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Metabolic engineering to improve ethanol production in *Thermoanaerobacter mathranii*

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Abstract *Thermoanaerobacter mathranii* can produce ethanol from lignocellulosic biomass at high temperatures, but its biotechnological exploitation will require metabolic engineering to increase its ethanol yield. With a cofactor-dependent ethanol production pathway in *T. mathranii*, it may become crucial to regenerate cofactor to increase the ethanol yield. Feeding the cells with a more reduced carbon source, such as mannitol, was shown to increase ethanol yield beyond that obtained with glucose and xylose. The *ldh* gene coding for lactate dehydrogenase was previously deleted from *T. mathranii* to eliminate an NADH oxidation pathway. To further facilitate NADH regeneration used for ethanol formation, a heterologous gene *gldA* encoding an NAD^+-dependent glycerol dehydrogenase was expressed in *T. mathranii.* One of the resulting recombinant strains, *T. mathranii* BG1G1 (*Δldh*, P_{xyl}GldA), showed increased ethanol yield in the presence of glycerol using xylose as a substrate. With an inactivated lactate pathway and expressed glycerol dehydrogenase activity, the metabolism of the cells was shifted toward the production of ethanol over acetate, hence restoring the redox balance. It was also shown that strain BG1G1 acquired the capability to utilize glycerol as an extra carbon source in the presence of xylose, and utilization of the more reduced substrate glycerol resulted in a higher ethanol yield.

Keywords Metabolic engineering · Ethanol production · Thermoanaerobacter · Glycerol dehydrogenase · Lactate dehydrogenase

Introduction

Improvements in the production of ethanol have been focused on fermentation of the carbohydrate fraction from lignocellulosic material. These so-called second-generation technologies require metabolically engineered production strains that possess a high degree of catabolic versatility and that are homoethanologenic. Mesophilic enteric bacteria, e.g., *Escherichia coli* and *Klebsiella oxytoca*, have been engineered to divert carbon flux toward ethanol synthesis (Ingram et al. 1999; Dien et al. 2003). *Saccharomyces cerevisiae* and *Zymomonas mobilis* have been engineered to acquire the metabolic capacity to ferment non-C6 sugars (Pronk et al. 2005; Yanase et al. 2005). Another interesting alternative approach would be to use thermophilic bacteria that are amenable to metabolic engineering, e.g., *Geobacillus thermoglucosidasius*, *Thermoanaerobacter mathranii*, and *Thermoanaerobacterium saccharolyticum* (Taylor et al. 2009).

Thermophiles are commonly able to readily ferment both pentose and hexose sugar fraction of biomass and even, in

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some cases, polymeric precursors or structurally complex polycarbohydrates, such as cellulose and hemicellulose (Zaldivar et al. 2001; Sommer et al. 2004). Cellulosic thermophiles such as *Clostridium thermocellum* and hemicellulolytic thermophiles of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* could be used to hydrolyze and ferment all the sugars in biomass (Shaw et al. 2008b). *T. mathranii* BG1 is such a xylanolytic, thermophilic, anaerobic bacterium with the ability to ferment all the sugars in the biomass at thermophilic temperature, resulting in high productivities and substrate conversion rates, low risk of contamination and facilitated product recovery (Mikkelsen and Ahring 2007). However, the production of high ethanol yields (>90% theoretical) and high ethanol tolerance (>40 g/L) are typically lacking in thermophilic ethanologens (Dien et al. 2003; Zaldivar et al. 2001).

Species of the genus *Thermoanaerobacter*, once classified as members of the genus *Clostridium*, are physiologically similar to the thermophilic saccharolytic *Clostridia* (Mitchell 1998). They have been reported to possess an Embden–Meyerhof glycolytic metabolism and produce organic acids in addition to ethanol (Lynd 1989; Shaw et al. 2008a). *T. mathranii* BG1 ferments glucose, xylose, arabinose, galactose, and mannose simultaneously and produce ethanol, acetate, lactate, CO_2, and H_2 as fermentation end-products (Mikkelsen and Ahring 2007). Genetic modification tools have been developed in *T. mathranii* BG1 and used for insertion, deletion, or over-expression of target genes (Yao 2008). The gene encoding lactate dehydrogenase (*ldh*) has been deleted, resulting a mutant strain *T. mathranii* BG1L1 with increased ethanol yield (Georgieva et al. 2008), which still however produce small amounts of acetate and hydrogen as fermentation by-products.

This study began with the intent to further improve the ethanol production in *T. mathranii* by metabolic engineering. We first use carbon sources with different reduction states to study their effects on ethanol production in *T. mathranii*. Furthermore, we have created different recombinant strains of *T. mathranii* by expressing a heterologous NAD^+-dependent glycerol dehydrogenase (GLDH), with and without concomitant deletion of a lactate dehydrogenase. One of the resulting recombinant strains, *T. mathranii* BG1G1 (*Δldh*, P_{xyl}GldA), produce ethanol at a high yield and surprisingly acquire the capability to utilize glycerol as an extra carbon source in the presence of xylose.

Materials and methods

Culture origin, maintenance, and cultivation

T. mathranii BG1 (Mikkelsen and Ahring 2007) was originally isolated from an Icelandic hot spring (Sonne-Hansen et al. 1993). *T. mathranii* BG1L1 is a mutant strain with a lactate dehydrogenase deleted (Mikkelsen and Ahring 2007). The *T. mathranii* BG1 strain and its derived mutants were cultured anaerobically at 70°C in synthetic BA medium as previously described (Larsen et al. 1997). The medium was further supplemented with 2 g/L yeast extract and 5 g/L xylose or glucose as the growth substrate. Single colonies were isolated using the anaerobic roll-tube technique (Hungate 1969; Bryant 1972) with medium solidified with Phytagel (from plant cells, Sigma) and $MgCl_2$. Colonies were picked with sterile needles, cultured, and stored in 25% glycerol and 75% growth medium at −80°C in 2-ml sealed vials under $80\%N_2/20\%CO_2$ atmosphere. Culture recovered from glycerol stocks were grown in liquid medium prior to use in experiments. For selection of antibiotic resistant strains, the medium was supplemented with 50 μg/ml kanamycin, and the culture was maintained anaerobically at 70°C.

