Exploring the reductive capacity of *Pyrococcus furiosus*

The reduction of carboxylic acids and pyridine nucleotides

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Exploring the reductive capacity of *Pyrococcus furiosus*

The reduction of carboxylic acids and pyridine nucleotides

Eyke Corrie Ditty van den Ban

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 19 oktober 2001
des namiddags te half twee in de Aula.

ISBN 90-5808-508-2

In tegenstelling tot wat Sapra *et al.* beweren, is het membraangebonden hydrogenase *wel* betrokken bij de directe oxidatie van ferredoxine in het waterstofmetabolisme van *Pyrococcus furiosus*.

dit proefschrift, hoofdstuk 4

Sapra R et al. (2000) J Bacteriol 182: 3423-3428

Ondanks het vele onderzoek aan geurverwijdering met biofilters is het effectieve gebruik ervan meer gebaseerd op ervaring dan op kennis verkregen door wetenschappelijk onderzoek.

Cox HHJ and Deshusses MA (1998) Curr Opin Biotechnol 9: 256-262

Pyrococcus furiosus hydrogenase is een uitstekende biokatalysator voor de productie en regeneratie van NADPH.

dit proefschrift, hoofdstuk 5

Het vaststellen van het molecuulgewicht van *Pyrococcus furiosus* ferredoxine is geen sinecure.

Aono S et al. (1989) J Bacteriol 171: 3433-3439

Johnson KA et al. (2001) Int J Mass Spec 204: 77-85

De diervoedernamen 'Kalver Smulmix' en 'Smikkels' suggereren dat landbouwhuisdieren menselijke eigenschappen bezitten.

AIO's (en OIO's) zouden aan teamsport moeten doen ter compensatie van hun vaak eenzame onderzoek.

RSI hoeft niet.

Intermediair, 11 januari 2001

Interdiciplinair onderzoek vereist niet alleen het hebben van de juiste kennis, maar ook het hebben van de juiste kennissen.

Stellingen behorend bij het proefschrift:
Exploring the reductive capacity of *Pyrococcus furiosus*
The reduction of carboxylic acids and pyridine nucleotides
Eyke van den Ban
Wageningen, 2001

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Aan mijn oma's

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Introduction

Aldehydes and alcohols are of great interest for pharmaceutical, food, and flavor industries. A possible method to obtain these compounds is the reduction of carboxylic acids (figure 1). However, the reduction of carboxylic acids to aldehydes is both chemically and enzymatically a difficult reaction, since the reduction requires a redox potential of about -600 mV [1]. Nevertheless, there are several chemical and biological processes described for carboxylic acid reduction [2-4].

Chemical processes are usually nonselective and not very environmentally friendly, because of the high temperatures and heavy metal-containing catalysts that are often used. An advantage of chemical reduction processes is the use of relatively cheap reductants. The main drawbacks of reductions by biological catalysts are the use of expensive enzymes and low overall yields. The advantages of biological reductions include relatively mild reaction conditions and specificity, particularly stereospecificity. The stereospecificity is important when the stereoisomers have different activities, for example, the difference in pharmaceutical activities of the acids *R* and *S*-ibuprofen. The *S*-enantiomer has an anti-inflammatory function, whereas the *R*-enantiomer has not [5]. Furthermore, biologically produced compounds can be labeled as 'natural', thereby increasing their commercial value [6]. This is especially true for food en flavor compounds.

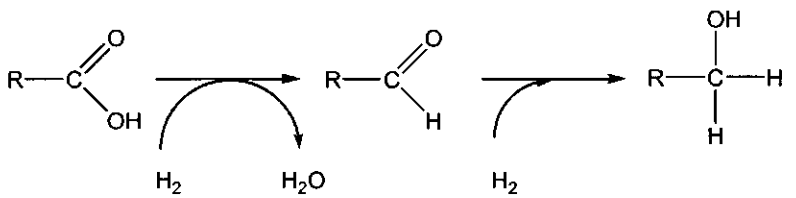


Figure 1 Reduction of carboxylic acid to the corresponding aldehyde and alcohol by dihydrogen (two electrons and two H^+).

CHEMICAL REDUCTION OF CARBOXYLIC ACIDS

The direct chemical reduction of carboxylic acids was not possible until 1946. The discovery of the powerful reducing agent lithium aluminum hydride ($LiAlH_4$) resulted in a method for reducing acids to primary alcohols. However, this reagent has some major disadvantages: it reacts violently with water [7], it also reduces the double bonds in unsaturated carboxylic acids, and it is expensive [3]. These features make it a less suitable agent for most industrial applications.

Since then, different methods and catalysts have been found to catalyze the chemical reduction of carboxylic acids. Several catalysts such as cadmium/rhenium [8], vanadium [9], and ruthenium [10, 11] are used together with dihydrogen. With these catalysts it is in principle possible to reduce only the carboxylic moiety and not the unsaturated part of the molecule [8, 12]. Typically, such reductions are performed at elevated temperatures (200 - 500°C) and high dihydrogen pressures (7 - 344 bar) [8, 11, 12], which make these processes rather expensive.

Some chemical reductions can also be performed at lower temperatures (< 50°C) For example, the reduction of carboxylic acids with sodium borohydride and a chloroformate ester [3]. These reactions are selective, result in a high yield, and are claimed to be relatively inexpensive but need organic solvents.

BIOLOGICAL REDUCTION OF CARBOXYLIC ACIDS

The ability to reduce carboxylic acids is described for eukaryotes, prokaryotes, and archaea. Carboxylic acid reduction and mainly the reduction of aromatic acids have been studied in several microorganisms [2]. The best-studied microbial systems are summarized in table 1.

Most studies on the reduction of carboxylic acids have been done on either whole cells or cell free extracts. Up till now three different ways to reduce carboxylic acids to aldehydes have been described as depicted in figure 2. In two out of three mechanisms the acid needs to be activated prior to reduction to the aldehyde (reactions A and B in figure 2). Activation of the acid means the formation of an energy-rich bond (acyl-AMP or acyl-CoA) and this is necessary, because it facilitates the thermodynamically difficult reduction [13]. Without activation, reductions of carboxylic acids are not possible with a

reductant like NAD(P)H since the (standard) free energy change, $\Delta G°$, of the redox reaction is not enough to allow carboxylic acid reduction [14].
In most microorganisms the aldehydes generated are further reduced to the corresponding alcohol. This reduction is catalyzed by alcohol dehydrogenases.

Table 1 A summary of microorganisms that are able to reduce (activated) carboxylic acids.

Source	Organism	Substrate type	Cofactor	References
Bacteria	*Actinobacter* sp. H01-N	acyl-CoA	NADH	[15]
	Clostridium butyricum	acyl-CoA	NADH	[16, 17]
	Clostridium formicoaceticum	aliphatic acid aromatic acid	unknown (TMV)[#], (MV)[¶]	[18-20]
	Clostridium thermoaceticum	aliphatic acid aromatic acid	unknown (MV)[¶], (CAV)[§]	[21, 22]
	Mycobacterium tuberculosis	acyl-CoA	ATP, NADPH	[23]
	Nocardia asteroides	acyl-AMP aromatic acid	ATP, NADPH	[24-26]
	Nocardia sp. strain NRRL 5646	aromatic acid	ATP, NADPH	[5, 27-29]
	Photobacterium phosphoreum	acyl-CoA acyl-AMP aliphatic acid	ATP, NADPH	[30-34]
	Vibrio harveyi	acyl-CoA	NADPH	[35]
Fungi	*Bjerkandera* sp. strain BOS55	aromatic acid	unknown	[36]
	Neurospora crassa	aromatic acid	ATP, NADPH	[37, 38]
	Pycnoporus cinnabarinus	aromatic acid	unknown	[6, 39]
	Trametes versicolor	aromatic acid	ATP, NADPH	[40, 41]
Archaea	*Thermococcus* strain ES-1	aliphatic acid	Fd[†], (MV)[¶]	[42]

[#] TMV = tetramethyl viologen, an artificial electron donor
[¶] MV = methyl viologen, an artificial electron donor
[§] CAV = 1,1'-carbamoylmethyl viologen, an artificial electron donor
[†] Fd = ferredoxin

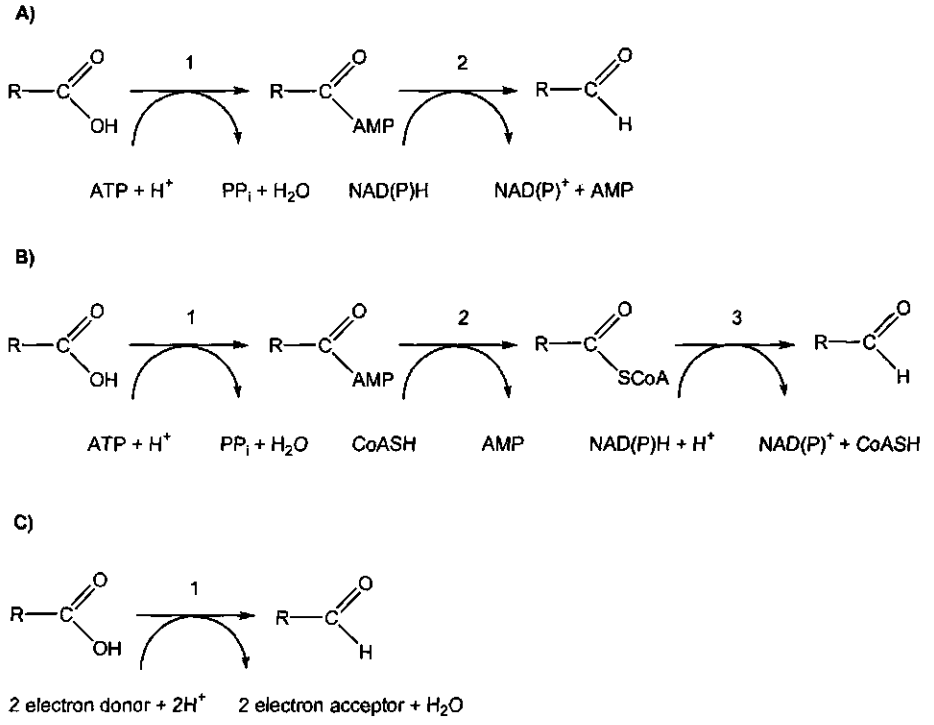


Figure 2 Three different routes described for carboxylic acid reduction.
A) Activation with ATP, reduction with NAD(P)H
B) Activation with ATP and CoA, reduction with NAD(P)H
C) Direct reduction

Carboxylic acid activation by ATP

The first step in the ATP-mediated reduction starts with the activation of the acid by ATP to generate acyl-AMP. Subsequently, acyl-AMP is reduced by NAD(P)H to the aldehyde as depicted in figure 2A. ATP-mediated reductions have only been found in bacteria [25-27, 32] and fungi [37, 38, 41], for example, in *Photobacterium phosphoreum* [32] and *Neurospora crassa* [37].

N. crassa, Nocardia asteroides and *Nocardia* sp. strain NRRL 5646 catalyze only the reduction of aromatic acids. This reaction is performed by an aryl-aldehyde:NADPH oxidoreductase and this enzyme catalyzes both the activating and reducing steps (reactions 1 and 2 in figure 2A) [25, 27, 38].

The substrate specificity of *P. phosphoreum* differs from the above-described ones. *P. phosphoreum* reduces aliphatic acids [32], whereas the *Nocardia* strains

only reduce aromatic acids [25, 27]. Moreover, the enzymes and mechanism involved in acid reduction in *P. phosphoreum* are different. The reduction of fatty acids is catalyzed by a fatty acid reductase complex. This complex contains fatty acyl-protein synthetase, fatty acyl transferase, and fatty acyl-CoA reductase subunits [33]. The synthetase subunit activates free fatty acids with ATP to produce a fatty acyl-AMP intermediate, which is shown as reaction 1 in figure 2A. Subsequently, the fatty acyl group is transferred to the reductase subunit by fatty acyl transferase and reduced to the aldehyde with NADPH. This reduction is catalyzed by fatty acyl-CoA reductase and depicted as reaction 2 in figure 2A [32].
As the name of the latter subunit already suggests it can also reduce fatty acyl-CoA (reaction 3, figure 2B) [30, 33], but the reduction of carboxylic acids in this organism does not proceed via acyl-CoA. The reduction is CoA-independent, moreover, there is no acyl-CoA synthetase activity described for this organism [34].
Finally, the fatty acid reductase complex from *P. phosphoreum* requires a flavin mononucleotide as a cofactor. This flavin requirement was not found in *Nocardia* species [27].

Carboxylic acid activation by ATP and CoA

The second bioreductive mechanism, like the first one, starts with the activation by ATP to acyl-AMP (reaction 1 in figure 2B). In a second step, acyl-AMP is converted to acyl-CoA (reaction 2 in figure 2B), which is subsequently reduced by acyl-CoA reductase to the aldehyde (reaction 3 in figure 2B) [13]. This mechanism is well documented. It is described for a range of organisms including bacteria and yeasts [2] as well as for mammalian mitochondria [14].
Well-studied systems include the fatty acyl-CoA synthetases of *Escherichia coli* [43], *Bacillus megaterium* [44], and *Rhodospirillum rubrum* [45]. These enzymes catalyze the ATP and CoA-dependent activation of carboxylic acids to acyl-CoA via acyl-AMP. The substrate specificity of these fatty acyl-CoA synthetases is, as expected, highest for fatty acids [13].

Reduction of non-activated acids

Some microorganisms are able to catalyze the reduction of aliphatic and aromatic carboxylic acids without the need for ATP and/or CoA. This third bioreductive mechanism is depicted in figure 2C and involves enzymes called

aldehyde oxidoreductases (AOR). AORs have been found in *Thermococcus* ES-1 [42], *Clostridium thermoaceticum* [22], and *C. formicoaceticum* [19, 20]. The enzymes contain either tungsten or molybdenum in their catalytic center. All three organisms appear to have a tungsten-containing AOR (W-AOR) [19, 22, 42], except *C. formicoaceticum* which also contains a molybdenum AOR (Mo-AOR) [20]. The pH optima of the enzymes range from pH 4.65 for *C. thermoaceticum* W-AOR [22] to pH 6.0 for the other AORs [19, 20, 42].
What the physiological cofactor is in the *Clostridia* strains remains to be established. Up till now the only described experiments, where non-activated acids were reduced, used artificial reductants with low redox potentials like methyl viologen and tetramethyl viologen [19, 20, 42]. A major difference with the other AORs is that *C. thermoaceticum* W-AOR can use artificial mediators with a less negative redox potential, like 1,1'-carbamoylmethyl viologen [22]. This might be explained by the low pH optimum of the enzyme. Under those conditions carboxylic acids are protonated, which makes them more susceptible to reduction. Therefore, it is suggested that undissociated acids are the real substrates of the *C. thermoaceticum* W-AOR [22].

Biological Functions of Carboxylic Acid Reductions

Carboxylic acids are reduced in microorganisms for several reasons. For example, acids are reduced as part of the biodegradation of lignin and humic acid. Furthermore, products of acid reduction such as long-chain aliphatic aldehydes are used for bioluminescence and fatty alcohols form building blocks for the synthesis of wax esters.

Degradation of lignin

Some of the fungi given in table 1 are white-rot fungi. They are involved in the degradation of lignin; a very complex polymer that is found in all higher plants. It is thought that a complex network of reactions like decarboxylation, oxidation, and reduction bring about the degradation of lignin [46]. Some of the intermediates generated are aromatic acids (figure 3), which are reduced intracellularly to the corresponding aldehydes and alcohols. These aldehydes and alcohols are oxidized by the extracellular aryl alcohol oxidases to generate H_2O_2, which is subsequently used by the extracellular lignolytic peroxidases [47, 48]. It is proposed that the aldehydes and acids thus formed are reduced intracellularly to close the physiological redox cycle [47, 49].

The reduction of ferulic acid, one of the acids formed during the degradation of lignin, is studied in detail. This compound can be reduced to the corresponding aldehyde and alcohol by several whole-cell cultures including *Phanerochaete chrysoporium* [50], *Sporotrichum pulverulentum* [51], *Trametes* sp. [52], and *Pycnoporus cinnabarinus* [39].
Alternatively, ferulic acid can be degraded to vanillic acid. Subsequently, vanillic acid is reduced to vanillin and vanillyl alcohol [39, 51, 53].

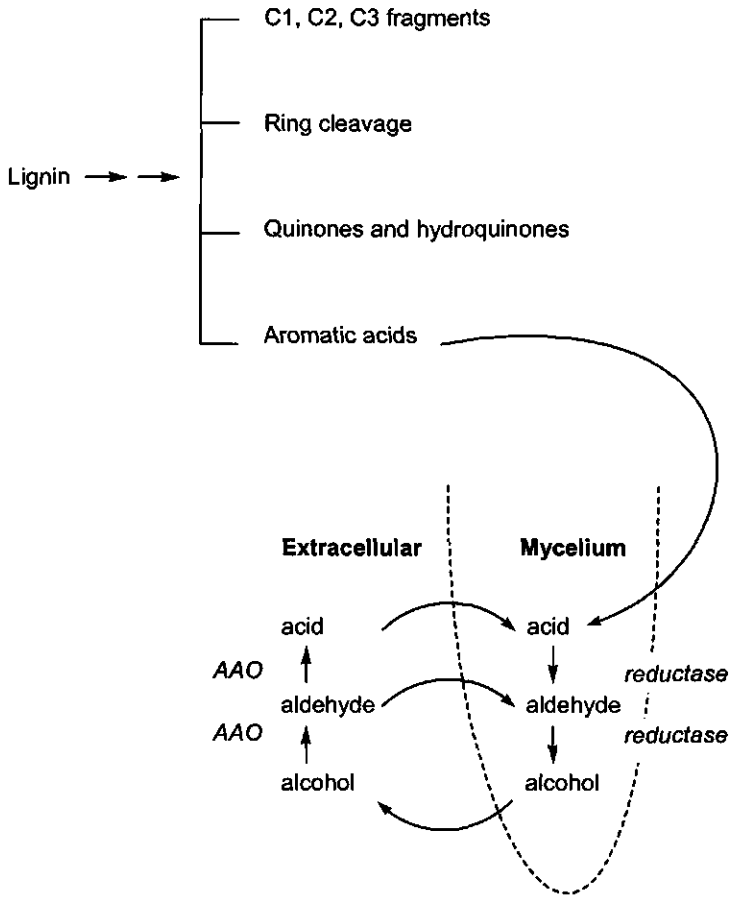


Figure 3 Schematic pathway for lignin degradation [49]. *AAO* = aryl alcohol oxidase.

Degradation of humic acid

Besides lignin, *Trametes versicolor* can also decompose humic acid in the presence of a carbon source that is easily assimilated [54, 55]. The chemical composition of humic acid is still unclear; it is difficult to identify all components and it is largely dependent on the type of soil it is extracted from. Vanillic acid, 3,5-dihydroxybenzoic acid, and *m*- and *p*-hydroxybenzoic acids are found in humic acid but only at low concentrations [54].

Several fungi, e.g. *Polyporus* sp. and *Trametes* sp. reduce the carboxylic group of *m*-hydroxybenzoic acid that is present in humic acid [54, 55]. For *T. versicolor* it has been shown that the acids are reduced in two steps, since the corresponding aldehyde could be detected prior to the appearance of the alcohol [54].

Bioluminescence

Bioluminescence is intensively studied in *Photobacterium phosphoreum* [33, 56] and *Vibrio harveyi* [35]. The key reaction is the luciferase-catalyzed oxidation of a long chain aliphatic aldehyde and $FMNH_2$. This results in the emission of blue-green light at 490 nm and the production of the corresponding carboxylic acid [56].

It was found that aldehydes involved in the bioluminescence reaction are generated by the reduction of aliphatic acids [56]. Tetradecanoic acid and tetradecanal are believed to be the physiological substrates for acid reduction and bioluminescence, respectively [31].

Synthesis of wax esters

Fatty alcohols that are generated by the reduction of fatty acids can be esterified with endogenous acyl groups to form wax esters. This is a common process in plants, where waxes are produced to form a protective layer on the leaves and fruits [2]. Less commonly is the production of waxes by microorganisms. One example is *Mycobacterium tuberculosis*, causal agent of tuberculosis in men [57], which forms waxes of unknown function. It is suggested that the formation of these waxes in cell free systems are caused by a combination of the abundance of endogenous acyl groups and the absence of enzyme(s) that can utilize the fatty alcohol [23].

Miscellaneous

In several cases when the physiological function of acid reduction is not clear, it is suggested that these reactions are involved in detoxification. An example of such a detoxification reaction is found in *Clostridium acetobutylicum* that produces and excretes acetic and butyric acids when grown under glucose-limiting conditions. At the end of growth, the acids pass back into the cells and are reduced to the corresponding alcohols. Since the alcohols are less toxic than the acids this is seen as a detoxification process in response to an unfavorable environment [58].

POTENTIAL COMMERCIAL APPLICATIONS OF CARBOXYLIC ACID REDUCTIONS

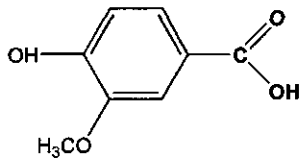
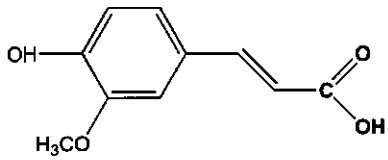
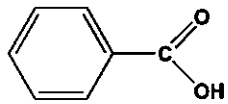
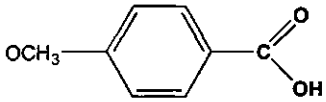
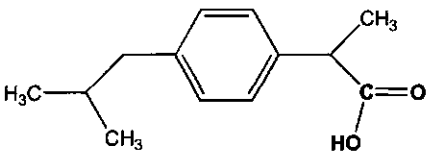
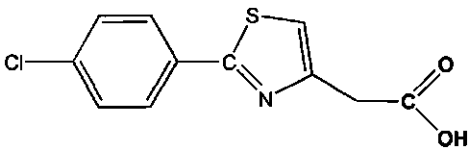
Despite the fact that there are many microorganisms described which are able to reduce carboxylic acids, there is hardly any information available about commercial applications of these bioreductions. Yet several reactions can be envisaged which might be of interest to the flavor, fragrance, and pharmaceutical industries (table 2).

The most intensively studied carboxylic acid reduction is the production of vanillin [59], a very important compound of vanilla. This alternative production method is interesting because the production of natural vanilla from *Vanilla* beans is laborious and therefore expensive [60]. Vanillin can, in principle, be obtained by the conversion of vanillic or ferulic acid and there are several microorganisms described that reduce these acids to vanillin [6, 28, 39, 53, 61, 62], for example, *Nocardia* sp. strain NRRL 5646 [29] and *Pycnoporus cinnabarinus* [6, 61]. However, despite several attempts to improve the yield, the amount of vanillin produced remains very low (10 - 25%). In addition, undesired byproducts are formed and the biotransformation times are long [28, 61].

Benzaldehyde, another flavor (almond like) compound can be obtained from the reduction of benzoic acid. *Nocardia* sp. strain NRRL 5646 [27], *N. asteroides* [24], and *Actinomyces* sp. [63] catalyze this reaction. Again the yields are not optimal, 50 - 69% [26, 63] after 60 hours of biotransformation [24]. As a result these bioreductions are not (yet) of interest from an industrial point of view.

For flavor and fragrance industry aldehydes are often more attractive than their corresponding alcohols, since they are better flavoring agents.

Table 2 Potential industrial applications of carboxylic acid bioreductions.

Starting carboxylic acid	Microorganism	References
	*Nocardia* sp. strain NRRL 5646	[28, 29]
	Pycnoporus cinnabarinus	[6, 39]
Vanillic acid		
	*Nocardia* sp. strain NRRL 5646	[28]
	Pycnoporus cinnabarinus	[6, 39]
Ferulic acid		
	*Nocardia* sp. strain NRRL 5646	[27]
	Nocardia asteroides	[25]
	Actinomyces sp.	[63]
Benzoic acid		
	*Bjerkandera* sp. strain BOS55	[36]
p-Anisic acid		
	*Nocardia* sp. strain NRRL 5646	[5]
Ibuprofen		
	several fungi e.g.	[64]
	Trichoderma sp.	
	Colletotrichum sp.	
Fenclozic acid		

Unfortunately, most whole cell and cell free systems described reduce aldehydes further to the corresponding alcohols resulting in low yields of the aldehyde. An exception is the white-rot fungus *Bjerkandera* sp. strain BOS55 that reduces a range of aromatic carboxylic acids, like *p*-anisic acid, mainly to the aldehyde with yields of 74 - 80%. However, when substrates with several functional groups, such as nitro, chloro, and fluoro substitutions, are used the yields of the aldehydes are much lower [36].

Nocardia sp. are very interesting microorganisms. Besides the production of vanillin and benzaldehyde, *Nocardia* sp. strain NRRL 5646 reduces (*R, S*)-ibuprofen, a pharmaceutical agent, enantioselective to yield optically pure *R*-ibuprofenol while the *S*-enantiomer remains intact [5]. The desired pharmacological activities of ibuprofen require almost exclusively the *S*-enantiomer and, therefore, this reduction is promising. At present, however, the enantiomeric excess and yields of both the acid and the alcohol are too low (ee 47.9 - 61.2%, yield 50%) to be economically attractive [5].

Another interesting pharmaceutical application might be the reduction of 2-(4-chlorophenyl)-thiazol-4-ylacetic acid (fenclozic acid), because the alcohol is a potent anti-inflammatory agent in rats and mice. Some microorganisms, mostly fungi, were found to reduce the acid to the alcohol [64].

Problems Involved in Carboxylic Acid Reduction Catalyzed by Whole Cells

Some of the problems associated with whole-cell reductions include a low product yield and the time needed for biotransformation. Furthermore, in many cases several side-products are generated, which decrease the yield even further and hinder down-stream processing. Another drawback that hampers the commercial application of acid bioreductions is the reduction of aldehydes to alcohols, because aldehydes are often more valuable than alcohols, especially in flavor industry.

When whole cell systems are used aldehydes are, in most cases, further reduced to the alcohols, thereby decreasing the overall aldehyde yield. Although the reduction of aldehydes is undesirable it is very difficult to avoid. The origin of this problem lies in the thermodynamics of the reduction of acids to aldehydes and subsequently to alcohols. The reduction of acids to aldehydes is thermodynamically unfavorable with a redox potential of about -600 mV [14]. To avoid oxidation of the aldehyde, i.e. the reversible reaction, an

aldehyde-scavenging reaction is necessary. In organisms aldehydes are scavenged conveniently by alcohol dehydrogenases. This reduction is thermodynamically favorable (redox potential of -200 mV) and functions as a sink of reducing equivalents, since the redox potential of $NAD(P)^+/NAD(P)H$ is -320 mV [14].

In order to retain higher concentrations of the aldehyde there are some options. Firstly, optimization of the reduction of acids to aldehydes catalyzed by whole cells. Secondly, elimination of the alcohol dehydrogenases to avoid the reduction of aldehydes to alcohols. However, elimination of alcohol dehydrogenases may result in non-viable microorganisms because the eliminated enzymes might have important metabolic functions. Moreover, the produced aldehydes can be oxidized back to the acid as described above in case the organism contains an aldehyde oxidoreductase. Thirdly, specific removal of the aldehyde from the medium during conversion by adsorbing agents, membranes, or on-line distillation in case of (hyper)thermophilic microorganisms. Finally, application of a two-incubations system can be envisaged: in the first incubation the acid is reduced to the alcohol (e.g. with metabolizing cells) and in the second incubation the generated alcohol is oxidized to the aldehyde (e.g. with resting cells).

IOP CATALYSIS

Industries producing fine chemicals form an important part of the Dutch economy. Different, cleaner, and more selective production methods need to be developed to maintain and strengthen the position of Dutch fine chemical industries.

The objective of the Innovation Oriented research Programs (IOP) is to strengthen the position and knowledge available for Dutch trade and industry. In order to reach that goal, innovative research at Dutch universities was set up in collaboration with the industry. IOP is financially supported by the Dutch Ministry of Economic Affairs and supports twelve fields of interest, among others, 'Catalysis'.

The main points of interest of the IOP Catalysis were to minimize side products produced during chemical reactions, to have more efficient production methods, and to reduce energy consumption. In order to reach these goals researchers from universities and industry from different disciplines collaborated.

IOP Catalysis consisted of several clusters and one of them is 'Reductases'. The three projects within this cluster dealt with the exploration of the reductive capacity of microorganisms:

IKA94039 Development of efficient enantioselective reductions by baker's yeast, carried out by ir. I. Chin-Joe, supervised by prof. dr. ir. J.J. Heijnen and dr. ir. A.J. Straathof.

IKA 94046 Production of chiral alcohols with the reductive system of *Phanerochaete chrysosporium*, carried out by ir. A. Hage, supervised by prof. dr. H.E. Schoemaker, prof. dr. R. Wever and dr. J.A. Field.

IKA 96001 Biocatalytic production of specialty chemicals from carboxylic acids, carried out by ir. E.C.D. van den Ban, supervised by prof. dr. N.C.M. Laane and dr. H.B.C.M. Haaker.

