticus* ST1 cultivated in MSM medium at 30°C and 150 rpm for 48 h

C-source (1%, wt/vol)	Dry cell weight, g/L	Final pH	EA, %	EI, %
Control**	0.15 ± 0.02	6.98 ± 0.01	2.57 ± 0.58^{d}*	0^{e}*
Glucose	2.59 ± 0.08	4.51 ± 0.07	30.51 ± 5.02^{c}	15.04 ± 5.02^{d}
Commercial sugar	2.70 ± 0.05	4.62 ± 0.05	28.70 ± 3.05^{c}	20.35 ± 6.14^{c}
Molasses	2.81 ± 0.03	4.63 ± 0.04	60.08 ± 4.25^{a}	40.40 ± 4.25^{a}
Soybean oil	1.21 ± 0.23	6.35 ± 0.06	32.74 ± 5.15^{c}	14.84 ± 3.17^{d}
Palm oil	1.31 ± 0.61	6.13 ± 0.08	55.01 ± 3.26^{b}	34.21 ± 4.25^{b}
Rice bran oil	1.40 ± 0.41	6.31 ± 0.05	29.78 ± 5.16^{c}	19.75 ± 3.06^{c}

* Different letters in the same column indicate significant different ($p < 0.05$)
** Control: no carbon source. Results represented mean ± standard deviation from 3 determinations.

examined. Table 2 represents the cell growth and emulsion activity produced by *M. hydrocarbonoclasticus* ST1 using different carbon sources. Comparison between water-soluble (glucose, commercial sugar and molasses) and water-immiscible carbon sources (soybean oil, palm oil and rice bran oil) was performed. The results showed that *M. hydrocarbonoclasticus* ST1 grew better in water-soluble carbon sources. This biomass was about 1–1.5-fold more of that obtained from water-immiscible carbon sources used. Molasses was the most appropriate carbon source with the maximum growth and emulsion activity. Growth also showed increase in dry cell weight, with the increased in concentration of molasses from 0–3% (wt/vol) (data not shown). This result can be compared with that obtained by Abbasi et al. [23] demonstrated that water-insoluble substrates were more effective on biosurfactant production in *Pseudomonas aeruginosa*. The biosurfactant production is the most effective in the presence of vegetable oils. Maximum biosurfactant yield was obtained when corn and soybean oils were used as carbon source. Moreover, there is evidence that nitrogen plays an important role in the production of biosurfactant compound by microorganisms. *M. hydrocarbonoclasticus* ST1 could utilize a wide range of nitrogen sources for growth. Among the nitrogen sources used, organic nitrogen sources better support the growth than inorganic nitrogen compounds. The highest growth occurred in the presence of peptone. However, $NaNO_3$ exhibited the highest emulsion activity (Table 2). Similar observations have been reported by Saimmai et al. [15] showed that

