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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Enhanced production of a diastereomer type of mannosylerythritol lipid-B by the basidiomycetous yeast *Pseudozyma tsukubaensis* expressing lipase genes from *Pseudozyma antarctica*

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Abstract Basidiomycetous yeasts in the genus *Pseudozyma* are known to produce extracellular glycolipids called mannosylerythritol lipids (MELs). *Pseudozyma tsukubaensis* produces a large amount of MEL-B using olive oil as the sole carbon source (> 70 g/L production). The MEL-B produced by *P. tsukubaensis* is a diastereomer type of MEL-B, which consists of 4-*O*-β-D-mannopyranosyl-(2*R*,3*S*)-erythritol as a sugar moiety, in contrast to the conventional type of MELs produced by *P. antarctica*, which contain 4-*O*-β-D mannopyranosyl-(2*S*,3*R*)-erythritol. In this study, we attempted to increase the production of the diastereomer type of MEL-B in *P. tsukubaensis* 1E5 by introducing the genes encoding two lipases, PaLIPAp (*PaLIPA*) and PaLIPBp (*PaLIPB*) from *P. antarctica* T-34. Strain 1E5 expressing *PaLIPA* exhibited higher lipase activity than the strain possessing an empty vector, which was used as a negative control. Strains of 1E5 expressing *PaLIPA* or *PaLIPB* showed 1.9- and 1.6-fold higher MEL-B production than the negative control strain, respectively, and oil consumption was also accelerated by the introduction of these lipase genes. MEL-B production was estimated using time course analysis in the recombinant strains. Strain 1E5 expressing *PaLIPA* produced 37.0 ± 1.2 g/L of MEL-B within 4 days of cultivation, whereas the strain expressing an empty vector produced 22.1 ± 7.5 g/L in this time. Overexpression of *PaLIPA* increased MEL-B production by *P. tsukubaensis* strain 1E5 from olive oil as carbon source by more than 1.7-fold.

Keywords Mannosylerythritol lipid · Basidiomycetous yeast · *Pseudozyma tsukubaensis* · *Pseudozyma antarctica* · Lipase

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Introduction

Biosurfactants (BSs) have received much attention in recent years because of their environmentally friendly properties such as biodegradability, biocompatibility, and mild production conditions. Several types of BSs have been reported, including sophorolipid, rhamnolipid, trehalose lipid, mannosylerythritol lipids (glycolipid type), surfactin, serrawettin (lipopeptide type), spiculisporic acid (fatty acid type), and emulsan (polymer type) (Kitamoto et al. 1990a, b; Maget-Dana and Ptak 1992; Matsuyama et al. 1992; Ban et al. 1998; Lang and Philp 1998; Rosenberg and Ron 1999; Maier and Soberón-chávez 2000; Ogawa and Ota 2000; Rau et al. 2001; Lang 2002). Mannosylerythritol lipids (MELs) are remarkable as they are not only excellent surfactants but also have several unique properties. MELs also show great potential as hair and skin care ingredients, antioxidants, and cell activators (Morita et al. 2010a; Morita et al. 2010b; Takahashi et al. 2012; Yamamoto et al. 2012). Recently, Toyobo Co., Ltd. (Japan) has commercialized a diastereomer

type of MEL-B (Ceramela, a skin care ingredient) based on its ability to repair damaged skin (Yamamoto et al. 2012).

MELs are categorized by the degree of acetylation and the chirality of erythritol. MEL-A is di-acetylate, and MEL-B and MEL-C are mono-acetylate (Fig. 1a). The basidiomycetous yeast *Pseudozyma* has been well studied as an MEL producer. Most strains of the genus *Pseudozyma* produce a conventional MEL, consisting of 4-*O*-β-D-mannopyranosyl-(2*S*,3*R*)-erythritol (*S*-form) as the hydrophilic moiety (Fig. 1a). On the other hand, *P. tsukubaensis*, which was isolated from the leaves of *Perilla frutescens*, produces a diastereomer type of MEL-B consisting of 4-*O*-β-D-mannopyranosyl-(2*R*,3*S*)-erythritol (*R*-form; Fig. 1b) (Fukuoka et al. 2008; Morita et al. 2010c). *Pseudozyma tsukubaensis* produces a large amount of the diastereomer type of MEL-B using olive oil as a sole carbon source, and its production can reach over 70 g/L (Morita et al. 2010c). *Pseudozyma tsukubaensis* is able to utilize several carbon sources, such as glucose, glycerol, and plant oils, and shows the highest production with olive oil. To date, the MEL biosynthesis pathway in *P. tsukubaensis* has been identified in a draft genome sequence (Saika et al. 2016), and we can improve upon the current understanding of the MEL biosynthesis pathway in *P. tsukubaensis* using genetic engineering techniques. To further increase production of the diastereomer type of MEL-B, enhancement of the MEL biosynthetic pathway is required.

