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Isolation and characterization of biosurfactant-producing bacteria isolated from agriculture area in Thailand

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Abstract

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Biosurfactant-producing bacteria were isolated from various soil and water in agricultural area of Thailand. Based on hemolytic activity, drop collapsing test, oil displacement test, as well as emulsification activity (%EA), 8 effective isolates from 234 isolates were selected. All selected isolates were tested for antimicrobial activities against plant pathogenic bacteria. It was revealed that all isolates can inhibit more than 2 tested-plant pathogenic bacteria. The crude extracts of selected isolates were tested for stability of pH, NaCl, $CaCl_2$, $MgCl_2$ and temperature by measuring %EA and showed stability over a broad range of these conditions. All interested isolates were identified by biochemical test and MALDI-TOF MS. The isolates were: 1 isolate *Alcaligenes faecalis* subsp. *faecalis*, 2 isolates *Proteus mirabilis*, and 1 isolate *Providencia alcalifaciens*. Overall, the strains found in this study were useful for the bioremediation of the agricultural area.

Keywords: biosurfactant; agricultural area; biodegradation; antimicrobial activity

Introduction

Biosurfactants or microbial surfactants are surface metabolites that are produced by microbial fermentation extracellularly or intracellularly secreted and contain hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases, thereby reducing surface and interfacial tension at surface and interface regions, respectively. Biosurfactant are produced by wide variety of microorganisms, including yeast and fungi. They vary in their chemical structures and properties (Desai and Banat, 1997; Ron and Rosenberg, 2001). Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids and phospholipids such as mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein and phospholipid. These polarities render surfactants capable of reducing surface and interfacial tension and forming micro emulsions where hydrocarbons can be solubilizing in water or where water can solubilize in hydrocarbons (Nitschke and Pastore, 2006).

Biosurfactants could be potential alternatives to chemically synthesized surfactants for a variety of applications because of their advantages in emulsification, detergency, wetting, dispersing or solubilization. Other advantages include lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, the ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Desai and Banat, 1997; Gautam and Tyagi, 2006; Mukherjee et al., 2006; Singh, 2012).

Biosurfactant-producing microorganisms are naturally present in oil-contaminated soil or in hydrocarbon contaminated environments. These microorganisms exhibit emulsifying activity by producing biosurfactants often mineralizing or converting contaminants into harmless products. Unlike

chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro-based substrates and waste materials (McInerney et al., 1990). Since they are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment, biosurfactant may be more suitable for a variety of environmental controls such as stabilization of industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and the bioremediation of contaminated soil (Kosaric, 2001).

The objective of this study was to screen hydrocarbon-degrading bacteria from agricultural areas in Thailand for their ability to produce biosurfactants and to optimize those physical parameters that enhance biosurfactant production.

Materials and Methods

Source of soil and water sample

Eighty-five samples (40 soil samples and 45 water samples) were collected from various agricultural areas in Thailand. To optimize geographical varieties of the samples, collections were gathered from three categories of agricultural areas. These included 22 samples from rice fields, 44 samples from vegetable fields and 19 samples from fruit gardens.

Isolation of biosurfactant-producing bacteria

Microorganisms from the samples were isolated by the dilution plate method. Briefly, 5 g of soil and 5 ml of water samples were serially diluted 10-fold in sterile 0.85% NaCl. Then 100 µl culture broths from serial dilution of 10^{-3}-10^{-5} in soil and 10^{-1}-10^{-3} in water were pipetted and spread on blood agar and incubated at room temperature for 24-48 h. Single colonies which produced β and α hemolysis were selected from plates which produced 30-300 colonies per plate and streaked on blood agar. These culture plates were incubated at room temperature to obtain pure cultures. Successfully grown cultures were maintained on blood agar stored at 4°C and activated in the same media for 24 h before experimental use.

Screening methods used for isolated biosurfactant-producing bacteria

Hemolytic activity

Isolated strains were screened as described by Carrillo et al. (1996) on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for 24 h. Hemolytic activity was evaluated according to patterns of β-hemolysis, α-hemolysis, or γ-hemolysis. The assay was performed in triplicate.

