Kill●Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D3.10 Knowledge of the parameters necessary to maximise biosurfactant production in selected microorganism

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SLP – Sophorolipid, RL – Rhamnolipid, MEL – Mannosylerythritol lipids

1 About this deliverable

Objective of WP3: “To explore the ability and availability of novel biosurfactants, dispersants and sorbent materials particularly those of biological origin for deployment on oil spills in marine and terrestrial environment as a mean to accelerate dispersion and/or rate of degradation and removal of such compounds. In addition, we aim to explore the requirements for up-scaled production for each product with the aim of achieving economic commercial large-scale production.” The contribution of Ulster University [UU] is to provide information for biosurfactant optimisation. This report aims to provide information on the most important parameters to consider for the optimisation of biosurfactant production. Deliverable 3.10 (Knowledge of the parameters necessary to maximise biosurfactant production in selected microorganism: [month 36]) has the purpose of providing the necessary information for the maximization of production of biosurfactants and bioemulsifiers to those groups willing to pursue production from the selected strains or from related strains.

2 Introduction

At UU we have produced and characterised the biosurfactant producing strains outlined in Table 1. Table 1 shows the biosurfactant producing strains that have protocols developed for production and purification in this project. The next stage is to optimise the fermentation process for maximum production yields. For all production and purification protocols please refer to deliverable **D3.4.** For chemical characterisation of the selected biosurfactants please refer to **D3.4**, **D3.7** & **D3.8.**

Table 1 Biosurfactant producing strain characterised in this project.

Microorganism	Main Congener*	Yield (g/L)	Surface tension (mN/m)	Emulsification Index (EI 24%)
Pseudomonas aeruginosa PAO1	Rhamnolipids (Rha-Rha-C10-C10)	2-3	24-29	53-64
Pseudomonas aeruginosa ST5	Rhamnolipids (Rha-Rha-C10-C10)	1.5-2.5	25-29	52-65
Pseudomonas aeruginosa DS10	Rhamnolipids (Rha-Rha-C10-C10)	1.5-3	25-31	52-61
Burkholderia thailandensis E264	Rhamnolipids (Rha-Rha-C14-C14)	2-4	26-33	67-70
Burkholderia glumae	Rhamnolipids (Rha-Rha-C14-C14)	1.3-2.2	28-30	65-72
Burkholderia plantarii	Rhamnolipids (Rha-Rha-C14-C14)	0.8-1.1	26-27	60-67
Burkholderia kururiensis	Rhamnolipids (Rha-Rha-C14-C14)	1.9-3.1	30-33	60-63
Candida bombicola	Sophorolipids	20-100	36-39	48-53
Pseudozyma aphidis	Di-acetlyated Mannosylerythritol (C18)	30-100	**	**
Bacillus sp.	Surfactin	0.2-0.8	24-31	58-65

*Most abundant congener produced by each strain.

** The production of mannosylerythritol lipids (MEL's) by *Pseudozyma aphidis* were detected by ESI-MS analysis after Solid Phase Extraction (SPE), though the measurement of the produced amount and the evaluation of their physicochemical properties is still difficult since MEL's are contained in the oil phase and the solvent extraction is not efficient.

3 Experimental approach in the identification of the critical parameters

[ACTY] have successfully produced rhamnolipids and sophorolipids from *Pseudomonas aeruginosa* and *Candida species* provide by **[ULSTER],** the process is fully detailed in D3.8 and **[ULSTER]** has provided all the LC-MS analysis. During the large scale production process **[ACTY]** has identified the following parameters as the most important within 36 months of this project, which include, but not in an exhaustive manner the following:

- carbon sources
- nitrogen sources
- oxygen concentration
- stirring or shaking
- use of antifoaming agents
- temperature
- pH

The production and purification of rhamnolipids and sophorolipids have been extensively described in deliverables 3.8 and 3.7 including details on how the critical parameters have been identified. **[ACTY]** has identified the critical parameters for the production of rhamnolipids and sophorolipids as outlined in Tables 2 and 3.

Table 2 *Pseudomonas aeruginosa* DS10. Parameters for production of rhamnolipids [ACTY]

Parameters	Type	Critical (Y/N)	Optimal value	Notes
Carbon sources	Glycerol (20-80 g/L) Soybean oil (10-50g/L)	Y Y	40-50 g/L 20 g/L	Glycerol feeding during fermentation increases the yield of rhamnolipids
Nitrogen sources	Corn steep liquor Peptone Soybean meal Yeast extract (20-50 g/L)	N N Y Y	20 g/L 46 g/L	
Oxygen concentration			> 5% pO2	
Airflow	2L/min	Y	2L/min	
Oxygen distribution	stirring or shaking		200 rpm in flask culture, 250 rpm in bioreactor (30Lt)	
Antifoam agents	Hodag	Y	Added on request	
Temperature	26-37°C	N	35	Temperatures from 26 to 30 are tolerable
pH	5-8	Y	6-7	

Table 3 ***Candida bombicola*. Parameters for the production of sophorolipids [ACTY]**

