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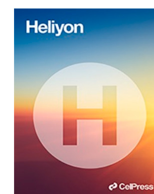

Research article

# Enhancement of mono-acylated MEL-D production in an acyltransferase gene-deleted strain of *Pseudozyma tsukubaensis* by supplementation with di-acylated MEL-B in culture medium

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ABSTRACT

Mannosylerythritol lipids (MELs) are glycolipid biosurfactants produced by various yeasts. MEL producers produce mainly di-acylated MELs (consisting of two fatty acid chains). Among them, *Pseudozyma tsukubaensis* is a di-acylated MEL-B (d-MEL-B) producer. In a previous study, we generated an acyltransferase-deleted strain of *P. tsukubaensis* (Δ*PtMAC2*), which selectively produced mono-acylated MEL-D (m-MEL-D, consisting of one fatty acid chain), but not d-MEL-B. However, m-MEL-D productivity in Δ*PtMAC2* was low, and oil consumption was significantly reduced compared to the parent strain. Based on these findings, we hypothesized that the d-MEL-B produced by the parent strain may act as an emulsifier in the culture medium, leading to easier utilization of the oil. By contrast, the m-MEL-D produced by Δ*PtMAC2* may not have the ability to emulsify oil, thus the oil is used inefficiently and productivity of m-MEL-D is low. Therefore, we expected that adding d-MEL-B to the culture medium during Δ*PtMAC2* cultivation would increase m-MEL-D production. To enhance the oil consumption and m-MEL-D production of Δ*PtMAC2*, d-MEL-B and chemical surfactants were added to the culture medium as emulsifiers during Δ*PtMAC2* cultivation. Adding d-MEL-B enhanced both the oil consumption and m-MEL-D production of Δ*PtMAC2*; Tween 20 and Triton X-100 also showed enhancement effects. As expected, d-MEL-B, Tween20 and TritonX-100, showed marked olive oil emulsification activity, whereas m-MEL-D did not. These results strongly support our hypothesis and significantly improve m-MEL-D productivity.

## 1. Introduction

Biosurfactants (BSs) are surfactants produced by microorganisms from renewable carbon sources, such as sugars and plant oils. Recently, BSs have received increasing attention due to their eco-friendly properties. BSs such as sophorolipids, rhamnolipids, surfactin, and mannosylerythritol lipids (MELs) have been commercialized over the past decade and are primarily used in cleaning, personal care, and cosmetic fields.

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Mannosylerythritol lipids, which are glycolipid BSs, are produced by basidiomycetous yeasts, such as genera *Ustilago*, *Moesziomyces*, *Pseudozyma*, and *Sporisorium*. They not only show excellent interfacial activity but also have unique properties, such as self-assembly, anti-bacterial, against cancer cell lines, anti-oxidative activities, and repairing properties of damaged human skin and hair [1–8]. Mannosylerythritol lipids are categorized based on their degree of acetylation (MEL-A, MEL-B, MEL-C, or MEL-D) and according to the number of fatty acid chains (mono- or di-acylated) (Fig. 1A). Most of MEL producers synthesize di-acylated MELs, which are poorly soluble in water (forming a cloudy dispersion at $\geq 1$ mg/mL), limiting their use in aqueous applications. Thus, the development of MELs with higher water solubility is required to expand their utilization. Fukuoka et al. [9] reported that *M*oesziomyces *antarcticus* and *M*oesziomyces *paraantarcticus* produced mono-acylated MEL-D (m-MEL-D) from glucose as a minor product in an MEL mixture (the main compound was di-acylated MEL-A; d-MEL-A). Because m-MEL-D has one fatty acid chain, it is expected to have higher water solubility than di-acylated MEL. In evaluations of the critical micelle concentration (CMC), CMC of m-MEL-D ($3.6 \times 10^{-4}$ M) was more than 100-fold higher than d-MEL-A ($2.7 \times 10^{-6}$ M), while surface tension at a CMC (γCMC) value was maintained >30 mN/m (28.4 mN/m for d-MEL-A). Generally, chemical surfactants with higher solubility in water have higher CMCs. Therefore, m-MEL-D is more suitable for use in aqueous applications than di-acylated MELs. However, the yield of m-MEL-D was insufficient (1.1–1.3 g/L) for industrial use, and its efficient production was the next challenge in mono-acylated MEL development.

In 2006, the complete genome of *U. maydis* was sequenced [10–12]; sequencing of the genomes of MEL-producing yeasts has since accelerated [13–19]. MELs are synthesized by a glycosyltransferase (*emt1p*), acyltransferases (*mac1p* and *mac2p*), and an acetyltransferase (*mat1p*). Mmf1p is a putative intracellular to extracellular transporter of MELs. Based on this genome-sequence information, tailor-made production of MELs has been attempted by genetic modification of genes involved in the MEL biosynthesis pathway. For example, di-acylated MEL-D (d-MEL-D) was selectively produced by a *mat1*-deleted strain [11,20,21], mono-acylated MELs by an *mmf1*-deleted strain [22,23], and customizable and predictable MELs were generated by complementing the *mac 1*, *mac 2*, and *mat1*-deleted strains [24,25].