The three projects studied the reductive capacity of yeast, fungi, and a hyperthermophilic archaeon, respectively. In all cases the aim was to produce (optically pure) alcohols and aldehydes. IKA94039 dealt with baker's yeast (*Saccharomyces cerevisiae*) and IKA94046 with white-rot fungi (*Bjerkandera* and *Merulius* species). In my project, IKA96001, the reductive capacity of the archaeon *Pyrococcus furiosus* was studied.
The anaerobic and hyperthermophilic *P. furiosus* was chosen as a 'biocatalyst', because it grows optimally at temperatures ranging from 90 to 100°C [65]. These high temperatures are useful because they help to prevent infectious mesophilic contaminations during growth of *P. furiosus*. Furthermore, this microorganism grows relatively fast and can use the cheap carbon and energy source (potato)-starch. Moreover, *P. furiosus* produces dihydrogen during growth on starch indicating potential reducing power [65]. Finally, *P. furiosus* is an archaeon and these organisms are not reported to be pathogenic. These features make *P. furiosus* a very interesting microorganism to study reductive reactions.

OUTLINE OF THIS THESIS

The aim of this research was to explore the bioreductive potential of *Pyrococcus furiosus* in producing interesting aldehydes and/or alcohols from carboxylic acids for the fine chemical industries. In order to obtain insight in the reductive capacity of *P. furiosus*, it is important to investigate the bioreductive scope of the reduction reactions and the factors that facilitate the bioreductive process. On the basis of this knowledge, the characteristics of the reductive reactions in *P. furiosus* can be understood and used for their potential application in industrially relevant conversions.

In chapter 2 we investigated the bioreductive scope of growing *P. furiosus* cells at 90°C. Both aromatic and aliphatic acids were used as substrates for the reduction to the corresponding aldehydes and alcohols. Successively, the bioreductive capacity was optimized for the production rate and the yield of the alcohol, using 3-phenylpropionic acid as substrate. The effects of, for example, pH and temperature on these two optimization terms are presented in chapter 3.
The reductive reactions described in chapters 2 and 3 showed to be dependent on dihydrogen. The role of dihydrogen during starch fermentation is presented in chapter 4.
One of the proposed functions of the dihydrogen metabolism is the regeneration of NADPH. This commercially interesting reduction reaction was further investigated for the production of NADPH and the regeneration of NADPH (chapter 5).
Chapters 2 and 3 showed that *P. furiosus* was able to reduce carboxylic acids but the mechanism involved is yet unknown. The research performed to elucidate this mechanism is described in chapter 6.

2

Bioreduction of carboxylic acids by *Pyrococcus furiosus* in batch cultures

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Published in Enzyme and Microbial Technology (1999) 25: 251-257

The reduction of aromatic and aliphatic (di)carboxylic acids to their corresponding aldehydes and alcohols by the hyperthermophilic organism *Pyrococcus furiosus* was investigated. The reduction was performed with *P. furiosus* cells growing in the presence of 1 mM acid with starch as a carbon and energy source at 90°C. The aromatic acids *t*-cinnamic and 3-phenylpropionic acid were reduced to their corresponding alcohols with the highest yields in the described batch cultures: 67% and 69% respectively. The aliphatic acid reduced with the highest yield was hexanoic acid (yield: 38%). No aldehydes were detected during the reduction of acids indicating that the reduction of aldehydes to alcohols is faster than the reduction of acids to aldehydes. Some aldehydes were both reduced to the corresponding alcohol and oxidized to the corresponding acid. Besides reduction of the unsaturated *t*-cinnamaldehyde to *t*-cinnamyl alcohol (63%), the double bond of *t*-cinnamaldehyde was also reduced by *P. furiosus*.

INTRODUCTION

The reduction of carboxylic acids to aldehydes and subsequently alcohols requires a redox potential of about -600 mV [1]. Because of its very low potential, this reduction is, biologically, a difficult process. For this reason, the reduction of non-activated carboxylic acids has been described only for a limited number of mesophilic microorganisms. *Nocardia* [5, 27], *Clostridium formicoaceticum* [18], *C. thermoaceticum* [21], and some fungi [66] reduce aromatic carboxylic acids to alcohols. *C. formicoaceticum* [18] and *C. thermoaceticum* [21] also reduce aliphatic carboxylic acids. The reduction of acids to alcohols is catalyzed by at least two enzymes. Firstly, acids are reduced to aldehydes by reversible aldehyde oxidoreductases (AORs) [19, 67, 68]. Secondly, the reduction of aldehydes to alcohols is catalyzed by alcohol dehydrogenases (ADHs) [69].

It is expected that non-activated carboxylic acids can also be reduced by some hyperthermophilic archaea since *Pyrococcus furiosus, Thermococcus litoralis* and *Thermococcus* strain ES-1 contain several aldehyde oxidizing enzymes [42, 70-72]. But, it has been suggested that the physiological role of aldehyde oxidoreductases in these organisms is the oxidative removal of aldehydes that are generated during amino acid catabolism [68, 69, 72].

A limited number of ADHs from hyperthermophilic archaea have been characterized. Three iron-containing ADHs have been isolated from *Thermococcus* strain AN1 [73], *T. litoralis* [74], and *Thermococcus* strain ES-1 [75]. Two of these enzymes are thought to be involved in aldehyde reduction [74, 75]. Recently, two nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ADHs from *P. furiosus* were expressed in *Escherichia coli*: AdhA and AdhB. AdhB oxidizes methanol, but its activity is rapidly lost. AdhA preferentially oxidizes secondary alcohols over primary alcohols. The enzyme had a higher affinity for aldehydes than for alcohols [76]. Therefore, it has been suggested that the physiological role of AdhA is aldehyde reduction [76].

The conversion of acids to their corresponding aldehydes and alcohols by microorganisms offers the possibility for the natural production of specialty chemicals. The previously described microorganisms that perform this reduction have several disadvantages: *Clostridia* sp. are not preferred as a production organism, especially for food applications; several fungi have poor yields [66]; and *Nocardia* sp. have low reaction rates [5, 24]. Yeasts, that are often used in the food industry, cannot reduce carboxylic acids. Instead, they are successfully applied in the reduction of prochiral alkenes and in that of aldehydes [77].

The reduction of acids catalyzed by *P. furiosus* could be an alternative for the production of specialty chemicals (aldehydes and alcohols). This anaerobic archaeon grows optimally at 100°C and has a doubling time of 37 minutes under optimal conditions. It can use potato starch, which is very cheap, as a carbon and energy source [65]. Furthermore, *P. furiosus* is not known to produce toxins and can be grown under non-sterile conditions which is an advantage over mesophiles.

We report here the capacity of *P. furiosus* to reduce carboxylic acids. Growing cells were used since the reduction equivalents (reduced ferredoxin and NADPH), generated during the catabolism of starch, are necessary for the aldehyde oxidizing enzymes and ADHs that are probably involved in these reductions. It will be shown that *P. furiosus* can reduce a variety of aliphatic and aromatic carboxylic acids to their corresponding alcohols. The substrate specificity of *P. furiosus* differs from the organisms described in literature: *P. furiosus* reduces longer aliphatic acids much better than the organisms characterized before.

MATERIALS AND METHODS

Chemicals

Acetic acid, acetaldehyde, and ethanol were purchased from Merck (Darmstadt, Germany). Hexanoic, decanoic, and palmitic acid and the corresponding aldehydes and alcohols were from Acros (Geel, Belgium). Myristic acid, tetradecanal, and 1-tetradecanol were from Fluka (Buchs, Switzerland). The other substrates and products were obtained from Aldrich (Steinheim, Germany). Potato starch (Honig, Koog aan de Zaan, The Netherlands) was obtained from the local grocery. Other chemicals and solvents were of the highest grade available.

Growth of *Pyrococcus furiosus*

P. furiosus (DSM 3638) was grown anaerobically at 90°C in a basic medium described previously [78]. Yeast extract, cysteine, vitamins, and trace elements were added as described earlier [79]. Potato starch (5 g/l) was used as a carbon and energy source. Cells were grown in 100 ml bottles containing 50 ml medium, which was flushed with N_2/CO_2 (80/20) before inoculation. During growth, the bottles were shaken continuously at 200 rev./min. The medium was inoculated with a 2% preculture. At the start of growth the pH was 6.8 - 7.0 and was not adjusted during the experiment. Growth was determined by the increase in

protein concentration and by dihydrogen production. Protein concentration was measured using the Coomassie Brilliant Blue-method with bovine serum albumin as standard [80]. Dihydrogen production was analyzed by gas chromatography with N_2 as internal standard.

Conversion experiments

The substrate concentration was 1 mM before inoculation. The experiment was stopped by cooling the medium to room temperature when no further growth was observed and the pH had decreased to 4.5 - 5.0 (after 18 - 25 hours). All samples [30 ml for GC analysis, 1 ml for high-performance liquid chromatography (HPLC) analysis] were centrifuged for 15 min at 18,000 *g*. In the case of aromatic compounds; the supernatant was directly analyzed by HPLC. Aliphatic compounds were analyzed by GC. Before this analysis, the pH of 25 ml supernatant was decreased to 2.5 with 5 N HCl, and 5 g NaCl was added to the supernatant. These samples were extracted with 2 ml di-ethylether containing 0.1% 1-pentanol as an internal standard. The ether phase was analyzed. All experiments were repeated three times.

Analytical procedures

Dihydrogen was analyzed on a Varian 3400 gas chromatograph equipped with a thermal conductivity detector. Argon was used as carrier gas. Dihydrogen was measured on a molesieve 5A 45/50 column (1.5 m x 1/4" SS, Chrompack, Bergen op Zoom, The Netherlands).

Aliphatic compounds were analyzed using N_2 as carrier gas and a flame ionization detector. Samples were analyzed on a packed Cromosorb WPH 100-120 mesh 10%CP-Sil 58 column (2 m x 1/8" x 2 mm, Chrompack).

Aromatic compounds were analyzed by HPLC on a Nucleosil 100 C18 5U (Alltech, Laarne, Belgium) column. A Waters 600 controller and pump and a Waters 996 photodiode array detector were used (all Millipore, Milford, USA). Methanol/1% acetic acid was used as eluent and the analysis was isocratic.

RESULTS AND DISCUSSION

The reduction of various substrates was performed in batch cultures at 90°C. During growth, *P. furiosus* metabolized starch to acetate, which acidified the medium. Within 18 - 25 h the pH decreased to 4.5 - 5.0 at which point metabolism was inhibited [65]. As a result, the generation of reductant was stopped, and

consequently, the capacity for bioreduction. When growth had stopped, no further conversion was observed.

The substrate concentration at the start of the experiment was 1 mM. Some substrates (butyric, hexanoic, decanoic, and tetradecanoic acid) were also added in higher concentrations (10 mM). Butyric or hexanoic acid (10 mM) had no effect on growth of *P. furiosus* but 10 mM decanoic or tetradecanoic acid completely inhibited growth. Even 2 mM of these acids inhibited growth. At 1 mM, the growth rate was only slightly inhibited (50 - 80% compared to a blank that contained no substrate). To be able to compare the reduction rates of the different acids, we used 1 mM substrate concentration in all experiments described here. The yield of the conversion is expressed as mole percent of the product formed relative to the starting concentration of the substrate. The yields are calculated after growth had stopped, which occurred under the described experimental conditions 18 - 25 h after inoculation.

Reduction of acids versus reduction of aldehydes

Several aliphatic and aromatic carboxylic acids were reduced to their corresponding alcohols during growth (tables 1 - 4). During these conversions no aldehydes were detected. This phenomenon was also observed for the reduction of acids by, e.g. *C. thermoaceticum* [21], *N. asteroides* JCM 3016 [24], and several fungi [66]. The lack of aldehydes indicated that the second reaction, the reduction of aldehydes, was faster than the reduction of acids. The reduction of aldehydes to alcohols by growing *P. furiosus* resulted in equal or higher yields than the reduction of acids to alcohols (tables 1 - 4). Therefore, it is very likely that, for most substrates, the reduction of acids to aldehydes is the rate-limiting step of the conversion of acids to alcohols. It was tested whether the aldehydes used were chemically stable under the experimental conditions at 90°C. This was the case for all aldehydes tested except phthalaldehyde and 2-carboxybenzaldehyde.

Reduction of aliphatic carboxylic acids

The range of aliphatic acids reduced to their corresponding alcohols is given in table 1. Acetic acid was not reduced. This was expected because *P. furiosus* metabolizes starch to acetic acid. Small amounts of ethanol were only found when glucose was fermented by concentrated cell suspensions [81]. The reduction of short aliphatic acids (C3 - C6) resulted in higher yields than those of longer aliphatic acids. Myristic and palmitic acid were hardly reduced.

Table 1 Yield of alcohols from various aliphatic acids and aldehydes reduced by *P. furiosus* in batch cultures.

Substrate[#]		Product	Yield (%)	
Acid	Aldehyde		Acid as substrate	Aldehyde as substrate
Acetic acid	Acetaldehyde	-	-	-
Propionic acid	Propanal	1-Propanol	16.9 ± 1.9	> 99
Butyric acid	Butyraldehyde	1-Butanol	26.1 ± 1.4	> 99
Hexanoic acid	Hexanal	Hexyl alcohol	38.1 ± 2.0	> 99
Decanoic acid	Decanal	Decyl alcohol	13.2 ± 2.2	> 99
Myristic acid	Tetradecanal	1-Tetradecanol	< 1	< 1
Palmitic acid	-[¶]	1-Hexadecanol	< 1	-

[#] Substrate concentrations were 1 mM

[¶] Aldehyde was not commercially available

The highest yield for these batch conversions was found for the reduction of hexanoic acid: 38%. These data can be interpreted as reversible aldehyde-oxidizing enzymes have a higher binding affinity for hexanoic acid than for the other tested aliphatic acids. A kinetic analysis of these enzymes can prove this hypothesis.

The *Clostridia* strains and enzymes described also reduced a range of aliphatic acids, but their substrate specificity differed from *P. furiosus*. They preferentially reduced small aliphatic acids (acetic and propionic acid) [18-21].

Reduction of aromatic carboxylic acids

Table 2 shows that *P. furiosus* reduced aromatic substrates with much higher yields than it reduced aliphatic substrates. In these batch conversions, 3-phenylpropionic acid was reduced with the highest yield (69%). This substrate has a length that corresponds to that of hexanoic acid. The length might be important for the rate of reduction of these carboxylic acids. The optimal length of the aliphatic part of this type of aromatic molecule is three C atoms. The yields of the reduction of C1 (benzoic acid) and longer chains (4-phenylbutyric, 5-phenylvaleric, and 6-phenylhexanoic acid) were lower. The yields of benzoic and 6-phenylhexanoic acid are comparable to that of butyric acid.

A crude extract of *C. formicoaceticum* reduced benzoic and butyric acid at rates proportionally similar to *P. furiosus*. However, the absolute rate of these

Table 2 Yield of alcohols from various aromatic acids and aldehydes reduced by *P. furiosus* in batch cultures.

Substrate[#]		Product	Yield (%)	
Acid	Aldehyde		Acid as substrate	Aldehyde as substrate
Benzoic acid	Benzaldehyde	Benzyl alcohol	26.7 ± 1.7	> 99
3-Phenylpropionic acid	3-Phenylpropanal	3-Phenyl-1-propanol	68.7 ± 4.2	61.4 ± 2.9
4-Phenylbutyric acid	-[¶]	4-Phenyl-1-butanol	38.9 ± 6.1	-
5-Phenylvaleric acid	-[¶]	5-Phenyl-1-pentanol	42.3 ± 5.7	-
6-Phenylhexanoic acid	-[¶]	6-Phenyl-1-hexanol	25.1 ± 1.2	-

[#] Substrate concentrations were 1 mM

[¶] Aldehyde was not commercially available

reductions catalyzed by the extract of *C. formicoaceticum* was about 25 times higher [18] than that of the whole cell system of *P. furiosus*. Butyric acid was reduced by both the tungsten-containing AOR and molybdenum-containing AOR of *C. formicoaceticum*. The reduction of benzoic acid was solely catalyzed by its tungsten-containing AOR [19, 20] A *Nocardia* species reduced benzoic acid (40 mM) to benzyl alcohol with a yield of up to 60%, but this reaction took 60 hours [24].

Reduction of unsaturated compounds

The unsaturated acids crotonic and *t*-cinnamic acid were reduced to their corresponding alcohols (table 3). Crotonic acid was reduced with a lower yield than the saturated analogue, butyric acid (17% vs. 26%). The α-β double bond seemed to have a negative effect on the reduction of the carboxylic group. The reduction of crotonaldehyde to crotyl alcohol was not negatively affected by the α-β double bond (yield: >99%). The unsaturated bond in crotonic acid, crotonaldehyde, and crotyl alcohol was not reduced.

The aromatic *t*-cinnamic acid was reduced to *t*-cinnamyl alcohol with a high yield (63%). The unsaturated bond in *t*-cinnamic acid had no negative effect on the yield compared to the yield of 3-phenylpropionic acid (yield: 69%). These reduction properties are also found in *C. formicoaceticum* [20] and *C. thermoaceticum* [22].

Table 3 Yield of alcohols from various unsaturated compounds reduced by *P. furiosus* in batch cultures.

Substrate[#]	Product	Yield (%)
Crotonic acid	Crotyl alcohol	16.8 ± 2.4
Crotonaldehyde[¶]	Crotyl alcohol	> 99
t-Cinnamic acid	*t*-Cinnamyl alcohol	67.4 ± 4.8
t-Cinnamaldehyde	*t*-Cinnamyl alcohol and	62.7 ± 1.7
	3-Phenyl-1-propanol	8.4 ± 0.4

[#] Substrate concentrations were 1 mM

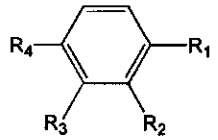
[¶] Substrate concentration was 0.1 mM because higher concentrations inhibited growth

When *t*-cinnamaldehyde, the intermediate of the reduction of *t*-cinnamic acid, was added to growing *P. furiosus* cells two types of reductions were observed: (1) reduction of the aldehyde to the alcohol and (2) saturation of the double bond. Most of the aldehyde was rapidly reduced to *t*-cinnamyl alcohol (yield: 63%). The reduction of the double bond, probably via 3-phenylpropanal, resulted in 3-phenyl-1-propanol (yield: 8%). The reduction of the double bond might be caused by mesomeric stabilization of the transition state with the aromatic ring. It is possible that the double bond of the aliphatic crotonaldehyde is not reduced because it has no adjacent ring to stabilize the transition state during this reduction. *P. furiosus* reduced neither the unsaturated bond of *t*-cinnamic acid nor that of *t*-cinnamyl alcohol.

Reduction of benzoic acid and benzaldehyde derivatives

The effect of substitutions on the aromatic ring on bioreduction was tested with benzoic acid and benzaldehyde analogous (table 4). Vanillic acid was reduced to vanillyl alcohol, but the yield was lower than for benzoic acid (10% vs. 27%). Phthalic, isophthalic, and terephthalic acid, and 4-carboxybenzyl alcohol were tested but no reduction was found. The extra carboxyl, hydroxymethyl, methoxy, and hydroxyl groups seemed to have a negative effect on the reduction of these substrates. On the other hand, the aldehyde analogs of isophthalic and terephthalic acid (terephthal aldehyde, isophthal aldehyde, 3-carboxybenzaldehyde, 4-carboxybenzaldehyde) and vanillin were oxidized to their corresponding acids. This indicates that aldehyde oxidoreductases, in principle, also can bind the acid analogs. Benzaldehyde, without additional

Table 4 Yield of products from various benzoic acid and benzaldehyde derivatives converted by *P. furiosus* in batch cultures.



Substrate[#]	R_1	R_2	R_3	R_4	Product	Yield (%)
Benzoic acid	-COOH	-H	-H	-H	Benzyl alcohol	26.7 ± 1.7
Benzaldehyde	-CHO	-H	-H	-H	Benzyl alcohol	> 99
Vanillic acid	-COOH	-H	$-OCH_3$	-OH	Vanillyl alcohol	10.2 ± 0.7
Vanillin	-CHO	-H	$-OCH_3$	-OH	Vanillic acid and	21.2 ± 1.1
					Vanillyl alcohol	12.0 ± 1.9
Phthalic acid	-COOH	-COOH	-H	-H	-	-
Isophthalic acid	-COOH	-H	-COOH	-H	-	-
3-Carboxybenzaldehyde	-CHO	-H	-COOH	-H	Isophthalic acid	60.9 ± 0.5
Isophthal aldehyde	-CHO	-H	-CHO	-H	Isophthalic acid and an	35.1 ± 0.9
					Unknown compound[¶]	(~60)[§]
Terephthalic acid	-COOH	-H	-H	-COOH	-	-
4-Carboxybenzaldehyde	-CHO	-H	-H	-COOH	Terephthalic acid and	11.9 ± 0.9
					4-Carboxybenzyl alcohol	75.5 ± 1.8
Terephthal aldehyde	-CHO	-H	-H	-CHO	4-Carboxybenzaldehyde	6.9 ± 0.3
4-Carboxybenzyl alcohol	$-CH_2OH$	-H	-H	-COOH	-	-

[#] Substrate concentrations were 1 mM

[¶] The unknown compound had an UV spectrum similar to that of 4-carboxybenzyl alcohol. Therefore, the unknown compound might be 3-carboxybenzyl alcohol

[§] Estimated yield: the area of the unknown compound was compared to the areas of the calibration curve of 4-carboxybenzyl alcohol

groups, was not oxidized probably because of the efficient reduction to the alcohol. That aldehyde-oxidizing enzymes can reduce carboxylated and hydroxymethylated benzoic acid was shown for the tungsten-containing AOR from *C. thermoaceticum*. With isolated enzyme and a strong chemical reductant, terephthalic acid and 4-carboxybenzyl alcohol were reduced, but at low rates [22]. Isophthal aldehyde was also converted to a still-unknown compound. This compound had an UV spectrum similar to that of 4-carboxybenzyl alcohol. Hence, the compound may be 3-carboxybenzyl alcohol. Besides oxidation, the aldehyde groups of vanillin and 4-carboxybenzaldehyde were reduced to vanillic alcohol (yield 12%) and 4-carboxybenzyl alcohol (yield 75%). Figure 1 shows the conversion of 4-carboxybenzaldehyde in time. 4-Carboxybenzaldehyde was converted to its two products in 22 h. The aldehyde was reduced and oxidized at the same time. The reduction rate was much higher than the oxidation rate. The conversion of the aldehyde vanillin was also studied in time. This conversion showed the same pattern as for 4-carboxybenzaldehyde but the yields were not as high: after 28 h only 50% of the aldehyde had been converted.

Because of the reactivity of the aldehydes towards two types of enzymes must be considered, the rate of reduction of the aldehyde by alcohol dehydrogenases and the rate of oxidation by aldehyde oxidoreductases, it is not possible to draw conclusions about the effect of substitutions on the rate of oxidation.

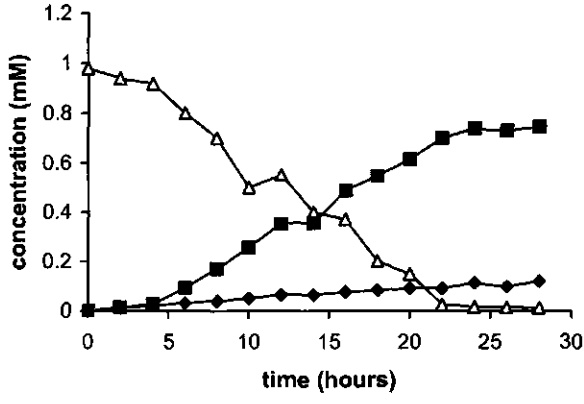


Figure 1 Conversion of 4-carboxybenzaldehyde by growing *P. furiosus* cells. -Δ- 4-carboxybenzaldehyde; -■- 4-carboxybenzyl alcohol; -◆- terephthalic acid.

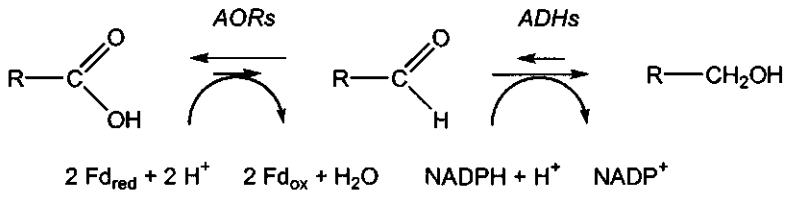
Enzymes involved in the bioreduction

Oxidation and reduction of the aldehyde group are thought to be catalyzed by two classes of enzymes: the aldehyde oxidoreductases, AOR and FOR, and the alcohol dehydrogenases, ADH [69]. This reaction scheme is shown in scheme 1. The reduction of acids requires two substrates: the acid and reduced ferredoxin. Either the concentration of the acid or the ratio reduced/oxidized ferredoxin is rate limiting. There are indications that the ratio reduced/oxidized ferredoxin is rate limiting for the bioreduction of acids. Reduced ferredoxin is generated during the metabolism of starch.

Normally, the disposal of reduced ferredoxin is suggested to be catalyzed by two enzymes, ferredoxin:$NADP^+$ oxidoreductase and sulfhydrogenase, generating dihydrogen or H_2S when S^0 is available. The involved enzymes catalyze these reactions reversibly [82]. A high concentration of dihydrogen inhibits the growth of *P. furiosus* and this is explained by a reversible inhibition of the suggested route for the disposal of reduced ferredoxin.

Initial experiments in a 2 l fermentor showed that dihydrogen is essential for the reduction of 3-phenylpropionic acid (data not shown). When *P. furiosus* was grown under a constant replacement of dihydrogen by N_2, no conversion of 3-phenylpropionic acid was found. The same experiment without the removal of dihydrogen resulted in reduction of this acid to the alcohol. We like to explain this result by assuming that a low dihydrogen concentration decreases the ratio reduced/oxidized ferredoxin. The opposite, a high ratio, is probably essential for the reduction of acids.

Furthermore, we think that the disposal of the formed aldehyde is also essential to obtain a significant reduction of acid. Firstly, the equilibrium of the reaction favors the oxidation of aldehydes over the reduction of acids because the redox potential of these reductions is low (e.g. acid/aldehyde: $E'_0 = \sim -600$ mV) [1].



Scheme 1 Reaction scheme for the oxidations and reductions catalyzed by aldehyde oxidoreductases (*AORs*) and alcohol dehydrogenases (*ADHs*) in *P. furiosus*.

This is demonstrated by the oxidation of some aldehydes, which had higher yields than the reduction of the corresponding acids (table 4). Secondly, *P. furiosus* could not reduce acetic, myristic (both table 1), terephthalic, isophthalic, and phthalic acid. This may be caused by the lack of an aldehyde-withdrawing system: acetaldehyde, tetradecanal (both table 1), and 3-carboxybenzaldehyde (table 4) were not reduced to the alcohols. Whether this explanation is relevant to phthalic acid is not known because the corresponding aldehydes were chemically unstable in control experiments (which were not inoculated with *P. furiosus*). Therefore, they could not be used as substrates. One exception was found: 4-carboxybenzaldehyde was reduced to 4-carboxybenzyl alcohol, indicating that an aldehyde-withdrawing system was present. Two explanations can be envisaged: 1) the corresponding terephthalic acid cannot be reduced at all, or 2) the oxidation of the aldehyde is much faster than the reduction of the acid. The importance of aldehyde removal has been described for *C. thermoaceticum* AOR: the specific activity for acid reduction was increased when an aldehyde-withdrawing system, such as ADH, was present [67].

CONCLUSIONS

Based on these experiments, it can be concluded that growing *P. furiosus* cells rapidly reduced a range of aliphatic and aromatic carboxylic acids to their corresponding alcohols. Aromatic acids were reduced with higher yields than aliphatic acids. In general, *P. furiosus* had a preference for substrates with a length of four to eight C-atoms. This substrate specificity differed from that of the previously described organisms [5, 18, 21, 27, 66].

The rate-limiting step is the reduction of acids to aldehydes. The presence of dihydrogen and an aldehyde-withdrawing system (ADH) are probably essential for the reduction of acids to alcohols.

The use of *P. furiosus* for the production of aromatic and aliphatic alcohols offers a new alternative to chemical synthesis. The results reported here for batch cultures are promising. Using continuous cultures and improvement of the yield are currently under investigation. Furthermore, our results show that stable, long-term bioreductions can be achieved near the boiling temperature of water.

ACKNOWLEDGMENTS

This research was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Dutch Ministry of Economic Affairs (project number IKA96001).

3

Optimization of the reduction of 3-phenylpropionic acid by *Pyrococcus furiosus*

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Submitted for publication (2001)

Factorial designs were used to optimize the reduction of 3-phenylpropionic acid to the corresponding alcohol 3-phenyl-1-propanol by the hyperthermophile *Pyrococcus furiosus*. The influences of pH, partial dihydrogen pressure, 3-phenylpropionic acid concentration, the amount of carbon and energy source, and temperature on both $Y_{\text{Production rate}}$ and $Y_{\text{Yield of conversion}}$ were investigated.

The $Y_{\text{Production rate}}$ was influenced by all variables tested and could be described by a linear model. An optimal $Y_{\text{Production rate}}$ (118.2 μM 3-phenyl-1-propanol/hour = 16 mg/l.hour) was found at pH 7.0, a 3-phenylpropionic acid concentration of 10 mM, and at 90°C. A linear model for the $Y_{\text{Yield of conversion}}$ was not sufficient; a quadratic model resulted in a more adequate description. The $Y_{\text{Yield of conversion}}$ was highest (83.7%) at pH 6.35, a 3-phenylpropionic acid concentration of 1 mM, and at 80°C.

It was not possible to optimize both $Y_{\text{Production rate}}$ and $Y_{\text{Yield of conversion}}$. Nevertheless, two optima were found for the sum of the normalized responses with response surface analysis.

