Harnessing the oxidative power of the yeast *Starmerella bombicola* for innovative biochemical production

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Abstract

Biosurfactants have been gaining interest as environmentally friendlier alternatives to the classical petroleum-based surfactants. *Starmerella bombicola* is an industrial relevant yeast due to its capacity of producing large amounts of glycolipid-type biosurfactants known as sophorolipids. However, the full exploitation of its potential is dependent on overcoming the difficulty in obtaining a standardised product, since this yeast produces a mixture of structurally related molecules whose composition varies according to fermentation conditions and medium composition. This lack of uniformity can be attributed to the versatile oxidative power of *S. bombicola*. In fact, this yeast possesses several enzymes which can take part of the hydroxylation system of hydrocarbon-assimilating yeasts such as *S. bombicola*. In this work, engineered strains, with knock-outs in different genes encoding for oxidising enzymes, were tested on the type of sophorolipids produced. A lower number of compounds was detected for strains with a knock-out in genes 1 or 3, while the deletion of gene 2 did not produce a difference comparing to the wild-type. However, the double deletion of genes 1 and 2 led to a lower variety of products comparing to the respective single knock-outs. Finally, the selective production of product A was intended to be used as a proof of concept that gene 2 encodes for the enzyme responsible for hydroxylation of a specific substrate in *S. bombicola*, but the results indicated that another enzyme must be capable of catalysing this step.

Keywords: *Starmerella bombicola*; sophorolipids; biosurfactants; oxidative power.

1. Introduction

1.1. (Bio)surfactants

Surfactants (surface-active agents) have the capacity of adsorbing onto interfaces, decreasing the surface tension between two phases. [1] This characteristic results from their chemical structure composed of two different functional groups: the hydrophobic or lipophobic group has very little attraction towards the solvent, whereas the hydrophilic or lyophilic group has a strong attraction. [2] Surfactants are versatile products which find several applications as cleaning, wetting, dispersing, emulsifying, foaming and anti-foaming agents. [3] However, environmental awareness raises the need to limit their use, due to their accumulation in waste water [4] and because most of them are produced from petroleum feedstocks [5]. Biosurfactants, on the other hand, are produced by microorganism, and present several advantages over the first ones, since they are environmentally friendlier, biodegradable [6] and less toxic [7]. Amongst them, sophorolipids, glycolipid-type biosurfactants, have been gaining commercial interest [8] and are already being used in detergent and cosmetic formulations [9]. Furthermore, their unique chemical structure can serve as the basis for synthesizing certain hydroxy fatty acids and other compounds, such as fatty amines. [10]

1.2. Sophorolipids

Sophorolipids (Fig. 1) have a structure composed of a ω/ω-1 hydroxy fatty acid tail β-glycosidically linked to a hydrophilic carbohydrate head, the sophorose. [10] Regarding its composition diversity, the fatty acid part of the molecule depends on the type of hydrophobic substrate used and varies in terms of chain

length (16 or 18 carbon atoms), number of unsaturations (between 0 and 2) and position of the hydroxyl group (terminal or sub-terminal) [11]. Furthermore, the sophorose unit can be mono- or diacetylated and the fatty acid tail can either be free (acid form) or esterified to the sophorose head (lactonic form) [10]. Besides, the sophorose head can even be present on both ends of the molecule, forming the so-called bola-sophorolipids [12].

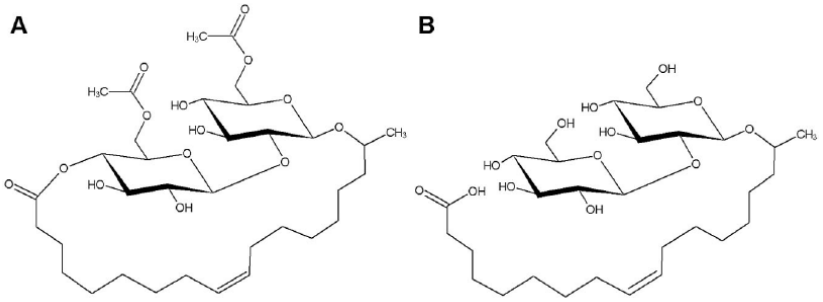

Figure 1: Sophorolipids produced by *S. bombicola*. **A)** diacetylated lactonic sophorolipid; **B)** non-acetylated open-chain (acidic) sophorolipid. [13]

