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Conversion of hemicellulose and D-xylose into ethanol by the use of thermophilic anaerobic bacteria

Mikrobiologiske undersøgelser af termofile anaerobe bakterier som omsætter hemicellulose til ethanol

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List of Papers

1. **Larsen L, Nielsen P, Ahring BK** *Thermoanaerobacter mathranii* sp. nov., an ethanol producing extremely thermophilic anaerobic bacterium from a hot-spring in Iceland (1997). Archieves of Microbiology 168:114-119

2. **Sommer P, Meinander, NQ, Hahn-Hägerdal B** Comparison of the cold methanol and perchloric acid methods for extraction of intracellular metabolites from recombinant, xylose fermenting *Saccharomyces cerevisiae*. prepared for submission to Applied Microbiology & Biotechnology

3. **Sommer P, Ahring BK** Measurements of intracellular metabolites and enzymes of the pentose phosphate pathway of *Thermoanaerobacter mathranii* A3M1 at varying D-xylose concentrations. Prepared for submission to Applied & Environmental Microbiology.

4. **Sommer P, Ahring BK** Measurements of intracellular metabolites and enzymes in the glycolysis of *Thermoanaerobacter mathranii* A3M1: The influence of the D-xylose concentration. Prepared for submission to Applied & Environmental Microbiology.

Sammenfatning

Ethanol er et CO_2-neutralt brændstof, der kan erstatte brugen af flydende fossile brændstoffer i transportsektoren og derved nedbringe udledningen af CO_2 til atmosfæren. Denne udledning betragtes som en væsentlig årsag til den globale opvarmning, drivhuseffekten. Substratet til produktion af ethanol skal være billigt og kunne produceres i store mængder. Til dette formål er lignocelluloseholdig biomasse, som f.eks. halm, piletræ og træflis, anvendelig. Deres komplekse kemiske natur kræver dog en lang række processer for at frigøre kulhydraterne (primært glukose og xylose) til ethanol-fermentering. Glukose kan fermenteres til ethanol ved brug af gærstammer, som ved fremstilling af vin og øl. Disse gærstammer kan ikke fermentere xylose, som udgør en væsentlig del af den samlede kulhydratmængde. For at nedbringe procesomkostningerne ved fremstilling af ethanol fra lignocellulose biomasse er det nødvendigt også at omsætte xylose til ethanol. Til denne fermentering er thermofile anaerobe bakterier et kvalificeret bud. De kan fermentere adskillige kulhydrater inklusiv de kulhydrater, der findes i lignocellulose biomasse. De har en høj væksthastighed (t_{gen} ca. 50 minutter) og producerer ethanol ved 70°C. Det er tæt på kogepunktet for ethanol, hvilket kan lette den efterfølgende destillering. Fysiologiske undersøgelser af disse bakteriestammer har imidlertid vist at ethanoludbyttet mindskes med stigende koncentrationer af kulhydrater. De biokemiske begrænsninger der forårsager dette fænomen kendes ikke i detaljer. Biokemiske undersøgelser af den manglende D-xylose tolerance blev undersøgt i en nylig karakteriseret termofil anaerob ethanolproducerende bakterie, *Thermoanaerobacter mathranii*. Dette studium påviste flere begrænsende trin i både glykolysen og pentose fosfat nedbrydningsvejene. Disse undersøgelser er essentiel viden for en senere genetisk modifikation af denne stamme med henblik på forøgelse af ethanoludbyttet og ethanolproduktiviteten, også ved høje xylosekoncentrationer.

Abstract

Ethanol is a CO_2 neutral liquid fuel that can substitute the use of fossil fuels in the transportation sector, thereby reducing the CO_2 emission to the atmosphere. CO_2 emission is suspected to contribute significantly to the so-called greenhouse effect, the global heating. Substrates for production of ethanol must be cheap and plentiful. This can be met by the use of lignocellulosic biomass such as willow, wheat straw, hardwood and softwood. However, the complexity of these polymeric substrates and the presence of several types of carbohydrates (glucose, xylose, mannose, galactose, arabinose) require additional treatment to release the useful carbohydrates and ferment the major carbohydrates fractions. The costs related to the ethanol-production must be kept at a minimum to be price competitive compared to gasoline. Therefore all of the carbohydrates present in lignocellulose need to be converted into ethanol. Glucose can be fermented to ethanol by yeast strains such as *Saccharomyces cerevisiae*, which, however, is unable to ferment the other major carbohydrate fraction, D-xylose. Thermophilic anaerobic ethanol producing bacteria can be used for fermentation of the hemicellulose fraction of lignocellulosic biomass. However, physiological studies of thermophilic anaerobic bacteria have shown that the ethanol yield decreases at increasing substrate concentration. The biochemical limitations causing this phenomenon are not known in detail. Physiological and biochemical studies of a newly characterized thermophilic anaerobic ethanol producing bacterium, *Thermoanaerobacter mathranii*, was performed. This study included extraction of intracellular metabolites and enzymes of the pentose phosphate pathway and glycolysis. These studies revealed several bottlenecks in the D-xylose metabolism. This knowledge makes way for physiological and genetic engineering of this strain to improve the ethanol yield and productivity at high concentration of D-xylose.

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1 Introduction

Ethanol is a versatile chemical, present in wine, beer and spirits, consumed for human pleasure. Ethanol is also a chemical feedstock (e.g. used in pharmaceuticals) and has an enormous potential for supplementing or even substituting fossil transportation fuels.

1.1 Fossil fuels

The fossil fuel, crude oil, constitutes the largest share in The Unites States of total energy used and has the highest fraction imported (Lynd et al. 1991b). A large part of the crude oil is refined. 60% of the total petroleum consumption in the U.S is consumed by the transportation sector (Lynd et al. 1991b). Furthermore, the transportation sector, which is highly dependent on petroleum, has actually not capacity to switch to other fuels in the event of a supply disruption (Lynd et al. 1991b).

1.2 Ethanol – a non-fossil fuel

Renewable feedstocks have national interest because they provide an almost endless supply of raw materials for production of ethanol, that are less vulnerable to disruption by foreign suppliers and could improve the international balance of payments (Wyman, Hinman, 1990). The application of ethanol as an octane booster, fuel extender, or neat liquid fuel has received considerably attention since the 1970's as a substitute for petroleum (Wyman, Hinman, 1990).

Brazil began a renewable energy program in the 1980´s to reduce the import of crude oil. Today, the program is the worlds largest renewable energy program. Four million vehicles use neat ethanol fuel and 22% ethanol is added as a fuel extender in all gasoline. Brazil produces ethanol equivalent to 200 thousand barrels of gasoline per day, constituting 20% of all transportation fuel consumed. The ethanol plants produce a surplus of bagasse and power. There is extensive research ongoing to use the bagasse as substrate for the ethanol production, to diminish the dependency upon sugar cane, which can be sold as a high value product. The renewable energy program has an annually sale of 7 billion US$ and a 30 billion US$ total savings in import of crude oil. In Brazil, up to 90% of all new automobiles have been designed to burn hydrous ethanol (Grayson, 1984).

Fuel ethanol is currently produced from sugar cane in Brazil. In The United States, ethanol is produced from corn. 10% ethanol blended with gasoline is called gasohol (Wyman, Goodman, 1993).

1.3 Environmental effect of fossil fuels contra non-fossil fuels

Air pollution is for instance an important factor, which motivates the interest in alternatives to fossil fuels (Lynd et al. 1991b). Carbon dioxide (CO_2) produced from the combustion of fossil fuels, is postulated to be partly responsible for global climate changes – The greenhouse effect. Transportation fuels account for 27% of the 3.3 billion tons of CO_2 released annually in the United States from combustion of fossil fuels. Vehicles account for 4.7% of total worldwide man made CO_2 emissions, with U.S. vehicles being responsible for 2.5% of total emissions (Lynd et al. 1991b). These data are from the early 1990's and the proportion has probably increased through this decade.

CO_2 is produced from fermentation of the carbohydrate fraction of biomass to ethanol, combusting of unfermentable biomass fractions to provide process energy and combustion of fuel ethanol. The quantity of CO_2 released, however, is the same as the quantity previously removed from the atmospheric pool by photosynthesis in the feedstock production. The lignocellulose ethanol fuel cycle thus involves cyclic carbon flow in contrast to CO_2 emission from fossil fuel (Lynd et al. 1991b; Wyman, Hinman, 1990; Wyman, Goodman, 1993). This means that no net CO_2 is released when ethanol produced from biomass is used as fuel, avoiding possible contribution to global climate change suspected to cause the greenhouse effect.

Airborne emissions, liquid effluents, and solid wastes from ethanol production processes appear to pose any problems that cannot be solved by conventional waste-treatment technology. Ethanol is substantially less toxic than methanol and gasoline at the same dosage levels (Lynd et al. 1991b). Carbon monoxide (CO) emission is caused by incomplete combustion of fossil fuels. Transportation sources account for over two-thirds of US CO emissions and automobiles account for 75% of all vehicle emissions (Wyman, Hinman, 1990). Older automobile engines often burn rich and produce excessive CO. A blend of 10% ethanol in gasoline reduces CO and unburned hydrocarbon emissions by ensuring a more complete combustion of gasoline because of the presence of oxygen in the ethanol molecule (Wyman, Goodman, 1993).

Nitrogen oxides (NO), unburned fuel and derivatives from vehicle exhaust are involved in the production of tropospheric ozone (ozone closest to the earth), which is the primary component of "smog" (Wyman, Hinman, 1990). Smog has been shown to impair respiratory function and damage vegetation. Ozone builds up when volatile organic compounds (VOC) and NO is released into the atmosphere. Transportation sources account for 30-50% of VOC emissions in The United States, emitted from the tailpipe, through evaporative loss in the fuel system and as evaporative loss during refueling (Wyman, Hinman, 1990).
Neat ethanol has the potential to reduce smog formation in cities substantially, such as London, Tokyo and Los Angeles, because ethanol has a lower volatility than gasoline and is less photochemically reactive (Wyman, Hinman, 1990; Lynd et al. 1991b).

1.4 Engine design for ethanol fuel

Ethanol is in many aspects superior to gasoline as fuels for spark-ignited engines. Combustion of ethanol in internal combustion engines designed for alcohols will give higher thermal efficiency and power than combustion of gasoline in conventional engines (Lynd et al. 1991b). This statement is somewhat contradictory to the finding that Volkswagen in Brazil has reported that VW engines designed for operation with hydrous ethanol consume about 22% (volume) more fuel than comparable VW gasoline engines (Grayson, 1984). Ford's experience indicates that approximately 15-25% more ethanol is used compared to gasoline to travel the same distance (Lynd et al. 1991b).

One of the major problems in application of neat fuel ethanol is the problem of starting the engine in cold weather. However, several suggestions have been made to solve this problem, including the addition of more volatile fuels to ethanol, the use of electric heaters to warm a small amount of fuel, or the use of a small catalytic alcohol reformer to produce enough hydrogen to start the engine (Wyman, Hinman, 1990).

1.5 Economics

Production of ethanol from lignocellulosic material with the technology currently available and under the present economic conditions in industrialized countries is not competitive with the cost of gasoline (Olsson, 1994). However, the global political awareness, manifested at the earth summit in Rio de Janeiro in 1992 and Kyoto in 1997, in actions against global CO_2 emission, display a growing political awareness to solve this environmental problem. Reduction of CO_2 emission could be met by tax reductions of non-fossil fuel production and more radically, increased tax on fossil fuel. This would, at once, make the non-fossil fuel price competitive to fossil gasoline.
Research for improvement of the production process from lignocellulose will gradually reduce the production cost of ethanol.

1.6 Conclusion

Excessive CO_2 emission from fossil fuel contribute to global heating and consequently melting of the ice poles, which will cause massive environmental changes to our planet. Reduction of CO_2 emission is therefore imperative. The transportation sector accounts for a significant part of the global CO_2 emission, released by combustion of liquid fuel, gasoline and diesel. However, at present, only a few fossil fuel alternatives are available. Ethanol is an excellent alternative for supplementing or replacement of liquid fossil fuel. Ethanol is a non-fossil fuel as no net emission of CO_2 is released into the atmosphere, because it is produced from photosynthesizing biomass, consuming the released CO_2. Ethanol may serve as a fossil fuel extender as 20% ethanol can readily be blended with gasoline and at the same time serve as an octane booster. By small adjustment of the engines, ethanol may serve as a neat fuel. Ethanol can be produced by biological conversion of lignocellululose biomass (e.g. softwood, hardwood, straws), however, presently at a price non-competitive compared to crude oil. Tax reduction of fuel ethanol would make the price competitive to various fossil fuels and thereby even more attractive for the consumers.

2 Thermophilic anaerobic ethanol producing bacteria

Thermophilic anaerobic ethanol producing species are found in three genera: *Clostridium*, *Thermoanaerobacter* and *Thermoanaerobacterium*, respectively. The most intensive investigated species are:

-*Thermoanaerobacter ethanolicus* (Wiegel, Ljungdahl, 1981; Kannan, Mutharasan, 1985)
-*Clostridium thermocellum* (Viljoen et al. 1926; McBee, 1954; Ng et al. 1981; Hörmeyer et al. 1988)
-*Clostridium thermohydrosulfuricum* (Wiegel et al. 1979; Dönmez, Özcelik, 1992; Cook, Morgan, 1994), (reclassified as *Thermoanaerobacter thermohydrosulfuricus* (Lee et al. 1993))
-*Clostridium thermosaccharolyticum* (McClung, 1935; Mistry, Cooney, 1989), (reclassified as *Thermoanaerobacterium thermosaccharolyticum* (Collins et al. 1994))

The species have been isolated from various geothermal habitats and man-made systems (sugar cane industry, pulping industry). Although they grow at thermophilic temperatures, they seem to be widely distributed in mesophilic environments. They are all rod-shaped bacteria, growing in a temperature range of 37-78°C, with optimal temperature around 70°C. They grow in a pH range of 4.4 to 9.9, with an optimum close to neutral pH, where *Thermoanaerobacter ethanolicus* has the broadest optimum growth range from pH 5.8 to 8.5 (table 2.1). They can ferment a variety of carbohydrates even of polymeric nature (table 2.1) (Wiegel, Ljungdahl, 1986; Slapack et al 1987). *Clostridium thermocellum* is the only species capable of fermenting cellulose and not D-xylose, while the remaining species ferment xylan and some species ferment hemicellulose as well. The end-fermentation product patterns are identical and the products are ethanol, acetate, lactate, CO_2 and H_2 (table 2.1).
These strains, except for *T. mathranii*, have mostly been studied for the conversion of cellulose or glucose as substrate. The strains have been examined during fermentation as a monoculture or as co-culture of cellulose- and D-xylose fermenting microorganisms.

2.1 Thermoanaerobacter mathranii

From previous studies on thermophilic, anaerobic xylan degrading bacteria in hot springs and man made systems, we have obtained a substantial culture collection of xylan and D-xylose degrading microorganisms (Mathrani, Ahring 1991; Sonne-Hansen et al 1993, Nielsen et al, 1993a; Nielsen et al 1993b). Several of these strains produced ethanol as the main end-fermentation product. This was investigated further in a screening test for selection of the best ethanol producing bacterium among our strains. Examination of growth and ethanol production in wet-oxidized hemicellulose hydrolysate and finally in high concentration of D-xylose were further included as success criteria. A total of 130 thermophilic strains were tested and *Thermoanaerobacter mathranii* fulfilled the success criteria defined for the screening test. This strain has been morphologically and physiologically characterized and examined further in our experimental work for conversion of D-xylose and pretreated hemicellulose hydrolysate into ethanol.

Table 2.1 Characteristics of selected thermophilic anaerobic ethanol producing bacteria.

	T.ethanolicus^T	*T. ethanolicus39E*	*T.thermohydrosulfuricus*^{T}	*T. thermosaccharolyticum*^{T}	*T. mathranii*^{T}
Gram staining	Variable	Variable	Variable	Negative	Variable
Cell size (µm)	0.5-0.8 x 4-100	0.5 x 2.7-5.0	0.4-0.6 x 1.8-8.0	0.4-0.7 x 2.4-16	0.5 x 2-8
Spores	No spores	Round terminal	Round terminal	Round/oval	Round terminal
Motility	+	+	+	+	+
Growth range (°C)	37-78	-	38-78	45-65	50-75
T_{Opt} (°C)	70	65	62-70	55-60	68-75
pH range	4.4-9.9	n.d.	4.8-9.7	n.d.	4.7-8.8
pH_{Opt}	5.8-8.5	n.d.	6.9-7.5	n.d.	6.8-7.8
Energy source:					
Cellulose	-	-	-	-	-
Hemicellulose	+	n.d.	-	-	+
Xylan	+	+	+	+	+
Glucose	+	+	+	+	+
Xylose	+	+	+	+	+
Arabinose	+	n.d.	-	n.d.	+
Galactose	+	n.d.	+	n.d.	-
Mannose	+	n.d.	+	+	+
Products:					
Ethanol	+	+	+	+	+
Acetate	+	+	+	+	+
Lactate	+	+	+	+	+
CO_2	+	+	+	+	+
H_2	+	+	+	+	+
Habitat	Hot spring USA	Hot spring USA	Hot spring, sewage plant USA	Industry processes	Hot spring Iceland
Reference	(Zeikus et al. 1980) (Wiegel, Ljungdahl, 1981)	(Ng et al. 1981) (Lovitt et al 1984)	(Wiegel et al. 1979) (Ljungdahl et al. 1981) (Cato et al. 1986)	(McClung, 1935) (Cato et al. 1986)	(Larsen et al. 1997)

+ = Yes, - = No, n.d. = Not determined.

3 Carbohydrate metabolism in thermophilic anaerobic bacteria

All of the examined saccharolytical thermophiles employ the Embden Meyerhof pathway for metabolism of glucose (Zeikus, 1980; Lamed, Zeikus, 1980a; Lamed, Zeikus, 1980b; Ben-Bassat, Zeikus, 1981) and the presence of a non-oxidative pentose phosphate pathway has been established in *C. thermohydrosulfuricum* (Ben Bassat, Zeikus, 1981).

3.1 Pentose phosphate pathway

D-xylose can be transported into the cell either by active transport or by facilitated diffusion as in *Saccharomyces cerevisiae* (Busturia, Lagunas, 1986; Van Zyl et al. 1989). Bacteria generally employ active transport mechanisms for the uptake of sugars and other nutrients, however, the mechanism is still unknown in *T. mathranii*. Inside the cell, D-xylose is isomerized to D-xylulose by the enzyme xylose isomerase.

Phosphorylation of D-xylulose to xylulose 5-phosphate marks the beginning of the non-oxidative part of the pentose phosphate pathway (Zubay, 1988) (figure 3.1). Xylulose 5-phosphate, ribulose 5-phosphate, ribose 5-phosphate, sedoheptulose 7-phosphate and erythrose 4-phosphate can undergo several inter-conversions catalyzed by two enzymes, transaldolase and transketolase, respectively (Zubay, 1988). They catalyze the conversion to the 3-carbon intermediary glyceraldehyde 3-phosphate and the six carbon intermediary fructose 6-phosphate, linking this pathway to the glycolysis (Zubay, 1988).

Transketolase catalyzes the reversible interconversion of four intermediates in the pentose phosphate pathway. Ribose-5-phosphate and xylulose-5-phosphate are converted to glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. Erythrose 4-phosphate and xylulose 5-phosphate are converted to fructose 6-phosphate and glyceraldehyde 3-phosphate (Zubay, 1988). Transketolase does not control the rate of xylose metabolism in metabolic engineered *S. cerevisiae* (Meinander et al. 1996).

Transaldolase catalyzes the reversible conversion of sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate. Transaldolase activity in recombinant *S. cerevisiae* fermenting D-xylose controls the xylose metabolism in aerobicly- but not anaerobicly grown *S. cerevisiae* cells (Meinander et al. 1996).
The non-oxidative part of the pentose phosphate pathway and its regulation has not been studied in detail in thermophilic anaerobic bacteria.

