Efficient production of tri-acetylated mono-acylated mannosylerythritol lipids by *Sporisorium* sp. aff. *sorghi* SAM20

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Abstract
Aims: The aim of this study was to isolate a novel yeast strain, evaluate biosurfactant production by the strain and characterize the major product.

Methods and Results: The strain SAM20, isolated from grass, identified as *Sporisorium* sp. aff. *sorghi* based on phylogenetic analyses. The strain produced approximately 32 g l⁻¹ glycolipid biosurfactants from 40 g l⁻¹ soybean oil after 7 days at 28°C. The glycolipids showed a unique pattern of mannosylerythritol lipids (MELs) on thin layer chromatography plate compared to those hitherto reported. Structural characterization of the major product, called GL-A, revealed that it was mainly tri-acetylated mono-acylated MELs (MEL-A2) with C₁₄:₀, C₁₆:₀, C₁₂:₀ or C₁₄:₁ as the hydrophobic chain. The critical micelle concentration (CMC), the surface tension at CMC and hydrophilic–lipophilic balance value for GL-A were estimated to be 20 mg l⁻¹, 30 mN m⁻¹ and 8.7, respectively.

Conclusions: A MEL-A2 with novel composition and surface activities was efficiently produced from a novel MEL producer. This is the first report on production of MEL-A2 as the major product and from soybean oil. The biosurfactant has potential application as a wetting agent and oil-in-water emulsifier.

Significance and Impact of the Study: Discovery of novel structures and novel strains is valuable for further commercial development and application of MELs. *Sporisorium* sp. aff. *sorghi* SAM20 can be considered as a potential candidate for commercial production of biosurfactants.

Introduction
Surfactants are amphiphilic molecules with a wide range of applications in almost every aspect of human daily life. They can reduce surface and interfacial tensions at the interface between different fluid phases; thereby show detergency, wetting, emulsifying, solubilizing, dispersing and foaming effects (Tadros 2005). Microbial biosurfactants comprise a group of diverse surface-active molecules synthesized by micro-organisms (Banat et al. 2010). They have attracted increasing interest as alternatives to chemical surfactants due to their advantageous properties, e.g. low toxicity, high biodegradability, effectiveness in extreme conditions, production from renewable sources and in mild conditions, widespread applicability and structural diversity (Santos et al. 2016). Among the biosurfactants, glycolipid biosurfactants are the most promising for commercial production because of high production yields from renewable resources and versatile interfacial and biochemical properties (Kitamoto et al. 2009; Ishaq et al. 2015).

Mannosylerythritol lipids (MELs) are one of the best studied glycolipid biosurfactants. These compounds are mainly secreted by fungi belonging to the order Ustilaginales (Morita et al. 2015). Regarding recently revised taxonomy of fungi in the order (Wang et al. 2015), the reported MEL-producing strains are species of
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Moesziomyces, Ustilago, Sporisorium, Dirkmeia, Kalmanozyma, Triodiomyces, Pseudozyma and Schizonella (Morita et al. 2015). Large amount of MELs (over 100 g l\(^{-1}\)) have been produced by Moesziomyces antarcticus, Moesziomyces aphidis, M. rugulosus and M. parantarcticus from vegetable oils by intermittent feeding of the substrates (Kitamoto et al. 2001; Rau et al. 2005a; Morita et al. 2006, 2008a). These glycolipids possess not only excellent surface activity, but also versatile biological and biochemical functions such as antimicrobial, antitumour, excellent surface activity, but also versatile biological and anti-inflammatory actions, differentiation-inducing biochemical functions such as antimicrobial, antitumour and anti-inflammatory actions, differentiation-inducing bioactivity towards different immunoglobulins (Kitamoto et al. 2009; Morita et al. 2015). Therefore, MELs have been increasingly attracting attention in various fields from environmental technology to advanced biomedical applications (Morita et al. 2015; Sanja et al. 2015).

MELs contain 4-O-\(\beta\)-d-mannopyranosyl-erythritol as the hydrophilic head and fatty acids as the hydrophobic tail. Different acylation and acetylation patterns create the structural variants of MELs. Also, there are two diastereomers of MELs (S-form and R-form) in respect to the erythritol moiety configuration (Morita et al. 2015). The conventional MELs produced by Moesziomyces species have two fatty acyl groups at the C-2’ and C-3’ positions of the mannose moiety. They are classified into four classes, MEL-A (di-acetylated), MEL-B and MEL-C (mono-acetylated at the C-4’ and C-6’ positions) and MEL-D (fully deacetylated), based on the order of appearance on the thin layer chromatography (TLC) plate (Arutchelvi et al. 2008).

During the two past decades, the researchers described several MEL-producing strains. The strains show different product compositions, production yield and substrate usage. In addition, various types of MELs have distinct interfacial and biological properties which expand their potential applications (Konishi et al. 2014; Morita et al. 2015). Therefore, investigation on novel MEL producers may advance the commercial development of these biosurfactants. In the present study, we report on the isolation and identification of a novel MEL-producing yeast strain and the characterization of its major product.

