Metabolic engineering of pyruvate metabolism in *Saccharomyces cerevisiae*

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Chapter 1

General Introduction

Industrial biotechnology and metabolic engineering

For ages, nature's yearly cycles provided mankind with fuel and many of the other things that make life comfortable. Although mismanagement of these renewables came to pass (21,38,70), feedstocks that could not be renewed in short-term, such as for instance peat, were still used only sparsely. This changed drastically with the discovery of vast amounts of coal and oil, bringing forth further development of organic-chemical synthesis. These reserves, stockpiled by nature over millions of years, are used rapidly by the petro-chemical industry, providing the expanding world population with both consumer goods and fuel for transportation, heating or other industrial activities. However, the fossil-fuel supplies are finite and awareness of the side effects of the large-scale release of carbon dioxide is growing. In addition, mainly in the USA, the dependence on imported oil has resulted in increasing geopolitical concerns (40,46). In search for more sustainable alternatives for the current oil-based industry, nature's renewable resources are explored once again. These natural short-term renewable resources, such as sugars derived from biomass, have the benefit of sustainability, closed carbon cycles and a world-wide distribution.

The area of industrial biotechnology that is nowadays fashionably referred to as white biotechnology (31), is devoted to the application of living cells or enzymes to industrial production of commodity chemicals using renewable resources. Such developments might eventually result in biorefineries, providing mankind with a wide variety of fuels and chemicals, but using biomass instead of oil (47,57). Evidently, microorganisms with desirable characteristics for substrate utilization and product formation under the relevant process- and economic constraints are indispensable for successful biotechnological production of commodity chemicals (47). Although certain interesting compounds, such as lactic acid and ethanol, are common products of microbial metabolism, naturally occurring microorganisms do not meet all the demands for large-scale biotechnological production of commodity chemicals. Improvements of production strains, using techniques such as natural selection or random mutagenesis, have resulted in many past en present advances in biotechnology. Such techniques, often referred to as 'classical' strain improvement, have for instance resulted in the more than 1000-fold increase in specific productivity of penicillin by the fungus *Penicillium chrysogenum* (72). Aided by the rapid developments in molecular genetics, a new scientific field, metabolic engineering, has

arisen that supplements classical strain improvement (5,47,54,69). Metabolic engineering is defined as the improvement of cellular activities by manipulations of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology (5).

The requirements for optimizing the performance of microorganisms by either classical strain improvement or metabolic engineering vary widely and clearly depends on the parental strain as well as on the demands of the production process. Common targets of strain improvement for industrial biotechnology (Fig. 1), more extensively described by Nielsen (54), are:

(i) Substrate range. Abundant biomass-derived resources, such as crop waste and wood, contain complex mixtures of sugars that not only include glucose, but also large fractions of xylose and arabinose (45). Many microorganisms, including those with desired product formation characteristics, do not optimally use such mixed feedstocks, thereby making strain improvement desirable.

(ii) Introduction of new product formation pathways. Interesting host microorganisms, having many characteristics that are beneficial for production processes, may lack the metabolic route to the desired product. In such cases the introduction of heterologous pathways can result in economically interesting microorganisms. In this way, *E. coli* was engineered for the production of 1,3-propanediol for use in the polymer industry (50). The products of choice do not have to originate from other microorganisms. Interesting examples of this are the production of spider silk with genetically modified *E. coli* (4) and the production of human insulin with genetically modified *S. cerevisiae* (23).

(iii) Reduction of by-product formation. The large scale production of commodity chemicals requires optimal use of the feedstock and therefore greatly benefits from a minimum formation of by-products. For instance, during microbial production of fuel ethanol by *S. cerevisiae* a substantial part of the sugar feedstock is directed towards the

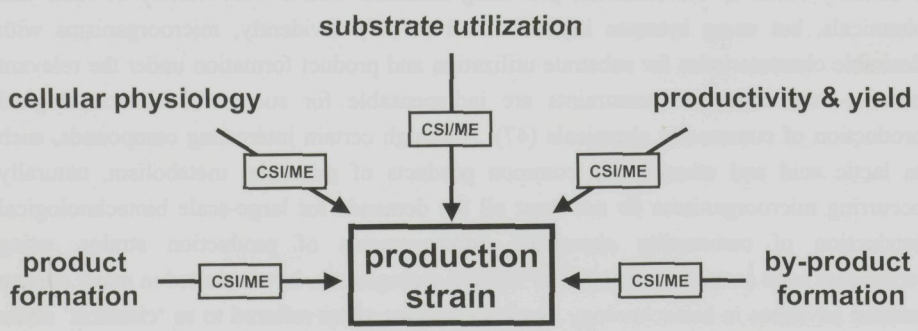

Fig. 1. Representation of the role of classical strain improvement and metabolic engineering in the manufacturing of production organisms for industrial biotechnology. Abbreviations: classical strain improvement (CSI) and metabolic engineering (ME).

formation of glycerol. It has been shown that this glycerol formation can be reduced by up to 40% by modification of the ammonia-assimilation pathways in *S. cerevisiae* (56). In addition to decreasing the yield of product on feedstock, certain by-products are toxic for the microorganism used or interfere with product purification.

(iv) Improvement of productivity or yield. If a microorganism is already established as producer of a certain compound, especially commodity chemicals, increasing the product yield or formation rate will still have a significant impact on process economy. Even when the maximum theoretical yield has been achieved, it might still be feasible to increase the productivity. Therefore improvement by either metabolic engineering or classical strain improvement will be an ongoing process.

(v) Improvement of cellular physiology. In addition to high product yields and productivity, microbial processes often benefit from either decreased substrate sensitivity or increased product tolerance. Also tolerance of microorganisms to environmental parameters, such as temperature or pH, can be interesting targets for strain improvement.

Whether defined as microbial physiology or as systems biology (55), qualitative and quantitative integral analysis of the cellular responses to different environmental conditions and/or genetic interventions, is indispensable for successful development of industrial biotechnology. These responses can and should be studied at all relevant information levels of cellular physiology. Quantitative analysis of messenger-RNA levels, also known as transcriptomics, can be used to study transcriptional responses and to obtain insight into the physiological state of cells concerning, for instance, stress conditions or nutrient limitation. Similarly, the study of the various proteins of living cells is often referred to as proteomics. Proteomics can yield valuable information on both translational responses and, depending on the type of analysis, post-translational modifications. The integral, system-wide study of these and other components of cellular physiology, such as for instance metabolite levels and enzyme activities, is popularly referred to as 'the omics'.

Combining classical strain improvement with emerging scientific fields such as metabolic engineering will be invaluable for industrial biotechnology. However, the replacement of the current oil-based refineries by bio-refineries also requires the availability of a wide range of biotechnological products having both desired properties and economic viability. Therefore, successful development of industrial biotechnology will also depend on the coalescence of the availability of cheap renewable feedstocks, increasing oil prices, consumer acceptance and political co-operation.

Saccharomyces cerevisiae: an introduction

Archaeological evidence indicates that the yeast *S. cerevisiae* was used for the production of wine at least as early as 3150 BC (14). Throughout the development of what is now regarded as human civilization, this yeast has not only been used in wine fermentation, but also for the production of beer and leavened bread. Even though

alcoholic fermentation was used for millennia and the first microscopic observations of yeast cells by Antonie van Leeuwenhoek date back to 1680 (14,74), it was not until the second half of the 19th century that Louis Pasteur unequivocally established the role of living yeast in alcoholic fermentation (10,61). This acceptance was preceded by decades of extensive, nearly hostile, scientific debate (thoroughly reviewed by J.A. Barnett (7,8,10)), including an anonymous parody that (translated from German) entitled: 'The riddle of alcoholic fermentation solved', describing yeast under the microscope as a tiny animal shaped like a distilling apparatus, swallowing sugar and excreting alcohol from an anus and carbonic acid from its genitals' (3).

The main characteristic of *S. cerevisiae*, used in all its classical applications, is the rapid anaerobic conversion of sugars into ethanol and carbon dioxide. During alcoholic fermentation glucose is taken up by the yeast and consecutively broken down by glycolysis, also known as the Emben-Meyerhof-Parnas pathway, to two molecules of pyruvate (Fig. 2). This results in the conversion of two ADP and two inorganic phosphate (P_i) into two ATP, along with the reduction of two NAD^+ to two NADH + H^+ (Fig. 2). This ATP, the free-energy currency of the cell, is subsequently converted back to ADP and P_i to drive various free-energy requiring processes, such as biosynthesis and cellular maintenance. In view of the limited total amount of the adenosine phosphates (AMP, ADP and ATP) in the cell, continuous regeneration of these compounds is required. Such interconvertable compounds, available in limited amounts and with high turnover rates, are termed 'conserved moieties'. Not only ATP, but also the pyridine-nucleotides (NAD^+ and NADH) are conserved moieties. Therefore, during alcoholic fermentation, pyruvate is converted to ethanol and carbon dioxide, via pyruvate decarboxylase and alcohol dehydrogenase, thus regenerating the glycolytically produced NADH to NAD^+ and maintaining overall redox balance (Fig. 2). The history of the elucidation of glycolysis, the alcoholic fermentation pathway and their component enzymes, with special attention to the important role of *S. cerevisiae*, has recently been reviewed by J.A. Barnett (9).

When oxygen is available, alcoholic fermentation is not the only possibility for the regeneration of glycolytic NAD^+ in *S. cerevisiae*. Instead, additional ATP can be formed by respiratory regeneration of NAD^+. After transfer of the electrons from NADH oxidation to the respiratory chain, a sequence of enzymes and enzyme complexes associated with the mitochondrial inner membrane, results in the reduction of an oxygen atom to water. During this process, the free energy available from this cascade of reactions is used for the generation of an electro-chemical gradient, the proton-motive force (49), by outward translocation of protons across the mitochondrial inner membrane. ATP synthase, a mitochondrial-membrane enzyme complex, can subsequently use this electro-chemical gradient for the conversion of ADP and P_i to ATP. Both the number of protons translocated per NADH oxidized by the respiratory chain and the number of protons required for the formation of ATP by ATP synthase varies between species. In *S. cerevisiae*, lacking the classical complex I-type NADH dehydrogenase (18), the coupling between the respiratory chain and ATP synthase results in the formation of

approximately one ATP per oxygen atom reduced (76). This ratio between the amount of ATP produced per oxygen atom reduced is termed the P/O-ratio.

Using respiratory NAD^+ regeneration, the pyruvate produced by glycolysis can be further dissimilated to carbon dioxide and water via the pyruvate-dehydrogenase complex and the tricarboxylic acid cycle. This results in the direct formation of one additional ATP equivalent and in the formation of five redox equivalents, defined as either NADH, NADPH or FADH, per pyruvate dissimilated. Assuming a P/O-ratio of 1.0 for all redox equivalents in *S. cerevisiae*, complete respiratory dissimilation yields 16 ATP per glucose, which thereby is 8-fold higher than the ATP yield of alcoholic fermentation.

Even though respiratory glucose dissimilation has far superior ATP yields, *S. cerevisiae* has a strong tendency towards alcoholic fermentation, even when oxygen is present in excess. Respiratory glucose dissimilation, without coinciding alcoholic fermentation, only occurs at low glucose concentrations and at low specific growth rates, such as in glucose-limited chemostat or fed-batch cultures (26). Under other aerobic

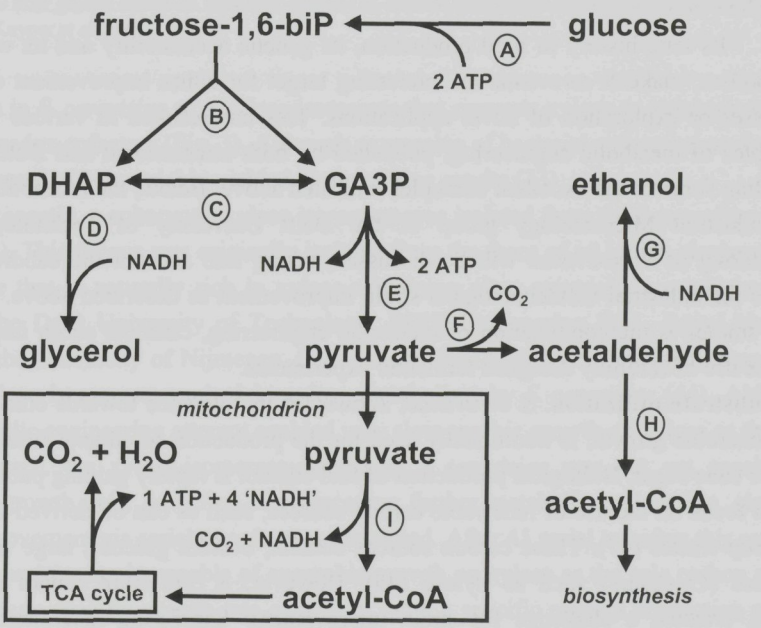

Fig. 2. Schematic overview of glucose metabolism of *Saccharomyces cerevisiae* as discussed in the context of this introduction. (A) the upper part of glycolysis, consisting of hexokinase, phosphoglucose isomerase and phosphofructokinase. (B) fructose-1,6-bisphosphate aldolase, splicing the C_6 molecule into two C_3 molecules. (C) triosephosphate isomerase, interconverting dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P). (D) the branch of metabolism responsible for the formation of glycerol consisting of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. (E) the lower part of glycolysis, consisting of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase. (F) pyruvate decarboxylase. (G) alcohol dehydrogenase. (H) acetaldehyde dehydrogenase and acetyl-CoA synthase. (I) the mitochondrial pyruvate dehydrogenase complex. Transport of substrates and metabolites is not indicated in this overview.

conditions, i.e. at high specific growth rates or under conditions where glucose is present in excess, *S. cerevisiae* exhibits mixed respirofermentative metabolism (17,26,73,77). Such respirofermentative glucose dissimilation also occurs under oxygen-limited conditions (16,79). Anaerobically, for instance during beer and wine fermentations, glucose is metabolised exclusively via alcoholic fermentation (61,75).

After being used for millennia and having proven invaluable in the elucidation of glycolysis (9,14), it is not surprising that the first chromosome and the first complete eukaryotic genome to be sequenced were from *S. cerevisiae* (34,35,59). Both man and *S. cerevisiae* are eukaryotes and share many common features including compartmentalized metabolism. Since the yeast is relatively easy to cultivate and has a high maximum specific growth rate, *S. cerevisiae* is often used as an eukaryotic model organism. This is not only limited to metabolic insights, but also holds for the study of certain hereditary diseases and the annotation of functions to many of the human genes.

Metabolic engineering of *S. cerevisiae* in Delft

Its long history in food production, its genetic accessibility and its well-known metabolism, make *S. cerevisiae* an interesting target for either improvement of existing processes or exploration of novel applications. This has resulted in various interesting examples of metabolic engineering, published by both international and Dutch authors, including some of the excellent examples indicated above. Below, the recent advances by the Industrial Microbiology group of the Delft University of Technology in the engineering of *S. cerevisiae* will be discussed, taking into account the subdivision into targets for industrial biotechnological strain improvement as described above. Central in these studies is the combination of metabolic engineering, classical strain improvement and the use of carefully designed fermenter experiments.

Substrate utilization. *S. cerevisiae*, known for its tolerance towards ethanol and its fast anaerobic growth, is traditionally used for the production of ethanol from grain and beet or cane sugar. Biological production of fuel ethanol is rapidly gaining public interest, with a focus on the use of renewable carbon sources, such as can be derived from wood and crop-wastes (47). These carbon sources contain, besides glucose, large fractions of pentoses (C_5-sugars) such as xylose (45). However, *S. cerevisiae* cannot metabolize xylose, creating a challenge for strain improvement. Expressing heterologous xylose reductases (XR) and xylitol dehydrogenases (XDH) in *S. cerevisiae* resulted in very low rates of xylose metabolism (39,42). A common problem with the use of heterologous XRs and XDHs is the occurrence of redox imbalance originating from different cofactor specificities of the enzymes (Fig. 3) (13). Combining the expression of these enzymes with strain improvement via guided evolution resulted in *S. cerevisiae* strains that were capable of extremely slow anaerobic growth on xylose (68). However, this anaerobic growth was accompanied by the production of large amounts of xylitol, exceeding that of ethanol, indicating severe redox restrictions (39). An alternative for XR and XDH is the

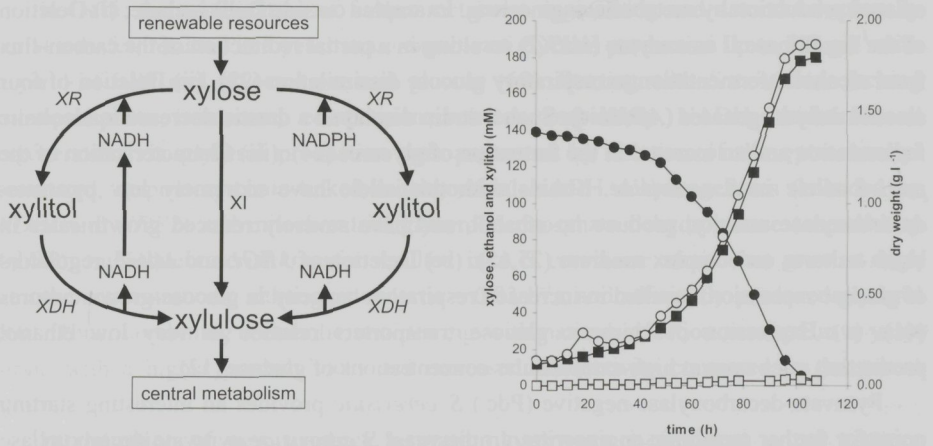

Fig. 3. Left panel. First steps of xylose metabolism. The combination of NADPH-dependent xylose reductase (XR) and NADH-dependent xylitol dehydrogenase (XDH) results in redox imbalance. This is avoided using a xylose isomerase (XI). Right panel. Growth and ethanol production on xylose of xylose-isomerase expressing *S. cerevisiae* after guided evolution. Symbols: xylose (λ), ethanol (ν), culture dry weight (μ) and xylitol (π). Data from Kuyper *et al.* 2004.

expression in *S. cerevisiae* of a xylose isomerase that converts xylose to xylulose without the use of redox cofactors (Fig. 3). Although expression of bacterial xylose isomerases has been attempted, this invariably yielded disappointing results (33).

Recently, a eukaryotic xylose isomerase was isolated from the fungus *Piromyces* sp. E2 (36). This fungus was originally isolated from the feces of an Indian elephant (71), a substrate that is naturally rich in xylose-containing plant material. In a collaboration between the Delft University of Technology, BIRD Engineering B.V., Royal Nedalco B.V. and the University of Nijmegen, it was shown that high-level functional expression of this xylose isomerase resulted in xylose metabolism by *S. cerevisiae* (44). Although this metabolic engineering attempt enabled very slow aerobic growth on xylose as the sole carbon source, the xylose-isomerase-expressing *S. cerevisiae* was not yet capable of anaerobic growth (44). As a first step preceding further metabolic engineering, classical strain improvement via serial transfer was attempted. After 45 serial transfers this resulted in an *S. cerevisiae* strain capable of anaerobic growth on xylose as the sole carbon source at a maximum specific growth rate of 0.03 h^{-1} with a specific ethanol production rate of 3.8 mmol ethanol g $biomass^{-1}$ h^{-1} (43) (Fig. 3).

Decreasing by-product formation. In the production of fuel ethanol, and classical applications of *S. cerevisiae*, alcoholic fermentation is highly desired. However, when *S. cerevisiae* is used for other industrial biotechnological applications, such as the production of heterologous proteins or low molecular-weight compounds, alcoholic fermentation decreases product yields and complicates process operation. Although alcoholic fermentation can be avoided by maintaining low glucose concentrations and relatively low growth rates, attempts have been undertaken at decreasing or eliminating

ethanol production by metabolic engineering. Examples outside Delft include: (i) Deletion of the hexokinase II isoenzyme (*HXK2*) resulting in a partial redirection of the carbon-flux from alcoholic fermentation to respiratory glucose dissimilation (22). (ii) Deletion of four alcohol dehydrogenases (*ADH1-4*). Such a strain displayed a drastic decrease in alcoholic fermentation and an increase in the formation of glycerol (24). (iii) Characterization of the *pdc1-8* allele in *S. cerevisiae*. Strains with this allele have extremely low pyruvate-decarboxylase activity, produce no ethanol, and have severely reduced growth rates in batch cultures on complex medium (25,65). (iv) Deletion of *MIG1* and *MIG2*, regulators of glucose repression, resulted in increased respiratory capacity in glucose-grown cultures (41). (v) Expression of chimeric glucose transporters resulted in very low ethanol production rates even at high extracellular concentrations of glucose (12).

Pyruvate-decarboxylase-negative (Pdc^-) *S. cerevisiae* provides an interesting starting point for further metabolic engineering. In the yeast *S. cerevisiae* pyruvate decarboxylase is encoded by three structural genes *PDC1*, *PDC5* and *PDC6* (37). When these three structural genes are deleted, *S. cerevisiae* has no detectable pyruvate decarboxylase activity and produces no ethanol. *S. cerevisiae* Pdc^- strains were further characterized in detail by the Industrial Microbiology group in Delft. Surprisingly, pyruvate decarboxylase, long considered a strictly catabolic enzyme, was found to have an essential biosynthetic function in the provision of cytosolic acetyl-CoA (27,28). This cytosolic acetyl-CoA requirement for the synthesis of lysine and fatty acids, can also be fulfilled by the provision of ethanol or acetate to the glucose-limited chemostat cultures (27). In addition Pdc^- *S. cerevisiae* is unable to grow in the presence of high glucose concentrations, even when C_2-compounds are provided (29). This growth defect, likely originating from glucose repression of biological functions that are essential in Pdc^- strains but not in the wild type, severely limits large-scale use of Pdc^- *S. cerevisiae* in industrial batch processes.

Improving yield and productivity. *S. cerevisiae* naturally produces glycerol, the main by-product of alcoholic fermentation, to maintain redox balance during anaerobic growth or in response to osmotic stress (53,73). Although mostly produced chemically, microbial glycerol production with yeast was used during world war I and II in nations with oil shortages (2). This process used bisulfite to bind acetaldehyde, thereby depriving the yeast of NAD^+ regeneration by alcoholic fermentation. To maintain redox balance the yeast was thus forced to produce glycerol (51). Although proven useful in extreme times, the low glycerol yield (in practice no more than 0.55 mol glycerol per mol glucose) of this process makes it an anachronism (2,78). Since the dawn of metabolic engineering many have attempted to improve microbial glycerol production by yeasts (24,48,52). The yield-wise most successful attempt resulted in 0.90 mol glycerol per mol glucose with non-growing *S. cerevisiae* lacking triosephosphate isomerase (encoded by *TPI1*) (15).

In Delft, the combination of further metabolic engineering and natural selection by serial transfer, resulted in a yeast strain capable of glycerol production up to 219 g l^{-1} with a yield of 0.99 mol glycerol per mol glucose (60). Starting with *S. cerevisiae* lacking triosephosphate isomerase, competition between respiratory NADH oxidation and regeneration via glycerol production was prevented by deletion of the cytosolically-orientated, respiratory-chain-linked mitochondrial NADH dehydrogenases (encoded by *NDE1* and *NDE2*) and mitochondrial glycerol-3-phosphate dehydrogenase (encoded by *GUT2*). Subsequent serial transfer resulted in a *S. cerevisiae* strain that grew at high glucose concentrations, although slowly (μ = 0.03 h^{-1}), and efficiently produced glycerol (60). Thus, combining metabolic engineering with natural selection resulted in a yeast strain with a higher product yield and increased productivity compared to the parent strain.

Introduction of new product formation pathways. Lactic acid is not a major product of wild-type *S. cerevisiae*. Within the context of this thesis a genetically engineered *S. cerevisiae* strain (TAM) was transformed with a bacterial lactate dehydrogenase. This strain was capable of lactic-acid production up to 110 g l^{-1} in aerated pH-controlled batch cultures on glucose (Fig. 4). Later in this thesis, in Chapters 3 and 5, both the TAM strain and lactic-acid production with *S. cerevisiae* will be discussed further.

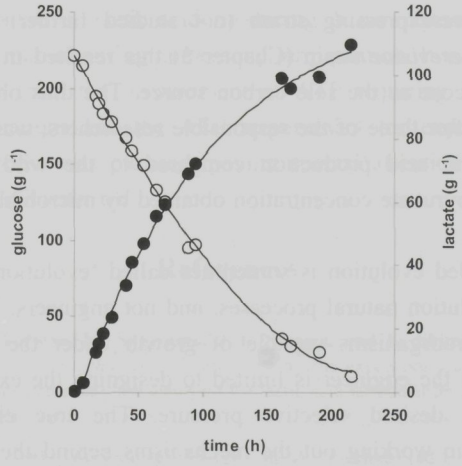

Fig. 4. Lactic-acid production with evolved Pdc⁻ *S. cerevisiae* expressing a bacterial lactate dehydrogenase. Symbols: glucose (μ) and lactate (λ). The pH of the aerated batch culture was controlled at pH 5.0 with a 10 M KOH solution. Data from van Maris, van den Bulk and de Hulster (unpublished results).

Scope of this thesis

For many years pyruvate metabolism of *S. cerevisiae* has been studied by the Industrial Microbiology group of the Delft University of Technology. Using the gathered knowledge, this thesis focuses on further 'metabolic engineering of pyruvate metabolism in *Saccharomyces cerevisiae*'. As mentioned above, Pdc$^-$ *S. cerevisiae* has two growth defects: a requirement for C_2 compounds and an inability to grow at high glucose concentrations. The first goal of this Ph.D. study was the improvement of cellular physiology of Pdc$^-$ *S. cerevisiae* by resolving these growth defects. Increasing the availability of cytosolic acetaldehyde by overexpression of threonine aldolase in Pdc$^-$ *S. cerevisiae*, was shown to circumvent the essential biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures (Chapter 2). An alternative approach was the use of natural selection in chemostat cultures to obtain a Pdc$^-$ *S. cerevisiae* strain capable of growth on glucose as the sole carbon source (Chapter 3). Both the selected Pdc$^-$ *S. cerevisiae* and the threonine aldolase overexpressing strain were still unable to grow in the presence of high concentrations of glucose.

As mentioned above, natural selection of individuals that thrive better than the parent organisms under a given set of conditions can be used to obtain microorganisms with desired attributes. This technique is especially valuable when knowledge on the exact origin of a growth defect is lacking. Since the exact origin of the inability of Pdc$^-$ *S. cerevisiae* to grow in the presence of high glucose concentrations is unknown, nature's ingenuity was used to obtain Pdc$^-$ strains that do not have this growth defect. For both the threonine aldolase overexpressing strain (not studied further) and the selected C_2-independent Pdc$^-$ *S. cerevisiae* strain (Chapter 3) this resulted in a strain that grows in batch cultures on glucose as the sole carbon source. The thus obtained double-selected strain, named TAM after three of the responsible researchers, was found to have a 100-fold increased pyruvic acid production compared to the wild type, thereby almost doubling the highest pyruvate concentration obtained by microbial fermentation (Chapter 3).

This type of guided evolution is sometimes called 'evolutionary engineering' (64). However, during evolution natural processes, and not engineers, are responsible for the accumulation of microorganisms capable of growth under the prevailing conditions. Therefore, the role of the engineer is limited to designing the experimental set-up, and thereby creating the desired selective pressure. The true challenge of industrial biotechnology arises in working out the mechanisms behind the selection of desirable phenotypes and re-applying this knowledge to the parent organism. Successful application of the thus obtained knowledge is also termed 'reverse- or inverse metabolic engineering' (6). Since the genetic changes behind the C_2-independent and glucose-tolerant phenotype of the selected Pdc$^-$ *S. cerevisiae* strain remain unknown, reverse metabolic engineering could not yet be applied to the parent Pdc$^-$ strain.

Not only deletion of pyruvate decarboxylase results in a redirection of the carbon-flux away from alcoholic fermentation. As has been described above for *HXK2* and *MIG1/MIG2* (22,41), altered expression of transcriptional regulators can change the distribution of carbon fluxes between respiration and alcoholic fermentation. Another transcriptional regulator, the Hap2/3/4/5p complex, activates many genes required for growth on non-fermentable carbon sources (19,32,58,63,66). The carbon-source responsive part of the Hap2/3/4/5p complex is encoded by *HAP4* (30). In collaboration with researchers at the University of Amsterdam the overexpression of *HAP4* was studied under a variety of growth conditions in which wild-type *S. cerevisiae* displays respiro-fermentative metabolism, including chemostat cultivation at high dilution rates or with ammonia limitation (Chapter 4 & (11)).

The high glycolytic capacity of *S. cerevisiae* and the availability of glycolytic NADH make Pdc$^-$ *S. cerevisiae* an interesting platform for the introduction of heterologous product formation pathways that require redox equivalents. The introduction of a bacterial lactate dehydrogenase in Pdc$^-$ *S. cerevisiae* adds the fifth and last category of metabolic engineering targets to the Delft portfolio. First attempted in 1994 (20), various other examples of the introduction of heterologous lactate dehydrogenases in wild-type *S. cerevisiae* are known in the literature (1,62,67). Mixed lactic- and alcoholic fermentation, as occurred in these strains, would be avoided by expression of a lactate dehydrogenase in Pdc$^-$ *S. cerevisiae*. However, industrial studies of Pdc$^-$ *S. cerevisiae* expressing bacterial lactate dehydrogenases indicated an oxygen requirement for this strain. In this thesis, a thorough quantitative physiological analysis of this phenomenon is presented (Chapter 5). From this study it was concluded that the oxygen requirement for growth of homofermentative, lactic-acid producing *S. cerevisiae*, originates from the absence of net ATP formation during homolactic fermentation. It is likely that this lack of ATP formation results from the involvement of ATP requiring lactic-acid export. To further study the limitations and the ATP requirement for export, a thermodynamic assessment of this process was pursued, paying special attention to low pH and high product concentrations (Chapter 6).

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Chapter 2

Overproduction of threonine aldolase circumvents the biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*

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Pyruvate-decarboxylase-negative (Pdc$^-$) mutants of *Saccharomyces cerevisiae* require small amounts of ethanol or acetate to sustain aerobic, glucose-limited growth. This nutritional requirement has been proposed to originate from: (i) a need for cytosolic acetyl-CoA for lipid and lysine biosynthesis, and (ii) an inability to export mitochondrial acetyl-CoA to the cytosol. To test this hypothesis and to eliminate the C_2-requirement of Pdc$^-$ *S. cerevisiae*, it was attempted to introduce an alternative pathway for synthesis of cytosolic acetyl-CoA. Addition of L-carnitine to growth media did not restore growth of a Pdc$^-$ strain on glucose, indicating that the C_2-requirement was not solely due to the inability of *S. cerevisiae* to synthesize this compound. The *S. cerevisiae GLY1* gene encodes threonine aldolase (EC 4.1.2.5), which catalyses the cleavage of threonine to glycine and acetaldehyde. Overexpression of *GLY1* enabled a Pdc$^-$ strain to grow under carbon limitation in chemostat cultures on glucose as the sole carbon source, indicating that acetaldehyde formed by threonine aldolase served as a precursor for the synthesis of cytosolic acetyl-CoA. Fractionation studies revealed a cytosolic localization of threonine aldolase. The absence of glycine in these cultures indicates that all glycine produced by threonine aldolase was either dissimilated or assimilated. These results confirm the involvement of pyruvate decarboxylase in cytosolic acetyl-CoA synthesis. The Pdc$^-$ *GLY1* overexpressing strain was still glucose-sensitive with respect to growth in batch cultivations. Like any other Pdc$^-$ strain it failed to grow on excess glucose in batch cultures and excreted pyruvate when transferred from glucose limitation to glucose excess.

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Introduction

Pyruvate decarboxylase (PDC), encoded by *PDC1*, *PDC5* and *PDC6*, catalyses the first, irreversible step of alcoholic fermentation by yeasts and has therefore long been considered a strictly catabolic enzyme. Consistent with this notion, pyruvate-decarboxylase-negative (Pdc^-) mutants of *Saccharomyces cerevisiae* exhibit a drastically reduced specific growth rate in batch cultures on complex medium with glucose, in which glucose metabolism of wild-type *S. cerevisiae* is predominantly fermentative (35).