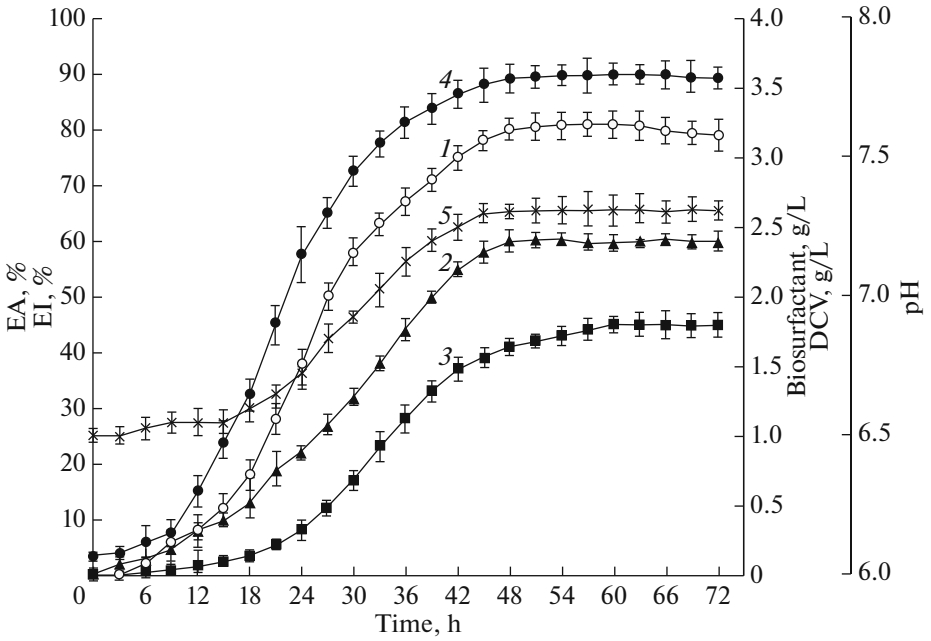

Fig. 1. Time course of growth, biosurfactant production and emulsion activity of *M. hydrocarbonoclasticus* ST1 under optimal medium conditions. *1*—EA, %; *2*—EI, %; *3*—biosurfactant, g/L; *4*—DCW, g/L and *5*—pH.

$NaNO_3$ was the most efficient nitrogen source for *Oleomonas sagaranensis* AT18 to produce biosurfactant.

The growth characteristics and biosurfactant production of *M. hydrocarbonoclasticus* ST1 were studied by using MSM medium containing 5% (vol/vol) of inocula, 2.5% molasses as a carbon source, 0.3% $NaNO_3$ as a nitrogen source (pH 7.0) during incubation at 30 ± 3°C and 150 rpm for 72 h. Bacterial growth (DCW), biosurfactant concentration (g/L), %EA, %EI and pH of culture broth were monitored during incubation time (Fig. 1). The biosurfactant production by this strain started during the exponential phase beginning after 6 h of growth and continued up to the stationary phase. The maximum growth and biosurfactant production occurred after 60 h of cultivation. The highest biomass yield was 3.60 g/L of DCW and the highest yield of biosurfactant production, as determined by acid precipitation, was found to be 1.85 g/L after 60 h of cultivation. Moreover, the culture was tested for the %EA and %EI. It can be seen that a cultivation time of 60 h gave the highest activity of 81% EA and 60% EI. It evidenced the growth associated pattern of biosurfactant production. The production profile of biomass and biosurfactant as a function of time revealed that it was primary metabolite. However, in other reports, a partial growth-associated biosurfactant production profile was observed, in which the biosurfactant production continued during the stationary growth phase. Panjiar et al. [4] reported growth kinetics and bioemulsifier production of *Lysinibacillus* sp. SP1025 and *Bacillus cereus* SP1035 isolated from oil-contaminated sites. In *Lysinibacillus* sp. SP1025, bioemulsifier production started after 12 h of incubation period and attained a maximum value after 24 h of incubation reaching 83.3% EI. However, in the case of *B. cereus* SP1035, bioemulsifier production started when the strain was in the middle of the log phase of its growth and reached a maximum 76.5% EI after 48 h of incubation. Gudina et al. [24] showed the kinetics of cell growth and bioemulsifier production by *Paenibacillus* sp. isolated from crude oil. The bioemulsifier was produced in the presence of sucrose with and without adding hydrocarbons (paraffin or crude oil) under aerobic and anaerobic conditions at 40°C. Bioemulsifier production was found to be growth-associated in both cases, as a parallel relationship was observed between the biomass production and emulsifying activity. Also, Kim [25] revealed that biosurfactant synthesis from *Bacillus pumilus* IJ-1 isolated from crude oil sample was growth-associated, producing mainly at the stationary phase.

Recovery of biosurfactant. The biosurfactant was precipitated or extracted from the culture supernatant of *M. hydrocarbonoclasticus* ST1. Among six methods of precipitation and extraction, chloroform:methanol (2 : 1) was the most efficient in biosurfactant recovery from culture supernatant of this strain (Table 3). A recovery yield of 1.85 g/L was obtained from *M. hydrocarbonoclasticus* ST1. Panjiar et al. [4] reported the amount of bioemulsifier produced, as recovered by the acid precipitation method. It was found to be 3.07 ± 0.62 and 3.90 ± 0.3 g/L for *Lysinibacillus* sp. SP1025 and *Bacillus cereus* SP1035, respectively.