*Pseudozyma tsukubaensis* is a selective producer of di-acylated MEL-B (d-MEL-B) [26]. In our previous report [27], we focused on the *PtMAC2* encoding acyltransferase in *P. tsukubaensis* (Fig. 2) and generated a *PtMAC2*-deleted strain (Δ*PtMAC2*) by homologous recombination with uracil auxotrophy as a selectable marker. Strain Δ*PtMAC2* selectively produced m-MEL-D but not d-MEL-B. However, the productivity of m-MEL-D is low (<0.5 g/L) using this strain. In addition, Δ*PtMAC2* is unable to consume even 50 g/L oil in 7 days during m-MEL-D production, whereas the parent strain shows a high capability for oil consumption during d-MEL-B production (>100 g/L oil in 7 days). Because Δ*PtMAC2* loses the ability to produce d-MEL-B, we speculated that the inability to use oil could be linked to the lack of d-MEL-B. That is, the produced d-MEL-B may have acted as an emulsifier in the medium, whereas m-MEL-D did not. The difference in emulsifying ability may be attributable to the greater hydrophilicity of m-MEL-D compared to d-MEL-B. Therefore, we hypothesized that when the parent strain produces d-MEL-B, the produced d-MEL-B may promote the emulsification of oil, making it easier for the strain to utilize the oil and produce more d-MEL-B. However, when Δ*PtMAC2* produces m-MEL-D, the lack of emulsification ability of m-MEL-D precludes the efficient use of oil, and thus productivity of m-MEL-D is low. Based on this hypothesis, we expected that adding d-MEL-B to the culture medium during Δ*PtMAC2* cultivation would increase m-MEL-D production.

In this study, to improve both the oil consumption and m-MEL-D production of Δ*PtMAC2*, d-MEL-B and chemical surfactants were added to the culture medium to act as emulsifiers during Δ*PtMAC2* cultivation. As expected, adding d-MEL-B enhanced m-MEL-D production from olive oil by Δ*PtMAC2*. Although less effective than d-MEL-B, some chemical surfactants, such as Tween 20 and Triton X-100, enhanced oil consumption and m-MEL-D production. Consequently, the m-MEL-D productivity of Δ*PtMAC2* was increased by more than 10-fold compared to without d-MEL-B addition.

## 2. Materials and methods

### 2.1. Strain and materials

The acyltransferase gene-deleted strain of *Pseudozyma tsukubaensis* 1E5 (Δ*PtMAC2*) was generated previously [27]. Purified

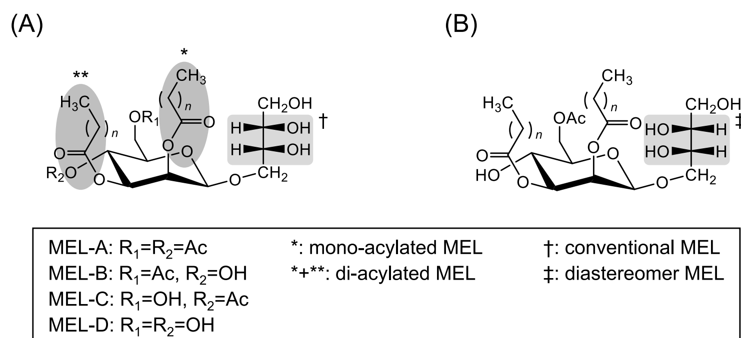


**Fig. 1.** Structures of MELs. Structural varieties of conventional MELs (A), and of diastereomer-type di-acylated MEL-B produced by *Pseudozyma tsukubaensis*.

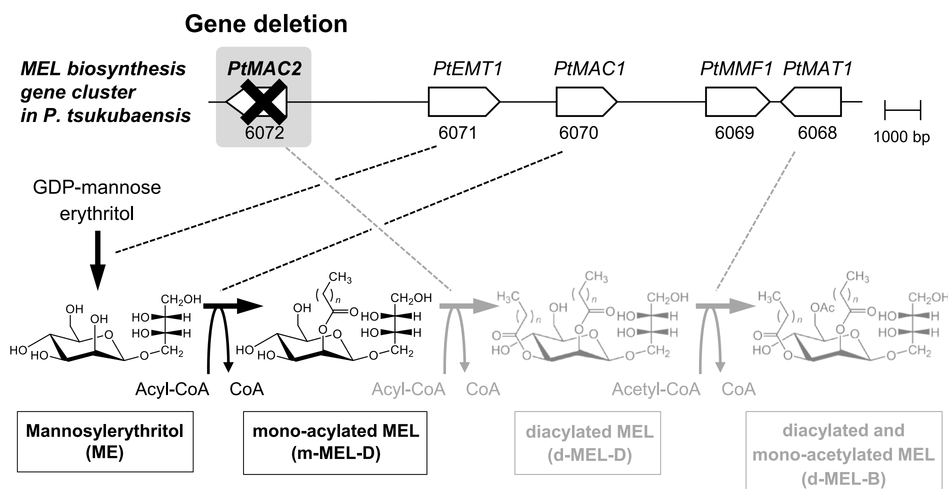


**Fig. 2.** Gene cluster and biosynthetic pathway of diastereomer-type di-acylated MEL-B in *P. tsukubaensis*. *PtEMT1*, erythritol/mannose transferase; *PtMAC1* and *PtMAC2*, acyltransferases; *PtMAT1*, acetyltransferase; and *PtMMF1*, putative transporter.

d-MEL-B (produced by *P. tsukubaensis* 1E5), purified d-MEL-D (produced by an acetyltransferase gene-deleted strain of *P. tsukubaensis* 1E5, Δ*PtMAT1*), polyoxyethylene (20) sorbitan monolaurate (Tween 20; Fujifilm Wako Pure Chemical Co., Osaka, Japan), polyethylene glycol mono-p-isooctylphenyl ether (Triton X-100; MP Biomedicals, Solon, OH, USA), polyoxyethylene (23) lauryl ether (Brij35; MP Biomedicals), sodium lauryl sulfate (SDS; Fujifilm Wako), and sodium laurate (C12Na; Fujifilm Wako) were used as additives to enhance m-MEL-D production.