INTRODUCTION

Aldehydes and alcohols are of interest for several industries. A possible method to obtain these compounds is by the reduction of carboxylic acids. Carboxylic acids are often cheap, abundantly available, and water-soluble [59]. The chemical reduction of carboxylic acids to the corresponding aldehydes and alcohols is difficult and often needs high temperatures. Biological reduction has the advantage that mild reaction conditions can be used. An interesting bioroute involves the hyperthermophilic *Pyrococcus furiosus* that can reduce a range of (un)-saturated aliphatic and aromatic carboxylic acids to their corresponding alcohols when it is grown at 90°C [83].

P. furiosus can use the very cheap potato starch as a carbon and energy source and grows optimally at 90-100°C with a doubling time of 37 minutes under optimal conditions [65]. Furthermore, *P. furiosus* is not known to produce toxins and can be grown under non-sterile conditions which is an advantage over mesophiles.

It was found that growing *P. furiosus* cells are essential to reduce the carboxylic acids to the corresponding alcohols. As a result, parameters influencing growth of *P. furiosus* have to be considered to optimize the reduction. Two factors negatively affecting growth are the metabolic end products acetate [84] and dihydrogen [65, 85, 86].

In this paper we describe the optimization of the reduction of an aromatic carboxylic acid: 3-phenylpropionic acid. This compound was chosen as a model substrate because this aromatic acid was reduced most efficiently by *P. furiosus* [83].

This system was optimized for two responses: the rate of conversion ($Y_{\text{Production rate}}$) and the yield of conversion ($Y_{\text{Yield of conversion}}$). To optimize this reduction, the effects of several variables on these two responses have to be known. The effects of five variables were investigated: pH, partial dihydrogen pressure, substrate concentration, carbon and energy source concentration, and temperature. Factorial and face-centered central composite designs were used to study these effects [87].

The pH was set to 7 and 6. A higher pH favors growth [65] but a lower pH favors reduction of carboxylic acids in *Clostridium formicoaceticum, C. thermoaceticum* [18, 21] and *Thermococcus* ES-1 [42].

Previous experiments in a 2 l fermentor showed that dihydrogen is essential for conversion of carboxylic acids [83] but that a high dihydrogen pressure inhibits growth [85, 86, 88].
Substrate concentrations were also varied to examine possible inhibition of growth or conversion.
The amount of starch was varied to examine carbon and energy source limitation. Since a growing system is essential, carbon source limitation can play a role.
The effects the five variables above discussed were examined. pH, substrate concentration, and temperature were found to have the largest but opposite effects on both $Y_{\text{Production rate}}$ and $Y_{\text{Yield of conversion}}$. Despite the opposite effects it will be showed that it was possible to find optima for the normalized responses resulting in a high $Y_{\text{Production rate}}$ or a high $Y_{\text{Yield of conversion}}$.

MATERIALS AND METHODS

Chemicals

3-Phenylpropionic acid, 3-phenyl-1-propanol, 1-propanol and benzaldehyde were obtained from Aldrich (Steinheim, Germany). Potato starch (Honig, Koog aan de Zaan, The Netherlands) was obtained from the local grocery. Other chemicals and solvents were of the highest grade available.

Growth of *Pyrococcus furiosus*

Precultures of *P. furiosus* (DSM 3638) were grown anaerobically in 100 ml bottles at 90°C in a basic medium as described previously [83]. Precultures were grown for 7 hours till a partial dihydrogen pressure of approximately 30 kPa was reached.
Conversion experiments were performed in a 2.8 l glass reactor with a working volume of 2.0 l controlled by a Prelude control system (Biolafitte, France). The agitation speed was 200 rpm and the gassing rate of N_2 was 0.16 vvm. The partial dihydrogen pressure was set to 12, 18 or 24 kPa by flushing the system with dihydrogen. The reactor was operated at atmospheric pressure.
The medium was as described above except that the starch concentration at t = 0 hours was 7.5 g/l. In some experiments an extra amount of starch (7.5 g/l) was added after 16, 24 or 39 hours when the experiments were performed at 90°C and 98°C, 85°C or 80°C, respectively. The extra starch was added after 16 to 39 hours to avoid Maillard reactions and caramelization [89]. The pH of the medium was

adjusted to 6.0, 6.5 or 7.0 with 2.0 M NaOH. Growth was followed by determining the protein concentration, which was measured using the Coomassie Brilliant Blue-method with BSA as standard [80].

Conversion experiments

The final 3-phenylpropionic acid concentrations in the reactor were 1, 5.5 or 10 mM. Samples were taken during the experiments. All samples for HPLC analysis were centrifuged for 15 minutes at 16.000 *g* and the samples for the conversion of 3-phenylpropionic acid were diluted (1:1) with 1 % (v/v) acetic acid containing 2 mM benzylalcohol as internal standard. The samples for acetate analysis were diluted (1:1) with 0.1 N H_2SO_4 containing 50 mM 1-propanol as internal standard.

Analytical procedures

Dihydrogen was analyzed on a Varian 3400 gas chromatograph equipped with a TCD detector. Argon was used as carrier gas. Dihydrogen was measured on a molesieve 5A 45/50 column (1.5 m x 1/4" SS, Chrompack, Bergen op Zoom, The Netherlands).

Aromatic compounds were analyzed by HPLC on a Nucleosil 100 C18 5U (Alltech, Laarne, Belgium) column. A Waters 600 controller and pump and a Waters 996 photodiode array detector were used (all Millipore, Milford, USA). 30% Methanol/70% 1% (v/v) acetic acid was used as eluent.

Acetic acid was analyzed by HPLC on a Polyspher OA HY column (Merck, Darmstadt Germany) at 60°C. A P1000 pump and a RefractoMonitor IV refractive index detector (all Spectra System, Thermo Separation Products, Riviera Beach, USA) with 0.01 N H_2SO_4 as eluent were used.

Experimental design and data analysis

To optimize the reduction of 3-phenylpropionic acid, the effects of several variables on that have to be known. This paper describes the effects of five variables: *pH, partial dihydrogen pressure, 3-phenylpropionic acid* concentration, carbon and energy source concentration, and temperature. The experiments were set up according to a fractional factorial design [87]. This system is easy to use and gives, in a limited number of experiments, information about the effects of the variables and of the interactions of these variables. Table 1 shows the selected variables and their levels. The experimental design is given in table 2 with the levels in coded units.

Table 1 Levels of the variables tested in the fractional factorial and face-centered central composite design.

		Coded level (x_i)		
	Variable	-1	0	+1
x_{pH}	pH	6.0	6.5	7.0
x_{pH2}	partial dihydrogen pressure (kPa)	12	18	24
$x_{Substrate}$	3-Phenylpropionic acid (mM)	1	5.5	10
x_{Starch}	Pulse of starch (g/l) at t = 16, 24 or 39 hours	0	3.75	7.5
x_T	Temperature (°C)	80	85	90

From the results of the experiments the estimates of effects are calculated and analyzed using analysis of variance (ANOVA) with the SAS system for Windows, version 6.12 TS. The F test was used to evaluate whether a variable had a significant effect ($p \leq 0.05$).
In the experiments, two responses were considered:

$Y_{\text{Production rate}}$ (μM/hour)
$Y_{\text{Yield of conversion}}$ (%)

The $Y_{\text{Production rate}}$ is the maximal amount of 3-phenyl-1-propanol formed divided by the time needed to reach this and the volume of the reactor. The $Y_{\text{Yield of conversion}}$ is the maximal amount of 3-phenyl-1-propanol formed divided by the initial amount of 3-phenylpropionic acid and expressed as a percentage.
The responses can be described by a function (response surface) of the levels of the variables with a significant influence on the response. A fractional factorial design results in a linear model:

$$Yx = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j \qquad \text{for } i \neq j$$

Where Yx = predicted response, β_0 = constant, β_i = coefficient for linear effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variables.
The linear model has to be checked for second-order curvature by analysis of variance of the $SS_{Pure\ quadratic}$. To calculate this value experiments at the center point (CP) have to be done. The coded levels of the variables are all set to zero at the center point. The data of the center point experiments also provide information about the error of the responses.

$$SS_{Pure\ quadratic} = \frac{n_F n_C (\bar{y}_F - \bar{y}_C)^2}{n_F + n_C}$$

Where n_F = the number of factorial design points, n_C = the number of center points, $\bar{y}_F$ = the average of the points in the factorial portion of the design, and $\bar{y}_C$ = the average of the points at the center.

If the value of $SS_{Pure\ quadratic}$ is significant, quadratic curvature is present and a second-order model might give a better description of the response:

$$Yx = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad \text{for } i \neq j$$

Where Yx = predicted response, β_0 = constant, β_i = coefficient for linear effect, β_{ij} = coefficient for the interaction effect, β_{ii} = coefficient for the quadratic effect, x_i and x_j = the coded level of variables.

The coefficients of the second-order model can be obtained by performing a face-centered central composite design (table 3).

RESULTS AND DISCUSSION

Growing cells were found to be essential for reduction of carboxylic acids, therefore, factors affecting growth were expected to be important. The following variables are known to have an effect on growth: pH, partial dihydrogen pressure, carboxylic acid concentration, carbon and energy source concentration, and temperature [65, 83]. The values of the variables are based on published results and experimental data.

Initially, temperatures were set at 90 and 98°C. When experiments with the same values of the other variables (pH, partial dihydrogen pressure, substrate and starch concentrations) were performed at 98°C, growth was similar to those at 90°C. This agrees with literature [65]. However, 3-phenylpropionic acid was not reduced at 98°C. Experiments performed at 95°C resulted in equal growth but the reduction of the acid was 15 to 50% lower compared to the experiments performed at 90°C under similar conditions. Since we do not know which enzymes are involved in the reduction of carboxylic acids no explanation can be given. To have reduction of 3-phenylpropionic acid and to have sufficient growth the second temperature was lowered to 80°C.

In the first optimization step a 2^{5-1} fractional factorial design was used [87]. Table 2 shows the matrix of the experimental set up and the results of the experiments for the two responses. From these data it is difficult to conclude which variables have an effect on the responses. Some effects can be seen easily: high substrate concentrations ($x_{Substrate}$ = +1) give better results for the $Y_{Production\ rate}$ than low values ($x_{Substrate}$ = -1). Nevertheless, statistical analysis is essential to reveal all effects of the variables.

Table 2 Fractional factorial design for five variables. Matrix for experiments and responses.

	Coded level					Response	
Experiment	x_{pH}	x_{pH2}	$x_{Substrate}$	x_{Starch}	x_T	$Y_{Production\ rate}$ #	$Y_{Yield\ of\ conversion}$ ¶
1	-1	-1	-1	-1	-1	13.4	80
2	+1	-1	-1	-1	+1	14.7	33
3	-1	+1	-1	-1	+1	16.3	36
4	+1	+1	-1	-1	-1	9.7	53
5	-1	-1	+1	-1	+1	35.6	8
6	+1	-1	+1	-1	-1	65.4	34
7	-1	+1	+1	-1	-1	35.4	26
8	+1	+1	+1	-1	+1	85.1	19
9	-1	-1	-1	+1	+1	18.2	35
10	+1	-1	-1	+1	-1	5.7	55
11	-1	+1	-1	+1	-1	9.8	89
12	+1	+1	-1	+1	+1	8.1	22
13	-1	-1	+1	+1	-1	25.4	19
14	+1	-1	+1	+1	+1	137.2	33
15	-1	+1	+1	+1	+1	19.8	7
16	+1	+1	+1	+1	-1	45.0	50
CP §	0	0	0	0	0	48.4	54
CP §	0	0	0	0	0	44.7	48
CP §	0	0	0	0	0	39.3	44
CP §	0	0	0	0	0	32.9	48
CP §	0	0	0	0	0	39.4	47

\# $Y_{Production\ rate}$ in µM/hour

¶ $Y_{Yield\ of\ conversion}$ in %

§ CP = Center point

Responses

The reduction of 3-phenylpropionic acid is optimized to two responses; both a high $Y_{\text{Production rate}}$ and a high $Y_{\text{Yield of conversion}}$ are essential. When a system is optimized to two responses it is important that both responses are not correlated. The results shown in table 2 are combined in correlation plots (figure 1) and from that it becomes clear that these two responses are not correlated.

The same results (table 2) are used to calculate the main effects and interactions for the two responses, assuming that three or four factor interactions are negligible [87]. Statistical analysis of these data showed which main effects and interactions are significant (F test, $p \leq 0.05$).

Analysis of $Y_{\text{Production rate}}$

From the analysis of variance we found the following linear model describing the $Y_{\text{Production rate}}$ as a function of the significant variables and interactions of variables:

$$Y_{\text{Production rate}} = 35.69 + 12.31x_{\text{pH}} - 5.40x_{\text{pH2}} + 22.06x_{\text{Substrate}} + 7.83x_{\text{T}} + 14.75x_{\text{pH}}x_{\text{Substrate}} + 7.09x_{\text{pH}}x_{\text{T}} - 7.57x_{\text{pH2}}x_{\text{Starch}} + 5.49x_{\text{Substrate}}x_{\text{T}}$$

Being significant at the 99% level and explaining 86.76% of the variance (R_{adj}^2), this model gives a good approximation of the true response. The standard error of the estimate was 2.001. Analysis of the second-order curvature for the $Y_{\text{Production rate}}$ showed that there is no significant quadratic effect. Testing for lack of fit also revealed that the model is appropriate. Thus, this linear model does give an adequate description of the $Y_{\text{Production rate}}$.

The response surface of the model is shown in figure 2. This model describes that the highest $Y_{\text{Production rate}}$ can be found at a temperature of 90°C ($x_{\text{T}} = +1$), a substrate concentration of 10 mM ($x_{\text{Substrate}} = +1$), pH 7 ($x_{\text{pH}} = +1$) and partial dihydrogen pressure of 12 kPa ($x_{\text{pH2}} = -1$). In figure 2, x_{pH2} and x_{Starch} are set at -1 and +1, respectively, since then the highest $Y_{\text{Production rate}}$ is found.

In the next paragraphs the possible biological backgrounds of the effects of the variables are discussed.

The $Y_{\text{Production rate}}$ is largely influenced by temperature, which also affects growth of *P. furiosus*. When growth and reduction of 3-phenylpropionic acid are plotted in time it is obvious that at 90°C, $x_{\text{T}} = +1$, *P. furiosus* grew faster and reduced 3-phenylpropionic acid faster compared with the experiment performed at 80°C, $x_{\text{T}} = -1$ (figure 3).

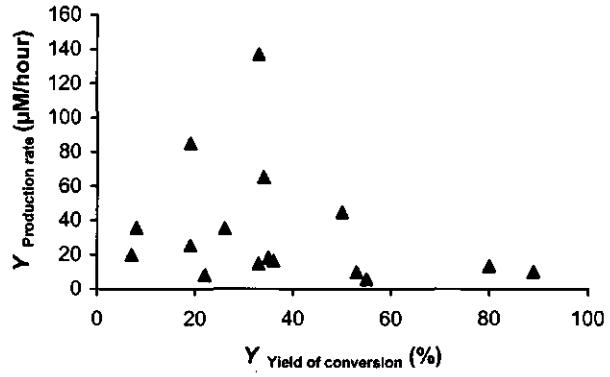

Figure 1 Correlation plot of $Y_{\text{Production rate}}$ and $Y_{\text{Yield of conversion}}$.

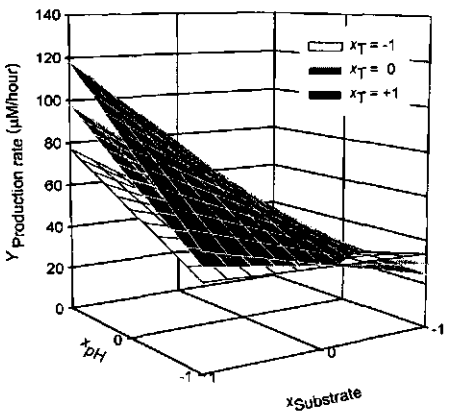

Figure 2 Theoretical response surfaces of the $Y_{\text{Production rate}}$ obtained from the fractional factorial design; predicted $Y_{\text{Production rate}}$ as a function of 3-phenylpropionic acid concentrations ($x_{\text{Substrate}}$) and pH levels (x_{pH}) in coded units at different levels of temperature (x_T). In all cases $x_{\text{pH2}} = -1$ and $x_{\text{Starch}} = +1$.

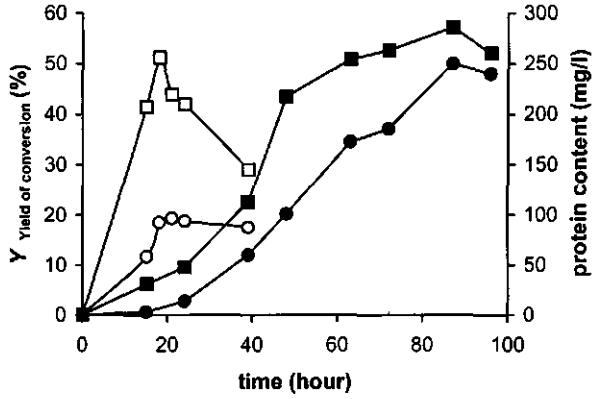

Figure 3 Examples of protein content and conversion of 3-phenylpropionic acid by *Pyrococcus furiosus*. Experiments 8 (a) and 16 (b) are taken from table 2. Experimental set up: pH = 7 (x_{pH} = +1), partial dihydrogen pressure = 24 kPa (x_{pH2} = +1) and concentration 3-phenylpropionic acid = 10 mM ($x_{Substrate}$ = +1) are the same for both experiments; (a) no extra addition of starch (x_{Starch} = –1) and T = 90°C (x_T = +1), -○- $Y_{Yield\ of\ conversion}$, -□- protein content, (b) extra addition of starch = 7.5 g/l (x_{Starch} = +1) and T = 80°C (x_T = –1), -●- $Y_{Yield\ of\ conversion}$, -■- protein content.

In these experiments the maximum yield of 3-phenyl-1-propanol at 80°C was obtained after about 40 hours, while the experiments performed at 90°C, x_T = +1, reached a maximum yield after about 18 hours.

On the other hand, at 98°C there is no reduction of 3-phenylpropionic acid but growth is similar to that at 90°C. Apparently, there is an optimum for $Y_{Production\ rate}$ in the range of 90°C. So, temperature had an impact on the time needed to reach a maximum yield. This is an important feature for optimizing the reduction of 3-phenylpropionic acid.

Growth and reduction are not only affected by temperature but also by pH. At pH 6.0 growth was much slower than at pH 7.0, therefore the reduction of the carboxylic acid is also retarded. These effects, pH and temperature, are considerable and also result in an interaction effect ($x_{pH}x_T$). Another variable that affects growth and therefore might affect the time needed to reduce a certain amount of 3-phenylpropionic acid is the substrate concentration ($x_{Substrate}$). Experiments in bottles under non-controlled conditions showed that 3-phenylpropionic acid concentrations ranging from 5 mM already inhibited growth. At 50 mM there is hardly any growth left (figure 4).

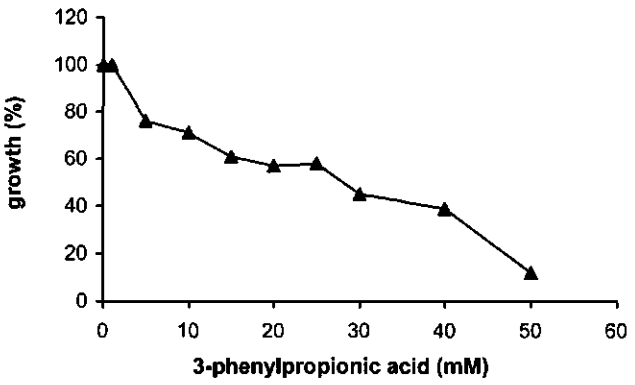

Figure 4 Effect of 3-phenylpropionic acid concentrations on growth of *Pyrococcus furiosus*. Experiments were performed under the same conditions as used for precultures.

Inhibition of growth was also found for other carboxylic acids with concentrations of 10 mM, like for decanoic acid [83]. However, in a 2 l reactor under controlled conditions the inhibitory effects of 10 mM 3-phenylpropionic acid are much less dramatic than in the bottle experiments. The protein content was similar to the experiments performed in a 2 l reactor with 1 mM of the acid but the growth rate was only slightly lower.

Fortunately, the 3-phenylpropionic acid concentration has a strong positive effect on the $Y_{\text{Production rate}}$. The effect of growth inhibition at a high substrate concentration is less than that of the rate of conversion (expressed as $Y_{\text{Production rate}}$).

The model of $Y_{\text{Production rate}}$ shows that a low partial dihydrogen pressure ($x_{pH2} = -1$) has a small positive effect and that might be explained by the inhibitory effect high partial dihydrogen pressure values have on growth [85, 86, 88]. However, growth was not negatively affected by partial dihydrogen pressure in the range studied. Because of an interaction effect ($x_{pH2}x_{Starch}$), the effect of the partial dihydrogen pressure is strongest when a high amount of starch is used ($x_{Starch} = +1$). This is about 7% of the highest $Y_{\text{Production rate}}$ that can be found in the range of variables used.

Analysis of $Y_{\text{Yield of conversion}}$

Statistical analysis of the data resulted in the following linear model describing $Y_{\text{Yield of conversion}}$:

$$Y_{\text{Yield of conversion}} = 40.00 - 12.94x_{\text{Substrate}} - 13.31x_T + 9.56x_{pH}x_{\text{Substrate}} + 5.56x_{\text{Substrate}}x_T$$

The response surface of this model is shown in figure 5a. Statistical testing of the model reveals that this model is significant at the 95% level and that the model explains 84.87% (R_{adj}^2) of the variation. The standard error of the estimate was 2.485.

The value of β_0 in the linear model is much lower than the average of the center points (CP): 40.00 versus 48.2. This might indicate that the given model does not give an adequate description of the $Y_{\text{Yield of conversion}}$. An analysis of the second-order curvature for the $Y_{\text{Yield of conversion}}$ showed there is a significant quadratic effect. Indeed, a linear model does not give an adequate description of the $Y_{\text{Yield of conversion}}$.

To gain information about this quadratic effect additional experiments, by a face-centered central composite design, are essential. For these experiments only three variables are used: pH (x_{pH}), substrate concentration ($x_{\text{Substrate}}$) and temperature (x_T). The remaining two variables had no effect on the $Y_{\text{Yield of conversion}}$ and were kept constant during the experiments. They were set to the values at which they resulted in a high $Y_{\text{Production rate}}$: partial dihydrogen pressure at 12 kPa ($x_{pH2} = -1$) and addition of starch ($x_{\text{Starch}} = +1$).

With the results of the experiments (table 3) an analysis of variance was performed to reveal the significant effects of variables. The same variables and interactions were found with the addition of one quadratic factor: x_{pH}^2. As can be seen from the new model describing the $Y_{\text{Yield of conversion}}$ the variables have almost the same values as in the linear model with the addition of one quadratic factor. The constant (β_0) has changed to a value that gives a much better value for the $Y_{\text{Yield of conversion}}$ at the center point (CP).

$$Y_{\text{Yield of conversion}} = 50.00 - 14.17x_{\text{Substrate}} - 12.50x_T + 9.56x_{pH}x_{\text{Substrate}} + 5.56x_{\text{Substrate}}x_T - 15.76x_{pH}^2$$

Statistical testing of the second-order model reveals that this model is significant at the 99% level and that the model explains 87.78% (R_{adj}^2) of the variation. This means that the model is an adequate predictor of the

experimental results. The standard error of the estimate was 1.829. A test for the lack of fit showed that this model is appropriate and describes the $Y_{\text{Yield of conversion}}$ better than the linear model does.

The response surface of this adapted model is shown in figure 5b. The effect of pH on the $Y_{\text{Yield of conversion}}$ can be seen clearly: in the used pH range there is an optimum. For the other two variables there are probably optima too but these are not in the ranges of temperature and substrate concentration that are used for these experiments.

Table 3 Face-centered central composite design for three variables. Matrix for experiments and response $Y_{\text{Yield of conversion}}$.

	Coded level			Response	
Experiment	x_{pH}	$x_{Substrate}$	x_T	$Y_{\text{Yield of conversion}}$ ¶	
1	-1	-1	-1	89 †	80 †
2	+1	-1	+1	22 †	33 †
3	-1	-1	+1	35 †	36 †
4	+1	-1	-1	55 †	53 †
5	-1	+1	+1	7 †	8 †
6	+1	+1	-1	50 †	34 †
7	-1	+1	-1	19 †	26 †
8	+1	+1	+1	33 †	19 †
9	-1	0	0	35	
10	+1	0	0	38	
11	0	-1	0	75	
12	0	+1	0	27	
13	0	0	-1	63	
14	0	0	+1	51	
CP §	0	0	0	54	
CP §	0	0	0	48	
CP §	0	0	0	44	
CP §	0	0	0	48	
CP §	0	0	0	47	

¶ $Y_{\text{Yield of conversion}}$ in %

† Data from the first eight experiments are the same as in table 2

§ CP = Center point

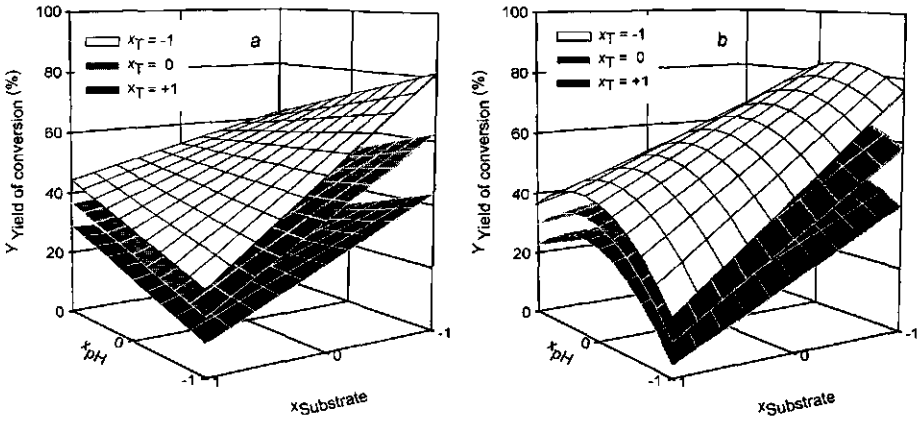

Figure 5 Theoretical response surfaces of the $Y_{Yield\ of\ conversion}$ obtained from (a) the fractional factorial design (b) the face-centered central composite design; predicted $Y_{Yield\ of\ conversion}$ as a function of 3-phenylpropionic acid concentrations ($x_{Substrate}$) and pH levels (x_{pH}) in coded units at different levels of temperature (x_T).

The optimal pH varies with substrate concentration:

$$\frac{d\,Y_{Yield\ of\ conversion}}{d\,x_{pH}} = 9.56x_{Substrate} - 31.52x_{pH}$$

At a low 3-phenylpropionic acid concentration ($x_{Substrate}$ = -1) the optimal pH is 6.35 and at a high concentration ($x_{Substrate}$ = +1) pH is 6.65.

As can be seen from figure 5b a high $Y_{Yield\ of\ conversion}$ was obtained at a temperature of 80°C (x_T = -1), a substrate concentration of 1 mM ($x_{Substrate}$ = -1) and pH 6.35 (x_{pH} = -0.3). The effect of temperature on $Y_{Yield\ of\ conversion}$ is consistent with the results of experiments performed at 98°C. In all cases an increasing temperature has a negative effect on $Y_{Yield\ of\ conversion}$.

A low pH had a positive effect on the $Y_{Yield\ of\ conversion}$. This was also found for reduction of carboxylic acids by several *Clostridia* strains and *Thermococcus* ES-1, a low pH (5.5 - 6.2) resulted in better reduction of the acid [18, 21, 42].

An extra amount of starch had no effect on the $Y_{Yield\ of\ conversion}$. The amount of starch that was added initially was not growth limiting. That could be seen from the results of the analysis of the protein content (results not shown). Growth was most likely to be limited by accumulation of acetate, the metabolic

end product of *P. furiosus*. Acetate is known to inhibit growth [84]. At the end of growth acetate concentrations of approximately 65 mM at pH 7.0 and 16 mM at pH 6.0 were found in our experiments.
Surprisingly, the dihydrogen pressure had also no effect. However, it is possible that the difference between the low and high value is not large enough to distinguish an effect. It is assumed that dihydrogen is produced to dispose off reducing equivalents (ferredoxin and NADPH) that are produced during fermentation [82]. It was expected that some back pressure on the disposal of reducing equivalents (a higher dihydrogen content) could have a positive effect on the maximal yield since reducing equivalents are needed to reduce the acids. On the other hand when the partial dihydrogen pressure is high it inhibits growth [85, 86, 88].

CONCLUDING REMARKS

When the results from the $Y_{\text{Production rate}}$ and the $Y_{\text{Yield of conversion}}$ are combined it is obvious that it is not possible to have both a high $Y_{\text{Production rate}}$ and a high $Y_{\text{Yield of conversion}}$. All important variables have opposite optima for both responses. To achieve a high $Y_{\text{Production rate}}$ (maximal 118.2 μM/hour = 16 mg/l.hour) experiments must be performed at pH 7.0 (x_{pH} = +1), 90°C (x_T = +1), and the 3-phenylpropionic acid concentration has to be 10 mM ($x_{Substrate}$ = +1). A high $Y_{\text{Yield of conversion}}$ (maximal 83.7 %) is obtained at 80°C (x_T = -1), a substrate concentration of 1 mM ($x_{Substrate}$ = -1), and pH 6.35 (x_{pH} = -0.3).
To be able to compare both responses they have to be normalized i.e. divided by the maximal value of that response (Yx / Yx_{max}). Therefore, $Y_{\text{Production rate}}$ / $Y_{\text{Production rate max}}$ and $Y_{\text{Yield of conversion}}$ / $Y_{\text{Yield of conversion max}}$ have a maximal value of 1 and the sum of these normalized responses ($\Sigma\ Yx$ / Yx_{max}) can not exceed a value of 2. The sum of the normalized responses has to be high in order to achieve our goal: a high $Y_{\text{Yield of conversion}}$ and a high $Y_{\text{Production rate}}$.
Figure 6 shows the values of the sum of the normalized responses. These plots show that there are two optima in the tested ranges of pH (x_{pH}), substrate concentration ($x_{Substrate}$), and temperature (x_T). One maximum ($\Sigma\ Yx$ / Yx_{max} = 1.24) is obtained at 80°C (x_T = -1), a substrate concentration of 1 mM ($x_{Substrate}$ = -1), and pH 6.25 (x_{pH} = -0.5) and the other ($\Sigma\ Yx$ / Yx_{max} = 1.27) at the same point as the maximal $Y_{\text{Production rate}}$, see figures 6a and 6c, respectively.