Figure 2 illustrates the pathway of MEL biosynthesis from plant oil. Saika et al. (2016) identified the gene cluster involved in MEL biosynthesis, which consists of four genes: an erythritol/mannose transferase (*PtEMT1*), two acyltransferases (*PtMAC1* and *PtMAC2*), and an acetyltransferase (*PtMAT1*) in *P. tsukubaensis* strain NBRC1940. Because MEL consists of mannose, erythritol, and fatty acid, the complex biosynthesis pathway should contribute to increasing MEL production.

To increase the carbon flux of the MEL biosynthetic pathway, we focused on the lipase that catalyzes the hydrolysis of triglyceride to form glycerol and fatty acids at the oil/water interface. To date, a large number of microbial lipases have been reported, with many industrial uses, including in food

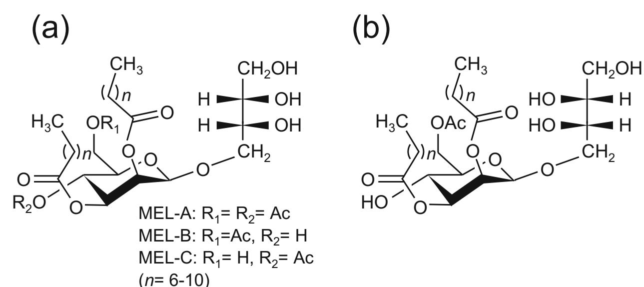

Fig. 1 Chemical structures of MELs (**a**) and the diastereomer type of MEL-B (**b**)

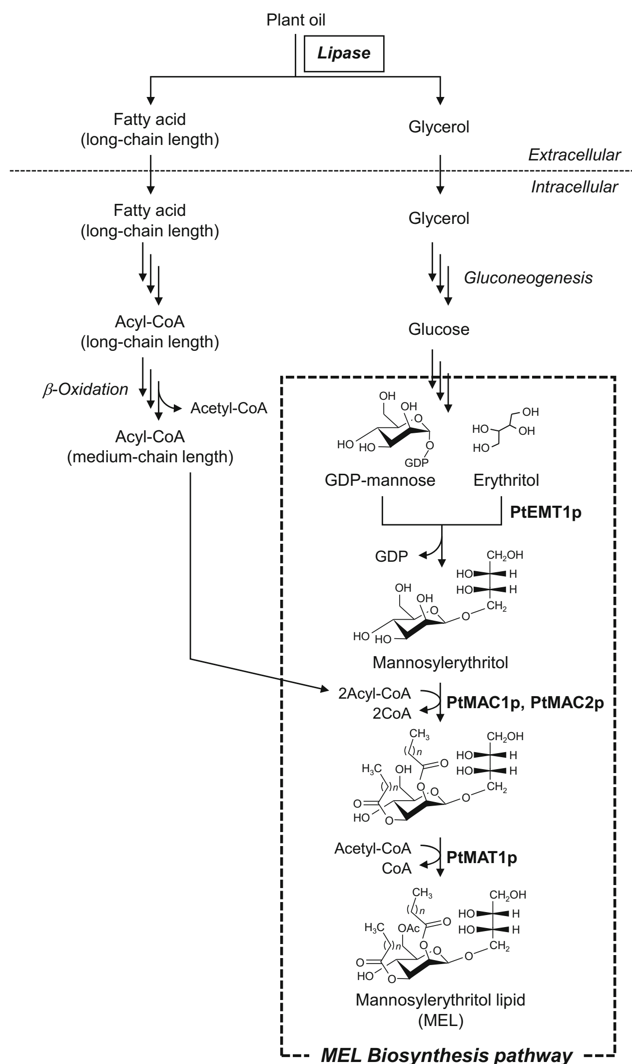

Fig. 2 The biosynthetic pathway of the diastereomer type of MEL-B produced in *P. tsukubaensis* from plant oil. PtEMT1p, erythritol/mannose transferase; PtMAC1p and PtMAC2p, acyltransferases; PtMAT1p, acetyltransferase