### 2.2. Production of mono-acylated MEL-D (m-MEL-D)

Δ*PtMAC2* was cultivated in YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose) containing 5 % (w/v) glycerol at 25 °C for 2 days with reciprocal shaking at 250 rpm. An aliquot (1 mL) of this culture was inoculated into 20 mL of MEL production medium (5 g/L yeast extract, 3 g/L $NaNO_3$, 0.3 g/L $KH_2PO_4$, 0.3 g/L $MgSO_4 \bullet 7H_2O$) containing 2 % (w/v) glycerol, 5 % (v/v) olive oil (3 % at day 0 and 2 % at day 3), and 0 %, 0.01 % or 0.1 % (w/v) surfactants (d-MEL-B, Tween 20, Triton X-100, Brij35, SDS, and C12Na) and cultivated at 25 °C for 5–7 days with rotary shaking at 250 rpm. The culture was centrifuged at 820×*g* for 2 min, and the supernatant was harvested and lyophilized for thin-layer chromatography (TLC). m-MEL-D in lyophilized supernatant was extracted into 5 mL of acetone overnight. For quantification of m-MEL-D, the culture was centrifuged at 820×*g* for 5 min, and the supernatant was harvested and analyzed by high-performance liquid chromatography (HPLC). Cell pellets were dried to measure cell growth. For jar fermentation, Δ*PtMAC2* was cultivated in YM medium at 25 °C for 2 days with reciprocal shaking at 250 rpm. For main cultures, 50 mL of preculture were added to 1 L of MEL production medium containing 2 % (w/v) glycerol, 9 % (v/v) olive oil (6 % at day 0 and 3 % at day 3), and 0.1 % (w/v) d-MEL-B in a 2 L jar fermenter (Labo-controller, MDL-8C, B. E. Marubishi Co., Ltd., Tokyo, Japan). Jar cultivation was performed at 25 °C with shaking at 580 rpm and 0.5 vvm.

### 2.3. Thin-layer chromatography (TLC)

Extracted m-MEL-D was detected by TLC [28] using chloroform, methanol, and 12 % $NH_4OH$ in a 55:25:2 (v:v:v) ratio as the eluent. m-MEL-D was detected on the TLC plate by spraying with 2 % anthrone-sulfate reagent and heating at 90 °C for 5 min.

### 2.4. Quantification by high-performance liquid chromatography (HPLC)

m-MEL-D in culture supernatant was quantified by HPLC using the Waters 2695 HPLC system (Waters, Milford, MA, USA) with a SH1011 column (Shodex, Tokyo, Japan) at 60 °C. The mobile phase was 5 mM $H_2SO_4$ at a flow rate of 1 mL/min. The differential refractive index detector (RI-8020) was used to detect m-MEL-D at 40 °C. The m-MEL-D standard for HPLC was prepared as follows: m-MEL-D was purified using silica gel (Wako-gel C-200; Fujifilm Wako) column chromatography with elution in chloroform/acetone (100:0, 20:80, and 0:100 v/v).

Residual oil quantity was quantified using HPLC with a Tosoh DP-8020 Pump (TOSOH, Tokyo, Japan) and low-temperature evaporative light scattering detector (ELSD) Model 300S (SofTA Corporation, Thornton, CO, USA) with a silica gel column (Inertsil SIL 100A, 5 μm, 4.6 × 250 mm; GL Science Inc., Japan) at room temperature [28]. Quantification of residual oil was based on a standard curve prepared using olive oil.

### 2.5. Structural analysis

The fatty-acid profile of m-MEL-D was characterized by liquid chromatography-mass spectrometry (LC-MS) as described previously [23]. An acetone extract of m-MEL-D was used for analysis. The adduct ions detected were the parental molecule plus $NH_4^+$ (+18) and $HCOO^-$ (+45).

### 2.6. Determination of the surface-active properties of m-MEL-D

The surface tension of purified m-MEL-D was determined by the pendant-drop method [9]. The results are averages of data from 10 independent experiments. The emulsifying activity of purified m-MEL-D was determined using a colorimetric method [9]. Olive oil was used as the hydrophobic phase. m-MEL-D produced by Δ*PtMAC2*, d-MEL-D produced by *P. tsukubaensis* strain *ΔPtMAT1* [21], and d-MEL-B produced by *P. tsukubaensis* [26], Tween 20, Triton X-100, Brij35, SDS, and C12Na were used as emulsifiers. Olive oil (0.1 mL) was added to 4 mL of distilled water containing 0.4 or 4 mg of surfactant (0.01 or 0.1 %) in a test tube. The test tubes were vortexed for 1 min and allowed to stand at room temperature for 3 h. The lower 1 mL of the aliquot was transferred to a cuvette, and its turbidity at 620 nm was measured. The results are averages of data from three independent experiments.

## 3. Results

### 3.1. Effects of di-acylated MEL-B and chemical surfactants on mono-acylated MEL-D production by ΔPtMAC2

To investigate the effect of supplementation of d-MEL-B to the culture medium on the production of m-MEL-D, Δ*PtMAC2* was cultivated in medium containing 5 % (v/v) olive oil and 0.01 % or 0.1 % (w/v) d-MEL-B for 7 days at 25 °C. Cultivation without d-MEL-B was used as the negative control. m-MEL-D was extracted with acetone and detected by TLC. By TLC (Fig. 3B), a very weak spot corresponding to m-MEL-D was detected in the extract of the negative control (without d-MEL-B), whereas a strong m-MEL-D spot was detected in the extract of 0.01 % and 0.1 % d-MEL-B. Furthermore, when d-MEL-B was added to the medium, the dry cell weights were higher compared to the negative control (Fig. 3A).

To analyze whether surfactants other than d-MEL-B enhanced the production of m-MEL-D, we supplemented medium with 0.01 % or 0.1 % (w/v) Tween 20, Triton X-100, Brij35, SDS, and C12Na during Δ*PtMAC2* cultivation. Under the same conditions as d-MEL-B supplementation, the addition of 0.1 % Tween 20 and 0.01 % Triton X-100 increased the production of m-MEL-D (Fig. 4B) and the dry cell weights (Fig. 4A). This indicates that surfactants other than d-MEL-B can increase the production of m-MEL-D by Δ*PtMAC2*. However, the addition of Brij35, SDS, and C12Na did not increase the production of m-MEL-D.

