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Production and Application of Biosurfactant Produced by *Agrobacterium rubi* L5 Isolated from Mangrove Sediments

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Abstract. An effective biosurfactant-producing bacteria, isolate L5, was isolated from mangrove sediments from both east coast and west coast of Southern of Thailand. Analysis of the 16S rRNA gene sequence confirmed that isolate L5 was *Agrobacterium rubi* with 100% homology. The biosurfactant production was performed using a mineral salt medium (MSM) with molasses as a carbon and commercial monosodium glutamate (MSG) as nitrogen sources. Under optimized conditions, *A. rubi* L5 was able to grow and produce biosurfactant with the yield of 4.62 g/l at 54 h of cultivation. It could reduce the surface tension of pure water from 72.0 to 25.5 mN/m and exhibit emulsification activity toward palm oil with 65.4%. The biosurfactant found to be stable even under extreme pH, temperature and salinity conditions. The results revealed the potential use of a biosurfactant produced by *A. rubi* L5 to enhance mobilization sorbed motor oil from environment in comparison with those of synthetic surfactants, i.e. a nonionic surfactant Triton X-100 and anionic surfactants sodium dodecyl sulfate.

Introduction

Mangrove ecosystem is a bridge between terrestrial and marine ecosystem and harbors unique microbial diversity. Mangrove sediments are rich in organic matter because of their high biodiversity. Microbes, being an important component of the mangrove environment, not only play a very critical role in creating and maintaining this biosphere but also serve as a source of biotechnologically valuable and important products [1]. Biosurfactants are surface-active compound (SAC) that are produced by a variety of microorganisms. They have gained biotechnologist interest for high diversity and they have numerous advantages compared to chemically synthesized [2]. Biosurfactants are becoming important biotechnology products due to their specific modes of action, lower toxicity, biodegradability, produced from renewable and cheaper substrates and widespread applicability [3]. They are interesting candidates in many fields such as environmental, food, biomedical and other industrial applications [4]. However, they remained several problems before more widespread commercial use including yield and cost of production [5]. Accordingly, several cheap renewable substrates from agro-industrial wastes have been intensively studied for microorganism cultivation and surfactant [6]. Molasses is a by-product of the sugar cane industry which widespread use compared to other sources. The current study was to investigate of using molasses as the substrate for the production of biosurfactant by *Agrobacterium rubi* L5 isolated from mangrove sediment and its possible application for motor oil removal from contaminated sand.

Materials and Methods

Potential biosurfactant producing strain

A promising biosurfactant-producing strain (isolate L5) was isolated from mangrove sediment in Southern of Thailand by enrichment culture technique with palm oil as the sole carbon source [7]. This strain was selected for further studies based on the best surface tension reduction and emulsification activity. A pure culture of the isolate was obtained by repeated sub-culturing monthly on nutrient agar (NA) (HiMedia, India) and maintained at 4ºC. The isolate was identified by 16S rRNA gene sequence analysis. 16S rRNA gene was amplified using universal primer 27F and 1492R (5'-AGAGTTTGATCATGGCTCAG-3'; 5'-GGTACCTTGTTACGACTT-3') [8]. The resulting sequence was compared with sequences in the GenBank database of NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) using the nucleotide-nucleotide blast (BLASTn) network service.

Kinetics of microbial growth and production of biosurfactant

For time course of biosurfactant production, *A. rubi* L5 was cultivated in MSM medium with optimal culture conditions (5.0% (w/v) molasses and 1.0% (w/v) MSG, 2.5% (v/v) inoculum, initial pH 7.0, 150 rpm at 30ºC) for 120 h with an interval of 6 h starting from the lag phase to stationary phase under batch culture conditions. Samples were taken at different time points during the fermentation to determine the biomass concentration and biosurfactant production.

For recovery of crude biosurfactant, *A. rubi* L5 was cultivated in 6 liters of MSM medium with optimal culture conditions for 30 h. Cells were separated from the cultures broth by centrifugation at 6000×g for 10 min at 4°C. The culture supernatant was extracted 2 times with equal volume of chloroform:methanol (2:1) and evaporated to dryness in rotary evaporator at 40°C under reduced pressure [9].

Study of biosurfactant stability

Stability studies were performed using crude biosurfactant prepared in distilled water at a concentration of 1 mg/l. To investigate the effects of pH, sodium chloride (NaCl) concentrations and temperature on biosurfactant activity, the pH stability was studied by adjusting the biosurfactant solutions to different pH values (2-12) using 1.0 N HCl or NaOH. Biosurfactant solutions were supplemented with different NaCl concentration to obtain the final concentrations of 0-12% (w/v). To evaluate the thermal stability, biosurfactant solution was incubated at 30-100°C for 1 h and 110 and 121°C for 15 min and cooled to 30°C. The remaining activity was then determined by surface tension measurement and emulsification activity.

