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Optimization of Ethanol Production in *Saccharomyces cerevisiae* by Metabolic Engineering of the Ammonium Assimilation

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Ethanol is still one of the most important products originating from the biotechnological industry with respect to both value and amount. In addition to ethanol, a number of byproducts are formed during an anaerobic fermentation of *Saccharomyces cerevisiae*. One of the most important of these compounds, glycerol, is produced by yeast to reoxidize NADH, formed in synthesis of biomass and secondary fermentation products, to NAD^+. The purpose of this study was to evaluate whether a reduced formation of surplus NADH and an increased consumption of ATP in biosynthesis would result in a decreased glycerol yield and an increased ethanol yield in anaerobic cultivations of *S. cerevisiae*. A yeast strain was constructed in which *GLN1*, encoding glutamine synthetase, and *GLT1*, encoding glutamate synthase, were overexpressed, and *GDH1*, encoding the NADPH-dependent glutamate dehydrogenase, was deleted. Hereby the normal NADPH-consuming synthesis of glutamate from ammonium and 2-oxoglutarate was substituted by a new pathway in which ATP and NADH were consumed. The resulting strain TN19 (*gdh1-Δ1 PGK1p-GLT1 PGK1p-GLN1*) had a 10% higher ethanol yield and a 38% lower glycerol yield compared to the wild type in anaerobic batch fermentations. The maximum specific growth rate of strain TN19 was slightly lower than the wild-type value, but earlier results suggest that this can be circumvented by increasing the specific activities of Gln1p and Glt1p even more. Thus, the results verify the proposed concept of increasing the ethanol yield in *S. cerevisiae* by metabolic engineering of pathways involved in biomass synthesis.

INTRODUCTION

In 1996 the world ethanol production reached an estimated 31.3 billion liters (Berg, 1998). Approximately 80% was produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae*. Thus, ethanol is still one of the most important products originating from the biotechnological industry with respect to both value and amount. Two-thirds of the production is located in Brazil and the United States with the primary objective of using ethanol as a renewable source of liquid fuels. This market is expected to result in a substantial growth in the ethanol production industry in the near future. There are therefore strong economic incentives to further improve the ethanol production process. The price of the sugar source is a very important process parameter in determining the overall economy of ethanol production (Wyman and Hinman, 1990), and it is of great interest to optimize the ethanol yield in order to ensure an efficient utilization of the carbon source. In addition to biomass and carbon dioxide, a number of byproducts are formed during an anaerobic fermentation of *S. cerevisiae* (Oura, 1977). Glycerol is the most important of these compounds, consuming up to 4% of the carbon source in industrial fermentations. Therefore, by eliminating formation of this compound it is potentially possible to increase the ethanol yield by 4%, which in principle would increase the world production of ethanol by 1.25 billion liters a year without any additional costs.

In this paper a new strategy is presented for optimization of the ethanol yield in *S. cerevisiae* through metabolic engineering. It is based on knowledge of the physiological roles of glycerol and ethanol in oxidation of surplus NADH and in formation of ATP, respectively, under anaerobic growth conditions. Furthermore, experimental results from anaerobic batch cultivations of strains developed on the basis of the strategy are presented.

THEORETICAL BACKGROUND

Glycerol formation has two physiological roles in *S. cerevisiae*. Synthesis of biomass and organic acids, i.e., succinic acid, acetic acid, and pyruvic acid, results in a net formation of intracellular NADH (Oura, 1977; van Dijken and Scheffers, 1986; Nissen *et al.*, 1997). This must be balanced by a mechanism in which NADH is reoxidized to NAD^+ in order to avoid a serious imbalance in the NAD^+/NADH ratio. Under anaerobic conditions the respiratory chain is not functioning. Instead, NADH is reoxidized to NAD^+ by formation of glycerol, and synthesis of 1 mol of glycerol from glucose leads to reoxidation of 1 mol of NADH.

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Furthermore, during growth under osmotic stress conditions glycerol is formed and accumulated inside the cell where it works as an efficient osmolyte that protects the cell against lysis (Ansell *et al*., 1997; Larsson *et al*., 1993).

The formation of glycerol occurs in two steps from dihydroxyacetone phosphate, catalyzed by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase. Attempts have been made to increase ethanol formation by elimination of glycerol synthesis through deletions of *GPD1* and *GPD2*, encoding the two existing isoenzymes of glycerol-3-phosphate dehydrogenase (Björkqvist *et al*., 1997). The double-deletion mutant is unable to grow under anaerobic conditions due to accumulation of intracellular NADH since under these growth conditions no alternative pathways exist in *S. cerevisiae* in which NADH is reoxidized to NAD^+. Also elimination of the possibility to form glycerol results in a strain with high sensitivity toward osmotic stress conditions which is ruinous for an industrial process which normally works with a medium with high concentrations of various carbon sources and salts. Deletion of one of the genes does not result in a significant reduction in glycerol formation or an increase in ethanol formation (Björkqvist *et al*., 1997; Nissen *et al*., 1999b). Thus, metabolic engineering of the glycerol synthesis has not yet proven to be a successful way to decrease glycerol production and increase ethanol production in *S. cerevisiae*.

