



**HAL**  
open science

# **Pycnopus cinnabarinus glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives**

Craig Faulds, Mariane Daou, Bassem Yassine, Saowanee Wikee, Eric Record, Françoise Duprat, Emmanuel Bertrand

► **To cite this version:**

Craig Faulds, Mariane Daou, Bassem Yassine, Saowanee Wikee, Eric Record, et al.. Pycnopus cinnabarinus glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives. Fungal Biology and Biotechnology, 2019, 6, 10.1186/s40694-019-0067-8 . hal-02622737

**HAL Id: hal-02622737**

**<https://hal.inrae.fr/hal-02622737v1>**

Submitted on 26 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

# Fungal Biology and Biotechnology

## Pycnoporus cinnabarinus glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives

--Manuscript Draft--

<b>Manuscript Number:</b>									
<b>Full Title:</b>	Pycnoporus cinnabarinus glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives								
<b>Article Type:</b>	Research								
<b>Funding Information:</b>	<table border="1"><tr><td>Ambassade de France en Thaïlande (na)</td><td>Dr Saowanee Wikee</td></tr><tr><td>H2020 European Research Council (720303)</td><td>Prof Craig Faulds Dr Marianne Daou</td></tr><tr><td>FP7 International Cooperation (KBBE-2013-7-613549)</td><td>Prof Craig Faulds Dr Marianne Daou Dr Eric Record</td></tr><tr><td>ECCOREV (na)</td><td>Mr Bassem Yassine Dr Françoise Duprat Dr Emmanuel Bertrand</td></tr></table>	Ambassade de France en Thaïlande (na)	Dr Saowanee Wikee	H2020 European Research Council (720303)	Prof Craig Faulds Dr Marianne Daou	FP7 International Cooperation (KBBE-2013-7-613549)	Prof Craig Faulds Dr Marianne Daou Dr Eric Record	ECCOREV (na)	Mr Bassem Yassine Dr Françoise Duprat Dr Emmanuel Bertrand
Ambassade de France en Thaïlande (na)	Dr Saowanee Wikee								
H2020 European Research Council (720303)	Prof Craig Faulds Dr Marianne Daou								
FP7 International Cooperation (KBBE-2013-7-613549)	Prof Craig Faulds Dr Marianne Daou Dr Eric Record								
ECCOREV (na)	Mr Bassem Yassine Dr Françoise Duprat Dr Emmanuel Bertrand								
<b>Abstract:</b>	<p><b>Background</b></p> <p>5-hydroxymethylfurfural (HMF), a major residual component of a lignocellulosic bio-refinery process, can be transformed into fundamental building blocks for green chemistry via oxidation. While chemical methods are well established, interest is also being directed into the enzymatic oxidation of HMF into the bio-plastic precursor 2,5-furandicarboxylic acid (FDCA).</p> <p><b>Results</b></p> <p>We demonstrate that three glyoxal oxidases (PciGLOX) isoenzymes from the Basidiomycete fungus Pycnoporus cinnabarinus were able to oxidize HMF, with PciGLOX2 and PciGLOX3 being the most efficient. The major reaction product obtained with the three isoenzymes was 5-hydroxymethyl-2-furancarboxylic (HMFCa) a precursor in polyesters and pharmaceuticals production, and very little conversion of this compound was observed. However, small concentrations of 2,5-furandicarboxylic acid, a substitute for terephthalic acid in the production of polyesters, were also obtained. The oxidation of HMF was significantly boosted in the presence of catalase for PciGLOX2 leading to 70% HMFCa yield. The highest conversion percentages were observed on 2,5-Furandicarboxaldehyde (DFF), a minor product from the reaction of PciGLOX on HMF. To bypass HMFCa accumulation and exploit the efficiency of PciGLOX in oxidizing DFF and FFCA towards FDCA production, HMF was oxidized in a cascade reaction with an aryl alcohol oxidase (UmaAAO). After 2 hours of reaction, UmaAAO completely oxidized HMF to DFF and further to FFCA, with FDCA only being detected when PciGLOX3 was added to the reaction. The maximum yield of 16% FDCA was obtained 24 hours after the addition of PciGLOX3 in the presence of catalase.</p> <p><b>Conclusions</b></p> <p>At least two conversion pathways for HMF oxidation can be considered for PciGLOX however the highest selectivity was seen towards the production of the valuable polyester precursor HMFCa. The three isoenzymes showed differences in their catalytic efficiencies and substrate specificities when reacted with HMF derivatives.</p>								
<b>Corresponding Author:</b>	Craig Faulds, PhD Aix-Marseille Université/INRA Marseille, FRANCE								
<b>Corresponding Author Secondary</b>									

<b>Information:</b>	
<b>Corresponding Author's Institution:</b>	Aix-Marseille Universite/INRA
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Craig Faulds, PhD
<b>First Author Secondary Information:</b>	
<b>Order of Authors:</b>	Craig Faulds, PhD
	Marianne Daou
	Bassem Yassine
	Saowanee Wikee
	Eric Record
	Françoise Duprat
	Emmanuel Bertrand
<b>Order of Authors Secondary Information:</b>	
<b>Opposed Reviewers:</b>	Angel Martinez Centro de Investigaciones Biologicas
	Competitor in this area
	Martin Hofrichter Technische Universitat Dresden
	Competitor in this area
	Marco Fraaije Rijksuniversiteit Groningen
	Competitor in this area
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
<b>Is this study a clinical trial?</b><hr><i>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.</i>	No

