American Journal of Biochemistry and Biotechnology 9 (4): 430-437, 2013
ISSN: 1553-3468

doi:10.3844/ajbbsp.2013.430.437 Published Online 9 (4) 2013 (http://www.thescipub.com/ajbb.toc)

Identification of *Pseudozyma hubeiensis* Y10BS025 as a Potent Producer of Glycolipid Biosurfactant *Mannosylerythritol lipids*

[1]Martha Sari, [2]Atit Kanti, [3]I. Made Artika and [1]Wien Kusharyoto

[1]Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI), Jalan Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, Indonesia
[2]Research Center for Biology-Indonesian Institute of Sciences (LIPI), Jalan Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, Indonesia
[3]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia

Received 2013-10-26; Revised 2013-11-10; Accepted 2013-11-27

ABSTRACT

Mannosylerythritol Lipids (MEL's) are glycolipid biosurfactants that contain 4-O-β-D-mannopyranosyl-meso-erythritol as a hydrophilic moiety and fatty acids as a hydrophobic moiety. MEL's are abundantly produced by several kinds of microorganism and are one of the most promising biosurfactants currently known. The search for a novel endogenous producer of MEL's was undertaken based on the available collection of the yeast strains from the genus *Pseudozyma*. Using thin layer chromatography and based on morphological and molecular taxonomic analysis using the D1/D2 domains of the large subunit 26S rRNA gene, *Pseudozyma hubeiensis* Y10BS025 was found to be a potential producer of MEL's from soybean oil. The structure of the major glycolipid produced by the strain was analyzed by ^{1}H and ^{13}C nuclear magnetic resonance and was found to be similar to those of well known MEL-A, -B and -C respectively. Under improved shaking culture conditions, using yeast extract as nitrogen source and soybean oil as substrate, a maximum yield of 115±3.2 $g.L^{-1}$ of MEL's for 8 days of fermentation was achieved. The major fatty acids of MEL's produced by *P. hubeiensis* Y10BS025 were C-18 acids, considerably different from those of MEL-C produced by other *Pseudozyma* strains such as *P. antarctica* and *P. shanxiensis*. The main product, MEL-C produced by *P. hubeiensis* Y10BS025 exhibited surface-tension-lowering activity. The results demonstrated that the newly isolated *P. hubeiensis* Y10BS025 provided high efficiency in MEL's production and would thus be highly advantageous in commercial production of promising biosurfactants.

Keywords: Biosurfactants, *Mannosylerythritol Lipids* (MEL's), *Pseudozyma*, Soybean Oil

1. INTRODUCTION

Biosurfactants are extracellular amphipathic compounds produced by a variety of microorganisms and have numerous applications in the food and pharmaceutical industries, as well as in environmental protection and energy-saving technology (Morita *et al*., 2009a; Bhangale *et al*., 2013). The application of biosurfactants in cosmetics has also been addressed (Morita *et al*., 2010; Yamamoto *et al*., 2012; Morita *et al*., 2013). In addition, biosurfactants are considered to play important roles in differentiation induction with respect to human leukemia, rat pheochromocytoma and mouse melanoma cells, as well as high affinity binding toward different immunoglobulins and lectins (Morita *et al*., 2009b).

In recent years, biosufactants have attracted considerable interest due to their biodegradability, mild production conditions and a variety of functions

Corresponding Author: Martha Sari, Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI), Jalan Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, Indonesia Tel: 021-8754587 Fax: 021-8754588

(Liu *et al*., 2011). However, the relatively low efficiency in their production and recovery has limited wide industrial use. It is therefore considered important to search for novel biosurfactant producers and to improve the systems for large-scale production of biosurfactants. Based on the structure of their hydrophilic moiety, biosurfactants are categorized into four major groups; glycolipid type, amino acid type, carboxylic acid type and polymer type (Morita *et al*., 2009a). One of the most promising glycolipid biosurfactants are the *Mannosylerythritol Lipids* (MEL's). MEL's contain 4-O-β-D-mannopyranosyl-meso-erythritol as a hydrophilic moiety and fatty acids as a hydrophobic moiety (Onghena *et al*., 2011; Morita *et al*., 2011).

