Engineering NADH metabolism in *Saccharomyces cerevisiae*: formate as an electron donor for glycerol production by anaerobic, glucose-limited chemostat cultures

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Received 16 February 2006; accepted 17 March 2006.
First published online 17 August 2006.

DOI:10.1111/j.1567-1364.2006.00124.x

Editor: Barbara M. Bakker

Keywords
anaerobic formate metabolism; glycerol; formate dehydrogenase; metabolic engineering; mixed substrate utilization; NADH metabolism.

Abstract

Anaerobic *Saccharomyces cerevisiae* cultures reoxidize the excess NADH formed in biosynthesis via glycerol production. This study investigates whether cometabolism of formate, a well-known NADH-generating substrate in aerobic cultures, can increase glycerol production in anaerobic *S. cerevisiae* cultures. In anaerobic, glucose-limited chemostat sultures ($D = 0.10\ h^{-1}$) with molar formate-to-glucose ratios of 0 to 0.5, only a small fraction of the formate added to the cultures was consumed. To investigate whether incomplete formate consumption was by the unfavourable kinetics of yeast formate dehydrogenase (high k_M for formate at low intracellular NAD^+ concentrations) strains were constructed in which the *FDH1* and/or *GPD2* genes, encoding formate dehydrogenase and glycerol-3-phosphate dehydrogenase, respectively, were overexpressed. The engineered strains consumed up to 70% of the formate added to the feed, thereby increasing glycerol yields to 0.3 mol mol^{-1} glucose at a formate-to-glucose ratio of 0.34. In all strains tested, the molar ratio between formate consumption and additional glycerol production relative to a reference culture equalled one. While demonstrating that that format can be use to enhance glycerol yields in anaerobic *S. cerevisiae* cultures, This study also reveals kinetic constraints of yeast formate dehydrogenase as an NADH-generating system in yeast mediated reduction processes.

Introduction

In the yeast *Saccharomyces cerevisiae*, glycerol is formed from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) in two reactions. DHAP is first reduced to glycerol-3-phosphate (G3P) by two isozymes of NADH-linked glycerol-3-phosphate dehydrogenase (GPD) (EC 1.1.1.8), encoded by the *GPD1* and *GPD2* genes (Gancedo *et al.*, 1968; Ansell *et al.*, 1997). Subsequently, G3P is dephosphorylated by two isozymes of glycerol-3-phosphatases (EC 3.1.3.21), encoded by the *GPP1* and *GPP2* genes (Tsuboi & Hudson, 1956; Bakker *et al.*, 2001).

Although currently not applied on an industrial scale, glycerol production by *S. cerevisiae* continues to attract interest (for a review, see Taherzadeh *et al.*, 2002). The classical sulfite process (Neuberg & Reinfurth, 1919), in which trapping of acetaldehyde results in an excess of NADH that must be reoxidized by the conversion of sugar into glycerol, has now been surpassed by metabolic engineering strategies. The highest glycerol yields on glucose hitherto achieved with *S. cerevisiae* have been obtained with triosephosphate isomerase-negative strains that contain additional modifications in mitochondrial NADH metabolism (Overkamp *et al.*, 2002a). A drawback of this metabolic engineering strategy, as well as of the classical sulfite process, is that the maximum carbon conversion of glucose into glycerol is limited to 50% by the stoichiometries of carbon and redox metabolism.

An alternative approach to steer glucose metabolism towards glycerol production might be the provision of an additional substrate that provides the NADH needed for DHAP reduction. Although, in batch cultures, the simultaneous utilization of substrates may be prevented by catabolite repression and/or kinetic interactions, substrate-limited cultivation generally allows for the simultaneous utilization of different carbon and energy sources by microorganisms (Babel *et al.*, 1993; de Jong-Gubbels *et al.*, 1995; Müller & Babel, 1996; Berrios-Rivera *et al.*, 2002; Egli & Zinn, 2003; Kaup *et al.*, 2003; Zinn *et al.*, 2004).

Co-utilization of formic acid and sugars by yeasts represents an interesting case of mixed substrate utilization. In aerobic cultures of yeasts, formate cannot serve as a carbon

source, but it can provide redox equivalents for respiratory dissimilation (van Dijken *et al.*, 1981; Hazeu & Donker, 1983; Babel *et al.*, 1993). In *S. cerevisiae* CEN.PK113-7D, two isozymes of NAD^+-linked formate dehydrogenase (FDH) (EC 1.2.1.2), encoded by the *FDH1* and *FDH2* genes, are induced on cultivation in the presence of formate (Overkamp *et al.*, 2002b). Hitherto, formate utilization by glucose-grown yeast cultures has only been investigated under aerobic conditions. In aerobic, glucose-limited cultures, co-utilization of formate replaces redox equivalents derived from glucose dissimilation to enable respiration. At optimal formate-to-glucose ratios, this results in a scenario in which glucose is used for carbon assimilation alone (Bruinenberg *et al.*, 1985; Gommers *et al.*, 1988; Verduyn, 1991).

Formate is a popular electron donor for cofactor regeneration in enzyme catalysis, because CO_2, the product of the FDH reaction, does not accumulate in solution (Wandrey, 2004). A similar role of FDH can be envisaged in whole-cell biotransformation processes (Fig. 1). Indeed, it has been demonstrated that formate oxidation by glucose-grown anaerobic cultures of *Escherichia coli* leads to a shift towards fermentation products that are more reduced than glucose (Neuberg & Reinfurth, 1919; Holzer *et al.*, 1963; Berrios-Rivera *et al.*, 2002). Furthermore, formate has been used as an electron donor for the production of mannitol by engineered *E. coli* strains (Kaup *et al.*, 2003).

