Check for updates

Advanced Synthesis & Catalysis

Accepted Article

Title: Formate dehydrogenase from Rhodococcus jostii (RjFDH) - A high-performance tool for NADH regeneration

Authors: Alexander Boldt and Marion Ansorge-Schumacher

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.202000536

Link to VoR: https://doi.org/10.1002/adsc.202000536

10.1002/adsc.202000536

Very Important Publication - VIP

FULL PAPER

DOI: 10.1002/adsc.202000536

Formate Dehydrogenase from *Rhodococcus jostii* (RjFDH) - A High-Performance Tool for NADH Regeneration

Alexander Boldt [a] and Marion B. Ansorge-Schumacher [a,*]

[a] Chair of Molecular Biotechnology, TU Dresden, Zellescher Weg 20b, 01217 Dresden
Fax: +49 351 463-39520; phone: +49 351 463-39518; e-mail: marion.ansorge@tu-dresden.de

Received: ((will be filled in by the editorial staff))

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. The use of formate dehydrogenases (FDHs) for regeneration of the important cofactor NADH in enzyme-catalysed synthetic reactions has several advantages over alternative systems. However, a major bottleneck for broad industrial applications is the low specific activity of the currently used FDHs. In this study, we introduce a novel NAD-dependent formate dehydrogenase from *Rhodococcus jostii* (RjFDH) with both high specific activity and stability. The enzyme was identified in a targeted database research and recombinantly obtained from *Escherichia coli*. RjFDH is a homodimer with a monomeric molecular mass of 44.7 kDa. The homology model shows that all amino acid residues of the NAD-dependent formate dehydrogenases are usually concerned with catalytic activity, substrate acceptance, and cofactor binding. The only substrate oxidised by these enzymes is formate. RjFDH had a specific activity of 19.9 U mg^{-1} at 22 °C along with unimpaired activity and high stability over a broad pH range. The K_m values for formate and NAD^+ were 7.3 and 0.098 mmol L^{-1}, respectively. The optimum temperature was found to be 50 °C, at which the enzyme activity increased to about 318%. Both activity and thermal stability were higher than those of the FDH from *Candida boidinii* (CbFDH), which is the standard enzyme currently in use for cofactor regeneration. Different solvents roughly had the same impact on the activity and stability of both RjFDH and CbFDH. The superior performance of RjFDH over CbFDH as a regeneration system for NADH was demonstrated for the synthesis of L-*tert*-leucine as well as (*S*)-1-phenylethanol. In both systems, the concentration of RjFDH used was only one-third of the concentration of CbFDH required to achieve comparable conversion rates. Rational designing provided a promising NADP-accepting variant. Thus, RjFDH has a great potential to serve as an alternative system for NADH regeneration in enzyme-catalysed synthetic reactions.

Keywords: Biocatalysis, Cofactors, Green chemistry, Oxidoreduction, Protein expression

Introduction

Cofactors including non-protein chemicals and metal ions are mandatory for the catalytic activity of many enzymes. Hence, their appropriate provision plays an important role in synthetic biocatalysis. Most prominently, nicotinamide adenine dinucleotide (phosphate), $NAD(P)^+$, or its reduced form, NAD(P)H, is involved in the production of important building blocks and fine chemicals employing oxidoreductases [1]. In fact, for chemical reactions with distinct substrates, $NAD(P)^+$ acts as a stoichiometric agent. However, due to its high cost, it is usually not economically feasible to use stoichiometric amounts of $NAD(P)^+$ in industrial processes [1,2]. Thus, efficient strategies for the regeneration of $NAD(P)^+$ or NAD(P)H are crucial for large-scale synthetic applications [3]; therefore, various chemical, electrochemical, microbial, and enzymatic methods have been developed for this purpose [3,4]. For industrial purposes, the enzymatic approach is often

This article is protected by copyright. All rights reserved.

Accepted Manuscript

preferred owing to its high selectivity and efficiency [1,2]. However, till date, only a few enzymes suitable for the enzymatic regeneration of NAD(P)H are known; among them are NAD-dependent formate dehydrogenases.

First described in 1950, NAD-dependent formate dehydrogenases (FDH, EC 1.2.1.2) catalyse the oxidation of formate to carbon dioxide [5,6,7]. This oxidation is coupled to the reduction of NAD^+ to NADH. During the catalytic step, direct transfer of hydride ion from formate to NAD^+ takes place [8]. Several advantages of FDHs over alternative enzymes make them particularly desirable for cofactor regeneration: 1. During formate oxidation and associated NAD^+ reduction, only inert CO_2 is produced as a by-product. The CO_2 produced can easily be removed from the medium pushing the reaction equilibrium towards product formation [1]. 2. The thermodynamics of formate oxidation are quite favourable because the back reaction i.e. fixation of CO_2 to formate, is prevented by thermodynamic barriers under standard conditions [1,9]. 3. Formate as a substrate is innocuous for most enzymes as well as inexpensive [1,10,11]. 4. NAD-dependent formate dehydrogenases do not incorporate sensitive metal ions or prosthetic groups and are known to have a wide pH optimum which enables coupling with a broad range of NADH-dependent oxidoreductases.

The widely employed enzyme for the regeneration of NADH is the formate dehydrogenase of the yeast *Candida boidinii* (CbFDH) [1]. A prominent industrial application of this enzyme is the production of L-*tert*-leucine [12,13,14]. Because of its bulky and inflexible *tert*-butyl side chain, L-*tert*-leucine is widely used in the pharmaceutical and cosmetic industry as a precursor molecule for various drugs such as HIV protease inhibitors or tumour-fighting agents [14,15,16]. Since chemical methods of L-*tert*-leucine production have major drawbacks, a biocatalytic approach has been established, yielding excellent enantioselectivity under mild conditions [16,17]. In this process, enantiopure L-*tert*-leucine is synthesised on a ton-scale through reductive amination of trimethylpyruvic acid catalysed by leucine dehydrogenase (LeuDH) from *Lysinibacillus sphaericus*, previously *Bacillus sphaericus* (Figure 1) [1]. The process, however, became industrially feasible only through the establishment of CbFDH-catalysed cofactor regeneration. Other applications of CbFDH include enzymatic synthesis of chiral aromatic α-keto esters or L-lactic acid, with xylose reductase and lactic acid dehydrogenase as catalysts, respectively [18,19].

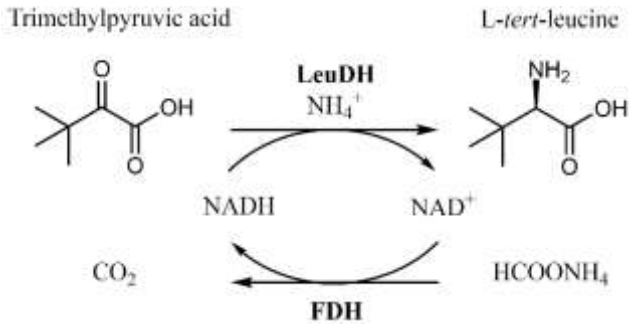


Figure 1. L-*tert*-leucine formation from trimethylpyruvic acid catalysed by leucine dehydrogenase (LeuDH) and NADH regeneration with formate dehydrogenase (FDH).