Parameters	Type	Critical (Y/N)	Optimal value	Notes
Carbon sources	Glucose (40-100 g/L) Soybean oil (60-100 g/L)	Y Y	100 g/L 100 g/L	A feeding of glucose during of fermentation increases the yield of Sophorolipids
Nitrogen sources	Yeast extract	N	2-4 g/L	
Oxygen concentration	1-5 pO2		<0.5% pO2	
Airflow	2L/min	Y	2L/min	
Oxygen distribution	stirring or shaking		200 rpm in flask culture 350 rpm in bioreactor (30Lt)	
Antifoam agents	Hodag	N	Added on request	
Temperature	25-30 °C	N	28	Temperatures from 26 to 30 are tolerable
pH	2.9-4.5	N	3.0	A fermentation at pH controlled improves the yield

4 Production of biosurfactants in bioreactors

Low yields and high production costs are the main obstacles for the large scale production of biosurfactants from microorganisms. Over the last few decades the production of biosurfactants (BSs) has received considerable attention in the biotechnology sector as sustainable replacements for current synthetically produced surfactants (Marchant and Banat, 2012). The structural diversity and unique properties of BS molecules is making these metabolites top of the sustainability agenda and have been widely studied for applications in pharmaceutical, biomedical, cosmetic food environmental and cleaning/detergency (Van Hamme et al., 2006).

Biosurfactants have compelling advantages over synthetic surfactants, they are in general less toxic, show higher biodegradability and can be sustainably produced from renewable/waste resources (Makkar et al., 2011). However, low product yields coupled with expensive production processes is one of the major hurdles for developing bioprocesses that are economically competitive with synthetic surfactants. In combination with traditional process development there has been a shift toward systems metabolic engineering to advance bioprocess development for BS bulk application. To date there is still a limited understanding of cellular metabolic and regulatory pathways controlling BS production. It is expected that systems metabolic engineering will develop a more cost effective and efficient production process for BSs. Industrial sustainability must not only be eco-efficient but also economically competitive to sustain a bio-based economy. This report highlights the biosynthetic pathways for some of the selected microorganisms that have already established a

production protocol within this project. It is expected that for efficient optimisation of the fermentation process understanding of the biosynthetic production and regulation of biosurfactants will be essential.

[ACTY] has in place processes for pilot scale production of rhamnolipids and sophorolipids and **[ULSTER]** has characterised biosurfactants produced by LC-MS (D3.4, D3.7, D3.8). With the production processes already established the focus is now to develop strategies for improving the production yields to develop efficient cost effect industrial processes.

4.1 Challenges for biosurfactant production

4.1.1 Foaming

The unique characteristics of biosurfactants such as low surface tension, emulsification and foaming properties which make them diverse natural products also create significant problems when it comes to production in bioreactors. Foaming issues are particularly significant with high foaming biosurfactants during aerated fermentations (e.g. rhamnolipids and surfactin) with hydrophilic substrates (i.e. glycerol). It is not recommended to use antifoaming agents for rhamnolipid fermentations as they interfere with quantitative measurements of biomass and is often co-extracted during solvent extraction which significantly overestimates the crude yield (data from Ulster not shown). Use of antifoam agents increase the cost of the production process and they can have significant effect on oxygen transfer rate. Often mechanical foam breakers are used to break up the foam however they are not very efficient, strategies to overcome this problem include optimising oxygen transfer using non-dispersive methods. Most commonly hydrophobic carbon substrate is steadily supplied during the fermentation which acts like an antifoam and does not affect production.

4.1.2 Excess hydrophobic carbon substrate

Excess unutilised hydrophobic carbon is a significant challenge for downstream purification of biosurfactants. This is a significant problem to consider when producing biosurfactants from the yeasts *Candida bombicola* and *Pseudozyma aphidis*. Production of biosurfactants in these organisms is only induced under excess hydrophobic carbon sources at the end of fermentation there is significant amount of unutilised fatty acids that are co-extracted. Attempts to remove the excess unutilised carbon source with polar solvents can affect the yields as these biosurfactant typically contain long chain fatty acids i.e. C_{18} and are often washed away with polar solvents. Purification of the excess carbon source is essential as it significantly overestimates the final yield. Overestimation of yields will compromise the optimisation process.

4.2 Bioprocess optimisation: important parameters for biosurfactant production in bioreactor

In general there are three main issues when it comes to optimising the biosurfactant production process.

4.2.1 Carbon Source

Most biosurfactants can be produced on hydrophilic carbon substrates and generally production is enhanced with hydrophobic substrates. For some yeast species a combination of hydrophilic and hydrophobic substrates is essential for production. Carbon chain length and degree of saturation can affect the composition of the biosurfactant produced. This is most common in biosurfactant production from the yeast species (i.e. sophorolipids (SLs) and mannosylerythritol lipids (MELs)) as

they use β-oxidation and chain shortening pathways to directly incorporate the fatty acid moiety of the biosurfactants.