The aim of the present study was to evaluate whether glycerol production by anaerobic, glucose-limited cultures of *S. cerevisiae* could be enhanced by the addition of formate as an additional electron donor. To this end, formate consumption and glycerol production were studied in anaerobic chemostat cultures of the reference strain *S. cerevisiae* CEN.PK113-7D, as well as in strains with increased expression levels of the *FDH1* and *GPD2* genes.

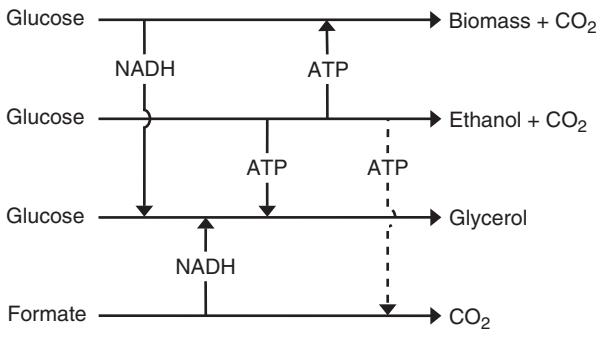

Fig. 1. Schematic representation of the predicted metabolism of carbon, NADH and ATP in anaerobic cultures of *Saccharomyces cerevisiae* grown on glucose–formate mixtures. The dotted line indicates the possible input of free energy for formate transport. Note that formate can provide redox equivalents, but cannot act as a carbon source for biomass formation.

Materials and methods

Construction and maintenance of yeast strains

The *Saccharomyces cerevisiae* strains used in this study (Table 1) originate from the CEN.PK strain family, which has been identified as a suitable genetic background for combined genetic/physiological studies (van Dijken *et al.*, 2000). Strains were grown to the stationary phase in shake-flask cultures on mineral medium supplemented with vitamins (Verduyn *et al.*, 1992), which was set to pH 6.0 using potassium hydroxide and contained 2% (w/v) glucose. After the addition of sterile glycerol (20% v/v), 2-mL aliquots were stored in sterile vials at −80 °C. These frozen stock cultures were used to inoculate precultures for batch and chemostat experiments.

The plasmids used in this study are listed in Table 2. In order to construct YEplac195P_{TPI}, the *CYC1* terminator was amplified using primers 5′-AA*CTGCAG*TAAACAGGCCCCTTTTCC-3′ and 5′-GAGAGTGTAAACTGCGAAGC-3′. The resulting fragment was digested with *Pst*I (introduced, italic) and *Hin*dIII and cloned into YEplac195 (Gietz & Sugino, 1988) cut with *Pst*I and *Hin*dIII, resulting in YEplac195T_{CYC}. Next, the *TPI1* promoter was amplified using primers 5′-G*GAATTC*AGGACGTTATGAAGAAGAGA-3′ and 5′-GG*GGTACC*TGTGTTTTTTGTAGTTATAGAT-3′. The fragment was cut at the introduced *Eco*RI and *Kpn*I sites (italic) and ligated to YEplac195T_{CYC} cut with *Eco*RI–*Kpn*I, resulting in YEplac195P_{TPI}. To obtain the *GPD2* overexpression plasmid YEplac195P_{TPI}-GPD2, the *GPD2* gene was first amplified by PCR from genomic DNA of CEN.PK113-13D (*MAT*α *ura3-52*) using two primers, GC*TCTAGA***ATG**CTTGCTGTCAGAAGATTA and TGA*CTCGAG*GAGTGT**CTA**TTCGTCATCGA. These primers introduced two restriction sites (italic): an *Xba*I site at the 5′ end and an *Xho*I site at the 3′ end of the PCR fragment. The resulting fragment was isolated from gel, digested with *Xba*I and *Xho*I, and ligated into YEplac195P_{TPI} digested with *Xba*I and *Sal*I. PCR was performed using $Vent^R$ DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer's specifications and in a Biometra TGradient Thermocycler (Biometra, Göttingen, Germany) with the following settings: 30 cycles of 1 min of annealing at 60 °C, 3 min of extension at 75 °C and 1 min of denaturation at 94 °C. Restriction endonucleases (New England Biolabs and Roche, Basel, Switzerland) and DNA ligase (Roche) were used according to the manufacturers' specifications. Plasmid isolation from *Escherichia coli* was performed with the Qiaprep spin miniprep kit (Qiagen, Hilden, Germany). DNA fragments were separated on a 1% agarose (Sigma, St Louis, MO) gel (Sambrook *et al.*, 1989). Isolation of fragments from gel was carried out with the Qiaquick gel extraction kit (Qiagen). Plasmids were amplified in *E. coli*

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

Strain	Relevant genotype	Source/reference
CEN.PK113-7D	*MATa MAL2-8^C SUC2*	(Wild-type reference strain)
FDH1 overexpression	*MATa trp1 MAL2-8^C SUC2* containing pRUL158	This study
GPD2 overexpression	*MATα ura3 MAL2-8^C SUC2* containing YEplac195P_{TPI}-GPD2	This study
GPP1 overexpression	*MATα ura3 MAL2-8^C SUC2* containing YEplac195P_{TPI}-GPP1	This study
FDH1/*GPD2* overexpression	*MATα ura3 trp1 MAL2-8^C SUC2* containing pRUL158 and Yeplac195P_{TPI}-GPD2	This study

FDH, formate dehydrogenase; GPD, glycerol-3-phosphate dehydrogenase; GPP, glycerol-3-phosphatase.

