CHEMICAL ENGINEERING TRANSACTIONS

VOL. 49, 2016

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet

Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz
Copyright © 2016, AIDIC Servizi S.r.l.,
**ISBN** 978-88-95608-40-2; **ISSN** 2283-9216

DOI: 10.3303/CET1649043

# Engineering *Ashbya Gossypii* for the Production of Ricinoleic and Linoleic Acid

Patricia Lozano-Martínez, Rodrigo Ledesma-Amaro, Jose Luis Revuelta*

Departamento de Microbiología y Genética, Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca, Spain
revuelta@usal.es

*Ashbya gossypii* is a natural overproducer of riboflavin or vitamin B2, and it is used nowadays in industrial scale for its production. This filamentous fungus is suitable for metabolic engineering approaches due to the development of a large number of molecular biology techniques, its fully sequenced genome and its high similarity to the model organism *Saccharomyces cerevisiae*. Additionally, it has been recently proposed as a paradigm in biolipid production, because of its highly active lipid metabolism and its ability to accumulate triacylglycerol.
We here propose the use of *A. gossypii* for the production of ricinoleic acid and linoleic acid, two high value lipids with different industrial applications. The first is widely used as a building block for biopolymers and the second is essential for human health. Interestingly, *A. gossypii* mainly accumulates oleic acid, which is the common substrate for the synthesis of ricinoleic and linoleic acid. With this purpose, we manipulated *A. gossypii* by the heterologous expression of a gene from *Claviceps purpurea*, *CpFAH12*, an enzyme than inserts either a hydroxyl group or a double bond in the twelfth carbon of oleic acid. In our conditions, ricinoleic acid is only synthesized in modest amounts in an engineered strain that achieves high lipid production. However, linoleic acid accumulation is produced up to 20 % with respect to total fatty acids in one of the engineered strains, 15-fold the wild type amount. The genetic background of this strain consists on the simultaneous overexpression of the two $\Delta 9$ desaturases identified in *A. gossypii*, which catalyse the insertion of the first double bond in palmitic and stearic acid, producing palmitoleic and oleic acid respectively. This strain presents an increased lipid accumulation with respect to wild type as this desaturation step is one of the rate limiting steps in lipid synthesis. Therefore, *CpFAH12* major contribution in *A. gossypii* lipid synthesis is related to its $\Delta 12$ desaturase function, enhancing linoleic acid synthesis and representing a great starting point for metabolic engineering of this fungus for producing omega-6 and omega-3 fatty acids.

## 1. Introduction

*A. gossypii* is a filamentous fungus which was firstly identified for its natural capacity to overproduce riboflavin (vitamin B2) and it is currently used for its industrial production (Stahmann et al., 2000). Apart from its suitability for large scale fermentations, *A. gossypii* is an excellent host for metabolic engineering since its genome is well annotated, and a wide range of genetic tools have been developed thanks to its phylogenetic relationship with the model organism *S. cerevisiae*. Moreover, *A. gossypii* is able to use oil, including low cost oils, as the sole carbon source in industrial scale fermentations. These unique features of *A. gossypii* have stimulated the use of this fungus for biolipid production (Ledesma-Amaro et al., 2015a) and nucleosides production (Buey et al., 2015).
The use of microorganisms for the synthesis of biofuels and other wide range of value-added oleochemicals is a promising alternative to fossil oils. The use of microorganisms has important advantages such as the use of renewable or even waste feedstocks, which do not compete with food. In the last years, studies unraveling the lipid metabolic pathways in microorganisms have boosted the application of systems metabolic engineering to produce biolipids (Beopoulos et al., 2014; Nunes et al., 2014).
Engineered strains of *A. gossypii* are able to accumulate up to 70 % of its dry cell weight as lipids when they were cultured on a medium supplemented with 2 % of oleic acid (Ledesma-Amaro et al., 2015a). The

Please cite this article as: Lozano Martinez P., Ledesma Amaro R. , Revuelta J.L., 2016, Engineering ashbya gossypii for the production of ricinoleic and linoleic acid, Chemical Engineering Transactions, 49, 253-258 DOI: 10.3303/CET1649043

understanding of the lipid metabolic pathway is the key to enhance lipid synthesis in *A. gossypii* and to redirect the metabolic fluxes to the production of valuable fatty acids such as ricinoleic and linoleic acid.