Materials and methods

Materials

Raw soybean oil was obtained from a local supplier and was analysed for some tests as shown in Table S1 following the American Oil Chemists’ Society official methods of analysis (Firestone 1994). All other chemicals used in this research were analytical reagent grade. Ultra-pure water for LC-MS analyses and surface tension measurements was obtained from a Direct-Q 3 water purification system (EMD Millipore Corporation, Bedford, MA, USA).

Micro-organism

The yeast strain SAM20 was isolated from a garden grass in Qom Province, Iran in April 2013. The strain was identified as a promising biosurfactant producer during a screening program on several yeast strains. To isolate the strain, the plant surface was washed with sterile distilled water, shredded and macerated in 20 ml saline solution for 30 min on a shaker. Aliquots of 100 ml from the suspension were spread on the surface of yeast extract peptone dextrose (YPD) agar (10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) dextrose; Seki et al. 1985) supplemented with 100 mg l\(^{-1}\) chloramphenicol. After incubation at 28°C for 7 days, yeast colonies were isolated and purified. The stock cultures on potato dextrose agar (PDA) were stored at 4°C and renewed every 4 weeks.

Molecular and phenotypic identification

Genomic DNA was extracted from the pure culture of the strain SAM20 grown in YPD broth for 24 h using the method described by Hanna and Xiao (2006). The sequences of the internal transcribed spacer (ITS) regions (including 5-8S rDNA) and the large subunit (LSU) rRNA gene D1/D2 domains were determined from PCR products amplified from the genomic DNA using the primers NL1 and NL4 (Kurtzman and Robnett 1998), and ITS1 and ITS4 (White et al. 1990), respectively. They were aligned with the sequences of related species retrieved from GenBank database using CLUSTAL W algorithm (Thompson et al. 1994). For phylogenetic analysis, the trimmed sequences of the ITS regions (including 5-8S rDNA) and LSU rDNA D1/D2 domains were concatenated and aligned. The phylogenetic tree was constructed from the combined ITS-D1/D2 dataset using the neighbour-joining algorithm (Saitou and Nei 1987) of MEGA software ver. 7.0.21 (Kumar et al. 2016). Confidence levels of the clades were estimated from bootstrap analysis based on 1000 replications (Felsenstein 1985). The strain SAM20 was characterized morphologically, biochemically and physiologically using standard methods currently employed in yeast taxonomy (Kurtzman et al. 2011). Assimilation tests and growth at various temperatures were conducted in liquid media.

Culture conditions for glycolipid production

Precultures were prepared by inoculating cells grown on PDA into test tubes containing 5 ml of YPD broth, followed by incubation at 28°C for 24 h. The cells were
harvested by centrifugation at 6000 g for 5 min and washed with saline solution. The washed cells were transferred to 100-ml Erlenmeyer flasks containing 20 ml of a basal medium (1 g l⁻¹ NH₄NO₃, 1 g l⁻¹ yeast extract, 0.5 g l⁻¹ KH₂PO₄ and 0.3 g l⁻¹ MgSO₄·7H₂O; pH 6) supplemented with 40 g l⁻¹ soybean oil as the main carbon source. The initial optical absorbance of the cultures was adjusted to 0.6 at the wavelength of 600 nm. The cultures were incubated on a rotary shaker at 28°C and 250 rpm for 7 days. Also, glycolipid production at the presence of 40 and 80 g l⁻¹ glucose as the carbon source was studied under the same conditions mentioned above.

Kinetics of growth and glycolipid production

Growth and glycolipid production by the strain SAM20 were monitored by harvesting samples at regular daily intervals up to 7 days. The cells were separated from the culture broth by centrifugation at 6000 g for 5 min and sequentially washed with ethyl acetate and distilled water to remove the oils and the medium components (Santos et al. 2013). Then, the cells were suspended in water and the absorbance was measured at 600 nm. Dry biomass concentration was determined using the correlation obtained from a calibration curve: dry biomass (g l⁻¹) = 0.19 × A600. The concentration of MELs in the culture broth was determined using the anthrone method as described by Kitamoto et al. (1998).

Isolation and purification of glycolipids

For product recovery, the whole culture (containing both cells and broth) was extracted with an equal volume of ethyl acetate (Kitamoto et al. 2001). After dehydration with MgSO₄ and filtration, the separated ethyl acetate extract was evaporated under vacuum and the remaining materials were washed with n-hexane : methanol (1 : 1, v/v) to remove the remaining oil and fatty acids (Fan et al. 2014). The extracted glycolipids were analysed by TLC using silica gel 60 plates (Merck, Darmstadt, Germany) and chloroform : acetone (from 7 : 3 to 4 : 6, v/v) as described previously by Kitamoto et al. (1990). Fractions of approximately 5 ml were collected and analysed by TLC using the conditions described above.

Structural analyses

Nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses were used to characterize the chemical structure of the purified glycolipid.