To assess the effects of surfactants on oil consumption, we quantified by HPLC the residual oil concentration in Δ*PtMAC2* cultures with added d-MEL-B, Tween 20, and Triton X-100. In the absence of surfactants, more than half of the added olive oil remained after 7 days (Fig. 5). By contrast, with the addition of 0.1 % d-MEL-B, 0.1 % Tween 20, or 0.01 % Triton X-100, almost all the added olive oil was consumed. Therefore, the addition of an appropriate concentration of d-MEL-B, Tween 20, or Triton X-100 promotes oil consumption, thereby enhancing the production of m-MEL-D.

### 3.2. Emulsifying activity of mono-acylated MEL-D

In this study, we hypothesized that when the parent strain produces d-MEL-B, the produced d-MEL-B may promote the

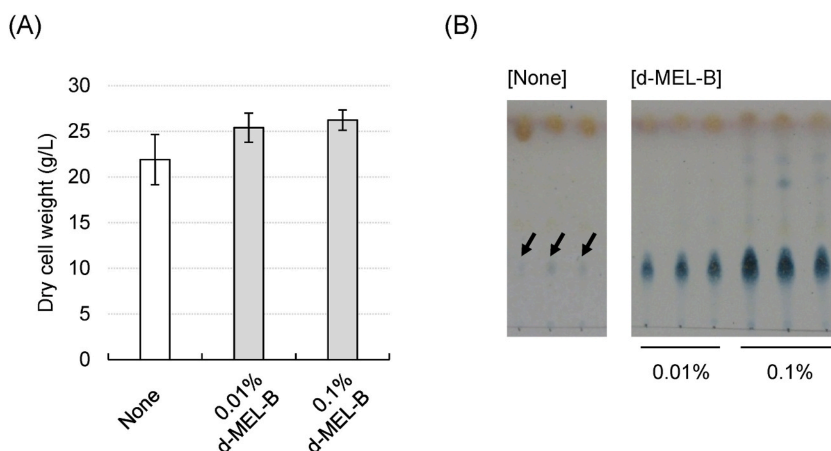


**Fig. 3.** m-MEL-D production by *P. tsukubaensis* Δ*PtMAC2* supplemented with 0.01 % or 0.1 % d-MEL-B. Cells were grown in MEL production medium containing 5 % (w/v) olive oil at 25 °C for 7 days. Dry cell weight (A) and m-MEL-D production by TLC (B). Arrows indicate weak spots of m-MEL-D. Data are averages of results from three independent cultivations.

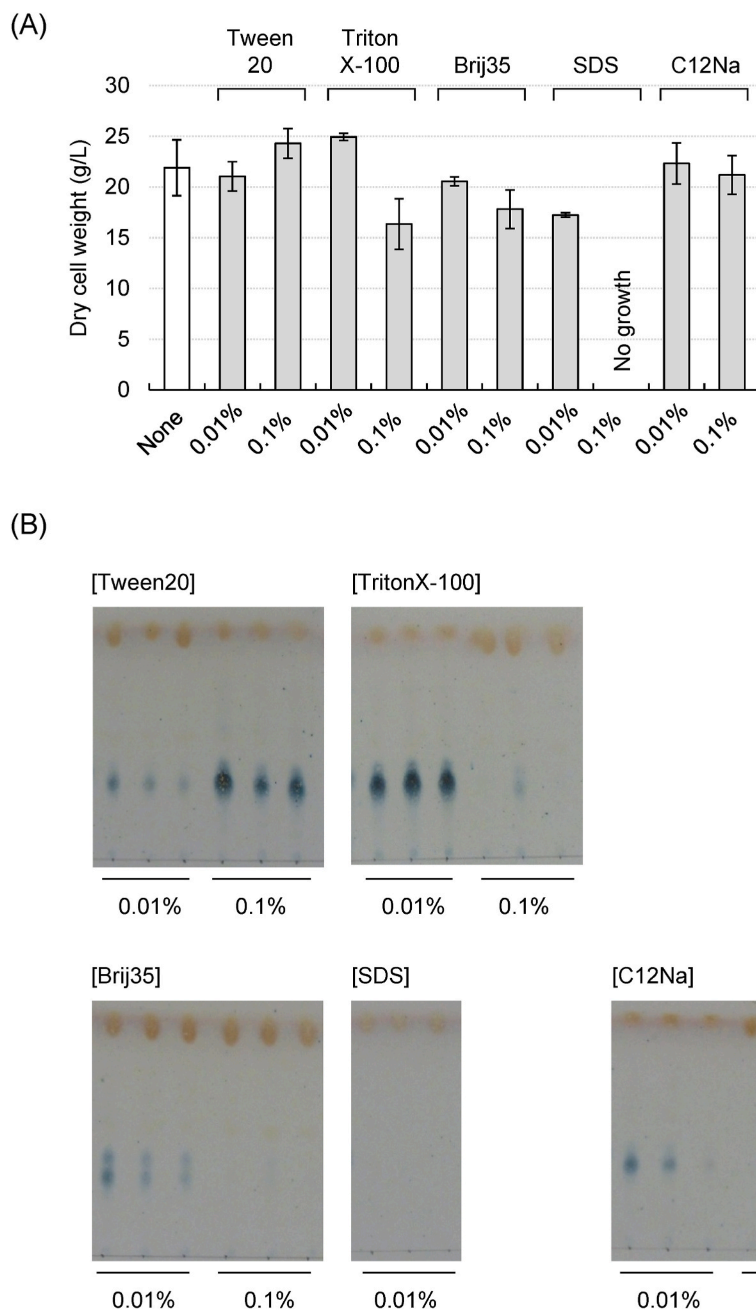


**Fig. 4.** m-MEL-D production by *P. tsukubaensis* Δ*PtMAC2* supplemented with 0.01 % or 0.1 % Tween 20, Triton X-100, Brij35, SDS, and C12Na. Cells were grown in MEL production medium containing 5 % (w/v) olive oil at 25 °C for 7 days. Dry cell weight (A) and m-MEL-D production by TLC (B). Data are averages of results from three independent cultivations.

