Engineering of Fatty Acid Production and Secretion in *Saccharomyces cerevisiae*

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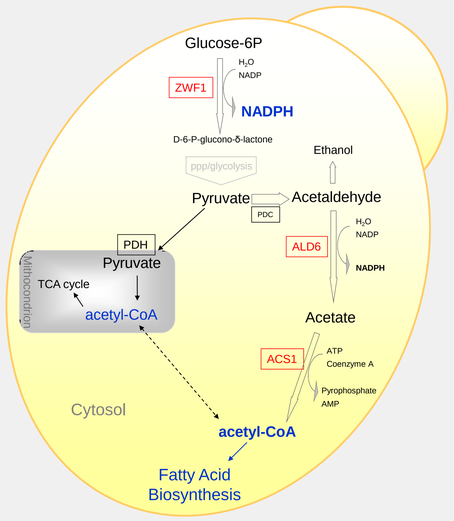
Introduction



Fatty acid biosynthesis is essentially a reversal of fatty acid beta-oxidation but is carried out in the cytosol. It is a demanding process in terms of reducing power in the form of NADPH and cytosolic acetyl-CoA.

The main source of cytoplasmic NADPH in *Saccharomyces cerevisiae* is the glucose-6-phosphate dehydrogenase (encoded by ZWF1) that catalyzes the first irreversible and rate-limiting step of the oxidative pentose phosphate pathway.

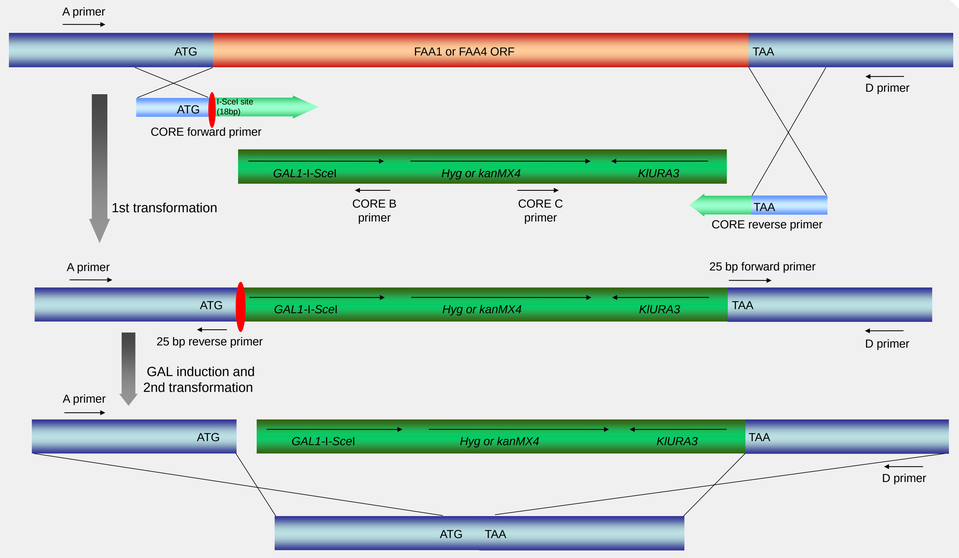
Conversion of pyruvate into mitochondrial acetyl-CoA is catalyzed by the concerted action of the catalytic subunits of the mitochondrial pyruvate dehydrogenase complex. Redirection of the pyruvate metabolism (a pyruvate dehydrogenase bypass) to the cytosol can be constructed by deleting the alpha subunit of the pyruvate dehydrogenase complex (encoded by PDA1). The pyruvate dehydrogenase bypass converts pyruvate into acetyl-CoA by the action of pyruvate decarboxylase, acetaldehyde dehydrogenase (ALD6), and acetyl-CoA synthetase (ACS1). Overexpression of the enzymes involved in the bypass, even without deletion of PDA1, has also been shown to support a high level of acetyl-CoA demanded for isoprenoid production in yeast (Shiba *et al*. 2007).

In this work genetic engineering was performed in order to enhance the supply of NADPH and acetyl-CoA to the fatty acid biosynthesis pathway by overexpression of ZWF1, ACS1 and ALD6 and by deleting PDA1 in a fatty acyl-CoA synthetase null mutant. The FAA1,4 double deletion has been shown to result is secretion of fatty acids (Scharnewski *et al*. 2008).