MEL's are abundantly produced by several kinds of microorganisms (Morita *et al*., 2009a). Previously, a number of yeast strains have been reported to secrete MEL's in a large amounts. These include *P. aphidis* (Onghena *et al*., 2011), *P. antarctica* (Bhangale *et al*., 2013), *P. parantarctica* (Morita *et al*., 2009b), *P. siamensis*, *P. graminicola*, *P. tsukubaensis*, *U. cynodontis* (Morita *et al*., 2009a), *U. scitaminea* (Morita *et al*., 2011), *U. maydis* (Liu *et al*., 2011) and *P. hubeiensis* SY62 (Konishi *et al*., 2013). The MEL's produced by *Pseudozyma* antarctica (ATCC 32657) are consisted of three major components, MEL-A, MEL-B and MEL-C. In addition, the structure can vary in the number of carbon atoms and unsaturation in fatty acid moiety (Bhangale *et al*., 2013). The aim of the present study, was to investigate for the first time, the production of MEL's using yeast strain *P. hubeiensis* Y10BS025 isolated in Indonesia. During the course of our study, *P. hubeiensis* Y10BS025, (a recently identified strain) was found to secrete a more hydrophilic glycolipids than the known MEL's. Here we describe the purification and structural analysis of MEL's produced with a varied ratio of glucose and glycerol as carbon sources. We also analyze the fatty acid composition and the surfactant activity of these glycolipids MEL's.

2. MATERIALS AND METHODS

2.1. Yeast Strain and Culture

An endophytic yeast (isolated from *sirih* leaf, *Piper betle* L.), *Pseudozyma* Y10BS025 was obtained from the *Biotechnology Culture Collections* (BTCC), Indonesian Institute of Sciences (LIPI). Cultivation of yeast seed culture was started by inoculating yeast cells grown on slants into a 100 mL flask containing 20 mL growth basal medium (4% w/v glucose, 0.3% w/v $NaNO_3$, 0.03% w/v $MgSO_4$, 0.03% w/v KH_2PO_4 and 0.1% w/v yeast extract) at 30°C on a rotary shaker (200 rpm) for 2 days. The yeast inoculums (10%, v/v) was added to the 200 mL production basal medium containing 8% v/v soybean oil and cultivation was performed by the submerged method with shaking for 8 days.

2.2. Molecular Phylogenetic Analysis

The genomic DNA of the strain of *Pseudozyma* Y10BS025 was prepared with a genomic DNA isolation kit (Go Beads, Japan) after cell rupture by vortexing for 3 min and heating it at 98°C for 10 min. The D1/D2 domain of the large ribosomal subunit (26S rRNA) gene was sequenced directly from PCR products generated using the primer NL-1 (5'-GCATATCAATAAGCGGAAAAG)-F and NL-4 (5'GGTCCGTGTTTCAAGACGG)-R.

The D1/D2 sequences of related taxa were retrieved from GenBank. The BLAST program was used for similarity search in the database available on the National Centre Biotechnology Information (NCBI). Multiple alignment was performed with the CLUSTAL W program. Phylogenetic analysis was performed using the neighbor-joining method with the program MEGA 5 (Tamura *et al*., 2011).

2.3. Isolation and Purification of Glycolipid MEL's

After cultivation, the post culture was harvested by centrifugation at 10000×g for 20 min at 10°C and the resulting supernatant, including glycolipid biosurfactant, was extracted using ethylacetate in 1:1(v:v) for 1 h (Morita *et al*., 2011). The organic layer (top phase) was separated from the water layer (bottom phase). Its volume was measured and concentrated using vacuum evaporation. The stepwise conventional extraction of the organic phase using different solvents was used to purify biosurfactant MEL's. A complete separation of residual soybean oil and fatty acids was achieved by using n-hexane, methanol and water (1:6:3, v/v) as solvent mixture with subsequent threefold extraction by n-hexane. The lyophilized aqueous phase resulted in a MEL's fraction.

The concentrated MEL's fraction were dissolved in chloroform and then purified by silica gel (Wako gel C-200) column chromatography using a gradient elution of chloroform/acetone (10:0 to 0:10, v/v) mixtures as solvent (Morita *et al*., 2011).

The pure MEL's were analyzed by thin layer chromatography on a silica gel plate G-60 (Merck). TLC was performed with chloroform-methanol-NH_4OH (65:15:2, v:v:v) as the solvent system.

Chromatograms were stained in the anthrone reagents (2%, w/v) in concentrated H_2SO_4 and by heating at 115°C for 5 min for visualization (blue spots). Purified MEL-A, MEL-B and MEL-C, prepared as reported previously, was used in the following experiments as the standards (Morita *et al*., 2011).