At the low residual glucose concentration in aerobic glucose-limited chemostat cultures, the glucose repression of respiratory enzymes is alleviated (4,37). When grown at low specific growth rates in such cultures, wild-type cells dissimilate glucose exclusively via respiration (4,37). Nevertheless, under these conditions, Pdc^- *S. cerevisiae* strains were unable to grow on glucose as the sole carbon source, but growth could be restored by addition of small amounts of ethanol or acetate to the medium (10,12). This C_2-compound requirement of Pdc^- *S. cerevisiae* has been proposed to reflect an essential role of PDC in the synthesis of cytosolic acetyl-CoA, which is required for the synthesis of lipids and lysine (10). PDC catalyses the first reaction of a pathway for the cytosolic conversion of pyruvate into acetyl-CoA, which also involves acetaldehyde dehydrogenase and acetyl-CoA synthetase (17,31,32). Consistent with this proposed biosynthetic role of PDC, the experimentally determined minimum requirement of Pdc^- mutants for C_2-compounds matched the theoretical demand for cytosolic acetyl-CoA (10).

An essential role of PDC in the synthesis of cytosolic acetyl-CoA is to some extent surprising, since several yeast species are known to grow rapidly on glucose in the absence of pyruvate decarboxylase. For example, Pdc^- strains of *Kluyveromyces lactis* grow rapidly on glucose as the sole carbon source (2,14). Furthermore, the lipid-accumulating yeast *Yarrowia lipolytica*, which lacks pyruvate decarboxylase, uses ATP-citrate lyase for the export of acetyl-CoA units to the cytosol from the mitochondrial matrix, where acetyl-CoA is formed by the pyruvate-dehydrogenase complex (8). ATP-citrate lyase does not occur in *S. cerevisiae*, thus precluding the involvement of this enzyme in cytosolic acetyl-CoA synthesis (33).

It is generally assumed that in eukaryotic cells, including *S. cerevisiae* (19,31) the carnitine shuttle plays a key role in the transport of acetyl-CoA across the mitochondrial inner membrane. Although *S. cerevisiae* contains the genetic information encoding carnitine transferases and acetyl-carnitine translocase (1,7,27,36,39), it has recently emerged that *S. cerevisiae* is unable to synthesize L-carnitine (39). Since it has not been investigated whether L-carnitine supplementation enables growth of Pdc^- strains of *S. cerevisiae* on glucose, it remains unclear whether the carnitine shuttle can catalyse the export of acetyl-CoA from the mitochondrial matrix.

The C_2 requirement of Pdc^- *S. cerevisiae* is not only of fundamental scientific interest. The absence of alcoholic fermentation in Pdc^- strains may be beneficial in biomass-directed applications. An other demonstrated application of such strains is the introduction

of lactate dehydrogenase, to utilize the glycolytic NADH to produce lactic acid (29), a chemical with commercial value. In all these applications, elimination of the C_2-compound requirement would facilitate process design.

The aims of the present study were to verify the hypothesis that PDC is essential for cytosolic acetyl-CoA synthesis in glucose grown *S. cerevisiae*, to investigate a possible role of the carnitine shuttle in the export of acetyl-CoA from the mitochondrial matrix and to eliminate the C_2-requirement of Pdc⁻ *S. cerevisiae* via metabolic engineering. The latter goal was pursued by overexpressing the *GLY1* gene encoding threonine aldolase, which catalyses the cleavage of threonine to glycine and acetaldehyde (21,25), in a Pdc⁻ strain.

Materials and Methods

Strains and maintenance. The *S. cerevisiae* strains used and constructed in this study (Table 1) are congenic members of the CEN.PK family (38). Stock cultures were grown at 30 °C in shake flasks containing 100 ml synthetic medium with 20 g l^{-1} glucose. When stationary phase was reached, 20 % (v/v) glycerol was added and 2-ml aliquots were stored at –80 °C.

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
CEN.PK 182	*MATa pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP*
CEN.PK 111-61A	*MATα ura3-52 leu2-112 his3-Δ1*
RWB882	*MATa pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP leu2-112 his3-Δ1*
RWB893	*MATa pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP leu2-112 his3::*pYX022-Aat

Plasmid construction. *GLY1* was cloned behind the constitutive *TPI1* promoter on the 2μ-based expression vector YEplac181 (15). To this end, the *GLY1* open-reading frame was isolated by performing a PCR amplification on chromosomal DNA from *S. cerevisiae* strain M5 (34) with the following oligonucleotide primers: 5'-GGAATTCTAGAATGACTGAGTTCGAATTGCCTCCAAAATATAC-3' and 5'-CCGCTCGAGACATGATGCAACTGGAACGC-3'.

The PCR mix was separated on an agarose gel, after which the desired fragment was isolated and digested with *EcoR*I and *Xho*I. The resulting fragment was ligated into pYX042-AatII, digested with *EcoR*I and *Xho*I. This pYX042-AatII plasmid is derived from pYX042 (R&D systems, Minneapolis USA) by digesting it with *Aat*II and inserting a linker which destroys the *Aat*II site and introduces 4 other restriction sites, *Xho*I, *BamH*I, *Sma*I and *Nhe*I. The resulting plasmid, pRWGLY1, was then digested with *Nhe*I and *Sac*I. The P_{TPI}-*GLY1* fragment thus obtained was ligated to YEplac181, cut with *Xba*I and *Sac*I. The result of this procedure was YEpGLY1.

Strain construction. RWB882 was derived from a cross between CEN.PK182 and CEN.PK111-61A (both provided by Dr. P. Kötter, Frankfurt, Germany). The resulting diploid was sporulated and the spore mixture was heated for 15 min to 56 °C. Subsequently the mixture was plated on YP medium with 0.2 % sodium acetate as the carbon source. The resulting colonies were checked for growth on glucose. Those that could not grow on glucose were tested by PCR for the presence of a disrupted *PDC6* gene. Subsequent selection on synthetic medium for the presence of the desired auxotrophic marker(s), in this case *leu2-112*, resulted in RWB882. To eliminate the histidine auxotrophy, RWB882 was first transformed with pYX022-Aat to give RWB893. Transformation of this strain with the plasmids YEpGLY1 and YEplac181 (15) resulted in the *GLY1*-overexpressing strain RWB893(YEpGLY1) and the corresponding empty-vector strain RWB893(YEplac181), respectively.

Polymerase-chain reaction (PCR). PCR was performed with Vent$_R$® DNA Polymerase (New England Biolabs) according to the manufacturers' specifications. The PCR was performed as follows: 30 cycles with the following settings: denaturation during 1 min at 94 °C, annealing for 1 min at 65 °C and an extension time of 3.5 min at 75 °C.

Media. The synthetic medium for chemostat cultivation contained per litre of demineralized water 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 0.05 ml silicon antifoam (BDH) and trace element concentrations according to Verduyn *et al.* (41). After heat sterilization of the medium for 20 min at 120 °C a filter-sterilized vitamin solution, prepared according to Verduyn *et al.* (41), was added. The concentration of substrate carbon in the reservoir medium was always 250 carbon mM. The carbon substrate consisted either of a mixture of glucose (6.75 g l^{-1}) and acetate (0.75 g l^{-1}), or of 7.5 g l^{-1} glucose as the sole carbon source. Glucose was added separately after heat sterilization at 110 °C. Pure acetic acid was added to the autoclaved medium without prior sterilization. Synthetic media for batch cultivation and precultures contained 1.5 % ethanol as the sole carbon source and were otherwise identical to the chemostat media. In cultures supplemented with L-carnitine, its final concentration was 0.4 g l^{-1}.

Chemostat cultivation. Aerobic chemostat cultivation was performed at 30 °C in 2-l fermenters (Applikon, Schiedam, the Netherlands) with a working volume of 1 l. The pH was controlled at 5.0 via automated addition of 2 M KOH (Applikon ADI 1030 biocontroller). A stirrer speed of 800 rpm and an air flow of 0.5 l min^{-1} were applied to keep the dissolved-oxygen concentration higher than 60 % of air saturation, measured with a oxygen electrode, in all chemostat cultivations performed. The addition of medium was regulated by a peristaltic pump. The working volume of the cultures was kept constant by means of an electric level sensor. Cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight, glucose concentration, carbon-dioxide production rate and oxygen consumption rate, changed by less than 2 % during one volume change. Sustained oscillations of the dissolved-oxygen concentration (20) were not observed. There was no significant difference (<1%) between the biomass concentrations in effluent and in samples taken directly from the cultures.

Glucose-pulse experiments. Glucose-pulse experiments were performed by adding glucose to steady-state glucose-limited chemostat cultures. Just before the start of the pulse experiment, the medium pump was switched off. To achieve a 50 mM glucose pulse, 36 ml of a 50 % (w/v) glucose solution was injected aseptically through a rubber septum. During glucose consumption and the subsequent consumption of metabolites, the OD_{660} of culture samples and the concentrations of glucose and metabolites in supernatant samples were determined at appropriate intervals.

Determination of culture dry weight. To determine biomass dry weight, a known culture volume containing 0.01-0.03 g dry weight was filtrated over predried nitrocellulose filters of known weight (pore size 0.45 µm, Gelman Sciences). The filters were washed with 20 ml demineralized water and dried for 20 minutes in a microwave oven at 360 W and the increase of the filter weight was measured. Duplicate samples varied by less than 1%.

Metabolite analysis. Acetate, glucose, glycerol and pyruvate concentrations in supernatants were determined by HPLC analysis with a Biorad Aminex HPX-87H column at 60 °C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Pyruvate and acetate were detected by a Waters 2487 Dual λ Absorbance Detector at 214 nm. Glucose and glycerol were detected by a Waters 2410 Refractive Index Detector. Glucose concentrations were confirmed enzymically with a commercial Roche Diagnostics kit (no 716 251).

Gas analysis. The exhaust gas of chemostat cultures was cooled and dried with a Permapure Dryer (Inacom Instruments, UK), before analysis of the O_2 and CO_2 concentrations with a Rosemount NGA 2000 analyzer. The gas-flow rate was determined with a Ion Science Saga digital flowmeter. Calculation of specific O_2 consumption and CO_2 production for chemostat cultures were performed according to van Urk *et al.* (40).

Enzyme-activity assays. Cell extracts for enzyme-activity assays were prepared as described previously (6). Subcellular fractionation was performed according to Luttik *et al.* (23). The marker enzymes, cytochrome *c* oxidase (EC 1.9.3.1; Douma et al. (5)) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Postma et al. (30)), used for the localization study were assayed at 30 °C in a Hitachi model 100-60 spectrophotometer according to previously published methods. Pyruvate decarboxylase was measured as described by Flikweert *et al.* (12). The assay mixture for threonine aldolase contained: 0.1 M HEPES buffer (pH 7.0) 50 µM pyridoxal-5-phosphate, 88 U ml^{-1} alcoholdehydrogenase (EC 1.1.1.1) and 150 µM NADH in demineralized water. The reaction was started by the addition of 10 mM threonine. Oxidation of NADH was followed by monitoring its absorbance at 340 nm with a Hitachi 100-60 spectrophotometer. The protein concentration of cell extracts was estimated by the Lowry method with bovine serum albumin as the standard (22).

Results

L-carnitine addition to chemostat cultures of Pdc⁻ *S. cerevisiae*. It has recently been shown that *S. cerevisiae* is unable to synthesize L-carnitine (39). If carnitine auxotrophy is the reason for the C_2 compound requirement of Pdc⁻ *S. cerevisiae*, growth in aerobic, glucose-limited chemostat cultures should be possible after L-carnitine supplementation. To investigate this possibility, duplicate chemostat cultures of the Pdc⁻ strain RWB893(YEplac181) on glucose and acetate as the carbon source were supplemented with 0.4 g l^{-1} carnitine. The addition of L-carnitine to the medium did not influence the biomass yield on carbon (14.1 g biomass $Cmol^{-1}$), nor the fluxes of glucose (1.07 mmol g $biomass^{-1}$ h^{-1}), acetate (0.4 mmol g $biomass^{-1}$ h^{-1}), O_2 (2.95 mmol g $biomass^{-1}$ h^{-1}) or CO_2 (3.00 mmol g $biomass^{-1}$ h^{-1}) compared to cultures on glucose and acetate without L-carnitine (Table 3). After a steady state was reached, the culture was switched to a medium with glucose as the sole carbon source, but still containing L-carnitine. On this medium, the Pdc⁻ strain washed out of the chemostat cultures. Apparently, L-carnitine supplementation could not rescue Pdc⁻ *S. cerevisiae* in chemostat cultures on glucose as the sole carbon source.

Overproduction of threonine aldolase in Pdc⁻ *S. cerevisiae*. In addition to PDC, the metabolic network of *S. cerevisiae* contains at least one alternative reaction that may provide cytosolic C_2-compounds. Threonine aldolase (EC 4.2.1.5), a key enzyme in glycine biosynthesis encoded by the *GLY1* gene, catalyses the cleavage of threonine to glycine and acetaldehyde (21,25). To investigate whether the threonine aldolase may replace PDC in its biosynthetic role, it was attempted to overexpress *GLY1* in a *pdc1Δ pdc5Δ pdc6Δ* strain.

To determine whether introduction of the *GLY1* expression vector resulted in a higher activity of threonine aldolase, activity of the enzyme was measured in cell extracts. The threonine-aldolase activity of a Pdc⁻ strain carrying the *GLY1* expression vector was 0.75 ± 0.01 U mg $protein^{-1}$, whereas the activity in the corresponding empty-vector strain was below the detection limit of 0.005 U mg $protein^{-1}$.

To investigate the subcellular localization of the overproduced Gly1p, threonine-aldolase activity was determined in both the soluble and particulate fractions of cell homogenate obtained from a glucose-limited chemostat culture. The cytosolic enzyme glucose-6-phosphate dehydrogenase was fully recovered in the soluble fraction of the homogenate. Activity of cytochrome c-oxidase, a mitochondrial marker enzyme, was almost exclusively located in the particulate fraction. Threonine-aldolase activity in the overproducing strain was almost exclusively found in the soluble fraction of cell homogenate (Table 2), indicating a cytosolic localization of Gly1p.

Table 2 Recovery of threonine aldolase and marker enzymes in the particulate and soluble fractions of homogenates of the *GLY1* overexpressing Pdc⁻ strain RWB893(YEpGLY1), harvested from aerobic glucose-limited chemostat cultures. Data presented are from a single representative experiment. A duplicate experiment yielded similar results.

Enzyme	Recovery of enzyme activity in fractions (%)	
	Particulate fraction	Soluble fraction
Threonine aldolase	1	94
Glucose-6P dehydrogenase	1	101
Cytochrome *c* oxidase	98	12

***GLY1* overexpression eliminates the C_2-compound requirement of Pdc⁻ *S. cerevisiae*.** Growth of Pdc⁻ *S. cerevisiae* in glucose-limited chemostat cultures requires the addition of small amounts of ethanol or acetate to the glucose media (10). Like other Pdc⁻ mutants the Gly1p-overproducing Pdc⁻ strain did not grow in batch culture on glucose, not even in the presence of small amounts of ethanol or acetate (data not shown). Therefore, to test the ability of the Gly1p-overproducing Pdc⁻ strain and the empty-vector reference strain to grow on glucose as the sole carbon source, aerobic mixed-substrate cultures grown at a dilution rate of 0.10 h^{-1} were switched to a medium containing glucose as the sole carbon source.

As expected, both strains were able to grow in chemostat cultures on a mixture of glucose and acetate (Table 3). Under these conditions, key physiological parameters of the cultures such as biomass yields and respiratory quotient (RQ) were not significantly different for the two strains (Table 3). Consistent with a complete (> 97 %) recovery of substrate carbon in biomass and carbon dioxide, no significant accumulation of metabolites, such as ethanol, acetate or glycerol, was observed in culture supernatants.

Table 3 Physiology of the threonine aldolase overproducing Pdc⁻ *S. cerevisiae* strain RWB893(YEpGLY1) and the empty- vector reference Pdc⁻ strain RWB893(YEplac181) in aerobic chemostat cultures. Averages and mean deviations were obtained from duplicate experiments with independent steady-state cultures on synthetic medium containing a mixture of glucose and acetate (0.25 M substrate carbon, 10 % acetate on a carbon basis) or only glucose (0.25 M substrate carbon). Calculations of the carbon recovery were based on a biomass carbon content of 48 % (w/w).

	Medium with acetate		Medium without acetate	
	Empty vector ($D = 0.10\ h^{-1}$)	*GLY1*↑ ($D = 0.10\ h^{-1}$)	*GLY1*↑ ($D = 0.10\ h^{-1}$)	*GLY1*↑ ($D = 0.15\ h^{-1}$)
Y_{sx} ($g_{biomass}$·$Cmol^{-1}$)	14.0 ± 0.1	14.0 ± 0.1	14.0 ± 0.2	14.7 ± 0.0
$q_{glucose}$ (mmol·$g_{biomass}$·h^{-1})	1.11 ± 0.02	1.08 ± 0.03	1.17 ± 0.03	1.66 ± 0.02
$q_{acetate}$ (mmol·$g_{biomass}$·h^{-1})	0.4 ± 0.0	0.4 ± 0.0	-	-
q_{CO2} (mmol·$g_{biomass}$·h^{-1})	3.09 ± 0.07	3.12 ± 0.09	2.85 ± 0.02	4.06 ± 0.05
q_{O2} (mmol·$g_{biomass}$·h^{-1})	3.01 ± 0.08	3.04 ± 0.09	2.82 ± 0.06	3.93 ± 0.05
RQ ($mmol_{CO2}$·$mmol_{O2}$)	1.03 ± 0.00	1.03 ± 0.00	1.02 ± 0.02	1.04 ± 0.00
Carbon recovery (%)	100 ± 0	97 ± 2	100 ± 0	98 ± 1

After switching to a medium with glucose as the sole carbon source, the empty-vector Pdc⁻ reference strain washed out of the chemostat cultures (Fig. 1). The exponential decrease of the biomass concentration, accompanied by the accumulation of glucose and pyruvate, was consistent with a low residual growth rate of 0.03 h^{-1}. It has previously been shown that a non-isogenic Pdc⁻ *S. cerevisiae* strain washed out of the chemostat cultures in a similar manner (12). Evidently, the reference strain was unable to sustain glucose-limited growth without acetate in the medium at a dilution rate of 0.10 h^{-1}.

Under identical conditions, the threonine-aldolase overproducing Pdc⁻ strain was capable of growth on glucose as the sole carbon source (Table 3), indicating that threonine aldolase could provide the cells with sufficient precursors for the synthesis of cytosolic acetyl-CoA. The obtained biomass yield on glucose of the Gly1p-overproducing strain (0.47 ± 0.01 g biomass g $glucose^{-1}$) was comparable to the yield obtained for the wild-type strain (0.48-0.49 g biomass g $glucose^{-1}$) under similar conditions. By-product formation in steady-state cultures of the Gly1p-overproducing strain was negligible. This indicated that the additional glycine produced by threonine aldolase was either dissimilated or assimilated by the glucose-limited chemostat cultures. The engineered strain was also capable of glucose-limited growth at a dilution rate of 0.15 h^{-1} (Table 3). However, attempts to further increase the dilution rate to 0.20 h^{-1} resulted in wash-out.

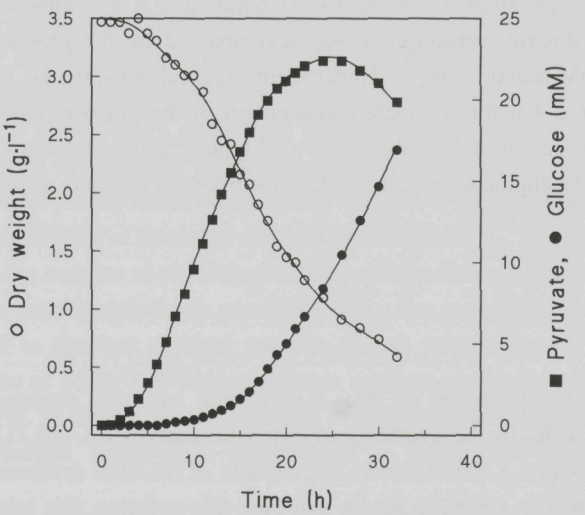

Fig. 1. Concentrations of glucose, metabolites and biomass after switching an aerobic chemostat culture (dilution rate 0.10 h^{-1}) of the *pdc1Δ pdc5Δ pdc6Δ* reference strain RWB893(YEplac181) from growth on synthetic medium containing a mixture of glucose and acetate (0.25 M substrate carbon, 10 % acetate on a carbon basis) to a synthetic medium containing glucose (0.25 M substrate carbon) as the sole carbon source. The graph shows the wash-out profile of a single representative culture. An independent replicate experiment yielded the same results.

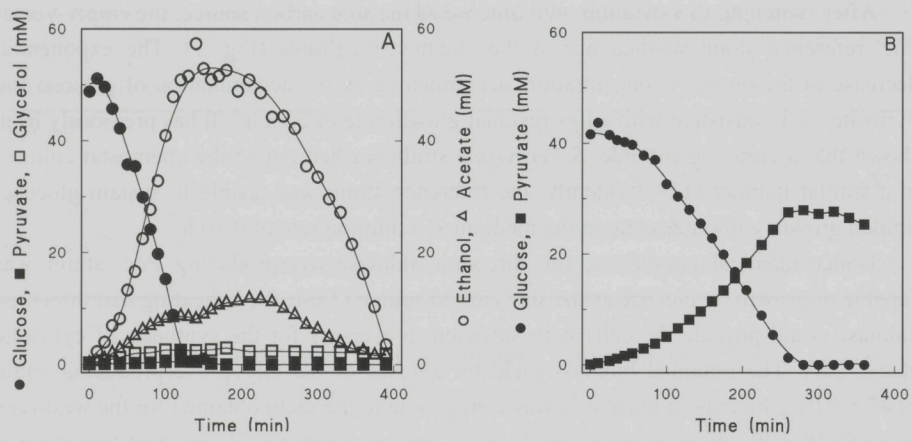

Fig. 2. Metabolic responses of aerobic, glucose-limited chemostat cultures (dilution rate 0.10 h^{-1}) to a 50 mM glucose pulse. (A) *S. cerevisiae* CEN.PK 113-7D (prototrophic wild-type strain). (B) *GLY1*-overproducing Pdc⁻ strain RWB893(YEpGLY1). The graphs show single representative glucose-pulse experiments for each strain. Independent replicate experiments yielded essentially the same results.

Byproduct formation upon exposure of glucose-limited cultures to excess glucose. To investigate the short-term response of the threonine-aldolase overexpressing Pdc⁻strain to excess glucose, a glucose pulse was administered to a steady-state, aerobic, glucose-limited chemostat culture. After a 50-mM glucose pulse was administered, the prototrophic reference strain CEN.PK113-7D required 2 h to completely consume the added glucose. Glucose consumption was accompanied by the production of 50 mM ethanol and 10 mM acetate (Fig. 2). Under identical conditions, the *GLY1*-overproducing Pdc⁻ strain required 4 h for complete consumption of the glucose pulse. No ethanol or acetate was produced but, in contrast to the wild-type strain and similar to a non-isogenic Pdc⁻ strain (13), the engineered strain produced substantial amounts of pyruvate (up to 30 mM, Fig. 2).

Discussion

The C_2-compound requirement of Pdc⁻ *S. cerevisiae* has been proposed to reflect an essential role of PDC in the synthesis of cytosolic acetyl-CoA (10,31). The available evidence indicates that *S. cerevisiae* is not capable of *de novo* synthesis of L-carnitine (39). During growth in synthetic media that lack this cofactor, this might preclude the involvement of the L-carnitine shuttle (39) in export of mitochondrial acetyl-CoA to the cytosol. Our results demonstrate that the C_2-requirement of Pdc⁻ *S. cerevisiae* is not caused by a simple L-carnitine auxotrophy. This does not necessarily imply that a mitochondrial carnitine shuttle in *S. cerevisiae* is unidirectional, as has earlier been proposed based on the phenotype of Pdc⁻ strains and on the assumption that this yeast was capable of L-carnitine biosynthesis (31). Instead, the absence of an effect of L-carnitine

addition might reflect a limitation in L-carnitine uptake over the yeast plasma membrane, as has recently been demonstrated in a different *S. cerevisiae* genetic background (37). Once the biochemistry and regulation of L-carnitine uptake and metabolism in *S. cerevisiae* are better understood, Pdc⁻ strains may be useful for studies into the role of the L-carnitine shuttle in mitochondrial acetyl-CoA transport.

The observation that, at low through moderate specific growth rates, threonine-aldolase overproducing Pdc⁻ strains were capable of growth on glucose as the sole carbon source (Table 3), is consistent with the proposed essential role of PDC in cytosolic acetyl-CoA biosynthesis (10,31). Synthesis of acetaldehyde via threonine aldolase overproduction is accompanied by the formation of equimolar amounts of glycine. The minimum cytosolic acetyl-CoA requirement for the lipid and lysine (3,9) biosynthesis during glucose- limited growth has previously been estimated at 1.05 mmol g biomass^{-1} (10). Therefore, at least 1.05 mmol glycine g biomass^{-1} will be produced if all cytosolic acetyl-CoA is produced via threonine aldolase. Multiple pathways may be involved in the metabolism of this glycine in the engineered Pdc⁻, *GLY1*-overexpressing strain. In addition to direct incorporation in cellular protein (the glycine content of yeast biomass is ca. 0.29 mmol g biomass^{-1} (26)), glycine may be used for the synthesis of serine via serine-hydroxymethyl transferase and the glycine-cleavage system (18,28). If all serine is produced in this way, consuming two molecules of glycine per serine produced, an additional 0.37 mmol glycine g biomass^{-1} can be incorporated in the biomass (26). Furthermore, additional glycine may be converted via the glycine-cleavage system in conjunction with either methionine biosynthesis or one-carbon metabolism (18,28).

The inability of Pdc⁻ strains to grow on glucose as the sole carbon source indicates that regulatory properties of the *GLY1* gene and/or the regulatory and kinetic properties of Gly1p prevent the native *GLY1* gene from meeting the cellular demand for cytosolic acetyl-CoA. In terms of regulatory properties, it seems likely that regulation of the native *GLY1* gene will be primarily based on its role in nitrogen metabolism. In terms of kinetic properties, the low affinity of threonine aldolase for threonine (K_m 55 mM (21)) may limit the flux through the enzyme at physiological intracellular threonine concentrations (5-10 mM (16,24)). We cannot exclude the possibility that a low expression level of *GLY1* may have contributed to the low residual specific growth rates observed upon switching chemostat cultures of a Pdc⁻ reference strain to a medium containing glucose as the sole carbon source (Fig. 1). It will be of interest to investigate whether threonine aldolase is involved in cytosolic acetyl-CoA biosynthesis in eukaryotes that lack pyruvate decarboxylase.

The aerobic production of ethanol and acetate by wild type *S. cerevisiae* is considered a substantial problem in biomass- and protein-directed industrial applications. The engineered Pdc⁻, *GLY1*-overexpressing strain combines the absence of this alcoholic fermentation with the ability to grow on glucose as the sole carbon source in aerobic carbon-limited chemostat cultures. However, several growth characteristics of this strain limit the industrial application as a host for the expression of heterologous proteins or as a

strain platform for the production of L-lactate (29). First, similar to a strain with reduced expression of pyruvate decarboxylase (11), the engineered strain exhibited a reduced maximum specific growth rate of 0.20 h^{-1} in glucose-limited chemostat cultures compared to 0.38 h^{-1} of the wild type. Secondly, like other strains of *S. cerevisiae* with reduced or zero pyruvate decarboxylase activity (11,13,35) it produced substantial amounts of pyruvate during exposure to glucose excess (Fig. 2). Thirdly growth of this strain on glucose in batch culture was not possible.

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Chapter 3

Directed Evolution of Pyruvate-Decarboxylase-Negative *Saccharomyces cerevisiae* Yielding a C_2-Independent, Glucose-Tolerant and Pyruvate-Hyperproducing Yeast

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The absence of alcoholic fermentation makes pyruvate-decarboxylase-negative (Pdc^-) strains of *Saccharomyces cerevisiae* an interesting platform for further metabolic engineering of central metabolism. However, Pdc^- *S. cerevisiae* strains have two growth defects: (i) growth on synthetic medium in glucose-limited chemostat cultures requires the addition of small amounts of ethanol or acetate, and (ii) even in the presence of a C_2-compound, they cannot grow in batch cultures on synthetic medium with glucose. We used two subsequent phenotypic selection strategies to obtain a Pdc^- strain without these growth defects. An acetate-independent Pdc^- mutant was obtained via (otherwise) glucose-limited chemostat cultivation by progressively lowering the acetate content in the feed. Transcriptome analysis did not reveal the mechanisms behind its C_2-independence. Further selection for glucose tolerance in shake flasks resulted in a Pdc^- *S. cerevisiae* mutant (TAM) that could grow in batch cultures (μ_{max} = 0.20 h^{-1}) on synthetic medium with glucose as the sole carbon source. Although the exact molecular mechanisms underlying the glucose-tolerant phenotype were not resolved, transcriptome analysis of the TAM strain revealed increased transcript levels of many glucose repressible genes relative to a isogenic wild type in nitrogen-limited chemostat cultures with excess glucose. In pH-controlled aerobic batch cultures, the TAM strain produced large amounts of pyruvate. By repeated glucose feeding, a pyruvate concentration of 135 g l^{-1} was obtained, with a specific pyruvate production rate of 6 - 7 mmol g $biomass^{-1}$ h^{-1} during the exponential growth phase and an overall yield of 0.54 g pyruvate g $glucose^{-1}$.

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Introduction

Traditionally, *Saccharomyces cerevisiae* has been used to rapidly ferment sugars to ethanol and carbon dioxide. More recently, developments in molecular biology have led to the application of *S. cerevisiae* as a host for therapeutical-protein production (13) and for the production of chemicals with commercial value via metabolic engineering (28,29,31). In view of the process economy of manufacturing bulk products, the yield of the desired product should be maximised. In case of yeasts as production organisms this necessitates the redirection of carbon fluxes away from alcoholic fermentation towards the desired product. (1,4,7,8,19).

Pyruvate decarboxylase (EC 4.1.1.1) is located at the branchpoint between fermentative and respiratory sugar catabolism and catalyzes the first step in the fermentative branch. *S. cerevisiae* contains three structural genes (*PDC1*, *PDC5* and *PDC6*) that encode active pyruvate decarboxylase isoenzymes (18). Pyruvate decarboxylase was long considered to be a strictly catabolic enzyme, but recently a biosynthetic function of the enzyme was discovered (8). Growth of pyruvate-decarboxylase-negative (Pdc^-) *S. cerevisiae* in aerobic glucose-limited chemostat cultures on synthetic media required a supply of acetate or ethanol corresponding to ca. 5% of the carbon fed to the cultures (6,8). This requirement for a C_2-compound probably reflects an essential function of pyruvate decarboxylase in the synthesis of cytosolic acetyl-CoA (Fig. 1), required for lysine and fatty-acid synthesis (6).

Overproduction of threonine aldolase, catalysing the cleavage of threonine to glycine and acetaldehyde, can circumvent the essential biosynthetic role of pyruvate decarboxylase (46). Even when the C_2-requirement of Pdc^- strains is met by overexpression of threonine aldolase or by inclusion of ethanol or acetate in the medium, Pdc^- strains can only grow on glucose when the glucose supply is growth limiting. When Pdc^- strains are exposed to the glucose concentrations normally applied in batch cultures, they excrete significant amounts of pyruvate but are completely unable to grow (9). This glucose sensitivity is a general characteristic of Pdc^- strains (6,46).

The exact cause of the glucose sensitivity of Pdc^- strains remains unknown. In the absence of alcoholic fermentation, which is blocked in Pdc^- *S. cerevisiae*, cells rely on respiration (Fig. 1) for the reoxidation of cytosolic NADH (33). However, respiration of wild-type *S. cerevisiae* in batch cultures on glucose is repressed but not blocked, judging from the significant oxygen consumption rate under these conditions (1,4). It is therefore unlikely that glucose repression of respiration is the sole cause for glucose sensitivity of Pdc^- *S. cerevisiae*.