Whereas biosurfactant production from bacteria use *de novo* fatty acid synthesis to supply the hydrophobic fatty acid chain. A variety of carbon sources can be used to produce biosurfactants however the best reported yields are with hydrophobic substrates (e.g. range of vegetable oils). **[ULSTER]** has previously shown that the best production of rhamnolipids was achieved with oleic acid (Figure 1). For production of SLs and MELs the best reported yields have been achieved with the use of two carbon sources (hydrophilic and hydrophobic substrates). Typically C_{18} carbon substrates are used which can be directly incorporated into the biosurfactant. However for both SLs and MELs the degree of acetylation and production of either Lactonic or acidic SLs depends mainly on the producer microorganism.

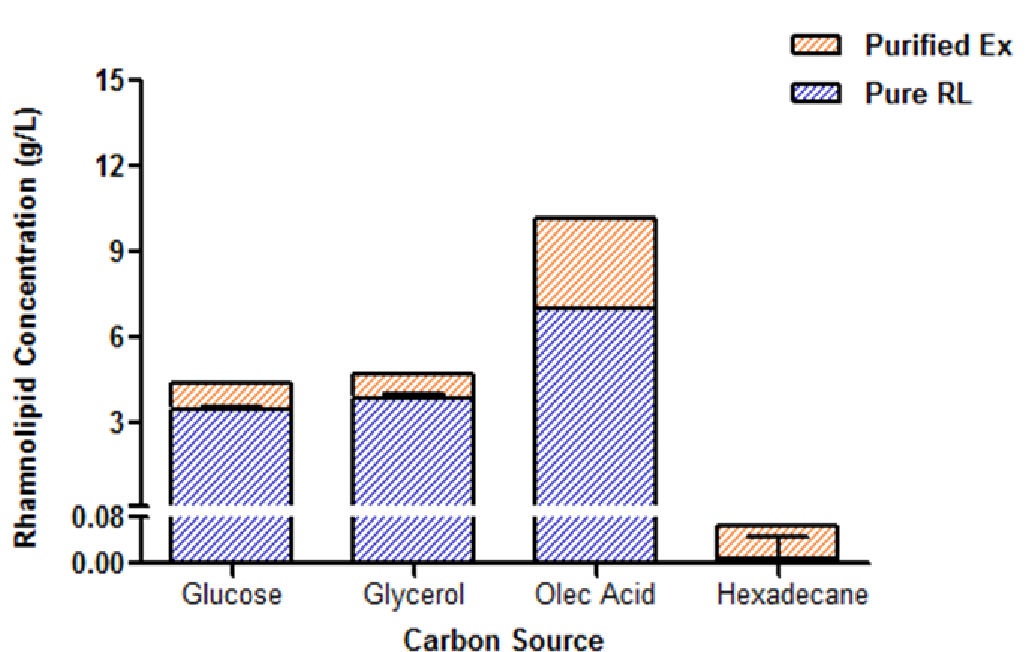

Figure 1 Comparison of carbon sources used for rhamnolipid production in *Pseudomonas aeruginosa* ST5

Glucose is the main carbon source used for the production of biomass for both SL and MEL production. **[ACTY]** has reported the best production of SLP when glucose and oil were both supplied in the fermentation (D3.4). While the use of hydrophobic substrates produces higher yields, excess fatty acids produces significant challenges for downstream process as previously mentioned (4.1.2) therefore some research has focused on the use of alternative hydrophilic substrates. Significant amounts of MELs have been produced using sucrose, fructose, glucose and mannose as well as using sugarcane juice supplemented with urea as a nitrogen source (Morita et al., 2009a 2009b). Sophorolipids have also been produced with a variety of hydrophilic carbon sources including sucrose, fructose, xylose, mannose, lactose and galactose (Bajaj et al., 2009; Rispoli et al., 2010). Both SLs and MELs are produced as complex mixtures of congeners that are often not easily separated. Figure 2 shows HPLC-MS chromatograms for both SLs and MEL-A fraction of MELs purified from *P. aphidis*. The complexity of congeners makes downstream purification more difficult and increases the production costs.

Figure 2 HPLC-MS of SLs from *Candida bombicola* and MEL-A HPLC-MS from *P. aphidis.*

SLs are produced mostly with C_{18} fatty acid chain lengths due to the specificity of the P450 monooxygenase (see Figure 5 for biosynthetic pathway), which hydroxylates the terminal or sub-terminal portion of the fatty acid. Typically stearic acid (C18:0) and oleic acid (C18:1) are preferred substrates for SL biosynthesis as they can be directly incorporated into the SLP pathway and are the preferred length for some enzymatic affinity in the SLP pathway.

4.2.2 Nitrogen Source

Biosurfactants are typically produced under nutrient limiting conditions most commonly nitrogen limitation. For rhamnolipid production from *P. aeruginosa* sodium nitrate is the best reported nitrogen source with high concentrations of rhamnolipids produced in a bioreactor from *P. aeruginosa* using sunflower oil as a carbon source and nitrogen limiting conditions (Müller et al., 2010). For biosurfactant production in yeast the best nitrogen source reported is yeast extract. Organic nitrogen (yeast extract) sources are best for production of biomass in yeast however for efficient glycolipid production nitrogen limitation must be achieved. Yeast extract typically contains high carbon to nitrogen ratios, which can sometimes be a limiting factor for glycolipid production. It is recommended that the optimisation process should look at varying concentrations of yeast extract.