Preparation of culture medium

An individual colony from each isolate was suspended in a solution of sterile 0.85% NaCl with turbidity measured by spectrophotometry (OD = 0.08-0.1 at 625 nm). Then, 5% (v/v) of this standardized inoculum was inoculated into 15 ml centrifuge tube containing 5 ml of nutrient broth and incubated at room temperature in a static condition for 3 days. Next, the culture medium was centrifuged at 5000 g at 4°C for 30 min. The supernatant was collected and used for preliminary screening of biosurfactant production using a variety of tests, including: drop-collapse test, oil spreading technique, calculation of the emulsification activities (EA) and emulsification index (EI24) and antimicrobial activities.

Distilled water and nutrient broth without inoculation were used as a negative control, while 1% (w/v) sodium dodecyl sulphate (SDS), tween 80 and triton X-100 were used as positive controls.

Drop collapsing test

A modified drop collapse test was used for measurement as described by the Youssef et al. (2004). Two µl of mineral oil was added to each well of a 96-well microliter plate lid (Nunc, Roskilde, Denmark). The lid was equilibrated for 1 h at room temperature, and then 5 µl of the cultural supernatant was added to the surface of the oil. The shape of the drop on the oil surface was inspected after 1 min and compared to those drops produced by negative controls of distilled water and culture medium. Biosurfactant-producing cultures producing flat drops were scored as positive '+'. Those cultures that resulted in rounded drops were scored as negative '-', indicative of the lack of biosurfactant production.

Oil displacement test (ODT)

The oil spreading assay was adapted from the method described by Bodour and Miller-Maier (1998). Twenty ml of distilled water were added to a petri dish (150 mm in diameter) followed by the placement of 15 µl of crude oil on the surface of water. Then, 10 µl of the culture supernatant were gently put on the center of the oil film. The diameter and area of clear halo formed on the oil surface was measured and calculated after 30 sec. Biosurfactant oil spreading (displacement) activity was defined as the area of oil displacement in cm^2.

Emulsification activity measurement (EA and E24)

Emulsification activity (EA) was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 1 ml of culture supernatant or biosurfactant crude extract (0.5%, w/v), 1 ml of various oils (diesel oil, soy bean oil, palm oil, and castle oil) were added and vortexed at high speed for 2 min. The mixture was allowed to stand for

10 min prior to measurement. EA is defined as the height of the emulsion layer divided by the total height. After 24 h, the emulsification index (E24) was calculated using following formula:

EA (%) = (Total height of the emulsified layer x 100) / Total height of the liquid layer

E24 (%) = (Total height of the emulsified layer after 24 h x 100) / Total height of the liquid layer after 24 h

The results were compared with the positive controls such as SDS and Tween 80.

Antimicrobial activities against plant pathology bacteria by agar well diffusion

Antimicrobial activity of secondary metabolites was tested by the method of agar well diffusion method given by Bharali et al. (2014) with slightly modification using plant pathogenic bacteria such as *Xanthomonas axonopodis* pv. citri, *Xanthomonas axonopodis* pv. glycines, *Xanthomonas campestris* pv. campestris and *Acidovorax avenae* subsp. *avenae.* Indicator bacteria were restreaked on nutrient agar and incubated at 37°C for 24 h to obtain pure cultures. Then an isolated colony of each indicator bacteria was suspended in a sterile 0.85% NaCl solution. The turbidity of microbial suspension was measured by spectrophotometry (OD = 0.08-0.1 at 625 nm) corresponding to $1x10^8$ CFU/ml. The microbial suspension was streaked by a three-way swab on Muller Hinton agar. The agar was then punched using a cork borer with a diameter of 6 mm. Finally, 50 µl of supernatant from each biosurfactant-producing bacterial isolate was added to each well and incubated at 37°C for 24 h with the experiment carried out in triplicate. Chemically synthesized surfactants e.g. 1% sodium dodecyl sulfate (SDS), Triton x-100 and Tween 80 were used as positive controls. Nutrient broth was used as a negative control. The plates were incubated at 37°C for 24 h. The diameter of inhibition zone around each well was measured.

Isolates possessing biosurfactant-producing activity, as evidenced by positive results in all of the following tests: the qualitative drop collapsing test, ≥ 1.5 cm of quantitative oil displacement, the presence of hemolytic activity, antimicrobial activity, and emulsification index in diesel oil, palm oil, soy bean oil, and machine oil of ≥ 20%, ≥ 50%, ≥ 50%, and ≥ 50% respectively were chosen for further investigation.