Methods

***Delitto perfetto* aproach to generate a *S. cerevisiae* FAA1,4 double mutant:**

The method used in this work to delete FAA1 and FAA4 in CEN.PK 113.5D is described by Storici F. and Resnick MA. 2006 with the following modifications:
25 bp oligonucleotides containing the sequence upstream, immediately followed by the sequence downstream, of the region to be knocked out were designed. One PCR was performed using primers A and D, and these 25 bp forward and reverse primers, producing 2 fragments that were then fused is a second PCR. The second step of the method was accomplished by transformation with the fusion product such that the CORE cassette was lost and the desired genetically clean deleted strain was generated.



Overexpression of ZWF1, ALD6 and ACS1

To overexpress genes, we constructed multicopy plasmids derived from p426GPD, p425GPD and p426TEF (Mumberg *et* al. 1995) where ZWF1, ALD6 and ACS1 were cloned.

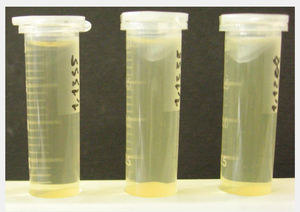
Deletion of PDA1

The PDA1 gene was deleted by inserting the *loxP-KanMX4-loxP* cassette, amplified from pUG6, into the genome of CEN.PK 113-5D ΔFAA1/ΔFAA4.

Cell growth and Lipid analytical methods

Yeast cultures were grown at 30ºC in YPD medium (1% yeast extract, 2% peptone and 2% dextrose).
At the time points indicated, 2 mL of the culture were collected and the extracellular fraction was extracted with 2 mL of n-hexane, after acidification, using pentadecanoic acid as internal standard. The lipids extract was dried under a stream of nitrogen and methylated in 500 uL of BF3/methanol, 30 min at 90ºC. The mixture was then extracted with 500 uL of n-hexane and the fatty acid composition of the supernatant was determined by GC of fatty acid methyl esters (FAMEs). FAMEs were identified by comparing their retention times with those of standards.

