Application of Yeast Glycolipid Biosurfactant, Mannosylerythritol Lipid, as Agrospreaders

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Abstract: The spreading property of mannosylerythritol lipids (MELs) was investigated in connection with our search for new application in agriculture. The wetting ability of MEL solutions for hydrophobic surfaces was evaluated based on contact angle measurements for several surfactant solutions on abiotic and biotic surfaces. The contact angle of MEL-A solution on a hydrophobic plastic surface at 100 s after placement decreased to 8.4°, and those of other MEL solutions decreased more significantly compared to those of commonly-used nonionic surfactants. In addition, the contact angle of MEL solutions also dropped down to around 10° on various plant leaf surfaces. MEL solutions, in particular, efficiently spread even on poorly wettable Gramineae plant surfaces on which general nonionic surfactant solutions could not. Moreover, the wetting ability of MEL solutions was found to be greatly affected by the structural difference in their carbohydrate configuration. Furthermore, surface pretreatment with MEL solution led to more efficient spreading and fixing of microbial cells onto plant leaf surface compared to several conventional surfactants used in this study. These results suggested that MELs have a potential to use as a natural bio-based spreading agent, particularly as agrochemical spreader for biopesticides.

Key words: biosurfactant, mannosylerythritol lipid, agrochemical spreader, wetting ability, biopesticide

1 INTRODUCTION

Surfactants have been exploited for many commercial purposes due to their several functional properties. There are numerous areas of agriculture which also require the use of surfactants. One of the well-known surfactant applications in agriculture is as agrochemical spreaders. Agrochemical spreaders are generally used by mixing them with agro-pesticides and insecticides to improve the adherability of these agrochemicals onto plant surfaces, leading to enhanced effectiveness of these chemicals on plants, which consequently results to reducing the amount of chemicals to be used1,2). Most spreaders currently used in agriculture in Japan are non-ionic synthetic surfactants, such as polyoxyethylene alkyl ethers, polyalkylene glycols alkyl ethers, and polyoxyethylene fatty acid esters2). On the other hand, microorganisms that affect target pests have been used recently as biopesticides to protect susceptible plants3). Suitable additives are necessary to spread these microorganisms efficiently on plant surface, however they are limited because they are expected not suppress the growth and activity of the microorganisms. Moreover, because these chemicals are directly sprayed to agricultural fields, several reports have highlighted the advantages of using less toxic and eco-friendly biosurfactants over the synthetic surfactants in view of environmental sustainability4).

Mannosylerythritol lipids (MELs) are glycolipid biosurfactants abundantly produced from renewable resources such as vegetable oils and sugars by fungal strains of the genus Pseudozyma and Ustilago5-7). To date, MELs are one of the most promising biosurfactants5-9) because they have excellent surface-active and self-assembling properties, as well as versatile biochemical actions5-7). We recently demonstrated that MELs show a ceramide-like moisturizing activity toward human skin cells9), cell activating properties toward fibroblast and papilla cells10), and hair care properties11). Taking these functions into consideration, a MEL...
homologue (MEL-B) is now commercially available from TOYOBO Co., Ltd. (Osaka, Japan). Thus, MELs can be used as environmentally friendly surfactants as well as advanced biomaterials for cosmetic use.

Reflecting on the above described situation, we have focused our attention on the utilization of MELs in agricultural fields aiming at extending the range of their applications. In this study, we investigated the wetting ability of MEL solutions for hydrophobic plastic and plant leaf surfaces using contact angle measurement. In addition, we evaluated the effect of MELs on spreading and adhesion of microbial cells on plant surfaces. The strain used is of the same species as the one commonly used as biopesticide.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

