Korean J. Chem. Eng., 39(4), 997-1003 (2022)
DOI: 10.1007/s11814-021-1020-0

pISSN: 0256-1115
eISSN: 1975-7220

Enhanced production of biosurfactants through genetic engineering of *Pseudozyma* sp. SY16

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(*Received 12 August 2021 • Revised 15 November 2021 • Accepted 19 November 2021*)

Abstract−Mannosylerythritol lipids (MELs) are natural glycolipids that possess biosurfactant properties and are abundantly produced by *Pseudozyma* sp. Due to their specific characteristics, such as biodegradability and low toxicity, MELs have attracted significant interest as an alternative to petroleum-based surfactants in medical and cosmetic fields. The present study describes a novel expression system and optimal transformation conditions of *Pseudozyma* sp. SY16 for producing MELs. The hygromycin resistance gene under the control of *Deinococcus radiodurans*-derived *Kat1* promoter was used as selection marker and the superfolder green fluorescent protein under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter was used for confirming successful expression. Using this expression system, several transformants overexpressing genes related to MEL production, including *emt1*, *mmf1*, and *mat1*, were generated. Among them, MMF1-2 strain exhibited an MEL yield of 27.9 g/L, which was 31.6% higher than that of the wild-type strain. Altogether, this study demonstrates that engineered yeast strains hold potential for large-scale production of MELs.

Keywords: Biosurfactant, MEL, *Pseudozyma* sp., Transformation, Yeast

INTRODUCTION

Surfactants are extensively used in several fields, including detergents, cosmetics, pharmaceuticals, and food [1]. Due to their high demand, most of the commercial surfactants are of synthetic origin, which may pose risks to the ecosystem and human health due to their persistency [2]. Therefore, eco-friendly products, such as biosurfactants, have attracted increasing attention [3]. In particular, glycolipid biosurfactants produced by various yeast and bacterial species have become the focus of research due to their unique properties (i.e., mild emulsifying conditions, higher biocompatibility, and versatile biological functions) [4]. The numerous advantages of biosurfactants have thus prompted their applications in various industries; for example, they are used for removing soil contaminants including heavy metals and other toxic pollutants [5,6]. Moreover, some biosurfactants are potential antimicrobial agents and are suitable alternatives to synthetic antibiotics [7].

Among the common glycolipid biosurfactants are mannosylerythritol lipids (MELs), which contain 4-*O*-[*β*-D-mannopyranosyl] meso-erythritol as the hydrophilic moiety and a fatty acid and/or an acetyl group as the hydrophobic moiety [8]. MELs are abundantly produced by *Pseudozyma* sp., acting as energy storage compounds similar to triacylglycerols [8]. Even though biosurfactants including MELs have a wide range of applications, they are not yet commercially viable, mainly due to their low yield and high production cost [9]. DKBIO, a Korean company, has successfully developed methods for the production and purification of MELs from *Pseudozyma* sp. [10]. Further improvement in MELs productivity is expected to be possible through genetic engineering, which could reduce the market price of the final products.

Among different strategies for genetic engineering, overexpression of endogenous genes has been effectively used to improve the production of desired products in multiple organisms [11]. In *Ustilago maydis* and *Pseudozyma antarctica*, clusters of genes related to the production of MELs were found to comprise *emt1*, *mac1*, *mac2*, and *mat1*, which are involved in MEL biosynthesis, and *mmf1*, which is involved in the export of MEL [12,13]. In this study, we designed and optimized a new expression system that can achieve enhanced large-scale production of MELs. First, we established a recombinant expression system using *Pseudozyma* sp. SY16 along with the superfolder green fluorescent protein (sfGFP) and optimized the transformation conditions. In addition, we identified the endogenous MEL biosynthesis genes of this species and successfully

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overexpressed *emt1* and *mmf1*. The *mmf1*-overexpressed strains exhibited increased MELs production, which suggests that this strategy could be used for yeast strain improvement for industrial production of the biosurfactant MELs.