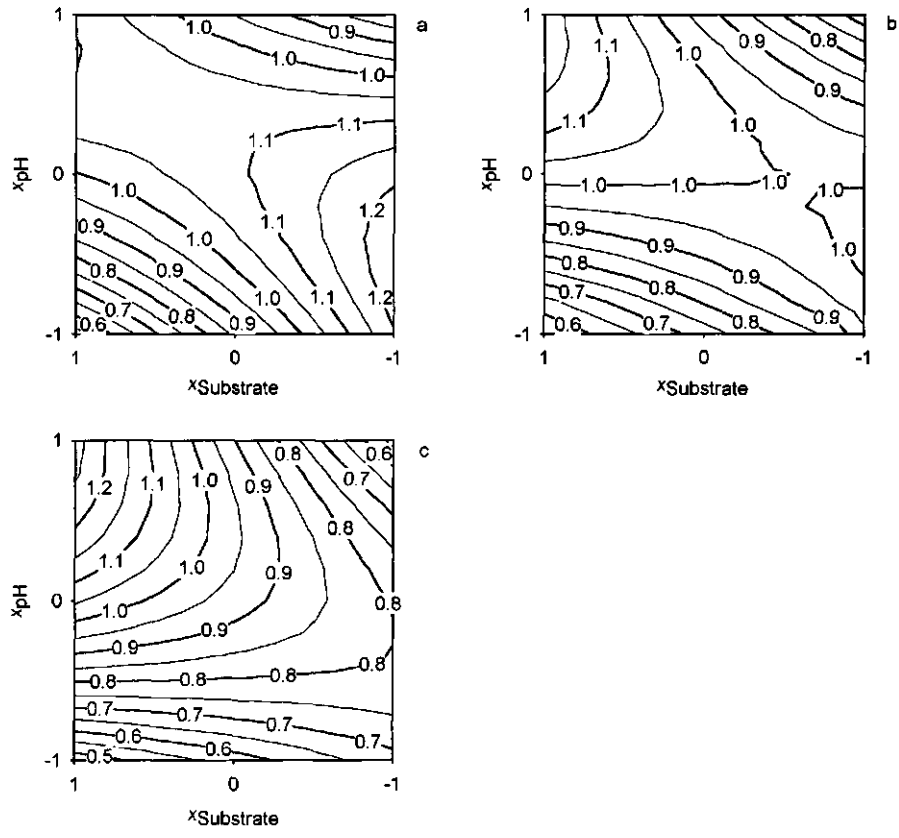

Figure 6 Theoretical contour plots obtained from the sum of the normalized responses ($\Sigma Yx / Yx_{max}$) $Y_{Production\ rate} / Y_{Production\ rate\ max}$ and $Y_{Yield\ of\ conversion} / Y_{Yield\ of\ conversion\ max}$ as a function of 3-phenylpropionic acid concentrations ($x_{Substrate}$) and pH levels (x_{pH}) in coded units at different levels of temperature (x_T), a: x_T = -1; b: x_T = 0; c: x_T = +1. In all cases x_{pH2} = -1 and x_{Starch} = +1.

Figure 7 shows the highest values of the sum of the normalized responses ($\Sigma Yx / Yx_{max}$ opt) dependent on either pH (x_{pH}) or substrate concentration ($x_{Substrate}$) for different temperatures (x_T). In these graphs, the highest values of the sum of the normalized responses ($\Sigma Yx / Yx_{max}$ opt) can be linked to the corresponding pH, substrate concentration and temperature. For example, $\Sigma Yx / Yx_{max}$ opt = 1.27 is only found for x_T = +1, this corresponds with one x_{pH} (= +1) and with one $x_{Substrate}$ (= +1) as can be seen in figures 7a and 7b respectively. The same is shown for $\Sigma Yx / Yx_{max}$ opt = 1.24.

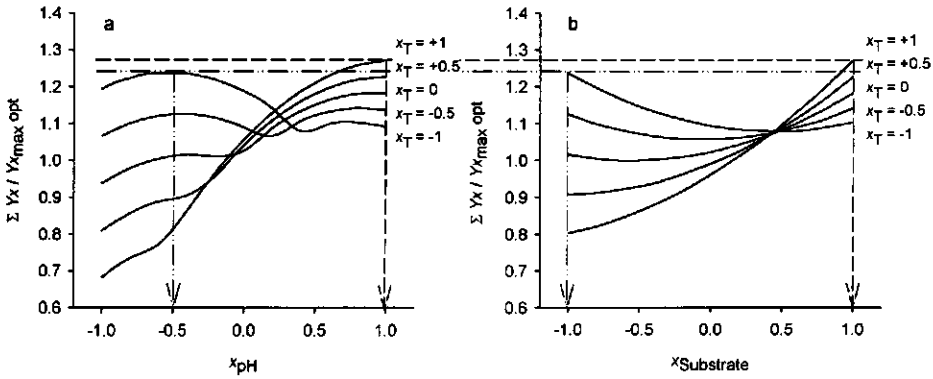

Figure 7 Theoretical plots obtained from the highest values of the sum of the normalized responses (Σ Yx / Yx_{max} opt) dependent on either (a) pH (x_{pH}) or (b) 3-phenylpropionic acid concentration ($x_{Substrate}$) for different temperatures (x_T). The arrows indicate at which pH (x_{pH}), 3-phenylpropionic acid concentration ($x_{Substrate}$), and temperature (x_T) the highest Σ Yx / Yx_{max} opt are found.

In addition, these graphs also show that the highest values of the sum of the normalized responses are only found for the two extreme temperatures used (x_T = -1 and x_T = +1). At low pH the highest values are found for a low temperature (x_T = -1), at pH 6.6 (x_{pH} = +0.2) it shifts towards a high temperature (x_T = +1) (figure 7a). Similar effects are found for the substrate concentration: the shift is at 7.53 mM ($x_{Substrate}$ = +0.45) (figure 7b).

Which of the two optima found for the sum of the normalized responses (Yx / Yx_{max} opt) is most suitable for a production process depends on other aspects e.g. costs of the process and substrates. These aspects need to be investigated.

ACKNOWLEDGMENTS

We thank Drs Albert Otten, Department of Mathematics, Wageningen University, for his help in the statistical analysis of the data and Wim Doesburg, Laboratory of Microbiology, Wageningen University, for his help in HPLC analysis of acetate. This research was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Ministry of Economic Affairs of the Netherlands (project number IKA96001).

4

Enzymes of hydrogen metabolism in *Pyrococcus furiosus*

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Published in European Journal of Biochemistry (2000) 267:6541-6551

The genome of *Pyrococcus furiosus* contains the putative *mbhABCDEFGHIJKLMN* operon for a 14-subunit transmembrane complex associated with a Ni-Fe-hydrogenase. Ten open reading frames (*mbhA-I* and *mbhM*) encode hydrophobic, membrane-spanning subunits. Four ORFs (*mbhJKL* and *mbhN*) encode putative soluble proteins. Two of these correspond to the canonical small and large subunit of Ni-Fe-hydrogenase, however, the small subunit can coordinate only a single iron-sulfur cluster, corresponding to the proximal [4Fe-4S] cubane. The structural genes for the small and the large subunits, *mbhJ* and *mbhL*, are separated in the genome by a third ORF, *mbhK*, encoding a protein of unknown function without Fe/S binding. The fourth ORF, *mbhN*, encodes a 2[4Fe-4S] protein. With the *P. furiosus* soluble [4Fe-4S] ferredoxin as electron donor the membranes produce dihydrogen, and this activity is retained in an extracted core complex of the *mbh* operon when solubilized and partially purified under mild conditions. The properties of this membrane-bound hydrogenase are unique. It is rather resistant to inhibition by carbon monoxide. It also exhibits an extremely high ratio of dihydrogen evolution to dihydrogen uptake activity compared with other hydrogenases. The activity is sensitive to inhibition by dicyclohexylcarbodiimide, an inhibitor of NADH dehydrogenase (complex I). EPR of the reduced core complex is characteristic for interacting iron-sulfur clusters with $E_m \approx -0.33$ V. The genome contains a second putative operon, *mbxABCDFGHH'MJKLN*, for a multisubunit transmembrane complex with strong homology to the *mbh* operon, however, with a highly unusual putative binding motif for the Ni-Fe-cluster in the large hydrogenase subunit. Kinetic studies of membrane-bound hydrogenase, soluble hydrogenase and sulfide dehydrogenase activities allow the formulation of a comprehensive working hypothesis of dihydrogen metabolism in *P. furiosus* in terms of three pools of reducing equivalents (ferredoxin, NADPH, dihydrogen) connected by devices for transduction, transfer, recovery and safety-valving of energy.

INTRODUCTION

Hydrogenases are a diverse family of enzymes catalyzing the reversible reaction $H_2 \leftrightharpoons 2\,H^+ + 2\,e^-$. A classification in two major groups is commonly accepted: iron-only and nickel-iron hydrogenases. Fe-only hydrogenases are usually monomeric enzymes that catalyze primarily dihydrogen evolution [90] and contain a novel 6Fe cluster composed of a [4Fe-4S] subcluster covalently linked by a cysteinate thiol to a 2Fe subcluster [91, 92]. Ni-Fe hydrogenases are considerably less active than Fe-hydrogenases but have a much higher affinity for dihydrogen. All Ni-hydrogenases contain at least a small (around 30 kDa) subunit, which harbors a variable number of Fe-S clusters, and a larger (around 60 kDa subunit) subunit where the active site, a cysteine-bridged dinuclear Ni-Fe cluster [93], is located. Other subunits may also be present, allowing the hydrogenase to interact with dedicated electron carriers (NAD^+, F_{420}, cytochrome *b*) [94]. Ni-Fe hydrogenases may catalyze dihydrogen oxidation, which is then coupled to energy conservation and reduction of electron acceptors, or reduce protons to dihydrogen as a way of disposing of excess reducing power [95].

P. furiosus is a hyperthermophilic archaeon originally isolated by Fiala and Stetter from geothermally heated marine sediments near Vulcano, Italy. It is a strictly anaerobic heterotroph, which grows near 100°C by fermentation of carbohydrates [65] through a modified Embden-Meyerhoff pathway, producing acetate, alanine, CO_2 and dihydrogen [81]. In the presence of elemental sulfur H_2S is formed instead of dihydrogen [65] and this has been proposed to be either a means of detoxification or an energy-conserving process [96]. Two soluble Ni-Fe hydrogenases have been purified from this organism [97, 98] and shown to possess also sulfur reductase activity [98, 99]. Upon increase of temperature the soluble hydrogenase I is reduced by an as yet unidentified internal substrate, and the Ni-Fe cluster becomes catalytically competent [100]. Besides the Ni-Fe cluster the enzyme contains up to six Fe-S clusters [100], of which three have been detected by electron paramagnetic resonance [79, 97]. Hydrogenase II is very similar to hydrogenase I, however, it is significantly less active in both the dihydrogen production and the sulfur reduction assays [98].

Both proteins can use NADPH as an electron donor for the reduction of protons to dihydrogen and polysulfide to H_2S. Polysulfide stimulates NADPH oxidation and reduces dihydrogen production, which may result from the

diversion of the electron flow to polysulfide rather than to protons [99]. Originally, a [4Fe-4S] ferredoxin was proposed to be the natural redox partner of (sulf)hydrogenase I in *P. furiosus* [97]. Later this idea was rejected in favor of a model in which ferredoxin gives electrons to a complex enzyme, which is not only a sulfide dehydrogenase (SuDH) but also has ferredoxin:$NADP^+$ oxidoreductase activity. The produced NADPH is then thought to reduce hydrogenase [101]. Recent genome analysis has provided evidence for two different SuDH-encoding operons [102]. The putative second sulfide dehydrogenase, SuDH II, has not yet been studied at the protein level.

It has been shown that the reduction, catalyzed by enzymes, of protons to dihydrogen by pyridine nucleotides is possible when the partial pressure of dihydrogen is kept below 0.1 kPa [103, 104]. *P. furiosus* is, however, able to grow under dihydrogen accumulating conditions (partial dihydrogen pressure ≈ 30 kPa) [65]. This apparent inconsistency prompted the present study on electron flow in pathways of dihydrogen-metabolism.

Recently, a novel membrane-bound hydrogenase complex in *P. furiosus* has been described in some detail [105]. Solubilization and purification apparently resulted in a heterodimeric protein with dihydrogen production activity from reduced artificial dye. Unfortunately, a physiological redox partner could not be identified. Remarkably, the protein was reported to contain 4.4 Fe and to exhibit Fe/S-cluster EPR, although the sequence of the two subunits does not contain any putative iron-sulfur cluster-binding motif. We have isolated the membrane-bound hydrogenase using milder extraction and purification procedures, which do not include the 4 M NaCl wash and the addition of 2 M urea used by Sapra *et al.* [105]. We have found that the previous subunit analysis of the purified complex is probably not correct. Furthermore, *P. furiosus* [4Fe-4S] ferredoxin is found to be a good electron donor for the membrane-bound hydrogenase also after solubilization. Re-evaluation of hydrogenase activities leads to an integrated working hypothesis of dihydrogen metabolism.

MATERIALS AND METHODS

Growth of *Pyrococcus furiosus* and sample preparation

Pyrococcus furiosus (DSM 3638) was grown as in Arendsen *et al.* [79] in a medium described in Kengen *et al.* [81]. Frozen cells of *P. furiosus* were lysed by a 2 h incubation at 30°C in 3 l of 20 mM Tris buffer, pH 8.0 in the presence of

DNase, RNase and $MgCl_2$ and centrifuged at 9,000 *g* for 120 min. Ferredoxin was isolated as described earlier [106]. Pyruvate:ferredoxin oxidoreductase (POR) [107], sulfide dehydrogenase (SuDH) [102], and sulfhydrogenase [79] were isolated according to published methods. Cytoplasmic membranes were isolated from the pellet obtained after centrifugation of the osmotically lysed cells for 1 h at 20,000 *g*. The pellet was suspended in 50 mM *N*-[2-hydroxyethyl]-piperazine-N'[3-propanesulfonic acid] (Epps), pH 8.0, and passed three times through a French press pressure cell at 70 MPa at room temperature. Cell debris was removed by centrifugation at 8,000 *g* for 10 min, and membranes were precipitated by centrifugation for 90 min at 200,000 *g*. The last traces of soluble proteins were removed by suspending the membranes in buffer and repeating the centrifugation procedure. The cell extract used to measure whole-cell activities was prepared from cells (5 g wet cells suspended in 30 ml Epps buffer, pH 8.0) broken by passing through a French pressure cell three times. The cell debris was removed by centrifugation at 8,000 *g* for 10 min.

Analytical procedures

Dihydrogen production activity was routinely measured at 80°C over a period of 15 min in 100 mM Epps buffer, pH 8.0 in the presence of 1 mM methyl viologen. The reaction was started by adding buffered dithionite (final concentration 20 mM) and the evolved dihydrogen was measured by GC as described by Adams and Mortenson [108]. One unit of hydrogenase catalyzes the production of 1 µmol dihydrogen/min. For measurements at different pH, and in order to minimize changes in pH brought about by the change in temperature, buffers with a low $\Delta pK/\Delta T$ (Ches, Mops, Epps or Mes, all at 100 mM) were used. Dihydrogen uptake was measured by following the dihydrogen dependent reduction of methyl viologen or benzyl viologen (1 mM) by the hydrogenase in dihydrogen saturated buffer after activating the hydrogenase with a small amount of dithionite. Studies with various inhibitors were performed. CO-inhibition assays were performed by adding CO to the headspace of the assay vials to yield from 0 to 100% CO in the headspace. *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibition was assayed by incubating 10 µl of a 50 mg/ml solution of DCCD in 100% ethanol and 20 µl membranes in 1.9 ml assay buffer for variable periods of time at room temperature and at 80°C and measuring the dihydrogen evolution activity as described above. The pyruvate oxidation driven, ferredoxin mediated, dihydrogen formation assay

mixture contained: 100 mM Epps pH 8.0, 0.1 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate, 10 mM pyruvate, 1.5 mM CoA, 15 mM dithiothreitol, 13 μM *P. furiosus* ferredoxin, 75 μg POR, 10 μg sulfhydrogenase (activated). The assays were performed at 80°C in a butyl rubber stoppered bottle (8 ml) with a reaction volume of 1 ml. Dihydrogen production was followed in time with gas chromatography. The sulfhydrogenase was activated by an incubation of 20 min at 80°C under a dihydrogen atmosphere. The dithionite oxidation driven dihydrogen formation assay mixture contained: 100 mM Epps, pH 8.0, 10 mM dithionite, 1 mM methyl viologen, 2 μg sulfhydrogenase (activated). With $NADP^+$/NADPH as oxidant/reductor for the sulfhydrogenase the POR reaction mixture (minus ferredoxin) was supplemented with 1 mM $NADP^+$ and 100% dihydrogen in the gas phase or with 1.5 mM NADPH. When SuDH was used as NADPH regenerating system, the POR system was supplemented with 0.3 mM $NADP^+$ and 30 μg SuDH. The kinetic parameters of SuDH were measured as described in Ma and Adams [109]. In view of the oxygen sensitivity of SuDH and the sulfhydrogenase during their activity measurements, the last traces of oxygen in the assay bottles or cuvettes were removed prior to the start of the reaction by reducing the viologen to 'slightly blue' with deazaflavin and light (10 μM deazaflavin and 50 mM *N*-[Tris(hydroxymethyl)methyl]glycine as electron donor). When no viologens were involved in the reaction, the last traces of oxygen were removed during the temperature equilibration by POR. When no standard deviation is given, the values have a standard deviation of 15% or less.

Partial purification of the membrane-bound hydrogenase

The membrane-bound hydrogenase was solubilized by mixing freshly prepared membranes (diluted to a final protein concentration of 10 mg/ml) with 2% sodium deoxycholate, incubating at room temperature for 30 min, and centrifuging at 14,000 *g* for 15 min. Protein was precipitated with ammonium sulfate (to 25% saturation) and resuspended in buffer A (10 mM Tris/HCl, pH 8.0, 0.1% (w/v) Triton X-100). Ammonium sulfate was eliminated by repeatedly concentrating the sample and exchanging the buffer with buffer A in an Amicon concentration cell with a 30 kDa filter. The sample was then loaded onto a 300 ml Q-Sepharose column and eluted with a 1250 ml gradient from 0 to 500 mM NaCl, followed by a steeper gradient of 100 ml from 500 to 1000 mM NaCl, and a column wash with 1000 mM NaCl. Hydrogenase containing fractions (eluting between 440 - 480 mM NaCl) were pooled and

concentrated to 4 ml, dialyzed against 1 liter buffer A, and loaded onto a 20 ml hydroxyapathite column equilibrated with 10 mM Tris buffer, pH 8.0. Under these conditions the membrane-bound hydrogenase flows though the column without binding. Samples with hydrogenase activity were pooled, concentrated and frozen in liquid N_2.

Protein analysis

Protein concentration was determined according to the microbiuret method [110]. SDS-PAGE was performed as described in Laemmli [111]. The gel was blotted onto a PVDF membrane, relevant bands were excised and their N-terminals were sequenced in the gas phase sequenator at the Sylvius Laboratories, Department of Medical Biochemistry, Leiden University.

Sequence analysis

The *P. furiosus* genomic sequence database was screened for Ni-Fe hydrogenase-coding regions using the amino acid sequence of the large subunit of hydrogenase-2 from *Escherichia coli*. Open reading frames in the relevant genome regions (with a minimum size around 80 amino acids, to eliminate statistical artifacts) were evaluated according to codon usage. Codon usage was compared to the average codon usage in genes coding for known *P. furiosus* proteins (data obtained from the Codon Usage Database at http://www.kazusa.or.jp/codon/). Similarity searches were performed using the BLAST set of programs (available at http://www.ncbi.nlm.nih.gov/BLAST/) with the non-redundant protein databases of the National Center for Biotechnology Information (Bethesda, MD, USA). Membrane spanning helices were predicted according to Tusnády and Simon [112] using the interface at http://www.enzim.hu/hmmtop/index.html. Ribosome-binding sites were identified by comparing the gene sequence to the 3'-terminal of the 16S rRNA small subunit rRNA (Achenbach, LA (1995) *Pyrococcus furiosus* 16S small subunit rRNA sequence, accession number U20163).

EPR Spectroscopy

Anaerobic redox titration of the membrane-bound hydrogenase was carried out as follows. A 17 mg/ml enzyme solution in 50 mM Tris/HCl buffer, pH 8.0, was poised at different redox potentials in the presence of redox mediators (40 μM). The mediators and their respective potentials were: tetramethyl-phenylene diamine (260 mV), 2,6-dichloro-4-[4-(hydroxyphenyl)imino]

(271 mV), N-methylphenazonium methosulfate (80 mV), thionine (56 mV), methylene blue (11 mV), indigo tetrasulfonate (-46 mV), indigo disulfonate (-125 mV), 2-hydroxy-1, 4-naphtoquinone (-152 mV), antraquinone-2-sulfonate (-225 mV), phenosafranin (-252 mV), safranin O (-280 mV), neutral red (-329 mV), benzyl viologen (-359 mV) and methyl viologen (-449 mV). The reductive redox titration was performed with sodium dithionite. After a suitable equilibration time samples were taken and frozen in liquid N_2. X-band EPR spectroscopy was done with a Varian E-9 spectrometer operating with a homemade He flow cryostat. The modulation frequency was 100 kHz and the modulation amplitude was 1.0 mT.

RESULTS

Kinetics of soluble sulfhydrogenase I and of sulfide dehydrogenase I

In table 1 the kinetic parameters of SuDH and the sulfhydrogenase are presented. The apparent K_m and V_{max} values of SuDH for the benzyl viologen reduction by NAD(P)H have been determined. The data are of the same order of magnitude as those published by Ma and Adams [109], but the K_m values for NADH and NADPH are significantly different. The V_{max} of the $NADP^+$ reduction by reduced *P. furiosus* ferredoxin is lower compared with the value reported by Ma and Adams [109]. The sulfhydrogenase catalyzes the dihydrogen oxidation by $NADP^+$ about 10 times faster than the reverse reaction: the H^+-reduction by NADPH (table 1). The proposed pathway from POR-reduced ferredoxin, via SuDH, $NADP^+$, sulfhydrogenase, to dihydrogen was also reconstituted with purified enzymes. 75 μg reduced POR, 14 μM Fd, 30 μg SuDH, 0.3 mM $NADP^+$ and 10 μg sulfhydrogenase were incubated in a total volume of 1 ml. After 5 min a linear dihydrogen production of 58 nmol/min was observed. This is an activity of 5.8 μmol dihydrogen produced/min.mg of sulfhydrogenase, which indicates that the maximal specific activity of the sulfhydrogenase with NADPH as electron donor can be obtained with SuDH as NADPH regenerating system.

Table 1 Reactivity of purified sulfide dehydrogenase and sulfhydrogenase with different electron donors and acceptors. Abbreviations as follows: Fd_{red}, Fd_{ox} the reduced or oxidized form of *P. furiosus* ferredoxin respectively; MV, methyl viologen; BV benzyl viologen. All enzyme activities were measured at pH 8.0, except in the experiments used to determine the kinetic parameters of the NAD(P)H reduction of BV catalyzed by SuDH. These experiments were performed with 50 mM (3[cyclohexylamino]-1-propanesulfonic acid) buffer at pH 10.3. Data in braces were taken from Ma and Adams [109] for comparison.

Enzyme	Electron donor (mM)	Electron acceptor (mM)	Apparent V_{max}[#] (unit/mg)	Apparent K_m (μM)
Sulfide dehydrogenase	Fd_{red} (0 - 0.02)	$NADP^+$ (0.3)	3.9 ± 0.3 {8}	1.8 ± 0.5 {0.7}
	Fd_{red} (0.014)	NAD^+ (0.3)	0.7	
	NADPH (0 - 0.25)	BV (1)	355 ± 24 {263}	77 ± 13 {11}
	NADH (0 - 0.25)	BV (1)	85 ± 9 {182}	23 ± 8 {71}
	NADH (0.3)	BV (1)	16	
	NADPH (0.3)	BV (1)	144	
Sulfhydrogenase	Fd_{red} (0 - 0.05)	H^+ (10^{-5})	0	
	NADPH (1.5)	H^+ (10^{-5})	6	
	dithionite (10)	MV (1) H^+ (10^{-5})	96	
	H_2[¶]	MV (1)	420	
	H_2[¶]	$NADP^+$ (1)	59	
	NADH (0.3)	BV (1)	3	
	NADPH (0.3)	BV (1)	81	

[#] When the electron donor concentration was varied the value is an apparent V_{max}, otherwise it is the activity at the indicated substrate concentration. Units (μmol) are the transfer of the equivalent of two electrons from the donor to the acceptor per min

[¶] Pure dihydrogen was used at a pressure of 120 kPa

Whole cell acetate fermentation and activities in dihydrogen metabolism

The activities of SuDH and sulfhydrogenase must be high enough to account for the flux of reductant observed in *P. furiosus* cells during starch fermentation. In the absence of elemental sulfur the major fermentation products in growing *P. furiosus* cells are acetate, CO_2, dihydrogen and alanine [65, 88]. The maximal alanine production at a partial dihydrogen pressure of 60 kPa was 40% of the acetate formation (data not shown), but the formation of alanine from pyruvate (derived from starch) is neutral with respect to reductant usage/production [88]. Inspection of the glucose fermentation

pathway shows that, for each molecule of acetate produced, four molecules of ferredoxin are reduced [82]. It is, therefore, possible to determine the flux of reductant towards H^+ in whole cells from the rate of acetate formation during growth. In growing *P. furiosus* cells the effect was studied of the partial dihydrogen pressure in the gas stream (0.17 l/min.l) on the rate of ferredoxin oxidation by H^+ reduction by measuring the acetate production. *P. furiosus* was grown at 90°C in a 2 liter fermentor without elemental sulfur in the medium. The maximum acetate production at a partial dihydrogen pressure of 0, 12, 24, and 60 kPa was 0.71 ± 0.14, 0.58 ± 0.13, 0.54 ± 0.16, and 0.14 ± 0.04 μmol acetate formed/min.mg protein, respectively. These data indicate that *P. furiosus* can metabolize starch under dihydrogen and they confirm the observation of Fiala and Stetter [65] that *P. furiosus* is capable of growing under dihydrogen accumulating conditions. The rate of acetate production at 80°C, the standard temperature of the enzymatic assays, was 0.19 μmol/min.mg protein (table 2). The cells were grown with 100% N_2 in the gas stream. These cells were used to prepare cell free extracts. Based on the acetate production, the activity of the enzymes involved in the disposal of catabolically generated reductant as dihydrogen must be at least 0.38 μmol electron-pairs/min.mg cell protein (table 2).

Table 2 Comparison of whole-cell acetate formation with cell-free extract activities of enzymes involved in the dihydrogen metabolism at 80°C. Abbreviations as follows: Fd_{red}, the reduced form of *P. furiosus* ferredoxin was produced by the POR catalyzed reaction; MV_{SQ}, methyl viologen semiquinone. The enzyme activities were measured at 80°C and are the average of at least three experiments. Starch catabolism is a whole cell activity. In cell extracts the substrates and/or cofactors necessary to detect the indicated enzyme activity were present in the assay mixture.

Substrate(s) or reductant	Activity (product formed min^{-1} mg $protein^{-1}$)	Enzymes involved
Starch	0.19 ± 0.04 μmol acetate	Whole cells
Pyruvate, CoA	4.0 ± 0.3 μmol MV_{SQ}	Cell extract: POR
NADPH	0.02 ± 0.003 μmol H_2	Cell extract: sulfhydrogenase
H_2 (120 kPa)	0.12 ± 0.02 μmol NADPH	Cell extract: sulfhydrogenase
Fd_{red}	0.62 ± 0.08 μmol H_2	Cell extract: hydrogenase
Fd_{red}	0.14 ± 0.01 μmol NADPH	Cell extract: SuDH, hydrogenase, sulfhydrogenase

The maximal activity of POR, a key enzyme of the glucose fermentation pathway, was found to be high enough. The rate of NADPH formation with reduced ferredoxin, an activity catalyzed by SuDH, is about the same as the observed disposal rate. However, the activity of the second enzyme in the proposed pathway, the sulfhydrogenase catalyzing the dihydrogen production with NADPH as electron donor, is only 5% of the disposal rate (0.02 versus 0.38 μmol dihydrogen/min.mg protein). The activity of the sulfhydrogenase in this reaction in cells is clearly too low to support acetate formation. Surprisingly, the overall reaction from reduced ferredoxin to dihydrogen is catalyzed at a rate of 0.62 units/mg protein. This rate even exceeds the estimated rate of catabolically generated reductant. Since the soluble sulfhydrogenase shows no activity with ferredoxin, another hydrogenase system must be active in the cells.