Application of the biosurfactant for motor oil removal from contaminated sand

Biosurfactant suitability for enhance oil recovery was investigated using 800.0 g of acid washed sand impregnated with 50.0 ml of motor oil. Fractions of 20.0 g of the contaminated sand were transferred to 250 ml flasks which were submitted to the following treatments: addition of 60.0 ml distilled water (control), and addition of 60.0 ml aqueous solutions of the SDS, Triton X-100, biosurfactant under the CMC and above the CMC of each compound. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 30°C and centrifuged at 5,000 rpm for 20 min for separation of the laundering solution and the sand. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane [10]. Percentage of oil recovery is amount of oil recovery by biosurfactant divided by the amount of oil recovery by hexane multiplied by 100.

Analytical methods

Biomass determination was done in terms of dry cell weight [10].

The surface tension was measured using a Model 20 Tensiometer (Fisher Science Instrument Co., Pittsburgh, PA, USA) at 25℃.

The emulsification activity was performed to evaluate the emulsifying ability of culture supernatant following the method described by Cooper and Goldberg [11].

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, Chicago, IL, USA).

Results and Discussion

Isolation and identification of potential bacteria

From the result of isolation and screening of biosurfactant producing bacteria, the bacterial isolate L5 showed the highest biosurfactant production. It showed the best surface tension reduction from 71.2 to 38.0 mN/m as well as an emulsification activity of 50%. Thus, bacterial isolate L5 was selected for further study.

The isolate L5 was identified as *Agrobacterium rubi* with 100% similarity. The 16S rRNA sequence was deposited in DDBJ/EMBL/GenBank as accession number MH359100. The phylogenetic tree was constructed by neighbor-joining method, using the bootstrap resampling method with 1000 replicates. Isolate L5 was clustered into genus *Agrobacterium rubi* by 100% bootstrap confidence based on 16S rRNA (data not shown). Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4. Therefore, this bacterial isolate was identified as *Agrobacterium rubi* L5. *Agrobacterium* can be isolated from a variety of environments usually isolated from hydrocarbon contaminated environments and have ability to degrade oil and degraded many types of contaminated hydrocarbon [12,13]. Some *Agrobacterium* are also produce biosurfactant or bioemulsifier which improved the solubilization of oil and hydrocarbon [13,14]. However, there is no biosurfactant production from *Agrobacterium rubi* which have been reported so far.

Kinetics of microbial growth and production of biosurfactant

Cultivation of *A. rubi* L5 in the 50 ml optimal medium 5.0% (w/v) molasses and 1.0% (w/v) MSG, 2.5% (v/v) inoculum, pH 7.0 in 250 ml flask at 150 rpm and 30ºC is depicted in Fig. 1. *A. rubi* L5 grew rapidly during 30 h of cultivation and slightly increased to the maximum growth (4.62 g/l) at 54 h of cultivation. Strain L5 produced biosurfactant after 6 h of cultivation and maximum biosurfactant activity was obtained at 66 h of cultivation. It could reduce the surface tension of supernatant from 72.0 to 25.5 mN/m and exhibited emulsification activity toward palm oil with 65.4%. In addition, the result showed that the biosurfactant production by *A. rubi* L5 was growth associated. Normally extracellularly secreted biosurfactants are growth associated [15]. There was an almost parallel relationship between cell growth, substrate utilization and biosurfactant production. The growth-associated production of biosurfactant has been reported from several other microorganisms [15,16,17].

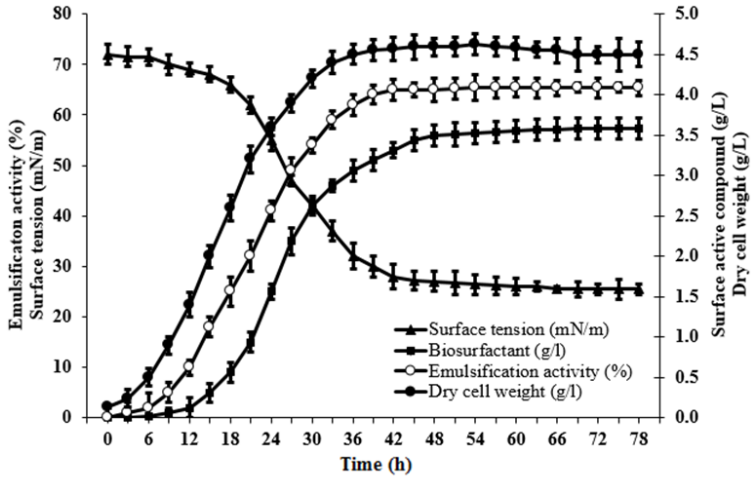

Fig. 1 Times course of growth and biosurfactant production by *Agrobacterium rubi* L5 in optimal medium (5% of molasses, 1% of MSG, initial pH 7.0, 2.5% of inoculation concentration) at 150 rpm and 30°C. Bars indicate that standard derivation from triplicate determinations.

