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Selective Production of Acid-form Sophorolipids from Glycerol by *Candida floricola*

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Abstract: Biosurfactants (BSs) are produced in abundance from various feedstocks by diverse microorganisms, and are used in various applications. In this paper, we describe a new yeast isolate that produces glycolipid-BSs from glycerol, with the aim of enhancing the utilization of the surplus glycerol produced by the oleo-chemical industry. As a result of the screening, strain ZM1502 was obtained as a potential producer of BS from glycerol. Based on TLC analysis, the strain produced glycolipid BSs. According to structural analyses (NMR, MALDI-TOF MS, and GC-MS), the main component of the glycolipids was 6',6"-di-*O*-acetylated acid-form sophorolipid (SL). Interestingly, the strain produced only acid-form SL, without lactone-form SLs, although the conventional SL-producing yeast, *Starmerella bombicola*, produces lactone-form SLs with small amounts of the acid-form. Based on taxonomy, the strain was identified as *Candida floricola*. It produced 3.5 g L^{-1} of acid-form SLs in 20% (w/v) glycerol. In addition, *C. floricola* CBS7290 and NBRC10700^T also produced only acid-form SLs from glycerol. These results suggest that *C. floricola* would enhance the utilization of waste glycerol as a fermentation feedstock and facilitate a broad range of applications for SLs.

Key words: sophorolipid, acid-form sophorolipid, glycerol, *Candida floricola*

1 INTRODUCTION

Shifting from petroleum to biomass will be necessary for a sustainable industrial society and effective management of greenhouse gas emissions[1]. Over the last decade, production of biodiesel fuel (BDF) based on fatty acid methyl esters from plant oils has increased dramatically. Production of BDF and some oleochemicals is based on a transesterification reaction between triglycerides and alcohol (e.g., methanol) under alkaline conditions, which results in the formation of a crude glycerol waste (raw glycerol) as a by-product[2]. Owing to increased BDF production worldwide, surplus glycerol derived from BDF production is used as an alternative feedstock, and many projects seek to convert glycerol into useful compounds[3-5], such as 1,3-propandiol[6-8], 2,3-butanediol[9], 3-hydroxypropionaldehyde[10], dihydroxyacetone[11, 12], succinic acid[13], and polyhydroxyalkanoate[14]. Habe *et al.* reported the production of lignoceric acid from glycerol by *Aspergillus fumigates*[15]. Therefore, glycerol has potential as a carbon source and substrate for the production of biomaterials.

Biosurfactants (BS) are promising biomaterials defined as extracellular surface-active compounds produced by microorganisms from biomass, and have potential for industrial use, not only as emulsifiers and detergents, but also for energy-saving technology and medicines[16-18]. Recently, production of glycolipid BSs—such as mannosylerythritol lipids (MELs)—from glycerol was reported[19]. Ashby *et al.* reported production of 65 g L^{-1} of lactone-form sophorolipids (SLs) as main product by *Starmerella* (*Candida*) *bombicola* from a biodiesel co-product stream, which com-

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prised 40% glycerol, 34% hexane-soluble substrates, and 26% water[20]. Therefore, glycerol should undergo further development as a fermentation substrate for BS production.

Here, we report a screening for yeasts that produce glycolipid BS from glycerol as the sole carbon source, and the determination of the structure of the main component of the glycolipid. We also demonstrate that the yeast *Candida floricola* produces the acid-form, but not the lactone-form, SL.

2 EXPERIMENTAL

2.1 Strains

Strain ZM1502 was isolated from withered leaf by means of the following procedure. *Starmerella bombicola* NBRC10243^T and *Candida floricola* NBRC 10700^T were purchased from the National Institute of Technology and Evaluation (NITE), Biological Resource Center (NBRC). *C. floricola* CBS 7290 was purchased from the Centraalbereau voor Schimmelcultures (CBS) of the Royal Netherlands Academy of Arts and Sciences.

