MINIREVIEW

Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges

Derek A. Abbott[1,2], Rintze M. Zelle[1,2], Jack T. Pronk[1,2] & Antonius J.A. van Maris[1,2]

[1]Department of Biotechnology, Delft University of Technology, Delft, The Netherlands; and [2]Kluyver Centre for Genomics of Industrial Fermentation, Delft, The Netherlands

Correspondence: Antonius J.A. van Maris, Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628BC Delft, The Netherlands. Tel.: +31 15 278 1616; fax: +31 15 278 2355; e-mail: a.j.a.vanmaris@tudelft.nl

Received 23 December 2008; revised 26 May 2009; accepted 1 June 2009.
Final version published online 29 June 2009.

DOI:10.1111/j.1567-1364.2009.00537.x

Editor: Teun Boekhout

Keywords
pyruvate; lactate; malate; succinate; transport; tolerance.

Abstract

To meet the demands of future generations for chemicals and energy and to reduce the environmental footprint of the chemical industry, alternatives for petrochemistry are required. Microbial conversion of renewable feedstocks has a huge potential for cleaner, sustainable industrial production of fuels and chemicals. Microbial production of organic acids is a promising approach for production of chemical building blocks that can replace their petrochemically derived equivalents. Although *Saccharomyces cerevisiae* does not naturally produce organic acids in large quantities, its robustness, pH tolerance, simple nutrient requirements and long history as an industrial workhorse make it an excellent candidate biocatalyst for such processes. Genetic engineering, along with evolution and selection, has been successfully used to divert carbon from ethanol, the natural endproduct of *S. cerevisiae*, to pyruvate. Further engineering, which included expression of heterologous enzymes and transporters, yielded strains capable of producing lactate and malate from pyruvate. Besides these metabolic engineering strategies, this review discusses the impact of transport and energetics as well as the tolerance towards these organic acids. In addition to recent progress in engineering *S. cerevisiae* for organic acid production, the key limitations and challenges are discussed in the context of sustainable industrial production of organic acids from renewable feedstocks.

Introduction

Decreasing oil reserves and concerns on climate change represent major drivers for the development of new, non-petrochemical production routes for bulk chemicals that are based on renewable feedstocks. Industrial biotechnology and in particular the microbial fermentation of carbohydrate feedstocks – which should ultimately be derived from nonfood crops or residues to avoid competition with food production – is one of the alternative approaches that are currently under development. Many petroleum-derived chemicals can be directly or functionally substituted with chemicals from renewable feedstocks. Among these compounds, several organic acids may fulfill a role as platform molecules using their (multiple) functional groups as a target for enzymic or chemical catalysis. The United States Department of Energy has identified 10 organic acids as key chemical building blocks that can potentially be derived from plant biomass (Werpy & Petersen, 2004). Similarly, the European focus group BREW identified 21 key compounds that can be produced from renewable feedstocks, a number of which were organic acids (BREW, 2006). These studies clearly indicate that while the current market size for microbially produced organic acids is still modest and often associated with food applications, its future economical potential is staggering (Sauer *et al.*, 2008).

In order to compete with petrochemical production, any microbial alternative must meet challenging demands in terms of product yield on substrate, productivity and robustness. In general, such demands are unlikely to match the selective pressures that microorganisms have faced during their long evolutionary history in natural environments. Consequently, strain improvement is an essential element in the design and optimization of microbial alternatives to replace conventional oil-based processes. Traditionally, microbial strain improvement has leaned heavily on

'classical' strain improvement [i.e. the use of nontargeted mutagenesis, combined with high-throughput analysis to select for better-performing mutants (Vinci & Byng, 1999)]. While classical strain improvement continues to be of great importance in industrial biotechnology, it is increasingly being complemented by metabolic engineering, which has been defined as 'the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology' (Bailey, 1991).

Presently, several organic acids are produced with prokaryotic organisms (for reviews, see Warnecke & Gill, 2005; Singh *et al.*, 2006; Song & Lee, 2006; McKinlay *et al.*, 2007). However, many of these prokaryotes, such as lactic-acid bacteria, have complex nutritional requirements because of their limited ability to synthesize B-type vitamins and amino acids (Chopin, 1993). These requirements increase the cost and complicate downstream processing (Benninga, 1990; Vaidya *et al.*, 2005). Furthermore, these prokaryotic organisms are generally unable to grow and produce organic acids at the low pH values where these compounds occur predominantly in their undissociated form. Production at these lower pH values with more acid-tolerant microorganisms, such as *Saccharomyces cerevisiae*, would reduce the cost for pH titrants and ensuing byproduct formation (e.g. gypsum). In addition, several species of filamentous fungi are known to naturally produce significant quantities of organic acids, including *Aspergillus niger*, which is applied for large-scale citric acid production (Papagianni, 2007), and *Aspergillus flavus*, which naturally produces high concentrations of malic acid (Peleg *et al.*, 1988; Battat *et al.*, 1991). However, these fungi can be difficult to grow because their morphology can strongly affect growth and production characteristics. Moreover, *A. flavus* can produce aflatoxins, which presents additional problems in process and product safety (Hesseltine *et al.*, 1966; Do & Choi, 2007). These restrictions provide an incentive to integrate and optimize pathways in organisms that have benefits over naturally occurring producers, such as higher robustness, better genetic accessibility or better compatibility with safety regulations in production.

The extensive knowledge of the molecular genetics, physiology and genomics of *S. cerevisiae* (bakers' yeast) and its excellent accessibility to genetic modification make it a very attractive platform for metabolic engineering (Ostergaard *et al.*, 2000; Nevoigt, 2008). *Saccharomyces cerevisiae* is already used in a wide array of industrial applications ranging from new and traditional food applications and production of primary metabolites to biomass-derived products (Walker, 1998; Donalies *et al.*, 2008). Many of these processes still rely on wild-type strains or strains that have been optimized via classical strain improvement. A huge international research effort is currently underway to optimize *S. cerevisiae* for the production of ethanol from lignocellulosic biomass by expanding its substrate range, reducing byproduct formation and improving robustness in plant biomass hydrolysates (as recently reviewed in van Maris *et al.*, 2006; Almeida *et al.*, 2007; Chu & Lee, 2007; Hähn-Hägerdal *et al.*, 2007). A few selected examples of other targets for yeast metabolic engineering include the expansion of its product range [e.g. by engineering *S. cerevisiae* for production of heterologous proteins or low-molecular-weight drugs (Szczebara *et al.*, 2003; Porro *et al.*, 2005; Ro *et al.*, 2006; Branduardi *et al.*, 2008)], quality improvement of alcoholic beverages [e.g. the degradation of malic acid in wine (Canonaco *et al.*, 2002), the degradation of diacetyl in beer (Blomqvist *et al.*, 1991)] and improvement of strain properties such as freeze tolerance, which is relevant for bread production (Tanghe *et al.*, 2002).

Although *S. cerevisiae* is currently not applied on an industrial scale for the production of simple organic acids, it has a number of potential advantages for such processes. *Saccharomyces cerevisiae* grows well under acidic conditions, even at pH values < 3. At such low pH values, many weak acids, including lactate ($pK_a = 3.86$), succinate ($pK_a = 4.21$, 5.67) and malate ($pK_a = 3.41$, 5.05), occur predominantly in their undissociated form. This is advantageous for industrial production, as it reduces the need for titration with alkali and allows for direct recovery of undissociated acids. Consequently, there is no need for large quantities of acidifying agents, and the formation of salt byproducts (e.g. gypsum) is strongly reduced. In addition, *S. cerevisiae* grows well on relatively simple chemically defined media, which may reduce the cost in production and downstream processing. Finally, the long history of the safe use of *S. cerevisiae* in the food and beverage industry may facilitate its approval for use in the production of organic acids destined for human consumption (Table 1).