On the one hand, ricinoleic acid (abbreviated as RA, (R)-12-hydroxyoctadec-cis-9-enoic acid, C18:1-OH) is a hydroxyl fatty acid with many specialized uses in the industrial chemistry. RA and its derivatives are used in food industry (additive), textile (surfactants, pigment wetting agents), paper, plastics, perfumes and cosmetics, electronics, pharmaceuticals, paints, inks and adhesives (Beopoulos et al., 2014). On the other hand, linoleic acid (LA, (9Z, 12z)-octadec-9,12-dienoic acid) is one of the denominated omega-6 fatty acids and it is essential in human diet. It is usually found in vegetable oils, eggs and chicken meat. Apart from its importance in food industry, RA is also used by the cosmetic industry.

RA is commonly extracted from castor bean (*Ricinus communis*), where it is accumulated in triacylglycerol (TAG) and it can reach up to 90 % of total fatty acids. However, ricin, a potent toxin, is also accumulated in these seeds, which is a serious health risk for the workers during harvesting and refining. The enzyme oleate hydroxylase (*RcFAH12*), responsible for the production of RA in *R. communis*, was identified thanks to its homology with fatty acid desaturases (Van de Loo et al., 1995). Afterwards, RA has been produced in *Arabidopsis thaliana* and in the model yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* by the heterologous expression of *RcFAH12*. The fact that these species are non-oleaginous has reduced the possibilities of producing RA in sufficient high quantities for industrial purposes. In *S. cerevisiae*, the expression of *RcFAH12* achieved 10 % of RA accumulation respect to total fatty acids. In *S. pombe*, the expression of this gene and the deletion of TAG lipases lead to a 53 % of RA accumulation respect to total fatty acids (Holic et al., 2012; Mavraganis et al., 2010). Apart from RA production from castor bean, *Claviceps purpurea*, a fungal pathogen that causes ergot in cereal crops, has been subject of scientific studies due to its ability to accumulate RA up to 50 % of total fatty acids. The hydroxylation of oleic acid at the 12 position is catalysed by an oleate 12-hydroxylase, *CpFAH12* (Mavraganis et al., 2010; Meesapyodsuk and Qiu, 2008). This hydroxylase has a double function, since it is either able to introduce a hydroxyl group in the twelfth carbon of oleic acid, or a double bound, converting oleic acid into LA. This enzyme had been heterologously expressed in the oleaginous yeast *Y. lipolytica* for producing RA with the simultaneous effect of increasing LA (Beopoulos et al., 2014).

Fatty acid (FA) composition in *A. gossypii* is determined by the desaturase and elongase system, which has been recently characterized (Ledesma-Amaro et al., 2014). The FAS complex synthesizes fatty acids up to sixteen and eighteen carbons, C16:0 and C18:0 (Figure 1). The elongase *AgElo624* adds up to two sets of two carbons to C18:0 while the second elongase *AgElo586* is also able to produce C20:0 and C22:0 and to add two more groups of two carbon backbone to get C24:0 and C26:0.The synthesis of monounsaturated forms of these fatty acids is catalysed by two Δ9 desaturases present in *A. gossypii*. Furthermore, modest amounts of linoleic acid are synthesized. Oleic acid is the main synthesized fatty acid in this fungus, representing more than 50 % of total fatty acids. As oleic acid is the substrate for ricinoleic and linoleic acid synthesis both in plants and microorganisms, we propose to use *A. gossypii* as a source of these two valuable products. The single overexpression of the Δ12 desaturase codified by the gene *AFR589C* led to a 5-fold production of LA with respect to wild type in a previous work (Ledesma-Amaro et al., 2014).

With the aim of producing ricinoleic acid and enhancing linoleic acid synthesis in *A. gossypii*, *CpFAH12* was heterologously expressed. This oleate hydroxylase enzyme has a well-established bifunctional hydroxylation/desaturation activity that has been corroborated in this work.