2.2 Isolation of BS-producing yeast

Leaves and flowers were sampled in Japan to isolate yeasts. The samples were incubated in a screening medium (200 g L^{-1} glycerol, 4 g L^{-1} $NaSO_4$, 1 g L^{-1} NH_4NO_3, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$ $7H_2O$, 1 g L^{-1} yeast extract, 50 mg L^{-1} streptomycin, and 50 mg L^{-1} chloramphenicol) in disposable test tubes at room temperature for 3 d. Cultures were then transferred to fresh screening medium and incubated for a further 3 d. After incubation for 1 week under identical conditions, 20 μL of the culture were dropped on Parafilm, and BS production was estimated based on the droplet diameter compared to sterile culture medium[19]. The culture medium of positive samples (100 μL) was plated on YM agar medium, and incubated at room temperature for several days until colonies were observed. The colonies were picked, inoculated into screening medium (5 mL), and incubated at room temperature with shaking at 200 rpm for 1 week. The glycolipids produced were extracted with 5 mL of ethyl acetate, and analyzed by thin-layer chromatography (TLC) with an 8:2 $CHCl_3$/CH_3OH mobile phase. Visualization was performed using 2% anthrone sulfate solution.

To purify the glycolipid components, the organic layer was evaporated and dissolved with ethyl acetate, and then purified by silica-gel chromatography using a stepwise gradient of chloroform-acetone (100:0 to 0:100).

2.3 Structural analysis

The purified glycolipid was characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) using a Varian INOVA 400 (400 MHz) instrument at 30℃ with CD_3OD solution.

To assess the saccharide composition, 50 mg of glycolipid were hydrated by 3 N HCl solution at 80℃ for 3 h. Fatty acid residues were removed by hexane extraction. The water phase (which contained the saccharides) was desalted by passage through a column containing Amberlite IRA-410 (Organo Co., Tokyo, Japan). The desalted sample was analyzed by high-performance liquid chromatography (HPLC) using an Shodex SUGAR SC1011 column (8.0×300 mm, Tosoh, Japan) with water as the mobile phase (85℃, 0.6 ml/min). The retention times of reagent-grade sugars were used as references.

The fatty-acid profile of the purified glycolipid was examined by a method described previously with some modification[21]. The methyl ester derivatives of fatty acids were prepared by mixing the above-purified glycolipid (10 mg) with 1 mL of 5% HCl-methanol reagent (Tokyo Kasei Kogyo, Tokyo, Japan). After heating at 80℃ for 3 h, the reaction was quenched with water (1 mL). The methyl ester derivatives were then extracted with *n*-hexane (2 mL) and analyzed by gas chromatography-mass spectrometry (GC-MS) (Agilent 5973 System, Agilent Technologies Inc., Palo Alto, CA, USA) with a TC-Wax column (GL-Science Ltd., Tokyo, Japan), the temperature being programmed from 80℃ (held for 4 min) to 250℃ at 10℃ min^{-1}, followed by a hold at 250℃ for 90 min.

The molecular weight of the purified glycolipid was measured by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS; Voyager system 6294) using α-cyano-4-hydroxycinnamic acid as the matrix.

2.4 Molecular phylogenetic analysis

DNA extraction was performed using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. puRe*Taq* Ready-To-Go PCR beads (Amersham Biosciences, NJ, USA) were used for PCR amplification with an initial denaturation at 95℃ for 7 min, followed by 35 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min 30 s, and a final step of 72℃ for 7 min. The sequences of the D1/D2 region of 26S rRNA genes were analyzed using the primers NL1, NL2, NL3, and NL4[22]. Sequencing reactions were performed using the ABI PRISM BigDye Terminator Kit v. 3.1 (Applied Biosystems, CA, USA) as directed by the manufacturer. The PCR products were sequenced using an ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems, CA, USA). The sequence has been deposited in the DNA Data Base of Japan (DBBJ) under the accession number DM065008.

The sequence of ZM1502 was compared with those obtained from the DDBJ (http://ddbj.nig.ac.jp) by BLAST search and alignment using the ClustalW software[23].