All reagents and solvents are commercially available and were used according to manufacturer’s instructions. Bio-surfactants used in this study are well-known MEL homologues. MEL-A, -B, and -C (Fig. 1a) were produced from soybean oil by the yeast strain Pseudomyza antarctica T-34 in our laboratory according to procedures used in our previous study[15]. The diastereomer type MEL-B[14] (isoMEL-B; Fig. 1b), which is registered as “SurfMellow”, was kindly supplied by TOYOBO Co., Ltd (Osaka, Japan). It was produced from olive oil by the yeast strain P. tsukubaensis NBRC 1940. MELs purified by silica gel column chromatography and the following synthesized surfactants, namely, polyoxyethylene (20)sorbitan monolaureate (Tween 20) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), polyoxyethylene (10) lauryl ether (Brij 35) (Wako Pure Chemical Industries, Ltd.), and polyalkyleneoxide modified heptamethyldisiloxane (Silwet-L77) (Dow Corning Toray Co. Ltd., Tokyo, Japan) were used. The concentration of these surfactants were individually adjusted to 0.1 % (w/w) with sterilized distilled water, and used for the following experiments after standing them for at least 24 h. Hydrophobic plastic film “GelBond®” (Takara Bio Inc., Otsu, Japan) was employed as a pseudo plant surface for the contact angle measurement. In the experiment concerning the spreading of microorganisms, we used a MEL mixture, which was extracted from culture broth of P. antarctica T-34 that contained MEL-A, -B, and -C (mixing ratio; 58 : 25 : 10)[16].

2.2 Measurement of contact angle of various surfactant solutions on hydrophobic plastic surface

Contact angle (θ) of droplet of each surfactant solution on an abiotic hydrophobic surface was evaluated according to Young’s equation[17]. The θ of a droplet varies inversely with the strength of its attraction to the solid surface. Small segments (2-3 cm square) of plastic film (GelBond) were used as the abiotic surface for the measurement. A 10 μL aliquot of each surfactant solution prepared as described above was dropped on the hydrophobic side of the film, and temporal dynamics of θ of the droplet was immediately measured every second until 100 s using Drop Master DM500 (Kyowa Interface Science Co., Ltd., Niiza, Japan). Sterilized distilled water was used as control. Measurement for each surfactant solution was independently replicated five times and the data obtained were averaged. Relative spreading ability of surfactant-containing water drops on the solid surface was evaluated based on θ at 100 s after dropping, and results were subjected to ANOVA followed by Scheffé’s test using KaleidaGraph 4.1J software (HULINKS Inc., Tokyo, Japan).

2.3 Measurements of contact angle of various surfactant solutions on plant leaf surfaces

The θ of each surfactant solution (0.1 %) on leaf surfaces of wheat, rice, mulberry, and strawberry was also measured by the same procedures as described above. To obtain wheat leaves, wheat seedlings (cv. Ayahikari) were grown in a glass house for 7 days after seed sowing; primary leaves were detached and cut approximately 10 cm long. Matured rice leaves at the 4th-5th stage (cv. Koshihikari) (approximately 10 cm long) were cut off the seedlings in a plastic nursery box (15 × 6 × 10 cm) grown in the glass house. Healthy matured mulberry leaves were detached from a mulberry tree (unknown cultivar) planted in NIAES, then divided into small segments (approximately 1.5 cm square). Similarly, intact strawberry leaves (cv. Nyohou) were detached from potted plants grown in the glass house, and cut out into approximately 1.5 cm-squared segments. On the adaxial surface of each leaf segment, 10 μL of each surfactant solution and sterilized distilled water as control was placed, and temporal dynamics of θ of each droplet was immediately measured as described above. Measurement was carried out independently in triplicate and the averaged data was used for evaluation of the temporal dynamics. Results at 100 s after dropping were subjected to ANOVA followed by Scheffé’s test as above described.

