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Isolation of Biosurfactant–Producing Bacteria with Antimicrobial Activity against Bacterial Pathogens

Siripun Sarin [a], Boonruang Khamsri [a] and Charoon Sarin [b]

[a] *Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand*
[b] *Department of Natural Resources and Environment, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Thailand*

Abstract

The aims of this research were to study biosurfactant producing bacteria isolated from soil and to determine their property and efficiency as biosurfactants in order to inhibit bacterial pathogens. The result showed that there were 8 bacterial isolates out of 136 isolates of the total biosurfactant producing bacteria screened that exhibited the diameter of clear zone more than 1.5 cm. in the oil spreading test. The highest potential of emulsifying activity (%EA_{24}) of 54.4 and the maximum additive concentration, (%MAC) of 24.2 was obtained from the fermentation broth of the G7 isolate which the G7 isolate was later identified as *Pseudomonas fluorescens, Escherichia coli, Staphylococcus aureus* and *Psuedomonas aeruginosa* were the tested bacterial pathogens that were most sensitive to the acid precipitated biosurfactant obtained from *P. fluorescens* G7 with the lowest minimum inhibitory concentration (MIC) of 41.6 mg/ml and minimum bactericidal concentration (MBC) of 41.6 mg/ml compared with the acid precipitated bisurfactants of the other isolates used in the antimicrobial activity test. The type of the separated crude biosurfactant produced by *P. fluorescens* G7 analyzed later by using the rhamose test, TLC and FT-IR techniques was rhamnolipid.

Keywords: biosurfactant-producing bacteria; antimicrobial activity

1. Introduction

Biosurfactants are produced by a wide variety of bacteria, yeast, and fungi. They include peptides, glycolipids, glycopeptides, fatty acids and phospholipids (Banat *et al*., 2000). Like chemical surfactants, these amphipathic compounds contain both hydrophilic and hydrophobic moieties and have ability to reduce surface and interfacial tension between different fluid phases which confer excellent detergency, emulsifying, foaming, wetting and dispersing or solubilizing traits (Holmberg, 2001; Makkar and Cameotra, 2002). Biosurfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH (Kosaric, 1992; Cameotra and Makkar, 1998), therefore these biomolecules have also emerged as potential agents in health care and food processing industries, apart from their potential applications in environmental protection and management and crude oil recovery (Desai and Banat, 1997). In recent years, these biomolecules were also found to possess several interesting properties of therapeutic and biomedical importance (Singh and Cameotra, 2004; Das *et al*., 2008). Biosurfactants may interact with the interfaces and affect the adhesion and detachment of pathogenic bacteria apart from acting as biocide agents (Rodrigues *et al*., 2006a). As an example, the positively charged biomaterial surfaces exerted an antimicrobial effect on adhering Gram-negative bacteria, but not on Gram-positive bacteria (Gottenbos *et al*., 2001). The probiotic strain, *Lactobacillus fermentum* RC-14, and its secreted biosurfactant reduced infection associated with surgical implants, which are mainly caused by *Staphylococcus aureus*, through inhibition of growth and reduction of adherence to surgical implants (Gen *et al*., 2002). In addition, biosurfactants may also be used for applications as emulsifying agents for drug transport to the infection site, as agents supplementing the pulmonary surfactant, and as adjuvants for vaccines (Kosaric, 1996). The potential application of various microbial surfactants in the field of biomedical sciences – is of particular importance at this point in time, when increasing numbers of drug-resistant microorganisms are being encountered and there is a need for alternative lines of therapy. This study was, therefore, aimed to isolate the biosurfactant-producing bacteria and study their antimicrobial potentials.