2.5 Morphological, physiological, and biochemical characterization

The morphological, physiological, and biochemical characteristics of the isolate were examined using the methods described[24, 25]. Strain ZM1502 has been deposited in the International Patent Organism Depositary, National Institute of Technology and Evaluation (IPOD-NITE), Japan, as FERM P-21133.

2.6 HPLC

To quantify glycolipids (sophorolipids, SLs), HPLC analysis was carried out using a HPLC system (SSPC; Tosoh, Tokyo, Japan) equipped with a silica gel column (Inertsil SIL-100A 5 μm, 4.6×250 mm; GL science, Japan) and a low-temperature evaporative light-scattering detector (ELSD-LT; Shimadzu, Kyoto, Japan) using a chloroform:methanol gradient (from 100:0 to 0:100) at a flow rate of 1 mL/min. SLs were quantified using standard curves generated from purified SL.

3 RESULTS

3.1 Glycolipid production by strain ZM1502

Strain ZM1502 was isolated as a novel BS-producing yeast through the above screening procedure. To estimate glycolipid production, strain ZM1502 was cultured in screening medium at room temperature at 150 rpm for 7 d. Strain ZM1502 produced the highest amounts of glycolipids (Fig. 1). The purified main glycolipid (GL-A) produced by ZM1502 was subjected to structural analysis.

3.2 Structural determination

HPLC analysis of the hydrolyzed glycolipid was undertaken to examine its saccharide composition, and GL-A (t_R = 13.64 min) resulted in the peak corresponding to glucose (t_R = 13.60 min). This result indicated that the saccharide portion includes only glucose residues.

Purified GL-A was identified by NMR analyses. The ^{1}H-NMR spectrum of GL-A was similar to that of previously reported SLs[26]. GL-A was a disaccharide, and H-1' and -1" protons were detected at 4.45 and 4.55 ppm, respectively. In addition, H-6' and -6" protons were shifted to a lower field (H-6'a and -6"a at 4.20 ppm, and H-6'b and -6"b at 4.36 ppm) by esterification of hydroxyl groups at the 6'- and 6"-positions. All proton peaks (except these derived from the two glucose units) in the molecule appeared at 3.2–3.7 ppm. There were no other proton peaks from 4.6 to 5.3 ppm, indicating that the proton was in the esterified carbon. Typical peaks at 2.05 and 2.06 ppm indicated the existence of two acetyl groups. Meanwhile, in the ^{13}C-NMR spectrum of this compound, a C-2' carbon was detected at 82.6 ppm. These data strongly indicated that the disaccharide of GL-A is sophorose.

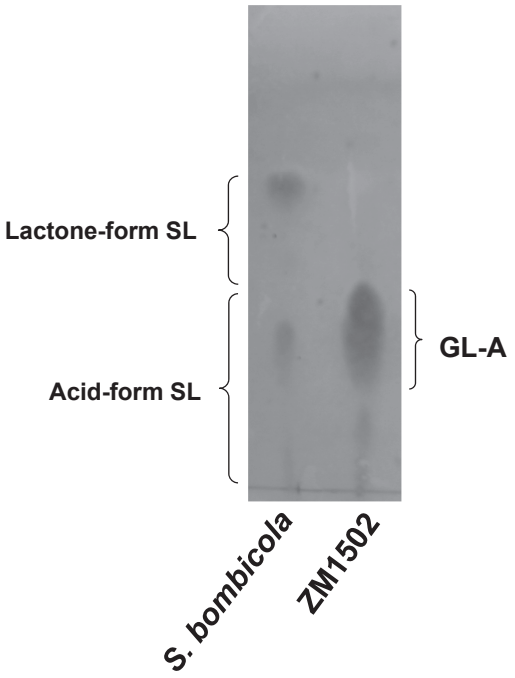

Fig. 1 Production of glycolipids by strain ZM1502 and *Starmerella bombicola* NBRC10243^T. Glycolipids were extracted from culture broth with ethyl acetate, and the organic solvent fraction was subjected to TLC analysis. Spots were visualized using anthrone-sulfuric regent. White arrow indicates the main glycolipid (GL-A).