Instead, a strategy was formulated to drain off the surplus formation of NADH by changing the cofactor requirement in amino acid synthesis. Ammonium is often used as nitrogen source in industrial fermentations of *S. cerevisiae*. Following transport across the membrane into the cytoplasm, ammonium is assimilated into glutamate by reaction with 2-oxoglutarate. In wild-type cells this reaction is catalyzed by an NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye *et al*., 1985):

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NADPH} \rightarrow \text{Glutamate} + \text{NADP}^+ \quad (1)$$

Two other glutamate dehydrogenases, encoded by *GDH2* and *GDH3*, are present in *S. cerevisiae*. Gdh2p uses NADH as cofactor but the reaction is normally in the opposite direction of Reaction (1) and NADH is formed (Miller and Magasanik, 1990, 1991; Coschigano *et al*., 1991; Courchesne and Magasanik, 1988). The Gdh2p catalyzed reaction is mainly of importance when other nitrogen sources than ammonium and glutamine are used, and it may be involved in the supply of ammonium needed in synthesis of various amino acids. In normal cells the activity of Gdh2p is 70 times smaller than the activity of Gdh1p when ammonium is used as nitrogen source (Nissen *et al*., 1997). The function of the NADPH-dependent Gdh3p is unknown but it does not function in *Δ gdh1* strains and it is therefore of no interest for practical applications since ammonium is the preferred nitrogen source (Avendanõ *et al*., 1997).

Another system that synthesizes glutamate exists in *S. cerevisiae*. This consists of two coupled reactions, catalyzed by glutamate synthase [Reaction (2)], encoded by *GLT1*, and glutamine synthetase [Reaction (3)], encoded by *GLN1* (Cogoni *et al*., 1995; Mitchell and Magasanik, 1983).

$$\text{2-Oxoglutarate} + \text{Glutamine} + \text{NADH} \rightarrow 2\ \text{Glutamate} + \text{NAD}^+ \quad (2)$$

$$\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i \quad (3)$$

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NADH} + \text{ATP} \rightarrow \text{Glutamate} + \text{NAD}^+ + \text{ADP} + P_i \quad (2+3)$$

In wild-type cells the activity of Glt1p is 80 times lower than the activity of Gdh1p, and the system is therefore likely to be of limited importance in ammonium assimilation in normal cells.

If *GDH1* is deleted in *S. cerevisiae*, the cell becomes unable to assimilate ammonium by the NADPH-dependent glutamate dehydrogenase. By overexpressing *GDH2* or both *GLT1* and *GLN1* in a *Δ gdh1* mutant it should be possible to convert NADH to NAD^+ in the synthesis of glutamate from ammonium and 2-oxoglutarate resulting in a reduced surplus formation of NADH and, thus, a lower glycerol yield. In the latter case, the genetically modified strain probably will have an additional requirement for synthesis of ATP since Reaction (3) requires ATP. Presumably the larger drain of ATP will be compensated by a higher ethanol production.

Earlier the anaerobic physiology in continuous cultivations of *S. cerevisiae* CBS8066 was analyzed by metabolic flux analysis (Nissen *et al*., 1997). It was shown that the flux through the reaction catalyzed by the NADPH-dependent glutamate dehydrogenase was 8.0 C-mmol/g biomass/h at a dilution rate of 0.3 h^{-1}, although the net flux from 2-oxoglutarate to glutamate was only 2.0 C-mmol/g biomass/h. This large difference is due to the role of glutamate as nitrogen donor in a number of biosynthetic reactions. If Glt1p and Gln1p catalyzed the reaction instead of Gdh1p, 1 mol NADH and ATP each would be consumed per mole of glutamate synthesized in the reaction instead of 1 mol NADPH. This would give a reduction in surplus formation of NADH leading to a decrease in formation of glycerol by 49 % from 9.8 to 5.0 C-mmol/g biomass/h. Furthermore, the

Metabolic Engineering **2**, 69–77 (2000)
Article ID mben.1999.0140

extra consumption of ATP would result in a 6% increase in ethanol formation from 54.9 to 58.2 C-mmol/g biomass/h.

MATERIALS AND METHODS

Microorganisms and their maintenance. *Escherichia coli* DH5α (GIBCO BRL, Gaithersburg, MD) was used for subcloning. All yeast strains were maintained at 4°C on selective plates, monthly prepared from a glycerol stock kept at −80°C.

Preparation of DNA. Plasmid DNA from *E. coli* and chromosomal DNA from *S. cerevisiae* were prepared as described earlier (Nissen *et al.*, 1999a). DNA primers were purchased from DNA Technology (Aarhus, Denmark).

Deletion of GDH1. Plasmid pGDH1del was kindly donated by Prof. F. K. Zimmermann (Boles *et al.*, 1993). In pGDH1del a 1.0-kb fragment of *GDH1* has been replaced by a 1.1-kb fragment containing the open reading frame of *URA3*. The construct was treated with *Cla*I and *Pvu*II prior to transformation of yeast to uracil independence in order to obtain one-step gene replacement. Correct deletion of *GDH1* was verified by PCR analysis and by measurements of GDH1p activity in protein extracts from transformants. No NADPH-dependent glutamate dehydrogenase activity could be detected in correct transformants.