Comment citer ce document :

Faulds, C., Daou, M. (Co-premier auteur), Yassine, B., Wikee, S., Record, E., Duprat, F. (Collaborateur), *Powered by Editorial Manager® and Production Manager® from Aries Systems Corporation*  
display differential catalytic efficiencies on 5hydroxymethylfurfural and its oxidized derivatives. *Fungal Biology and Biotechnology*. 6. 4. . DOI : <https://doi.org/10.1186/s40694-019-0067-8>

[Click here to view linked References](#)

1                    ***Pycnoporus cinnabarinus* glyoxal oxidases display**  
2                    **differential catalytic efficiencies on 5-hydroxymethylfurfural**  
3                    **and its oxidized derivatives**

4  
5                    **Marianne Daou<sup>1</sup>, Bassem Yassine<sup>1</sup>, Saowanee Wikee<sup>1</sup>, Eric Record<sup>1</sup>,**  
6                    **Françoise Duprat<sup>2</sup>, Emmanuel Bertrand<sup>1</sup>, Craig B. Faulds<sup>1</sup> #**

7                    *<sup>1</sup>Aix Marseille Université, INRA UMR1163 Biodiversité et Biotechnologie*  
8                    *Fongiques (BBF), 13009 Marseille, France*

9                    <sup>2</sup>Aix-Marseille Univ, CNRS, Centrale Marseille, M2P2, Marseille, France

10                    # Address correspondence to [craig.faulds@univ-amu.fr](mailto:craig.faulds@univ-amu.fr)

11

12

13

14

15

16

17

18

19

Comment citer ce document :

Faulds, C., Daou, M. (Co-premier auteur), Yassine, B., Wikee, S., Record, E., Duprat, F. (Collaborateur), Bertrand, E. (Collaborateur) (2019). *Pycnoporus cinnabarinus* glyoxal oxidases display differential catalytic efficiencies on 5hydroxymethylfurfural and its oxidized derivatives. *Fungal Biology and Biotechnology*. 6. 4. . DOI : <https://doi.org/10.1186/s40694-019-0067-8>

20

21 **Abstract**

22 **Background:** 5-hydroxymethylfurfural (HMF), a major residual component of  
23 a lignocellulosic bio-refinery process, can be transformed into fundamental  
24 building blocks for green chemistry via oxidation. While chemical methods are  
25 well established, interest is also being directed into the enzymatic oxidation of  
26 HMF into the bio-plastic precursor 2,5-furandicarboxylic acid (FDCA).

27 **Results:** We demonstrate that three glyoxal oxidases (*PciGLOX*) isoenzymes  
28 from the Basidiomycete fungus *Pycnoporus cinnabarinus* were able to oxidize  
29 HMF, with *PciGLOX2* and *PciGLOX3* being the most efficient. The major  
30 reaction product obtained with the three isoenzymes was 5-hydroxymethyl-2-  
31 furancarboxylic (HMFCFA) a precursor in polyesters and pharmaceuticals  
32 production, and very little conversion of this compound was observed.  
33 However, small concentrations of 2,5-furandicarboxylic acid, a substitute  
34 for terephthalic acid in the production of polyesters, were also obtained. The  
35 oxidation of HMF was significantly boosted in the presence of catalase for  
36 *PciGLOX2* leading to 70% HMFCFA yield. The highest conversion percentages  
37 were observed on 2,5-Furandicarboxaldehyde (DFF), a minor product from  
38 the reaction of *PciGLOX* on HMF. To bypass HMFCFA accumulation and  
39 exploit the efficiency of *PciGLOX* in oxidizing DFF and FFCA towards FDCA  
40 production, HMF was oxidized in a cascade reaction with an aryl alcohol  
41 oxidase (*UmaAAO*). After 2 hours of reaction, *UmaAAO* completely oxidized  
42 HMF to DFF and further to FFCA, with FDCA only being detected when  
43 *PciGLOX3* was added to the reaction. The maximum yield of 16% FDCA was  
44 obtained 24 hours after the addition of *PciGLOX3* in the presence of catalase.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
**Conclusions:** At least two conversion pathways for HMF oxidation can be considered for *Pci*GLOX however the highest selectivity was seen towards the production of the valuable polyester precursor HMFCA. The three isoenzymes showed differences in their catalytic efficiencies and substrate specificities when reacted with HMF derivatives.

51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
**Keywords:** 5-Hydroxymethylfurfural, bio-catalysis, glyoxal oxidase, 2,5-furancarboxylic acid, furan derivatives

## 66 **Background**

67 With the growing concerns about the depleting supply of fossil fuel and the  
68 global problem of climate change, the demands for sustainable substitutes for  
69 petroleum-based products are increasing. Being one of the most abundant  
70 renewable natural materials on earth, plants, especially those low-cost  
71 residues from agro-industrial processing, have become a major candidate for  
72 this role. Approximately 75% of annual production of agro-industrial residues  
73 is in the form of carbohydrates, making this material highly exploitable [1].  
74 Efforts are being directed for the conversion of plant carbohydrates into  
75 valuable chemicals and fuels. Among the valuable platform chemicals that  
76 can be obtained from biomass carbohydrates are 5-hydroxymethylfurfural  
77 (HMF) and furfural [2], produced by triple dehydration of hexoses [3] and acid  
78 hydrolysis of pentosans respectively [4].