MATERIALS AND METHODS

1. Strains and Flask Cultivation

Pseudozyma sp. SY16 strain was obtained from DKBIO (Daejeon, South Korea). In flask cultivations, YM medium (BD, Franklin Lakes, NJ, USA) was used for the gene transformation, strain maintenance, and inoculum preparation. For MEL production, baffled flasks with 180 mL of a growth medium (21 g/L glycerin, 50 g/L oleic acid, 2.5 g/L K_2HPO_4, 2 g/L NH_4NO_3, 0.1 g/L NaH_2PO_4, 1 g/L yeast extract, 0.5 g/L $MgSO_4·7H_2O$, 0.1 g/L $CaCl_2·H_2O$, 0.02 g/L $MnSO_4·H_2O$) were prepared and 20 mL of the seed culture was inoculated into the flasks. The cells were incubated for 5 d at 30 °C with agitation of 200 rpm. For gene cloning, as well as plasmid maintenance and replication, *Escherichia coli* DH5α and LB medium (BD) were used.

2. Plasmid Construction

The pTopHPH plasmid harboring *aph7* (the hygromycin B resistance gene) under the control of *Deinococcus radiodurans*-derived *Kat1* promoter was used as backbone. The homologous arms HA1 and HA2 were amplified from the endogenous ribosomal RNA sequence using the F/R-HA1 and F/R-HA2 primer sets, respectively (Table 1). Gene fragments were cloned into the backbone plasmid using *Hin*dIII and *Kpn*I for HA1, plus *Not*I and *Apa*I for HA2, resulting in pTOP-HA. The expression cassette comprising the promoter, target gene (*sfGFP*), and terminator was digested from the pRS424_GPD-sfGFP plasmid using *Sac*I and *Kpn*I (Fig. 1(b)), which was then cloned into pTOP-HA, resulting in pTOP-sfGFP (Fig. 1(a)). Using the obtained plasmid, expression vectors for genes related to MEL production (*emt1*, *mmf1*, *mac1*, *mac2*, and *mat1*) were created. The target genes were amplified from cDNA of *Pseudozyma* sp. SY16 using the primers listed in Table 1, and replaced the *sfGFP* gene using *Xba*I-*Xho*I or *Xba*I-*Nhe*I restriction enzymes. To avoid the use of *Xho*I, of which a restriction site occurs within *emt1*, *mac2* and *mat1* sequences, a *Nhe*I restriction site was generated during the construction of the *mmf1* expression vector and it was used as the backbone for cloning the other plasmids (Fig. 3(b)).

3. Electroporation of *Pseudozyma* sp. SY16

Cells were grown in 50 mL of YM medium and harvested when the optical density at 660 nm reached 0.8-1.3. The harvested cells were washed with ice-chilled distilled water, and then suspended in 250 μL of ice-chilled 1 M sorbitol solution. The cell solution (80 μL) was mixed with 5-10 μg of DNA, then transferred to a 2-mm gap cuvette (Bio-Rad, Hercules, CA, USA) and incubated on ice for 10 min. A Bio-Rad Gene Pulser electroporation system was used at 1.5-2.5 kV/cm, 25 μF, and 200 Ω After electroporation, 1 mL of ice-chilled 1 M sorbitol solution was immediately added, the cell