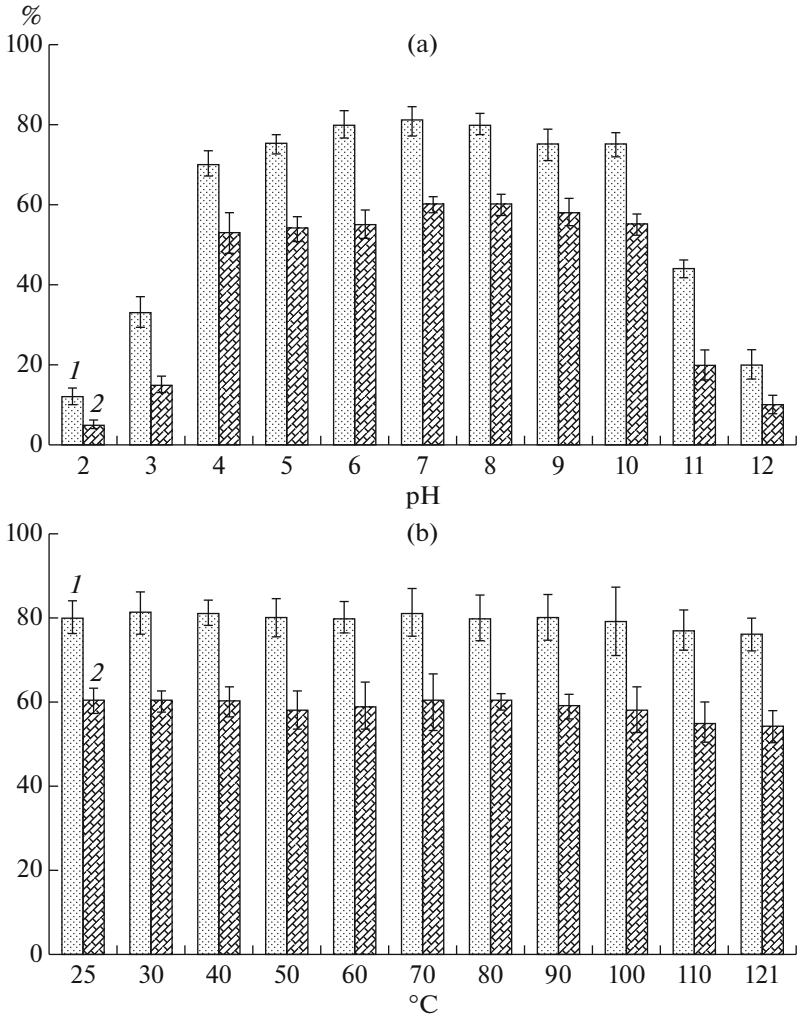

Fig. 2. Effect of pH (a) and temperature (b) on emulsion activity of crude biosurfactant produced by *M. hydrocarbonoclasticus* ST1. *1*—EA, %; *2*—EI, %.

Characterization of biosurfactant. *Stability of biosurfactant.* Crude biosurfactant was used to study the effect of pH, temperature and salinity on emulsion stability. The effects of various pHs in the range of 2.0–12.0 on the emulsion activity (%EA and %EI) of biosurfactant are presented in Fig. 2a. Biosurfactant from *M. hydrocarbonoclasticus* ST1 showed broad range of emulsion activity between pH from 4.0 to 10.0. The lowest emulsion activity was observed under acidic (pH < 4.0) and alkaline (pH > 11.0) conditions

Table 3. Effect of nitrogen source on biosurfactant production by *Marinobacter hydrocarbonoclasticus* ST1 *M. hydrocarbonoclasticus* ST1 cultivated in MSM medium at 30°C and 150 rpm for 48 h (carbon source: 2.5% molasses)