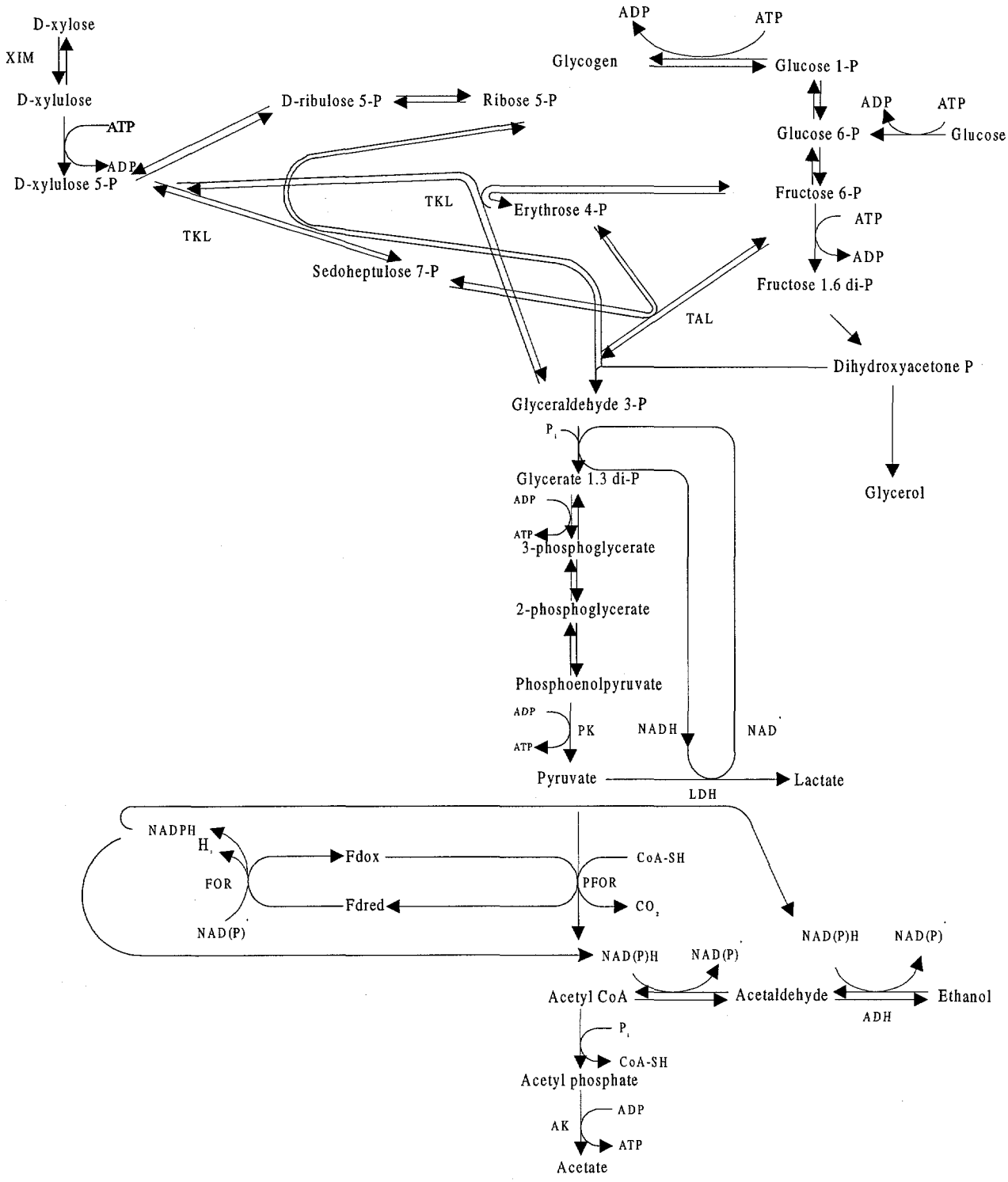

Figure 3.1 Model of anaerobic D-xylose and glucose metabolism in thermophilic anaerobic ethanol producing bacteria. XIM = Xylose isomerase, TKL = Transketolase, TAL = Transaldolase, LDH = Lactate dehydrogenase, FOR = NADPH-ferredoxin oxidoreductase, PFOR = Pyruvate-ferredoxin oxidoreductase, ADH = Alcohol dehydrogenase, AK = Acetate kinase, PK = Pyruvate kinase

The oxidative pentose phosphate pathway for generation of NADPH and ribose 5-phosphate for reductive biosynthesis, does not seem to be present in thermophilic anaerobic bacteria (Lamed, Zeikus, 1980a; Jones, Woods, 1991). Ribose 5-phosphate, however, is needed for reductive biosynthesis of histidine, pyridine and pyrimidine (Brock et al. 1984). The generation of this intermediate metabolite can be achieved in the non-oxidative part of the pentose phosphate pathway. D-xylulose-5-phosphate can be epimerized to D-ribulose-5-phosphate catalysed by D-ribulose 5-phosphate epimerase and can subsequently be isomerized to ribose-5-phosphate, catalysed by D-ribose 5-phosphate ketol-isomerase.

D-ribulose 5-phosphate epimerase was found to be essential for xylulose metabolism, while deletion of the D-ribose 5-phosphate ketol-isomerase-gene, with the same function, was lethal for *S. cerevisiae* (Miosga, Zimmermann, 1996). These findings indicate a crucial role of the xylulose 5-phosphate ribose 5-phosphate interconversion in the pentose phosphate pathway (Meinander et al. 1996).

3.2 Glycolysis

The metabolic flux of the pentose phosphate pathway is channeled into the upper and middle part of the Embden-Meyerhof pathway as fructose 6-phosphate and glyceraldehyde 3-phosphat, respectively (Zubay, 1988). Fructose 6-phosphate can be converted to glucose 6-phosphate by phosphohexoisomerase and, during anabolism, to glucose-1-phosphate and glycogen (figure 3.1). Fructose 6-phosphate and glyceraldehyde 3-phosphate are, during catabolism, converted via pyruvate to the different end fermentation products. This intermediate is a key metabolic branch point, determining the distribution of end-fermentation products. Ferredoxin is present in thermophilic anaerobic bacteria (Lovitt et al. 1988; Jones, Woods, 1991). The reduction and oxidation of this molecule is achieved by decarboxylation of pyruvate to acetyl-CoA, production of H_2 or generation of NAD(P)H, respectively. This key metabolic branch point can be controlled by the activities of four oxidoreductases (Jones, Woods, 1991).

Pyruvate ferredoxin oxidoreductase catalyzes the conversion of pyruvate to acetyl-CoA and CO_2 with a concomitant reduction of ferredoxin. The re-oxidation of reduced ferredoxin can be catalyzed by three different enzymes. H_2-ferredoxin oxidoreductase (hydrogenase) catalyzes the production of H_2, NADH and NADPH ferredoxin oxidoreductases, catalyses the production of NADH and NADPH, respectively. The activity of NAD^+ ferredoxin oxidoreductase (oxidation of ferredoxin and concomitant generation of NADH) seems to be low in thermophilic anaerobic bacteria and is strongly inhibited by NADH (Jungermann et al. 1973). *T. mathranii* A3M1 cannot grow on pyruvate as a sole carbon source. This indicated the absence of NAD^+-ferredoxin- oxidoreductase.

The NADPH oxidoreductase is believed to substitute the oxidative part of the pentose phosphate pathway for generation of NADPH for reductive biosynthesis (Jones, Woods, 1991). The generation of NAD^+ and $NADP^+$ by NAD^+ oxidoreductase and $NADP^+$ oxidoreductase, respectively, seem to be absent or very low due to generation of a redox potential by these reactions, unfavorable for generation of reduced ferredoxin (Jones, Woods, 1991). These oxidoreductases and their regulation have not been studied in general in anaerobic thermophiles (Jones, Woods, 1991).

The second branch point of the carbohydrate degradation is the conversion of acetyl-CoA to either acetate via acetyl-phosphate and the generation of extra ATP or ethanol and oxidation of NAD(P)H.

The formation of the final end-fermentation products and their mutual distribution is determined by the response of the cells to a given metabolic state, their need for energy, metabolites for reductive biosynthesis and reducing equivalents (Zeikus et al. 1981).

3.3 Product formation

Acetate and hydrogen production. The phosphoroclastic reaction converting pyruvate to acetylphosphate by the action of coenzyme A leads to production of extra ATP in the glycolysis. The ability to divert acetyl-CoA to regenerate ATP with acetate production is directly linked to the ability of the cell to produce H_2 (Jones, Woods, 1991). To divert all of the Acetyl-CoA to acetate, the NADH produced during glycolysis must be regenerated by the transfer of H_2 to ferredoxin. It can then be released as H_2 by hydrogenase. However, the equilibrium of this reaction is unfavorable as it involves a change to a more negative redox potential of H_2 and can only occur if the partial pressure of H_2 is kept very low (Jones, Woods, 1991). *T. mathranii* and related species can maintain the redox balance of the cell by production of other end-fermentation products like ethanol and lactate.

Lactate and carbon dioxide production. Conversion of pyruvate to lactate maintains the $NADH/NAD^+$ redox-balance in the glycolysis, as *T. mathranii* has an $NADP^+$-dependent alcohol dehydrogenase at low D-xylose concentrations. At high D-xylose concentration, *T.mathranii* switches to a NADH-dependent alcohol dehydrogenase. That could be a response to a low concentration of glyceraldehyde 3-phosphate, which influence the generation of NADH. Conversion of pyruvate to acetyl-CoA is a decarboxylation reaction, resulting in generation of CO_2. *T. mathranii* does not have a pyruvate decarboxylase enzyme, as in yeast, which converts pyruvate to acetaldehyde.

Ethanol production. Acetyl-CoA, which is not converted to acetyl phosphate, can be converted into acetaldehyde and subsequently to ethanol. Production of ethanol maintains the NADPH redox balance in *T. mathranii* as the cell performs reductive biosynthesis. Pyruvate has been found to be an allosteric *in vitro* activator of alcohol dehydrogenase in *T. ethanolicus* (Bryant, Ljungdahl, 1981) and in *T. thermohydrosulfuricum* (Carreira et al. 1982) and a high concentration of pyruvate in the cell activates the alcohol dehydrogenase.

3.4 Conclusion

The carbohydrate metabolism in thermophilic anaerobic bacteria is less studied than the metabolism of e.g. *E. coli* and *S. cerevisiae*. The non-oxidative pentose phosphate pathway is present in thermophiles, however, the presence of the oxidative pentose phosphate pathway for generation of NADPH for reductive biosynthesis seems to be absent. Thermophilic anaerobic ethanol producing bacteria produce a mixture of end-fermentation products, besides ethanol, in quantities regulated by the metabolic state caused by the particular fermentation conditions. The Embden Meyerhof pathway for generation of pyruvate is present in thermophiles. The *in vivo* regulatory mechanisms of both pathways are almost unknown and more knowledge of metabolic bottlenecks is needed for subsequent physiological and genetic engineering to improve the ethanol yield and productivity.

4 Measurements of intracellular enzymes of *T. mathranii* during continuous fermentation

Previous experiment with continuous fermentation of *T. mathranii* has established the optimal conditions for production of ethanol from D-xylose (EFP-94-0003 report, 1997). The optimal operational conditions were pH 6.0 and a retention time of 12 hours (table 4.1; table 4.2). Cells were extracted from the reactor for determination of specific enzymatic activities of selected intracellular glycolytic enzymes during varied operational conditions (table 4.3). These enzyme measurements showed that the optimal pH for the alcohol dehydrogenase was at pH 6.0.

Table 4.1. Steady state values for *Thermoanaerobacter mathranii* A3M1 grown on 35 mM xylose in a CSTR reactor under various pH conditions.

pH	OD_{578}-blind	Ethanol (mM)	Acetate (mM)	Lactate (mM)	Yield as ethanol/ acids	Inlet xylose (mM)	Outlet xylose (mM)	Consumed xylose (mM)	Consumed xylose (%)	Ethanol yield (mol ethanol/ mol xylose consumed)
5.0	0.46±0.07	21.1±1.1	**7.5 ± 0.8**	**1.6 ± 0.6**	**2.32**	46	11.8±0.9	**33**	72	0.62
5.5	0.60±0.07	15.5±1.2	9.0 ± 0.5	5.2 ± 0.4	1.09	28	4.1±0.9	24	86	0.65
6.0	0.59±0.03	**27.0±1.0**	9.4 ± 0.6	2.8 ± 0.5	2.21	37	**5.3±1.5**	32	**86**	0.84
6.5	0.62±0.01	20.3±1.3	6.3 ± 0.4	8.2 ± 2.1	1.40	36	4.5±0.7	31	86	0.65
7.0	0.58±0.03	19.3±0.6	8.9 ± 0.6	4.1 ± 1.1	1.48	30	10.8±0.5	19	63	**1.01**
7.5	0.50±0.03	9.0± 1.1	13.6 ± 0.4	0.5 ± 0.0	0.64	27	10.2±2.0	17	63	0.52
8.0	0.37±0.02	6.3± 0.7	8.6 ± 0.1	8.6 ± 0.1	0.36	19	13.4±3.7	6	32	1.12

(Measured by Dorte Licht)

Table 4.2. Steady state values for *Thermoanaerobacter mathranii* A3M1 under various pH conditions. The ethanol yield is calculated from an inlet xylose concentration at 35 mM

pH	Ethanol (mM)	Outlet xylose (mM)	Theo. Consumed xylose (mM)	Ethanol productivity (mmol/l/h)	Ethanol yield (mol ethanol/ mol xylose consumed)
5.0	21.1±1.1	11.8±0.9	23	**1.75**	**0.92**
5.5	15.5±1.2	**4.1±0.9**	**31**	1.29	0.50
6.0	**27.0±1.0**	**5.3±1.5**	**30**	**2.24**	**0.90**
6.5	20.3±1.3	**4.5±0.7**	**31**	1.68	0.65
7.0	19.3±0.6	10.8±0.5	24	1.60	0.80
7.5	9.0± 1.1	10.2±2.0	25	0.75	0.36
8.0	6.3± 0.7	13.4±3.7	22	0.52	0.28

(Measured by Dorte Licht)

Table 4.3. Measurements of intracellular specific enzyme activities of *T. mathranii* A3M1.

Ret. time reaktor (h)	pH	Ethanol (mM)	Alkohol Dehydrog (U/mg)	Acetat (mM)	Acetat kinase (U/mg)	Lactat (mM)	Lactat Dehydrog (U/mg)
10.0	6.0	25.0±2.0	0.091±0.0006	4.6±2.0	0.047±0.006	9.1±0.3	0.019±0.0001
11.0	6.0	13.9±1.1	1.2±0.0012	14.3±0.5	0.054±0.005	1.4±0.2	0.051±0.0001
12	6.0	30.0±0.8	2.20±0.07	12.2±0.8	0.021± 0.001	3.3±1.5	0.076±0.004
14.3	6.0	29.6±1.4	0.099±0.0008	11.0±0.6	0.061± 0.008	1.9±0.4	0.035±0.0002
17.5	6.0	22.7±1.5	0.094±0.005	12.1±1.4	0.023±0.003	2.5±1.8	0.029±0.0003
12	8.0	6.3±0.7	0.1±0.002	8.6±0.1	0.02 ± 0.003	0.2±0.1	0.12±0.0009
12	7.5	9.0±1.1	0.21±0.03	13.6±0.4	n.d.	0.5±0.03	0.030±0.001
12	7.0	19.3±0.6	0.81±0.05	8.9±0.7	0.053± 0.005	4.1±1.1	0.13 ± 0.002
12	6.5	20.3±1.3	0.33±0.06	6.3±0.4	n.d.	9.2±0.03	0.043±0.0001
12	6.0	27.0±1.0	2.42±0.13	9.4±0.7	0.020 ± 0.001	2.8±0.5	0.33 ± 0.03
12	5.5	15.5±1.2	0.3±0.01	9.0±0.5	0.043 ± 0.001	5.2±0.4	0.029±0.0003
12	5.0	21.2±1.1	0.2±0.01	7.5±0.8	n.d.	1.6±0.6	0.031± 0.001

n.d. Not determined

5 Extraction of metabolites from thermophilic anaerobic bacteria

Knowledge of the biochemical mechanisms responsible for decreased ethanol yield at increasing substrate concentration is scarce in thermophilic anaerobic ethanol-producing bacteria. Moreover, there is very limited knowledge of the intracellular enzyme activities and intracellular metabolite concentrations and their regulation during carbohydrate metabolism in this bacterial group (Zeikus et al. 1981).

In vivo measurements of intracellular metabolites along with *in vitro* measurements of intracellular enzymes have wide perspectives in the fundamental knowledge of the metabolism in thermophiles and hyper-thermophiles. Especially the regulation of different metabolic pathways has been difficult to interpret from measurements of the *in vitro* specific intracellular enzymes alone, with no knowledge of the intracellular pool of metabolites being catalyzed by the measured enzymes. Measurements of intracellular metabolites in addition to enzyme activity can reveal bottlenecks in a particular pathway and give way for genetic modification of the pathway, to improve biotechnological utilization of thermophiles and hyperthermophiles.

Extraction of intracellular metabolites from thermophilic anaerobic ethanol producing bacteria has only been conducted to a limited extent. Lovitt et al. (1988) acid extracted cells of *T. thermohydrosulfuricus* and measured the co-factors NAD^+/NADH in response to ethanol inhibition. Lamed and Zeikus (1980a) and Germain et al. (1986) applied the perchloric acid method (Skoog, Hahn-Hägerdal, 1989) for extraction of intracellular metabolites from *C. thermocellum*, *T. brockii* and *T. thermohydrosylfuricum*, respectively. Both groups measured only the intracellular concentration of fructose 1.6 di-phosphate.

5.1 Application

The biomass production is significantly higher in *S. cerevisiae*, an organism used for most metabolic studies (Jeppsson, et al, 1996). This is quite the opposite of thermophilic anaerobic bacteria, whereby the amount of withdrawn fermentation broth has to be significantly higher. When applying the extraction method on thermophilic anaerobic bacteria, this volume has to be adjusted to be able to measure the concentrations of intracellular metabolites. The application of continuous fermentation is also important to avoid metabolic response caused by pressure and broth volume changes when applying batch culturing. We extracted 100 ml fermentation broth from a Continuous Stirred Tank Reactor, equivalent to 26-40 mg dry weight cells. This quantity is sufficient for successful measurements of glycolytic intracellular metabolites by spectrofluorometric methods.

5.2 Conclusion

Extraction of intracellular metabolites from thermophilic anaerobic bacteria has only been done to a limited extent, but is a valuable tool for studying the metabolism of these microorganisms. The extraction of intracellular metabolites from *Thermoanaerobacter mathranii* has been successful in this thesis as a tool for identification of bottlenecks in the metabolic flux at elevated D-xylose metabolism. *In vitro* studies of the specific intracellular enzyme activities give only limited information of the *in vivo* specific activity of a given enzyme. Intracellular enzymes can be regulated by the concentration of the intracellular pool of the particular intracellular metabolites being catalyzed by the examined enzyme. The perspective of extracting intracellular metabolites from thermophiles is the identification and regulation of pathways and within the scope of

biotechnology, the first step in the application of metabolic and physiological engineering to improve yields of desirable product produces by these microorganisms.

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PAPER 1

Thermoanaerobacter mathranii sp. nov., an ethanol producing extremely thermophilic anaerobic bacterium from a hot-spring in Iceland

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ORIGINAL PAPER

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Thermoanaerobacter mathranii sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland

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Abstract The extremely thermophilic ethanol-producing strain A3 was isolated from a hot spring in Iceland. The cells were rod-shaped, motile, and had terminal spores; cells from the mid-to-late exponential growth phase stained gram-variable but had a gram-positive cell wall structure when viewed by transmission electron microscopy. Strain A3 used a number of carbohydrates as carbon sources, including xylan, but did not utilize microcrystalline cellulose. Fermentation end products were ethanol, acetate, lactate, CO_2, and H_2. The temperature optimum for growth was between 70 and 75°C, and growth occurred in the range of 50–75°C. The pH range for growth was 4.7–8.8, with an optimum at pH 7.0. Strain A3 was sensitive to tetracycline, chloramphenicol, penicillin G, neomycin, and vancomycin at 100 mg/l but was not sensitive to chloramphenicol and neomycin at 10 mg/l, which indicates that strain A3 belongs to the eubacteria. Addition of 50.66 kPa H_2 or 2% NaCl did not affect growth. The isolate grew in the presence of exogenously added 4% (w/v) ethanol. The G+C ratio was 37 mol%. 16S rDNA studies revealed that strain A3 belongs to the genus *Thermoanaerobacter*. Genotypic and phenotypic differences between strain A3 and other related species indicate that strain A3 can be assigned to a new species, and the name *Thermoanaerobacter mathranii* is proposed.