Results

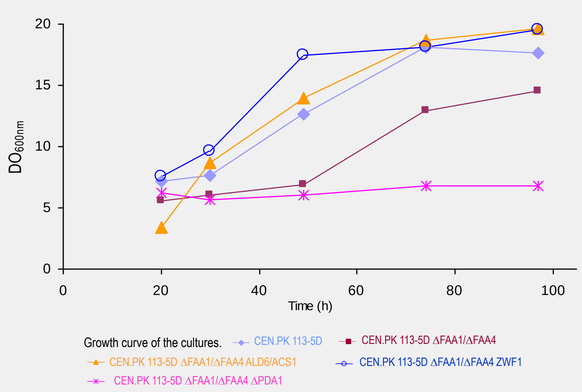


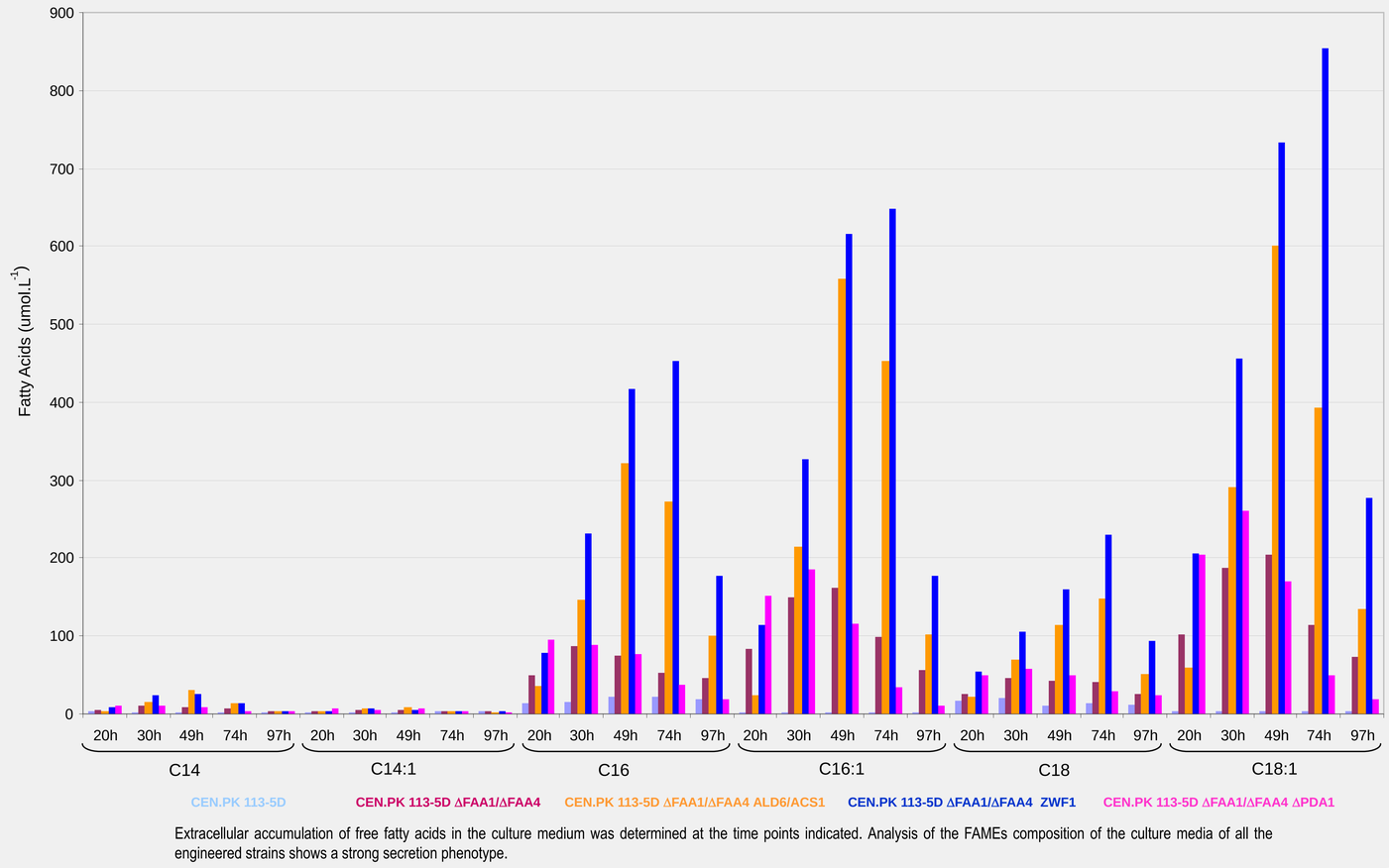
Example of the visual aspect of the fatty acids in the extracellular media after 30 hours of incubation.

Aspect of the supernatant after centrifuging 5 min, 14000 rpm. Left: CEN.PK 113-5D; centre: CEN.PK 113-5D ΔFAA1/ΔFAA4; right: CEN.PK 113-5D ΔFAA1/ΔFAA4 ALD6/ACS1.

Strain	G6PDH activity (umol/mg protein min)
CEN.PK 113-5D ΔFAA1/ΔFAA4	0,22
CEN.PK 113-5D ΔFAA1/ΔFAA4 ZWF1	1,66

Glucose-6-phosphate dehydrogenase activity measured for selected strains





Extracellular accumulation of free fatty acids in the culture medium was determined at the time points indicated. Analysis of the FAMEs composition of the culture media of all the engineered strains shows a strong secretion phenotype.

Final Remarks

The "delitto perfetto" method was successfully applied to delete two fatty acyl-CoA synthetases, creating genetically clean strains without markers or bacterial DNA.

Deletion of FAA1 and FAA4 increased the amount of secreted palmitoleic acid (C16:1) up to 120 times and the amount of secreted oleic acid (C18:1) up to 60 times in accordance with the findings by Scharnewski *et al.* 2008.

The overexpression of ZWF1 causes a 7,7 fold increase in the enzymatic activity.

The amount of secreted fatty acids increased up to 8 fold when ALD6, ACS1 or ZWF1 were overexpressed in the FAA1,4 deleted strain.

The average ratio of secretion of palmitoleic/palmitic and oleic/stearic acids was 1,6 and 4,1 for the analyzed strains.

A fatty acid re-import phenotype was observed, in agreement with results already obtained by Scharnewski *et al*. 2008, for all the mutant strains tested, indicating fatty acid uptake during stationary phase.

The flocculent phenotype observed in other strains carrying deletions in both FAA1 and FAA4 (Faergeman *et al.* 2001, Scharnewski *et al.* 2008) was never observed in our deleted strain.

References

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Acknowledgments

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