2.4. Optimation of MEL's Production

In order to determine optimum medium formulation for MEL's production, the yeast strain was grown on minimum basal medium with varied ratios of glucose and glycerol as the carbon source and with different vegetable oils as an inducer. The ratios of glucose: Glycerol tested were (100:0, 75:25, 50:50, 25:75 and 0:100, w/w). Two different vegetable oils were tested as an inducer, soy bean oil and olive oil. Glycolipid biosurfatants produced from each medium formulation were extracted and quantified using high performance liquid chromatography.

2.5. Quantification of MEL's by High Performance Liquid Chromatography (HPLC)

The glycolipid quantification was carried out by subjecting the extracts to normal phase HPLC analysis on a silica gel column (i.d., packed with Zorbax Rx-SIL, 4,6 mm ID×250 mm, 5µm, Shimadzu, Kyoto, Japan) using a gradient solvent program using isocratic elution consisting of mixed acetonitrile and 2-propanol (95:5, v/v). The eluent was monitored over 20 min period at 206 nm, followed by regeneration of column for the next analysis for 10 min at a flow rate of 1 mL min^{-1}. The quantification of MEL was carried out by HPLC based on a standard curve using the pure MEL fraction, which was prepared by *P. antarctica* (Morita *et al*., 2009b). All measurements reported here are calculated values from at least three independent experiments.

2.6. Structure Analysis of the Purified Glycolipids, MEL's

The structures of the purified glycolipids were identified by using spectroscopy analysis, ^{1}H & ^{13}C Nuclear Magnetic Resonance (NMR) using a Varian INOVA 500 MHz at 30°C using $CdCl_3$ solution (Morita *et al*., 2011). NMR spectra were recorded under conditions as indicated on a JEOL JNM ECA-500 spectrometer.

2.7. Analysis of MEL's Glycolipid Fatty Acids

The methyl esters derivatives from the fatty acids were prepared by mixing the purified glycolipids (10 mg) with 5% v/v HCl-methanol reagent (1 mL). After the reaction was quenched with water (1 mL), the methyl ester derivatives were extracted with n-hexane and separated using gas chromatography (Shimadzu GC 2010 Plus, Cyanopropil-methyl-sil, 230°C detector temperature, 200°C injector temperature, 190°C column temperature (held for 15 min) to 230°C at 10°C min^{-1}, carrier gas-helium. The methyl esters of the fatty acids were prepared with a modified method.

2.8. Surface Tension Measurement

The surface tension of the aqueous solution at different surfactant concentrations was measured by the Du Nouy method using an interfacial tensionmeter (Dziegielewska and Adamzak, 2013). The surface measurement was carried out at 25±1°C at the end of incubation. Each measurement was repeated 3×to give an average value.

3. RESULTS

3.1. Selection of *Pseudozyma* Y10BS025 as a MEL's Producer and Molecular Phylogenetic Analysis

Two yeast strains belonging to genus *Pseudozyma*, Y10BS016 and Y10BS025, were tested for ability to secrete MEL's. Results showed that only *Pseudozyma* Y10BS025 produces MEL's in significant amounts. This strain was selected as a MEL producer and was subjected to further analysis. Molecular phylogenetic analysis based on the gene encoding the D1/D2 domain of the 26S rRNA (**Fig. 1**), placed the *Pseudozyma* Y10BS025 close to *Pseudozyma hubeiensis*. Nucleotide-sequence-alignment showed that the *Pseudozyma* Y10BS025 has 98-100% identity with other *Pseudozyma* strains and 100% identity with *Pseudozyma hubeiensis*, thus confirming its identity.

3.2. Separation of Glycolipids Produced by *P. hubeiensis* Y10BS025

On TLC, the *P. hubeiensis* Y10BS025 extract gave spots of glycolipids corresponding to MEL. The anthrone-reagent-positive spots having the same Rf values as those of the purified MEL standard (**Fig. 2**). The glycolipids produced by *P. hubeiensis* Y10BS025 showed nearly the same spots as the purified MEL standard corresponded to MEL-C and yielded higher amounts of glycolipids than the standard.

