ENVIRONMENTAL BIOTECHNOLOGY 9 (1) 2013, 14-18

Free fatty acids and a high initial amount of biomass in the medium increase the synthesis of mannosylerythritol lipids by *Pseudozyma antarctica*

Ewelina Dzięgielewska, Marek Adamczak

Department of Food Biotechnology, University of Warmia and Mazury in Olsztyn, Heweliusza 1, 10-718 Olsztyn, Poland
Corresponding author: E-mail: marek.adamczak@uwm.edu.pl; Phone and Fax: +48 89 5233838

Key words: biosurfactants, lipases, mannosylerythritol lipids, *Pseudozyma antarctica*

Received in December 2012. Published in September 2013.

ABSTRACT

It was shown that the addition of lipase to a YPG cultivation medium containing 5% (v/v) of rapeseed oil increased the yield of mannosylerythritol lipids synthesized by *P. antarctica* from 12.69 to 26.97g·L^{-1} compared to the control culture. As a renewable resource of free fatty acids for *P. antarctica* cultivation, post-refining fatty acids (Post-FFA) were used and after 192h a 85.43g·L^{-1} of biosurfactant was obtained. The experiments indicated that the biosurfactant synthesis could depend on carbon source availability and biomass concentration in the cultivation medium. The supplementation of the medium after 8 days of cultivation (fed-batch culture), with glucose to 2% (w/v) and Post-FFA to 20% (v/v), did not increase the concentration of the biosurfactant in the medium but did increase the total amount of the product from about 170 to 232g. By increasing the initial concentration of the biomass to 500g·L^{-1}, the concentration of biosurfactant was increased to 120.50g·L^{-1} after 168h.

INTRODUCTION

Biosurfactants are amphiphilic molecules which have both hydrophilic and hydrophobic parts. They are an important class of chemical products because of their great variety of applications, including the cosmetic, detergent, bioremediation, food and medical industries. Moreover, they can be used as emulsifiers, de-emulsifiers, wetting and foaming agents. These molecules are characterized by a low toxicity and biodegradability. Some of them exhibit antibacterial, antifungal and antiviral activities . According to their chemical structure and biological functions glycolipids are classified as sophorolipids, mannosylerythritol lipids, rhamnolipids or trehalose lipids.

One of the most promising groups of biosurfactants are mannosylerythritol lipids (MEL) because of their unique properties, possible pharmaceutical applications and relatively high productivity. MEL are extracellular biosurfactants mostly synthesized by yeast of the *Pseudozyma* genus, e.g. *P. antarctica*, *P. aphidis, P. parantarctica, P. tsukubaensis, P. rugulosa* or *Ustilago maydis*. MEL (MEL-A, MEL-B, MEL-C and MEL-D) contains a hydrophilic part of 4-O-β-D-mannopyranosyl-D-erythritol and a hydrophobic part of two fatty acid chains. Each homologue possesses no (MEL-D), one (MEL-B and MEL-C) or two acetyl groups (MEL-A) located on C-4' and/or on the C-6' mannose moiety. Hydrophobic parts of MEL consist of C2:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:0 or C18:1 fatty acids. Microorganisms synthesize mixtures of these four MEL but most of them are MEL-A and MEL-B. The fatty acid composition of glycolipids synthesized by *P. antarctica* depends on the composition of fatty acids present in a medium.

MEL reduce the surface tension of water from 72mN·m^{-1} to 33.8mN·m^{-1} and are characterized by a critical micelle

concentration (cmc) of $3.6 \cdot 10^{-4}$M . Mannosylerythritol lipids have also a high antimicrobial activity against Gram-positive bacteria and weak activity against Gram-negative bacteria. Moreover, MEL-A and MEL-B particularly induce the differentiation of several carcinoma cell lines.

Because of the high costs and the small-scale of biosurfactant synthesis, their use is currently limited. An economically reasonable solution could be the synthesis of biosurfactants in a medium containing waste products, i.e. a hydrophobic or hydrophilic carbon source and selecting a strain capable of producing high yields of biosurfactants.

The aim of the study was to determine factors which influence the yield of biosurfactant synthesis by *Pseudozyma antarctica* (*Candida antarctica*).