emulsification of oil, making it easier for the strain to utilize the oil and produce more d-MEL-B. By contrast, when Δ*PtMAC2* produces m-MEL-D, because m-MEL-D may not have the ability to emulsify oil, the oil is not used efficiently, and productivity of m-MEL-D is low.

To verify this hypothesis, the emulsifying activity of purified m-MEL-D was compared to synthetic surfactants and MELs. As expected, m-MEL-D showed no emulsifying activity towards olive oil, because optical density at 620 nm was the same as the negative control (Fig. 6, none; no added any surfactant). By contrast, d-MEL-B, Tween 20, and Triton X-100, which increased m-MEL-D production, showed increased emulsifying activity compared to m-MEL-D. This result strongly supports our hypothesis that m-MEL-D lacks the ability to emulsify oil, resulting in inefficient oil utilization and low m-MEL-D productivity.

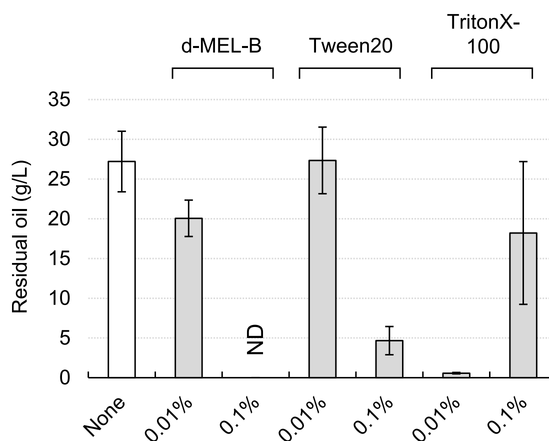


**Fig. 5.** Oil consumption by *P. tsukubaensis* Δ*PtMAC2* supplemented with 0.01 % or 0.1 % d-MEL-B, Tween 20, and Triton X-100. Data are averages of results from three independent cultivations.

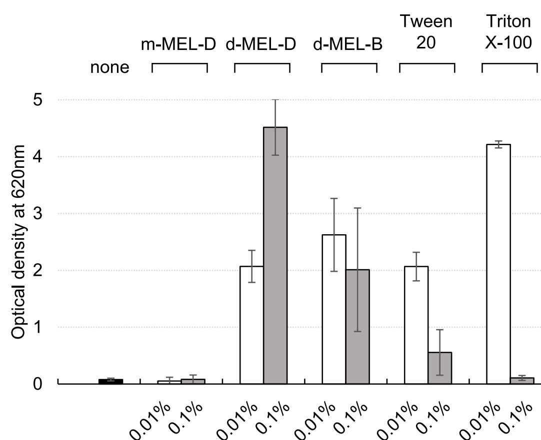


**Fig. 6.** Emulsifying activities of MELs and chemical surfactants. Olive oil was used as the hydrophobic phase. Data are averages from three independent experiments.

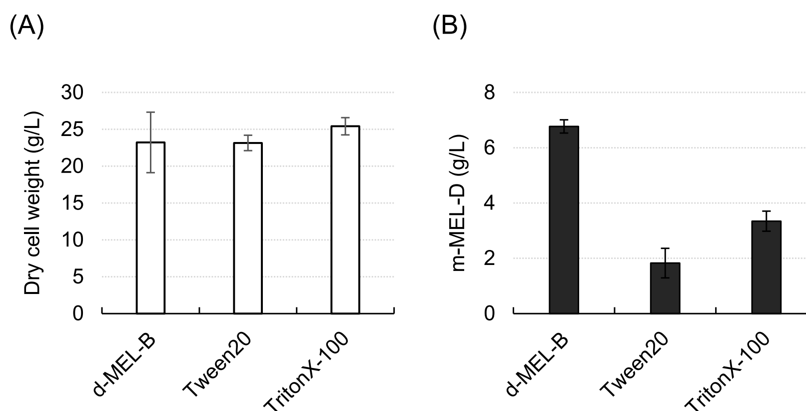


**Fig. 7.** m-MEL-D production by *P. tsukubaensis* Δ*PtMAC2* supplemented with 0.1 % d-MEL-B, 0.1 % Tween 20, and 0.01 % Triton X-100. Cells were grown in MEL production medium containing 5 % (w/v) olive oil at 25 °C for 5 days. Dry cell weight (A) and m-MEL-D production (B). Data are averages of results from three independent cultivations.

### 3.3. Production of mono-acylated MEL-D with di-acylated MEL-B, Tween 20, and Triton X-100

Because the addition of d-MEL-B, Tween 20 and TritonX-100 significantly enhanced the m-MEL-D productivity of Δ*PtMAC2*, quantitative evaluation, structural analysis, and various property evaluations were performed.

