Isolation and Characterization of Biosurfactant Produced by Lactic Acid Bacteria from Indigenous Thai Fermented Foods

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***Abstract*—Biosurfactant-producing lactic acid bacteria were isolated from the indigenous Thai fermented foods. MRS-oil agar and surface tension measuring methods were used as primary and secondary screening, respectively. There were 78 LAB isolates from 24 samples of the indigenous Thai fermented foods showed halo zone on MRS-oil agar. PS001 isolate derived from pickled fish (*Pla Som*) represented the lowest surface tension with 54.33 mN/m of PBS supernatant. PS001 produced cell-bound biosurfactant as growth associated product. For biosurfactant extraction, PBS supernatant was acid precipitated and followed by extraction with a mixture of chloroform:methanol (2:1). Its chemical structure of purified biosurfactant from PS001 was identified by TLC and FTIR spectroscopy as glycoprotein.**

***Index Terms*—indigenous Thai fermented foods, lactic acid bacteria, biosurfactant, surface tension, glycoprotein**

I. Introduction

Fermented foods play an important role in the Thai diet and in the life of Thai people. Most Thais eat an indigenous fermented product in one or another every day. This indigenous Thai fermented food is normally concerned with a wide diversity of lactic acid bacteria (LAB).

LAB are group of Gram-positive, acid-tolerant, non-spore forming, either rod- or cocci-shaped bacteria. These groups of bacteria produce lactic acid as the product of carbohydrate fermentation [1]. LAB are generally recognized as safe (GRAS) and they play the important role in various industries due to their contributions to the healthy microflora of human mucosal surfaces.

Biosurfactants are surfactants produced by organisms, including animals, plants and especially, microorganisms. These biosurfactants contain with hydrophobic and hydrophilic moieties in molecules. According to their structures, biosurfactants are able to reduce surface and/or interfacial tensions and also enhance the emulsification properties [2]. Biosurfactants have several advantages over the chemical surfactants such as low toxicity, environmental compatibility, high biodegradability and temperature and pH tolerance [3]. Biosurfactants are widely applied in several industries including agriculture, food, cosmetics, pharmaceutical and petroleum industry [4].

Biosurfactants produced by lactic acid bacteria (LAB) are alternative materials to use in food applications. Many researchers have been reported the biosurfactant production by LAB. *Lactobacillus paracasei* isolated from a Portuguese dairy industry was found to produce biosurfactant [5]. *Lactobacillus delbrueckii* cultured with peanut oil cake as the carbon source also produced biosurfactant [6]. Gudina *et al.* revealed that biosurfactant was produced by *L. paracasei* ssp. *paracasei* A20 [7]. *Lactobacillus fermentum* B54 and *L. acidophilus* RC14 were also reported as cell-bound biosurfactant strains [8]. Shokouhfard *et al*. reported the cell-bound biosurfactant production from *L. acidophilus* ATCC 4356 [9]. Furthermore, cell-bound biosurfactants from *L. fermenti* 126 and *L. rhamnosus* CCM 1825 were also characterized their molecules by Fourier transform infrared spectroscopy (FTIR) [10].

The objectives of this study were to screen biosurfactant producing lactic acid bacteria from the indigenous Thai fermented foods. A selected isolate was then studied biosurfactant production and extractions. Finally, biosurfactant was purified and analyzed its chemical component.

II. Matterial and Method

A. Samples and LAB Isolation

Indigenous Thai fermented foods including fermented meat (*Nham*), pickled fish (*Pla Som*), fermented sour sausage (*Sai Krok Preaw*), fermented soybean (*Thua Nao*) and pickled shallots (*Horm Dong*) form local markets were purchased to isolate LAB. Thai people are familiar with these fermented foods.

Twenty five grams of each food sample was added with 250 ml normal 0.85% saline solution (NSS) and blended by a commercial blender. Mixed suspension was performed by ten-fold dilution and spread on De Man, Rogosa and Sharpe (MRS) (Difco, USA) agar supplemented with 0.5% (w/v) $CaCl_2$ granule. Culture was incubated at 37 ℃ for 72h under oxygen limitation.

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Strains with halo zone on MRS agar with $CaCl_2$ were observed the morphology and individually streaked onto MRS agar to isolate a pure culture. Each strain was given its code and stored at -18°C for the further experiments.