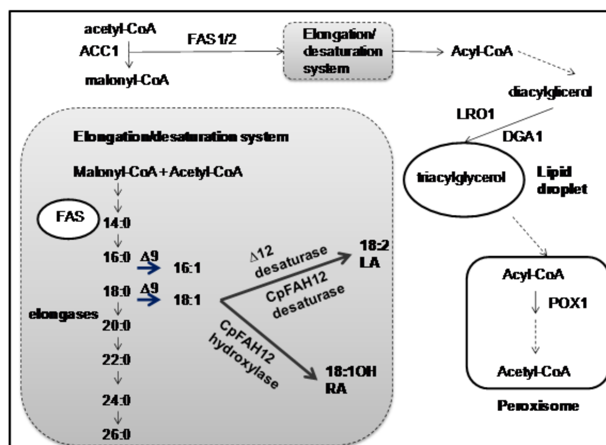


*Figure 1: Schematic representation of lipid metabolic pathway in A. gossypii*

## 2. Materials and methods

### 2.1 Genetic manipulation of *A. gossypii*

Gene deletion and overexpression were carried out by the construction of recombinant integrative cassettes (Choi et al., 1988; Ledesma-Amaro et al., 2014). For gene deletion a replacement cassette with selection marker (*loxP-KanMX-loxP* module for *G418* resistance) was used. This selection marker is flanked by the repeated inverted sequences *loxP* which enable the elimination of the selection marker by a Cre recombinase (Güldener et al., 1996). In the case of overexpression constructs, a module containing the selection marker followed, the *GPDp* constitutive strong promoter from *A. gossypii* and recombination flanks was integrated. Genomic integration of the deletion and overexpression modules was confirmed by analytical PCR and DNA sequencing.

### 2.2 Golden Gate cloning system for heterologous expression of genes in *A. gossypii*

The Golden Gate method allows the assembly of multiple DNA fragments into a recipient vector in a single step. The method is based on the properties of type IIs restriction enzymes (i.e.: *Bsa*I) to cleave DNA outside of the recognition sequence and leave user-defined sticky ends. This property and the design of the primers with a sequence of cohesive nucleotides allow the removal of the restriction sequences and the ligation of the different fragments in a specific order. DNA fragments flanked by appropriate *Bsa*I sites and *Bsa*I-cut recipient vector were assembled by incubation at 37 ºC for 3 min., then 5 minutes at 16 ºC and finally 50 cycles of 50 ºC for 5 min. followed by 80 ºC for 5 min in the presence of *BsaI* enzyme, T4 ligase and Golden Gate buffer as described elsewhere (Enger et al., 2008). The ligation product was used to transform *Escherichia coli* DH5$\alpha$ cells and correct constructs used to obtain the corresponding *A. gossypii* integration transformants.

### 2.3 Lipid extraction and quantification

Triacylglycerols were extracted and transmethylated from lyophilized biomass using a modification of the method described by Bligh and Dyer. Approximately two hundred micrograms of dried mycelia was mixed with 1 ml of 97.5 % methanol 2.5 % sulfuric acid. The samples were incubated at 80 ºC for 90 minutes. The transesterification reaction was stopped by the addition of 1 ml of distilled water. The extraction was made with 0.5 ml de hexane and after mixing, the upper phase was recovered by centrifugation. Fatty acid methyl esters dissolved in the hexane were analysed by gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS was carried out using an Agilent 7890A gas chromatograph with an Agilent MS200 mass spectrometer. A VF50 column (30 m long, 0.25 mm internal diameter and 25 µm film) was used. The conditions for the analysis were as follows: helium was used as the carrier at 1 ml/minute, with a split ratio of 1:20. The oven program was as follows: an initial temperature of 90 ºC for 5 min, a ramp of 12 ºC/min up to 190 ºC, and a ramp of 4 ºC/minute up to 290 ºC. Mass spectrometric detection was from 50 to 400 Da. Fatty acids were identified by comparison with commercial fatty acid methyl ester standards (FAME32; Supelco) and total quantification of fatty acids, expressed as total fatty acids (TFA) was performed by using an internal standard: 50 µg of heptadecanoic acid C17:0 (Sigma).