Table 1. List of primers used in the study

Primers for cloning homologous arms	
F_HA1	gcaagcttgaagagggaagagcccaa
R_HA1	gcggtaccatgccctcgttcacttaagt
F_HA2	gcgcggccgcgtggttagggcctttgtt
R_HA2	gcgggccctcagtagggtaaaactaacctg
Primers for cloning MEL-related genes	
F_XbaI_Mmf1	atgcatgctctagaatggacgagaaattctcctc
R_Mmf1_6His_NheI_XhoI	gcatgcatctcgaggctagctcattaatggtgatggtgatgatgaggcattgtgcttggaa
F_XbaI_Emt1	atgcatgctctagaatgaaagtggcactgcttt
R_Emt1_FLAG_NheI	gcatgcatgctagctcattacttgtcgtcgtcgtccttgtagtcgcaggtagtgatggtcg
F_XbaI_Mat1	atgcatgctctagaatgacagcccgaacc
R_Mat1_HA_NheI	gcatgcatgctagctcattaagcgtaatctggaacatcgtatgggtactgcacaaatgggtatcgt
F_XbaI_Mac1	atgcatgctctagaatgtcatccgtcgatcag
R_Mac1_FLAG_XhoI	gcatgcatctcgagtcattacttgtcgtcgtcgtccttgtagtcgagacgagctgcaacag
F_XbaI_Mac2	atgcatgctctagaatgctaggagatcaagtttgg
R_Mac2_6His_NheI	gcatgcatgctagctcattaatggtgatggtgatgatgcagcttggcctcggg
Primers for RT-qPCR analysis	
F_Emt1_RT	caacagtcattgctggcacc
R_Emt1_RT	gcgaagggtaagcttgaggt
F_Mmf1_RT	tggatcccacttgttgtcgg
R_Mmf1_RT	aacgccgtaaaagacaacgc
F_Mac1_RT	atccaggctcgatacttgcg
R_Mac1_RT	ctccgagacgtatcaccacg
F_Actin_RT	gttctctcgctctacgcctc
R_Actin_RT	gggtagcggtaagcatctcc

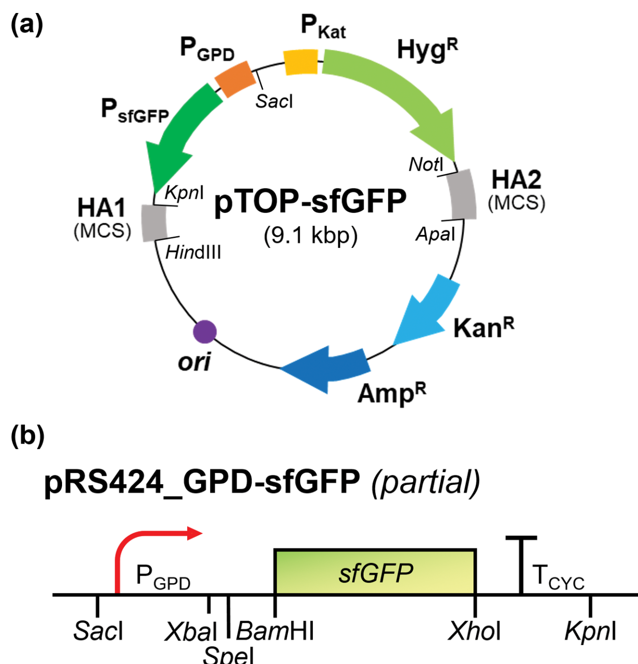

Fig. 1. Structure of the plasmids and strategy for vector construction. (a) Schematic map of the pTOP13-HA (sfGFP) plasmid. (b) Expression cassette of *sfGFP* comprising the promoter, terminator, and sites of the relevant restriction enzymes.

Table 2. Optimization of hygromycin concentration and electroporation conditions

Hygromycin B concentration (ppm)	Voltage (kV)	Colony counts*
0	-	Lawn
20	-	+++++
20	1.5	+++++
20	2.5	++++
50	-	+
50	1.5	++
50	2.5	+++
100	-	+
100	1.5	++
100	2.5	+++
200	-	No
200	1.5	++
200	2.5	+++

*+, ++, +++, ++++, and +++++ indicate <10, 10-50, 51-500, 501-5,000, and >5,000 colonies, respectively. "Lawn" and "no" indicate, literally, lawn plate formation and no colonies, respectively.

suspension was transferred to 14 mL of YM medium, and left to recover for 1 h. Recovered cells were then spread on YM agar medium containing appropriate concentration of hygromycin B (Sigma-Aldrich, St. Louis, MO, USA) (Table 2).