Table 2. Plasmids used in this study

Plasmid	Description	Source/reference
pRUL158	YEplac112 containing native *Saccharomyces cerevisiae FDH1*	H.Y. Steensma (Leiden University, The Netherlands) (van den Berg & Steensma, 1997)
Yeplac195P_{TPI}-GPD2	YEplac195 containing p_{TPI} (promoter sequence) fused to *GPD2* gene at ATG codon	This study
Yeplac195P_{TPI}-GPP1	YEplac195 containing p_{TPI} (promoter sequence) fused to *GPP1* at ATG codon	This study

FDH, formate dehydrogenase; GPD, glycerol-3-phosphate dehydrogenase; GPP, glycerol-3-phosphatase.

strain XL-1 blue (Stratagene, La Jolla, CA). Transformation was performed according to Inoue *et al.* (1990). *Escherichia coli* was grown on Luria–Bertani (LB) plates or in liquid Terrific Broth (TB) medium for the isolation of plasmids (Sambrook *et al.*, 1989). Transformation of *S. cerevisiae* with plasmids was performed following the lithium acetate method as described by Gietz & Woods (2002).

Chemostat cultivation

Anaerobic chemostat cultivations were carried out in 2 L laboratory fermenters (Applikon, Schiedam, The Netherlands) thermostatically controlled at 30 °C and with a stirrer speed of 800 r.p.m. The working volume was kept at 1 L by a Masterflex® peristaltic effluent pump (Barrington, IL) coupled to an electric level sensor. As a compromise between high undissociated-acid availability ($pK_a = 3.75$), increased transmembrane ΔpH for higher diffusion, and the detrimental effect of growth at a low pH on the biomass yield, continuous cultivations were carried out at pH 4.0. The pH was kept constant at 4.0 by an Applikon ADI 1030 biocontroller via automatic addition of 2- M potassium hydroxide. Defined anaerobic continuous culture media were prepared as described previously (Verduyn *et al.*, 1992), containing 25 g L^{-1} glucose as the carbon source. When used, formate was aseptically added to the media as concentrated acid. To ensure anaerobicity, the fermenter was sparged with nitrogen (≥99.995% v/v) at a flow rate of 0.5 L min^{-1} and the medium vessel was sparged with nitrogen (≥99.995% v/v) at a flow rate of 0.1 L min^{-1} using Brooks 5876 mass-flow controllers. To minimize oxygen diffusion, the fermenter system was fitted with Norprene® tubing (Saint-Gobain Performance Plastics, Charny, France) and Viton® O-rings (Eriks, Alkmaar, The Netherlands). The dissolved-oxygen tension was continuously monitored with an oxygen electrode (Ingold, model 34 100 3002, Mettler, Utrecht, The Netherlands), and was below the detection limit throughout the fermentation. The dilution rate (in steady-state cultures equal to the specific growth rate) was set to 0.1 h^{-1}. Steady state was defined as the situation in which at least five volume changes had passed since the last change in culture parameters, and in which the biomass concentration, as well as all other specific production or consumption rates, had remained constant (< 2% variation) for at least two volume changes. Steady-state cultures did not exhibit detectable metabolic oscillations. Chemostat cultures were routinely checked for purity by phase-contrast microscopy.

Culture dry weight determination

Culture samples (20 mL) were passed through preweighed nitrocellulose filters (pore size 0.45 μm; Pall, Ann Arbor, MI) and washed twice with an equal volume of demineralized water. Filters were then dried in a microwave oven at 360 W for 20 min. The increase in filter weight was measured. Duplicate determinations varied by <1%. Biomass dry weight concentrations of samples taken directly from the culture differed by <1% from those of samples taken from the effluent line (Postma *et al.*, 1989).

Gas analysis

Fermenter exhaust gas was cooled in a condenser (2 °C) and dried with a Perma Pure Dryer (model MD 110-48P-4; Inacom Instruments, Veenendaal, The Netherlands) prior to

the analysis of oxygen and carbon dioxide concentrations with a Rosemount NGA 2000 analyser (Baar, Switzerland). The exact gas flow rate was determined using a Saga digital flow meter (Ion Science, Cambridge, UK). Carbon dioxide production rates were determined as described previously (van Urk *et al.*, 1988).

Substrate and metabolite analysis

Culture supernatants were analysed by high-performance liquid chromatography (HPLC) mounted with a dual-wavelength absorbance detector (Waters 2487, Milford, MA) and a refractive-index detector (Waters 2410). An Aminex HPX-87H (Biorad, Hercules, CA) column was used and eluted with sulphuric acid (5 mM; 0.6 mL min^{-1}) at 60 °C. Glycerol and formate concentrations were confirmed by enzymatic analysis. Carbon recoveries were only $95 \pm 2\%$. This is probably because no correction was made for ethanol evaporation.

Enzyme assays

Cell extracts were prepared according to Luttik *et al.* (2000) and used to determine enzyme activities with a Hitachi model 100-60 spectrophotometer (Tokyo, Japan) at 30 °C. FDH activity was assayed as described by Overkamp *et al.* (2002b), in which NADH production was measured at 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$). For the purpose of k_M determination, formate concentrations ranged from 1.25 to 100 mM and NAD^+ concentrations varied from 0.04 to 2 mM. For GPD (EC 1.1.1.8) and glycerol-3-phosphatase (EC 3.1.3.21) activity determination, cell extracts were prepared as described above, except that the phosphate buffer was replaced by triethanolamine buffer (10 mM, pH 7.5) (Albertyn *et al.*, 1992) for washing and sonication. GPD activities were assayed according to Blomberg & Adler (1989) by measuring the consumption of NADH at 340 nm. Glycerol-3-phosphatase activity was assayed off-line as described by Norbeck *et al.* (1996); the released inorganic phosphate concentration was measured according to Ames (1966) using a spectrophotometer (Amersham Pharmacia Novaspec II, Freiburg, Germany). Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951).