Membrane-bound hydrogenase activity

Fractionation of the cell extract demonstrated that the ferredoxin dependent hydrogenase activity was associated with the cytoplasmic membranes. The apparent K_m for ferredoxin is 36 ± 7 μM. Surprisingly, the membranes showed a very low dihydrogen oxidation activity with 1 mM methyl viologen or 20 μM oxidized *P. furiosus* ferredoxin as acceptor. When combined with the soluble (sulf)hydrogenase I an effective NADPH producing system can be reconstituted (table 3). Both hydrogenases are necessary for the reduction of $NADP^+$ by reduced ferredoxin. In both cases, the maximal activity of each hydrogenase was obtained when the other hydrogenase was present in excess.

Partial purification

After cell lysis a significant amount of dihydrogen production activity is found in the membrane fraction of *P. furiosus*. This activity remains even after extensive washing (2 times 60 min at 200,000 *g*) indicating that the enzyme responsible is firmly associated with the membrane. The activity remains largely associated with the membrane after treatment with Triton X-100, but it can be quantitatively solubilized by incubating the membranes with 2% sodium deoxycholate. Long exposure to deoxycholate, however, leads to loss of activity. Therefore, the protein was immediately precipitated with 25% ammonium sulfate, which is enough to precipitate >90% of the solubilized membrane proteins and leads to minimum precipitation of soluble proteins still remaining in the sample.

Table 3 Reduction of $NADP^+$ by ferredoxin via dihydrogen. Reduced *P. furiosus* ferredoxin (14 μM) was generated with POR (75 μg/ml). The sulfhydrogenase was activated in 20 min at 80°C under a dihydrogen atmosphere.

Membranes (μg/ml)	Sulfhydrogenase (μg/ml)	NADPH formation (nmol/min.ml)
0	1	0
1	1	15
2	1	25
4	1	45
20	1	65
2	0	0
2	0.5	12
2	1	25
2	2	23
2	5	30

The pellet was resuspended in 0.1% Triton X-100 containing buffer and dialyzed in order to remove sodium deoxycholate and ammonium sulfate. The results of a typical purification are shown in table 4. We have consistently found membrane-associated hydrogenase activities one order-of-magnitude less than those reported by Sapra *et al.* [105]. These values are not due to lower amounts of hydrogenase in our membrane preparations: the purified core complex also has an activity (4.18 units/mg) one order-of-magnitude less than that reported by Sapra *et al.* (30 units/mg), although the protein recoveries and purification factors are very similar (compare table 4 with table 2 in Sapra *et al.* [105]). Also the dihydrogen evolution rate by sulfhydrogenase I and the dihydrogen evolution/dihydrogen uptake ratio reported by these authors are about ten times greater than the values determined by us (see below). It has been previously argued [113] that the dihydrogen production assay is very sensitive to changes in pH, electron donor concentration, buffer composition, manometric vs. gas chromatography detection, etc. It is, therefore, possible that these differences arise from unreported differences in the method used.

Table 4 Purification of the membrane-bound hydrogenase from *P. furiosus*. The core complex was purified from 100 g cells (wet mass). For the details of the purification see Materials and Methods.

Step	Protein (mg)	Activity (units)	Specific act. (units/mg)	Purification (fold)	Recovery (%)
Membrane extract	1936	880	0.45	1.0	100
DOC extraction	1128	730	0.65	1.4	83
Q-Sepharose	157	280	1.78	3.9	32
Hydroxyapatite	32	135	4.18	9.2	15

Sequence analyses

A TBLASTN search with the large subunit of *E. coli* hydrogenase-2 revealed three open reading frames in the *P. furiosus* genome with a high similarity score. One of these three sets of genes belongs to the gene cluster encoding the soluble sulfhydrogenase [114] and the other to soluble sulfhydrogenase II [98]. The third detected open reading frame encodes a protein very similar to the large subunits of some membrane-bound hydrogenases. Using a combination of codon usage analysis and ORF searches, a total of 14 contiguous genes were detected in this putative operon (figure 1 and table 5). The operon has been recently identified by Sapra *et al.* to encode the membrane-bound hydrogenase of *P. furiosus;* it has been named *mbh* [105].

All of these genes are preceded by ribosome-binding regions. Several subunits show high similarity to subunits from the CO-induced hydrogenase from *Rhodospirillum rubrum* [115], hydrogenase-3 [116] and -4 [117] from *E. coli*, the Ech hydrogenase from *Methanosarcina barkeri* [118, 119], and two putative membrane-bound hydrogenases from *Methanobacterium thermoautotrophicum* [120].

Striking homology is also observed with subunits of proton-translocating NADH:quinone oxidoreductase (complex I) from many sources. Below, comparison with complex I subunits is made with reference to the nuo operon (NADH:ubiquinone oxidoreductase) of *E. coli* [121, 122]. A very similar operon (13 contiguous genes) is present ca. 6 kb downstream of mbh. We have named this operon mbx (figure 1, and see below).

Table 5 Predicted properties of the subunits of the membrane-bound hydrogenase from *P. furiosus*.

Gene (ortholog in *E. coli* complex I)	Translation product			
	Size		Predicted pI	Membrane-spanning helices
	(aa)	(Da)		
mbhA	167	18736	6.6	3
mbhB	84	9060	5.9	3
mbhC	124	13504	9.6	3
mbhD	96	10413	8.1	3
mbhE	99	11140	4.8	2
mbhF	148	15518	9.9	4
mbhG	117	12773	9.4	3
mbhH (nuoL)	510	54980	8.9	14
mbhI	115	13043	6.7	2
mbhJ (nuoB)	167	18284	6.0	0
mbhK (nuoC)	173	20184	5.0	0
mbhL (nuoD)	427	47934	6.6	0
mbhM (nuoH)	321	35388	9.5	8
mbhN (nuoI)	139	15686	8.7	0

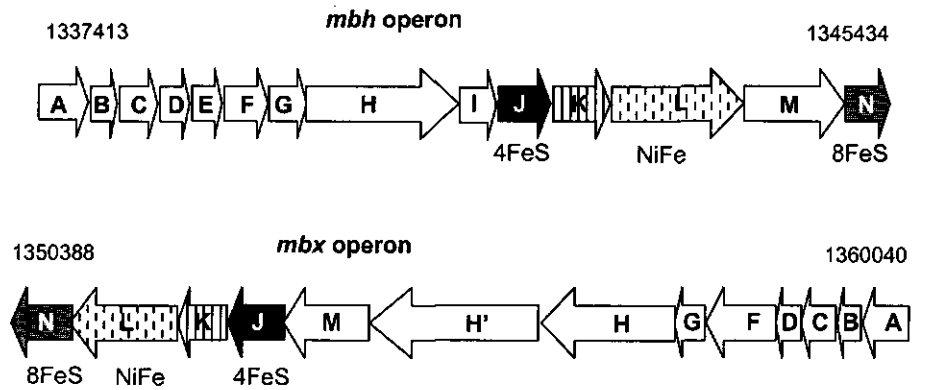

Figure 1 Organization of the putative operons in the genome of *P. furiosus* containing the genes that encode the membrane-bound hydrogenase and its "mirror" complex. The single-letter names of the open reading frames in the *mbx* operon indicate their high similarity with ORFs in the *mbh* operon.

Description of the mbh gene products

MbhA-G and MbhI

These putative gene products present no relevant similarities to any purified proteins; however, the usage of "preferred codons" in these ORFs is close to the average in identified *P. furiosus* proteins. Furthermore, the prediction of membrane-spanning helices in all of these ORFs would be unlikely if they were mere statistical artifacts. Very close homologs exist in the genomes of *Pyrococcus horikoshii* (*mbhACEFGI*), *Pyrococcus abyssi* (*mbhABCEFGI*) and *Thermotoga maritima* (*mbhABG*). These subunits may have a role in anchoring the complex to the membrane.

MbhH

This subunit shows 28% identity and 44% similarity to the CooM subunit of the CO-induced hydrogenase from *R. rubrum*. A similar level of homology is found with the B subunit of hydrogenase-4 from *E. coli*. The function of these subunits is unknown. MbhH is also homologous to NADH dehydrogenase NuoL (*E. coli* labeling [121, 122]) chains from many organisms.

MbhJ

The translated protein sequences of *mbhJ-N* are given in figure 2. Unlike the other ORFs (which use ATG as the start codon) *mbhJ* is predicted to start with TTG. The gene encodes a protein with high similarity to the small subunits of the CO-induced hydrogenase from *R. rubrum*, hydrogenases-3 and -4 from *E. coli* and to the Ech hydrogenase from *M. barkeri* [119]. It is considerably shorter than most small subunits from Ni-Fe hydrogenase, but it contains the four conserved cysteine residues predicted to bind the 'proximal' Fe-S cluster [93] (figure 3).

MbhK

MbhK shows moderate homology to the N-terminal of HyfG (large subunit of hydrogenase-4 from *E. coli*) and to NADH dehydrogenase NuoC chains. The level of homology is much lower than that observed for MbhH.