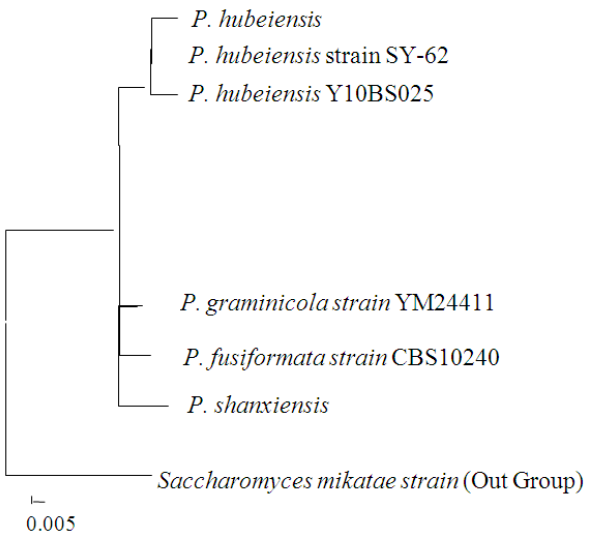

Fig. 1. Phylogenetic tree of the genus *Pseudozyma*. molecular phylogenetic tree constructed using molecular taxonomic D1/D2 domains of the large subunit 26S rRNA gene

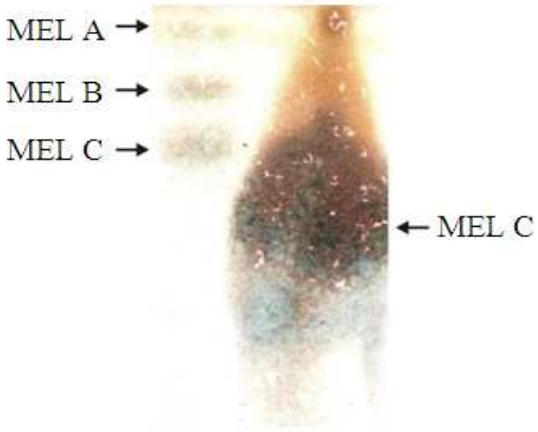

Fig. 2. TLC pattern of glycolipids produced by *P. hubeiensis*. The purified MEL's standard prepared from soybean oil by *P. antarctica* was used as a reference

3.3. Optimum Medium Formulation for MEL's Production by *P. hubeiensis* Y10BS025

The effects of the carbon source on MEL's production by *P. hubeiensis* Y10BS025 were studied using varied ratios of glucose and glycerol in the minimal basal medium and the effects of the composition of vegetable oil used as an inducer were studied by supplementing either soy bean oil or olive oil. Results showed that glycolipid biosurfactants were produced by *P. hubeiensis* Y10BS025 on all medium formulations tested. The optimum medium for MEL's production was the minimal basal medium with glucose: Glycerol ratio of 75:25, w/w using soybean oil as an inducer. The yield achieved was 115±3.2 $g.L^{-1}$ of MEL's for 8 days of fermentation (**Fig. 3**). In all medium formulations tested, medium supplemented with soybean oil gave a better yield compared to that supplemented with olive oil.

3.4. Structure Analysis of Purified MELs Produced by *P. hubeiensis* Y10BS025

To identify the structure of MEL's produced by *P. hubeiensis* Y10BS025, the purified major MEL's was further studied by ^{1}H and ^{13}C NMR spectroscopy (**Table 1**). All the spectra obtained from the major glycolipid corresponded well with those of the previously reported MEL-C produced by *P. antarctica* (**Fig. 4**): The chemical shift of the doublet at~δ 4.88 (mannose H-1′), the double doublet at~δ 5.03 (mannose H-3′), the triplet at~δ 5.13 (mannose H-4′), the double doublet at~δ 5.51 (mannose H-2′), the multiplet around~δ 3.56-3.61 (mannose H-5′), the multiplet around~δ 3.56-3.71(mannose H-6′) and multiplet from~δ 4.03 to 3.56 (erythritol H-1~H4). The same result was also obtained by ^{13}C NMR (data not shown).

3.5. Fatty-Acid Composition of MELs Produced by *P. hubeiensis* Y10BS025

The fatty-acid composition of the present MEL-C from soybean oil was also analyzed by the GC-MS method and compared with that of MEL-C produced by the other strains (**Table 2**). The fatty-acid composition of the MEL-C produced by *P. hubeiensis* Y10BS025 was different from that by *P. antarctica*, which mainly possess two medium chain acids (C_8 to C_{10}), while those of MEL-C produced by *P. shanxiensis* were C_{16} acids. The major fatty acids of *P. hubeiensis* Y10BS025 consisted of C_{10}, C_{14} and C_{18}, with the hydrophobic structure of the present MEL-C being considerably different from those of MEL-C produced by other *Pseudozyma* strains. The present MEL-C produced by *P. hubeiensis* Y10BS025 from soy-bean oil possessed a short chain (C_2 or C_4) at the C-2' position and a long chain (C_8 to C_{18}) at the C-3' position of the mannose moiety. *P. hubeiensis* Y10BS025 was found to be a novel MEL's producer and was able to produce MEL-C with a different fatty acid composition.