Key words Anaerobe · Thermophile · Xylose · Hemicellulose · Ethanol · Hot spring · Iceland · *Thermoanaerobacter mathranii*

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Introduction

Lignocellulosic material is an abundant substrate for the production of fuel ethanol. The hemicellulose fraction of the lignocellulose is dominated by pentoses, most significantly by D-xylose, which is not metabolized by conventional ethanol-producing microorganisms such as the yeast *Saccharomyces cerevisiae* (Hahn-Hägerdal et al. 1993). Thermophilic bacteria have been considered for ethanol production from biomass. The wide range of carbohydrates utilized by this group of bacteria is important in the conversion of complex substrates such as plant material, and also fermentation at elevated temperatures may be advantageous in the downstream processing of ethanol (Lynd 1989). Besides pentose sugars, hemicellulosic hydrolysates contain components such as sugar products and lignin degradation products that may have an inhibitory effect on the fermentation (Frazer and McCaskey 1989). In an initial screening for ethanol production on synthetic medium and hemicellulosic hydrolysates, strain A3 was superior to other isolates (Ahring et al. 1996).

The species considered for thermophilic ethanol production fall into cluster V and VII in the phylogenetic interrelationships according to Collins and coworkers (1994) and correspond to cluster A and C proposed by Rainey and coworkers (1993), respectively, of the thermophilic Clostridia. The taxonomy of the group was revised by Lee et al. (1993). The most investigated species for ethanol production from hemicellulose-derived sugars (mainly D-xylose and arabinose) are *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl 1981), *Thermoanaerobacter thermohydrosulfuricus* [Klaushofer and Parkkinen 1965; formerly *Clostridium thermohydrosulfuricum* (Lee et al. 1993)], and *Thermoanaerobacterium thermosaccharolyticum* [Collins et al. 1994; formerly *Clostridium thermosaccharolyticum* (McClung 1935)]. They all ferment pentoses and hexoses at 60–70°C with ethanol as the main product, but show mixed-acid fermentation with acetic acid as the most prominent side product.

This paper reports on the characterization of strain A3 based on morphological, physiological, and phylogenetic

studies. It is concluded that the strain belongs to the genus *Thermoanaerobacter*, and the name *Thermoanaerobacter mathranii* is proposed.

Materials and methods

Origin of the isolate and culture conditions

Strain A3 was found in a sediment sample at 70° C (pH 8.5) from an Icelandic hot spring in the Hverðagerdi-Hengil area and was isolated at 68° C (pH 8.4) on beechwood xylan as described by Sonne-Hansen and coworkers (1993).

The strain was isolated and cultured anaerobically in medium as previously described (Angelidaki et al. 1990) with the following modifications: yeast extract (1 g/l; Difco, Detroit, Mich., USA) was added to the medium, but cysteine was omitted; medium was dispensed (8.5 ml in 26-ml glass tubes) and autoclaved at 140° C for 20 min; D-xylose and vitamin solution DSM-141 (German Collection of Microorganisms and Cell Cultures) were added from filter-sterilized stock solutions prior to inoculation, and the medium was reduced with $Na_2S \cdot 7–9\ H_2O$ to a final concentration of 0.5 g/l. The final xylose concentration was 5 g/l, and cultures were incubated in the dark without shaking at 70° C (pH 7.0) unless otherwise stated. Media were inoculated with a culture (10%, v/v) in the late exponential growth phase to a final volume of 10 ml.

Morphological and physiological tests

Hucker's method (Doetsch 1981) was applied for Gram staining. Flagella were stained according to the method of Gray (Doetsch 1981). Catalase activity was tested with 3% (v/v) hydrogen peroxide on slants of liquid medium solidified with $MgCl_2 \cdot 6\ H_2O$ (0.9 g/l) and Gelrite (11.0 g/l; Merck, Darmstadt, Germany). H_2S production from the addition of a mixture of ferrous sulfate, sodium thiosulfate, and cysteine-HCl was measured photometrically as colloidal CuS by reaction with a copper reagent (Smibert and Krieg 1981; Cord-Ruwisch 1985). The latter cultures were reduced with ascorbic acid and sodium thioglycolate. Utilization of various carbon sources was examined by addition of 2 g/l of the substrate tested. Growth and substrate utilization were determined by measurement of optical density and, in the case of turbid substrates, by ethanol and fatty acid production. Media for pH experiments were modified by varying the amount of HCO_3^- in the medium and of CO_2 in the head-space gas. Growth was measured early in the exponential growth phase and pH was measured to verify that it had not changed significantly. For determination of tolerance to ethanol, various concentrations were added to media before autoclaving. Cultures were allowed to adapt once at each temperature, pH, or ethanol concentration and were transferred in mid-exponential growth phase. Experiments were performed at least in triplicate and were repeated once.

The bacteria were observed with a light microscope (Olympus BH-2), and photomicrographs were taken with an Olympus (Om-2) camera attached to the microscope.

G+C content and 16S rRNA sequencing analysis

The DNA base composition (mol% G+C) was determined by HPLC (Mesbah et al. 1989). 16S rRNA sequence analysis was performed at the DSM.

Analytical techniques

Ethanol, acetate, and propionate were analyzed by gas chromatography with flame ionization detection (FID). Gasses were identified and quantified by gas chromatography with thermal conductivity detection. High-performance liquid chromatography (HPLC) was employed for the measurement of lactate. Reducing sugars were measured with dinitrosalicylic acid using a method slightly modified from that of Miller (1959). Unless otherwise stated, growth was monitored by measuring the increase in turbidity at 578 nm in tubes inserted directly into a spectrophotometer.

Results

Colony and cell morphology

After 7 days surface colonies appeared white and irregular with a diameter of 1 mm when grown on 2 g/l beechwood xylan. The surface was granulated with top formation (Sonne-Hansen et al. 1993). Liquid cultures in the exponential growth phase showed straight, rod-shaped single cells (Fig. 1). Vegetative cells were 0.7 µm to 1.8–3.9 µm long. Sporulating cells were longer (6.4–8.2 µm) and spores were terminal and spherical. At suboptimal conditions, such as a temperature of 75° C, chains and long single cells were observed. The reaction to Gram stain was variable. Transmission electron micrographs of late-exponential-phase cultures showed a gram-positive cell wall structure (not shown).

Physiological and biochemical properties

Optimal temperature for growth was between 70 and 75° C (Fig. 2); no growth was observed at 47 or 78° C. The highest growth rate was obtained at pH 6.8–7.8, 4.7 and 8.8 being the most extreme pH values. The generation time of strain A3 on 2 g/l xylose was 74 min at 69° C and pH 7.0; after termination of growth, the pH of the medium was 5.9. A time course of xylose fermentation, growth, and product formation is shown in Fig. 3. The production of ethanol and lactate was slightly delayed as compared to the growth of the bacterium. The fermentation balance on

Fig. 1 Photomicrograph of strain A3 after 18 h of incubation at 70° C (pH 7) (*bar* 5 µm)

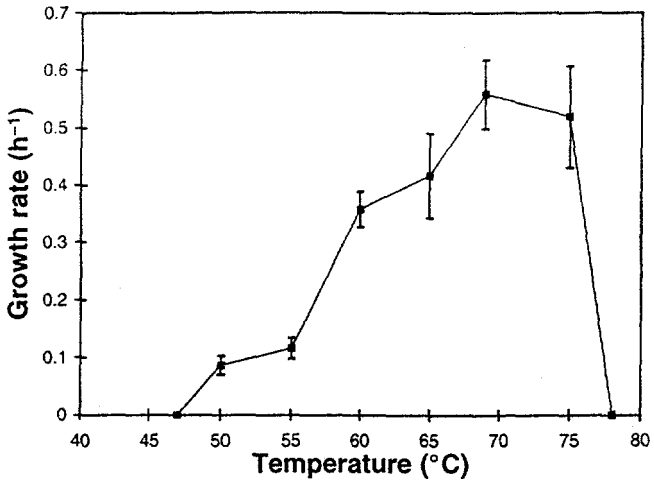

Fig. 2 The effect of temperature on the growth rate of strain A3. *Bars* indicate the standard deviations of at least six replicates

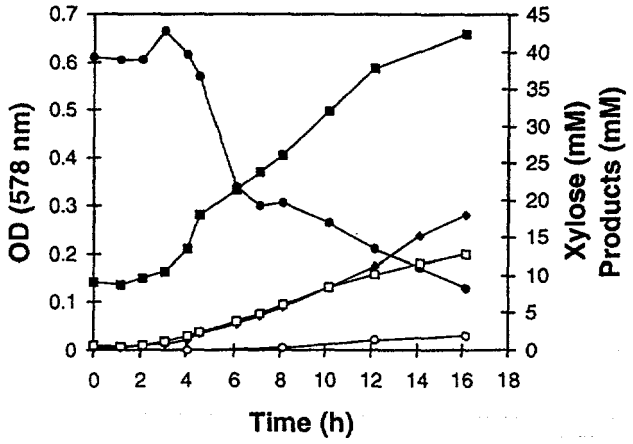

Fig. 3 Relationship between growth and product formation during xylose fermentation. The experiment was performed in a media volume of 250 ml, and 3 ml were taken out at each time point. Samples for measurement of growth were reduced with crystals of $Na_2S \cdot 7$–$9\ H_2O$ and measured in cuvettes. ■ Optical density, ● D-xylose, ◆ ethanol, □ acetate, ○ lactate

13.3 mM D-xylose of a culture grown to the stationary phase can be described as follows (in mol quantities):

$$C_5H_{10}O_5 + H_2O \rightarrow 1.1\ C_2H_5OH + 0.4\ CH_3COOH + 0.06\ CH_3CHOHCOOH + 1.81\ CO_2 + 0.9\ H_2$$

The carbon recovery was 99.9% and the O/R was 1.2.

The following carbohydrates were utilized by the bacterium: amygdalin, L-arabinose, cellobiose, D-fructose, D-glucose, glycogen, lactose, maltose, D-mannitol, mannose, melibiose, melizitose, raffinose, D-ribose, sucrose, trehalose, xylan, and D-xylose. However, avicel, casein-peptone, cellulose, D-galactose, glycerol, inulin, pectin, L-rhamnose, salicin, sorbitol and yeast extract (Difco) were not fermented. Yeast extract was required as a cofactor for growth at the vitamin concentration applied, and at 1 g/l of yeast extract the addition of vitamins had no effect on growth. Sulfide production from casein-peptone, sulfate, and thiosulfate was detected, but catalase activity was not observed.

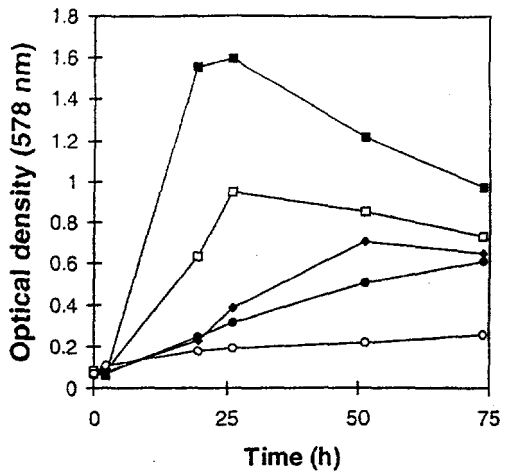

Fig. 4 The effect of exogenously added ethanol (w/v) on the growth of strain A3. ■ 0%, □ 1%, ◆ 2%, ● 3%, ○ 4%

Sensitivity to antibiotics, H_2, NaCl, and ethanol

Strain A3 was effectively inhibited by tetracycline, chloramphenicol, penicillin G, neomycin, and vancomycin, all at 100 mg/l. However, at 10 mg/l the isolate grew in the presence of chloramphenicol and neomycin. This indicates that strain A3 belongs to the eubacteria (Böck and Kandler 1985). Overpressure (50.66 kPa) of H_2 and 2% NaCl did not affect growth.

Figure 4 shows the sensitivity of strain A3 towards exogenous ethanol. The isolate grew in the presence of 4% (w/v) added ethanol. Growth was observed in one culture with 5% ethanol added. Adaptation was evident in the third subculture (data not shown).

Phylogenetic analysis

The G+C content of the total DNA of strain A3 was 37 mol%. The results of the analysis of the complete sequence of 16S rDNA (EMBL accession No: Y11279) are presented as a similarity matrix (Table 1) and as a phylogenetic dendrogram (Fig. 5) showing the position of strain A3 within the radiation of the members of the genus *Thermoanaerobacter*. Strain A3 and *Thermoanaerobacter thermocopriae* form a subcluster within the genus that branches off below other species of the *Thermoanaerobacter*. Intracluster 16S rDNA similarity values for strain A3 within the *Thermoanaerobacter* ranged between 93.3 and 98.2%.

Discussion

Examination of the complete 16S rDNA sequence showed that strain A3 belongs to cluster V of the clostridia, as described by Collins and coworkers (1994). Strain A3 had a similarity of 94.9–98.2% with the closest species *T. thermocopriae* (formerly *Clostridium thermocopriae*; Jin et al. 1988; Collins et al. 1994), *Thermoanaerobacter*

Table 1 16S rDNA similarity values for strain A3 and representatives of the thermophilic clostridia

Strains	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1. Strain A3	–											
2. *Thermoanaerobacter thermocopriae*	98.2	–										
3. *Thermoanaerobacter acetoethylicus*	93.5	93.3	–									
4. *Thermoanaerobacter ethanolicus*	93.4	93.2	99.1	–								
5. *Thermoanaerobacter thermohydrosulfuricus*	93.3	93.2	96.9	97.2	–							
6. *Thermoanaerobacter brockii*	95.5	95.0	95.8	95.3	95.8	–						
7. *Thermoanaerobacter finnii*	94.9	94.4	95.3	94.8	95.5	98.9	–					
8. *Thermoanaerobacter ethanolicus* 39E	95.6	95.1	96.0	95.5	95.5	99.3	98.7	–				
9. *Thermoanaerobacter kivui*	93.6	93.4	94.3	94.2	95.5	95.2	94.7	95.5	–			
10. *Moorella thermoacetica*	87.4	87.4	87.4	87.3	87.5	88.4	88.1	88.0	88.1	–		
11. *Thermoanerobacterium thermosulfurigenes*	85.7	85.7	86.4	86.4	87.4	86.6	86.6	86.4	87.3	86.4	–	
12. *Caldocellosiruptor saccharolyticus*	84.4	84.9	83.8	83.5	83.3	84.2	83.9	84.5	84.0	83.5	82.7	–
13. *Desulfotomaculum australicum*	83.5	83.3	83.5	83.4	83.8	83.8	83.5	84.1	84.0	84.9	81.2	78.8

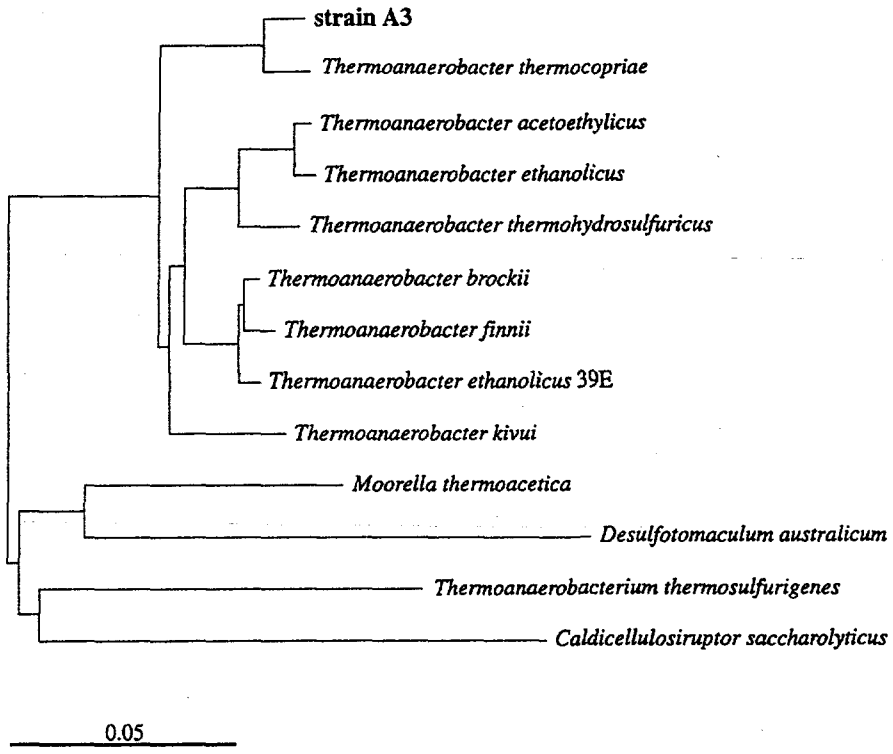

Fig. 5 Phylogenetic tree based on 16S rDNA sequence analysis showing the position of strain A3 among cluster V of the Clostridia (Collins et al. 1994), the genus *Thermoanaerobacter*. *Thermoanaerobacter thermocopriae* was formerly *Clostridium thermocopriae*, and *Thermoanaerobacter kivui* was formerly *Acetogenium kivui* (Collins et al. 1994) (*bar* 5% nucleotide substitutions)

ethanolicus 39E (formerly *Clostridium thermohydrosulfuricus* 39E; Zeikus et al. 1980; Lee et al. 1993), *Thermoanaerobacter brockii* (formerly *Thermoanaerobium brockii*; Zeikus et al. 1979; Lee et al. 1993), and *Thermoanaerobacter finnii* (Schmid et al. 1986). Characteristics of the species are listed in Table 2. These strains are all thermophilic, rod-shaped bacteria that form round-to-oval terminal spores. As far as sensitivity to antibiotics has been investigated, no differences in sensitivity patterns have been found.

T. thermocopriae was the closest related species according to the 16S rDNA analysis. As illustrated in Table 2, *T. thermocopriae* differs from strain A3 with respect to both the physiology and the carbohydrates utilized. The former stains gram-negative and has a temperature optimum at 60° C, 10° C lower than strain A3. They also differ in cellulolytic ability and in the utilization of six other carbohydrates examined, and *T. thermocopriae* has butyric acid, which is not produced by strain A3, among its major products.

T. ethanolicus, *T. brockii*, and *T. finii* differed significantly from strain A3 with respect to 16S rDNA similarity values, and *T. brockii* and *T. finii* have a much lower G+C content. Furthermore, as opposed to strain A3, both *T. brockii* and *T. finii* ferment galactose and neither ferment arabinose.

When comparing *T. ethanolicus*^{T} with strain A3, some major differences were obvious. *T. ethanolicus*^{T} does not

Table 2 Characteristics of *Thermoanaerobacter* species closely related to strain A3 (*nd* not determined)

	Strain A3	*T. thermocopriae*^{T}	*T. ethanolicus* 39E	*T. brockii*^{T}	*T. finnii*^{T}	*T. ethanolicus*^{T}
16S rDNA similarity with A3 (%)		98.2	95.6	95.5	94.9	93.4
G+C (mol%)	37.0	37.2	nd	31.0	32.0	32/37–39
Gram staining	Variable	Negative	Variable	Positive	Variable	Variable
Cell size (μm)	0.5 × 2–8	0.4–0.7 × 2–8	0.5 × 2.7–5.0	0.8–1.0 × 2–20	0.4–0.6 × 1–4	0.5–0.8 × 4–100
Spores	Round terminal	Round terminal	Round terminal	Round terminal	Oval terminal	No spores
Motility	+	+	+	–	+	+
Temperature optimum	70° C	60° C	65° C	70° C	65° C	69° C
pH optimum	6.8–7.8	6.5–7.3	nd	7.5	6.5–6.8	5.8–8.5
Energy source						
Cellulose	–	+	–	–	–	–
Galactose	–	+	nd	+	+	+
Salicin	–	+	nd	nd	nd	nd
Sorbitol	–	+	nd	nd	nd	–
Arabinose	+	+	nd	–	–	nd
Mannose	+	+	nd	–	+	+
Mannitol	+	–	nd	nd	+	–
Melibiose	+	–	nd	nd	nd	–
Melizitose	+	–	nd	nd	nd	–
Products						
Ethanol	+	+	+	+	+	+
Acetate	+	+	+	+	+	+
Lactate	+	+	+	+	+	+
Butyrate	–	+	–	–	–	–
CO_2	+	+	+	+	+	+
H_2	+	+	+	+	+	+
Habitat	Hot spring Iceland	Cattle dung, hot-spring, Japan	Hot spring, Yellowstone National Park	Hot spring, Yellowstone National Park	Lake sediment, East Africa	Hot spring, Yellowstone National Park
Reference	Our isolate	Jin et al. (1988)	Zeikus et al. (1980) Ng et al. (1981)	Zeikus et al. (1979) Cook et al. (1991)	Schmid et al. (1986)	Wiegel and Ljungdahl (1981)

produce terminal endospores, produces lactate in high concentrations (inhibited by H_2 added), and has a broader growth range with respect to pH (4.4–9.8) and temperature (37–78° C). Furthermore, *T. ethanolicus*^{T} does not ferment amygdalin, mannitol, melibiose, melizitose, raffinose, or trehalose, as strain A3 does; in addition, it grew on galactose, which was not used by strain A3. Finally, 16S rDNA differences between strain A3 and *T. ethanolicus*^{T} are significant (93.4% similarity). These morphological and physiological differences and the large phylogenetic distance clearly justify the separation of the two strains as two separate species.

