HMF oxidase
Dijkman, Willem Pieter

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Discovery and characterization of a 5-(hydroxymethyl)furfural oxidase from Methylovorus sp. Strain MP688

Willem P. Dijkman and Marco W. Fraaije

This chapter is based on:  
Applied and environmental microbiology, 2014, 80: 1082-1090
Abstract

In the search for useful and renewable chemical building blocks, 5-(hydroxymethyl)-furfural (1) has emerged as a very promising candidate as it can be prepared from sugars. 5-(hydroxymethyl)furfural (1) can be oxidized to 2,5-furandicarboxylic acid (4) and used as a substitute for petroleum-based terephthalate in polymer production. Based on a recently identified bacterial degradation pathway for 5-(hydroxymethyl)furfural (1), candidate genes responsible for selective 5-(hydroxymethyl)furfural (1) oxidation have been identified. Heterologous expression of a protein from Methylovorus sp. Strain MP688 in Escherichia coli and subsequent enzyme characterization showed that the respective gene indeed encodes an efficient 5-(hydroxymethyl)furfural oxidase (HMFO). HMFO is an FAD-containing oxidase and belongs to the GMC-type flavoprotein oxidase family. Intriguingly, activity of HMFO is not restricted to 5-(hydroxymethyl)furfural (1), as it is active with a wide range of aromatic primary alcohols and aldehydes. The enzyme was shown to be relatively thermostable and active at a broad pH range. This makes HMFO a promising oxidative biocatalyst that can be used for the production of 2,5-furandicarboxylic acid (4) from 5-(hydroxymethyl)furfural (1), a reaction involving both alcohol and aldehyde oxidations.
Introduction

To switch from petroleum-based chemicals to biologically based, and therefore renewable chemicals, new technologies and processes have to be developed.\textsuperscript{[1]} One promising bio-based chemical is 2,5-furandicarboxylic acid (4). This chemical building block has been mentioned for many years as having great potential in future applications.\textsuperscript{[1–2]} Recently, new technologies in the polymer industry have already led to the production of an 2,5-furandicarboxylic acid (4) based polymer: polyethylene furanoate (PEF). This polyester displays similar characteristics when compared with the well-known and widely applied polyethylene terephthalate (PET) but is based on 2,5-furandicarboxylic acid (4) instead of terephthalate. The application of 2,5-furandicarboxylic acid (4) is, however, not restricted to PEF and other polyesters as it can be used in the preparation of polyamines and polyurethanes as well.\textsuperscript{[2]}

2,5-furandicarboxylic acid (4) is a renewable compound as it can be produced from fructose and other sugars. The production of 2,5-furandicarboxylic acid (4) starts from fructose and proceeds via the formation of 5-(hydroxymethyl)furfural (1) as an intermediate. In the first step, 5-(hydroxymethyl)furfural (1) is formed from fructose. This intramolecular dehydration step is acid catalyzed and takes place at relatively high temperatures.\textsuperscript{[3]} In the second part of the process 5-(hydroxymethyl)furfural (1) is oxidized to 2,5-furandicarboxylic acid (4) (Scheme 1). The reaction from 5-(hydroxymethyl)furfural (1) to 2,5-furandicarboxylic acid (4) is a six electron oxidation, in which the alcohol group is oxidized to the corresponding aldehyde. This aldehyde group and the aldehyde group already present in 5-(hydroxymethyl)furfural (1) are further oxidized to the corresponding carboxylic acids, resulting in 2,5-furandicarboxylic acid (4). The existing, chemical methods for this reaction require the use of stoichiometric quantities of strong oxidants or involve metal salt catalysts in organic solvent and high temperature and pressure.\textsuperscript{[4,5]} Thus a gentler and less expensive, enzymatic procedure is desirable.

\begin{equation}
\begin{array}{c}
\text{Scheme 1. HMFO-catalyzed oxidation of 5-(hydroxymethyl)furfural (1) into 5-formylfuran-2-carboxylic acid (3) and 2,5-furandicarboxylic acid (4). The reaction of 5-(hydroxymethyl)furfural (1) to 5-formylfuran-2-carboxylic acid (3) is a double oxidation, which involves the transfer of four electrons from the substrates to the enzyme. In the reaction of 5-formylfuran-2-carboxylic acid (3) to 2,5-furandicarboxylic acid (4), the aldehyde group is oxidized to the carboxylic acid.}
\end{array}
\end{equation}

Unlike chemical oxidation, enzymatic or whole cell-catalyzed conversions can be performed at lower temperature and pressure. However, enzymes active on 5-(hydroxymethyl)furfural (1) are scarce. To date, only few enzymes were shown to be active towards 5-(hydroxymethyl)furfural (1).\textsuperscript{[6–8]} of which non is able to form 2,5-furandicarboxylic acid (4). One of these enzymes is chloroperoxidase CPO from \textit{Caldariomyces fumago}, a heme-containing peroxidase producing mainly the single oxidized products furan-2,5-dicarbaldehyde (2) and 5-(hydroxymethyl)furan-2-carboxylic acid (6) (74% and 20% respectively) from 5-(hydroxymethyl)furfural (1).\textsuperscript{[7]} Similarly, aryl alcohol oxidase (AAO, EC 1.1.3.7) from \textit{Pleurotus eryngii} can form 5-formylfuran-2-carboxylic acid (3), but not 2,5-furandicarboxylic acid (4).\textsuperscript{[8]}