Study of biosurfactant stability

The effect of NaCl concentration on biosurfactant stability. The effect of NaCl concentration on activity of crude biosurfactant produced by *A. rubi* L5 is shown in Fig. 2a. The activity of the biosurfactant was decreased when the concentration of NaCl increasing to 6%. According to this result, the crude biosurfactant was suitable for application in seawater which contains NaCl 3.02%. Thimon et al. [18] reported the ion of salts had effect on the structure of biosurfactant. Salt ions will bind the carboxylic group of biosurfactant and then the surface tension reduction area between water/air was lost. The effect of seawater (pH 8.0) on biosurfactant activity of crude biosurfactant obtained from *A. rubi* L5 was also investigated. Crude biosurfactant could reduce the surface tension of seawater from 72 mN/m to 31 mN/m. It indicated that combination of salts (NaCl, $MgCl_2$, $CaCl_2$, etc.) in seawater did not affect biosurfactant from *A. rubi* L5.

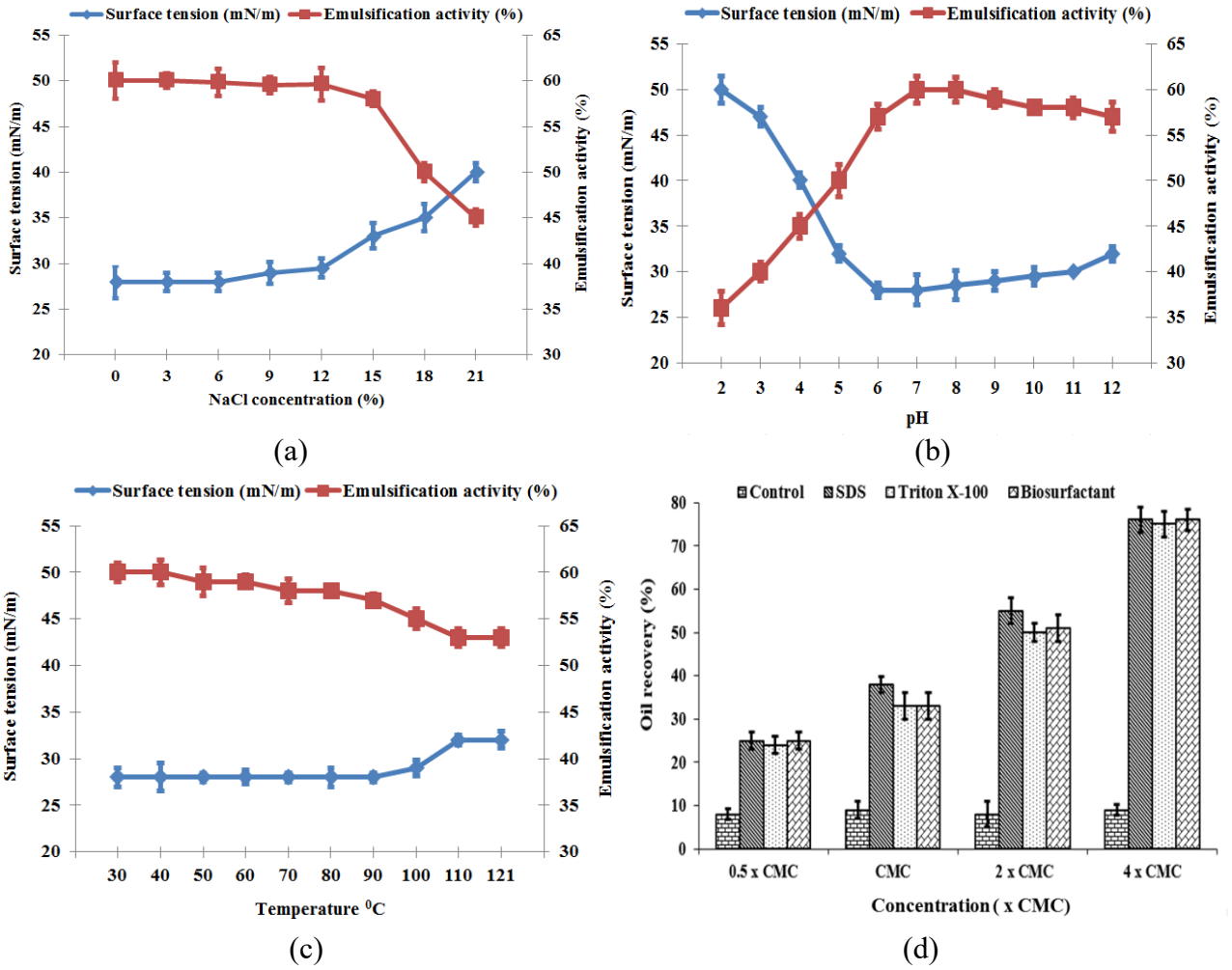

Fig. 2 Effect of NaCl concentration (a), pH (b), temperature (c) on activity of crude biosurfactant and effect of biosurfactants on efficiency in used motor oil removal from contaminated sand (d). Bars represent the standard derivation from triplicate determinations.

Effect of pH on biosurfactant stability. The effect of pH on activity of crude biosurfactant produced by *A. rubi* L5 is shown in Fig. 2b. The solution of the crude extract was adjusted to various pH ranging from 2.0 to 12.0 by 1.0 N HCl or 1.0 N NaOH and kept for 24 h at 4°C. Biosurfactant activity decreased dramatically when decreasing pH below 6 due to precipitation of biosurfactant [19]. However, the slight change in activity were noticeable in the pH range from pH 6.0-11.0. Similar result had been reported for biosurfactant produced by *Pseudomonas aeruginosa* SP4 which stable in the range of pH 4.0 to 11.0 [20,21].

Effect of temperature on biosurfactant stability. The temperature stability of the crude biosurfactant was examined by incubating the crude extract at various temperatures (30-100°C for 1 h and at 110 and 121°C for 15 min). Temperatures ranging from 30-80°C did not show any

influence on biosurfactant activity (Fig. 2c). The similar results have been reported for biosurfactant produced by *Pseudomonas fluorescens* [22] and *Pseudomonas aeruginosa* MR01 [23]. Heat treatment of some biosurfactant caused no appreciable change in surfactant properties i.e. lowering of surface tension and interfacial tension [24]. While the temperature at 90 to 121°C the biosurfactant activity produce by *A. rubi* L5 slightly decreased.