The low tolerance to ethanol found among thermophilic bacteria [generally below 1% (w/v)] may be an impediment to the application of this group of organisms for industrial ethanol production due to the high cost of distillation at low ethanol concentrations (Lynd 1989). The problem may be overcome by continuous ethanol removal by specialized systems such as membrane distillation (Mulder and Smolders 1986) or by adaptation of the microorganisms to ethanol. In this work, strain A3 grew at 4% ethanol (w/v) without previous adaptation. *T. ethanolicus* 39E growing on glucose has previously been adapted to an ethanol concentration of 3.3% (w/v) at 68° C (Lovitt et al. 1984). At 45° C the latter grew well even at 8% ethanol.

Strain A3 has previously been reported to grow and produce ethanol in pretreated hemicellulosic hydrolysates from wheat straw (Ahring et al. 1996). The high tolerance to ethanol further verifies the potential of this strain.

Description of *Thermoanaerobacter mathranii* sp. nov.

Thermoanaerobacter mathranii (sp. nov.) ma.thra(ni.i. M.L.gen.n., of Mathrani, in honor of the late Indra M. Mathrani, who contributed greatly to our understanding of thermophilic anaerobes from hot springs during his short career.

Gram-variable, straight, rod-shaped cells. Vegetative cells in exponential growth phase are 0.7 × 1.8–3.9 μm. Terminal spherical spores that swell the cells. Cells are

longer when sporulating or at suboptimal conditions. Occur singly or, at sub-optimal conditions, in long chains.

Catalase-negative. Hydrogen sulfide is produced. Grows on amygdalin, L-arabinose, cellobiose, D-fructose, D-glucose, glycogen, lactose, maltose, D-mannitol, mannose, melibiose, melizitose, raffinose, D-ribose, sucrose, trehalose, xylan, and D-xylose, but not on avicel, casein-peptone, cellulose, D-galactose, glycerol, inulin, pectin, L-rhamnose, salicin, sorbitol, or yeast extract (Difco). Yeast extract is required for growth when a vitamin solution is added at 0.1 ml/10 ml culture. Inhibited by tetracycline, chloramphenicol, penicillin G, neomycin, and vancomycin at 100 mg/l. Grows in the presence of chloramphenicol and neomycin at 10 mg/l. Insensitive to 50.66 kPa overpressure of H_2 and 2% NaCl.

DNA base composition of 37 mol% G+C.

Isolated from biomat and sediment from a slightly alkaline hot spring in Hverðagerdi-Hengil, Iceland.

The type strain, *Thermoanaerobacter mathranii* A3, has been deposited in the DSM culture collection under accession no. 11426.

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PAPER 2

Comparison of the cold methanol and the perchloric acid methods for extraction of intracellular metabolites from recombinant, xylose metabolising *Saccharomyces cerevisiae*

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Comparison of the cold methanol and the perchloric acid methods for extracting intracellular metabolites from recombinant, xylose metabolising *Saccharomyces cerevisiae*

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Summary

Metabolic engineering can change the metabolic pathways of a microorganism, increasing the metabolic flux at key points and changing the product pattern. In order to perform metabolic engineering on a microorganism, knowledge and quantification of the different enzymes and intracellular metabolites are needed. This requires a method ensuring extraction of all the metabolites from the cells, representing the *in vivo* condition of the ongoing experiment. The cell metabolism should be stopped instantaneously, in order to obtain i*n vivo* measurements of intracellular metabolites. We compared the traditionally perchloric acid extraction method and the newly developed cold methanol extraction method on recombinant *Saccharomyces cerevisiae* S104. The extractions were conducted on the same series of batch experiments (within 30 minutes interval), and we measured glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and pyruvate extracted by the two methods. We found that the cold methanol method was the most reliable method having the best reproducibility between repeated experiments. We also observed that when glucose was depleted in a xylose/glucose fermentation with recombinant *S. cerevisiae* S104, the intracellular fructose 6-phosphate concentration decreased below the detection limit and the concentration of fructose 1,6-diphosphate decreased markedly.

Key words: Recombinant *Saccharomyces cerevisiae*; extraction of intracellular metabolites; perchloric acid; cold methanol; glycolysis; xylose metabolism

Introduction

The yeast *Saccharomyces cerevisiae* is well established for industrial ethanol production. It can tolerate high concentration of inhibitors, which makes it a suitable candidate for ethanol production from lignocellulosic substrate (Olsson, Hahn-Hägerdal, 1993). However, it cannot convert the major pentose of lignocellulose, D-xylose, into ethanol (Wang et al. 1980), a prerequisite for an economically viable ethanol production from lignocellulose (Wright, 1988), (Lynd, 1990), (von Sivers, Zacchi, 1995). Metabolic engineering can change the metabolic pathways of a microorganism, increasing the metabolic flux at key points and changing the product pattern. The intracellular metabolites and enzymes affected by the metabolic engineering have to be quantified, but in order to obtain information about the *in vivo* metabolic state at the time of sampling, several conditions have to be fulfilled. The metabolic activity of baker´s yeast is high, a typical half-life of metabolites being in the order of seconds or less, requiring rapid inactivation of the enzymes in the sampling step (Saez, Lagunas, 1976), (De Koning, van Dam, 1992), (Gancedo, Gancedo, 1973). To obtain reliable results, the metabolite concentrations must remain constant throughout the extraction, storage and measurement period (Klein et al., ?). Thus, an extraction method that inactivates the metabolism instantaneously and prevent subsequent breakdown or modification of metabolites is required, in order to measure the *in vivo* metabolite concentrations.

Extraction of intracellular metabolite with perchloric acid has been used in several studies (Skoog, Hahn-Hägerdal, 1989), (Kötter, Ciriacy, 1993), (Senac, Hahn-Hägerdal, 1990), (Ciriacy, Porep, 1986). In this method, cells are filtered, washed and inactivated by immersion in liquid nitrogen. The advantage of this method is a concentration of the cells in the filtration step, increasing the concentration of the metabolites in the final extract. The

major disadvantages are extraction at extreme pH, causing degradation of some metabolites, and the long time needed for filtration and washing, which cannot be done in less than 10 seconds. This time scale is believed to influence the metabolite concentrations in the cells (De Koning, van Dam, 1992). In a newly developed extraction method (de Koning & van Dam, 1992), the cold methanol method, the cell metabolism is inactivated in 0.1 second by immersion in -40°C methanol without filtration of the cells. The extraction procedure is conducted at -20°C to -40°C and at neutral pH. In contrast to the perchloric acid method, rapid inactivation of metabolic activity and prevention of the pH dependent co-factors like NAD(P)/NAD(P)H is achieved. The disadvantage of this method is the need for up-concentration of the metabolites from the dilute extraction broth in an evaporation step. This operation is conducted at above zero degrees for several hours, which can degrade some of the labile metabolites.

In this work the perchloric acid method and the cold methanol method was applied to the same series of batch experiments, within 30 minutes intervals, with the purpose of comparing the concentrations of intracellular metabolites extracted by the two methods. The recombinant *Saccharomyces cerevisiae* S104 transformed with the genes *XYL1* and *XYL2* from *Pichia stipitis* encoding the enzymes xylose reductase and xylitol dehydrogenase, respectively (Walfriedsson et al. 1995)was used in the experiments. The recombinant strain was able to utilise xylose, but the consumption rate was low and the formation of by-products reduces the ethanol yield. In experiments with a mixed sugar fermentation of xylose and glucose, it was shown that the xylose consumption rate decreased when glucose was depleted (Walfriedsson et al. 1995)(Meinander et al., 1997). We compared the intracellular concentrations of some key intermediary metabolites during the simultaneous xylose and glucose consumption phase, and at the time point of glucose depletion, when the cells metabolise only xylose.

Materials & Methods

Microorganism

The recombinant *S. cerevisiae* strain S104 (Walfriedsson et al. 1995), based on the strain H158 GPY55-15Bα (leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir^+) obtained from Gregg Payne (University of California, Berkeley) containing the plasmid pHWY10 expressing the genes encoding xylose reductase and xylitol dehydrogenase was used in the experiments.

Media & inoculum

SC-leu plates (Sherman et al. 1983) contained per litre: Yeast Nitrogen without amino acids base (DIFCO Laboratories, Detroit Michigan, USA), 6.7 g; BACTO AGAR (DIFCO Laboratories, Detroit Michigan, USA), 20 g; Glucose, 20 g; histidine, tryptophan and uracil, 0.05 g.

A selective synthetic medium (Verduyn et al. 1992) supplemented with 0.05 g/L histidine, tryptophan, and uracil was used for all liquid cultures. 0.01 g/L ergosterol and 0.42 g/L Tween 80 (Sigma) was added in anaerobic fermentations. A frozen stock suspension of *S. cerevisiae* S104, stored at -80°C, was inoculated on SC-leucine plates, and incubated at 30°C for 72 hours. For the aerobic batch cultivations, a 250 ml baffled flask containing 50 ml liquid media with 30 g/L glucose as carbon source was inoculated from the plate, and incubated until mid-exponential growth phase (24-48 hours, depending on lag phase). The culture was used as such to inoculate aerobic batch cultivations. For the anaerobic fermentations, 1 litre flasks containing 500 ml media were inoculated from a plate and incubated for 48-72 hours. The inoculum was centrifuged at 4000 x g for 15 minutes, washed and resuspended in 100 ml 0,9% (w/v) NaCl, before inoculation of the anaerobic batch cultivation.

Batch fermentations

The aerobic batch cultivations were conducted in 1 litre baffled flasks containing 500 ml media with 30 g/L glucose as carbon source incubated at 30°C at 140 rpm. Metabolite extractions were conducted at mid exponential growth phase, determined to be at an optical density of around 4. Prior to extraction of the metabolites, 50 ml cultivation broth was removed for protein and enzyme activity determinations, and centrifuged at 4°C for 15 minutes at 4000 x g. The anaerobic batch fermentations were conducted as previous described (Meinander et al. 1997) with 15 g/L xylose and 30 g/L glucose as carbon source. Metabolite extractions were conducted at two sampling points...

Sampling of intracellular metabolites

Aerobic growth. A water-bath containing the culture was placed in immediate vicinity of the cold methanol bath and the equipment for percloric acid extraction in order to ensure fast transfer of cells. 5 or 10 ml culture broth was transferred from the baffle flask, with a 5 ml pipette, into the cold methanol and to the filter dish for the perchloric acid.

Anaerobic growth. 5 or 10 ml culture broth was withdrawn from the fermentor with 10 ml syringes, and rapidly sprayed into the cold methanol or onto the filter dish for the perchloric acid. For both extraction methods in each experiment, at least triple samples were taken.

Extraction methods

In the cold methanol method, the cells were sprayed into -40°C methanol and after five minutes, the cells were centrifuged (4000 x g) at -20°C for 20 minutes. The methanol was

removed and the cells were resuspended in a -40°C methanol/buffer (Pipes/EDTA)/ chloroform mixture. The metabolites were extracted by shaking (2200 rpm) at -20°C for 45 minutes. After collection of the methanol/buffer phase containing the metabolites, fresh methanol/buffer was added and the extraction was repeated. The extracts were pooled and traces of fatty acid were removed by ether extraction. The extracts were vacuum evaporated to a volume below 1 ml in a Speedvac (Savant Instruments, Hicksville, NY) for 2-4 hours at 0-4°C. The protocol of the original cold methanol method (de Koning & van Dam, 1992) was used with following modifications: Methanol bath without circulation was used and the metabolites were extracted at -20°C instead of -35°C. Broth volume of 5 ml and 10 ml were extracted (5 ml broth volume contained 1.0-1.8 mg/ml dry weight biomass), whereas 10-15 ml broth volumes containing 50 mg/ml wet weight biomass were used in the original method.

The perchloric acid method was conducted as previous described (Skoog, Hahn-Hägerdal, 1989). The cells were rapidly filtered, washed with ice cold Milli-Q-water, and inactivated by immersion of the filter in liquid nitrogen. The filter was broken apart in a dry ice cooled mortar, and transferred to 1.2 M perchloric acid. The metabolites were extracted into the perchloric acid by vigorous shaking (2200 rpm, 20 minutes at room temperature) and neutralised with potassium bicarbonate. The supernatant was collected after centrifugation (+4°C) and kept at -80°C until analysis.

Analyses

The extracellular concentrations of the substrates xylose and glucose and the fermentation products acetate, ethanol, xylitol and glycerol in the broth were analysed by HPLC as previous described (Meinander et al. 1997). Biomass concentration was analysed by optical density (OD) measurements on an UV-120-02 spectrophotometer (Shimadzu, Kyoto, Japan)

at 620 nm, and by dry weight determinations (Yu et al. 1995), respectively. Xylose reductase and xylitol dehydrogenase activites were measured as previous described (Walfriedsson et al. 1995), from cell lysates prepared from harvested cells by shaking with glass beads (Meinander et al. 1996). The protein concentration of the cell lysates was measured according to (Bradford, 1976), using bovine serum albumin as standard. Glucose 6-phosphate and fructose 6-phosphate concentrations in the metabolite extracts was analysed spectrophotometrically (Bergmeyer, 1974), using a Shimadzu UV-120-02 (Kyoto, Japan). Fructose 1,6-diphosphate and pyruvate were analysed by a fluorometric method (Jeppsson et al. 1996).

Results

Two identical aerobic baffled flask cultivations with glucose as carbon-source and two anaerobic fermentations, with xylose and glucose as carbon sources were conducted in order to determine the inter-experimental deviations. At each sample point for determination of intracellular metabolites, at least triple samples were taken in order to determined the intra-experimental deviations. In order to check the recovery, a standard solution of 2.2 mM glucose 6-phosphate was extracted by both methods. The recovery was 2.1–2.2 mM with the cold methanol method and 2.1 mM with the perchloric acid method. Different final methanol concentrations (40, 48, 53 and 64% v/v) were tested as medium for inactivation of metabolic activity. When comparing the concentration of extracted metabolites at each methanol concentration, no significant differences could be observed (table 1).

TABLE 1

Amount of sample volume

With the perchloric acid extraction method, the concentration of glucose 6-phosphate and fructose 6-phosphate extracted from 5 ml sample volumes (5.2-8.0 mg d.w. cells), were in most cases below the detection limit of a spectrophotometer (table 1).

Using the cold methanol method, the metabolite concentrations were, with a few exceptions, above the detection limit with both sample volumes, and no significant intra-experimental differences could be observed (table 1). The data in table 1 was simplified by taking the average of the results obtained with different final methanol concentrations and different sample volumes for each fermentation (table 2).

The sample points were taken in mid exponential growth phase determined to be at an optical

density of four. The differences in extraction times between the four batch experiments were due to different lag-phases of up to 26 hours. Sample for the cold methanol method was taken at the indicated sampling times and samples for the perchloric acid extractions, 30 minutes later.

TABLE 2

Cold methanol methodCold methanol method

Using the cold methanol method glucose 6-phosphate, fructose 1,6-diphosphate and pyruvate were detectable in both 5 ml and 10 ml sample volumes (table 1). Fructose 6-phosphate could not be detected in the first aerobic cultivation experiment and was only detectable in 10 ml sample volumes in the anaerobic fermentations. In the first aerobic cultivation, a lower value of glucose 6-phosphate was measured compared to the second experiment.

Perchloric acid methodPerchloric acid method

With the perchloric acid method glucose 6-phosphate was not detectable with 5 ml sample volumes, with an exception in the second anaerobic fermentation experiment. Fructose 6-phosphate could not be detected in the anaerobic fermentation experiments (table 2). The inter-experimental reproducibility of the measured values of glucose 6-phosphate and fructose 6-phosphate were fairly poor. Fructose 1,6-diphosphate and pyruvate could be detected in both sample volumes (table 2).

Diskussion af intra-eksperimentelle forskelle

Based on these observations, it can be concluded that the application of the cold methanol method for extracting intracellular metabolites are more accurate and reproducible as opposed to the perchloric acid method.

Intracellular metabolite concentrations during xylose/glucose fermentationIntracellular metabolite concentrations during xylose/glucose fermentation

The simultaneous fermentation of xylose and glucose (figure 1) was a repetition of a previously conducted identical experiment (Meinander *et al.* submitted). The extracellular product pattern and XR and XDH activities obtained in the present fermentation agreed well with those previously reported. In the previous investigation, a marked decrease in xylose consumption rate and the increase in the excretion of xylitol was noted, after depletion of glucose. This could be due to that glucose metabolism sustains higher glycolytic metabolite concentrations than xylose metabolism, ensuring efficient activation and induction of glycolytic and ethanologenic enzymes. In the present investigation intracellular metabolite concentrations were analysed at two different sampling points during the xylose/glucose fermentation, indicated in Fig 1. Firstly, during exponential growth when glucose and xylose were simultaneously consumed and secondly, at the point of glucose depletion, i.e. the transition between mixed consumption of glucose and xylose and consumption of xylose alone. This results show that the intracellular concentration of glucose 6-phosphate remained constant at the point of glucose depletion, whereas fructose 6-phosphate concentration decreased below the detection limit and fructose 1,6-diphosphate and pyruvate concentrations decreased markedly (table 3).

Discussion

Intracellular metabolites can be extrated by several methods, all with the objective of displaying the *in vivo* metabolic state of the cell at the time of extraction. Saez & Langunas (1976) among others, have argued that the critical point of obtaining the *in vivo* condition, is the time needed for inactivation of the cell metabolism. de Koning and van Dam (1992) argue further, that working at low temperatures during extraction is equally essential, to minimize degradation of labile metabolites. The metabolic activity of baker´s yeast is high, a typical half life of metabolites being in the order of seconds or less (Saez, Lagunas, 1976), (De Koning, van Dam, 1992), (Gancedo, Gancedo, 1973). The time for inactivation of metabolism and extraction temperature, are the two main differences between the traditional perchloric acid extraction and the newly developed cold methanol method.

A disadvantage of the perchloric acid method is that inactivation of metabolism cannot be accomplished in less than 10 seconds, due to the filtration step. On this time scale the concentration of metabolites can change significantly. In the cold methanol method the quenching of metabolism is performed in 1-2 seconds, and with special sampling equipment, in less than one second (de Koning & van Dam, 1992).

The temperature could be important in terms of minimising the degradation of labile metabolites during the extraction procedures. The temperature varies substantial between the two methods. The perchloric acid extraction is conducted at above zero degrees, and the cold methanol at -20°C to –40°C.

The perchloric acid method has the advantages that the cells are separated from the growth medium before the extraction, increasing the concentration of metabolites. In the cold methanol method, cells and media are not separated, giving a dilute solution of metabolites at the end of the extraction. Vacuum evaporation of excess methanol and water is therefore

needed. This procedure is conducted at above zero degrees for 4-6 hours, a critical step in terms of the stability of some of the labile metabolites.

In order to compare these two extraction methods, we sampled recombinant yeast cells from the same batch experiment, and applied the two different extraction protocols for recovery of intracellular metabolites. Spectrophotometric measurements of glucose 6-phosphate and fructose 6-phosphate from cells extracted in cold methanol, were more constant and reproducible, compared to the perchloric acid method (table 1 and 2). These metabolites were detectable even in 5 ml cell volumes (5.2-7.0 mg dry weight) (table 1). de Koning and van Dam (1992) measured all of their metabolites spectrophotometrically. However, they extracted 10-15 ml sample volumes of 50 mg/ml wet weight (approx. 10 mg/ml dry weight) in comparison to 5-10 ml sample volumes of 1.04-1.6 mg/ml dry weight in our experiments, i.e. almost 6-10 times higher cell concentration.

Fluorophotometric measurements of fructose 1,6-diphosphate and pyruvate from extracts obtained by the cold methanol method were more reproducible than from the perchloric acid extracts.

The difference in reproducibility between the two methods could indicate that the extraction with perchloric acid was inadequate to extract all of the metabolites or that metabolites were degraded during the extraction. However the main reason for this discrepancy is probably due to the extra time needed for filtrating, washing and inactivation of the cells in the perchloric acid method protocol. This procedure cannot be standardised and the time needed for the operation will vary between each experiment. This leads to poor reproducibility when applying the perchloric acid method. On the other hand, the cold methanol method protocol for cell inactivation is a one step operation, and if conducted by hand, still possible to carry out within a few seconds. Therefore the "true" *in vivo* situation of the cell metabolism is met

more accurate with the cold methanol method.