\textbf{Chapter 2}
Recently, 5-(hydroxymethyl)furfural (1) oxidase activity was identified in the bacterium *Cupriavidus basilensis*.[9] This microbe harbors a gene cluster involved in 5-(hydroxymethyl)furfural (1) metabolism, and is able to grow on 5-(hydroxymethyl)furfural (1) as a sole carbon source. One of the genes encodes a putative oxidoreductase, HmfH, which presumably oxidizes 5-(hydroxymethyl)furfural (1). The actual substrates used and products formed by HmfH are, however, unclear. The HmfH-encoding gene has been introduced into a *Pseudomonas putida* host which could subsequently be used in a fermentative process to produce 2,5-furandicarboxylic acid (4) from 5-(hydroxymethyl)furfural (1). The *Pseudomonas* strain itself is able to produce 5-(hydroxymethyl)furan-2-carboxylic acid (6) but not 2,5-furandicarboxylic acid (4) from 5-(hydroxymethyl)furfural (1). Only when HmfH is expressed in *Pseudomonas*, is 2,5-furandicarboxylic acid (4) formed.[10] Based on these findings it has been suggested that HmfH can perform both alcohol and aldehyde oxidations and therefore may be able to 2,5-furandicarboxylic acid (4) from 5-(hydroxymethyl)furfural (1). However, this has not yet been confirmed experimentally.

HmfH is member of the glucose-methanol-choline (GMC) oxidoreductase protein family.[11] Members of the GMC-oxidoreductase family have two conserved domains. The N-terminal GMC domain (pfam 00732) is involved in binding the FAD as prosthetic group. The FAD cofactor in GMC-type oxidases provides the oxidative power needed in the reactions and is reduced to FADH₂ upon oxidation of the substrate. The reduced cofactor is reoxidized by molecular oxygen, resulting in the formation of hydrogen peroxide along with the product. In some GMC flavoprotein oxidases, the FAD is covalently linked to a histidine side chain in this domain of the enzyme. The second conserved domain is the C-terminal GMC domain (Pfam 05199). This roughly 150 amino acid long domain contains the active site residues including a strictly conserved histidine.[12]

The substrates for GMC oxidoreductases are diverse, ranging from small alcohols like methanol and choline[13] to more complex alcohols like glucose[14] and aromatic alcohols.[15] While most GMC oxidases convert an alcohol to ketone or aldehyde, some are able to perform a double oxidation, converting a primary alcohol to the aldehyde and subsequently to the carboxylic acid. Overall, the substrate donates 4 electrons to the enzyme in such a reaction. However, only a few GMC oxidoreductases can oxidize alcohols to the corresponding carboxylic acids.[15,16] Although hydroxylations and amine oxidations have been described for other types of flavoprotein oxidases,[17] thus far no GMC flavoprotein oxidases have been identified with such activities.[11]

In this paper we describe the cloning of a gene from *Methylovorus* sp. Strain MP688, encoding an HmfH homologue. The respective enzyme could be overexpressed in *Escherichia coli* and purified by affinity chromatography. This enabled us to explore the biocatalytic properties of this enzyme which turned out to be an effective FAD-containing oxidase which is able to oxidize 5-(hydroxymethyl)furfural (1) to 2,5-furandicarboxylic acid (4). Therefore we have named the enzyme HMFO (5-(hydroxy)methyl)furfural (1) oxidase). Intriguingly, activity of HMFO is not restricted to 5-(hydroxymethyl)furfural (1) and many other, often aromatic, substrates are efficiently oxidized as well.
Results

For a more detailed study on the 5-(hydroxymethyl)furfural (1) oxidizing enzyme from *Cupriavidus basilensis*, we first tried to express HmfH in *E. coli*. For this, several pBAD- and pET-based expression plasmids were used while testing a variety of *Escherichia coli* strains, growth temperatures and inducer concentrations. In addition to the native *hmfH*-gene, several expression optimized genes were included in the expression tests. However, no soluble expression of HmfH could be achieved, even when using MBP or SUMO as fusion partners to boost expression of soluble protein (results not shown). Therefore, we decided to look for homologous genes for which the corresponding enzymes are expected to fulfill a similar metabolic role. A sequence database BLAST search revealed several homologues of HmfH, all with unknown function. The closest homologues are mainly from *Burkholderia* species (32-69% sequence identity) while also genes from other bacteria were identified. We decided to continue with two homologous genes, one from *Burkholderia phytofirmans* PsJN (WP_012433179.1) and one from *Methylovorus* sp MP688 (WP_013440946.1). The encoded proteins showed 69% and 46% sequence identity with the HmfH protein sequence. The genes encoding these homologues were ordered as expression-optimized genes in a pJexpress 404 vector. Like HmfH, the protein from *Burkholderia phytofirmans* PsJN could not be expressed in *E. coli*. The *Methylovorus* homologue could be functionally expressed. Because expression was limited using this construct, a pET-SUMO fusion construct was generated resulting in a 20-fold increase of expression.

From one liter culture with an OD$_{600}$ of 20, it was possible to purify 88 mg yellow-colored SUMO-HMFO. The purified protein runs as a single band on SDS-PAGE, with a mass of around 70 kDa. This is in agreement with the predicted mass 70.4 kDa of the His$_6$-SUMO-HMFO fusion protein. Upon soaking the SDS-PAGE gel in 5% acetic acid, no UV-fluorescent band could be detected, suggesting that the flavin cofactor is not covalently bound to the protein. A close homologue with a known structure is choline oxidase (EC 1.1.3.17) from *Arthrobacter globiformis* (32% sequence identity). In this protein the FAD cofactor is covalently linked via an 8-α-histidyl bond. A pair wise sequence alignment shows that the respective histidine responsible for the protein-FAD bond, H99, is not conserved in HMFO (V101 in this position). To verify that the flavin cofactor is dissociable, HMFO was subjected to TCA treatment and subsequent centrifugation. This indeed resulted in formation of a white protein pellet and a yellow supernatant.

The UV-Vis spectrum of native enzyme revealed a typical flavoprotein absorbance spectrum with absorbance maxima at 385 nm and 456 nm. Upon unfolding by SDS, a flavin spectrum was obtained that was identical to commercially available FAD with an absorbance maximum at 450 nm. Using the known extinction constant for FAD ($\varepsilon_{450} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$), the extinction constant for HMFO ($\varepsilon_{456} = 10.7 \text{ mM}^{-1} \text{ cm}^{-1}$) could be determined (Figure 1).