## 3. Results and discussion

### 3.1 Heterologous expression of *CpFAH12* in *A. gossypii*

Two genetic constructions were carried out using Golden Gate methodology for the heterologous expression of *CpFAH12*. The difference between the two constructions resides on the promoter used: *GPDp* a strong constitutive promoter, and *ICLp* an inducible promoter activated by acetate (Umemura et al., 1995). This latter promoter was proposed because of the cell toxicity to high amounts of ricinoleic acid described in certain yeasts. Most widely used media for *A. gossypii* is MA2, which consists in 2 % of glucose, 0.2 % of yeast extract, 2 % of bactopeptone and 0.06 % of myo-inositol. We optimized media composition by increasing carbon-nitrogen ratio in order to enhance lipid accumulation. Thus, we used MA2 8G with 8 % of glucose to grow the strains overexpressing both constructions. For *ICPp* induction, strains were firstly grown in MA2 8G and cells were then passed to media with 2 % of sodium acetate.

Interestingly we observed a slight improvement in total accumulated fatty acids, which increased 10-19 % comparing to the wild type (Table 1). We also observed that the fatty acid profile of the engineered strains remains invariable to the wild type (Figure 2). Furthermore, the strain A537, which expresses *CpFAH12* under the strong promoter *GPDp* is enriched in LA content, up to 40 % more than the wild type. Nonetheless, no RA was produced with the two overexpressions of *CpFAH12*. Although the only expression of *CpFAH12* produced RA in *S. pombe* (Holic et al., 2012), in agreement with our results in *Y. lipolytica* additional modifications were needed to produce RA (Beopoulos et al., 2014). Therefore, we decided to test these two constructions in different backgrounds of *A. gossypii* enhanced for lipid production.

*Table 1: Total fatty acid (TFA) accumulation in the engineered A. gossypii expressed as percentage of dry cell weight (DCW). Cultures were grown in MA2 with 8 % glucose after 3 days. The results are the means of two independent experiments performed in duplicate. The errors represent the standard deviations.*

| Strain | Genotype | % TFA/DCW | % RA/TFA | % LA/TFA |
|---|---|---|---|---|
| A4 | Wild type (ATCC 10895) | 4.9 ± 0.1 | 0 | 1.6 ± 0.5 |
| A537 | *loxP-KanMX4-loxP-*$P_{GPD}$*-CpFAH12* | 5.4 ± 0.2 | 0 | 2.3 ± 0.1 |
| A538 | *loxP-KanMX4-loxP-*$P_{ICL}$*-CpFAH12* | 5.8 ± 0.1 | 0 | 1.5 ± 0.4 |

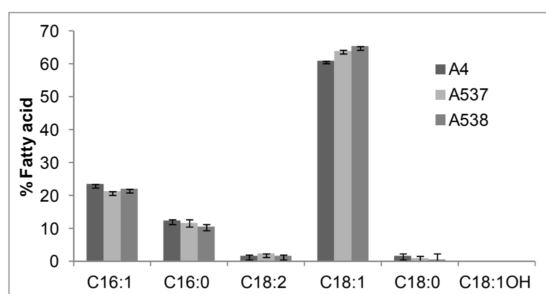


*Figure 2: FA profile in the engineered A. gossypii. Cultures were grown in MA2 with 8 % of glucose for 3 days. The results are the means of two independent experiments performed in duplicate. The error bars represent the standard deviations.*

**3.2 Heterologous expression of *CpFAH12* in different genetic backgrounds of *A. gossypii***

Three different genetic backgrounds were used for the heterologous expression of *CpFAH12*, with the aim of either producing RA or increasing LA production. Firstly, we selected a strain blocked for β-oxidation of fatty acids (strain A355 with *AgPOX1* disruption, Figure 1), which show high lipid production yield. By enhancing total fatty acid accumulation we expected to increase total RA and LA content after the heterologous expression of *CpFAH12*. Secondly, we used a strain with the simultaneous overexpression of the two identified ∆9 desaturases of *A. gossypii* (Jose Luis Revuelta, unpublished results). This first desaturation step is necessary for the conversion of palmitic and stearic acids, which is an essential function in eukaryotic cells since unsaturated fatty acids are necessary for cell membrane formation. Additionally, this enzymatic step has been recently associated with increased lipid accumulation in other yeasts, such as *Y. lipolytica* (Qiao et al., 2105). In *A. gossypii*, two ∆9 desaturases have been identified, *AgOLE1* and *AgOLE2* and therefore a strain with both enzymes overexpressed under *GPDp* promoter was used for expressing *CpFAH12*. Thirdly, *CpFAH12* was expressed in the engineered strain with the deletion of the gene *AgDGA1*. There are two enzymes involved in the last step of TAG formation: the diacylglycerol acyltransferase (DGA1) and the phospholipid:diacylglycerol acyltransferase (*LRO1*) (Figure 1). We identified both enzymes in *A. gossypii* by its homology with the *S. cerevisiae* corresponding genes. *LRO1* is involved in the accumulation of hydroxilated fatty acids such as RA (Dahlqvist et al., 2000). Accordingly, *LRO1* was identified in *Y. lipolytica* as the acyltransferase with certain specificity for the RA (Beopoulus et al., 2014). For this reason, we deleted *AgDGA1* to force all triacylglycerides in *A. gossypii* to be formed by *AgLRO1*.