processing, high-grade chemical production, cosmetics, and pharmaceuticals (Sharma et al. 2001; Hasan et al. 2006). In particular, *P. antarctica* lipase A (PaLIPAp) and *P. antarctica* lipase B (PaLIPBp), known as CALA and CALB, are widely utilized in several fields. PaLIPAp and PaLIPBp encoded by *PaLIPA* and *PaLIPB* contain 462 and 342 amino acids, respectively. Both have an a/b hydrolase-fold, and the active-site triad is located at Ser184, Asp334, and His366 in PaLIPAp and Ser105, Asp187, and His224 in PaLIPBp (Gotor-Fernandez et al. 2006; Ericsson et al. 2008). PaLIPAp exhibits *Sn*-2 preference in the hydrolysis of triglycerides, whereas PaLIPBp prefers the *Sn*-3 position (Kirk and Christensen 2002). Both lipases can retain their activity through immobilization and exhibit broad specificity, and thus PaLIPAp and PaLIPBp are quite useful in industrial applications.

In this study, we demonstrated that overexpression of lipase leads to increased MEL-B production using olive oil as a carbon source. Two lipase genes from *P. antarctica* T-34, *PaLIPA* and *PaLIPB* encoding PaLIPAp and PaLIPBp, were overexpressed in the diastereomer type of MEL-B producer, *P. tsukubaensis* strain 1E5. MEL production by the recombinant strain harboring *PaLIPA* improved more than 1.9-fold (23.3 ± 0.8 g/L) compared to the control strain (12.4 ± 0.6 g/L).

Materials and methods

Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *Pseudozyma tsukubaensis* strain 1E5 (Japan Collection of Microorganisms, JCM16987) was employed as a host strain for MEL production. The genomic DNA of *P. antarctica* T-34 (formerly *Candida antarctica* T-34 and now renamed as *Moesziomyces antarcticus*; Kitamoto et al. 1990b) was used as a PCR template to amplify the genes *PaLIPA* (DDBJ Accession No. LC215917) and *PaLIPB* (DDBJ Accession No. LC215918), encoding PaLIPAp and PaLIPBp, respectively. The expression vectors pUC_neo-PaLIPA and pUC_neo-PaLIPB harboring *PaLIPA* and *PaLIPB* from *P. antarctica* strain T-34 were transformed into the host strain by electroporation.

Plasmid construction

The plasmids pUC_neo-PaLIPA and pUC_neo-PaLIPB (Fig. 3) were constructed as follows. The primers used in this study are listed in Table 1. An autonomously replicating sequence from *Ustilago maydis* (UARS) and neomycin resistance gene with the heat shock protein 70 (hsp70) promoter from *U. maydis* were excised from pUXV1_neo using *Ssp*I and *Hin*dIII-*Nde*I restriction enzymes, respectively, and inserted at the corresponding site in pUC18, yielding pUC_UARSneo. The 1.4- and 1.0-kb *PaLIPA* and *PaLIPB* gene fragments were amplified with *Kpn*I-*Eco*RI sites by PCR using *P. antarctica* strain T-34 genomic DNA as a template, and were introduced at the same site in pUC_UARSneo. Subsequently, the glyceraldehyde-3P-dehydrogenase (gap) promoter from *P. antarctica* strain T-34 was amplified with *Sal*I-*Xba*I sites by PCR using the genomic DNA of *P. antarctica* strain T-34 as a template, and inserted at the same site, yielding pUC_neo-PaLIPA and pUC_neo-PaLIPB.

Transformation

The plasmids pUC_neo-PaLIPA and pUC_neo-PaLIPB were introduced into the chromosome of *P. tsukubaensis* strain 1E5 by non-homologous recombination using electroporation. Competent cells of *P. tsukubaensis* strain 1E5 were prepared according to Watanabe et al. (2016). Approximately 3 μg each