According to the above results, we presumed that GL-A has two acetyl groups at the C-6' and C-6" positions in the sophorose backbone. Moreover, it is likely different from a lactone-type SL because of the lack of other ester groups. To confirm the structure in detail, the positions of esterified carbons were determined by hetero-nuclear multiple bond correlation (HMBC) spectrometry (Fig. 2). Carbonyl carbons (C=O) were detected at 171.5 and 176.5 ppm. The former peak (171.5 ppm) was identified as acetyl groups because it was correlated with the H-6' and -6" protons and the proton derived from acetyl groups (at 2.05 and 2.06 ppm). In contrast, the latter peak (176.5 ppm) was correlated with only the next-to-methylene protons (–C$\underline{H_2}$C=O), not correlated with protons on the saccharides. This carbon was identified as a free carboxylic acid, not an ester group. We thus identified GL-A as an acid-form 6',6"-di-*O*-acetylated SL. The molecular structure is shown in Fig. 3A.

Based on the GC-MS fatty-acid composition analysis, the acid-form SL was composed of ω-1-hydroxy-fatty acids; C18:1 (52.6%), C18:0 (23.5%), C16:0 (15.3%), and C16:1 (8.5%) (Table 1). The retention time of the main peak corresponded to the methyl 17-hydroxy octadecenoate of SLs produced by *S. bombicola* (data not shown). MALDI-TOF-MS analysis of GL-A showed four main peaks of $[M+Na]^+$ ions [(*m/z*) = 730, 732, 704, and 702, respectively],

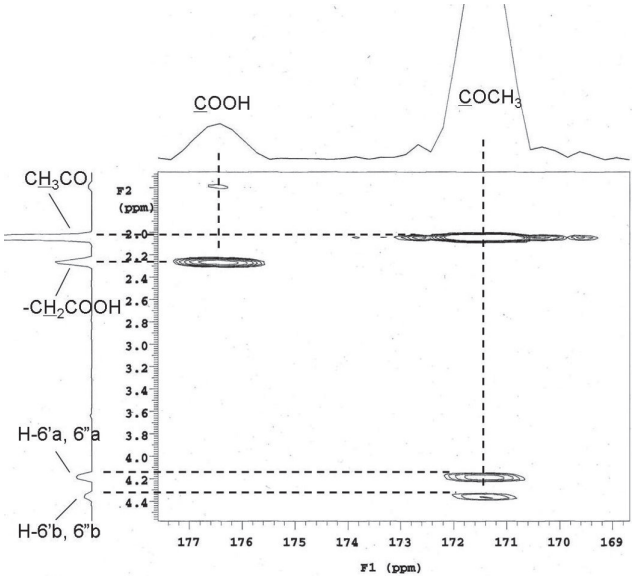

Fig.2 HMBC analysis of purified major glycolipid.

in agreement with the fatty acid composition (Table 1).

Based on the detailed structural analysis, the glycolipid, GL-A, produced by strain ZM1502 was identified as an acid-form 6',6"-di-*O*-acetylated SL bearing mainly 17-hydroxy octadecenoic acid (C18:1). The ^{1}H- and ^{13}C-NMR chemical shifts of GL-A are summarized in Table 2.

3.3 Taxonomic analysis

To identify strain ZM1502 taxonomically, we sequenced the D1/D2 regions of 26S ribosomal DNA. Figure 4 shows the phylogenetic tree of ZM1502 and related strains. The partial 26S ribosomal DNA sequence of strain ZM1502 showed 100% similarity to that of *Candida floricola* NRRL Y-17676. Furthermore, we examined the morphological and biochemical characteristics of strain ZM1502. The cells are of a coccal form, and are propagated by multipolar budding with no formation of a spore, sexual reproductive organ, or pseudomycelium on YM agar medium at 25℃ after 1 week. A micrograph is shown in Fig. 5. The biochemical characteristics of strain ZM1502 were corresponded with those of the type strain of *C. floricola* (Table 3)[24, 25], with the exception of ethanol assimilation and growth at 37℃. Based on these results, strain ZM1502 was identified as *C. floricola* (named *C. floricola* ZM1502).