Fig. 1 Chemical structures of mannosylerythritol lipids (MELs).
2.4 Evaluation of spreading of microorganism on surfactant-treated wheat leaf surfaces

*Bacillus subtilis* strain MAFF302084 was obtained from NIAS Genebank in the National Institute of Agrobiological Sciences, Japan. The strain was cultured on a potato-peptone-glucose-agar (PPGA) medium plate (200 g potato, 5 g peptone, 5 g glucose, 3 g NaHPO₄·12H₂O, 0.5 g KH₂PO₄, 6H₂O, 3 g NaCl, and 15 g agar per L) for 2 days at 25°C; then bacterial cell suspension (OD₆₀₀ = 0.5) was prepared as inoculum. Wheat leaf segments (approximately 3 cm long) were cut from glass house-grown seedlings (cv. Norin No.61), and then were fixed on the outer surface of a 96 well plastic microplate lid with the adaxial surface facing up using a double-sided adhesive tape. On the surface of each segment, 5 μL each of Tween 20, Silwet-L77 or MELs solution (0.08% [v/v]) was applied. Four replicated segments were used for each treatment. After air drying the surface, 1 μL of 100 fold-diluted bacterial inoculum was placed at the center of each surfactant-treated segment as previously reported(7). The inoculated leaf segments were kept in a moist chamber for 30 min at 25°C (Fig. 4a). The inoculated surface of leaf segments was then transferred onto the surface of a plated PPGA medium containing 50 μg/mL cycloheximide for 5 min. Subsequently, the leaf segments were carefully removed from the culture plate, and the plate was incubated for 3 days at 25°C (Fig. 4b). After the incubation, the maximal distance of the appearing bacterial colonies from the initial point, where bacterial suspension was placed, was measured. Additionally, the area occupied by the bacterial colonies that appeared on the medium transferred with each leaf segment was also measured according to the procedure given in a previous report using Adobe Photoshop CS4 and Image J software(7).

![Fig. 2 Temporal dynamics of contact angle of each surfactant droplet placed on plastic (GelBond).](image)

3 RESULTS AND DISCUSSION

3.1 Contact angle of various surfactant solutions on hydrophobic plastic surface

In order to evaluate the wettability of MEL containing solution onto plant surfaces, we measured the contact angles(θ) of MELs and several surfactants solutions. Decreasing values of θ reflect an improvement in the extensibility of the biosurfactant solution on the surface. First, hydrophobic plastic (GelBond®) was used as a pseudo plant surface. Temporal dynamics of contact angle of each surfactant solution were shown in Fig. 2, and the θ values of the surfactant solutions measured at 100 s after droplet placement are summarized in Table 1.

The θ of all surfactant solutions immediately decreased after dropping onto the plastic, whereas that of the water

### Table 1 Contact angle (θ) of surfactant solutions on abiotic and biotic surfaces at 100 s after droplet placement

| Surfactant | Plastic film | Plant leaf |
|------------|--------------|------------|
|            | GelBond      | Wheat      | Rice       | Mulberry   | Strawberry |
| Water      | 78.5 ± 0.3   | ND³         | ND³        | 76.9 ± 2.2 | 94.2 ± 3.0 |
| MEL-A      | 8.4 ± 1.1    | 14.1 ± 2.1  | 17.1 ± 0.8 | 10.3 ± 2.0 | 18.8 ± 4.3 |
| MEL-B      | 10.2 ± 0.7   | 17.2 ± 0.8  | 13.8 ± 1.4 | 8.4 ± 1.2  | 22.0 ± 2.9 |
| MEL-C      | 13.1 ± 0.5   | 10.5 ± 0.8  | 17.5 ± 2.3 | 6.5 ± 0.3  | 29.4 ± 2.2 |
| isoMEL-B   | 29.1 ± 0.6   | 56.4 ± 1.9  | 72.4 ± 2.0 | 33.3 ± 2.7 | 41.1 ± 2.1 |
| Tween 20   | 43.1 ± 0.3   | 113.2 ± 1.5 | 125.8 ± 2.6 | 51.6 ± 1.6 | 55.4 ± 0.7 |
| Brij 35    | 42.2 ± 0.4   | 113.1 ± 1.4 | 121.1 ± 1.1 | 43.0 ± 0.7 | 49.3 ± 0.1 |
| Silwet-L77 | -⁴          | -⁴         | -⁴         | -⁴         | -⁴ |

¹ Measurements were carried out using Drop Master DM500.
² Data represent average ± standard error of the mean.
³ Not determined since measurement failed.
⁴ Not determined since contact angle immediately decreased to less than 1° in seconds.