MATERIAL AND METHODS

The yeast culture (*Pseudozyma antarctica* ATCC 20509; *Candida antarctica*) was stored at 4°C in a YPG agar-medium (yeast extract 1%, peptone 2%, glucose 2%, agar 2% (w/v)), pH 4.5. Inoculum was prepared in 100mL of the YPG medium, sterilized at 121°C for 20min, in 500mL Erlenmeyer flask. The cultivation was performed at 30°C in 100mL of the medium in a 500mL Erlenmeyer flask, with shaking at 300rev·min^{-1} for 24h in an Innova 44 shaker (New Brunswick, USA). The cultivation was started by adding 10% (v/v) of inoculum to the cultivation medium.

Synthesis of biosurfactants in a shake-flask culture

The batch cultivation of *P. antarctica* was performed in an Erlenmeyer flask containing 100mL of the medium at 30°C with shaking at 300rev·min^{-1} for 168h. Based on the preliminary experiments, a 100mL of YPG medium for cultivation of *P. antarctica* was supplemented with 5% (v/v) rapeseed oil (Kruszwica, Poland) and 100μL Lipozyme TL100L (Novozymes).

In the next stage of experiments, the synthesis of biosurfactants was performed in conditions as presented above but in a YPG medium supplemented with post-refining fatty acids (Post-FFA, Elstar Oils, Poland) (20%, v/v) for 240h.

Fed-batch synthesis of biosurfactant in fermenter

P. antarctica was cultivated at 30°C and 300rev·min^{-1} using BioFlo 115 (New Brunswick, USA), in 3L of the YPG medium (initial pH 4.5) containing 20% (v/v) Post-FFA. After 8 days of cultivation the medium was supplemented with glucose and Post-FFA (20%, v/v) to obtain an initial concentration of carbon sources of 2% (w/v) and 20% (w/v), respectively. The cultivation was performed for 20 days.

The influence of the initial amount of biomass in the cultivation medium on the synthesis of biosurfactant by *P. antarctica* was performed by adding: 5, 50, 100 or 500g·L^{-1} of wet biomass to the YPG medium containing 20% (v/v) Post-FFA (acid value 34.8mg of KOH per g; lipid content 527.1g·L^{-1}; fatty acid composition (%): 18:0, 0.73; 18:1, 68.08; 18:2, 18.71; 18:3, 9.01). The cultivation was performed in conditions as above for 168h.

During the cultivation experiments, the following parameters were determined: pH value of the medium, surface tension (σ, mN·m^{-1}), diameter of the medium drop (DMD, mm), biomass concentration (Y, $g_{dry\ matter} \cdot L^{-1}$), yield of the glycolipids (B, g·L^{-1}) and glucose concentration (GLU, g·L^{-1}).

Biomass concentration assay

The biomass was separated from the medium by centrifugation (5000×g, 10min, 4°C), washed with ethanol:butanol:chloroform mixture (10:10:2, v/v/v) and de-ionized water. The biomass concentration was determined gravimetrically after overnight drying at 105°C.

Determination of glucose concentration

Glucose (GLU, g·L^{-1}) content was determined by a standard method using 3,5-dinitrosalicylic acid (DNS).

Glycolipid concentration assay

The glycolipid assay was carried out by triple extraction with ethyl acetate from the cultivation medium, without biomass separation. The excess lipidic carbon sources were removed by washing the extract three times with n-hexane. The solvents were removed in a rotor vacuum evaporator at 50°C, 240mbar. The crude biosurfactants containing mainly extra-cellular (but also intra-cellular) compounds were determined gravimetrically.

Surface activity determination

The surface tension was quantified at 25°C with the Du Noüy ring method using a K-9 tensiometer (Krüss).

The surface activity of the medium was determined by the ability to collapse medium droplets on the hydrophobic surface of Parafilm M® laboratory film. The diameter of the medium drop (DMD) (50μL) on parafilm was determined using AnalySIS software (Soft Imaging System).

The results are the mean of triplicate experiments and the standard deviation did not exceed 8% of the recorded value.

RESULTS AND DISCUSSION

The efficient synthesis of biosurfactants usually requires a hydrophobic and hydrophilic carbon-source in the cultivation medium. In our earlier studies, we demonstrated that waste products could be used as a carbon source for surface active compound synthesis. However, the influence of the lipase activity and biomass amount on the synthesis of biosurfactants was controversial and ambiguous.