79 HMF is particularly important due to its biodegradability and its versatility as a  
80 precursor for a wide selection of furan-based products, and has been  
81 recognized as top value-added molecule in biotechnology [5]. Important  
82 molecules derived from HMF include dimethylfuran, levulinic acid, 2,5-  
83 furancarboxylic acid (FDCA), 2,5-diformylfuran (DFF), 3,5-  
84 dihydroxymethylfuran, 5- hydroxy-4-keto-pentenoic acid and 5-hydroxymethyl-  
85 2-furancarboxylic acid (HMFCFA) [6]. DFF is a stable derivative of HMF. This  
86 molecule is considered important for the synthesis of pharmaceutical  
87 compounds [7], antifungal products [8], electroconductors [9] and polymeric  
88 materials [10]. Another important product from the oxidation of the aldehyde  
89 group of HMF is HMFCFA. This is particularly important for the production of  
90 polyesters [11] and interleukin inhibitors [12], and to have *in vivo* antitumor

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

91 activity against Sarcoma 180 cells [13]. Furan-based derivatives also include  
92 2,5-furandicarboxylic acid (FDCA), which was considered among the 12 most  
93 promising sugar-based molecules for the production of bio-based material  
94 [14]. FDCA is particularly important because it can replace fossil-based  
95 terephthalic acid for the production of bio-based polyesters [15]. Chemically,  
96 HMF derivatives are produced using metals as catalysts under extreme  
97 conditions such as high temperature, high pressure and in the presence of  
98 toxic solvents. The development of bio-catalytic processes is therefore  
99 advantageous since enzymes work under mild conditions which can reduce  
100 the energy required and the production cost.

101

102 Among the enzymes that were previously tested and used for the oxidation of  
103 HMF are three alcohol oxidases (EC 1.1.3.13) and one galactose oxidase  
104 (GAO; EC 1.1.3.9) that have been reported to oxidize HMF leading to the  
105 formation of DFF as the sole product following the reaction presented in  
106 **scheme 1** [16]. Synthesis of HMFCA by bio-catalysis remained limited due to  
107 the need of high reaction selectivity in order to oxidize the aldehyde group and  
108 leave the hydroxyl group intact [17]. Xanthine oxidase (EC 1.17.3.2) from  
109 *Escherichia coli* was used for the selective oxidation of HMF to HMFCA [16].  
110 Oxidative conversion of HMF to FDCA was also performed following a two-  
111 steps reaction using fungal aryl-alcohol oxidase (AAO; EC 1.1.3.7) and an  
112 unspecific heme peroxygenase (UPO; EC 1.11.2.1) [18]. Full HMF conversion  
113 with high FDCA yields was also achieved using an FAD-dependent oxidase,  
114 named HMF oxidase (HMFO; EC 1.1.3.47) [19, 20].



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

115 Although HMF derivatives are highly important, limited examples describing  
116 the synthesis of these compounds via enzymatic reactions were previously  
117 described and the pursuit for new enzymes that can be involved in this  
118 process is still highly interesting. Promising candidates are enzymes with  
119 versatile substrate specificity able to perform the successive oxidation steps  
120 required in the process by recognizing HMF and its derivatives as substrates.

121 Glyoxal oxidases (GLOX; EC 1.2.3.15) are metalloenzymes containing one  
122 copper metal ion, and belong to a class of enzymes called radical copper  
123 oxidases (CRO) [21]. These enzymes have been grouped within the AA5  
124 family in the CAZymes (Carbohydrate Active Enzymes database;  
125 <http://www.cazy.org/>) classification system [22, 23]. GLOX catalyzes the  
126 oxidation of aldehydes to their corresponding carboxylic acids, generating  
127 simultaneously hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [24, 25]. This enzyme was first  
128 isolated in ligninolytic cultures of the white-rot Basidiomycete *Phanerochaete*  
129 *chrysosporium* under restricted nitrogen supply, suggesting a role in lignin  
130 modification and peroxidase H<sub>2</sub>O<sub>2</sub>-dependent action [26]. Subsequent  
131 sequencing of the *P. chrysosporium* genome has indicated the presence of  
132 only one gene encoding GLOX in this fungus [27]. The genome of another  
133 Polyporale fungus, *Pycnoporus cinnabarinus* [28] indicated that this fungus  
134 contained 3 *glox* genes. *In vitro* characterization of these GLOX revealed their  
135 ability to act on a broad range of substrates including toxic and inhibitory  
136 aldehydes [24, 25]. In addition, GLOX were able to recognize and oxidize the  
137 alcohol group of glycerol extending further the specificity range of these  
138 enzymes [29, 25]. Although the utilization of GLOX for biotechnological  
139 applications had not previously been elaborated upon, the reactions catalyzed

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

140 by this enzyme made it a promising candidate, especially for the oxidation of  
141 HMF and its derivatives.

142 In this work, the ability of two previously produced and characterized GLOX  
143 from *P. cinnabarinus* BRFM 137 (*PciGLOX1* and *PciGLOX2*) [25] and a third  
144 isoform from this fungus (*PciGLOX3*) to oxidize HMF and its derivatives was  
145 investigated.