Overexpression of GLT1. Primers Glt1start (5′-GCG CGG GAT CCT CTA GAA TGC CAG TGT TGA AAT CAG AC-3′), containing restriction enzyme sites for *Bam*HI and *Xba*I in front of nucleotides 1 to 21 of *GLT1*, and Glt1stop (5′-CGC GCG GAT CCC CGC GGG CTG GAC CAT CCC AAG GTT CC-3′), containing restriction enzyme sites for *Bam*HI and *Sac*II in front of nucleotides 1149 to 1169 of *GLT1*, were used to clone parts of the structural gene of *GLT1* by PCR with the pfu polymerase (New England Biolabs). The fragment was digested with *Bam*HI and ligated into the unique *Bgl*II digestion site of plasmid Yep24-pPGK1 behind the *PGK1* promoter and in front of the *PGK1* terminator (Walfridsson *et al.*, 1997), resulting in plasmid Yep24-pPGK1-GLT1. A 2.5-kb *Sma*I/*Sac*II DNA fragment, consisting of the *PGK1* promoter and the cloned part of *GLT1*, was isolated from Yep24-pPGK1-GLT1. The fragment was ligated into plasmid pFA6A-kanMX3 (Wach *et al.*, 1994), digested with *Eco*RV and *Sac*II, resulting in plasmid pPGK1-GLT1. The plasmid was linearized by digestion with *Eco*RV prior to transformation of yeast to G418 resistance. Correct insertion of the plasmid into the *GLT1* locus on chromosome IV was verified by PCR. For this purpose primers PGK1verif, consisting of nucleotides 420 to 400 upstream of the *PGK1* start codon, and GLT1verif, consisting of nucleotides 1243 to 1260 of *GLT1*, were used. Loop-out of the geniticin resistance gene by homologous recombination of the two direct repeats flanking the gene was obtained by cultivating correct transformants for 100 generations in nonselective YPD medium followed by plating of approximately 50,000 colonies on YPD plates. The colonies were then replica plated to YPD plates containing 150 mg/liter geniticin and transformants without resistance toward geniticin were isolated. It was verified by PCR analysis that the *PGK1* promoter was introduced in front of *GLT1*.

Overexpression of GLN1. Primers Gln1start (5′-GCG CGG GAT CCT CTA GAA TGG CTG AAG CAA GCA TCG AA-3′), containing restriction enzyme sites for *Bam*HI and *Xba*I in front of nucleotides 1 to 21 of *GLN1*, and Gln1stop (5′-CGC GCG GAT CCC CGC GGT TAT GAA GAT TCT CTT TCA AA-3′), containing restriction enzyme sites for *Bam*HI and *Sac*II in front of nucleotides 1093 to 1113 of *GLN1*, were used to clone *GLN1* by PCR with the pfu polymerase (New England Biolabs). The obtained DNA fragment was used to construct plasmid pPGK1-GLN1, containing *GLN1* behind the promoter of *PGK1* inserted into pFA6A-kanMX3, as described above for plasmid pPGK1-GLT1. pPGK1-GLN1 was linearized by digestion with *Kpn*I prior to transformation of yeast to G418 resistance. Correct insertion of the plasmid into the *GLN1* locus on chromosome XVI was verified by PCR analysis. For this purpose primers PGK1verif, consisting of nucleotides 420 to 400 bp upstream of the *PGK1* start codon, and GLN1verif, consisting of nucleotides 52 to 70 downstream of *GLT1*, were used.

Overexpression of GDH2. Primers Gdh2start (5′-GCG CGA GAT CTT CTA GAA TGC TTT TTG ATA ACA AAA AT-3′), containing restriction enzyme sites for *Bgl*II and *Xba*I in front of nucleotides 1 to 21 of *GDH2*, and Gdh2stop (5′-CGC GCA GAT CTC CGC GGA GAG CCT AAA CGA TTA ACA AA-3′), containing restriction enzyme sites for *Bgl*II and *Sac*II in front of nucleotides 1221 to 1201 of *GDH2*, were used to clone parts of the structural gene of *GDH2* by PCR with pfu polymerase (New England Biolabs). The obtained DNA fragment was used to construct plasmid pPGK1-GDH2, containing *GDH2* behind the promoter of *PGK1* inserted into pFA6A-kanMX3, as described above for plasmid pPGK1-GLT1. pPGK1-GDH2 was linearized by digestion with *Tth*III1 prior to transformation of yeast to G418 resistance. Correct insertion of the plasmid into the *GDH2* locus on chromosome IV was verified by PCR analysis. For this purpose primers PGK1verif (5′-GTC ACA CAA CAA GGT CCT A-3′), consisting of nucleotides −420 to −400 upstream of the *PGK1* start codon, and Gdh2verif (5′-GGT TTT CTA CAA

TCT CCA AAA GAG-3′), consisting of nucleotides 1294 to 1271 of the *GDH2* open reading frame, were used.