4. Flow Cytometry and Confocal Microscopic Analyses

To confirm the expression of sfGFP, flow cytometric analysis was performed using a FACSCalibur system (BD). Yeast cells were analyzed after washing with phosphate-buffered saline. GFP was excited by the 488 nm laser and detected at 530 nm. A minimum of 10,000 cells per sample were recorded for fluorescence analysis. Confocal microscopic analysis was performed using an LSM510 meta-laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100× objective and a camera (Nikon, Tokyo, Japan). Excitation/emission maxima of 485/530 nm was used to acquire GFP fluorescence.

5. Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using GoScript Reverse Transcription System (Promega, Madison, WI, USA) with oligo dT primers after RNA samples were treated with RQ1 RNase-Free DNase kit (Promega) to eliminate genomic DNA contamination. The generated cDNA was used as template for RT-qPCR using iQ SYBR Green Supermix (Bio-Rad) with the primers listed in Table 1. Three technical and two biological replicates were performed for each gene. Relative expression levels were calculated using housekeeping gene (act1) levels for normalization. Data represent the average standard deviation of two biological replicates.

6. MEL Extraction and Quantification

MEL extraction and quantification were performed based on the total sugar analysis method (phenol-sulfuric acid method) [14]. Briefly, 0.5 mL of culture solution was mixed with 2 mL of ethyl acetate and vortexed vigorously. After resting for 10 min, 100 μL of supernatant was collected, 1 mL of 0.4 N NaOH was added, and the mixture was vortexed. The solution was incubated at 50 °C for 30 min to dissolve fat-soluble ingredients. After centrifugation at 10,000 rpm for 15 min, the supernatant was collected and diluted to an appropriate concentration. Diluted sample (0.2 mL) was transferred into a test tube and mixed with 0.2 mL of 5% phenol solution. Then, 1 mL of H_2SO_4 was added and incubated for 30 min. Total sugar content was determined by measuring absorbance at 490 nm and calculated using the following equation: $y=(0.2374x-0.0003)\times 40\times F$, where 40 is the conversion factor when 0.5 mL of culture was used for analysis and F is the dilution factor. Data represent the average standard deviation of three biological replicates.

RESULTS AND DISCUSSION

1. Development of Expression Plasmid and Optimization of the Transformation Conditions

To develop an efficient expression vector for *Pseudozyma* sp. SY16, the pTopHPH plasmid, which was successfully transformed into *P. antarctica*, was employed as a backbone plasmid for vector construction. The *DrKat1* constitutive promoter was used to drive the expression of the hygromycin resistance gene. In addition, sfGFP was selected as the model recombinant protein as its fluorescent intensity was less affected by intracellular location or solubility [15]. The constitutive glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter was selected to express *sfGFP* as it was reported to achieve relatively high expression of recombinant proteins in yeast [16-18]. The ribosomal RNA coding sequence was used as homologous arms, due to the ease of sequence identifica-

tion using universal primers and the abundance of copy number [19,20].

After construction of the model plasmid pTop-sfGFP (Fig. 1(a)) we examined different hygromycin concentrations and voltage to obtain the optimal electroporation conditions for *Pseudozyma* sp. SY16 (Table 2). At 20 ppm of hygromycin B, more than 10,000 colonies were obtained that had not been electroporated. With 50-100 ppm of hygromycin B, most non-electroporated cells were dead, but some colonies still survived. When hygromycin B concentration was increased to 200 ppm, all non-transformed cells were dead (Table 2). Next, the model plasmid was transformed using different voltages (1.5 and 2.5 kV) and the electroporated cells were screened using different concentrations of hygromycin B. Under 20 ppm of hygromycin B, fewer colonies were obtained when applying 2.5 kV. However, over 50 ppm of hygromycin B, which confers a certain level of antibiotic selection pressure, similar or more colonies were obtained in 2.5 kV-applied samples than in those exposed to 1.5 kV. This result implies that a higher voltage (2.5 kV) kills more competent cells due to physical damage, but increases the chance of plasmid delivery into the cells. Therefore, it was concluded that 200 ppm of hygromycin B and 2.5 kV were the best options for the transformation of *Pseudozyma* sp. SY16 among the conditions examined. These conditions were used for the following experiments.