As a result, RA was only synthesized in the strain blocked in the fatty acids degradation pathway although the accumulated amounts are still low and far from a commercial requirement (Table 2 and Figure 3). We also checked that RA was not present in culture media by supernatant lipid extraction (data not shown) as it was described that RA was secreted to media in *S. pombe* strains showing a high RA production inside the cell (Uemura, 2013). As expected, lipid accumulation in the *AgDGA1* disrupted mutant reached 6.8 % of dry cell weight, similarly to the *AgPOX1* disrupted strain which produced 6.5 % after 3 days of culture. In addition, fatty acid composition was not significantly affected by these modifications (Figure 3).

In the other two backgrounds tested no RA was found, but LA was highly increased. On the one hand, LA production was higher in the strain with disruption of the gen *AgDGA1* and overexpression of *CpFAH12*, where about 12 % of TFA was LA, while in the wild type and parental strain LA was 0.8 % and 1.6 % respectively. This suggests that when *AgLRO1* is the only enzyme capable of converting diacylglycerol into triacylglycerol, LA is a preferred substrate. The main drawback of this strain is that when *AgDGA1* is deleted, total lipids are diminished when compared to wild type. *DGA1* has been identified as one of the main lipidogenic genes in *Y. lipolytica* (Qiao et al., 2015) and its disruption in *A. gossypii* showed the predicted decrease in total fatty acid accumulation (3.5 % of dry cell weight).

On the other hand, in the strain overexpressing both Δ9 desaturases and *CpFAH12*, LA accumulation reached about 20 % of total fatty acids (Figure 3). These results suggest that this strain shows fluxs enhancements from stearic and to oleic acid from oleic to linoleic acid due to the overexpression of both *AgOLE1* and *AgOLE2*, and *CpFAH12* genes, respectivelyt. We also found in both strains overexpressing *AgOLE1* and *AgOLE2*, A570 and A586, an increase in lipid accumulation. This is in accordance to the results in *Y. lipolytica* where the first desaturation step has been described as one of the main bottlenecks in lipid production (Qiao et al., 2015).
We therefore obtained strains of *A. gossypii* able to produce RA and LA. Although we show here the proof of concept that RA can be produced in *A. gossypii*, the produced amounts are still very low and more experiments are required to make the bioprocess economically feasible. In the case of LA, our modified strains were able to produce up to 20 % (15-fold that of wild type) of LA, the precursor or the high valuable omega-3 and omega-6 fatty acids. Thus, this strain can serve as starting point for the additional overexpression of elongases and desaturases to produce EPA and DHA, as it has been previously achieved in *Y. lipolytica* (Xue et al., 2013).

*Table 2: Total fatty acid (TFA) accumulation in the engineered A. gossypii expressed as percentage of dry cell weight (DCW). Cultures were grown in MA2 with 8 % glucose after 3 days. The results are the means of two independent experiments performed by duplicate. The errors represent the standard deviations.*