1.3. *Starmerella bombicola*

Amongst the species able to produce sophorolipids, *Starmerella bombicola* has been the most studied due to its non-pathogenic nature and ability to produced large amounts of this biosurfactant. [14] Sophorolipids appear to be produced by this yeast as secondary metabolites in conditions of nitrogen limitation [15]. Therefore, it is hypothesised that sophorolipids are used by cells as extracellular carbon storage material to safeguard the consumption by other organisms, which is complemented by their antimicrobial effect. [13]

In order to have an efficient sophorolipid production, yeast cells should be provided with both hydrophilic (glycidic) and a hydrophobic (lipidic) types of carbon sources. In most cases, glucose is used as the hydrophilic carbon source, whereas several substrates can act as the hydrophobic carbon source: oils, fatty acids, and their corresponding esters, alkanes, etc. [16] High yields of production are achieved when vegetable oils, namely the ones which contain oleic acid, are used, since they consist of saturated or unsaturated fatty acids with the optimal chain length for direct incorporation (16 or 18 carbon atoms) [17].

The sophorolipid biosynthetic pathway, together with the genes involved, has already been established for *S. bombicola*. The first step in sophorolipids biosynthesis consists of terminal (ω) or subterminal (ω-1) hydroxylation of a fatty acid performed by the cytochrome P450 monooxygenase CYP52M1. After, two glucosylstransferases, which act in two distinct steps, are responsible for a subsequent glycosylation of the hydroxylated fatty acid: UGTA1 catalyses the transfer of a first glucose molecule from UDP-glucose, generating a glucolipid, which then receives a second glucose molecule transferred by UGTB1 from UDP-glucose. This results in the formation of an acidic, non-acetylated sophorolipid molecule. However, the majority these molecules are subsequently acetylated resulting in mono or di-acetylated sophorolipids. The acetylation at the 6′- and/or 6′′-position is carried out by an acetyl-coenzyme A (CoA) dependent acetyltransferase. Finally, the molecule is secreted by MDR proteins, which are membrane integrated transporters, to the extracellular space, where lactonic sophorolipids are formed. This final step is performed by a lactone esterase which catalyses the esterification of the carboxyl group of the hydroxy fatty acid with a hydroxyl group of sophorose at the 4”-position. [13]

1.4. Industrial potential of *S. bombicola*

The InBio.be group has started the pioneering work of creating genetic manipulation tools for the yeast *S. bombicola*, which allowed the creation of several new strains via knock-in’s and knock-out’s in the wild type. [18] This enabled the generation of a battery of yeast strains whose production is directed towards glucolipids [19], bola-sophorolipids [12], non-acetylated sophorolipids [20], among others. Furthermore, the production of the bioplastic polyhydroxyalkanoate (PHA) and of a cellobioselipid biosurfactant was used as a proof of concept that *S. bombicola* could be used as a platform organism for the production of ”new-to-nature” molecules. The production of these compounds was done by introducing the respective expression cassettes into the *S. bombicola* genome using a knock-out/knock-in strategy at the *locus* of one of the genes of the sophorolipid biosynthetic pathway. [5]

S. bombicola is also capable of producing alkyl polyglucosides (APGs) in a single step starting from secondary alcohols. However, if grown on primary alcohols, which are less costly and commercially available, the strong activity of a fatty acid oxidase (FAO1) will convert them into fatty acids. In [21], production of APGs from primary alcohols was possible by using a *S. bombicola* strain with the fatty alcohol oxidase gene (*fao1*) knocked-out. However, an expected high production of diolpolyglucosides (DPGs) was also observed, which indicates that *S. bombicola* has ω and ω-1 activity towards alcohols.