146

## 147 **RESULTS**

### 148 ***PciGLOX3* Characterization**

149 *PciGLOX3* shared 84% and 89% protein sequence identity with *PciGLOX1*  
150 and *PciGLOX2*, respectively, and the amino acids at the active site were  
151 highly conserved (**Fig. 1**). *PciGLOX3* showed a generally lower activity on the  
152 tested substrates compared to the other two *PciGLOX* enzymes, however, the  
153 specificity range for all three isoenzymes was similar except in the case of  
154 glycerol, which was only oxidized by *PciGLOX2* and *PciGLOX3* (**Table 1**).  
155 One major difference observed was that highest activity detected on  
156 formaldehyde for *PciGLOX3* while the two other enzymes were most active on  
157 glyoxylic acid. Differences in the kinetic constants were also observed for  
158 *PciGLOX3* that showed the highest specificity and catalytic efficiency on  
159 glycerol compared to the other enzymes (**Table 2**). In addition, *PciGLOX3*  
160 was less efficient in oxidizing glyoxylic acid compared to the two other GLOX.

161

### 162 **Enzyme Stability under the Conditions of Reaction**

163 The standard reaction conditions for the oxidation of HMF and its derivatives

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

164 were chosen based on the stability and performance of the enzymes under  
165 these conditions. The optimal pH was 6 and the enzymes were active in  
166 dimethylsuccinate and tartrate buffers [25]. The reactions were performed in  
167 tartrate buffer, as dimethylsuccinate could not be resolved from the substrates  
168 and expected products of the enzymatic reaction in the HPLC analysis. The  
169 stability of *PciGLOX* enzymes under these conditions and over a prolonged  
170 period of time were assessed. *PciGLOX2* was the least stable of the three  
171 isoenzymes and lost around 90% of its activity after 2 hours of incubation  
172 (**Figure 2a**). On the other hand, *PciGLOX1* and *PciGLOX3* retained 70% and  
173 40% of their activities after 48 hours of incubation, respectively. The stability  
174 of the three enzymes was also assessed in the presence of 3 mM HMF. In the  
175 presence of this substrate, no activity was detected for *PciGLOX2* after 2  
176 hours of incubation (**Figure 2b**). On the other hand, the activities of  
177 *PciGLOX1* and *PciGLOX3* were relatively stable with *PciGLOX3* showing  
178 increased residual activity after 48 hours. The stability of the three *PciGLOX*  
179 enzymes was further investigated in the presence of 3 mM HMFCa the major  
180 theoretically expected product of the reaction on HMF. In the presence of this  
181 molecule, the significant loss in activity after 24 hours of incubation was  
182 comparable between the three isoforms (**Figure 2c**). HRP was highly stable  
183 under the tested conditions (**Additional file 1**).

184

### 185 **Stability towards H<sub>2</sub>O<sub>2</sub>**

186 The stability of the three *PciGLOX* isoenzymes was investigated in the  
187 presence of varying concentrations of H<sub>2</sub>O<sub>2</sub>. *PciGLOX1* was the most stable  
188 enzyme in the presence of this reaction product, retaining 60% of its activity

189 when incubated with 10 mM H<sub>2</sub>O<sub>2</sub> for 24 hours (**Figure 3**). Although more  
190 stable than *PciGLOX3*, *PciGLOX2* significantly lost activity with increasing  
191 concentrations of H<sub>2</sub>O<sub>2</sub> and lost more than 95% of its activity after incubation  
192 in the presence of 8 mM H<sub>2</sub>O<sub>2</sub>. *PciGLOX3* on the other hand lost 60% of its  
193 activity with only 2 mM H<sub>2</sub>O<sub>2</sub> and was completely inactive with 10 mM H<sub>2</sub>O<sub>2</sub>  
194 after 24 hours of incubation.

195

### 196 **Oxidation of HMF and its derivatives by *PciGLOX***

197 The three *PciGLOX* isoenzymes were found to be active on HMF and this  
198 became evident by the decrease in HMF concentration and the appearance of  
199 the oxidation products over time (**Figure 4, Additional file 2**). Although the  
200 catalytic efficiencies of the three enzymes were comparable on HMF (**Table**  
201 **3**), the conversion percentage of this substrate varied between the *PciGLOX*  
202 enzymes (14% for *PciGLOX1*, 34% for *PciGLOX2* and 28% for *PciGLOX3*). In  
203 addition, the three isoenzymes noticeably differed in their products' patterns.

204 The major product of the reactions of the *PciGLOX* isoenzymes on HMF was  
205 HMFCFA with *PciGLOX2* and *PciGLOX3* producing the highest yields (39%  
206 and 41%, respectively; **Figure 4b, c**). By adding catalase to the reaction of  
207 *PciGLOX2*, the amount of produced HMFCFA was considerably increased to  
208 76 % (**Figure 4b right**). This increase was coupled to a significant boost in  
209 HMF conversion. Interestingly, the addition of catalase seemed to shift the  
210 reaction more towards the production of HMFCFA and decreased the amount  
211 of FFCA produced from 5% to 0.7%. This activity-enhancing effect in the

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

212 presence of catalase was also observed with *PciGLOX3* but not with  
213 *PciGLOX1*.