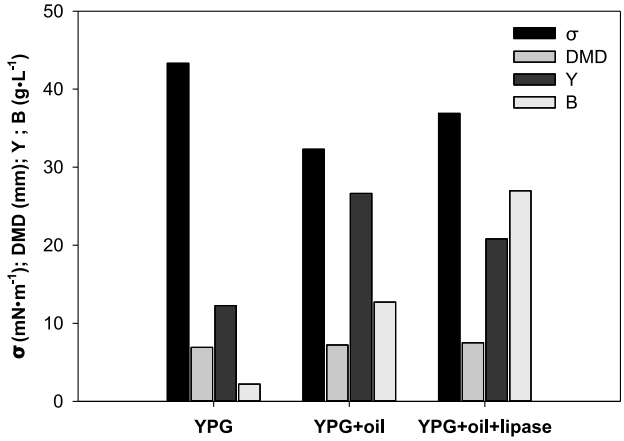

Figure 1. The influence of medium composition on biosurfactant synthesis by *Pseudozyma antarctica* (30°C, 168h). Surface tension (σ, mN·m^{-1}), diameter of the medium drop (DMD, mm), biomass concentration (Y, $g_{dry\ matter}$·L^{-1}), yield of the glycolipids (B, g·L^{-1}).

The synthesis of biosurfactant by *P. antarctica* was detected in the medium containing only glucose as a carbon source (Figure 1) but Morita et al. (2007) did not observe the synthesis of MEL by *Pseudozyma aphidis* when the only substrate was glucose. The addition of 5% (v/v) of rapeseed oil increased the synthesis of surface active compounds from 2.20 to 12.69g·L^{-1} (Figure 1). The addition of rapeseed oil to the cultivation medium decreased the surface tension and increased both DMD and the biomass concentration (Figure 1).

Fungi can degrade free fatty acids and triacylglycerols to provide a carbon and energy source. The uptake of fatty acids is possible after its release from acylglycerols and it is well-known that fatty acids are utilized by microorganisms (degraded by β-oxidation or stored into lipid bodies) and some fatty acids, e.g. medium chain fatty acids inhibit the growth of fungi. *P. antarctica* is a well-known producer of lipases that split the acylglycerols into free fatty acids and glycerol enables their transport into the cell usually by a specific multi-component complex. In addition to lipases, some microorganisms, e.g. *Yarrowia lipolytica*, synthesize surfactants which promote the surface-mediated transport of hydrophobic substrates by reducing the size of hydrophobic droplets.

One of the major components of fatty acids of rapeseed oil and Post-FFA is oleic acid (C18:1) which has a beneficial effect on the growth of yeast during the synthesis of biosurfactants. Felse et al. (2007). have shown that fatty acids with more than one unsaturated bond reduce the synthesis of biosurfactants by *Candida bombicola*, while medium and long chain fatty acids composed from 10 up to 14 carbons are metabolized by yeast in a path of de novo synthesis of C16 or C18 fatty acids.

The addition of Lipozyme TL 100L (Novozymes) to a YPG medium containing 5% (v/v) of rapeseed oil increased the synthesis of biosurfactant by *P. antarctica*. It was shown that the addition of the enzyme probably increased the bioavailability of fatty acids by reducing the size of hydrophobic droplets and produced a more than 2-fold increase in the synthesis of biosurfactants. After 168 hours of cultivation, the content of biosurfactants was 26.97g·L^{-1} (Figure 1).

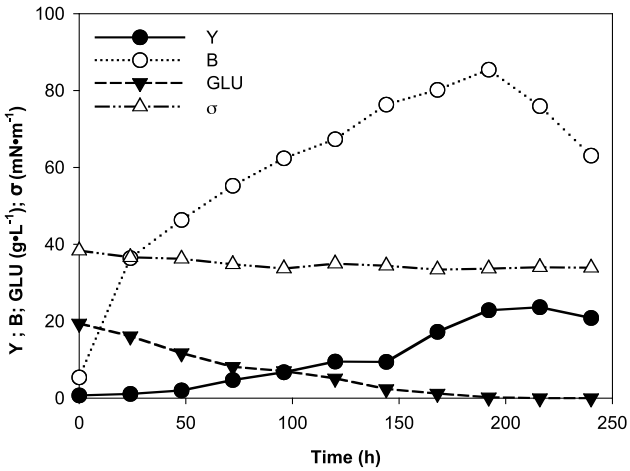

Figure 2. The synthesis of biosurfactants in a shake-flask culture by *Pseudozyma antarctica* (YPG medium, 20% (v/v) Post-FFA, 30°C, 240h). Biomass concentration (Y, $g_{dry\ matter}$·L^{-1}), yield of the glycolipids (B, g·L^{-1}), glucose concentration (GLU, g·L^{-1}), surface tension (σ, mN·m^{-1}).