2. Confirmation of the Model Protein (*sfGFP*) Expression

Among the hundreds of colonies obtained by electroporation under the optimal conditions, 48 colonies were randomly selected and the insertion of *sfGFP* was examined through PCR analysis. More than 50% of the colonies presented the band with the expected size (Fig. 2(a)). To confirm the expression of the model protein (sfGFP), four clones that showed a relatively thicker band were selected for sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis. However, the cells were not well disrupted, even with harsh sonication or bead beating, and we could not obtain a reliable result. As an alternative, since the target protein was sfGFP, protein expression could be confirmed by measuring its fluorescence. First, the fluorescence of wild-type SY16 and four mutants (named as M1, M2, M3, and M4) was compared using confocal microscopy. As expected, all mutants exhibited fluorescence, whereas the wild-type SY16 strain showed no fluorescent signal (Fig. 2(b)). Accordingly, flow cytometric analysis further

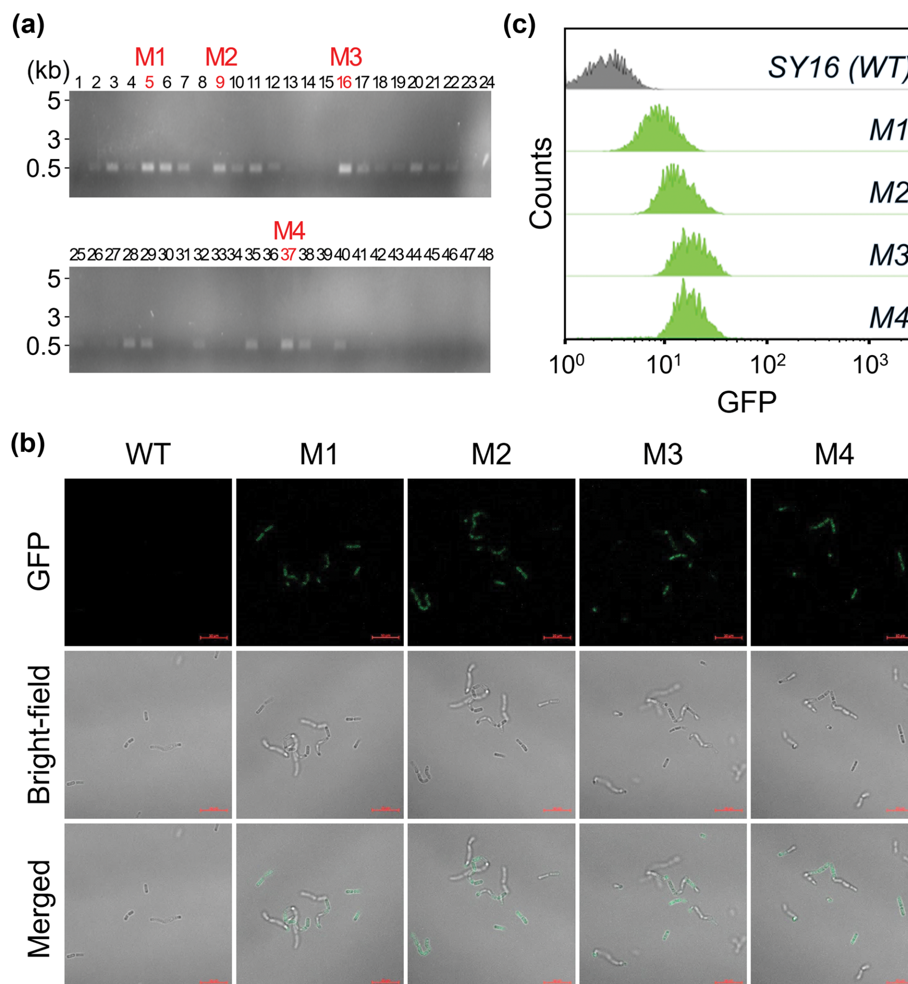

Fig. 2. Characterization of sfGFP-transformed *Pseudozyma* sp. SY16. (a) Polymerase chain reaction analysis of *sfGFP* in putative transformants. Numbers indicate the independent transgenic strains. (b) Confocal imaging of yeast cells expressing sfGFP. (c) Flow cytometry analysis of transgenic strains. M, mutants; WT, wild-type. Scale bar=10 μm.

confirmed that all mutants showed a fluorescent signal, whereas the wild-type SY16 did not.

