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Burapha University International Conference 2015

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ST-P-022

Biosurfactant act as antimicrobial properties produced by *Candida mucifera* NJP25

Jamroonsri Poomtien[a*], Thayawee Thanasukhonphat[a], Napawee Songmark[a], Sasitorn Jindamorakot[b], and Jiraporn Thaniyavarn[c]

[a]*Department of Biological Science, Faculty of Sciece and techology, Huachiew ChalermprakietUniversity.Samutprakarn 10540, Thailand*
[b]*Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani 12120, Thailand*
[c]*Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10300, Thailand*
E-mail: jamroonsri@yahoo.com

Abstract

Biosurfactants are important bioactive compounds which have ability to reduce surface tension and increase the solubility. In this present study, yeast strain NJP 25 isolated from the rotten oil palm bunches was found to be a biosurfactant-producing yeast, conspecific identified as *Candida mucifera*. This work aimed to study the yeast growth and biosurfactant activities and test the antibacterial activity of crude biosurfactants produced from yeast using agar well diffusion and micro-dilution method in order to determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentration (MBC). As a result, *Candida mucifera* NJP25 was the first reported as a biosurfactant producing yeast, showed the best performance of antibacterial activity. The cultivation for yeast NJP25 was performed at 30°C in shake flask at 200 rpm with a medium containing 2% glucose and 2% palm oils as carbon sources, 0.4% $NaNO_3$ and 0.1 % yeast extracts as nitrogen source with an initial pH of 5.5. The maximum growth was obtained on day 9 at 3.78 g L^{-1}. The maximum biosurfactant activities in cell free supernatant was on day 7 which significantly reduced from 53.3 mN m^{-1} to 34.2 mN/m of medium surface tension (ΔST value 19.1 mN/m) . Their antimicrobial properties by using agar well diffusion showed that it had maximum effects against Gram positive and Gram negative bacteria. MIC values against the growth of *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus were* 1.953 mg/ml and *Pseudomonas aeruginosa* at 3.906 mg/ml. In addition MBC values could destroy bacterial *E. coli* and *S. aureus* at 3.906 mg/ml, *B. subtilis* and *Ps. aeruginosa* at 7.81 mg/ml. This preliminary study exhibited that the biosurfactants derived from yeast, *Candida mucifera* NJP25, has the advantage that it uses in cosmetics and pharmaceuticals application.

Keywords: biosurfactant-producig yeast; agar well diffusion; Minimum Inhibitory Concentration (MIC)

Introduction

Biosurfactants are surface active compounds some were synthesized by microorganisms such as bacteria, yeast and fungi. They are totally or partially extracellular substances with amphiphillic compounds containing both polar and nonpolar moieties then can soluble in both liquid and oil phases. With diverse properties, new value adding opportunities will result from the identification of new biosurfactants with different specific activities, especially in relation to their biological effects where they have potential application as surface active agents, bioemulsifiers, solubilizers, cleansing agents, antiadhesives and antimicrobial agents (Deleu and Paquot, 2004; Mulligan, 2005). Biosurfactant have demonstrated that they could have a wide range of application in pharmaceutical fields such as gene delivery, immunological adjuvants, inhibition the adhesion of pathogen and antimicrobial activity (Fathabad, 2011). Several authors reported on biosurfactants applications have been focusing on antimicrobial activities against bacteria, fungi, algae and viruses (Nitschke and Costa, 2007). With the interesting among yeasts, *Candida* species have been widely employed for soluble and insoluble carbon sources for fermentation and have been reported to produce surface-active agents (Amarall et al., 2008). Many researches have emphasized the economical factors affecting the efficiency of biosurfactant production in terms of higher yields and lower production costs (Makkar et al., 2011). Palm oil is a common cooking ingredient in the many countries of Africa, Southeast Asia and Brazil. Its use in the commercial food industry in other parts of the world is widespread because of its lower cost. Thus it has attractive to be a hydrophobic carbon source for biosurfactant production. The potentials of biosurfactants with antimicrobial properties have certainly enormous values. However biosurfactants production for use as antimicrobial agents has quite a few claimed, there are increasing search on microbial sourcing, strain, improvement and production processes. Therefore this preliminary study had objectives to investigated abilities of yeast strain NJP25 produce biosurfactant in fermentation medium supplemented glucose and palm oil as carbon sources and evaluated significantly the antimicrobial properties of the crude biosurfactant.

