

DSM Science & Technology Awards 2005

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Summary of the PhD research project of Dr. A.J.A. van Maris

1. Scope of the project

1.1 Industrial context

The PhD project of Ton van Maris is a typical example of ‘White Biotechnology’; the production of bulk and fine chemicals from renewable carbohydrate feedstocks with the aid of genetically engineered microorganisms. More specifically, the project aimed at investigating the potential of *Saccharomyces cerevisiae* as a host for the production of L-lactic acid from glucose. This target was provided by the industrial partner in this project, Tate & Lyle North America. The world market for L-lactic acid is rapidly expanding, mainly due to the increasing popularity of polylactate (PLA) as a sustainable and biodegradable polymer.

Currently, the world market for L-lactic acid (ca. 300,000 tonnes per annum) is virtually completely covered by fermentation processes that are based on lactic acid bacteria. The idea to look beyond lactic acid bacteria as the production system for L-lactic acid was based on two problems associated to the conventional processes:

- Lactic acid bacteria cannot grow at pH values below the pK of lactic acid (3.8). As a result, fermentation processes based on lactic acid bacteria invariably produce the lactate anion. Consequently, production of free lactic acid, which is the precursor for PLA production, requires extensive down-stream processing. Yeasts, including *Saccharomyces cerevisiae*, grow well at pH values below 3 and might therefore be able to produce free lactic acid. This would tremendously simplify product recovery and, therefore, process economics.
- Polymerization of L-lactic acid requires a high product purity. Lactic acid bacteria have complex nutritional requirements and therefore require the addition of complex medium components which may be difficult to remove from the product.

In contrast, *Saccharomyces cerevisiae* can be grown in completely defined synthetic media.

In summary, development of a yeast-based alternative for L-lactic acid production might improve process economics for the production of a biopolymer from renewable carbohydrate feedstocks.

1.2 Scientific context

At first glance, the metabolic engineering strategy for turning *S. cerevisiae* into a lactic-acid producing microorganism seems very straightforward. Alcoholic fermentation (the normal mode of sugar dissimilation in *S. cerevisiae*) and lactate fermentation are equivalent in terms of ATP yield and redox balance. Therefore, it would seem to suffice to simply exchange pyruvate decarboxylase (the key enzyme of alcoholic fermentation) for a bacterial lactate dehydrogenase. Despite this apparent simplicity, it was clear from the outset of the project that at least two serious challenges would have to be met:

- Deletion of all three structural genes for pyruvate decarboxylase (a prerequisite for the simple strategy outlined above) in *S. cerevisiae* completely abolishes growth on glucose. While it had been shown previously that this was due to a role of pyruvate decarboxylase in the synthesis of cytosolic acetyl-coenzyme A, a precursor for lipid biosynthesis, no metabolic engineering strategies were available to address this problem.
- For unknown reasons, strains of *S. cerevisiae* that lack pyruvate decarboxylase are extremely sensitive to high glucose concentrations. This glucose sensitivity – which can only be circumvented by growth under glucose limitation - is not compatible with large-scale lactic acid production.
- Pilot experiments at the laboratories of the industrial partner suggested that engineered, lactate-producing strains exhibited an unexpected requirement for molecular oxygen. Since respiratory glucose dissimilation would clearly go at the expense of lactate yields on glucose (and would complicate reactor design) this problem required a follow-up.

During the PhD project of Ton van Maris, DNA-microarray technology was implemented at the Industrial Microbiology Section of the Delft University of Technology. This provided the opportunity to use this genomics tool in the context of this application-oriented project.

2. Original goals of the project

1. Develop metabolic engineering strategies to address the acetyl-Coenzyme A requirement of pyruvate-decarboxylase-negative strains
2. Develop metabolic engineering strategies to address the glucose-sensitivity of pyruvate decarboxylase-negative strains
3. Quantify the requirement of engineered 'homolactate-fermenting' strains of *S. cerevisiae* and investigate its physiological background

3. Results

3.1 Elimination of the acetyl-CoA requirement of pyruvate-decarboxylase-negative *S. cerevisiae*

After a thorough analysis of the metabolic network of *S. cerevisiae*, Ton van Maris identified an alternative pathway for the production of cytosolic acetyl-Coenzyme A. This pathway involves the enzyme threonine aldolase (Gly1p), which splits the amino acid threonine into acetyl-coenzyme A and glycine. Under relevant cultivation conditions, the level of threonine aldolase in *S. cerevisiae* is too low to meet the cellular demand for cytosolic acetyl-Coenzyme A. However, overexpression of the *GLY1* gene from a strong constitutive promoter turned out to be sufficient to overcome the cytosolic acetyl-Coenzyme deficiency of pyruvate-decarboxylase-negative *S. cerevisiae*.