The last example illustrates the major drawback for the industrialisation of the sophorolipids produced by *S. bombicola*, related with the variety of products obtained with this yeast [22]. For commercialisation is necessary to obtain a standardised product, since the physicochemical properties of the final product cannot vary according to the composition of the produced mixture. [23]

Genetic engineering of *S. bombicola* can be used has a strategy to selective produce certain molecules, which has already been done to produce either lactonic or acidic sophorolipids [23]. However, the full exploitation of this technique depends on fully understanding the versatile oxidative power of *S. bombicola*.

1.5. Conclusion

The natural mixture produced by *S. bombicola* can vary in terms of acidic, lactonic or bolaform sophorolipids, with different degrees of acetylation, number of unsaturations and position of the hydroxyl group. The composition of the produced mixture is influenced by the culturing conditions (pH, presence of citrate, aeration, etc.), but the complex regulation of sophorolipid biosynthesis is not yet understood. In this master's thesis, the influence of the enzymes responsible for oxygenation reactions in *S. bombicola* was studied in an attempt to understand the versatile oxidative power of this yeast. A more particular objective was using the production of product A as a proof of concept to find the gene responsible for the hydroxylation of a specific substrate in *S. bombicola*.

Therefore, the first part of the experimental work consisted in genetic engineering *S. bombicola*. In the second part, engineered strains were tested by performing shake flask growth trials, in order to determine the type of sophorolipids produced by them.

2. Material and Methods

2.1. Strains, media and culture conditions

Strains: *S. bombicola* (SB) ATCC 22214 was used as the wild-type (WT) strain. The engineered strains were PT36 strain, an *ura3* deficient strain, and strains with knock-outs (KO) (single or in combination) in genes 1, 2, 3 and 6. These strains will be designated using the Δ symbol followed by the gene deleted. The *Escherichia coli* Top10 strain was used for the *E. coli* transformations and to harbour plasmids.

Culture conditions: SB was grown at 30°C and *E. coli* at 37°C. When liquid media was used, both cells were cultured at 200 rpm.

Non-selective media: YPD medium (20 g/L glucose.H_2O; 10 g/L yeast extract; 20 g/L bacto peptone; 20 g/L agar) was used for the growth of SB, whereas *E. coli* strains were grown on LB medium (10 g/L tryptone; 5 g/L yeast extract; 5 g/L NaCl; 15 g/L agar). To count Colony Forming Units (CFU), a 3C-agar medium (110 g/L glucose.H_2O; 10 g/L yeast extract; 1g/L urea; 20 g/L agar) was used.

Selective media: *E. coli* was selected on LB medium supplemented with 50 mg/mL ampicillin. For SB, the selective medium depends on the marker present on the KO cassette: strains with the *ura3* marker were selected on solid SD+CSM-U medium (6.7 g/L YNB without amino acids; 20 g/L glucose.H_2O; 0.77 g/L CSM-ura; 20 g/L agar), whereas strains with the hygromycin resistance gene were selected on YPD medium supplemented with 1000 mg/L hygromycin B from *Streptococcus hygroscopus*.

Production media: during shake flask growth trials, Lang medium, described by *Lang et al.* (2000) [24], was used for sophorolipid production. The substrate was added after 48 h.

2.2. Molecular methods

Plasmid extraction: plasmids were extracted from *E. coli* Top10 strains using the Analytik Jena miniprep plasmid kit.

Genomic DNA extraction: a mixture of 200 μL of breaking buffer (50 mM Tris; 100 mM NaCl; 1% SDS; 2% Triton X-100; 1 mM EDTA) and 200 μL of glass beads was used to break the cell wall and nuclear envelope and 400 μL of Phenol:Chloroform:Isoamyl alcohol saturated with TE buffer were used for the precipitation and removal of proteins. After centrifugation (13k rpm, 10 min), the aqueous top layer, which contains the DNA, was recovered, and absolute ethanol was used to precipitate DNA. Two final washing steps using ethanol 75% were performed to remove all traces of organic solvents. In the end, after the pellet was left to dry by contact with air, the DNA was dissolved in 50-100 μL of TE buffer.