*J. Oleo Sci.*
droplet showed only a slight change. The $\theta$ of MELs at 100 s (around $10^5$) were remarkably lower than those of conventional nonionic surfactants, Tween 20 (43.1) and Brij 35 (42.2), although that of Silwet-L77, a well-known "super spreader", was the lowest (less than 1 in seconds). In addition, the slopes of $\theta$ of MELs at approximately 10 s after dropping were steeper than those of Tween 20 and Brij 35, but were lower than that of Silwet-L77. These results suggested that MEL solutions exhibit higher spreading properties onto a hydrophobic surface compared to conventional nonionic surfactant solutions.

Among MELs, MEL-A, -B, and -C showed similar spreading behavior as evidenced by their $\theta$ changes. Although these three MEL homologues have different degrees of acetylation of mannose (Fig. 1a) and showed different interfacial and self-assembling properties, contrary to our expectation, we did not find any marked difference in their spreading property. Thus, it could be inferred that the number and position of acetyl groups in mannose did not significantly affect the extensibility of MELs on hydrophobic surface.

On the other hand, in the case of isoMEL-B, a diastereomer type of MEL-B, which has a different carbohydrate configuration produced by *P. tsukubaensis* (Fig. 1b), the decreases in the $\theta$ at 100 s (to 29.1) were lower and the slope of $\theta$ within 10 s after dropping were gradual compared to those of the other MELs. Between isoMEL-B and the conventional MELs produced by *P. antarctica*, particularly MEL-B, the only structural difference in the hydrophilic domain is in the configuration of erythritol moiety, which is located opposite each other. In addition, the fatty acid compositions are slightly different between these compounds; while the conventional MELs are composed of two random fatty acids around C10, isoMEL-B has mainly a combination of C8 and C14 fatty acids. These results indicate that the difference in their spreading properties depend on the configuration of erythritol moiety and/or the combination of the fatty acids in MEL-B.

Over the last two decades, a lot of literature on wetting and spreading mechanism of aqueous surfactant solutions on hydrophobic surfaces have been published. The mechanism is complex involving various processes of different time scales that combine synergistically. An important feature of spreading is assumed that the transfer of surfactant molecules onto the hydrophobic solid interface takes place from liquid-vapor interface of the drop. We have previously reported that the differences in MEL carbohydrate configurations significantly affect their self-assembly and hydration ability by comparison of them among four types of MEL optical isomers. Previous study suggested that isoMEL-B shows more hydrophilic and has higher hydration force of the hydrophilic group compared to the conventional MEL-B. Therefore, we speculate that the direct adsorption of isoMEL-B molecule from liquid-vapor interface to the contact line of hydrophobic surface is slower than that of MEL-B. We thus think that these structural differences in MEL-B change the glycolipid orientation at the liquid-vapor and solid-liquid interface, and this behavior has an effect on its wetting ability for solid surfaces.

### 3.2 Contact angle of various surfactant solutions on plant leaf surface

The wetting ability of surfactant solutions for several plant leaves was also investigated in the same way as in hydrophobic plastics (Fig. 3). The $\theta$ values of the surfactant solutions on abiotic and biotic surfaces at 100 s are summarized in Table 1.