One of the factors limiting the widespread utilization of biosurfactants is the high cost of their synthesis and downstream processing. The application of waste products in the synthesis of biosurfactants can be an economically attractive and environmentally-friendly method of their bioutilization. Based on the preliminary experiments, a YPG medium containing 20% (v/v) of Post-FFA was used for synthesis of MEL by *P. antarctica*. The highest content of biosurfactant (85.43g·L^{-1}) was achieved after 192h of cultivation. After 216h of cultivation a decrease in the yield of biosurfactant was observed (Figure 2), probably because of an insufficient amount of hydrophilic and/or hydrophobic carbon sources in the medium. However, in some cases, the biosurfactants from the medium could be either inactivated or incorporated into other metabolites, e.g. with no surface activity. It was also shown that the decrease in biosurfactant concentration could be a result of its uptake by the cells.

The surface changes in the cultivation medium were controlled by using two methods, i.e. the Du Noüy ring method (which is accurate, but requires about 50mL of the sample) and a drop-collapse assay (which is simple and quick and is very often used for screening purposes). During the

cultivation, a reduction in the surface tension of the medium from 38.33 to 33.40mN·m^{-1} was observed (Figure 2). The highest DMD value of 8.40mm was measured after 96h of cultivation and no relationship was found between the DMD and the synthesis of biosurfactant. Morita et al. (2007) did not find a relationship between DMD and the yield of biosurfactant. It is probable that, besides the presence of the synthesized biosurfactant, other components of growth medium influenced the size of the droplet diameter as well as the results of surface tension obtained by the ring method.

During the study it was found that the intensive synthesis of biosurfactant caused an increase in the pH of a medium to pH 6.1 as a result of the transformation of free fatty acids present in the medium, similar to the results presented by Adamczak and Bednarski (2000b).

A significant increase in the biomass concentration during cultivation of *P. antarctica* was observed and the highest concentration of biomass of 23.63g·L^{-1} was achieved after 216h (Figure 2). A reduction in the biomass concentration was then recorded which was related to the depletion of the carbon source.

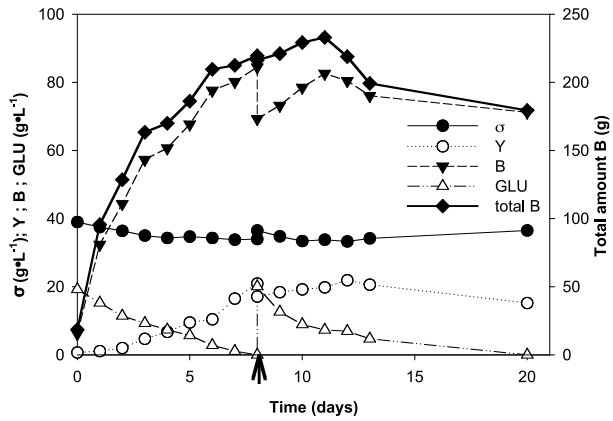

Figure 3. The synthesis of biosurfactants in a fed-batch culture by *Pseudozyma antarctica* in the YPG medium containing 20% (v/v) Post-FFA. The arrow indicates the time when the medium was supplemented with Post-FFA and glucose (30°C, 20 days). Surface tension (σ, mN·m^{-1}), biomass concentration (Y, $g_{dry\ matter}$·L^{-1}), yield of the glycolipids (B, g·L^{-1}), glucose concentration (GLU, g·L^{-1}).

In the following steps of the experiment the synthesis of biosurfactant was stimulated by fed-batch cultivation of *P. antarctica*. A mixture of a hydrophobic and hydrophilic carbon source after depletion of the carbon source on day 8 of cultivation was added to obtain an initial glucose and Post-FFA concentration of 2% (w/v) and 20% (v/v), respectively (Figure 3). The addition of substrates increased the concentration of MEL to about 85g·L^{-1} and substantially increased the total amount of biosurfactant from about 170g (batch culture) to 232g (Figure 3). The higher concentration of biosurfactant resulted in a higher decrease in surface tension from 39.0 to 33.3mN·m^{-1} (Figure 3).

The mannosylerythritol lipids have many unique properties, and applications and the yield of synthesis is usually about 40g·L^{-1}, although it could also reach 165g·L^{-1}.