B. Screenning of Biosurfactant Producing LAB

1) Primary screening

Primary screening was performed by using oil agar technique [11]. Briefly, MRS agar was spread with 40 µl sterilized lubricant oil and left to dry. Each LAB strain was 9 points spotted on MRS-oil agar and incubated at 37 ℃ for 72h under oxygen limitation. The strains with dispersion zone on MRS-oil agar were selected for secondary screening.

2) Secondary screening

Secondary screening was evaluated by surface tension measurement using Tensiometer K6 (Kruss, Germany) with Du-Nouy Ring method [12]. Each strain was cultured in MRS broth at 37 ℃ for 72h under oxygen limitation. Microbial biosurfactants are produced both of extra-cellular and cell-bound biosurfactants, therefore cell suspension and cell free culture broth (CFCB) were measured their surface tensions [8], [12]. Culture broth was centrifuged at 6,500g, 4 ℃ for 15 min to collect cells and CFCB. Cells were washed twice with 0.85% NSS and re-suspended in phosphate buffer saline (PBS, pH 7) for 24 h with gentle stirring. Then, cell suspension was centrifuged with the same conditions above to collect and measure surface tension of PBS supernatant. Controls were sterile MRS broth and phosphate buffer saline. Experiment was performed in triplicate. A lactic acid bacteria strain with the lowest surface tension was selected for the next experiments.

C. Growth Curve of Selected LAB Strain

Selected isolate was cultured in MRS broth at 37 ℃ for 72 h. Culture broth was collected every 4h to investigate variable cells and surface tension. Viable cells were determined by 10-fold dilution and using 0.85% NSS as a diluent. Pour plate technique in MRS agar was used in this experiment. Inoculated MRS agar was then incubated at 37 ℃ for 72h. The surface tension of PBS supernatant was monitored during cultivation. The triplicate experiments were individually performed in this experiment.

D. Biosurfactant Extractions

Biosurfactant from PBS supernatant was derived from both extraction and precipitation. Extraction was performed by ethyl acetate [13], chloroform [14] and a mixture of chloroform: methanol (2:1) [15]. Cold acetone precipitation [16] and combination of acid precipitation and chloroform: methanol (2:1) extraction [12] were also used to extract biosurfactant.

PBS supernatant (200 ml) was 3 times extracted by each solvent with the equal volume. Organic phase of each solvent was collected and evaporated by vacuum rotary evaporator at 40 ℃ to get crude biosurfactant.

For cold acetone precipitation, 200 ml PBS supernatant was added with same volume of cold acetone. Crude biosurfactant was collected by centrifugation with 8,000g, 4 ℃ for 10 min.

Acid precipitation coupled with chloroform: methanol extraction was also performed to collect crude biosurfactant. The PBS supernatant (200 ml) was acidified to be pH 2 by 6 M HCl and stored at 4 ℃ for overnight. Acidified PBS suspension was centrifuged at 8,500g 4 ℃ for 15 min. The precipitate was re-dissolved in sterile water and its pH was adjusted to 7. Neutral solution was then extracted 3 times by chloroform: methanol (2:1). Organic phase was collected and evaporated by vacuum rotary evaporator at 40 ℃.

The yield of crude biosurfactant from each method was determined. The oil displacement method [12] was used to evaluate surface active activity of crude biosurfactant by measuring diameter of clear zone on oil film. The crude biosurfactant was kept in glass wear at 4^oC for the further experiments. The experiments were individually triplicate performed.

E. Chemical Structure Identification of Biosurfactant

1) Thin layer chromatography

To purify crude biosurfactant, silica gel plate (Merck) and a mixture of chloroform: methanol: water (65:25:4) were used as stationary and mobile phases [17], respectively.

Iodine vapor was used to visualize bands on TLC. Each band on silica gel was scraped and re-suspended by chloroform: methanol (2:1). After centrifugation, supernatant of each band was concentrated by gentle N_2 blowing. Residues were individually tested their surface active activities by the oil displacement method [12].

Visualization was performed by 1% ninhydrin solution, Molish reagent, and 0.1% bromothymol blue in sodium hydroxide solution for amino acid, sugar [18], and lipid detections [17], respectively.

2) Fourier Transform Infrared Spectrometer (FTIR)

The band with the highest oil displacement activity from TLC was analyzed its chemical structure by FTIR (Perkin Elmer, USA). FTIR spectrum was carried out in transmittance mode by using 4 cm^{-1} of resolution with the wave number range of 400 to 4,000 cm^{-1}[10].