2. Material and Methods

2.1. Isolation of bacteria

The biosurfactant producing strains were isolated from agricultural soil and contaminated soil, by the method described by Bento (Bento *et al*., 2005). Five

gram of soil was inoculated into 100 ml of mineral salt medium (MSM) containing 2% of hexadecane. The MSM was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g $NaNO_3$, 0.4 g $MgSO_4.7H_2O$, 1.0 g NaCl, 1.0 g KCl, 0.05 g $CaCl_2.2H_2O$, and 10 ml concentrated phosphoric acid (85%). This solution was adjusted to pH 7.2 with KOH. Solution B contained (per liter) 0.5 g $FeSO_4.7H_2O$, 1.5 g $ZnSO_4.7H_2O$, 1.5 g $MnSO_4.H_2O$, 0.3 g K_3BO_3, 0.15 g $CuSO_4.5H_2O$, and 0.1 g $Na_2MoO_4.2H_2O$. One milliliter of solution B was added to 1,000 ml of solution A to form the MSM (Bodour *et al*., 2003). The broth culture was incubated with shaking (200 rpm) at 30°C for 48 h. An aliquot was transferred every 2 days to a fresh medium to ensure growth on this carbon source. After three cycle of enrichment, the bacterial suspension in the cultural flask was diluted serially and spread plated on nutrient agar (NA, Merck) plates. The bacteria were purified by cross-streaking on NA plates and incubated at 30°C for further subculturing on NA slants.

2.2. Screening of biosurfactant-producing bacteria by oil spreading test

To screen for biosurfactant-producing bacteria, a loopful of subculture strain was grown on NB with shaking (200 rpm) at 30°C for 18 h. The seed culture at 10% (v/v) was used as an inoculum into 5 ml of mineral salt medium (MSM) containing 2% of glucose in a 25 ml test tube. The broth culture was incubated with shaking (200 rpm) at 30°C for 3 days. The oil spreading test measures the diameter of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil-water surface. For the oil spreading test, 30 ml of distilled water was added to a petri dish (9.5 cm diameter) followed by addition of 10 µl of lubricant oil to the surface of water, 10 µl of fermentation broth was then added to the oil surface (Rodrigues *et al*., 2006b). The diameters of clear zones of triplicate assays from the same sample were determined. The biosurfactant-producing bacterial isolates that exhibited a diameter of clear zone more than 1.5 cm in the oil spreading test were further analyzed for maximum additive concentration, (%MAC) and emulsifying activity (%EA_{24}).

2.3. Analysis of the maximum additive concentration, (%MAC) and emulsifying activity (%EA_{24})

To measure the MAC, the test tube containing 1 ml of fermentation broth (V1) and 1 ml of 0.02 M Tris-HCl buffer pH 7.3 was supplemented with 50 µl of vegetable oil. The mixture was then vortexed for 30 s and allowed to stand for 5 min. After each addition of 50 µl of the vegetable oil, the mixture was again vortexed for 30 s and allowed to stand for 5 min. This procedure was repeated by adding the vegetable oil until the emulsifier layer collapsed and the adding volume of the vegetable oil was finally summed up (V2) (Ghurye and Vipulanandan, 1994). The MAC is defined as percentage of oil volume of the V2 (ml) divided by the V1 (ml). The EA_{24} of fermentation broth was determined by adding 2 ml of vegetable oil to the same amount of fermentation broth in a test tube (1.5X15 cm), mixed at maximum speed for 2 min with a vortex mixer and allowed to stand for 24 h. The EA_{24} is defined as percentage of height of emulsified layer (cm) divided by the total height of the liquid column (cm) (Cooper and Glodenberg, 1987).

2.4. Biosurfactant production, isolation and partial purification

After 5 days, the fermentation broth obtained was centrifuged at 10,000g for 15 min at 4°C to remove the bacterial cells. The supernatant pH was adjusted to 2.0 with 6 M HCl, and then left overnight at 4°C for precipitation. The acid precipitated biosurfactant was washed 3 times with distilled water (pH 2.0) and dissolved in the distilled water (pH 7.0). The acid precipitated biosurfactant was dried in the oven at 37°C for 2 days and kept for antimicrobial study. For partial purification, the supernatant pH was adjusted to 2.0 with 6 N H_2SO_4, and an equal volume of $CHCl_3/CH_3OH$ (65:15) was added. The mixture was vigorously shaken for 5 min and allowed to set until phase separation occurred. For crude biosurfactant extraction, the bottom solvent phase was removed and the upper aqueous phase was re-extracted as before. The crude biosurfactant was then concentrated from the pooled solvent phases using a rotary evaporator at 40°C (Costa *et al*., 2006) and was collected for chemical analysis of the components.