Transformation of E. coli and S. cerevisiae. *E. coli* DH5α was transformed by electrotransformation using the Bio-Rad electroporation equipment (Bio-Rad Laboratories, Richmond, U.S.A.). Transformants were selected on L broth plates containing 100 mg/ml ampicillin. *S. cerevisiae* cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylene glycol (Schiestl and Gietz., 1989). Transformants were suspended in YPD at 10^9/ml at 30 °C for 24 h prior to plating on YPD, containing 150 mg geniticin/liter, in order to allow expression of the G418 resistance gene.

Medium in the batch cultivations. The strains of *S. cerevisiae* were cultivated in a minimal medium prepared according to Verduyn *et al.* (1990). Vitamins were added by sterile filtration following heat sterilization of the medium. The concentrations of glucose and $(NH_4)_2SO_4$ initially in the batch cultivations were 25 and 3.75 g/liter, respectively. Growth of *S. cerevisiae* under anaerobic conditions requires the supplementary addition to the medium of ergosterol and unsaturated fatty acids, typically in the form of Tween 80 (Andreasen and Stier, 1953). Ergosterol and Tween 80 were dissolved in hot 96% (v/v) ethanol. The final concentrations of ergosterol and Tween 80 were 4.2 and 175 mg/g dry wt, respectively. Antifoam (Sigma A-5551) was added at 75 ml/liter.

Experimental setup for the batch cultivations. Anaerobic batch cultivations were performed at 30°C and at a stirring rate of 600 rpm in in-house-manufactured bioreactors. The working volume of the batch reactors was 4.5 liters. pH was kept constant at 5.00 by addition of 2 M KOH. The bioreactors were continuously sparged with N_2 containing less than 5 ppm O_2, obtained by passing N_2 of a technical quality (AGA 3.8), containing less than 100 ppm O_2, through a column (250×30 mm) filled with copper flakes and heated to 400°C. A mass flow controller (Bronkhorst HiTec F201C) was used to keep the gas flow into the bioreactors constant at 0.50 liters nitrogen/min/liter. Norprene tubing (Cole–Parmer Instruments) was used throughout in order to minimize diffusion of oxygen into the bioreactors. The bioreactors were inoculated to an initial biomass concentration of 1 mg/liter with precultures grown in unbaffled shake flasks at 30°C and 100 rpm for 24 h. Ethanol evaporation from the bioreactors was minimized by off-gas condensers cooled to 2°C. The anaerobic batch cultivations of strains TN1, TN9, TN15, TN17, TN19, and TN22 were each carried out three times with identical results.

Analysis of product formation and determination of dry weight. The content of glucose, ethanol, glycerol, acetic acid, pyruvic acid, and succinic acid in filtered samples withdrawn from the anaerobic batch cultivations was determined by HPLC as described earlier (Nissen *et al.*, 1997). The CO_2 concentration in the off gas was determined using a Brüel & Kjær 1308 acoustic gas analyzer (RSD = 0.02%) (Christensen *et al.*, 1995). The biomass concentration in the medium was measured gravimetrically as described earlier (Nissen *et al.*, 1997).

Measurement of enzyme activities. The total protein pool was extracted from cell samples withdrawn from the batch cultivations as described earlier (Nissen *et al.*, 1997). Glutamate dehydrogenase (NAD^+ and $NADP^+$) (EC 1.4.1.5 and EC 1.4.1.4, respectively) was assayed as described by Bruinenberg *et al.* (1983). Glutamine synthetase (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.1.14) were assayed as described by Holmes*et al.* (1989). Reaction rates, corrected for endogenous rates, were proportional to the amount of extract added. Enzyme activities are expressed as micromoles of substrate converted per minute per milligram of total cellular protein as determined by the Lowry method.

RESULTS

Construction of S. cerevisiae *Strains with an Altered Cofactor Consumption in the Ammonium Assimilation*

In an earlier study, *URA3* was deleted in the haploid wild-type strain *S.* cerevisiae TN1 (*MATα ho-Δ*)), derived from the diploid industrial model strain CBS8066, whereby strain TN2 (*MATα ho-Δ1 ura3-Δ20*::*SUC2*) was obtained (Nissen *et al.*, 1999a). In the present study, *GDH1*, encoding the NADPH-dependent glutamate dehydrogenase, was deleted as described earlier (Boles *et al.*, 1993) in TN2. The resulting strain was named TN9 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3*). To obtain new strains with a stable, constitutive overexpression of *GLN1*, encoding glutamine synthetase, *GLT1*, encoding glutamate synthase, or*GDH2*, encoding the NADH-dependent glutamate dehydrogenase, the strong, constitutive promoter of *PGK1*, encoding phosphoglycerate kinase, was integrated in front of the start codons of the three genes on chromosomes XVI, IV, and IV, respectively. This was achieved by homologous recombination into the three loci of plasmids pPGK1-GLN1, pPGK1-GLT1, and pPGK1-GDH2, respectively (see Materials and Methods). TN9 was transformed with plasmids pPGK1-GLN1, pPGK1-GLT1, and pPGK1-GDH2 after linearization with restriction enzymes *Kpn*I, *Eco*RV, and *Tth*III1, respectively, resulting in strains TN15 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3 gln1*::(*G418^r-PGK1p-GLN1*)), TN17 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3 glt1*::(*G418^r-PGK1p-GLT1*)), and