F. Experimental Design and Data Analysis

One way Analysis of variance (ANOVA) was used to analyze the results with a significant level of 0.05 by Statistical Package for the Social Science for Windows (SPSS) version 17.0.

III. Results and Discussions

A. Isolation of Lactic Acid Bacteria

In this study, bacteria were isolated from 24 samples indigenous Thai fermented foods of fermented meat (*Nham*), pickled fish (*Pla Som*), fermented sour sausage (*Sai Krok Preaw*), fermented soybean (*Thua Nao*) and pickled shallots (*Horm Dong*). Only 102 isolates showed the halo zone around colony by using MRS agar supplemented with $CaCl_2$ granule. The number of lactic acid bacteria from samples was showed in Table I.

TABLE I. THE NUMBER OF LAB ISOLATE FROM INDIGENOUS THAI FERMENTED FOODS

Sample	Abbreviation	Number of sample	Number of isolate
Fermented meat (*Nham*)	MN	6	34
Pickled fish (*Pla Som*)	PS	3	32
Fermented sour sausage (*Sai Krok Preaw*)	SK	6	26
Fermented soybean (*Thua Nao*)	TN	6	7
Pickled shallots (*Horm Dong*)	HD	3	3

B. Screening of Biosurfactant Producing LAB

After primary screening, there were 78 LAB isolates that showed dispersion zone on MRS-oil agar. These isolates were selected for secondary screening.

In secondary screening, tensiometer was used to measure both CFCB and PBS supernatant from selected isolates. Five isolates with the lowest surface tension were showed in Table II. The surface tensions of all isolates were lower than controls of MRS broth (49.5 mN/m) and PBS (70.5 mN/m).

However, the more difference surface tension between PBS supernatant and PBS control was observed in all 5 isolates. It indicated that PBS supernatant contained with surface active compound. The similar results were reported by many researchers. Velraeds *et al*. [8] revealed that *Lactobacillus acidophilus* and *L*. fermentum B54 produced cell-bound biosurfactants. In addition, Brzozowski *et al*. reported that *L. fermenti* 126 and *L. rhamnosus* CCM 1825 were also produced cell-bound biosurfactants [10].

PBS supernatant of PS001 isolate showed the lowest surface tension of 54.33mN/m. It also showed the highest value of surface tension reduction (16.17mN/m) when compared with PBS control. Therefore, cell-bound biosurfactant of PS001 isolate was focused in this study.

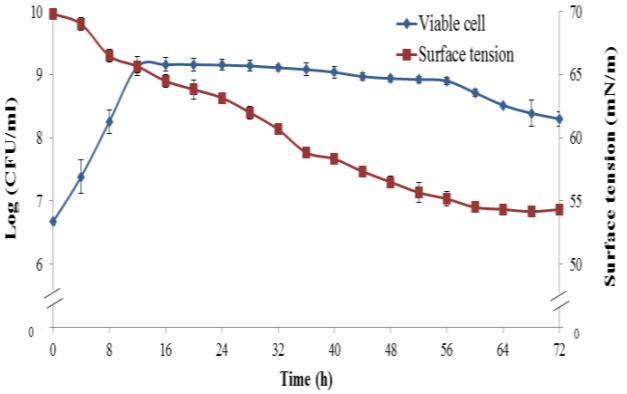
TABLE II. THE SURFACE TENSION OF FIVE LAB ISOLATES

Isolate	Surface tension (mN/m)	
	CFCB*	PBS supernatant**
PS001	45.33 ±0.47^{a}	54.33 ±0.94^{a}
MN003	45.50 ±1.28^{a}	59.50 ±0.12^{b}
MN019	46.83 ±0.24^{b}	60.00 ±1.63^{b}
PS009	47.00 ±0.41^{bc}	61.00 ±2.94^{b}
MN005	47.17 ±0.62^{c}	60.00 ±8.64^{b}
Controls	49.33 ±0.28^{d}	70.50 ±0.50^{c}

Values with the same letter within a column are not significantly different at $p > 0.05$.

C. Growth Curve

During PS001 cultivation in MRS broth for 72 h, the surface tension of PBS supernatant was significantly decreased at the logarithmic growth phase (Fig. 1). After 56 h of cultivation, the surface tension was mostly constant at 54 mN/m. According to the result, it indicated that cell-bound biosurfactant of PS001 was growth associated product. The result was agreed with *Lactobacillus delbrueckii* [6]. Rodrigues *et al.* also reported that *L. casei* CECT-5275 and *L. rhamnosus* CECT-288 produced cell-bound biosurfactants during exponential growth phase [19].