Strains, plasmids, and DNA manipulation

Strains and plasmids used in the study are listed in Table 1 with their relevant characteristics and sources. *E. coli* Top10 (Invitrogen A/S, Taastrup, Denmark) was used as a cloning host and grown under standard conditions (Sambrook and Russell 2001). *Thermotoga maritima* MSB8 (DSM accession number 3109) is used as the DNA source for amplifying glycerol dehydrogenase gene and grown anaerobically at 80°C in DSM medium 343 supplemented with 5 g/L glucose (Huber et al. 1986). Plasmids of p3CHPT (developed from pUC19, Invitrogen) and pYPA (developed from pYES2, Invitrogen) were used as cloning vectors. Plasmid preparation and manipulation (kits from A&A Biotechnology, Poland), genomic DNA preparation (kit from A&A Biotechnology), and transformation were carried out using standard procedures (Sambrook and Russell 2001) or the suppliers' instructions. Restriction enzymes were obtained from Fermentas (GmbH, Germany) and New England Biolabs (Ipswich, England). DNA modification enzymes including T4 polynucleotide kinase (PNK), calf intestinal phosphatase (CIP), and T4 DNA ligase were purchased from Fermentas. *Taq*, *Pwo*, and *Phi29* DNA polymerase were purchased from A&A Biotechnology and Fermentas. Oligonucleotides were synthesized, and DNA sequencing was conducted by MWG (MWG-BIOTECH AG, Ebersberg, Germany).

Construction of GLDH expression vectors

A GLDH regulative expression vector was constructed by inserting the *gldA* gene derived from *T. maritima* (NCBI Gene ID 897429 *gldA*; Brinen et al. 2003) into a *T. mathranii ldh* knockout vector (p3CHPT plasmid, Electronic Supplementary Material). Genomic DNA of *T. maritima* was extracted and used as a template to amplify the *gldA* gene

Table 1 Strains and plasmids used in this study

	Relevant characteristic(s)	Source or reference
Strains		
E. coli Top10	*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Δ*lac*X74, *deo*R, *rec*A1, *ara*D139Δ(*ara-leu*)7697, *gal*K, *rps*L, *end*A1, *nup*G	Invitrogen A/S, Denmark
T. maritima MSB8	Wild-type strain DSM3109	(Huber et al. 1986)
T. mathranii BG1	Wild-type strain	(Mikkelsen and Ahring 2007)
T. mathranii BG1L1	*ldh* deletion mutant	(Mikkelsen and Ahring 2007)
T. mathranii BG1G1	*ldh* deletion, GldA regulative expression mutant	This study
T. mathranii BG1G2	GldA constitutive expression mutant	This study
Plasmids		
pUC19	Cloning vector	Invitrogen A/S, Denmark
p3CHPT	pUC19 containing *ldh* upstream, *htk* gene, *xyl* promoter, *ldh* downstream	Mikkelsen (unpublished)
p3CHgld	p3CHPT containing *gld*A gene inserted after the promoter	This study
pYES2	Cloning vector	Invitrogen A/S, Denmark
pYPA	pYES2 containing *gap* gene fragment, *htk* gene, *pgk* gene	Slawomir (unpublished)
pYPAgld	pYPA containing *gld*A gene	This study

using primer of gldAF1 (5′gcggcgaggaggataattcatgataacaac caccatatttccag3′) and primer of gldAR1 (5′cggaaggatga gaaaaaacctcacttgacgccgc3′). PCR amplification was performed with *Pwo* polymerase. The resulting PCR fragments were treated with T4 PNK (Fermentas) and ligated with *Sma*I digested and CIP treated p3CHPT plasmid, where the *Sma*I restriction site is placed following the *xyl* promoter. Following the standard subcloning procedure, the constructed plasmid of p3CHgld (Fig. 1a) was obtained. Construct A aims at expressing the *gldA* gene under the regulation of the xylose induced P_{xyl} promoter and concomitantly knocking out the *ldh* gene in *T. mathranii*.

A GLDH constitutive expression vector was constructed by inserting the *gldA* gene derived from *T. maritima* into a *gap-pgk* expression cassette derived from *T. mathranii* on a pYPA plasmid (supplementary material). A DNA fragment of *gld*A gene was amplified by PCR using genomic DNA of *T. maritima* as a template and primers of gldAF1 and gldAR1. The resulting PCR fragment was treated with T4 PNK (Fermentas) and ligated with *Ecl136*II digested and CIP treated pYPA plasmid, where the *Ecl136*II restriction site is placed between *gap* and *htk* region in the pYPA plasmid. Following the standard subcloning procedure, the constructed plasmid of pYPAgld (Fig. 1b) was obtained. Construct B aims at expressing the *gldA* gene constitutively in *T. mathranii* by integrating it into the *gap-pgk* expression cassette without influencing the enzyme functions.