For SLP production yeast extract is the optimal nitrogen source (Casas 1997) however the optimal concentration has still to be determined with concentrations ranging from 1-5g.L^{-1} (Van Bogaert et al., 2007). The optimum concentration may depend of the producer strain and carbon source used. Sodium nitrate is the best reported nitrogen source for production of MELs from *Pseudozyma spp.* **[UNIBO]** have investigated the effect of the C/N ratio and nitrogen source in *Bacillus subtilis* R39 (D3.4) and reported that a C/N ratio of 13 with sodium nitrate as the nitrogen source and glucose as the main carbon source. Urea is not a suitable nitrogen source as other nutrients that are usually supplied with the yeast extract (such as thiamine, pantothenic acid and pyridoxine) are not present which can limit cell growth.

4.2.3 pH

pH is an important parameter for biosurfactant production. For production of rhamnolipids from *P. aeruginosa* the optimal pH range is 6-7.2 (Zhu et al., 2012). This pH range has been used in all rhamnolipid fermentations previously carried out in **[ULSTER].** [UNIBO] showed that the use of a strongly buffered medium (high in phosphates) is preferred to control pH for *Bacillus* fermentations (D3.4). The effect of pH is a significant for glycolipid production from the yeasts. In SLP production during the exponential phase the pH can significantly drop from 6.0 to 2-4.0. Consumption of the nitrogen source for biomass production and the production of fatty acids is thought to cause the pH drop however the pH must be at 6.0 in the beginning of fermentation to promote growth. It is reported that along with the antimicrobial effect of SLPs the low pH protects the culture from contamination when SLP can have long fermentation runs (up to one week). **[ULSTER]** have reported similar drops in pH during MEL production in *P. aphidis* (Figure 3). pH is an critical for the production process.

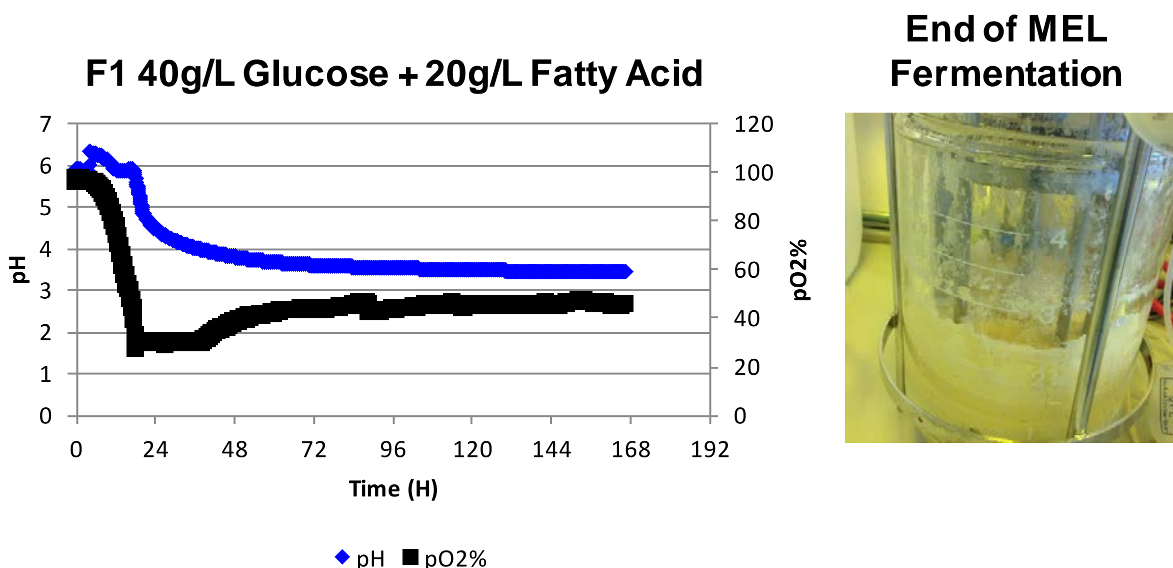

Figure 3 Fermentation profile for MEL production in *Pseudozyma aphidis* [ULSTER]

4.2.4 Aeration and Oxygenation

Oxygen is essential during the whole fermentation process but especially during exponential growth as yeast cells are sensitive to oxygen limitation. Good aeration is also required for SLP biosynthesis in stationary phase due the requirements of cytochrome P450 needing molecular oxygen for activity.

For SLP production it has been reported that high oxygenation can influence the structure produced where high aeration promotes the formation of diacetlyated forms of SLP (Ratsep and Shah, 2009).

4.2.5 Fermentation Process

Biosurfactants can be produced by a variety of fermentation processes including batch, fed batch and continuous. Shake flask fermentation is suited for screening new biosurfactant strains and also for optimising culture parameters i.e. varying concentrations of carbon source, nitrogen, yeast extract etc. the best reported yields for any biosurfactant is reported with aerobic fermentation in bioreactors, with yields reported for SLPs in the range of 300->400g.L^{-1} (Pekin et al., 2005). For the MELs Rau et al., 2005 reported the highest yield of 165g.L^{-1}. Müller et al., 2010 has reported maximum rhamnolipid yield of 39g.L^{-1} in a 30-L bioreactor.