Nuclear magnetic resonance spectroscopy

Structure determination of the purified glycolipid (GL-A) was carried out by ¹H-NMR, ¹³C-NMR, distortionless enhancement by polarization transfer using a 135 degree decoupler pulse (DEPT 135) and two-dimensional NMR analyses including ¹H–¹H correlation spectroscopy (COSY), heteronuclear single quantum correlation and heteronuclear multiple bond correlation (HMBC). NMR spectra were recorded on a Bruker AVANCEIII 400 spectrometer (Bruker Corporation, Billerica, MA, USA), at 400 MHz (for ¹H-NMR and 2D NMRs) or 100 MHz (for ¹³C and DEPT 135) at 22–24°C using CDCl₃ as solvent. The spectra were analysed using ACD/NMR Processor software ver. 12.01 (Advanced Chemistry Development Inc., Toronto, Canada) and compared to the previously reported data.

Liquid chromatography-mass spectrometry

The GL-A molecules were separated by Agilent 1200 series LC system (Agilent Technology Inc., Palo Alto, CA, USA) using a C18 column (4.6 mm × 250 mm × 5 μm; Macherey-Nagel, Düren, Germany). The compounds were eluted at a flow rate of 0.4 ml min⁻¹ with a linear gradient of 25% 0.01 mol l⁻¹ ammonium acetate (A) and 75% acetonitrile (B) within 25 min followed by an increase of B up to 100% within 5 min. The effluent was connected to a Finnigan LCQD²CA ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) with electrospray ionization (ESI) set to the positive mode to scan from m/z range of 300–1300. The ESI–MS experiment was carried out using the following settings: sheath gas flow rate of 60 ml min⁻¹, aux gas flow rate of 20 ml min⁻¹, spray voltage of 4.5 kV, a capillary voltage of 20 V and a capillary temperature of 170°C. The injection volume was 20 μl.

Gas chromatography–mass spectrometry

Fatty acid methyl esters derivatives of the purified GL-A were prepared by HCl-catalysed methanolyis as described by Ichihara and Fukubayashi (2010). Two microlitres of the hexane phase containing the methyl esters was injected (split ratio 1 : 2) into a GC–MS system (Agilent 6890 N gas
chromatograph with a 5973 N mass spectrometer; Agilent Technology) equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The oven temperature was programmed from 100°C (held for 2 min) to 180°C at 15°C min⁻¹, and maintained for 1 min, and finally raised to 290°C at 5°C min⁻¹ (held for 10 min). A source temperature of 250°C and electron impact at 70 eV with a scan range of 32–800 Da were applied. Helium gas was used at a flow rate of 1 ml min⁻¹. The fatty acid methyl esters were identified based on their mass spectra and retention indices using NIST (National Institute of Standards and Technology) libraries. Temperature-programmed Kovats retention indices were determined with reference to homologous series of n-alkanes (C9–C24; Polyscience Corporation, Niles, IL, USA) under the same experimental conditions. Retention indices were calculated by applying the equation proposed by Van den Dool and Kratz (1963).

Determination of surface activities

Different concentrations (0–200 mg l⁻¹) of the purified GL-A in ultra-pure water were prepared and allowed to equilibrate overnight (Imura et al. 2013). The surface tension of the aqueous solutions was measured at 29°C using a calibrated Krüss K100MK2 tensiometer (Krüss GmbH, Hamburg, Germany) by applying the Wilhelmy plate method. Each measurement was repeated eight times to assure repeatability. The platinum plate and the glassware were cleaned by rinsing sequentially with acetone and ultra-pure water, followed by flaming. The critical micelle concentration (CMC) was determined by plotting surface tension as a function of surfactant concentration and considering the breakpoint as CMC (Tadros 2005). Surface excess (adsorption per unit area) of GL-A molecules was calculated using Gibbs adsorption equation and then occupation area of the molecules at the air–water interface was obtained from surface excess concentration (Attwood and Florence 2008).

Estimation of hydrophilic–lipophilic balance value

The hydrophilic–lipophilic balance (HLB) value of the GL-A molecules was calculated using Griffin’s formula for nonionic surfactants: HLB = 20 × Mw/M where Mw represents the molecular weight of the hydrophilic moiety and M represents the molecular weight of the entire molecule (Griffin 1954).

Results

Identification of yeast strain

The ITS region (including 5.8S rDNA) and the LSU rRNA gene D1/D2 domains of the strain SAM20 were amplified and sequenced (accession numbers: KM269191 and KM269189). Based on pairwise sequence alignments, the strain showed the highest sequence identity value to the authentic strains of Sporisorium sorghi, S. cruentum, S. foveolati and S. monilferum (99-7%) in D1/D2 domains and to the authentic strains of S. sorghi (99-4%; difference: 2 nt + 1 gap from 661 nt) and S. cruentum (99-4%; difference: 2 nt + 2 gaps from 729 nt) in the ITS region (Table S2). In the phylogenetic tree constructed from the combined ITS-D1/D2 dataset (Fig. 1), the strain SAM20 was located in the Sporisorium clade within Ustilaginales at the same position as the four mentioned species. These species are indistinguishable at molecular level based on ribosomal genes but can be separated from each other using soral morphology (Stoll et al. 2005). McTaggart et al. (2012) studied taxonomy of the Ustilaginaceae using morphological characters, host specificity and phylogenetic analysis of four nuclear loci and concluded that the Sporisorium clade must be described explicitly to prevent ambiguity for future taxonomic placement of new species. The strain SAM20 is an anamorphic fungus and no information about its host specificity or soral morphology can be obtained. Therefore, the strain SAM20 is temporarily designated as Sporisorium sp. aff. sorghi until the taxonomy of the interrelated species is revised.