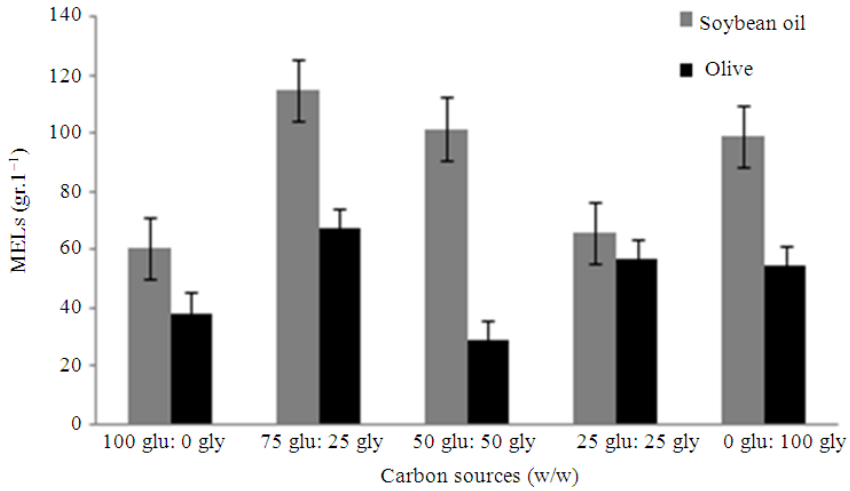

Fig. 3. MEL's formation by *P. hubeiensis* Y10BS025 at 30°C for 8 days with variation of carbon sources used. MEL's was quantified by HPLC with three independent experiments. Vertical bars show the standard error of the mean based on three independent measurements

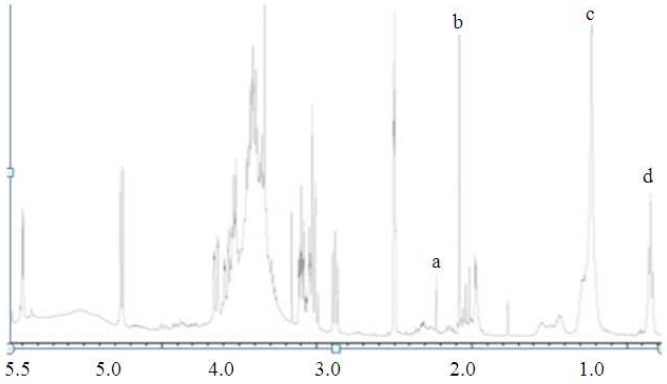

Fig. 4. ^{1}H-NMR ($CdCl_3$, 500 MHz) spectra of MEL-C produced by *P. hubeiensis*. Peak a, acetyl group ($-CH_3$) at C-2' position of mannose moiety; peak b, acetyl group ($-CH_3$) at C-4' position; peak c, butanoyl group ($-CH_3$) at C-2' position; peak d, fatty acid ($-CH_3$) at C-3' position

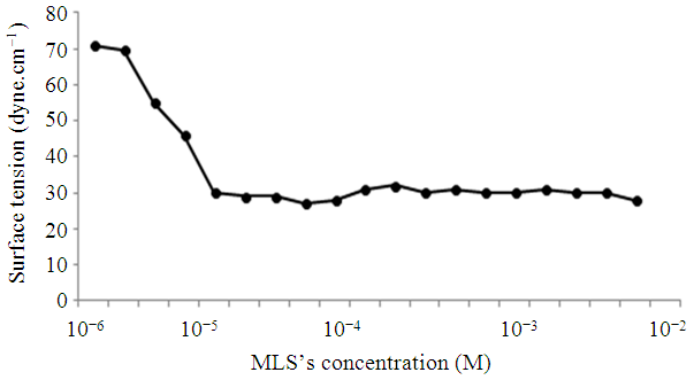

Fig. 5. Surface tension-concentration plots of purified MEL-C using tensionmeter at 30°C