To probe whether the SUMO fusion tag has an effect on the properties of HMFO, the N-terminal SUMO part was cleaved of by proteolytic treatment of the fusion enzyme with SUMO protease. The generated recombinant wild-type HMFO was compared with the fused variant by measuring the UV-Vis spectra, determining the steady-state kinetic parameters for 5-(hydroxymethyl)furfural (1), and measuring the thermostability. These results show that neither the flavin spectra (indicative for an identical microenvironment around the flavin cofactor), nor the activity of HMFO (differences between $K_M$ and $k_{cat}$ values <10%), nor the thermostability (apparent melting temperatures differed <0.5 °C) are affected by fusing it to
SUMO at the N-terminus. Therefore we decided to use the SUMO-fused HMFO for the rest of our study.

HMFO was identified with a BLASTp search as a homologue (46% seq. identity) of HmfH from *Cupriavidus basilensis*. The latter protein is thought to be active on 5-(hydroxymethyl)furfural (1). Hence, purified HMFO was assayed for activity with this compound. An enzyme-dependent decrease of molecular oxygen showed HMFO is indeed active on 5-(hydroxymethyl)furfural (1). This matches the prediction of HMFO being a member of the GMC-type oxidase family. In most oxidases, molecular oxygen is used the reoxidized the flavin cofactor, resulting in the formation of $\text{H}_2\text{O}_2$. For HMFO, hydrogen peroxide formation was experimentally confirmed by adding catalase after incubation of HMFO and cinnamyl alcohol. During catalysis, 0.08 mm molecular oxygen was consumed. Adding 150 units of catalase resulted in the formation of 0.04 mm molecular oxygen, showing that HMFO is an oxidase.

Enzymatic oxidation of 5-(hydroxymethyl)furfural (1) is of interest for the production of 2,5-furandicarboxylic acid (4). As shown in Scheme 1, this conversion consists of three oxidation steps. Therefore, the activity of HMFO on the intermediates (furan-2,5-dicarbaldehyde (2), 5-(hydroxymethyl)furan-2-carboxylic acid (6) and 5-formylfuran-2-carboxylic acid (3)) was also measured. Surprisingly, HMFO was also found to convert 5-formylfuran-2-carboxylic acid (3) and 5-(hydroxymethyl)furan-2-carboxylic acid (6). Although chemically related, all these furans have different properties. This is clearly reflected in the $k_{\text{cat}}$ and $K_M$ values obtained (Table 1).[A] The affinity of HMFO for 5-(hydroxymethyl)furfural (1) is 50 fold higher than for 5-(hydroxymethyl)furan-2-carboxylic acid (6), possibly due to the negative charge introduced by the carboxylic acid. Intriguing is the activity of HMFO on furan-2,5-dicarbaldehyde (2), an aldehyde. Oxidation of aldehydes is not a common feature of GMC-type oxidases. Several alcohol-aldehyde couples were analyzed. For furan-2,5-dicarbaldehyde (2), the $k_{\text{cat}}/K_M$ value is only 7 times lower than for the analogous alcohol [5-(hydroxymethyl)furan-2-yl]methanol (5).

---

[A] The $k_{\text{cat}}$ and $K_M$ values are in fact apparent values ($k_{\text{cat,app}}$ and $K_M,\text{app}$), as all experiments are performed at atmospheric oxygen concentration. However, throughout the thesis the designations $k_{\text{cat}}$ and $K_M$ are used for simplicity.
Table 1. Steady state kinetic parameters for HMFO.<sup>a</sup>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s⁻¹ mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfuryl alcohol</td>
<td></td>
<td>11.9</td>
<td>7.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Furfural</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt; 0.010</td>
</tr>
<tr>
<td>5-(Hydroxymethyl)-furan-2-yl methanol (5)</td>
<td></td>
<td>22</td>
<td>5.7</td>
<td>3.9</td>
</tr>
<tr>
<td>5-(Hydroxymethyl) furfural (1)</td>
<td></td>
<td>9.9</td>
<td>1.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Furan-2,5-dicarbaldehyde (2)</td>
<td></td>
<td>1.6</td>
<td>1.7</td>
<td>0.94</td>
</tr>
<tr>
<td>5-(Hydroxymethyl)furan-2-carboxylic acid (6)</td>
<td></td>
<td>8.5</td>
<td>73</td>
<td>0.12</td>
</tr>
<tr>
<td>5-Formylfuran-2-carboxylic acid (3)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt; 0.010</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td></td>
<td>13</td>
<td>1.3</td>
<td>10</td>
</tr>
<tr>
<td>4-(Hydroxymethyl)phenyl methanol (7)</td>
<td></td>
<td>21</td>
<td>1.4</td>
<td>15</td>
</tr>
<tr>
<td>4-(Hydroxymethyl)benzaldehyde (8)</td>
<td></td>
<td>8.6</td>
<td>0.15</td>
<td>57</td>
</tr>
<tr>
<td>Terephthaldehyde (9)</td>
<td></td>
<td>1.3</td>
<td>0.15</td>
<td>57</td>
</tr>
<tr>
<td>4-(Hydroxymethyl)benzoic acid (10)</td>
<td></td>
<td>12</td>
<td>1.0</td>
<td>12</td>
</tr>
<tr>
<td>1,3-Benzenedimethanol</td>
<td></td>
<td>14</td>
<td>1.5</td>
<td>9.3</td>
</tr>
<tr>
<td>4-Hydroxybenzyl alcohol</td>
<td></td>
<td>7.2</td>
<td>0.30</td>
<td>24</td>
</tr>
</tbody>
</table>