In this paper, we will review the progress in the metabolic engineering of *S. cerevisiae* for the production of the monocarboxylic acids pyruvate and lactate and the dicarboxylic acids malate and succinate (see Fig. 1 for an overview). Although *S. cerevisiae* produces all these compounds during growth on glucose, wild-type strains only excrete them in very small amounts. While the present paper focuses on four products (pyruvate, lactate, malate and succinate; Table 1), it is hoped that the concepts discussed in this paper will also be useful for *S. cerevisiae* as a metabolic-engineering platform for the production of other biotechnologically interesting carboxylic acids. The challenge in metabolic engineering of *S. cerevisiae* for the production of these compounds involves at least four levels: (1) elimination of alcoholic fermentation, which, irrespective of the availability of oxygen, is the major route of sugar dissimilation in batch cultures of wild-type *S. cerevisiae* (Verduyn *et al.*, 1984; Van Dijken & Scheffers, 1986;

Table 1. Current production and application status of organic acids that could potentially be produced on an industrial scale with *Saccharomyces cerevisiae*

	Pyruvate	Lactate	Malate	Succinate
Properties/description	C_3 carboxylic acid with a ketone group	C_3 carboxylic acid with a hydroxyl group	C_4 dicarboxylic acid with a hydroxyl group	C_4 symmetrical dicarboxylic acid
Current applications	Pharmaceuticals, polymers, cosmetics, food additives, agrochemicals	Polylactic acid, food preservative	Acidulant and flavor-enhancer in food and beverages	Acidulant, surfactant, ion chelator, antibiotics, pharmaceuticals
Possible applications	Flavoring agent	Polyesters, acrylates	Maleic anhydride, biodegradable polymers	Maleic anhydride, bionelle (biodegradable polyester), butanediol, biodegradable polymers
Key organisms	*Torulopsis glabrata*, *E. coli*	Lactic acid bacteria	*Aspergillus flavus*	Ruminant bacteria (*Actinobacillus succinogenes, Mannheimia succiniciproducens*), *E. coli*
Current production method	Chemically from tartaric acid or microbial conversion with *Torulopsis*	Bacterial conversion of sugars	Chemical conversion from petrochemically derived maleic anhydride	Chemical conversion from petrochemically derived maleic anhydride

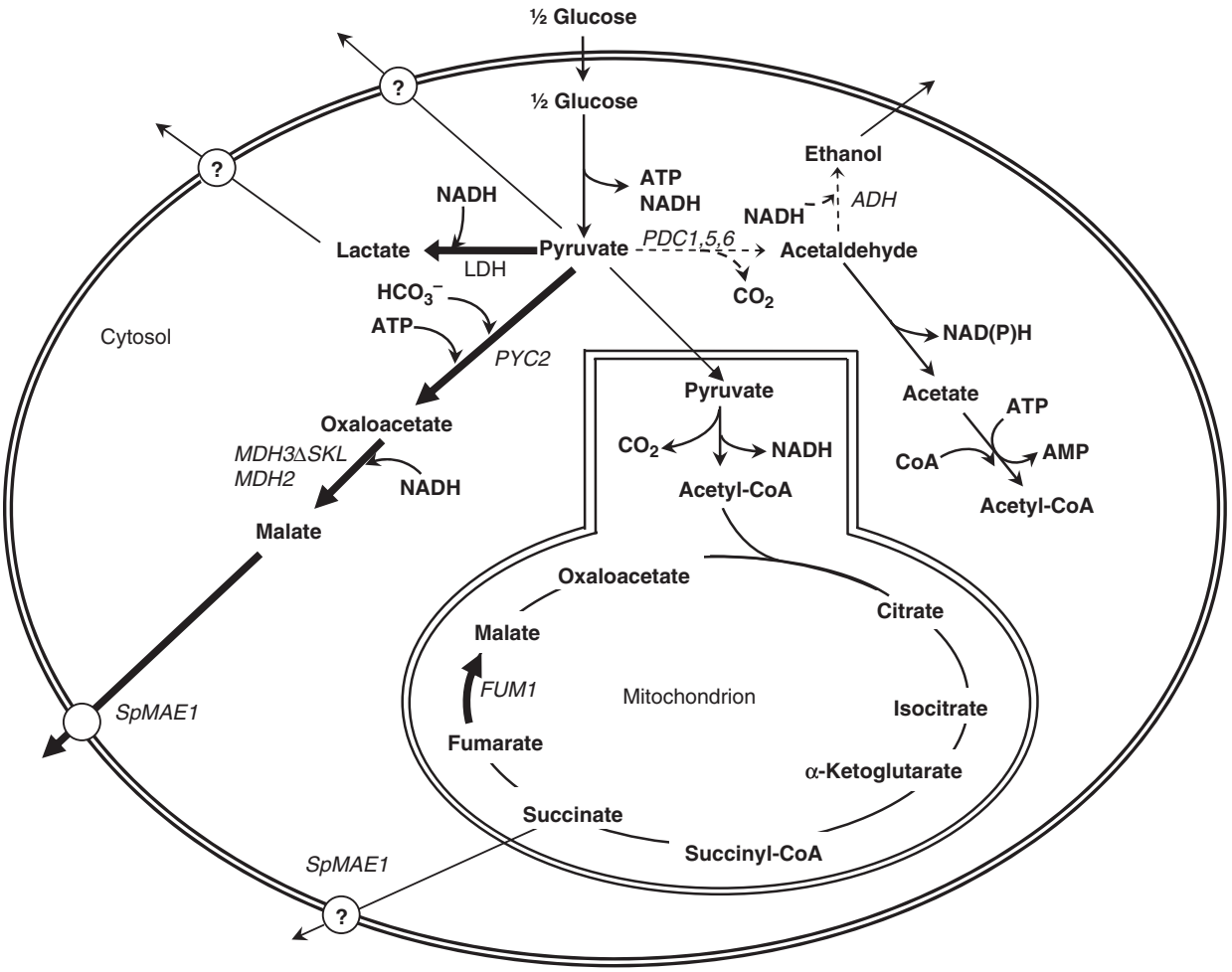

Fig. 1. Schematic representation of various genetic alterations used to enhance the production of organic acids in *Saccharomyces cerevisiae*. Heterologously expressed or overexpressed native genes are denoted by bold arrows and limitation/elimination of gene expression is denoted by dashed arrows.

Postma *et al.*, 1989); (2) engineering fast and efficient metabolic pathways that link the high-capacity glycolytic pathway in *S. cerevisiae* to the product of choice, taking into account redox and free-energy constraints; (3) engineering of product export; (4) engineering of product, substrate and/or environment tolerance.

Studies on the expansion of the product range of *S. cerevisiae*, as described in this review, are almost exclusively based on glucose as the carbon and energy source. However, one can envision a future combination of these concepts with strategies on the expansion of the substrate range of *S. cerevisiae* towards the consumption of the other sugars derived from lignocellulosic biomass (van Maris *et al.*, 2006).

Pyruvate production: elimination of alcoholic fermentation

When organic acid production is the goal, ethanol is an undesired byproduct that leads to a decrease in the product yield by loss of carbon and/or competition for cofactors. However, even under fully aerobic conditions, high glycolytic fluxes in wild-type *S. cerevisiae* strains are intrinsically linked to alcoholic fermentation (Verduyn *et al.*, 1984; Postma *et al.*, 1989). To avoid reduced product yields as a result of ethanol coproduction, any metabolic-engineering strategy for high-yield production of organic acids with *S. cerevisiae* will therefore first have to eliminate ethanol formation. Besides attempts to limit the ethanol formation in response to glucose excess by intervening in cellular regulation and hexose transport (Klein *et al.*, 1999; Boles *et al.*, 2001; Diderich *et al.*, 2001), there are only two reactions in the pathway between pyruvate and ethanol that can be targeted to eliminate ethanol production: pyruvate decarboxylase and alcohol dehydrogenase.

Initial attempts to eliminate alcoholic fermentation in *S. cerevisiae* focused on alcohol dehydrogenase. Deletion of four structural genes for alcohol dehydrogenase (*ADH1-4*) did not result in the complete elimination of ethanol production and caused the accumulation of large amounts of glycerol and toxic acetaldehyde (Drewke *et al.*, 1990). In addition, Skory (2003) described the deletion of the *ADH1* gene encoding the major alcohol dehydrogenase in a *S. cerevisiae* strain overexpressing lactate dehydrogenase (LDH). However, despite lower ethanol titers, the deletion also resulted in lower lactate yields (when compared with the control strain expressing an LDH and the native *ADH1*) in favor of glycerol production, which indicated a redox cofactor imbalance (van Dijken & Scheffers, 1986). Furthermore, the *adh1* strain grew poorly, which was attributed to toxic intracellular accumulation of acetaldehyde (Skory, 2003).

An alternative approach is to block ethanol formation one step upstream of alcohol dehydrogenase by eliminating pyruvate decarboxylase activity. *Saccharomyces cerevisiae* contains three structural genes, *PDC1, 5* and *6*, that encode functional pyruvate decarboxylase isozymes (Hohmann, 1991). Deletion of all three *PDC* genes completely eliminates alcoholic fermentation. In complex media, pyruvate-decarboxylase-negative (Pdc^-) strains grow poorly and produce large amounts of pyruvate (Flikweert *et al.*, 1997). In synthetic media, *pdc1,5,6* deletion mutants even fail to grow on glucose as the sole carbon source and are hypersensitive to high glucose concentrations (Flikweert *et al.*, 1996, 1997). When aerobic, glucose-limited chemostat cultures were used to circumvent the glucose sensitivity of Pdc^- strains, growth on glucose could be restored by the addition of small amounts of ethanol or acetate (Flikweert *et al.*, 1996, 1999). Based on these observations, the inability to grow on glucose as a sole carbon source was attributed to a biosynthetic role of pyruvate decarboxylase in the synthesis of cytosolic acetyl-CoA (Flikweert *et al.*, 1996), which is essential for lysine and lipid synthesis. Intriguingly, this suggests that mitochondrial acetyl-CoA cannot be transported to the cytosol in *S. cerevisiae*. Consistent with this hypothesis, overexpression of the *GLY1*-encoded threonine aldolase, which splits threonine into glycine and acetaldehyde and thus provides an alternative route to cytosolic acetyl-CoA, restored growth of Pdc^- *S. cerevisiae* on glucose as the sole carbon source (van Maris *et al.*, 2003).