To quantitatively evaluate the effects of d-MEL-B and other surfactants on the production of m-MEL-D, Δ*PtMAC2* was cultivated at 25 °C for 5 days in medium supplemented with 5 % (v/v) olive oil and 0.1 % (w/v) d-MEL-B, 0.1 % (w/v) Tween 20, or 0.01 % (w/v) Triton X-100. The production of m-MEL-D was evaluated by HPLC. The dry cell weight of Δ*PtMAC2* under the above culture conditions ranged from 20 to 25 g/L (Fig. 7A). HPLC analysis revealed that the yields of m-MEL-D were 6.8 ± 0.2 g/L, 1.8 ± 0.5 g/L, and 3.3 ± 0.4 g/L with the additions of 0.1 % d-MEL-B, 0.1 % Tween 20, and 0.01 % Triton X-100, respectively (Fig. 7B). The highest yield occurred with the addition of 0.1 % d-MEL-B, indicating the d-MEL-B is the most suitable to enhance the production of m-MEL-D.

To further enhance the production of m-MEL-D, jar fermentation was performed with 0.1 % d-MEL-B supplementation. Δ*PtMAC2* was cultivated at 25 °C for 7 days in medium supplemented with 9 % (v/v) olive oil and 0.1 % (w/v) d-MEL-B. The dry cell weight of Δ*PtMAC2* was ~40 g/L and the added oil was completely consumed in 7 days (Fig. 8). The m-MEL-D yield with 0.1 % d-MEL-B supplementation was 16.9 g/L.

### 3.4. Characterization of the fatty-acid profiles of mono-acylated MEL-D

We previously reported that m-MEL-D produced by Δ*PtMAC2* is MEL acylated at the C-2′ position of the mannose moiety [27]. To assess the variation in the length of the fatty acid chain of m-MEL-D, we performed selected ion monitoring (SIM) mode analyses of m-MEL-D using LC-MS. Peaks were identified by matching with mass spectra. The total ion chromatogram (TIC) and SIM chromatogram are shown in Fig. 9. The mass spectrum of m-MEL-D produced at a retention time (RT) of 13.8 min is shown in Fig. 9A. In SIM mode (Fig. 9B), signals at $m/z$ = 428 ($[M + NH_4]^+$) and 455 ($[M + HCOO]^-$), corresponding to m-MEL-D with C8:0, were dominant (molecular weight of ME is 284). Weak signals corresponding to m-MEL-D with C6:0 ($m/z$ = 400 $[\{M + NH_4\}^+]$ and 427 $[\{M + HCOO\}^-]$) and m-MEL-D with C10:0 ($m/z$ = 456 $[\{M + NH_4\}^+]$ and 483 $[\{M + HCOO\}^-]$) were also detected. No m-MEL-D with longer fatty-acid chains, such as C12:0 ($m/z$ = 584 $[\{M + NH_4\}^+]$ and 511 $[\{M + HCOO\}^-]$), C12:1 ($m/z$ = 482 $[\{M + NH_4\}^+]$ and 509 $[\{M + HCOO\}^-]$), C14:0 ($m/z$ = 512 $[\{M + NH_4\}^+]$ and 539 $[\{M + HCOO\}^-]$), C14:1 ($m/z$ = 510 $[\{M + NH_4\}^+]$ and 537 $[\{M + HCOO\}^-]$), or C14:2 ($m/z$ = 508 $[\{M + NH_4\}^+]$ and 535 $[\{M + HCOO\}^-]$), was detected. From these results, strain Δ*PtMAC2* dominantly produced m-MEL-D with C8:0 at the C-2' position of the mannose moiety (Fig. 9C).

### 3.5. Surface-active properties of mono-acylated MEL-D

We determined the surface tension of m-MEL-D by the pendant-drop method. We created a surface (air–water) tension *versus* concentration plot of m-MEL-D in distilled water (Fig. 10). d-MEL-B at ≥ 1 mg/mL formed a cloudy dispersion in water, whereas m-MEL-D dissolved in water at > 50 mg/mL and remained transparent. The CMC and γCMC of m-MEL-D produced by Δ*PtMAC2*, which consisted dominantly of C8:0 fatty acids, were $5.8 \times 10^{-2}$ M and 40.8 mN/m, respectively.

## 4. Discussions

We previously successfully generated Δ*PtMAC2*, which selectively produced m-MEL-D from olive oil; however, the m-MEL-D

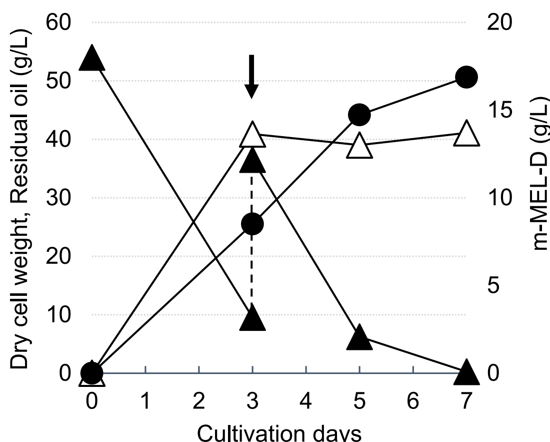


**Fig. 8.** Cultivation profile of jar fermentation. *P. tsukubaensis* strain Δ*PtMAC2* was grown in MEL production medium containing 9 % (w/v) olive oil at 25 °C for 7 days. Dry cell (open triangles), m-MEL-D (closed circles), and residual oil (closed triangles) contents are averages of three independent cultivations. Arrow indicates addition of olive oil.