A critical step in the cold methanol method could be the time needed for vacuum evaporation of excessive methanol and water, in order to concentrate the metabolites. We observed total recovery of glucose 6-phosphate after extraction and vacuum evaporation of a standard solution, indicating that at least this metabolite is not degraded during the evaporation step.

The time span in between the two extraction methods was 30 minutes, in all four fermentations, starting with the cold methanol method. This time difference should not influence the aerobic growth experiments as it was confirmed that the cells remained in mid exponential growth phase during the time interval. However, the amount of medium was reduced from 500 ml to 440 ml after taking samples for the cold methanol method, a factor that could change the oxygenation in the baffled flasks and therefore the concentration of intracellular metabolites. In the anaerobic growth experiments at the second extractions, some change in the metabolite levels could have occured as the cells were at a switching point from glucose + xylose metabolism to xylose metabolism only. The concnetrations of fructose 1,6-diphosphate and pyruvate significantly differed between the two methods, which could be partly ascribed to this time difference. The high concentration of pyruvate could also be due to insufficient extraction of metabolites in the cold methanol method, although this was not observed by de Koning and van Dam (1992).

The decrease in xylose consumption rate and increase in xylitol production observed after glucose depletion in previous fermentations (Meinander *et al.*, submitted), conducted similary to the present anaerobic fermentation of xylose/glucose mixture, was in the present investigation found to be connected with a drastic decrease in the concentration of several glycolytic intermediates (Table 3). This validates the hypothesis that one of the reasons for the positive effect of simultaneous glucose metabolism on xylose consumption (Meinander *et al.*, submitted) is the maintenance of higher concentrations of key intermediates, effecting induction and activation of glycolytic and ethanologenic genes. Fructose 6-phosphate is required for full induction of pyruvate kinase and pyruvate decarboxylase (Boles *et al.*, 1993; Boles and Zimmermann, 1993) and, together with other hexose phosphates for the inactivation of the gluconeogenic enzyme fructose 1,6-bisphosphatase (Boles *et al.*, 1993). The concentration of glucose 6-phosphate, which is involved in the induction of enolase II, pyruvate kinase and pyruvate decarboxylase (Müller *et al.*, 1995; Boles *et al.*, 193; Boles and Zimmermann, 1993), did not decrease at the point of glucose depletion according to the current measurements. However, a low concentration of glucose still remained in the broth at the second sampling point (Fig. 1), implying that a further decrease in metabolite levels may occur after complete depletion of glucose.The decrease in xylose consumption rate and increase in xylitol production observed after glucose depletion in previous fermentations (Meinander *et al.*, submitted), conducted similary to the present anaerobic fermentation of xylose/glucose mixture, was in the present investigation found to be connected with a drastic decrease in the concentration of several glycolytic intermediates (Table 3). This validates the hypothesis that one of the reasons for the positive effect of simultaneous glucose metabolism on xylose consumption (Meinander *et al.*, submitted) is the maintenance of higher concentrations of key intermediates, effecting induction and activation of glycolytic and ethanologenic genes. Fructose 6-phosphate is required for full induction of pyruvate kinase

and pyruvate decarboxylase (Boles *et al.*, 1993; Boles and Zimmermann, 1993) and, together with other hexose phosphates for the inactivation of the gluconeogenic enzyme fructose 1,6-bisphosphatase (Boles *et al.*, 1993). The concentration of glucose 6-phosphate, which is involved in the induction of enolase II, pyruvate kinase and pyruvate decarboxylase (Müller *et al.*, 1995; Boles *et al.*, 193; Boles and Zimmermann, 1993), did not decrease at the point of glucose depletion according to the current measurements. However, a low concentration of glucose still remained in the broth at the second sampling point (Fig. 1), implying that a further decrease in metabolite levels may occur after complete depletion of glucose.

Table 4

When the present results are compared with those obtained by others (table 4), differences could be observed. When recombinant xylose metabolising *S. cerevisiae* was grown on glucose, slightly higher concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate were observed (Kötter, Ciriacy, 1993). When recombinant *S. cerevisiae* was grown on xylose (corresponding to the second extraction in the present anaerobic experiments), the concentration of fructose 1,6-diphosphate and pyruvate data were in the same range. When the present results are compared to those of (Senac, Hahn-Hägerdal, 1990), higher concentrations of fructose 1,6-diphosphate and especially pyruvate were found even when comparing data obtained by the same extraction method. Data from Jeppsson et al. (1996) are from a glucose limited continuous fermentation, and this differs markedly from the present results obtained in batch cultivation.

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Table 1. Extraction of intracellular metabolites from *S. cerevisiae* S104 by the cold methanol method and the perchloric acid method. The effect of methanol concentration and sample volume.

Extraction method	Cultivation conditions	Final [MeOH] (% v/v)	Sample volume (ml)	Cell dry weight (mg/ml)	[G6P] (nmole/mg dw)	[F6P] (nmole/mg dw)	[FDP] (nmole/mg dw)	[PYR] (nmole/mg dw)
Methanol	O_2-limited	48	5	1.3	0.8 ± 0.01	N.D.	8.7 ± 0.6	16.8 ± 1.5
		64	5	1.3	1.1 ± 0.3	N.D.	10.4 ± 0.5	13.0 ± 0.2
		40	10	1.3	1.7 ± 0.02	N.D.	9.4 ± 1.9	13.1 ± 0.1
		53	10	1.3	1.7 ± 0.03	N.D.	10.5 ± 1.2	n.d.
$HClO_4$	O_2-limited	-	5	1.5	N.D.	N.D.	9.2 ± 1.0	21.1 ± 1.3
		-	10	1.5	1.3 ± 0.2	0.6 ± 0.2	8.5 ± 1.1	22.0 ± 0.3
Methanol	O_2-limited	48	5	1.4	3.4 ± 0.2	1.0 ± 0.6	10.2 ± 1.6	9.4 ± 0.4
		64	5	1.4	3.2 ± 0.02	0.2 ± 0.06	9.8 ± 0.6	16.5± 0.9
		40	10	1.4	3.1 ± 0.3	0.4 ± 0.04	10.5 ± 2.0	16.0 ± 0.03
		53	10	1.4	3.6 ± 0.0	0.4 ± 0.04	6.7 ± 2.2	11.6 ± 0.04
$HClO_4$	O_2-limited	-	5	1.6	N.D.	N.D.	6.4 ± 1.9	15.6 ± 0.7
		-	10	1.6	0.7 ± 0.1	0.3 ± 0.03	5.3 ± 0.7	13.3 ± 1.6
Methanol	Anaerobic	48	5	1.2	2.0 ± 0.04	N.D.	9.2 ± 0.2	10.8 ± 1.9
		64	5	1.2	1.9 ± 0.1	N.D.	11.5 ± 0.5	8.1 ± 0.7
		40	10	1.2	2.8 ± 0.2	0.3 ± 0.1	10.0 ± 1.1	6.5 ± 0.7
		53	10	1.2	2.5 ± 0.3	0.3 ± 0.1	12.1 ± 1.1	7.9 ± 0.2
$HClO_4$	Anaerobic	-	5	1.28	N.D.	N.D.	13.2 ± 1.5	14.1 ± 1.7
		-	10	1.28	0.3 ± 0.00	N.D.	11.5 ± 2.1	12.1 ± 0.1
Methanol	Anaerobic	48	5	1.04	2.0 ± 0.2	N.D.	10.8 ± 0.7	6.9 ± 1.2
		64	5	1.04	1.11 ± 0.4	N.D.	9.1 ± 0.5	7.0 ± 1.1
		40	10	1.04	2.42 ± 0.3	0.1 ± 0.06	10.4 ± 0.9	6.2 ± 1.0
		53	10	1.04	1.73 ± 0.1	0.2 ± 0.05	7.4 ± 1.9	6.6 ± 0.9
$HClO_4$	Anaerobic	-	5	1.2	0.73 ± 0.1	N.D.	16.3 ± 1.8	25.2 ± 3.6
		-	10	1.2	0.84 ± 0.2	N.D.	16.7 ± 0.8	19.1 ± 2.0

N.D. = Not detectable. n.d. = Not determined. G6P = glucose 6-phosphate. F6P = fructose 6-phosphate. FDP = fructose 1,6-diphosphate. PYR = pyruvate. G6P and F6P where measured spectrophotometrically. FDP and PYR were measured fluorophotometrically.

Table 2. Comparison between the metabolite concentrations obtained in mid-exponential growth phase using the perchloric acid method and the cold methanol method of metabolite extraction.

Cultivation conditions	Substrate	Sampling time	G6P (nmole/mg d.w.)		F6P (nmole/mg d.w.)		FDP (nmole/mg d.w.)		PYR (nmole/mg d.w.)	
		(hours)	Methanol	$HClO_4$	Methanol	$HClO_4$	Methanol	$HClO_4$	Methanol	$HClO_4$
O_2-limited	Glucose	44.5	1.3 ± 0.5	1.3 ± 0.2[1]	N.D.	0.6 ± 0.2[1]	9.8 ± 0.9	8.9 ± 0.5	14.3 ± 2.1	21.6 ± 0.6
O_2-limited	Glucose	36	3.3 ± 0.2	0.7 ± 0.1[1]	0.4 ± 0.1[2]	0.3 ± 0.03[1]	9.3 ± 1.7	5.9 ± 0.8	13.4 ± 3.4	14.5 ± 1.7
Anaerobic	Xyl./gluc.	23	2.3 ± 0.4	0.3 ± 0.0[1]	0.3 ± 0.04[1]	N.D.	10.7 ± 1.4	12.4 ± 1.3	8.3 ± 1.8	13.1 ± 1.5
Anaerobic	Xyl./gluc.	18.5	1.8 ± 0.6	0.8 ± 0.1	0.2 ± 0.1[1]	N.D.	9.4 ± 1.5	16.5 ± 0.3	6.7 ± 0.4	22.1 ± 4.4

N.D. = Not detectable neither in 5 ml nor 10 ml sample volumes.

[1]Not detectable at 5 ml sample volumes. Values from 10 ml sample volumes are reported.

[2]Data using 48% methanol has been omitted due to a very large standard deviation.

Xyl.= xylose. gluc.= glucose. d.w. = dry weight cells.

Table 3. Intracellular metabolite concentration in *S. cerevisiae* S. 104 during anaerobic fermentations of 15 g/L xylose and 30 g/L glucose, obtained using the cold methanol method.

Substrate	Sampling time (hours)	[Glucose-6-P] (nmole/mg dw)	[Fructose-6-P] (nmole/mg dw)	[FDP] (nmole/mg dw)	[Pyruvate] (nmole/mg dw)
Glucose +	23	2.3 ± 0.44	0.3 ± 0.04[1]	10.7 ± 1.4	8.3 ± 1.8
xylose	18.5	1.8 ± 0.6	0.2 ± 0.10[1]	9.4 ± 1.5	6.7 ± 0.4
Average	-	2.1 ± 0.5	0.2 ± 0.09	10.1 ± 1.5	7.5 ± 1.5
Xylose[2]	33	1.9 ± 0.1	N.D.	2.8 ± 0.5	4.4 ± 0.5
	27	2.1 ± 0.04	N.D.	2.6 ± 0.3	4.7 ± 0.3
Average	-	2.0 ± 0.4	-	2.7 ± 0.7	4.5 ± 0.6

N.D. = Not detectable either with 5 ml and 10 ml sample volumes.

[1]Not detectable in 5 ml sample volume. Values from 10 ml sample volume are reported.

[2]Point of glucose depletion.

Table 4. Comparison of intracellular metabolite concentrations obtained in the present study and other investigations This table is an extension of a table presented in Jeppsson et al. (1996).

Strain	Cultivation conditions	Carbon source(s)	G6P[1]	F6P[1]	FDP[1]	PYR[1]	Extraction method	Reference
ATCC 24860	Batch	Glucose	n.d.	nd.	6.5	2.36	PCAM	Senac & Hahn-Hägerdal, 1990
ATCC 24860	Batch	Xylulose	n.d.	n.d.	0.78	1.38	PCAM	Senac & Hahn-Hägerdal, 1990
PUA6-9	Batch	Glucose	3.8	0.9	12.4	12.4	Boiling eth.	Kötter & Ciriacy, 1993
PUA6-9	Batch	Xylose	1.1	0.8	1.7	2.8	Boiling eth.	Kötter & Ciriacy, 1993
ATCC 24860	Chemostat	Glucose	n.d.	n.d.	2.4	0.7	CMM	Jeppsson et al., 1996
ATCC 24860	Chemostat	Xylulose& glucose	n.d.	n.d.	3.3	0.8	CMM	Jeppsson et al., 1996
S. cerevisiae S104	O_2-limited batch	Glucose	1.3	N.D.	9.7	14.3	CMM	Present study
S. cerevisiae S104	O_2-limited batch	Glucose	3.3	0.4	9.3	13.4	CMM	Present study
S. cerevisiae S104	O_2-limited batch	Glucose	1.3	0.6	8.1	21.6	PCAM	Present study
S. cerevisiae S104	O_2-limited batch	Glucose	0.7	0.3	5.9	14.5	PCAM	Present study
S. cerevisiae S104	Anaerobic Batch 1. extract.	Xylose & Glucose	2.3	0.3	10.7	8.3	CMM	Present study
S. cerevisiae S104	Anaerobic Batch 1. extract.	Xylose & Glucose	1.8	0.2	9.4	6.7	CMM	Present study
S. cerevisiae S104	Anaerobic Batch 2. extract.	Xylose	1.9	N.D.	2.8	4.4	CMM	Present study
S. cerevisiae S104	Anaerobic Batch 2. extract.	Xylose	2.1	N.D.	2.6	4.7	CMM	Present study

n.d. = Not determined. N.D. = Not detectable. CMM = Cold methanol method. PCAM = Perchloric acid method. Extract. = extraction. Eth = ethanol. [1]nmole/mg cell dry weight.

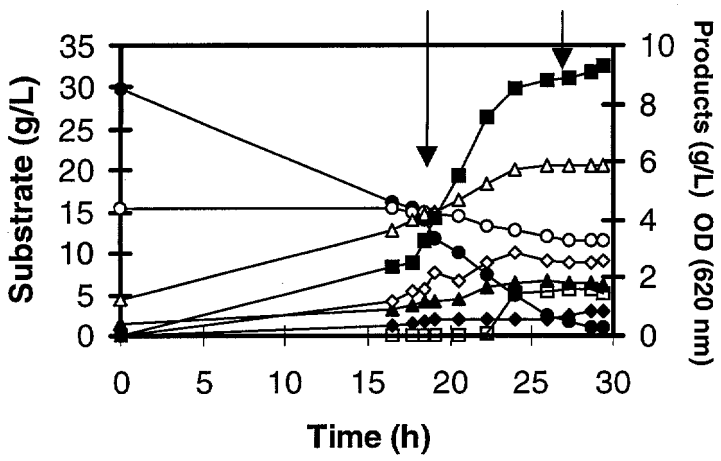
Substrate (g/L)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (h)
Products (g/L) OD (620 nm)
10
8
6
4
2
0

Figure legend.

Figure 1. Anaerobic batch fermentation of xylose and glucose. Arrow's indicates sampling times for intracellular metabolite extractions. Symbols. Glucose (●) Xylose ( ) Acetate ( ) Ethanol (■) Dry weight (▲) Xylitol (◆) Glycerol ( ) Optical density (△)

PAPER 3

Measurements of intracellular metabolites and enzymes of the pentose phosphate pathway of *Thermoanaerobacter mathranii* A3M1 at varying D-xylose concentrations

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Measurements of intracellular metabolites and enzymes of the pentose phosphate pathway in *Thermoanaerobacter mathranii* A3M1 at varying D-xylose concentrations

Abstract Knowledge is limited of the biochemistry in the D-xylose metabolism and of the regulatory effect on distribution of end-fermentation products in thermophilic anaerobic ethanol-producing bacteria. There is limited knowledge of the intracellular enzyme activities and almost no knowledge of *in vivo* intracellular metabolite concentrations during carbohydrate metabolism in these microorganisms. Intracellular metabolites may act both as inductors or repressors of enzyme synthesis and as affectors with enzymes already present in the cell. Identification, quantification and regulation of intracellular metabolites and, more importantly, the bottlenecks in the metabolism is therefore essential knowledge, for physiological and genetic engineering to improve ethanol yield and productivity.
The present study examines the physiological and biochemical responses in the pentose phosphate pathway of *T. mathranii* during continuous fermentation at fluctuating concentrations of D-xylose. Substrate consumption, product formation, two intracellular enzymes and two intracellular metabolites from the pentose phosphate pathway have been quantified. The cold methanol method (de Koning, van Dam, 1992), for extraction of intracellular metabolites at neutral pH, has been applied in this study. We found a markedly decreased activity through the pentose phosphate pathway at elevated D-xylose concentration with a high level of intracellular D-xylose accumulation. The restriction of this pathway was the isomerization of intracellular D-xylose.

Keywords: Ethanol, Pentose phosphate pathway, Intracellular metabolites, Intracellular enzymes, Thermophilic, Bacteria

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Introduction

Lignocellulosic biomass is a low-cost carbohydrate source produced in vast unexploited quantity (Lynd, 1990), which may serve as substrate for production of the non-fossil fuel-ethanol (Lynd, 1989). The combustion of bio-ethanol as transportation fuel is CO_2-neutral and could contribute to a feasible solution for a global decrease in CO_2 emission from ground- and air transportation (Hall et al. 1991). Saccharolytic thermophilic anaerobic bacteria have been considered as candidates for production of bio-ethanol from lignocellulosic biomass (Lynd, 1989). This is primarily because they can metabolize several carbohydrates even of polymeric nature (Wiegel, Ljungdahl, 1986). However, these microorganisms lack industrial importance mainly because of low substrate and ethanol tolerance and organic acid production (Lynd, 1989).

Fermentation experiments with thermophilic anaerobic ethanol producing bacteria have, in most cases, been restricted to physiological evaluation of consumption and end-fermentation product formation during batch- and continuous fermentation at various D-xylose concentrations (Lacis, Lawford, 1985; Lacis, Lawford, 1988b; Lacis, Lawford, 1991). Thermophilic anaerobic strains fermenting D-xylose, produce ethanol yields close to the theoretical ethanol yield of 1.67 mole ethanol per mole xylose (0.51 g ethanol per g xylose) (Hinman et al. 1989). These yields have, however, only been achieved at concentrations less than or equal to 67 mM (10 g/L) D-xylose (Carreira et al. 1983; Lacis, Lawford, 1988a; Lacis, Lawford, 1989; Cook, Morgan, 1994). At exceeding concentrations of D-xylose, decreased ethanol yield was often observed (Wiegel et al. 1983; Lynd, 1989; Mistry, Cooney, 1989; Lacis, Lawford, 1991).

Knowledge is scarce of the biochemistry causing these metabolic limitations in thermophilic anaerobic ethanol-producing bacteria. There is

limited knowledge of the intracellular enzyme activities (Zeikus et al. 1981) and almost no knowledge of *in vivo* intracellular metabolite concentrations during carbohydrate metabolism in these microorganisms. Intracellular metabolites may act both as inductors or repressors of enzyme synthesis and as affectors with enzymes already present in the cell (Lynd et al. 1991; Jeppsson et al. 1996). Identification, quantification and regulation of intracellular metabolites, and more importantly, the bottlenecks in the metabolism is essential knowledge, for physiological and genetic engineering to improve ethanol yield and productivity.

The present study examines the physiological and biochemical variations in the pentose phosphate pathway of *T. mathranii* during continuous fermentation in response to varying concentrations of D-xylose. D-xylose consumption rate, intracellular concentration of D-xylose, accumulation of an unidentified D-xylose derivate, erythrose 4-phosphate, glyceraldehyde 3-phosphate, xylose isomerase and transaldolase activities have been quantified.

The cold methanol method (de Koning, van Dam, 1992) for extraction of intracellular metabolites at neutral pH has been applied in this study. This method has, to our knowledge, not previously been applied in any biochemical studies of the carbohydrate metabolism in thermophilic anaerobic bacteria. We extracted 100 ml fermentation broth (10 times 10 ml) equivalent to 26-40 mg dry weight cells enough for successful measurements of intracellular metabolites by spectrofluorometric methods.

The investigated strain, *Thermoanaerobacter mathranii*, is a newly characterized extreme thermophilic anaerobic bacterium metabolizing D-xylose, xylan as well as D-glucose and L-arabinose to ethanol, acetate, lactate, CO_2 and H_2 (Larsen et al. 1997).

Materials & Methods

Microorganism

Thermoanaerobacter mathranii was isolated from an Icelandic hot spring as described by Sonne-Hansen et al. (1993). The bacterium has been identified and characterized by Larsen et al. (1997). *T. mathranii* A3M1 strain originated from experiments for adaptation to wet oxidized hemicellulose hydrolysate from wheat straw (Ahring et al. 1996).