To date, the main bottleneck of cofactor regeneration employing CbFDH is its low specific activity, of about 6 U mg^{-1} [20]. Alternative NAD-dependent FDHs capable of replacing CbFDH in cofactor regeneration have not been described in the literature, to the best of our knowledge. Although enzymes with higher formate-oxidising activity were discovered, they considerably lacked stability. A recent example is the formate dehydrogenase of *Ancylobacter aquaticus* [9].

In the present study, we report a novel NAD dependent formate dehydrogenase with both high catalytic activity and stability. This enzyme was derived from a rational database research based on the amino acid sequences of three NAD-dependent FDHs with the highest reported activities so far. The coding gene was cloned and expressed in *Escherichia coli*, and the resulting enzyme was described. The cofactor regeneration performance of this enzyme was investigated for the synthesis of L-*tert*-leucine as well as (*S*)-1-phenylethanol, and benchmarked against the established formate dehydrogenase from *C. boidinii* (CbFDH). Rational designing provided a promising NADP-accepting variant with high cofactor regeneration ability.

Results and Discussion

Based on the assumption that FDHs with increased formate-oxidising activity will be structurally related to already known FDHs having high activity, a database research was performed using the amino acid sequences of the highly active NAD-dependent FDHs

This article is protected by copyright. All rights reserved.

Accepted Manuscript

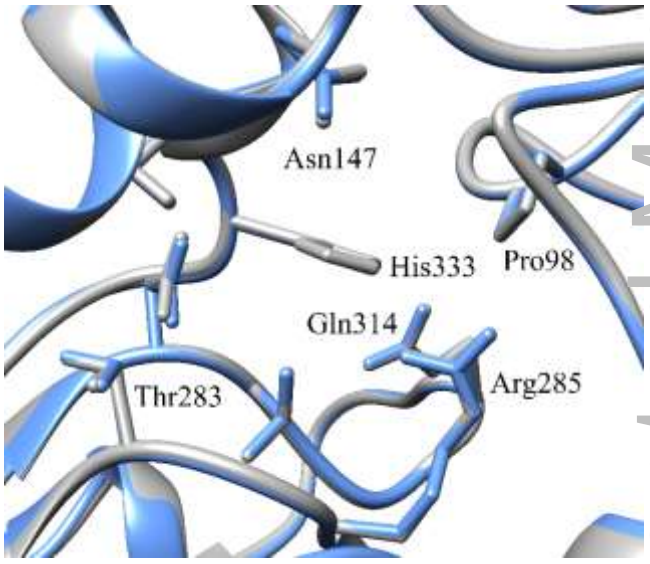
from *A. aquaticus*, *Thiobacillus* KNK65M, and *Pseudomonas* sp. 101 [8,9,21,22] as templates. The three sequences were aligned against various NAD-dependent FDHs using the BLASTP database (Figure S1, supplementary material). The search was restricted to bacterial FDHs because bacterial FDHs have been reported to have overall higher specific activities and superior stabilities as compared to eukaryotic FDHs [8]. Bacterial FDHs are often identified by a prolonged N-terminal loop, which is considered to contribute to its stability [23]. In addition, only candidates with more than 385 amino acid residues were taken into consideration assuming that the additional C-terminal extension that was found in many highly active FDHs might also play a role in its activity [24]. Among the resulting hits, seven FDH candidates with 90–60% amino acid sequence identity to the primary sequences were chosen. Their genes were expressed in *E. coli* and the activity of the recombinant enzymes was determined in the crude cell extracts (Table 1). FDH from
R. jostii showed the highest enzyme activity. Notably, it also exceeded the documented maximum activity of FDH from *C. boidinii* [20], even in its crude form. To the best of our knowledge, this FDH has not been described in the literature so far.

Table 1. Amino acid identities with templates from *A. aquaticus*, *Thiobacillus* sp. KNK65M, and *Pseudomonas* sp. 101, and chain length and specific catalytic activities of FDH candidates.

Donor organism	Sequence identity with *A. aquaticus* (%)	Sequence identity with *Thiobacillus* sp. KNK65M (%)	Sequence identity with *Pseudomonas* sp.101 (%)	Chain length [aa]	Specific activity [U mg^{-1}]
Cupriavidus necator	61	64	65	386	0.03
Granulicella mallensis	74	76	78	386	1.6
Hyphomicrobium denitrificans	75	77	77	399	2.5
Methylohalobius crimeensis	72	72	76	402	1.3
Methylotenera versatilis	74	74	75	402	0.4
Rhodococcus jostii	84	86	84	400	6.3
Sciscionella marina	64	67	68	396	0.8

RjFDH, having a chain length of 400 amino acids, belongs to the longest NAD-dependent FDHs described so far. The sequence of this enzyme is 84%, 86%, and 84% identical to the very active template FDHs from *A. aquaticus*, *Thiobacillus* sp. KNK65M and *Pseudomonas* sp. 101, respectively. However, sequence identity to the CbFDH was only 41%. One subunit of this recombinant enzyme corresponds to a molecular weight of 44.7 kDa (including the adjusted His-Tag; Figure S2, supplementary material). A homology model of RjFDH (Figure S3, supplementary material) built with the SWISS-MODEL algorithm [25] on the crystal structure of *Pseudomonas* sp. 101 FDH (RCSB ID: 2NAD) shows a particularly high identity between the active centres of the two enzymes in terms of spatial structure as well as amino acid composition. Figure 2-A illustrates the highly conserved amino acids Arg285 and Asn147, which are essential for formate binding, and Gln314 and His333, which are crucial for catalysis [26]. Thr283, Asp309, Ser335, and Gly336 stabilise the nicotinamide ring in the active centre and are conserved in the FDHs from *C. boidinii*, *Moraxella* sp., *A. aquaticus*, and *Thiobacillus* sp. KNK65M (Figure S1, supplementary material).

A



Accepted Manuscript

This article is protected by copyright. All rights reserved.