Results and discussion

Theoretical evaluation of formate-driven glycerol production

During the anaerobic growth of *Saccharomyces cerevisiae* on glucose, biomass synthesis and the excretion of oxidized metabolites lead to a net production of NADH (van Dijken & Scheffers, 1986). To maintain a redox balance, glycerol is produced. For a theoretical prediction of the impact of formate consumption on the stoichiometry of growth and product formation by anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D, we used published yields of biomass, glycerol, ethanol and carbon dioxide obtained with this strain at a dilution rate of 0.10 h^{-1} (Aguilera *et al.*, 2005; Tai *et al.*, 2005). On the basis of these measured yields, the anaerobic growth stoichiometry of this strain is described by the following equation:

$$58\ \text{mmol glucose} \rightarrow 1\ \text{g biomass} + 88\ \text{mmol ethanol} + 100\ \text{mmol}\ CO_2 + 7.7\ \text{mmol glycerol} \quad (1)$$

In this scenario, 6.8% of the glucose carbon is recovered as glycerol (Eq. 1). For each mole of glycerol produced from glucose, 1 mol of ATP is consumed. This ATP consumption is balanced by redox-neutral alcoholic fermentation of glucose, which yields 2 mol of ATP, 2 mol of CO_2 and 2 mol of ethanol per mole of glucose. Thus, in anaerobic cultures of *S. cerevisiae*, 13.6% of the total glucose carbon consumed is devoted to redox balancing of the $NADH/NAD^+$ couple (Verduyn *et al.*, 1990). The limited ATP yield from alcoholic fermentation and the need to balance ATP consumption and production together restrict the theoretical maximum glycerol yield in anaerobic cultures to 1 mol glycerol · $(\text{mol glucose})^{-1}$, even if the availability of NADH were to be increased. When anaerobic cultures oxidize formate via an NAD^+-dependent FDH, the additional NADH formed has to be balanced by glycerol formation

$$1\ \text{glucose} + 1\ \text{formate} \rightarrow 1\ \text{glycerol} + 1\ \text{ethanol} + 2CO_2 \quad (2)$$

Although, especially at low pH values, passive diffusion may contribute to formate uptake, energy-dependent, carrier-mediated formate transport has been reported to occur in *S. cerevisiae* (Cássio *et al.*, 1987; Casal *et al.*, 1996; Overkamp *et al.*, 2002b). If formate is transported via proton symport (Cássio *et al.*, 1987; Casal *et al.*, 1996), the expulsion of the cotransported proton via the plasma membrane ATPase will cost one ATP molecule per molecule of formate (recently reviewed by van Maris *et al.*, 2004). In that case, additional ethanol must be produced to allow for the oxidation of formate

$$1.5\ \text{glucose} + 1\ \text{formate} \rightarrow 1\ \text{glycerol} + 2\ \text{ethanol} + 3CO_2 \quad (3)$$

It follows from Eqs. (1) and (2) that, on oxidation of n mmol of formate, the overall stoichiometric equation for glycerol formation in the absence of active formate uptake is described by the following equation:

$$(58 + n)\ \text{glucose} + n\ \text{formate} \rightarrow 1\ \text{g biomass} + (88 + n)\ \text{ethanol} + (100 + 2n)\ CO_2 + (7.7 + n)\ \text{glycerol} \quad (4)$$

Similarly, when formate uptake requires one ATP equivalent, combination of Eqs. (1) and (3) yields Eq. (5):

$$(58 + 1.5n)\ \text{glucose} + n\ \text{formate} \rightarrow 1\ \text{g biomass} + (88 + 2n)\ \text{ethanol} + (100 + 3n)\ CO_2 + (7.7 + n)\ \text{glycerol} \quad (5)$$

Theoretically, this implies that, in the absence of energy-dependent formate uptake, the glycerol yield on glucose in anaerobic, glucose-limited chemostat cultures will steadily increase from 0.13 to 1.0 mol glycerol · (mol glucose)$^{-1}$ as the formate-to-glucose ratio increases from zero to unity (Fig. 2a). However, this situation cannot be attained in practice. To reach the theoretical maximum glycerol yield [1.0 mol glycerol · (mol glucose)$^{-1}$], the biomass yield has to decrease to zero (Fig. 2a). Even at suboptimal formate-to-glucose ratios, the reduced biomass yields necessitate extremely high specific rates of glucose consumption and alcoholic fermentation (Fig. 2b). The maximum specific rates of glucose consumption in exponentially growing batch cultures of this strain are *c.* 15 mmol g^{-1} h^{-1} (van Hoek *et al.*, 2000), which corresponds to a maximum formate-to-glucose ratio of *c.* 0.6 (Fig. 2b).

When Eq. (5) is used to calculate the maximum yield of glycerol, it becomes clear that the maximum glycerol yield, as well as the maximum formate-to-glucose ratio, that can be attained in the cultures will be severely limited when formate is exclusively transported via an energy-dependent transport mechanism (Fig. 2).

This theoretical analysis illustrates that, in attempts to enhance glycerol formation by formate addition, kinetic as well as stoichiometric constraints need to be taken into account. Moreover, it illustrates that the energetics of formate uptake may have a strong impact on maximum conversion yields.

Formate oxidation by anaerobic chemostat cultures of *Saccharomyces cerevisiae*

The theoretical predictions of anaerobic formate oxidation were experimentally verified by growing the reference strain *S. cerevisiae* CEN.PK113-7D in anaerobic, glucose-limited chemostat cultures (dilution rate 0.10 h^{-1}). At a constant glucose concentration in the feed of 25 g L^{-1}, steady-state cultures were established at concentrations of 0–61.8 mM formate in the feed (representing molar formate-to-glucose ratios of 0–0.46 mol mol^{-1}). A further increase in the formate concentration in the feed reproducibly led to washout of the cultures, probably as a result of weak-acid uncoupling by residual formate (Verduyn *et al.*, 1992).