5 Biosynthesis of Biosurfactants

Although glycolipid production in yeast and fungi has been long established and well reported in the literature, the genetic basis and physiological function of their production remains to be fully elucidated.

5.1 Biosyntesis of Rhamnolipids (RLs)

RLs are low molecular weight glycolipids that consist of a hydrophilic rhamnose (Rha) head glycosidically linked to one or more β-hydroxy fatty acids. RLs are synthesised as a heterogeneous mixture of congeners of two main classes; mono- and di-rhamnolipids. The structural diversity of RLs arises from either one or two rhamnose sugar rings glycosidically linked to various β-hydroxy fatty acids varying in chain length and degree of saturation. The RL biosynthetic pathway has largely been elucidated on carbohydrate substrates such as glucose (Campos-Garcia et al., 1998; Rahim et al., 2001; Hauser and Karnovsky, 1954, 1958,) and it is separated into three main parts namely the synthesis of the hydrophilic rhamnose, aliphatic chain and rhamnolipid. Figure 4 shows the biosynthesis of RLs in *P. aeruginosa*.

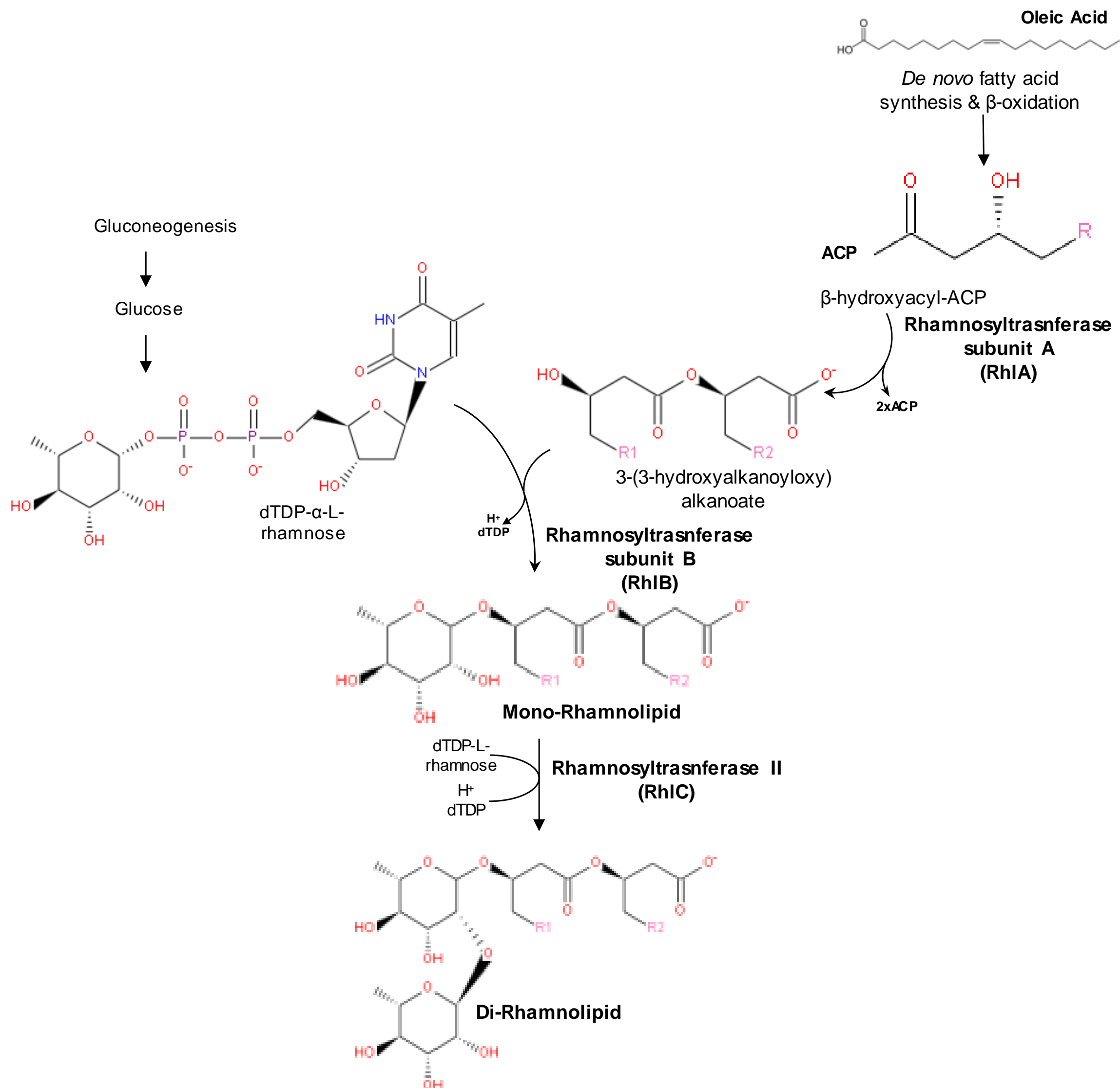

Figure 4 Rhamnolipid biosynthesis in *Pseudomonas aeruginosa* (Figure Adapted from Zhang et al., 2012).