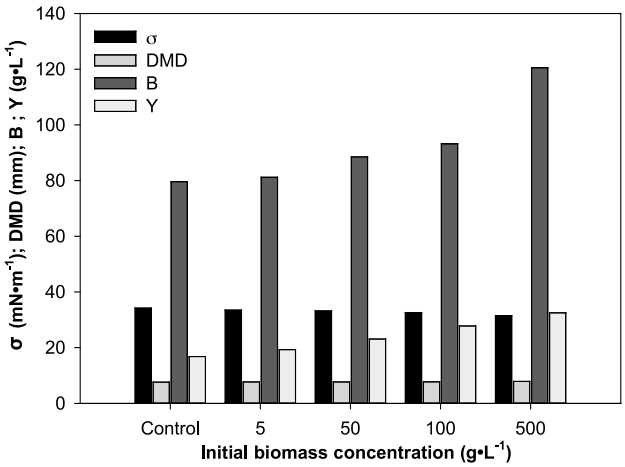

Figure 4. The influence of initial biomass concentration on the synthesis of biosurfactants by *Pseudozyma antarctica* (30°C, 168h). Surface tension (σ, mN·m^{-1}), diameter of the medium drop (DMD, mm), yield of the glycolipids (B, g·L^{-1}), biomass concentration (Y, $g_{dry\ matter}$·L^{-1}).

The initial concentration of biomass was chosen as the second parameter that could influence the synthesis of MEL by *P. antarctica*. To the YPG medium containing 20% (v/v) of Post-FFA, wet biomass in the amount of 5, 50, 100 or 500g·L^{-1} was added (Figure 4). Following the addition of standard inoculum, 79.60g·L^{-1} of biosurfactant was obtained after 168h of cultivation. After the addition of 5, 50, 100 or 500g·L^{-1} (w/v) of biomass at the beginning of cultivation the following concentrations of biosurfactant were obtained: 81.2, 88.5, 93.2 and 120.5g·L^{-1}, respectively. It should be noted that despite the different amount of biomass at the beginning of cultivation, the concentration of biomass after 168h did not substantially differ (Figure 4). The lowest value of the surface tension (31.5mN·m^{-1}) was obtained for the highest yield of MEL while the largest value of DMD was 7.9mm.

CONCLUSIONS

The application of waste products in the synthesis of biosurfactants may be an attractive method for the environmentally-friendly conversion of waste into valuable compounds. Lipids present in the waste, especially free fatty acids, are preferred substrates for the synthesis of MEL by *P. antarctica*. The synthesis of MEL is usually performed in a shake flask while very few attempts have been made to

produce it in a fermenter. It is necessary to optimize MEL synthesis in a bioreactor and analyze the possibility of using a high cell density culture for its synthesis. MEL are attractive because of their varied biochemical functions and potential pharmaceutical applications. Another major issue in the widespread use of MEL is the need to analyze the structural and functional relationship in the structural diversity of this group of surface active compounds.

REFERENCES

Adamczak, M. 2003. Synthesis, properties, and application of lipase from *Candida antarctica* for high yield monoacylglycerol biosynthesis. Polish Journal of Food and Nutrition Sciences 12/53: 3-8.

Adamczak, M., W. Bednarski. 2000a. Properties and yield of synthesis of mannosylerythritol lipids by *Candida antarctica*. In: Food Biotechnology (ed. S. Bielecki, J. Tramper, J. Polak), pp. 229-234. Elsevier, Amsterdam.

Adamczak, M., W. Bednarski. 2000b. Influence of medium composition and aeration on the synthesis of biosurfactants produced by *Candida antarctica*. Biotechnology Letters 22: 313-316.

Arutchelvi, J., S. Bhaduri, P. Uppara, M. Doble. 2008. Mannosylerythritol lipids: a review. Journal of Industrial Microbiology & Biotechnology 35: 1559-1570.

Bednarski, W., M. Adamczak. 2008. Production, properties, and application of biosurfactants. Na pograniczu chemii i biologii 20: 451-472 (in Polish).

Bednarski, W., M. Adamczak, J. Tomasik, M. Plaszczyk. 2004. Application of oil refinery waste in the biosynthesis of glycolipids by yeast. Bioresource Technology 95: 15-18.

Bednarski, W., M. Narwojsz, M. Adamczak, R. Nawotka. 2006. Carbon-source-dependent synthesis and composition of biosurfactant synthesized by *Pseudozyma antarctica*. Environmental Biotechnology 2: 31-36.