continued on page 38
Table 1. continued

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-aminobenzyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>17</td>
<td>1.4</td>
<td>12</td>
</tr>
<tr>
<td>4-chlorobenzyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>9.5</td>
<td>0.08</td>
<td>120</td>
</tr>
<tr>
<td>4-nitrobenzyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>4.5</td>
<td>0.078</td>
<td>58</td>
</tr>
<tr>
<td>4-butylbenzyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>10</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>1-phenylethanol</td>
<td><img src="image" alt="Structure" /></td>
<td>n.a.[c]</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>17</td>
<td>0.07</td>
<td>260</td>
</tr>
<tr>
<td>2-(hydroxymethyl)quinoline</td>
<td><img src="image" alt="Structure" /></td>
<td>5.3</td>
<td>0.04</td>
<td>132</td>
</tr>
<tr>
<td>2,4-hexadien-1-ol</td>
<td><img src="image" alt="Structure" /></td>
<td>13</td>
<td>0.59</td>
<td>23</td>
</tr>
<tr>
<td>Glycerol</td>
<td><img src="image" alt="Structure" /></td>
<td>n.s.[d]</td>
<td>&gt; 2.5 · 10$^3$</td>
<td>0.016</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>21</td>
<td>0.73</td>
<td>29</td>
</tr>
<tr>
<td>HMFO-H467A:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td><img src="image" alt="Structure" /></td>
<td>4.7 · 10$^{-3}$</td>
<td>0.82</td>
<td>0.0057</td>
</tr>
</tbody>
</table>

[a] The kinetic parameters of HMFO are determined in a 50 mM potassium phosphate buffer of pH 8.0 at 25 °C with REDFLASH sensor spots and a Firesting O$_2$ detector and light source on all substrates unless stated otherwise. [b] n.d., not determined. [c] n.a., no activity. [d] Kinetic parameters were determined by H$_2$O$_2$ formation in a coupled assay. [e] n.s., no saturation. [f] Kinetic parameters were calculated from the increase in the product’s absorbance at 340 nm.
A similar alcohol-aldehyde couple is furfuryl alcohol and furfural. The kinetic parameters for furfuryl alcohol are comparable to those for [5-(hydroxymethyl)furan-2-yl]methanol (5). Furfural on the other hand is not a substrate.

We discovered that the substrate acceptance profile of HMFO is not restricted to furans. Benzylic alcohols are readily accepted by the enzyme too. In fact, HMFO is active on all tested primary alcohols (both furan or benzyl). The $k_{cat}$ on all these alcohols are rather similar (Table 1).

Like other GMC oxidoreductases, HMFO seems to be restricted to carbon oxygen bond oxidation. For instance, the amine benzylamine is not a substrate whereas benzyl alcohol is. In addition, hydroxylation of a double bond, as performed by the FAD-containing vanillyl alcohol oxidase (VAO),[18] cannot be performed by HMFO. To establish the identity of HMFO-formed products, high-performance liquid chromatography (HPLC) analysis was performed. After 5 hours incubation, 2 mM 5-(hydroxymethyl)furfural (1) was fully converted by 5 µM HMFO (Table 2). The main product of the reaction was 5-formylfuran-2-carboxylic acid (3) (92%) but also a considerable amount of 2,5-furandicarboxylic acid (4) was present: 8%. In a control reaction without HMFO, 5-(hydroxymethyl)furfural (1) remained the main component (>99%) which implies that HMFO is needed for the conversion of 5-(hydroxymethyl)-furfural (1). To exclude the spontaneous oxidation of the intermediates (5-(hydroxymethyl)-furan-2-carboxylic acid (6), furan-2,5-dicarbaldehyde (2) and 5-formylfuran-2-carboxylic acid (3)) in time, similar control reactions were performed for these compounds. In all cases, over 99% of all 5-(hydroxymethyl)furfural (1) derivatives detected were the starting compound. This excludes the spontaneous formation of 2,5-furandicarboxylic acid (4) and confirms that HMFO can produce 2,5-furandicarboxylic acid (4).

To establish the optimal conditions for HMFO, pH and temperature optima have been determined. Activity was determined between pH 6.5 and pH 10 in a Britton-Robinson buffer. The optimal pH value of HMFO for activity was 8.0, while retaining almost 80% of its peak activity between pH 6.5 and 9.0. At pH 10.0, the activity decreases to 30% (Figure 2A). The Britton-Robinson buffer reduces the activity of HMFO by 10% compared to phosphate buffer at pH 8.0.

Table 2. Product formation by HMFO. The products formed by 5 µM HMFO starting from 2 mM 5-(hydroxymethyl)furfural (1) as substrate. 5-(Hydroxymethyl)furfural (1) is fully converted into 5-formylfuran-2-carboxylic acid (3) and 2,5-furandicarboxylic acid (4). In the control experiments without enzyme 5-(hydroxymethyl)furfural (1) and all intermediates no conversion is observed. All experiment were performed in duplicate, the average is shown.

<table>
<thead>
<tr>
<th>compound</th>
<th>% detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-(hydroxymethyl)furfural (1)</td>
<td>n.d.[a]</td>
</tr>
<tr>
<td>furan-2,5-dicarbaldehyde (2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>5-(hydroxymethyl)furan-2-carboxylic acid (6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>5-formylfuran-2-carboxylic acid (3)</td>
<td>92 ± 0.4</td>
</tr>
<tr>
<td>2,5-furandicarboxylic acid (4)</td>
<td>8.1 ± 0.4</td>
</tr>
</tbody>
</table>

[a] n.d. = not detected
The temperature optimum was determined between 25 °C and 70 °C. HMFO is most active at 55 °C. At 25 °C, HMFO has 60% of the activity when compared to 55 °C, and at 70 °C only 10% activity is left (Figure 2B). By using the ThermoFAD method we determined the apparent melting temperature: 48.5 °C. This shows that the oxidase is reasonably thermotolerant.