The combination of a requirement for C_2 compounds and a high glucose sensitivity represented major impediments for the use of Pdc^- strains for the production of organic acids. Although glucose sensitivity has been observed in Pdc^- strains constructed in different *S. cerevisiae* genetic backgrounds (Flikweert *et al.*, 1999; van Maris *et al.*, 2003), its molecular basis so far remains unknown. However, by applying evolutionary engineering – which does not require a direct insight into the molecular basis of a selectable trait (Sauer, 2001) – both undesirable phenotypes of Pdc^- *S. cerevisiae* could be addressed (van Maris *et al.*, 2004c). First, the C_2 dependence was successfully eliminated by progressively reducing the acetate feed during prolonged glucose-limited chemostat cultivations. Subsequently, spontaneous mutants with decreased glucose sensitivity were selected by serial transfers in shake flasks, with increasing concentrations of glucose as the sole carbon source. This two-stage evolutionary engineering strategy eventually yielded a Pdc^- *S. cerevisiae* single-cell isolate, denoted TAM, with the ability to grow on synthetic medium with glucose as the sole carbon source (Fig. 2) at a specific growth rate of 0.20 h^{-1}. Aerobic batch fermentation of this strain at pH 5 with repeated glucose feeding, without any medium or process optimization, yielded pyruvate concentrations of up to 135 g L^{-1} at a yield of 0.54 g pyruvate g^{-1} glucose (van Maris *et al.*, 2004c).

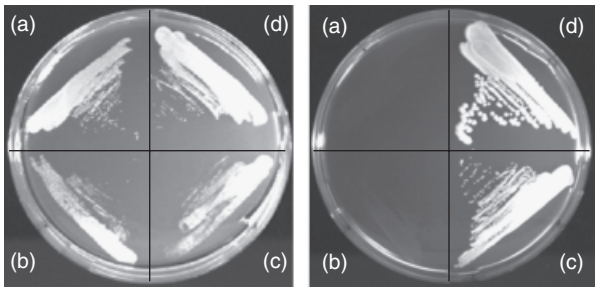

Fig. 2. Growth of (a) Pdc$^-$ *Saccharomyces cerevisiae*, (b) Pdc$^-$ *S. cerevisiae* selected for C_2 independence, (c) Pdc$^-$ *S. cerevisiae* selected for C_2 independence and glucose tolerance (TAM) and (d) CEN.PK 113-7D (*S. cerevisiae* reference strain) on media containing ethanol (2% v/v, left panel) or glucose (2% w/v, right panel) as the sole carbon source. Figure reprinted from van Maris *et al*. (2004c) with permission from American Society for Microbiology.

The high pyruvate titers and yields generated by Pdc$^-$ *S. cerevisiae* strains highlight their potential for the production of organic acids. Although all three remaining organic acids discussed in this review share pyruvate as an intermediate, the challenges that need to be overcome to achieve efficient production differ significantly for each acid.

Lactate production: a deceptively difficult challenge

The many traditional applications of lactic acid include its use as a preservative and iron chelator in food products, cosmetics and pharmaceuticals (Benninga, 1990). However, the main driver for research on microbial lactic acid production is currently the production of polylactic acid, a biodegradable polymer of lactic acid (Datta *et al*., 1995). Most lactic acid is now produced with lactic acid bacteria (Benninga, 1990), which require complex media compositions and pH control (Chopin, 1993). The use of calcium carbonate ($CaCO_3$) as a buffering agent results in the production of large amounts of the byproduct gypsum ($CaSO_4 \cdot 2H_2O$) during the acidification that is required for the recovery of undissociated lactic acid (Benninga, 1990; Vaidya *et al*., 2005). Because *S. cerevisiae* is acid tolerant, grows in simple synthetic media and is capable of anaerobic growth, it is an interesting alternative platform for production of pure lactic acid (Liu & Lievense, 2005).

Wild-type *S. cerevisiae* strains only produce trace amounts of D-lactate, presumably as a result of the operation of the methylglyoxal bypass (Pronk *et al*., 1996; Martins *et al*., 2001). Already over a decade ago, metabolic engineering of *S. cerevisiae* for lactic acid production has been proposed (Dequin & Barre, 1994; Porro *et al*., 1995). The basic strategy consisted of two steps. First, one or more of the three functional genes encoding pyruvate decarboxylase (Hohmann, 1991) were deleted to reduce or eliminate alcoholic fermentation. Then, a heterologous LDH was introduced. This approach resulted in strains that produced lactic acid from glucose and, depending on the degree to which pyruvate decarboxylase activity had been reduced, lactate was either the main fermentation product or produced in combination with ethanol (Adachi *et al*., 1998; Porro *et al*., 1999; van Maris *et al*., 2004b; Saitoh *et al*., 2005; Ishida *et al*., 2006a, b). Lactate production by these strains was observed under fully aerobic conditions with excess glucose (Porro *et al*., 1999; van Maris *et al*., 2004b), at approximately half the rate observed for ethanol formation by wild-type strains under similar conditions. However, the apparent simplicity of this approach has proven to be deceptive.

Stoichiometrically, conversion of glucose to lactate is equivalent to the production of ethanol and carbon dioxide (CO_2) via the native yeast pathway. In both cases, conversion of 1 mol glucose via glycolysis yields 2 mol pyruvate, which is coupled to the formation of 2 mol ATP and NADH. The NADH generated in glycolysis is then reoxidized to NAD^+ by the formation of either lactate or ethanol plus CO_2. Based on these considerations alone, it seems possible to simply replace alcoholic fermentation by lactate fermentation and thereby enable the efficient anaerobic, homolactic growth of *S. cerevisiae*. However, engineered 'homolactic' *S. cerevisiae* strains could not sustain high rates of lactate production under anaerobic conditions and failed to grow unless cultures were aerated (van Maris *et al*., 2004b). This was exemplified by the introduction of an LDH expression vector in the Pdc$^-$ TAM strain, which circumvented the C_2-requirement and glucose sensitivity of nonevolved Pdc$^-$ *S. cerevisiae* strains. Lactate titers of up to $110\,g\,L^{-1}$ could be obtained with the resulting strain in 1 L batch fermentations, but only if the culture was aerated (Fig. 3; A.J.A. van Maris *et al*., unpublished data). Pdc$^-$ *S. cerevisiae* has been shown to produce $> 50\,g\,L^{-1}$ of lactic acid in aerobic shake-flask cultures at the low final pH of 3 (Fig. 3; right panel). At this pH, most lactic acid will be predominantly in its undissociated form.

Based on an analysis of the growth energetics of oxygen-limited chemostat cultures of an engineered lactate-producing *S. cerevisiae* strain, it has been proposed that energy-dependent export of lactic acid (or of the lactate anion and a proton) uses the ATP formed in glycolysis, thereby reducing the net ATP yield of homolactic fermentation to 0 (van Maris *et al*., 2004a, b). An energy requirement for the export of lactate would represent a clear difference with ethanol, which is generally accepted to exit yeast cells via passive diffusion (Guijarro & Lagunas, 1984). Because ATP is required for cellular maintenance, the absence of net synthesis of ATP in anaerobic, homolactic cultures can be expected to result in depletion of intracellular ATP (van Maris *et al*., 2004b). Indeed, measurements confirmed a

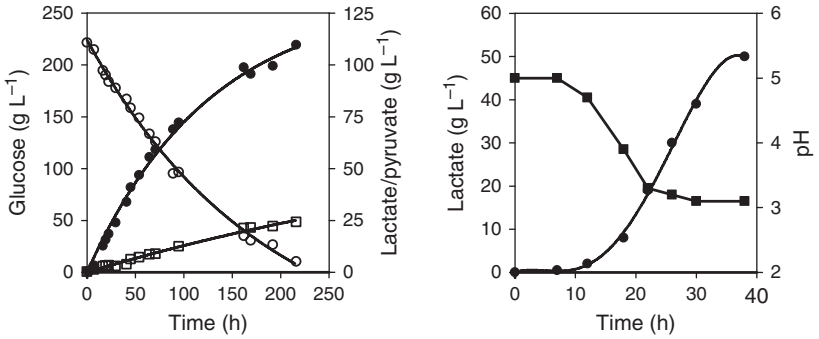

Fig. 3. Left panel: lactate (●) and pyruvate (□) production and glucose consumption (○) in *Saccharomyces cerevisiae* TAM strain expressing a bacterial LDH. The pH of the aerated batch culture was controlled at pH 5.0 with automated addition of 10 M KOH (A.J.A. van Maris *et al.*, unpublished data). Right panel: lactate production (●) and pH (■) in shake flask cultures of an evolved Pdc⁻ *S. cerevisiae* expressing a bacterial LDH (J. Lievense, pers. commun.).

rapid decrease in the intracellular ATP concentration and, coupled to this, a decreasing energy charge after a switch to anaerobic conditions (Abbott *et al.*, 2009).