In the anaerobic chemostat cultures, formate was incompletely consumed (Fig. 3a). At a formate concentration in the feed of up to 20 mM (representing a formate-to-glucose ratio of 0.14 mol mol^{-1}), *c.* 50% of the formate fed to the cultures was consumed. This decreased to *c.* 15% at the highest formate-to-glucose ratio tested. When the specific rate of glycerol production by the cultures was plotted as a function of the actual specific rates of formate consumption, a linear relationship was observed (Fig. 3b) with a slope of 1.0 mol glycerol produced per mole of formate consumed. This confirmed that the cytosolic NADH formed during anaerobic formate oxidation was indeed quantitatively re-oxidized by glycerol formation. At very low formate-to-glucose ratios, formate consumption did not lead to a linear increase in glycerol production rates (Fig. 3b). This may reflect changes in biomass composition and/or redox metabolism at low formate consumption rates.

In view of the incomplete oxidation of formate in the anaerobic chemostat cultures, a comparison with the theoretical analysis was based on consumed formate rather than on the formate provided in the reservoir medium.

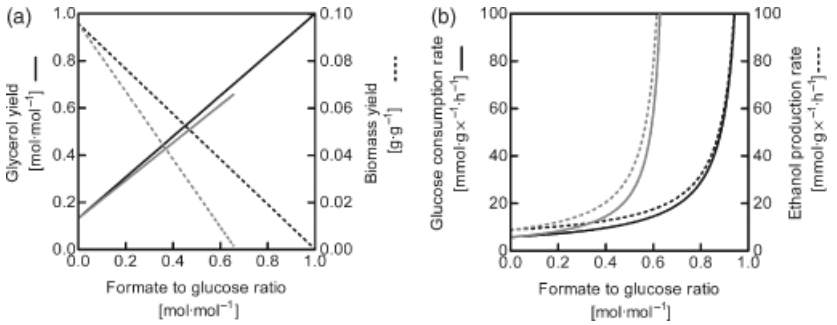

Fig. 2. (a) Glycerol (full line) and biomass (broken line) yields on glucose. (b) Biomass-specific glucose consumption (full line) and ethanol production (broken line) rates. Theoretical values were calculated according to Eqs (4) and (5), as a function of the formate-to-glucose ratio at a fixed dilution rate ($D = 0.1\ h^{-1}$), taking ATP loss resulting from glycerol formation into account. Two possible situations for formate transport were evaluated: no free-energy input (black) and one ATP per translocated formate (grey).

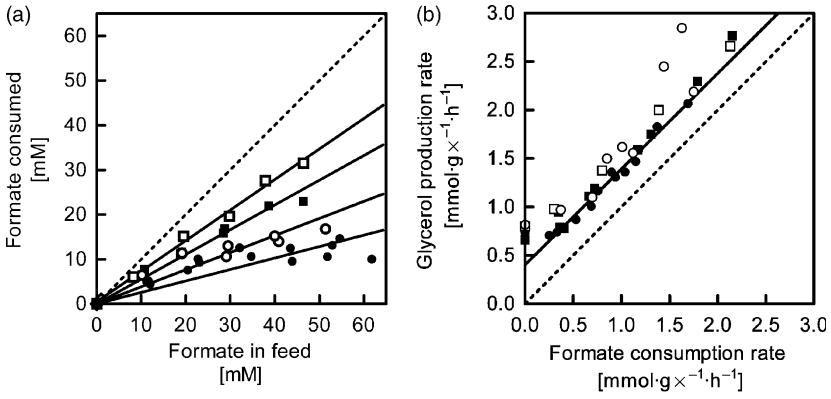

Fig. 3. (a) Formate consumption in anaerobic chemostat cultures ($D = 0.1\ h^{-1}$), as a function of the formate concentration in the feed, for Gpd2p (○), Fdh1p (■) and combined Fdh1p and Gpd2p (□) overexpression strains, in comparison with the CEN.PK113-7D reference strain (●). (b) Equimolar formate consumption and glycerol production in all strains. Each data point is derived from an independent chemostat culture.

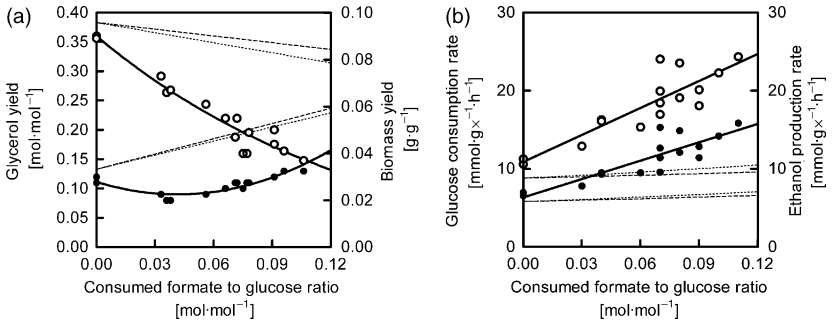

Fig. 4. (a) Glycerol (●) and biomass (○) yields on glucose of the wild-type strain, as a function of the formate-to-glucose ratio, in comparison with theoretical glycerol and biomass yields on glucose. (b) Biomass-specific glucose consumption (●) and ethanol production (○) rates, as a function of the formate-to-glucose ratio, in comparison with the specific glucose consumption and ethanol production rates. Each data point is derived from an independent chemostat culture. Theoretical values were calculated according to Eqs (4) and (5) at a fixed dilution rate ($D = 0.1\ h^{-1}$). Taking ATP loss resulting from glycerol formation into account, and assuming no free-energy input for formate transport (broken line) or assuming one ATP per formate transported (dotted line).

Compared with the theoretical prediction, the biomass yield decreased much more strongly with increasing formate-to-glucose ratios than expected on the basis of the model prediction, even when formate uptake was assumed to require one ATP equivalent (Fig. 4a). The strong decrease in the biomass yield was accompanied by a strong increase in the specific rate of ethanol production (Fig. 4b), indicating that the consumption of formate was accompanied by an enhanced ATP requirement that could not be explained by the ATP requirement for glycerol production or formate uptake alone. As a result of the decreased biomass yield, biosynthesis-associated glycerol production decreased. As a consequence, no substantial increase in the glycerol yield on glucose was observed (Fig. 4a).