One key feature of GMC-type oxidases is the presence of an absolutely conserved histidine in the C-terminal domain. The role of this histidine has been investigated in well-studied GMC members such as glucose oxidase, choline oxidase and AAO.\cite{14,19,20} Replacing this histidine by an alanine resulted in a drastic reduction of catalysis of all these oxidases. A homology model of HMFO was made by using the crystal structures of pyranose oxidase (3T37) and choline oxidase (3NNE). Inspection of this model revealed H467 as the active site histidine in HMFO. As expected, catalysis of HMFO H467A is severely reduced compared to the wild type enzyme. Using vanillyl alcohol as a substrate, the $k_{cat}$ value of the mutant is 4400 times lower than for the wild type enzyme. The affinity for the substrate is, however, hardly affected (Table 1). The microenvironment of the flavin is also altered in the mutant as can be seen from the UV-Vis spectrum (Figure 1). The absorbance maximum has shifted from 456 nm to 464 nm ($\varepsilon_{464} = 9.9 \text{ mm}^{-1} \text{ cm}^{-1}$). It is clear that the histidine does not play an important role in substrate binding while it is essential for catalysis. Whether H467 is involved in the oxidative or reductive half reaction remains to be determined for HMFO.

Conclusions and discussion

HMFO was identified as a homologue of HmfH from *Cupriavidus basilensis*\cite{9}. The latter protein is thought to be active on 5-(hydroxymethyl)furfural (1). Although being only 45% identical to HmfH and lacking a 35 amino acid long part near the C-terminus of the protein, HMFO is...
active on 5-(hydroxymethyl)furfural (1) as well. The substrate profile of HMFO is however not restricted to 5-(hydroxymethyl)furfural (1). Many other furans and also benzylic compounds are accepted by the enzyme. From the substrates tested the enzyme shows activity on all aromatic compounds with a methanol substituent. This shows that in addition to aromaticity, the position and nature of the alcohol are important. This strict specificity for primary alcohols is likely due to the specific architecture of the active site. The most supported reaction mechanism for GMC oxidases involves abstraction of a proton from the alcohol group as initial step of catalysis.[21] If the substituent is more bulky, the alcohol moiety may not be positioned close enough to facilitate hydride transfer to the flavin cofactor. Alternatively, deprotonation of the alcohol by the active site base is obstructed.

Another requirement is the presence of a conjugated system within the molecule, illustrated by for instance cinnamyl alcohol and 2,4-hexadien-1-ol. The planar structure caused by the double bond system might be a requirement for productive binding of the substrate. Alternatively, the conjugated system is extended when the alcohol is oxidized to the aldehyde and might thereby facilitate alcohol oxidation.

The enzyme is not active on secondary alcohols, and therefore we could not demonstrate enantiospecificity for secondary alcohols with the oxidase. This does not mean the enzyme is not stereospecific, it will most likely abstract a hydride in a preferred stereospecific manner. The Cα atom of the alcohol moiety contains two hydrogens, of which one will be selectively abstracted. For P. eryngii AAO it has been shown using mono-deteurated p-methoxybenzyl alcohol that the enzyme abstracts only the 'R'-hydrogen as a hydride, which is closest to the flavin N5.[22] The active site architecture and selectivity of HMFO will be presented into detail in chapter 4.

The rates of catalysis on all identified substrates are fairly similar, regardless of the substituents on the aromatic ring. With the para-substituted, electron donating groups of 4aminobenzyl alcohol and 4-hydroxybenzyl alcohol, similar $k_{cat}$ values were obtained when compared with an electron withdrawing halide like in 4-chlorobenzyl alcohol. All of these rates are comparable with the non-substituted benzyl alcohol. The ring substituents are however of great importance for the affinity of HMFO for its substrate. The negatively charged carboxylic acid of 5-(hydroxymethyl)furan-2-carboxylic acid (6) reduces affinity >10 fold compared to most non-substituted substrates. 4-Chlorobenzyl alcohol on the other hand has an 16 fold reduces $K_M$ value compared to the non-substituted benzyl alcohol.

The relaxed substrate profile of HMFO makes it an interesting biocatalyst for the oxidation of aromatic alcohols. Moreover, HMFO not only oxidizes a wide variety of aromatic alcohols, it can also perform aldehyde oxidations. Aldehyde oxidation is not a general feature of GMC-oxidases. A notable exception is choline oxidase.[23] but for fungal AAOs aldehyde oxidation has been demonstrated as well.[16,24] For these GMC-oxidases it was shown that the hydrated aldehyde, the gem-diol, is the actual substrate. As a consequence, the reactivity of aldehydes as substrates is greatly affected by the nature of the substituents on the aromatic ring. Electron withdrawing groups, like carbonyl substituents, both deactivate and activate the aldehyde. Hydride transfer is hampered because electrons are withdrawn from the aldehyde. On the other hand, the aldehyde is more easily hydrated when an electron withdrawing group is present. Having more substrate in the hydrated form could therefore lead to more rapid oxidation.[24] Because of the deactivating and activating effects of a single substituent, it is hard to predict which effect a substituent will have on catalysis.

For the full oxidation of 5-(hydroxymethyl)furfural (1) to form 2,5-furandicarboxylic acid (4), both alcohol and aldehyde groups have to be oxidized. Product analysis revealed that
2,5-furandicarboxylic acid (4) is formed by HMFO. 5-formylfuran-2-carboxylic acid (3) is the only intermediate observed, whereas the single oxidized intermediates were not detected (Table 2). This illustrates the effect of the activating or deactivating groups on aldehyde oxidation as described above. In 5-formylfuran-2-carboxylic acid (3), the carboxylic acid on the 2 position might hamper oxidation of the aldehyde on the 5 position. However, when the furan ring is substituted with an additional aldehyde instead of a carboxylic acid the reactivity increases (Table 1). The formation of 5-formylfuran-2-carboxylic acid (3) as main product is in agreement with the rates listed in Table 1, as it is the only intermediate between 5-(hydroxymethyl)furfural (1) and 2,5-furandicarboxylic acid (4) on which catalysis was poor.