214 Interestingly, small amounts of DFF, the alternative oxidation product of HMF,  
215 were also detected. The concentration of detected DFF slightly increased over  
216 time in the reaction with *PciGLOX1* (0.017 mM at 24 hours). In the cases of  
217 *PciGLOX2* and *PciGLOX3*, smaller DFF concentrations were detected early in  
218 the reaction. The substrate specificity and catalytic efficiencies of *PciGLOX2*  
219 and *PciGLOX3* on DFF as a substrate ( $K_m = 0.2$  mM;  $K_{cat}/K_m = 23403$  s<sup>-1</sup> M<sup>-1</sup>  
220 and  $K_m = 0.2$  mM;  $K_{cat}/K_m = 7267$  s<sup>-1</sup> M<sup>-1</sup>, respectively) were significantly  
221 better than that of *PciGLOX1* ( $K_m = 4.3$  mM;  $K_{cat}/K_m = 124.3$  s<sup>-1</sup> M<sup>-1</sup>). This  
222 was further supported in the results obtained when DFF was used as the  
223 initial substrate and 80% to 84% converted by *PciGLOX2* and *PciGLOX3*,  
224 respectively (**Figure 5a**).

225 The three *PciGLOX* were also reacted with HMFCFA as the initial substrate.  
226 Although this compound was produced as the major product in HMF  
227 oxidation, the three *PciGLOX* showed very weak activity towards HMFCFA,  
228 with *PciGLOX3* having the best conversion rate (2%; **Figure 5b**). The extent  
229 of HMFCFA to FFCA conversion by *PciGLOX* was not sufficient to calculate  
230 catalytic constants. In addition, Higher concentrations of FFCA and FDCA  
231 were obtained when the enzymes were reacted with DFF and FFCA as  
232 substrates, respectively (**Figure 5a and c**).

233

## 234 **GLOX and AAO Cascade Reactions**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

235 A cascade reaction where *UmaAAO* was used to oxidize HMF followed by the  
236 addition of *PciGLOX* was tested. *PciGLOX3* was used in this experiment  
237 since this enzyme showed the highest conversion percentage on DFF (84%  
238 after 24 hours) and the highest FDCA yields (14% after 24 hours on FFCA).  
239 Two conditions were tested: (1) *PciGLOX3* was added after 2 hours of  
240 *UmaAAO* reaction on HMF, and (2) *PciGLOX3* was added after 24 hours of  
241 *UmaAAO* reaction on HMF. Under condition (1) HMF was completely oxidized  
242 by *UmaAAO* and the major product before the addition of *PciGLOX3* was DFF  
243 whereas in condition (2) FFCA was the predominant product (**Figure 6**;  
244 **Additional file 2**).

245 After 4 hours of adding *PciGLOX3*, FFCA was the major product (2.6 mM) in  
246 the reaction and a little amount of DFF (0.5 mM) was detected (**Table 4**).  
247 Twenty hours later, DFF was almost completely consumed and the  
248 concentration of FDCA increased (0.03 mM). In the presence of catalase,  
249 DFF was completely oxidized after 4 hours of reaction and the concentration  
250 of FDCA obtained was 6 folds higher after 24 hours (**Table 4**). The yield of  
251 FDCA was further increased in condition (2) to reach 14% and 16% after 24  
252 hours in the absence and presence of catalase, respectively (**Table 4**). The  
253 obtained yields in both tested conditions were comparable to the ones  
254 obtained when *PciGLOX3* was reacted with DFF and FFCA as the initial  
255 substrates (**Figure 5**).

## 257 Discussion

258 As mentioned in the introduction, HMF, a residue of the 2G lignocellulosic

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

259 biorefinery, is of particularly industrial importance due to its biodegradability  
260 and its versatility as a precursor for a wide selection of furan-based products,  
261 and as such has been recognized as top value-added molecule in  
262 biotechnology. Currently, large chemical plants are being constructed to  
263 produce HMF, but a number of biological conversions have been identified in  
264 recent years, including the use of glyoxal oxidases as described in this paper.  
265 Although the three GLOX from *P. cinnabarinus* have a high protein sequence  
266 similarity and a conserved copper center, significant catalytic differences were  
267 observed and it was therefore interesting to compare the activity of all three  
268 enzymes on the conversion of HMF and its derivatives. The observed catalytic  
269 differences, in addition to the fact that *PciGLOX3* has not yet been found to be  
270 secreted during fungal growth on different substrates [28], suggests different  
271 physiological roles of these enzymes during fungal growth.  
272 While enzymatic assays with *PciGLOX* were performed at 30°C, the  
273 temperature at which fungi usually grows and secretes these enzymes *in*  
274 *vitro*, the pH and buffer system were also previously found to be critical  
275 factors for activity [25]. *PciGLOX1* and *PciGLOX3* were very stable under the  
276 reaction conditions used, while *PciGLOX2* lost significantly activity over time,  
277 especially in presence of its substrate, HMF. The stability of AAO from *P.*  
278 *eryngii*, which can also catalyze the oxidation of HMF to DFF, was previously  
279 determined in the presence of 3 mM HMF, and the enzyme was found to lose  
280 30% of its activity after 24 hours [18]. Similarly to HMF, the major reaction  
281 product HMFCA seems to alter considerably the catalytic properties of the  
282 *PciGLOX* enzymes. However, it is difficult to determine in the presence of  
283 HMF and HMFCA if the observed effect is related to changes in stability or if it

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

284 is due to these molecules remaining in or close to the active sites of the  
285 enzymes, especially in the case of *PciGLOX2* that was completely inactive on  
286 GA in the presence of HMF.