Polymerase Chain Reaction (PCR): high fidelity polymerases - PrimeSTAR HS DNA Polymerase (Takara, Clontech), PrimeSTAR GXL DNA Polymerase (Takara, Clontech) and Q5 High-Fidelity DNA Polymerase (NEB) - were used when the aim was to amplified DNA to be used in transformation and plasmid assembly. To identify correctly transformed cells (colony PCR), Taq DNA polymerase (NEB) and OneTaq Quick-Load 2X Master Mix with Standard Buffer (NEB) were used. The appropriate primers for each PCR reaction were chosen using the *Clone Manager* Sci-Ed software, which also allows to determine the optimal annealing temperature between the primers and the template.

Gel eletrophoresis: it was run in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 25 minutes under a voltage of 120 V. The gel was composed of 1% agarose in 1x TAE buffer.

PCR product purification: it was performed using innuPREP PCRpure Kit from Analytik Jena.

Circular polymerase extension cloning (CPEC): it was used to create circular plasmids from linear DNA fragments. Since it requires the existence of overlapping regions between the pieces to be assembled, the primers used to linearise the fragments contained approximately 20 bps at the 5' end which overlap with the other piece. The CPEC was performed using Q5 High-Fidelity DNA Polymerase with its standard protocol.

Sequencing: DNA Sanger sequencing of constructed plasmids was done by Macrogen inc. DNA solutions.

2.3. Transformation techniques

E. coli: it was transformed using heat shock or eletroporation. For eletroporation, 2 μL of the CPEC mixture were added to 50 μL of eletrocompetent cells and transferred to a cold 0.2 cm sterile biorad gene pulser cuvette. Eletroporation was performed in biorad gene pulser with a resistance of 200 Ω and a voltage of 2.5 kV. In the heat sock protocol, the culture of cells was centrifuged and 100 μL of cold sterile $CaCl_2$ 100 mM was added to the pellet. After, 3μL of the CPEC product were added to this suspension and stirred. After a period of 20-30 minutes on ice, the eppendorf was put in a bath at 42°C for 45 seconds and then on ice again for 2 minutes. After each transformation technique, the mixture was incubated for 1 hour with LB medium before plating on selective medium.

S. bombicola: the transformation technique used was eletroporation. To obtain competent cells, an overnight culture of the strain indented to be transformed was centrifuged (4000 rpm, 4°C, 5 minutes) and washed two times with sterile *Milli-Q* water. The pellet was then sequentially dissolved and centrifuged with sorbitol 1 M, Li-acetate 0.1 M + DTT 2.5 mM, and again with sorbitol 1 M. In the end, the pellet was ressuspended in 250 μL of sorbitol 1 M and divided into volumes of 50 μL which were put in sterile eppendorfs, together with 50 μL of glycerol 70%, and stored in the -80°C freezer. Before performing the eletroporation, the previously prepared cells were washed with sorbitol 1 M and the pellet ressuspended in 50 μL of sorbitol 1 M. The suspension was mixed with 500-1000 ng of linearised DNA. Eletroporation was performed as describe for *E. coli*, but with a voltage of 1.7 kV. The cells were incubated for 1 hour in YPD before being plated in the selective medium.

2.4. Analytical techniques

Nanodrop: spectrophotometer Nanodrop 2000 from Thermo scientic was used to quantify and assess purity of DNA. The concentration of DNA was determined by measuring the absorbance at 260 nm. To analyse purity absorbance at 280 nm and 230 nm was also measured. A 260/280 ratio < 1.8 indicated protein pollution, whereas a 260/230 < 2 indicated solvent pollution.

pH: it was measured with a Five easy F20 Mettler Toledo pH/mV meter.

Optical Density (OD): it was measured with a Jasco V-630 Bio Spectrophotometer at a wavelength of 600 nm.

Thin layer chromatography (TLC): samples were spotted on the bottom of a TLC silica plate, which was placed in a glass chamber together with an eluent, composed of chloroform, methanol and water (65:15:2, V:V:V), at a lower level. After migration of the eluent through the plate, this was removed from the chamber and dried to remove the solvents. Lastly, a detection step was performed by immersing the plate in a 10% H_2SO_4 solution and heating it with a paint dryer until spots appeared on the plate.