Materials and methods

1. Microorganisms

1.1. Yeast strains. The biosurfactant-producing yeasts in this study were isolated from the rotten oil palm bunches as oils rich samples. They collected from palm oil tree garden in Pakjan District, Ranong in Thailand (Poomtien et al., 2013). Strain NJP25 was obtained by using dilution plating technique on YM agar (pH5.5) supplemented with 1% (v/v) palm oil, 100 µg/ml chloramphenicol, and 0.2% (w/v) sodium propionate incubated at 30 °C for 3 days. This yeast strain was selected as efficient biosurfactant-producing yeast by determining surface tension reduction that showing surface tension values lower than 40 mN m^{-1}.

1.2 Bacterial strains. Four bacterial cultures were used to test the antimicrobial properties of the biosurfactant. *Bacillus subtilis* TISTR 6633 *Escherichia coli* TISTR 8739 *Pseudomonas aeruginosa* TISTR 781 and *Staphylococcus aureus* TISTR 1466, were kept in Trypticase Soy Broth (TSB) added 20% sterile glycerol and stored in deep freeze at – 20ºC. Whenever required, frozen stocks were streaked on TSA agar plates and incubated overnight at the optimum growing temperature for each strain for further culturing. Working stock cultures were kept at 4°C for up to 2 weeks.

2. DNA sequencing and phylogenetic analysis. Nuclear DNA was isolated and purified according to the procedure reported by Nakase and Suzuki, 1985. For the PCR protocol, the D1/D2 domain of LSU rDNA were amplified using the primers F63 and LR3 according to the methods of Kurtzman and Robnett, 1998. The D1/D2 domains of yeast strains were identified by comparing with these of the yeast reference sequences databases using the BLAST sequence analysis tool. For phylogenetic tree analysis, sequence data were aligned with rDNA sequences of representative related species, type strain, obtained from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). These DA sequences were aligned using the CLUSTAL X ver1.81 (Thompson et al., 1997) and constructed phylogenetic trees using Neighbor-Joining analysis (Saitou and Nei, 1987). Bootstrap analyses were performed 1,000 random resamplings (Felsenstein, 1985).

3. Media for cultivation. The medium used for cultivation of efficient biosurfactant production was a modified Hua's medium (Hua et al., 2003) at an initial pH 5.5 containing 0.4% (w/v) $NaNO_3$, 0.02% (w/v) $MgSO_4.7H_2O$, 0.02%(w/v) KH_2PO_4, 0.1% (w/v) yeast extract and supplemented with 2% (w/v) glucose and 2% (v/v) palm oil as hydrophilic and hydrophobic carbon sources, in respectively. The culture was incubated at 30 ºC with shaking at 200 rpm for 7 days. The inoculums having OD 1.0 at wavelength 600 nm (10% v/v) was introduced to a growth medium. This experiment having three sets of flasks was determined at interval at 1, 3, 5, 7 and 9 days for biomass and biosurfactant activities. Time-course study of the biosurfactant production was performed by determination of yeast dry cell weight (DCW), pH change, biosurfactant activity from surface tension (ST) values (mN/m) and oil displacement areas (ODA).

4. Cell biomass measurement. Biomass as cell dry weight was determined by centrifugation of the culture at 8,000 rpm, 4^{o}C for 20 min. The pellet was washed once time with hexane and twice with distilled water, placed on a pre-weighed foil-plate and dried at 105^{o}C to obtain constant weight.