Table 1 Strains, plasmids, and primers used in this study

Strains, plasmids, primers	Description	Reference/source
Strain		
Pseudozyma tsukubaensis 1E5 (JCM16987)	Diastereomer type of MEL-B producer	Morita et al. (2010c)
P. antarctica T-34	Conventional type of MELs producer, source of *PaLIPA* and *PaLIPB*	Kitamoto et al. (1990b)
Escherichia coli JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Takara Bio Inc.
Plasmid		
pUC18	Expression vector, Amp^R	Takara Bio Inc.
pUXV1_neo	Neomycin/G418 selection cassette (Neo^R) under control of the *P. antarctica* T-34 (P_{LYS20}) promoter	Watanabe et al. (2016)
pUC_UARSneo	Inserted UARS and Neo^R with P_{hsp70} from pUXV1_neo into pUC18	This study
pUC_neo	Inserted P_{gap} from *P. antarctica* T-34 into pUC_UARSneo	This study
pUC_neo-LIPA	Inserted *PaLIPA* from *P. antarctica* T-34 into pUC_neo	This study
pUC_neo-LIPB	Inserted *PaLIPB* from *P. antarctica* T-34 into pUC_neo	This study
Primer		
Pgap_F1	GTAGTCGACGTCGCCTCGGAAAGATC	This study
Pgap_R1	CTGTCTAGAGATGATGGATGGGGAGTGTG	This study
PaLIPA_F1	TTTGGTACCATGCGAGTGTCCTTG	This study
PaLIPA_R1	GCAGAATTCCTAAGGCGGTGTG	This study
PaLIPB_F1	CGAGGTACCATGAAGCTACTCTC	This study
PaLIPB_R1	TGAGAATTCTCAGGGGGTGACG	This study

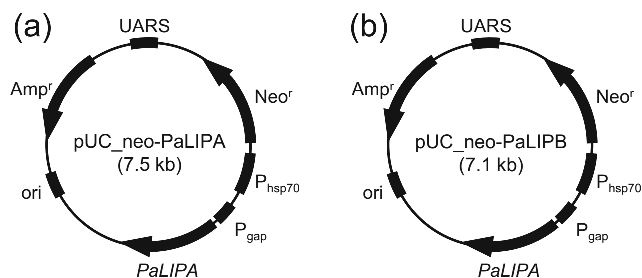

Fig. 3 Plasmids used in this study. *PaLIPA* (**a**) and *PaLIPB* (**b**) are derived from *P. antarctica* strain T-34

of plasmid pUC_neo-PaLIPA and pUC_neo-PaLIPB were linearized by *Ssp*I digestion (removed the UARS region) and mixed with 100 μL of competent cells. Electroporation was performed using a Gene Pulser II with Pulse Controller Plus (Bio-Rad, Tokyo, Japan) with 2-mm gap electroporation cuvettes. The pulse setting was 1,500 V and 8.0 ms, and under these conditions, the capacitance and resistance were 25 μF and 200 Ω, respectively. The electroporated cells were immediately diluted in 1 mL of chilled 1 M sorbitol, and incubated at 25 °C for 2 h. After incubation, an aliquot (200 μL) of cell suspension was spread onto a plate with YM medium (3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, and 10 g L^{-1} glucose) containing 500 μg mL^{-1} G418, and the resulting colonies were grown at 25 °C for 3 days. Three different clones for each recombinant strains were selected and used following experiments.

Lipase activity assay

Seed cultures of the recombinant *P. tsukubaensis* strain 1E5 harboring *PaLIPA* and *PaLIPB* were cultivated in 2 mL of YM medium containing 5% (*w*/*v*) glycerol at 25 °C for 3 days with 250 rpm shaking. One milliliter of seed culture was inoculated into 20 mL of MEL production medium (5 g L^{-1} yeast extract, 3 g L^{-1} $NaNO_3$, 0.3 g L^{-1} KH_2PO_4, and 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$) containing 1% (*v*/*v*) olive oil and 2% (*w*/*v*) glycerol at 25 °C for 3 days with 250 rpm shaking. After cultivation, the supernatant from the culture was recovered by centrifugation at 12,000 rpm for 3 min at 4 °C. Lipase activity of *PaLIPA* and *PaLIPB* in the supernatant was determined using a Lipase Activity Assay Kit (Cayman Chemical, Ann Arbor, Michigan) following the manufacturer's instructions. One unit is defined as the amount of lipase required to convert 1 μmol of substrate per minute in the culture supernatant. To confirm enzyme expression, culture supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Any KD Mini-PROTEAN TGX Precast Gels (Bio-Rad, Tokyo, Japan) following standard procedures. Proteins were visualized using the Quick-CBB PLUS stain (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

MEL production

Pseudozyma tsukubaensis 1E5 expressing *PaLIPA* and *PaLIPB* were cultivated in 2 mL of YM medium containing 5% (*w*/*v*) glycerol at 25 °C for 3 days with 250 rpm shaking. An aliquot of this seed culture (1 mL) was inoculated into 20 mL of MEL production medium containing 6% (*v*/*v*) olive oil and 2% (*w*/*v*) glycerol, and cultivated at 25 °C for 3–5 days while shaking at 250 rpm. The MEL produced by the cell culture was extracted with an equal volume of ethyl acetate. After recovery of the ethyl acetate layer, 10 mL of methanol was added to the residual water layer containing cell debris, and the mixture was shaken vigorously. Cells were harvested by centrifugation at 10,000 rpm for 2 min and dried to measure cell growth.