Table 1. NMR data for MEL-C by *P. hubeiensis*

Functional groups (ppm)	C-NMR δ (ppm)		H-NMR δ
D-Mannose			
C-1’	96.96	H-1’	4.88 d
C-2’	70.3	H-2’	5.51 dd
C-3’	73.1	H-3’	5.03 dd
C-4’	63.4	H-4’	5.13 t
C-5’	76.7	H-5’	3.56-3.61 m
C-6’	61.2	H-6’	3.56-3.71 m
Meso-erythritol			
C-1	63	H-1	3.56-3.70 m
C-2	70.6	H-2	3.60-3.70 m
C-3	72	H-3	3.56-3.61 m
C-4	70.6	H-4a	3.70 dd
		H-4b	4. 03 dd
Acetyl groups	19.6	$-CH_3$ (C-2’)	2.20 s
	19.6	(C-4’)	2.05 s
	169.6		
	170.2	-C = O (C-2’) (C-4’)	
Acyl groups	33.8	$-CO-CH_2-$ (C-2’)	2.40 m
	33.8	(C-3’)	2.23 m
	22.3-31.7	$-(CH_2)n-$	1.23-1.38 b

s: singlet; d: doublet; dd: double doublet; t: triplet; m: multiplet; b: broad

Table 2. The fatty acid profile of MEL-C produced by *P. hubeiensis* Y10BS025, *P. shanxiensis* CBS 10075 and *P. antarctica* T-34

	Fatty acid composition (%)		
Fatty acid	*P. hubeiensis* Y10BS025	*P. shanxiensis* CBS 10075[a]	*P. antarctica* T-34[a]
C8:0	3.70	-	22.8
C8:1	-	-	-
C9:0	-	-	-
C10:0	22.60	-	26.2
C10:1	-	-	28.4
C10:2	-	-	2.9
C12:0	1.07	-	5.2
C12:1	-	-	5.1
C14:0	-	3.7	-
C14:1	-	17.6	-
C14:2	20.21	-	4.7
C14:3	4.70	-	1.1
C16:0	3.01	41.8	-
C16:1	2.71	21.7	-
C16:2	-	10.3	-
C18:2	36.49	4.8	-
unknown	5.50	0.1	3.6

[a]All data are from Morita *et al*. (2008) for comparison

3.6. Surface Active Properties of Purified MEL-C

The surface tension of MEL-C was determined by the Du Nouy method. **Fig. 5** shows the surface (air-water) tension Vs concentration plot of MEL-C in distilled water. This shows MEL reduces the surface tension of water to 30.80 dyne.cm^{-1}

4. DISCUSSION

Here, we show for the first time that *P. hubeiensis* Y10BS025 is a novel MEL-C producer. Indeed, the genus *Pseudozyma* has been well known to be MEL’s producer (Morita *et al*., 2009a) and more specifically, a number of *P. hubeiensis* strains have also been reported

to produce glycolipid MEL's (Konishi *et al*., 2013). These facts indicate that the genes involved in MEL's biosynthesis should be conserved among these strains. The molecular phylogenetic tree based on the sequence of the D1/D2 domain of the 26S rRNA demonstrated that *P. hubeiensis* Y10BS025 is closely related to *P. prolifica*, *Ustilago maydis* and *Ustilago vetiveriae*. It was therefore necessary to test the ability of these microorganisms to produce glycolipids MEL's. A number of strains of *Pseudozyma* and *Ustilago* isolated from leaves and smuts of sugarcane plants have recently been identified as MEL's producers. The strains showed the ability to form abundant of MEL's using sugarcane juice as a sole nutrient source (Morita *et al*., 2012).

In this study, an optimum medium for MEL's production by *P. hubeiensis* Y10BS025 has been developed. This was achieved by optimizing the ratio of glucose and glycerol as carbon sources and evaluating effects of the type of vegetable oil used as an inducer. A wide range of carbon sources such as glucose, pentose, hexose, glycerol, triglycerides, fatty acids and ethanol, has been reported to support cell growth and MEL's production. Glucose (Morita *et al*., 2009b) and glycerol (Bhangale *et al*., 2013) has been used for efficient production of MEL's. To overcome the expensive cost constraints associated with MEL's production, the use of inexpensive and waste substrates for the formulation of fermentation media has been suggested (Saharan *et al*., 2011). Accorsini *et al*. (2012) used soybean oil and glycerol as low cost substrates for biosurfactant production by yeast. The use of olive oil for MEL's production has also been reported (Morita *et al*., 2009c). Similarly, Dziegielewska and Adamzak (2013) found that addition of 5% (v/v) of rapeseed oil increases the synthesis of MEL's from 2.20 to 12.69 $g.L^{-1}$. They also found that addition of rapeseed oil to the cultivation medium decreases the surface tension and increases both the Diameter of the Medium (DMD) and the biomass concentration.