5. Biosurfactant activity test. After centrifugation of the culture broth, the supernatant was harvested, through roughly filtration to remove oil and examined the oil displacement test to form an oil displacement area and the ST value for its ability to reduce the surface tension. The oil displacement test was performed by measuring the diameter of the clear zone formed on the surface of an oil-water phase (15 µL oil/ 153.9 cm^2 surface area) after dropping 10 µL of the culture supernatant and evaluating the displacement area, as previously reported (Morikawa et al., 1993). The ST value was measured by the Du Nouy Ring method (Tensiometer, K6; Kruss, Hamberg, Germany). The surface measurement was carried out at 25 ± 1 ºC after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. For calibration of the instrument, the surface tension of the pure water was measured before each set of experiment.

6. Production and isolation of crude biosurfactants produced by yeast strain NJP25. Biosurfactant production was performed by culturing in a medium supplemented with 2% (w/v) glucose, 2% (v/v) palm oil (GP medium) with shaking at 200 rpm, 30°C for 7 days. The supernatant was harvested after oil removing and then extracted 3 times with ethyl acetate. The ethyl acetate extracts were pooled, evaporated, to dryness in a vacuum at 40°C, and weighed to determine the crude biosurfactant yield.

7. Determination of antimicrobial properties of crude biosurfactant NJP25

7.1 Agar well diffusion method. Fresh bacterial inoculum reading OD625 at 0.08-0.1 was uniformly swabbed on a Muller Hinton Agar (MHA) plate. The wells were punched over the agar plates using sterile cork borer with diameter 0.6 mm. Crude biosurfactant at various concentration (250, 500, 750 and 1,000 mg/ml) were added to the wells. The plates were incubated for 24 hours at 37 °C. After incubation the diameter of inhibiton zones around each well were measured in mm and recorded. Tests were performed in duplicate.

7.2 Micro-dilution method. The antibacterial activity of crude biosurfactant was analyzed by a micro-dilution method in 96-well microtiter plates. For this experiment, 100 ul of sterile Muller Hinton Broth (MHB) were filled into 12 wells in a row. After that 100 µl of biosurfactant solution (500 mg/ml) were added to the first column of the microplate and mixed well. This result in the first well was a biosurfactant concentration of 250 mg/ml and then diluted in two-folded serial dilution by transferring 100 µl to the subsequent wells until the 11th column. The 12th column had not filled biosurfactant and served as positive (growth) controls. All the wells were inoculated with 5 µl of an overnight culture having OD625 at 0.08 -0.1 and added 5 µl of 0.3% resazurin solution as indicator. Microplates were covered and incubated 37 C for 48 h. The experiments were performed in duplicate in each bacterial strain. To observe whether bacterial growth occurred, indicator changed from blue to pink. However we further to find out the minimal bactericidal concentration (MBC) of crude biosurfactant, broth from MIC well including upward and backward of MIC were streaked on to TSA agar plate, incubate 37 °C for 48 h. No colonial bacteria growth was recored as MBC value indicated the lethal effect of the antimicrobial agent (bactericidal) on the test organism. The results were expressed in mg/ml.

Results and Discussions

1. Isolation and identification of a biosurfactant producing yeast strain NJP25. Yeast strain NJP25 was isolated from the rotten oil palm bunches. They collected from palm oil tree garden in Pakjan District, Ranong in Thailand (Poomtien et al., 2013). The rotten palm bunches were agricultural wastes with abundantly plant oil as hydrophobic carbon source for microbial growth and biosurfactant production.
From screening the biosurfactant - producing yeast, strain NJP25 showed the biosurfactant producing ability, as possessed minimum surface tension of 27.33 mN/m of cell free broth after growth in acidified YM broth containing 1% (v/v) palm oil for 7 days. A good surfactant is capable of reducing the surface tension (ST) of water from 72 to 35 mN/ml (Mulligan, 2005). Therefore strain NJP25 had gain interested in biosurfactant production. Based on D1/D2 region of the large subunit (LSU) 26S rRNA gene, strain NJP25 was identified as *Candida mucifera*, showing similarity 99.70% with type strain of *Candida mucifera* CBS 7409^{T} (AJ508572). As the guideline of yeast taxonomy (Kurtzman and Robnett 1998), strain with difference less than 1% nucleotide substitution of the D1/D2 region usually conspecific species. The phylogenetic tree constructed by neighbor-joining method of yeast NJP25, it was the closet relationship to *Candida mucifera* and showed relationships among the *Stephanoascus ciferrii* complex. It located closely to *Stephanoascus ciferrii* CBS4856 ^{T} and *Candida allociferrii* IFO 10194 ^{T} as shown in figure 1. The phylogram construction in this paper is in agreement with other previous reports (Nishimura and Mikata, 2002) by depicting relationships among the *Stephanoascus ciferrii* complex and related species analysed by 26S rDNA domain D1/D2.