Construction of *T. mathranii* GLDH expression mutants

Plasmid DNA (10–100 ng) of p3CHgld and pYPAgld were used as templates for multiple displacement amplification

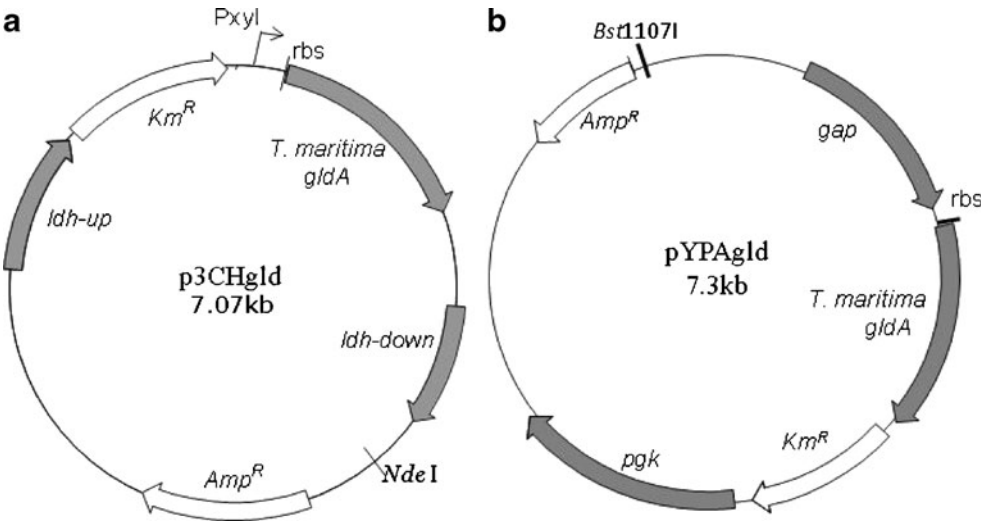

Fig. 1 Construction of the carbon source regulated expression vector p3CHgld (**a**) and constitutive expression vector pYPAgld (**b**). *Km*, kanamycin marker gene; *Amp*, ampicillin marker gene; *ldh-up* and *ldh-down*, *ldh* upstream and downstream DNA fragments; *gap*, truncated glyceraldehyde 3-phosphate dehydrogenase gene; *pgk*, phosphoglycerate kinase gene; *gldA*, glycerol dehydrogenase gene; *rbs*, ribosome binding site

(MDA) using a final concentration of 1 U/ml of yeast pyrophosphatase and 300 U/ml *Phi*29 DNA polymerase in the presence of 0.4 mM dNTPs and 50 μM exonuclease-resistant hexamer according to the Fermentas' protocol. Three micrograms of amplified DNA products from MDA reaction using p3CHgld and pYPAgld as templates were later digested with *Nde*I and *Bst1107*I, respectively (Fig. 1). Digested DNA products from MDA reaction were transformed into strain BG1 using electroporation as previously described (Tyurin et al. 2004). Electropulsed cell suspensions were recovered and cultured in liquid BA medium with 5 g/L glucose for 24 h and later transferred into the same medium supplemented with 50 μg/ml kanamycin. The cultures grown with kanamycin were later transferred into roll tubes for single colony isolation. Gene disruptions were verified by PCR using the primers annealing to the kanamycin cassette or inserted *gld*A region or outside of p3CHgld and pYPAgld plasmid sequences (Electronic Supplementary Material). The PCR products are confirmed by digestion with restriction enzyme and sequencing analysis.

Glycerol dehydrogenase assay

The GLDH activity of the tested strain was determined as described below. The tested strains were cultivated in 100 ml of BA media using 5 g/L glucose or xylose and further provided with 2.5 g/L glycerol at 70°C under anaerobic conditions. Cultures at an OD578 of ~0.5 were harvested by centrifugation of 50 ml of the culture at 4,000 rpm and 4°C for 30 min. The pellet was re-suspended in 2 ml of ice chilled extraction buffer composed of 50 mM Tris–HCl, 10% glycerol, and 1 mM $MgCl_2$ at pH 8.0. The cells were sonicated at 10% ultrasound amplitude for 2 min in an ice bath (Digital Sonifier, Model 250; Branson Ultrasonics Corporation, Danbury, USA). The sonicated cells were centrifuged at 20,000×*g* and 4°C for 30 min. The supernatant was used for GLDH activity assay at 70°C and pH 8.0 using continuous spectrophotometric rate determination methods as previously described (Burton 1955). One unit was defined as the amount of enzyme that produced 1 μmol of NADH per minute at 70°C and pH 8.0. Total protein concentration in the cell extracts was routinely measured by the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

Fermentation

All batch fermentations were performed in 26-ml serum tubes with 10 ml of BA medium supplemented with the tested carbon source under strictly anaerobic conditions using 10% (*v*/*v*) inoculum from the overnight culture. The cultures were grown at 70°C, and the samples were collected for analysis after 48 h of growth. In the carbon source comparison experiment, the BA medium is supplemented with 5 g/L of the tested carbon source. In the genotype comparison experiment, the BA medium is supplemented with 5 g/L glucose or xylose and 2.5 g/L glycerol.

Continuous fermentation was performed in an immobilized upflow bioreactor with working volume of 200 ml of BA medium supplemented with 2 g/L yeast extract and the tested substrates. The initial pH of the medium was adjusted to 7.4–7.7. To ensure anaerobic condition, medium was flushed for 45 min with a mixture of N_2/CO_2 (4:1), and finally, Na_2S was injected into the reactor to give a final concentration of 0.25 g/L. The reactor was started with initial xylose and glycerol at concentrations of 17.5 and 9.7 g/L, respectively. The reactor was started up in a batch mode by inoculation with 10 ml of cell suspension with an optical density (OD578) of 0.9–1.0 and running for 48 h to allow cells to attach on the carrier matrix in the reactor. After the batch run, the system was switched to a continuous mode by applying a hydraulic retention time of 24 h and an upflow velocity of 1 m/h. The pH in the reactor was maintained at 7.0 by addition of NaOH (1–2 M). The reactor setup is further described in Mikkelsen and Yao (2010).