Malate and succinate production: towards net CO_2 fixation

Malate and succinate, two dicarboxylic acids with chemically versatile structures, have been recognized as potential platform molecules for sustainable production of a wide range of chemicals (Werpy & Petersen, 2004). High-yield production of these compounds requires large fluxes through the carboxylating anaplerotic pathways, converting the three-carbon intermediates of glycolysis into the desired four-carbon backbones of malate and succinate. As such, malate and succinate can, at least in theory, be produced from glucose with a net consumption of CO_2. In the case of redox-neutral malate production, this would result in a maximum theoretical yield of 2 mol malate mol^{-1} glucose. Whereas bacteria contain a large variety of anaplerotic pathways, as reviewed by Sauer & Eikmanns (2006), this function in *S. cerevisiae* is fulfilled by either the glyoxylate cycle or by the higher yielding route via pyruvate carboxylase (Stucka *et al.*, 1991; Brewster *et al.*, 1994; Pronk *et al.*, 1996; de Jong-Gubbels *et al.*, 1998).

Initial efforts to produce malate via biotechnological routes focused on the filamentous fungus *A. flavus* (Abe *et al.*, 1962; Peleg *et al.*, 1988; Battat *et al.*, 1991). *Aspergillus flavus* is a natural malate producer and closely related to *A. niger*, which is currently used for citrate production. After medium optimization, a high titer of 113 g L^{-1} malate was obtained in $CaCO_3$-buffered synthetic medium at a yield of 1.26 mol malate mol^{-1} glucose (Battat *et al.*, 1991). However, *A. flavus* has not been used for industrial production, presumably due to the risk of aflatoxin production (Hesseltine *et al.*, 1966), which is incompatible with process safety and with the major role of malate as a food additive. In addition to *A. flavus*, many other microorganisms have been shown to be able to produce malate (listed by Zelle *et al.*, 2008), although not at industrially significant yields and titers.

Perhaps as a result of *S. cerevisiae*'s specialization in ethanolic fermentation, wild-type *S. cerevisiae* strains are poor malate producers, with maximum titers of only 1–2 g L^{-1} (Fatichenti *et al.*, 1984; Schwartz & Radler, 1988). However, significant progress has been made in metabolic-engineering studies aimed at malate production by *S. cerevisiae*. In an early effort, malate titers of up to 6 g L^{-1} were obtained by overexpressing fumarase (Pines *et al.*, 1996), although this improvement was attributed to increased malate dehydrogenase (Mdh) activities. The role of Mdh activity was studied further, and overexpression of the cytosolic isozyme Mdh2 resulted in titers of 12 g L^{-1} malate (Pines *et al.*, 1997). These experiments were performed in galactose-grown cultures, as glucose is known to inactivate Mdh2p and to repress *MDH2* transcription (Minard & McAlister-Henn, 1992; van den Berg *et al.*, 1998). However, despite numerous attempts in different strain backgrounds, we have been unable to reproduce the described effect of Mdh2 overexpression on malate production in our laboratory (J.T. Pronk & J.P. van Dijken, unpublished data). To avoid possible complications with glucose inactivation of the native cytosolic malate dehydrogenase (Mdh2p), Zelle *et al.* (2008) retargeted and overexpressed the peroxisomal malate dehydrogenase (Mdh3p) in the *S. cerevisiae* TAM strain. Retargeting to the cytosol was accomplished by deletion of the C-terminal SKL peroxisomal-targeting sequence (McAlister-Henn *et al.*, 1995). Despite confirmed overexpression of this Mdh3ΔSKL protein, malate titres increased by only approximately threefold in the engineered strain (see Fig. 4).

Malate degradation by wine yeast is often desirable to reduce wine acidity and to improve the taste profile (Davies *et al.*, 1985). However, based on the genome sequence of *S. cerevisiae* and the results of physiological studies, it was concluded that *S. cerevisiae* does not contain a specialized malate plasma membrane transporter (Ansanay *et al.*, 1998; Volschenk *et al.*, 2003). A malate transporter gene from the yeast *Schizosaccharomyces pombe*, Sp*MAE1* (not to be confused with the *MAE1* gene in *S. cerevisiae*, which encodes malic enzyme), was shown to facilitate malate import and conversion in *S. cerevisiae* (Volschenk *et al.*, 1997). Based on

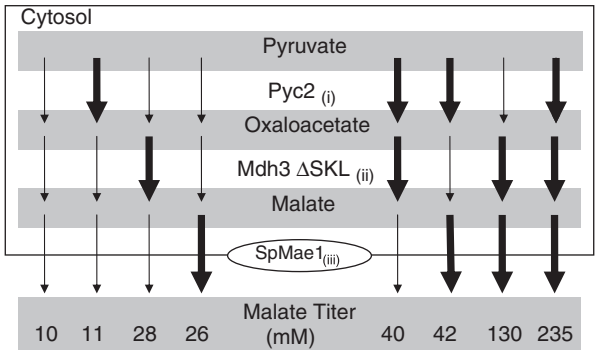

Fig. 4. Malate titers in glucose-grown (100 g L^{-1}) shake flask cultivations of *Saccharomyces cerevisiae* TAM expressing various combinations of pyruvate carboxylase (*PYC2*), cytosolically retargeted malate dehydrogenase (*MDH3ΔSKL)* and malate permease (Sp*MAE1*) (Zelle *et al.,* 2008). Thin arrows indicate activities present in *S. cerevisiae* TAM: (i) pyruvate carboxylase naturally present in the cytosol, (ii) endogenous malate dehydrogenase activity or (iii) background malate export activities or diffusion. Heavy arrows indicate overexpression of Pyc2, Mdh3ΔSKL or SpMae1.

this result, malolactic fermentation was successfully engineered into a wine strain of *S. cerevisiae* and the resulting strain has been commercialized (Husnik *et al.*, 2006).

The importance of malate import in metabolic-engineering strategies for malate consumption suggested that malate export might be similarly important in strategies for malate production. In a recent study, the effects of pyruvate carboxylase overexpression, malate dehydrogenase overexpression and expression of the *S. pombe* malate transporter gene Sp*MAE1* on malate production by Pdc$^-$ *S. cerevisiae* were systematically evaluated (Zelle *et al.*, 2008). While individual overexpression of the Pyc2p pyruvate carboxylase isozyme, the cytosolically retargeted Mdh3ΔSKL malate dehydrogenase or the SpMae1p malate transporter had only small effects, combined overexpression yielded a strong increase of malate titres and yields. The highest malate production was observed in a strain with all three genetic modifications (Fig. 4; Zelle *et al.*, 2008). In shake flasks with synthetic, $CaCO_3$-buffered medium, malate titers of up to 59 g L^{-1} were reached at a malate yield of 0.42 mol mol^{-1}-glucose (Zelle *et al.*, 2008). Although insufficient for industrial application, this result placed *S. cerevisiae* firmly on the list of potential candidates for malate production.

The similarities between malate and succinate go beyond their structure as dicarboxylic acids and the importance of anaplerosis. The effects that the energetics of export of these weak organic acids can have on industrial production are also comparable. Just as with lactic acid, export of these compounds can become a challenge and might require the investment of free energy, especially at low pH. In this light, it is worth mentioning that, in contrast to alcoholic fermentation, malate or succinate formation from glucose via pyruvate carboxylase does not generate net ATP (see Fig. 1). Therefore, if malate or succinate is produced via this pathway, some glucose will have to be respired to meet the energy requirements of the cell. For succinate, however, there is another complication, which is its higher degree of reduction as compared with malate.

The fact that succinate is more reduced than malate, and therefore requires the net input of two electrons when produced via an anaplerotic pathway, makes metabolic engineering of *S. cerevisiae* for high-yield succinate production intrinsically more difficult. The maximum theoretical yield for succinate production from sugar therefore depends not only on the pathways used but also on the boundary conditions that are set for the provision of CO_2 and reducing equivalents (electrons or NADH). When both are provided in addition to the sugar supply, the maximum theoretical yield equals that of malate at 2 mol succinate mol^{-1} glucose. If only CO_2 is provided and electrons have to be derived from glucose, the maximum theoretical succinate yield becomes 1.71 mol mol^{-1} glucose (1.12 g g^{-1}). To reach this yield, part of the carbon has to be redirected to the oxidative route through the citric acid cycle to produce redox equivalents (for simplicity, we assume that only NADH is formed), thereby counterbalancing the consumption of NADH in the conversion of fumarate to succinate. Although, in *Escherichia coli*, yields of up to 1.6 mol mol^{-1} have already been demonstrated (Jantama *et al.*, 2008a, b), achieving these very high succinate yields in *S. cerevisiae* is expected to require extensive metabolic engineering including cofactor engineering and rerouting of metabolism.