Morphological, physiological and biochemical characteristics have been conventionally used for the description of the ustilaginomycetous yeasts (Kurtzman et al. 2011). Thus, these properties were determined for the strain SAM20. On 5% malt extract agar after 5 days at 17°C, the colonies of the strain SAM20 measured about 1 mm in diameter and were cream to pale yellow, opaque, butyrous, depressed in the centre to ring-like, and occasionally wrinkled or covered with fine hairs (Fig. S1). The cells of the strain SAM20 were narrowly ellipsoidal, cylindrical or fusiform, and variable in size, 1.2–4.3 × 6.5–40.2 μm. Conidiogenesis was multilateral on short denticles (Fig. S1). In Dalmat plate culture on corn meal agar, hyphae with septa were formed and blastoconidia were observed on the hyphae. The results of growth reactions on carbon and nitrogen sources and other phenotypic characteristics of the strain are presented in Table 1.

Glycolipid production and purification

Sporisorium sp. aff. sorghi SAM20 was cultivated in basal media containing different carbon sources at 28°C for 7 days. The amount of the glycolipids was determined using the anthrone method. The strain did not produce any detectable glycolipids using glucose as the carbon source but 32 ± 1.2 g l⁻¹ glycolipids was obtained from the medium containing 40 g l⁻¹ soybean oil (Fig. 2).
Interestingly, TLC analysis of the extracted glycolipids showed three anthrone-positive spots, GL-A (Rf = 0.58), GL-B (Rf = 0.46), and GL-C (Rf = 0.43), with the Rf values lower than those of conventional MELs produced by *M. antarcticus* SA006 (Fig. 3). The amount of GL-A seems to be greater than that of two other compounds.
Silica gel column chromatography was used for purification of glycolipid components produced by *Sporisorium* sp. aff. *sorghii* SAM20. TLC analysis of the collected fractions showed that the pure GL-A was eluted with chloroform : acetone 7 : 3 to 6 : 4 (v/v). Then, beside GL-A, GL-B was eluted with chloroform : acetone 6 : 4 to 4 : 6 (v/v) but GL-C was not eluted from the column. Although an increasing ratio of GL-B to GL-A was observed in the fractions, GL-B was not obtained as a pure compound. The fractions containing the pure GL-A, as major glycolipid produced by the strain SAM20, were combined and the solvent was evaporated. The purified GL-A was obtained as a yellow viscous liquid and used for further studies.

**Table 1** Biochemical and physiological properties of *Sporisorium* sp. aff. *sorghii* SAM20

| Characteristic          | Reaction |
|-------------------------|----------|
| Fermentation of D-glucose | –        |
| Assimilation of D-glucose, sucrose, raffinose, maltose, cellobiose, D-arabinose and D-ribose | +        |
| Assimilation of α-α-trehalose, soluble starch, L-sorbose, β-mannitol, succinate, D-gluconate and N-acetyl-D-glucosamine | s        |
| Assimilation of inulin, melezitose, salicin, glycerol, myo-inositol, nitrate, ethylamine, L-lysine and cadaverine | w        |
| Assimilation of melibiose, D-galactose, lactose, D-ribose, D-xylose, L-arabinose, erythritol, ribitol, D-lactate, citrate, D-gluconate and nitrate | –        |
| Growth in vitamin-free medium | +        |
| Growth in 10% NaCl+5% glucose, 50% glucose, 0-0.1% cycloheximide and 1% acetic acid | –        |
| Growth at 37°C | w        |
| Growth at 40°C | –        |
| Diazonium Blue B | +        |
| Urease | +        |
| Gelatinase | +        |
| Acid production from glucose | –        |
| Starch formation | –        |

+: positive, –: negative, w: weak, s: slow.

Silica gel column chromatography was used for purification of glycolipid components produced by *Sporisorium* sp. aff. *sorghii* SAM20. TLC analysis of the collected fractions showed that the pure GL-A was eluted with chloroform : acetone 7 : 3 to 6 : 4 (v/v). Then, beside GL-A, GL-B was eluted with chloroform : acetone 6 : 4 to 4 : 6 (v/v) but GL-C was not eluted from the column. Although an increasing ratio of GL-B to GL-A was observed in the fractions, GL-B was not obtained as a pure compound. The fractions containing the pure GL-A, as major glycolipid produced by the strain SAM20, were combined and the solvent was evaporated. The purified GL-A was obtained as a yellow viscous liquid and used for further studies.