2.5. Antimicrobial assay

The minimum inhibitory concentrations (MIC) were determined in 100 µl of Muller Hinton broth (MHB, Merk) with various concentrations of crude biosurfactant solutions and 100 µl of each bacterial suspension tested; *Escherichia coli, Staphylococcus aureus* and *Psuedomonas aeruginosa*, (set to McFarland Standard No.0.5) in round-bottomed 96-well microplates. After incubation at 30°C for 24 h, the tested microplates were assayed by tetrazolium salt (Singh, 2008). To each well, 50 µl of 2,3,5-triphenyltetrazolium chloride (0.5% w/v) was added. Cultures were then incubated at 37°C for 24 h. A change in color from yellow to red indicated growth of bacteria and the MIC was interpreted

visually. The MIC was estimated as the first dilution which completely inhibits bacterial growth in MHB medium. The minimum bactericidal concentrations (MBC) were determined by inoculation of yellow cultural medium from 96-well microplates of the MIC test (which indicates that the organisms did not grow in these wells) on Muller Hinton agar (MHA, Merck). The MBC was the lowest concentration of a crude biosurfactant that results in more than 99.9% killing of the bacteria being tested.

2.6. Analysis of the components of the partially purified biosurfactant

The partially purified biosurfactants were separated by TLC using aluminum sheets silica gel 60 F_{254} plates (Merck) with various solvent systems. The components were observed under UV light (wavelength 254 and 360 nm) and visualized by staining with ninhydrin, rhodamine B, alkali potassium permanganate, and iodine vapor in the presence of amino acids, lipids, organic compounds/sugar and sugar/lipids, respectively (Prommachan, 2002). To determine the carbohydrate groups in the biosurfactants, the rhamnose test was assayed (Abouseoud *et al*., 2008). These samples were also analyzed for functional groups using a FTIR spectroscopy (Perkin Elmer, Spectrum GX) at the Department of Chemistry, Naresuan University.

2.7. Identification of isolates

The bacterial isolates giving the best results in antimicrobial assay were identified by biochemical tests (Thailand Institute of Scientific and Technological Research, Pathum Thani).

3. Results and Discussion

There were 8 bacterial isolates (NS1, NS2, NS3, NS4, NS5, NS6, G7 and TP8) out of 136 isolates of the total biosurfactant producing bacteria screened that exhibited the diameter of clear zone more than 1.5 cm in the oil spreading test. The highest potential of emulsifying activity (%EA_{24}) of 54.4 and the maximum additive concentration, (%MAC) of 24.2 were obtained from the fermentation broth of the G7 isolate (Table 1). An approach for screening potential biosurfactant-producing bacteria in this study is the estimation of the emulsification activity (EA_{24}), therefore the results observed over 50% were selected for further antimicrobial activity tests. Most data published in the literature reported that bacteria with high potential of emulsifying activity are promising microbial candidates for biosurfactant production (Banat *et al*., 2000; Bento *et al*., 2005).

Table 1. MAC and EC_{24} of fermentation broth of the biosurfactant-producing bacteria

Isolate	% MAC	% EA_{24}
NS1	45.0	40.0
NS3	44.0	46.7
NS4	90.0	46.7
NS5	12.5	40.0
NS6	27.5	50.0
NS7	85.0	50.0
G7	24.2	54.4
TP8	31.7	48.1

The best potential of acid precipitated biosurfactant inhibiting all 3 tested bacterial pathogens (*Escherichia coli, Staphylococcus aureus* and *Psuedomonas aeruginosa*) was also obtained from the G7 isolate with the lowest minimum inhibitory concentration (MIC) of 41.6 mg/ml and minimum bactericidal concentration (MBC) of 41.6 mg/ml compared with the acid precipitated bisurfactants of other isolates used in the antimicrobial activity test (Table 2). These biocontrol results were similar to the experimental results of Abouseoud *et al*. (2008) where the biosurfactant produced from *Pseudomonas florescens* Migula 1895-DZMZ had a good emulsifying and antimicrobial activity against *Bacillus subtilis*, and the results of Hultberg *et al*. (2008) in which the biosurfactant produced from *Pseudomonas fluorescens* had the ability to inhibit the growth of plant pathogens *Pythium ultimum, Phytophthora cryptogea* and *Fursarium oxysporum*.