10.1002/adsc.202000536

B

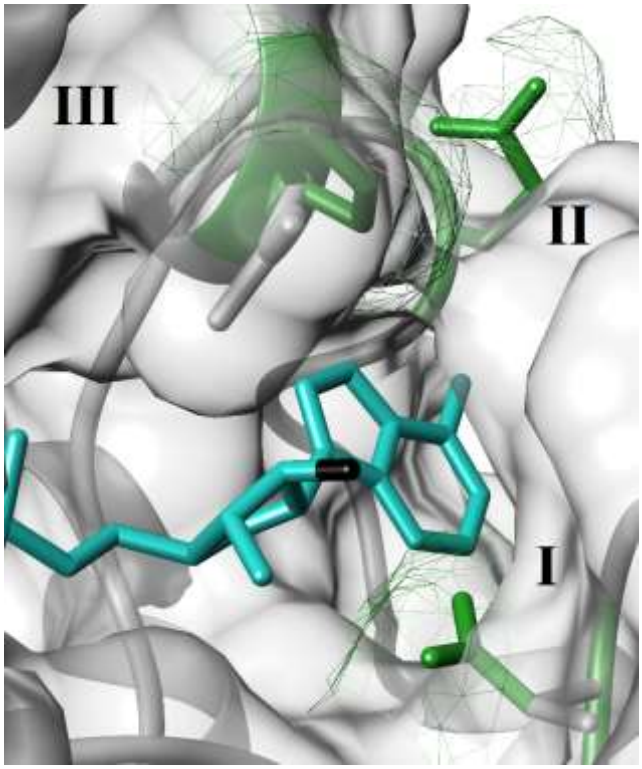


Figure 2. Active centre (**A**) and cofactor-binding pocket (B) of RjFDH. A: Superposition of the homology model of RjFDH (white) with the crystal structure of FDH from *Pseudomonas* sp. 101 (blue). **B**: Spatial enlargement of the cofactor-binding pocket due to the exchange of three amino acids. **I**: Asp222Ser, **II**: Glu261Gly, **III**: His380Arg. NAD^+ is illustrated in turquoise, and the position of the bulky phosphate residue is shown in black. The green mesh illustrates the wild-type cofactor-binding pocket

As typical for most FDHs, this enzyme also accepted only formate as a substrate; glycerol, 2-propanol, acetaldehyde, ethanol, methanol, and formaldehyde at a concentration of 25 mmol L^{-1} (pH 7,0; 22 °C) were not converted by RjFDH. When $NADP^+$ was used as cofactor, RjFDH activity was approximately 4% of the activity when NAD^+ was used. The enzyme activity was lost after 96 h of incubation in the presence of 1 mmol L^{-1} Cu^{2+}, but remained unaffected by other metal ions (Ni^{2+}, Fe^{2+}, Fe^{3+}, Li^+, Mn^{2+}, and Mg^{2+}) under the same conditions.

For reduction of NAD^+ by RjFDH, a V_{max} of 19.9 ± 2.1 U mg^{-1} (22 °C, pH 7.0) and a catalytic efficiency of 117.4 mmol L^{-1} min^{-1} was obtained. This exceeds the reported values of CbFDH by a factor of 3 and 4.5, respectively (Table 2). RjFDH activity obtained was also higher than that reported for NAD-dependent FDHs from *Paracoccus* sp. 12-A, *Pseudomonas* sp. 101, *Moraxella* sp., and *A. aquaticus* [8,9,27]. On the other hand, K_m values for both NAD^+ and formate were in the same range as those of other FDHs [8]. This makes RjFDH the most efficient enzyme among the non-metal–containing NAD-dependent FDHs described till date.

Table 2. Kinetic parameters for formate oxidation and NAD^+ reduction by RjFDH and CbFDH. Activity was measured at 22 °C and pH 7.0.

FDH	V_{max} [U mg^{-1}]	K_m NAD^+ [mmol L^{-1}]	K_m $HCOO^-$ [mmol L^{-1}]	k_{cat} / K_m $HCOO^-$ [mmol L^{-1} min^{-1}]
RjFDH	19.9 ± 2.1	0.098 ± 0.05	7.2 ± 1.8	117.4
CbFDH	6.0 [20]	0.05 [20]	5.6 [20]	25.4 [28]

An explanation for the differences in the activity of NAD-dependent FDHs has not yet been established. However, considering the high degree of conservation, the differences in activity certainly cannot be related to the amino acid composition which is directly involved in the catalytic reaction. For the reverse reaction, i.e. reduction of CO_2, it was assumed that high activity was related to the presence of an elongated C-terminus. In FDH from *Thiobacillus* sp., which is highly active, this elongation comprises 26 amino acids. The additional amino acids seem to mask the active site of the enzyme, thus excluding solvent molecules [24]. Computer simulations show that this constellation lowered the activation energy of CO_2 reduction by 10 kcal mol^{-1} as compared to reactions catalysed by enzymes without an elongated C-terminus, similar to CbFDH.

Preliminary experiments in our lab indicate that the C-terminal elongation might also benefit formate oxidation. We created variants of RjFDH, in which the C-terminus was truncated by 5, 15, 25, and 35 amino acids, and determined their catalytic activity. Deletion of 5 to 15 amino acids decreased the activity to about 90%, while truncation of 25 amino acids decreased the activity to 10%, and truncation of 35 amino acids resulted in complete loss of enzyme activity. From the homology model, it can be deduced that this effect is not due to the loss of amino acids from the active centre. The C-terminal elongation can be found in many NAD-dependent FDHs having better formate-oxidising activity than CbFDH, including FDHs from *Paracoccus* sp-12-A, *Pseudomonas* sp. 101, and *A. aquaticus*. On the other hand, these enzymes show only half of the specific activity of RjFDH, which suggests the contribution of factors other than the C-

Accepted Manuscript

This article is protected by copyright. All rights reserved.

10.1002/adsc.202000536

terminal length to catalytic activity. One such factor might be the composition of the N-terminal region of the enzymes, as it has been suggested that a special loop on the prolonged N-terminus of bacterial FDHs contributes to the enzymatic reaction [8,23].

The pI of RjFDH was calculated to be 5.4, and the highest formate-oxidising activity was obtained at pH 7.0 (Figure 3-A). The pH optimum for this enzyme was wide and ranged from 6.0 to 9.0, which complies with the features of other NAD-dependent FDHs. According to the findings of Tishkov et al. (1996), this correlates with the presence of glutamine 97 and 342 in close proximity to the active centre [29]. Nearly no loss of activity was observed when RjFDH was incubated at pH 6.0–9.0 for 24 h (Figure 3-B). However, the activity dropped to 75% after 24 h of incubation at pH 10.0, and to 16.6% and 0.75% at pH 5.0 and 4.0, respectively. The wide pH optimum and high stability of RjFDH in that pH range ensure broad applicability for cofactor regeneration [30].