Although several sake strains of *S. cerevisiae* have been identified as natural succinate producers, the succinate concentrations obtained are low (*c.* 1 g L^{-1}) (Arikawa *et al.*, 1999; Song & Lee, 2006). Furthermore, despite the pursuit of several industrial and academic research groups to metabolically engineer *S. cerevisiae* for succinate production, few results have so far been published in scientific journals. In an *in silico* optimization study with very strict boundary conditions and without CO_2 addition, Patil *et al.* (2005) calculated that a set of five deletions (the SDH-complex, *ZWF1*, *PDC6*, *U133*, and *U221*) would allow for a succinate yield of 0.60 mol mol^{-1} glucose. This prediction has not yet been verified *in vivo*.

As for malate production, export of succinate from the cytosol to the production broth will be another challenge in metabolic engineering of *S. cerevisiae* for succinate production. Interestingly, the *S. pombe* malate transporter also seems to be able to mediate succinate transport in *S. cerevisiae*, as expression of Sp*MAE1* in the Pdc$^-$ TAM background overexpressing pyruvate carboxylase and malate dehydrogenase increased succinate titers from 1.5 to 4.8 g L^{-1} when this strain was grown on 100 g L^{-1} glucose. In batch cultures on 200 g L^{-1} glucose, this strain was shown to produce 8.0 g L^{-1} succinate (Zelle *et al.*, 2008).

Transport and energetics

The pH- and pK_a-dependent equilibrium between organic acid anion(s) (A^-, HA^- and A^{2-}) and undissociated acid (HA and HHA), as described by the Henderson–Hasselbalch equation (Fig. 5), impacts both production and recovery of organic acids in biotechnological processes. In general, the undissociated species that predominate at low pH are desirable for efficient recovery of organic acids from biotechnological processes. However, their relatively high membrane permeability facilitates entry into the cell via passive diffusion. The near-neutral pH of the yeast cytosol (pH 6–7) then causes dissociation of the acid. This dissociation results in release of protons and anions, which cannot readily diffuse across the membrane, and, in the absence of export systems, accumulate intracellularly. *Saccharomyces cerevisiae* harbors an array of membrane transporters that maintain pH and ion homeostasis (Casal *et al.*, 2008). The energetics of monocarboxylate export and its impact on metabolic-engineering strategies for the production of these compounds has recently been reviewed elsewhere (van Maris *et al.*, 2004a).

While the principles of monocarboxylate transport also apply to dicarboxylic acids, the energetics of dicarboxylate export warrant special attention. For compounds such as malate and succinate, the equilibrium between the undissociated acid, the monovalent anion and the divalent anion needs to be considered (Fig. 5). Whereas uniport of the monovalent anion is energetically equivalent to uniport of the anions of monocarboxylic acids, primary or secondary transport of the divalent species requires the export of an additional proton to maintain charge and pH gradients. Consequently, additional ATP is required. Malate transport via Sp*MAE1*, the transporter used in metabolic engineering of *S. cerevisiae* for malate production (Zelle *et al.*, 2008), has been shown to involve symport of protons and monovalent acid anions (Camarasa *et al.*, 2001).

Export energetics can have a huge impact on the net ATP yield and maximum product titers, and thereby impose upper limits on the maximum yields of organic acids in microbial fermentations, especially at low pH and high product titers. Rational engineering of transport energetics is still complicated by a lack of knowledge and understanding of organic acid exporters in *S. cerevisiae*. Even identification of the genetic determinants of (often inefficient) endogenous transport is complicated by the redundancy of proteins and transport systems (e.g. 20 genes encode for hexose transporters; Özcan & Johnston, 1999). Furthermore, expression of heterologous transport proteins, specifically those of prokaryotic origin, in the yeast plasma membrane presents an additional hurdle due to differences in protein targeting (Rapoport, 2007) and folding (Gárdonyi & Hahn-Hägerdal, 2003). Hence, engineering of export mechanisms probably represents the single most important challenge for the commercialization of organic acid production with *S. cerevisiae*.

Product toxicity and tolerance

Classical 'uncoupling' of the plasma membrane proton gradient (Krebs *et al.*, 1983; Salmond *et al.*, 1984; Russell, 1991) contributes to the antimicrobial action of several weak organic acid preservatives in acidic foods and beverages. The acids considered here are less hydrophobic and therefore less membrane permeable than food preservatives such as benzoate and sorbate. However, at high product concentrations and/or low pH, they are nevertheless likely to diffuse across membranes at significant rates, as exemplified by the common use of high-concentration lactic acid as a food preservative. Intracellular pH acidification can be prevented by the ATP-dependent export of protons (Eraso & Gancedo, 1987; Pampulha & Loureiro-Dias, 1989). The enzyme responsible, the plasma membrane H^+-ATPase (Pma1p), is the most abundant protein in yeast plasma membranes. Consistent with its key role in protection against organic acid stress, Pma1p activity increases in response to organic acid stress (Viegas & Sá-Correia, 1991; Holyoak *et al.*, 1996). This increased activity can, at least transiently, lead to a reduction in intracellular ATP levels (Holyoak *et al.*, 1996) and contributes to a partial uncoupling of dissimilation and biomass formation, resulting in reduced biomass yields (Verduyn *et al.*, 1990, 1992). At high acid concentrations,

Monocarboxylic acids

$$HA \leftrightarrow A^- + H^+$$

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

$$[HA] = \frac{[\text{Total acid}]}{10^{(pH-pKa)} + 1}$$

Dicarboxylic acids

$$HHA \leftrightarrow HA^- + H^+ \leftrightarrow A^{2-} + 2H^+$$

$$pK_{a1} + \log\frac{[HA^-]}{[HHA]} = pH = pK_{a2} + \log\frac{[A^{2-}]}{[HA^-]}$$

$$[HHA] = \frac{[\text{Total acid}] \times (10^{-pH})^2}{((10^{-pH})^2 + 10^{(-pH-pKa1)} + 10^{(-pKa1-pKa2)})}$$

Fig. 5. The distribution between undissociated acid (HA or HHA) and dissociated anions (A^-, HA^- and A^{2-}) and protons (top equations) is determined by the pH of the environment and the pK_a of the carboxylate groups as described by the Henderson–Hasselbalch equation (middle equations). By reformulating these equations, the proportion of, for instance, undissociated acid can be determined at any pH (bottom equations).

ATP depletion, cytosolic acidification and even cell death may occur.

Although the identity of the transporters involved is dependent on the organic acid studied, knowledge of typical weak-acid food preservatives is highly relevant for engineering of acid tolerance in organic acid-producing strains. The *S. cerevisiae* plasma membrane transporter Pdr12p is regulated by the transcriptional regulator War1p (Kren *et al.*, 2003; Gregori *et al.*, 2007). Pdr12p facilitates ATP-dependent efflux of moderately lipophilic acid anions (e.g. benzoate and sorbate) and is a key determinant of tolerance to these compounds (Piper *et al.*, 1998, 2001; Holyoak *et al.*, 1999). The transcriptional regulator Haa1p regulates a complementary set of genes, whose deletion results in hypersensitivity to acetic, propionic and butyric acids, but not to more lipophilic compounds (Fernandes *et al.*, 2005). The Haa1 regulon includes the H^+ antiporters Tpo2p and Tpo3p, which couple export of organic acids to the proton motive force (Sá-Correia & Tenreiro, 2002). Deletion of *TPO2* leads to increased accumulation of intracellular acetate (Fernandes *et al.*, 2005). A recent study confirmed the involvement of the *S. cerevisiae* Haa1 regulon, but, interestingly, not of the Tpo2p and Tpo3p transporters, in lactic acid tolerance (Abbott *et al.*, 2008).

In addition to export of organic acids, their entry into yeast cells can be modulated to combat toxicity. Transient activation of the Hog1p MAP kinase in response to acetic acid stress leads to phosphorylation of the plasma membrane aquaglyceroporin Fps1p. Endocytosis of Fps1p then decreases influx of acetic acid via diffusion through this porin (Mollapour & Piper, 2007; Mollapour *et al.*, 2008). The same or similar mechanisms may apply to other organic acids.

Organic acid toxicity can involve multiple mechanisms other than classical uncoupling. Depending on the efficiency of organic acid export, toxicity mechanisms that act on intracellular targets may be more pronounced when the acids are produced intracellularly than when toxicity is screened by their external addition. Specific acid anions have been shown to inhibit specific intracellular enzyme activities. Krebs *et al.* (1983) showed that phosphofructokinase is inhibited by benzoate and enolase inhibition was demonstrated for acetate (Pampulha & Loureiro-Dias, 1990). A bovine LDH expressed in *S. cerevisiae* was severely inhibited by lactic acid, with obvious implications for high-level lactate production (Branduardi *et al.*, 2006). Metabolomics approaches are likely to be required to systematically elucidate such highly specific enzyme–acid interactions.