3. Identification of MEL-biosynthesis Genes in *Pseudozyma* sp. SY16

In previous studies, five key genes related to the MEL biosynthesis pathway (*emt1*, *mac1*, *mac2*, *mat1*, and *mmf1*) were identified in *U. maydis* and *P. antarctica* [12,13]. In this study, whole genome sequencing of *Pseudozyma* sp. SY16 was accomplished by next generation sequencing performed by an external company. The full *Pseudozyma* sp. SY16 genome sequence is available online at http://web.seeders.co.kr/sy16/. By comparing the sequences of the MEL biosynthesis genes previously reported, we found that these genes were conserved in *Pseudozyma* sp. SY16 (Table 3). In addition, all five genes were located in the same contig and arranged in the same order and direction as in *U. maydis* (Fig. 3(a)). The *Pseudozyma* sp. SY16 DNA sequences for *emt1* (erythritol/mannose transferase), *mmf1* (putative transporter), *mac1* (acyl transferase 1), *mac2* (acyl transferase 2), and *mat1* (acetyl transferase) were obtained and amplified by PCR, and were cloned into the backbone plasmids to generate the overexpression vectors (Fig. 3(b)).

4. Overexpression of MEL-biosynthesis Genes Improve MEL Production in Transformants

Overexpression vectors containing target genes related to MEL production were transformed into *Pseudozyma* sp. SY16 using the

Table 3. Similarity analysis of MEL-biosynthesis genes in *Pseudozyma* sp. SY16 and other species

Protein	*Pseudozyma* sp. SY16	*U. maydis* UM521	*P. antarctica* T-34
EMT1	LOCUS_003204	um03117 (69%)	19c00001 (69%)
MAC1	LOCUS_003205	um03116 (58%)	19d000003 (61%)
MAC2	LOCUS_003203	um10636 (60%)	19d00002 (56%)
MAT1	LOCUS_003207	um03114 (53%)	19c00002 (51%)
MMF1	LOCUS_003206	um03115 (71%)	19d00004 (66%)