3.4 SL production from glycerol

SL production by *S. bombicola* NBRC 10243^{T}, *C. floricola* ZM1502 and CBS 7290, and NBRC 10700^{T} was evaluated.

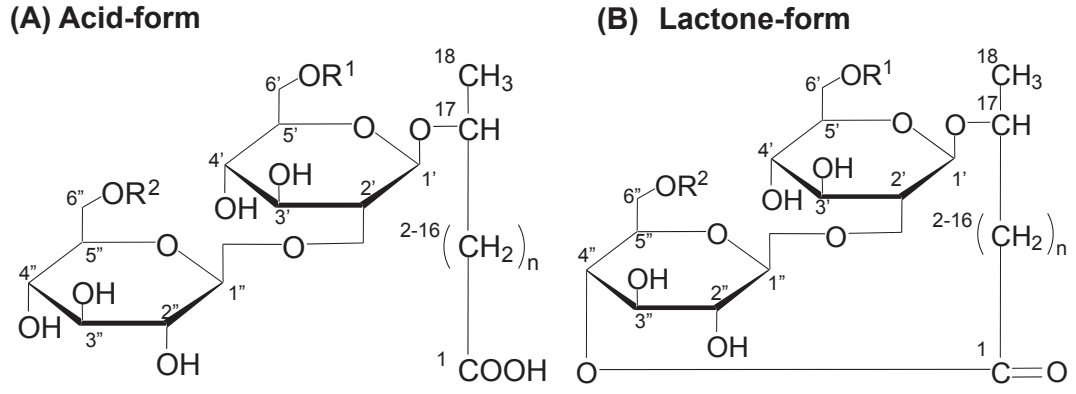

Fig. 3 Molecular structures of acid-form SLs (A) and lactone-form SLs (B). R^1 and R^2 = acetyl or H.

Table 1 Hydroxy fatty acid composition and estimated molecular weights of diacetylated SL acid-forms.

Hydroxy fatty acid	Composition (%)[*1]	MW[*2]	MW[*3]
C16:0	15.3	680.78	681
C16:1	8.5	678.76	779
C18:0	23.5	708.83	709
C18:1	52.6	706.82	707

[*1)] Composition was calculated from peak areas of GC-MS analysis.
[*2)] Molecular weight of diacetylated SLs acid-forms calculated from the estimated molecular structures by CS ChemDraw Ultra software (Hulinks Co., Tokyo).
[*3)] Molecular weight of diacetylated SLs acid-forms estimated from the MALDI-TOF-MS data.

Table 2 ^{1}H- and ^{13}C-NMR data of GL-A.

	^{13}C-NMR		^{1}H-NMR	
Functional group	δ (ppm)		δ (ppm)	*J* (Hz)
Saccarides				
C-1'	101.4	H-1'	4.45 *d*	7.6
C-1"	104.5	H-1"	4.55 *d*	8.0
C-2'	82.6	H-2'~5', 2"~5"	3.2-3.7 *m*	
C-2"	74.9			
C-3', 3"	76.3, 76.6			
C-4', 4"	70.2, 70.3			
C-5', C-5"	73.7, 74.4			
C-6', 6	63.6, 63.7	H-6', 6"	4.20 *dd*	6.4, 5.6
			4.36 *dd*	1.8, 10.0
Acetyl groups				
$CH_3 \times 2$	19.6, 19.8	CH_3	2.05, 2.06 *s*	
$C=O \times 2$	171.5			
Acyl moiety				
CH_3				
C-18	20.7	H-18	1.20 *d*	6.4
-CH_2-				
C-17	77.4	H-17	3.75 *m*	
C-3, 16	24.9	H-3, 16	1.55-1.64 *m*	
C-4-15	23.1, 25.0, 29.0-29.8	H-4-15	1.26-1.44 *br*	
-CH=CH-	129.6		5.35 *m*	
-CH_2CH=CH-	27.0		2.0-2.08 *m*	
-CH_2COOH				
C-2	33.8	H-2	2.27 *t*	8.6
COOH				
C-1	176.5			

s, singlet; *d*, doublet; *dd*, double doublet; *t*, triplet; *m*, multiplet; *br*, broad