From the time of their invention, chemostats have been associated with selection of spontaneous mutants (25,26). The first chemostat studies already describe the selection of an *Escherichia coli* strain with a higher affinity for the growth-limiting nutrient (26). Subsequent review articles cite a variety of other examples of selection in chemostats (5,37) and elaborate on the theory of selection during chemostat cultivation (17).

Similarly, extended cultivation of microorganisms in shake flasks can be used to select for spontaneous mutants that grow under conditions in which the original strain would not grow (10,37).

The first goal of this study was to apply selection pressure in batch and chemostat cultures to obtain a Pdc⁻ *S. cerevisiae* strain capable of growth in batch culture on synthetic medium, containing high concentrations of glucose as the sole carbon and energy source. Prolonged chemostat cultivation on glucose with progressively decreasing acetate feeds was used to select for C_2-independent Pdc⁻ *S. cerevisiae*. A subsequent round of selection was performed in batch cultures to select for C_2-independent Pdc⁻ *S. cerevisiae* that could grow on high concentrations of glucose. A second goal was to physiologically characterize the selected strain and to gain insight in the molecular mechanisms underlying the selected phenotype. To this end, biomass and product yields were analysed in batch and chemostat cultures and genome-wide transcriptome analysis was performed using nitrogen-limited chemostat cultures grown under glucose-excess conditions.

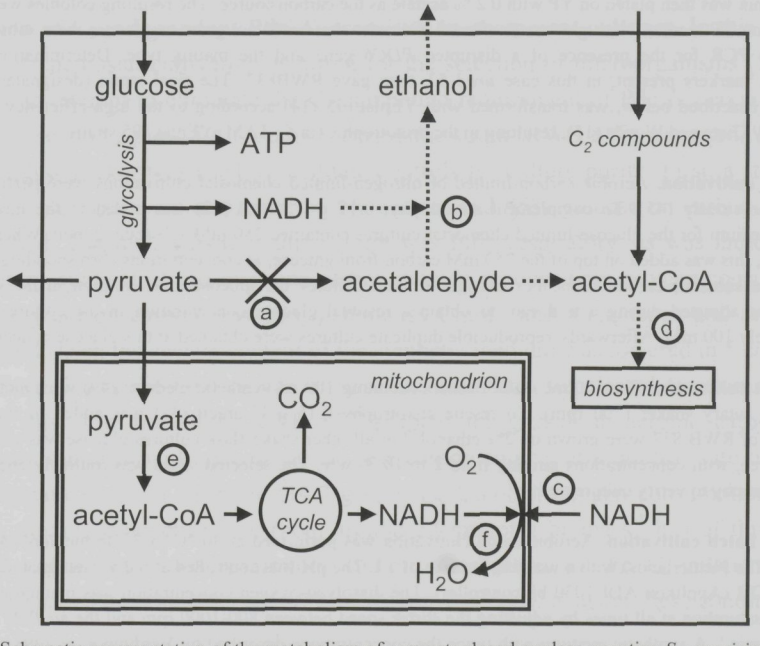

Fig. 1. Schematic representation of the metabolism of pyruvate-decarboxylase-negative *S. cerevisiae* growing on glucose. By deletion of all genes encoding for pyruvate decarboxylase (reaction a) two important processes (dotted lines) are impaired: Firstly, reoxidation of cytosolic NADH via alcohol dehydrogenase (reaction b) is blocked. Cytosolic NADH must therefore be oxidized by the mitochondria via external NADH dehydrogenase (reaction c) or redox shuttle systems. Secondly, the formation of cytosolic acetyl-CoA from acetaldehyde is blocked. Instead, the C_2-compounds required for the cytosolic acetyl-CoA for lysine and fatty-acid biosynthesis (reaction d) must be taken up from the environment. When oxygen consumption exceeds the amount of oxygen necessary for oxidation of glucose to pyruvate, mitochondrial oxidation of pyruvate, via pyruvate dehydrogenase (reaction e) and the tricarboxylic acid cycle (TCA cycle) can occur, resulting in CO_2 formation and the oxidation of NADH via internal NADH dehydrogenase (reaction f).

Materials and Methods

Strains and maintenance. All *S. cerevisiae* strains used in this study (Table 1) were derived from the congenic CEN.PK family (41). Stock cultures were prepared from shake-flask or chemostat cultures, by addition of 20 % (v/v) glycerol to cultures and storage of 2 ml aliquots in sterile vials at –80 °C.

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
CEN.PK 113-7D	MATa *URA3 PDC1 PDC5 PDC6*
CEN.PK182	MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP*
CEN.PK111-61A	MATα *ura3-52 leu2-112 his3-Δ1*
RWB837	MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP ura3-52*
RWB837*	'MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP ura3-52*', selected for C_2-indepence in glucose-limited chemostats
TAM	'MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP ura3-52*', selected for C_2-indepence in glucose-limited chemostats and glucose-tolerant-growth in batch.

Strain construction. RWB837 was obtained from a cross between CEN.PK182 and CEN.PK111-61A (constructed by Dr. P. Kötter, Frankfurt, Germany and obtained from Dr. Jefferson C. Lievense, Tate and Lyle North America). The resulting diploid was sporulated and the asci heated for 15 min at 56 °C. This random dissection mix was then plated on YP with 0.2 % acetate as the carbon source. The resulting colonies were tested for growth on YP medium with glucose or ethanol. Colonies that could not grow on glucose were subsequently checked by PCR for the presence of a disrupted *PDC6* gene and the mating type. Determination of the auxotrophic markers present, in this case *ura3-52*, then gave RWB837. The final strain (designated TAM), selected as described below, was transformed with YEplac195 (14) according to the high-efficiency protocol described by Gietz and Woods (15), resulting in the prototrophic (ura^+) TAM+YEplac195 strain.

Chemostat cultivation. Aerobic carbon-limited or nitrogen-limited chemostat cultivations were performed as described previously (45). To complement auxotrophy, 0.15 g l^{-1} uracil (32) was added to the media. The synthetic medium for the glucose-limited chemostat cultures contained 250 mM substrate carbon. When acetate was present, this was added on top of the 250 mM carbon from glucose, at concentrations corresponding to 0-5% acetate on a substrate-carbon basis. For nitrogen-limited cultures the glucose concentration in the synthetic medium was adjusted during a trial run, to obtain a residual glucose concentration in the culture broth of approximately 100 mM. Afterwards, reproducible duplicate cultures were obtained at this glucose concentration.

Shake-flask cultivation. The 500 ml shake flasks, containing 100 ml synthetic medium (49) were incubated at 30 °C in a rotary shaker (200 rpm). To rescue auxotrophy 0.15 g l^{-1} uracil (32) was added to the media. Precultures of RWB 837 were grown on 2% ethanol. For all other shake-flask cultures glucose was used as the carbon source, with concentrations ranging from 2 to 10 % w/v. The selected strain was routinely checked for uracil auxotrophy to verify culture purity.

Fermenter batch cultivation. Aerobic batch cultivation was performed at 30 °C in 2 l fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1 l. The pH was controlled at 5.0 via automated addition of 10 M KOH (Applikon ADI 1030 biocontroller). The dissolved-oxygen concentration was maintained above 10% of air saturation at all times by adjusting the stirrer speed between 800-1000 rpm and the air flow between 0.50-0.75 l min^{-1}. A synthetic medium with twice the concentrations described by Verduyn *et al.* (49) was used. The initial glucose concentration was 100 g l^{-1}. During the repeated batch, 100 g of non-sterile solid glucose was added twice at 32 and 48 h after inoculation. Antifoam (BDH) was added to the fermenters when required. Culture purity was checked microscopically at the end of the fermentation and no contaminants were observed.

Microarray analysis. Sampling of cells from chemostats, probe preparation and hybridisation to Affymetrix GeneChip® Microarrays was performed as described previously (30). The results were derived from two independent replicate cultures for the selected Pdc^- strain and from three independently cultured replicates for the wild type.

Microarray data acquisition and analysis. Acquisition and quantification of array images and data filtering were performed using the Affymetrix software packages: Microarray Suite v5.0, MicroDB v3.0 and Data Mining Tool v3.0. For further statistical analysis Microsoft Excel running the Significance Analysis of Microarrays (SAM; v1.12) add-in was used, with a delta value that corresponded with the minimum expected median false-positive rate and a minimum change of 2-fold (40). In our experience, these criteria establish a data set able to be reproduced by an independent laboratory (30).

Before comparison, all arrays were globally scaled to a target value of 150 using the average signal from all gene features using Microarray Suite v5.0. From the 9,335 transcript features on the YG-S98 arrays a filter was applied to extract 6,383 yeast open reading frames of which there were 6,084 different genes. This discrepancy was due to several genes being represented more than once when sub-optimal probe sets were used in the array design. Since the lowest 900 transcripts could not be reliably measured, their level was set to a value of 12 for the comparison analyses.

Promoter analyses were performed using the web-based software 'Regulatory Sequence Analysis Tools' (42) as was described previously (2).

Analytical procedures. Dry weight determination, glucose, acetate and metabolite analysis, off-gas analysis and pyruvate decarboxylase and threonine aldolase assays were performed as described previously (46). The protein content of whole cells was determined by a modified biuret method (48).

Results

Selection of C_2 independent Pdc⁻ *S. cerevisiae* in chemostat cultures. In this study, the power of chemostat cultivation as a tool for the selection of microorganisms (5,17), was used in an attempt to eliminate the C_2-compound requirement of Pdc⁻ *S. cerevisiae* (6,8). For the selection a *pdc1,5,6Δ ura3Δ S. cerevisiae* strain (RWB 837) was used. The *ura3Δ* auxotrophic marker was used to facilitate controls for culture purity. First, a steady state of Pdc⁻ *S. cerevisiae* on a mixture of 5% acetate and 95% glucose on carbon basis was established. The metabolism of this culture was fully respiratory, as was indicated by a respiratory quotient of just over one carbon dioxide produced per oxygen consumed. The biomass yield on carbon was 14.6 g biomass $Cmol^{-1}$ and all glucose and acetate was consumed. Then the acetate content of the synthetic medium was lowered in 5 consecutive steps from 5% of the total carbon content to zero. Each step lasted 5 volume changes. During this slow transition, RWB 837 adapted for growth in aerobic carbon-limited chemostat cultures, with glucose as the sole carbon source, at a dilution rate of 0.10 h^{-1}. The biomass yield on substrate (14.7 g biomass $Cmol^{-1}$), oxygen consumption rate and carbon dioxide production rate (both around 2.9 mmol g $biomass^{-1}$ h^{-1}) of this glucose-limited culture indicated respiratory carbon metabolism of the C_2-independent Pdc⁻ *S. cerevisiae* culture, as observed with wild-type *S. cerevisiae* under these conditions (43).

Transcriptome analysis of the C_2-independent Pdc⁻ *S. cerevisiae* strain. Transcriptome analysis of the glucose-limited chemostat culture of the C_2-independent *S. cerevisiae* strain was performed to study the genetic changes responsible for the C_2-independence. The C_2-independent Pdc⁻ strain was compared to glucose-limited chemostat cultures of the wild type (30). Of the genes with a known function, only 18 were upregulated and only 16 genes were downregulated in the selected strain. These upregulated genes included 11 genes involved in meiosis or sporulation (*HOP2*, *IME2*, *REC102*, *REC104*, *RED1*, *SLZ1*,

SPO13, *SPO16*, *SPR1*, *YER179W* and *ZIP1*). The other seven upregulated genes were *CAR1*, *ECM1*, *HXT3*, *HXT4*, *IRE1*, *NUF1* and *NUF2*. The downregulated genes included four expected genes (*PDC1*, *PDC5*, *PDC6* and *URA3*) and in addition *ALP1*, *AQY1*, *GND2*, *FUI1*, *HSP30*, *HXT5*, *MEP2*, *MLS1*, *PDR12*, *PHO4*, *SSA3*, *SSA4*. None of these genes had a clear link to the C_2-independence of the selected mutant. Transcript levels of the *GLY1* gene, overexpression of which alleviates the C_2 requirement of Pdc$^-$ *S. cerevisiae* (46), were not significantly changed in the selected strain.

Selection for glucose tolerance in shake-flask cultures. After the selection for C_2 independence, a small aliquot of the chemostat culture was transferred to a shake flask with synthetic medium containing uracil and 20 g l^{-1} glucose. As was expected from previous results, neither the original Pdc$^-$ *S. cerevisiae* nor the C_2-independent Pdc$^-$ strain grew on agar plates with synthetic medium, uracil and 2% glucose (Fig. 2 right), whereas both strains did grow on agar plates with synthetic medium, uracil and 2% ethanol (Fig. 2 left). In agreement with this, no growth was observed during the first seven days of the initial shake-flask culture of the C_2-independent Pdc$^-$ strain on 2% glucose. Prolonged cultivation of the C_2-independent Pdc$^-$ strain, however, resulted in significant biomass formation, indicating an accumulation of spontaneous glucose-tolerant mutants. The observed growth rate was well below 0.01 h^{-1}. After growth had ceased, which occurred at relatively low biomass density due to acidification of the culture by pyruvic acid accumulation, one ml of the culture was transferred to a new shake flask with identical synthetic medium.

The process of serial transfer was repeated 27 times in total. The specific growth rate of the Pdc$^-$ strain after the sixth transfer was already 0.10 h^{-1} on 20 g l^{-1} glucose. After 14 shake-flask cultivations and an obtained specific growth rate of 0.18 h^{-1}, the glucose content of the medium was raised to 32, 54, 69 and 100 g l^{-1} in consecutive cultures. At 100 g l^{-1} of glucose, the finally obtained C_2-independent, glucose-tolerant Pdc$^-$ *S. cerevisiae* culture grew at a specific growth rate of 0.20 h^{-1}.

The culture, possibly consisting of a mixture of different spontaneous mutants, was streaked on agar plates with synthetic medium, glucose and uracil. Four of the resulting colonies were tested for growth in shake flasks on glucose and no significant differences in specific growth rate were observed. One of these cultures was chosen for further study and this C_2-independent glucose-tolerant Pdc$^-$ *S. cerevisiae* strain will be referred to as TAM in this and future work.

The differences in growth between the original Pdc⁻ strain (RWB 837), the C_2-independent Pdc⁻ strain, the TAM strain and the isogenic wild-type strain, on synthetic medium with glucose or ethanol as the sole carbon source were clearly demonstrated by agar-plate growth as depicted in Fig. 2. Although the TAM strain displayed a three-day-longer lag phase, all four strains grew on plates with ethanol as the carbon source. As described above, when glucose was the carbon source the original Pdc⁻ strain (RWB837) and the C_2-independent Pdc⁻ strain did not grow. Consistent with the growth in shake flasks on glucose, the selected TAM strain, and of course the wild type, proliferated well on the agar plates with glucose (Fig. 2).

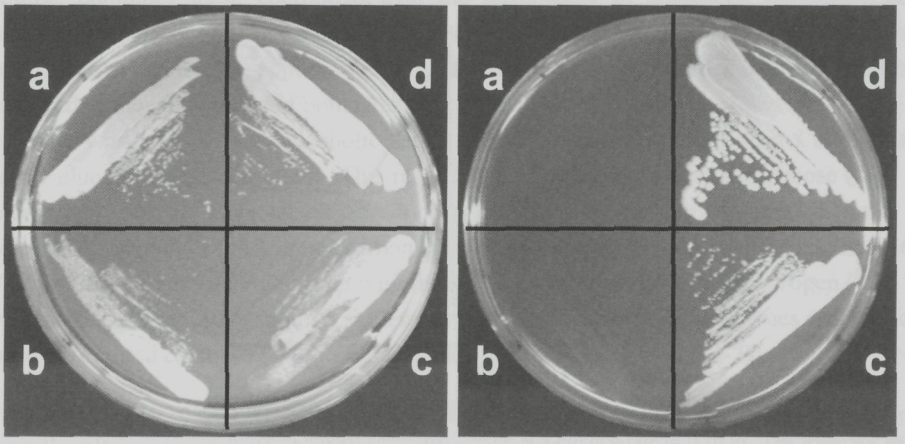

Fig. 2. Growth of three Pdc⁻ *S. cerevisiae* strains and wild-type *S. cerevisiae* on synthetic-medium agar plates with ethanol (left plate) or glucose (right plate) as the carbon source. Both plates were supplemented with uracil to alleviate the auxotrophy of the Pdc⁻ *S. cerevisiae* strains. Ethanol plates were incubated for 7 days, glucose plates were incubated for 3 days. The strains used were: a; RWB837 (Pdc⁻ *S. cerevisiae*), b; RWB837* (selected C_2-independent *S. cerevisiae*), c; TAM (selected C_2-independent and glucose-tolerant Pdc⁻ *S. cerevisiae*), and d; CEN.PK 113-7D (wild type).

Pyruvate production by the selected TAM strain in fermenter cultures. During the selection for glucose tolerance in shake flasks, a rapid acidification of the culture due to pyruvate excretion was observed. To study growth and pyruvate production of the TAM strain under controlled conditions, aerobic batch cultivations on 100 g l^{-1} glucose were performed in fermenters at a constant pH of 5.0. During the exponential growth phase (Fig. 3) the maximum specific growth rate of the TAM strain was 0.20 h^{-1}, which equalled the maximum specific growth rate in shake flasks. Consistent with the observations in shake flasks, large amounts of pyruvate were produced in fermenter cultures. The rate of pyruvate production during the exponential growth phase was 6-7 mmol g biomass^{-1} h^{-1}. In the first 40 h of this batch, starting with a low biomass concentration (an OD_{660} of 0.1), a pyruvate concentration of 50 g l^{-1} was obtained with a yield of 0.55 g pyruvate g glucose^{-1}.

To further assess the potential of the TAM strain for the production of pyruvate the fermentation was continued as a repeated batch by the addition of solid glucose to the fermenter (Fig. 3). During this repeated batch phase the specific growth rate gradually decreased and growth ultimately ceased, probably due to nutrient limitations in the medium. The pyruvate concentration in the supernatant exceeded 100 g l^{-1} within 60 hours after inoculation of the fermenter. The final concentration of pyruvate obtained after 100 hours was 135 g l^{-1}, with an overall yield of 0.54 g pyruvate g $glucose^{-1}$.

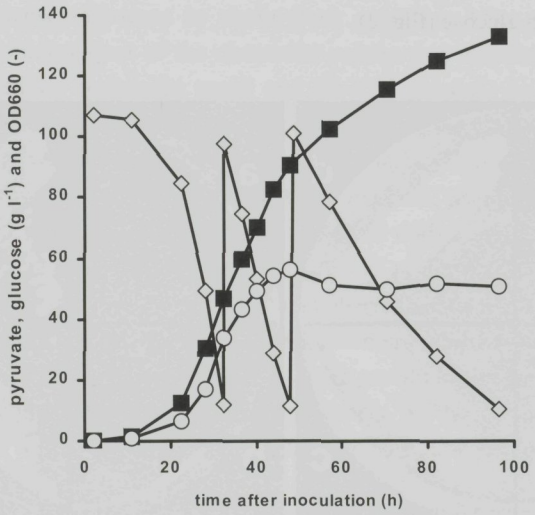

Fig. 3. Growth and pyruvate production during an aerobic repeated batch culture on glucose of the selected TAM strain. The results shown are from one representative batch experiment. Biomass and pyruvate concentrations in independent replicate experiments varied by less than 5 %. The closed squares indicate pyruvate concentration, open symbols refer to the glucose concentration (diamonds) or OD_{660} (circles).

Glucose-limited chemostat cultivation of the TAM strain. The obtained maximum specific growth rate of the TAM strain in batch on glucose was 0.20 h^{-1}. Under the same conditions wild-type *S. cerevisiae* CEN.PK 113-7D grew with a higher maximum specific growth rate of 0.37 h^{-1} (data not shown). To further study this deviation in growth, glucose-limited chemostat cultures of the TAM strain were performed at increasing dilution rates. At a dilution rate of 0.10 h^{-1}, glucose dissimilation by the TAM strain was fully respiratory, without accumulation of metabolites. Except for a lower biomass yield of the selected strain (0.43 g biomass g $glucose^{-1}$ compared 0.48 g biomass g $glucose^{-1}$ for the wild type), physiological parameters were comparable to those of the wild type (43). At a dilution rate of 0.15 h^{-1} the biomass yield of the TAM strain had increased to 0.47 g biomass g $glucose^{-1}$, which is still lower than the 0.50 g biomass g $glucose^{-1}$ for the wild type at this dilution rate. The TAM strain was capable of growth at a dilution rate of 0.20 h^{-1} in glucose-limited chemostat cultures, but this growth was accompanied by a variable pyruvate production rate (0.25-0.45 mmol pyruvate g $biomass^{-1}$ h^{-1}). At a dilution rate of 0.23 h^{-1} the TAM strain washed out of the chemostats, indicating a maximum specific

growth rate of this strain between 0.20 h^{-1} and 0.23 h^{-1} in glucose-limited chemostat cultures. After this prolonged glucose-limited chemostat cultivation an aliquot of the culture was transferred to a shake flask with 100 g l^{-1} of glucose. In this shake flask rapid growth was observed, indicating that the culture had maintained its glucose-tolerant phenotype.

Nitrogen-limited chemostat cultivation of the TAM strain. Comparison of the selected TAM strain with wild-type *S. cerevisiae* CEN.PK 113-7D is best performed at high glucose concentrations and in the absence of C_2-compounds in the medium. Nitrogen-limited chemostat cultivation at the same dilution rate with glucose as the sole carbon source, combines these conditions with the advantages of chemostat cultivation for reproducible physiological studies. The glucose concentrations in the synthetic medium were chosen such that approximately the same residual glucose concentration was obtained in the cultivations of both strains (Table 2).

The wild type showed alcoholic fermentation, as is characteristic for *S. cerevisiae* under glucose-excess conditions. This resulted in a low biomass yield on glucose (0.09 g biomass g $glucose^{-1}$), an ethanol production rate of 8.0 mmol g $biomass^{-1}$ h^{-1} and a respiratory quotient of 4.5 mmol carbon dioxide produced per mmol oxygen consumed. The protein content (0.29 g protein g $biomass^{-1}$) and the biomass yield on nitrogen (18.8 g biomass g $nitrogen^{-1}$) are in good agreement with previously published values (44,45) for nitrogen-limited chemostat cultures of the wild-type strain CEN.PK113-7D.

Table 2. Physiology of TAM (C_2-independent, glucose-tolerant Pdc⁻ *S. cerevisiae*) and the isogenic wild-type CEN.PK 113-7D in aerobic nitrogen-limited chemostat culture at a dilution rate of 0.10 h^{-1}. The wild-type data are obtained from the same cultures as used by Boer *et al.* (2003). Averages and mean deviations were obtained from duplicate (TAM) and triplicate (wild type) experiments, respectively, with independent steady-state cultures. Calculations of the carbon recovery were based on a carbon content of biomass of 48 % (w/w). Y_{sx} and Y_{nx} are the biomass yields on glucose and nitrogen.

Characteristic	Wild type	TAM
Reservoir glucose concentration ($g \cdot l^{-1}$)	58.8 ± 0.1	35.1 ± 0.1
Residual glucose concentration ($g \cdot l^{-1}$)	16.7 ± 0.7	20.4 ± 0.1
Y_{sx} ($g_{biomass} \cdot g_{glucose}^{-1}$)	0.09 ± 0.00	0.21 ± 0.00
Y_{nx} ($g_{biomass} \cdot g_N^{-1}$)	18.8 ± 0.1	14.7 ± 0.1
Protein content ($g_{protein} \cdot g_{biomass}^{-1}$)	0.29± 0.01	0.33± 0.01
RQ	4.5 ± 0.2	0.70 ± 0.01
$q_{glucose}$ ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	5.8 ± 0.1	2.6 ± 0.1
$q_{ethanol}$ ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	8.0 ± 0.1	<0.01
$q_{pyruvate}$ ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	**0.1 ± 0.0**	**2.8 ± 0.0**
$q_{glycerol}$ ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	0.08 ± 0.00	<0.01
$q_{acetate}$ ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	0.06 ± 0.02	<0.01
q_{CO2} ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	12.1 ± 0.2	2.8 ± 0.0
q_{O2} ($mmol \cdot g_{biomass}^{-1} \cdot h^{-1}$)	2.7 ± 0.1	4.0 ± 0.1
Recovery of consumed carbon (%)	94.0 ± 1.0	97.4 ± 0.7
Total carbon recovery (%)	95.6 ± 0.7	98.6 ± 0.4

Under the same conditions, the TAM strain, that fully depends on respiration in the absence of alcoholic fermentation, had a higher biomass yield on glucose (0.21 g biomass g glucose^{-1}) and produced pyruvate as the only major by-product at a rate of 2.8 mmol g biomass^{-1} h^{-1} (Table 2). The oxygen consumption rate was 4.0 mmol g biomass^{-1} h^{-1} compared to 2.7 mmol g biomass^{-1} h^{-1} for the wild type. The respiratory oxidation of the NADH formed during pyruvate formation lowered the respiratory quotient to 0.70 mmol carbon dioxide produced per mmol oxygen consumed. The protein content of the biomass was slightly higher for the TAM strain (0.33 g protein g biomass^{-1}) than it was for the wild type (0.29 g protein g biomass^{-1}). This higher protein content of the cells partially explains the significantly lower yield on nitrogen of the TAM strain (14.7 g biomass g nitrogen^{-1}) compared to the wild type (18.8 g biomass g nitrogen^{-1}).

Transcriptome analysis of the TAM strain. Central in transcriptome analysis is the choice of adequate culture conditions for the comparison. In the case of the selected TAM strain the absence of C_2 compounds and the presence of high levels of glucose in the broth are typical for uncovering its phenotype. To combine the benefit of chemostat cultures in microarray studies (30) and the requirement for glucose excess, the nitrogen-limited chemostat cultures of the TAM strain and the isogenic wild-type CEN.PK 113-7D, were chosen for the transcriptome analysis.

The comparison of the nitrogen-limited chemostat cultures revealed 305 genes of which the mRNA level was significantly changed and at least two-fold higher in the TAM strain than in the wild type. The mRNA abundance of 168 genes was significantly changed and at least two-fold lower in the TAM strain than in the wild type. In total, these changed genes comprise almost 8% of the total *S. cerevisiae* genome. Of these changed genes, 273 (58%) have an unknown function, which is higher than the percentage of not fully annotated genes in the whole *S. cerevisiae* genome (47%).

Sequence analysis of the upstream regions of genes upregulated in the selected strain showed an overrepresentation of possible Mig1p-binding sites amongst these genes, indicating a (partial) alleviation of Mig1p-mediated repression. Although the transcript level of the primarily posttrancriptionally (12) regulated *MIG1* is not changed, the transcript level of its close homologue *MIG2* was almost 11-fold downregulated. Many genes required for growth on carbon sources other than glucose, were upregulated in the TAM strain. This included genes involved in gluconeogenesis and ethanol utilisation (*ACS1*, *ADH2*, *ADR1*, *CAT8*, *FBP1*, *SIP4*), fatty acid metabolism (*CAT2*, *CRC1*, *ECI1*, *FAA2*, *FOX2*, *PEX11*, *POT1*, *POT1*, *YAT2*), galactose metabolism (*GAL2*, *GAL3*, *GAL4*), maltose metabolism (*MPH2, MPH3, YFL052W*) and pyruvate and lactate metabolism (*DLD1*, *JEN1*).

A striking observation was the change in expression of the genes coding for the hexose transporters. Despite the high glucose concentrations under nitrogen limitation the low-affinity transporters (*HXT1* and *HXT3*) were downregulated 50-fold in the TAM strain compared to the wild type (Fig. 4). The known high-affinity transporters (*HXT6* and *HXT7*) were also downregulated (4 fold) in this strain (Fig. 4). As a result, the summed transcript abundance of all *HXT* genes represented on the arrays (*HXT1-10*, *HXT12*, *HXT14* and *HXT16*) is four times lower in the TAM strain under nitrogen limitation than in the wild type. In the glucose-responsive regulatory network of the *HXT* genes (35), the only significant transcriptional change was the 12-fold downregulation of *STD1*, a glucose-concentration-dependent modulator of expression, in the TAM strain compared to the wild type.

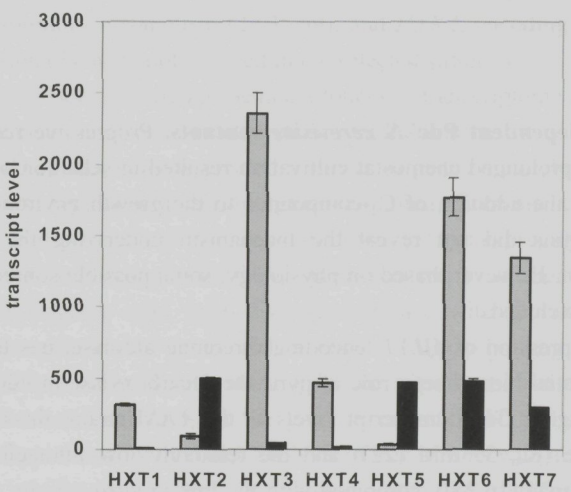

Fig. 4. Transcript-level comparison between the selected TAM strain and its isogenic wild type in nitrogen-limited chemostat cultures with glucose as the carbon source, of the main hexose transporter genes (*HXT1-7*). The wild-type data were obtained from the same cultures as used by Boer *et al.* (2003). The data represented were obtained from independent duplicate (TAM) or triplicate (wild type) chemostat cultivations. The grey bars correspond to the wild type, whereas the black bars corresponds to the TAM strain. Error bars represent mean deviation.

Since the TAM strain was not only glucose tolerant but also independent of C_2-compounds for growth on glucose, it is of interest to know to what extent transcriptional changes are responsible for the obtained C_2 independence. The transcript abundance of *GLY1*, a previously demonstrated source of cytosolic C_2-compounds (46), was upregulated 2.5 fold in the TAM strain. However, the threonine aldolase activity in the TAM strain was still below the detection limit of 0.005 U mg protein^{-1}. The derepressed genes of fatty acid metabolism can also be interpreted as a modification in the metabolism of cytosolic C_2-compounds. In addition two genes (*CAR1* and *CAR2*) of the arginine

metabolism, which includes reactions involving acetyl-CoA, were upregulated 6-fold in the TAM strain. Thus, no immediate and complete explanation for the C_2-independence of this strain could be established via transcriptome analysis.

A surprisingly large group of upregulated genes in the selected Pdc$^-$ strain were involved in mating (3 genes), meiosis (17 genes) and sporulation (8 genes). Since both the original strain (RWB837) and the selected Pdc$^-$ strain are confirmed haploids, the mechanism behind and the origin of the expression of these genes, including the early meiotic transcription factor *IME1*, remains unknown. Sequence analysis of the upstream regions of the upregulated genes in the selected Pdc$^-$ strain, also clearly showed an overrepresentation of the binding sites for *UME6* and *IME1*, both involved in regulation of meiosis.

Discussion

Selection of C_2-independent Pdc$^-$ *S. cerevisiae* mutants. Progressive reduction of the acetate feed during prolonged chemostat cultivation resulted in selection of a Pdc$^-$ strain that did not require the addition of C_2-compounds to the growth environment (Fig. 1). Transcriptome analysis did not reveal the mechanism underlying the physiological changes in this strain. However, based on physiology, some possible sources of cytosolic acetyl-CoA can be excluded.

Massive overexpression of *GLY1*, encoding threonine aldolase, has been shown to circumvent the essential biosynthetic role of pyruvate decarboxylase in Pdc$^-$ *S. cerevisiae* (45). Despite the higher *GLY1* transcript levels in the TAM strain, the low affinity of Gly1p for threonine (K_m 55 mM (22)) and the relatively low intracellular threonine concentration (5-10 mM (16,23)), combined with the low *in vitro* activity (<0.005 U mg protein^{-1}), make it unlikely that threonine aldolase is responsible for its C_2-independent phenotype. In addition, the absence in *S. cerevisiae* of *de novo* carnitine biosynthesis (47) and of ATP-citrate lyase (34) eliminate the carnitine shuttle and citrate efflux from the mitochondria as possible sources of cytosolic acetyl-CoA. *In vitro* pyruvate-decarboxylase activity was not observed, nor was acetate or ethanol detected under any of the tested conditions.