287 When incubated with different concentrations of H<sub>2</sub>O<sub>2</sub> over time, the three  
288 isoenzymes again showed differences in stability. The inhibition of *PchGLOX*  
289 by exogenous H<sub>2</sub>O<sub>2</sub> has previously been reported [30]. However, the three  
290 *PciGLOX* isoenzymes were found to be more stable than *PchGLOX*, which  
291 retained only 25% of its initial activity in the presence of 2.1 mM exogenous  
292 H<sub>2</sub>O<sub>2</sub> in the reaction mixture [30]. Furthermore, the detection of the reaction  
293 product of *PchGLOX* on glycerol was only possible when the activity was  
294 extended by adding catalase [29], which is not the case for *PciGLOX* [25].  
295 H<sub>2</sub>O<sub>2</sub> can oxidize proteins and alter the functional groups of certain amino  
296 acids leading to cleavage of the polypeptide chain and protein aggregation  
297 [31]. Therefore, one could postulate that the differences in stability observed  
298 between the three *PciGLOX* in the presence of H<sub>2</sub>O<sub>2</sub> could be explained by  
299 structural variations between the three isoenzymes.

300 Significant difference in the kinetic parameters and products' yields for the  
301 oxidation of HMF were observed between the three enzymes, with the highest  
302 catalytic efficiency on HMF obtained with *PciGLOX3* ( $K_{cat}/K_m = 118.35 \text{ s}^{-1} \text{ M}^{-1}$ )  
303 <sup>1</sup>). The oxidation of HMF using a phylogenetically different GLOX from  
304 *Myceliophthora thermophila* M77 (*MtGLOX*) has been recently published [32].  
305 Similarly to *PciGLOX* described in this paper, low conversion levels were  
306 observed with HMF. However *MtGLOX* showed a higher catalytic efficiency  
307 and specificity towards HMF ( $K_{cat}/K_m = 787 \text{ s}^{-1} \text{ M}^{-1}$ ,  $K_m = 0.02 \text{ M}^{-1}$ )



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
308 compared to *Pci*GLOX. Interestingly MtGLOX oxidized the alcohol group of  
309 HMF leading to the formation of DFF.

310 Higher specificity and two-fold higher catalytic efficiency of AAO from *P.*  
311 *eryngii* were reported on HMF ( $K_{cat}/K_m = 210 \text{ s}^{-1} \text{ M}^{-1}$ ) compared to that  
312 recorded in this study on *Pci*GLOX [18]. In a more recent study using AAO for  
313 the oxidation of HMF, Karich and co-workers reported varying catalytic  
314 constants for the homologously produced AAO from *P. eryngii* on HMF with  
315 catalytic efficiency comparable to that obtained for *Pci*GLOX [33]. However, *P.*  
316 *eryngii* AAO was less specific on HMF. Another enzyme used for the oxidation  
317 of HMF is the FAD-dependent oxidase, HMFO, which was more specific on  
318 HMF compared to *Pci*GLOX and has a significantly higher catalytic efficiency  
319 ( $K_{cat}/K_m = 7000 \text{ s}^{-1} \text{ M}^{-1}$ ) [19]. A 95% conversion yield of HMF to FDCA was  
320 achieved using HMFO, however the concentration used was 60 times higher  
321 than the one used for *Pci*GLOX in this work. HMFO also requires oxygen as  
322 an electron acceptor.

323 The major product of the *Pci*GLOX reaction on HMF was HMFCA. This result  
324 was expected since GLOX recognizes the aldehyde group of its substrate  
325 [25], HMF in this case (**Scheme 1**). The need for this specificity has limited  
326 the number of described bio-catalytic pathways for the production of HMFCA.  
327 A previously described enzyme for this purpose is the bacterial xanthine  
328 oxidase (XO), which recognizes the formyl group of HMF [16]. Using 2.2  $\mu\text{M}$   
329 of this enzyme, HMFCA was obtained with a yield of 94% after 7 hours of  
330 reaction on 26 mM HMF. The amount of *Pci*GLOX used in this work was  
331 comparable to XO, but the yield of HMFCA obtained with *Pci*GLOX2 after 24  
332 hours of reaction was significantly lower. However, a two folds increase in

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

333 HMFCA yields was observed when catalase was added to *Pci*GLOX2  
334 reaction. This was in agreement with the results showing that *Pci*GLOX2 was  
335 very sensitive to accumulating H<sub>2</sub>O<sub>2</sub> concentrations. Catalase converts H<sub>2</sub>O<sub>2</sub>  
336 to O<sub>2</sub>, protecting the enzymes from oxidative damage and supplying O<sub>2</sub>. This  
337 enzyme was previously used to eliminate the accumulating H<sub>2</sub>O<sub>2</sub> from the  
338 action of alcohol oxidases on HMF [16]. In addition, catalase was found to  
339 boost HMF conversion in the coupled reaction of GAO and HRP, which is  
340 similar to the effect observed in this work [16].