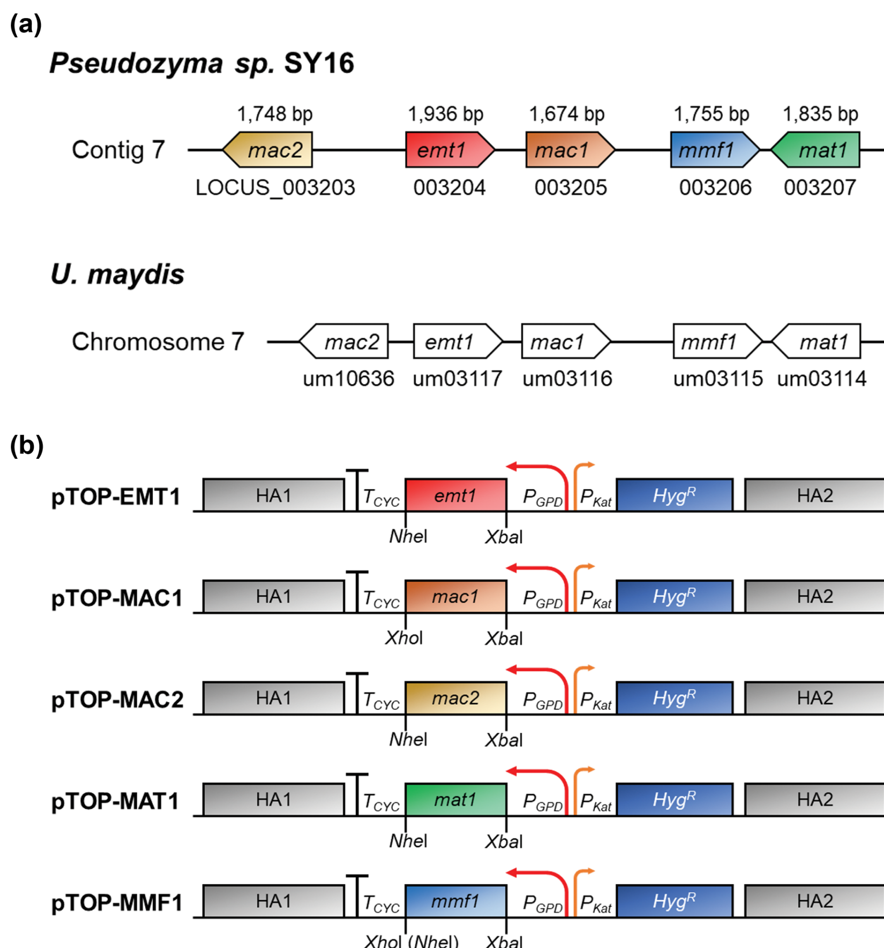

Fig. 3. Mannosylerythritol lipid (MEL) biosynthesis-related genes in *Pseudozyma* sp. SY16 and construction of the expression plasmids. (a) Organization of the complete MEL biosynthesis gene cluster in *Pseudozyma* sp. SY16 and *Ustilago maydis*. (b) Construction of the vectors for gene overexpression.

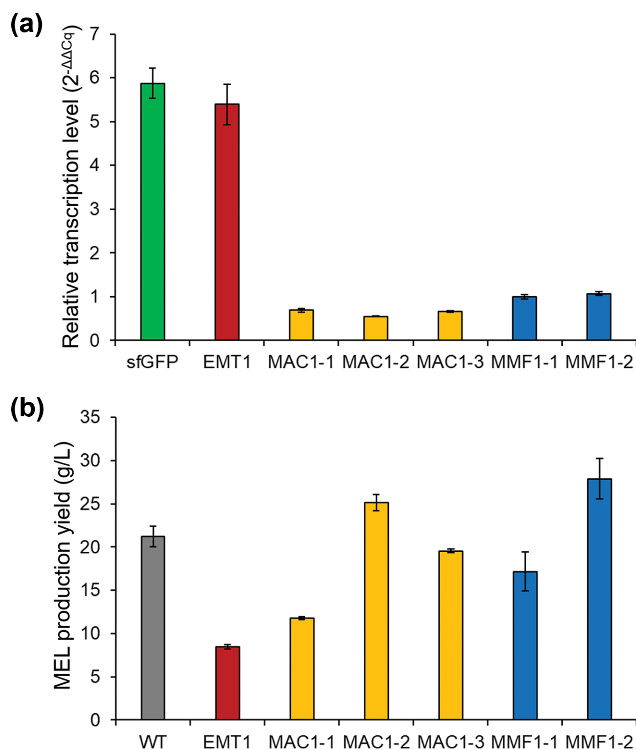

Fig. 4. Characterization of mannosylerythritol lipid (MEL) biosynthesis genes-overexpressing transformants. (a) Reverse transcription-quantitative polymerase chain reaction for analyzing the transcript levels of *sfGFP* and MEL biosynthesis-related genes in yeast transformants. (b) MEL content in yeast transformants and wild-type strain.