Mineral medium

The bacterium was cultivated in anaerobic medium as previously described (Angelidaki et al. 1990) amended with 0.2 g yeast extract per g D-xylose added (Difco) but with no addition of cysteine, vitamins and bicarbonate. The medium was autoclaved at 134°C for 1½ hour and flushed with sterilized nitrogen before addition of 3.2 mM sodium sulfide and either 33 mM or 133 mM D-xylose from sterile stock solutions.

Chemostat conditions

The chemostat was operated as follows: Reactor volume, 2000 ml; working volume, 1200 ml; flow, 100 ml/h; dilution rate, 0.083; retention time, 12 hours; agitation speed, 40 rpm; temperature, 69°C; pH 5.7-6.0. The chemostat was operated until steady state and kept for four retention times before sampling. During transitions between different D-xylose concentrations, the chemostat was operated for two retention times before sampling. The pH in the culture broth was controlled by a pH controller (Consort, Turnhout, Belgium) and automatically adjusted by addition of 1.0 M KOH. The chemostat was flushed with sterile nitrogen to secure strictly anaerobic condition during start-up of the reactor. The temperature was regulated by circulation of water from a waterbath (Heto DT Hetotherm). The reactor was inoculated with 5% *T. mathranii* A3M1 pre-grown on 33 mM D-xylose in 100 serum vials and kept as a batch culture for 12 hours before operation of the chemostat.

Continuous fermentation

The inlet D-xylose concentration was initially 33 mM, then raised to 133 mM, and finally decreased to 33 mM D-xylose. Steady state conditions were obtained twice at 33 mM and at 133 mM D-xylose, respectively, before initializing transitional phases between the two inlet concentrations.
The first and second steady state values at 33 mM D-xylose are denoted steady state 1. The transition period between 33 mM and 133 mM D-xylose is denoted transition 1. The first and second steady state values at 133 mM D-xylose are denoted steady 2. The transition period between 133 mM and 33 mM D-xylose is denoted transition 2. The reestablished steady state at 33 mM D-xylose is denoted steady state 3.

Preparation of crude cell extract

For protein and enzyme activity determinations, 2 x 50 ml fermentation broth was sampled from the chemostat immediately after extraction of the

metabolites. The withdrawn cultivation broth was cooled to 0°C in vials placed in –25°C ethanol and maintained anaerobic by flushing with nitrogen gas. The cells were centrifuged at 4000 g (Sorvall RC-5B) at +4°C for 20 minutes and the pellet was washed twice in anaerobic and sterile 0.9% NaCl. The cells were sonicated (Sonifier 250, Struers Denmark) for 2-3 minutes while cooled on ice. The degree of cell lysis was monitored by use of a phase contrast microscope (Olympus OM-2). The supernatant was centrifuged at 4000 g at +4°C for 20 minutes and then transferred to several Eppendorf tubes and kept at -80°C until analysis.

Determination of intracellular enzyme activities

Protein concentrations were determined according to Bradford (1976), with bovine serum albumin as standard. The crude cell extracts and assay mixture were kept anaerobic by flushing cuvettes (Hellma precision quartz cuvettes) with nitrogen gas and sealed with rubber stoppers. The activities were measured spectrophotometrically (Pharmacia, Ultrospec 3000) at 340 nm at 70°C. The temperature was maintained by using a heated cell changer (Pharmacia Biotech 80-2106-03) connected to a waterbath (Pharmacia Biotech Multitemp III). Because of the high temperature when conducting the assays, double amount of enzymes (when needed) and co-factors were applied. Xylose isomerase activity was determined by measuring the conversion of fructose to glucose as described by Gong et al. (1980). Transaldolase was measured as previously described (Bruininberg et al. 1983). Specific enzyme activities in the extracts were expressed in unit milligram of protein^{-1}. 1 U is equivalent to the conversion of 1 µmol of substrate min^{-1}.
Enzymatic assays were performed in at least triplicates. Controls were assayed where assay mixture without cell-free extract was analyzed, and subtracted from the sample assay.

Sampling of intracellular metabolites

10 x 10 ml culture broth was withdrawn from the chemostat with 10 ml syringes and rapidly sprayed into 13 ml -25°C 60% (v/v) methanol. Medium stored in the sample port tube was discharged in between sub-sampling. The total sampling time was 2-4 minutes.

Extraction of intracellular metabolites

Intracellular metabolites were extracted by the use of the cold methanol method (de Koning, van Dam, 1992). 10 ml cells were sprayed into 13 ml -25°C (60% v/v) methanol. After a resting period of five minutes, the cells were centrifuged (4000 x g) at -20°C for 20 minutes. The methanol was removed and the cells were re-suspended in a -25°C methanol buffer (Pipes/EDTA)/chloroform mixture. The metabolites were extracted by shaking at 2200 rpm at -20°C for one hour. After collection of the methanol/buffer phase containing the metabolites, fresh 100% (v/v) methanol buffer was added and the extraction was repeated. Traces of fatty acid were removed by dimethyl-ether. The extracts from the same experiment were pooled, centrifuged at 4°C, for 15 minutes at 10000 rpm for removal of traces of Pipes. The extract was weighed and evaporated under vacuum (Maxi Dry Plus) for 8-10 hours at 0-4°C. The extracts were then weighed again and kept at -80°C until analysis. The final volumes of the extract were between 2-7 ml. The cold methanol method was modified as follows: Cooling-bath without circulation containing 96% ethanol instead of methanol was used and the metabolites were extracted at -20°C instead of -40°C.

Determination of intracellular metabolite concentrations

The concentration of the intracellular metabolites was determined with an enzymatic assay in which the oxidation or reduction of $NADP^+$ was measured as end point analysis. This was done with a fluorescence spectrophotometer (Hitachi F-2000) at an emission of 450 nm after excitation at 340 nm (Jeppsson et al. 1996).

Erythrose-4-phosphate (E4P) was measured according to Bergmeyer (1983) and modified from spectrophotometric to spectrofluorometric determination as follows: 50 mM imidazol (grade III) pH 7.7 was used as buffer, 0.01% (w/w) Bovine Serum Albumin (BSA), 20 µM $NADP^+$, 0.25 mM $MgCl_2$, 5 µM thiamine pyrophosphate, 20 µM hydroxypuruvate or 2,5 mM fructose, 0.08 U/ml phosphoglucose isomerase, 0.08 U/ml glucose-6-phosphate dehydrogenase and 0.025 U/ml transketolase.

Glyceraldehyde-3-phosphate (G3P) was measured by a modified FDP assay (Lowry, Passonneau, 1972), where G3P was isomerised to dihydroxyacetone phosphate (DHP) and DHP converted to glycerophosphate by a concomitant oxidation of NADH to NAD^+. The assay mixture contained: 50 mM imidazol (grade III) pH 7.5 was

used as buffer, 0.01% (w/w) BSA, 20 µM NADH, 1.6 U/ml triosephosphate isomerase and 0.11 U/ml glycerophosphate dehydrogenase.

The concentration of the intracellular metabolites was expressed in nmol mg of dry weight cells^{-1}. The total assay volume was 1 ml. Assays were performed in triplicates.
Intracellular D-xylose concentration was measured by HPLC as described for excreted D-xylose.
All chemicals and enzymes used for the determination of enzyme activities and intermediary metabolite concentrations were from Sigma C0. (St Louis, Mo, USA).

Analyses

Steady state values were determined by optical density with a variance below 10% between four retention times. Samples were analyzed at 578 nm by withdrawing 10 ml fermentation broth into a anaerobic tube. This was kept anaerobic by addition of 20 µl (0.3 M) sodium sulfide and measured directly in a spectrophotometer (Milton Ron, Spectronic 301).

D-xylose was quantified by high performance liquid chromatography on an organic acid analysis column (AminexR 10N exclusion HPX87-H 300 x 7.8 mm) at 65°C with Refractive Index Detection (LDC Analytical refracto monitor IV). The mobile phase was 0.01 N H_2SO_4.
Dry weight determinations were conducted as described in Yu et al. (1995) by withdrawing 3 x 20 ml culture broth from the chemostat after extraction of intracellular metabolites.

Results

Fermentation

The metabolism of *T. mathranii* A3M1 was examined during continuous fermentation with varying D-xylose concentrations (figure 1). The optimal condition for continuous ethanol production on 33 mM D-xylose was applied, established by Licht *et al.* (manuscript in preparation) which was at 70°C, pH 6.0 and a retention time of 12 hours.

Specific D-xylose consumption rate and volumetric biomass productivity

Steady state 1 As shown in figure 1, most of the inlet D-xylose is transported into the cells. The concentrations of end-fermentation products are described in Sommer, Ahring (1998). The volumetric biomass productivity was 21.0 mg $l^{-1}h^{-1}$

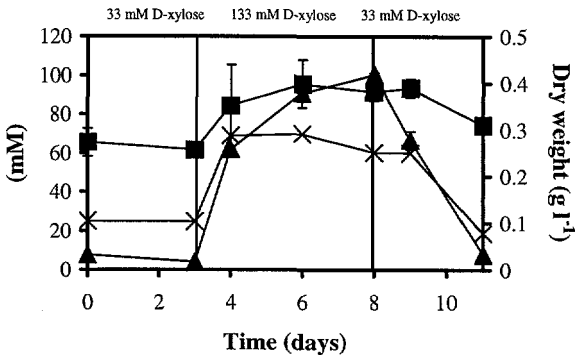

Fig. 1 Time course of D-xylose and biomass production in the chemostat culture of *T. mathranii* A3M1. The inlet D-xylose concentration was initially 33 mM D-xylose at 0 hour, then increased to 133 mM at day 3 and then changed back to 33 mM at day 8. Continuous fermentation, 70°C, pH 5.7-6.0, retention time 12 hours. Effluent D-xylose (▲), Consumed D-xylose (×), Dry weight of cells (■).

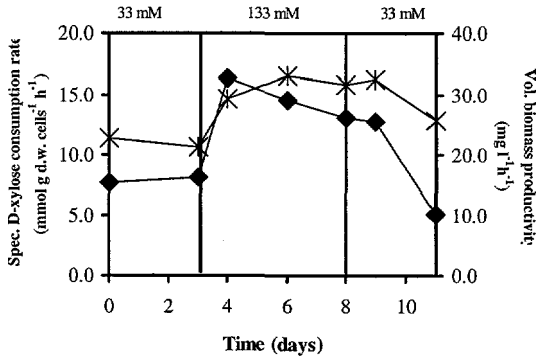

Fig. 2. Specific consumption rate and volumetric biomass productivity rate of *T. mathranii* A3M1 with varying D-xylose concentrations. Vertical lines marks the transition periods in D-xylose concentration. Continuous fermentation, 70°C, pH 5.7-6.0, retention time 12 hours. D-xylose (◆), Biomass (✳).

and the specific D-xylose consumption rate was 7.9 mmol $g^{-1}h^{-1}$ (figure 2).

Transition 1 The specific D-xylose consumption rate increased drastically by 52% and the volumetric biomass productivity increased by 25% compared to steady state 1 (figure 2).

Table 1 Measurements of intracellular enzymes and metabolites of *T. mathranii* A3M1 during varying inlet D-xylose concentrations. Continuous fermentation, 70°C, pH 5.7-6.0, retention time 12 hours.

Inlet [xylose]	Xylose isomerase	Transaldolase	Intracellular D-xylose	Intracellular [E4P]	Intracellular [G3P]
(mM)	U mg d.w. cells^{-1}		µmol mg. d.w. cells^{-1}	nmol mg d.w. cells^{-1}	
33.3	0.10 ± 0.02	0.15 ± 0.02	3.7 ± 0.1	2.3 ± 0.4	18.3 ± 4.3
33.3	0.11 ± 0.01	0.04 ± 0.01	0	0.7 ± 0.2	29.8 ± 0.2
Trans.	0.034 ± 0.002	0.26 ± 0.02	19.0 ± 0.9	1.5 ± 0.1	5.0 ± 0.5
133	0.064 ± 0.01	0.30 ± 0.01	54.5 ± 0.6	ND	ND
133	0.024 ± 0.004	0.30 ± 0.01	91.5 ± 0.6	ND	ND
Trans.	0.058 ± 0.008	0.28 ± 0.00	13.0 ± 0.3	1.8 ± 0.02	0.9 ± 0.03
33.3	0.11 ± 0.01	0.13 ± 0.00	1.1 ± 0.2	1.2 ± 0.3	12.0 ± 1.0

ND = Not detected. Trans. = transition period between steady states. E4P = Erythrose 4-phosphate. G3P = Glyceraldehyde-3-phosphate

Steady state 2 The physiological transition from steady state 1 and transition 1 was still evident. The specific D-xylose consumption rate increased by 43% compared to steady state 1, but decreased compared to transition 1. The volumetric biomass productivity continued to increase through transition 1 by 32% compared to steady state 1 (figure 2).

Transition 2 The specific D-xylose consumption rate increased by 38% compared to steady state 1, then decreased through transition 1 and steady state 2 by 22% and 8%, respectively (figure 2). The volumetric biomass productivity remained unchanged between steady state 2 and transition 2 (figure 2).

Steady state 3 The physiological conditions was not reestablished as at steady state 1. The specific consumption rate of D-xylose was decreased by 36% compared to steady state 1 (figure 2). The volumetric biomass productivity finally decreased at steady state 3, however, remained 15% higher than at steady state 1 (figure 2).

Specific *in vitro* activities of xylose isomerase and transaldolase

As shown in table 1, the specific xylose isomerase activity decreased significantly as the inlet D-xylose concentration was raised at transition 1. But the specific activity increased slightly at steady state 2 before the activity was the same at steady state 3, compared to steady state 1.

The specific transaldolase activity increased significantly by 63% at transition 1 compared to steady state 1. It remained more or less constant at steady state 2 and transition 2. Finally it decreased to the same specific activity at steady state 3 compared to steady state 1 (table 1).

Levels of intracellular D-xylose, E4P and G3P

Steady state 1 As shown in table 1, the intracellular concentrations of D-xylose were low (0 and 3.7 µmol mg dry weight cells^{-1} ; 0-3.6 mM) and showed an almost complete conversion of the inlet D-xylose to D-xylulose. The intracellular concentration of E4P varied for unknown reasons between 0.7 and 2.3 nmol mg dry weight cells^{-1} (table 1). The intracellular concentration of G3P was significantly higher than the concentration of E4P.

Transition 1 Five times higher intracellular concentration of D-xylose was measured at increasing inlet concentration of D-xylose (table 1). The intracellular concentration of E4P was not significantly affected by the increased D-xylose concentration, however, the concentration of G3P decreased significantly.

Steady state 2. The intracellular concentration of D-xylose was accumulated and peeked at 91.5 µmol mg dry weight cells^{-1} (202.9 mM). Concentrations of E4P and G3P were not detectable (table 1).

Transition 2. A seven-fold decrease in the accumulated intracellular D-xylose concentration was measured. The intracellular concentration of E4P and G3P were again detectable compared to steady state 2, but the concentration of G3P was significantly lower compared to steady state 1 and transition 1.

Steady state 3 The intracellular D-xylose was not accumulated to the same extent, as observed for one of the measured values at steady state 1 (table 1). The intracellular concentration of E4P was two times lower compared to steady state 1 (table 1). The intracellular concentration of G3P was again in high concentration as seen at steady state 1.

Discussion

The successful application of the cold methanol method (De Koning, van Dam, 1992) in our study, has to our knowledge, not previously been done for studies of the carbohydrate metabolism in thermophilic anaerobic bacteria. The selected extraction method has several advantages opposed to other extraction methods, such as the perchloric acid extraction. The metabolism of the cells is rapidly inactivated by immersion in -40°C methanol without filtration. The extraction procedure is conducted at -20°C to -40°C and at neutral pH. In contrast to the perchloric acid method, rapid inactivation of metabolic activity and preservation of the pH sensitive co-factors like NAD(P)/NAD(P)H is achieved. The disadvantage of this method is the need for up-concentration of the metabolites from the dilute extraction broth in a vacuum-evaporation step This makes the method somewhat tedious. The method has previously been used for extraction of intracellular metabolites in *Saccharomyces cerevisiae* (Sommer et al., manuscript in preparation). The biomass production is significantly higher in *S. cerevisiae* as opposed to thermophilic anaerobic bacteria. The amount of withdrawn fermentation broth could be reduced to 5-10 ml, equivalent to 1-1.5 mg d.w. cells/ml. When using the method for extraction of intracellular metabolites from thermophilic anaerobic bacteria, the amount of withdrawn fermentation broth must be adjusted to measure the concentrations of intracellular metabolites. We extracted 100 ml fermentation broth (10 times 10 ml) equivalent to 26-40 mg d.w. cells, to achieve successful measurements of intracellular metabolites by spectrofluorometric methods.

The specific D-xylose consumption rates increased two-fold when the D-xylose concentration increased from 33 mM to 133 mM. Although a slight linear decrease was shown through the steady state 2, transition 2, the increased consumption rate indicated that an inadequate D-xylose fermentation at high concentration, was not restricted by D-xylose transport into the cells. It was evident that ascending accumulation of D-xylose occurred, peeking at the second sample point at steady state 2. An approximately two-fold higher concentration was measured compared to the surrounding medium indicating an active transport of D-xylose into the cells. The difference in substrate concentration could indicate that some sort of osmotic stress situation had arised, towards lysis of the cells, affecting the overall metabolism of the cell. The accumulation of D-xylose at an inlet D-xylose concentration of 133 mM, indicated a deficiency of the cells to isomerize D-xylose to D-xylulose, catalyzed by xylose isomerase. This was confirmed by the decreased *in vitro* specific xylose isomerase activity. The *in vitro* specific xylose isomerase activity only increased to the level of steady state 1, when the steady state was reestablished at steady state 3. The increasing specific D-xylose consumption rate observed at transition 1 did not lead to a concomitant increased D-xylose conversion rate in the non-oxidative part of the pentose phosphate pathway.
Another carbohydrate or sugar phosphate not presently identified, which was not D-xylulose or glucose however, with a retention time almost identical with glucose, accumulated in the cells at a concentration of D-xylose of 133 mM (data not shown).
This indicated a further deficiency in the pentose phosphate pathway when channeling the intracellular metabolites through the pathway.

The Phosphorylation of D-xylulose to xylulose 5-phosphate marks the beginning of the non-oxidative part of the pentose phosphate pathway (Zubay, 1988) (figure 3). Through a series of interconversions, involving ribulose 5-phosphate, ribose 5-phosphate, sedoheptulose 7-phosphate and erythrose 4 phosphate catalyzed by two enzymes, transaldolase and transketolase, respectively, glyceraldehyde 3-phosphate and fructose 6-phosphate are channeled into the glycolysis (figure 3) (Zubay, 1988).

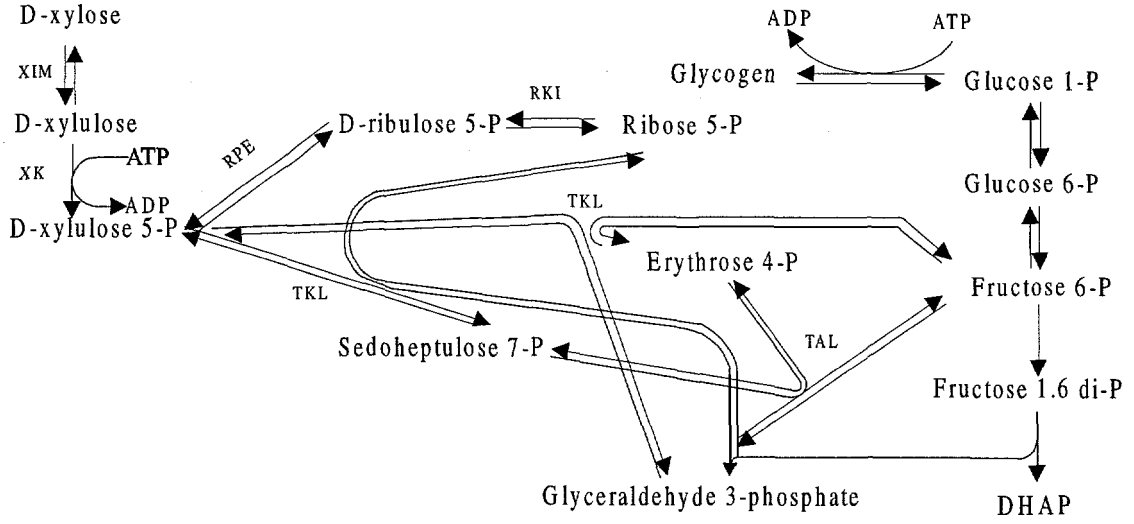

Fig 3 Model of the pentose phosphate pathway of thermophilic anaerobic ethanol producing bacteria. XIM = D-xylose isomerase, RPE = Ribulose 5-phosphate epimerase, RKI = Ribose 5-phosphate ketol-isomerase, TKL, Transketolase, TAL = Transaldolase. DHAP = dihydroxyacetone phosphate

Examination of some clostridia strains did not show any presence of an oxidative pentose phosphate pathway (Lamed, Zeikus, 1980; Jones, Woods, 1991). Generation of ribose-5-phosphate and NADPH, respectively, must then be accomplished by other metabolic pathways. Epimerization of xylulose 5-phosphate and subsequently isomerization of ribulose 5-phosphate could generate ribose 5-phosphate in the non-oxidative part of the pentose phosphate pathway. However, the existence of this pathway has not been confirmed.