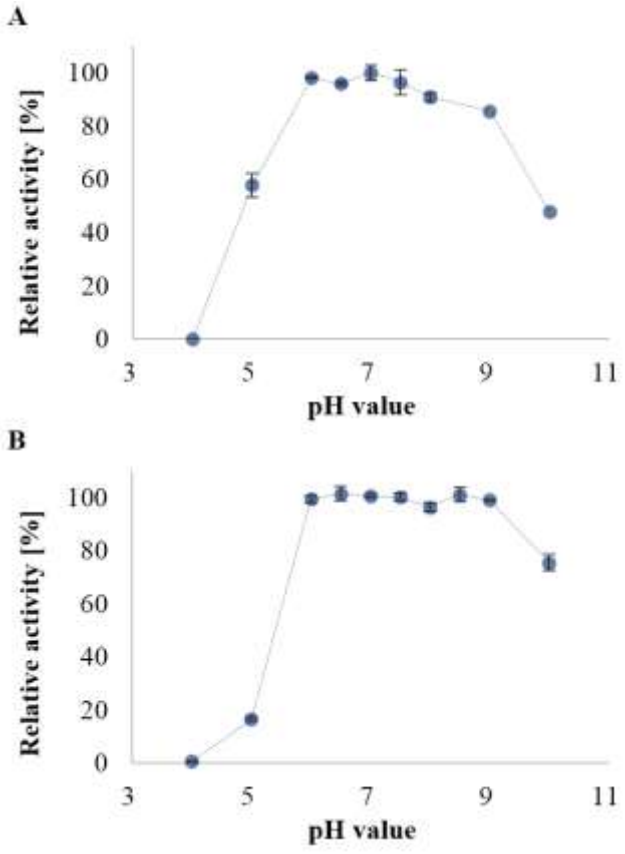


Figure 3. Effect of pH on formate oxidation (**A**) and stability (**B**) of RjFDH. Enzyme activity was determined using 3.5 µg mL^{-1} RjFDH at 25 °C in 100 mmol L^{-1} buffer at different pH. Enzyme activity of fresh enzyme measured at pH 7.0 was used as reference (100% relative activity refers to 20.4 U mg^{-1} at 25 °C). Each result represents the average of three individual measurements. A: Residual activity at different pH values. B: Residual activity after 24 h of incubation.

Maximum activity of RjFDH was obtained at 50 °C. At this temperature, the activity was about 320% of the activity at 22°C (Figure 4-A). The temperature maximum is in the same range as that of the FDH from *A. aquaticus* and slightly lower than that of the FDHs from *Thiobacillus* sp. KNK65M and *C. boidinii* [9,20,22].

Thermal stability was investigated for molecular unfolding as well as deactivation at increasing temperatures. The unfolding experiments utilised the changes in intrinsic fluorescence of tryptophan and tyrosine residues within the enzyme at an absorption wavelength of 350 nm and an excitation wavelength of 330 nm. Transitions in the overall folding state of RjFDH were observed at an inflation temperature of 73.0 ± 0.19 °C. For CbFDH, a slightly lower inflation temperature of 70.8 ± 0.52 °C was observed. Deactivation experiments revealed that the thermal stability of RjFDH and CbFDH diverged at 50 °C and above (Figure 4-B). At 55 °C, the residual activity of RjFDH was about 70% after 20 min of incubation, and 50% activity was observed after 20 min of incubation at 60 °C. In contrast, CbFDH only showed 20% residual activity after 20 min of incubation at 60°C. This is in accordance with previous studies, which reported 50% residual activity of CbFDH after 20 min of incubation at 55 °C [20,31]. Alternatively, FDHs from *A. aquaticus*, *Moraxella* sp., and *Thiobacillus* sp. KNK65M lost 40, 50, and 55% activity, respectively, after only 10 min of incubation at 55 °C [9,22,32].

The half-life time of the RjFDH was determined to be 20.4 hours at a temperature of 50 °C (k_d = 0.034). Under comparable conditions, a half-life time of only 4.7 hours was reported for CbFDH [33]. All results indicate that RjFDH is a suitably stable NAD-dependent FDH for synthetic application.

Accepted Manuscript

This article is protected by copyright. All rights reserved.

10.1002/adsc.202000536

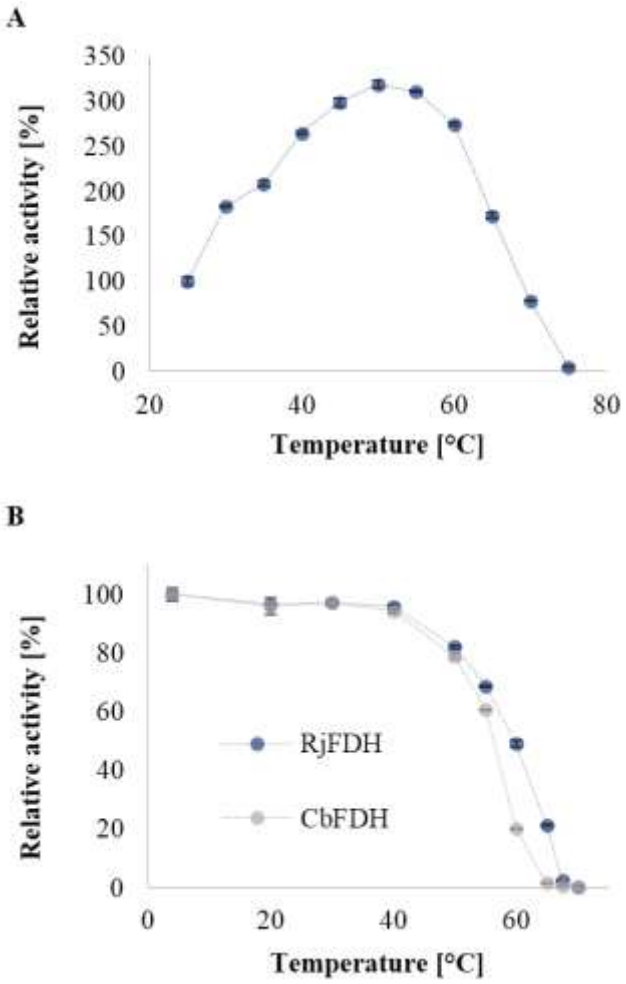


Figure 4. Effect of temperature on activity (**A**) and stability (**B**) of RjFDH. Enzyme activity was determined using 7 µg mL^{-1} RjFDH at pH 7.0 after 20 mins of incubation at the selected temperature. Each measurement was performed with fresh enzyme. The activity was related to the maximum activity at 22 °C (100% relative activity refers to a specific activity of 19.9 U mg^{-1}). Each result represents the average of three individual measurements.

Considering the synthetic application of enzyme, the impact of organic solvents on RjFDH in mono- and biphasic reaction systems was determined. Both these systems are frequently applied in biocatalysis for the conversion or production of compounds with low water solubility [34,35,36].

It was found that addition of water-miscible organic solvents, including acetone, dimethyl sulfoxide, ethylene glycol, ethanol, and acetonitrile, to the standard reaction set-up had little impact on RjFDH activity up to a solvent concentration of 5% (v/v) (Figure 5). At 25% (v/v) solvent concentration, the enzyme activity dropped to a varying extent, except in the presence of DMSO, which increased the activity of RjFDH to about 134%. This is consistent with the report by Demchenko et al. (1990), which demonstrated a possible increase in FDH activity by organic solvents [37]. A complete loss of RjFDH activity was observed in 50% (v/v) acetone as well as ethanol. Altogether, the relative activity of RjFDH was very similar to that of CbFDH in different solvents (Figure 5). However, the RjFDH appears to be a bit more tolerant towards acetonitrile, but slightly more sensitive towards ethanol and ethylene glycol.