**Figure 3** Thin layer chromatogram of glycolipids produced by *Sporisorium* sp. aff. *sorghii* SAM20 from soybean oil (b) as compared with conventional MELs produced by *Moesziomyces antarcticus* SA006 from soybean oil and glucose (a). TLC was carried out by the development of silica gel 60 plates with the solvent system of chloroform:methanol:7 mol l⁻¹ ammonium hydroxide (6:5:1:5:0:2, v/v/v). The spots were visualized using anthrone reagent.

**Figure 2** Time course of MEL production (●) and cell growth (○) of *Sporisorium* sp. aff. *sorghii* SAM20 in batch culture. The strain was cultivated in 100-ml Erlenmeyer flasks containing 20 ml of the production medium containing 40 g l⁻¹ soybean oil as the carbon source and incubated on a rotary shaker at 28°C and 250 rpm.

**Figure 3** Thin layer chromatogram of glycolipids produced by *Sporisorium* sp. aff. *sorghii* SAM20 from soybean oil (b) as compared with conventional MELs produced by *Moesziomyces antarcticus* SA006 from soybean oil and glucose (a). TLC was carried out by the development of silica gel 60 plates with the solvent system of chloroform:methanol:7 mol l⁻¹ ammonium hydroxide (6:5:1:5:0:2, v/v/v). The spots were visualized using anthrone reagent.

Structural characterization of the purified glycolipids

NMR spectra of GL-A were taken in CDCl₃ (Fig. S2–S9) and the chemical shifts are presented in Table 2. The spectra showed a similar peak pattern to that of the conventional MEL-A produced by *M. antarcticus* (Fig. S2; Fukuoka *et al.* 2008a) but had some significant variations in the hydrophobic moiety (Fig. S3). The HMBC spectrum showed that one sharp peak at 2.2 ppm in the ¹H-NMR spectrum correlated with a peak at 170.8 ppm in the ¹³C-NMR spectrum. These resonances were assigned as the acetyl group at the C-2₀ position of the

**Table 1** Biochemical and physiological properties of *Sporisorium* sp. aff. *sorghii* SAM20

| Characteristic                  | Reaction |
|--------------------------------|----------|
| Fermentation of D-glucose       | –        |
| Assimilation of D-glucose, sucrose, raffinose, maltose, cellobiose, D-arabinose and D-ribose | +        |
| Assimilation of α-α-trehalose, soluble starch, L-sorbose, β-mannitol, succinate, D-gluconate and N-acetyl-D-glucosamine | s        |
| Assimilation of inulin, melezitose, salicin, glycerol, myo-inositol, nitrate, ethylamine, L-lysine and cadaverine | w        |
| Assimilation of melibiose, D-galactose, lactose, D-ribose, D-xylose, L-arabinose, erythritol, ribitol, D-lactate, citrate, D-gluconate and nitrate | –        |
| Growth in vitamin-free medium   | +        |
| Growth in 10% NaCl+5% glucose, 50% glucose, 0-0.1% cycloheximide and 1% acetic acid | –        |
| Growth at 37°C                  | w        |
| Growth at 40°C                  | –        |
| Diazonium Blue B                | +        |
| Urease                          | +        |
| Gelatinase                      | +        |
| Acid production from glucose    | –        |
| Starch formation                | –        |

+: positive, –: negative, w: weak, s: slow.
Table 2  NMR data for GL-A produced by Sporisorium sp. aff. sorghi SAM20 (CDCl₃, 400 MHz)

| Functional group       | ¹H-NMR δ (ppm) | ¹³C-NMR δ (ppm) |
|------------------------|----------------|-----------------|
| o-Mannose              |                |                 |
| H-1'                   | 4.78 s         | C-1' 99.2       |
| H-2'                   | 5.52 bs        | C-2' 68.9       |
| H-3'                   | 5.09 dd        | C-3' 70.7       |
| H-4'                   | 5.27 t         | C-4' 65.9       |
| H-5'                   | 3.72 m*        | C-5' 72.5       |
| H-6'a                  | 4.19 dd        | C-6' 62.4       |
| H-6'b                  | 4.29 dd        |                 |
| meso-Erythritol         |                |                 |
| 3100                    |                |                 |
| 2490                    |                |                 |
| 2340                    |                |                 |
| 2100                    |                |                 |
| 1980                    |                |                 |
| 1810                    |                |                 |
| 1650                    |                |                 |
| 1300                    |                |                 |
| 1080                    |                |                 |
| 980                     |                |                 |
| s: singlet, d: doublet, dd: double doublet, t: triplet, sxt: sextet, m: multiplet, b: broad. *These peaks are not distinguishable in ¹H-NMR and the data are based on 2D NMR analyses.