At pH 4.0 used for these experiments, over half of the formate ($pK_a = 3.75$) present in the medium will be in the undissociated form. Therefore, the observed decrease in the biomass yield (Fig. 4a) and the increased rate of alcoholic fermentation (Fig. 3a) are likely to be caused by classical weak-acid uncoupling of the plasma membrane pH gradient (Verduyn *et al.*, 1992). The resulting acidification of the cytosol must be balanced by an enhancement of the rate of proton export by the plasma membrane ATPase, which, in turn, requires strongly increased rates of alcoholic fermentation.

In vivo and *in vitro* kinetics of formate oxidation

In a previous study, the addition of formate to aerobic glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D was reported (Overkamp *et al.*, 2002b). In aerobic cultures, formate was quantitatively (>99%) oxidized even at a molar formate-to-glucose ratio of 2.5. This is in sharp contrast with the formate consumption under anaerobic conditions, in which approximately 85% of the formate remained unused at a formate-to-glucose ratio in the medium feed of only 0.46 mol mol^{-1} (Fig. 3a).

A clear positive correlation was observed between the specific rate of formate consumption and the residual formate concentration in the anaerobic chemostat cultures (Fig. 5a). This increased rate of formate consumption could not be attributed to an induction of FDH synthesis as, with the exception of the cultures grown without formate in the feed, FDH activities were similar at all formate-to-glucose ratios.

In principle, the poor *in vivo* kinetics of formate consumption in the anaerobic cultures might be caused by formate transport. However, in view of the uncoupling effect of formate, such a limitation of formate uptake is highly unlikely, which implies that formic acid readily enters the cells. To assess whether, instead, formate oxidation by the anaerobic cultures might be limited by the *in vivo* kinetics of FDH, we first analysed the *in vitro* kinetic properties of the enzyme in cell extracts.

Under standard assay conditions (see 'Materials and methods'), cell extracts of anaerobic chemostat cultures fed with formate exhibited an FDH activity of *c.* 0.1 μmol (mg protein)$^{-1}$ min^{-1} (Table 3). Although threefold lower than the FDH activities found in aerobic cultures (Table 3), this activity should be sufficient for complete formate oxidation, provided that the *in vitro* assay conditions mimic the *in vivo* situation. Yeast FDHs are known to have high k_M values for formate (3–40 mM) (Blanchard & Cleland, 1980; Popov & Lamzin, 1994; Serov *et al.*, 2002). The enzyme activity assays were performed at saturating substrate concentrations at pH 7.0. Decreasing intracellular pH values (e.g. as a result of uncoupling by excess formate) may negatively affect enzyme activity as yeast FDHs exhibit rather high pH optima (Blanchard & Cleland, 1980). In addition, FDH catalyses a two-substrate reaction. Therefore, not only the formate concentration, but also the cytosolic concentration of NAD^+ may affect enzyme activity. Kinetic analysis of FDH activity in cell extracts demonstrated that the k_M value for formate was strongly dependent on the NAD^+ concentration. A fivefold increase in the k_M value for formate (up to 26 mM) was observed when the NAD^+ concentration in the assay was decreased from 2.0 to 0.04 mM (Fig. 5b). The convergent lines in Lineweaver–Burk plots measured at different NAD^+ concentrations (Fig. 5c) confirmed that FDH from *S. cerevisiae* obeyed sequential bi-bi two-substrate kinetics (Peacock & Boulter, 1970; Blanchard & Cleland, 1980; Popov & Lamzin, 1994; Tishkov & Popov, 2004).

The *in vivo* and *in vitro* kinetics of formate oxidation by anaerobic cultures of *S. cerevisiae* are consistent with a scenario in which low intracellular NAD^+ concentrations negatively affect the *in vivo* k_M of FDH for formate. The superior *in vivo* kinetics of aerobic cultures could then be attributed to their higher NAD^+/NADH ratios than in