After isolation and partial purification of the crude biosurfactant produced from the G7 isolate, the pale yellow powder product obtained from the extraction and purification processes was defined as a crude biosurfactant. For detection of partially purified biosurfactants by TLC study, the solvent system used was toluene/acetone (1/1). The TLC plate showed the presence of sugar/lipids when the components were stained with developing agents, iodide vapor. The rhamnose test was positive which indicates that the partially purified biosurfactant of the G7 isolate could be glycolipid type, rhamnolipid. The IR spectroscopy revealed that the biosurfactant showed absorption bands, indicating the presence of aliphatic chain as indicated by the R-C-H saturated alkanes and alkyl groups at 1350-1480 cm^{-1}. The presence of a peptide component at 1500-1650 cm^{-1} resulting from the R-N-H, N-H bend with the C=O stretch in amide. The band at 1630-1690 cm^{-1} was due to carbonyl absorption. At 1000-1400 cm^{-1}, the mode of C-N stretch was observed. The presence of fatty acid was indicated by the O-H board mode found in carboxylic acid at 3000-3400 cm^{-1}. These results confirm that the biosurfactant produced contains aliphatic hydrocarbon

Table 2. Growth inhibition of acid precipitated biosurfactants produced by different isolates on bacterial strains tested

Isolate	MIC (mg/ml)			MBC (mg/ml)		
	E. coli	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*	*P. aeruginosa*
NS1	57.9	28.9	57.9	57.9	28.9	57.9
NS3	43.5	43.5	43.5	43.5	43.5	43.5
NS4	90.8	90.8	181.6	90.8	90.8	181.6
NS5	59.1	59.1	59.1	59.1	59.1	59.1
NS6	96.2	96.2	96.2	96.2	96.2	96.2
NS7	47.5	95.0	95.0	47.5	95.0	95.0
G7	41.6	41.6	41.6	41.6	41.6	41.6
TP8	>173.0	86.6	86.6	>173.0	>173.0	86.6

as well as peptide and carbonyl moieties. The biosurfactant producing strain G7 isolated from oil contaminated soil was later identified as *Psedomonas fluorescens* by morphological and biochemical tests (Table 3). These results also support by those of biosurfactant produced by *Psudomonas fluorescens*, which usually produced glycolipid surfactant, rhamnolipid (Desai and Banat, 1997; Abouseoud *et al*., 2008; Hultberg *et al*., 2008).

Table 3. Characteristic of *Pseudomonas fluorescens* G7

Characteristics	Reaction *
Gram reaction	- ve
Reduction of nitrate	+
Indole production of tryptophane	-
Fermentative of acid fromglucose	-
Arginine dihydrolase	+
Urease production	-
Hydrolysis of esculin	-
Hydrolysis of gelatin	+
β-galactosidadase production (ρ-nitro phenyl-β-galactopyramnoside)	-
Glucose	+
Arabinose	+
Mannose	+
Mannital	+
N-acetyl-glucosamine	+
Maltose	-
Gluconate	+
Caprate	+
Adipate	-
Malate	+
Citrate	+
Phenyl-acetate	+
Cytocrome oxidase	+

* -ve = gram negative bacteria + = positive reaction
- = negative reaction % identification = 99.4%

Although the acid precipitated biosurfactant of *Psedomonas fluorescens* G7 had a potential to inhibit the growth of some bacterial pathogens, the MIC index obtained from this isolate was still quite high compared to that of other purified biosurfactants, where very low concentrations leveling (µg/ml) were MIC expressed. This may be due to the impurity of the acid precipitated biosurfactant. Most of the biosurfactants used as antimicrobial agents are required in very low concentrations as expressed by their MIC index (Cameotra and Makkar, 2004) and this factor makes biosutfactants highly sought biomolecules for present and future applications as fine specialty chemicals, biological control agents, and new generation molecules for pharmaceutical, cosmetic and health care industries. Further studies, including, *i.a*., the separation and purification steps of the biosurfactant, and process optimization at the biological and engineering levels are, therefore, required for biosurfactant production.

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Correspondence to
Associate Professor Dr. Siripun Sarin
Department of Microbiology and Parasitology,
Faculty of Medical Science,
Naresuan University,
Thailand
E-mail: schidburee@hotmail.com