3.2 Elimination of the glucose sensitivity of pyruvate-decarboxylase-negative *S. cerevisiae* by evolutionary engineering

The successful elimination of the acetyl-CoA requirement of a pyruvate-decarboxylase-negative mutant still left its glucose sensitivity as a problem to be dealt with. Here, Ton van Maris successfully applied 'evolutionary engineering'. By gradually increasing the glucose concentration in batch cultures, he was able to select a highly glucose-tolerant, acetyl-CoA-independent strain. This strain overproduced pyruvate to record levels. In a non-optimized aerobic batch fermentation, pyruvate titers of 135 g/L were reached. The evolved strain was characterized by DNA-microarray analysis. This suggested a role of *MIG1*-mediated glucose repression in glucose sensitivity. This lead is now being pursued by Ton's successor as a PhD student. This work is unique in that it has enabled a complete redirection of glycolytic flux towards a product (pyruvate) that is not normally produced at high concentrations by *S. cerevisiae*. The strain platform developed in this work has been patented and forms the basis for a range of yeast-based 'white biotechnology' projects.

3.3 Production of L-lactate by engineered *S. cerevisiae* strains: energetic consequences of lactate export

Introduction of a bacterial lactate dehydrogenase in the constructed strain background (see 3.3) allowed for production of > 100 g/L L-lactate in aerobic batch cultures. However, contrary to expectation, no product was formed under anaerobic conditions. Ton van Maris then set up a chemostat with preprogrammed oxygen feeding profiles, which enabled him to quantify the oxygen requirements and bio-energetics of the engineered 'homolactic' *S. cerevisiae* strain. This stoichiometric analysis convincingly demonstrated that lactate production by the engineered yeast strain did not lead to a net yield of ATP. This was explained from an ATP requirement for lactate export. Given the apparent relevance of the energetics of product export in this and other processes, Ton van Maris proceeded with a rigorous theoretical analysis of

the thermodynamics/energetics of the export of organic acids by microorganisms. This analysis convincingly demonstrates that, especially under industrial process conditions (high product concentration, low pH) improving the energetics for product export is a key challenge for this type of fermentation processes.

3.4 Research activities on subjects other than lactate fermentation by *S. cerevisiae*

Whereas the main emphasis in the PhD research of Ton van Maris was on metabolic engineering of *S. cerevisiae* for L-lactic acid production, he has made decisive contributions to other projects as well. As a PhD student, he was a co-author of a well-cited review paper on the compartmentation of redox metabolism. Moreover, in collaboration with researchers at the University of Amsterdam, he completed a study on the modification of metabolic fluxes in *S. cerevisiae* by engineering transcriptional regulation. In this study, the transcriptional regulator protein Hap4 was overexpressed. This substantially increased the contribution of respiratory glucose metabolism in *S. cerevisiae*, leading to improved biomass yields on glucose.

4. Scientific and applied impact

The scientific impact of the PhD research of Ton van Maris is twofold:

- *Glycolysis and product formation*

Glycolysis is the main 'conveyor belt' for most white biotechnology processes. The work of Ton van Maris has increased our fundamental understanding of the metabolism of pyruvate, the final product of this pathway. His experimental and theoretical analysis of the energetics of product export has identified a new and very important area of research in metabolic engineering. Whereas previous research almost exclusively focused on kinetic aspects of product export, it is now clear that energy-coupling of exporters can be absolutely vital in the design of new production processes and strains.

- *Methodology of metabolic engineering research*

The research of Ton van Maris highlights how successful metabolic reprogramming of an industrial microorganism can be accomplished by a combination of knowledge-based metabolic engineering and evolutionary approaches. In the latter case, he has demonstrated the potential, but also the clear limitations of transcriptome analysis for the analysis of selected phenotypes.

In an applied sense, the PhD research of Ton van Maris has, for the first time, demonstrated how it is possible to divert the majority of the glycolytic flux in *S. cerevisiae* to novel products. Both in terms of yields and in terms of achieved product titers, his work presents a clear shift in comparison with earlier studies on the engineering of *S. cerevisiae*. As such, his work is a major incentive for the Delft group and, it is hoped, the wider scientific community to move forward with attempts to develop this yeast into a multifunctional cell factory for 'white biotechnology'.