above described optimal electroporation protocol. All five plasmids were introduced into yeast cells, but no or only few colonies were obtained during repeated experiments using pTOP-MAC2 and pTOP-MAT1. In contrast, tens of colonies were obtained from each of the other plasmids (pTOP-EMT1, pTOP-MAC1, and pTOP-MMF1). To confirm the gene insertion, colony PCR was performed, and one clone for *emt1* expression (named as EMT1), three clones for *mac1* expression (named as MAC1-1, MAC1-2, and MAC1-3), and two clones for *mmf1* expression (named as MMF1-1 and MMF1-2) were isolated. With these selected transformants, RT-qPCR was performed to examine the transcript levels of the target genes (Fig. 4(a)). In the case of the EMT1-expressing strain, 5.39-fold increase in transcription was observed, which was similar to the outcome of the sfGFP model. However, for the MAC1-expressing strains, rather lower transcript levels were detected, although gene insertion was confirmed. In the MMF1-1- and MMF1-2-expressing strains, similar (1.00-fold) or slightly higher (1.07-fold) transcript levels were observed (Fig. 4(a)). Noteworthy that MEL production yields were not proportional to the transcript levels. For example, EMT1-expressing strain exhibited a dramatic increase in the transcript level, but the MEL production was less than half (40.1%) of that of the wild-type strain. The relative *mac1* level in MAC1-2 was 0.55, but MEL production was 18.6% higher than in the wild-type strain. In the case of MMF1-2, transcript level of the target gene was only increased by 7%, but a 31.5% increase in MEL yield was observed. In this study, the homologous arms were designed to direct the target genes into the loci of ribosomal DNAs; thus, the number of copies and locus of the integrated gene may differ among clones. Therefore, transcript and protein levels may have varied, although the same backbone plasmid was used or the same gene was transformed into the cells. In addition, transcript levels are not always perfectly correlated with the protein expression [21]. Overexpression of a recombinant gene may affect the transcription of its endogenous counterpart in a feedback-like manner. To clarify this, time-course of qPCR or protein analysis (e.g. SDS-PAGE, Western blot) should be performed; however, we could not obtain enough mRNA or protein extracts due to the difficult disruption of *Pseudozyma* sp. SY16. Nevertheless, we believe that the transformation method described in this study is still valid, since both gene insertion and improvement of MEL productivity were successfully demonstrated.

Emt1 is an erythritol/mannose transferase that plays an important role in the synthesis of the polar head of MEL. Thus, we expected that overexpression of *emt1* would improve the production yield of MEL; however, the yield was rather dramatically decreased. It seems that a metabolic burden due to excessive transcription (5.39-fold increase) of *emt1* occurred in the transformant, which may in turn have affected the synthesis of the desired metabolite [22]. Moreover, a 7% increase of *mmf1* (the putative MEL transporter) transcription resulted in 31.5% improvement of MEL production. This result suggests that reducing the partial concentration of MEL in the intracellular compartment through its secretion is one of the most important factors to induce MEL biosynthesis. *Mac1* encodes an acyl transferase that confers a non-polar tail to the polar head of the MEL; therefore, regulation of MAC1 expression may affect the isomerization and productivity of MEL [12]. Hence, structural evaluation of MEL would be required to further elucidate the effect of each gene.

CONCLUSION

Wew report the first transformation system for *Pseudozyma* sp. SY16. The recombinant plasmid pTOP-sfGFP was successfully transformed into SY16, with overexpression of sfGFP being demonstrated by confocal microscopy and flow cytometry. In addition, overexpression of *mmf1* was found to be associated with enhanced productivity of MELs (by 31.5%) in *Pseudozyma* sp. SY16. Although further studies are needed to improve the transformation efficiency and elucidate some theoretical questions, these results suggest that genetic engineering can enhance the industrial application of *Pseudozyma* sp. SY16 for MEL production. Furthermore, the draft genome and genome browser of this strain have been established, which will be helpful for several relevant fields.

ACKNOWLEDGEMENTS

This work was supported by Carbon to X project (2020M3H7A1098291) and by Basic Science Research Program (2021R1C1C1003425) through the National Research Foundation (NRF) funded by the Ministry of Science and ICT, Republic of Korea; and was also supported by the Korea Research Institute of Biosci-

ence and Biotechnology (KRIBB) Research Initiative Programs (KGS1382113 and KGM5252113).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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