Black, P.N., C.C. DiRusso. 2003. Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. Microbiology and Molecular Biology Reviews 67: 454-472.

Desai, J.D., I.M. Banat. 1997. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews 61: 47-64.

Dzięgielewska, E., M. Adamczak. 2012. The application of by-products and waste for synthesis of sophorolipids by *Candida bombicola*. Nauki inżynierskie i technologia 3: 37-48 (in Polish).

Dzięgielewska, E., M. Adamczak. 2013. Evaluation of waste products in the synthesis of surfactants by yeasts. Chemical Papers 67: 1113-1122.

Dzięgielewska, E., M. Adamczak, W. Bednarski. 2011. Application of waste products in the synthesis of biosurfactants by *Candida aphidis*. Zeszyty Problemowe Postępów Nauk Rolniczych 566: 41-48 (in Polish).

Felse, P.A., V. Shah, J. Chan, K.J. Rao, R.A. Gross. 2007. Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues. Enzyme and Microbial Technology 40: 316-323.

Fukuoka, T., T. Morita, M. Konishi, T. Imura, H. Sakai, D. Kitamoto. 2007. Structural characterization and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*. Applied Microbiology and Biotechnology 76: 801-810.

Hubert, J., K. Plé, M. Hamzaoui, G. Nuissier, I. Hadef, R. Reynaud, A. Guilleret, J.-H. Renault. 2012. New perspectives for microbial glycolipid fractionation and purification processes. Comptes Rendus Chimie 15: 18-28.

HuÀková, Z., A. Fencl. 1978. Toxic effects of fatty acids on yeast cells: Possible mechanisms of action. Biotechnology and Bioengineering 20: 1235-1247.

Kinderlerer, J.L. 1993. Fungal strategies for detoxification of medium chain fatty acids. International Biodeterioration & Biodegradation 32: 213-224.

Kitamoto, D., H. Yanagishita, T. Shinbo, T. Nakane, C. Kamisawa, T. Nakahara. 1993. Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. Journal of Biotechnology 29: 91-96.

Konishi, M., T. Morita, T. Fukuoka, T. Imura, K. Kakugawa, D. Kitamoto. 2007. Production of different types of mannosylerythritol lipids as biosurfactants by the newly isolated yeast strains belonging to the genus *Pseudozyma*. Applied Microbiology and Biotechnology 75: 521-531.

Kuiper, I., E.L. Lagendijk, R. Pickford, J.P. Derrick, G.E.M. Lamers, J.E. Thomas-Oates, B.J.J. Lugtenberg, G.V. Bloemberg. 2004. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Molecular Microbiology 51: 97-113.

Marchant, R., I.M. Banat. 2012. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends in Biotechnology 30: 558-565.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428.

Morita, T., M. Konishi, T. Fukuoka, T. Imura, D. Kitamoto. 2007. Physiological differences in the formation of the glycolipid biosurfactants, mannosylerythritol lipids, between *Pseudozyma antarctica* and *Pseudozyma aphidis*. Applied Microbiology and Biotechnology 74: 307-315.

Onghena, M., T. Geens, E. Goossens, M. Wijnants, Y. Pico, H. Neels, A. Covaci, F. Lemiere. 2011. Analytical characterization of mannosylerythritol lipid biosurfactants produced by biosynthesis based on feedstock sources from the agrofood industry. Analytical and Bioanalytical Chemistry 400: 1263-1275.

Smyth, T.J.P., A. Perfumo, R. Marchant, I. Banat. 2010. Isolation and analysis of low molecular weight microbial glycolipids. In: Handbook of Hydrocarbon and Lipid Microbiology (ed. K.N. Timmis), pp. 3705-3723. Springer-Verlag, Heidelberg, Germany.

Thevenieau, F., A. Beopoulos, T. Desfougeres, J. Sabirova, K. Albertin, S. Zinjarde, J.M. Nicaud. 2010. Uptake and assimilation of hydrophobic substrates by the oleaginous yeast *Yarrowia lipolytica*. In: Handbook of Hydrocarbon and Lipid Microbiology (ed. K.N. Timmis), pp. 1513-1527. Springer-Verlag, Heidelberg, Germany.

Van Bogaert, I., W. Soetaert. 2011. Sophorolipids. In: Biosurfactants (ed. G. Soberón-Chávez), pp. 179-210. Springer-Verlag, Heidelberg, Germany.