N-source (0.1%)	Dry cell weight, g/L	Final pH	EA, %	EI, %
Control	1.24 ± 0.27	5.98 ± 0.02	20.58 ± 3.78^{g*}	10.41 ± 4.05^{f*}
Peptone	4.80 ± 0.38	8.27 ± 0.04	36.81 ± 4.51^{f}	25.04 ± 3.25^{e}
Yeast extract	4.29 ± 0.44	4.60 ± 0.05	52.27 ± 5.15^{d}	40.57 ± 4.67^{d}
Urea	3.85 ± 0.12	7.94 ± 0.02	47.58 ± 4.81^{e}	35.47 ± 5.24^{b}
$(NH_4)_2SO_4$	3.61 ± 0.31	4.23 ± 0.04	68.34 ± 2.05^{b}	48.85 ± 2.18^{a}
NH_4Cl	3.20 ± 0.21	4.81 ± 0.03	56.80 ± 5.73^{c}	42.61 ± 6.04^{c}
$NaNO_3$	3.58 ± 0.54	7.51 ± 0.41	72.15 ± 5.02^{a}	56.88 ± 5.02^{a}

* Different letters in the same column indicate significant different ($p < 0.05$).
Control: No nitrogen source.
Results represented mean ± standard deviation from 3 determinations.