2. Growth, biosurfactant activity and production of a biosurfactant producing yeast strain NJP25.
Biosurfactant production by yeast NJP25 was examined by cultivating in defined medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil and 0.4% (w/v) $NaNO_3$ at an initial pH 5.5 with agitation speed of 200 rpm under controlled at 30°C for 7 days. Biomass growth profile and biosurfactant activity were obtained in order to establish the relations between cell growth and production of biosurfactants as surface tension reduction values monitoring in time as shown in Fig. 2. Yeast NJP25 could grow increasingly within 3 days when easily consumed the simplest carbon source, glucose. It displayed diauxic growth that it subsequently consumed another carbon source, palm oil after day 5. The yield of biosurfactant steadily increased even after the culture reached the stationary phase. The surface tension of the cell free broth was lowered from 53.3mN/m to 34.2 mN/m as minimum surface tension (min ST) value. The ΔST value was derived from the ST of the supernatant minus the ST of the initial medium was used as followed by Thaniyavarn et al., 2008. Under cultivation conditions, the Δ surface tension (ΔST) of the glucose and palm oil culture showed the highest value as 19 mN/m when grew on day 7 (Fig. 2). This result is consistent with the finding of other previous studies that showed ST reduction activity of *Pichia anomala* PY1 at 21 mN/m (Thaniyavarn et al., 2008) and *Cyberlindnera samutprakarnensis* JP52^{T} at 19 mN/m when grew on day 7 (Poomtien et al., 2013).

The production yield of the crude biosurfactant of yeast NJP25 obtained from cultivation in 2% (w/v) glucose plus 2% (v/v) palm oil (GP) medium for 7 days was found to be 3.32 g/L. It was oily- deep brownish color.