Extraction of the biosurfactant

Bacteria isolates that displayed high biosurfactant and bioemulsifier production were selected for large scale crude extraction according to technique of George and Jayachandran (2009). A single colony of each isolate was suspended in a sterile 0.85% NaCl solution and turbidity measured by spectrophotometry (OD = 0.08-0.1 at 625 nm). Microbial suspensions were added into 300 ml of NB equivalent to 5%. Cultures were incubated at room temperature for 72 h. Samples were then centrifuged at 5,000 rpm for 30 min. Supernatant was collected in sterile flask and adjusted to pH 2 using 6 M HCL. The extraction was performed twice with chloroform-methanol (2:1) by ratio of supernatant and solvent = 1:1, and repeated 3 times. Pools of extract in solvent were concentrated using a rotary evaporator (EYELA SB-651, Tokyo, Japan) under reduced pressure at 60-70ºC and weighed to reconstitute the crude extract. Crude biosurfactant was stored at -20°C.

Characterization of biosurfactant crude extract

Crude extract from bacterial isolates that displayed high biosurfactant production (0.5%, w/v) in 50 mM potassium phosphate buffer, pH 7.0 were characterized. To investigate the effect of varying pH, salt concentrations (NaCl, $CaCl_2$ and $MgCl_2$) and temperature on the emulsification activity of crude extract, the extract was adjusted with 1 N HCl or NaOH to obtain pH of 3, 5, 7, 10, and 12. NaCl was added to the sample to obtain the final percent concentrations of 0, 2, 5, 10, 15, and 20 (w/v). $CaCl_2$ and $MgCl_2$ were also added to the sample to obtain the final concentrations of 0, 2, 5, 10, 15, 20 mM and 0, 0.1, 0.3, 0.5, 0.7, 1 mM respectively. To study thermal stability, bacterial crude extract was incubated for 1 h at different temperatures (4, 25, 30, 45, 100°C) and cooled to 30°C. Remaining activity was then determined by emulsification activity (EA). Chemically synthetic surfactants (1% SDS and Triton X-100) and distilled water were used to compare the activity with biosurfactant crude extract.

Identification of promising bacterial isolates

Those bacterial isolates capable of biosurfactant-production were tentatively identified from culture characteristics, gram stain, motility and biochemical-characteristics, according to Bergey's Manual of Determinative Bacteriology. All isolates were confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as previously described by Seng et al. (2009).

Optimization of cultivation conditions

To identify each bacterial isolate's optimal growth conditions, a range of abiotic factors and their optimized range were selected. These factors include temperature, pH and % NaCl concentration. The experiments were performed in 50 ml flasks containing 30 ml of nutrient broth and inoculated with 10% of suspension of isolate in order to test the optimal growth conditions for biosurfactant-producing bacteria. These flasks were then incubated at 30°C for 24 h. Bacte-

rial growth was determined by measuring optical density at 625 nm (A1) and taking 3 ml samples from each tube. There were 3 experimental conditions. First, the bacterial mixtures were supplemented with 0.5, 1.0, and 2.0% w/v of NaCl. Second, the bacterial mixtures were incubated at different temperatures (4, 37 and 45ºC). Third, the bacterial mixtures were adjusted to different initial pH (6.0, 7.0, and 8.0) and incubated at 30°C for 24 h. Finally, all conditions determined growth by measurement of the turbidity at 625 nm using a spectrophotometer (A2).

Statistical analysis

All of the experiments were carried out three times and studied in triplicate. Results represent the mean ± standard deviation. One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was conducted to determine the significant differences in microbial efficacy of the different bacterial strain. SPSS ver.18 software (Chicago, IL) was used to carry out the statistical analysis.

Results and Discussion

In this study, we carried out isolation and screening of biosurfactant-producing bacteria from 85 samples (40 soil samples and 45 water samples) originally gathered from a variety of agricultural areas. A total of 234 colonies were isolated using hemolytic screening. Out of 234 isolates screened, only 8 isolates showed an effective result in all screening experiments (Table 1, Table 2).

Maneerat and Phetrong (2007) achieved similar isolation rates from biosurfactant-producing marine bacteria in oil-spilled seawater collected from harbors and docks in Songkhla Province, Thailand. They found only 8 out of 200 strains with high biosurfactant-producing activity.