A general link between organic acid toxicity and reactive oxygen species (ROS) was first observed when a mutation of *SOD1* (encoding superoxide dismutase) rendered aerobic cultures of *S. cerevisiae* sensitive to organic acids (Piper, 1999). Catalase has also been implicated in organic acid tolerance (Giannattasio *et al.*, 2005; Wei *et al.*, 2008). Two mechanisms may contribute to organic acid-mediated generation of ROS. Firstly, exposure of respiring cultures to organic acids induces an increased rate of respiration, which provides ATP for export of protons and/or organic acids via Pma1p and acid transporters, respectively (Verduyn *et al.*, 1990, 1992; Verduyn, 1991). Increased respiration rates have been linked to increased ROS generation (Tarrío *et al.*, 2008) via electron leakage from the bc_1 complex, resulting in superoxide formation (Gille & Nohl, 2001; Sun & Trumpower, 2003). Secondly, several organic acids stimulate iron-mediated generation of hydroxyl radicals via the Fenton reaction (Ali & Konishi, 1998; Ali *et al.*, 2000). Lactate tolerance was recently shown to be improved in a yeast strain engineered for production of ascorbate, a well-known scavenger of ROS (Branduardi *et al.*, 2007). Benzoate and acetate have also been implicated in inhibition of autophagy (Hazan *et al.*, 2004) and induction of apoptosis (Ludovico *et al.*, 2001), respectively. It is as yet unclear whether acetate-induced apoptosis is an indirect consequence of ROS formation.

Conclusions and outlook

Research conducted over the past decade has demonstrated the potential of *S. cerevisiae* for organic acid production at low pH. The inherent characteristics of tolerance to low pH and simple nutritional requirements provide an economical advantage over similar processes with less robust prokaryotic organisms. Three completely different (potential) benefits discriminate *S. cerevisiae* from the naturally organic acid-producing filamentous fungi. Firstly, *S. cerevisiae* has the benefit of its generally recognized as safe status, allowing for faster governmental approval and public acceptance. Secondly, its simple morphology makes *S. cerevisiae* very suitable for large-scale fermentation. Finally, the well-established metabolic-engineering toolbox for *S. cerevisiae* allows for comparatively easy genetic manipulation.

Although carbon has been successfully rerouted from ethanol to the various acids described in this review, it is necessary to increase rates, titers and, in some cases, yields, before industrial processes based on *S. cerevisiae* become economically feasible. In addition to screening culture collections, access to biodiversity via metagenomic strategies is a promising approach to screen for improved enzymes and/or transport proteins. Further modification of these proteins by targeted or random approaches in combination with high-throughput selection methods may also prove useful for improving enzyme kinetics and specificity.

Engineering efficient transport systems, allowing high productivity and product tolerance, is crucial to optimize production rates and titers. Although the metabolic pathways leading to product formation may be ATP neutral

(e.g. previously described malate production) or may even result in net ATP formation (e.g. lactate production), energy requirements for active transport of the acid anion and/or the dissociated proton ultimately result in an overall process that is either ATP neutral or, even worse, requires a net energy input. Even in nongrowing cultures, viable cell populations cannot be sustained when ATP formation is insufficient to drive the ongoing process of cellular maintenance. Consequently, optimization of the most efficient transport systems that operate within the restrictions of the production system, with conditions that allow for cellular maintenance and (limited) growth, is crucial for successful engineering of biocatalysts for production of organic acids.

Despite the rapidly growing body of information on genome-wide regulation in response to organic acids (Schüller *et al.*, 2004; Abbott *et al.*, 2007, 2008) and on organic acid tolerance of whole-genome mutant libraries (Mollapour *et al.*, 2004; Schüller *et al.*, 2004), the complexity of organic acid tolerance continues to present a daunting challenge for rational metabolic engineering aimed at reaching high product titers. Classical strain improvement and application of evolutionary engineering will therefore continue to play a key role in improvement of this incompletely understood and likely multigenic aspect of yeast physiology. Modification of components of global transcription machinery provides a new, promising approach for enhancing stress tolerance. For example, transcriptional engineering resulted in increased ethanol tolerance and production in *S. cerevisiae* (Alper *et al.*, 2006) and *E. coli* (Alper & Stephanopoulos, 2007). Similarly, screening of σ factor mutants in *Lactobacillus plantarum* resulted in increased lactic acid tolerance and productivity (Klein-Marcuschamer & Stephanopoulos, 2008). However, just as with classical methods of strain improvement, these techniques often do not contribute to our understanding of stress responses as the underlying changes that are responsible for the phenotype are not easily identified.

Despite the substantial progress that has been made in metabolic engineering of *S. cerevisiae* for production of organic acids, many problems remain to be solved. However, the ever-growing need to replace petrochemically derived chemicals demands that academic and industrial research in this field continues. Clearly, as a workhorse of industrial microbiology, *S. cerevisiae* will continue to contribute to the development of a more sustainable, environment-friendly industrialized society.

Acknowledgements

The PhD research of D.A.A. and R.M.Z. is financed by Tate & Lyle Ingredients Americas Inc. The research group of J.T.P. is part of the Kluyver Center for Genomics of Industrial Fermentation and is supported by the Netherlands Genomics Initiative. We thank Stefan de Kok for discussions on transport and energetics and critical reading of the manuscript.

References

Abbott DA, Knijnenburg TA, de Poorter LMI, Reinders MJT, Pronk JT & van Maris AJA (2007) Generic and specific transcriptional responses to different weak organic acids in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7**: 819–833.

Abbott DA, Suir E, van Maris AJA & Pronk JT (2008) Physiological and transcriptional responses to high concentrations of lactic acid in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microb* **74**: 5759–5768.

Abbott DA, van den Brink J, Minneboo IMK, Pronk JT & van Maris AJA (2009) Anaerobic homolactate fermentation with *Saccharomyces cerevisiae* results in depletion of ATP and impaired metabolic activity. *FEMS Yeast Res* **9**: 349–357.

Abe S, Furuya A, Saito T & Takayama K (1962) Method of producing L-malic acid by fermentation. US Patent 3063910.

Adachi E, Torigoe M, Sugiyama M, Nikawa JI & Shimizu K (1998) Modification of metabolic pathways of *Saccharomyces cerevisiae* by the expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH value. *J Ferment Bioeng* **86**: 284–289.

Ali MA & Konishi T (1998) Enhancement of hydroxyl radical generation in the Fenton reaction by alpha-hydroxy acid. *Biochem Mol Biol Int* **46**: 137–145.

Ali MA, Yasui F, Matsugo S & Konishi T (2000) The lactate-dependent enhancement of hydroxyl radical generation by the Fenton reaction. *Free Radical Res* **32**: 429–438.

Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G & Gorwa-Grauslund MF (2007) Increased tolerance and conversion on inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J Chem Technol Biot* **82**: 340–349.

Alper H & Stephanopoulos G (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. *Metab Eng* **9**: 258–267.

Alper H, Moxley J, Nevoigt E, Fink GR & Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science* **314**: 1565–1568.

Ansanay V, Dequin S, Camarasa C, Schaeffer V, Grivet JP, Blondin B, Salmon J-M & Barre P (1998) Malolactic fermentation by engineered *Saccharomyces cerevisiae* as compared with engineered *Schizosaccharomyces pombe*. *Yeast* **12**: 215–225.

Arikawa Y, Kobayashi M, Kodaira R, Shimosaka M, Muratsubaki H, Enomoto K & Okazaki M (1999) Isolation of sake yeast strains possessing various levels of succinate- and/or malate-producing abilities by gene disruption or mutation. *J Biosci Bioeng* **87**: 333–339.

Bailey JE (1991) Toward a science of metabolic engineering. *Science* **252**: 1668–1675.

Battat E, Peleg Y, Bercovitz A, Rokem JS & Goldberg I (1991) Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermenter. *Biotechnol Bioeng* **37**: 1108–1116.

Benninga H A (1990) *The History of Lactic Acid Making*. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Blomqvist K, Suihko ML, Knowles J & Penttilä M (1991) Chromosomal integration and expression of two bacterial α-acetolactate decarboxylase genes in brewer's yeast. *Appl Environ Microb* **57**: 2796–2803.

Boles E, Bill R, Gustafsson L, Hohmann S, Larsson C & Otterstedt K (2001) Recombinant *Saccharomyces cerevisiae* expressing chimeric glucose transporters. Patent WO02/00880.

Branduardi P, Sauer M, De Gioia L, Zampella G, Valli M, Mattanovich D & Porro D (2006) Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. *Microb Cell Fact* **5**: 4–15.

Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D & Porro D (2007) Biosynthesis of vitamin C by yeast leads to increased stress resistance. *PLoS One* **2**: e1092.

Branduardi P, Smeraldi C & Porro D (2008) Metabolically engineered yeasts: 'potential' industrial applications. *J Mol Microb Biotech* **15**: 31–40.

BREW (2006) Medium and long-term opportunities and risks of the biotechnological production of bulk chemicals from renewable resources – final report. Prepared under the European Commissions GROWTH program, Utrecht, September 2006.

Brewster NK, Val DL, Walker ME & Wallace JC (1994) Regulation of pyruvate carboxylase isozyme (*PYC1, PYC2*) gene expression in *Saccharomyces cerevisiae* during fermentative and nonfermentative growth. *Arch Biochem Biophys* **311**: 62–71.