The strains were cultivated on screening medium including 20% glycerol at 28℃ at 250 rpm for 1 week. SL production patterns were examined by TLC analysis (Fig. 6A). *C. floricola* ZM1502, NBRC 10700^{T}, and CBS 7290 selectively produced acid-form SLs, while *S. bombicola* NBRC 10243^{T} (a conventional SL producer) produced mainly lactone-form SLs, as reported previously[20]. The chemical structures of SL produced from glycerol were confirmed by NMR after purification (data not shown) Based on quantification of acid-form SLs, ZM1502 and CBS 7290 produced acid-form SL (containing >95% 6',6"-di-*O*-acetylated derivatives) with a yield of >3.5 g L^{-1} (Fig. 6B), compared to <0.9 g L^{-1} for the type strain NBRC 10700^{T}. These results indicated that *C. floricola* produces acid-form SL from glycerol.

4 DISCUSSION

We isolated an acid-form-SL producer, *C. floricola* ZM1502, using glycerol as the sole carbon source, and revealed the main components to be 6',6"-di-*O*-acetylated acid-form SL.

SLs are promising glycolipid-type BSs produced from glucose and/or hydrophobic materials—including vegetable oils, fatty acids, fatty acid methyl esters (FAMEs), and alkanes—by yeasts such as *S. bombicola*[27], *Candida apicola*[28, 29], and *Wickerhamiella domercqiae*[30]. *Candida batistae* CBS 8550 produces SLs, consisting mainly of the acid forms[31].

SLs comprise sophorose (2'-*O*-β-D-glucopyranosyl-β-D-glucopyranose) linked to a hydroxyl group at the penultimate position of, most frequently, a monounsaturated C18 fatty acid. SLs generally exist as a mixture of eight compo-

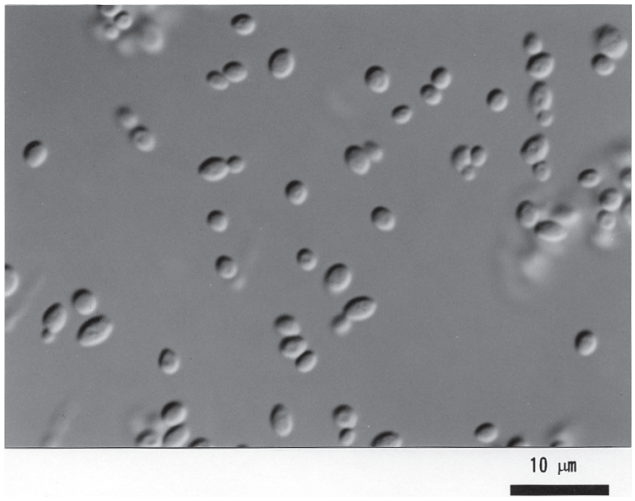

Fig. 4 Micrograph of strain ZM1502.

nents (lactone and acid forms) with varying degrees of acetylation at the 6'- and 6''- positions of the sophorose moiety (Fig. 3)[32, 33]. Hydrophobic lactone-form SLs accumulate in the growth medium, while hydrophilic acid-form SLs accumulate at markedly lower levels[20]. The large quantity of lactone-form SLs reduces the water solubility of the SL mixture and limits their applications[34].