The antimicrobial activity of culture supernatant from 4 bacterial plant pathogens such as *X. axonopodis* pv. citri (Xac), *X. axonopodis* pv. glycines (Xag), *X. campestris* pv. campestris (Xcc) and *A. avenae* subsp. *avenae* (Aaa) were tested using the agar well diffusion method. All 8 isolates demonstrated antimicrobial activity on at least 2 indication bacteria. Isolate RSS8 (6) showed the widest inhibition zone to Xcc and Xac when compared with chemical surfactants, which were used as controls (Table 2).

Patowary et al. (2017) have shown that bacterial isolated from hydrocarbon contaminated soil samples can produce the biosurfactant contained antibacterial properties which can inhibit the growth of *B. subtilis, S. aureus, K. pneumonia*, and *E. coli* .

From the above results, biosurfactants from 8 biosurfactant-producing bacteria were chosen for large scale

Table 1
Summary of the screening methods for biosurfactant producing bacteria from 3 independent experiments ± standard deviation (mean ± SD)

No. Isolate		Source	EA (%)				E24 (%)				Oil displacement	Drop collapsing test
			Palm oil	Soy bean oil	Diesel oil	Machine oil	Palm oil	Soy bean oil	Diesel oil	Machine oil		
1	FWS41(2)	Water from banana garden	41.2±0.0	74.5±3.4	37.0±3.2	83.3±0.0	29.4±0.0	33.3±3.4	22.2±5.6	74.0±3.2	2.8±0.4	+
2	RSS7(1)	Soil from rice field	51.0±1.4	39.2±3.4	35.4±3.6	58.3±3.6	19.6±3.0	13.8±3.4	6.3±3.6	42.0±3.6	1.2±0.2	++
3	RSS8(6)	Soil from rice field	54.2 ± 3.6	64.7±5.9	43.8±0.0	64.6±7.2	27.1±4.0	13.8±3.4	0±0.0	39.9±6.3	1.3±0.1	+
4	RWNY13(2)	Water from rice field	25.0±0.0	31.1±3.9	25.0±6.3	54.2±3.6	16.8±0.0	9.0±3.9	12.6±0.0	33.6±0.0	1.2±0.1	++
5	VSNY5(6)	Water from chilli garden	66.7±0.0	66.7±0.0	11.8±0.0	100.0±0.0	24.1±3.0	22.4±3.2	0±0.0	62.3±4.1	1.5±0.1	++
6	VSNY5(8)	Water from chilli garden	94.1±0.0	88.2±0.0	41.2±0.0	83.3±0.0	37.3±3.0	4.0±0.0	0±0.0	52.3±3.2	0.5±0.1	+
7	VSNY5(9)	Soil from chilli garden	49.0±3.4	77.8±0.0	14.8±3.2	75.4±3.0	21.6±3.0	13.1±3.2	1.9±3.2	46.0±3.0	1.5±0.2	+
8	VWNY14(3)	Water from vegetables field	15.7±6.8	43.1±3.4	15.7±6.8	53.3±6.7	17.8±3.0	11.9±0.0	5.9±3.4	38.0±3.9	1.7±0.1	0

Symbol means: (0) = no result; (+) = good result, (++) = high result

Table 2
Antimicrobial activities against 4 plant pathogenic bacteria using the agar diffusion method

No.	Isolate	Inhibition zone (mm)			
		Xanthononas axonopodis pv. *glycines* (Xaa)	*Xanthomonas campestris* pv. *campestris*(Xcc)	*Xanthomonas axonopodis* pv. *citri* (Xac)	*Acidovorax avenae* subsp. *avenea* (Aaa)
1	FWS41(2)	15.3±0.6	–	17.7±2.5	–
2	RSS7(1)	8.0±5.7	15.3±6.7	20.0±0.0*	11.3±1.5
3	RSS8(6)	14.7±1.5	27.7±14.0*	28.3±2.9*	15.7±3.8
4	RWNY13(2)	–	12.7±1.53	–	15.3±2.3
5	VSNY5(6)	14.3±2.5	9.7±2.5	24.3±9.3*	23.3±5.8
6	VSNY5(8)	17.0±4.2	8.3±0.6	17.0±2.0	20.0±5.0
7	VSNY5(9)	10.0±1.7	20.3±10.7*	25.3±4.2*	17.0±2.7
8	VWNY14(3)	11.0±1.4	–	–	12.7±0.6
control	1% SDS	18.3±2.1	12.0±3.5	21.0±3.6	9.7±0.6
control	Tween 80	12.3±2.5	13.7±2.9	18.7±1.2	12.3±2.5
control	Triton X 100	13.7±0.6	13.3±0.6	21.0±7.9	15.7±4.5