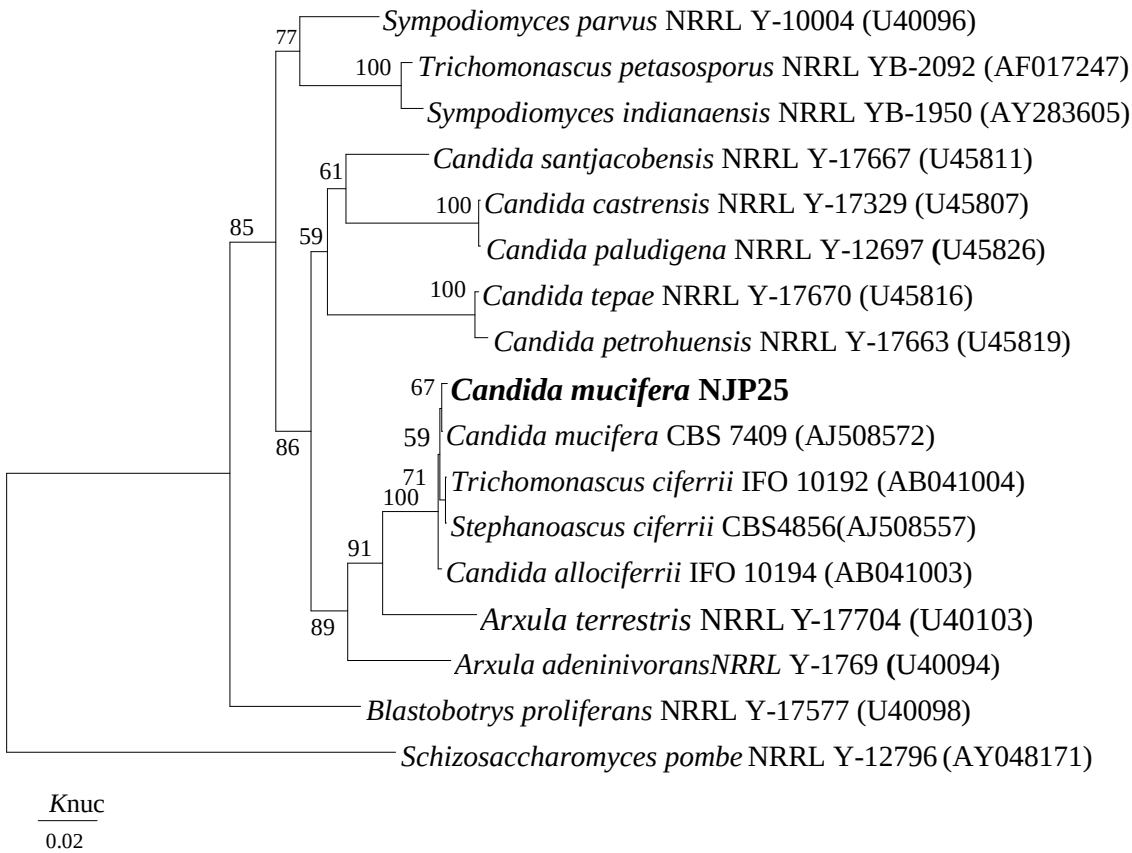

Fig. 1 NJ-based phylogenetic tree for *Candida mucifera* (NJP25) based on the D1/D2 region of the LSU rRNA gene sequence. *Schizosaccharomyces pombe* NRRL Y-12796^{T} is used as the outgroup. The numerals at each node represent the percentages from 1,000 replicate bootstrap resamplings (excluded when < 50%). Sequences were retrieved from the NCBI Genbank databases and CBS database (*). *Bar* 0.01 substitutions per nucleotide position.