Table 3  Detected masses for GL-A obtained from LC–ESI–MS analysis

| Molecular mass (Da) | [M+NH₄]⁺* | Retention time (min) | Possible fatty acids chain combinations (C-2'–C-3') |
|---------------------|-----------|---------------------|-------------------------------------------------|
| 592                 | 610       | 13.9                | 2/12:0, 4/10:0                                   |
| 614                 | 632       | 10.6                | 2/14:3, 4/12:3                                   |
| 616                 | 634       | 12.5                | 2/14:2, 4/12:2                                   |
| 618                 | 636       | 15.8                | 2/14:1, 4/12:1                                   |
| 620                 | 638       | 20.3                | 2/14:0, 4/12:0                                   |
| 644                 | 662       | 16.5                | 2/16:2, 4/14:2                                   |
| 646                 | 664       | 21.8                | 2/16:1, 4/14:1                                   |
| 648                 | 666       | 25.2, 30            | 2/16:0, 4/14:0                                   |
| 676                 | 694       | 36                  | 2/18:0, 4/16:0                                   |

mannose moiety. The conventional MEL-A has only two acetyl groups at C-4' and C-6' positions (Fukuoka et al. 2008a). In the ¹H-NMR spectrum, two weak resonances at 0.99 ppm (triplet) and 1.62 ppm (sextet) were assigned as the butanoyl group at the C-2' position (Fukuoka et al. 2007a). By integrating the multiple peaks at 1.21–1.41 ppm, average length of the fatty acyl group at the C-3' position was estimated to be 14 carbons. The resonances at 5.30–5.45 ppm indicated the existence of double bond in the fatty acyl group. Based on the NMR data, GL-A was identified as 4-O-β-D-mannopyranosyl-(2S,3R)-erythritol with an acetyl (C2) or butanoyl (C4) group at the C-2' position and a fatty acyl group (mainly C14) at the C-3' position. Therefore, the GL-A molecules mainly are tri-acetylated mono-acylated MEL or MEL-A2, the name proposed by Morita et al. (2011).

The results of LC–ESI–MS analysis of the purified GL-A are summarized in Table 3. The GL-A molecules were detected as adducts with ammonia, [M + NH₄]⁺, according to those reported by Onghena et al. (2011) and Goossens et al. (2016). The mass range of 592–676 Da and possible fatty acid compositions including C10–C18 were predicted for the GL-A molecules. The most abundant chromatographic peak corresponding to the mass of 620 Da (Fig. S10) was presumed to be due to the MEL-A2 molecule containing C14:0 at the C-3' position or the MEL-A molecule containing C4:0 and C12:0 at the C-2' and C-3' positions, respectively. This consists well with the structure proposed by the NMR analyses.

The fatty acid profile of the GL-A molecules was determined by GC–MS analysis. It was revealed that the GL-A molecules were mainly composed of C14:0, C16:0, C12:0 and C14:1 fatty acyl groups, in descending order by relative content (Table 4, Fig. S11). These data were in agreement with the results obtained from the NMR and LC–MS analyses. However, no structure with three double bonds was detected in the GC–MS experiment but they were predicted in the LC–MS results. Similar results have been reported by Fan et al. (2014) and Onghena et al. (2011) and it is likely due to low levels of these compounds to be detectable by GC–MS or changes in their structure during the experiment. Overall, the schematic chemical structures proposed for the GL-A molecules are shown in Fig. 4.

Surface activities
To evaluate the surface activities of GL-A, the surface tension of its aqueous solutions at different concentrations was measured using the Wilhelmy plate method. As shown in Fig. 5, the estimated CMC and surface tension at CMC (γCMC) of GL-A at 29°C were 20 mg l⁻¹ and 30.0 mN m⁻¹, respectively. Average molecular mass of the GL-A molecules was estimated using their relative content.
The HLB value of GL-A was calculated to be 8.7 by the Griffin’s formula, based on the average molecular mass of 610 Da. This was consistent with a milky solution of the surfactant in water.

**Discussion**

In this study, a novel promising biosurfactant-producing yeast strain was isolated and identified as *Sporisorium* sp. aff. *sorghii* SAM20. Biosurfactant production by *S. sorghi* and its closely related species has not yet been reported (Fig. 1). According to our knowledge, only two MEL-producing species, *S. graminicola* and *S. setiforme*, had been described in the *Sporisorium* clade (Morita et al. 2008b, 2009b). The strain produced approximately 32 g l⁻¹ glycolipids from 40 g l⁻¹ soybean oil after 7 days. It seems that the efficiency of glycolipid production in batch culture by the strain is comparable to that of the known efficient producers. A maximum MEL production of 75 g l⁻¹ has been reported by *M. aphidis* DSM 70725 using 80 g l⁻¹ soybean oil and 40 g l⁻¹ erythritol after 10 days in batch-mode flask culture (Rau et al. 2005b). The yield of biosurfactant production by the strain SAM20 has been remained to be further improved using optimization methodologies.