Selection of glucose-tolerant Pdc$^-$ *S. cerevisiae* mutants. The exact reason for the inability of Pdc$^-$ strains to grow on high concentrations of glucose, even in the presence of C_2-compounds or with a threonine aldolase overproduction construct, is still not known. Respiration is obviously essential for the reoxidation of cytosolic NADH formed by glycolysis (Fig. 1). However, the significant respiration rate of wild-type *S. cerevisiae* (1,4,20) under conditions with excess glucose suggests that oxidative regeneration of cytosolic NADH, and therefore ATP synthesis via glycolysis and oxidative phosphorylation, should also be possible in Pdc$^-$ *S. cerevisiae* under these conditions.

Furthermore, any surplus respiratory capacity can be used for oxidative dissimilation by the mitochondria. What then causes the inability of Pdc⁻ *S. cerevisiae* to grow under conditions with excess glucose?

This study does not answer this question satisfactory. However, transcriptome analysis indicated a partial relief of repression of genes with a possible Mig1p-binding box in the upstream sequence and a 4-fold decrease in transcript levels of genes involved in glucose uptake. Combined deletion of *MIG1* and *MIG2* has been reported to increase the respiratory capacity and specific growth rate of *S. cerevisiae* in aerobic glucose-grown cultures (19). In addition it has been shown before, during a study of phosphoglycerate-mutase-negative *S. cerevisiae*, that partial alleviation of carbon catabolite repression in a suppressor mutant coincided with a decreased glucose uptake capacity (11). Interestingly, transcript levels of *HAP4*, encoding a known positive transcriptional regulator of genes involved in respiratory metabolism (27,36,39), and *HXK2*, encoding a central protein in glucose repression (3), were not affected in the selected strain.

Recently, Boer *et al.* (2) presented a four-way transcriptome analysis of glucose-, nitrogen-, phosphate- and sulfur-limited chemostat cultivations of wild-type *S. cerevisiae* CEN.PK 113-7D. Their analysis yielded a subset of genes uniquely up- (164 genes) or down- (62 genes) regulated in aerobic glucose-limited chemostat cultivations. 57% of these genes were also found up- (94 genes) or down- (35 genes) regulated in the nitrogen-limited chemostat cultures of the TAM strain relative to nitrogen-limited chemostat cultures of the isogenic wild type. This resemblance with glucose-limited wild-type cultures, the upregulation of genes with a possible Mig1p-binding box and the 4-fold lowering of *HXT*-transcript levels, all support the hypothesis that a release of glucose catabolite repression contributes to the glucose tolerance of the selected Pdc⁻ strain. Testing of this hypothesis by thorough analysis of Pdc⁻ strains with additional deletions in transcriptional regulators involved in glucose catabolite repression is currently in progress.

DNA microarrays as a diagnostic tool for yeast strain improvement. To maximize the quality of the transcriptome data, the TAM strain and an isogenic reference strain were grown in replicate chemostat cultures under identical, carefully defined conditions. Although a substantial number of genes yielded a significantly altered transcript level, this did not yield a clear insight into the molecular mechanisms responsible for C_2-independence. In the case of glucose tolerance only a possible correlation with glucose catabolite repression was observed. This demonstrates that application of high-information-density analytical tools will not always yield clearcut answers to physiological questions. DNA microarrays, however valuable for identifying changes in transcript level, cannot identify many other relevant changes, such as point mutations or changes in post-transcriptional regulation. The absence of a clearly established biological function for many of the differentially transcribed genes further complicates interpretation of transcriptome analysis. Taking into account that similar constraints exist for proteome

and metabolome analysis, it has to be concluded that the full analysis of complex phenotypes continues to require time- and labour-intensive genetic dissection of the genotype of selected strains.

Metabolic fluxes in the TAM strain and wild-type *S. cerevisiae*. During carbon-limited growth at a dilution rate of 0.10 h^{-1}, both the TAM strain and wild-type *S. cerevisiae* displayed full respiratory glucose metabolism, although the biomass yield of the TAM strain was almost 10% lower. In nitrogen-limited chemostat cultures at a dilution rate of 0.10 h^{-1}, a more suitable environment to investigate the selected glucose-tolerant phenotype, the oxygen consumption rate of the TAM strain (4.0 mmol g biomass^{-1} h^{-1}) was higher than that of the wild type (2.7 mmol g biomass^{-1} h^{-1}) (Table 2). Interestingly, the increase in oxygen consumption rate (4.0 - 2.7 = 1.3 mmol g biomass^{-1} h^{-1}) almost equals the oxygen required to regenerate the cytosolic NADH formed during pyruvate production (0.5 x 2.8 = 1.4 mmol g biomass^{-1} h^{-1}). The rate of oxidative pyruvate dissimilation by the mitochondria is apparently identical in nitrogen-limited chemostat cultures of the TAM strain and of wild-type *S. cerevisiae*.

Production of pyruvate by the selected TAM strain. The excretion of pyruvate by *S. cerevisiae* with reduced or absent pyruvate-decarboxylase activity has been observed before (9,38). The TAM strain, however, displays rapid growth on synthetic medium with glucose as the sole carbon source, whereas other Pdc$^-$ *S. cerevisiae* strains completely fail to grow under these conditions. The TAM strain has the additional benefit of the complete absence of ethanol as a byproduct and, in contrast to many other pyruvate-producing microorganisms, does not require the addition or omission of specific compounds in the media (21). The final pyruvate concentration of 135 g l^{-1} (Fig. 3) is almost 2-fold higher than the previously highest concentration obtained by fermentation (24). The high specific rate of pyruvate production (6-7 mmol pyruvate g biomass^{-1} h^{-1}) resulted in 100 g l^{-1} of pyruvate, starting with a low density inoculum (OD_{660} of 0.1), in less than 60 h. The amount of pyruvate obtained per gram of glucose consumed was 0.54 g pyruvate g glucose^{-1} for both the repeated batch and the nitrogen-limited chemostat cultures of the TAM strain. This yield is lower than the highest reported yield in the public domain (0.68 g pyruvate g glucose^{-1}) (24). Engineering of the respiratory pyruvate degradation, either by decreasing internal mitochondrial respiration or decreasing the activity of the pyruvate-dehydrogenase complex, might result in improvement of the pyruvate yield on glucose of the TAM strain. Even allowing for its suboptimal pyruvate yield, the high final pyruvate concentration (135 g l^{-1}) and pyruvate production rate (6-7 mmol g biomass^{-1} h^{-1}) already make the TAM strain a serious contender for industrial pyruvate production via microbial fermentation (21).

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Chapter 4

Modulating the distribution of fluxes among respiration and fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*

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The tendency of *Saccharomyces cerevisiae* to favour alcoholic fermentation over respiration is a complication in aerobic, biomass-directed applications of this yeast. Overproduction of Hap4p, a positive transcriptional regulator of genes involved in respiratory metabolism, has been reported to positively affect the balance between respiration and fermentation in aerobic glucose-grown batch cultures. In this study, the effects of *HAP4* overexpression have been quantified in the prototrophic *S. cerevisiae* strain CEN.PK113-7D under a variety of growth conditions. In aerobic glucose-limited chemostat cultures, overexpression of *HAP4* increased the specific growth rate at which aerobic fermentation set in by about 10% relative to the isogenic wild type. Upon relief of glucose-limited conditions, the *HAP4*-overexpressing strain produced slightly less ethanol than the wild-type strain. The effect of Hap4p overproduction was most drastic in aerobic, glucose-grown chemostat cultures in which ammonium was limiting. In such cultures, the biomass yield on glucose was double that of the wild type.

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Introduction

The two main modes of aerobic sugar dissimilation in the yeast *Saccharomyces cerevisiae* are alcoholic fermentation and respiration. Alcoholic fermentation results in a lower biomass yield per sugar consumed than respiratory dissimilation and is accompanied by accumulation of metabolites (35,36). The ability of *S. cerevisiae* to rapidly ferment sugars is used in metabolite-directed applications, such as beer, bread and wine production and in the production of fuel ethanol. *S. cerevisiae* is also applied in biomass-directed processes, including the production of bakers' yeast, yeast extract and heterologous proteins. Low by-product formation and a high biomass yield on sugar are prerequisites for economical viability of biomass-directed applications. In *S. cerevisiae* the main respiratory pathway via the pyruvate-dehydrogenase complex and the TCA-cycle yields approximately 16 ATP per glucose consumed (23,38). This results in a yield of a respiratory culture of *S. cerevisiae* of 0.50 g biomass per g glucose (23,38). The fermentation of glucose to ethanol plus CO_2 yields only 2 molecules ATP per molecule glucose. Consequently, the biomass yield of purely fermentatively growing cells is only 0.10 g biomass per g glucose (36). The observation that the biomass yield on glucose is not proportional to the ATP yield on glucose is due to the fact that glucose is not only catabolized, but also incorporated into biomass. Because of the low biomass yield of fermenting cultures, alcoholic fermentation should be avoided in biomass-directed processes.

In *S. cerevisiae* fermentation is not restricted to oxygen-limited conditions (40). Even under fully aerobic conditions alcoholic fermentation occurs if the glucose concentration exceeds a certain threshold value and at high specific growth rates (22,39). The specific growth rate at which alcoholic fermentation sets in, is called the critical specific growth rate. In industrial, biomass-directed applications alcoholic fermentation is avoided by glucose-limited fed-batch cultivation and intensive aeration and mixing. Yet, in large-scale fermentors and particularly during high-cell-density cultivation, high glucose concentrations can occur locally due to inadequate mixing. This then leads to a decrease of the biomass yield and an increase of by-product formation.

To construct a *S. cerevisiae* strain in which the metabolic flux is diverted from alcoholic fermentation to respiration, one could either block the fermentative pathway or stimulate the respiratory pathway. Flikweert *et al.* have focussed on the first option and deleted all structural genes encoding pyruvate decarboxylase isoenzymes, *PDC1*, *PDC5* and *PDC6* (7). Growth of this strain, however, required addition of a C_2 compound (acetate or ethanol) to the medium, presumably to meet the requirement of the cell for cytosolic acetyl CoA (7). A *pdc2Δ* strain with a decreased pyruvate decarboxylase activity could grow on glucose as the sole carbon source, but it accumulated pyruvate and its maximal specific growth rate was decreased by almost twofold compared to that of the wild type (8). In this study we will focus on increasing the respiratory flux.

In many cases it has been attempted to increase pathway capacity by overexpression of a single, supposedly rate-limiting enzyme (26). Such an approach is usually bound to

fail, because a metabolic flux is hardly ever controlled by a single enzyme. Rather, flux control is distributed among several or all of the enzymes in the pathway (6,14). Moreover, even an enzyme that exerts substantial control over the flux, often loses control when it is overexpressed. A much more successful approach may be to overproduce all enzymes in the pathway of interest (13,19). In case of the respiratory pathway of *S. cerevisiae* this approach is complicated, because of the large number of genes involved.

An attractive alternative is modulation of the expression of a global regulator, which in turn regulates the expression of many genes involved in respiration (2,15). The onset of alcoholic fermentation at high glucose concentrations is, at least partially, caused by glucose catabolite repression of the synthesis of respiratory enzymes. When glucose is present at high concentrations, many genes involved in respiration, gluconeogenesis and utilization of non-glucose carbon sources are expressed at low levels or not at all (10,24). An important regulatory system is the Hap2/3/4/5p-complex, which activates transcription of genes encoding respiratory-chain components (*e.g.* Qcr8p and Cyt1p of the bc_1 complex) and enzymes of the TCA cycle (*e.g.* citrate synthase Cit1p) in the presence of non-fermentable carbon sources such as ethanol and acetate (3,4,10,20,25,27). Expression of *HAP4* is repressed to a low level in the presence of glucose and induced when only non-fermentable carbon sources are available, while *HAP2* and *HAP3* are expressed constitutively (9). This suggests that Hap4p, necessary for activity of the Hap2/3/4/5p-complex, is the regulatory part of the complex. Hence, it is to be foreseen that overproduction of Hap4p will result in an increased expression of respiratory genes and a stimulation of the respiratory pathway. Indeed this has been shown to be the case. The overproduction of Hap4p in *S. cerevisiae* strain Dl1 in batch cultures resulted in a 40% increased biomass yield on glucose in aerobic batch cultures, from 0.10 g biomass per g glucose to 0.14 g biomass per g glucose, coinciding with an increased respiratory capacity and *in vivo* oxygen consumption flux (2).

Hitherto the physiological characterization of *HAP4*-overexpressing strains has been limited to studies on glucose-grown batch cultures. The aim of this study was to quantify the effects of *HAP4* overexpression in *S. cerevisiae* on respiration rates and biomass yield under a variety of conditions where wild-type cells tend to produce ethanol.

Materials and Methods

Strains and maintenance. The *HAP4* overexpressing strain 436 GH was constructed by chromosomal integration of the *HAP4* gene fused to the glyceraldehyde3-phosphate dehydrogenase (*TDH3*) promoter. A 2621 bp BspHI-PstI fragment from pSLF406 (9), containing the complete coding and terminator region of the *HAP4* gene was SmaI/PstI-cloned in vector 425GPD, containing the *TDH3* promoter (18). A 3359 bp Ecl136II-SalI *TDH3-HAP4* fragment was subsequently recloned into the SmaI and SalI sites of the multiple cloning site present in the *SPR3* gene on the integration vector pKSPO2 (constructed at and provided by Gistbrocades), containing the AmdS gene as dominant selectable marker (11). Strain CEN.PK 113-7D was transformed according to the LiAc method described by Ito *et al.* (12) with 10 µg PKSPO2-*TDH3-HAP4* plasmid linearised at the Sfi locus in the *SPR3* gene. Transformants were selected on medium containing 1.8% nitrogen-free agar (Oxoid), 1.17% Yeast Carbon Base (Difco), 30 mM phosphate buffer pH 6.8 and 5 mM acetamide (Sigma).

To obtain transformants which recombined the pKSPO2 plasmid sequences out of the genome, counterselection was carried out on plates containing 1.8% nitrogen-free agar, 1.17% Yeast Carbon Base, 30

mM phosphate buffer, 60 mM fluoro-acetamide (Fluka) and 0.1% $(NH_4)_2SO_4$. Transformants still containing the *TDH3-HAP4* fusion were selected by PCR and Southern blot analysis.

It was verified that the use of the *TDH3* promoter for *HAP4* overexpression - in single copy - did not lead to a decrease of the expression of *TDH3* itself. Such effects have been observed with other genes when their promoters were introduced in multicopy (17,30). Genome-wide mRNA analysis of samples from nitrogen-limited cultures, however, showed no significant effect of *HAP4* overexpression on the concentration of *TDH3* mRNA (not shown).

Stock cultures of CEN.PK 113-7D and 436 GH (Table 1) containing a final concentration of 20% (v/v) glycerol were stored at –80 °C. Cultures used for the inoculation of the precultures were maintained on YPD agar (10 g l^{-1} Difco Yeast Extract, 20 g l^{-1} Difco Peptone, 18 g l^{-1} agar and 20 g l^{-1} glucose).

Table 1 Strains used in this study

Strain	Trivial name	Genotype
CEN.PK 113-7D	wild-type strain	*MATa MAL2-8^c SUC2*
436GH	*HAP4* overexpressing strain	*MATa MAL2-8^c SUC2 spr3::TDH3p-HAP4*

Media. The mineral medium for glucose-limited chemostat cultivation contained per litre of demineralized water 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 0.05 ml silicon antifoam (BDH) and trace element concentrations according to Verduyn *et al.* (37). After heat sterilization of the medium for 20 min at 120 °C a filter-sterilized vitamin solution, prepared according to Verduyn *et al.* (37), was added. The concentration of glucose (separately heat sterilized for 20 min at 110 °C) in the reservoir medium was 7.5 g l^{-1}.

To assure nitrogen limitation in the nitrogen-limited chemostat cultures the $(NH_4)_2SO_4$ content of the medium was decreased five fold to 1 g l^{-1} and the glucose concentration was increased five fold to 37.5 g l^{-1} compared to the glucose-limited cultivations. To maintain similar sulfate concentrations, 5.28 g l^{-1} K_2SO_4 was added.

The media used for batch cultivation differed from the glucose-limited chemostat medium in a four fold increased (0.2 ml l^{-1}) antifoam concentration and an increased glucose concentration (20 g l^{-1}).

Batch cultivation in fermenters. Aerobic batch cultivation was performed in 7 l fermenters (Applikon, Schiedam, the Netherlands) with an initial working volume of 4 l. The temperature was kept constant at 30 °C. The pH was kept constant at 5.0 by an ADI 1030 biocontroller via addition of 2 M KOH. An airflow of 4 l min^{-1} and a stirrer speed of 800 rpm were applied to maintain a dissolved oxygen tension above 40% of air saturation.

Chemostat cultures. Aerobic chemostat cultivation was performed in 2 l fermenters (Applikon, Schiedam, the Netherlands) at a working volume of 1 l and at 30 °C. The ADI 1030 biocontroller kept the pH at 5.0 by titration with 2 M KOH. A stirrer speed of 800 rpm and an air flow of 0.5 l min^{-1} kept the dissolved oxygen tension higher than 40% of air saturation in all chemostat cultivations performed. The influx of medium was regulated by a peristaltic pump. The working volume of the cultures was kept constant by means of an electric level sensor. Cultures were assumed to be at steady state when, after at least five volume changes, the relevant parameters, such as culture dry weight, glucose concentration, ethanol concentration and respiratory quotient (i.e. the ratio between carbon-dioxide production and oxygen consumption) changed by less than 2% during one volume change, and showed no downward or upward trend in the last three measurements, at least one volume change apart. Sustained oscillations of the dissolved oxygen concentration were not observed. There was no significant difference in dry weight (<1%) between effluent and culture liquid.

Determination of culture dry weight. To determine biomass dry weight, a known culture volume containing 0.01-0.03 g dry weight was filtrated over dried nitrocellulose filters of known weight (pore size 0.45 μm, Gelman Sciences). The filters were washed with 20 ml demineralized water and dried for 20 min in a microwave oven at 360 W and the increase of the filter weight was measured. Duplicate samples varied by less than 1%.

Metabolite analysis. Acetate, ethanol, glucose, glycerol and pyruvate concentrations in supernatants were determined by HPLC analysis with a Biorad Aminex HPX-87H column at 60 °C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Pyruvate and acetate were detected by a Waters 2487 Dual λ Absorbance Detector at 214 nm. Ethanol, glucose and glycerol were detected by a Waters 2410 Refractive Index Detector. Acetate, ethanol and glucose concentrations were confirmed enzymically. Acetate and glucose were determined with commercial Boehringer kits (no 148 261 and 761 251). Ethanol concentrations were determined with an assay based on *Hansenula polymorpha* alcohol oxidase (39). The alcohol oxidase was a kind gift of BIRD Engineering, Schiedam, the Netherlands. Glucose concentrations in the mineral medium were determined on HPLC.

Protein determination. The protein content of the cells was determined by a modified Biuret method (36).

Gas analysis. The exhaust gas was cooled and dried before analysis of the O_2 and CO_2 concentrations. The O_2 and CO_2 concentrations in the gas exhaust from the chemostat cultures were determined with a Servomex 1100 analyser and a Rosemount Analytical model 870 infrared detector, respectively. The gas-flow rate was determined according to Weusthuis *et al.* (43). Calculation of specific O_2 consumption and CO_2 production for chemostat cultures were performed according to van Urk *et al.* (34).

mRNA isolation and Northern analysis. Messenger RNA was isolated from 2 ml culture samples according to Diderich *et al.* (5). The isolated RNA was used for Northern analysis. The gels contained 0.8 g agarose and 1% formaldehyde in NBC buffer (0.5 M boric acid, 10 mM sodium citrate, 50 mM sodium hydroxide, 0.1% diethyl pyrocarbonate). Approximately 10 µg RNA was dissolved in 5 µl 10 x NBC, 7.5 µl formaldehyde plus 25 µl formamide and RNAse free water was added to 50 µl. This was incubated at 65 °C for 5 min. After adding 2 µl of loading buffer (15% Ficoll, 0.1 M Na_2EDTA and 0.25% bromophenol blue) and 0.5 µl 2 mg ml^{-1} ethidiumbromide, the gel was loaded and run at 100 V for about 90 min with NBC as running buffer. Afterwards the RNA was transferred overnight by capillary electrophoresis to a Hybond-N-filter (Amersham Life Science), cross-linked to the membrane by ultraviolet irradiation and baked for 1 h at 80 °C.

Prehybridization was performed in hybridization buffer (0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1mM EDTA) at 65 °C. DNA fragments used as probes in this study include a 840 bp HindIII-SalI fragment from pJH1 containing the yeast gene *QCR8* (3); a 1333 bp NcoI-HindIII fragment of from pAZ6 containing the yeast gene *PDA1* (42); a 488 bp *HAP4* PCR fragment obtained from chromosomal DNA using forward primer 5'AAATGTTTCACGCAACCACCC-3' and a backward primer 5'-GCCCTGAAGATACGGTTTCC-3'; a 638 bp *CYT1* PCR fragment obtained from chromosomal DNA using the forward primer 5'-AAGCTCGTTACAGCGGGTG-3' and the backward primer 5'TTGAGTGTCGTTGCGGGG-3'; and a *COX1* PCR fragment obtained from mitochondrial DNA using the forward primer 5'CGCAGGATGAAATTATGTCG-3' and the backward primer 5'AAATGAGTGTACAGCTGGTGGA-3'.

For random labelling, DNA was denatured by heating for 10 min at 100 °C and cooled on ice. 25 ng DNA, 3 µl dNTP mix (a mixture of 0.5 mM of each dCTP, dGTP and dTTP), 2 µl hexanucleotide mix (Boehringer Mannheim), 2 µl = 20 µCi (α-^{32}P) dATP, 300 Ci $mmol^{-1}$, and 1 µl = 2 U Klenow enzyme were added to a final volume of 20 µl and incubated for 30 minutes at 37 °C. The free label was removed with a Sephadex G-50 column. The probe was denatured for 3 minutes at 100 °C before addition to the hybridization buffer.

After overnight hybridization at 65 °C the filter was washed three times with 2 SSC, 0.1% SDS; 1 SSC, 0.1% SDS; 0.5 SSC, 1% SDS each for 20 minutes at 65 °C. The filter was then exposed to a Bas-MS 2025 imaging plate (Fujifilm). Stripping of the label from the filter was performed at 75 °C in 0.1 SSC, 0.1% SDS for three times 30 min.

Results

***HAP4* overexpression increased the critical specific growth rate**. In glucose-limited chemostat cultures at dilution rates below 0.30 h^{-1}, wild-type *S. cerevisiae* CEN.PK113-7D exhibited completely respiratory growth (Fig. 1). Ethanol was absent from culture supernatants, the respiratory quotient (*i.e.* the specific CO_2 production rate divided by the specific O_2 consumption rate) was close to unity and the biomass yield was high (0.49 ± 0.01 g biomass per g glucose). At dilution rates of 0.30 h^{-1} and higher, metabolism became respirofermentative (Fig. 1). Above this 'critical dilution rate' the oxygen-consumption rate decreased with increasing dilution rate, while the ethanol and carbon-dioxide production rates increased with increasing dilution rate. As a consequence, the biomass yield on glucose decreased with increasing dilution rate. At dilution rates above 0.38 h^{-1} wild-type cells washed out of the culture.

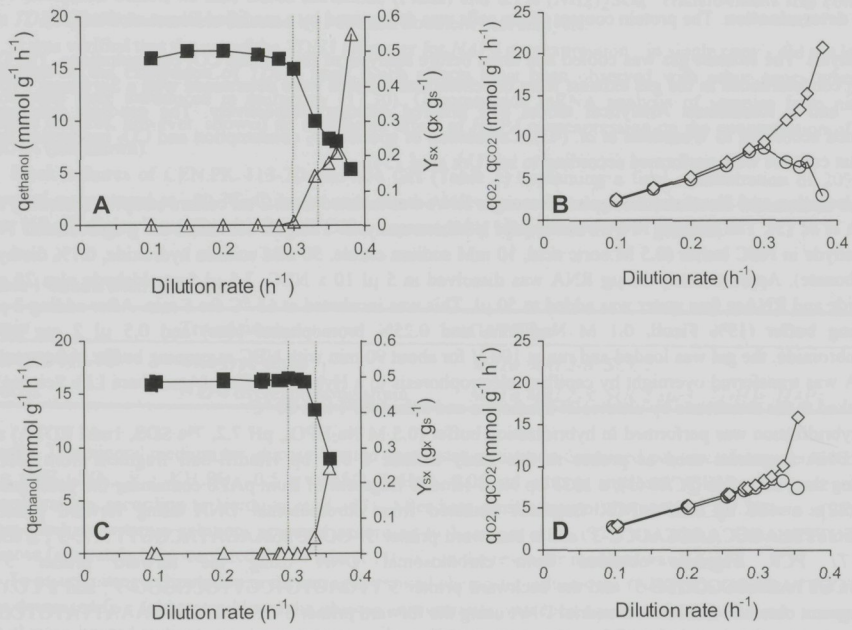

Fig. 1. Physiology of wild type CEN.PK 113-7D (A and B) and Hap4p-overproducing strain 436 GH (C and D) in glucose-limited chemostat cultures at increasing dilution rates. Each data point represents the average of two independent cultures. Open symbols refer to the primary axis and indicate $q_{ethanol}$ (triangles), q_{O2} (circles) and q_{CO2} (diamonds). Closed symbols refer to the secondary axis and indicate the biomass yield on glucose Y_{sx} (squares). The data for the wild type CEN.PK113-7D are from van Hoek *et al.* (1998) The vertical dashed lines indicate the critical specific growth rate at which respiro-fermentative metabolism sets in.

Subsequently the *HAP4*-overexpressing strain was grown in aerobic, glucose limited chemostat cultures at increasing dilution rates. Overexpression of *HAP4* increased the critical specific growth rate by 10%. The biomass yield on glucose, the absence of ethanol from culture supernatants, the oxygen consumption rate and the carbon-dioxide production rate all indicated that metabolism was respiratory at dilution rates below 0.33 h^{-1} (Fig. 1). At 0.33 h^{-1} ethanol appeared in culture supernatants and the biomass yield on glucose started to decrease. Above a dilution rate of 0.35 h^{-1} the cells washed out of the culture, indicating a slightly lower maximum specific growth rate of the *HAP4*-overexpressing strain as compared to that of the isogenic wild type.

Throughout the range of dilution rates, the *HAP4* mRNA level of the *HAP4* overexpressing strain was increased by approximately twofold compared to that of the wild-type strain (Fig. 2). The mRNA of the integrated *HAP4* gene can be distinguished from that of the endogenous *HAP4* gene since its non-translated sequences are shorter, resulting in a faster migration in gels. The increased *HAP4* level has no detectable effect on its target gene *QCR8*, which is induced in both wild type and Hap4p overproducer (Fig. 2).

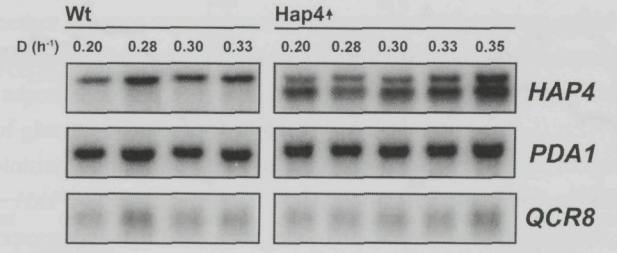

Fig. 2. Northern blot analysis of *HAP4* overexpression in aerobic glucose-limited chemostat cultures of the wild type strain (WT) and its Hap4p overproducing derivative (Hap4) at dilution rates ranging from 0.20 to 0.35 h^{-1}. Total RNA was hybridized with probes specific for *HAP4*, *PDA1* or *QCR8* mRNA. The *HAP4* overexpression construct lacks part of the non-coding region and, therefore, its mRNA is somewhat smaller than the native *HAP4* mRNA. The constitutively expressed gene *PDA1* (Wenzel *et al.* (1995)), encoding subunit E1α of the pyruvate-dehydrogenase complex, is used as a measure of the amount of mRNA loaded.

Effect of *HAP4* overexpression on the response to a glucose pulse. To investigate the short-term response of the *HAP4*-overexpressing strain to excess glucose, a pulse of glucose was given to a steady-state, aerobic, glucose-limited chemostat culture. At a dilution rate of 0.10 h^{-1}, growth of the wild type and the *HAP4*-overexpressing strain was fully respiratory. At the start of the experiment the *HAP4* mRNA level of the overexpressing strain was approximately fourfold higher than that of the wild-type strain and it remained unaltered during the course of the experiment (result not shown). Immediately after addition of glucose (50 mM, final concentration) to these cultures the medium pumps were switched off and it was observed that wild-type CEN.PK 113-7D started to produce ethanol and acetate (Fig. 3, panels A and B) (8). Ethanol accumulated up to 60 mM and acetate up to 10 mM (Table 2). After 130 min the wild-type cells had consumed all glucose. Consumption of the produced ethanol was complete after 390 min. The response of the *HAP4*-overexpressing strain to the glucose pulse differed slightly, but significantly from that of the wild-type culture (Fig. 3, panels C and D).

Also in the *HAP4*-overexpressing culture, alcoholic fermentation started immediately after the glucose pulse, but the maximum ethanol concentration was slightly lower in the *HAP4*-overexpressing culture (50 mM) than in the wild-type culture (Table 2). As a result of this, the Hap4p overproducer had consumed all ethanol an hour before the wild-type strain. (Fig. 3, Table 2). The difference between the strains was exclusively due to a slower specific production of ethanol by the Hap4p-overproducing strain. The consumption of ethanol was equally fast in the two strains (on average 0.24 mM min^{-1} at similar biomass densities, cf. Table 2).

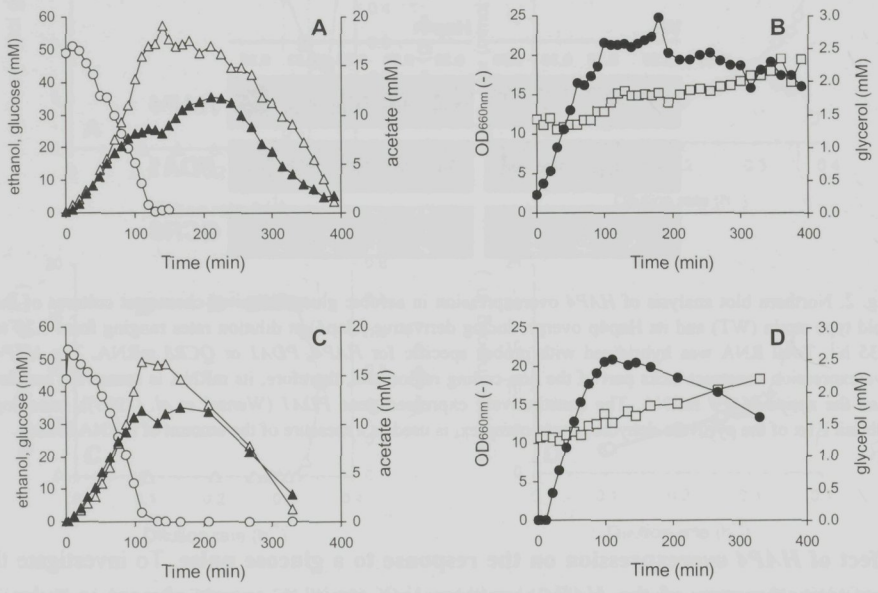

Fig. 3. Metabolic response of wild-type CEN.PK 113-7D (A and B) and the HAP4-overexpressing strain 436 GH (C and D) upon a transition from glucose limitation to glucose excess. The graphs show the results of a single representative glucose-pulse experiment per strain. Duplicate experiments yielded essentially the same results (cf. Table 2). Open symbols refer to the primary axis and represent glucose (circles), ethanol (triangles) and $OD_{660\ nm}$ (squares). Closed symbols refer to the secondary axis and represent acetate (triangles) and glycerol (circles). The data of wild type CEN.PK 113-7D are from Flikweert *et al.* (1999b) and they were obtained according to the same procedures.

Table 2 Maximum and minimum metabolite concentrations after a glucose pulse to a glucose-limited chemostat culture (cf. Fig. 2). Averages and standard deviations (σ_{n-1}) were obtained from duplicate experiments with independent cultures. The wild-type data are from Flikweert *et al.* (1999b).