The intracellular concentration of E4P and G3P, respectively, decreased significantly and were not detectable at high inlet D-xylose concentration. The specific *in vitro* transaldolase activity, however increased at increased inlet D-xylose concentration, which indicated that an activity for an increased flux through the non-oxidative part of the pentose phosphate pathway, was present. The transaldolase and transketolase are symmetrical enzymes with an equilibrium constant close to 1 (Zubay, 1988). Therefore, these observations indicated that the interconversion of xylulose-5-phosphate to ribose 5-phosphate could be a rate-limiting step in the flux through the non-oxidative part of the pentose phosphate pathway. It could also be explained by an increased conversion of ribose 5-phosphate for pyrine and pyrimidine synthesis because of the increased biomass production, or a combination. A decreased concentration of ribose-5-phosphate would decrease the flux through the pentose phosphate pathway and an increased competition between glycolysis and pentose phosphate pathway for G3P could have arised.

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PAPER 4

Measurements of intracellular metabolites and enzymes in the glycolysis of *Thermoanaerobacter mathranii* A3M1: The influence of the D-xylose concentration

Sommer P and Ahring BK

Prepared for submission to Applied & Environmental Microbiology.

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Measurements of intracellular metabolites and enzymes in the glycolysis of *Thermoanaerobacter mathranii* A3M1: The influence of the D-xylose concentration

Abstract Saccharolytical thermophilic anaerobic bacteria have been considered as candidates for production of non-fossil fuel, ethanol, from lignocellulosic biomass. This is primarily because they can metabolize several carbohydrates even of polymeric nature. However, these microorganisms lack industrial importance mainly because of low substrate- and ethanol tolerance and organic acid production. Knowledge is limited of the biochemical mechanisms responsible for the distribution of end-fermentation products in thermophilic anaerobic ethanol-producing bacteria. Identification and regulation of metabolic fluxes and more importantly, the bottlenecks in the metabolism is an essential basis for physiological and genetic engineering to improve ethanol yield and productivity. The present study examines the physiological and biochemical variations in the glycolysis of *T. mathranii*, in response to varying concentrations of D-xylose during continuous fermentation. Substrate consumption and product formation pattern, several intracellular enzymes and intracellular metabolites from the glycolysis have been quantified. The cold methanol method (de Koning, van Dam, 1992) for extraction of intracellular metabolites at neutral pH has been applied in this study.

Keywords: Ethanol, Glycolysis, Intracellular metabolites, Intracellular enzymes, Thermophilic, Bacteria

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Introduction

Lignocellulosic biomass is a low- cost carbohydrate source, produced in vast unexploited measure (Lynd, 1990). Production of bio-ethanol from lignocellulosic biomass has received much attention as substitution for non-fossil transportation fuel (Lynd, 1989). The combustion of bio-ethanol as transportation fuel is CO_2-neutral and could contribute to a feasible solution for a global decrease in CO_2 emission from ground- and air transportation (Hall et al. 1991). Saccharolytic thermophilic anaerobic bacteria have been considered as candidates for production of bio-ethanol from lignocellulosic biomass (Lynd, 1989). This is primarily because they can metabolize several carbohydrates even of polymeric nature (Wiegel, Ljungdahl, 1986). However, these microorganisms lack industrial importance mainly because of low substrate- and ethanol tolerance and organic acid production (Esser, Karsch, 1984; Lynd, 1989).

Examination of thermophilic anaerobic species for production of ethanol have mostly been restricted to physiological evaluations of consumption- and end-fermentation product formation during batch and continuous fermentation at varying D-xylose concentrations (Lacis, Lawford, 1985; Lacis, Lawford, 1988b; Lacis, Lawford, 1991; Mollah, Stuckey, 1992). When fermenting D-xylose, thermophilic anaerobic strains can produce ethanol yields close to the theoretical ethanol yield of 1.67 mole ethanol per mole xylose (0.51 g ethanol per g xylose) (Hinman et al. 1989). These yields have however, only been achieved at concentrations of 66.7 mM (10 g/L) D-xylose or less, (Carreira et al. 1983; Lacis, Lawford, 1988a; Lacis, Lawford, 1989; Cook, Morgan, 1994). At D-xylose concentrations exceeding 66.7 mM decreased ethanol yield were observed (Wiegel et al. 1983; Lynd, 1989; Mistry, Cooney, 1989; Lacis, Lawford, 1991). There is very limited knowledge of the intracellular enzyme activities and intracellular metabolite concentrations during carbohydrate metabolism in thermophilic

anaerobic ethanol-producing bacteria (Zeikus et al. 1981). Intracellular metabolites may act both as inductors or repressors of enzyme synthesis and as affectors with enzymes already present in the cell (Lynd et al. 1991; Jeppsson et al. 1996). Identification and regulation of metabolic fluxes during carbohydrate metabolism and more importantly, the bottlenecks in the metabolism are a fundamental basis for physiological and genetic engineering to improve ethanol yield and productivity of these microorganisms.

Examined saccharolytical thermophiles employ the Embden Meyerhof pathway for metabolism of glucose (Lamed, Zeikus, 1980; Zeikus, 1980; Ben-Bassat, Zeikus, 1981).
The metabolic activities of the pentose phosphate pathway are channeled into the upper and middle part of the Embden-Meyerhof pathway as fructose 6-phosphate and glyceraldehyde 3-phosphat, respectively (Zubay, 1988). Fructose 6-phosphate can be converted to glucose 6-phosphate by phosphohexoisomerase and during anabolism, be converted to glucose-1-phosphate and glycogen. Fructose 6-phosphate and glyceraldehyde 3-phosphate will, during catabolism, be converted to pyruvate. This intermediate is a key metabolic branch point, determining the distribution of end-fermentation products (Ljungdahl et al. 1985). Ferredoxin is present in thermophilic anaerobic bacteria (Lovitt et al. 1988; Jones, Woods, 1991) and the reduction and oxidation of this molecule is achieved by decarboxylation of pyruvate to acetyl-CoA and production of H_2, NAD(P)H, respectively.

T. mathranii converts D-xylose into ethanol, acetate, lactate, CO_2 and H_2. The final formation of end-fermentation products and their mutual distribution and determined by the cells response to a given metabolic state, their needs for energy, metabolites for reductive biosynthesis, and reducing equivalents (Zeikus et al. 1981).

T. mathranii employ the Embden-Meyerhof pathway for production of pyruvate and has a non-oxidative pentose phosphate pathway. The presence of an oxidative pentose phosphate pathway or a ferredoxin cycle was not examined. However, the existence of the latter was indicated by production of H_2.

The present study examines the physiological and biochemical variations in the glycolysis of *T. mathranii*, in response to varying concentrations of D-xylose during continuous fermentation. Substrate and product formation patterns, several intracellular enzymes and intracellular metabolites from the glycolysis have been quantified.
The cold methanol method (de Koning, van Dam, 1992) for extraction of intracellular metabolites at neutral pH has been applied in this study as described in Sommer, Ahring (1998). To our knowledge, no comprehensive biochemical studies of saccharolytical thermophiles have previously been conducted with continuous fermentation of varying D-xylose concentrations. The investigated strain, *Thermoanaerobacter mathranii*, is a newly characterized extreme thermophilic anaerobic bacterium metabolizing D-xylose, xylan as well as D-glucose and L-arabinose to ethanol, acetate, lactate, CO_2 and H_2 (Larsen et al. 1997).

Materials & Methods

Microorganism

Thermoanaerobacter mathranii was used in this experiment as described in Sommer, Ahring (1998).

Mineral medium

The bacterium was cultivated in anaerobic medium as previously described (Sommer, Ahring, 1998).

Chemostat conditions and fermentation

The operation conditions of the chemostat and the fermentation conditions were as described in Sommer, Ahring (1998).

Preparation of crude cell extract

Methods used for protein and enzyme activity determinations were as described in Sommer, Ahring (1998).

Determination of intracellular enzyme activities

The handling of the crude extract and performance of the assays were as described in Sommer, Ahring (1998). Pyruvate decarboxylase was determined as previously described (Hoppner, Doelle, 1983). Acetate kinase activity was determined as previous described (Bergmeyer, 1983). NAD^+ and $NADP^+$-dependent alcohol dehydrogenase activities were determined as previous described (Vallee, Hoch, 1955), with ethanol as substrate. Lactate dehydrogenase was determined according to Bergmeyer, Bernt (1974).
Specific enzyme activities in the extracts were expressed in unit milligram of protein^{-1}, where 1 U is equivalent to the conversion of 1 µmol of substrate min^{-1}.

Sampling and extraction of intracellular metabolites

The procedures for sampling and extraction of intracellular metabolites were as described in Sommer, Ahring (1998).

Determination of intracellular metabolites

The concentration of intracellular metabolites was determined with enzymatic assays in which the oxidation or reduction of NAD(H) or $NADP^+$ was measured as end point analysis. This was done with a fluorescence spectrophotometer (Hitachi F-2000) at an emission of 450 nm after excitation at 340 nm (Jeppsson et al. 1996).

The standards and sample assay mixtures were incubated at room temperature (22-25°C) in the dark for one hour, before measurements.

Fructose 1,6 di-phosphate (FDP), pyruvate (PYR), and phosphoenol pyruvate (PEP) were measured according to Lowry, Passonneau (1972).

Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were measured in a coupled assay according to Bergmeyer (1983). The assay was modified to spectrofluorometric determination as follows: 50 mM imidazol (grade III) pH 7.6 was used as buffer, 0.01% (w/w) BSA, 50 µM $MgCl_2$, 40 µM $NADP^+$, 0.17 U/ml glucose-6-phosphate dehydrogenase, and 0.7 U/ml phosphoglucose isomerase.

Glyceraldehyde-3-phosphate (G3P) was measured by a modified FDP assay (Lowry, Passonneau, 1972). G3P was isomerised to dihydroxyacetone phosphate (DHP) and DHP converted to glycerophosphate by a concomitant oxidation of NADH to NAD^+. The assay mixture contained: 50 mM imidazol (grade III), pH 7.5 was used as buffer, 0.01% (w/w) BSA, 20 µM NADH, 1.6 U/ml triosephosphate isomerase, and 0.11 U/ml glycerophosphate dehydrogenase.

Reduced form of Nicotinamide Adenine Dinucleotide (NADH) was measured according to (Lowry, Passonneau, 1972). Pyruvate was converted to lactate by lactate dehydrogenase and concomitant NADH oxidation to NAD^+. The assay mixture contained: 50 mM imidazole (grade III), pH 7.8, 0.01% (w/w) BSA, 20 µM pyruvate, and 0.11 U/ml lactate dehydrogenase.

Nicotinamide Adenine Dinucleotide (NAD^+) was measured according to Vallee, Hoch (1955). Ethanol was converted to acetaldehyde by NAD^+-dependent alcohol dehydrogenase and concomitant reduction of NAD^+ to NADH. The assay mixture contained: 50 mM imidazole (grade III), pH 7.8, 0.01% (w/w) BSA, 200 µM 96% ethanol, and 0.55 U/ml NAD^+-dependent alcohol dehydrogenase.

Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) was measured by a modification of the assay described by Bergmeyer (1983). Glucose-6-phosphate was converted to gluconolactone-6-P with concomitant reduction of $NADP^+$ to NADPH . The assay contained: 50 mM imidazole (grade III), pH 7.8, 0.01% (w/w) BSA, 20 µM glucose-6-phosphate, 0.3 mM $MgCl_2$, and 0.17 U/ml glucose-6-phosphate dehydrogenase.

Intracellular acetate concentrations were measured by gas chromatography as described for excreted acetate. Intracellular lactate concentration was measured by HPLC as described for excreted lactate. Intracellular ethanol could not be determined due to vacuum evaporation treatment of the samples.

The concentration of intracellular metabolites was expressed in nmol mg of dry weight $cells^{-1}$.

All chemicals and enzymes used for the determination of enzyme activities and intermediary metabolite concentrations were from Sigma C0. (St Louis, Mo, USA).

Analyses

Steady state values were determined by a variance below 10% between four retention times. Samples were analyzed for optical density as described in Sommer, Ahring (1998). Ethanol and acetate were quantified by gas chromatography (HP5890) with flame ionization detection, with a fused silica capillary column (cross-linked polyethylene glycol-TPA; 30 m x 0.53 mm).
Lactate was quantified by high performance liquid chromatography on an organic acid analysis column (AminexR 10N exclusion HPX87-H 300 x 7.8 mm) at 65°C with UV detection at 190 nm, respectively (Waters Lambda max model 480). The mobile phase was 0.01 N H_2SO_4. Dry weight determinations were conducted as described in Yu et al. (1995).
CO_2 and H_2 were quantified with gas chromatography (column, 2 m poropak Q) with thermal conductivity detection (Mikrolab, Aarhus Denmark).

Results

Fermentation

The D-xylose metabolism of *T. mathranii* A3M1 was examined during continuous fermentation with varying D-xylose concentrations as described in Sommer, Ahring (1998).

Product formation rates

As shown in table 1, most of the inlet D-xylose is transported into the cells and converted to ethanol, acetate, lactate, CO_2 and H_2. However at increasing inlet concentration, an increasing D-xylose effluent concentration was measured.

Ethanol

At an inlet concentration of 33.3 mM (steady state 1), the ethanol yield was 1.15 mol ethanol/mol xylose, and the specific ethanol productivity rate was 8.9 mmol $g^{-1}h^{-1}$ (figure 1). The transition period between an inlet D-xylose concentration of 33.3 mM and 133 mM (transition 1), caused a decreased ethanol yield and specific ethanol productivity of 65% and 28%, respectively, compared to steady state 1 (figure 1). At the new steady state at an inlet D-xylose concentration of 133 mM (steady state 2), the ethanol yield and specific ethanol productivity, decreased further compared to transition 1. Not as drastically as compared to steady state 1, however (figure 1). By re-introduction of 33 mM D-xylose in the chemostat (transition 2), the same drastical physiological change as seen at transition 1 was not observed. The ethanol yield and specific ethanol productivity increased by 19% and 12%, respectively, compared to steady state 2 (figure 1). Reestablished steady state at 33 mM D-xylose gave an ethanol yield and specific ethanol productivity increase of 76% and 38%, respectively, compared to transition 2 (figure 1).

Acetate and lactate

The specific acetate and lactate productivities at steady state 1 were 2.9 and 2.0 mmol $g^{-1}h^{-1}$, respectively. The specific acetate productivity decreased slightly by 8%, at transition 1, however, the specific lactate productivity increased significantly by 67% compared to steady state 1. The specific acetate productivity decreased by 21% at steady state 2 compared to transition 1. The specific lactate productivity decreased by 64% compared to transition 1, the same productivity rate as at steady state 1 (figure 1).

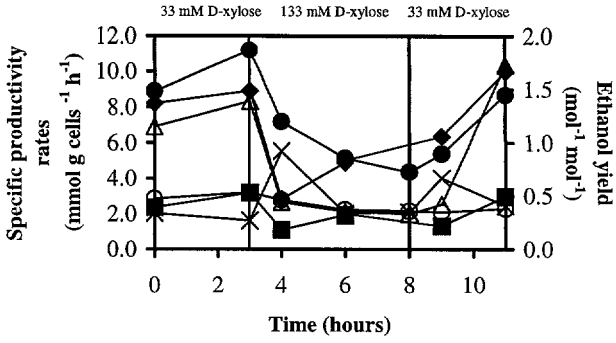

Fig. 1 Ethanol yields and specific productivity rates of *T. mathranii* A3M1 with varying D-xylose concentrations during continuously fermentation, 70°C, pH 6.0, retention time,12 hours. $q_{ethanol}$ (●), $q_{acetate}$ (○), $q_{lactate}$ (×),q_{CO2} (◆), q_{H2} (■),$Y_{E/X}$ ( ). g cells are expressed in g dry weight.

The specific productivities of acetate and lactate decreased by 4% and increased by 49% at transition 2, respectively, compared to steady state 2 (figure 1). The specific productivities of acetate and lactate increased by 8% and decreased by 39%, respectively at steady state 3, compared to transition 2 (figure 1).

CO_2 and H_2

The specific CO_2 and H_2 productivity rates were 8.6 and 2.8 mmol $g^{-1}h^{-1}$, respectively at steady state 1. The specific CO_2 and H_2 productivities decreased 67% and 60%, respectively, at transition 1, compared to steady state 1 (figure 1). The specific CO_2 and H_2 productivities increased both by 42%, at steady state 2 compared to transition 1 (figure 1). At transition 2, the specific CO_2 productivity was unchanged, while the specific H_2 productivity increased by 31%, both compared to steady state 2 (figure 1), the specific CO_2 and H_2 productivities increased by 50% and 55% at steady state 3 compared to transition 2 (figure 1).

Specific *in vitro* activities of intracellular enzymes in glycolysis

The specific pyruvate kinase activity decreased by 69% at transition 1, steady state 2 and transition 3, before regaining similar specific activity at steady state 3 compared to steady state 1 (figure 2). The specific acetate kinase activity increased significantly at increased D-xylose concentration and peaked by a 53% increase at steady state 2 (figure 2). The specific activity decreased when the D-xylose concentration was restored at 33 mM at steady state 3 (figure 2). The specific lactate dehydrogenase activity increased by 50% at transition 1 compared to steady state 1. Then dropped drastically at

Table 1 D-xylose consumption and product formation of *Thermoanaerobacter mathranii* A3M1 of varying D-xylose concentrations. Continuous fermentation, 70°C, pH 6.0, retention time,12 hours.

Inlet xylose (mM)	Dry weight (g/l)	Xylose consum .(mM)	Ethanol (mM)	Acetate (mM)	Intra acetate[1]	Lactate (mM)	Intra lactate[1]	CO_2 (mM)	H_2 (mM)	Carbon recovery (%)	O/R
33.3	0.27	25.1	28.9	9.3	8.4	6.6	5.4	35.9	10.3	132	1.05
33.3	0.26	24.8	34.4	9.8	15.2	5.0	0.6	34.0	12.1	140	0.84
trans.	0.35	69.0	30.5	11.7	3.7	23.5	4.5	34.1	13.6	77.4	0.92
133	0.40	69.8	24.7	10.7	4.5	9.7	7.1	27.4	10.8	63.3	0.91
133	0.38	60.2	20.1	9.9	4.3	9.6	8.3	n.d.	n.d.	-	-
trans.	0.39	60.0	25.3	9.9	6.2	18.9	4.2	28.1	7.6	83.3	0.97
33.3	0.31	18.8	32.4	8.5	0	9.1	6.3	37.7	11.2	214	0.99

[1] µmol mg dry weight cells^{-1}. Intra = Intracellular. n.d. = not determined O/R Oxidation/Reduction ratio.

steady state 2, after which the specific activity increased significantly at transition 2 also seen at transition 1,before regaining similar specific activity at steady state 3. The specific $NADP^+$-dependent alcohol dehydrogenase activity showed significant fluctuation during the experiment. The specific activity was high at steady state 1, approximately 13 times higher than the activity of the other measured intracellular enzymes. However, when the D-xylose concentration was raised, the specific activity dropped by 99%. Only a partly recovery of the activity was regained at steady state 3 compared to steady state 1 (figure 2). The specific NAD^+-dependent alcohol dehydrogenase activity was low at steady state 1. When the D-xylose concentration was raised at transition 1, the activity increased significantly as opposed to the $NADP^+$ dependent alcohol dehydrogenase (figure 2).

Levels of intramediary metabolites in the glycolysis in *T. mathranii* A3M1

Steady state 1. In the upper part of the glycolysis, intracellular concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1.6-di-phosphate varied (table 2). A two- to three fold difference was seen in concentrations of glucose 6-phosphate and fructose 6-phosphate. Fructose 1.6 di-phosphate was found in very high concentration of 2.5-6 times higher compared to glucose 6-phosphate and fructose 6-phosphate, respectively (table 2).