The substrate profile and the ability to oxidize aromatic alcohols and aldehydes reveals a striking resemblance between HMFO and the fungal AAOs. Both enzyme types belong to the same flavoprotein oxidase family. Yet, the sequence similarity between these oxidases is not indicative of a strong functional relationship (sequence identity < 30%). The physiological roles of both oxidase types are also clearly different. AAOs are secreted by fungi to assist in the degradation of lignin while the bacterial HMFO is an intracellular enzyme. This prohibits a direct role in lignin degradation. In view of the sequence similarity of the HMFO-encoding gene with the hmfH gene of C. basilensis that is involved in 5-(hydroxymethyl)furfural (1) degradation, it is tempting to conclude that also HMFO is part of a 5-(hydroxymethyl)furfural (1) degradation pathway. The typical gene cluster found in 5-(hydroxymethyl)furfural (1) degrading bacteria is however not present in Methylovorus sp. Strain MP688.

To identify catalytically important residues in HMFO, an homology model of HMFO was prepared by using the Yasara software. The best studied close homologue of HMFO with a known crystal structure is choline oxidase. Comparing the HMFO model structure with the structure of choline oxidase reveals that several catalytically important residues of choline oxidase are not conserved. The negatively charged E312 of choline oxidase, for instance, positions the positively charged amine of choline and is therefore not conserved because the substrates of HMFO do not contain a positive charge. In addition, choline is not a substrate for HMFO. Other catalytically important residues, like histidine 307, valine 465 and histidine 467 (H310, V464, H466 in choline oxidase) are conserved. For choline oxidase it is has been shown that mutating the active site histidine 466 to alanine decreases the reaction rate 60 fold and increases the Michaelis constant 17-fold. In our study the analogous mutation was introduced in HMFO. This resulted in a 4400 fold decrease of the $k_{cat}$ whereas the $K_M$ was hardly affected, confirming the importance for catalysis of this residue. In studies on AAO from Pleurotus eryngii, H502 was replaced by an alanine, resulting in a decrease of $k_{cat}$ of almost 3000 fold, similar to the effect seen in the HMFO mutant. The mutation also increased the $K_M$ value 80 fold, an effect much more severe than observed in HMFO. Studies on choline oxidase and AAO from Pleurotus eryngii suggest that this histidine is the active site base. The role of H467 in HMFO is likely to be similar.

The identification and characterization of a 5-(hydroxymethyl)furfural (1) oxidase adds a new tool to develop a biocatalytic process for the production of 2,5-furandicarboxylic acid (4). HMFO is the first characterized enzyme able to oxidize 5-(hydroxymethyl)furfural (1) and produce 2,5-furandicarboxylic acid (4). In addition, the enzyme can perform selective oxidations on many benzylic substrates. The substrate profile resembles the substrate acceptance of fungal AAOs to a large extend. Production of these fungal oxidases in a recombinant form has been shown to be difficult. Therefore HMFO may also develop as a biocatalytic alternative for these oxidases. Overall, HMFO is a suitable catalyst for applications involving selective alcohol and aldehyde oxidations.
Materials and methods

Expression, purification and spectral analysis of HMFO

The gene encoding HMFO (WP_013440946.1), HmfH (ADE20408.1) and WP_012433179.1 from *Burkholderia phytofirmans* PsJN were purchased from DNA2.0 (Menlo Park, CA, USA). The synthetic genes were optimized for expression in *Escherichia coli* and the HMFO and *Burkholderia phytofirmans* PsJN genes included a sequence that encodes a C-terminal TEV-protease cleavage site followed by a Strep-tag facilitating affinity chromatography purification. The genes were delivered in a pJexpress 404 plasmid of DNA 2.0.

To increase expression levels, a SUMO-HMFO fusion construct was created. The *hmfo*-gene was amplified from the pJexpress 404 plasmid using the following primers: forward, 5'-ATGACTGATACGATTTTTGACTACGTG-3'; and reverse, 5' TTAAAGCCTGGGTCAAATCGC-3'. The underlined bases introduce a stop codon behind the last residue of HMFO. This stop codon was introduced because it was not present in the ordered gene since it contained a C-terminal TEV-protease site and a Strep-tag for purification. The PCR product (2.9 µg) was incubated with 5 units Taq polymerase and 0.6 mM dATP (both New England Biolabs) in a total volume of 40 µL for 15 minutes at 72 °C to introduce 3'-A overhangs. This DNA was ligated into the pET-SUMO vector as described in the Champion pET SUMO Protein Expression System user manual (Invitrogen).

For HMFO expression, an overnight culture of *Escherichia coli* BL21 (DE3) cells with the SUMO-HMFO coding plasmid was diluted 1:100 in 1 L Terrific Broth containing 50 µg·mL⁻¹ kanamycin and grown at 37 °C until it reached an OD₆₀₀ density of 0.5. Cells were induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for 68 hours at 17 °C. Cells were harvested by centrifugation at 3730 g for 15 minutes (JLA 10.500 rotor, 4 °C) and resuspended in 80 mL 100 mM Tris/HCl pH 8.0 supplemented with 10% v/v glycerol, 150 mM NaCl and 10 µM FAD. The cell-free extract was obtained after cell disruption at 25,000 Psi (Constant systems cell disrupter, Daventry, UK) and subsequently centrifuged for 1 hour at 29,100 g (JA 17 rotor, 4 °C). All subsequent steps were performed at 4 °C. The soluble fraction was mixed with 2 mL pre-equilibrated Ni-Sepharose resin (GE-Healthcare) for 1.5 hour. The flow through was removed and the column was washed with 5 CV of 50 mM Tris/HCl with 150 mM NaCl and subsequently washed with 3 CV of 50 mM Tris/HCl with 150 mM NaCl containing 5 mM imidazole. The protein was eluted with 4 CV of 50 mM Tris/HCl with 150 mM NaCl containing 500 mM imidazole. The eluate was desalted on a 10 DG Biorad column.