Thin layer chromatography analysis

MELs were detected using thin layer chromatography (TLC) (Morita et al. 2007a). The extracted MELs were analyzed by TLC using chloroform, methanol, and 12% NH_4OH in a 65:15:2 (*v*:*v*:*v*) ratio as an eluent. MELs were detected on the TLC plate by spraying the plate with 2% anthrone-sulfate reagent and heating at 90 °C for 5 min. A purified MEL-B was used as a reference.

Quantification of MEL and residual oil

MEL production and the residual oil quantity were analyzed using high-performance liquid chromatography (HPLC) with a Tosoh DP-8020 pump (TOSOH, Tokyo, Japan) and low-temperature evaporative light scattering detector (ELSD) Model 300S (SofTA Corporation, Thornton, CO) with a silica gel column (Inertsil SIL 100A 5 μm, 4.6 × 250 mm; GL Science Inc., Japan) at room temperature. MEL and residual oil were eluted with chloroform and methanol as a mobile phase at a flow rate of 1 mL/min. The methanol gradient program was 0–15 min, linear gradient of 0–100%; 15–25 min, 100%; 25–25.1 min, linear gradient of 100–0%; 25.1–35 min, 0%. Quantification of MEL and residual oil were based on the standard curves of purified MEL-B and olive oil, respectively.

Results

Gene expression and lipase activity of recombinant *P. tsukubaensis* strain 1E5 expressing *PaLIPA* or *PaLIPB*

To increase the carbon flux involved in MEL biosynthesis, two lipase genes, *PaLIPA* and *PaLIPB* from *P. antarctica* strain T-34, were introduced into *P. tsukubaensis* strain 1E5. Recombinant strains of *P. tsukubaensis* 1E5

possessing lipase gene expression vectors, the plasmids pUC_neo-PaLIPA and pUC_neo-PaLIPB, were cultivated in MEL production medium containing 1% (*v*/*v*) olive oil as a carbon source for 3 days, and the gene products were analyzed via SDS-PAGE (Fig. 4a). The empty vector pUC_neo was used as a negative control (without overexpressing exogeneous lipase). The protein bands corresponding to PaLIPAp and PaLIPBp were detected at 45 and 33 kDa, respectively (Kirk and Christensen 2002) in each recombinant strain transformed with lipase gene expression vectors, whereas these bands were not detected in the negative control strain. On the basis of genome annotation, *P. tsukubaensis* 1E5 has two conserved genes encoding lipases (PtLIPAp and PtLIPBp), and the putative molecular weight of the gene products are 49 and 36 kDa, respectively; however, it exhibits no protein bands corresponding to the sizes of PtLIPAp and PtLIPBp (Fig. 4a). Cell growth of the recombinant strains was not affected by gene expression (Fig. 4b).

Lipase activity of the culture supernatant is shown in Fig. 4c. The lipase activity in the recombinant strain expressing *PaLIPA* and *PaLIPB* was 105.1 ± 17.9 and 50.3 ± 6.9 U/mL. On the other hand, lipase activity in the culture supernatant of the strains possessing the empty vector was only 2.4 ± 0.2 U/mL. It was confirmed that the overexpression of *PaLIPA* and *PaLIPB* lead to enhance the lipase activity in *P. tsukubaensis* 1E5.

MEL-B production of *P. tsukubaensis* 1E5 expressing *PaLIPA* or *PaLIPB*

To estimate the effect of lipase overexpression on MEL production, cultures of *P. tsukubaensis* strain 1E5 harboring pUC_neo-PaLIPA or pUC_neo-PaLIPB were grown in MEL production medium containing 6% (*v*/*v*) olive oil and 2% (*w*/*v*) glycerol for 3 days at 25 °C. The growth of strain 1E5 expressing *PaLIPA* (34.0 ± 2.1 g/L) was slightly higher than that of strains with the empty vector or *PaLIPB* (30.2 ± 2.1 and 32.1 ± 0.5 g/L, respectively; Fig. 5a).

On TLC analysis, each recombinant strain produced MEL-B (Fig. 5b). The small amount of tri-acylated MEL was detected above MEL-B spot. Tri-acylated MEL was produced by esterification with presence of excess amount of oil in culture medium (Morita et al. 2008). Strain 1E5 expressing *PaLIPA* and *PaLIPB* showed higher MEL-B productivity, and also, the amount of residual oil is less than expressing empty vector.