Metabolic Engineering **2**, 69–77 (2000)
Article ID mben.1999.0140

TN22 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3 gdh2*::(*G418ʳ-PGK1p-GDH2*)), respectively. TN17 was cultivated in nonselective YPD medium for 100 generations in order to remove the gene encoding resistance against geniticin from chromosome IV by homologous recombination of the direct repeats flanking the resistance gene. This resulted in isolation of strain TN18 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3 glt1*::(*PGK1p-GLT1*)) which had lost the geniticin resistance, but maintained the strong promoter in front of *GLT1*. TN18 was transformed with pPGK1-GLN1 as described above, resulting in strain TN19 (*MATα ho-Δ1 ura3-Δ20*::*SUC2 gdh1-Δ1*::*URA3 glt1*::(*PGK1p-GLT1*)*gln1*::(*G418ʳ-PGK1p-GLN1*)) with the *PGK1* promoter inserted in front of both *GLN1* and *GLT1* on chromosomes XVI and IV, respectively.

TABLE 1

The Specific Activities of the NADPH-Dependent and NADH-Dependent Glutamate Dehydrogenases, Glutamine Synthetase, and Glutamate Synthase in Protein Extracts from Biomass Samples Withdrawn in the Exponential Growth Phases of Strains TN1 (Wild Type), TN9 (Δ*gdh1*), TN15 (Δ*gdh1 PGK1p-GLN1*), TN17 (Δ*gdh1 PGK1p-GLT1*), TN19 (Δ*gdh1 PGK1p-GLN1 PGK1p-GLT1*), and TN22 (Δ*gdh1 PGK1p-GDH2*) in Anaerobic Batch Cultivations

	TN1	TN9	TN15	TN17	TN19	TN22
Gdh1p	1.522	None	None	None	None	None
Gdh2p	0.020	0.055	0.045	0.028	0.033	0.625
Gln1p	0.011	0.009	0.068	0.009	0.062	0.009
Glt1p	0.030	0.045	0.045	0.195	0.211	0.029

Anaerobic Batch Cultivations

The anaerobic physiology of the genetically engineered *S. cerevisiae* strains was studied in anaerobic batch cultivations with glucose as carbon source and ammonium as nitrogen source. This was done to quantify the effect of the genetic changes on the specific enzyme activities of Gdh1p, Gdh2p, Glt1p, and Gln1p, the maximum specific growth rate, μ_{max}, and the product yields.

The specific enzyme activities were measured in protein extracts from biomass samples withdrawn from the bioreactors when the cells were in the exponential growth phase (Table 1). The activity of the NADPH-dependent glutamate dehydrogenase in strain TN1 was 50–100 times higher than the other enzymes involved in assimilation of ammonium. This clearly indicated the importance of this enzyme in wild-type cells during growth on ammonium as nitrogen source. No activity of Gdh1p could be detected in extracts from the strains where *GDH1* had been deleted. The activity of the NADH-dependent glutamate dehydrogenase, encoded by *GDH2*, was approximately 2.5 times higher in strain TN9 than observed in the wild type (TN1). It has been shown that a high intracellular level of glutamine results in strong repression of *GDH2* expression at the transcriptional level (Miller and Magasanik, 1991). Thus, the increase in Gdh2p activity in TN9 is interpreted to be a consequence of relief of glutamine repression of *GDH2*. This could be caused by a reduced synthesis rate of glutamate in cells deleted in *GDH1* leading to a lower intracellular concentration of glutamine. A similar increase in Gdh2p activity was observed in strain TN15, indicating that overexpression of the structural gene for glutamine synthetase only resulted in a small increase in the intracellular concentration of glutamine. In strains TN17 and TN19 the Gdh2p activity was reduced to a level close to that observed in TN1, suggesting that an overexpression of glutamate synthase leads to an increase in the intracellular glutamine concentration that represses expression of *GDH2*. A strong induction of the NADH-dependent glutamate dehydrogenase was observed in strain TN22, containing the *PGK1* promoter in front of *GDH2* on chromosome IV. The specific activity of the enzyme in TN22 was 0.625 U/mg total cellular protein (TCP) which was 30-fold higher than measured in the wild type (TN1). Insertion of the strong constitutive promoter of *PGK1* in front of *GLN1* into the chromosome of TN15 and TN19 resulted in an increase in the specific activity of glutamine synthetase from approximately 0.010 to 0.068 and 0.062 units/mg TCP in strains TN1 and TN9, respectively. Insertion of the promoter in front of *GLT1* resulted in a fivefold increase in the activity of glutamate syntase in strains TN17 and TN19 compared with the three other strains.