Analytical methods

Samples used for sugar and fermentation product determination were prepared and analyzed using HPLC as previously described (Georgieva et al. 2008). Hydrogen production was determined by gas chromatography. The gas chromatograph (GC Microlab, Århus, Denmark) was equipped with a thermal conductivity detector. Gasses were separated by a molecular sieve (0.5 nm). The temperatures of oven, injection port, and the detector were at 110°C. The carrier gas was N_2 at a flow rate of 30 ml/min.

Calculation of carbon recovery

Carbon recovery was calculated exclusive of cell synthesis based on yields of organic fermentation products plus stoichiometrically associated CO_2 production accompanying the production of ethanol and acetic acid (Lynd et al. 2002; Mikkelsen and Ahring 2007) using the formula

$$\%\ \text{Carbon recovery} = \frac{3(\text{mM ethanol} + \text{mM acetate} + \text{mM lactate})}{n \times (\text{mM substrate})} \times 100$$

where n is 5 for pentose and 6 for hexose.

Results

Fermentation study on different carbon sources

Batch fermentation experiments were performed to study the effect of different carbon sources on ethanol production by *T. mathranii* BG1 wild-type strain and BG1L1 (Δ*ldh*) mutant strain. Mannitol was used as a more reduced carbon source in comparison with glucose and xylose. Figure 2a highlights the differences in the catabolism of the tested carbon source as they enter the glycolytic pathway. Table 2 summarizes the results of the experiments. When the BG1 wild-type strain was examined, ethanol yields with mannitol were 22.6% and 9.1% higher than with the use of glucose and xylose, respectively, based on moles of carbon (Cmol/Cmol). When the BG1L1 mutant strain was exam-

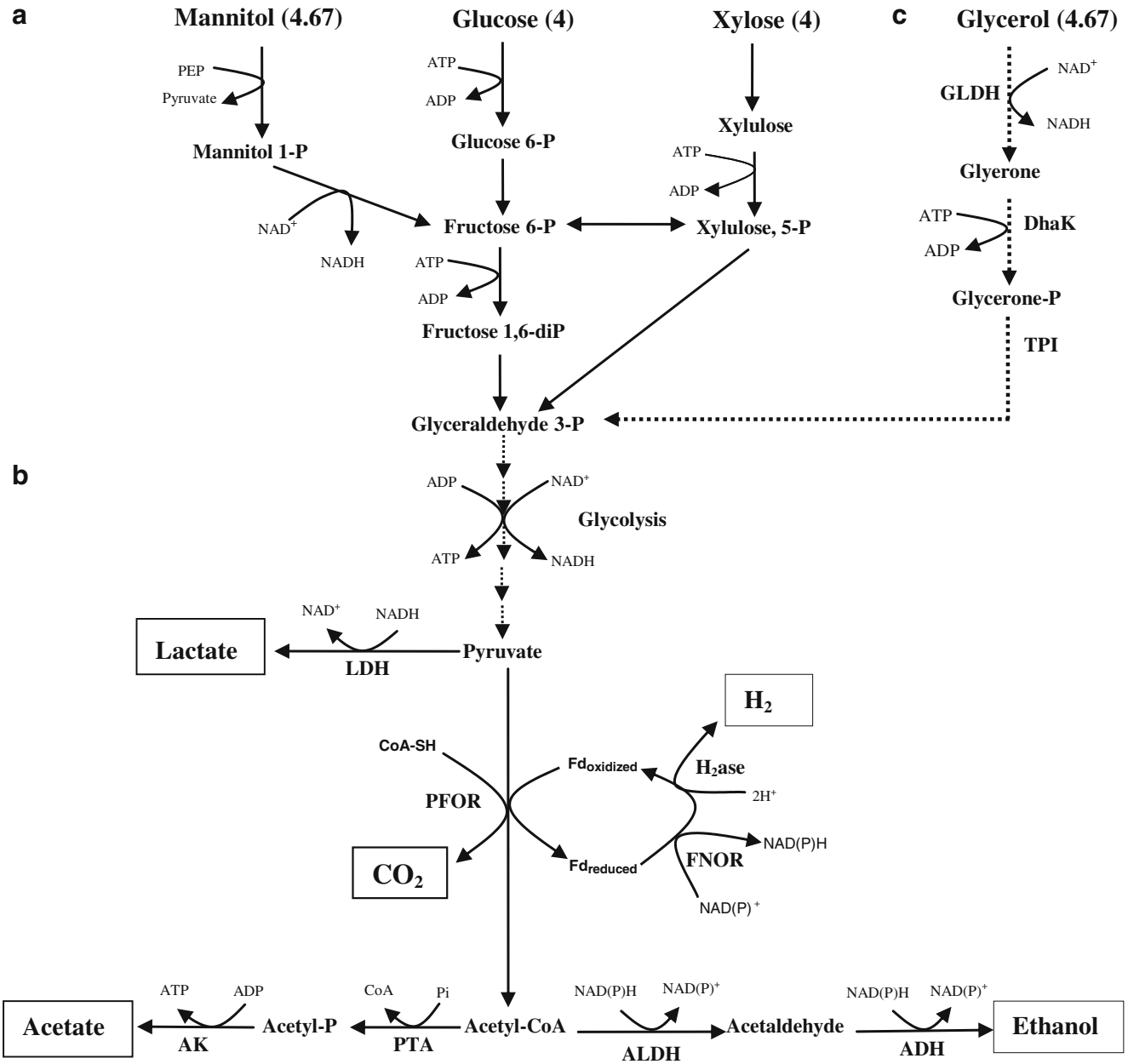

Fig. 2 Schematic representation of the metabolism of *Thermoanaerobacter* spp. **a** Schematic showing the differences of catabolism of mannitol, glucose, and xylose as they enter the glycolysis pathway; **b** end-product fermentation pathways; **c** newly introduced glycerol degradation pathway. *LDH* lactate dehydrogenase, *PFOR* pyruvate ferredoxin oxidoreductase, *Fd* ferredoxin, *H_2ase* hydrogenase, *FNOR* ferredoxin/NAD(P)H oxidoreductase, *ALDH* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase, *PTA* phosphate acetyltransferase, *AK* acetate kinase, *GLDH* glycerol dehydrogenase, *DhaK* dihydroxyacetone kinase, *TPI* triosephosphate isomerase. The *numbers* shown in parenthesis are the reduction degree per carbon of the carbon sources (Nielsen et al. 2003)