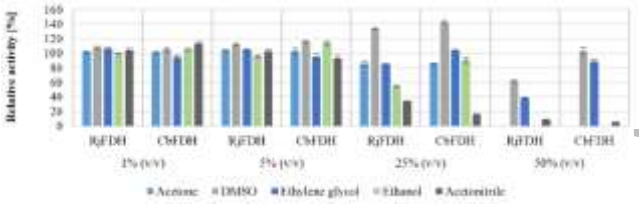
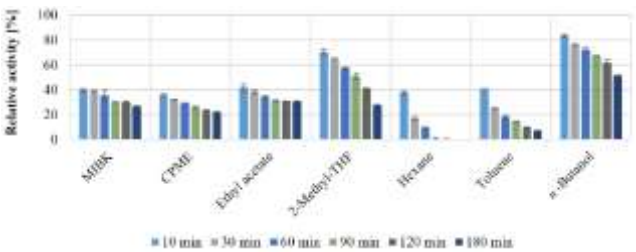


Figure 5. Activity of RjFDH and CbFDH in the presence of water-miscible organic solvents. The solvents were added to the assay mixture and enzyme activity was immediately measured at standard conditions. The activity was related to the maximum enzyme activity in a solvent-free system. Each result represents the average of three individual measurements.

After incubation of RjFDH in biphasic systems consisting of water and methyl isobutyl ketone (MIBK), cyclopentyl methyl ether (CPME), ethyl acetate, 2-methyltetrahydrofuran (2-methyl-THF) hexane, toluene, or *n*-butanol, at a fixed ratio of 1:1 (v/v) at 22 °C, a decrease in activity was observed in all systems (Figure 6). High residual activities were obtained in systems containing *n*-butanol and 2-methyl-THF, reaching 72% and 58%, respectively, after 60 min. With MIBK, CPME, and ethyl acetate systems, a very high initial activity drop of about 60% was observed; however, the activity continued to decrease gradually over time. With hexane and toluene systems, the activity not only dropped to 40% within the first 10 min, but continued to decrease rapidly even after that. Interestingly, the solvent-tolerant NAD-dependent FDH from *Bacillus* sp. F1 behaved similar in hexane, although the residual activity in *n*-butanol system was slightly higher (94%) [38].



Accepted Manuscript

This article is protected by copyright. All rights reserved.

10.1002/adsc.202000536

Figure 6. Residual activity of RjFDH after exposure to non–water-miscible organic solvents in a biphasic system. The ratio of water to organic solvent was 1:1. The enzyme was incubated in the system at *22 °C*. Samples were collected from the aqueous phase at defined time intervals and transferred to a standard assay for activity measurements. The activity was related to the maximum activity in a solvent-free system. Each result represents the average of three individual measurements.

The performance of RjFDH as a regeneration system for NADH was demonstrated for the synthesis of *L-tert*-leucine and (*S*)-1-phenylethanol, and compared to the regeneration capacity of the commercially established CbFDH under the same conditions.

Synthesis of *L-tert*-leucine was accomplished with LeuDH from *L. sphaericus*, which complies with the industrially established system [1,39]. LeuDH was added in excess over FDH to ensure that NADH regeneration by FDH was the rate-limiting step in the reaction. The decrease in substrate concentration was monitored, which, due to the documented selectivity of LeuDH [14,16,17,40], directly reflects *L-tert*-leucine production. Figure 6-A illustrates the rapid substrate consumption (initial substrate concentration: 10 mmol L^{-1}) by RjFDH at a concentration of 25 µg mL^{-1}. After 60 min, 95% of trimethylpyruvic acid (TMP) was converted to *L-tert*-leucine. However, with the same concentration of CbFDH, the substrate consumption rate was considerably lower, and only 47% was converted after 60 min, demonstrating the superior catalytic efficiency of RjFDH. In fact, a three-fold higher concentration of CbFDH was required to obtain cofactor regeneration equal to that obtained by RjFDH.

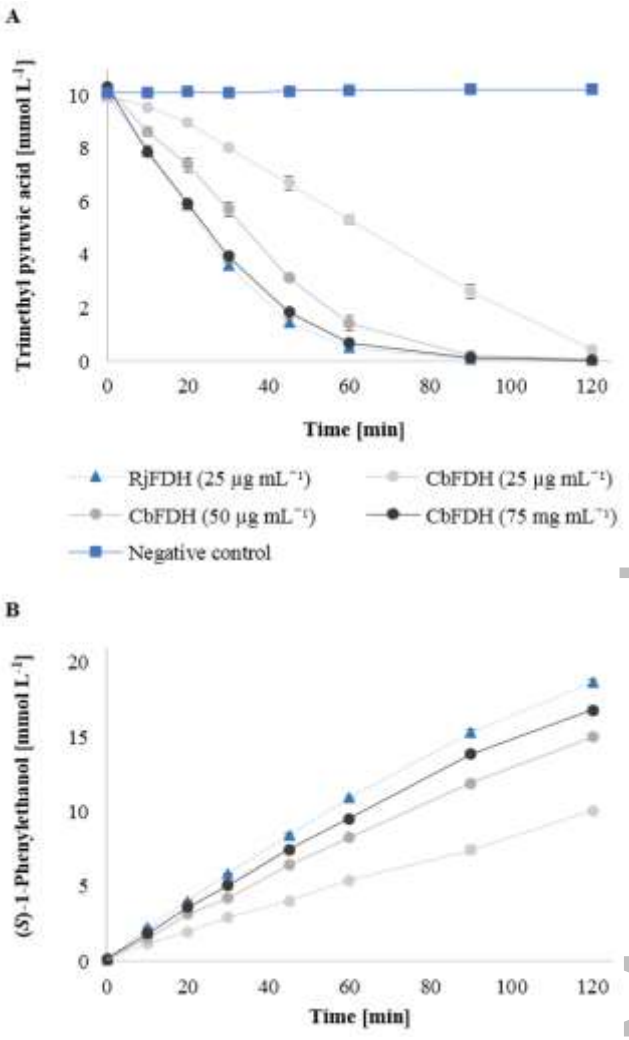


Figure 7. Catalytic performance of NADH-dependent dehydrogenases with FDHs for cofactor regeneration. All calculations were based on the average of three individual measurements. **A**: TMP concentration in a combined system of LeuDH and FDHs at different concentrations. **B**: (*S*)-1-phenylethanol concentration in a combined system of ADH-A and FDHs at different concentrations.

Very similar results were obtained when RjFDH and CbFDH were coupled with the NAD-dependent secondary ADH-A from *R. ruber*. ADH-A is a versatile catalyst for the asymmetric reduction of ketones [41] and was used for the synthesis of (*S*)-1-phenylethanol. Like LeuDH, it was added in excess over FDH. Figure 6-B illustrates the increasing (*S*)-1-phenylethanol concentration over time (initial substrate concentration: 25 mmol L^{-1}). The use of 25 µg mL^{-1} RjFDH yielded about 75% of the product within 120 min. In contrast, only 40% (*S*)-1-phenylethanol was obtained with the same amount of CbFDH within the same time period. Moreover, even with 75 µg mL^{-1} CbFDH, the product yield was only

Accepted Manuscript

This article is protected by copyright. All rights reserved.