Ultra performance liquid chromatography (UPLC): it was performed on a ACQUITY UPLC system (Waters) and Evaporative Light Scattering (ELS) was used as the detection method. *Glucose analysis*: glucose samples were run on a Waters Acquity UPLC BEH Amide 2.1x100 mm column (Hydrophilic Interaction Chromatography). The eluent, 0.25% triethylamine (TEA), 75% acetonitrile (ACN) and 24.75% *Milli-Q* water, was used in isocatric mode with a flow rate of 0.6 mL/min during a period of 4 min for each sample. *Sophorolipid analysis*: product samples were run on Acquity UPLC CSHTM C18 1.7 μm, 2.1x50 mm column (Reversed Phase Chromatography). A gradient elution with two solvents, 0.5% acetic acid in *Milli-Q* water and 100% ACN, was used to separate the components. For each sample, the gradient started at 5% ACN and linearly increased to 95% in 10 min with a flow rate of 0.6 mL/min.

Liquid chromatography – mass spectrometry (LC-MS): the analysis was performed in a Exactive™ Plus Orbitrap Mass Spectrometer (Thermo Scientific) with the purpose of identifying the type of sophorolipids present in a product sample. The column used was the same used for UPLC sophorolipid analysis. The MS had an m/z range of 215-1800 and Heated-Electrospray Ionization in negative mode was used for ion source.

2.5. Shake flask growth trials

Growth trials with *S. bombicola* strains were performed in triplicate in 250 mL Erlenmeyers during 10 days at 30°C and 200 rpm. The cells were grown in Lang Medium and the substrate was added after 48h at a concentration of 37 g/L.

During the period of incubation, samples were taken to measure OD, pH, CFU, glucose concentration and sophorolipid production. To measure pH and glucose, a sample of 500 μL of culture was centrifuged for 5 min at 13K rpm and the supernatant divided into two tubes: 50 μL to measure glucose, which was diluted 20 times, and the rest to measure pH. To prepare the samples to detect sophorolipid production, 1 mL of absolute ethanol was added to 500 μL of culture. After shaking for 5 minutes, the mixture was centrifuged for 5 min at 13K rpm and the supernatant recovered. In order to count CFU, 90 μL of saline solution (0.9% NaCl) and 10 μL of culture were added into a well of a 96-well microplate and successive 10 time dilutions were applied until a dilution of 10^{-8} was obtained. The appropriate dilutions were plated on 3C medium and incubated at 30°C until individual colonies were formed.

3. Results and Discussion

3.1. Engineering *S. bombicola*

The first part of this master's thesis consisted in genetic engineering SB strains by knocking-out genes encoding for oxidising enzymes. These enzymes are involved in alkane metabolism and some of them have also been identified as responsible for hydroxylation of other substrates.

The mechanism used to knock-out genes in *S. bombicola* strains was homologous recombination. Therefore, SB was transformed with a KO cassette which contains the selective marker (*ura3* or hy-

gromycin resistance gene) flanked at the 5' and 3' side by 1000 bps of the sequence upstream and downstream the target gene, respectively - homologous regions (HRs). The KO cassette is part of a plasmid assembled in *E. coli* cells. During this work, the plasmid which contains the gene 4 KO cassette was constructed, which was then used to linearise the cassette and transform the PT36 and Δgene6 SB strains. The same attempt was made using the gene 5 KO cassette. However, the transformations were not successful, since random integration might have occurred in the genome, instead of the indented homologous recombination. In fact, *S. bombicola* has a low transformation efficiency, which can be due to the fact that it prefers non-homologous end joining instead of homologous recombination as the repair mechanism of DNA double-strand breaks. The attempt to KO gene 1 on the Δgene3, Δgene3Δgene6 and Δgene2Δgene6 strains was also not successful. For this transformation, an overgrow of cells was always observed after each transformation attempt, which was later on attributed to lack of effectiveness of the hygromycin antibiotic used for selection.