Table 2 Comparison of product profile of *T. mathranni* BG1 and BG1L1 strains grown on different carbon sources

Strain	Wild type[a]			BG1L1[a]		
	Glucose	Xylose	Mannitol	Glucose	Xylose	Mannitol
Sugar (mM)	28.9±1.0	36.7±2.5	24.1±0.3	27.1±2.5	34.6±0.8	24.7±0.4
Lactate (mM)	9.4±2.2	1.9±0.2	3.7±0.7	ND[e]	ND	ND
Acetate (mM)	3.4±0.2	6.1±0.7	0.8±0.3	5.7±0.4	7.6±0.6	1.5±0.1
Ethanol (mM)	42.5±0.8	50.7±1.7	43.5±1.6	47.6±3.9	48.4±0.5	47.1±1.1
Hydrogen (mM)	24.0±2.8	33.4±2.4	54.5±3.6	29.0±3.9	36.0±2.6	63.4±3.5
$Y_{Lactate}$[b]	0.16	0.03	0.08	0.00	0.00	0.00
$Y_{Acetate}$[b]	0.04	0.07	0.01	0.07	0.09	0.02
$Y_{Ethanol}$[b]	0.49	0.55	0.60	0.59	0.56	0.64
$Y_{Hydrogen}$[b]	0.14	0.18	0.38	0.18	0.21	0.43
$T_{Ethanol}$ (%)[c]	73.5	82.5	90.0	88.5	84.0	96.0
CN (%)[d]	96±2.9	96±2.6	100±1.3	98±1.7	97±2.3	98±0.9

[a] Results of batch experiments using 5 g/L of the carbon source in BA medium after 48 h of incubation, and the values shown are averages of triplicate experiments ± standard deviations

[b] $Y_{Lactate}$, $Y_{Acetate}$, $Y_{Ethanol}$, $Y_{Hydrogen}$ values are product yield based on moles of carbon (Cmol/Cmol)

[c] Percentage of ethanol produced relative to the theoretical maximum

[d] Carbon recovery

[e] Not detected

ined, ethanol yields with mannitol were 8.5% and 13.8% higher than with the use of glucose and xylose, respectively. Furthermore, the increased ethanol production with the use of mannitol was accompanied by increased hydrogen production and decreased acetate formation, for both of the tested strains. In addition, strain BG1L1 showed no lactate production and overall higher ethanol yields than the wild-type strain grown on all of the tested carbon sources.

Isolation of *T. mathranii* mutant strains

T. mathranii BG1 wild-type strain transformed with either linearized p3CHgld or linearized pYPAgld vector was recovered in the selected medium after 2 days. Five of each presumptive chromosomal integrant isolates were picked as single colonies from the roll tubes. All picked isolates grew in liquid medium containing kanamycin and yielded identical results following the PCR analysis (data not shown). Several independent clones were chosen for further analysis and identified as *T. mathranii* BG1G1 and BG1G2 strains transformed with construct A and B, respectively. Gene disruption in both constructed mutant strains were verified and found identical to the predicted sequences of the recombinant clones (Electronic Supplementary Material).

Enzymatic study of different genotypes

To confirm the GLDH expression in *T. mathranii* BG1G1 and BG1G2 strain, specific GLDH activity was determined from the cells grown on glucose or xylose with glycerol present in the medium. Table 3 shows the specific NAD^+-dependent GLDH activity from the cell extracts of different *T. mathranii* strains in units per milligram of total protein. One unit is defined as the amount of enzyme that produced 1 μmol of NADH per minute at 70°C and pH 8.0. The results show that the specific NAD^+-dependent GLDH activity was detected in strain BG1G1 grown on xylose, but no activity was detected in the same strain grown on glucose. When strain BG1G2 was tested, NAD^+-dependent GLDH activity is seen in the cells grown on both glucose and xylose. In contrast, BG1 wild-type and BG1L1 strain showed no detectable NAD^+-dependent GLDH activity from the cells grown on either glucose or xylose.

Fermentation study with different genotypes

Batch fermentation studies were performed with different genotypes of *T. mathranii* to study their metabolic patterns using glucose or xylose with glycerol present in the medium. Table 4 summarizes the results of the experiments calculated based on glucose or xylose as the sole carbon source. A comparison of the results with different genotypes shows the effect of eliminating the LDH from strain BG1L1, or the effect of expressing an NAD^+-dependent GLDH in strain BG1G2, or the combined effect of both

Table 3 Specific NAD^+-dependent glycerol dehydrogenase activity from cell extracts of different *T. mathranii* strains in units per milligram of total protein

Strain (genotype)	Activity (U/mg)[a]	
	Glucose	Xylose
T. mathranii wild type	ND[b]	ND
T. mathranii BG1L1 (*Δldh*)	ND	ND
T. mathranii BG1G1 (*Δldh*, P_{xyl}GldA)	ND	0.438±0.04
T. mathranii BG1G2 (GldA)	0.396±0.02	0.287±0.06

[a] Values shown are average of triplicates from anaerobic cultures ± standard deviation (one unit is defined as the amount of enzyme that produced 1 μmol of NADH per minute at 70°C and pH 8.0)