341 In addition to HMFCA, small amount of DFF were detected at early stages of  
342 the reaction especially with *Pci*GLOX1. This result shows that *Pci*GLOX are,  
343 to a smaller extent, able to act on the alcohol group of HMF. This is the  
344 second example of alcohol oxidation by these enzymes, which have been  
345 previously found to be active on glycerol [25, 29]. HMF oxidation to DFF as  
346 the only product was recently observed with *Mt*GLOX; however this enzyme  
347 was unable to further oxidize DFF in the reaction [32]. Enzymes known to  
348 convert HMF to DFF include also AAO, alcohol oxidase, GAO, and HMFO  
349 [16, 18, 34, 19, 33] and these enzymes showed higher yields of DFF  
350 compared to *Pci*GLOX. However, the substrate specificity and catalytic  
351 efficiencies of *Pci*GLOX2 and *Pci*GLOX3 on DFF as a substrate ( $K_m = 0.2$   
352 mM;  $K_{cat}/K_m = 23403 \text{ s}^{-1} \text{ M}^{-1}$  and  $K_m = 0.2 \text{ mM}$ ;  $K_{cat}/K_m = 7267 \text{ s}^{-1} \text{ M}^{-1}$ ,  
353 respectively) were significantly better than that of AAO from *P. eryngii* ( $K_m$   
354  $= 3.3 \text{ mM}$ ;  $K_{cat}/K_m = 158 \text{ s}^{-1} \text{ M}^{-1}$ ) [18] and HMFO ( $K_m = 1.7 \text{ mM}$ ;  $K_{cat}/K_m =$   
355  $940 \text{ s}^{-1} \text{ M}^{-1}$ ) [19]. *Pci*GLOX2 and *Pci*GLOX3 were also more efficient than  
356 *Pci*GLOX1 in oxidizing DFF which explains the presence of constant  
357 concentrations of DFF with *Pci*GLOX1. On the other hand, in the cases of

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
358 *PciGLOX2* and *PciGLOX3*, it is more likely that DFF was produced and  
359 consumed very fast in the reaction.

360 On the contrary, the major reaction product, HMFCa was very weakly  
361 oxidized by *PciGLOX*. In addition, although the three enzymes and especially  
362 *PciGLOX2* and *PciGLOX3* were able to oxidize HMF, higher concentrations of  
363 FFCA and FDCA were obtained when the enzymes were reacted with DFF  
364 and FFCA as substrates, respectively.

365 In the reactions of *PciGLOX* on HMF, very little DFF is produced compared to  
366 the “dead-end product” HMFCa which might be strongly altering the stability  
367 of the enzymes at early stages of the reaction preventing further oxidation  
368 steps. In addition, the obtained results suggest that similarly to AAO, HMFO  
369 and chloroperoxidase [18, 19, 34], *PciGLOX* enzymes produce FFCA mainly  
370 via the oxidation of DFF (**Scheme 1**), which is a minor product in the oxidation  
371 of HMF by *PciGLOX*. However, *PciGLOX* are more specific and catalytically  
372 efficient than these enzymes in oxidizing DFF. For these reasons a cascade  
373 reaction, where *UmaAAO* was used to oxidize HMF followed by the addition  
374 of *PciGLOX*, was tested. AAO was previously used in tandem reactions with  
375 an unspecific oxidase for the oxidation of HMF [18, 33]. Similarly to AAOs  
376 from *Pleurotus eryngii*, and *Pleurotus ostreatus* AAO, the AAO from *U. maydis*  
377 (*UmaAAO*) generates FFCA via the oxidation of HMF to DFF [18, 33].  
378 *UmaAAO* was also found as efficient as *P. eryngii* AAO in oxidizing HMF  
379 leading to full conversion after 2 hours of reaction. In contrary to *P. eryngii*  
380 AAO, *UmaAAO* seems less efficient in converting DFF to FFCA in the  
381 reaction. However, the used concentration of *UmaAAO* in this study is  
382 significantly lower and a kinetic study is needed to determine the efficiency.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

383 In the current *Pci*GLOX tandem reaction, the yields of obtained FDCA were  
384 lower compared to the ones obtained in the cascade reactions with UPO [18,  
385 33]. A limiting step in the reaction of *Pci*GLOX seems to be the conversion of  
386 FFCA to FDCA and this was observed when FFCA was used as initial  
387 substrate. This effect is most possibly due to exogenous factors affecting the  
388 reaction since the enzymes exhibit relatively high specificity and catalytic  
389 efficiency towards FFCA. A probable factor could be the inhibition of the  
390 enzymes by the substrate and/or the product of the reaction. A second  
391 possible factor affecting this reaction is the accumulation of H<sub>2</sub>O<sub>2</sub> throughout  
392 the first oxidation steps inhibiting the progress of the reaction in the FFCA  
393 oxidation stage. The sensitivity of *Pci*GLOX enzymes in the presence of H<sub>2</sub>O<sub>2</sub>  
394 supports this hypothesis. It is also interesting to mention that this effect was  
395 previously reported for *Post*AAO where the sensitivity of FFCA oxidation  
396 reaction to H<sub>2</sub>O<sub>2</sub> was 300-times higher than that of the initial reaction [33].