As mentioned above, raw soybean oil was efficiently used for glycolipid production by *Sporisorium* sp. aff. *sorghii* SAM20. Vegetable oils are reported as the best substrate for the maximum MEL production yield (Morita et al. 2009a). Using these cheap and renewable agro-industrial wastes is advantageous to decrease the production cost and subsequently to expand the industrial application of the biosurfactants. The strain was not able to produce the glycolipids from glucose and the glycolipids production was dependent on the presence of vegetable oil. This characteristic also has been observed in *M. aphidis* ATCC 32657 (Morita et al. 2007a). Günther et al. (2015) analysed the transcriptomic profile of the strain under MEL-inducing and noninducing conditions and concluded that induction of MEL pathway was concurrent with the adjustment of the strain to growth-limiting conditions in the presence of plant oil. Thus, inducible MEL production by strain SAM20 may create the opportunity to study the role of MELs in these smut fungi using plant oil-dependent expression analysis.

The glycolipids produced by the strain SAM20 showed a unique pattern of MELs on TLC plate compared to those hitherto reported. The three MEL components (GL-A, GL-B and GL-C) have lower RI values than those of the conventional MELs. Previous studies demonstrated that the more hydrophilic compounds such as mono-acylated MELs and R-form diastereomers of MELs appear at lower RI on TLC plate compared to the conventional di-acylated MELs (Fukuoka et al. 2007b, 2008a,b). Higher hydrophilic structure of GL-B and GL-C is a probable reason for unsuccessful purification of the components by the column chromatography. Onghena et al. (2011)
purified the conventional MEL-A and MEL-B using silica gel column chromatography under similar condition applied in this study but MEL-C and MEL-D were not eluted from the column. GL-B and GL-C are more hydrophilic than MEL-B (Fig. 3). So, most likely the polarity of the last solvent was not strong enough to elute GL-C from the column.

GL-A is the major component of the MELs produced by that *Sporisorium* sp. aff. *sorghii* SAM20. Structural characterization of the purified GL-A revealed that it was mainly tri-acetylated mono-acylated MELs (MEL-A2). As shown in Fig. 1, two other MEL producers in the *Sporisorium* clade i.e. *S. graminicola* and *S. scitamineum* synthesize the conventional MEL-C and MEL-B as the major product, respectively (Morita et al. 2008b, 2009b). These data suggest the diversity of MELs can be produced by *Sporisorium* species.

Regarding the Rf values of GL-B and GL-C compared to those obtained from the conventional MELs (Fig. 3), it seems that GL-B and GL-C are likely derivatives of MEL-C and MEL-D, with an acetyl group instead an acyl group at the C-2’ position, respectively. However, further studies are required to determine the chemical structure of these compounds.

Morita *et al.* (2011) reported that *Dirkmeia churashimaensis* OK96 produced a mixture of MEL-A and MEL-A2 using glucose as the carbon source but it produced only MEL-A when cultivated in a medium containing vegetable oils. In the same manner, *M. antarcticus* strains T-34 and JCM 10317\(^T\) and *M. parantarcticus* JCM 11752\(^T\) produced di-acetylated mono-acylated MEL-A only from glucose but not from hydrophobic substrates such as vegetable oils (Fukuoka *et al.* 2007b). In contrast, *Trichodinmyces crassus* CBS 9959\(^T\) was able to produce the R-form diastereomer of tri-acetylated mono-acylated MEL-A at a very low concentration from oleic acid and glucose (Fukuoka *et al.* 2008a). Thus, *Sporisorium* sp. aff. *sorghii* SAM20 is the first efficient producer of mono-acylated MELs from vegetable oil.

The hydrophobic moiety of the MEL-A2 molecules was mainly composed of C14:0, C16:0, C12:0 or C14:1 fatty acyl groups. The major fatty acyl groups of MEL-A2 molecules produced by *D. churashimaensis* OK96 were C16:0 (53%), C16:1 (17-5%) and C14:0 (14-5%) creating somewhat longer hydrophobic part compared to that of GL-A (Morita *et al.* 2011). This is indicated that *Sporisorium* sp. aff. *sorghii* SAM20 produces a MEL-A2 mixture different from those previously reported.

The presence of fatty acyl groups with 12–18 carbons at the C-3’ position and acetyl or butanoyl at the C-2’ position of GL-A is indicative of regioselective alkylation by two different acyltransferases. Two acyltransferases in the MEL biosynthesis pathway were reported previously in *Ustilago maydis*, *M. antarcticus* and *M. aphidis* (Hewald *et al.* 2006; Morita *et al.* 2014; Günther *et al.* 2015). Hewald *et al.* (2006) demonstrated that these enzymes transfer the acyl groups specifically to the hydroxyl groups of mannose at the C-2’ or the C-3’ positions.

Kitamoto *et al.* (1998) showed that most of the fatty acids in MELs produced by *M. antarcticus* were direct β-oxidation intermediates of the fatty acids supplied as the carbon source. The fatty acid composition of the soybean oil used in this study was mainly C18:2 and C18:1. Fatty acids with even number of carbons at the C-3’ position may be originated from the soybean oil via the mentioned pathway but the acetyl or butanoyl groups at the C-2’ position are likely synthesized via a different pathway. Also, fatty acids with odd number of carbons were detected in GL-A by the GC–MS analysis as previously reported for some other MELs (Morita *et al.* 2006, 2007b, 2008b). The fatty acids are generated by α-oxidation (Kitamoto *et al.* 1993). Clarifying the biosynthetic pathway involved in MEL production in different species can be useful for the development of diverse structures from these compounds.