[b] ND=not detected (less than 0.001 U/mg)

Table 4 Comparison of product profiles of different *T. mathranii* strains

Strain	Glucose[a]				Xylose[a]			
	Wild type	BG1L1	BG1G1	BG1G2	Wild type	BG1L1	BG1G1	BG1G2
Sugar (mM)	25.4±0.1	25.7±2.2	22.9±0.2	27.4±0.2	30.2±1.6	30.1±1.7	31.2±1.9	29.0±1.5
Lactate (mM)	9.2±1.3	ND [e]	ND	41.0±1.0	2.8±0.3	ND	ND	23.6±3.5
Acetate (mM)	6.2±1.3	6.4±0.6	5.6±0.7	10.6±0.5	10.2±0.8	7.4±0.2	3.3±0.5	12.5±3.9
Ethanol (mM)	33.4±1.3	43.6±1.0	38.5±0.5	3.0±0.6	34.3±1.2	40.0±2.0	49.0±2.6	14.7±2.0
Hydrogen (mM)	13.4±1.2	19.4±3.8	18.0±1.1	32.3±1.8	11.7±0.6	18.5±1.0	5.8±0.9	37.1±3.0
$Y_{Lactate}$ [b]	0.18	0.00	0.00	0.75	0.06	0.00	0.00	0.49
$Y_{Acetate}$ [b]	0.08	0.08	0.08	0.13	0.13	0.10	0.04	0.18
$Y_{Ethanol}$ [b]	0.44	0.56	0.56	0.037	0.45	0.53	0.63	0.20
$Y_{Hhydrogen}$ [b]	0.09	0.13	0.13	0.23	0.08	0.12	0.04	0.22
$T_{Ethanol}$ (%)[c]	66.0	84.0	84.0	5.6	67.5	79.5	94.5	30.0
CN (%)[d]	96±2.1	97±0.8	96±1.9	99±1.0	94±3.0	95±1.0	101±1.3	105±2.7

[a] Results of batch experiments using 5 g/L of tested sugar supplemented with 2.5 g/L glycerol in BA medium after 48 h of incubation, and the values shown are averages of triplicate experiments ± standard deviations

[b] $Y_{Lactate}$, $Y_{Acetate}$, $Y_{Ethanol}$, $Y_{Hydrogen}$ values are product yield based on moles of carbon (Cmol/Cmol)

[c] Percentage of ethanol produced relative to the theoretical maximum

[d] Carbon recovery calculated based on glucose or xylose

[e] Not detected

genetic manipulations in strain BG1G1. Strain BG1L1 and BG1G1 showed no detectable lactate production and higher ethanol yield compared with the wild type on both of the tested substrates. With glucose as a substrate, strain BG1L1 and BG1G1 showed similar metabolic patterns. With xylose as a substrate, an increased ethanol yield by 18.9% was observed with strain BG1G1 as compared with strain BG1L1. In comparison, a drastic decrease in ethanol yield was observed with strain BG1G2, accompanied with increased organic acid production, especially the production of lactate. Furthermore, the hydrogen production from different genotypes of *T. mathranii* is also different. Strain BG1G1 grown on xylose produced the lowest amount of hydrogen while strain BG1G2 produced the highest amount of hydrogen among all the genotypes.

Ethanol production of *T. mathranii* BG1G1

A physiological characterization of *T. mathranii* wild-type strain and BG1G1 mutant strain was performed in batch fermentation. Figure 3 presents the batch fermentation results with the tested strains. The results indicated that the time required to achieve substrate exhaustion is longer, and final optical densities achieved in stationary phase are lower by strain BG1G1 as compared with the wild type. Final optical density at the end of exponential phase was 0.93±0.04 for the wild type and 0.42±0.03 for strain BG1G1. Strain BG1G1 showed overall higher ethanol concentration and lower acetate concentration without lactate production at the end of fermentation as compared with the wild type. Around 3 mM of glycerol is consumed during the fermentation using strain BG1G1 as compared with the wild-type strain, which does not consume glycerol. Carbon recovery of the fermentation products based on xylose consumption is 100%.

In a continuous immobilized cell reactor using strain BG1G1 for ethanol production, growth rates and ethanol productivities were comparable to those obtained with the wild-type strain. Figure 4 presents the result of a continuous fermentation using strain BG1G1. The result indicates that the steady state of the continuous reactor was obtained after 27 days. Less than half of the xylose was consumed at 24-h hydraulic retention time (HRT) after 5 days, and 95% of the xylose was consumed at the same HRT after 17 days. The highest volumetric ethanol productivity of 0.25 g/L/h was observed in the final part of the continuous fermentation with more than 95% of xylose consumed. At steady state, almost all the sugars and around 30% of the glycerol were consumed, and no lactate was detected. The highest ethanol yield of 0.55 g ethanol per grams consumed xylose, corresponding to 108% of the maximal theoretical yield, was obtained after 32 days during growth on 12.8 g/L of xylose and 7.2 g/L of glycerol. The carbon recovery of fermentation products based on xylose consumption is 108%. If the ethanol yield is based on both consumed xylose and glycerol, a yield of 0.47 g ethanol per gram of consumed xylose and glycerol, corresponding to 92% of the maximal theoretical yield, was