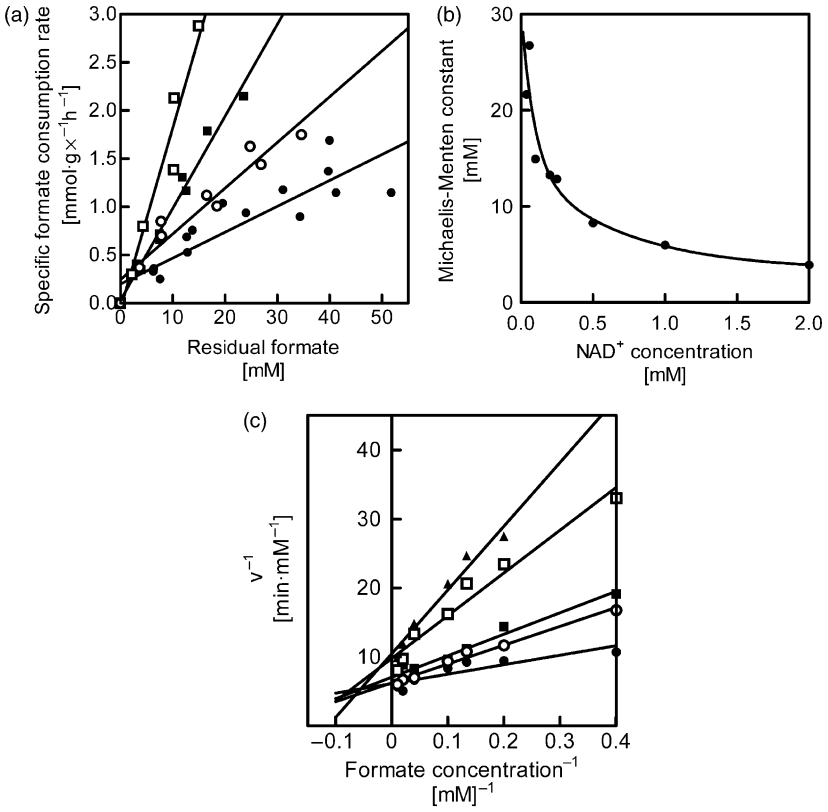

Fig. 5. (a) Specific rate of formate consumption, as a function of residual formate, for the Gpd2p (○), Fdh1p (■) and combined Fdh1p and Gpd2p (?) overexpression strains, in comparison with the CEN.PK113-7D reference strain (●). (b) Michaelis–Menten constant (k_M) of formate dehydrogenase for formate in cell-free extract as a function of NAD^+ concentration. (c) Lineweaver–Burk plot of formate dehydrogenase in cell-free extract as a function of formate concentration at varying NAD^+ concentrations [2 mM (●), 1 mM (○), 0.5 mM (■), 0.2 mM (□) and 0.1 mM (▲)], showing bi-bi kinetics.

Table 3. Growth parameters and average formate dehydrogenase (FDH) and glycerol-3-phosphate dehydrogenase (GPD) activities, with mean deviations, of aerobic and anaerobic, glucose-limited chemostat cultures at $D = 0.1\ h^{-1}$ of *Saccharomyces cerevisiae* CEN.PK113-7D wild-type and overexpression mutants

Strain number	Formate in feed (mM)	Residual formate (mM)	Y_{XS} [g_x (g glucose)$^{-1}$]	$q_{formate}$ (mmol g_x^{-1} h^{-1})	FDH [μmol (mg protein)$^{-1}$ min^{-1}]	GPD [μmol (mg protein)$^{-1}$ min^{-1}]
Aerobic carbon-limited cultures						
CEN.PK113-7D	0	0	0.48	0	0.03 (±0.00)	ND
CEN.PK113-7D	23.1	0.2	0.50	0.6	0.28 (±0.02)	ND
CEN.PK113-7D	45.8	0.2	0.52	1.2	0.30 (±0.02)	ND
Anaerobic carbon-limited cultures						
CEN.PK113-7D	0	0	0.09	0	0.00 (±0.00)	ND
CEN.PK113-7D	22.1	13.1	0.06	0.7	0.14 (±0.01)	ND
CEN.PK113-7D	43.7	32.7	0.05	1.0	0.13 (±0.01)	0.03 (±0.00)
FDH1 overexpression	0	0	0.09	0	0.00 (±0.00)	ND
FDH1 overexpression	28.6	12.2	0.06	1.7	0.50 (±0.05)	ND
FDH1 overexpression	46.5	23.5	0.05	2.2	0.49 (±0.06)	ND
GPD2 overexpression	0	0	0.09	0	0.00 (±0.00)	ND
GPD2 overexpression	19.1	7.80	0.05	0.9	0.11 (±0.01)	0.22 (±0.02)
GPD2 overexpression	40.0	24.8	0.04	1.6	0.13 (±0.01)	0.24 (±0.03)
FDH1/GPD2 overexpression	0	0	0.09	0	0.01 (±0.00)	ND
FDH1/GPD2 overexpression	29.8	10.2	0.06	1.4	0.32 (±0.01)	ND
FDH1/GPD2 overexpression	46.5	15.0	0.05	2.9	0.45 (±0.03)	0.23 (±0.02)

Data shown are representative of a series chemostat cultures.
ND, not determined.

anaerobically grown cells (Oura, 1972), which is probably caused by the activity of mitochondrial, respiratory-chain-linked systems for NADH oxidation under aerobic conditions (Bakker *et al.*, 2001). Accurate measurement of free cytosolic concentrations of NAD^+ and NADH is difficult as a result of metabolic compartmentalization (Bakker *et al.*, 2001) and of the binding of substantial amounts of NAD^+ and NADH to highly expressed enzymes, such as glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase (Sies, 1982; Lin & Guarente, 2003). Therefore, it was decided to investigate this hypothesis by modifying the expression level of key enzymes.

Metabolic engineering of anaerobic formate oxidation

To improve *in vivo* anaerobic formate oxidation, two genetic modifications were evaluated. GPD, encoded by the *GPD2* gene, was overexpressed sevenfold to facilitate NADH reoxidation (Table 3). The Gpd2 isozyme was chosen because of its known contribution to redox balancing (Ansell *et al.*, 1997). Indeed, increased GPD activity in the cells resulted in an increased consumption of formate (Fig. 3a) and a stronger increase in the specific rate of formate oxidation relative to increasing residual formate concentrations (Fig. 5a). Overexpression of glycerol-3-phosphatase (Gpp1p) did not result in increased formate oxidation (data not shown). This is consistent with the observation by Remize *et al.* (2001) that glycerol-3-phosphatase is not rate limiting for glycerol formation, in contrast with GPD.

In an attempt to stimulate *in vivo* formate oxidation at suboptimal concentrations of NAD^+ and/or formate, we overexpressed the *FDH1*-encoded FDH isozyme. This isoenzyme is highly homologous to Fdh2p (Overkamp *et al.*, 2002b), which was not tested separately. The *c.* fourfold increase in FDH activity (Table 3) resulted in a substantially higher formate consumption (Fig. 3a) and improved *in vivo* kinetics of formate oxidation (Fig. 5a). In cell extracts of the FDH-overproducing strain, the specific activity of FDH was *c.* 0.5 μmol (mg protein)$^{-1}$ min^{-1} (Table 3). Based on the reported activity of purified *S. cerevisiae* FDH of 10 μmol (mg protein)$^{-1}$ min^{-1}, this means that *c.* 5% of the soluble protein in the overexpressing strain consisted of FDH. The low turnover number (*c.* 6.5 s^{-1}) and high k_M (5.5 mM) of *S. cerevisiae* FDH (Tishkov & Popov, 2004) may be a major problem in attempts to further increase *in vivo* FDH activities.