The efficiency of a surfactant is determined by CMC and its effectiveness is defined by the minimum value to which it can lower the surface tension (Santos *et al.* 2016). The CMC and γCMC values for GL-A were estimated to be 20 mg l\(^{-1}\) and 30-0 mN m\(^{-1}\), respectively. Various MELs reported in the literature reduce the surface tension of water to 24-2–33-8 mN m\(^{-1}\) and their CMC is in the range of 1.7 × 10\(^{-6}\) mol l\(^{-1}\) to 3.6 × 10\(^{-4}\) mol l\(^{-1}\) (Morita *et al.* 2015). As expected, the CMC and γCMC values obtained for GL-A were intermediate values between the values reported for the conventional MEL-A (2.7 × 10\(^{-6}\) mol l\(^{-1}\) and 28.4 mN m\(^{-1}\); Kitamoto *et al.* 1993) and the mono-acylated MELs (3.6 × 10\(^{-4}\) mol l\(^{-1}\) and 33.8 mN m\(^{-1}\); Fukuoka *et al.*
2007b). Most of the GL-A molecules have only one fatty acyl group as hydrophobic part, while the conventional MEL-A has two fatty acid chains. In addition, GL-A was appeared in a lower Rf value on TLC plate compared to the conventional MEL-A. These data indicated that the water solubility as well as hydrophilicity of GL-A is higher than the conventional MEL-A but the effectiveness of the surfactants is comparable. Therefore, GL-A can be highly advantageous for some industrial applications because of its high water solubility.

Morita et al. (2011) reported the CMC of $1.7 \times 10^{-6} \text{ mol l}^{-1}$ and $\gamma\text{CMC}$ of 29.2 mN m$^{-1}$ at 25°C for MEL-A2 produced by $D.\ churashimaensis$ OK96. The MEL-A2 sample mainly contained molecules with C16 fatty acyl groups as the hydrophobic chain, while the average length of hydrophobic chain in the GL-A molecules was 14 carbons. Thus, the significant difference between the CMC values of the two MEL mixtures can be somewhat explained by Traube’s rule. Traube’s rule states that in dilute aqueous solutions of surfactants packing at the interface leads to the greater reduction in surface tension. Occupation area of the surfactant molecules was estimated to be 85 Å$^2$. This value was greater than those reported for the conventional MEL-A produced by $Moesziomyces antarcticus$ (60 Å$^2$; Kitamoto et al. 1993) and the R-form diastereomer of MEL-A produced by $T.\ crassus$ (43 Å$^2$; Fukuoka et al. 2008a) and verified the higher $\gamma\text{CMC}$ of GL-A compared to the above MEL-A samples.

The HLB value is an empirical scale developed for categorizing surfactants. It represents the geometric ratios of the hydrophilic and the hydrophobic parts of surfactants. The higher HLB value indicates the greater water solubility (Tadros 2005). The HLB value of GL-A was estimated to be 8.7 by the Griffin’s method. The HLB values of about 6, 8 and 12 have been reported for tri-acylated, di-acylated and mono-acylated MELs, respectively (Fukuoka et al. 2007b; Goossens et al. 2016). Surfactants with HLB of 7–9 and 8–18 are considered as wetting agents and oil-in-water emulsifiers, respectively (Tadros 2005). These properties of GL-A should be further characterized to evaluate its potential applications in various industries.

In conclusion, a MEL-A2 with novel composition and surface activities was reported here from a novel MEL producer. $Sporisorium$ sp. aff. $sorghi$ SAM20 can be considered as a potential candidate for commercial production of the biosurfactants. This is the first report on production of MEL-A2 as the major product and from soybean oil. These results expand the diversity of MELs and MEL producers and are valuable for further commercial development and application of them.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Colonies (a) and cells (b) of *Sporisorium* sp. aff. *sorghii* SAM20 grown on 5% malt extract agar for 5 days at 17°C.

**Figure S2** Partial 1H-NMR spectra of GL-A produced by *Sporisorium* sp. aff. *sorghii* SAM20 (a) and MEL-A produced by *Moesziomyces antarcticus* T-34 (b).

**Figure S3** 1H-NMR spectrum of GL-A produced by *Sporisorium* sp. aff. *sorghii* SAM20.

**Figures S4**–**S9** NMR spectra of GL-A produced by *Sporisorium* sp. aff. *sorghii* SAM20.

**Figure S10** LC–ESI–MS chromatogram of GL-A produced by *Sporisorium* sp. aff. *sorghii* SAM20.

**Figure S11** GC–MS total ion current of GL-A produced by *Sporisorium* sp. aff. *sorghii* SAM20.

**Table S1** Analysis of soybean oil.

**Table S2** Similarity between the sequences of the LSU rDNA D1/D2 domains and ITS region of *Sporisorium* sp. aff. *sorghii* SAM20 and the closely related species.