Although from the point of view of MEL's production yield, vegetable oil has been reported as the best substrate (Morita *et al*., 2009b), in the present study glucose and glycerol were used as the main carbon source for MEL's production. This was intended to facilitate purification of MEL's and circumvent the need for removal of the residual oil and resulting lipase degradation products. It has been reported that when soybean oil is employed as a substrate for MEL's production, additional complicated process is required to remove the residual oil and lipase degradation products such as monoacylglycerols, diacylated glycerols and non-esterified fatty acids (Morita *et al*., 2012). To improve the efficiency of MEL production, therefore, the use of watersoluble carbon sources, such as glucose and glycerol, instead of vegetable oils is highly desirable (Morita *et al*., 2009c). It has been reported that the purity of glucose and glycerol-derived MEL's is higher than that of soybean oil-derived MEL's. In the extract from the culture with glucose and glycerol, by-products such as the residual oil and its degradation products were not detected on TLC and HPLC analysis (Morita *et al*., 2009b). From an environmental perspective, the successful conversion of carbon glycerol to glycolipid biosurfactants by *P. hubeiensis* Y10BS025 in the present study has the potential to facilitate the utilization of waste glycerol.

As presented in **Table 2**, the fatty acid composition of MELs produced by *P. hubeiensis* Y10BS025 is different from those produced by *P. antarctica and P. shanxiensis*. This suggests that there may be different kinds of fatty acid biosynthetic pathways among MEL's producers. Differences in the steps for acetylation and fatty acid synthesis may lead to different fatty acid composition of MEL's (Morita *et al*., 2009b). Differences in MEL's fatty acid composition were also reported by Morita *et al*. (2009c). They found that the major fatty acids of the MEL-B produced from olive oil by *U. scitaminea* NBRC 32730 were C_8 and C_{10} acids. On the other hand, the main fatty acids of MEL-B produced from olive oil by *P. tsukubaensis* were C_8 and C_{14}. Similarly, strains of *Pseudozyma* producing mainly MEL-C showed different lengths of fatty acid in comparison with one another: *P. hubeiensis* was C_6, C_{10}, C_{12} and C_{16}, *P. graminicola* was C_6, C_8, C_{12} and C_{14} and *P. shanxiensis* and *P. siamensis* were C_2, C_4, C_{14} and C_{16} (Morita *et al*., 2009a). Differences in fatty acid composition shown by the present study suggest that these are novel types of MEL's. Further characterization on these glycolipids biosurfactants, therefore, may provide us with a better undestanding of the structure-function relationships of biosurfactants.

The observed structural analyses demonstrated that the glycolipids produced from soybean oil by *P. hubeiensis* Y10BS025 are MEL's. However these have a different chirality on the carbohydrate moiety compared to those from *P. siamensis* CBS 9960 which corresponded well to the previously reported MEL-C, in which there is a different peak structure at 5.0 -5.5 ppm. The present MEL-C possesses a hydrophobic structure different from those of conventional MEL's. Accordingly, our results show that *P. hubeiensis* Y10BS025 has considerable potential as a MEL's producer and exhibits excellent surface activity. This study expands our knowledge on the variety of glycolipid biosurfactants and provides useful information on their structure relationships.

5. CONCLUSION

We conclude that the *P. hubeiensis* Y10BS025 is a novel MEL's producer that produces MEL-C as a majority product with mainly C-18 acids. The MEL-C produced by *P. hubeiensis* Y10BS025 exhibits surface-tension-lowering activity. Hence the newly isolated *P. hubeiensis* Y10BS025 provides high efficiency in MEL's production and would thus be highly advantageous in commercial production of promising biosurfactants.

6. ACKNOWLEDGEMENT

We would like to thank Dr. Zainal A. Mas'ud of Bogor Agricultural University, Indonesia for instruction on HPLC analysis. We also thank Mr. A. Darmawan from Research Centre for Chemistry-LIPI, Serpong, Jakarta for NMR technical analysis.