While plastic surfaces are artificial and relatively flat, natural plant surfaces are generally composed of cuticular layers and show complicated and indented structures. The leaf surfaces of Gramineae plants (wheat and rice) have been categorized as a poorly wettable species because of their repellency property based on hydrophobicity and irregularity. In fact the water droplets placed on the leaves of wheat and rice slide down, and hence their $\theta$ could not be measured. On the other hand, the $\theta$ of Tween 20 and Brij 35 solutions on these leaves hardly decreased. However, the $\theta$ of MEL solutions dramatically decreased close to $10^5$ after dropping (Fig. 3a and b). Meanwhile, Silwet-L77 showed excellent spreading properties on all plant surfaces tested. On mulberry leaf which is relatively flat and hydrophilic compared to leaves of Gramineae plants, $\theta$ of all surfactant solutions gradually decreased after dropping on the leaf surface. Among them, the $\theta$ of MEL solutions (almost below $10^5$) were also lower than those of the other surfactant solutions, except for Silwet-L77 (Fig. 3c). Similar decreasing behavior of $\theta$ was observed with strawberry leaf surface although the differences in the final angles among the tested surfactants were small (Fig. 3d).

In each case, similar to the result of GelBond, the decreases in the $\theta$ of isoMEL-B were lower than those of the conventional MELs.

These results demonstrated that MEL solutions efficiently spread onto various kinds of plant surfaces, thus MELs could potentially be used as a natural bio-based spreading agent in agriculture. In addition, the conventional MELs show better spreading property compared to the diastereomer type of MEL.

### 3.3 Spreading of microorganism on surfactant-treated wheat leaf surfaces

Agrochemical spreaders are used to spread and fix agricultural active components onto plant body. Thus, we have tried to investigate the effectivity of MELs as agrochemical spreader for agrochemically active microorganisms onto plant leaf surfaces. Here, we added *B. subtilis* cell suspension onto wheat leaves pretreated with several surfactant solutions...
solutions (Fig. 4a) and observed the diffusion behavior of cells on leaves (Fig. 4b and 5).

The evaluation of cell diffusion on wheat leaf surface was indirectly performed by measuring the area occupied by the bacterial colonies that grew on agar plate after having transferred with wheat leaf. Figure 4b clearly shows that the colonies on surfactant-pretreated leaf spread more widely compared to on untreated leaf. In particular, using a MEL mixture caused the colonies to spread most widely among the tested surfactants. The diffusion length and area of colonies on treatments using MELs were confirmed to have spread over sixfold than those on control treatment (water only) (Fig. 5). On the other hand, surprisingly, the use of Silwet-L77 could not spread the colonies very well despite its markedly high spreading property. These results suggested that, in the presence of MELs, bacterial cell suspension can be transferred far away and attach cells on plant surface, whereas the use of Silwet-L77 could not. We have recently reported that MEL-producing yeasts adhere to plant surfaces very well and the area colonized by the cells became enlarged in the presence of MELs(17). Therefore, it is considered that MELs could be effectively used

Fig. 3 Temporal dynamics of contact angle of each surfactant droplet placed on leaf surfaces: a, wheat; b, rice; c, mulberry; and d, strawberry.

Fig. 4 The diffusion of B. subtilis cell colonies on wheat leaf treated with surfactant solutions.
for the spreading and fixing of microbial cells onto plant surfaces.

The present study demonstrated therefore that MEL is a good agrospreader because it did not only enhance the wettability of hydrophobic surfaces, but was also found to effectively spread the microbial cells on plant surfaces.

4 CONCLUSION

In this study, we investigated the spreading properties of MEL as part of our search for a novel application of MEL in agriculture. MEL solutions spread significantly a wide variety of surfaces, including hydrophobic plastic and plant leaves compared to known chemical nonionic surfactants. In particular, MEL solutions showed good wetting ability even on poorly wettable Gramineae plant surfaces. Furthermore, using the droplet method, cell suspension of B. subtilis, which is selected as the model bacterial species for biopesticide, was found to be most efficiently spread and fixed on wheat leaf surface treated with MEL solutions among several surfactants tested.

This study demonstrated that MEL is a promising for agrochemical spreader, especially for biological pesticides. We will further investigate its other functional properties for agricultural applications and confirm the actual effectiveness of such beneficial characters in field conditions.

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Application of mannosylerythritol lipid to agrochemical spreaders

J. Oleo Sci.

7

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