67%, that is, lower than that obtained by 25 µg mL^{-1} RjFDH.

Both model systems demonstrated superior performance of RjFDH as compared to the standard enzyme. Thus, RjFDH shows great potential to replace the current standard enzyme in cofactor regeneration.

Studies on the homology model suggested the possibility of improving RjFDH activity with $NADP^+$ as the cofactor by decreasing the steric hindrance of the binding pocket for the phosphate moiety. Suitable amino acid residues for this purpose and appropriate substitutions were predicted using the online tool CSR-SALAD [42]. An RjFDH variant with the amino acid exchanges Asp222Ser, Glu261Gly, and His380Arg was created, in which the cofactor-binding pocket was spatially enlarged, while all electrostatics were maintained (Figure 2-B). The specific activity of this variant with $NADP^+$ as the cofactor was 3.73 U mg^{-1}, which was over 400-fold higher than that of the wild-type enzyme (Table S4, supplementary material). The activity increase corresponded to a 10-fold decrease in the K_m value for $NADP^+$ to 0.15 mmol L^{-1}. Thus, an $NADP^+$-accepting RjFDH variant with an activity almost similar to that of CbFDH in NADH regeneration was obtained, indicating a potential for further improvement.

Conclusion

NAD-dependent FDH from *Rhodococcus jostii* (RjFDH) was found to have high formate-oxidising activity over a broad pH and temperature range with good thermal stability. Thus, RjFDH can serve as a suitable alternative system for NADH regeneration. As only low concentrations of this enzyme are required for efficient NAD^+ reduction in a coupled synthetically active reaction system, RjFDH can reduce production costs and contribute to the expansion of biocatalytic processes. This could be further enhanced with improvement of the enzyme through molecular engineering Moreover, we obtained a first variant of RjFDH with a promising acceptance of $NADP^+$ as a cofactor.

Experimental Section

Materials

Unless stated otherwise, all chemicals and oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth GmbH & Co. KG (Karlsruhe, Germany) or abcr GmbH (Karlsruhe, Germany). Media components were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and enzymes for molecular cloning were obtained from New England Biolabs Inc. (Ipswich, USA).

Construction of expression vectors

The genes coding for formate dehydrogenase (FDH) in *Rhodococcus jostii* (RjFDH), *Cupriavidus necator*, *Granulicella mallensis*, *Hyphomicrobium denitrificans*, *Methylohalobius crimeensis*, *Methylotenera versatilis*, *Sciscionella marina*, and leucine dehydrogenase (LeuDH) in *Lysinibacillus sphaericus* were obtained via polymerase chain reaction (PCR) of genomic DNA purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The sequences of the primers used are mentioned in supplementary material (Table S1). The PCR mixture contained 1-× Phusion HF buffer, 0.5 µmol L^{-1} of each primer, 0.2 mmol L^{-1} of each dNTP, 5% (v/v) dimethyl sulfoxide (DMSO), 44–100 ng genomic DNA, and 0.05 U $µL^{-1}$ Phusion® high-fidelity DNA polymerase (New England Biolabs Inc., USA). Gibson assembly was used to insert the genes into a pET-22b(+) expression vector (Merck, Darmstadt, Germany) according to the standard protocol [43] to create proteins with a C-terminal polyhistidine tag. The insertion was carried out under the following conditions: 60 min at 50 °C followed by 15 min at 72 °C. After Gibson assembly, *E. coli* TOP10 cells were transformed with the gene construct and positive clones were screened by colony PCR. Plasmids were isolated from the positive clones, and sequenced by GATC Biotech (Konstanz, Germany). The plasmids pET22b_ADH-A, harbouring the alcohol dehydrogenase A (ADH-A) gene from *Rhodococcus ruber*, and pET22b_CbFDH harbouring the FDH gene from *C. boidinii*, were obtained from our laboratory stock.

Recombinant expression of the FDH and LeuDH genes

Vectors harbouring the FDH and LeuDH genes were transformed into *E. coli* BL21 (DE3) for expression. The bacteria were cultured overnight at 37 °C in 5 mL lysogeny broth (LB) containing 100 mg mL^{-1} ampicillin. A total volume of 500 mL of the main culture was inoculated with 50 µL of preculture and

Accepted Manuscript

This article is protected by copyright. All rights reserved.

the cells were grown at 37 °C until an optical density of 0.6 was reached at 600 nm (OD_{600}). Gene expression was induced by addition of 0.1 mmol L^{-1} Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the temperature was decreased to 20 °C. After 40 h of incubation, the cells were harvested by centrifugation (4000 rpm for 15 min at 4°C) and stored in a freezer at −20 °C.

Enzyme purification

Cell pellets obtained by centrifugation were resuspended in 50 mmol L^{-1} potassium phosphate buffer (pH 7.0 for FDH-containing cells and pH 8.0 for LeuDH-containing cells). Phenylmethylsulfonyl fluoride (PMSF) was added to the suspension at a final concentration of 1 mmol L^{-1}. A French press was applied for cell lysis, utilizing three cycles at a pressure of 12000 psi. After lysis, cell debris were removed by centrifugation (20000 rpm, 90 min and 4°C) and the clear lysate was used for protein purification by immobilised metal affinity chromatography (IMAC). IMAC involved a HisTrap FF Crude 5 mL column (GE Healthcare Life Sciences, Chicago, USA) in an ÄKTA FPLC system (GE Healthcare Life Sciences, Chicago, USA). The column was equilibrated with 25 mL of washing buffer (500 mmol L^{-1} NaCl, 50 mmol L^{-1} potassium phosphate at pH 7.0) and 4% (v/v) elution buffer (500 mmol L^{-1} NaCl, 500 mmol L^{-1} imidazole, 50 mmol L^{-1} potassium phosphate, pH 7.0). The lysate was applied to the ÄKTA system, the flow rate was set to 1 mL min^{-1}, and the step gradient was set until the concentration of imidazole reached 500 mmol L^{-1}. The whole flow-through was fractionated and the fractions containing the recombinant FDH or LeuDH were tested for their respective activities. Fractions with the highest activity were pooled and dialysed against potassium phosphate buffer (50 mmol L^{-1}, pH 7.0) for 20 h at 6 °C. ADH-A was purified by heat precipitation at 70 °C for 50 min followed by centrifugation at 20.000 rpm for 45 min at 4°C. Protein concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at a wavelength of 280 nm and a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to the method of Laemmli [44] on a SERVA BlueVertical™ PRiME™ (SERVA Electrophoresis GmbH, Heidelberg, Germany) using 12% (v/v) acrylamide [44]. Samples were incubated in Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% (m/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.025% bromophenol blue) at 95°C for 10 min (16 µL total volume) and loaded onto the gel after cooling on ice. The protein standard used was 5 µL of protein colour standard (New England Biolabs Inc., Ipswich, MA, USA). Electrophoresis was performed for 80 min at 180 V. Proteins were visualised by Coomassie brilliant blue R-250 staining.