MbhL

Analysis of the deduced MbhL protein sequence indicates moderate similarity to the large subunit of some Ni-Fe hydrogenases. The closest identified protein sequence is EchE, the large subunit of the Ech hydrogenase from *M. barkeri*. Alignment of the two sequences shows 41% identity and 59% similarity. The protein is more closely related to NuoD subunits of complex I of various organisms than to subunits of other Ni-Fe hydrogenases, however, all four

```
MbhJ
0     LTNNSERKRL EKRIAQLCKF IGRSPWVFHV NSGSCNGCDI EIIAALTPRY
50    DAERFGVKLV GSPRHADILL VTGPVTNQSL ERVKLVYEQT PDPKIVIAIG
100   ACPTGGSVFY ESPFTNAPLD RIIPVDVFVP GCPPRPEAIL HGVVLALEKL
150   AKMIKGEVPP EEGEENE

MbhK
0     MSKAEMVANK IKERFPNAEV VVKTNKWGRE RVWVRISREE YKELMKFIRE
50    LDPEAHYSIG IEQDWGDELG FLNHILLFYD EPPGVSLLID VHAPKDNPVL
100   PDTSDIFPIS LQFEREGMEM VGLDFEGAPD KRRLFLPDDF PEGIYPLRTD
150   EKGVPEEMVK NAGHPYLLRR EKK

MbhL
0     MKKVEYWVKI PFGPIHPGLE EPEKFIITLD GERIVNVDVK LGYNLRGVQW
50    IGMRRNYVQI MYLAERMCGI CSFSHNHTYV RAVEEMAGIE VPERAEYIRV
100   IVGELERIHS HLLNLGVVGH DIGYDTVLHL TWLARERVMD VLEAVSGNRV
150   NYSMVTIGGV RRDIGEKQKR LILDMIKYYR EVLPQIEDVF LHDSTIEARL
200   RDVAVVPKKL AIEMGAVGPT ARGSGIKEDS RWSEQLGVYP DLGIKPVTPE
250   DVTGEKARGD VYDRMAVRIG ELWMSLDLLE HALDQMPEGK IKTFPKDNIL
300   VAKLKLLGDG EGIGRYEAPR GELVHYVRGQ KGRDGPVRWK PREPTFPNLF
350   TIAKALEGNE LADLVVAIAS IDPCLSCTDR VAIVKEGKKV VLTEKDLLKL
400   SIEKTKEINP NVKGDPTPTG IGCSRGV

MbhM
0     MKIVYGVIGL ILIYIYVSVV SLLFSGIDRK LVARMQRRIG PPILQPFYDF
50    LKLMSKETII PKTANFMFKA APILMLATVI ALLAYTPLGF PPIFGTKGDI
100   IVFIYLLTLA DFFLVVGVMS SGSPYGRIGA ARGIALLVSR EPAMMLGVFA
150   VMWAISKLGV EKPFSLSSLY EHTIWDFGPV AWVAGVVLIY VFMAWLASEI
200   EVGFFNIPEA EQEIAEGTLV EYSGRYLGII KLAESIKEFI AASLVVAVLF
250   PWQLNIPGVQ GYLINLLLHT LKVFIVLLVS KTIFRTITGR LKISQAVNLL
300   WTRVFTASVI GALLLAIGVM L

MbhN
0     MIRLPLLPTV IKNLFKKPAT NPFPKTEPVP VPEDFRGKLV YNVDKCVGCR
50    MCVTVCPAGV FVYLPEIRKV TLWIGRCVMC KQCVDVCPTA ALQMSDEFLL
100   ASYDKYDAKL IYLTPEEAED IKKKLEEANK AKAEKQASK
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Figure 2 Amino acid sequences of the Mbh JKLMN subunits. N-termini identified by Edman degradation in MbhK and MbhL are underlined; predicted membrane-spanning helices in MbhM are boxed; charged residues in MbhM that are conserved in other membrane-bound hydrogenases and complex I proteins are shown in bold (italic denotes conservation only within hydrogenases); metal-coordinating cysteine residues are shaded; the highly conserved [4Fe-4S] cluster binding-motifs in MbhN are underlined.

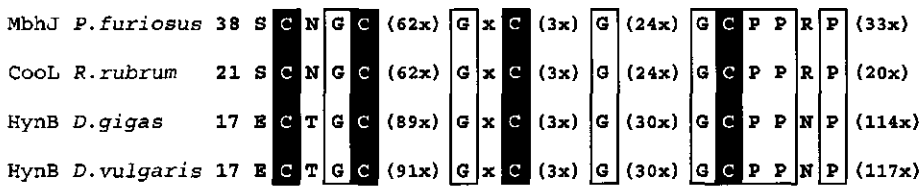

Figure 3 Sequence comparisons of iron-sulfur cluster binding motifs in *P. furiosus* membrane-bound hydrogenase small subunit. The putative [4Fe-4S] cluster motif of *P. furiosus* MbhJ and of *Rhodospirillum rubrum* CooL [115] are compared with a similar $(Cys)_4$-containing motif in typical Ni-Fe hydrogenase small subunits *viz* from *Desulfovibrio gigas* [125] and from *Desulfovibrio vulgaris* Miyazaki F [126].

cysteines involved in the coordination of the active site Ni-Fe cluster are present.

MbhM

MbhM shows high homology to CooK from the CO-induced hydrogenase from *R. rubrum*, HycD and HyfC from *E. coli* hydrogenase-3 and -4, respectively, and to the NADH dehydrogenase NuoH subunit from several organisms, which have been proposed to be the site of proton translocation and energy coupling [123, 124]. The high number of strictly conserved charged residues present in these proteins (figure 2) argues in favor of such a role.

MbhN

This subunit is similar to CooX from the CO-induced hydrogenase from *R. rubrum* and to the complex I NuoI subunit from several organisms. The sequence comparisons show two conserved, canonical, ferredoxin-like CxxCxxCxxxCP motifs, strongly suggesting the presence of two regular [4Fe-4S] cubane clusters in this subunit. A T-rich region (possibly a transcription termination signal) is present 6 bp downstream from the stop codon of this subunit.

An additional lone open-reading frame is present 121 bp downstream from the stop codon of *mbhN*. The C-terminal of this ORF shows 43% similarity (22% identity) to 3-phosphoadenosine-5-phosphosulfate (PAPS) sulfotransferase from *Synechocystis* sp. The key KXECG(I/L)H conserved amino-acid cluster involved in the catalysis [127] is, however, absent. It is unlikely that this ORF belongs to the membrane-bound hydrogenase gene, since all other subunits are consecutive or even overlapping. The absence of a ribosome-binding site in the

sequence just upstream of the starting codon and the possible transcription termination signal after *mbhN* give credence to this hypothesis.
Assuming 1:1 stoichiometry of all 14 subunits, the assembled complex is expected to have a molecular mass of 297 kDa and to contain three [4Fe-4S] clusters and one dinuclear [Ni-Fe] cluster.

N-terminal sequencing

The N-terminal sequences of two subunits of the partially purified membrane-bound hydrogenase were determined after they were separated by SDS-polyacrylamide electrophoresis. These are as follows (where X is an unknown residue): MKKVEYWVKIPFGPIHPGLEEPEKFIITLD- and KAEMVANKIXERFPNAEVVVGDN-. The first sequence corresponds exactly to the N-terminal of the MbhL subunit, the Ni-Fe-cluster containing 'large' subunit. The second sequence is almost identical (two misidentified amino acids) to the N-terminus of MbhK (figure 2). Several other subunits were apparent in the gel. Some of those were too close to be excised without contamination from neighboring bands. The N-terminal sequencing of four of these proteins showed sequences that are not present in the proposed membrane-bound hydrogenase operon. A search of the available genome using these N-terminal as probes, allowed confirmation of their origin as contaminating *P. furiosus* proteins. One is a putative leucine-responsive element, and the other three have no homology with any protein with an established function.

Catalytic properties

Dihydrogen formation was assayed with dithionite-reduced methyl-viologen as an electron donor. This activity was found to be insensitive to a three-hour oxygen exposure of the sample and to have a pH optimum around 6.8. The characteristics of the membrane-bound hydrogenase have proven to be quite interesting. Even though the enzyme appears to be a Ni-Fe hydrogenase based on its large-subunit sequence, its properties differ significantly from those of most other Ni-Fe hydrogenases. The ratio of dihydrogen evolution activity to dihydrogen uptake activity, in crude extracts, as well as in the partially purified enzyme, is very large compared to that of other enzymes. Most Ni-Fe hydrogenases favor dihydrogen uptake over evolution and exhibit a dihydrogen evolution / dihydrogen uptake ratio of much less than unity. The 250:1 ratio of evolution to uptake for the *P. furiosus* enzyme (measured at

pH 7.4) is one order of magnitude larger than the one observed for the membrane-bound hydrogenase from *Hydrogenovibrio marinus*, the most extreme ratio observed so far [128]. Dihydrogen uptake activity does not show a significant increase at physiological pH when using benzyl viologen instead of methyl viologen as electron acceptor.

The isolated core complex was able to evolve dihydrogen from reduced *P. furiosus* ferredoxin (figure 4). Dihydrogen evolution did not occur if POR, ferredoxin, or purified hydrogenase were absent. Under the conditions used the activity increased linearly with the amount of ferredoxin.

CO inhibition

Most hydrogenases are extremely sensitive to inhibition by CO. Dihydrogen evolution by the Mbh hydrogenase is, however, only slightly inhibited by CO. The inhibition curve shows approximately 40% inhibition at 100% CO (≈ 860 μM), whereas, for example, the *D. gigas* enzyme is 50% inhibited with only 35 μM CO. This membrane-bound hydrogenase thus joins the soluble sulfhydrogenase from *P. furiosus* [97], the CO-induced hydrogenase from *R. rubrum* [129] and the soluble hydrogenase from *Alcaligenes eutrophus* [130] in the restricted group of CO-tolerant hydrogenases. A possible physiological relevance of this is not evident, since no reports on the metabolism of

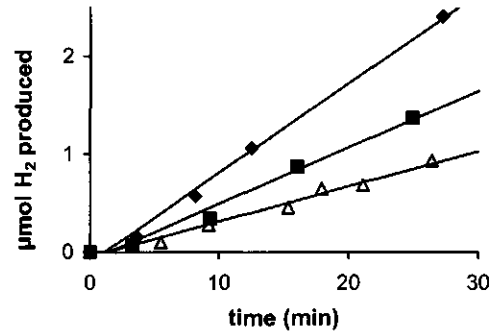

Figure 4 Dihydrogen evolution from ferredoxin-reduced Mbh-core complex. (Δ) 7 nmol Fd; (■) 10 nmol FD; (◆) 20 nmol Fd. Ferredoxin was reduced *in situ* by the POR system (10 mM pyruvate, 1 mM $MgCl_2$, 0.9 mM CoA, 0.9 mM dithiothreitol, 60 μg POR), and dihydrogen evolution was measured by gas chromatography. The partially purified hydrogenase complex was 170 μg protein/ml.

P. furiosus mention a role of CO. On the other hand, since dihydrogen and CO are predicted to bind at the same site, it is possible that the low affinity for CO is simply a reflection of the low affinity of the membrane-bound hydrogenase for dihydrogen.

Inhibition by *N,N'*-dicyclohexylcarbodiimide

N,N'-dicyclohexylcarbodiimide, DCCD, is a covalently carboxyl-modifying reagent that attacks specifically acidic amino acids in hydrophobic domains [131]. It has been shown to inhibit the methyl viologen-dependent dihydrogen evolution by the CO-induced hydrogenase from *R. rubrum* [129]. The similarity of this hydrogenase to the membrane-bound hydrogenase from *P. furiosus* suggested that the later might also be inhibited by DCCD. Indeed, upon pre-incubation of the protein at room temperature in the presence of DCCD a significant decrease of the activity was observed. Moreover, incubation of the protein in the presence of DCCD at 80°C leads to a much faster and more drastic inactivation (figure 5), possibly due to increased accessibility of the relevant amino acids as a result of increased backbone flexibility. The likely target for inhibition by DCCD is MbhM, which is similar to the *R. rubrum* CooK

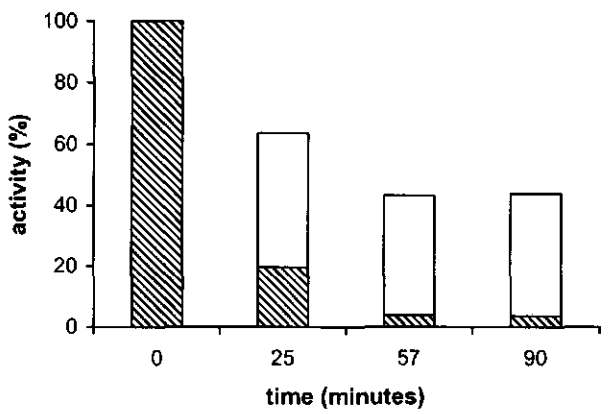

Figure 5 Effect of *N,N'*-dicyclohexylcarbodiimide on the dihydrogen evolution activity in *P. furiosus* membranes. Shaded: membranes incubated with 1.25 mM *N,N'*-dicyclohexylcarbodiimide (10 µl of a 50 mg/ml solution in ethanol) at 80°C; White: membranes incubated with ethanol (10 µl) at 80°C.

and to the subunit-1(bovine)/NuoH(E. coli) family of NADH dehydrogenase (complex I). Proteins of this family are probably involved in proton translocation and energy coupling [123, 124], and have been shown to be inhibited by DCCD. It is likely that MbhM is also involved in similar processes. The membrane-bound complex is quite thermostable (with a half-life at 80°C of approximately 3 hours) which rules out the possibility that the faster decrease in activity upon incubation with DCCD at 80°C is due to protein denaturation. The isolated core complex is significantly less thermostable, with a half life at 80°C of only 35 min, which suggests that some of the subunits lost during the purification may have a role in protecting the protein against denaturation. The isolated core complex is also susceptible to inhibition by DCCD.

EPR spectroscopy

From +200 mV to -250 mV (vs. standard hydrogen electrode, SHE) no signals were detected in the Mbh hydrogenase by EPR spectroscopy. Upon reduction of the protein to potentials below -300 mV (vs. SHE) redox equilibrium proved impossible to attain. This is probably due to H+ reduction by the hydrogenase, as observed with other hydrogenases [113]. At these potentials, a weak signal characteristic for two interacting [4Fe-4S] clusters [132] was observed. Reduction of the hydrogenase with excess dithionite at room temperature in the absence of redox mediators allowed the observation of this signal with a significantly higher intensity (figure 6). The higher stability of the signal under these conditions is probably due to the low dihydrogen production activity from dithionite in the absence of redox mediators. No additional signals were detected upon reduction of the protein with deazaflavin and light. The signal disappeared upon anaerobic incubation of the protein at 60°C, which is consistent with the clusters putative role in relaying electrons to the active site in order to reduce protons to dihydrogen. In spite of the inability to obtain a fully reduced sample in the redox titration, comparison of the results obtained with a dithionite-reduced sample allowed the estimation of a potential of approximately -0.33 V for the reduction of the two interacting clusters, i.e. within the usual potential range for these centers.

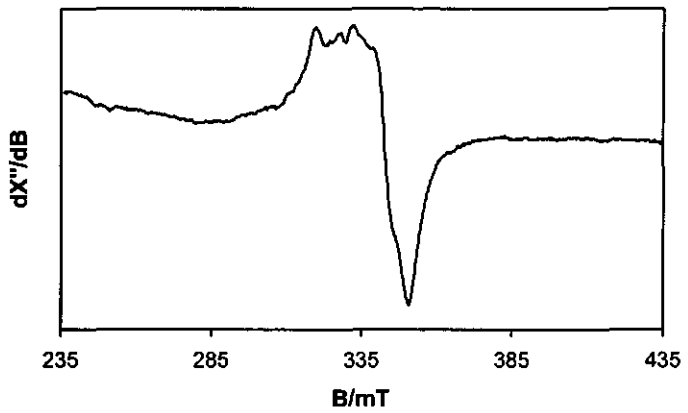

Figure 6 EPR spectrum of dithionite-reduced Mbh hydrogenase core complex from *P. furiosus*. The sample contained 34 mg/ml hydrogenase in 20 mM Tris/HCl, pH 8.0. The conditions of the measurement were: microwave frequency, 9.22 GHz; microwave power, 50 mW; temperature, 19 K.

A putative fourth hydrogenase in *P. furiosus*

Using the MbhL amino-acid sequence as a probe, a fourth hydrogenase-containing operon was found in the genome of *P. furiosus* about 6 kb downstream from the *mbhABCDEFGHIJKLMN* operon (see figure 1). Like the membrane-bound hydrogenase, this complex is expected to coordinate three [4Fe-4S] clusters. Almost every subunit in this operon is more similar to the corresponding subunit in the *mbh* operon than to any other protein (except homologs in other *Pyrococcus* species).

However, in MbxL (the homolog to the hydrogenase large subunit) the Ni-Fe binding motif deviates from the commonly observed consensus (see figure 7): in each of the two Ni-Fe coordinating CxxC motifs the second cysteine (which normally provides the ligands bridging between the nickel and the iron ions) is replaced by an acidic residue. Additional proline residues are also present in the motifs. These residues may have a role in distorting the polypeptide chain in order to allow metal coordination by the carboxylato residues. The putative carboxylato-bridged Ni-Fe cluster is not unique to *P. furiosus*. Genes coding for similar binding motifs are also present in the genomes of *P. abyssi, P. horikoshii,* and *T. maritima* (figure 7).

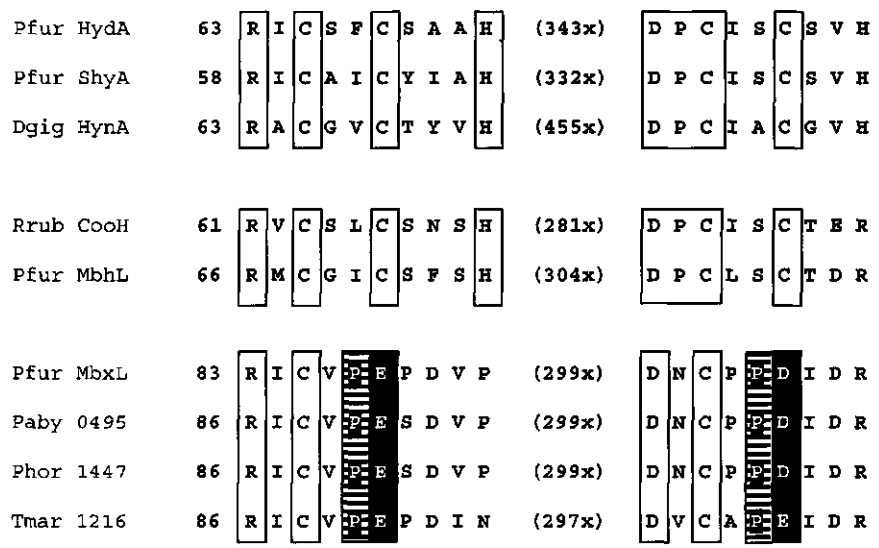

Figure 7 Sequence comparisons of the Ni-Fe cluster binding motifs in *P. furiosus* membrane-bound hydrogenase large subunit MbhL and in MbxL with the well-characterized Ni-Fe binding motifs in Ni-Fe hydrogenase large subunits from *Rhodospirillum rubrum* [129], *Desulfovibrio gigas* [93, 125], and *P. furiosus* sulfhydrogenases I [100, 114] and II [98], and with genes in *P. abyssi* (PAB0495), *P. horikoshii* (PH1447), and *Thermotoga maritima* (TM1216).

Discussion

The genome of the archaeal *P. furiosus* has four different and separate open reading frames with significant homology to the large subunit of well characterized bacterial Ni-Fe-hydrogenases: *hydA* [114], *hydP/shyA* [98, 102], *mbhL*, and *mbxL*. The first two each encode a Ni-Fe-cluster carrying α-subunit of a αβγδ-heterotetrameric soluble sulfhydrogenase, i.e. a complex iron-sulfur flavoprotein with both sulfur reductase and hydrogenase activity [98, 114]. The expression product of the third ORF, *mbhL*, is presumably part of a very different complex, namely a large, multi-subunit transmembrane system, which we hypothesize to act as an energy transducting system coupled to proton-based respiration. This hypothesis is presently based on the following still limited information: the essentially unidirectional nature of the hydrogenase as a 'dihydrogen synthetase', the inhibition of this activity by

DCCD, the predicted extensive membrane spanning of the product of the *mbhA-N* operon, and the homology of individual subunits, in particular MbhM, to subunits of the proton-translocating NADH:quinone oxidoreductase complex.

Several multi-subunit membrane-bound hydrogenases with high similarity to NADH:quinone oxidoreductase have been proposed previously to be involved in energy transduction under certain growth conditions. Proton translocation has been suggested to occur through the CO-oxidizing, dihydrogen producing complex of *R. rubrum* when grown CO-dependent in the dark [129]. The dihydrogen evolution activity was also shown to be inhibited by DCCD [115]. Similarly, the Ech hydrogenase of *M. barkeri* has been proposed to function as a proton pump when producing dihydrogen during acetate-dependent growth [119]. Two gene groups in the genome of *M. thermoautotrophicum* each encoding a putative multi-subunit membrane-bound hydrogenase have been suggestively named *eha* and *ehb* for energy converting hydrogenase A and B [120]. Energy converting hydrogenase B has been suggested to be involved in reverse electron flow, and several of its subunits show homology to Mbx subunits ABDFGHJLMN and to MbhAEFGJL. Eha homology is, however, limited to the JL subunits of both hydrogenases, and to MbxN (which encodes a 2 [4Fe-4S] ferredoxin).

The product of the fourth ORF, *mbxL*, has not been characterized yet beyond the level of genomic inspection. ORF *mbxL* is part of a putative large operon *mbxABCDFGHH'MJKLN*, which is a 'mirror' of the *mbhA-N* operon, therefore, it putatively codes for another energy transducing membrane complex.

The MbhL-containing complex was isolated from the membranes and partially purified under mild conditions in an attempt to maintain integrity with a view to future studies on its bioenergetics. The exact subunit composition and stoichiometry of the complex is not known. However, it presumably contains at least the MbhJKLMN core: the N-terminal sequence of MbhK and MbhL were identified on extracted subunits after SDS-PAGE. The two [4Fe-4S] cubanes of MbhN were putatively identified in EPR spectroscopy. The MbhJ subunit with the 'proximal' iron sulfur cluster for electron transfer to the Ni-Fe-cluster should be present, because the complex catalyses dihydrogen production. At least one membrane-spanning subunit is expected to be present because detergent is required to keep the complex in solution; the MbhM subunit is a reasonable candidate, because it would - by analogy with NADH dehydrogenase - confer DCCD sensitivity to the complex.

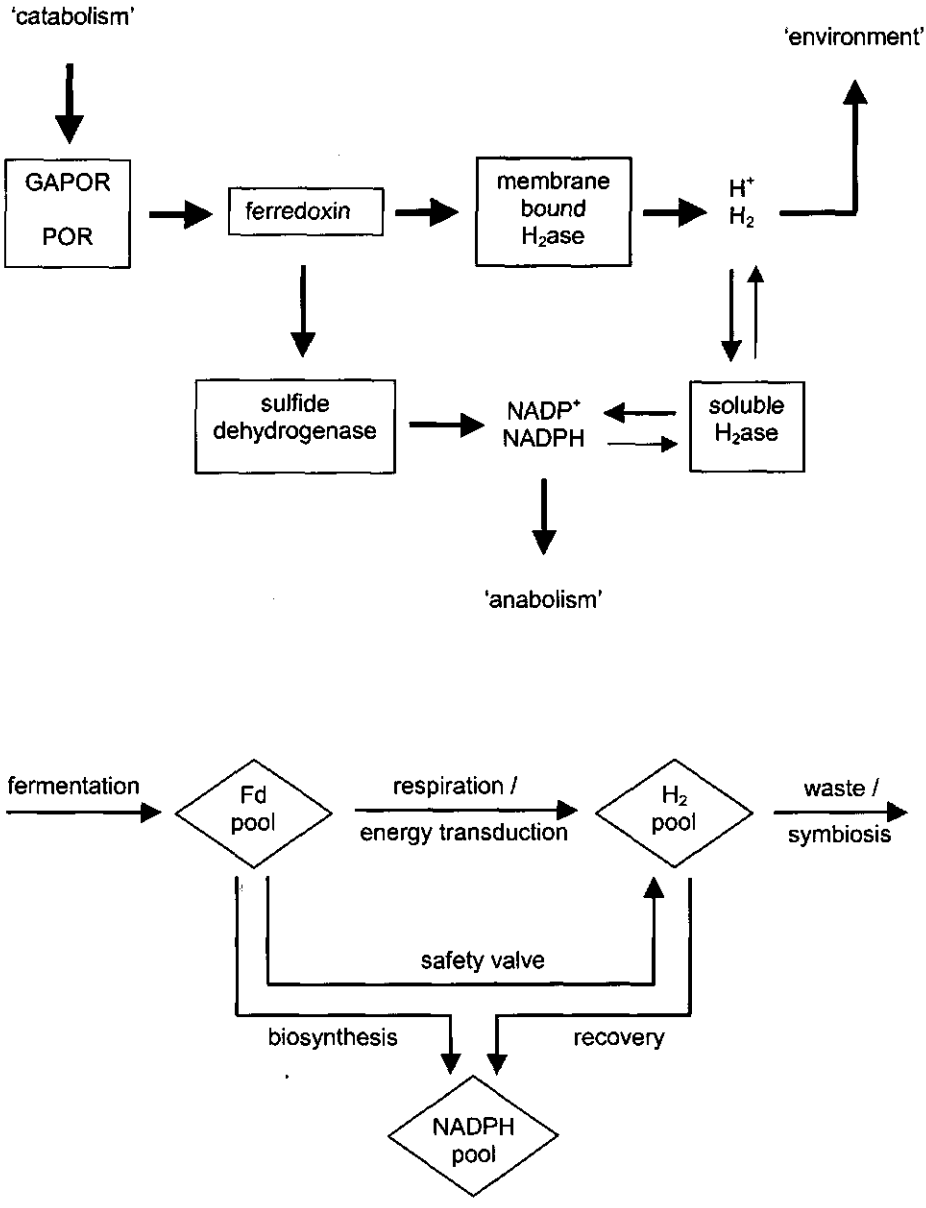

Figure 8 A working hypothesis of dihydrogen metabolism in *P. furiosus* formulated in terms of electron-transfer steps (upper) or as a scheme of energy devices (lower).

Isolation of a different core complex of membrane-bound *P. furiosus* hydrogenase has recently been described by Sapra *et al.* [105]. Extraction from the membrane after repeated washing with up to 4 M NaCl and chromatography in the presence of 2 M urea (to prevent loss of activity by aggregation) resulted in a complex that has lost the ability to evolve dihydrogen using reduced ferredoxin as electron donor. The purified isolate was claimed to be a stoichiometric complex of MbhK and the Ni-Fe-cluster binding MbhL. However, this appears to be incompatible with the observation by the same authors of an EPR signal characteristic for iron-sulfur cluster(s). Thus, the composition and functionality of this complex is not clear.

A soluble electron-transfer chain in *P. furiosus* has been proposed by Ma *et al.* [101] for the generation of molecular dihydrogen from reducing equivalents derived from fermentation: pyruvate oxidoreductase, POR, reduces ferredoxin, Fd, which reduces, sulfide dehydrogenase, SuDH, which reduces $NADP^+$, which diffuses to (sulf)hydrogenase I to produce dihydrogen. The kinetic data presented in the present paper argue against this hypothesis: the overall activity of the soluble chain is too low to sustain fermentation of glucose to acetate. As an alternative hypothesis we propose proton respiration coupled to energy transduction in a transmembrane 'dihydrogen synthetase' complex as outlined in figure 8. The originally proposed route through SuDH and hydrogenase I could then function as a safety valve for the disposal of excess reducing equivalents as dihydrogen, an option that would not be available via the tightly coupled energy transduction system. Furthermore, the SuDH branch can produce NADPH for biosynthetic purposes. Additionally, the soluble hydrogenase can save energy by also producing NADPH from the 'waste' product of respiration, dihydrogen. In figure 8 this working hypothesis has been formulated in two formats, one in terms of electron transfer between proteins/enzymes and an equivalent one in terms of downstream processes coupled through pools of redox buffers.

ACKNOWLEDGEMENTS

This work was supported by the Fundação para a Ciência e Tecnologia (Lisbon, Portugal) through a PRAXIS XXI Ph.D. grant (BD/9557/96) to PJS, by the Ministry of Economic Affairs of the Netherlands within the framework of the Innovation Oriented Research Program (IOP) Catalysis (IKA96001) to ECDvdB, and by grants from the National Science Foundation, NATO, and the Wallenburg Foundation (to FTR).

5

NADPH production and regeneration using *Pyrococcus furiosus* hydrogenase

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Colja Laane and Huub Haaker

Submitted for publication (2001)

Hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* catalyzed the reduction of pyridine nucleotides by dihydrogen. It will be shown that crude hydrogenase preparations can be applied as an efficient and stable NADPH regenerating system. Hydrogenase was used together with *P. furiosus* glutamate dehydrogenase to reduce high concentrations of 2-ketoglutarate to glutamate. Next to that, hydrogenase was used together with *Thermoanaerobium brockii* alcohol dehydrogenase to reduce 2-pentanone and butyraldehyde to 2-pentanol and 1-butanol, respectively. Since hydrogenase was not inhibited by high concentrations of $NADP^+$ or NADPH (110 mM), it was also used for NADPH production: 110 mM $NADP^+$ (100 mg/ml) was reduced in 4 hours by 2.6 units hydrogenase/ml. Hydrogenase was applied in the temperature range between 20 and 50°C. Higher temperatures are possible because hydrogenase is stable at 80°C, but due to the instability of pyridine nucleotides the maximum temperature in our experiments was 50°C. The enzyme is still very active at 50°C and relatively high total turnover numbers (320 - 500) were obtained.

INTRODUCTION

Several methods have been studied for the production and regeneration of pyridine nucleotides. These include electrochemical methods [133, 134], light-driven (bio)chemical systems [133, 134], chemical reactions [133, 134], and a range of enzymatic methods [133, 135, 136]. In all cases the motive is to reduce the cost of the expensive cofactor NADPH in biosynthetic applications.
In the last decade a number of efficient NAD(P)H regenerating systems have been developed. These include protein-engineered formate dehydrogenase [137], glucose dehydrogenase from *Gluconobacter scleroides* [138], and alcohol dehydrogenase from *Thermoanaerobium brockii* [133, 139, 140]. In addition hydrogenases are being employed. These enzymes use dihydrogen as reductant, which is inexpensive, has a high reducing power, and leaves no harmful byproducts after reaction [141-143]. However, the hydrogenases applied so far suffered from a low operational stability, mainly due to the fact that they were oxygen sensitive [141, 143]. Furthermore, some require redox dyes [143, 144] and are only selective for NADH [141, 145, 146].
It will be shown that a crude preparation of *P. furiosus* can be used in the preparative production of reduced pyridine nucleotides and for NADPH regeneration in several coupled systems.

MATERIALS AND METHODS

Chemicals and enzymes

Dithionite, methyl viologen, 2-pentanol, 2-pentanon, 1-butanol, butyraldehyde, 1-propanol, and 1-hexanol were obtained from Sigma. DNase, RNase, glutamate, 2-ketoglutarate, $NADP^+$, NADPH, NADH, NAD^+, AMP, and nicotinamide were obtained from Boehringer Mannheim.
Purified *Pyrococcus furiosus* glutamate dehydrogenase (GDH) was a kind gift of Dr. J. Lebbink, Laboratory of Microbiology, Wageningen University. *Thermoanaerobium brockii* alcohol dehydrogenase (ADH) was obtained from Sigma. One unit of enzyme activity is defined as the amount of enzyme that produces one μmol of product per minute. Specific activities were as described for GDH at 25°C [147] and *T. brockii* ADH at 40°C [148].

Growth of *Pyrococcus furiosus*

P. furiosus was grown in a 200 l reactor in a standard medium [83] with starch as carbon source. Cells were harvested by continuous centrifugation (Sharples) and stored at -80°C until used. About 200 - 300 g cell paste (wet weight) was obtained after 1 night.

Fractionation of hydrogenase

Ion exchange chromatography

P. furiosus cells were disrupted by autolysis: about 100 g cell paste was suspended in 500 ml 50 mM Tris/HCl pH 8.0 containing 10 μg/ml DNase and RNase and was stirred under argon. After 5 hours at room temperature the mixture was centrifuged for one hour at 30,000 *g* to remove whole cells and cell debris. The supernatant was used to obtain two different preparations. One part was dialyzed for 16 hours against 50 mM Tris/HCl, pH 8.0 and concentrated on an Amicon YM10 filter to about 30 mg/ml (cell free extract, CFE). The preparation was stored in liquid nitrogen.

The supernatant obtained after cell lysis was also loaded on a Source 15Q column (Pharmacia) under anaerobic conditions and eluted with a gradient from 0 to 500 mM NaCl in 50 mM Tris/HCl pH 8.0.

All fractions were analyzed for dihydrogen:methyl viologen oxidoreductase activity. The ones having high activities (eluted at 200 - 300 mM NaCl) were concentrated anaerobically using an Amicon YM10 filter and were stored in liquid nitrogen (SQ fraction).

60% Ammonium sulfate precipitation

P. furiosus cells were disrupted and centrifuged as described above. The supernatant was treated with 10% ammonium sulfate. This was centrifuged for 30 min and the supernatant was saturated with 60% ammonium sulfate. After centrifugation for another 30 min the precipitate was dissolved in 50 mM Tris/HCl pH 8.0 and dialyzed for 16 hours against the same buffer. The protein preparation was stored in liquid nitrogen. This fraction was called 60% fraction.

Assays and analytical procedures

Dihydrogen:NADP$^+$ oxidoreductase activity

A cuvette was filled with 50 mM EPPS pH 8.0 containing 0.4 mM NADP$^+$, made anaerobic and saturated with dihydrogen. The cuvette was incubated at 40°C and after 5 min 20 μM dithionite (to remove the last traces of oxygen) and

activated hydrogenase were added (total volume 1 ml). The extinction was measured at 340 nm. One unit of enzyme activity is the amount of protein needed to produce one μmol NADPH per minute.

Dihydrogen:methyl viologen oxidoreductase activity

Hydrogenase activity was also measured via the reduction of methyl viologen. A 1.5 ml vessel containing 50 mM EPPS pH 8.0 with 1 mM methyl viologen was made anaerobic and saturated with dihydrogen. The vessel was incubated at 40°C and after 5 min 20 μM dithionite was added. When the solution colored slightly blue (due to partial reduction of methyl viologen) anaerobicity was obtained and activated hydrogenase was added (total volume 1 ml). Absorption was measured at 600 nm and an extinction coefficient of 13,600 $M^{-1}.cm^{-1}$ for methyl viologen semiquinone was used [149]. One unit of enzyme activity is the amount of protein needed to produce two μmol methyl viologen (semiquinone) per minute.

Dithionite:H^+ oxidoreductase activity

Dihydrogen evolution was measured in 100 mM EPPS pH 8.0 and 1 mM methyl viologen. The incubation was flushed with argon and incubated at 40°C. After 5 min 10 mM dithionite and hydrogenase were added (total volume 1 ml). The dihydrogen evolution was detected by GC analysis. One unit is the amount of protein needed to produce one μmol dihydrogen per minute.

Phosphatase activity

The incubation mixture contained 50 mM EPPS pH 8.0, 0.5 mM NADPH, and protein. The total volume was 1.5 ml and the bottles were kept at 40°C under argon. At different time intervals samples (200 μl) were taken and analyzed by HPLC for NADPH, $NADP^+$, NADH, and NAD^+.

Stability experiments

To test stability of hydrogenase preparations, samples were incubated with several materials. 2.8 units of hydrogenase/ml (dihydrogen:$NADP^+$ oxidoreductase activity) were incubated in 50 mM EPPS pH 8.0 with stainless steel, silicon or teflon tubing, and three types of membranes: Centricon YM10, Microcon YM3, and Amicon YM10. The amount of membranes added was adjusted to obtain the same surface areas for all types in the test system (6.3 cm^2/ml). The total volume was 1 ml and the bottles were shaken at 100 rpm at 40°C. Samples were taken and assayed for dihydrogenase:methyl viologen oxidoreductase activity.

Stability of hydrogenase was also tested in an ultrafiltration membrane reactor. In these experiments a peek filtration unit [150], a flow-switching valve

(right angle, Upchurch Scientific), and a circulation pump (P-500, Pharmacia) were used. All tubing was of stainless steel or peek. The filtration unit with a total internal volume of 3 ml was stirred with a magnetic stirrer at 300 rpm. The membrane, an Amicon YM10 filter, was located above the liquid. The circulation pump had an internal volume of 25 ml.

The filtration unit was kept at 23°C and a supply vessel was kept at 40°C. The supply vessel was continuously flushed with water-saturated dihydrogen. To achieve anaerobicity in the system, it was flushed for one day with 500 ml 100 mM Tris/HCl pH 8.0. Subsequently, the system was flushed in the following order: 200 ml 100 mM Tris/HCl pH 8.0 containing 0.1 mM dithionite to scavenge traces of oxygen, 200 ml 100 mM Tris/HCl pH 8.0 to remove dithionite, 200 ml 100 mM Tris/HCl pH 8.0 containing 5 mg/ml BSA to saturate the system with protein, and 200 ml 100 mM Tris/HCl pH 8.0 to remove non-absorbed protein. The final solution was saturated with dihydrogen and 56 units hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity) in 100 ml buffer were added. The flow rate was kept at 0.5 ml/min. The liquid was concentrated twofold and subsequently diluted to the volume at start of the experiment. Samples were taken to determine dihydrogen:methyl viologen oxidoreductase activity and protein content.

NADPH production

NADPH production was performed in 1M EPPS buffer pH 8.0. The total volume was 3 ml. When the system was made anaerobic and flushed with dihydrogen the last traces of oxygen were removed by the addition of 20 μM dithionite followed by the hydrogenase preparation. All experiments were performed at 40°C. The NADPH and $NADP^+$ concentrations were determined by HPLC.

Regeneration experiments

The reaction mixture contained 50 mM Tris/HCl buffer, pH 7.5 for experiments with GDH and pH 8.0 for experiments with ADH. The reaction mixture was put in a 100 ml capped bottle. Subsequently, the bottles were made anaerobic and filled with dihydrogen followed by hydrogenase, substrates, and an NADPH using enzyme (total volume 1 ml). The GDH experiments were performed with 50 mM 2-ketoglutarate, 50 mM ammonia, 0.2 units GDH (25°C), 2.8 units hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity), and $NADP^+$ as indicated in table 2. Hydrogenase was not activated prior to addition.

The ADH experiments were performed with 0.2 mM $NADP^+$, 2 units ADH, 100 mM butyraldehyde, and varying amounts of hydrogenase. Hydrogenase was activated prior to the addition. The total turnover number is the total number of moles product formed per mole $NADP^+$ initially added (-), the turnover number is the total turnover number per unit time (hr^{-1}).

Activation of hydrogenase

To measure the dihydrogen oxidation activity ($NADP^+$ or methyl viologen reduction) the hydrogenase preparations needed to be activated [151]. This was done by an incubation of the sample for 5 min at 80°C under dihydrogen. Activation was not necessary when the dihydrogen evolution activity was measured.

Determination of protein content

The protein content was determined via the microbiuret method with bovine serum albumin (BSA) fraction V as standard [80].

GC analysis

Dihydrogen was analyzed on a Varian 3400 gas chromatograph equipped with a thermal conductivity detector. Argon was used as carrier gas. Dihydrogen was measured on a molesieve 5A 45/50 column (1.5 m x 1/4" SS, Chrompack).

2-Pentanol, 2-pentanon, 1-butanol, and butyraldehyde were analyzed by GC equipped with a flame ionization detector and N_2 was used as carrier gas. Samples were analyzed on a packed Cromosorb WPH 100-120 mesh 10%CP-Sil 58 column (2 m x 1/8" x 2 mm, Chrompack). Samples for GC analysis were extracted with di-ethylether (1:1) containing 1-hexanol (for 1-butanol and butyraldehyde) or 1-propanol (for 2-pentanon and 2-pentanol) as internal standards.