Camarasa C, Bidard F, Bony M, Barre P & Dequin S (2001) Characterization of *Schizosaccharomyces pombe* malate permease by expression in *Saccharomyces cerevisiae*. *Appl Environ Microb* **67**: 4144–4151.

Canonaco F, Schlattner U, Pruett PS, Wallimann T & Sauer U (2002) Functional expression of phosphagen kinase systems confers resistance to transient stresses in *Saccharomyces cerevisiae* by buffering the ATP pool. *J Biol Chem* **277**: 31303–31309.

Casal M, Paiva S, Queirós O & Soares-Silva I (2008) Transport of carboxylic acids in yeasts. *FEMS Microbiol Rev* **32**: 974–994.

Chopin A (1993) Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiol Rev* **12**: 21–38.

Chu BCH & Lee H (2007) Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnol Adv* **25**: 425–441.

Datta R, Tsai SP, Bonsignore P, Moon SH & Frank JR (1995) Technological and economic potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiol Rev* **16**: 221–231.

Davies CR, Wibowo W, Eschenbruch R, Lee TH & Fleet GH (1985) Practical implications of malolactic fermentation: a review. *Am J Enol Viticult* **36**: 290–301.

de Jong-Gubbels P, Bauer J, Niederberger P, Stückrath I, Kötter P, van Dijken JP & Pronk JT (1998) Physiological characterisation of a pyruvate-carboxylase-negative *Saccharomyces cerevisiae* mutant in batch and chemostat cultures. *Antonie van Leeuwenhoek* **74**: 253–263.

Dequin S & Barre P (1994) Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. *Biotechnology* **12**: 173–177.

Diderich JA, Raamsdonk LM, Kruckeberg AL, Berden JA & van Dam K (2001) Physiological properties of *Saccharomyces cerevisiae* from which hexokinase II has been deleted. *Appl Environ Microb* **67**: 1587–1593.

Do AH & Choi DK (2007) Aflatoxins: detection, toxicity and biosynthesis. *Biotechnol Bioproc E* **12**: 585–593.

Donalies UEB, Nguyen HTT, Stahl U & Nevoigt E (2008) Improvement of *Saccharomyces* yeast strains used in brewing, wine making and baking. *Adv Biochem Eng Biot* **111**: 67–98.

Drewke C, Thielen J & Ciriacy M (1990) Ethanol formation in *adh0* mutants reveals the existence of a novel acetaldehyde-reducing activity in *Saccharomyces cerevisiae*. *J Bacteriol* **172**: 3909–3917.

Eraso P & Gancedo C (1987) Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Lett* **224**: 187–192.

Fatichenti F, Farris GA, Deiana P & Ceccarelli S (1984) Malic acid production and consumption by selected of *Saccharomyces cerevisiae* under anaerobic and aerobic conditions. *Appl Microbiol Biot* **19**: 427–429.

Fernandes AR, Mira NP, Vargas RC, Canelhas I & Sá-Correia I (2005) *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. *Biochem Bioph Res Co* **337**: 95–103.

Flikweert MT, van der Zanden L, Janssen WMTM, Steensma HY, Van Dijken JP & Pronk JT (1996) Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. *Yeast* **12**: 247–257.

Flikweert MT, van Dijken JP & Pronk JT (1997) Metabolic responses of pyruvate decarboxylase-negative *Saccharomyces cerevisiae* to glucose excess. *Appl Environ Microb* **63**: 3399–3404.

Flikweert MT, de Swaaf M, van Dijken JP & Pronk JT (1999) Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **174**: 73–79.

Gárdonyi M & Hahn-Hägerdal B (2003) The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. *Enzyme Microb Tech* **32**: 252–259.

Giannattasio S, Guaragnella N, Corte-Real M, Passarella S & Marra E (2005) Acid stress adaptation protects *Saccharomyces cerevisiae* from acetic acid-induced programmed cell death. *Gene* **354**: 93–98.

Gille L & Nohl H (2001) The ubiquinol/bc_1 redox couple regulates mitochondrial oxygen radical formation. *Arch Biochem Biophys* **388**: 34–38.

Gregori C, Bauer B, Schwartz C, Kren A, Schüller C & Kuchler K (2007) A genetic screen identifies mutations in the yeast *WAR1* gene, linking transcription factor phosphorylation to weak-acid stress adaptation. *Eur J Biochem* **274**: 3094–3107.

Guijarro JM & Lagunas R (1984) *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. *J Bacteriol* **160**: 874–878.

Hähn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I & Gorwa-Grauslund M (2007) Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biot* **74**: 937–953.

Hazan R, Levine A & Abeliovich H (2004) Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in *Saccharomyces cerevisiae*. *Appl Environ Microb* **70**: 4449–4457.

Hesseltine CW, Shotwell OL, Ellis JJ & Stubblefield RD (1966) Aflatoxin formation by *Aspergillus flavus*. *Microbiol Mol Biol R* **30**: 795–805.

Hohmann S (1991) Characterization of PDC6, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *J Bacteriol* **173**: 7963–7969.

Holyoak CD, Stratford M, McMullin Z, Cole MB, Crimmins K, Brown AJ & Coote PJ (1996) Activity of the plasma membrane H^+-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. *Appl Environ Microb* **62**: 3158–3164.

Holyoak CD, Bracey D, Piper PW, Kuchler K & Coote PJ (1999) The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. *J Bacteriol* **181**: 4644–4652.

Husnik JI, Volschenk H, Bauer J, Colavizza D, Luo Z & van Vuuren HJJ (2006) Metabolic engineering of malolactic wine yeast. *Metab Eng* **8**: 315–323.

Ishida N, Saitoh S, Ohnishi T, Tokuhiro K, Nagamori E, Kitamoto K & Takahashi H (2006a) Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of pure l-(+)-lactic acid. *Appl Biochem Biotech* **129–132**: 795–807.

Ishida N, Suzuki T, Tokuhiro K, Nagamori E, Onishi T, Saitoh S, Kitamoto K & Takahashi H (2006b) D-Lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. *J Biosci Bioeng* **101**: 172–177.

Jantama K, Haupt MJ, Svoronos SA, Zhang XL, Moore JC, Shanmugam KT & Ingram LO (2008a) Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. *Biotechnol Bioeng* **99**: 1140–1153.

Jantama K, Zhang XL, Moore JC, Shanmugam KT, Svoronos SA & Ingram LO (2008b) Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. *Biotechnol Bioeng* **101**: 881–893.

Klein CJL, Rasmussen JJ, Rønnow B, Olsson L & Nielsen J (1999) Investigation of the impact of *MIG1* and *MIG2* on the physiology of *Saccharomyces cerevisiae*. *J Biotechnol* **68**: 197–212.

Klein-Marcuschamer D & Stephanopoulos G (2008) Assessing the potential of mutational strategies to elicit new phenotypes in industrial strains. *P Natl Acad Sci USA* **105**: 2319–2324.

Krebs HA, Wiggins D, Stubbs M, Sols A & Bedoya F (1983) Studies on the mechanism of the antifungal action of benzoate. *Biochem J* **214**: 657–663.

Kren A, Mamnun YM, Bauer BE, Schüller C, Wolfger H, Hatzixanthis K, Mollapour M, Gregori C, Piper P & Kuchler K (2003) War1p, a novel transcription factor controlling weak acid stress response in yeast. *Mol Cell Biol* **23**: 1775–1785.

Liu C L & Lievense J C (2005) Lactic acid producing yeast. Patent WO2005052174.

Ludovico P, Sousa MJ, Silva MT, Leão C & Côrte-Real M (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology* **147**: 2409–2415.

Martins AM, Cordeiro CA & Ponces Freire AM (2001) *In situ* analysis of methylglyoxal metabolism in *Saccharomyces cerevisiae*. *FEBS Lett* **499**: 41–44.

McAlister-Henn L, Steffan JS, Minard KI & Anderson SL (1995) Expression and function of a mislocalized form of peroxisomal malate dehydrogenase (MDH3) in yeast. *J Biol Chem* **270**: 21220–21225.

McKinlay J, Vieille C & Zeikus J (2007) Prospects for a bio-based succinate industry. *Appl Microbiol Biot* **76**: 727–740.

Minard KI & McAlister-Henn L (1992) Glucose-induced degradation of the MDH2 isozyme of malate dehydrogenase in yeast. *J Biol Chem* **267**: 17458–17464.

Mollapour M & Piper PW (2007) Hog1 MAP kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol* **27**: 6446–6456.

Mollapour M, Fong D, Balakrishnan K, Harris N, Thompson S, Schüller C, Kuchler K & Piper P (2004) Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. *Yeast* **21**: 927–946.

Mollapour M, Shepherd A & Piper PW (2008) Novel stress responses facilitate *Saccharomyces cerevisiae* growth in the presence of the monocarboxylate preservatives. *Yeast* **25**: 169–177.

Nevoigt E (2008) Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol Mol Biol R* **72**: 379–412.