The strains which contain a deletion in gene 6 were intended for the particular objective of this master's thesis of producing product A from a specific substrate. For this to be possible, there cannot be activity of enzyme 6, since this enzyme oxidises this substrate. However, the isolated production of product A has not yet been possible, since there is also an enzyme with hydroxyation activity towards this substrate, which has not yet been identified. Based on past transcriptomics results, the enzyme encoded by gene 2 is a good candidate, so a different strategy was used to create the Δgene1Δgene2Δgene6 SB strain.

3.2. Shake flask growth trials

3.2.1 Growth trial with WT, Δgene1, Δgene2 and Δgene3 SB strains

In this growth trial, strains with single knock-outs in genes which encode for oxidising enzymes were tested in order to evaluate the type of sophorolipids produced in comparison with the wild type. The goal of this experiment was to understand which oxidising enzymes are involved in sophorolipid biosynthesis and what is their influence on the type of products obtained.

The UPLC chromatograms for sophorolipid analysis, showed that the Δgene1 and Δgene3 strains were affected in terms of production since the peak corresponding to the substrate was practically the only one visible. However, LC-MS showed that sophorolipids are still being produced by these strains, but there is less variety in the sophorolipid mixture, comparing with the WT and Δgene2 strains. In Table 1 it can be seen the summary of the compounds identified on the LC-MS.

Table 1: Summary of the compounds identified on the LC-MS chromatograms of the WT, Δgene1, Δgene2 and Δgene3 SB strains from the tenth day of the growth trial.

Compounds	Strains
Product F	WT, Δgene2
Product C	All
Product E	Δgene1
Product B	All
Product D	WT, Δgene2

Product F could only be identified in the WT and Δgene2 strain, which could be due to a regulatory effect caused by the KO of the genes 1 and 2, which makes the activity of the enzymes involved in the sophorolipid biosynthesis more specific.

Finally, the presence of products D and E for the WT, Δgene2 and Δgene1 strains indicates that the enzyme encoded by gene 3 could be the one with a preference for accepting a substrate with a different degree of saturation. However, these products are probably also being produced at a quicker rate for the WT and Δgene2 strains, since these products shift to the form of product D sooner in these strains, comparing with the Δgene1 strain. This could mean that both enzymes encoded by gene 1 and 3 are involved in the conversion of the substrate with a different degree of saturation. Therefore, the lack of products D or E in the Δgene3 strain could be due to the fact that the enzyme encoded by gene 1 is more selective towards the specific substrate used than the enzyme encoded by gene 3.

3.2.2 Growth trial with Δgene1, Δgene2 and Δgene1Δgene2 SB strains

In the previous growth trial was demonstrated that there must be one or more oxidising enzymes capable of catalysing the hydroxylation of the substrate. Even though the enzyme encoded by gene 2 does not seem do play a role in sophorolipid biosynthesis, based on the previous results, the effect of a double KO (Δgene1Δgene2 strain) might lead to different conclusions. The activity of this enzyme might only become evident in the absence of the hydroxylation activity of the enzyme encoded by gene 1, which will be investigated in this experiment.

Regarding sophorolipid production, the summary of the compounds identified in this growth trial by LC-MS can be found in Table 2.

Table 2: Summary of the compounds identified on the LC-MS chromatograms of the Δgene1, Δgene2 and Δgene1Δgene2 SB strains from the tenth day of the growth trial.

Compounds	Strains
Product F	Δgene2
Product C	All
Product E	Δgene1
Product B	All
Product D	WT, Δgene2

The only compounds which could be identified for the Δgene1Δgene2 strain are products B and C, so there might be a tendency towards a selective production of these compounds when the sophorolipid biosynthetic pathway is under stress due to the absence of two oxidising enzymes.

This experiment allowed to conclude that the Δgene1Δgene2 strain is producing a sophorolipid mixture with less variety than the Δgene1 strain. Therefore, the enzyme encoded by gene 2 might play a role in the production, even though the previous growth trial did not show a difference between the WT and Δgene2 strain, in terms of types of compounds identified. However, one fact that remains is that the production still occurs with both of these genes knocked-out, which means that another enzyme can secure the production. A transcriptomic analysis on this strain could allow to identify this enzyme.