Values are expressed as mean ± SD (n = 3).
Symbol means: (-) = noinhibition zone; * represent significant different from control ($p < 0.05$ by One way Anova)

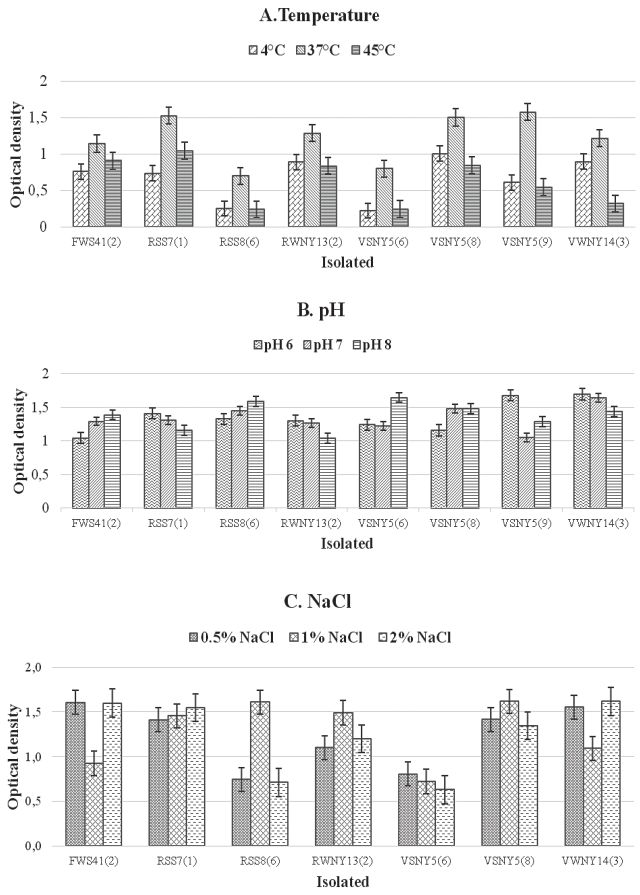

Fig. 1. Optimal growth conditions of selected biosurfactant-producing bacteria

crude extraction by methanol-chloroform (1:1). The 0.5% w/v crude extract of each isolate was then investigated for the effects of pH, salt concentrations (NaCl, $CaCl_2$ and $MgCl_2$) and temperature on emulsification activity. All crude biosurfactants extracted still found to have good emulsification activity. In other words, factors such pH, salt concentrations (NaCl, $CaCl_2$ and $MgCl_2$) and temperatures were found to have no influence on the emulsification of soybean oil by all crude biosurfactants we extracted (Table 3).

Our results were in contrast to Maneerat and Phetrong who found that crude biosurfactants extracted from biosurfactant-producing marine bacteria were stable over a broad range of pH, and salt concentrations, as well as temperatures from 80 to 100°C, but showed an adverse effect on emulsifying activity upon exposure to $CaCl_2$ (Maneerat and Phetrong, 2007).

Horowitz et al. (1990) reported that *Bacillus licheniformis*, the selected biosurfactant bacteria, showed wide tolerance and grew well at temperatures ranging from 25 to 120°C. However, the emulsification activity of its biosurfactant was decreased with increased temperature.

Optimal growth conditions of selected biosurfactant-producing bacteria are shown in Figure 1. Each isolate was cultured in Nutrient broth at various temperatures, pH, and NaCl concentrations. It was found that the majority of biosurfactant-producing bacteria showed optimal growth at 37-45°C, at pH of 6-8 and from 0.5% to 2% w/v NaCl concentrations.