5.2 Biosynthesis of Sophorolipids (SLPs)

Sophorolipids are produced by yeasts of the genus *Candida*, particularly *C. bombicola* and *C. apicola,* and are produced in mixtures comprising usually 8 major and up to 15 minor components (van Bogaert et al. 2007). Sophorose, a 1,2-disaccharide of glucose, forms the hydrophilic head of the molecule that may or may not be acetylated with one or two acetyl groups. The fatty acid chain typically has 16 or 18 carbon atoms with different degrees of saturation (none, one or two double bonds). Sophorolipid molecules exist either in the acidic or lactonic form; in the latter, the carboxylic end of the fatty acid is esterified at the C4, or less frequently at the C6 or C6” position, of the sophorose unit. The possible variants make the sophorolipid mixture produced by *Candida* species very complex, although lactonic sophorolipid with 17-hydroxy-octadecanoic acid is reported to be the predominant congener (Van Bogaert et al. 2007). The biosynthesis of SLPs begins in the stationary phase. The first step of SLP biosynthesis is the hydroxylation of the fatty acids by the enzyme CYP52M1 (cytochrome P450 monooxygenase). The next step is carried out by the

glucosyltransferases I and II (UgtA1 and UgtA2), which sequentially add glucose molecules producing the deacetylated form of SLP. The glucose precursors are supplied via the glycolytic pathways and are not directly incorporated from the medium. The next steps synthesis the structural variants of SLP by acetylation and lactonisation of the unacetlyated form of SLP. As with the MELs SLPs are acetylated at the C6' and C6'' positions on the sophorose molecule by an acetyltransferase enzyme. A lactonase enzyme carries out the lactonisation process. An overview of SLP biosynthesis is given in Figure 5.