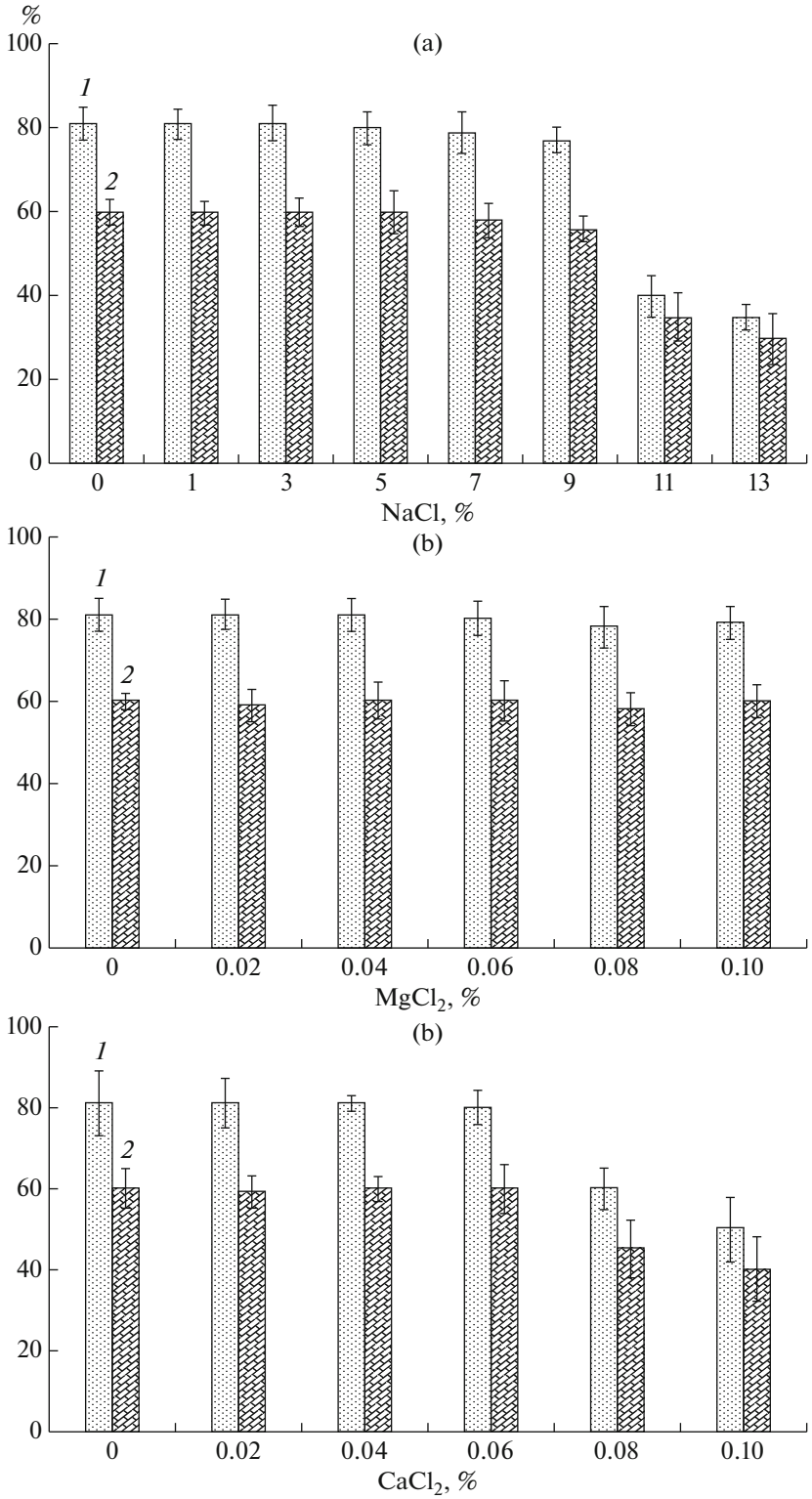

Fig. 3. Effect of NaCl (a), $MgCl_2$ (b) and $CaCl_2$ (c) on emulsion activity of crude biosurfactant produced *M. hydrocarbonoclasticus* ST1. *1*—EA, %; *2*—EI, %.

due to precipitation of the bioemulsifier [26]. Thermal stability analysis over a wide range of temperatures (25–121°C) showed that the biosurfactant had considerable stability under studied conditions (Fig. 2b). Furthermore, study on the effect of salts and salt concentrations on emulsion activity was performed (Fig. 3). Emulsion stability retains up to 9% NaCl, 0.1% $MgCl_2$ and 0.06% $CaCl_2$ and thereafter a considerable reduc-

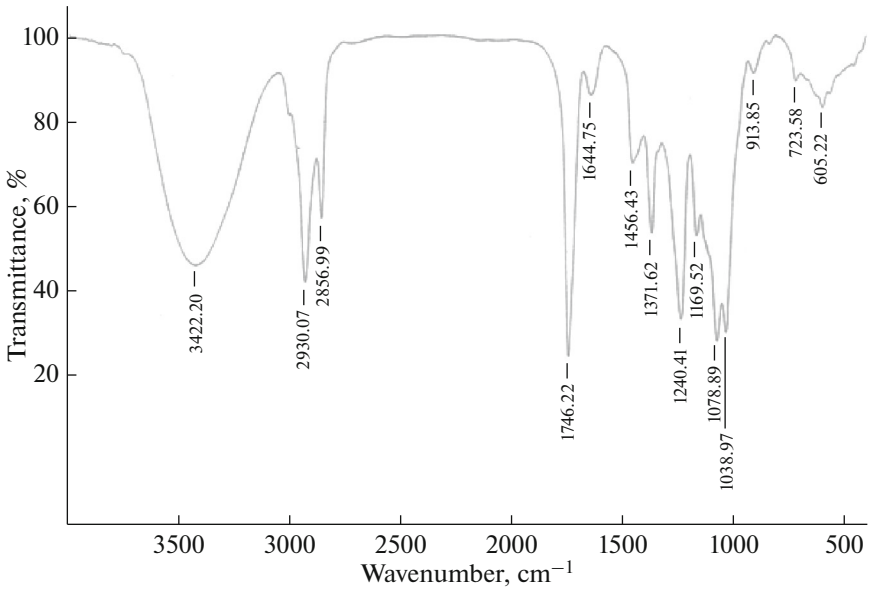

Fig. 4. FT-IR spectrum of the biosurfactant produced by *M. hydrocarbonoclasticus* ST1.

tion occurred at higher concentration. Biosurfactants were reported stable at various temperatures, pH and salinity. Gudina et al. [24] reported novel bioemulsifier produced by a *Paenibacillus* strain isolated from crude oil. Its emulsifying ability was not affected by exposure to high salinities (up to 300 g/L), high temperatures (100–121°C) or a wide range of pH values (2.0–13.0). Similarly, the bioemulsifier from *Solibacillus silvestris* AM1 was to be thermalstable and active in the pH from 5.0 to 9.0 and 0–5 M NaCl range [11]. Also, an *Ochrobactrum pseudintermedium* strain C1 that secrets an exopolysaccharide as a bioemulsifier showed its properties over a wide range of pH (2.0–8.0), at moderate salinity (4–6% NaCl), and during exposure to elevated temperatures (100°C) [27]. These interesting properties offer the opportunities for the biosurfactants to be investigated in extreme environment for microbial enhanced oil recovery and *in situ* biodegradation of oil sludge. In addition, their use is possible in industrial processes for food and pharmaceutics frequently involving exposure to extremes of temperature, pressure, pH and ionic strength [9].