Petsuriyawong et al. (2012) found that the most effective biosurfactant-producing bacteria, *B. amyloliquefaciens* B5,

Table 3
Emulsification activity (%EA) of crude extract from selected biosurfactant-producing bacteria from 3 independent experiments (mean±SD)

	conc.	SDS	FWS41(2)	RSS7(1)	RSS8(6)	RWNY13(2)	VSNY5(6)	VSNY5(8)	VSNY5(9)	VWNY14(3)
$CaCl_2$ (mM)	**0**	95.8±0.0	72.1±3.6	74.5±0.0	75.2±3.6	85.9±3.6	79.2±3.6	72.6±3.4	77.2±3.6	76.1±3.6
	2	100.0±0.0	43.1±3.4	54.9±3.4	40.7±3.2	85.2±3.2	62.5±6.3	54.9±3.4	60.4±3.6	51.0±3.4
	5	100.0±0.0	54.9±3.4	49.0±3.4	45.1±3.4	66.7±3.6	66.7±3.4	56.3±0.0	58.3±3.6	64.6±3.6
	10	100.0±0.0	55.6±3.9	56.9±3.4	53.3±0.0	60.0±0.0	56.3±0.0	60.4±3.6	66.7±0.0	56.9±3.4
	15	70.4±3.2	49.0±3.4	48.2±3.2	64.7±0.0	51.1±3.4	44.4±3.9	41.7±3.6	64.4±3.9	52.1±7.2
	20	58.8±0.00	49.0±3.4	50.0±0.0	43.8±0.0	35.4±3.6	52.9±0.0	51.0±3.4	58.3±3.6	56.9±3.4
$MgCl_2$ (mM)	**0**	95.8±0.0	79.9±3.6	74.5±0.0	80.0±3.6	70.8±3.6	80.7±3.6	74.5±3.4	81.9±3.6	81.3±3.6
	0.1	100.0±0.0	66.7±3.4	58.8±0.0	58.8±0.0	45.8±3.6	71.1±3.9	62.8±3.4	68.6±3.4	64.6±3.6
	0.3	100.0±0.0	60.8±3.4	60.8±3.4	70.6±0.0	51.0±3.4	64.6±3.6	62.8±3.4	62.8±3.4	75.6±3.9
	0.5	100.0±0.0	60.8±3.4	58.8±0.0	68.6±3.4	51.0±3.4	64.6±3.6	70.8±3.6	88.2±0.0	59.5±4.1
	0.7	100.0±0.0	64.7±0.0	49.0±3.4	92.2±3.4	62.2±3.9	64.6±3.6	58.8±0.0	78.4±3.4	68.9±3.9
	1	100.0±0.0	60.8±3.4	72.2±0.0	84.3±6.8	74.5±3.4	66.7±3.4	82.4±0.0	60.4±3.6	47.1±0.0
NaCl (% v/w)	**0**	93.9±0.0	77.9±3.6	72.6±0.0	80.0±3.6	73.9±3.6	75.9±3.6	69.6±3.4	77.1±3.6	78.1±3.6
	2	94.1±0.0	58.8±0.0	51.0±3.4	60.8±3.4	54.9±3.4	58.8±0.0	54.2±3.6	58.3±3.6	54.9±3.4
	5	80.4±3.4	62.8±3.4	56.3±0.0	52.9±0.0	51.0±3.4	52.9±0.0	62.5±0.0	51.0±3.4	54.9±3.4
	10	58.3±3.6	60.0±0.0	50.0±0.0	54.2±3.6	54.9±3.4	58.3±3.6	52.9±0.0	60.8±3.4	54.9±3.4
	15	64.7±0.0	52.9±0.0	47.1±0.0	52.9±0.0	56.9±3.4	57.4±3.2	58.8±0.0	51.0±3.4	58.8±0.0
	20	60.4±3.6	56.3±0.0	41.2±0.0	52.9±0.0	74.5±3.4	54.9±3.4	54.9±3.4	52.1±3.6	52.1±3.6
T (°C)	**4**	79.0±0.0	58.3±3.6	49.0±3.4	83.3±3.6	55.6±3.9	82.4±0.0	50.0±0.0	57.8±3.9	62.2±3.9
	25	88.8±0.0	77.1±3.6	70.6±0.0	87.5±3.6	76.3±3.6	83.7±3.6	69.6±3.4	76.1±3.6	80.4±3.6
	30	93.8±0.0	89.6±3.6	82.4±0.0	91.7±3.6	85.4±3.6	83.3±3.6	78.4±3.4	85.4±3.6	89.6±3.6
	45	100.0±0.0	62.5±0.0	42.6±3.2	51.0±3.4	52.9±0.0	56.9±3.4	74.5±3.4	62.8±3.4	83.3±3.6
	100	88.2±0.0	54.2±3.6	52.9±5.9	54.9±3.4	66.7±3.4	60.8±3.4	54.9±3.4	79.2±3.6	68.6±3.40
	control	95.8±0.0	77.9±3.6	70.6±0.0	80.0±3.6	75.0±3.6	73.9±3.6	73.8±3.4	77.9±3.6	78.1±3.61
pH	**3**	100.0±0.0	56.9±3.4	47.1±0.0	62.8±3.4	56.3±0.0	58.8±5.9	66.7±3.6	56.9±3.4	52.9±0.00
	5	100.0±0.0	60.4±7.2	81.5±16.0	54.9±3.4	56.3±0.0	60.4±3.6	60.8±3.4	50.0±0.0	47.1±0.00
	7	100.0±0.0	64.6±3.6	61.1±0.0	51.0±3.4	54.9±3.4	58.8±0.0	66.7±0.0	56.9±3.4	54.9±3.40
	10	96.1±3.4	88.2±0.0	35.3±0.0	80.4±3.4	68.6±3.4	56.9±3.4	100.0±0.0	79.6±3.2	75.0±0.00
	12	100.0±0.0	72.2±0.0	37.0±3.2	48.2±3.2	80.4±3.4	75.9±3.2	78.6±0.0	72.6±3.4	78.4±3.40