| Strain | Genotype | % TFA/DCW | % RA/TFA | % LA/TFA |
|---|---|---|---|---|
| A4 | wild type (ATCC 10895) | 4.9 ± 0.1 | 0 | 0.8 ± 0.0 |
| A355 | *pox1Δ* | 6.5 ± 0.2 | 0 | 0.5 ± 0.1 |
| A570 | $P_{GPD}$-*OLE1*, $P_{GPD}$-*OLE2* | 6.4 ± 0.2 | 0 | 2.6 ± 0.0 |
| A534 | *dga1Δ* | 3.5 ± 0.1 | 0 | 1.6 ± 0.1 |
| A549 | *pox1Δ, loxP-KanMX4-loxP-*$P_{GPD}$-*CpFAH12* | 6.8 ± 0.1 | 0.5 ± 0.1 | 1.0 ± 0.0 |
| A586 | $P_{GPD}$-*OLE1*, $P_{GPD}$-*OLE2*, *loxP-KanMX4-loxP-*$P_{ICL}$-*CpFAH12* | 5.9 ± 0.6 | 0 | 20.0 ± 1.1 |
| A588 | *dga1Δ, loxP-KanMX4-loxP-*$P_{ICL}$-*CpFAH12* | 4.6 ± 0.2 | 0 | 11.6 ± 3.2 |

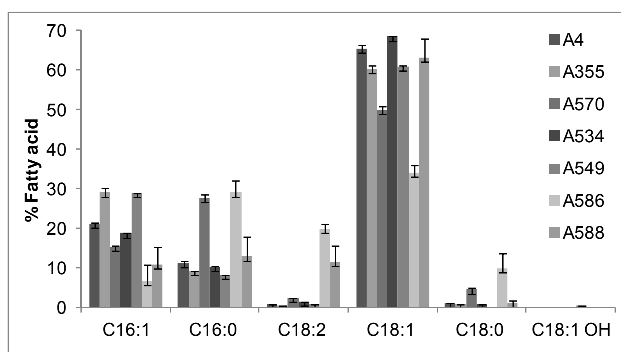


*Figure 3: FA profile in the engineered A. gossypii. Cultures were grown in MA2 with 8 % of glucose within 3 days. The results are the means of two independent experiments performed by duplicate. The error bars represent the standard deviations.*

## 4. Conclusions

*A. gossypii* presents a substantial potential for industrial production of different valuable products such as riboflavin, nucleosides and lipids. In an attempt to increase total fatty acids synthesis in *A. gossypii* and to redirect lipid metabolism towards value-added products such as ricinoleic and linoleic acid, a heterologous gene from *C. purpurea*, *CpFAH12*, was expressed in different genetic backgrounds of *A. gossypii*. Ricinoleic acid is only synthetized in modest amounts in the strains with maximum yield in lipid accumulation, when degradation of fatty acids is abolished. However, linoleic acid is produced in a higher amount reaching up to 20 % or 12 % of total fatty acids in the strain with simultaneous overexpression of the two Δ9 desaturases and the strain deleted for *AgDGA1* respectively. These strains are interesting by themselves since linoleic acid is one of the denominated omega-6 fatty acids, necessary in human diet because humans and mammals are not able to synthesize it. Moreover, the strain with a 15-fold increase in LA could be used for further engineering in order to produce essential omega-3 fatty acids for which linoleic acid is the precursor, such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). We also have found that some genetics

modifications provoke increased total fatty acid synthesis in *A. gossypii* and make the production of lipids in this filamentous fungus a more feasible industrial bioprocess.

**Acknowledgments**

This work was supported by grant BIO2014-56930-P from the Spanish Ministerio de Economía y Competitividad

**Reference**

Beopoulos A., Verbeke J., Bordes F., Guicherd M., Bressy M., Marty A., Nicaud J.M., 2014, Metabolic engineering for ricinoleic acid production in the oleaginous yeast *Yarrowia lipolytica*, Applied Microbiology Biotechnology, 98(1), 251-62, DOI: 10.1007/s00253-013-5295-x

Buey R.M., Ledesma-Amaro R., Balsera M., de Pereda J.M., Revuelta J.L., 2015, Increased riboflavin production by manipulation of inosine 5'-monophosphate dehydrogenase in *Ashbya gossypii*, Applied Microbiology Biotechnology, 99(22), 9577-9589, DOI: 10.1007/s00253-015-6710-2

Choi H.T., Revuelta, J.L., Sadhu, C., Jayaram, M., 1988, Structural organization of the *TRP1* gene of *Phycomyces blakesleeanus*: implications for evolutionary gene fusion in fungi, Gene, 71(1), 85-95, DOI: 10.1016/0378-1119(88)90080-7