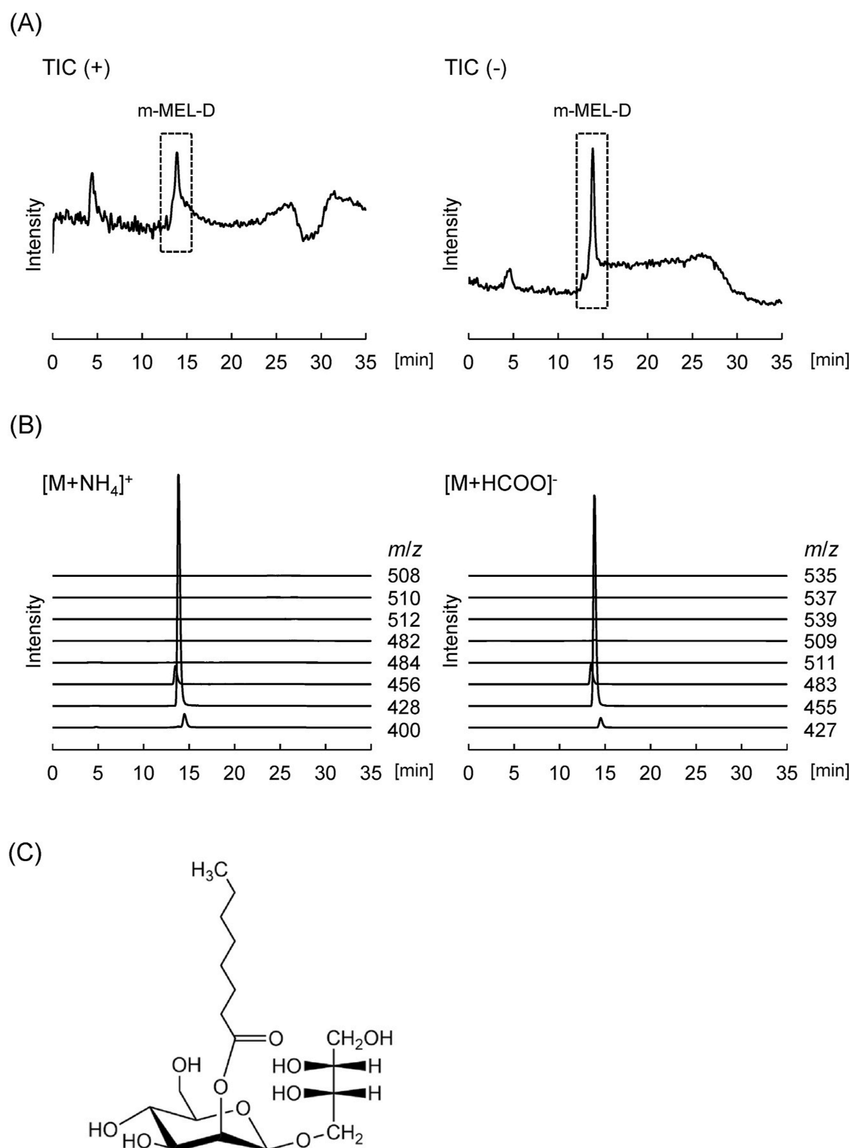


**Fig. 9.** LC-MS analyses of m-MEL-D produced by *P. tsukubaensis* Δ*PtMAC2*. TIC (A), SIM mode chromatogram of the peak at RT 13.9 min (B). $m/z$ = 400, 428, 456, 484, 482, 512, 510, and 508 correspond to the ammonium ion adducts of m-MEL-D containing C6:0, C8:0, C10:0, C12:0, C12:1, C14:0, C14:1, and C14:2. $m/z$ = 427, 455, 483, 511, 509, 539, 537, and 535 correspond to the formate ion adducts of m-MEL-D containing C6:0, C8:0, C10:0, C12:0, C12:1, C14:0, C14:1, and C14:2. The structure of m-MEL-D is shown (C).

productivity of this strain was low (<0.5 g/L). Thus, in this study, we aimed to enhance the m-MEL-D production of Δ*PtMAC2*. We hypothesized that the inability of m-MEL-D to emulsify oil resulted in inefficient use of the substrate and low productivity of m-MEL-D. Thus, we expected that addition of d-MEL-B as an emulsifier to the culture medium during Δ*PtMAC2* cultivation would increase m-MEL-D production. To verify this hypothesis, d-MEL-B and chemical surfactants were added to the culture medium as emulsifiers during Δ*PtMAC2* cultivation. As shown in Figs. 3–5, with the additions of 0.1 % d-MEL-B, 0.1 % Tween 20, and 0.01 % Triton X-100, the supplemented olive oil was almost completely consumed, and the m-MEL-D productivity of Δ*PtMAC2* was significantly enhanced. As surfactants, d-MEL-B, Tween 20 and Triton X-100, showed marked olive oil emulsification activity, while m-MEL-D did not (Fig. 6). These results strongly support our hypothesis. Emulsification of olive oil in culture medium by d-MEL-B, Tween 20 and Triton X-100 increased the olive oil consumption and m-MEL-D production of Δ*PtMAC2*.

Of the three surfactants that were shown to be effective in increasing m-MEL-D production, d-MEL-B was the most effective, followed by Triton X-100, then Tween 20. The reason for this difference is currently unknown. Brij35 and SDS did not increase m-MEL-D production. Because the dry cell weight decreased when Brij35 or SDS were added (Fig. 4), these surfactants likely inhibited the growth of Δ*PtMAC2*. In order to enhance m-MEL-D production in Δ*PtMAC2*, it is important to select surfactant that does not inhibit cell growth. As no negative effects on cell growth were observed with C12Na, the reason for C12Na not showed enhancement of m-MEL-D

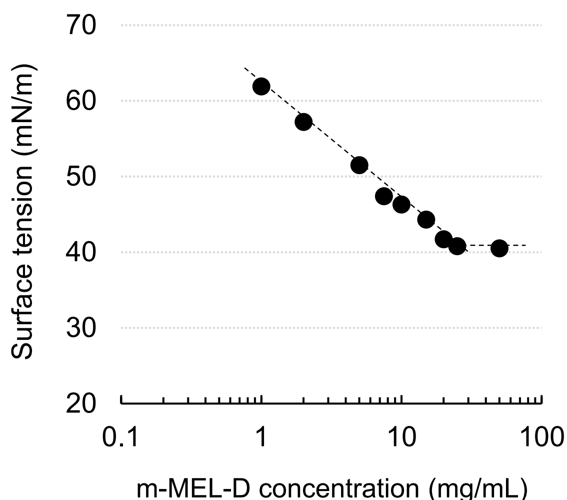


**Fig. 10.** Surface tension-concentration plot of m-MEL-D. Data are averages of 10 independent experiments.

production remains unclear.