	Wild type CEN.PK 113-7D		Hap4p overproducer 436 GH	
Metabolite	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)
Glucose	0	130 ± 0	0	125 ± 13
Ethanol	60 ± 4	140 ± 0	50 ± 1	123 ± 16
Ethanol	0	390 ± 0	0	335 ± 0
Acetate	10 ± 2	233 ± 32	11 ± 1	167 ± 0
Glycerol	2.6 ± 0.6	160 ± 28	2.6 ± 0.1	139 ± 40

***HAP4* overexpression increased the biomass yield on glucose in nitrogen-limited chemostat cultures.** In aerobic, nitrogen-limited chemostat cultures of *S. cerevisiae*, excess glucose is present and alcoholic fermentation is observed (16,33). It was investigated whether glucose repression in nitrogen-limited cultures could be relieved by *HAP4* overexpression. The concentrations of glucose and ammonium in the medium reservoir were adjusted such that the biomass densities of the cultures were independent of the amount of glucose present (not shown).

Northern blotting confirmed that the expression of *HAP4* was increased more than fourfold in the *HAP4*-overproducer 436 GH, compared to wild-type CEN.PK 113-7D (Fig. 4). The expression of *CYT1*, a targets of the Hap2/3/4/5p complex, was increased twofold, demonstrating that the overproduced Hap4p was functional (Fig. 4).

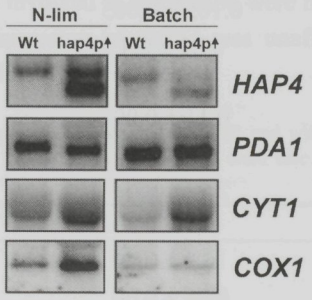

Fig. 4. Northern blot analysis of *HAP4* overexpression in aerobic batch cultures on glucose (lanes 1 and 2) and nitrogen-limited chemostat cultures (lanes 3 and 4) of wild-type strain CEN.PK113-7D (lanes 1 and 3) and the *HAP4* overexpressing strain (lanes 2 and 4). Total RNA was hybridized with probes specific for *HAP4*, *PDA1*, *CYT1*, or *COX1* mRNA. The *HAP4* overexpression construct lacks part of the non-coding region and, therefore, its mRNA is somewhat smaller than the native *HAP4* mRNA. The constitutively expressed gene *PDA1* (41), encoding subunit E1α of the pyruvate-dehydrogenase complex, is used as a measure of the amount of mRNA loaded.

Metabolism of the Hap4p overproducer was more respiratory than that of the wild type, as was evident from a decreased specific rate of ethanol production, a decreased respiratory quotient and a doubling of the biomass yield on glucose (Table 3). As a consequence of the higher biomass yield on glucose, the residual glucose concentration was higher in the *HAP4*-overexpressing culture than in the wild-type culture. One might expect that this should cause stronger glucose repression in *HAP4*-overexpressing cultures. Apparently, however, the overexpression of *HAP4* overruled glucose repression, resulting in a more respiratory metabolism in these cultures.

The observed shift to a more respiratory metabolism is likely to require a coordinate upregulation of all components of the mitochondrial respiratory chain. Several subunits of respiratory complexes are, however, encoded on the mitochondrial genome and not regulated directly by the Hap2/3/4/5 complex. Nevertheless, *COX1*, one of those mitochondrial genes, was threefold upregulated in nitrogen-limited cultures of the Hap4p-

overproducing strain (Fig. 4), indicating that a coordinate upregulation occurs of mitochondrial and nuclear genes involved in respiration.

Interestingly, the *HAP4*-overexpressing strain produced fourfold more glycerol than the wild-type strain. The Hap4p overproducer and the wild type had similar biomass yields on nitrogen (Table 3) and similar protein contents (30% of the cell dry weight). For comparison, at a dilution rate of 0.10 h^{-1} the protein content of wild-type cells grown in glucose-limited chemostat cultures is approximately 40% (33).

Table 3 Physiology of the Hap4p-overproducing strain and the wild type in aerobic nitrogen-limited chemostat culture. Averages and standard deviations (σ_{n-1}) were obtained from duplicate experiments with independent steady-state cultures. Calculations of the carbon recovery were based on a carbon content of biomass of 48% (w/w). Y_{sx} and Y_{nx} are the biomass yields on glucose and nitrogen, respectively.

	Wild type CEN.PK 113-7D	Hap4p overproducer 436 GH
Dilution rate (h^{-1})	0.105 ± 0.005	0.104 ± 0.001
Reservoir glucose (g l^{-1})	37.3 ± 0.4	36.9 ± 0.3
Residual glucose (g l^{-1})	2.4 ± 0.4	17.6 ± 1.0
Y_{sx} ($g_{biomass}$ $g_{glucose}^{-1}$)	0.11 ± 0.00	0.23 ± 0.01
Y_{nx} ($g_{biomass}$ g_{N}^{-1})	18.2 ± 0.2	20.5 ± 0.6
RQ	2.92 ± 0.08	1.24 ± 0.01
$q_{glucose}$ (mmol $g_{biomass}^{-1}$ h^{-1})	5.3 ± 0.2	2.6 ± 0.1
$q_{ethanol}$ (mmol $g_{biomass}^{-1}$ h^{-1})	6.2 ± 0.4	0.9 ± 0.0
$q_{glycerol}$ (mmol $g_{biomass}^{-1}$ h^{-1})	0.05 ± 0.00	0.20 ± 0.01
$q_{acetate}$ (mmol $g_{biomass}^{-1}$ h^{-1})	0.09 ± 0.02	0.05 ± 0.00
q_{CO2} (mmol $g_{biomass}^{-1}$ h^{-1})	10.5 ± 0.3	7.7 ± 0.1
q_{O2} (mmol $g_{biomass}^{-1}$ h^{-1})	3.6 ± 0.0	6.2 ± 0.2
$q_{ethanol}$/ $q_{glucose}$ (-)	1.17	0.36
Carbon recovery (%)	91.9 ± 1.0	97.1 ± 1.8

***HAP4* overexpression did not increase the biomass yield on glucose in batch cultures.** In aerobic batch cultures, glucose metabolism of prototrophic *S. cerevisiae* strains is mixed respirofermentative, *i.e.* in such cultures the biomass yield on glucose (0.13 g g^{-1}) is higher than that in strictly anaerobic cultures (0.1 g g^{-1}), yet much lower than that in completely respiratory cultures (0.5 g g^{-1}) (1). It was investigated whether the biomass yield of *S. cerevisiae* in aerobic batch fermenters increased above that of wild-type CEN.PK 113-7D by overexpression of *HAP4*.

During exponential growth on glucose in aerobic batch fermenters, strain 436 GH (the Hap4p overproducer) had a 1.5-fold higher level of *HAP4* mRNA than wild-type CEN.PK 113-7D (Fig. 4). The mRNA level of *CYT1*, a target of the Hap2/3/4/5p complex, increased 2.5-fold in the Hap4p overproducing strain (Fig. 4, lanes 3 and 4). The maximum specific growth rate (μ_{max}) of the wild-type strain was 0.37 h^{-1}, while the Hap4p-overproducing strain grew slightly slower at a μ_{max} of 0.35 h^{-1} (Table 4). This is consistent with the finding that the Hap4p overproducer washed out from glucose-limited

chemostat cultures above a dilution rate of 0.35 h^{-1}, while the wild type maintained growth at slightly higher dilution rates (see above). The biomass yields of the two strains on glucose were similar, being 0.13 ± 0.01 g g^{-1} for the wild type and 0.14 ± 0.01 g g^{-1} for the *HAP4*-overexpressing strain (Table 4). During the exponential growth phase the wild type and the *HAP4*-overexpressing strain produced simultaneously ethanol, acetate and glycerol (Fig. 5). The specific rates of glucose consumption and ethanol and glycerol production of the Hap4p overproducer were slightly lower than those of the wild-type strain, but their ratio was unaltered (Table 4). The rates of acetate and pyruvate production were low and did not differ between the strains (Table 4). Clearly, overexpression of *HAP4* hardly influenced the distribution of fluxes among respiration and fermentation in aerobic batch cultures, if at all.

A possible explanation for the lack of a physiological effect of *HAP*4 overexpression is that the mitochondrial genes involved in respiration were not upregulated in the aerobic batch cultures. Indeed, the expression of *COX1* was unaffected by overexpression of *HAP4* under these conditions (Fig. 4, lanes 3 and 4).

Table 4 Physiology of the *HAP4*-overexpressing strain (436 GH) and wild-type strain (CEN.PK 113-7D) in aerobic batch cultures. Averages and standard deviations (σ_{n-1}) were calculated from four independent batch cultures.

	Units	Wild type	Hap4p overproducer
μ_{max}	h^{-1}	0.37 ± 0.01	0.35 ± 0.02
Y_{sx}	$g_x\ g_s^{-1}$	0.13 ± 0.01	0.14 ± 0.01
$q_{glucose}$	mmol $g_x^{-1}\ h^{-1}$	14.8 ± 1.1	12.2 ± 0.5
$q_{ethanol}$	mmol $g_x^{-1}\ h^{-1}$	22.0 ± 0.5	17.0 ± 0.8
$q_{ethanol}/q_{glucose}$	-	1.5	1.4
$q_{glycerol}$	mmol $g_x^{-1}\ h^{-1}$	1.1 ± 0.2	0.7 ± 0.2
$q_{acetate}$	mmol $g_x^{-1}\ h^{-1}$	0.4 ± 0.1	0.5 ± 0.2
$q_{pyruvate}$	mmol $g_x^{-1}\ h^{-1}$	0.1 ± 0.0	0.1 ± 0.0

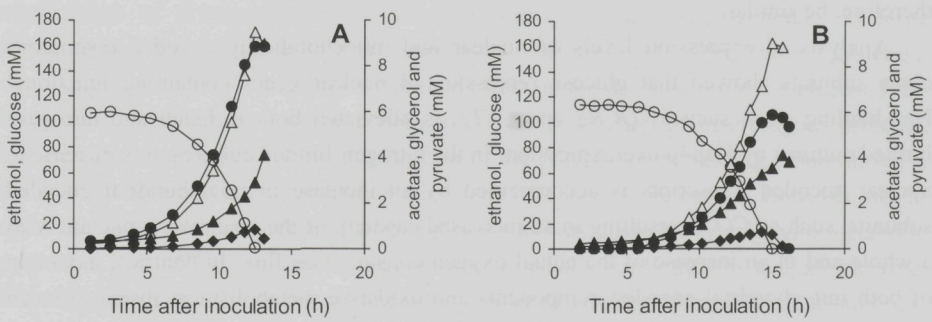

Fig. 5. Metabolite formation in aerobic batch cultures. The results of one representative batch experiment per strain are shown. Replicate experiments yielded essentially the same results (cf. Table 4). A: wild-type CEN.PK 113-7D. B: *HAP4*-overexpressing strain 436 GH. Open symbols refer to the primary axis and denote glucose (circles) and ethanol (triangles). Closed symbols refer to the secondary axis and denote acetate (triangles), glycerol (circles) and pyruvate (+).

Discussion

Modulating the distribution of fluxes by *HAP4* overexpression. This study confirms that modified expression of a global regulator, in this case *HAP4*, is a powerful tool to change the distribution of metabolic fluxes in a micro-organism. We previously showed that *HAP4* overexpression increased the biomass yield on glucose of the auxotrophic *S. cerevisiae* strain Dl1 by increasing the respiratory capacity of this strain in aerobic batch cultures on glucose (2). In the present work various conditions of glucose excess were studied to investigate under which conditions *HAP4* overexpression relieved glucose repression of respiration. In nitrogen-limited chemostat cultures *HAP4* overexpression redirected a large part of the glucose flux from alcoholic fermentation to respiration. This led to a strong increase of the biomass yield on glucose. In glucose-limited chemostat cultures a small positive effect of *HAP4* overexpression was observed with respect to the critical growth rate. Also ethanol production after a glucose pulse to a glucose-limited culture was weakly suppressed by *HAP4* overexpression. Finally, the physiology of aerobic batch cultures on glucose was hardly affected by overexpression of *HAP4.*

From the above results it is clear that regulation of metabolism by Hap2/3/4/5p is complex. Our working hypothesis was that glucose repression of genes involved in the respiratory pathway should be relieved by overexpression of *HAP4* and this in turn should lead to an increased rate of respiration and an increased biomass yield. The finding that the dynamics of a glucose pulse were not affected strongly by *HAP4* overexpression, may be consistent with this working hypothesis: glucose repression is a long-term effect that works at the time scale of transcription and translation, and during a transient glucose pulse its impact may be negligible. The hypothesis does not explain, however, why *HAP4* overexpression increased the biomass yield of *S. cerevisiae* in nitrogen-limited chemostat cultures, but not in batch cultures on glucose. The glucose concentration in the batch cultures (20 g l^{-1}) was similar to the residual concentrations in the nitrogen-limited cultures overexpressing *HAP4* (Table 3). The strength of glucose repression should, therefore, be similar.

Analysis of expression levels of nuclear and mitochondrially encoded respiratory chain subunits showed that glucose repression of nuclear genes containing functional Hap-binding sites, such as *QCR8* and *CYT1*, is alleviated both in batch and nitrogen-limited cultures by Hap4p overexpression. In the nitrogen-limited cultures, this increase in nuclear encoded transcripts is accompanied by an increase in mitochondrial encoded subunits, such as *COXI*, resulting in an increased capacity of the respiratory machinery as a whole and in an increase of the actual oxygen consumption flux. In contrast, induction of both mitochondrial encoded components and oxidative metabolism is absent in batch cultures. The mechanism by which Hap4p could overrule repression of mitochondrial genes is yet unclear, but it is likely to be an indirect effect that is apparently under control of other factors as well, causing the observed differences between nitrogen-limited cultures and batch cultures. Our current research focuses on the parameters that control a

coordinate upregulation of mitochondrial components and the role of Hap4p in nucleo-mitochondrial signalling pathways.

Glycerol production and free-energy metabolism of nitrogen-limited cultures. A surprising feature of the nitrogen-limited cultures was that *HAP4* overexpression led to a fourfold increase of the specific rate of glycerol production under these conditions. Glycerol production often serves as a redox sink, when excess NADH formed in anabolic pathways cannot be regenerated by the respiratory chain at a sufficiently high rate (21,31). Since the respiratory chain is stimulated in the Hap4p overproducer, this strain should easily get rid of excess NADH by respiration and therefore it is unlikely that the increased glycerol production indicates a redox problem in this strain.

Another explanation for the high glycerol production by the Hap4p overproducer is that it may serve as an overflow pathway to compensate for an imbalance between the upper and the lower part of glycolysis. The design of glycolysis is such that ATP is invested first, before net production takes place. If the [ATP]/[ADP] ratio is excessively high in the Hap4p-overproducer, it may stimulate the upper part of glycolysis where ATP is invested, and inhibit the lower part of the pathway where ATP is synthesized, which leads to an imbalance between the upper and lower part of glycolysis (29). This could lead to an accumulation of intermediates such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. It has been shown before that under such conditions glycerol production may serve as an overflow pathway and restore the balance between the upper and the lower part of glycolysis (28).

Industrial applications of Hap4p overproduction. An aim of our work was to further investigate whether Hap4p overexpression can improve biomass-directed applications of *S. cerevisiae*. We have shown that manipulation of this regulatory gene caused a substantial redistribution of the glucose flux and thereby an increase of the biomass yield during nitrogen limitation. Under conditions that are more relevant for the industrial applications of *S. cerevisiae*, such as a glucose pulse (cf. heterogeneities in large fermenters) and glucose-limited chemostat cultures (cf. fed-batch systems), overexpression of *HAP4* had only a marginal effect on the distribution of the glucose flux. Even these small effects may be beneficial for the production of heterologous proteins. For example, the higher critical growth rate of the *HAP4*-overexpressing strain allows a faster sugar feed and a shorter fermentation time. We have to be careful, however, not to make the same mistake with regulatory pathways as was made previously with metabolic pathways. It is well known that a metabolic flux is usually not limited by a single enzyme. Instead, control of a flux is distributed among many or all enzymes and the distribution of control may depend on the conditions (6,14). Likewise, it may be foreseen that in general regulatory circuits do not contain a single key regulator switching metabolic pathways on and off. Regulation is probably much more subtle. We should not be surprised if a regulator that strongly controls expression of a whole metabolic route under one

condition, such as Hap4p under nitrogen limitation, is not the sole controlling factor under another condition. If control of regulatory pathways is also distributed among various components, then manipulation of the expression of different regulatory genes simultaneously seems a very promising strategy to redirect metabolic fluxes.

To further improve the biomass yield of *S. cerevisiae* grown at excess glucose, it will be interesting to combine Hap4p overproduction with modulation of expression of other regulatory genes. For example, *HAP4* could be overexpressed in a *mig1Δ mig2Δ* deletion strain. *MIG1* and *MIG2* are involved in glucose repression. Like the Hap4p-overproducing strain, a *mig1Δ mig2Δ* deletion strain had a slightly but significantly higher critical specific growth rate than the wild type in glucose-limited cultures (15). It remains to be seen whether combination with *HAP4* overexpression can further improve the biomass yield of *S. cerevisiae* at high sugar concentrations.

Acknowledgements

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Chapter 5

Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: a possible consequence of energy-dependent lactate export

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Due to a growing market for the biodegradable and renewable polymer poly-lactic acid, the world demand for lactic acid is rapidly increasing. The tolerance of yeasts to low pH can benefit the process economy of lactic-acid production by minimizing the need for neutralizing agents. *Saccharomyces cerevisiae* (CEN.PK background) was engineered to a homofermentative lactate-producing yeast via deletion of the 3 genes encoding pyruvate decarboxylase and the introduction of a heterologous lactate dehydrogenase (LDH; EC 1.1.1.27). Like all pyruvate-decarboxylase-negative (Pdc^-) *S. cerevisiae* strains, the engineered strain required small amounts of acetate for the synthesis of cytosolic acetyl-CoA. Exposure of aerobic glucose-limited chemostat cultures to excess glucose resulted in the immediate appearance of lactate as the major fermentation product. Ethanol formation was absent. However, the engineered strain could not grow anaerobically and lactate production was strongly stimulated by oxygen. In addition, under all conditions examined, lactate production was slower than alcoholic fermentation by the wild type. Despite the equivalence of alcoholic fermentation and lactate fermentation with respect to redox balance and ATP generation, studies on oxygen-limited chemostat cultures showed that lactate production does not contribute to the ATP economy of the engineered yeast. This absence of a net ATP production is probably due to a metabolic-energy requirement (directly or indirectly in the form of ATP) for lactate export.

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Introduction

Traditional uses of L(+)-lactic acid, nowadays predominantly produced via microbial fermentation, comprise applications in food, cosmetics and pharmaceuticals (6,25). The world-wide production of lactic acid (currently an estimated 220,000 tonnes per year) is rapidly increasing, mainly as a result of the growing market for polylactic acid (7,14). It is expected that this biodegradable polymer, produced from renewable resources, will replace various petrochemistry-based polymers in applications ranging from packaging to fibres (34).

Lactic acid is currently mainly produced with lactic-acid bacteria, such as various *Lactobacillus* species (6,25). Due to the pH sensitivity of these organisms, industrial lactic-acid production requires large amounts of $CaCO_3$ or other neutralizing agents. This complicates downstream processing and yields large amounts of gypsum as a by-product (6). Development of alternative production organisms that are more tolerant to low pH may strongly decrease the requirement for neutralizing agents and lower the cost of down-stream processing.

Yeasts, including *Saccharomyces cerevisiae*, are known for their ability to grow at low pH. In addition, *S. cerevisiae* can grow anaerobically on glucose in the presence of the essential anaerobic growth factors nicotinic acid, oleic acid and ergosterol (2,3,17,27). These attributes, combined with its genetic accessibility (21), make *S. cerevisiae* an interesting microorganism for the production of lactic acid (16,30). Expression in *S. cerevisiae* of an NAD^+-dependent lactate dehydrogenase (LDH; EC 1.1.1.27) from either mammalian, bacterial or fungal origin, results in simultaneous formation of ethanol and lactate (1,16,30,33). To minimize ethanol formation, LDH has been expressed in *S. cerevisiae* strains with strongly reduced pyruvate-decarboxylase or alcohol-dehydrogenase activities (33). Although this indeed resulted in reduced ethanol formation, the lactate productivity or yield was not drastically increased. Complete elimination of alcoholic fermentation in *S. cerevisiae* can be achieved by deletion of all three structural genes encoding for pyruvate decarboxylase (*PDC1*, *PDC5* and *PDC6*) (22). Construction of a pyruvate-decarboxylase-negative (Pdc^-) *S. cerevisiae* strain expressing bovine lactate dehydrogenase has been reported previously (30) but its physiology has not been studied in detail.

The conversion of glucose to either ethanol or lactic acid is equivalent both in terms of redox balance and ATP yield (Fig. 1). Nevertheless, a *Kluyveromyces lactis* strain engineered for homolactic fermentation required oxygen for efficient lactate production (28). In our own research, we encountered a similar phenomenon in engineered homolactic *S. cerevisiae* strains (unpublished).

This study aims to resolve the role of oxygen in metabolically engineered homofermentative lactate-producing *S. cerevisiae*. To this end, we quantitatively analysed growth, metabolite production and energetics of a homofermentative *S. cerevisiae* strain (RWB850-2) in batch and continuous cultures under various defined aeration regimes.

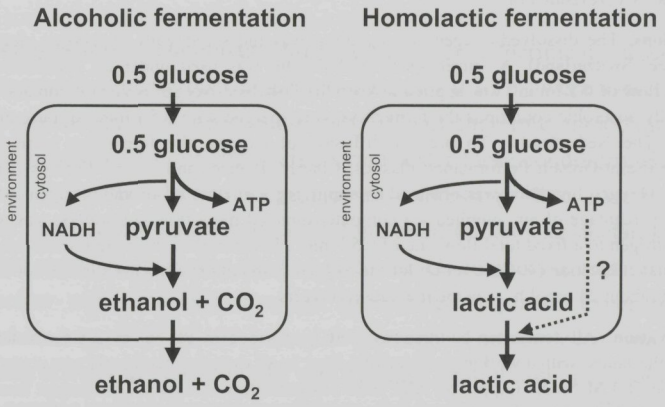

Fig. 1. Schematic comparison of ethanol and lactic-acid fermentation. Glucose uptake by *S. cerevisiae* occurs via facilitated diffusion. In both situations, the conversion of glucose by glycolysis results in the formation of 1 ATP per pyruvate produced. The excess NADH formed by glycolysis is either regenerated by pyruvate decarboxylase and alcohol dehydrogenase (alcoholic fermentation) or lactate dehydrogenase (lactate fermentation). Ethanol is known to rapidly diffuse through the cell membrane of *S. cerevisiae*. The mechanism(s) of lactic-acid export in *S. cerevisiae* are, as yet, unknown, but may involve ATP hydrolysis.

Materials and Methods

Strains and maintenance. All *S. cerevisiae* strains used in this study (Table 1) were derived from the isogenic CEN.PK family (35). Stock cultures were prepared from shake-flask cultures (100 ml synthetic medium in 500 ml flasks), by addition of 20 % (v/v) glycerol and storage of 2 ml aliquots in sterile vials at –80 °C.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
CEN.PK182	MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP*
CEN.PK111-61A	MATα *ura3-52 leu2-112 his3-Δ1*
RWB837	MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP ura3-52*
RWB850-2	MATa *pdc1*(-6,-2)::*loxP pdc5*(-6,-2)::*loxP pdc6*(-6,-2)::*loxP ura3-52* + YEpLDH#1

Strain and plasmid construction. RWB837 was obtained from a cross between CEN.PK182 and CEN.PK111-61A (constructed by Dr. P. Kötter, Frankfurt, Germany and obtained from A.E. Staley Manufacturing Company) (Table 1). The resulting diploid was sporulated and the asci heated for 15 minutes at 56 °C. This spore mix was then plated on YP with 0.2% acetate as the carbon source. The resulting colonies were tested for growth on YP medium with glucose or ethanol. Colonies that could not grow on glucose were subsequently checked by PCR for the presence of a disrupted *PDC6* gene and the mating type. Determination of the auxotrophic markers present, in this case *ura3-52*, then gave RWB837. The plasmid YEpLDH#1 was constructed by cloning an *Nhe*I fragment, containing the *TPI1* promoter and *Lactobacillus casei LDH* gene, from plasmid pLC5 (9) into YEplac195 (20) cut with *Xba*I. RWB837 was then transformed with YEpLDH#1, resulting in RWB850. Transformant number 2 (RWB850-2) was selected for this work. RWB850-2 is intellectual property A.E. Staley Manufacturing Company.

Media. The synthetic medium contained per liter of demineralized water 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 0.15 ml silicon antifoam (BDH) and trace-elements at concentrations given by Verduyn *et al.* (39). After heat sterilisation of the medium for 20 min at 120 °C a filter-sterilized vitamin solution, prepared according to Verduyn *et al.* (39), was added. Synthetic media for precultures and start-up batch cultivation contained 1.5 % (v/v) ethanol as the sole carbon source. For chemostat cultivation glucose (10.0 g l^{-1}) was added separately after heat sterilization at 110 °C. In addition, without prior sterilization acetic acid was added to the

autoclaved medium to a final concentration of 19 mM (10% of total substrate carbon) to rescue the C_2-requirement of Pdc$^-$ *S. cerevisiae* (18).

Aeration conditions. The dissolved-oxygen concentration was measured with an oxygen electrode (Mettler Toledo, Greifensee, Switzerland). A stirrer speed of 800 rpm was used under all conditions. For aerobic conditions, an air flow of 0.5 l min^{-1} was applied to keep the dissolved-oxygen concentration above 60 % of air saturation. For fully anaerobic conditions the fermenters were sparged with 0.5 l min^{-1} of pure nitrogen (Hoek-Loos, Schiedam, The Netherlands). To prevent diffusion of oxygen, the fermenters were equipped with Norprene® tubing (Saint-Gobain Performance Plastics, Charny, France) and Viton® O-rings (Eriks, Alkmaar, The Netherlands). Oxygen limitation was obtained by supplying a mixture of air and nitrogen to the fermenters (13). The desired percentage of air, supplied via computer-directed mass-flow controllers, was topped up with technical-grade nitrogen to a fixed total flow rate of 0.5 l min^{-1}. In contrast to the experimental set-up for strictly anaerobic chemostat cultivation (40), the set-up for the oxygen-gradient experiments allowed for entry of a small amount of oxygen (about 24 μmol h^{-1}) via the medium reservoirs.

Fermenter cultivation. All fermenter cultivations were performed at 30 °C in 2-l fermenters (Applikon, Schiedam, the Netherlands) with a working volume of 1 liter. In all experiments the pH was controlled at 6.0 via automated addition of 4 M NaOH (Applikon ADI 1030 biocontroller). At this pH the largest fraction of acetic- (pK=4.74, The Merck Index 11th edition) and lactic acid (pK=3.87, the most common handbook value) is in the dissociated form, thereby minimizing uncoupling by these weak acids (39). For chemostat cultivation, the addition of medium was controlled by a peristaltic pump. The working volume of the cultures was kept constant by means of an electrical level sensor. Chemostat cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight, specific carbon-dioxide production rate and oxygen-consumption rate, changed by less than 2% during 24 h. Spontaneous synchronization of the cell cycle, a phenomenon sometimes encountered with chemostat cultures of wild-type *S. cerevisiae* (24), was not observed with the engineered *S. cerevisiae* strain.

Analytical procedures. Dry weight determination, substrate- and metabolite analysis, in- and off-gas analysis, preparation of cell-free extract, enzyme-assays and protein determination were performed as described previously (37). Lactate dehydrogenase was assayed at 30 °C with a reaction mixture of: 2 mM $MnCl_2$, 1 mM fructose-1,6-diphosphate and 0.15 mM NADH in 0.1 M imidazole-HCl at pH 6.5. The reaction was started by addition of potassium pyruvate (10 mM).

Calculation of physiological parameters during defined aeration gradients. Defined, continuously decreasing oxygen feeds were applied to carbon-limited chemostat cultures by supplying a mixture of nitrogen and air to the cultures. During these 200-h experiments, the physiological parameters were continuously changing. Therefore, calculations similar to those used by Costenoble *et al.* (13) were used. The specific growth rate throughout this experiment was calculated from a mass balance. With constant volume, absence of biomass in the feed and a known dilution rate (D), the specific growth rate (μ) can be calculated according to: $\mu = D + (dC_x/dt) \cdot C_x^{-1}$, with C_x being the culture dry weight. Between 60 and 140 h, (dC_x/dt) was easily determined by linear regression (see Fig. 4A).

Expressions for the specific substrate-consumption and product formation rates were derived analogously. The specific substrate-consumption rate (q_s) can be calculated from: $q_s = (D \cdot (C_{s,0} - C_s) - (dC_s/dt)) \cdot C_x^{-1}$, with $C_{s,0}$ representing the reservoir substrate concentration and C_s the residual substrate concentration in the culture. To calculate q_p (specific product-formation rate), $q_p = ((dC_p/dt) + D \cdot C_p) \cdot C_x^{-1}$, was used. In this equation, C_p represents the product concentration in the culture supernatant. Since the turnover in the gas phase is much higher than in the liquid phase, the specific oxygen consumption and carbon-dioxide production rates in these transient-state cultures were calculated according to the standard procedures for steady-state cultures (38). The biomass yield on substrate was calculated by dividing the specific growth rate by the specific substrate consumption rate. Accordingly, the biomass yield on oxygen was calculated by dividing the specific growth rate by the specific oxygen consumption rate (q_{O2}).

Results

Aerobic glucose-limited chemostat cultivation. In all experiments described, carbon-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1} were used to obtain well-defined and reproducible starting conditions. It was therefore important to know the physiological behaviour of our engineered strain under these conditions. Similar to other Pdc$^-$ strains (18), the lactate-dehydrogenase-expressing Pdc$^-$ *S. cerevisiae* strain (RWB850-2) required the addition of a C_2-compound (ethanol or acetate) to the growth media. In this study, acetate was used to fulfil the C_2-compound requirement. The data described below were obtained from six independent replicate experiments, with an average carbon recovery of 95.3 ± 1.9 percent. The *in vitro* measured lactate dehydrogenase activity in these cultures was 25.3 ± 1.2 μmol min^{-1} mg $protein^{-1}$.

Under aerobic carbon-limited conditions, the metabolism of RWB850-2 was fully respiratory, as indicated by the high biomass yield on carbon (13.8 ± 0.1 g biomass $Cmol^{-1}$) and the absence of fermentation products. The specific glucose-consumption rate was 1.09 ± 0.01 mmol g $biomass^{-1}$ h^{-1} and acetate was consumed at a rate of 0.37 ± 0.01 mmol g $biomass^{-1}$ h^{-1}. The specific oxygen-consumption rate (2.78 ± 0.11 mmol g $biomass^{-1}$ h^{-1}) and the specific carbon-dioxide production rate (2.91 ± 0.14 mmol g $biomass^{-1}$ h^{-1}) resulted in a respiratory quotient (RQ) close to unity (1.04 ± 0.01 mol CO_2 produced per mol O_2 consumed). The biomass yield on oxygen was 36.0 ± 1.5 g biomass mol $oxygen^{-1}$. These values were similar to those obtained with the isogenic wild type grown under the same conditions (data not shown).

Aerobic versus anaerobic lactate formation. To assess the effect of oxygen on lactate production by *S. cerevisiae* RWB850-2, cells pregrown in an aerobic, glucose-limited chemostat culture were exposed to excess glucose under alternating anaerobic and aerobic conditions. After stopping the medium pumps and the air supply of the chemostat culture, it was sparged with nitrogen to achieve and maintain anaerobicity. Immediately after anaerobicity was established, a glucose pulse was administered to the culture (Fig. 2). During the first two (anaerobic) hours, lactate was produced at a rate of 0.8 ± 0.0 mmol g $biomass^{-1}$ h^{-1} (Fig. 2). After these two hours, normal aeration was resumed and the dissolved-oxygen concentration increased to above 80 % of air saturation. As a result, the specific lactate-production rate increased by 2.5-fold (to 2.0 ± 0.0 mmol g $biomass^{-1}$ h^{-1}) compared to the first (anaerobic) phase of the experiment. After two hours of aerobic incubation, aeration was stopped again and anaerobicity was re-established. In this third phase of the experiment, the specific lactate-production rate (0.8 ± 0.0 mmol g $biomass^{-1}$ h^{-1}) was the same as during the first anaerobic phase.