3.2.3 Growth trial with Δgene1Δgene2, Δgene1Δgene6 and Δgene1Δgene2Δgene6 SB strains

The Δgene1Δgene2Δgene6 SB strain was created in order to investigate if the enzyme encoded by gene 2 is responsible for the hydroxylation of a specific substrate. The selective production of product A from this specific substrate using this strain was intended to be used as a proof of concept of this hypothesis.

The experience did not produce the desire results since there is no significant difference between the UPLC chromatograms of the Δgene1Δgene6 and Δgene1Δgene2Δgene6 strains (Fig. 2).

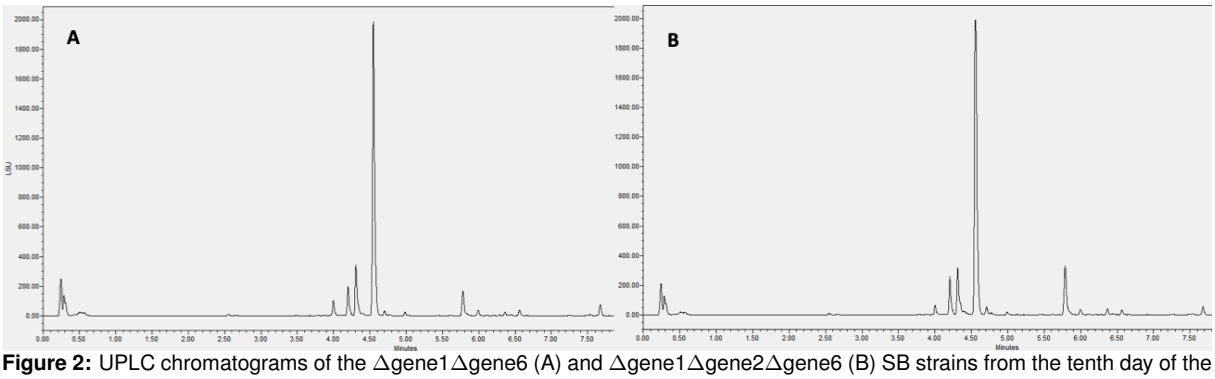

Figure 2: UPLC chromatograms of the Δgene1Δgene6 (A) and Δgene1Δgene2Δgene6 (B) SB strains from the tenth day of the growth trial.

Both of these strains are producing product H, which correspond to the peaks between 4 and 4.60 minutes. The only peak identified as the desired product is the one that appears at minute 6.0. The results are sufficient to conclude that the enzyme responsible for hydroxylation of the specific substrate is not yet identified, since its activity is leading to the formation of product H. The next attempt to identify this enzyme should be performing a RNA seq analysis of the Δgene1Δgene2Δgene6 strain.

4. Conclusion and Future Perspectives

This experimental work allowed for a preliminary investigation of the compounds produced using different engineered SB strains. The single KO growth trial, in which the WT, Δgene1, Δgene2 and Δgene3 strains were tested, allowed to conclude that when genes 1 and 3 are knocked-out, there is a lower variety of the compounds produced by *S. bombicola*, while no significant difference was encountered for the WT and Δgene2 strain. However, when the deletion of gene 2 was combined with a KO in gene 1, there was a lower number of compounds being produced in comparison with the Δgene1 strain. The compounds which were common to all strains in these growth trials were products B and C, while product F was only detected for the WT and Δgene2 strain. Furthermore, these strains are also producing a compound, which corresponds to the highest peak in the LC-MS analysis, that could not be identified, which might implicate performing a LC-MS/MS analysis.

Future efforts to understand the versatile oxidative power of *S. bombicola* include the engineering of more strains. Strains combining the deletion of gene 6 with genes that encode for other oxidising enzymes should be created, since the gene responsible for the hydroxylation of the specific substrate, used to produce product A, was not yet identified. Another candidate is gene 5, but the attempts to knock-out this gene were not yet successful. Furthermore, as a future perspective, it could also be included transcriptomic analysis of relevant strains, quantification information about the formed products and the analysis of the effect of fermentation conditions on the type of sophorolipids being produced by the engineered strains.

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