Fegure 1. Growth curve and surface tension of PS001 PBS supernatant

D. Biosurfactant Extractions

All extraction methods were able to extract biosurfactant from PBS supernatant of PS001 as shown in Table III. These results were similar to the previous reports [12]-[16]. The highest crude biosurfactant yield was 0.32 g/L by using chloroform extraction. However, the biggest of oil displacement activity with 4.2 cm was derived from the acid precipitation coupled with chloroform:methanol (2:1) extraction. Siriwong Na Ayudhya *et al*. was also reported that this method was able to extract biosurfactant [12].

TABLE III. YIELD AND OIL DISPLACEMENT ACTIVITY OF CRUDE BIOSURFACTANTS EXTRACTED WITH VARIOUS METHOD

Method	Yield (g/L)	Oil displacement activity (cm)
Chloroform extraction	0.32 ±0.01^{c}	0.9 ±0.10^{b}
Chloroform:methanol (2:1) extraction	0.28 ±0.01^{b}	1.5 ±0.25^{c}
Ethyl acetate extraction	0.24 ±0.02^{a}	2.8 ±0.10^{d}
Cold acetone precipitation	0.32 ±0.00^{c}	0.4 ±0.23^{a}
Acid precipitation coupled with chloroform:methanol (2:1) extraction	0.27 ±0.03^{b}	4.2 ±0.25^{e}

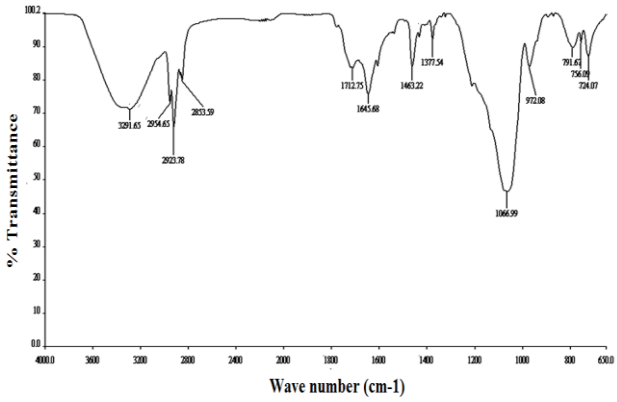
Values with the same letter within a column are not significantly different at $p > 0.05$.

E. Chemical Structure Identification of Biosurfactant

PS001 crude biosurfactant was purified by silica gel TLC plate. After visualization by I_2 vapor on TLC plate, there were 8 bands. Only one band with R_f of 0.8 showed surface active property by the oil displacement method. A band with the same R_f value was also detected by ninhydrin and Molish reagent with reddish and red spots, respectively. Furthermore, there was no detected band by

bromothymol blue reagent. It indicated that PS001 biosurfactant contained with amino and sugar.

These results were confirmed by FTIR spectrometer as showed in Fig. 2. The presence of 3,200-3,500 cm^{-1} peak was indicated the presence of OH and NH groups. Moreover, a presence of 1,712 and 1,645 cm^{-1} peaks was indicated the presence of C=O carbonyl group and NH peptide linkage. A peak at 1,000-1,200 cm^{-1} presented C-O stretching in sugar [20]. Purified biosurfactant from pattern of glycoprotein biosurfactant FTIR spectrum was reported by Moldes *et al*. [21]. Also, Gudina *et al*. reported that *Lactobacillus agilis* CCUG31450 produced glycoprotein biosurfactant [22].



Fegure 2. FTIR spectrum of biosurfactant isolated from PS001

IV. Conclusions

The 102 lactic acid bacteria were isolated from 24 samples of indigenous Thai fermented foods. The primary screening of biosurfactant producing LAB was performed by MRS-oil agar plates. There were 78 LAB isolates that showed halo zone on MRS-oil agar. For the secondary screening, the lowest surface tension of 54.33 mN/m was observed in PS001 PBS supernatant. The biosurfactant produced from PS001 was cell-bound and growth associated product. Acid precipitation coupled with chloroform: methanol extraction was the suitable extraction method on this experiment. The chemical structure of PS001 purified biosurfactant was analyzed as a glycoprotein. Furthermore, the PS001 isolate will be carried out for molecular identification and studying on biological activity of its biosurfactant.

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