Simultaneous overexpression of *FDH1* and *GPD2* showed that the two approaches were synergistic. Anaerobic cultures

of a strain overexpressing both genes consumed *c.* 70% of the formate supplied in the feed (Fig. 3a). Similar to the reference strain, the engineered strains exhibited a linear increase in glycerol production rate with increasing formate consumption rate (Fig. 3b).

Biomass and glycerol yields of the combined GPD/FDH-overproducing strain were higher than those of the CEN.PK 113-7D reference strain. At the highest formate-to-glucose ratio observed with the engineered strain, the glycerol yield was 0.28 mol mol^{-1}, which was 2.4-fold higher than the maximum yield observed in the reference strain (Fig. 6a). The engineered strain was not able to grow at a dilution rate of 0.10 h^{-1} at formate-to-glucose ratios in the feed exceeding 0.34 mol mol^{-1}. This may well be a consequence of limited ATP generation, which is dictated by the maximum glycolytic flux, as, under anaerobic conditions, ATP is only generated by glycolysis. The specific rate of glucose consumption (Fig. 6b) observed at the highest formate-to-glucose molar ratio that could be reached in steady-state cultures (12.7 mmol g^{-1} h^{-1}) amounted to *c.* 80% of the maximum specific glucose consumption rate of the reference strain CEN.PK113-7D in batch cultures (15.4 mmol g^{-1} h^{-1}; van Hoek *et al.*, 2000).

Conclusions

The present study demonstrates that the oxidation of formate by anaerobic, glucose-limited *Saccharomyces cerevisiae* cultures leads to an increased glycerol yield on glucose. Formate is not only of interest as a primary electron donor, but is also an intermediate in the linear oxidation pathway of methanol to CO_2 that is widespread in nature (Fujii & Tonomura, 1972; Kato *et al.*, 1979; van Dijken *et al.*, 1981; Arfman *et al.*, 1991), and can yield up to 3 mol of NADH per mole of methanol (Arfman *et al.*, 1991, 1997). Although *S. cerevisiae* harbours NAD$^+$-dependent formaldehyde dehydrogenase (Rose & Racker, 1962) and FDH (Overkamp *et al.*, 2002b), it lacks a methanol dehydrogenase. Thus, efficient use of methanol as an electron donor requires the functional expression of a heterologous NAD$^+$-linked methanol dehydrogenase, as has recently been successfully demonstrated in *Corynebacterium glutamicum* (Takeshita & Yasueda, 2004). Our results show that formate-driven glycerol production is strongly influenced by kinetic constraints. In anaerobic cultures of the reference strain CEN.PK113-7D, these kinetic constraints appear to arise primarily from the *in vivo* kinetics of NAD$^+$-dependent FDH. In particular, the low specific activity and bi-bi kinetics of FDH for NAD$^+$ and formate result in low *in vivo* activities when cytosolic NAD$^+$ concentrations are low. As a result, formate consumption is incomplete, leading to weak-acid uncoupling effects. These kinetic constraints can be partially compensated for by enhancing NAD$^+$/NADH turnover via the overexpression of FDH and NAD$^+$-dependent GPD. However, even in engineered strains, anaerobic formate oxidation remains incomplete. The low turnover number and high k_M of *S. cerevisiae* FDH present a major challenge in further optimization of the *in vivo* kinetics of formate oxidation through metabolic engineering. The kinetic constraints identified in the present paper are relevant for the application of FDH for *in vivo* cofactor generation in other systems that involve a low intracellular NAD$^+$/NADH ratio and, potentially, for cofactor regeneration via other oxidoreductases under such conditions.

Acknowledgements

The PhD research of J.M.A.G. was financed by Tate & Lyle Ingredients Americas, Inc. The research group of J.T.P. is part of the Kluyver Centre for Genomics of Industrial

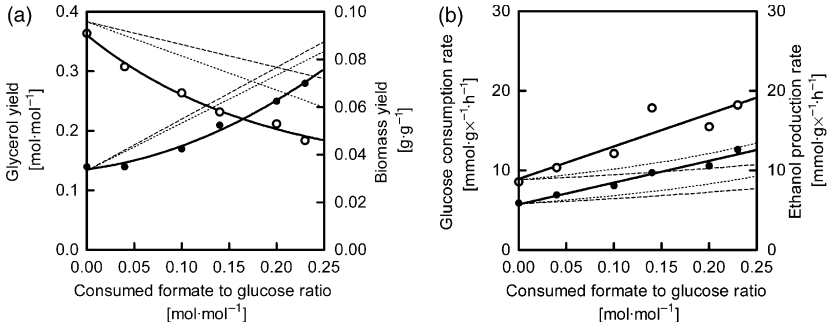

Fig. 6. (a) Glycerol (•) and biomass (○) yields on glucose of the combined Gpd2p and Fdh1p overexpression strain, as a function of the formate-to-glucose ratio, in comparison with theoretical glycerol and biomass yields on glucose. (b) Biomass-specific glucose consumption (•) and ethanol production (○) rates, as a function of the formate-to-glucose ratio, in comparison with the specific glucose consumption and ethanol production rates. Each data point is derived from an independent chemostat culture. Theoretical values were calculated according to Eqs (4) and (5) at a fixed dilution rate ($D = 0.1$ h^{-1}), taking ATP loss resulting from glycerol formation into account, and assuming no free-energy input for formate transport (broken line) or assuming one ATP per formate transported (dotted line).

Fermentation, which is supported by The Netherlands Genomics Initiative. We thank Drs A.A. Winkler, P. Kötter and H.Y. Steensma for their help in strain construction, Dr J.A. Jongejan for advice on the kinetics of formate dehydrogenase, and Dr P. Daran-Lapujade for fruitful discussions. MSc student E.A. Pinedo Vivero and BSc students P. van de Nes, J. Metsaars and P. Velthof contributed to this work as part of their studies.

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