We quantified the MEL production based on HPLC analysis. Strain 1E5 expressing *PaLIPA* exhibited the highest MEL-B production (23.3 ± 0.8 g/L), and this strain's production was 1.9-fold higher than that of the strain with the empty vector (12.4 ± 0.6 g/L; Fig. 5c). In strain 1E5 expressing *PaLIPB*, MEL-B production also increased to 20.2 ± 0.5 g/L.

The amount of residual olive oil was estimated using HPLC analysis (Fig. 5d). Oil consumption was significantly

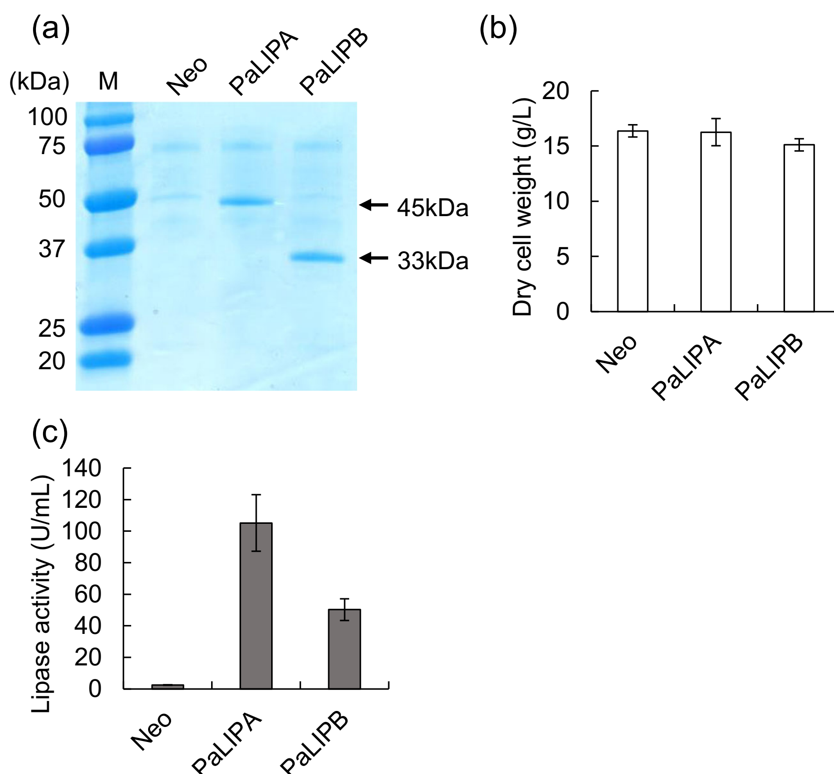

Fig. 4 Overexpression of *PaLIPA* and *PaLIPB* in *P. tsukubaensis* strain 1E5 using the expression vector pUC_neo. Cells were grown in MEL production medium containing 1% (*v*/*v*) olive oil at 25 °C for 3 days. SDS-PAGE analysis of culture supernatant (**a**), dry cell weight (**b**), and lipase activity assay (**c**) results for strain 1E5 expressing *PaLIPA* or *PaLIPB*. Neo, strain 1E5/pUC_neo (negative control); PaLIPA, strain 1E5/pUC_neo-PaLIPA; PaLIPB, strain 1E5/pUC_neo-PaLIPB; M, protein marker; ND, not detected. The results shown are averages of data from three independent cultivations