Dahlqvist A., Stahl U., Lenman M., Banas A., Lee M., Sandager L., Ronne H., Stymne S., 2000, Phospolipid:diacylglycerol acyltransferase: An enzyme that catalyses the acyl-CoA-independent formation of triacylglycerol in yeast and plants, PNAS, 97(12), 6487-6492

Enger C., Kandzia R., Marillonet S., 2008, A one pot, one step, precision cloning method with high throughput capability, Plos One, 3(11), DOI: 10.1371/journal.pone.0003647

Güldener U., Heck, S., Fiedler T., Beinhauer J., Hegemann J. H., 1996, A new efficient gene disruption cassette for repeated use in budding yeast, 24(13), 2519-2524

Holic R., Yazawa H., Kumagai H., Uemura H., 2012, Engineered high content of ricinoleic acid in fission yeast *Schizosaccharomyces pombe*, Applied Genetics and Molecular Biotechnology, 95(1), 179-187, DOI: 10.1007/s00253-012-3959-6

Qiao K., Imam Abidi S.H., Liu H., Zhang H., Chakraborty S., Watson N., Kumaran Ajikumar P., Stephanopoulos G., 2015, Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*, Metabolic engineering, 29, 56-65, DOI: 10.1016/j.ymben.2015.02.005

Ledesma-Amaro R., Santos M.A., Jiménez A., Revuelta J.L., 2014, Tuning single-cell oil production in *Ashbya gossypii* by engineering the elongation and desaturation systems, Biotechnology and bioengineering, 111(9), 1782-1791, DOI: 10.1002/bit.25245

Ledesma-Amaro R., Lozano-Martínez P., Jiménez A., Revuelta J.L., 2015a, Engineering *Ashbya gossypii* for efficient biolipid production, Bioengineered, 6 (2), 119-123, DOI: 10.1080/21655979.2015.1011525

Ledesma-Amaro R., Serrano-Amatriain C., Jiménez A., Revuelta J.L., 2015b, Metabolic engineering of riboflavin production in *Ashbya gossypii* through pathway optimization, Microbial Cell Factory, 14(1), DOI: 10.1186/s12934-015-0354-x

Mavraganis I., Meesapyodsuk D., Vrinten P., Smith M., Qiu X., 2010, Type II diacylglycerol acyltransferase from *Claviceps purpurea* with ricinoleic acid, a hydroxil fatty acid of industrial importance, as preferred substrate, Applied and Environmental Microbiology, 76(4),1135-1142, DOI: 10.1128/AEM.02297-09

Meesapyodsuk D., Qiu X., 2008, An oleate hydroxylase from the fungus *Claviceps purpurea*: cloning, functional analysis, and expression in *Arabidopsis*, Plant Physiology, 147(3), 1325-1333, DOI: 10.1104/pp.108.117168

Nunes P., Martins A., Brigida A., da Rocha-Leao M., Amaral P., 2014, Intracellular lipase production by *Yarrowia lipolytica* using different carbon sources, Chemical Engineering Transactions, 38, 421-426, DOI: 10.3303/CET1438071

Stahmann K.P., Revuelta J.L., Seulberger H., 2000, Three biotechnical processes using *Ashbya gossypii*, Candida famata or Bacillus subtilis compete with chemical riboflavin production, Applied Microbiology Biotechnology, 53(5), 509-516

Uemura H., 2012, Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives, Applied Microbiology Biotechnology, 95(1), 1-12, doi: 10.1007/s00253-012-4105-1

Umemura K., Atomi H., Kanai T., Teranishi Y., Ueda M., Tanaka A., 1995, A novel promoter, derived from the isocitrate lyase gene of Candida tropicalis, inducible with acetate in *Saccharomyces cerevisiae*, Applied Microbiology Biotechnology, 43(3), 289-492

Van de Loo F.J., Broun P., Turner S., Somerville C., 1995, An oleate 12-hydroxylases from *Ricinus communis* L. is a fatty acyl desaturase homolog, Proceedings of the National Academy of Sciences, 92(15), 6743-6747

Xue Z., et al., 2013, Production of omega-3 eicosapentanoic acid by metabolic engineering of *Yarrowia lipolytica*, Nature Biotechnology, 31(8), 734-740, DOI: 10.1038/nbt.2622