To remove the N-terminal His₆-SUMO fusion from the HMFO protein, 4.3 mg purified SUMO-HMFO was cleaved with 0.2 mg SUMO-Protease in 50 mM Tris/HCl of pH 8.0 in a total volume of 3.0 mL. The enzymes were incubated for 16 hours at 4 °C and afterwards the solution was incubated with 0.4 mL pre-equilibrated Ni-Sepharose for 1.5 hours at 4 °C. HMFO was present in the flow through. The protease and the His-SUMO fragment were eluted with 50 mM Tris/HCl with 150 mM NaCl containing 500 mM imidazole. As a negative control SUMO-HMFO was treated the same as described above but no protease was added.

The UV-Vis spectrum of the purified protein was recorded between 240 and 650 nm. HMFO was denatured using a final concentration of 0.1% w/v SDS to obtain the spectrum of the free flavin cofactor. The spectra of folded and denatured HMFO were used to calculate both the concentration and the extinction coefficient of HMFO.
**H467A mutation**

To introduce the H467A mutation into HMFO, a whole plasmid PCR was performed. For the H467A mutation the following primers were used:

- **forward**, 5'- CGGCGGTGTTTGCCGGCAGGGCCAGCAGC-3';
- **reverse**, 5'-CCTGCGGCTCCAAGCACCCGACCCGACG-3' (underlined bases are mismatches introducing alanine (GCT) instead of histidine (CAT)). Template DNA was cleaved with DpnI (New England Biolabs). The plasmid was purified using a PCR-purification kit (Qiagen) and transformed into *E. coli* TOP10 cells. Introduction of the mutations was confirmed by sequencing. Purification and spectral analysis of this mutant were performed like for the wild type enzyme.

**Temperature and pH optimum of HMFO**

To determine the temperature optimum of HMFO, 2.0 mM 4-hydroxybenzyl alcohol was oxidized in 50 mM phosphate buffer, pH 8.0. Observed rates were calculated based on the increase of absorbance at 330 nm caused by the formation of 4-hydroxybenzaldehyde ($\varepsilon_{330}=13.7$ mM$^{-1}$·cm$^{-1}$ at 25 °C). The cuvette containing the substrate dissolved in buffer was heated along with the spectrophotometer prior to the measurement, from 25 to 70 °C. To start the measurement 100 nm enzyme was added.

The pH optimum was determined using 100 nM HMFO and 2.0 mM 4-hydroxybenzyl alcohol in a 12 fold diluted Britton-Robbinson buffer of 200 mM boric acid, 200 mM acetic acid and 200 mM phosphoric acid adjusted to the correct pH using NaOH. Activity was assayed from pH 6.5 to pH 10. Observed rates were calculated using the formation of 4-hydroxybenzaldehyde as described above. The extinction coefficient of 4-hydroxybenzaldehyde was determined for all pH values measured.

**ThermoFAD on HMFO variants**

The unfolding temperatures of purified HMFO, SUMO-HMFO and the SUMO-HMFO H467A mutant were analyzed using the ThermoFAD method.[29] With this method, the release of the flavin cofactor is monitored while heating the enzyme. An real-time-PCR thermocycler was used to denature 20 µL of 10 µM enzyme. The temperature gradient was set from 20 to 90 °C while measuring fluorescence at every 0.5 °C.

**Activity measurements**

The substrate range of HMFO was determined by using a peroxidase-coupled assay to detect H$_2$O$_2$ formed upon oxidation or by using a direct method through measuring oxygen consumption upon oxidation of the substrate. Assayed alcohols are 2-furanmethanol (furfuryl alcohol), 5-(hydroxymethyl)furfural, (S)-5-hydroxymethyl-2(5H)-furanone, D-fructose, (1R,2S)-cyclohexane-1,2-diol, propane-1,2,3-triol (glycerol), (25,4R)-pentaan-1,2,3,4,5-pentol (d-xylitol), 2-hydroxy-N,N,N-trimethylethanaaminium (choline), (2E,4E)-hexa-2,4-dien-1-ol, benzyl alcohol, 4-hydroxybenzylalcohol, 4-aminobenzyl alcohol, 4-chlorobenzyl alcohol, 4-(hydroxymethyl)-2-methoxyphenol (vanillyl alcohol), 2-methoxy-4-(2-propenyl)phenol, 1,3-dihydroxymethylbenzene, (S)-1-phenylethanol, (R)-1-phenylethanol, 2-phenylethanol, 4-butylbenzyl alcohol, (S)-phenyl-1,2-ethanediol, (R)-phenyl-1,2-ethanediol, 4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol, 4-(hydroxymethyl) benzaldehyde, 4-(hydroxymethyl)benzoic acid, terephthaldehyde, 2-(hydroxymethyl) quinolone, 4-(hydroxymethyl)benzoic acid, terephthalaldehyde (all purchased from Sigma), [29]
5-hydroxymethyl-2-furfuryl alcohol (TCI), (2E)-3-phenylprop-2-en-1-ol (cinnamyl alcohol) (Acros), (4-hydroxymethyl)benzaldehyde (Manchester Organics) and 5-hydroxymethyl-2-furoic acid (Matrix Scientific). Because the latter was highly esterified, prior to use, 300 mM 5-(hydroxymethyl)furan-2-carboxylic acid was boiled in 2 mM H₂SO₄ for 2 hours. After hydrolysis, the pH was adjusted to 8.0 with NaOH and diluted to the proper concentration in 50 mM phosphate buffer of pH 8.0.