During this 6 h experiment, no increase in OD_{660} was observed, indicating that there was no biomass growth. Apart from lactate (40 mM), glycerol and pyruvate (the latter only during the aerobic phase) were formed, each to a maximum concentration of 1 mM. In the aerobic phase, part of the glucose was converted to carbon dioxide via respiratory metabolism (data not shown).

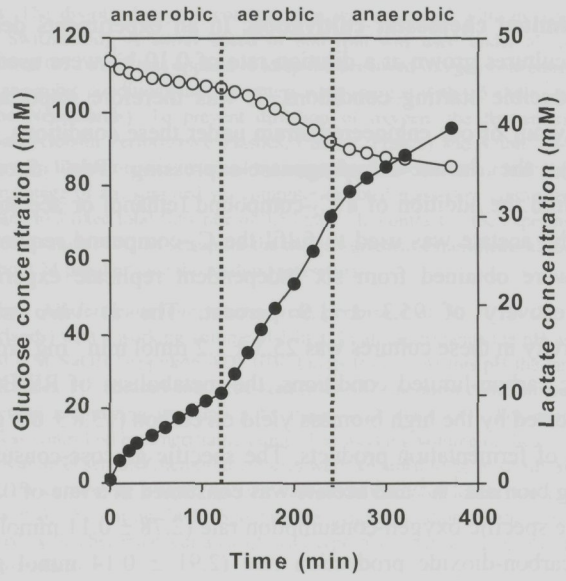

Fig. 2. Lactate formation upon exposure of an aerobic glucose-limited chemostat culture of *S. cerevisiae* RWB850-2 to excess glucose. During this experiment the conditions were changed from anaerobic to aerobic and back to anaerobic. The dashed lines indicate a change in the aeration regime. The open and closed circles indicate the glucose concentration, and lactate concentration, respectively. The results depicted are the average of independent duplicate experiments. The experimental variation was below 2 % for all measurements.

Long-term exposure to excess glucose under anaerobic conditions. The glucose-pulse experiment described above demonstrated that anaerobic lactate production is possible for at least two hours, although at a low rate. To further study anaerobic lactate production by *S. cerevisiae* RWB850-2, cells pregrown in aerobic, glucose-limited chemostat cultures were incubated for 96 h under anaerobic conditions in the presence of excess glucose. A few hours after the beginning of the experiment, the specific lactate-production rate was 0.63 ± 0.01 mmol g biomass^{-1} h^{-1} (Fig. 3), in good agreement with the short-term experiment discussed above (Fig. 2). This rate decreased to 0.34 ± 0.00 mmol g biomass^{-1} h^{-1} after 48 h and at the end of the 96-h anaerobic experiment, the specific lactate-production rate had even decreased further to 0.22 ± 0.02 mmol g biomass^{-1} h^{-1}. The overall yield of lactate on glucose during this 96 h anaerobic experiment was 1.66 ± 0.04 mol lactate mol glucose^{-1}. Glycerol (0.09 mol glycerol mol glucose^{-1}) and carbon dioxide (0.07 mol CO_2 mol glucose^{-1}) were the main by-products of the anaerobic fermentation. During the experiment, the biomass concentration decreased from 4.99 ± 0.02 g biomass l^{-1} to 3.23 ± 0.08 g biomass l^{-1}. The relatively low concentration of storage carbohydrates (around 5 % w/w) in aerobic chemostat cultures at a dilution rate of 0.1 h^{-1} (23), makes it

unlikely that only their consumption was responsible for the large decrease in the biomass concentration. Apparently also other cell constituents were subject to turnover.

To minimize the complexity of the experimental system, C_2-compounds (to meet the C_2-requirement of Pdc$^-$ *S. cerevisiae*) or oleate and ergosterol (to meet the anaerobic growth requirements of *S. cerevisiae*) were not included in these long-term anaerobic incubations. It was anticipated that endogenous stores of C_2-compounds and anaerobic growth factors should allow for at least one biomass doubling after the switch to anaerobic conditions. Indeed, combined addition of ethanol, Tween-80 (as a source of oleate) and ergosterol in anaerobic control experiments did not result in growth or enhanced lactate production after up to 7 days of incubation.

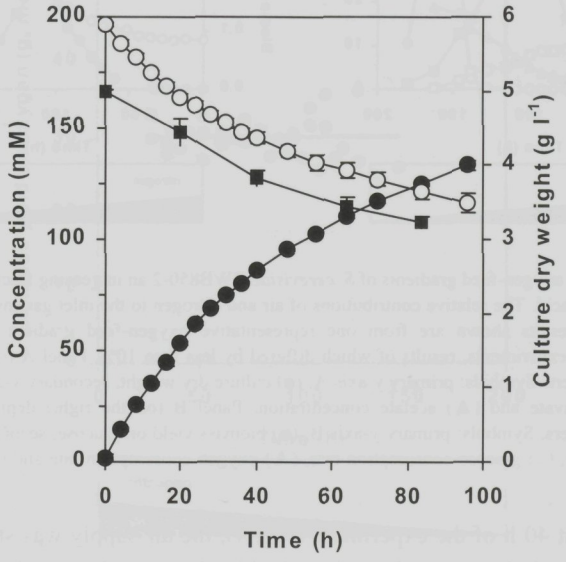

Fig. 3. Metabolic response of *S. cerevisiae* RWB850-2, pregrown in aerobic, glucose-limited chemostat cultures, to a 200-mM glucose pulse under strictly anaerobic conditions. The graph shows the average and mean deviation of two independent replicate experiments. The open circles indicate glucose (○) concentration. The closed symbols indicate the lactate concentration (●) and the culture dry weight (■).

Physiology during a gradual change from aerobic growth to a severely limited oxygen supply. The experiments described above demonstrate that lactate production is stimulated by oxygen, whereas growth of the engineered strain is totally oxygen dependent. To quantify the relation between oxygen availability and growth, *S. cerevisiae* RWB850-2 was subjected to a range of oxygen supplies. Since analysis of steady-state cultures under different degrees of oxygen limitation is laborious, an alternative approach was used. A defined, continuously decreasing oxygen feed-regime was applied to carbon-limited continuous cultures by changing the mixture of nitrogen and air in the inlet gas (see Materials and Methods). During the 200 h experiment the air supply was linearly

decreased from 200 ml min^{-1} air per 500 ml min^{-1} total gas at the start to 0 ml min^{-1} air per 500 ml min^{-1} total gas at 200 h.

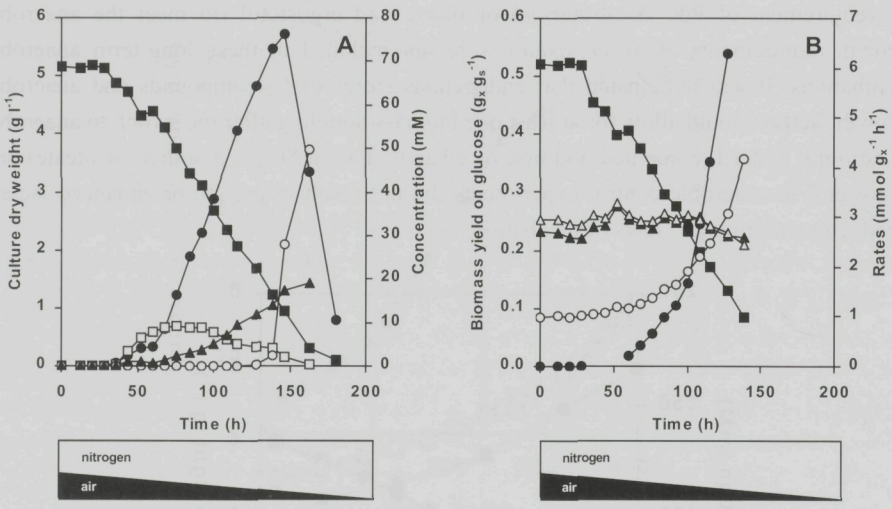

Fig. 4. During defined oxygen-feed gradients of *S. cerevisiae* RWB850-2 an increasing fraction of the glucose is fermented to lactic-acid. The relative contributions of air and nitrogen to the inlet gas mixture are depicted below the graphs. Results shown are from one representative oxygen-feed gradient of a set of four independent replicate experiments, results of which differed by less than 10%. Panel A (on the left) depicts the measured parameters. Symbols: primary y-axis A; (■) culture dry weight, secondary y-axis A; (●) lactate, (○) glucose, (□) pyruvate and (▲) acetate concentration. Panel B (on the right) depicts the calculated physiological parameters. Symbols: primary y-axis B; (■) biomass yield on glucose, secondary y-axis B; (●) lactate production rate, (○) glucose consumption rate, (▲) oxygen consumption rate and (Δ) carbon dioxide production rate.

During the first 40 h of the experiment (Fig. 4), the air supply was still sufficient for fully respiratory metabolism, as indicated by the high biomass dry weight and the absence of lactate production. After 40 h, the dissolved-oxygen concentration decreased below the detection limit. In agreement with the onset of oxygen limitation, lactate production immediately set in and the culture dry weight started to decrease (Fig. 4A). During the remaining 160 h of the oxygen gradient, the air supply was further decreased to zero. After some initial fluctuations, caused by small amounts of foam affecting the culture volume and therefore the oxygen transfer, the culture dry weight decreased linearly (Fig. 4A). In line with the decreasing culture dry weight, the consumption of acetic acid decreased with decreasing oxygen supply, resulting in accumulation of acetate in the supernatant. Pyruvate was produced throughout the experiment, probably reflecting a competition for glycolytic NADH between mitochondrial respiration and the heterologous LDH. No formation of glycerol was observed throughout the experiment.

Starting with the first appearance of lactate, the biomass yield on glucose decreased linearly with decreasing oxygen feed (Fig. 4B). The increasing specific glucose-consumption rate also reflected the decreasing biomass yield on glucose. As the culture

became progressively more oxygen limited, the specific lactate-production rate increased to above 6 mmol g biomass^{-1} h^{-1} (Fig. 4B). Surprisingly, the specific oxygen-consumption rate of RWB850-2 remained high, ranging between 3.1 and 2.6 mmol g biomass^{-1} h^{-1}, as long as no residual glucose was detected in the supernatant (Fig. 4B). Consequently, the biomass yield on oxygen was constant throughout the oxygen-limited phase of the four independent replicate experiments (30.1 ± 2.8 g biomass per mol oxygen) and therefore independent of the air supply (Fig. 5). Since homofermentative lactate production does not

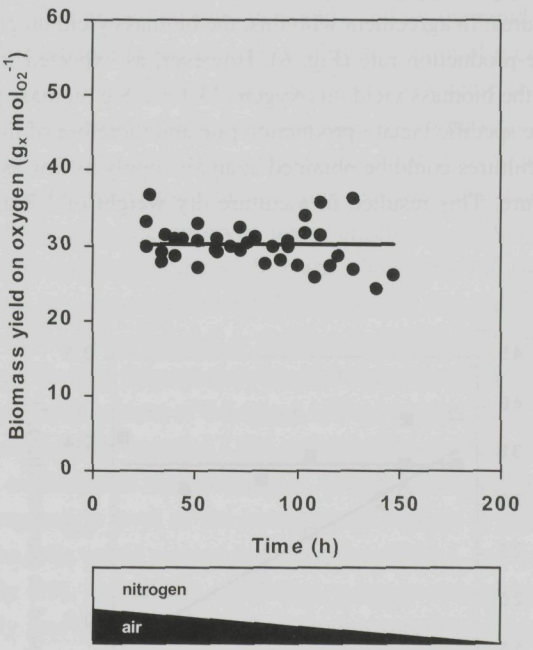

Fig. 5. Biomass yields on oxygen during defined oxygen-limitation gradients of *S. cerevisiae* RWB850-2. The relative contributions of air and nitrogen to the inlet gas mixture are depicted below the graphs. Measurements were obtained from 4 independent replicate experiments. The average of these values is indicated by the line. The larger deviation of the mean at the end of the experiment (more severe oxygen limitation) is due to measurement errors as a result of low volumetric oxygen consumption rates and biomass concentrations.

lead to carbon-dioxide formation, the respiratory quotient was 1.04 mol CO_2 produced per mol O_2 consumed.

Between 140 and 150 h after the start of the gradient, residual glucose was detected in the supernatant of the culture (Fig. 4A). The resulting nonlinearity hampered the calculation of the rates for the remainder of the experiment. Shortly after this appearance of glucose in the supernatant, the air supply finally dropped to zero and RWB850-2 washed out completely from the culture. The isogenic wild type still grew in the absence of aeration, with a biomass yield on glucose of 0.09 g biomass g glucose^{-1}. Apparently,

under these experimental conditions, entry of oxygen into the fermenters was sufficient for synthesis of the anaerobic growth factors oleate and ergosterol.

Steady-state analysis of cultures with a limited oxygen supply. Oxygen-limited steady-state chemostat cultivation of RWB850-2 was used to further investigate the apparent independence of the biomass yield on oxygen on the aeration rate and thereby on the specific lactate-production rate. As seen in the oxygen-gradient cultures, an inverse relationship between oxygen supply and specific rate of lactate fermentation was observed in the steady state cultures. In agreement with this, the biomass yield on glucose decreased with increasing lactate-production rate (Fig. 6). However, as expected from the oxygen-gradient experiments, the biomass yield on oxygen (34.1 ± 2.8 g biomass per mol oxygen) was independent of the specific lactate-production rate and therefore of the oxygen supply (Fig. 6). Steady-state cultures could be obtained at an air supply as low as 80 ml of air per 500 ml total gas mixture. This resulted in a culture dry weight of 1.7 g l^{-1}. Attempts to

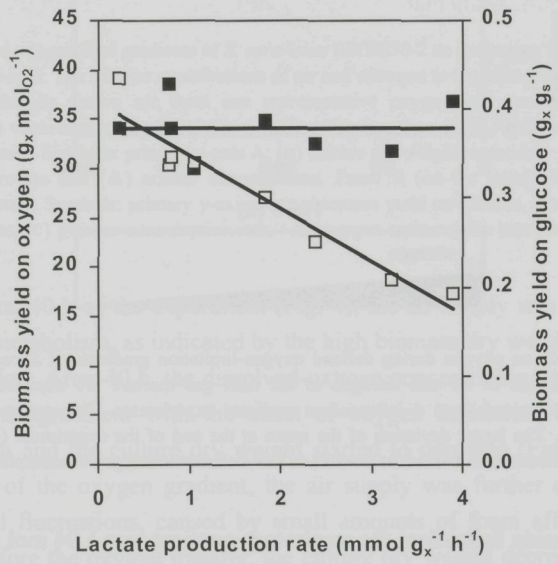

FIG. 6. Biomass yields on oxygen and glucose of steady-state chemostat cultures of *S. cerevisiae* RWB850-2 with different regimes of limited oxygen supply (in all cases the dissolved oxygen concentration was below 0.1 % of air saturation). The cultures were run at a dilution rate of 0.10 h^{-1} on a mixture of glucose and acetate (10% of total substrate carbon). With decreasing air supply to the fermenter, the specific lactate-production rate (x-axis) increased. The biomass yield on glucose (□) is linearly correlated to the specific lactate-production rate. In contrast, the biomass yield on oxygen (■) is independent of the specific lactate-production rate.

reduce the air supply below 80 ml of air per 500 ml total gas mixture, irrevocably resulted in complete wash out of the culture. Apparently, the engineered strain was not capable of oxygen-limited growth at elevated residual glucose concentrations.

Discussion

Fermentation rates in wild-type and homofermentative lactate-producing *S. cerevisiae*. Among yeasts, *S. cerevisiae* is known both for its unique ability to grow fast under aerobic as well as anaerobic conditions (40), and for the rapid conversion of glucose to ethanol. Specific ethanol production rates of up to 29 mmol g biomass^{-1} h^{-1} have been reported for wild-type *S. cerevisiae* CEN.PK 113-7D (isogenic to the engineered strain used in this study) in anaerobic batch fermentations, with a maximum specific growth rate of 0.33 h^{-1} (5). In contrast, our results demonstrate that Pdc^{-} *S. cerevisiae* expressing a heterologous lactate dehydrogenase cannot grow anaerobically. Furthermore, the highest specific lactate-production rate observed under anaerobic conditions in the presence of excess glucose (0.8 mmol g biomass^{-1} h^{-1}) was almost 36-fold lower than the specific ethanol production rate described above. If homolactic fermentation were energetically equivalent to alcoholic fermentation (i.e. yielding 1 mole of ATP per mole of fermentation product) this rate of lactate fermentation would be barely sufficient to meet the estimated maintenance energy requirement of 0.5 mmol ATP g biomass^{-1} h^{-1} (31,41). Enzyme assays in cell extracts consistently revealed LDH activities above 25 μmol.min^{-1}.(mg protein)$^{-1}$, which renders it unlikely that the low rates of anaerobic lactate fermentation were due to an insufficient expression level of the heterologous protein. A shortage of fructose-1,6-diphosphate in the cells would prevent full activation of the *L. casei* lactate dehydrogenase used in this study (19). To test this possibility, a steady-state chemostat cultivation with limited air supply was repeated with a Pdc^{-} strain expressing a *Lactobacillus plantarum* lactate dehydrogenase that does not require fructose-1,6-diphosphate (19). Since the results were the same as with RWB850-2 (data not shown), a shortage of fructose-1,6-diphosphate is probably not the cause of the low lactate-formation rate.

The immediate increase of the lactate fermentation rate to 2.0 mmol lactate g biomass^{-1} h^{-1} upon aeration of the anaerobic cell suspensions indicates that the complete absence of anaerobic growth was not due to an intrinsically limited capacity of the lactate fermentation pathway. Indeed, the highest specific lactate-production rate observed with RWB850-2, isogenic to CEN.PK 113-7D, was 6.3 mmol g biomass^{-1} h^{-1} during the defined aeration gradient experiments (Fig. 4B). This was, however, not a sustainable rate, since the highest specific lactate-production rate observed in oxygen-limited steady-state chemostat cultures was only 3.9 mmol g biomass^{-1} h^{-1} (Fig. 6).

Stimulation of fermentation rates by oxygen. Stimulation of glucose fermentation by oxygen is a common phenomenon among yeasts, but is absent in wild-type *S. cerevisiae* (32). In *S. cerevisiae* stimulation of alcoholic fermentation by oxygen is observed, however, when it is engineered for xylose fermentation via introduction of heterologous

xylose reductase and xylitol dehydrogenase (4). In the cases examined thus far, stimulation of sugar fermentation by oxygen in wild type or engineered yeasts was shown to be due to a redox imbalance (10,32). In such cases oxygen serves as a redox acceptor, a function that can also be fulfilled by other compounds such as acetoin (32,36). However, during anaerobic alcoholic fermentation on glucose, wild-type *S. cerevisiae* does not display a redox imbalance. When NADH is in excess it is channelled to glycerol production (36). Since lactic-acid fermentation via NAD^+-dependent LDH is redox-wise identical to alcoholic fermentation (Fig. 1) and the glycerol pathway was not affected in RWB850-2, it is highly unlikely that a redox imbalance is the cause of the low lactate-production rate of the engineered strain. Instead, the stimulatory effect of oxygen on lactate fermentation is probably reflecting an energetic constraint.

Energetics of homofermentative lactate production by engineered *S. cerevisiae*: absence of net ATP formation. The biomass yield on oxygen of wild-type *S. cerevisiae* CEN.PK 113-7D, which is isogenic to RWB850-2, is approximately 36 g biomass mol $oxygen^{-1}$ under aerobic glucose-limited conditions (8). Under conditions with a limited oxygen supply in otherwise glucose-limited chemostat cultures, wild-type *S. cerevisiae* displays respiro-fermentative metabolism (42). At all but extremely low air supplies oxygen has a mainly catabolic role in *S. cerevisiae* and, as the oxygen supply decreases, a larger fraction of the glucose is fermented to ethanol. Alcoholic fermentation yields ATP via substrate-level phosphorylation. Therefore, wild-type *S. cerevisiae* requires less oxygen to form a certain amount of ATP (and thus biomass) as more glucose is fermented to ethanol. Consequently, a decreasing oxygen supply leads to a decrease of the specific rate of oxygen consumption and an increase of the biomass yield on oxygen. A graphical representation of this phenomenon has been presented by Fiechter *et al.* (17). Experimental data from Costenoble *et al.* (13) on microaerobic glycerol formation of by *S. cerevisiae* CBS 8066 allow for the calculation of the biomass yields on oxygen. These range from 92 g biomass mol $oxygen^{-1}$, under moderate oxygen limitation, up to values as high as 540 g biomass mol $oxygen^{-1}$, under stringently oxygen-limited conditions. Similar extreme values can be calculated from a study by Weusthuis *et al.* (42).

The relationship between oxygen supply and biomass yield found with engineered *S. cerevisiae* under conditions of limited oxygen supply differed drastically from that of wild-type *S. cerevisiae*. Instead of steadily increasing with decreasing oxygen supply, the biomass yield on oxygen of RWB850-2 (30.1 ± 2.8 g biomass per mol oxygen in the oxygen-gradient cultures, 34.1 ± 2.8 g biomass per mol oxygen in steady-state cultures) was independent of the oxygen supply (Fig. 5, Fig. 6).

How can this constant biomass yield on oxygen be explained? A varying biomass yield on ATP which, at every oxygen feed tested, exactly balances the ATP generated by lactate fermentation to result in a constant biomass yield on oxygen, is highly unlikely. Furthermore, the biomass yield on oxygen was constant over a wide range of specific lactate production rates and lactate concentrations. Especially the lack of a correlation

with the extracellular lactate concentration strongly argues against the involvement of lactate toxicity (as a result of either weak-acid uncoupling or intracellular accumulation). The remaining and plausible explanation is that, in the engineered strain, the overall conversion of glucose into extracellular lactate does not yield ATP. Since the biochemical pathways for the uptake of glucose and its subsequent conversion into either intracellular lactic acid or ethanol are identical in both redox- as well as ATP balances (Fig. 1), the difference has to be sought in product export. Ethanol diffuses freely through the yeast plasma membrane. However, at the near-neutral intracellular pH in yeast, lactate will be predominantly in the anionic form (the most commonly cited pK_a value for lactic acid in chemical handbooks is 3.87). It is unlikely that this polar molecule will diffuse through the cell membrane of *S. cerevisiae* (12). The importer of lactic acid is known to be the proton-symporter *JEN1* (11). However, the transporter and the mechanism for export of this acid are still unknown. Deletion of *JEN1* and the putative monocarboxylate transporters *MCH1-5* did not affect lactate excretion in wild-type *S. cerevisiae* (26). Particularly since lactic-acid production is possible at pH values well below the pK of the acid (29), it seems likely that in *S. cerevisiae* the export of lactic acid from the cell requires ATP. This ATP requirement may be direct, involving an ATP-driven primary transport mechanism. Such a mechanism, involving the ABC-type transporter encoded by the *PDR12* gene (15), is involved in export of the organic acids benzoate and sorbate. Alternatively, an ATP requirement for lactate export may involve a secondary transport mechanism in combination with the plasma-membrane ATP-ase, in which case ATP indirectly supplies the energy for the translocation process via the generation of a proton-motive force across the plasma membrane. A net requirement of one ATP per molecule of lactate would exactly compensate for the single ATP produced per lactate in glycolysis.

A zero net ATP yield from lactate fermentation explains the physiology of the engineered strain under anaerobic conditions. When fermentation does not result in a positive ATP balance, cells cannot meet the ATP required for maintenance, thus explaining the massive turnover of biomass in the long-term experiments (Fig. 3). A zero net ATP yield offers a plausible explanation for the low lactate-production rates under anaerobic conditions: the resulting low cytosolic ATP concentration will limit the activity of the kinases in the upper part of glycolysis. Furthermore, the stimulatory effect of oxygen can be adequately explained from the alleviation of this restriction by the provision of ATP through oxidative phosphorylation.

Implications for industrial lactic-acid production. This study demonstrates that the current homofermentative lactate-producing *S. cerevisiae* strains require oxygen for the generation of ATP. This ATP is needed either for growth and, even under non-growing conditions, for meeting maintenance-energy requirements. In any industrial application of homofermentative lactate-producing *S. cerevisiae* strains, their requirement for aeration will have serious consequences for both reactor design and process economics. This

creates an incentive for the construction of homofermentative lactate-producing *S. cerevisiae* strains that do not require respiration.

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Chapter 6

Microbial Export of Lactic and 3-Hydroxypropanoic Acid: Implications for Industrial Fermentation Processes

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Lactic acid and 3-hydroxypropanoic acid are industrially relevant microbial products. This paper reviews the current knowledge on export of these compounds from microbial cells and presents a theoretical analysis of the bioenergetics of different export mechanisms. It is concluded that export can be a key constraint in industrial production, especially under the conditions of high product concentration and low extracellular pH that are optimal for recovery of the undissociated acids. Under these conditions, the metabolic energy requirement for product export may equal or exceed the metabolic energy yield from product formation. Consequently, prolonged product formation at low pH and at high product concentrations requires the involvement of alternative, ATP-yielding pathways to sustain growth and maintenance processes, thereby reducing the product yield on substrate. Research on export mechanisms and energetics should therefore be an integral part of the development of microbial production processes for these and other weak acids.

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Introduction

Microbial production of large-volume chemicals has gained renewed interest from industry and science (82). Bifunctional molecules such as the weak organic acids 2-hydroxypropanoic acid (lactic acid) and 3-hydroxypropanoic acid (β-lactic acid or 3-hydroxypropionic acid) are of special interest in view of their application as platform chemicals and building blocks for 'biobased' polymers (17,73). Compared to production of chemicals from petrochemical feedstocks, microbial processes have several advantages: microorganisms can use renewable carbohydrate feedstocks, the products are produced in a closed carbon cycle and are generally stereospecific (24,51,82).

In current microbial processes for the industrial production of weak organic acids, such as lactic acid, the optimum pH for microbial productivity is often different from those of product recovery and purification. Since the microorganisms generally require a pH well above the dissociation constant of the weak acid (pK; lactic acid 3.86, 3-hydroxypropanoic acid 4.51 (71)), large quantities of base titrant are needed to compensate for the acid production during industrial fermentation. To obtain the undissociated acid, which is the desired end product, a low pH and therefore the consumption of large volumes of acid is required. As a result, large quantities of by-products, often gypsum ($CaSO_4.2H_2O$), are formed (5). This inefficient use of resources could be avoided if microbial production were possible at a pH well below the pK of the acid.

Metabolic pathways and their implications for product formation have been investigated in detail. Comparatively little is known about the export of many industrially relevant compounds, such as citric acid and lactic acid. In this review, the impact of extracellular pH and product concentration on the bioenergetics and feasibility of export will be evaluated for two industrially relevant monocarboxylates: lactic acid and 3-hydroxypropanoic acid. Product export should also be considered in the design of engineered microorganisms or modified fermentation processes. Several conclusions drawn for export of these compounds will be generally applicable to production of intracellularly produced weak organic acids. However, the production of acids, like acetate in some microorganisms (1,2), that are produced in the periplasmic space or extracellularly will be subject to drastically different considerations.

The feasibility of weak organic acid production at low pH does not only depend on the thermodynamics of product export. Especially at low pH, lactic acid and other organic acids can also act as antimicrobial agents. This antimicrobial action is at least partly due to uncoupling of the pH gradient over the cytoplasmic membrane and, consequently, perturbation of intracellular pH homeostasis. For these inhibitory effects, that may also be relevant for selection and/or construction of production strains and in the design of processes, the reader is referred to other papers (13,21,26,41,50,55,62,78).

Hydroxy-propanoic acids: applications and production

Lactic acid. Traditionally, lactic acid and its derivatives are used as preservatives or flavour enhancers in food, for moisturizing and emulsifying in cosmetics, and in the synthesis of optically pure pharmaceuticals (5,38). The world-wide production of lactic acid (currently estimated at 220,000 tonnes per year) is increasing, mainly due to the growing market of polylactic acid (6,17). It is expected that this biodegradable polymer, produced from renewable resources, will partially replace various petrochemistry-based polymers in applications ranging from packaging to clothing (9,73).

The main commercial lactate-producing organisms are various fastidious lactic-acid bacteria, such as homolactic *Lactobacillus* species. These bacteria require relatively complex growth media and strict control of the pH, mostly at values between 6 and 8 (5,24,31,38). These factors complicate down-stream processing. In particular, the requirement for a near-neutral pH results in vast amounts of gypsum as a by-product (5). Therefore, attempts have been made to produce lactic acid with nutritionally less demanding, more acid-tolerant microorganisms, such as fungi from the genus *Rhizopus* (20) or genetically engineered *Saccharomyces cerevisiae* (19,61). For an overview of microbial lactic-acid production the reader is referred to several comprehensive reviews (5,10,24,31,38,79).

The maximum theoretical yield of the redox-neutral glycolytic conversion of glucose to lactic acid is 2 mol of product per mol of substrate consumed (equivalent to 1 g product per g substrate), resulting in one ATP from substrate-level phosphorylation per molecule of lactic acid produced (Fig. 1). Typical yields obtained with homofermentative lactic-acid bacteria are above 90% of this maximum theoretical figure (38). Depending on the organism, or enzyme in case of genetically engineered organisms, the D(-) or the L(+) enantiomer of lactic acid can be produced either in mixtures or at enantiomeric purities well in excess of 95 % (10,38). Already half a century ago, lactate concentrations of 135 $g.l^{-1}$ were achieved in anaerobic 100,000-liter fermentations at pH 6.0 (28). It is likely that current industrial processes operate at higher lactate concentrations. At laboratory scale (5 litres) a fed-batch fermentation of *Lactobacillus lactis*, controlled at a pH of 6.2, resulted in 210 $g.l^{-1}$ of lactate at a 97% yield (4).

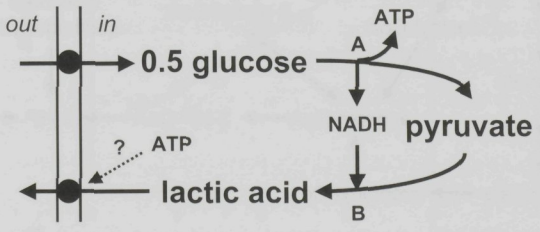

Fig. 1. Schematic representation of homolactic fermentation. Glucose is first taken up from the environment, either via facilitated diffusion or via active uptake. Glucose is converted by glycolysis (A), yielding one NADH and one ATP per pyruvate produced. To maintain redox balance the pyruvate is then converted to lactate by a lactate dehydrogenase (B).

3-hydroxypropanoic acid. Currently the high price of 3-hydroxypropanoic acid, which is only produced via organic-chemical synthesis, limits its use (68). However, the potential applications of this platform molecule are enormous, ranging from its direct use as a monomer for (co)-polymerization, to its use as a precursor for the synthesis of other commercially valuable chemicals, such as 1,3-propanediol, acrylic acid or acrylamide (9). This provides a strong incentive to develop microbial processes for production of 3-hydroxypropanoic acid (65,68).

In 2001, formation of low concentrations of 3-hydroxypropanoic acid (0.2 g l^{-1}) from glycerol, via 3-hydroxypropanal, was demonstrated with genetically modified *Escherichia coli* expressing a glycerol dehydratase from *Klebsiella pneumoniae* and a non-specific aldehyde dehydrogenase (*ALD4*) from *S. cerevisiae* (68). Recently, five other possible biosynthetic routes were proposed for the formation of 3-hydroxypropanoic acid from sugars or intermediates of central carbon metabolism (Fig. 2) (65). These routes result in the redox-neutral conversion of glucose into two 3-hydroxypropanoic-acid molecules with a maximum theoretical yield of 1 gram product per gram of glucose consumed. Due to the involvement of ATP-requiring carboxylases and/or co-enzyme A (CoA) ligases, most of the proposed routes probably do not result in net ATP formation or will even cost ATP equivalents. Only when, instead, the corresponding CoA transferase reactions are used, some of the proposed pathways, e.g. the route via lactoyl-CoA (Fig. 2), can result in net generation of ATP. As will be discussed below, absence of net ATP generation in the product formation pathways can have a significant impact on product export. Although high production levels of 3-hydroxypropanoic acid have not been reported so far, it is to be expected that its production is affected in a similar way as described for the microbial production of lactic acid.