HPLC analysis

NADPH, $NADP^+$, NADH, NAD^+, AMP, and nicotinamide were analyzed by HPLC using a Nucleosil 100 C18 5U column (Alltech), 10 mM phosphate buffer pH 7.0 was used as eluent. All samples (50 µl) were diluted in 450 µl 10 mM NaOH and filtered with an Amicon YM10 filter to separate protein from the nucleotide mixture.

Glutamate and 2-ketoglutarate were analyzed by HPLC on an ion-exchange column (Amino acid resin BP-AN6, Benson). They were detected at 570 nm after reaction with ninhydrin. Nor-leucine was used as internal standard.

RESULTS AND DISCUSSION

Hydrogenase preparation

P. furiosus contains two soluble hydrogenases and both are able to reduce $NADP^+$ by dihydrogen oxidation [98]. To use these hydrogenases for NADPH production and regeneration active preparations are preferred. To increase the activity of a preparation concentration and/or purification can be applied. But, the concentration and purification methods need to be simple (1 or 2 steps) and easily applicable on a large scale (industrial processes). Three different hydrogenase preparations were prepared and tested: concentrated cell free extract, fractions obtained after ion exchange chromatography, and a preparation obtained by a selective ammonium sulfate precipitation procedure. The latter method proved to be a simple method for partial purification and concentration of hydrogenase but seems less suitable for industrial applications. A purification step using a column seems more practical.

The three preparations were tested for dihydrogen:methyl viologen oxidoreductase activity and dihydrogen:$NADP^+$ oxidoreductase activity. Since we are not dealing with purified proteins, it is important to test the phosphatase and phosphodiesterase activity. These enzyme activities degrade NADPH or $NADP^+$ to NADH, NAD^+, AMP, and nicotinamide.

As is shown in table 1, the concentration step applied to obtain CFE resulted in a loss of only 5 % of the number of units. The fractionation with the Source 15Q

Table 1 Fractionation of *P. furiosus* cell extract. Fractionation started in all cases with 4860 units (dihydrogen:$NADP^+$ oxidoreductase activity). Hydrogenase and phosphatase activities were determined at 40°C.

Fraction	Units	Specific activity (µmol/min.mg)		
	(µmol/min)	Hydrogenase		Phosphatase
	NADPH #	MV ¶	NADPH #	
CFE §	4631	0.84	0.37	1.6 E-4
SQ †	1863	1.81	1.55	1.1 E-4
60% ‡	3018	2.15	0.69	8.9 E-5

\# NADPH = Dihydrogen:$NADP^+$ oxidoreductase activity
¶ MV = Dihydrogen:methyl viologen oxidoreductase activity
§ CFE = cell free extract
† SQ = fraction obtained after Source 15Q column
‡ 60% = fraction obtained after 60% ammonium sulfate precipitation

column resulted in higher specific activities for hydrogenase, measured with the dihydrogen:$NADP^+$ oxidoreductase assay, than the fraction obtained with 60% ammonium sulfate precipitation. On the other hand, the number of units of the SQ fraction was much lower than of the 60% fraction. This might be explained by 1) the type of fractionation method used: ion-exchange chromatography is a more specific fractionation method and, therefore, it results in higher specific activities, and 2) the types of soluble hydrogenases present. Both hydrogenases are present in the CFE and are not separated by the applied ammonium sulfate precipitation. This is concluded from the observation that the supernatant of the 60% ammonium sulfate precipitation does not show any hydrogenase activity (data not shown). However, the two hydrogenases are separated by ion-exchange chromatography. We used the fraction with the highest activity. The fraction that eluted at a higher salt concentration contained the less active hydrogenase II [98] and this fraction was discharged.

The different ratios between the dihydrogen:methyl viologen oxidoreductase and dihydrogen:$NADP^+$ oxidoreductase activities (for the three fractions shown in table 1) can be explained by the differences in specific activities for both substrates of hydrogenase I and hydrogenase II [98]. Hydrogenase II is relatively more active with methyl viologen compared to its activity with $NADP^+$ than hydrogenase I. When hydrogenase II is removed, the $NADP^+$ reduction activity will increase relative to the methyl viologen reduction activity.

The three fractions did not have measurable phosphodiesterase activities. Phosphatase activity was present in all fractions, but it was very low compared to the hydrogenase activity. It is of great importance to use Mg^{2+}-free incubation mixtures for NADPH production, since phosphatase activity increased about ten times in the presence of Mg^{2+} (not shown).

In principle all three fractions yield hydrogenase preparations which can be used in practice.

NADPH production

The conversion of 12 and 110 mM $NADP^+$ to NADPH is depicted in figure 1. It is clear from the data that there was no effect of the increasing concentration of NADPH on the activity of hydrogenase. After four hours reaction with 2.6 units/ml (dihydrogen:$NADP^+$ oxidoreductase activity) 110 μmol/ml (100 mg/ml) was converted. This is about 17% of the maximal rate. In the used

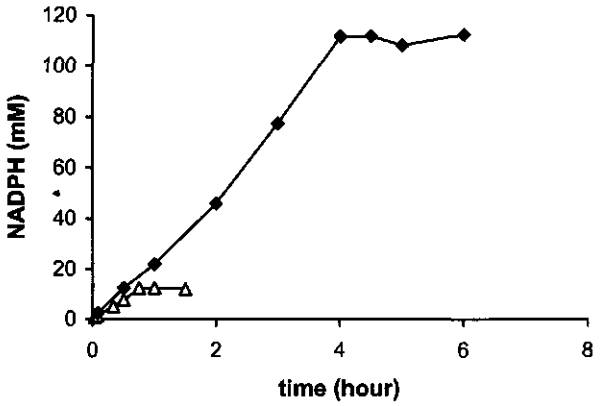


Figure 1 Conversion of $NADP^+$ by 2.6 units/ml *Pyrococcus furiosus* hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity) at 40°C. -Δ- 12 mM $NADP^+$; -◆- 110 mM $NADP^+$.

system, the gas exchange between medium and gas phase was not optimized and probably limited the activity.

The yield with our enzyme preparation is higher than reported for *Gluconobacter suboxydans* glucose dehydrogenase or *Achromobacter parvulus* malate dehydrogenase that produced 73 mg NADPH/ml [152] and 10 mg NADPH/ml [153], the conversion ratio was about 100% for both. However, it is difficult to compare these methods with ours, since all reaction conditions were different with respect to temperature, pH and the number of units used.

A very important advantage of hydrogenase is the reducing power of dihydrogen. It can be calculated that the midpoint potentials of the redox couples H_2/H^+ and $NADPH/NADP^+$ are -497 and -361 mV at 40°C and pH 8.0 [154] respectively, resulting in $\Delta G^{\circ\prime}$ = -26.24 kJ/mol for the reduction of $NADP^+$ by dihydrogen. The corresponding K_{eq}' = 23800 (1 atm dihydrogen, pH 8.0, 40°C) strongly favors the complete reduction of pyridine nucleotides and coupled reactions. This is observed in all experiments described in this paper (figure 1 etc).

The operational stability of hydrogenase was tested by sequentially adding $NADP^+$ (8 mM) to a hydrogenase preparation. In all cases $NADP^+$ was rapidly reduced to NADPH but the formed NADPH was not stable in time. As can be seen in figure 2, the half-life time of NADPH in this experiment was approximately 20 hours with or without enzyme present.

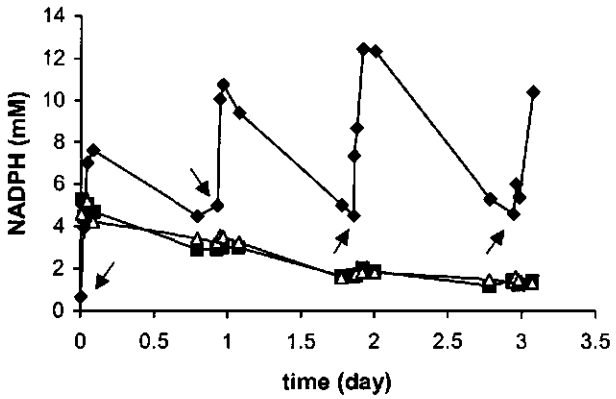


Figure 2 Operational activity of 0.63 units/ml *Pyrococcus furiosus* hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity) and stability of NADPH at 40°C. -Δ- NADPH (5 mM) and hydrogenase; -■- NADPH (5 mM); -◆- $NADP^+$ and hydrogenase, the arrows indicate the time of an addition $NADP^+$ (8 mM).

This (thermal) instability of NADPH has been described earlier. It has been shown that the enzyme-independent half-life time values of NADPH at 41°C varied from 2.5 to 28 hours depending on the anion concentration of the buffer [155]. A half-life time of 20 hours found in our experiments (pH 8.0 and 40°C) is in agreement with these published data concerning anion concentration, pH, and temperature.

HPLC analysis of samples at t = 2 hours and 48 hours showed no degradation products from phosphatase or phosphodiesterase activity. However, other compounds appeared at t = 48 hours: one major and two minor peaks with absorption maximums at 266 nm and one major peak with an adsorption maximum at 269 nm. In addition, the spectra of the major peaks both had shoulders. However, the maximums of the spectra did not correspond with the peaks that are described by Wu *et al.* at high pH (7.5 and 9.1). These degradation compounds of NADPH have maximums at 260 and 280 nm [155]. Furthermore, the compounds found in our experiments cannot be degradation products that are characterized by a positive or small cotton effect, because these 'cotton' compounds have absorption maximums at 290 nm [156].

The origin of the peaks found remains unclear but they are most probably degradation products of NADPH. As shown in figure 2, the degradation

products had little or no inhibitory effect on the reduction of $NADP^+$ by hydrogenase. Hydrogenase repeatedly reduced $NADP^+$ at about 0.20 μmol/min.mg during the first three additions and decreased to 0.11 μmol/min.mg at the fourth addition. These rates were similar to the rates found for the conversion of 12 and 110 mM $NADP^+$, 0.15 and 0.19 μmol/min.mg, respectively (figure 1).

From figures 1 and 2 it is not clear if high concentrations $NADP^+$ inhibited the initial rate of hydrogenase. The initial rates at 40°C were determined with 0.4, 1.0 or 20 mM $NADP^+$, respectively. The concentrations used were much higher than the K_m value of hydrogenase for $NADP^+$, which is 0.04 mM [101]. Concentrations up to 20 mM $NADP^+$, 500 times the K_m value, did not hamper the initial activity of hydrogenase. All concentrations used resulted in the same rates.

The stability of hydrogenase was tested for several materials (see Materials and Methods) that could be used in a final system for NADPH production and regeneration. The dihydrogen oxidation activity decreased mainly during the first 5 days but from that point on it remained relatively stable (40 - 55% of initial activity) for at least 37 days, an exception was silicon tubing. The partial inactivation was found in all experiments including the blank ones. It indicates that the inactivation was not caused by the materials that were added or by oxygen that might be present in pores of the materials, e.g. in membranes or tubing.

When the hydrogenase activity was measured by dihydrogen evolution no inactivation was observed in time. A possible explanation might be that when time passes hydrogenase is converted to an unready or ready state [151]. Both states are catalytically inactive. As described in the literature, activation of both states is possible with dithionite or dihydrogen although the activation of the unready state has a long lag phase [151]. In our case the hydrogenase might be partially active and this state could not be activated by dihydrogen but still by dithionite.

Subsequently, the stability of hydrogenase was tested in a membrane reactor. During a four days experiment, the hydrogenase preparation was concentrated two-fold (Amicon YM10 filter) for two times: after two days and after three days. Again, a decrease in dihydrogen oxidation activity was found during the first 24 hours. After this initial decrease in activity, no further decrease in activity was observed under operational conditions.

Our results give a protocol for the preparative production of NADPH. The best way is a batch-wise production process in stead of a continuous flow system. After a relative short reaction time allowing complete reduction, the enzyme preparation is separated from the reaction mixture (permeate) by ultrafiltration. Subsequently, the enzyme preparation is transferred back to the reaction vessel and replenished with buffer, $NADP^+$, and dihydrogen to start another batch. The NADPH-containing permeate can be processed further to obtain pure NADPH.

NADPH regeneration

Hydrogenases have already been applied for the regeneration of NADH [141, 144-146]. Examples of these coupled reactions are reductive amination catalyzed by glutamate dehydrogenases [146] and reduction of ketones and aldehydes catalyzed by alcohol dehydrogenases [136, 143, 145]. The described reactions sometimes have several disadvantages: the need for the redox dyes methyl viologen and soluble flavin analogues [143, 144] or low turnover numbers (tn), 1 hr^{-1}, combined with low substrate concentrations, 0.8 - 12 mM [143, 145, 146].

In order to explore the properties of the regeneration system by *Pyrococcus furiosus* hydrogenase two systems were investigated. The first one focused on the production of glutamate by *P. furiosus* glutamate dehydrogenase (GDH) and the second one dealt with the reduction of 2-pentanon and butyraldehyde by *Thermoanaerobium brockii* alcohol dehydrogenase (ADH).

Coupling NADPH regeneration to reductive amination

The results of the reduction of 2-ketoglutarate to glutamate catalyzed by GDH are presented in table 2. Under the experimental conditions all 2-ketoglutarate was reduced to glutamate with 0.2 and 0.5 mM $NADP^+$ during a 16 hours experiment at 50°C.

Relatively high total turnover numbers (ttn = 100 to 320) could be calculated and they were significantly higher than the ttn = 17 found for the combination of partially purified *Alcaligenes eutrophus* hydrogenase (0.7 units/ml) coupled to beef liver GDH (10 units/ml), that only reduced 0.68 mM 2-ketoglutarate [146].

Table 2 Conversion of 2-ketoglutarate to glutamate at 50°C in 16 hours catalyzed by 0.2 units/ml *P. furiosus* GDH using NADPH regeneration catalyzed by 2.8 units/ml *P. furiosus* hydrogenase.

2-Ketoglutarate (mM)	Glutamate (mM)	$NADP^+$ (μM)	ttn #
50	32	100	320
50	47	200	235
50	52	500	100

ttn = total turnover number

Coupling NADPH regeneration to ketone or aldehyde reduction

An important aspect of the enzymatic reduction of ketones is that chiral alcohols can be produced. Lamed and co-workers [139] described the reduction of 2-pentanon by the thermophilic *T. brockii* ADH; 110 mM of the 180 mM of the substrate added was reduced in 12 hours at 37°C. At 60°C even more substrate could be converted in the same period of time (144 mM). ADH had a dual role in this system: regeneration of NADPH via the oxidation of 2-propanol, the co-substrate [139]. The reduction of ketones by *T. brockii* ADH was also coupled to *Desulfovibrio vulgaris* hydrogenase. This system was, however, not efficient. A small amount of 2-pentanon (1.5 mM) was reduced in 8 hours [143].

With our regenerating system 25 mM 2-pentanol was produced in two hours at 40°C with 2.8 units/ml hydrogenase and 2 units/ml *T. brockii* ADH. Unfortunately, the substrate 2-pentanon appeared to be very unstable in aqueous environments, also in incubations without enzyme. When no enzyme was present only 30% of the initial amount added (200 mM) could be detected after four hours at 40°C. This is contrary to the results given previously [139].

To test the capacity of *Pyrococcus furiosus* hydrogenase as an NADPH regenerating system for ADH a more stable substrate was used. The reduction of 100 mM butyraldehyde was performed in an incubation mixture containing 0.2 mM $NADP^+$, 2.8 units/ml of hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity), and 2 units/ml *T. brockii* ADH. The time needed to reduce the aldehyde, resulting in a ttn of 500, was only 130 minutes. With 0.7 units/ml hydrogenase a ttn of 500 was reached in four hours. That shows again the efficient reduction of $NADP^+$ by this enzyme.

The reduction of butyraldehyde with 2.8 units/ml of hydrogenase (dihydrogen:$NADP^+$ oxidoreductase activity) and 2 units/ml *T. brockii* ADH

was also performed at 20°C; the reduction of 100 mM aldehyde took 9 hours. It was expected that for these (hyper)-thermophilic enzymes involved the reaction rate was lower than at 40°C. This shows that *P. furiosus* hydrogenase can also be used at low temperatures (20°C).

CONCLUDING REMARKS

The use of *P. furiosus* enzyme preparation for the production and regeneration of NADPH has several advantages over other systems. It was not necessary to use purified enzymes; crude preparations had a relatively high hydrogenase activity and the activity of interfering reactions was low (phosphatase activity). It was shown that *P. furiosus* hydrogenase could be used over a broad temperature range and showed to be very stable under the applied experimental conditions. Furthermore, the hydrogenase remains active at high substrate and product concentrations.

The reduction rate remained constant during all NADPH producing or regenerating experiments and the yields were 100%. Maximal conversion is caused by the reductive power of dihydrogen, which forces the reduction of pyridine nucleotides and the coupled reactions to completeness. The total turnover numbers for the reactions studied were similar or higher to the reported regenerating systems using hydrogenases.

In conclusion, *P. furiosus* hydrogenase offers a very elegant method to produce and regenerate NADPH.

ACKNOWLEDGMENTS

We thank Wim Doesburg for his help in HPLC analysis of 2-ketoglutarate and Joyce Lebbink for providing glutamate dehydrogenase. This research was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Ministry of Economic Affairs of the Netherlands (project number IKA96001).

6

ATP and dihydrogen dependent reduction of carboxylic acids in *Pyrococcus furiosus*

Eyke C.D. van den Ban, Hans Wassink and Huub Haaker

The mechanism involved in carboxylic acid reduction in *Pyrococcus furiosus* was investigated in a cell free extract. The route, catalyzed by a ferredoxin-dependent aldehyde oxidoreductase, could not be demonstrated by the used direct electrochemical or enzymatic techniques. However, the reduction was shown to be dependent on ATP and dihydrogen. The role of $NADP^+$ was ambiguous and coenzyme A inhibited the reduction. A possible process for carboxylic acid reduction in *P. furiosus* involves the following steps. Firstly, the acid is activated with ATP to acyl-AMP, which is subsequently reduced to the aldehyde. Finally, the aldehyde is further reduced to the corresponding alcohol. The reductive reactions were only partially NADPH-dependent, since the reductions were also catalyzed without $NADP^+$. This suggested that another compound could replace NADPH. A potential candidate to replace NADPH is reduced ferredoxin.

INTRODUCTION

Whole cells of *Pyrococcus furiosus*, a hyperthermophilic archaeon, have been shown to reduce a range of carboxylic acids to their corresponding alcohols [83]. 3-Phenylpropionic acid was reduced with the highest yield in these experiments. Recently, this reaction has been optimized for both yield and production rate in a 2 l reactor [157].

The enzymes catalyzing these reductions in *P. furiosus* are not known. In general, three routes have been described for biological systems that are involved in the reduction of carboxylic acids to the aldehydes. In most microorganisms, the aldehydes generated are subsequently reduced to the corresponding alcohols by alcohol dehydrogenases.

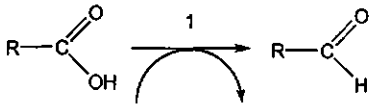
The first route involves the reduction of non-activated acids (scheme 1A). *Clostridium thermoaceticum* [22] and *Clostridium formicoaceticum* [19, 20] are examples of microorganisms, where it was shown that acids could be reduced to aldehydes and alcohols. In the first step, the acid is reduced to the aldehyde catalyzed by aldehyde oxidoreductases (AOR). Subsequently, NADPH-dependent alcohol dehydrogenases catalyze the reduction of the aldehydes to the alcohols [22]. The physiological cofactor for AOR is not known and cell free reductions were performed with artificial electron donors, like reduced methyl viologen and tetramethyl viologen [19, 20, 22].

In the second route, the carboxylic acid is activated with ATP to form acyl-AMP and pyrophosphate (reaction 1 in scheme 1B). Subsequently, the formed acyl-AMP is reduced by NADPH to the aldehyde (reaction 2 in scheme 1B). In *Neurospora crassa* [37, 38] and *Nocardia* strains [25-27], both reactions are catalyzed by one enzyme: aryl aldehyde oxidoreductase [25, 27, 38].

Photobacterium phosphoreum exhibits different substrate specificity than *Nocardia* sp. strains [25-27] and *N. crassa* [37]. In *P. phosphoreum* the enzyme complex reduces aliphatic acids and no aromatic acids [34]. Furthermore, its fatty acid reducing enzyme complex requires a flavin mononucleotide as a cofactor, which was not found in the other organisms [27].

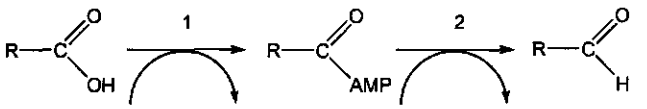
The third process also involves activation of the carboxylic acid by ATP to acyl-AMP (reaction 1 in scheme 1C). Next, the acyl-AMP reacts with the sulfhydryl group of coenzyme A to form acyl-CoA (reaction 2 in scheme 1C). Finally, acyl-CoA is reduced to the aldehyde (reaction 3 in scheme 1C) [13].

A)



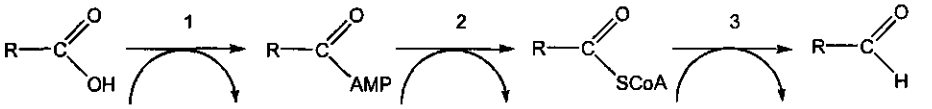
2 electron donor + $2H^+$ 2 electron acceptor + H_2O

B)



ATP + H^+ PP_i + H_2O NAD(P)H $NAD(P)^+$ + AMP

C)



ATP + H^+ PP_i + H_2O CoASH AMP NAD(P)H + H^+ $NAD(P)^+$ + CoASH

Scheme 1 Three different routes described for carboxylic acid reduction.
A) Direct reduction of carboxylic acids to the corresponding aldehyde.
B) Reduction of carboxylic acids to the corresponding aldehyde via acyl-AMP.
C) Reduction of carboxylic acids to the corresponding aldehyde via acyl-CoA.

This mechanism for the reduction of fatty acids has been described for a range of organisms including bacteria, like *Escherichia coli* [43], *Bacillus megaterium* [44], and *Rhodospirillum rubrum* [45], yeasts [2], and mammals [2].

The aim of this paper is to investigate which mechanism is involved in the reduction of carboxylic acids catalyzed by *P. furiosus* cells. The most obvious mechanism would be reduction catalyzed by AOR because *P. furiosus* contains an AOR [70]. However, the results show that the reduction of acids in *P. furiosus* cell free extracts differs from the ATP-dependent carboxylic acid activation mechanism (scheme 1B) with respect to reducing agents.

MATERIALS AND METHODS

Chemicals and enzymes

Methyl viologen was obtained from Sigma and hexanoic acid was from Acros. Benzoic acid, crotonic acid, 3-phenylpropionic acid and 3-phenyl-1-propanol were obtained from Aldrich. Mineral oil was from Fluka. Tetramethyl viologen was prepared as described [20]. Pyruvate kinase (PK) and lactate dehydrogenase, muscle type (LDH), were purchased from Boehringer Mannheim. *Pyrococcus furiosus* aldehyde oxidoreductase was a kind gift of Peter-Leon Hagedoorn. Ferredoxin [106] and pyruvate oxidoreductase [107] were purified as described.

Growth of *Pyrococcus furiosus* and fractionation

P. furiosus was grown in a 200 l reactor in a standard medium [83] with starch as carbon source. Cells were harvested by continuous centrifugation (Sharples) and stored at -80°C until used. About 200 - 300 g cell paste (wet weight) was obtained after 1 night.

P. furiosus cells were disrupted by autolysis: about 100 g cell paste was suspended in 500 ml 50 mM Tris/HCl pH 8.0 containing 10 µg/ml DNase and RNase. This was stirred under argon for 5 hours, and centrifuged for one hour at 30,000 *g* to remove cell debris. Subsequently, the supernatant was concentrated by a 30 kDa filter (Amicon) and dialyzed against 50 mM Tris/HCl pH 8.0 to remove cofactors like ATP and NADP(H). The dialysis tubing had a cut-off of 14 kDa. The protein preparation was called cell free extract (CFE) and was stored in liquid nitrogen. The protein content was 30 mg/ml.

A cell lysate was treated with 10% ammonium sulfate, centrifuged for 30 min and the supernatant was saturated with 60% ammonium sulfate. After centrifugation for another 30 min the precipitate was dissolved in 50 mM Tris/HCl pH 8.0 and dialyzed (cut-off: 14 kDa) against the same buffer. The protein preparation was stored in liquid nitrogen. This fraction was called 60% fraction and the protein content was 30 mg/ml.

Analytical procedures

The protein content was determined via the microbiuret method with bovine serum albumin (BSA) fraction V as standard [80].

3-Phenylpropionic acid, and 3-phenyl-1-propanol were analyzed by HPLC using a Nucleosil 100 C18 5U column (Alltech), methanol/0.1% trifluoro acetic acid was used as eluent.

Direct electrochemical measurements on a hollowed glassy carbon (ferredoxin and methyl viologen) and gold foil (tetramethyl viologen) electrode were performed as described by Hagen [158]. The system was flushed with water-saturated argon. The temperature ranged from 30 to 80°C and pH 6.3 - 6.5 were applied. The reaction mixture had a volume of 25 µl and contained the following (final concentrations): 100 mM MES buffer solution, 6 mM 3-phenylpropionic, hexanoic or crotonic acid, and 7 mM neomycine (only needed as promotor for ferredoxin). A second droplet was placed next to the above-mentioned droplet (without touching each other or the reference and counter electrodes) to measure the temperature inside the system. When the signal was stable one of the following electron donors was added 0.15 mM *P. furiosus* ferredoxin, 3 mM methyl viologen, or 5 mM tetramethyl viologen. Subsequently, the droplets were covered with mineral oil to prevent evaporation. When the signal was stable and the droplet had the proper temperature 5 µl aldehyde oxidoreductase or CFE was added. The scan rates were 2 or 5 mV/s and the range was -250 to -850 mV. The electron carriers were reduced by the working-electrode.

Acid reduction experiments were performed at pH 6.5 (50 mM MES buffer solution) and pH 8.0 (100 mM Tris/HCl buffer solution) at 60°C. The solution for the reduction of 3-phenylpropionic acid (1 mM) contained the following compounds when added (final concentrations): 5 mM Mg^{2+}-ATP, ADP or AMP, 0.1 mM $NADP^+$ or NAD^+, 10 mM DTT, and 1 mM Coenzyme A (CoA). The total volume was 1 ml and the experiments were performed in 100 ml gas tight bottles. Prior to the addition of enzyme the bottles were filled with dihydrogen. Either CFE (6 mg/ml) or the 60% fraction (7.5 mg/ml) was added. The pyruvate driven, ferredoxin mediated, dihydrogen production was measured according to Silva *et al.* [21].

The formation of acyl-CoA was detected spectrophotometrically. Experiments were performed at pH 8.0 (100 mM Tris/HCl buffer solution) and at 60°C. The formation of acyl-CoA compounds was detected according to the following scheme:

carboxylic acid + CoA + ATP	→	acyl-CoA + ADP + P_i	(1)
ADP + phosphoenolpyruvate	→	ATP + pyruvate	(2)
pyruvate + NADH + H^+	→	lactate + NAD^+	(3)

Reaction (1) is catalyzed by acetyl-CoA synthetase present in *P. furiosus* CFE, reaction (2) is catalyzed by PK, and reaction (3) by LDH. The reaction mixture, 1 ml, contained the following (final concentrations): 2 mM ATP, 10 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 0.12 mM NADH, 4 units PK (at 25°C), 55 units LDH (at 25°C), and 10 µl CFE. Prior to the addition of the enzymes the cuvette was filled with argon. As substrates were used: sodium acetate (10 mM), hexanoic acid (10 mM) and 3-phenylpropionic acid (1 mM). First the NADH oxidation was measured followed by the addition of ATP (ATP-ase activity); adding CoA (0.5 mM final concentration) started the actual reaction. The extinction was measured at 340 nm.

Results and Discussion

Reduction of carboxylic acids catalyzed by aldehyde oxidoreductase

As described above, three different mechanisms to reduce carboxylic acids can be distinguished. One process involves the reduction of non-activated acids (scheme 1A), catalyzed by aldehyde oxidoreductases (AOR) [19, 20, 22]. This class of enzymes reacts with electron carriers like viologens and ferredoxins.
Because *Pyrococcus furiosus* contains an AOR [70], this enzyme might be involved in the reduction of acids. To investigate this hypothesis direct electrochemical experiments were performed with purified AOR and three electron donors: the artificial methyl viologen and tetramethyl viologen and the physiological electron carrier ferredoxin. In all experiments no reduction of 3-phenylpropionic, hexanoic, or crotonic acid could be detected.
Next to that, experiments with CFE in the presence of reduced ferredoxin, showed no acid reduction. Even in the presence of dihydrogen (to maintain high ratios of NADPH/$NADP^+$, favorable for the reduction of aldehyde to alcohol by alcohol dehydrogenases) no acid reduction was observed.
Based on these results it seems not likely that carboxylic acids are reduced by reduced ferredoxin and catalyzed by AOR in *P. furiosus*.

Reduction of carboxylic acids after activation of the acid

Since the reduction of carboxylic acids is probably not catalyzed by AOR, other reactions must be involved in the observed whole cell reductions. Therefore, the route via the acyl-AMP and acyl-CoA activated intermediates was explored (scheme 1B and C). Table 1 shows the dependency of the different substrates necessary for a dialyzed CFE to catalyze the reduction of 3-phenylpropionic acid. The data clearly show that ATP and dihydrogen were essential for reduction of 3-phenylpropionic acid and that CoA had a negative effect. The role of $NADP^+$ was ambiguous.

Effects of ATP, ADP and AMP on carboxylic acid reduction

The reduction of carboxylic acids was dependent on ATP. AMP could not replace ATP. Only ADP could replace ATP although the rate of reduction was lower indicating that ADP was less efficient than ATP.

ADP probably needed to be converted to ATP prior to its use in carboxylic acid reduction. The most likely way of ATP production from ADP is the action of adenylate kinase. This enzyme catalyzes the formation of ATP and AMP using two ADPs and is present in *P. furiosus* [159].

Table 1 Dependency of *P. furiosus* cell free extract on substrates to catalyze the reduction of 1 mM 3-phenylpropionic acid at pH 8.0 and 60°C in one hour. The complete incubation contains CFE, dihydrogen, ATP and $NADP^+$.

Omission	Addition	Conversion (%)
-	-	11
dihydrogen	-	0
ATP	-	0
CFE	-	0
ATP	ADP	8
ATP	AMP	0
$NADP^+$	-	6
$NADP^+$	NAD^+	6
-	CoA	2

Effects of dihydrogen and NADP$^+$ on carboxylic acid reduction

Next to ATP, dihydrogen was essential for the reduction (table 1). During dihydrogen oxidation in the presence of NADP$^+$, *P. furiosus* hydrogenases generate NADPH, which is the reductant for aldehyde reduction catalyzed by the alcohol dehydrogenases [76, 160]. A second reason to explain the NADPH-dependency is the need for NADPH in the ATP-dependent reduction of carboxylic acids to aldehydes as found in other systems [25, 27, 37].

However, in table 1 can be seen that 3-phenylpropionic acid was also reduced without NADP$^+$, although the yield of conversion was lower. The replacement of NADP$^+$ by NAD$^+$ did not result in higher yields. Therefore NAD$^+$ could not replace NADP$^+$. These results suggest that another cofactor was present in the dialyzed CFE, which is capable of reducing the (activated) acid to the aldehyde followed by reduction to the alcohol.

Use of NADPH versus ferredoxin in carboxylic acid reduction

A possible candidate for the replacement of NADPH is ferredoxin. Firstly, because *P. furiosus* ferredoxin has a similar redox potential, Fd_{red}/Fd_{ox}: -345 mV [161], as NADPH/NADP$^+$ (-320 mV) [14]. Secondly, ferredoxin can be reduced by purified hydrogenases during dihydrogen oxidation. *P. furiosus* contains at least three hydrogenases: a membrane-bound hydrogenase [21] and two soluble hydrogenases [98]. The purified soluble hydrogenases are able to oxidize dihydrogen coupled to the reduction of NADP$^+$, NAD$^+$ and ferredoxin, although the ferredoxin reducing activity is very low [98]. The dihydrogen dependent reduction of ferredoxin was also tested in CFE. The ferredoxin reduction activity was 1.14 nmol/min.mg protein and this is about three times higher than the 3-phenylpropionic acid reduction activity (table 2).

In addition reduced ferredoxin could act as electron donor for *Thermoanaerobium brockii* alcohol dehydrogenase indicating that it could replace NADPH in redox reactions (data not shown).

Table 2 Specific activities for 3-phenylpropionic acid, ferredoxin, and NADP$^+$ reduction by dihydrogen oxidation catalyzed by *P. furiosus* CFE at 60°C and pH 8.0.

	Specific activity (nmol/min.mg protein)
3-phenylpropionic acid	0.30
ferredoxin	1.14
NADP$^+$	880