m-MEL-D showed no emulsifying activity towards olive oil (Fig. 6). By contrast, d-MEL-D (which has one more fatty acid chain than m-MEL-D) and d-MEL-B (which has one more fatty acid chain and acetyl group than m-MEL-D) showed greater emulsifying activities. Because m-MEL-D has only one fatty acid chain, it has higher hydrophilicity than d-MEL-D and d-MEL-B. Therefore, m-MEL-D is soluble in water and lacks emulsifying activity.

Fukuoka et al. [29] evaluated the CMC of the d-MEL-B produced by *P. tsukubaensis* (parent strain of Δ*PtMAC2*). The CMC of d-MEL-B, produced by *P. tsukubaensis*, was $3.1 \times 10^{-6}$ M and it consisted of C8:0 (33.5 %), C12:0 (12.5 %), C12:1 (16.4 %), and C14:1 (18.9 %) fatty acids. In general, chemical surfactants that are highly soluble in water typically have high CMCs. Because m-MEL-D has higher water solubility (>50 mg/mL) than d-MEL-B (≥1 mg/mL), it is expected to have a high CMC. As expected, m-MEL-D had a higher CMC ($5.8 \times 10^{-2}$ M) than d-MEL-B, retaining the surface tension (40.8 mN/m). This result indicates that the number of fatty acid chains influences the CMC.

Before the 2018 report on m-MEL-D production by Δ*PtMAC2* [27], there was only one report on m-MEL-D production by *M. antarcticus* with glucose supplementation in the culture medium [9]. In that study, m-MEL-D was produced as a minor product. The CMC of m-MEL-D produced by *M. antarcticus* (consisting of C8:0 (11.6 %), 10:0 (24.6 %), 12:0 (19.1 %), and 14:0 (10.6 %) fatty acids) was $3.6 \times 10^{-4}$ M. In this study, the CMC of m-MEL-D produced by Δ*PtMAC2* (consisting dominantly of C8:0 fatty acids) was $5.8 \times 10^{-2}$ M; two orders of magnitude higher than m-MEL-D produced by *M. antarcticus*. This is thought to be a result of the shorter fatty acid chain length in m-MEL-D produced by Δ*PtMAC2* compared to m-MEL-D produced by *M. antarcticus*. m-MEL-D production by Δ*PtMAC2* has significantly higher solubility in water because it consists of shorter fatty acid chains, therefore, it has a higher CMC than m-MEL-D produced by *M. antarcticus*. *M. antarcticus* produces conventional MELs, which contain 4-O-β-D-mannopyranosyl-(2*S*, 3*R*)-erythritol as the sugar moiety. By contrast, *P. tsukubaensis* produces diastereomer type of MEL, which contain 4-O-β-D-mannopyranosyl-(2*R*,3*S*)-erythritol (Fig. 1B). Diastereomer-type MELs have higher hydrophilicity than conventional-type MELs [29]. Based on this stereoisomeric difference and the shorter fatty acid chains, m-MEL-D produced by Δ*PtMAC2* showed higher hydrophilicity than m-MEL-D produced by *M. antarcticus*.

Kondo et al. [4] evaluated the correlation between the critical aggregation concentration (CAC) of di-acylated MEL-D and its fatty acid chain length by using chemically synthesized di-acylated MEL-D. When the fatty acid chain length of di-acylated MEL-D was reduced by C2, the CAC increased approximately one order of magnitude (MEL-D (C14), $5.9 \times 10^{-7}$ M; MEL-D (C12), $3.9 \times 10^{-6}$ M; MEL-D (C10), $2.6 \times 10^{-6}$ M ($CAC_I$) and $1.7 \times 10^{-5}$ M; MEL-D (C8), $4.4 \times 10^{-5}$ M; and MEL-D (C6), $8.0 \times 10^{-5}$ M). Between m-MEL-D (CMC $5.8 \times 10^{-2}$ M; mainly C8:0 fatty acids) and the m-MEL-D produced by *M. antarcticus* (CMC $3.6 \times 10^{-4}$ M; mainly C10:0 fatty acids), there was a difference of two orders of magnitude in CMC because of the difference in chain length. Therefore, the CMC of mono-acylated MELs is strongly influenced by the fatty-acid chain length.

In this study, we successfully enhanced the m-MEL-D productivity of Δ*PtMAC2* via supplementation of d-MEL-B or other chemical surfactants in the culture medium. m-MEL-D productivity was increased more than 10-fold (from 0.5 g/L to 6.7 g/L), and structural analysis and evaluation of various properties, such as emulsifying activity, water solubility and surface-active properties, have also become available. m-MEL-D was highly soluble in water and had a significantly higher CMC than di-acylated MELs, which are major products of most MEL producers. Our results may help promote the use of m-MEL-D in aqueous applications and lead to expanded use of MELs.

## CRediT authorship contribution statement

**Azusa Saika:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Tokuma Fukuoka:** Writing – review & editing, Methodology, Investigation. **Shuhei Yamamoto:** Writing – review & editing, Investigation. **Tomohiro Sugahara:** Writing – review & editing, Investigation. **Atsushi Sogabe:** Writing – review & editing, Investigation. **Tomotake Morita:** Writing – review & editing, Methodology, Investigation, Conceptualization.

## Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

## Data availability statement

All data accessed and analyzed in this study are available in the article.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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