The tested aldehydes were 2-furancarboxaldehyde (furfural), 2,5-diformylfuran (both Sigma) and 5-formyl-2-furancarboxylic acid (TRC). The screen further included, 1-phenylmethylamine (Sigma) and 2-methoxy-4-(2-propenyl)phenol (Sigma).

In the coupled H₂O₂ detection assay, horseradish peroxidase (HRP (Sigma), 20 or 50 units/mL) oxidizes 4-aminoantipyrine (AAP, 0.1 mM) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS, 1 mM) to form a pink product which was measured at 515 nm (ε₅₁₅ = 26 mM⁻¹ cm⁻¹).[30] Oxygen concentrations were determined using REDFLASH sensor spots and a Firesting O₂ detector and light source (Pyroscience, Aachen, Germany). For calibration, a 100 % oxygen solution was prepared by stirring MilliQ-water at 2500 rpm for 15 minutes while exposed to air. This resulted in a 274 µM solution (23 °C, pH 7.0, atmospheric pressure). The water was flushed with argon for 30 minutes to make the anoxic standard. Activity of 0.1 to 15 µM HMFO was assayed using 0.5 to 50 mM of substrate, depending on the substrate used. All reactions were performed at 25 °C in a 50 mM phosphate buffer, pH 8.0.

The kᵦ and Kᵢ values for most substrates of SUMO-HMFO were determined by monitoring the oxygen consumption during catalysis. A final concentration of 100-500 nM HMFO (depending on the substrate) was added to the substrate (0.01-100 mM) in 50 mM phosphate buffer of pH 8.0 at 25°C. The substrates used were furfuryl alcohol, furfural, [5-(hydroxymethyl)furan-2-yl]methanol, 5-(hydroxymethyl)furfural, furan-2,5-dicarbaldehyde, 5-(hydroxymethyl)furan-2-carboxylic acid, 5-formylfuran-2-carboxylic acid, terephthalaldehyde, 4-(hydroxymethyl)benzoic acid, benzyl alcohol, 1,3-dihydroxymethylbenzene, 1,4-dihydroxymethylbenzene, 4-butylbenzyl alcohol and 4-hydroxybenzyl alcohol, 4-aminobenzyl alcohol, 4-chlorobenzyl alcohol, cinnamyl alcohol and (2E,4E)-hexa-2,4-dien-1-ol, 4-(hydroxymethyl)benzaldehyde, 4-(hydroxymethyl)benzoic acid, terephthaldehyde, 2-(hydroxymethyl)quinoline. Oxygen concentrations were determined using REDFLASH sensor spots as mentioned above. The rates of oxygen decrease were converted to observed rates, and the Michaelis-Menten kinetic parameters were obtained by curve fitting (formula: v = kᵦ[S]/Kᵢ + [S]) using Sigmaplot. The kᵦ and Kᵢ values for vanillyl alcohol were determined for both the wild type enzyme and the H467A mutant. Product formation was monitored spectrophotometrically by measuring the absorption increase at 340 nm (ε₃₄₀ = 14 mM⁻¹ cm⁻¹). Substrate concentrations were as µM. For the wild type enzyme, 200 mM enzyme was used, while for the H467A mutant 5.0 µM was used. Oxidase activity of the wild type enzyme (5.0 µM) on glycerol was monitored using the coupled HRP assay mentioned above. Initial rates were measured on glycerol concentrations ranging from 1.0 mM to 1.0 M.

**Formation of hydrogen peroxide**

The production of H₂O₂ as by-product of catalysis was detected using catalase (Sigma). For this, 100 µM cinnamyl alcohol was oxidized by 0.2 µM HMFO. When the oxygen level no longer decreased, 150 U of catalase was added to monitor the reappearance of oxygen. Oxygen levels were monitored using REDFLASH sensor spots and a Firesting O₂ detector and light source (Pyroscience, Aachen, Germany). For calibration, a 100 % oxygen solution was prepared.
by stirring MilliQ-water at 2500 rpm for 15 minutes while exposed to air. This resulted in a 270 µm solution (23 °C, pH 7.0, 1.0·10^5 Pa). The water was flushed with argon for 5 minutes to make the anoxic standard.

**Product identification**
The products formed by HMFO using 5-(hydroxymethyl)furfural (1) as a substrate were analyzed using a Zorbax Eclipse XDB-C8 column, 5 µm (Agilent). As mobile phase, 12 mM phosphate buffer of pH 7.0 (A) and acetonitrile (B) were used at a flow rate of 1.2 mL min⁻¹. After 1 min 100% A, B was increased to 5 % in 3.5 min and subsequently to 40 % in 2.5 min. After 0.5 min at 40 % B, the eluent returned to 100% A in 0.5 min and this level was maintained for 2 minutes. Detection was done at 268 nm. The retention times of 5-(hydroxymethyl)furfural (1), furan-2,5-dicarbaldehyde (2), 5-(hydroxymethyl)furan-2-carboxylic acid (6), 5-formylfuran-2-carboxylic acid (3) and 2,5-furandicarboxylic acid (4) were 6.4, 6.0, 1.6, 2.1 and 1.2 minutes respectively. Calibration curves were made using 0.1, 0.5, 1.0 and 2.0 mM solutions.

Product formation was analyzed after 5 hours of conversion of 2 mM 5-(hydroxymethyl)furfural (1) by 5 µm HMFO (25°C, 600 rpm). The reactions were heated at 80°C for 5 minutes to inactivate the enzyme and protein aggregates were subsequently removed by centrifugation. As negative controls, solutions of 5-(hydroxymethyl)furfural (1), furan-2,5-dicarbaldehyde (2), 5-(hydroxymethyl)furan-2-carboxylic acid (6) and 5-formylfuran-2-carboxylic acid (3) were treated in the same way, but in the absence of enzyme, to monitor spontaneous oxidation of the substrate and the intermediates by air.
References