FT-IR. For identification of the main functional groups present in the biosurfactant produced by *M. hydrocarbonoclasticus* ST1, it was submitted to FT-IR. Figure 4 shows the FT-IR spectrum of biosurfactant. The presence of a range broad band at 3422.20 cm^{-1} characteristic of -OH group assigned to the carboxylic group of sugar moiety [28] and a set of intense bands within the 1200–1000 cm^{-1} region (1169.52, 1078.89 and 1038.97 cm^{-1}) were assigned to the vibration of the C–O and C–O–C glycosidic bands, demonstrating the occurrence of carbohydrates [29]. In addition, the C–O stretching bands at 1456.43 cm^{-1} and 1371.62 cm^{-1} confirm the presence of the bands formed between carbon atom and hydroxyl groups in the chemical structures of the glycoside part [3]. On the other hand, it was observed the presence of some characteristic band of fatty acids. The band at 1746.22 cm^{-1} was assigned to C=O stretch band related to α-ß-unsaturated esters [30].

Table 4. Effect of recovery method on yield, EA and EI of the biosurfactant produced by *M. hydrocarbonoclasticus* ST1

Recovery method	Yield, g/L	EA, %	EI, %
Acid precipitation	$6.25 \pm 1.45^{a*}$	$45.11 \pm 5.50^{a*}$	$35.17 \pm 3.50^{a*}$
Acetone precipitation	5.12 ± 1.5^{a}	55.54 ± 3.64^{a}	45.57 ± 4.78^{a}
$(NH_4)_2SO_4$ precipitation	4.44 ± 0.79^{a}	56.90 ± 5.87^{a}	40.08 ± 3.59^{a}
MeOH precipitation	2.15 ± 0.58^{b}	64.75 ± 6.48^{b}	54.58 ± 3.68^{b}
EtOH precipitation	2.16 ± 0.89^{b}	71.89 ± 5.45^{b}	51.97 ± 5.75^{b}
CH_3Cl : MeOH extraction	1.85 ± 0.18^{b}	81.00 ± 1.38^{b}	60.01 ± 4.63^{b}

* Results represented mean ± standard deviation from 3 determinations.