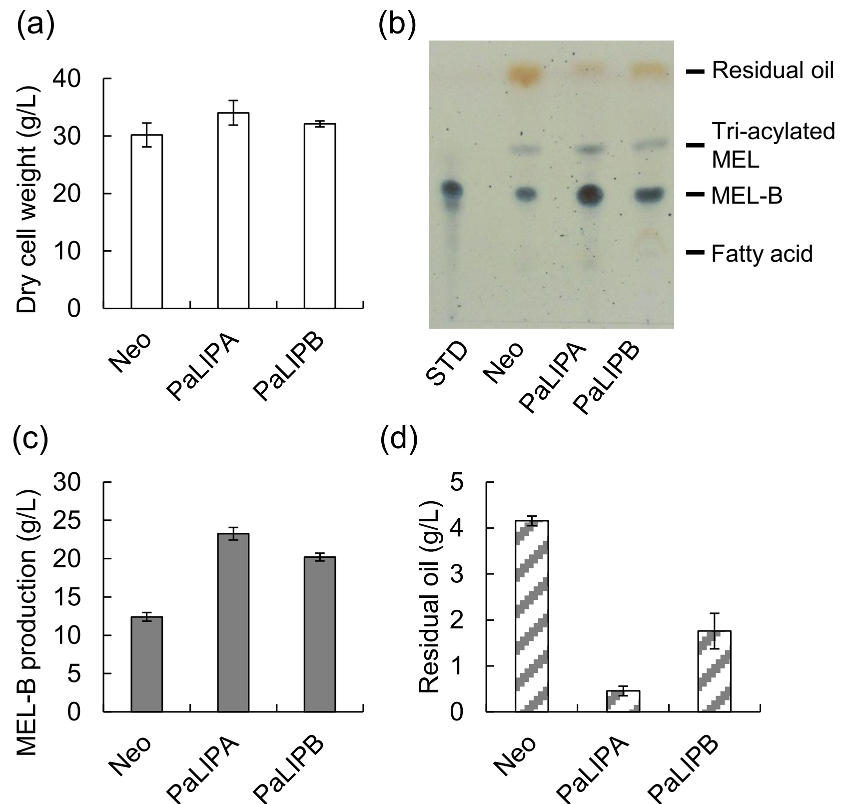

Fig. 5 MEL-B production and oil consumption by *P. tsukubaensis* strain 1E5 expressing *PaLIPA* or *PaLIPB*. Cells were grown in MEL production medium containing 6% (*v*/*v*) olive oil at 25 °C for 3 days. Dry cell weight (**a**), TLC analysis (**b**), MEL-B production (**c**), and residual oil (**d**). Neo, strain 1E5/pUC_neo (negative control); PaLIPA, strain 1E5/pUC_neo-PaLIPA; PaLIPB, strain 1E5/pUC_neo-PaLIPB. The concentrations of MEL-B and olive oil were determined using HPLC. The data shown are the averages of results from three independent cultivations

greater in strains expressing *PaLIPA* and *PaLIPB*. The residual oil concentrations in these two cultures were 0.5 ± 0.1 and 1.8 ± 0.4 g/L, respectively. In contrast, over 4 g/L oil remained in the supernatant of strain 1E5 with the empty vector. Based on these results, *PaLIPA* expression was the most effective method to accelerate carbon consumption and MEL-B production.

Time course of MEL-B production in strain 1E5 expressing *PaLIPA*

To further evaluate the efficiency of MEL-B production from plant oil by a lipase-expressing strain, we performed a time course study of MEL-B production in the strain expressing *PaLIPA* (Fig. 6). Cultures of strain 1E5 with an empty vector or pUC_neo-PaLIPA (*PaLIPA*-expressing) were grown in MEL production medium with 6% (*v*/*v*) olive oil. After 2 days of cultivation, 6% (*v*/*v*) olive oil was added directly to the culture flask. Strain 1E5 expressing *PaLIPA* consumed the olive oil completely within 4 days (1.9 ± 1.0 g/L), whereas 7.3 ± 5.9 g/L of olive oil remained after 5 days in the culture with the empty vector. Also, the strain expressing *PaLIPA* exhibited higher cell growth, and reached stationary phase at 3 days, while the strain expressing the empty vector was still in the exponential growth phase at that time. The strain expressing *PaLIPA* reached 37.0 ± 1.2 g/L production at 4 days of cultivation, whereas strain 1E5 with the empty vector had produced 22.1 ± 7.5 g/L of MEL-B within 4 days. The total amount of MEL-B was about 40 g/L in the cultures expressing the empty vector and *PaLIPA* at 5 days. These results indicated that *PaLIPA* expression led to increased rates of MEL-B production and oil consumption. Overexpression of *PaLIPA* is an efficient method to shorten the cultivation time, and thus reduce the cost of MEL-B production using olive oil as a carbon source.

Furthermore, we evaluated the stability of linearized plasmid integrated in *P. tsukubaensis* 1E5 chromosome, and stability was calculated by G418 resistance as a selection marker (Fig. S1). The result shows stability of linearized plasmid after 5 days cultivation with or without 500 μg mL^{-1} G418. Regardless of whether culture medium contained G418 or not, the stability of linearized plasmid of *PaLIPA* and *PaLIPB* were approximately 80% after 5 days cultivation. The integrated gene was maintained without antibiotics supplementation during cultivation.