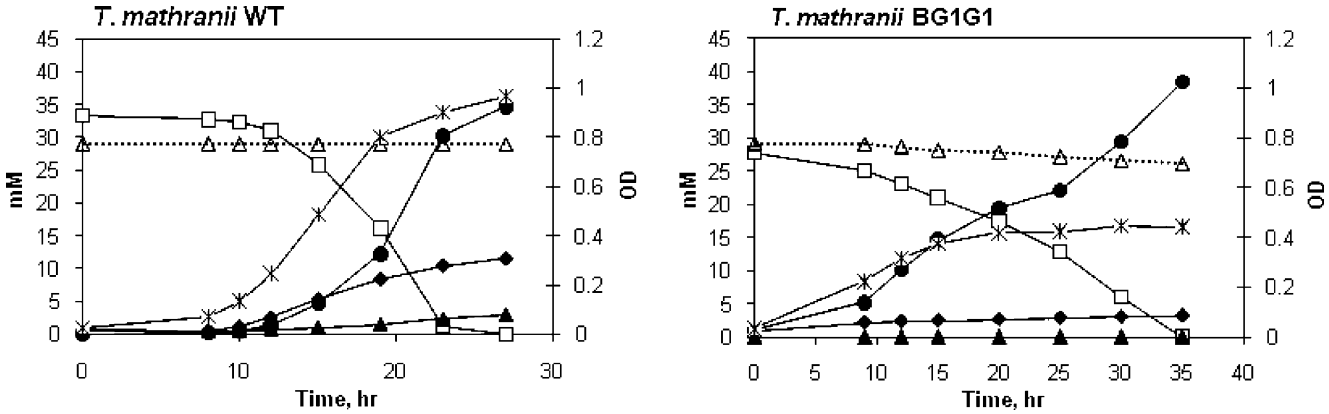

Fig. 3 Product profile of *T. mathranii* BG1 wild-type and BG1G1 strains during batch fermentation; xylose (*empty square*), glycerol (*empty triangle*), lactate (*filled triangle*), acetate (*filled diamond*), ethanol (*filled circle*), and OD (*x*)

obtained with a carbon recovery of 91% for the fermentation products. Ethanol evaporation and cell mass production are expected to account for approximately 5% and 4% of the consumed carbon, respectively, based on previous reactor fermentation experiment with strain BG1L1.

Discussion

The carbon metabolism of *Thermoanaerobacter* species is inextricably linked to cofactor oxidation and reduction reactions (Fig. 2) as previously reviewed (Zeikus et al. 1981; Lynd 1989; Jones and Woods 1991; Mitchell 1998; Shaw et al. 2008a). Glycolysis of carbohydrates leads to the production of ATP, NADH, and the key intermediate pyruvate (Fig. 2b). A critical oxidation occurs as pyruvate is converted to acetyl-CoA. The enzyme responsible, pyruvate ferredoxin oxidoreductase (PFOR), generates reduced ferredoxin (Fd_{red}), and subsequent fate of the electron is a major determinant of the fermentation pattern. The re-oxidation of Fd_{red} is catalyzed by different oxidoreductases. NADH and NADPH ferredoxin oxidoreductases catalyze the production of NADH and NADPH, respectively. Hydrogenase (H_2-ferredoxin oxidoreductase) forms hydrogen, and when reducing equivalents are transferred to ferredoxin and released as hydrogen, pyruvate may be metabolized to acetate with the generation of additional ATP. If no or only limited amount of H_2 is developed, electrons must be channeled toward the formation of the reduced products, i.e., ethanol and lactate.

Supplying the bacteria with a more reduced carbon source, mannitol, directed the metabolic pathway to ethanol production in both *T. mathranii* BG1 wild type and BG1L1 strains. Compared with mannitol, the use of glucose or xylose clearly limits the ethanol yields resulting from a shortage of reducing equivalents. Deletion of *ldh* in the *T.*

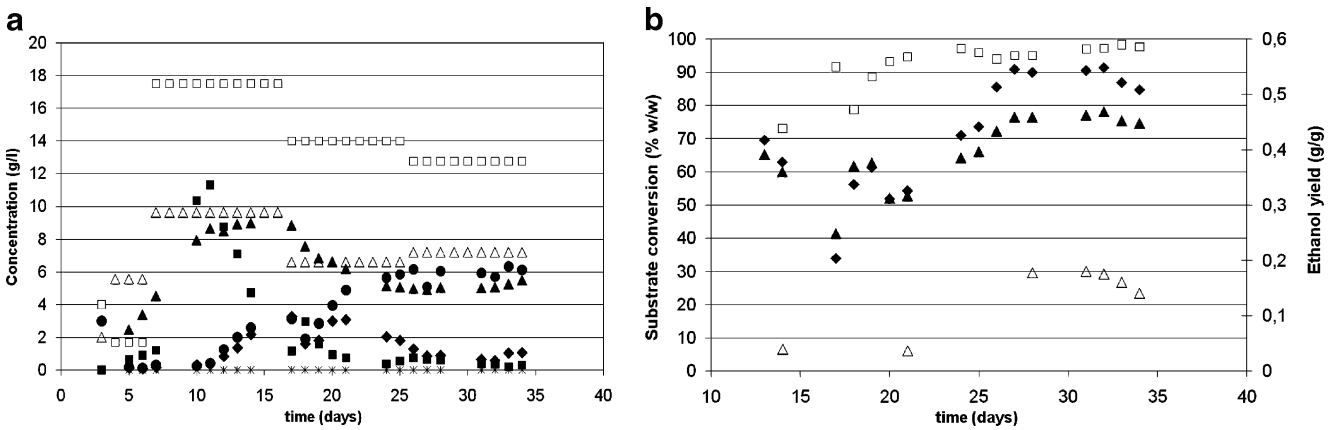

Fig. 4 Product profile of *T. mathranii* BG1G1 during a continuous fermentation of mixtures of xylose and glycerol in an immobilized upflow reactor. **a** Concentrations of different compounds in the influent (*empty symbols*) and inside the reactor (*closed symbols*): xylose (*empty and filled square*), glycerol (*empty and filled triangle*), lactate (*x*), acetate (*filled diamond*), ethanol (*filled circle*); **b** substrate conversions and ethanol yields: xylose conversion (*empty square*), glycerol conversion (*empty triangle*), ethanol yield based on xylose consumption (*filled diamond*), ethanol yield based on both xylose, and glycerol consumption (*filled triangle*)

mathranii BG1L1 strain eliminates an NADH oxidation pathway and the lactate formation. This leads to increases in both carbon flux and cofactor available for the ethanol formation pathway, as evidenced by the overall higher ethanol yields in the BG1L1 strain as compared with the wild-type strain. Furthermore, both of the tested strains showed higher hydrogen production with mannitol as substrate than with the other two sugars, suggesting that the excess reducing equivalent is also oxidized via H_2 production in addition to being utilized to produce ethanol. Overall, these results indicate that mannitol shifts the carbon balance toward more reduced products due to the increased formation of NADH during mannitol catabolism.