Application of the biosurfactant for motor oil removal from contaminated sand

Petroleum hydrocarbon compounds bind to soil components are difficult to remove and degrade [10]. Biosurfactants can emulsify hydrocarbons enhancing their water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles [6]. The ability of biosurfactant from *A. rubi* L5 to enhance motor oil removal from contaminated sand was examined in comparison with those of synthetic surfactants, i.e. a nonionic surfactant Triton X-100 and anionic surfactants SDS. The results obtained from the present study demonstrated that the surfactants enhanced motor oil removal from contaminated sand (Fig. 2d).

The efficiency of oil removal from sand packed column is shown to be surfactant dosage-dependent. At CMC level, the efficiency of oil removal from sand packed column of biosurfactant and synthetic surfactants was not significant difference ($p>0.05$) (85, 86 and 84% for biosurfactant, SDS and Triton X-100, respectively). An increase in the biosurfactant concentration higher than CMC 2 folds resulted an increase in the oil removal ($p<0.05$) (85 to 92%). Although the concentration of biosurfactant was lower than CMC 2 times, the efficiency of oil removal still higher than control ($p<0.05$) (63%). According to the result, at the concentration below its CMC, the biosurfactant from *A. rubi* L5 could increase oil removal from sand packed column higher than distilled water ($p<0.05$). This characteristic was actually not predicted by the conventional theory because the surfactant at the concentration below its CMC generally showed no enhancement on sorbed hydrocarbon [25]. These results have implications on the potential use of a biosurfactant produced by *A. rubi* L5 to enhance sorbed motor oil from environment. However, it is important not to rule out that the removal efficiency could be deviated depending on the characteristic of the contaminants and site characteristics.

Conclusions

An efficient biosurfactant-producing bacterium *Agrobacterium rubi* L5 isolated from mangrove sediment using molasses as a sole carbon and commercial MSG as nitrogen sources. This strain was able to produce biosurfactant which reduce the surface tension of pure water from 72.0 to 25.5 mN/m and exhibited emulsification activity toward palm oil with 65.4%. The stability of the biosurfactant was effective over a wide range of pH, temperature and salinity. The properties of the biosurfactant obtained have potential application for motor oil removal from contaminated sand.

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