In Fig. 1, the biomass concentrations during the exponential growth phases of TN1, TN9, TN17, TN19, and TN22 are depicted as functions of time. Deletion of *GDH1* resulted in a reduction in the maximum specific growth rate from 0.41 h^{-1} in strain TN1 to 0.21 h^{-1} in strain TN9. This was probably due to a reduction in the synthesis rate of glutamate since the total specific activities of the enzymes that potentially could substitute the physiological role of Gdh1p, namely Gdh2p and Glt1p, were 15 times lower than the specific activity of Gdh1p in TN1. Overexpression of *GLN1* in TN9, resulting in strain TN15, gave only a small increase in the maximum specific growth rate, to 0.24 h^{-1} (results not shown). The very limited effect of the increase in Gln1p activity was probably due to a slightly higher flux toward synthesis of glutamine. When *GDH1* is deleted, glutamine is a substrate in the formation of glutamate and overexpression of *GLN1* in TN15 probably removes a limitation in the glutamine supply to the reaction catalyzed by Gltp which results in the observed increase in μ_{max}. Overexpression of *GLT1* in TN17 had a significant effect

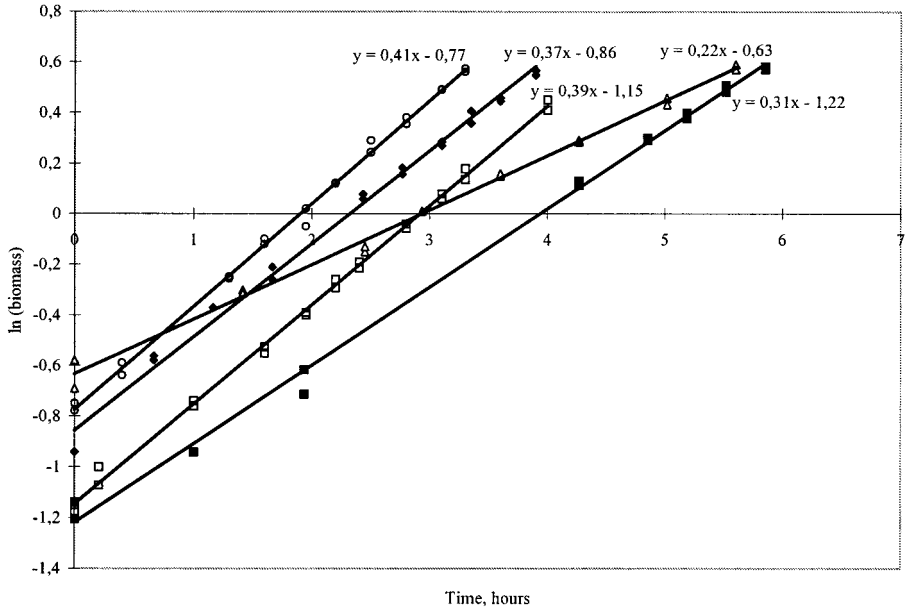

FIG. 1. Semilogarithmic plot of the biomass concentrations of strains TN1 (○), TN9 (△), TN17 (■), TN19 (◆), and TN22 (□) versus time during exponential growth in the anaerobic, glucose-limited batch cultivations. The equations show the slopes and intersections with the second axis of the straight lines through the measured values. The slopes are equal to the maximal specific growth rates of the strains.

on the maximum specific growth rate which increased to 0.31 h^{-1}. This clearly demonstrates that the reduction in the specific growth rate of TN9 is caused by a low synthesis rate of glutamate and that this limitation can be partly removed by constructing a strain with a high specific activity of glutamate synthase. When both *GLT1* and *GLN1* were overexpressed (TN19), there was a further increase in the maximum specific growth rate to 0.37 h^{-1} which is close to that of the wild-type strain (TN1). Thus, the increase in the specific activity of glutamine synthetase had a more pronounced effect on the specific growth rate in a strain where the specific activity of glutamate synthase was high compared to that in a strain with a wild-type level of activity. This indicated that overexpression of *GLT1* alone probably resulted in depletion in the intracellular pool of glutamine which limited the effect of the increase in the specific enzyme activity of Glt1p in TN17. This limitation was apparently removed by overexpression of the structural gene encoding glutamine synthetase. Overexpression of *GDH2* in a *gdh1Δ* background resulted in an even more pronounced effect on the maximum specific growth rate, since a μ_{max} of 0.39 h^{-1} was measured in the anaerobic cultivation of TN22 (Fig. 1). This indicated that the synthesis rate of glutamate was almost identical in TN1 and TN22. As described earlier, the specific enzyme activities of the glutamate producing enzymes in TN19 and TN22 were significantly lower than the activity of Gdh1p in the wild type (Table 1). Thus, if a higher expression level of Glt1p/Gln1p and Gdh2p could be obtained in TN19 and TN22, respectively, it probably would be possible to substitute the physiological role of Gdh1p in the wild type without any reduction in the maximum specific growth rate.