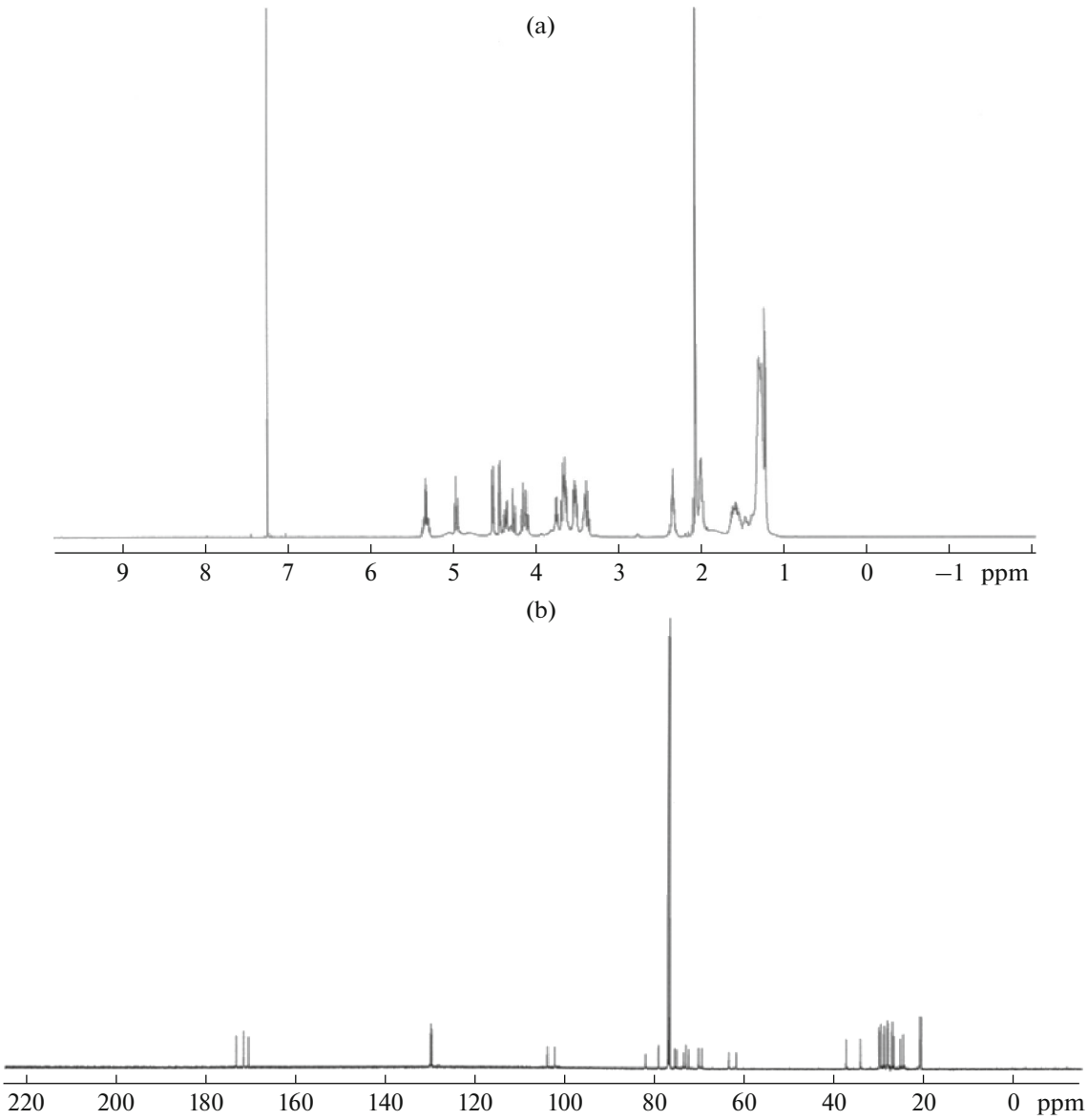

Fig. 5. ^{1}H (a) and ^{13}C (b) nuclear magnetic resonance spectra of the biosurfactant produced by *M. hydrocarbonoclasticus* ST1.

Aliphatic components of the lipid moiety were visualized by two bands at 2930.07 and 2856.91 cm^{-1} assigned to C–H asymmetric stretch of CH_2 and CH_3 groups, respectively [31]. The asymmetric vibrations of aliphatic stretching of C–H bands confirmed the presence of alkanes in the biosurfactant. The C=O stretching band of unsaturated ether was formed at the intense band at 1644.75 cm^{-1} and an absorption band at 913.85 cm^{-1} showed stretching mode of the $CH=CH_2$ which evidenced the presence of alkenes [32]. Overall, the FT-IR spectrum suggested that the biosurfactant produced by *M. hydrocarbonoclasticus* ST1 has been classified as a glycolipid with carbohydrate and lipid combination. The FT-IR spectra of this biosurfactant displayed a significant similarity in adsorption of other glycolipids [1, 2, 23, 24, 32, 33].

^{1}H-NMR and ^{13}C NMR. The structural characterization of the biosurfactant produced by *M. hydrocarbonoclasticus* ST1 was confirmed by ^{1}H-NMR and ^{13}C NMR spectra. ^{1}H-NMR spectrum revealed a number of proton signals corresponding to different functional groups assigned to lipid (fatty acid) and sugar (carbohydrate) moieties (Fig. 5a). The signals at 0.2–2.2 ppm are characteristic of CH_3, CH_2 and CH groups present in long chains of aliphatic fatty acids [23, 29]. The signal at 3.4–5.4 revealed the presence of glycosidic linkage of sugar molecules [31, 32]. The presence of characteristic of aliphatic and hydroxyl groups in the oligosaccharide-lipid complex was also confirmed by ^{13}C NMR (Fig. 5b). The resonances at 20–40 ppm assigned to aliphatic $CH_3/CH_2/CH$ moi-