Determination of enzyme activity

FDH activity was determined by measuring the reduction of NAD^+ to NADH in 1 mL reaction mixture at 22 °C using a Hach-Lange DR-3900 spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany) at a wavelength of 340 nm. The standard assay was composed of 100 mmol L^{-1}phosphate buffer (pH 7.0), 100 mmol L^{-1} sodium formate, and 2.5 mmol L^{-1} NAD^+. The reaction was started by adding RjFDH to the assay. For kinetic measurements, the enzyme concentration was set to 3.5 µg mL^{-1}. K_m and V_{max} were calculated from the measurements and various concentrations of NAD^+ and formate were analysed using hyperbolic regression with Hyper32 software (https://hyper32.software.informer.com/). One unit was defined as the reduction of 1 µmol NAD^+ per minute. To measure the pH dependency of enzyme activity, different buffers (pH 4.0-5.0: sodium acetate, pH 6.0-8.0: potassium phosphate, pH 9.0: triethanolamine (TEA), pH 10.0: 3-(Cyclohexylamino)-1-propanesulfonic acid) were used at a final concentration of 100 mmol L^{-1}. LeuDH activity was determined by measuring the oxidation of NADH to NAD^+ in 1 mL of reaction mixture at 22 °C using a Hach-Lange DR-3900 spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany) at a wavelength of 340 nm. The standard assay was composed of 100 mmol L^{-1} TEA buffer (pH 8.5), 200 mmol L^{-1} ammonium formate, 20 mmol L^{-1} trimethylpyruvic acid (TMP), and 0.5 mmol L^{-1} NADH. All activities were calculated as the average of three independent experiments.

Determination of optimum temperature and thermal stability

This article is protected by copyright. All rights reserved.

Accepted Manuscript

To determine thermal stability, 30 µL of purified RjFDH was incubated at different temperatures for 20 min in a thermocycler (Mastercycler personal; Eppendorf AG, Hamburg). The residual activity after 20 min of incubation was measured photometrically, as described previously. RjFDH incubated on ice was used as a reference. Thermal unfolding experiments were performed on a Tycho spectrofluorometer (Nanotemper Technologies, Munich) with concentrated enzyme (as obtained after purification) as well as 1:4 diluted enzyme. The optimum temperature was investigated in the range of 30–75 °C using a Cary 60 UV-Vis spectrophotometer. Cuvettes were preheated at the respective temperatures and the reaction was carried out under standard conditions.

Determination of solvent impact on enzyme activity

Impact of the water-miscible polar solvents DMSO, acetonitrile, ethanol, acetone, and ethylene glycol on the activity of RjFDH was measured by addition of the respective solvents in different concentrations (1–50% [v/v]) to the reaction mixture. The effect of a two-phase system consisting of water and the immiscible organic solvents methyl isobutyl ketone (MIBK), cyclopentyl methyl ether (CPME), ethyl acetate, 2-methyltetrahydrofuran, hexane, toluene, and *n*-butanol, on RjFDH activity was investigated by mixing 500 µL of 1:10 diluted RjFDH (50 mmol L^{-1} phosphate buffer, pH 7.0) and 500 µL of organic solvent at 22 °C in an overhead shaker (60 rpm). Ten microlitres of the aqueous phase was collected at defined time-points and subjected to standard activity assay.

Determination of cofactor regeneration

ADH-A from *R. ruber* [23] and different concentrations of RjFDH or CbFDH, were added to 25 mmol L^{-1} acetophenone, 100 mmol L^{-1} potassium formate, and 0.25 mmol L^{-1} NAD^+ in 100 mmol L^{-1} phosphate buffer (pH 7.0) to a total volume of 5 mL. ADH-A was always added in excess. Samples were collected at defined time-points and analysed for (*S*)-1-phenylethanol using gas chromatography. Product formation was measured using a Shimadzu GC2010-Plus (Kyoto, Japan) equipped with a Hydrodex-γ-DiMOM (0.25 mm, 50 m) column at 115 °C. (*S*)-1-phenylethanol was detected at a retention time of 7.83 min. Likewise, LeuDH and RjFDH were added to 200 mmol L^{-1} ammonium formate, 10 mmol L^{-1} TMP, and 0.25 mmol L^{-1} NAD^+ in 100 mmol L^{-1} TEA buffer (pH 8.5) to a total volume of 5 mL. LeuDH was always in excess. Each set-up was stirred on a magnetic stirrer at 22 °C. Samples were taken at defined time-points and analysed for TMP consumption using a KNAUER Smartline HPLC (Berlin, Germany) system equipped with a LiChrosorb RP-18 column (250 mm × 4 mm, 5 µm) at 30 °C. An isocratic mobile phase consisting of 25 mmol L^{-1} ammonium dihydrogen phosphate with 10% (v/v) acetonitrile (pH 6.0) was applied. UV detection was carried out at 205 nm for 14 min, and TMP was detected at a retention time of 6.60 min.

Acknowledgements

We thank Dr. Tobias Wager (NanoTemper Technologies GmbH) for providing the opportunity to use Tycho spectrofluorometer for measurement.

References

[1] A. Weckbecker, H. Gröger, W. Hummel, in Biosystems Engineering I (Eds.: C. Wittmann, R. Krull), Springer Berlin Heidelberg, Berlin, Heidelberg, **2010**, pp. 195.

[2] X. Wang, T. Saba, H. H. P. Yiu, R. F. Howe, J. A. Anderson, J. Shi, *Chem* **2017**, *2*, 621.

[3] S. K. Spaans, R. A. Weusthuis, J. van der Oost, S. W. M. Kengen, *Front. Microbiol.* **2015**, *6*: 742.

[4] H. K. Chenault, G. M. Whitesides, *Appl. Biochem. Biotechnol.* **1987**, *14*, 147.

[5] M. B. Mathews, B. Vennesland, *J. Biol. Chem.* **1950**, *186*, 667.

[6] C. Vinals, E. Depiereux, E. Feytmans, *Biochem. Biophys. Res. Commun.* **1993**, *192*, 182.

[7] M. Jormakka, S. Törnroth, B. Byrne, S. Iwata, *Science* **2002**, *295*, 1863.

[8] V. I. Tishkov, V. O. Popov, *Biochemistry* **2004**, *69*, 1252.

[9] H. Nanba, Y. Takaoka, J. Hasegawa, *Biosci., Biotechnol., Biochem.* **2003**, *67*, 720.

[10] W. A. van der Donk, H. Zhao, *Curr. Opin. Biotechnol.* **2003**, *14*, 421.