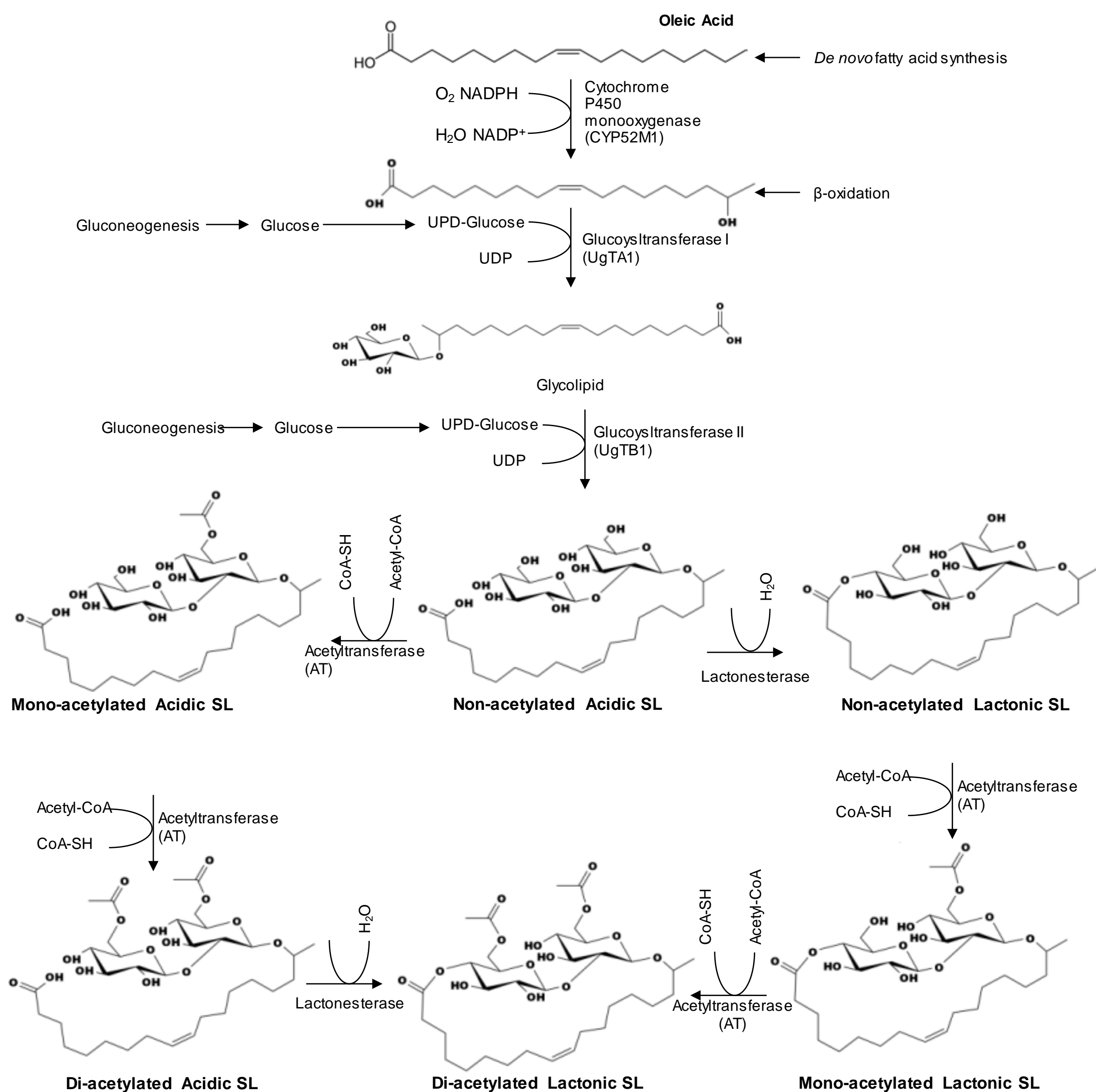

Figure 5 Sophorolipid biosynthesis in *Candida bombicola* Figure Adapted from Saerens et al., 2015).

5.3 Biosynthesis of Mannosylerythritol lipids (MELs)

The biosynthetic gene cluster for MEL biosynthesis was first described in the smut fungus Ustilago maydis (Hewald et al., 2006), which contains five genes coding for four enzymes involved in MEL biosynthesis and a potential transporter gene. The MEL biosynthesis gene cluster comprises the mat1 acetyltransferase gene, the mmf1 gene, which specifies a member of the major facilitator family,

mac1 and mac2, encoding putative acyltransferases, and the glycosyltransferase gene emt1. The first step is catalysed the glycosyltransferase Emt1 which is responsible for the transfer of a GDP-mannose to erythritol. Mac1 and Mac2 are both acyltransferase which are responsible for the acylation of the mannose group at C2 and C3 respectively for diacylated MEL-D. Mat1 catalyses the final acetylation step which stratifies MELs to MEL-A, MEL-B or MEL-C based on whether C4 or C6, or both positions have been acetylated. Finally MELs are extracellularly secreted by the major facilitator protein Mmf1. Figure 6 gives an overview of the MEL biosynthetic pathway.