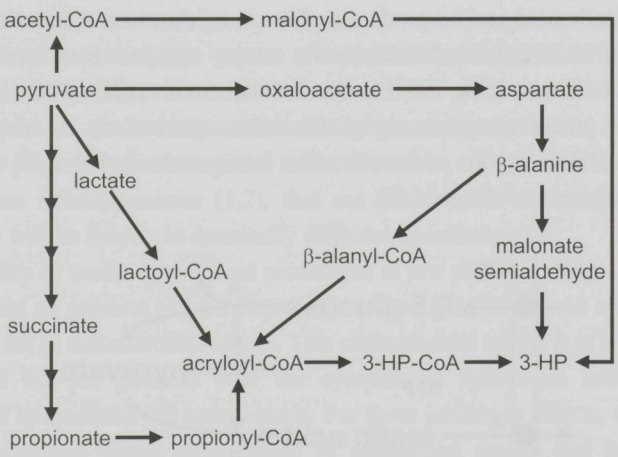

Fig. 2. Overview of the possible biosynthetic routes for the formation of 3-hydroxypropanoic acid (3-HP) starting at pyruvate as described by Cargill Inc. in patent application PCT WO 02/42418 A2.

Energetics of solute export

Cells maintain an electrochemical proton gradient across their cytoplasmic membrane (47). This gradient consists of two components: a pH gradient ($\Delta pH = pH_{in}-pH_{out}$) with the inside of the cell usually alkaline relative to the outside, and an electrical potential difference ($\Delta\psi = \psi_{in}-\psi_{out}$, in V) with the inside of the cell usually negatively charged relative to the outside. The sum of these components, the proton-motive force (pmf), equals: $pmf = \Delta\psi - Z\Delta pH$ (V), with Z representing $\ln 10 \cdot R \cdot T/F$. A $\Delta\psi$ can be generated by net translocation of positive- (cations or protons) or negative charges. A ΔpH, inside alkaline, can be formed by the net outward translocation of protons (or the net inward translocation of OH^-). These translocation processes can be mediated by a variety of free-energy coupled mechanisms, including membrane-associated proton-pumping ATPases, respiration-driven proton translocation and solute export systems. The resulting proton-motive force can be applied to drive a variety of metabolic-energy -requiring, membrane-associated cellular processes. Metabolic energy is defined as the free-energy stored in the ATP pool and in the electrochemical proton gradient across the cytoplasmic membrane.

Over a broad range of extracellular pH values, most microorganisms maintain a strict homeostasis, at near-neutral values, of the intracellular pH (7,32). In addition, most cells also have a tendency to keep the pmf almost constant, as for example has been demonstrated for acidophilic bacteria grown at a wide range of extracellular pH values (Fig. 3) (45). To reconcile these two homeostatic responses, $\Delta\psi$ is adjusted when the extracellular pH changes, and can even become inside positive at very low pH (14,27,43,45,74). For extremophiles, such as *Thermoplasma acidophilum* and *Picrophilus oshimae*, inside positive $\Delta\psi$'s of up to +120 mV have been reported (43,74).

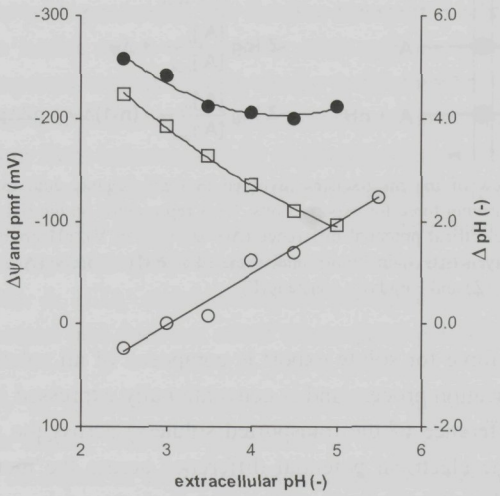

Fig. 3. The effect of extracellular pH on the proton motive force (pmf, λ) and its constituents, ΔpH (□) and $\Delta\psi$ (μ), in the acidophile *Bacillus acidocaldarius* (reproduced from Michels and Bakker, 1985).

At intracellular pH values between 6 and 8, the acids discussed in this paper are predominantly in the dissociated form, according to pH = pK + log(A^-/HA). Passive diffusion of solutes from or into the cell, especially (an)ions, is limited by their solubility in and their permeability across the cytoplasmic membrane (80). High export rates of most solutes can only be achieved by specific transport proteins in the membrane. Most transport systems of weak acids belong to two different classes (Fig. 4):

1. **Primary transport** uses free energy derived from biochemical reactions for the generation of solute gradients (34). Examples of primary transport include the export of drugs and organic acids by ATP-Binding-Cassette (ABC) transporters and proton transport by ATPases in the cytoplasmic membrane (25,35).
2. **Secondary transport** uses free energy stored in (electro-)chemical gradients as the driving force for the uptake or export of solutes (34,35). The secondary transport systems can be divided into three groups: uniporters, symporters and antiporters (35).

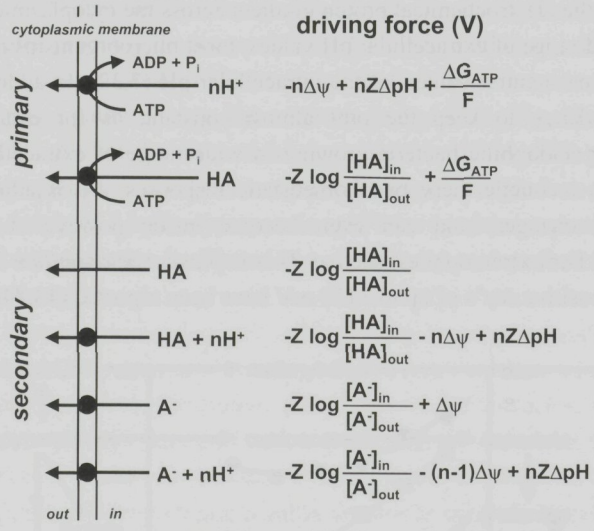

Fig. 4. Schematic overview of the mechanisms involved in weak organic acid export via primary and secondary transport. The driving force for export (Volts, V) is represented on the right side. The symbols in these equations indicate: electrical potential difference ($\Delta\psi=\psi_{in}-\psi_{out}$, in V), pH gradient ($\Delta pH=pH_{in}-pH_{out}$), free-energy of ATP hydrolysis (ΔG_{ATP} in J/mol), undissociated acid (HA), anion (A^-), number of protons co-transported (n), ln10·R·T/F (Z) and Faraday's constant (F).

The total driving force for solute export is composed of all solute and ion gradients involved in the translocation process and is conventionally expressed in V (Fig. 4): (i) the chemical potential difference of the transported solute(s) across the membrane (Z · log ([A]in/[A]out)), (ii) the electrical potential difference across the membrane (net-charge translocation · Δψ), when the net transport reaction is electrogenic, and (iii) the chemical components of the proton motive force (number of protons exported · (ZΔpH)), when protons are involved in the net transport reaction. In transport processes catalysed by ABC

transporters, also the free energy of ATP hydrolysis (ΔG_{ATP} in J/mol, or $\Delta G_{ATP}/F$ in V) contributes to the driving force of the translocation process. When the overall driving force is negative, as calculated from the formulas in Fig. 4, net transport via that mechanism is thermodynamically feasible. A driving force of zero indicates thermodynamic equilibrium.

In organic-acid producing microorganisms, intracellular pH homeostasis requires the net export of the undissociated acid. This export can be accomplished in two, energetically equivalent, ways: (1) uniport of the undissociated acid, or (2) symport of the anion with a proton. To maintain electroneutrality and intracellular pH homeostasis, anions and protons need not necessarily be transported by the same transporter. For example, when the anions are exported via a uniport system, the resulting surplus of protons can be expelled via proton-pumping ATPases. Alternatively, when secondary transport is coupled to the export of additional protons, the resulting pmf has to be used for pmf- driven processes, such as the generation of ATP by ATPase.

Mechanisms and energetics of lactic-acid export in microorganisms

To assess the impact of product export, the transport mechanisms involved should be known and understood. Since hardly anything is known about the mechanisms of export for 3-hydroxypropanoic acid, this section will focus on the use of known and proposed import and export systems as mechanisms for lactic-acid export. These transport systems are: symport of the lactate anion with protons, antiport with substrates and ATP-driven export of undissociated acid by ABC-type transporters (8,12,23,33,34,40,46,56,58,59).

Symport of lactate with proton(s). The familiar transport mechanism for lactic acid is the symport of the lactate anion with one or more protons. Although best studied for lactate transport (import as well as export), similar symporters have been implicated in the transport of pyruvate and propionate (12,58). Lactate-proton symporters have been found in organisms ranging from microorganisms (including lactic-acid bacteria) to mammals (including *Homo sapiens*) (34,46,58). In many organisms these symporters have a stoichiometry of one proton per anion, as has for instance been shown for the human monocarboxylate transporters (MCTs) and for the Jen1p monocarboxylate importer in *S. cerevisiae* (12,58). A H^+/A^- stoichiometry of one implies that the overall transport process is electroneutral and does not affect intracellular pH homeostasis in lactic-acid-producing cultures.

The possibility of a proton/lactate stoichiometry higher than one, resulting in electrogenic lactate export, was first postulated in 1979 by Michels *et al.* (46). They calculated that the co-export of the lactate anion with more than one proton, resulting in the generation of a proton-motive force, can significantly contribute to the available metabolic energy under fermentative conditions (33,46). Indeed, experiments with

membrane vesicles of *Lactococcus lactis* (then termed *Streptococcus cremoris*) and *E. coli* demonstrated electrogenic proton-lactate symport (53,69).

Due to thermodynamic constraints, the number of protons that can be co-exported with a lactate anion strongly depends on the lactate gradient and the ΔpH (Fig. 4). Physiological studies with *L. lactis* indeed showed a decreasing apparent proton/lactate stoichiometry with either increasing ΔpH or decreasing ratio between intra- and extracellular lactate concentrations (70). A contribution of electrogenic lactate-proton symport to the cellular energy budget at low extracellular lactate concentrations was shown by the increased biomass yield of *L. lactis* upon removal of extracellular lactate by co-cultivation with *Pseudomonas stutzeri* in anaerobic chemostat cultures (52). Although this 'end-product efflux mechanism' is of physiological significance for *L. lactis* growing in natural environments, the requirement for a low extracellular lactate concentration implies that it has little or no significance for industrial lactate production at high extracellular product concentrations.

The possibility exists that in certain lactic-acid producing microorganisms, lactate-proton export occurs with proton/anion stoichiometries below one. Such an export mechanism will result in acidification of the cytosol. To maintain intracellular pH homeostasis, compensating proton extrusion is required. Such active proton pumping requires additional sources of metabolic energy, and will therefore have an impact on overall metabolism.

Antiport with substrates. In many anaerobic microorganisms, uptake of a substrate is coupled to the export of a product via substrate/product antiport mechanisms (36). The best studied substrate/lactate-antiport system is found in malolactic fermentation by for example *Leuconostoc oenos* or *Lb. lactis* (40,59). During malolactic fermentation, $malate^{2-}$ is taken up by microorganisms in exchange for $lactate^-$. The electro-chemical gradients of both the substrate and the product contribute to the driving force of this transport process. This process is electrogenic and results in the generation of a $\Delta\psi$ (Fig. 5). Malate is subsequently decarboxylated to pyruvate and CO_2, resulting in the formation of NADH. In this decarboxylation reaction a cytoplasmic proton is consumed resulting in an increase of the cytoplasmic pH and generation of a ΔpH, inside alkaline. To maintain redox balance, the pyruvate is reduced to lactate. The electrogenic $malate^{2-}/lactate^-$ antiport together with the enzymatic conversion of malate to lactate thus results in the generation of both components of a pmf (15,16), which can subsequently drive ATP synthesis by the F_0F_1ATPase in *Lb. lactis* (59). Malolactic fermentation is a naturally occurring process in wine fermentations where it modifies acidity and contributes to the organoleptic properties of wine (18,40,81). However, since sugars rather than malate are the preferred feedstocks for large-scale fermentation, this antiport mechanism for lactate export is not relevant for industrial lactate production.

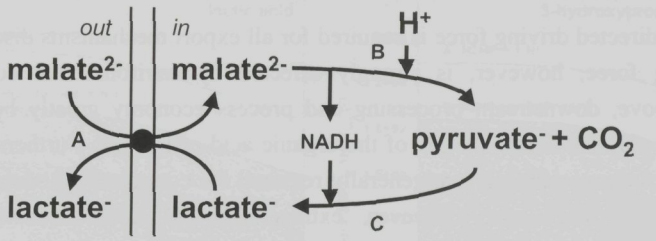

Fig. 5. Schematic representation of malolactic fermentation. Malolactic fermentation starts and ends with a substrate/product antiport system (A), importing malate^{2-} and exporting lactate^{-}. The malate^{2-} is converted to lactate^{-} via decarboxylating malate dehydrogenase (B, also known as malic enzyme), and lactate dehydrogenase (C).

Acid export via primary transport. Cells that do not possess a proton- translocating respiratory chain in their cytoplasmic membrane (i.e., in eukaryotes and anaerobic prokaryotes) require primary, ATP-driven export of protons via membrane-bound ATPase to maintain intracellular pH homeostasis when lactate is excreted by proton/lactate symport with stoichiometries below one. In addition to protons, other substrates can be exported by primary, ATP-driven transport. The transport proteins involved often belong to the ATP-Binding-Cassette (ABC) transporter family (23). ABC transporters have been found for the export of a wide variety of substrates, ranging from antibiotics to weak organic acids and proteins (8,23,57). For instance, in yeast, the ABC transporter *PDR12* has been directly shown to lower the intracellular levels of benzoate by catalyzing an active efflux from the cell (56). ABC transporters have not (yet) been reported to export lactic- or 3-hydroxypropanoic acid, but in view of the broad substrate range of the ABC-transport family their involvement in these export processes in certain microorganisms is not unlikely.

A wide variety of ATP/solute stoichiometries of ABC transport has been reported in the literature, ranging from 1 to 50 (54). However, only ABC transport with an ATP/solute stoichiometry of one, as has for instance been reported for maltose import in *E. coli* (49), or lower, can theoretically be responsible for lactic-acid export in homofermentative lactic-acid producing microorganisms. With lactic-acid export requiring one ATP and lactic-acid production resulting in the formation of only one ATP from substrate-level phosphorylation, homolactic fermentation would not yield any net ATP and, consequently, would not contribute to the metabolic energy budget of the cells. Since, even in non-growing cultures, some metabolic energy is required for maintenance, the absence of net ATP formation will have a strong impact on the energetics of the overall process. As will be discussed below, this situation may arise when either extreme conditions require the involvement of ATP-driven transport or when genetically-engineered microorganisms are used that lack more energy-efficient export systems.

Thermodynamic constraints to weak organic acid export

An outward-directed driving force is required for all export mechanisms discussed above. This driving force, however, is strongly affected by environmental conditions. As discussed above, downstream processing and process economy greatly benefit from a fermentation pH well below the pK of the organic acid of interest. Furthermore, product concentrations above 100 g l^{-1} are generally required for commercially viable production of large-volume chemicals. However, extracellular pH and extracellular product concentration both have a direct and strong impact on the thermodynamics of weak organic acid export. Therefore, simultaneous realization of both targets may not be achievable in practice.

The driving force of weak acid export can consist of the weak acid concentration gradient, $\Delta\psi$, ΔpH and, in the case of primary transport, the free-energy of ATP hydrolysis (see above and Fig. 4). To assess the impact of extracellular pH, we calculated the concentration ratio of intracellular over extracellular total-acid (i.e., undissociated acid + dissociated acid) that is required to enable a specific mode of transport (Fig. 6). For these calculations, it was assumed that the intracellular pH remained constant at 7.0, and that also the proton-motive force was constant at –150 mV. As a consequence, $\Delta\psi$ was dependent on the extracellular pH. Indeed, inside- positive $\Delta\psi$ values of up to +120 mV have been reported for acidophilic microorganisms (43,74). High $\Delta\psi$ values can probably also be expected during citric-acid production by the acid-tolerant yeast *Yarrowia lipolytica*, which is known to maintain a ΔpH of circa 5 at low extracellular pH (44). For the two-dimensional representation in Fig. 6, a $\Delta\psi$ value of +150 mV, which in these calculations occurred at pH 2.0 (ΔpH –300 mV), was assumed to be physiologically possible. Alternatively, microorganisms might slightly decrease either their intracellular pH or the proton motive force in response to very low extracellular pH.

Passive diffusion (without involvement of a transport protein) or facilitated diffusion (via a transporter) of undissociated acid is electroneutral, with neither $\Delta\psi$ nor ΔpH contributing to the driving force (Fig. 4). Thus, only the gradient of the undissociated acid itself can drive such an export process. With decreasing extracellular pH, the fraction of undissociated acid will increase, according to $pH = pK + \log(A^-/HA)$. Consequently, an increasing ratio of intra- over extracellular total acid is required to enable net export. The threshold ratio above which net export of the undissociated acid is possible is indicated in Fig. 6 by line B. At first glance, the equation that describes the driving force for proton/anion symport differs from that for export of the undissociated acid (Fig. 4). However, when acid-base dissociation is substituted into the symport equation, the two equations become identical, confirming that these two mechanisms are thermodynamically equivalent (line B in Fig. 6).

If the concentration ratio of total acid exceeds the threshold indicated by line B in Fig. 6, the driving force is sufficient for net export of the undissociated acid with additional proton(s). In this situation, positive charge and protons are exported with the acid and the

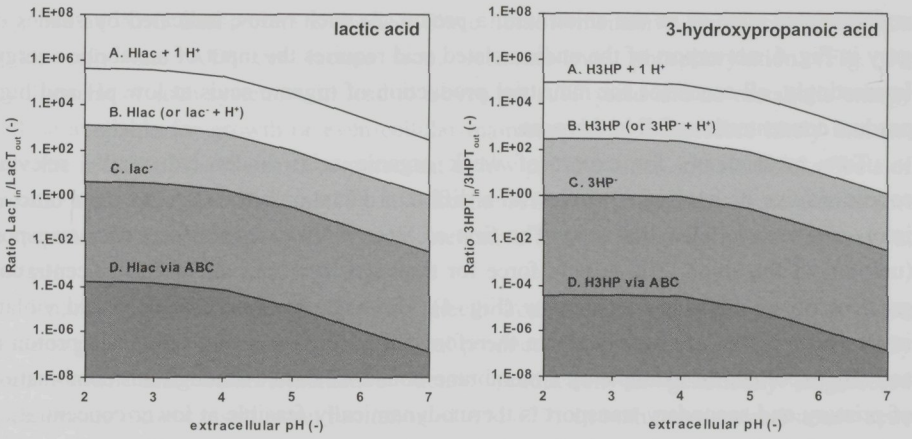

Fig. 6. The effect of the extracellular pH and the ratio of intra- and extracellular total-acid ($HA+A^-$) on the energetics of weak-organic-acid export. The top panel represents calculations for lactic acid (pK=3.86) and the bottom panel for 3-hydroxypropanoic acid (pK=4.51). For this 2-dimensional overview, we assumed a constant intracellular pH of 7.0 and a constant proton-motive force (pmf) of –150 mV. The free energy of ATP hydrolysis (assumed to be -40 kJ/mol, which is equivalent to –414 mV) was taken to be perfectly conserved during organic-acid export by primary transport. Different values for the intracellular pH, pmf and free energy of ATP hydrolysis will quantitatively change these figures. The lines, numbered A through D, indicate the conditions under which the designated export mechanisms are in thermodynamic equilibrium, i.e. the driving force equals zero. All export mechanisms are thermodynamically feasible for which the lines are below a point determined by a concentration ratio and an extracellular pH. An example: below the line 'B. Hlac (or $lac^- + H^+$)' facilitated diffusion of the anion and export of the undissociated acid via ABC transport are both thermodynamically feasible. The abbreviations used for lactic acid are: undissociated (Hlac), anion (lac^-) and total acid (LacT). The abbreviations used for 3-hydroxypropanoic acid are: undissociated (H3HP), anion ($3HP^-$) and total acid (3HPT). For both acids, export via an ATP-binding-cassette transporter with a stoichiometry of one ATP per transported molecule is indicated by ABC.

process contributes to the generation of a proton-motive force (46). The thermodynamic equilibrium for the co-export of one additional proton with the undissociated acid is indicated by line A in Fig. 6. In view of physical and biological limitations to intracellular total lactic-acid (anion plus undisscociated acid) concentrations, this clearly demands both low extracellular total lactic-acid concentrations and an extracellular pH close to the intracellular pH as has been found experimentally (53,70).

Although the patterns shown in Fig. 6 will differ quantitatively for other organic acids, with different pK values, extreme concentration ratios are invariably required to enable net export of the undissociated acid via secondary transport at pH values below the pK of the acid. This becomes evident when the net export of lactic acid is considered at an extracellular pH of 2.86 (one pH unit below the pK of lactic acid and therefore an interesting pH from the industrial viewpoint). To enable net export under these conditions, the intracellular concentration of total lactic acid has to be over 1000-fold higher than the extracellular concentration (Fig. 6). Clearly, such gradients are unachievable at external product concentrations exceeding 100 $g.l^{-1}$.

When the concentration gradient of total-acid is lower than indicated by line B in Fig.

6, the driving force is insufficient to facilitate the export of the acid, either in the undissociated state or as the anion with a proton. At such ratios, indicated by shades of grey in Fig. 6, net export of the undissociated acid requires the input of metabolic energy. Interestingly, all scenarios for industrial production of organic acids at low pH and high product concentrations fall in this area.

Two mechanisms for export of weak organic acids under industrially relevant conditions can be envisaged, each with a different demand on the concentration ratio of intra- and extracellular total acid. The first of these is electrogenic secondary transport (uniport) of the anion. The driving force for transport then consists of the concentration gradient of the anion and of the $\Delta\psi$ (Fig. 4). However, this mechanism would violate intracellular pH homeostasis and can therefore only occur when the associated proton is actively exported, for example by a membrane-bound ATPase. Although this combination of primary and secondary transport is thermodynamically feasible at lower concentration ratios, as is indicated in Fig. 6 by line C, due to the reversed $\Delta\psi$ at low pH, it still requires a ratio of 4 between intra- and extracellular total lactic acid at a pH of 2.86. An alternative mechanism for the export of weak acids is ABC-type transport with a stoichiometry of one ATP/acid molecule. Since the free energy of ATP hydrolysis is used to drive the export of acids via these transporters, the concentration ratios between intra- and extracellular total acid that can be achieved are extremely low (line D Fig. 6). Theoretically, at an extracellular pH of 2.0 and assuming that the free energy of ATP hydrolysis under the prevailing physiological conditions is –40 kJ/mol, the acid accumulation ratio in the broth compared to the cytoplasm can be as high as 5,000 for total lactic acid and even 25,000 for total 3-hydroxypropanoic acid, assuming perfect conservation of the free energy of ATP hydrolysis as concentration gradient (Fig. 6). Clearly, ABC-transporters can theoretically export acids even at the high product concentrations and low pH that are desired by industry. At lower concentration ratios of intra- and extracellular total acid, falling below line D in Fig. 6, the net export of the undissociated acid requires more free energy than that available from the hydrolysis of one ATP.

Physiological consequences of export thermodynamics

The metabolic energy requirement for the export of weak organic acids at low pH and/or high product concentrations can have drastic effects on the physiology of acid-producing microorganisms. Already under relatively mild conditions (the area between lines B and C in Fig. 6), excretion of acids needs to be accompanied by proton excretion via the proton-translocating membrane-bound ATPase or, in aerobic prokaryotes, via respiratory proton extrusion. The number of protons translocated per ATP hydrolyzed varies per microorganism (72). Typically, the H^+/ATP stoichiometries reported for *E.coli* are between 3 and 4 (3,29), whereas those reported for the eukaryotes *S. cerevisiae* and *Neurospora crassa* are 1 (11,66).

Under more severe conditions (below line C in Fig. 6) ATP- driven acid excretion (e.g. via an ABC transporter) is needed. Since homofermentative lactic-acid production yields only one ATP per lactate from substrate-level phosphorylation (Fig. 1), involvement of such systems will lead to a zero net ATP yield and no metabolic energy will be available for growth or even cellular maintenance. It is therefore likely that not only the increased maintenance requirement at low pH and other antimicrobial effects of organic acids (13,21,41,50,55,62,78), but also the ATP requirement for lactic-acid export is responsible for the absence of homofermentative lactic-acid bacteria that can grow at low pH.

Unpredictable situations arise when metabolically engineered microorganisms are made to produce an acid that is not a natural product of their metabolism. It has recently been shown that homofermentative lactate production in genetically engineered *S. cerevisiae* did not result in net formation of ATP (77). In both transient and steady-state experiments it was demonstrated that the engineered *S. cerevisiae* strain had a constant biomass yield on oxygen, independent of the specific rate of lactate production (Fig. 7), indicating that it required respiration to provide ATP for growth. Since these experiments were controlled at pH 6.0, an intra- over extracellular total lactic acid concentration ratio of just below 10 is required to drive net, electroneutral export of the undissociated acid (line B in Fig. 6). With total extracellular lactate concentrations between 15 and 75 mM, as reported in that study, the export of lactic acid does not necessarily require additional metabolic energy. However, since involvement of the proton/lactate symporter (*JEN1*) in export has not been demonstrated in *S. cerevisiae* (42) and lactic acid is not a major end

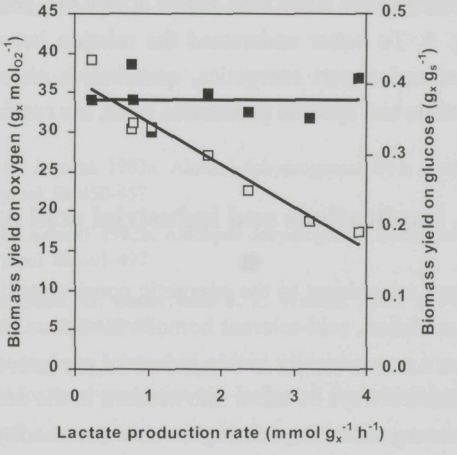

Fig. 7. Biomass yields on oxygen and glucose of steady-state chemostat cultures of *S. cerevisiae* RWB850-2 with different regimes of limited oxygen supply (in all cases the dissolved oxygen concentration was below 0.1 % of air saturation). The cultures were run at a dilution rate of 0.10 h^{-1} on a mixture of glucose and acetate (10% of total substrate carbon). With decreasing air supply to the fermenter, the specific lactate-production rate (x-axis) increased. The biomass yield on glucose (□) is linearly correlated to the specific lactate-production rate. In contrast, the biomass yields on oxygen (■) are independent of the specific lactate-production rate. Data from van Maris et al. (2004b).

product of fermentation in wild-type *S. cerevisiae*, it is possible that efficient export mechanisms are not available. This can necessitate the use of ATP requiring mechanisms, such as ABC transport or (facilitated) diffusion of the anion combined with proton extrusion by the plasma-membrane ATPase, which in *S. cerevisiae* has a proton/ATP stoichiometry of 1 (66). Consistent with the involvement of metabolic energy-requiring lactic acid export in yeasts, an oxygen requirement for efficient production of lactic acid has also been found in genetically engineered, homofermentative lactic-acid producing strains of the yeast *Kluyveromyces lactis* (60).

During aerobic microbial production of acids that are more oxidized than the substrate, oxidative phosphorylation can supply additional ATP. An example is the aerobic production of pyruvic acid from glucose in yeasts (37,48,76), that results in net ATP production even when the export would require one ATP. In this respect, pyruvate production bears some similarities with the production of citric acid. Citric acid is produced from glucose at high concentrations and at pH values below 2.0 (30,44,63). Apparently, this oxidative process yields sufficient ATP via oxidative phosphorylation to enable efficient active export of citric acid as well as cellular maintenance.

Under aerobic conditions, the redox-neutral production of lactic acid from glucose by *Rhizopus oryzae* is accompanied by respiration. When oxygen is not available or limiting, alcoholic fermentation becomes a more prominent additional pathway for glucose dissimilation (67). It seems plausible that, also in this fungus, alternative modes of sugar dissimilation are required to meet a metabolic energy requirement for lactate export. Lowering of the amount of neutralizing agent, in this case Na_2CO_3, has also been shown to increase the production of ethanol by *R. oryzae* (39). This may reflect an increased metabolic energy requirement for lactic acid export at low pH, consistent with the general patterns shown in Fig. 6. To better understand the relation between the mixed-product formation of *R. oryzae* and export energetics, quantitative physiological data, such as biomass yields on substrate and specific production rates, are required.

Outlook, implications and industrial acid production

Since all microorganisms are subject to the energetic constraints described in this review, the use of genetically modified, acid-tolerant homofermentative microorganisms does not necessarily result in more economically viable industrial processes. Therefore, the impact of export energetics should always be taken into account in the design of both the process and the producing microorganism for existing or new commodity chemicals. This issue becomes even more important when the metabolic pathways involved have a zero or marginal net ATP yield, as is the case for several of the proposed pathways leading to 3-hydroxypropanoic acid .

When the microbial production of lactic acid and 3-hydroxypropanoic acid at high product concentrations and low pH requires additional metabolic energy, this can be

provided by net ATP-yielding processes, such as co-product formation or respiratory substrate dissimilation. If the costs associated with the lower yield on sugar or the need for aeration are not economically acceptable, two alternative options remain open, although subject themselves to incremental operating costs. Firstly, down-stream processing procedures for the current near-neutral-pH processes may be optimized with respect to salt economy (5,75). Alternatively, production at low pH may be combined with efficient *in situ* product removal (22,64). Thus, low external product concentrations can be maintained, thereby allowing for acid export without the need for additional metabolic energy (Fig. 6).

In this paper, emphasis has been put on the stoichiometric aspects of organic acid export. We have not discussed possible kinetic implications of product export. Especially in engineered microorganisms that produce non-natural compounds (for example lactate production by engineered yeasts or production of 3-hydroxypropanoic acid by engineered microorganisms) the capacity of export can be an important factor in controlling productivity. Therefore, irrespective of the pH at which commercial fermentation processes are run, research into the mechanism and substrate-specificity of organic-acid transporters is highly relevant for successful engineering of microorganisms for organic-acid production.

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Summary

... of the thesis: 'metabolic engineering of pyruvate metabolism in *Saccharomyces cerevisiae*' by Ton van Maris.

Industrial biotechnology is rapidly gaining interest in the chemical industry. One area of biotechnology, recently termed 'white biotechnology', is devoted to the large-scale production of commodity chemicals from renewable feedstocks, such as sugars derived from biomass. These chemicals can, just as chemicals derived from oil, be used for the production of plastics and fuels. However, bio-based products often have the benefit of improved biodegradability and, due to closed carbon cycles, theoretically do not contribute to the greenhouse effect.

Saccharomyces cerevisiae, commonly known as brewers' or bakers' yeast, is a microorganism that has a long history in industrial biotechnology. This yeast can consume sugars in two different ways. The first is the characteristic alcoholic fermentation, resulting in the formation of ethanol and carbon dioxide. A second possibility is aerobic sugar dissimilation combined with respiration. Although used by mankind for millennia, *S. cerevisiae* still has attributes that require improvement, especially in view of (possible) new applications. This can sometimes be achieved by natural selection, also known as evolutionary engineering or directed evolution. Another approach, using targeted recombinant-DNA technology to alter the physiology of the microorganism, can be used when natural selection is not feasible or not desirable. This scientific field is also known as 'metabolic engineering'.

Using *S. cerevisiae* for the production of 'new' products requires optimum product yield on feedstock, and therefore minimizing alcoholic fermentation. This can be achieved by elimination of pyruvate decarboxylase, the first enzyme of alcoholic fermentation. Indeed, pyruvate-decarboxylase-negative (Pdc^-) bakers' yeast no longer produces ethanol. Surprisingly, it has been found that Pdc^- *S. cerevisiae* strains have a growth requirement for ethanol or other C_2-compounds. This has been suggested to be due to a cytosolic acetyl-CoA requirement for the biosynthesis of lysine and fatty acids. Moreover, Pdc^- *S. cerevisiae* is only capable of growth at very low glucose concentrations, even under fully aerobic conditions that should promote respiration. One of the goals of the research described in this thesis was to remove one or both of these problems, and thus enable large-scale use of Pdc^- *S. cerevisiae* as a platform for further metabolic engineering.