[11] V. I. Tishkov, V. O. Popov, *Biomol. Eng.* **2006**, *23*, 89.

[12] H. Schütte, J. Flossdorf, H. Sahm, M.-R. Kula, *Eur. J. Biochem.* **1976**, *62*, 151.

Accepted Manuscript

This article is protected by copyright. All rights reserved.

[13] A. S. Bommarius, M. Schwarm, K. Stingl, M. Kottenhahn, K. Huthmacher, K. Drauz, *Tetrahedron: Asymmetry* **1995**, *6*, 2851.

[14] A. S. Bommarius, M. Schwarm, K. Drauz, *J. Mol. Catal. B: Enzym.* **1998**, *5*, 1.

[15] J. Z. Jin, D. L. Chang, J. Zhang, *Appl. Biochem. Biotechnol.* **2011**, *164*, 376.

[16] J. Li, J. Pan, J. Zhang, J. H. Xu, *J. Mol. Catal. B: Enzym.* **2014**, *105*, 11.

[17] W. Liu, H. Ma, J. Luo, W. Shen, X. Xu, S. Li, Y. Hu, H. Huang, *Biochem. Eng. J.* **2014**, *91*, 204.

[18] R. Kratzer, M. Pukl, S. Egger, B. Nidetzky, *Microb. Cell Fact.* **2008**, *7*, 37.

[19] R. Jayabalan, M. Sathishkumar, E. S. Jeong, S. P. Mun, S. E. Yun, *Bioresour. Technol.* **2012**, *123*, 686.

[20] H. Slusarczyk, S. Felber, M. R. Kula, M. Pohl, *Eur. J. Biochem.* **2000**, *267*, 1280.

[21] V. Tishkov, A. Galkin, G. Marchenko, Y. Tsygankov, A. Egorov, *Biotechnol. Appl. Biochem.* **1993**, *18*, 201.

[22] H. Nanba, Y. Takaoka, J. Hasegawa, *Biosci., Biotechnol., Biochem.* **2003**, *67*, 2145.

[23] V. V. Fedorchuk, A. G. Galkin, I. E. Yasny, L. B. Kulakova, A. M. Rojkova, A. A. Filippova, V. I. Tishkov, *Biochemistry* **2002**, *67*, 1145.

[24] H. Choe, J. M. Ha, J. C. Joo, H. Kim, H. J. Yoon, S. Kim, S. H. Son, R. M. Gengan, S. T. Jeon, R. Chang, et al., *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2015**, *71*, 313.

[25] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T. A. P. de Beer, C. Rempfer, L. Bordoli, et al., *Nucleic Acids Res.* **2018**, *46*, 296.

[26] R. Castillo, M. Oliva, S. Martí, V. Moliner, *J. Phys. Chem. B* **2008**, *112*, 10012.

[27] M. Iida, K. Kitamura-Kimura, H. Maeda, S. Mineki, *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1966.

[28] W. Jiang, P. Lin, R. Yang, B. Fang, *Appl. Microbiol. Biotechnol.* **2016**, 100, 8425.

[29] V. I. Tishkov, A. D. Matorin, A. M. Rojkova, V. V. Fedorchuk, P. A. Savitsky, L. A. Dementieva, V. S. Lamzin, A. V. Mezentzev, V. O. Popov, *FEBS Lett.* **1996**, *390*, 104.

[30] T. Watanabe, T. Hattori, S. Tengku, M. Shimada, *Enzyme Microb. Technol.* **2005**, *37*, 68.

[31] K. Schirwitz, A. Schmidt, V. S. Lamzin, *Protein Sci.* **2007**, *16*, 1146.

[32] Y. Asano, T. Sekigawa, H. Inukai, A. Nakazawa, *J. Bacteriol.* **1988**, *170*, 3189.

[33] S. Felber, PhD thesis **2001**.

[34] E. Andersson, B. Hahn-Hägerdal, *Enzyme Microb. Technol.* **1990**, *12*, 242.

[35] H. J. Heipieper, G. Neumann, S. Cornelissen, F. Meinhardt, *Appl. Microbiol. Biotechnol.* **2007**, *74*, 961.

[36] A. M. Klibanov, *Nature* **2001**, *409*, 241.

[37] A. P. Demchenko, O. I. Rusyn, A. M. Egorov, V. I. Tishkov, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1990**, *1039*, 290.

[38] H. T. Ding, D. F. Liu, Z. L. Li, Y. Q. Du, X. H. Xu, Y. H. Zhao, *J. Appl. Microbiol.* **2011**, *111*, 1075.

[39] I. Ahmed, A. Yokota, A. Yamazoe, T. Fujiwara, *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 1117.

[40] L. Zhu, Z. Wu, J. M. Jin, S. Y. Tang, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 5805.

[41] B. Kosjek, W. Stampfer, M. Pogorevc, W. Goessler, K. Faber, W. Kroutil, *Biotechnol. Bioeng.* **2004**, *86*, 55.

[42] J. K. B. Cahn, C. A. Werlang, A. Baumschlager, S. Brinkmann-Chen, S. L. Mayo, F. H. Arnold, *ACS Synth. Biol.* **2017**, *6*, 326.

[43] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison III, H. O. Smith, *Nat. Methods* **2009**, *6*, 343.

[44] U. K. Laemmli, *Nature* **1970**, *227*, 680.

Accepted Manuscript

This article is protected by copyright. All rights reserved.

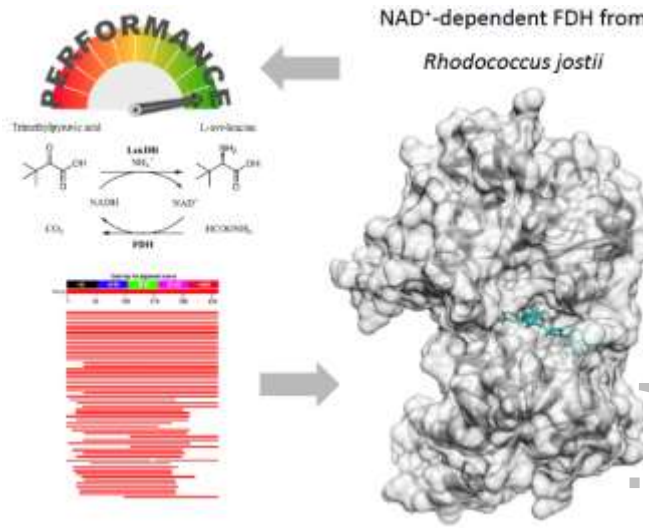
10.1002/adsc.202000536

FULL PAPER

Formate Dehydrogenase from *Rhodococcus jostii* (RjFDH) - A High-Performance Tool for NADH Regeneration

Adv. Synth. Catal. **2020**, *362*, Page – Page

Alexander Boldt and Marion B. Ansorge-Schumacher*



Accepted Manuscript

This article is protected by copyright. All rights reserved.