Table 2 Measurements of intracellular metabolites of strain *T. mathranii* A3M1.

Inlet [xylose]	[G6P]	[F6P]	[FDP]	[G3P]	[PEP]	[PYR]	[NAD^+]	[NADH]	[$NADP^+$]
(mM)	(nmol mg dry weight cells^{-1})								
33	3.7 ± 0.09	1.9 ± 0.05	9.2 ± 2.9	18.3 ± 4.3	13.5 ± 0.6	14.3 ± 0.3	83.3 ± 0.8	95.7 ± 3.6	3.9 ± 0.01
33	3.8 ± 0.1	1.1 ± 0.08	8.4 ± 2.2	29.8 ± 0.2	12.5 ± 2.7	10.6 ± 1.2	62.8 ± 0.8	89.2 ± 0.3	3.8 ± 0.1
Trans.	2.2 ± 0.1	1.7 ± 0.2	ND	5.0 ± 0.5	1.2 ± 0.4	8.5 ± 0.3	52.5 ± 0.2	n.d.	n.d.
133	2.7 ± 0.02	1.6 ± 0.2	1.1 ± 0.4	ND	4.8 ± 0.7	16.6 ± 0	65.2 ± 0.7	25.9 ± 0	2.6 ± 0.1
133	3.1 ± 0.05	1.8 ± 0.1	1.6 ± 0	ND	3.0 ± 0.4	19.1 ± 0.2	74.9 ± 3.7	36.2 ± 1.3	2.8 ± 0.1
Trans.	2.4 ± 0.01	1.3 ± 0.02	1.5 ± 0.3	0.9 ± 0	3.2 ± 0.5	7.1 ± 0.05	57.8± 2.1	20.3 ± 0.2	2.4 ± 0.01
33	2.7 ± 0.2	1.0 ± 0.01	7.6 ± 1.9	12.0 ± 1.0	6.6 ± 0.2	4.0 ± 0.3	75.3 ± 2.0	61.1 ± 0	2.0 ± 0

ND = Not detected, n.d. = Not determined, Intracell. = Intracellular concentration, G6P = Glucose-6-phosphate, F6P = Fructose-6-phosphate, FDP = Fructose 1,6-di-phosphate, G3P = Glyceraldehyde-3-phosphate, PEP = Phosphoenolpyruvate, PYR = Pyruvate, $NADP^+$, NADH, NAD

In the middle part of the glycolysis, the intracellular concentration of glyceraldehyde 3-phophate was significantly higher than the other measured metabolites (table 2). In the lower part of the glycolysis, the intracellular concentrations of phosphoenol pyruvate and pyruvate were similar, in high concentrations (table 2).
The concentrations of NAD^+ and NADH were very high compared to the other measured intracellular metabolites. The NADH /NAD^+ ratio was 1.15 to 1.40. The intracellular concentration of $NADP^+$ was 18.9 to 24.5 times lower compared to NAD^+ and NADH, respectively. In spite of several attempts, we were not successful in measuring reproducible intracellular concentrations of NADPH

Transition 1. The concentrations of all the measured intracellular metabolites decreased significantly compared to steady state 1, except for fructose 6-phosphate which remained unchanged (table 2). The concentrations of the co-enzymes NAD^+, NADH and $NADP^+$ also indicated a severe redox imbalance in the cells compared to steady state 1, as NADH and $NADP^+$ no longer could be measured.

Steady state 2. The intracellular concentrations of glucose-6-phosphate and fructose 6-phosphate had more or less the same concentrations as in steady state 1. The intracellular concentrations of fructose 1.6 di-phosphate, glyceraldehyde 3-phosphate and phosphoenol pyruvate were significantly decreased compared to steady state 1. The intracellular concentration of pyruvate increased compared to steady state 1. Compared to steady state 1, the NADH/NAD^+ratio was 2.5 times lower and the concentration of $NADP^+$ decreased 1.4 times.

Transition 2. The intracellular concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1.6 di-phosphate and phosphoenolpyruvate did not change significantly compared to steady state 2 (table 2). However, glyceraldehyde 3-phosphate increased and pyruvate decreased. The NADH/NAD^+ imbalance was still significant compared to steady state 2. The intracellular concentration of $NADP^+$ decreased slightly compared to steady state 2.

Steady state 3. The intracellular concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1.6 di-phosphate were all found in lower concentrations compared to steady state 1. The concentration of glyceraldehyde 3-phosphate increased significantly compared both to transition 2 and steady state 2. In the lower part of glycolysis, the intracellular concentration of phosphoenol pyruvate increased to twice the concentration compared to transition 2, however, did not reach the same concentrations measured at steady state 1. The concentration of pyruvate decreased significantly compared to the other measured metabolites at steady state 1 and 2 and transition 1 and 2 (table 2). The NADH/NAD^+ ratio was in balance at 1.0

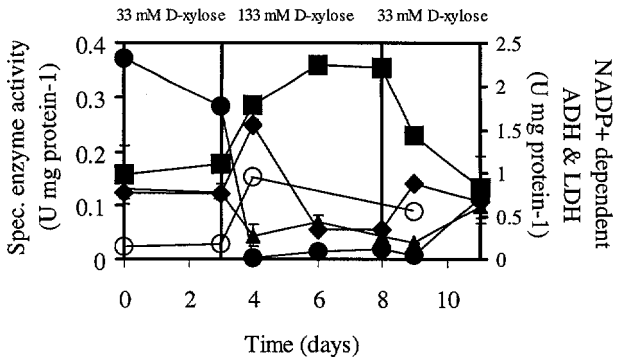

Fig 2 *In vitro* specific enzyme activity of *T. mathranii* A3M1 during continuous fermentation of varying D-xylose concentrations. Pyruvate kinase (▲), Acetate kinase (■), Lactate dehydrogenase (◆), $NADP^+$-dependent alcohol dehydrogenase (●), NAD^+-dependent alcohol dehydrogenase (○).

compared to transition 1, steady state 2 and transition 2. The intracellular concentration of $NADP^+$ was slightly decreased compared to steady state 2 and transition 2 (table 2).

Discussion

Thermophilic anaerobic ethanol producing bacteria have a lower substrate tolerance and ethanol productivity, compared to e.g. ethanol producing yeast and the bacteria, *Zymomonas mobilis* and *Escherichia coli* (Jones, Woods, 1991; Lovitt et al. 1988; Lynd et al. 1991). The physiological and biochemical mechanisms responsible for these metabolic insufficiencies are scarce in thermophilic anaerobic ethanol-producing bacteria. Identification and regulation of metabolic fluxes, i.e. the bottlenecks in the metabolism, is a fundamental basis for physiological and genetic engineering to improve ethanol yield and productivity. Thauer et al. (1977) states that fluxes in different catabolic branches are adjusted so that the ATP gain and metabolic efficiency are optimal for the respective growth conditions. As Lynd et al. (1991) points out, this statement of a control strategy is not enough to specify product distributions. Roos et al. (1985) bring the theory further, by stating that products of metabolic branches, having a higher ATP yield, are preferred when ATP is scarce and products of metabolic branches having a lower ATP yield are preferred when ATP is plentiful. During increased substrate concentration under constant growth rate conditions, the concentrations of added substrate may act as an inhibitor, influencing the metabolic flux though the appropriate pathways (Lynd et al. 1991).

The presence of fructose 6-phosphate and fructose 1.6 di-phosphate indicate that *T. mathranii* A3M1 employ the Embden-Meyerhof pathway (figure 3). The presence of an oxidative pentose phosphate pathway or a ferredoxin cycle has not been established in this study. Production of H_2, however, indicates the presence of a ferredoxin-H_2 oxidoreductase (hydrogenase).

In the upper part of the glycolysis, the response to an increased D-xylose concentration was less drastic. The concentration of fructose 6-phosphate was unchanged and only a small decrease in the concentration of glucose 6-phosphate was noted. This indicated an unaltered flux through the upper part of the glycolysis and possibly an indication of anabolic activity.
In the middle part of the glycolysis, the intracellular concentrations of glyceraldehyde 3-phophate and fructose 1.6 di-phosphate were high at steady state 1. This indicated a high flux from the pentose phosphate pathway into the upper and middle part of glycolysis. At increased inlet concentration of D-xylose, at transition 1, the flux was drastically altered. Fructose 1.6-di-phosphate was undetectable and a marked decreased concentration of glyceraldehyde 3-phosphate was observed. At steady state 2, the reverse situation emerged. Fructose 1.6-di-phosphate has been shown to function as an activator of lactate dehydrogenase in *C. thermohydrosulfuricum* (Germain et al. 1986; Turunen et al. 1987). The absence and low concentration of fructose 1.6-diphosphate at transition 1 and transition 2 and the increased *in vitro* specific lactate dehydrogenase activity and specific lactate productivity, respectively, indicated that fructose 1.6-di-phosphate does not have this regulative role in *T. mathranii in vivo*.

In the lower part of the glycolysis, the intracellular concentrations of phosphoenolpyruvate and pyruvate were similar, in high concentrations, which indicated a high flux through the glycolysis. However, at increasing inlet D-xylose concentrations, decreased levels of both metabolites were observed at transition 1. Phosphoenol pyruvate and pyruvate decreased significantly. This was supported by a decreased specific *in vitro* pyruvate kinase activity by 66%. At steady state 2, the intracellular concentration of phosphoenolpyruvate was still significantly lower than at steady state 1 indicating a lowered flux through the glycolysis. The specific *in vitro* pyruvate kinase activity decreased significantly at steady state 2 compared to steady state 1, but this did not result in a concomitant decrease in the concentration pyruvate. The accumulation of pyruvate indicated a decreased activity of the pyruvate ferredoxin oxidoreductase. This observation is supported by the significantly decreased in the specific CO_2 productivity rate and the increased lactate dehydrogenase activity at transition 1. The diversion to lactate production was observed in thermophilic clostridia . The cleavage of pyruvate is limited by the rate of ferredoxin regeneration (Ng, Zeikus, 1982; Germain et al. 1986). The decreased activity of the ferredoxin cycle of *T. mathranii* is also supported by the decreased specific H_2 productivity. Pyruvate is an allosteric *in vitro* activator of alcohol dehydrogenase in *T. ethanolicus* (Bryant, Ljungdahl, 1981) as well as in *T. thermohydrosulfuricum* (Carreira et al. 1982). This seems not to be the case with *T. mathranii* as pyruvate accumulation did not cause increased specific alcohol dehydrogenase activity nor increased ethanol production. However, if the conversion of acetyl-CoA to acetaldehyde is NADH dependent instead of NADPH dependent, the low concentration of NADH observed at high inlet concentration of D–xylose, could cause a decreased production of ethanol. At low inlet D-xylose concentration, the ratio of NADH/NAD^+ was maintained in balance however, was in imbalance at increasing D-xylose. The cells reached at threshold value whereby the increased substrate concentration cannot be utilized for increasing growth and growth inhibition emerges. The metabolism could be switched into anabolism, producing e.g. glygogen or polyhydroxyacetate. At the gas chromatographic analysis for short chain fatty acids, another peak was evident (not presently identified) at high D-xylose concentration The retention time was almost similar as that of acetate. This product was not identified nether as oxaloacetate, acetaldehyde nor acetyl CoA. However, it could be acetyl phosphate, indicating deficiency in acetate formation or phophorylated acetate, e.g. polyhydroxyacetate, an indication of anabolism.

The intracellular concentration of $NADP^+$ was 18.9 to 24.5 times lower compared to NAD^+ and NADH, respectively. $NADP^+$ was not detectable at the initial increase in D-xylose concentration in the chemostat, indicating that the co-enzyme was reduced to NADPH for reductive biosynthesis. The concentration was again detectable at steady state 2, however, in lower concentrations than at steady state 1. This could be explained by the significant decreased specific *in vitro* activity of the $NADP^+$-dependent alcohol dehydrogenase, whereby oxidation of NADPH was reduced.

Product formation

The final formation of end-fermentation products and their mutual distribution are determined by the cells response to a given metabolic state, their needs for energy, metabolites for reductive biosynthesis, and reducing equivalents. Evident from our experiment, at least some of the end-fermentation products fluctuated significantly while others remained more or less constant, as a response to the fluctuated D-xylose concentration.

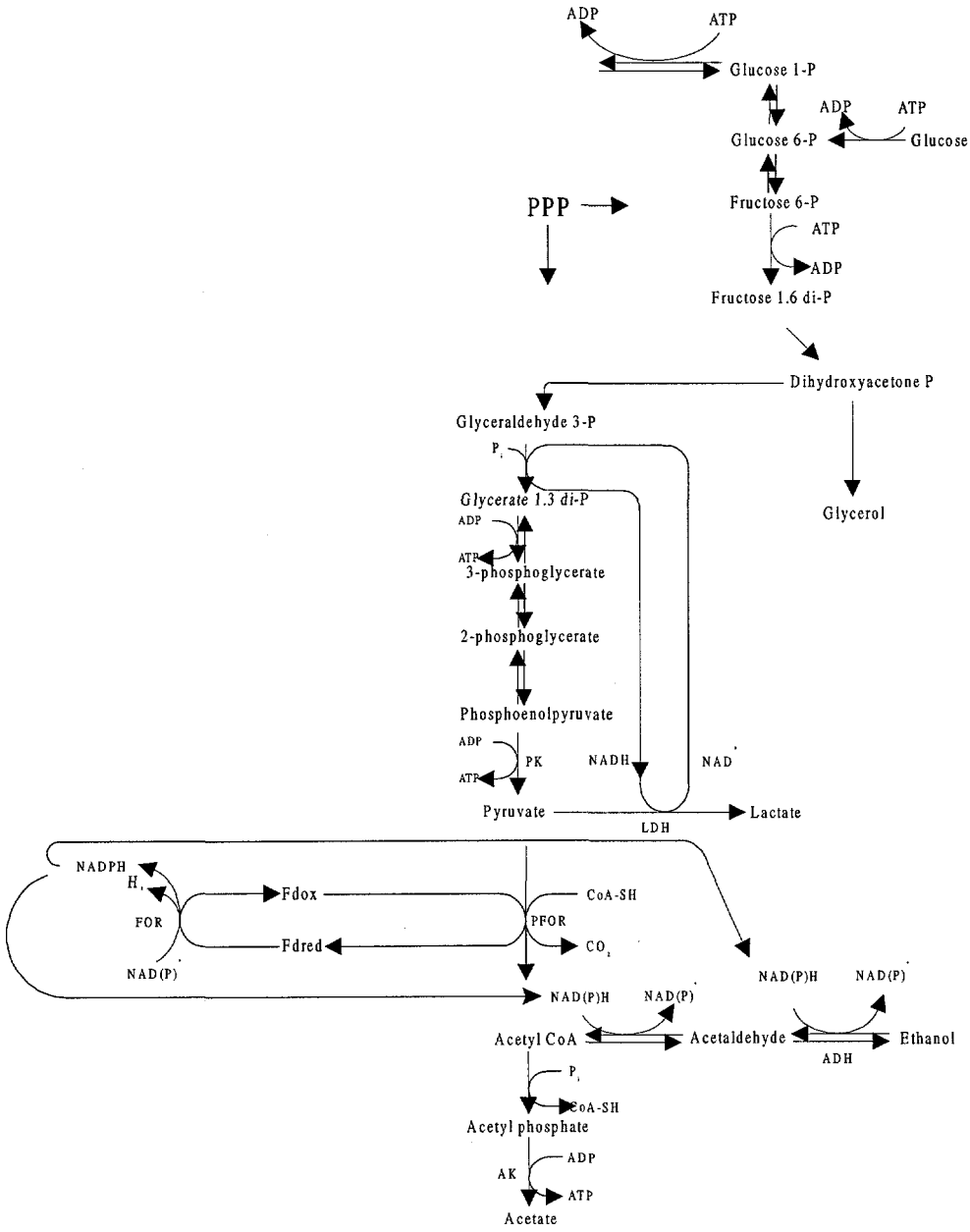

Fig 3 Model for glycolysis and product formation of *T. mathranii* A3M1 PPP = Pentose phosphate pathway, PFOR = Pyruvate ferredoxin oxidoreductase, ADH = Alcohol dehydrogenase, AK = Acetate kinase, LDH = Lactate dehydrogenase, PK = Pyruvate kinase, FOR = NAD(P)H Ferredoxin oxidoreductases.

Acetate production. The specific acetate productivity showed modest fluctuations through the experiment. The productivity decreased slightly at elevated D-xylose concentration at transition 1 and steady state 2, before regaining the productivity found at steady state 1.
The measured intracellular concentration of acetate did not show any accumulation at elevated D-xylose concentration. The specific *in vitro* acetate kinase activity, however, increased through transition 1 and steady state 2. As production of acetate is directly linked to H_2 production, this indicates that the H_2-ferredoxin oxidoreductase and pyruvate ferredoxin oxidoreductase activities, respectively, are tightly regulated by unfavorable redox potential at elevated H_2 production (Jones, Woods, 1991).

Lactate and CO_2 production. T. mathranii does not have a pyruvate decarboxylase enzyme as does yeast, which

converts pyruvate to acetaldehyde. The specific productivity of lactate showed a significant fluctuation through the experiment. The productivity increased markedly at both transition points before it reached the same level at steady state 2 as found in steady state 1. The productivity was slightly increased at steady state 3. The fluctuating production of lactate was confirmed in the specific *in vitro* activity pattern of lactate dehydrogenase, however, the activity being lower at steady state 2 than steady state 1. The increased conversion of glyceraldehyde 3-phosphate to 1.3 di-phosphoglycerate caused an elevated production of lactate at transition 1, balancing the $NADH/NAD^+$ ratio. However, at steady state 2, the reduced flux through the glycolysis decreased the reduction of NAD^+ to NADH by glyceraldehyde 3-phosphate, a reaction that could be more efficiently regulated than the activity of the lactate dehydrogenase. This could have caused the decreased $NADH/NAD^+$ratio at steady state 2 and transition 2. The $NADH/NAD^+$-balance was almost regained at steady state 3, caused by an increased flux through the glycolysis with concomitant reduction of NAD^+ to NADH.

Ethanol and biomass production. The ethanol yield, volumetric and specific productivities decreased significantly at increasing D-xylose concentration. This was confirmed by the significantly decreased specific *in vitro* $NADP^+$-dependent alcohol dehydrogenase activity. The decreased ethanol yield at transition 1 is probably caused by a decreased activity of pyruvate ferredoxin oxidoreductase and a concomitant diversion of pyruvate to lactate. However, at steady state 2 this situation did no longer exist, indicating that the increased biomass production balanced the NADP/NADPH ratio, so no surplus of NADPH should be oxidized by the $NADP^+$-dependent alcohol dehydrogenase. This coincide well with the biomass productivity, which did not decrease at transition 2 This was because of a large amount of intracellular D-xylose being catabolized. At steady state 3 the productivity started to decrease when the intracellular D-xylose pool was used. Only at steady state 3, was the $NADP^+$-dependent alcohol dehydrogenase activity recovered, however, not to the same activities as found at steady state 1. The reason for switching between an $NADP^+$ and a NAD^+-dependent alcohol dehydrogenase is not fully understood in this experiment. However, having this option gives the microorganisms more versatility at a metabolic stress situation.

Conclusion

The following observations were seen as a response in intracellular metabolite concentration and *in vitro* specific enzyme activities of the glycolysis by increasing inlet D-xylose concentration from 33 mM to 133 mM.

Upper part of glycolysis. An almost unchanged metabolic flux towards glucose-6-phosphate, indicating either anabolism or NADPH generation through an oxidative PPP.

Middle and lower part of glycolysis. Decreased flux towards and accumulation of pyruvate. A significant $NADH/NAD^+$ imbalance.

Product formation. Decreased ethanol yield and specific-ethanol productivity. Severe decreased *in vitro* $NADP^+$-dependent alcohol dehydrogenase activity, however, an increased *in vitro* NAD^+-dependent alcohol dehydrogenase activity. An increased *in vitro* specific acetate kinase activity, however, an almost unchanged specific acetate production rate. Accumulation of an unidentified acetate derivate (not identified as oxaloacetate, acetaldehyde, or acetyl CoA). It could be acetyl phosphate, indicating an insufficient phosphoryclastic system or the compound could be polyhydroxyacetate, indicating anabolism. The specific lactate productivity and the *in vitro* specific lactate dehydrogenase were significantly higher at the transition between D-xylose concentrations of 33 mM and 133 mM and visa versa, however, most notably at increased D-xylose concentration. This indicated a reduced activity of the ferredoxin oxidoreductase cycle at elevated D-xylose concentration.

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