In this research, it was shown that the shortage of cytosolic acetyl-CoA in Pdc^- *S. cerevisiae* can be circumvented by metabolic engineering. As described in chapter 2, over-production of threonine aldolase, an enzyme that catalyses the cleavage of the amino-acid threonine into acetaldehyde and glycine, enables growth of Pdc^- *S. cerevisiae* in glucose-limited chemostat cultures. Apparently, the yeast can produce sufficient cytosolic acetyl-CoA from this acetaldehyde. However, even with one problem solved by

over-production of threonine aldolase, Pdc⁻ *S. cerevisiae* is still not able to grow on high concentrations of glucose.

Using directed evolution as an alternative approach also resulted in Pdc⁻ *S. cerevisiae* without one or both of the growth defects described above, as is discussed in chapter 3 of this thesis. Applying an appropriate selection pressure resulted in a Pdc⁻ *S. cerevisiae* strain that could grow in glucose-limited chemostat cultures without the addition of C_2 compounds. After subsequent selection by serial transfer, a strain was obtained that was capable of rapid growth on high concentrations of glucose in batch cultures. Genome-wide analysis of the messenger-RNA levels using microarrays ('DNA-chips') indicated changes in the expression of glucose-transporter genes and glucose-repressible genes. Amongst the latter, many of the observed transcripts originated from target genes of the transcriptional regulator Mig1p. This 'genomics' approach gave a first impression of the changes in the selected strain. However, the exact molecular mechanisms underlying the selected phenotype were not revealed and (so far) remain unknown.

Pdc⁻ *S. cerevisiae*, able to grow on high concentrations of sugar without the addition of C_2-compounds, offers many interesting opportunities for industrial biotechnology. For example, the selected strain can be used for the production of pyruvic acid, a raw material in the production of cosmetics and medicines, among other things. The evolved strain not only produces 100-fold more pyruvate than wild-type *S. cerevisiae*, but has also doubled the previously known maximum pyruvate concentration obtained by fermentation, resulting in a concentration of 135 gram pyruvate per liter. Improving the pyruvate yield on sugar, now 0.54 gram pyruvate per gram glucose, provides an interesting challenge for future research. This can possibly be achieved by reducing intra-mitochondrial respiration or decreasing the activity of the pyruvate-dehydrogenase complex.

Likewise, the selected Pdc⁻ *S. cerevisiae* can be used for lactic-acid production. The commercial demand for lactic acid is increasing, mainly because of the growing market for polylactic acid. It is expected that this biodegradable polymer, produced from renewable resources, will partially replace various petrochemistry-based polymers in products ranging from packaging to clothing. Since lactic acid is not a normal product of fermentation in *S. cerevisiae*, the Pdc⁻ strain was first transformed with bacterial lactate dehydrogenase, an enzyme that reduces pyruvate to lactic acid. With this enzyme, the selected strain was capable of lactate production up to a concentration of 110 gram per liter (see introduction). During these experiments and comparable tests in industry, it was found that *S. cerevisiae* requires oxygen for growth and efficient lactate production. This was unexpected, since wild type bakers' yeast can grow anaerobically and the production of lactic acid and ethanol were thought to be equivalent in both redox- and ATP balances.

This mystery of homofermentative lactic-acid producing yeast was studied under oxygen-limited cultivation conditions. These experiments, described in chapter 5, showed that the amount of oxygen required for the synthesis of one gram of biomass remained constant, independent of the production of lactic acid. Apparently, lactic-acid production does not yield any net ATP (the free-energy currency of the living cell). Intracellular

conversion of glucose into lactic acid results in the formation of one ATP per molecule of lactic acid. Therefore the most plausible explanation for this phenomenon is that export of a molecule of lactic acid from the cell to the environment requires exactly one ATP. Consequently, the production of lactic acid does not result in net ATP formation, necessitating respiration to provide all ATP required for growth.

But why does the export of lactic acid in *S. cerevisiae* require ATP? It is commonly accepted that ethanol freely diffuses across the plasma membrane. However, export of lactic- and other organic acids is more complicated. A theoretical overview of the possible mechanism for lactic acid export is included in chapter 6 of this thesis. It might be that systems that do not use metabolic energy, such as those used by lactic-acid bacteria at near neutral pH, are absent in *S. cerevisiae*, thereby forcing the yeast to use ATP-consuming export systems. In that case, two export systems might operate in *S. cerevisiae*, both of which consume exactly one ATP per lactic acid. The first is (facilitated) diffusion of the anion combined with proton extrusion by the plasma-membrane ATPase, which in *S. cerevisiae* has a stoichiometry of one ATP per proton. A second option is the export of the undissociated acid by ATP-Binding-Cassette (ABC) transporters with a stoichiometry of one ATP per molecule of lactic acid.

The final product of industrial lactic-acid production is most often the undissociated acid. Optimally this requires a high product concentration and a low pH for both the fermentative production and the down-stream processing of lactic acid. Thermodynamic analysis showed that of the various mechanisms for lactic-acid export, only the ATP-requiring mechanisms are feasible at high product concentrations and low pH. In that case, the production of lactic acid does not result in net formation of ATP and other ATP yielding processes, such as respiration, are required. If microbial acid production at low pH is not economical due to the lower product yield on feedstock, other alternatives such as the use of *in situ* product removal or improvement of the current product-recovery processes might provide alternatives.

Not only alternative fermentation products such as lactic acid, but also for example, pharmaceutical proteins can be made with *S. cerevisiae*. A well-known example is the production of human insulin with bakers' yeast. To produce such heterologous proteins, the yeast needs large amounts of ATP. Aerobic glucose dissimilation combined with respiration yields more ATP per molecule of glucose than alcoholic fermentation. Consequently, during the production of proteins and other biomass-derived products it is important to maximize respiratory glucose dissimilation and to minimize alcoholic fermentation. A way to achieve this is by changing the expression of transcriptional regulators. One example of this can be found in chapter 4 of this thesis: overexpression of *HAP4*, an activator of many genes involved in respiratory metabolism. The effect of this overexpression has been tested under various conditions where wild-type *S. cerevisiae* displays mixed respiro-fermentative metabolism. Overexpression of *HAP4,* indeed, resulted in modulation of the fluxes between respiration and fermentation, with the most

predominant effect under ammonia-limited conditions in chemostat cultures, when it resulted in a doubling of the biomass yield on glucose.

Both the possibilities and the limitations of the combination of directed evolution and metabolic engineering have been shown in this thesis, paying special attention to the broadening of the product range of *S. cerevisiae*. Such progress is not exclusive to *S. cerevisiae*. Progress has also been made with other microorganisms and products. It is expected that these developments in industrial biotechnology will eventually result in biorefineries, producing a similar broad range of products to those of the current oil-based refineries, but using renewable resources instead of oil.

Ton van Maris

Samenvatting

... bij het proefschrift: 'Metabolic engineering van het pyruvaat metabolisme van *Saccharomyces cerevisiae*' door Ton van Maris.

Industriële biotechnologie staat momenteel in de middelpunt van de belangstelling. Binnen dit vakgebied richt één onderdeel, tegenwoordig modieus 'witte biotechnologie' genaamd, zich op de omzetting van hernieuwbare grondstoffen, zoals suikers uit planten, in nuttige chemicaliën. Uit deze chemicaliën kan de industrie, net als uit olie, plastics en brandstoffen maken. Het grote verschil is echter dat de plastics van de industriële biotechnologie vaak beter biologisch afbreekbaar zijn en, door een gesloten koolstofkringloop, in principe niet bijdragen aan het broeikaseffect.

Een van de werkpaarden van de industriële biotechnologie is *Saccharomyces cerevisiae*, bij veel mensen beter bekend als brouwers- of bakkersgist. Suikers kunnen door deze gist op twee manieren worden afgebroken. De eerste is de karakteristieke alcoholische fermentatie, resulterend in ethanol en koolstofdioxide. De tweede mogelijkheid is aërobe suikerafbraak gecombineerd met ademhaling. Hoewel de mensheid *S. cerevisiae* al millennia gebruikt voor de productie van bier, brood en wijn zijn er, vooral voor nieuwe toepassingen, vaak nog verbeteringen aan dit micro-organisme nodig. In sommige gevallen kan dit met natuurlijke selectie, ook wel gestuurde evolutie genoemd. Als dit niet kan of niet wenselijk is kunnen de micro-organismen ook met behulp van doelgerichte recombinant-DNA technieken verbeterd worden. Dit vakgebied staat ook wel bekend als 'metabolic engineering'.

Om met gist op een economische manier nieuwe producten te maken, moet zo min mogelijk suiker omgezet worden naar alcohol. Dit kan bereikt worden door het enzym pyruvaatdecarboxylase, het eerste enzym van de alcoholische gisting, uit te schakelen. Hoewel pyruvaatdecarboxylase-negatieve (Pdc^-) bakkersgist inderdaad geen ethanol meer maakt, kwam het als een verrassing dat zo'n Pdc^- gist nu ineens alcohol of andere C_2-verbindingen nodig heeft om cytosolisch acetyl-CoA te maken voor de biosynthese van vetten en het aminozuur lysine. Bovendien kan Pdc^- *S. cerevisiae* alleen groeien bij lage suiker concentraties, zelfs onder aërobe condities wanneer ademhaling mogelijk is. Hierdoor worden de mogelijkheden voor toepassing van een Pdc^- stam in de industrie beperkt. Eén van de doelen die ten grondslag lagen aan dit proefschrift was het wegnemen van deze obstakels voor grootschalig gebruik van Pdc^- *S. cerevisiae* als platform voor 'metabolic engineering'.

Allereerst is tijdens dit onderzoek aangetoond dat het tekort aan cytosolisch acetyl-CoA in Pdc^- *S. cerevisiae* opgeheven kan worden via aanvullende, doelgerichte genetische modificatie. In hoofdstuk 2 van dit proefschrift is beschreven hoe een Pdc^- bakkersgist door overproductie van threonine-aldolase, een enzym dat het aminozuur threonine omzet in glycine en aceetaldehyde, kan groeien in glucose-gelimiteerde chemostaatcultures. Kennelijk kan de gist uit het zo gevormde aceetaldehyde voldoende cytosolisch acetyl-

CoA maken. Hoewel nu één van de problemen is opgelost, kan Pdc$^-$ *S. cerevisiae*, ook na overproductie van threonine-aldolase, nog steeds niet groeien bij hoge suikerconcentraties.

Dat ook een alternatieve benadering, met gestuurde evolutie, kan resulteren in een Pdc$^-$ *S. cerevisiae* stam zonder een of beide van de genoemde groeiproblemen is beschreven in hoofdstuk 3. Door het opleggen van sterk selecterende kweekcondities is eerst geselecteerd voor een Pdc$^-$ *S. cerevisiae* die in glucose-gelimiteerde chemostaten zonder C_2-verbindingen kon groeien en vervolgens, in schudkolven, voor een stam die kon groeien bij hoge suikerconcentraties. Uit een genoombrede analyse van de boodschapper-RNA-niveaus met microarrays ("DNA-chips") bleek dat de expressie van suikertransporter-genen en van een flink aantal glucose-represseerbare genen, waaronder targets van de transcriptionele regulator Mig1p, veranderd was. Hoewel deze "genomics"-benadering een eerste indruk geeft van de wijzigingen in de geselecteerde celllijn, is nog niet precies bekend welke moleculaire veranderingen verantwoordelijk zijn voor de waargenomen fysiologische eigenschappen.

Een Pdc$^-$ stam van *S. cerevisiae* die zonder toevoeging van C_2-verbindingen en bij hoge suikerconcentraties kan groeien, biedt vele mogelijkheden voor toepassing in de industriële biotechnologie. Een eerste directe toepassing, de productie van pyrodruivenzuur (pyruvaat), een grondstof voor de productie van medicijnen en cosmetica, is reeds mogelijk met de geselecteerde stam. Deze maakt namelijk ruim honderd keer meer pyruvaat dan wildtype stammen van *S. cerevisiae*. Bovendien is de behaalde concentratie van 135 gram pyruvaat per liter bijna twee keer hoger dan de tot dan toe in de literatuur vermeldde hoogste concentratie. Het verhogen van de opbrengst van pyruvaat op suiker, nu 0.54 gram pyruvaat per gram suiker, biedt een interessant doel voor de toekomst. Dit zou bijvoorbeeld bereikt kunnen worden door verminderde ademhaling aan de binnenkant van het mitochondrion of door verminderde activiteit van het pyruvaatdehydrogenase-complex.

Met de geselecteerde Pdc$^-$ *S. cerevisiae*-stam kan eveneens melkzuur gemaakt worden. De verwachting is dat de vraag naar melkzuur de komende jaren sterk zal toe nemen, vooral door de productie van polymelkzuur, een afbreekbare kunststof die sommige op olie gebaseerde plastics kan vervangen. Normaal maakt bakkersgist geen melkzuur. Om dit wel mogelijk te maken werd de giststam eerst getransformeerd met een bacterieel melkzuurdehydrogenase, een enzym dat pyruvaat reduceert tot melkzuur. Met dit enzym bleek de geselecteerde stam wel in staat te zijn om grote hoeveelheden melkzuur te maken, tot wel 110 gram per liter (zie introductie). Tijdens deze en vergelijkbare proeven in de industrie bleek echter dat *S. cerevisiae* zuurstof nodig heeft om efficiënt melkzuur te maken. Dit is vreemd omdat gewone bakkersgist prima zonder zuurstof kan groeien en er, qua redox- en ATP balans, geen verschillen verwacht werden tussen alcoholische fermentatie en de productie van melkzuur.

De gekozen benadering om de zuurstofbehoefte van homofermentative melkzuurproducerende gist uitvoerig te analyseren was zuurstofgelimiteerde kweek in

chemostaten. Deze experimenten, beschreven in hoofdstuk 5, lieten zien dat ongeacht de omvang van de melkzuurproductie altijd dezelfde hoeveelheid zuurstof nodig was om een gram biomassa te maken. Kennelijk levert melkzuurproductie in deze Pdc$^-$ *S. cerevisiae* dus geen ATP op. Aangezien de omzetting van glucose in melkzuur, die zich binnen de cel afspeelt, wel moet resulteren in de productie van één molecuul ATP per molecuul melkzuur, is de meest waarschijnlijke verklaring voor dit fenomeen dat in *S. cerevisiae* de export van melkzuur uit de cel naar de omgeving één ATP kost. Hierdoor zou melkzuurproductie netto geen ATP opleveren en moet alle ATP, die nodig is voor de groei, van ademhaling en dus van zuurstofverbruik komen.

Maar waarom kost melkzuurexport in homofermentatieve *S. cerevisiae* ATP? Van alcohol weten we dat het vrij over de plasmamembraan kan diffunderen. Voor organische zuren zoals melkzuur ligt dit echter anders. Om de vraag goed te kunnen beantwoorden is in hoofdstuk 6 van dit proefschrift een theoretisch overzicht van de mogelijkheden voor melkzuurtransport opgenomen. Een mogelijkheid is dat exportsystemen die geen metabole energie verbruiken, zoals die bij bijna neutrale pH door melkzuurbacteriën gebruikt worden, ontbreken in bakkersgist, waardoor *S. cerevisiae* noodgedwongen ATP verbruikende systemen aanwendt. In dat geval zijn twee melkzuurexportsystemen denkbaar die, in *S. cerevisiae*, één ATP per melkzuur nodig hebben. De eerste is (gefaciliteerde) diffusie van het anion gecombineerd met export van het proton via het plasmamembraan ATPase, dat in *S. cerevisiae* een stoichiometrie van 1 ATP per proton heeft. Een tweede mogelijkheid is export van het ongedissocieerde zuur met ATP-Bindings-Cassette (ABC) transporters met een stoichiometrie van 1 ATP per getransporteerd melkzuurmolecuul.

Omdat niet het zout of het anion, maar het ongedissocieerde melkzuur het gewenste eindproduct is, heeft bij een optimaal proces zowel de fermentatie als de opwerking een lage pH en zijn de product concentraties hoog. Uit thermodynamische analyse bleek echter dat van de diverse mogelijkheden voor melkzuurexport, bij hoge concentraties extracellulair melkzuur en lage pH, sowieso alleen ATP-verbruikende export systemen mogelijk zijn. Er kan dus geconcludeerd worden dat, voor microbiële productie van organische zuren bij lage pH en bij hoge productconcentraties, altijd ATP-leverende metabole reacties, zoals ademhaling, nodig zijn om export van het product mogelijk te maken. Indien dit oneconomisch is, kunnen eventueel *in situ* productverwijdering of verbetering van de huidige opwerkingsprocessen een alternatief bieden.

Niet alleen alternatieve fermentatieproducten, maar bijvoorbeeld ook farmaceutische eiwitten kunnen met *S. cerevisiae* gemaakt worden. Een bekend voorbeeld is de productie van menselijk insuline met bakkersgist. Voor het maken van deze zgn. heterologe eiwitten heeft de gist vooral veel ATP nodig. Aërobe suikerafbraak gecombineerd met ademhaling levert per glucosemolecuul veel meer ATP op dan alcoholische gisting. Daarom is het voor de productie van eiwitten en andere van biomassa afgeleide producten van belang dat de gist zoveel mogelijk suiker verademt en zo min mogelijk fermenteert. Een manier om dit te beïnvloeden is het veranderen van de expressie van transcriptionele regulatoren. Een

voorbeeld hiervan is te vinden in hoofdstuk 4 van dit proefschrift: overexpressie van *HAP4*, een activator van verschillende genen die met het respiratoire metabolisme te maken hebben. Het effect hiervan is getest onder condities waar bakkersgist zowel ademhaling als gisting gebruikt. Inderdaad bleek dat op deze manier de bijdrage van ademhaling aan het metabolisme van de gist vergroot kan worden. Dit effect was het sterkst in ammonia-gelimiteerde chemostaatculturen, waar een verdubbeling van de biomassaopbrengst op suiker optrad.

In dit proefschrift zijn voorbeelden gegeven van zowel de mogelijkheden als de beperkingen van 'metabolic' engineering en gestuurde evolutie bij het uitbreiden van het productscala van bakkergist. Ook met andere micro-organismen en producten wordt op dit gebied vooruitgang geboekt. De verwachting is dat dergelijke ontwikkelingen in de industriële biotechnologie uiteindelijk zullen leiden tot bioraffinaderijen. Net als de huidige olieraffinaderijen zullen deze uiteindelijk een breed scala aan producten leveren, maar dan gebaseerd op hernieuwbare grondstoffen.

Ton van Maris

Lijst van publicaties/List of publications

Flikweert, M. T., M. Kuyper, A. J. A. van Maris, P. Kötter, J. P. van Dijken, and J. T. Pronk. 1999. Steady-state and transient-state analysis of growth and metabolite production in a *Saccharomyces cerevisiae* strain with reduced pyruvate-decarboxylase activity. Biotechnology and Bioengineering **66**:42-50.

Bakker, B. M., K. M. Overkamp, A. J.A. van Maris, M. A. H. Luttik, P. Kötter, J. P. van Dijken, and J. T. Pronk. 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiology Reviews **25**:15-37.

van Maris, A. J. A., B. M. Bakker, M. Brandt, A. Boorsma, M. J. Teixeira de Mattos, A. R. Grivell, J. T. Pronk, and J. Blom. 2001. Modulating the distribution of fluxes among respiration and fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*. FEMS Yeast Research **1**:139-149.

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van Maris, A. J. A., W. N. Konings, J. P. van Dijken, and J. T. Pronk. 2004. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metabolic Engineering. Manuscript accepted for publication.

Patent application

van Maris, A. J. A., J. P. van Dijken, and J. T. Pronk. 2003. Pyruvate producing yeast.

Curriculum Vitae (Nederlands)

Antonius (Ton) Jeroen Adriaan van Maris werd geboren op 31 mei 1976 te Haarlem. Geslaagd voor zijn VWO diploma aan het Ichthus College te Driehuis, begon hij in september 1994 met de studie Scheikundige Technologie aan de Technische Universiteit Delft. Na een stage bij Shell Chemicals in Moerdijk en een petrochemische ontwerp studie, koos hij vervolgens voor de afstudeerrichting Biotechnologie. Onder de bezielende leiding van Barbara Bakker en Jack Pronk werd het effect van *HAP4* overexpressie in de gist *Saccharomyces cerevisiae* onderzocht in de vakgroep Microbiologie van prof. Gijs Kuenen. In december 1999 behaalde hij het doctoraal examen voor de opleiding Scheikundige Technologie. Aangestoken door het wetenschappelijke vuur besloot hij vervolgens een promotie plaats bij, dan inmiddels professor, Jack Pronk en Hans van Dijken aan de Technische Universiteit van Delft te aanvaarden. Het onderwerp van dit onderzoek was het karakteriseren, en indien mogelijk wegnemen, van enkele physiologische eigenaardigheden van een gist die na uitvoerige genetische modificatie geen alcohol maar in plaats daarvan melkzuur maakte. De resultaten van dit onderzoek zijn onder andere in dit proefschrift beschreven. Na dit onderzoek zal Ton de universiteit en gist voor anderhalf jaar verlaten om te gaan werken aan de omzetting van mais in 1,3-propaandiol bij Tate and Lyle North America in Decatur, Illinois.

Curriculum Vitae (English)

Antonius (Ton) Jeroen Adriaan van Maris was born on the 31st of May 1976 in Haarlem, The Netherlands. Having finished his pre-university education at the Ichthus College in Driehuis, he commenced a study Chemical Engineering at the Delft University of Technology in September 1994. After a training period at Shell Chemicals and a petrochemical design study, he chose to specialize in biotechnology. Under the zealous supervision of Barbara Bakker and Jack Pronk, the effect of *HAP4* overexpression in the yeast *Saccharomyces cerevisiae* was thoroughly investigated within the microbiology group of prof. Gijs Kuenen. In December 1999 he was awarded the M.Sc. degree in Chemical Engineering. With the scientific fire awakened he started his Ph.D. research at the section industrial microbiology of, by then Professor, Jack Pronk and Hans van Dijken at the Delft University of Technology. The subject of this research was the characterization of, and possible resolving, a few physiological peculiarities of genetically modified yeast that produced lactic acid instead of ethanol. The most interesting results of this research make up this thesis. After completion of this research Ton will leave both the university and yeast for one-and-a-half year to work at the conversion of corn into 1,3-propanediol at Tate and Lyle North America in Decatur, Illinois.

Dankwoord

Als ik dit aan het schrijven ben is het al weer ruim zes jaar geleden dat ik, na een babbeltje met de mij toen nog volstrekt onbekende Jack, voor het eerst voet zette in de gistgroep kelder. In de hierop volgende drie maanden hebben Marko, toen zelf nog afstudeerder, en Marcel mij ingewijd in de microbiologie en het aseptisch werken. Na een onderbreking met negen maanden petrochemie deed de critische begeleiding van Barbara tijdens mijn scriptie en afstudeeronderzoek het wetenschappelijke vuur in me ontbranden. Hoewel deze tijd *sensu stricto* niet tot mijn promotie hoort: Jack, Marko, Marcel en Barbara, hartstikke bedankt !

En toen promoveren. Ik ben nog vergeten te vertellen dat er ook een wervelwind is die af en toe door de gistgroep waait, snel het koffiefilter volschudt, en terwijl de koffie doorloopt op de hoogte wordt gebracht van recente ontwikkelingen en daarbij zijn verfrissende mening geeft: Hans. Het gevoel bij de begeleiding van zowel Jack en Hans is moeilijk onder woorden te brengen, maar ik weet wel dat ik van de som van deze twee wetenswaardige mensen meer geleerd heb dan ik ooit van de delen had op kunnen steken. Hack en Jans ... !

The role of Tate and Lyle North America (i.e. A.E. Staley Manufacturing Company) ranged beyond the role as financial sponsor. During the work meetings with Jeff, Chi-Li and the many other participants, I learned much about many (often confidential) topics. I have always enjoyed these insights beyond the scientific world. Therefore: Jeff, Chi-Li thank you very much and I hope that the our collaboration continues after the end of this Ph.D. research.

Dan eerst mijn kantoorgenoten: Pascale (babbelen), Marijke (buitengewoon Marijke) en eerst Matt (link-to-the-real-world) en later Marinka (lekker direct) ik vond het gezellig, jullie ook ? En uiteraard de avonden doorzakken met Mickel, Viktor en de rest van de Yeastieboys (Marko, Dennis, Alex, Johan, Frank, Arjen en ook wel een beetje Preben). Voor Erik een korte: vergroeid met de gistgroep en da's maar goed ook en natuurlijk ook nog een vrijwel onuitputtelijke fantasie bibliotheek! Ook 'mijn' studenten (Alexander, Marino, Matthijs, Ha (Thanks !) en Susan) waren onmisbaar door hun gezelligheid en experimentele hulp. Zonder giststammen weinig onderzoek en die kwamen meestal bij Ron en Miranda vandaan. Zo zijn er nog vele anderen die deel uitmaken of deel uitgemaakt hebben van de gistgroep en daarmee een substantiele bijgedrage geleverd hebben aan een buitengewone tijd: bedankt en obrigado (om Jack voor te zijn, hier staat dus niet obrigada).

Niet alleen de gistgroep, maar ook de mensen die de infrastructuur en/of sfeer van het Kluyverlab bepalen (Sjaak, Jos, Herman, Ronald, Astrid, Theo e.v.a.), mij geholpen hebben met mijn gas africhting (Joop, Dirk en Rob) en het systeem beheerden (Hans, Marcel en Jochem) waren onmisbaar bij het tot stand komen van dit boekje.

Ondanks dat ik nooit veel problemen gehad heb met het van me af laten glijden van de arbeidsbeslommeringen, zijn vrienden, kennissen en familie zeker net zo belangrijk als collega's. Hoewel sommigen het misschien one-step-beyond vinden, is met zes vrienden om een tafel zitten (en met vijf bier drinken) en afdwalen in een wereld met 'evil steden', 'neutral units' en 'chaotic pipo-de-clowns' bijzonder ontspannend en dat had niet gekund zonder Jochem, Bart, Jilles, Remon, Sammy, Niels en Ulrika. Hoewel de laatste jaren een verre vriend, bracht een bezoekje aan Maciek altijd een goede gelegenheid voor een arbeidsonderbreking. Ook het afd(w)alen met Steven, Casper, Ivo, Robbert en Martijn lijkt me een traditie die we in stand moeten zien te houden, al hebben we het dit jaar al moeten laten schieten. Uiteraard is het, thuisgekomen van een dag werken, ook wel eens lekker om gewoon op de bank onderuit te zakken met je huisgenoten (Peter en Sietse). En als laatste maar zeker niet minste mijn ouders: pap, mam al hebben jullie mij nooit echt hoeven sturen qua school, studie of werk bedankt voor de mooie basis en ik hoop nog lang van jullie te kunnen genieten !

Ton van Maris
Delft, 2004

P.S. Having reached the end of this thesis, some of you might wonder about the almost total lack of fantasy-linked quotes or pictures in this thesis (no red dragon on the cover !). I will try to compensate for this by citing 'The Wizard's First Rule', which describes multiple occasions in the past four years of my own research with terrifying accuracy (i.e. mainly unpublished results):

"People are stupid; given proper motivation, almost anyone will believe almost anything. Because people are stupid, they will believe a lie because they want to believe it's true, or because they are afraid it might be true. People's heads are full of knowledge, facts, and beliefs, and most of it is false, yet they think it all true. People are stupid; they can only rarely tell the difference between a lie and the truth, and yet they are confident they can, and so are all the easier to fool. "

The Wizard's First Rule, Terry Goodkind

Stellingen bij het proefschrift:
"Metabolic engineering of pyruvate metabolism in *Saccharomyces cerevisiae*"
door Ton van Maris.

1. 'Evolutionary Engineering' is een *contradictio in terminis*.

2. Expressie van xylose isomerase is een inherent betere strategie voor introductie van het vermogen tot xylosefermentatie in *Saccharomyces cerevisiae* dan gecombineerde introductie van xylosereductase en xylitoldehydrogenase.
   Kuyper, M., A.A. Winkler, J.P. van Dijken and J.T. Pronk. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. Yeast. 4:655-664.

3. De duurzaamheid van wetenschappelijke publicaties met on-line beschikbare data kan ernstig beperkt worden door de vergankelijkheid van internetsites.

4. Competitieve microbiologische productie van 1,3-propaandiol biedt, in vergelijking tot petrochemische productie, voordelen qua zowel duurzaamheid als productzuiverheid.
   Jefferson C. Lievense. 2003. Fermentation production of polymer-grade 1,3 propandiol from carbohydrates. Fifth conference on Recent Advances in Fermentation Technology. Society for Industrial Microbiology.

5. *Saccharomyces cerevisiae* kent geen CO_2-stress in chemostaatculturen onder anaërobe condities bij atmosferische druk.
   J. Aguilera en J.T. Pronk, ongepubliceerde waarnemingen.

6. Sharpe en Milligan (2003) maken een fout bij het berekenen van de standaarddeviatie van bolvolumes.
   Sharpe, R.L. and C.L. Milligan. 2003. Lactate efflux from sarcolemmal vesicles isolated from rainbow trout *Oncorhynchus mykiss* white muscle is via simple diffusion. J. Exp. Biol. 206:543-549.

7. Introductie van het alaninedehydrogenase van *Bacillus subtilis* is onvoldoende om *Saccharomyces cerevisiae* te veranderen in een alanine-overproducerend microorganisme.
   M. Morais en J.T. Pronk, ongepubliceerde waarnemingen.

8. Het gebruik van kleuren voor het aanduiden van de verschillende toepassingsgebieden van de biotechnologie, zoals 'witte biotechnologie', draagt bij aan de publieke verwarring omtrent de biotechnologische wetenschappen.

9. Duurzame homomelkzuurfermentatie bij hoge productconcentraties en lage pH is een zure bezigheid.
   Dit proefschrift.

10. Gist is makkelijker te cultiveren dan de mens.

Deze stellingen worden verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotor Prof. Dr. Jack T. Pronk.

Propositions with the thesis:
"Metabolic engineering of pyruvate metabolism in *Saccharomyces cerevisiae*"
by Ton van Maris.

1. 'Evolutionary Engineering' is a *contradictio in terminis*.

2. Expression of xylose isomerase is an inherently better strategy for the introduction of the ability to ferment xylose into *Saccharomyces cerevisiae* than the combined introduction of xylose reductase and xylitol dehydrogenase.
   Kuyper, M., A.A. Winkler, J.P. van Dijken and J.T. Pronk. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. Yeast. 4:655-664.

3. The durability of scientific papers with on-line available data can be strongly affected by the limited life span of internet sites.

4. Competitive microbiological production of 1,3-propanediol not only provides te benefit of sustainability, but also results in a product with increased purity compared to petrochemical synthesis.
   Jefferson C. Lievense. 2003. Fermentation production of polymer-grade 1,3 propandiol from carbohydrates. Fifth conference on Recent Advances in Fermentation Technology. Society for Industrial Microbiology.

5. Under anaerobic conditions and at atmospheric pressure there is no CO_2 stress in chemostat cultures of *Saccharomyces cerevisiae*.
   J. Aguilera and J.T. Pronk, unpublished observations.

6. Sharpe and Milligan erroneously calculate the standard deviation of spherical volumes.
   Sharpe, R.L. and C.L. Milligan. 2003. Lactate efflux from sarcolemmal vesicles isolated from rainbow trout *Oncorhynchus mykiss* white muscle is via simple diffusion. J. Exp. Biol. 206:543-549.

7. Introduction of the alanine dehydrogenase from *Bacillus subtilis* is not sufficient to convert *Saccharomyces cerevisiae* into an alanine-overproducing microorganism.
   M.Morais and J.T. Pronk, unpublished observations.

8. The use of colors to discriminate the various areas of biotechnology, such as 'white biotechnology', contributes to the public confusion regarding the biotechnological sciences.

9. Sustained homolactic fermentation at low pH and high product concentrations is a sour enterprise.
   This thesis.

10. Yeast is easier to cultivate than man.

These propositions are considered defendable and as such have been approved by the supervisor, Prof. Dr. Jack T. Pronk.