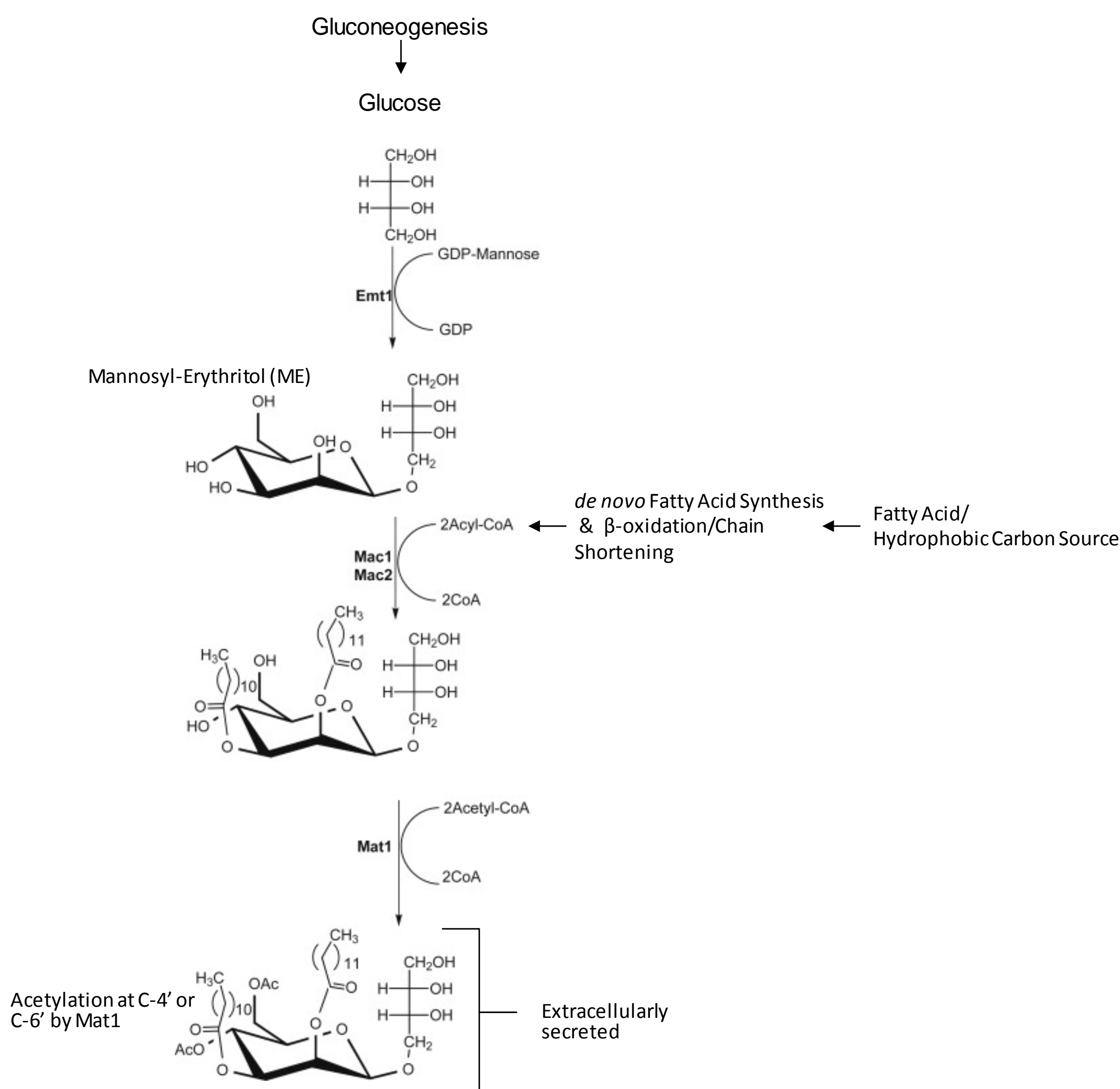

Figure 6 Mannosylerythritol lipid biosynthesis in *Pseudoyzyma aphidis* Figure Adapted from Günther et al., 2015)

MELs are produced by *Ustilago* and *Pseudozyma* spp. with the latter producing abundant amounts of MELs on various vegetable oils with yields reported in excess of 100g/L (Morita et al., 2009; Yamamoto et al., 2009; Morita et al., 2007). MELs are produced using hydrophobic carbon sources, typically almost all vegetable carbon sources can be used for production with soybean oil the best reported carbon source for production of MELs (Konishi et al., 2008; Rau et al. 2005a; Kim et al., 2006: Kitamoto et al., 2001). Substrate carbon chain length and saturation can affect the number of

acylation and unsaturation carbon chain length of MELs. MELs are one of the most promising microbial glycolipids because of their relatively high yield, yet despite over 60 years of research there are still not commercially viable due to high production costs. Most production of MELs is carried out on shake flasks usually trying to identify novel producers or structural variants. With only a few studies reporting the large scale production of MELs in a bioreactor. To develop a commercially viable MEL production process effort need to be focused on bioprocess optimisation. The highest reported yield for MEL production is 165g/L in *P. aphidis* with soybean oil and glucose as carbon sources and additional substrate feeding (glucose, nitrogen, yeast extract) (Rau et al., 2005). To date this is the best reported production yield for MELs in the literature. One of the major hurdles for MEL production is the excess unutilised hydrophobic substrate at the end of fermentation (Figure 7).

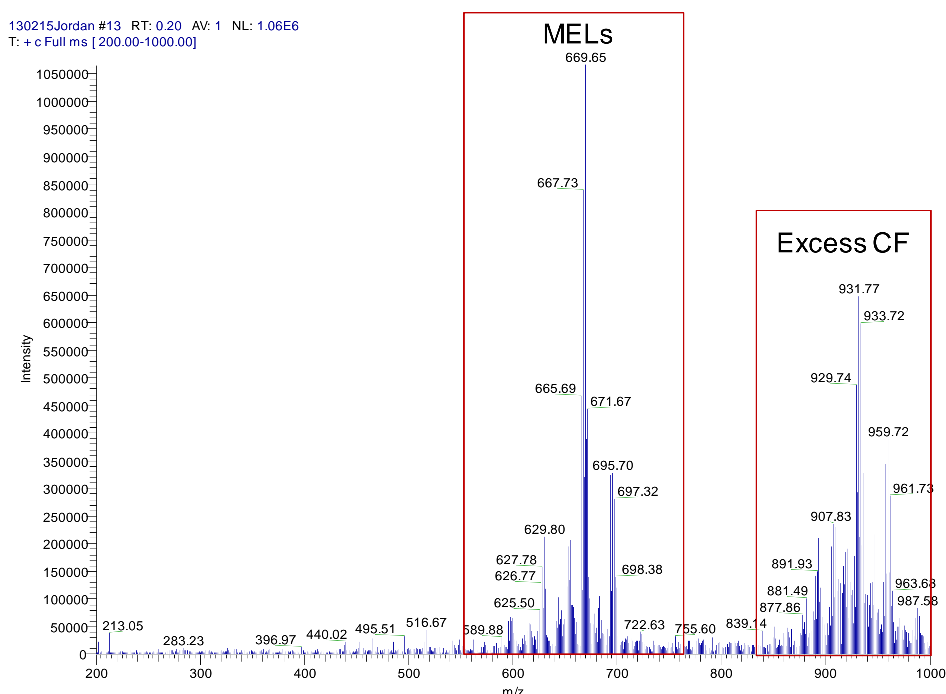

Figure 7 ESI-MS+ showing MEL composition for both *P. aphidis*. MEL fermentation was carried in [ULSTER]. ESI-MS shows excess fatty acids in the extract which were unused at the end of fermentation.

6 Conclusions and Future Recommendations

- [ULSTER] has produced and characterised all types of biosurfactants from the selected microorganisms (D3.4)
- [ULSTER] provided strains to Actygea for production of rhamnolipids and sophorolipids
- Actygea have produced pilot scale production of rhamnolipids and sophorolipids (D3.4)
- Ulster has analytical methods (LC-MS) in place for all biosurfactants and has characterised both rhamnolipids and sophorolipids from [ACTY] fermentations
- Industrial production of selected microorganisms has been demonstrated. The next steps is bioprocess optimisation to refine production processes making them economically feasible.

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