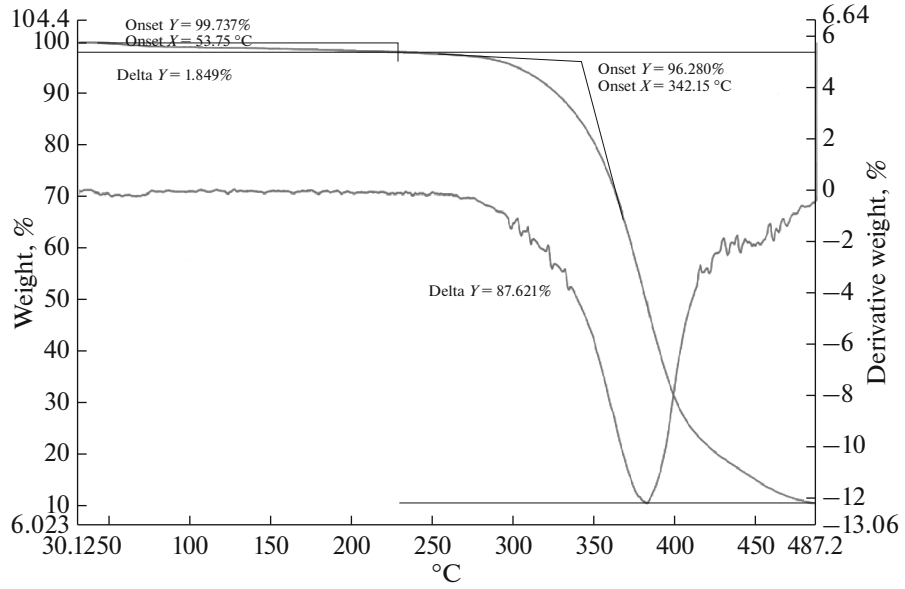

Fig. 6. Thermogravimetric analysis and differential scanning calorimetry of the biosurfactant produced by *M. hydrocarbonoclasticus* ST1 using simultaneous thermal analyzer STA 8000 (Perkin Elmer, USA). The substance was subjected to a temperature in the range of 30–480°C under nitrogen atmosphere at a rate of 10°C/min.

eties, and the resonances at 170.5, 171.6, 173.3 ppm assigned to carbon of carboxylic groups, indicating an eventual major contribution of fatty acids. A series of resonances at 60–110 ppm may be assigned to oligosaccharides [24]. The resonances at 60–80 ppm is primary/secondary carbons (C2–C6), along with two signals at 102.3 and 103.9 ppm indicates carbohydrate molecules are present [33].

Thermogravimetric analysis. The thermal stability of the biosurfactant was assessed by simultaneous thermal analyzer (measuring both thermalgravimetric and differential temperature signal, TG/DTA) (Fig. 6). The curve showed that the biosurfactant degraded in two steps. In the first stage of degradation, 1.84% weight loss of the biosurfactant was recorded at 53.75°C, which may be associated with the loss of moisture [34]. Such water loss is in evidence through an endothermic peak at 69.5°C in the differential thermogravimetric curve (DTA). Thereafter, 87.62% weight loss observed at 342.15°C corresponding to the thermal degradation of the molecule with an endothermic peak observed at 318.30°C in the DTA. Finally, the complete loss of the biosurfactant was detected at approximately 480°C. The results obtained suggest that the biosurfactant from *M. hydrocarbonoclasticus* ST1 is highly thermostable and can be applied for various industrial applications where heating to achieve sterility is of paramount importance [35].

In this study, the biosurfactant produced by *M. hydrocarbonoclasticus* ST1 exhibited the highest emulsion activity after grown in MSM supplemented with molasses as a carbon source and $NaNO_3$ as nitrogen source under the optimized cultivation conditions. The biosurfactant found to be stable even under extreme pH, temperature and salinity condition. The biosurfactant from *M. hydrocarbonoclasticus* ST1 was extracted and characterized through FT-IR, ^{1}H- and ^{13}C-NMR which indicated it was glycolipid. The features of this biosurfactant make it an interesting biotechnological product for many environmental and industrial applications.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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