which is in the mesophilic bacteria group, had the highest growth and produced the greatest amount of biosurfactant after 48 h, at temperatures ranging from 25-37ºC, and at a pH range of 5-8.

All effective isolates were characterized using biochemical techniques and MALDI-TOF MS revealed that these were *Alcaligenes faecalis* subsp. *faecalis*, *Proteus mirabilis*, and *Providencia alcalifaciens* (Table 4). All of the bacterial isolates were gram negative. This may be the characteristic of gram negative that contributes to the majority of the populations surviving such stressful conditions. It has previously study revealed that most bacteria isolated from hydrocarbon- or oil-contaminated environments are gram negative (Bodour et al., 2003; Batista et al., 2006).

Our report is similar to that of Bharali et al. (2011) who isolated biosurfactant-producing bacteria from crude oil contaminated soil of Upper Assam, India and screened for their ability to produce biosurfactant. Only one isolate was found to have that ability. This isolated was identified as *Alcaligenes faecalis*. Another report from Brazil, has characterized one isolate of biosurfactant-producing bacteria from contaminated soils with oily wastes and identified as *Pseudomonas aeruginosa* (Gámez et al., 2017).

Table 4
Identification of selected biosurfactant-producing bacteria

No.	Isolated	Gram stain	Biochem	MALDI-TOF MS
1	FWS41(2)	Gram negative bacilli	*Alcaligenes* spp.	*Alcaligenes faecalis* subsp. *faecalis*
2	RSS7(1)	Gram negative bacilli	*Alcaligenes* spp.	*Alcaligenes faecalis* subsp. *faecalis*
3	RSS8(6)	Gram negative bacilli	*Alcaligenes* spp.	*Alcaligenes faecalis* subsp. *faecalis*
4	RWNY13(2)	Gram negative bacilli	*Proteus* spp.	*Proteus mirabilis*
5	VNSY5(6)	Gram negative bacilli	*Alcaligenes* spp.	*Alcaligenes faecalis* subsp. *faecalis*
6	VSNY5(8)	Gram negative bacilli	*Providencia* spp.	*Providencia alcalifaciens*
7	VSNY5(9)	Gram negative bacilli	*Alcaligenes* spp.	*Alcaligenes faecalis* subsp. *faecalis*
8	VWNY14(3)	Gram negative bacilli	*Proteus* spp.	*Proteus mirabilis*

Conclusion

In conclusion, the present study revealed bacterial isolates, which were examined using ODT, EA, and E24 tests to evaluate whether they had high activity against various types of oils in order to identify potential candidates for bioremediation of polluted sites and resources for surface-active molecules of industrial importance. Moreover, as these strains have been shown to have antimicrobial activity against plant pathogens, therefore they may have the capability to produce effective biosurfactants that could be used in waste management and bioremediation. This information should therefore enable us to assess biosurfactant activity for further exploitation in specific applications such as environmental pollution management.

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