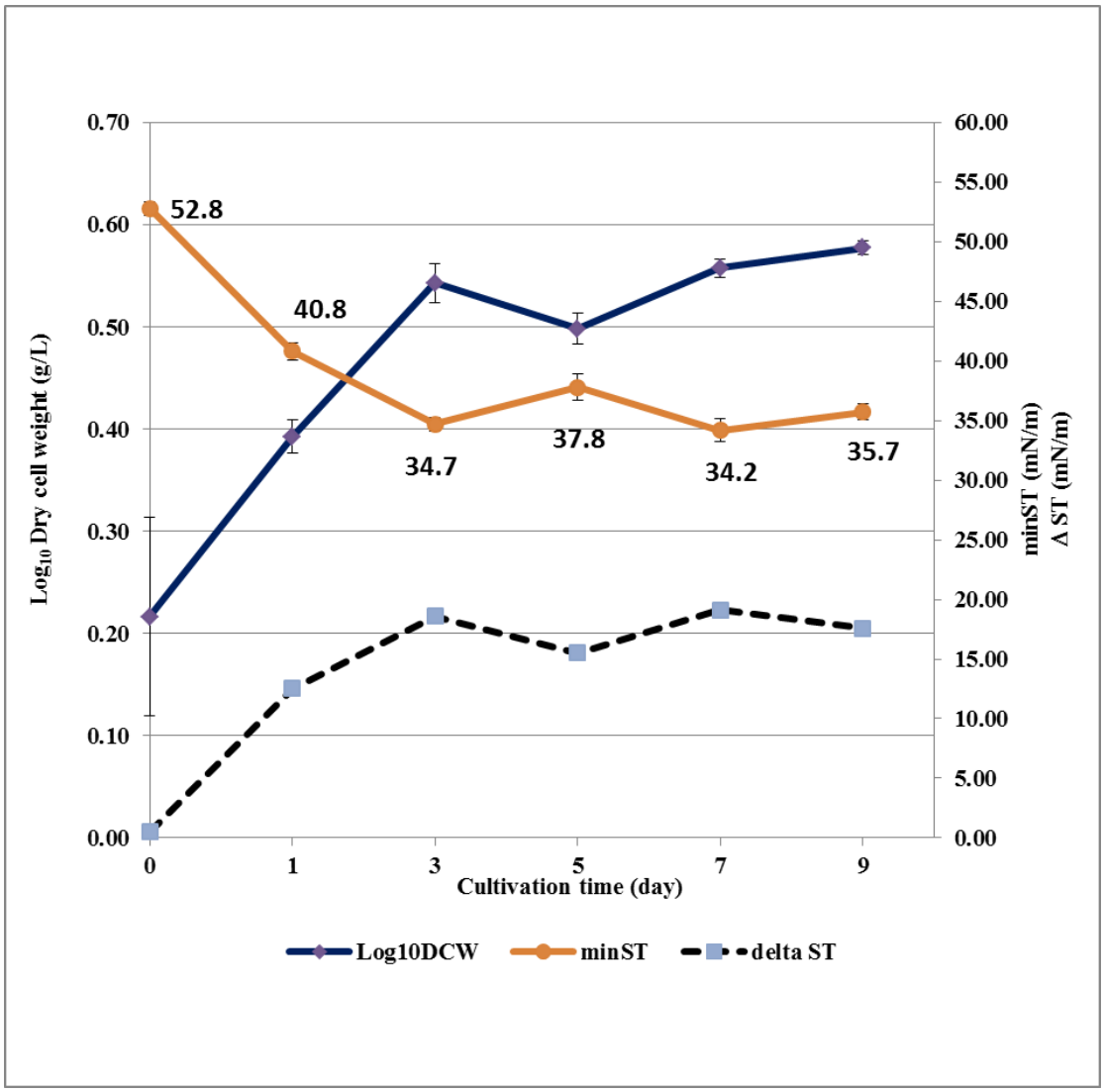

Fig. 2 Profile of cell growth and biosurfactant activities of yeast strain NJP25 when cultured in media supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil.

3. Antimicrobial properties of crude biosurfactant NJP25

3.1 Agar well diffusion

The samples of crude biosurfactant extracts at varied 250-1000 mg/ml dissolved in 50 mM Tris-HCl were tested against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonnas aeruginosa* by agar well diffusion. At volumes of 30 microliters of crude extract solution contained 7.5-30 µg /well. With regards to diameters of the inhibition zones, all crude biosurfactant NJP25 solution tests demonstrated effective inhibition on the growth of these bacterial strains as illustrated in Table.1. Their antimicrobial tests showed that it had maximum effect against Gram positive and negative bacteria.

The results were compared with results obtained using standard antibiotic disc (gentamicin 1 ug/disc and erythromycin 1 ug/disc). Their zone of inhibition of crude extracts at 500 mg/ml against all bacterial tests showed higher lethal effects than gentamicin. Gram bacteria, *E. coli* and *P. aeruginosa*, were found to be sensitive at 20.5 and 35.5 mm diameter in respectively. *S. aureus* and *B. subtilis* also exhibited clear zone at 30.0 and 29.5 mm diameter in respectively.