The consumption of glucose and the production of ethanol, glycerol, acetate, pyruvate, succinate, biomass, and carbon dioxide were measured in filtered samples withdrawn from the batch cultivations of TN1, TN9, TN15, TN17, TN19, and TN22. Figure 2 shows the consumption of glucose and the production of ethanol, glycerol, and carbon dioxide as functions of time in the cultivations of strains TN1 (wild type) and TN19 (*gdh1-Δ1 PGK1p-GLT1 PGK1p-GLN1*). In all cultivations formation of ethanol, glycerol, and carbon dioxide stopped immediately after depletion of glucose in the medium. No consumption of the products was detected as long as the bioreactors were sparged with nitrogen, which demonstrated that anaerobic growth conditions had been obtained in the cultivations.

No significant differences were detected in the formation of the individual organic acids between the six strains and, thus, only the total yield of the organic acids is listed in Table 2. The ethanol yield was increased by 8% in the cultivations of TN9 compared with TN1 while the glycerol yield was reduced by 38%. This was probably due to formation of glutamate by the NADH- and ATP-consuming reactions, catalyzed by glutamate synthase and glutamine synthetase, in TN9 in contrast to formation of glutamate by the NADPH-consuming Gdh1p in TN1. As described in the Introduction, the reoxidation of NADH to NAD^+ in glutamate synthesis was expected to result in a lower

Metabolic Engineering **2**, 69–77 (2000)
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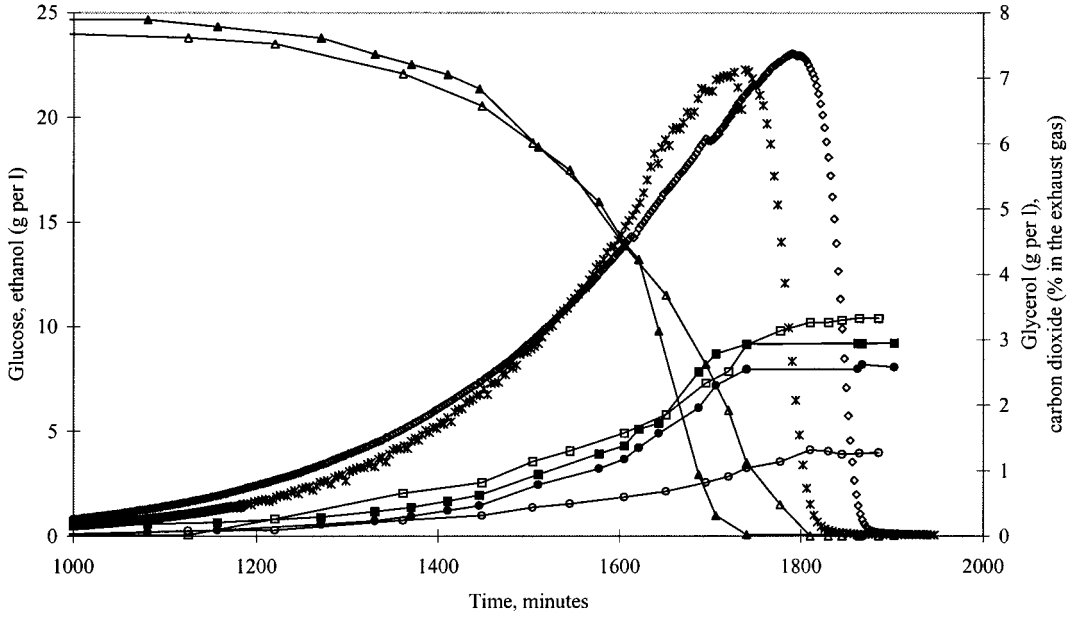

FIG. 2. Consumption of glucose (▲, △) and production of ethanol (■, □), glycerol (●, ○), and carbon dioxide (X, ◇) in anaerobic batch cultivations of strain TN1 (wild type, filled symbols) and strain TN19 (*Δgdh1 PGK1p-GLN1 PGK1p-GLT1*, open symbols). Units: glucose, ethanol, and glycerol, g/liter; carbon dioxide, percentage in the exhaust gas from the bioreactors.

glycerol yield, since one of the physiological roles of this compound in *S. cerevisiae* is to balance the surplus formation of NADH in the synthesis of biomass and secondary metabolites. Similarly, ethanol formation was expected to increase due to the higher ATP requirement in glutamate synthesis, since it is well known that ethanol formation is the primary route for ATP generation in *S. cerevisiae*. The increase in ATP consumption for biomass synthesis (glutamate is an important precursor in biomass synthesis) resulted in a reduction of the biomass yield in TN9 by 6%.

TABLE 2

Product Yields of Strains TN1 (Wild Type), TN9 (Δ*gdh1*), TN15 (Δ*gdh1 PGK1p-GLN1*), TN17 (Δ*gdh1 PGK1p-GLT1*), TN19 (Δ*gdh1 PGK1p-GLN1 PGK1p-GLT1*), and TN22 (Δ*gdh1 PGK1p-GDH2*) in Anaerobic Batch Cultivations

	TN1	TN9	TN15	TN17	TN19	TN22
Ethanol	0.480	0.520	0.521	0.530	0.531	0.495
Glycerol	0.097	0.060	0.059	0.061	0.060	0.067
Biomass	0.121	0.114	0.114	0.104	0.104	0.136
Carbon dioxide	0.261	0.275	0.272	0.271	0.273	0.262
Organic acids	0.009	0.009	0.010	0.009	0.008	0.008
Total	0.968	0.978	0.976	0.975	0.976	0.968

Note. Unit, C-moles product per C-mole glucose.