In order to shift the carbon balance toward the more reduced product, ethanol, without using an expensive substrate like mannitol, an NAD^+-dependent GLDH was expressed in *T. mathranii* and the resulting strains being BG1G1 (*Δldh*, P_{xyl}GldA) and BG1G2 (GldA). As expected, the expression of GLDH, as detected by NAD^+-dependent GLDH activity, was only induced by xylose in strain BG1G1 due to the presence of the glucose repressed xylose promoter. In strain BG1G2, constitutive expression of GLDH was seen during the growth on both glucose and xylose. With the expression of GLDH in the mutant strains, one NADH is formed per glycerol converted to glycerone, thereby increasing the NADH/NAD^+ ratio in the cells.

Batch fermentation performed with different genotypes revealed that the metabolite product profiles are strongly affected by the tested genotypes of *T. mathranii*. As discussed above, deletion of *ldh* in strain BG1L1 eliminates an NADH oxidation pathway in lactate formation and resulted in an increased ethanol yield as compared with the wild type. Moreover, GLDH-mediated NADH production in strain BG1G2 leads to a drastic shift in the metabolic pattern that favors the production of lactate instead of ethanol. This could be due to the reduced flux in glycolysis caused by the decreased NAD^+/NADH ratio in strain BG1G2, leading to the accumulation of a glycolysis intermediate, fructose 1,6-biphosphate, that could activate the lactate dehydrogenase activity in Clostridia (Germain et al. 1986; Mitchell 1998). However, when the lactate formation pathway is inactivated as in strain BG1G1, GLDH-mediated NADH production in the cells grown on xylose affects the partitioning at the acetyl-CoA node to favor the production of ethanol over acetate, hence restoring the redox balance. As hydrogen production is decreased in strain BG1G1, this suggests that the generated reducing equivalent excess is oxidized via ethanol production system rather than hydrogen formation. This could be due to the increased NADH/NAD^+ ratio in strain BG1G1, leading to the induction of ferredoxin/NAD^+ reductase and higher expression of alcohol dehydrogenase as observed in *Clostridium acetobutylicum* (Vasconcelos et al. 1994), which in turn directs the pathway to the ethanol formation. Similar redirection of electron flow associated with increased ethanol production is also seen in other thermophiles such as *Thermoanaerobacter tengcongensis*, *T. saccharolyticum*, and *C. acetobutylicum* (Soboh et al. 2004; Shaw et al. 2008a, b; Vasconcelos et al. 1994).

The physiological study of *T. mathranii* BG1G1 showed overall higher ethanol production as compared to the wild type, but this was accompanied by a slower growth rate, lower cell density, and longer fermentation time to deplete the substrate. This negative effect reflects the imbalanced metabolic changes, possibly due to the decreased ATP yield from acetate formation or to the depletion of NAD^+, which is essential for glycolysis. Ethanol fermentation of *T. mathranii* BG1G1 in a continuous immobilized cell reactor demonstrated the capability of the strain to increase ethanol productivity by long-term adaptation. The increase in the xylose consumption rate during the fermentation reflected an increased catabolic rate of the strain during the adaptation phase. In contrast to the batch experiments, the ethanol yield obtained based on xylose consumption was above the theoretical maximum using strain BG1G1 in the continuous reactor. Moreover, the carbon recovery based on xylose consumption is higher than 100%, even if carbon consumed for ethanol evaporation or production of cell mass is not accounted. This infers that *T. mathranii* BG1G1 could utilize glycerol as an extra carbon source through expression of a glycerol dehydrogenase. A closer look into the glycerolipid and glycolysis metabolism of *T. mathranii* BG1 revealed that the expression of GLDH catalyzes the oxidation of glycerol into glycerone, an intermediate that could be channeled into glycolysis by two sequential activities of dihydroxyacetone kinase (DhaK) and triosephosphate isomerase (TPI; Fig. 2c). Genes encoding both of these activities are present in the genome of *T. mathranii* BG1 or closely related *Thermoanaerobacter* species such as in *Thermoanaerobacter pseudethanolicus* (NCBI-GeneID 5873988 and 5874368 encoding DhaK and TPI, respectively). In this case, an endogenous glycerol metabolic pathway could possibly be constructed through expression of GLDH, which is not present in *T. mathranii* BG1 or in *T. pseudethanolicus*. Conversion of glycerol into the glycolytic intermediate pyruvate generates twice the amount of reducing equivalents produced by the metabolism of glucose or xylose (Yazdani and Gonzalez 2007). Fermentative metabolism of both xylose and glycerol consequently enables a higher yield of reduced product, i.e., ethanol. Although regulated expression of GLDH is informative, strains in which the glycerol dehydrogenase is constitutively expressed should

be constructed to facilitate ethanol production from all the sugars present in the lignocellulosic biomass including glucose. Glycerol not consumed in the fermentation can favorably be converted to biogas, thereby further increasing the value of the process.

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