These data show that, in theory, it is possible that ferredoxin can couple between dihydrogen and carboxylic acid reduction in *P. furiosus* when no $NADP^+$ is present. However, the molecular weight of ferredoxin is 7.5 kDa [162], implying that this protein would have been removed by dialysis with the used dialysis tubing (14 kDa cut-off). This appeared not to be the case. For example, a dissolved and dialyzed 60% ammonium sulfate pellet that was passed through a DEAE column resulted in a significant amount of ferredoxin, next to ferredoxin-dependent enzymes like glyceraldehyde-3-phosphate oxidoreductase, pyruvate oxidoreductase, and aldehyde oxidoreductase. This indicates that ferredoxin binds strongly to proteins and, therefore, is difficult to remove from crude extracts by dialysis only [163].
Another experiment that suggests that ferredoxin was not removed from CFE by dialysis, comes from pyruvate-dependent dihydrogen production. Pyruvate was oxidized by pyruvate oxidoreductase in the presence of CoA. In this reaction ferredoxin was reduced, and the reduced ferredoxin was subsequently oxidized by the membrane-bound hydrogenase under the formation of dihydrogen. When no ferredoxin is present there will be no pyruvate-driven dihydrogen production. Since this activity was found in dialyzed CFE and no stimulation was observed when 12.5 μM ferredoxin was added, it is clear that dialyzed CFE still contained significant amounts of ferredoxin.
To investigate the role of ferredoxin in the process of acid reduction experiments with purified proteins are necessary.

Effect of CoA on carboxylic acid reduction
It is shown in table 1 that the presence of CoA lowered the rate of reduction of 3-phenylpropionic acid. It is not likely that CoA itself caused the decrease in yield of acid reduction. It has been demonstrated that *P. furiosus* CFE contains acyl-CoA synthetases (ADP-forming). Two isoenzymes of acyl-CoA synthetases (ACS) are present in *P. furiosus*: ACS I and ACS II [164]. Both enzymes convert acetyl-CoA, but ACS I is specific for aliphatic acyl-CoAs and ACS II for aromatic acyl-CoAs [164].
We tested the activity of these enzymes in CFE of *P. furiosus*. The enzyme activity was measured as an acid and CoA-dependent ADP formation from ATP. The specific activity for 3-phenylpropionic acid was 0.16 μmol ADP/min.mg at 60°C. The activities with acetate and hexanoic acid at 60°C were 0.70 and 0.55 μmol ADP/min.mg, respectively. The results show that CFE was able to generate 3-phenylpropionyl-CoA when ATP and CoA were

present. Apparently, *P. furiosus* has an enzyme able to activate carboxylic acids to acyl-CoA. In addition it can be concluded that *P. furiosus* probably has no enzyme(s) to reduce this intermediate to the corresponding aldehyde and subsequently to the alcohol, since CoA inhibits alcohol production also in the presence of $NADP^+$ and dihydrogen (NADPH).

CONCLUDING REMARKS

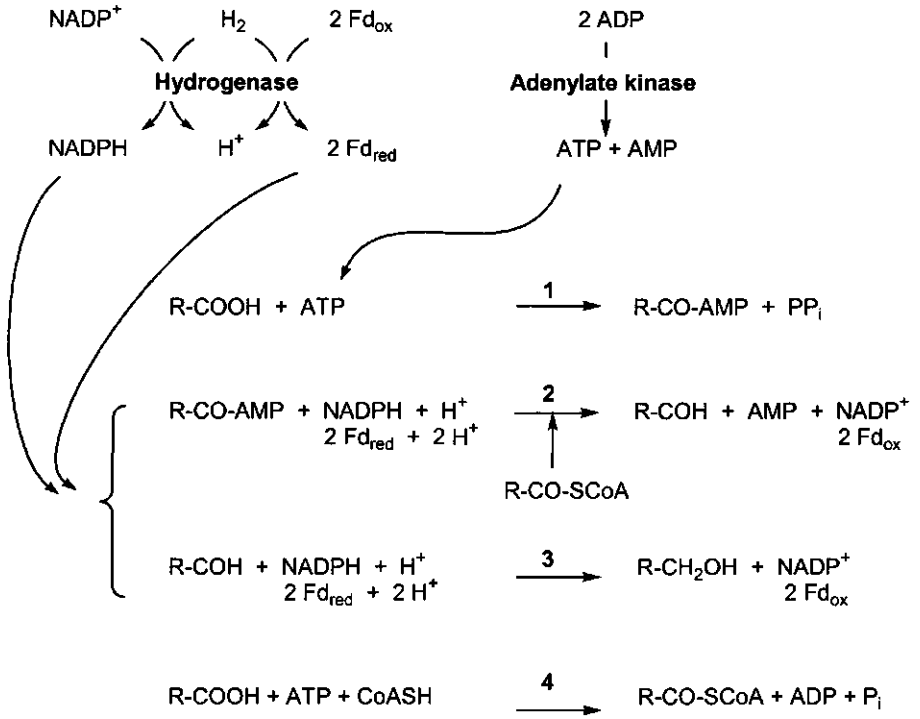
We were not able to show that carboxylic acid reduction occurs via reduced ferredoxin catalyzed by AOR. None of the carboxylic acids tested could be reduced electrochemically in the presence of both artificial and physiological electron carriers, not with cell free extract or with purified AOR. Yet we were able to show that the reduction of carboxylic acids in crude extract was possible with ATP and dihydrogen and that CoA inhibited the process. Based on these results we propose that *P. furiosus* catalyzes the reduction of acids via the intermediate acyl-AMP (scheme 2, reaction 1), which is subsequently reduced to the aldehyde (scheme 2, reaction 2).

A remarkable result was that the reduction was still possible without $NADP^+$, suggesting that another cofactor could replace NADPH. This finding is important because there are no experiments described in literature in which carboxylic acid reducing activity was observed without NAD(P)H [26, 31, 37].

Although we were not able to prove which cofactor could replace NADPH, we propose that reduced ferredoxin is able to replace NADPH in both the reduction of the activated acid (acyl-AMP) to the aldehyde and the aldehyde to the alcohol (scheme 2, reactions 2 and 3).

It is unknown which enzyme or enzymes catalyze(s) reactions 1 and 2. The third reaction, the reduction of the aldehyde to the alcohol, is probably catalyzed by an alcohol dehydrogenase. The fourth reaction is most likely catalyzed by ACS I and II, when aliphatic acids are used, and by ACS II in case of aromatic acids. The product of this reaction, acyl-CoA, is thought to inhibit the conversion of acyl-AMP to the aldehyde as shown in reaction 2. The inhibitory effect of CoA was not found in cell free extracts of *Neurospora crassa* [37], the only experimental data about the effects of CoA on the reduction of acids.

Finally, the enzymes adenylate kinase and hydrogenases are believed to be involved; adenylate kinase for the production of ATP in case only ADP is present and the hydrogenases for the reduction of $NADP^+$ or ferredoxin.



Scheme 2 Proposed routes involved in the reduction of carboxylic acids in *P. furiosus* CFE. The enzymes catalyzing reactions 1 and 2 are unknown. An alcohol dehydrogenase and an acyl-CoA synthetase probably catalyze reactions 3 and 4, respectively.

ACKNOWLEDGMENTS

We thank Peter-Leon Hagedoorn for supplying AOR and for his help with the electrochemical experiments and Ton Marcelis and Arie Koudijs for their help with the synthesis of tetramethyl viologen. This research was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Ministry of Economic Affairs of the Netherlands (project number IKA96001).

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Summary

This Ph.D. project started in 1997 and its main goal was to obtain insight in the reductive capacity of the hyperthermophilic archaeon *Pyrococcus furiosus*. The research was focused on the biocatalytic reduction of carboxylic acids.

Reductions of carboxylic acids are interesting reactions, since the generated products, aldehydes and alcohols, are potentially applicable in the fine-chemical industry. However, the reduction of carboxylic acids to the corresponding aldehydes is a thermodynamically difficult reaction, both chemically and biologically, because the reaction needs strong reducing agents. Nevertheless, there are several microorganisms able to catalyze the reduction of acids. For some microorganisms, the mechanism catalyzing the acid reduction has been elucidated. Two of the proposed mechanisms proceed via activation of the acid to an acyl-AMP or acyl-CoA intermediate followed by reduction of the activated acid to the corresponding aldehyde. The third one is reduction of the acid without activation.

P. furiosus is an anaerobic hyperthermophilic organism growing optimally at temperatures ranging from 90 to 100°C. The advantage of these high temperatures is that infectious mesophilic contaminations during culturing of this organism do not occur. Other advantages of this organism are the relatively fast growth and the usage of inexpensive carbon and energy sources like (potato)-starch. Next to that, *P. furiosus* is an archaeon and these organisms are non-pathogenic to men. These features and the fact that *P. furiosus* produced dihydrogen during growth make *P. furiosus* a very useful organism to study the reduction of acids.

First, an inventory was made of which carboxylic acids can be reduced by *P. furiosus* to the corresponding aldehydes and subsequently to the alcohols (chapter 2). Both aliphatic and aromatic acids were tested and *P. furiosus* was able to catalyze the reduction of compounds from both groups. The best results were obtained with aromatic compounds, of which the reduction of 3-phenylpropionic acid resulted in the highest yield: 69% of the acid was reduced to the corresponding alcohol.
The aldehyde, intermediate in this reduction, was not detected. This could be explained by the thermodynamically unfavorable reduction of the acid to the aldehyde followed by the thermodynamically favorable reduction of the aldehyde to the alcohol. It means that the 'difficult-to-generate' aldehyde is immediately converted into the corresponding alcohol. The experiments also showed that growing *P. furiosus* cells and dihydrogen are essential to reduce carboxylic acids.

Based on the results described in chapter 2, it was investigated which factors influenced the bioreduction and how the reduction rate as well as the yield of the alcohol could be optimized (chapter 3). Five factors were studied: 1) pH, 2) partial dihydrogen pressure, 3) substrate concentration, 4) carbon and energy concentration, and 5) temperature. To study the effects of these factors on the reduction of acids, experiments were performed according to the principle of 'factorial design'. 3-Phenylpropionic acid was used as model substrate.
The yield of the alcohol was found to be optimal at pH 6.3, a substrate concentration of 1 mM, and a temperature of 80°C. The reduction rate, however, was optimal at pH 7.0, 10 mM substrate and 90°C. Both the yield and the reduction rate showed to be dependent on the same variables, but the dependencies were opposite. As a result, it was not possible to maximize both terms at the same time within the limits of these experiments.

Dihydrogen showed to be essential for the reduction of carboxylic acids and *P. furiosus* is able to produce this gas. Chapter 4 describes the functions of several hydrogenases. It was demonstrated that previous hypotheses do not sustain, since particularly sulfhydrogenase has insufficient capacity to dispose off the reducing equivalents (reduced ferredoxin and NADPH), generated during starch fermentation, as dihydrogen. However, a membrane-bound hydrogenase was able to catalyze the disposal of catabolically generated

reducing equivalents as dihydrogen. This membrane-bound hydrogenase was partially purified. The complex consists of 14 subunits and the N-terminal sequences of two subunits were determined.
Based on the results, four functions were proposed for the dihydrogen metabolism: 1) the generation of dihydrogen coupled to a proton pump, 2) the production of NADPH for biosynthetic purposes, 3) an additional route to generate NADPH, and 4) a safety valve to dispose off a surplus of reducing equivalents.

One of the functions of the, in chapter 4 mentioned, cytosolic hydrogenase is the regeneration of NADPH. This reaction is very interesting from a commercial point of view, since NADPH is an expensive cofactor applied in numerous biosynthetic reactions. The potential applications of this hydrogenase for the production and regeneration of NADPH are described in chapter 5.
The enzyme showed an operational stability of at least 37 days (hydrogen oxidation activity) and proved to be active even at lower temperatures (20°C).
One of the most important results of the NADPH production and regeneration experiments was that hydrogenase was not inhibited by high concentrations $NADP^+$ and NADPH. Moreover, substrates and products present in the NADPH-dependent reduction of the model substrates 2-ketoglutarate, 2-pentanone, or butyraldehyde did not inhibit NADPH regeneration either. Furthermore, hydrogenase catalyzed the production of 100 mg/ml NADPD in a relative short period of time (4 hours). Finally, the NADPH-regeneration experiments resulted in 'total turnover' numbers of 500.

Chapters 2 and 3 described that *P. furiosus* was able to catalyze the reduction of carboxylic acids during growth. These experiments, however, did not provide indications by which mechanism the reduction was catalyzed. The research to elucidate this is described in chapter 6.
The most obvious route, the reduction of the acid to the aldehyde catalyzed by ferredoxin:aldehyde oxidoreductase, could not be demonstrated by the used electrochemical and enzymatic methods. Yet, it could be shown that the reduction was dependent on ATP, dihydrogen, and partially dependent on NADPH. Based on these results it was proposed that the reduction of carboxylic acids in *P. furiosus* proceeds by the activation of the acid with ATP to form an acyl-AMP intermediate and that the reduction of the acyl-AMP

intermediate is NADPH-dependent. It is remarkable that NADPH was not essential, but that it could be replaced by an unknown protein factor, possibly ferredoxin.

The results described in this thesis showed that *P. furiosus* is well able to catalyze several reductive reactions. Carboxylic acids as well as $NADP^+$ can be reduced and provide a potential alternative for chemical reductions. As yet, the production and regeneration of NADPH by *P. furiosus* hydrogenase seem to have the best chances to a successful commercial application.

Samenvatting

Dit promotieonderzoek is in 1997 gestart met als doel inzicht te krijgen in de reductieve eigenschappen van het hyperthermofiele archaeon *Pyrococcus furiosus*. Het accent van dit onderzoek ligt op de biokatalytische reductie van carbonzuren.

De reductie van carbonzuren is interessant omdat de gevormde producten, aldehyden en alcoholen, potentieel toepasbaar zijn in de fijn-chemicaliën industrie. Reductie van carbonzuren naar de corresponderende aldehyden is echter een thermodynamisch moeilijke reactie, zowel chemisch als biologisch, omdat voor de reactie sterke reductiemiddelen nodig zijn. Desondanks zijn er verschillende micro-organismen die deze reducties kunnen katalyseren. Van een aantal micro-organismen is het mechanisme van de zuurreductie opgehelderd. Er zijn drie mechanismen beschreven. Twee verlopen via activatie van het zuur tot een acyl-AMP of acyl-CoA intermediair en de derde verloopt zonder activatie van het zuur.

P. furiosus is een anaëroob hyperthermofiel organisme dat optimaal groeit bij temperaturen van 90 - 100°C. Deze hoge temperaturen hebben als voordeel dat het kweken van dit organisme geen problemen oplevert met betrekking tot infecties door mesofielen. Andere voordelen van dit organisme zijn dat het relatief snel groeit en het goedkope koolstof- en energiebronnen zoals (aardappel)zetmeel kan gebruiken. Daarnaast is *P. furiosus* een archaeon en deze organismen zijn niet pathogeen voor mensen. Deze kenmerken gecombineerd met de eigenschap dat *P. furiosus* waterstofgas vormt tijdens de groei maken *P. furiosus* tot een zeer bruikbaar organisme om (zuur)reducties mee te bestuderen.

Er is gestart met het inventariseren van welke carbonzuren gereduceerd kunnen worden door *P. furiosus* tot de overeenkomstige aldehyden en vervolgens tot de alcoholen (hoofdstuk 2). Er zijn zowel alifatische als aromatische zuren getest en *P. furiosus* is in staat verbindingen uit beide groepen te reduceren. De beste resultaten zijn behaald met aromatische verbindingen, waarvan de reductie van 3-fenylpropionzuur de hoogste opbrengst gaf: 69% van het zuur werd omgezet in de overeenkomstige alcohol. Het aldehyde, intermediair in deze reductie, werd niet gevonden. Dit kan verklaard worden, doordat de thermodynamisch ongunstige reductie van het zuur tot het aldehyde wordt gevolgd door de thermodynamisch gunstige reductie van het aldehyde tot de alcohol. Eén en ander betekent dat het 'moeizaam' gevormde aldehyde onmiddellijk wordt omgezet in de overeenkomstige alcohol. Uit de experimenten bleek ook dat om carbonzuren te kunnen reduceren groeiende *P. furiosus* cellen en waterstofgas essentieel zijn.

Op basis van de resultaten, die in hoofdstuk 2 beschreven staan, is onderzocht welke factoren invloed hebben op de bioreductie en hoe deze geoptimaliseerd kan worden naar zowel de reductiesnelheid als de opbrengst van de alcohol (hoofdstuk 3). Er zijn vijf factoren onderzocht: 1) pH, 2) partiële waterstofgasdruk, 3) substraatconcentratie, 4) koolstof- en energiebron concentratie en 5) temperatuur. Om de effecten hiervan op de zuurreductie te onderzoeken zijn er experimenten uitgevoerd volgens het 'factorial design' principe en is 3-fenylpropionzuur als modelsubstraat gebruikt.
Gevonden werd dat de opbrengst van de alcohol optimaal is bij pH 6.3, een substraatconcentratie van 1 mM en een temperatuur van 80°C. De reductiesnelheid is echter optimaal bij een pH van 7.0, 10 mM substraat en 90°C. Zowel de opbrengst als de reductiesnelheid zijn afhankelijk van dezelfde variabelen, maar de afhankelijkheden zijn tegengesteld. Hierdoor is het niet mogelijk om, binnen de grenzen van deze experimenten, beide termen tegelijkertijd te maximaliseren.

Waterstofgas bleek essentieel voor de reductie van carbonzuren en *P. furiosus* kan dit gas zelf vormen. In hoofdstuk 4 wordt ingegaan op de functies van de verschillende hydrogenases. Aangetoond werd dat eerdere hypothesen hierover geen stand houden, omdat met name het sulfhydrogenase onvoldoende capaciteit heeft om de, tijdens zetmeelfermentatie gegenereerde, reductie-equivalenten (gereduceerd ferredoxine en NADPH) in de vorm van

waterstofgas kwijt te raken. Daartoe beschikt *P. furiosus* over een membraangebonden hydrogenase. Dit membraangebonden hydrogenase is gedeeltelijk gezuiverd. Het complex bestaat uit 14 subunits en van twee subunits zijn de N-termini sequenties deels opgehelderd.
Op basis van de resultaten worden er vier functies voor het waterstofmetabolisme voorgesteld: 1) de vorming van waterstof gekoppeld aan een proton-pomp, 2) de productie van NADPH voor biosynthese, 3) een extra route om NADPH te vormen en 4) een veiligheidsroute om een overschot aan reductie-equivalenten kwijt te raken.

Eén van de functies van het, in hoofdstuk 4 beschreven, cytosolische hydrogenase is de regeneratie van NADPH. Deze reactie is vanuit een commercieel oogpunt erg interessant, omdat NADPH een kostbare co-factor is, die in veel biosynthetische reacties gebruikt wordt. De potentiële toepassingsmogelijkheden van dit hydrogenase voor de productie en regeneratie van NADPH zijn beschreven in hoofdstuk 5.
Het enzym bleek tot tenminste 37 dagen operationeel stabiel (waterstofgas oxidatie activiteit) en het bleef ook nog bij lagere temperaturen (tot 20°C) actief. Eén van de belangrijkste uitkomsten uit de NADPH productie en regeneratie experimenten is dat het hydrogenase niet geremd wordt door hoge concentraties $NADP^+$ en NADPH. Bovendien wordt het niet geremd door de substraten en producten aanwezig in de NADPH-afhankelijke reductie van de modelsubstraten 2-ketoglutaraat, 2-pentanon of butyraldehyde. Daarnaast kan het hydrogenase in relatief korte tijd (4 uur) 100 mg/ml NADPH produceren. Tenslotte leverden de NADPH-regeneratie experimenten 'total turnover' getallen tot 500 op.

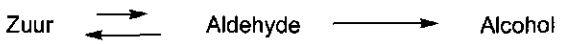
In de hoofdstukken 2 en 3 is beschreven dat *P. furiosus* tijdens de groei in staat is om carbonzuren te reduceren. Deze experimenten geven echter geen aanwijzing volgens welk mechanisme de reductie gekatalyseerd wordt. Hoofdstuk 6 beschrijft het onderzoek hiernaar. De meest voor de hand liggende route, de reductie van het zuur tot het aldehyde gekatalyseerd door het enzym ferredoxine:aldehyde oxidoreductase, kon niet aangetoond worden met de gebruikte elektrochemische en enzymatische technieken. Wel kon worden aangetoond dat de reductie afhankelijk is van ATP, waterstofgas en gedeeltelijk afhankelijk van $NADP^+$. Op basis hiervan wordt voorgesteld dat de reductie van carbonzuren in *P. furiosus* verloopt door activatie van het zuur

met ATP tot een acyl-AMP intermediair en dat de reductie van het acyl-AMP intermediair NADPH afhankelijk is. Het bijzondere is dat NADPH niet absoluut noodzakelijk is, maar vervangen kan worden door een onbekende eiwitfactor, mogelijk ferredoxine.

De resultaten die in dit proefschrift beschreven staan, laten zien dat *P. furiosus* goed in staat is om diverse reductiereacties te katalyseren. Zowel carbonzuren als $NADP^+$ kunnen worden gereduceerd en leveren daarmee een potentieel alternatief op voor chemische zuurreducties. Vooralsnog lijkt de productie en regeneratie van NADPH door *P. furiosus* hydrogenase de meeste kans te bieden op een succesvolle commerciële toepassing.

Samenvatting in gewoon Nederlands

Het doel van mijn onderzoek was het bestuderen van de omzetting van organische zuren in aldehyden en alcoholen (niet de 'drank'alcohol). Deze aldehyden en alcoholen zijn belangrijk voor verschillende industrieën waaronder de geur- en smaakstoffen industrie. Een voorbeeld van een aldehyde is vanilline, deze stof is de belangrijkste component van vanille.
De omzettingen van zuren werden uitgevoerd door *Pyrococcus furiosus*. *Pyrococcus furiosus* is een micro-organisme. Micro-organismen zijn de kleinste levensvorm en die bestaan uit één cel. Je kunt deze eencelligen alleen zien met een microscoop. In deze ene cel worden alle processen uitgevoerd die nodig zijn om te leven (opname en gebruik van voedingsstoffen om te leven, beweging, vermenigvuldiging en tal van anderen taken).
Pyrococcus furiosus (vrij vertaald: ziedende vuurbal) leeft bij hoge temperaturen, 90 tot 100°C, in de zee bij het eilandje Vulcano in Italië. Het leeft daar onder zuurstofloze omstandigheden en produceert onder andere het gas diwaterstofsulfide (dat ruikt naar rotte eieren).
Ik ben begonnen met het testen van de omzetting van een hele serie verschillende organische zuren in de overeenkomstige aldehyden en alcoholen (hoofdstuk 2). Het bleek dat *Pyrococcus furiosus* de zuren voornamelijk omzet in de alcoholen en nauwelijks in de aldehyden. Dat was een tegenvaller, omdat de aldehyden het meest belangrijk zijn voor de geur- en smaakstoffen industrie. De verklaring voor dit resultaat is dat de omzetting van zuur naar aldehyde zo moeilijk is (vergelijk het met een berg op fietsen). De omgekeerde reactie is veel makkelijker (helling af fietsen) en om te voorkomen dat de reactie van aldehyde naar zuur gaat, moet het aldehyde omgezet worden in een andere stof, in dit geval in het alcohol (zie figuur 1).



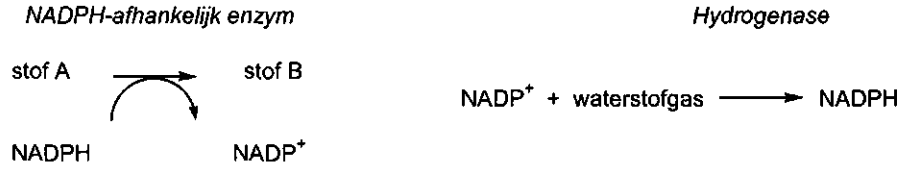
Figuur 1 De omzetting van zuren in aldehyden en alcoholen door *Pyrococcus furiosus* (hoe langer de pijl, hoe makkelijker de reactie verloopt).

Ondanks dit resultaat was het toch interessant genoeg om te proberen de meest optimale omstandigheden te bepalen om een zo hoog mogelijke opbrengst van het alcohol te krijgen en om deze reactie in een zo kort mogelijke tijd te laten plaatsvinden (hoofdstuk 3). Om dat te onderzoeken zijn er series van experimenten uitgevoerd waarin een aantal omstandigheden zijn gevarieerd. Denk daarbij aan temperatuur en zuurgraad (pH). Na de nodige statistische analyses blijkt dat een zo hoog mogelijke opbrengst van het alcohol in een zo kort mogelijke tijd niet samen gaan. Een hoge opbrengst krijg je bijvoorbeeld bij een lage temperatuur en de reactie is het snelst bij een hoge temperatuur. Hierbij moet ik wel opmerken dat een lage temperatuur een relatief begrip is: voor *Pyrococcus furiosus* is 80°C aan de frisse kant!

De reactie van zuren naar alcoholen verloopt alleen als er waterstofgas aanwezig is. *Pyrococcus furiosus* kan dit gas zelf maken en bezit hiervoor specifieke enzymen. Enzymen zijn in staat verschillende reacties te katalyseren. Katalyse betekent dat reacties versneld, efficiënt en gecontroleerd verlopen die zonder enzymen niet of nauwelijks plaatsvinden.

De enzymen die betrokken zijn bij de vorming en omzetting van waterstofgas heten hydrogenases (vanwege de Engelse benaming voor waterstofgas: hydrogen). Deze hydrogenases zijn in een breder opgezet onderzoek bestudeerd en er is onder andere gekeken naar de functies van deze hydrogenases (hoofdstuk 4).

Eén van de functies is het terugwinnen van NADPH. NADPH is een hulpstof die in veel biologische reacties (ook in cellen van de mens) een belangrijke rol speelt. Verschillende enzymen zijn NADPH-afhankelijk en als er geen NADPH aanwezig is, verloopt de omzetting van stof A in stof B niet. Tijdens zo'n reactie wordt NADPH omgezet in $NADP^+$ (figuur 2). Omdat er in biologische systemen maar kleine hoeveelheden NADPH aanwezig zijn, is de terugwinning (de omgekeerde reactie, zie figuur 2) een erg belangrijke reactie.



Figuur 2 Het gebruik van de hulpstof NADPH door een NADPH-afhankelijk enzym (linker reactie) en de terugwinning van NADPH met waterstofgas gekatalyseerd door een hydrogenase (rechter reactie).

NADPH is niet alleen belangrijk voor biologische reacties maar ook voor verschillende toepassingen in bijvoorbeeld de farmaceutische industrie. Deze industrie gebruikt NADPH-afhankelijke enzymen voor de productie van medicijnen of grondstoffen daarvoor. Echter, NADPH is behoorlijk duur en daarom is ook voor dit soort toepassingen de terugwinning van NADPH interessant.

Uit het onderzoek van hoofdstuk 4 is al gebleken dat enzymen van *Pyrocossus furiosus* deze terugwinning kunnen uitvoeren. Ik heb de reactie met het hydrogenase in meer detail bekeken (hoofdstuk 5) en de omzetting van $NADP^+$ in NADPH blijkt goed te werken, het wordt volledig omgezet. Het gebruik van hydrogenase heeft nog andere voordelen: De terugwinning kan uitgevoerd worden met waterstofgas en dit is een 'schone reactie': het levert geen afvalproducten op (figuur 2). Hierdoor wordt het makkelijker om zuiver NADPH te produceren. Daarnaast is het hydrogenase ook erg stabiel zodat het enzym lang gebruikt kan worden.

Ik ben mijn onderzoek begonnen met de omzetting van organische zuren in aldehyden en alcoholen. *Pyrococcus furiosus* is in staat om deze reactie uit te voeren maar hoe... dat is niet bekend. Om daar achter te komen heb ik de cellen kapot gemaakt en uit de vloeistof een aantal hulpstoffen gehaald (zoals NADPH). Wanneer er bepaalde hulpstoffen weer toegevoegd worden, verloopt de reactie en krijg je een idee hoe de reactie biochemisch gezien verloopt (hoofdstuk 6).

Uit mijn onderzoek blijkt dat *Pyrococcus furiosus* goed in staat is om organische zuren om te zetten in alcoholen en om de terugwinning van NADPH uit te voeren. Deze terugwinning heeft de grootste kans om commercieel toegepast te worden en of dat inderdaad zo is, zal in de toekomst blijken.

Curriculum vitae

Eyke Corrie Ditty van den Ban werd op 24 januari 1973 te Hellendoorn geboren. Ze behaalde het VWO diploma aan College Noetsele te Nijverdal in juni 1991. In september dat jaar startte zij met de studie Bioprocestechnologie aan de Landbouwuniversiteit te Wageningen. Haar studie werd afgesloten met onderzoek bij de Vakgroep Levensmiddelentechnologie (sectie Industriële Microbiologie, Prof. J.A.M. de Bont) en bij de Vakgroep Biochemie (Prof. N.C.M. Laane & Prof. W.R. Hagen) gecombineerd met de afdeling Enzyme Research van Quest International te Naarden (Dr. D.J.M. Schmedding). Haar stage liep ze bij het Australian Starter Culture Reseach Centre te Werribee, Australië (Dr. P. Bruinenberg). In november 1996 behaalde zij haar doctoraal diploma. Per januari 1997 is zij gestart met een promotieonderzoek bij het Laboratorium voor Biochemie aan de Wageningen Universiteit onder begeleiding van Prof. N.C.M. Laane en Dr. H.B.C.M. Haaker. De resultaten van het onderzoek zijn beschreven in dit proefschrift. Naast haar promotieonderzoek is ze sinds november 2000 parttime werkzaam bij Bioway bv te Ede. Vanaf augustus 2001 is ze daar voltijds werkzaam.

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Dankwoord

Een proefschrift is nooit het werk van een persoon alleen en daarom is een persoonlijk woord van dank voor de vele mensen die mij met raad en daad bijgestaan hebben zeker op zijn plaats. Daarbij moet ik wel opmerken dat het leeuwendeel van het werk is uitgevoerd door mijn vurige Italiaanse vriendjes.
Allereerst wil ik mijn co-promotor Huub Haaker bedanken. Je enthousiasme voor dit *Pyrococcus* onderzoek was enorm aanstekelijk. Je was een onuitputtelijke bron van ideeën en theorieën en ook al waren we het niet altijd met elkaar eens over de te volgen weg, het resultaat mag er zijn.
Ook mijn promotor Colja Laane heeft een belangrijke rol gespeeld. Eerst in Wageningen en later vanuit Delft heb je met veel belangstelling gewaakt over de voortgang van mijn onderzoek. Colja, bedankt voor het snelle nakijken van mijn artikelen en je kritische commentaar daarop.
In het lab heeft Hans Wassink een grote rol gespeeld, niet alleen bij het labwerk maar ook als de relativerende factor en de verzorger van onze goudvisjes. Hans, bedankt voor àl die zuiveringen en proeven die je gedaan hebt. Je stond altijd klaar om nog een conversie in te zetten of om monsters voor mij te nemen als ik weer eens op pad was!
Hendra, Koen, Roelco en Hanny -'mijn studenten'- bedankt voor jullie belangrijke en enthousiaste bijdragen aan mijn onderzoek. Koen, helaas sta je niet vermeld in dit proefschrift maar jouw werk heeft de basis gelegd voor hoofdstuk 5. Ik vond het begeleiden van jullie ontzettend leuk en leerzaam.
Naast Hans en 'mijn studenten' waren er nog andere 'bewoners' in lab 1-2. Fred, Peter-Leon, Pedro, Maria, Ilse, Robert, Marieke, Daniël, Petra en Juul: de gezelligheid en de labborrels op vrijdag zullen me bijblijven. Het is wel stil geworden na jullie vertrek naar Delft. Fred, ook bedankt voor de EPR experimenten. Peter-Leon en Pedro wil ik nog speciaal bedanken voor de

ferredoxine, AOR en andere enzymen die ik van jullie mocht gebruiken en voor de uitleg en hulp bij de electrochemie proeven.
Albert van der Padt, mijn dank is groot voor je bijdrage aan hoofdstuk 3. Door je kritische houding heb ik veel geleerd over optimaliseren en de statistiek die daarbij komt kijken.
Verschillende mensen bij Organische Chemie hebben mij geholpen met analyses en syntheses: Elbert van der Klift en Harm Niederlander bij de analyses op GC en HPLC en Ton Marcelis en Arie Koudijs bij de diverse syntheses.
Wim Doesburg ben ik dank verschuldigd voor de verschillende analyses die hij voor mij heeft uitgevoerd op de HPLC's bij Microbiologie.
De leden van de IOP begeleidingscommissie, onder bezielend voorzitterschap van Tom Kieboom, wil ik bedanken voor de belangstelling, de suggesties en de samenwerkingsverbanden die hieruit voortgekomen zijn. Op deze plaats bedank ik ook Annemarie en Ifoeng voor de samenwerking, tips en analyses van mijn monsters. Daarnaast waren Matthew, Robert, Annemarie, Ifoeng en Gerwin vrijwel altijd aanwezig bij de IOP-vergaderingen of de IOP-cursussen en dat was erg gezellig.
Professor Wandrey, Andreas Liese, Lasse Greiner and Rita Mertens, the collaboration was always on a distance but fruitfull. I enjoyed our discussions by e-mail and during our mutual visits. It has been a very pleasant experience.
Ik heb met veel plezier bij Biochemie gewerkt. Ik ga jullie niet allemaal bij naam noemen maar een aantal mensen wil ik speciaal bedanken. Laura en Martin, voor alle regeldingetjes, administratieve zaken en de praatjes. Sjef, voor alles wat met HPLC te maken heeft en de NMR experimenten. Volgens mij is er nu een grootverbruiker van HPLC-kolommen minder op de vakgroep. Adrie, voor alle hulp met eigenzinnige computers en printers. AIO's en niet te vergeten ex-AIO's van Biochemie, bedankt voor de gezelligheid, de kletspraatjes, pannenkoeken eten op dinsdag, de AIO-etentjes en de AIO-weekenden. Maarten, paranimf, fijn dat je mijn kamergenoot was. We hebben heel wat afgelachen en gepraat en dat zal ik zeker missen.
Collega's bij Bioway, bedankt dat jullie me ruim twee maanden konden missen om mijn proefschrift af te kunnen maken. Nu ga ik er full-time tegenaan en ik heb er zin in.
Suzanne en Mariëlle, het is mij gelukt, nu jullie nog. De eet-, kroeg- en filmafspraakjes moeten we maar weer oppakken als jullie in wat rustiger

vaarwater zijn beland en dan hebben we het over wat anders dan proefschrifen en promoveren.
Torsten, dankzij jou is er een omslag van dit boekje gekomen. Ik vind de 'Biempjes' geweldig mooi.

Op deze plaats wil ik ook mijn vrienden, familie en familie van Titus bedanken voor de belangstelling, steun en het 'even met iets anders bezig zijn'. Lieve mam en pap, ik wil jullie bedanken voor de ruimte om te leren, de liefde en belangstelling. Pap, het 'Pyromecoccus' tijdperk is nu teneinde maar we vinden wel een andere naam voor die beestjes waar ik nu mee werk. Jaap, ik ben blij met jou als paranimf aan mijn zijde. Nu staat er in elk geval één persoon in de houding op het podium.

Lieve Titus, de laatste regels van dit dankwoord zijn voor jou. Je hebt ze meer dan verdiend maar eigenlijk zijn er geen woorden voor, behalve 'Cliché'.

Eyke C.D. van den Ban - Exploring the reductive capacity of *Pyrococcus furiosus* - The reduction of carboxylic acids and pyridine nucleotides
Dutch: Verkenning van het reducerend vermogen van *Pyrococcus furiosus* - De reductie van carbonzuren en pyridine nucleotiden
Thesis Wageningen University - with summary in Dutch

Cover: Pictures of Porto di Levante, Vulcano Island, Italy; cartoons of *Pyrococcus furiosus* ('raging fireball')

The research described in this thesis was carried out in the framework of the Innovation Oriented Research Program on Catalysis (IOP Katalyse, IKA 96001) of the Dutch Ministry of Economy Affairs.