Ostergaard S, Olsson L & Nielsen J (2000) Metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol Mol Biol R* **64**: 34–50.

Özcan S & Johnston M (1999) Function and regulation of yeast hexose transporters. *Microbiol Mol Biol R* **63**: 554–569.

Pampulha ME & Loureiro-Dias MC (1989) Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Appl Microbiol Biot* **31**: 547–550.

Pampulha ME & Loureiro-Dias MC (1990) Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in the presence of acetic acid. *Appl Microbiol Biot* **34**: 375–380.

Papagianni M (2007) Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnol Adv* **25**: 244–263.

Patil K, Rocha I, Förster J & Nielsen J (2005) Evolutionary programming as a platform for *in silico* metabolic engineering. *BMC Bioinformatics* **6**: 308–319.

Peleg Y, Stieglitz B & Goldberg I (1988) Malic acid accumulation by *Aspergillus flavus*. *Appl Microbiol Biot* **28**: 69–75.

Pines O, Even-Ram S, Elnathan N, Battat E, Aharonov O, Gibson D & Goldberg I (1996) The cytosolic pathway of L-malic acid synthesis in *Saccharomyces cerevisiae*: the role of fumarase. *Appl Microbiol Biot* **46**: 393–399.

Pines O, Shemesh S, Battat E & Goldberg I (1997) Overexpression of cytosolic malate dehydrogenase (MDH2) causes overproduction of specific organic acids in *Saccharomyces cerevisiae*. *Appl Microbiol Biot* **48**: 248–255.

Piper P (1999) Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. *Free Radical Bio Med* **27**: 1219–1227.

Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P & Kuchler K (1998) The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J* **17**: 4257–4265.

Piper P, Calderon CO, Hatzixanthis K & Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147**: 2635–2642.

Porro D, Brambilla L, Ranzi BM, Martegani E & Alberghina L (1995) Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. *Biotechnol Progr* **11**: 294–298.

Porro D, Bianchi MM, Brambilla L *et al.* (1999) Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. *Appl Environ Microb* **65**: 4211–4215.

Porro D, Sauer M, Branduardi P & Mattanovich D (2005) Recombinant protein production in yeasts. *Mol Biotechnol* **31**: 245–259.

Postma E, Verduyn C, Scheffers WA & Van Dijken JP (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microb* **55**: 468–477.

Pronk JT, Steensma HY & van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**: 1607–1633.

Rapoport TA (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature* **450**: 663–669.

Ro DK, Paradise EM, Ouellet M *et al.* (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**: 940–943.

Russell AD (1991) Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. *J Appl Bacteriol* **71**: 191–201.

Sá-Correia I & Tenreiro S (2002) The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. *J Biotechnol* **98**: 215–226.

Saitoh S, Ishida N, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K & Takahashi H (2005) Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. *Appl Environ Microb* **71**: 2789–2792.

Salmond CV, Kroll RG & Booth IR (1984) The effects of food preservatives on pH homeostasis in *Escherichia coli*. *J Gen Microbiol* **130**: 2845–2850.

Sauer M, Porro D, Mattanovich D & Branduardi P (2008) Microbial production of organic acids: expanding the markets. *Trends Biotechnol* **26**: 100–108.

Sauer U (2001) Evolutionary engineering of industrially important microbial phenotypes. *Advances in Biochemical Engineering/Biotechnology* (Scheper Th., ed), pp. 129–169. Springer-Verlag, Heidelberg.

Sauer U & Eikmanns BJ (2006) The PEP–pyruvate–oxaloacetate node as the switch point for carbon flux distribution in bacteria. *FEMS Microbiol Rev* **29**: 765–794.

Schüller C, Mamnun YM, Mollapour M, Krapf G, Schuster M, Bauer BE, Piper PW & Kuchler K (2004) Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. *Mol Biol Cell* **15**: 706–720.

Schwartz H & Radler F (1988) Formation of l (-) malate by *Saccharomyces cerevisiae* during fermentation. *Appl Microbiol Biot* **27**: 553–560.

Singh SK, Ahmed SU & Pandey A (2006) Metabolic engineering approaches for lactic acid production. *Process Biochem* **41**: 991–1000.

Skory C (2003) Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. *J Ind Microbiol Biot* **30**: 22–27.

Song H & Lee SY (2006) Production of succinic acid by bacterial fermentation. *Enzyme Microb Tech* **39**: 352–361.

Stucka R, Dequin S, Salmon JM & Gancedo C (1991) DNA sequences in chromosomes 11 and VII code for pyruvate carboxylase isoenzymes in *Saccharomyces cerevisiae*: analysis of pyruvate carboxylase-deficient strains. *Mol Gen Genet* **229**: 307–315.

Sun J & Trumpower BL (2003) Superoxide anion generation by the cytochrome bc_1 complex. *Arch Biochem Biophys* **419**: 198–206.

Szczebara FM, Chandelier C, Villeret C *et al.* (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat Biotechnol* **21**: 143–149.

Tanghe A, Van Dijck P, Dumortier F, Teunissen A, Hohmann S & Thevelein JM (2002) Aquaporin expression correlates with freeze tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains. *Appl Environ Microb* **68**: 5981–5989.

Tarrío N, García-Leiro A, Cerdan ME & González-Siso MI (2008) The role of glutathione reductase in the interplay between

oxidative stress response and turnover of cytosolic NADPH in *Kluyveromyces lactis*. *FEMS Yeast Res* **8**: 597–606.

Vaidya AN, Pandey RA, Mudliar S, Suresh Kumar M, Chakrabarti T & Devotta S (2005) Production and recovery of lactic acid for polylactide – an overview. *Crit Rev Env Sci Tec* **35**: 429–467.

van den Berg MA, de Jong-Gubbels P & Steensma HY (1998) Transient mRNA responses in chemostat cultures as a method of defining putative regulatory elements: application to genes involved in *Saccharomyces cerevisiae* acetyl-coenzyme A metabolism. *Yeast* **14**: 1089–1104.

van Dijken JP & Scheffers WA (1986) Redox balances in the metabolism of sugars by yeast. *FEMS Microbiol Rev* **32**: 199–224.

van Maris AJA, Luttik MAH, Winkler AA, van Dijken JP & Pronk JT (2003) Overproduction of threonine aldolase circumvents the biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microb* **69**: 2094–2099.

van Maris AJA, Konings WN, van Dijken JP & Pronk JT (2004a) Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. *Metab Eng* **6**: 245–255.

van Maris AJA, Winkler AA, Porro D, van Dijken JP & Pronk JT (2004b) Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. *Appl Environ Microb* **70**: 2898–2905.

van Maris AJA, Geertman JM, Vermeulen A, Groothuizen MK, Winkler AA, Piper MDW, van Dijken JP & Pronk JT (2004c) Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C_2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. *Appl Environ Microb* **70**: 159–166.

van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, Wisselink HW, Scheffers WA, van Dijken JP & Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie van Leeuwenhoek* **90**: 391–418.

Verduyn C (1991) Physiology of yeasts in relation to biomass yields. *Antonie van Leeuwenhoek* **60**: 325–353.

Verduyn C, Zomerdijk TPL, van Dijken JP & Scheffers WA (1984) Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. *Appl Microbiol Biot* **19**: 181–185.

Verduyn C, Postma E, Scheffers WA & van Dijken JP (1990) Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J Gen Microbiol* **136**: 405–412.

Verduyn C, Postma E, Scheffers WA & van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeast: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**: 501–517.

Viegas CA & Sá-Correia I (1991) Activation of the plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid. *J Gen Microbiol* **137**: 645–651.

Vinci V I & Byng G S (1999) *Strain Improvement by Non-Recombinant Methods*. Manual of Industrial Microbiology and Biotechnology, American Society for Microbiology, Washington, DC.

Volschenk H, Viljoen M, Grobler J, Petzold B, Bauer F, Subden RE, Young RA, Lonvaud A, Denayrolles M & van Vuuren HJJ (1997) Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. *Nat Biotechnol* **15**: 253–257.

Volschenk H, van Vuuren HJJ & Viljoen Bloom M (2003) Malo-ethanolic fermentation in *Saccharomyces* and *Schizosaccharomyces*. *Curr Genet* **43**: 379–391.

Walker GM (1998) Yeast Technology. *Yeast Physiology and Biotechnology*, pp. 265–320. John Wiley & Sons Ltd, Chichester, UK.

Warnecke T & Gill RT (2005) Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. *Microb Cell Fact* **4**: 25–32.

Wei P, Li Z, He P, Lin Y & Jiang N (2008) Genome shuffling in the ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. *Biotechnol Appl Bioc* **49**: 113–120.

Werpy T & Petersen G (2004) *Top Value Added Chemicals from Biomass: Volume I – Results of Screening for Potential Candidates from Sugars and Synthesis Gas*. US Department of Commerce, Springfield, VA. Available at http://dx.doi.org/10.2172/15008859.

Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, Geertman JM, van Dijken JP, Pronk JT & van Maris AJA (2008) Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Appl Environ Microb* **74**: 2766–2777.