In this study, a newly isolated *C. floricola* strain was found to produce acid-form, but not lactone-form, SLs from glycerol (Figs. 1 and 6). According to the structural analysis (Table 2), the major component was 6',6''-di-*O*-acetylated acid-form SL. The absence of the lactone forms in the metabolites of *C. floricola* is likely due to lack of a specific lactone esterase catalyzing internal esterification at the 4''-position of the sophorose and carboxyl groups of the hydroxy fatty acid[34]. *C. floricola* ZM1502 and CBS7290 produced larger amounts of SLs from glycerol compared to the type strain NBRC 10070^{T} (Fig. 6).

In conclusion, the present yeast species, *C. floricola*, would have a great potential for selective production of acid-form SLs from waste glycerol as a low-cost feed-stock, which will increase the use of glycolipid-BSs in various fields of industry.

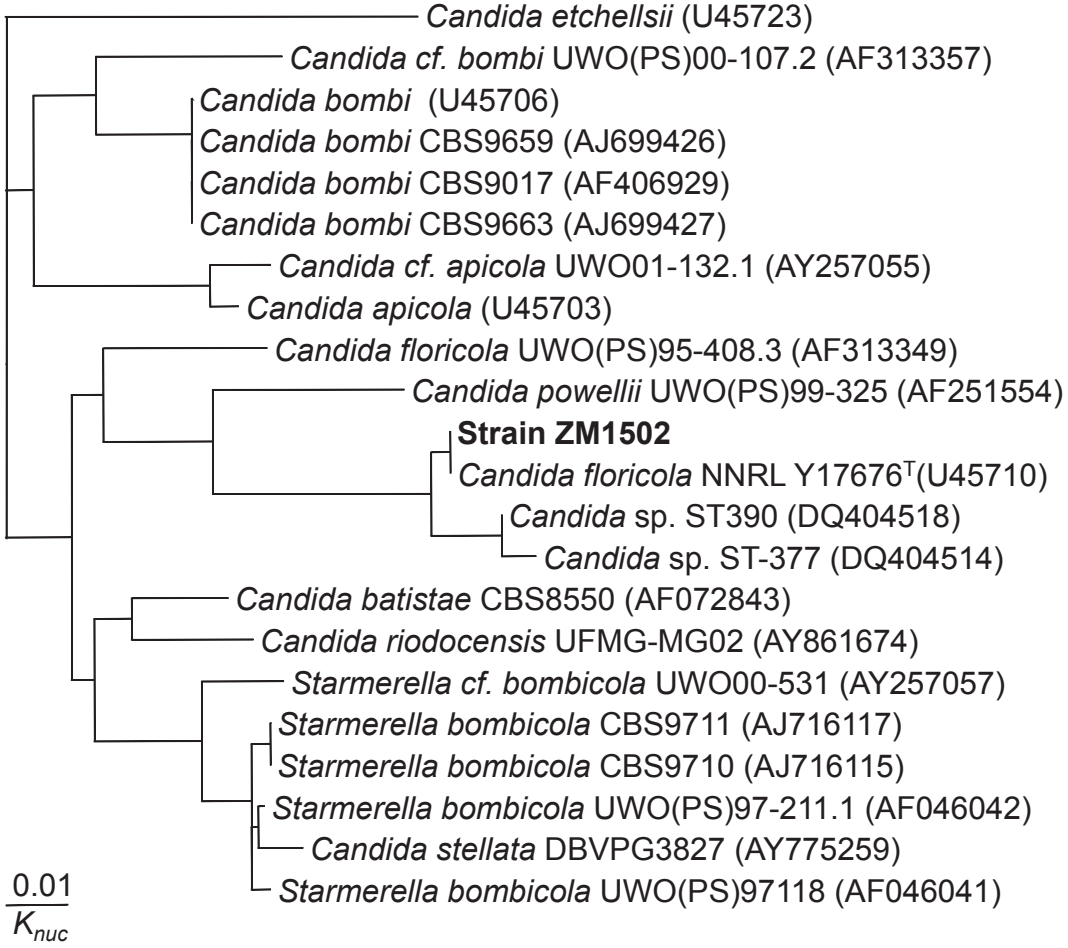

Fig. 5 Phylogenetic tree of ZM1502 and related strains based on the partial 26S ribosomal DNA sequences.