Discussion

In this study, we overexpressed two lipase genes, *PaLIPA* and *PaLIPB* (encoding PaLIPAp and PaLIPBp) from *P. antarctica* strain T-34, in the diastereomer type of MEL-B producer *P. tsukubaensis* strain 1E5 to enhance MEL synthesis using olive oil as a carbon source. The lipase activity of recombinant

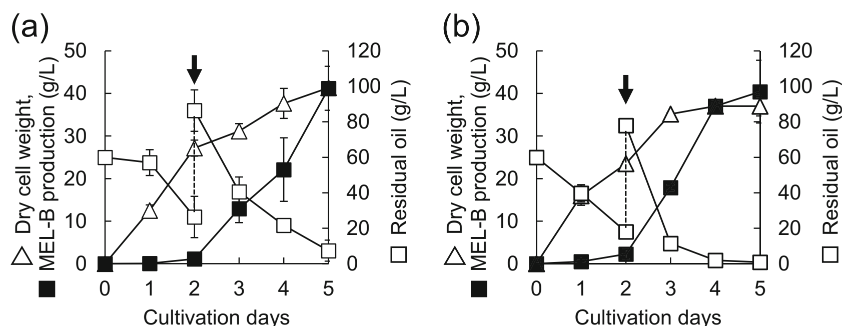

Fig. 6 Time course of MEL-B production and oil consumption in *P. tsukubaensis* strain 1E5 expressing *PaLIPA*. Strain 1E5/pUC_neo (**a**) and strain 1E5/pUC_neo-PaLIPA (**b**) were grown in MEL production medium containing 12% (*v*/*v*) olive oil at 25 °C for 5 days. Data for dry cell (open triangles), MEL-B (closed squares), and residual oil (open squares) contents are the averages of data from three independent cultivations. The concentrations of MEL-B and olive oil were determined using HPLC. The arrow indicates an addition of olive oil

strain 1E5 expressing *PaLIPA* increased, and the rates of MEL production and olive oil consumption were accelerated compared with strain 1E5 expressing an empty vector.

Based on the results shown in Figs. 5 and 6, overexpression of PaLIPAp and PaLIPBp leads to enhanced MEL production arising from enhanced oil consumption. Aside from *P. tsukubaensis*, many strains belonging to the genus *Pseudozyma* are reported to produce MELs from plant oil, including *P. antarctica*, *P. parantarctica* (renamed to *Moesziomyces parantarcticus*), *P. rugulosa* (renamed to *Moesziomyces rugulosus*), *P. aphidis* (renamed to *Moesziomyces aphidis*), and *P. hubeiensis* (Rau et al. 2005a; Konishi et al. 2007; Morita et al. 2007b). The lipase overexpression method may be an efficient way to increase MEL production by these strains.

We used *PaLIPA* and *PaLIPB* to increase the carbon flux for MEL-B production. Because *P. tsukubaensis* is in the same genus as *P. antarctica*, we expected that *PaLIPA* and *PaLIPB*, which encode PaLIPAp and PaLIPBp, would be compatible for expression by *P. tsukubaensis* strain 1E5. In addition, PaLIPAp and PtLIPAp share 77% amino acid sequence identity, and PaLIPBp and PtLIPBp also exhibited high identity (78%) based on BLASTP analysis. As expected, both lipases were expressed in *P. tsukubaensis* strain 1E5 (Fig. 4a).

Strain 1E5 expressing *PaLIPA* completely consumed the olive oil in the medium within 4 days of cultivation (Fig. 6b), and thus MEL production plateaued at 5 days. Strain 1E5 expressing the empty vector exhibited a linear increase in MEL production between 4 and 5 days (21.7 ± 1.7 g/L olive oil remained at 4 days), and thus, MEL production by strain 1E5 expressing *PaLIPA* may be effectively enhanced with fed-batch cultivation. In addition, Rau et al. (2005b) demonstrated that the production rate of MEL in a bioreactor was faster than via shake-flask cultivation. Applying fed-batch cultivation with a jar fermenter may lead to further increases of the MEL production rate.

In conclusion, we demonstrated that overexpression of *PaLIPA* and *PaLIPB* was an efficient method to increase diastereomer type of MEL-B production and oil consumption by *P. tsukubaensis* strain 1E5. This strategy may be applicable to other *Pseudozyma* strains that produce MELs from plant oils, and thus contribute to the expansion of MEL utilization in industrial applications.

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Compliance with ethical standards

Conflict of interest SY and TK are employees of Toyobo Co., Ltd., but Toyobo Co., Ltd. did not have any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. AS, HK, TF, SY, TK, and TM are inventors of submitted patent which related to this study (JP2016-112087). The authors have declared that no other competing interests exist.

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

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