Overexpression of *GLN1* alone had no significant influence on the product formation of strain TN15 compared with TN9. The fivefold increase in the specific glutamate synthase activity that was obtained by overexpression of *GLT1* in strain TN17 resulted in a small increase in the ethanol yield and a small reduction of the biomass yield. This indicated that the total flux through the reactions catalyzed by Glt1p and Gln1p probably was slightly higher in TN17 compared with TN9 which resulted in an increase in the ATP cost of biomass synthesis. No further decrease in glycerol formation was detected. Thus, the difference in ethanol formation of TN17 and TN9 could be an artifact caused by small errors in the measurement of ethanol. The standard deviation in the ethanol yields obtained in anaerobic cultivations of the same strain was 3–4%. Overexpression of both *GLN1* and *GLT1* in strain TN19 did not result in any changes in product formation compared with TN17.

In the anaerobic cultivations of TN22 (*gdh1-Δ1 PGK1p-GDH2*), the product formation was slightly different than in the cultivations of the other strains in which the cofactor consumption in the ammonium assimilation had been modified. The increase in the ethanol yield was significantly lower than observed in TN9, TN15, TN17, and TN19, which probably was due to the absence of a requirement for ATP in glutamate synthesis in TN22. Instead, a higher biomass yield was measured. This could be due to the lower requirement for NADPH in biomass synthesis, since the formation of this cofactor in the pentose phosphate pathway results in formation of CO_2. In TN22 where less NADPH is

required, this carbon flux toward CO_2 probably is redirected toward biomass formation. This effect on biomass formation was not observed in the cultivations of TN9, TN15, TN17, and TN19, since biomass formation in these strains requires more ATP, whereby the reduction in the carbon flux toward CO_2 in the pentose pathway is redirected toward ethanol instead. The higher biomass yield in the cultivations of TN22 also resulted in a slightly higher glycerol yield due to an increase in the amount of surplus NADH formation in biomass synthesis.

DISCUSSION

The cultivations of TN9 demonstrated that deletion of *GDH1*, encoding the NADPH-dependent glutamate dehydrogenase I, in a wild-type strain of *S. cerevisiae* resulted in an increase in ethanol formation and a decrease in glycerol formation. Unfortunately, the ethanol productivity was significantly affected by the deletion since the maximum specific growth rate of the *Δ gdh1* mutant was approximately half that of the wild type. Simultaneous overexpression of *GLN1*, encoding glutamine synthetase, and *GLT1*, encoding glutamate synthase, in the *Δ gdh1* mutant resulted in strain TN19 with almost the same maximum specific growth rate as the wild type and with a 10% higher ethanol yield. Thus, this new pathway for synthesis of glutamate could substitute the NADPH-dependent glutamate dehydrogenase in conversion of ammonium and 2-oxoglutarate to glutamate. The maximum specific growth rate of strain TN19 was 90% of the maximum specific growth rate of the wild type and consequently the increase in the specific ethanol productivity that was obtained by the genetic manipulations was relatively small. This problem could probably be solved by increasing the specific activities of Gln1p and Glt1p even more. In this study only a 5-fold increase in the specific activity of glutamate synthase was obtained by insertion of the *PGK1* promoter into chromosome IV in front of the structural gene of the enzyme. This level of activity could probably be increased significantly by inserting more copies of the gene into the chromosome. In the cultivations of TN22 where *GDH2*, encoding the NADH-dependent glutamate dehydrogenase, was overexpressed in a *Δ gdh1* background, an increase in the maximal specific growth rate from 0.22 to 0.39 h^{-1} was observed. The specific activity of Gdh2p in this strain was 0.625 units/mg TCP, 10-fold higher than in the wild type, which illustrates that the activity of Glt1p and Gln1p should be increased further in order to achieve the same maximum specific growth rate as the wild type. The small increase in the fermentation time of TN19 compared with TN1 may, however, not be a serious problem in an industrial process. As described in the Introduction, the substrate cost is a primary factor in determining the overall economy of ethanol production, which means that the observed increase in the ethanol yield of TN19 far outweighs a relatively small reduction of the maximum specific growth rate.

Furthermore, the results obtained in this study verified the proposed concept of increasing the ethanol yield in *S. cerevisiae* by metabolic engineering of pathways involved in biomass synthesis. The results from the cultivations of TN22 showed that reduction of glycerol formation through an increase in the reoxidation rate of NADH to NAD^+ is insufficient to obtain a higher flux toward ethanol. The reduction must be combined with a higher consumption of ATP in biomass formation in order to redirect carbon flux from glycerol toward ethanol.

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