Table 1 Antibacterial activity of crude biosurfactant NJP25 by agar well diffusion method

Sample at Concentration (mg/ml)	Amount of sample (µg /well)	Zone of innhibition (mm.)			
		E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*
Crude biosurfactant NJP25 at					
1000	30	27.5	36.5	34.5	43.5
750	22.5	23.0	34.5	34.5	40.0
500	15	20.5	30.0	29.5	35.5
250	7.5	16.0	24.5	24.5	32.0
Gentamicin	1 µg/disc	20.0	25.0	27.0	27.5
Erythromycin	1 µg/disc	12.5	26.0	31.5	10.0

3.2 Microdilution method

The antibacterial activity of crude biosurfactant was analyzed by a microdilution method in 96-well microtiter plates. The first well in this study was a biosurfactant concentration of 250 mg/ml and then serial diluted in two-fold dilution. Antimicrobial properties were indicated by observing the change color of rezasurin as indicator. The minimal inhibition concentration (MIC) of biosurfactant in the blue-well completely inhibited microbial growth. Whilst no antimicrobial activity was seen in the pink-well. Table 2 showed the MIC value of crude biosurfactant NJP25 obtained from broth microdilution, they were tested against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonnas aeruginosa*. These results illustrated that MIC value of crude biosurfactant NJP25 inhibited the all tested bacterial cultures of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were 1.953 mg/ml and also sensitivity test against *Pseudomonnas aeruginosa* at 3.906 mg/ml.

Table 2 Determination of the minimum inhibitory concentration (MIC) of crude biosurfactant NJP25

Bacteria	Biosurfactant concentration (mg/ml) in each well											Growth control
	250.0	125.0	62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488	0.244	
E.coli	-	-	-	-	-	-	-	-	+	+	+	+
P.aerugiosa	-	-	-	-	-	-	-	+	+	+	+	+
B.subtilis	-	-	-	-	-	-	-	-	+	+	+	+
S.aureus	-	-	-	-	-	-	-	-	+	+	+	+

\+ ----- Indicates the growth
\- ----- Absence of growth
Growth control: Bacterial culture without extract.

Further studies, broth from MIC well including upward and backward of MIC were investigated bacterial growth on TSA plates. No colonial bacteria growth was recored as MBC value. The minimal bactericidal

concentration (MBC) of crude biosurfactant NJP25 against *Escherichia coli* and *Staphylococcus aureus* were 3.906 mg/ml and *Bacillus subtilis* and *Pseudomonnas aeruginosa* at 7.813 mg/ml (Table 3). This finding was in accordance with those reported of Rufino et al. (2013) reported antimicrobial potential of a biosurfactant rufisan produced by *Candida lipolytica* UCP 0988 at concentration 0.75-12 mg/ml.

Table 3 Determination of the minimal bactericidal concentration (MBC) of crude biosurfactant NJP25

Bacteria	Biosurfactant concentration (mg/ml) in each well							Growth control
	31.25	15.625	7.813	3.906	1.953	0.977	0.488	
E.coli	-	-	-	-	+	+	+	+
P.aerugiosa	-	-	-	+	+	+	+	+
B.subtilis	-	-	-	+	+	+	+	+
S.aureus	-	-	-	-	+	+	+	+

+ ----- Indicates the growth
- ----- Absence of growth
Growth control: Bacterial culture without extract.

Conclusion

In summary, a yeast isolated from the rotten oil palm bunches as oils rich samples, was identified to be *Candida mucifera* that was able to grow effectively on glucose and palm oil as mixed carbon sources to produce biosurfactants. It showed significantly antimicrobial properties against both Gram positive and negative bacteria. The crude biosurfactants of yeast NJP52 had particularly potency to inhibit and destroy *E. coli*, *S. aureus*, *B. subtilis* and *P. aeruginosa* in a few amounts of substances of MIC and MBC values. Moreover bioactive compounds from yeast *Candida mucifera* has not been reported as biosurfactant and antimicrobial agent. Overall, this study has suggested *Candida mucifera* NJP25 is biosurfactant-producing yeast that produces potentially attractive biosurfactants for pharmaceutical applications.

Acknowledgements

We thank the department of Microbiology, Faculty of Science, Chulalongkorn University for using Tensiometer. This work was supported financially by Huachiew Chalermprakiet University.

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