Table 3 Physiological and biochemical characteristics of *C. floricola* ZM1502.

Strain	ZM1502	NBRC10700[T 25)] (=CBS7289)
Fermentation:		
Glucose	+	+
Galactose	−	−
Sucrose	+	+
Assimilation of carbon sources (25℃):		
Glucose	+	+
Galactose	+	+
L-Sorbose	S	−
D-Gulucosamine	−	−
D-Ribose	L	L
D-Xylose	L	−/L
L-Rhamnose	−	−
Sucrose	+	+
Maltose	+	+
Trehalose	−	−
Cellobiose	−	−
Melibiose	−	−
Lactose	−	−
Raffinose	S	+
Melezitose	−	−
Soluble starch	−	−
Glycerol	+	+
Erythritol	−	−
Xylitol	S	−/L
Inositol	−	−
2-Keto-D-gluconate	−	−
DL-Lactate	−	−
Citrate	−	−
Ethanol	−	+/L
Assimilation of nitrogen sources (25℃):		
Nitrate	−	−
Nitrite	−	−
Ethylamine	+	+
Additional assimilation tests and other growth characteristics		
Growth at 37℃	L	−
Growth at 40℃	−	−
0.01% Cyclohexiamide	−	−
Vitamine free	−	−
Starch formation	−	−
Urease	−	−

Abbreviation: +, positive; L, latent growth; S, slowly positive; w, weakly positive; −, negative.

*) Kurtzuman and Fell, 1998

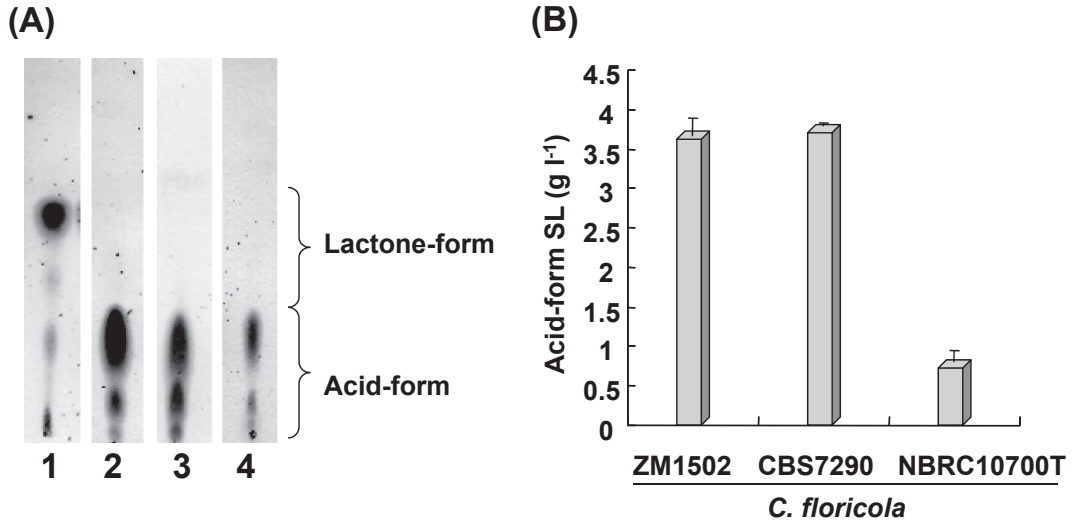

Fig. 6 SL production patterns (A). Lane 1, *Starmerella bombicola* NBRC10243^T; Lane 2, *Candida floricola* ZM1502; Lane 3, *C. floricola* CBS7290; Lane 4, *C. floricola* NBRC10700^T. TLC spots were visualized with anthrone-sulfuric acid regent. Production of acid-form SLs by the three *C. floricola* strains from 20% (w/v) glycerol at 28℃ with 250 rpm shaking for 7 d (B). Acid-form SL concentrations were determined by HPLC. Error bars indicate standard deviations.

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