7. REFERENCES

Accorsini, F.R., M.J.R. Mutton, E.G.M. Lemos and M. Benincasa, 2012. Biosurfactants production by yeasts using soybean oil and glycerol as low cost substrate. Brazil. J. Microbiol. DOI: 10.1590/S1517-83822012000100013

Bhangale, A., S. Wadekar, S. Kale and A. Pratap, 2013. Optimization and Monitoring of Water Soluble Substrate for Synthesis of *Mannosylerythritol Lipids* by *Pseudozyma* antarctica (ATCC 32657). Biotechnol. Bioprocess Eng., 18: 679-685. DOI: 10.1007/s12257-012-0647-4

Dziegielewska, E. and M. Adamzak, 2013. Free fatty acids and a high initial amount of biomass in the medium increase the synthesis of *Mannosylerythritol lipids* by *Pseudozyma* antarctica. Environ. Biotechnol., 9: 14-18.

Konishi, M., Y. Hatada and J.I. Horiuchia, 2013. Draft genome sequence of the basidiomycetous yeast-like fungus *Pseudozyma hubeiensis* SY62, which produces an abundant amount of the biosurfactant *Mannosylerythritol Lipids*. Genome Announc. DOI: 10.1128/genomeA.00409-13

Liu, H.H., S. Schmidt, H. F. Poulsen, A. Godfrey and Z.Q. Liu *et al*., 2011. Three-dimensional orientation mapping in the transmission electron microscope. Science, 332: 833-834. DOI: 10.1126/science.1202202

Morita, T., M. Konishi, T. Fukuoka, T. Imura and D. Kitamoto, 2008. Production of glycolipid biosurfactants, *Mannosylerythritol lipids*, by *Pseudozyma* siamensis CBS 9960 and their interfacial properties. J. Biosci. Bioeng., 105: 493-502. PMID: 18558340

Morita, T., T. Fukuoka, T. Imura and D. Kitamoto, 2009a. Production of glycolipid biosurfactants by basidiomycetous yeasts. Biotechnol. Applied Biochem., 53: 39-49. PMID: 19341364

Morita, T., T. Fukuoka, M. Konishi, T. Imura and S. Yamamoto *et al*., 2009b. Production of a novel glycolipid biosurfactant, mannosylmannitol lipid, by *Pseudozyma* parantarctica and its interfacial properties. Applied Microbiol. Biotechnol., 83: 1017-1025. PMID: 19296097

Morita, T., Y. Ishibashi, T. Fukuoka, T. Imura and H. Sakai *et al*., 2009c. Production of glycolipid biosurfactants, *Mannosylerythritol lipids*, by a smut fungus, Ustilago scitaminea NBRC 32730. Biosci. Biotechnol. Biochem., 73: 788-792. PMID: 19270362

Morita, T., M. Kitagawa, S. Yamamoto, A. Sogabe and T. Imura *et al*., 2010. Glycolipid biosurfactants, *Mannosylerythritol lipids*, Repair the damaged hair. J. Oleo Sci., 59: 267-272. PMID: 20431244

Morita, T., T. Fukuoka, T. Imura, N. Hirose and D. Kitamoto, 2012. Isolation and screening of glycolipid biosurfactant producers from sugarcane. Biosci. Biotechnol. Biochem., 76: 1788-1791. PMID: 22972331

Morita, T., T. Fukuoka, T. Imura and D. Kitamoto, 2013. Production of *Mannosylerythritol lipids* and their application in cosmetics. Applied Microbiol. Biotechnol., 97: 4691-4700. PMID: 23584242

Onghena, M., T. Geens, E. Goossens, M. Wijnants and Y. Pico *et al*., 2011. Analytical characterization of *Mannosylerythritol lipid* biosurfactants produced by biosynthesis based on feedstock sources from the agrofood industry. Anal. Bioanal. Chem., 400: 1263-1275. PMID: 21318245

Saharan, B.S., R.K. Sahu and D. Sharma, 2011. A review on biosurfactants: Fermentation, current developments and perspectives. Gen. Eng. Biotechnol. J., 29: 1-14.

Yamamoto, S., T. Morita, T. Fukuoka, T. Imura and S. Yanagidani *et al*., 2012. The moisturizing effects of glycolipid biosurfactants, *Mannosylerythritol lipids*, on human skin. J. Oleo Sci., 61: 407-412. PMID: 22790172

Tamura, K., D. Peterson, N. Peterson, G. Stecher and M. Nei *et al*., 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739. PMID: 21546353