## 398 **Conclusion**

399 The potential of *Pci*GLOX for the production of valuable furan derivatives from  
400 HMF was investigated in this work. Interestingly, the three GLOX belonging to  
401 the same organism and sharing high sequence similarity of 84-89%, showed  
402 differences in their catalytic properties and product patterns on the same  
403 substrates. Although known for their activity on aldehydes, these proteins  
404 were also able to act on the alcohol group of the substrate. Previously, this  
405 activity has been explained by the hypothesis that enzymes such as HMFO  
406 for example recognizes the hydrated form of the aldehyde in the reaction

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

407 which explains their activity on DFF that rapidly forms hydrate in buffer pH 5-8  
408 [19]. When the substrate has a carboxylic acid group in addition to the  
409 aldehyde group such as in the case of FFCA, the formation of hydrate is  
410 highly unfavorable and this explain the low activity of HMFO on FFCA [35,  
411 19]. However, *PciGLOX* were active on FFCA and were found to efficiently  
412 oxidize the aldehyde group of HMF. HMF was previously reported not to form  
413 gem-diol under conditions similar to the ones used in this work [18]. This  
414 shows that *PciGLOX* are able to recognize and oxidize both, aldehyde and, in  
415 certain cases, alcohol groups. HMFCA remains the major product of the  
416 reaction preventing the production of FDCA. However, the specificity of  
417 *PciGLOX* towards producing HMFCA itself can be considered of high  
418 biotechnological significance. The addition of catalase, to remove the  
419 influence of self-inhibition through the production of H<sub>2</sub>O<sub>2</sub>, improved reaction  
420 yields, while the addition of an AAO prior to the addition of *PciGLOX*, shifted  
421 the reaction pathway through DFF and towards the production of FDCA, the  
422 desired precursor for bioplastic synthesis.

## 423 **MATERIALS AND METHODS**

### 424 **Chemicals and Enzymes**

425 All chemicals were of analytical grade. HMF, DFF, HMFCA, 5-formyl-2-furan  
426 carboxylic acid (FFCA), FDCA, 2,2'-azino-bis(3-ethylbenzothiazoline-6-  
427 sulphonic acid (ABTS) and glyoxylic acid (GA), together with the enzymes  
428 horseradish peroxidase (HRP) and catalase were purchased from Sigma  
429 Aldrich (Lyon, France). *PciGLOX1* and *PciGLOX2* enzymes were  
430 heterologously produced in *Aspergillus niger* as described previously [25].

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

431 The same protocol was used to produce the third isoform, *PciGLOX3*, and is  
432 reported here for the first time. AAO from *Ustilago maydis* was produced as  
433 previously reported in our laboratory [36]. The sequences of *PciGLOX1*,  
434 *PciGLOX2* and *PciGLOX3* are available in GenBank under accession  
435 numbers KU215437, KU215438 and MK268804, respectively.

436

### 437 **Enzyme Activity Assay**

438 The activity of the three *PciGLOX* enzymes was assayed in a coupled  
439 reaction with HRP following the protocol previously described [25]. When  
440 purified, GLOX enzymes are inactive and their oxidative activation was found  
441 to be possible in the presence of lignin peroxidase or HRP, or by the addition  
442 of strong oxidants such as molybdicyanide ( $K_3Mo(CN)_8$ ), hexachloroiridate  
443 ( $Na_2IrCl_6$ ), or  $Mn^{3+}$  EDTA [24, 37]. HRP was used in this study since it is  
444 readily obtained commercially, highly stable under the conditions used, and  
445 does not act on any of the substrates under investigation for oxidation by  
446 GLOX. The reaction mixture consisted of 50 mM sodium tartrate buffer (pH 6)  
447 containing HRP (8 U), 0.1 mM ABTS, *PciGLOX* (1  $\mu$ g) and GLOX substrate at  
448 varying concentrations depending on the reaction in 1 mL final volume. The  
449 lag period was eliminated by adding 5  $\mu$ M  $H_2O_2$ . The reaction was initiated by  
450 the addition of the GLOX substrate and the oxidation of ABTS was followed at  
451 436 nm for 1.5 minute. The standard assay was performed at 30 °C. All  
452 assays were performed in triplicate.

453

### 454 ***PciGLOX3* Characterization**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

455 The substrate specificity of *Pci*GLOX3 on 10 mM of previously tested  
456 molecules (**Table 1**) with *Pci*GLOX1 and *Pci*GLOX2 was investigated  
457 following the activity test described above. The same standard assay was  
458 used to determine the kinetic constants for *Pci*GLOX3 on methylglyoxal (0.02  
459 to 5 mM), glyoxal (0.1 to 7 mM), glyoxylic acid (0.1 to 10 mM), and glycerol  
460 (0.3 to 80 mM) as substrates. Lineweaver-Burk plots, obtained using the  
461 GraFit (version 4) program [38], were used to calculate the kinetic  
462 parameters.

463 The three *Pci*GLOX sequences were aligned using the ClustalW program  
464 within the MegAlign module (version11.0.0) of DNASTar software (Madison,  
465 WI).

466

### 467 **Enzyme Stability**

468 The three *Pci*GLOX were incubated at 30 °C and 800 rpm agitation for 48  
469 hours in 50 mM sodium tartrate buffer pH6. Samples were taken after 2, 4, 24  
470 and 48 hours and the residual activity was measured using GA as substrate  
471 and calculated as a percentage of the activity at time zero. The same  
472 experiment was also performed in the presence of 3 mM HMF or HMFCA to  
473 determine the stability of the enzymes in the presence of these molecules.  
474 The stability of HRP was also assessed under the same conditions. All  
475 measurements were performed in triplicates.

476

### 477 **Kinetic Studies**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

478 The coupled reaction with HRP and ABTS under standard conditions was  
479 used to determine the steady-state kinetic parameters for *Pci*GLOX oxidation  
480 of HMF (0.3-50 mM), DFF (0.01-2 mM), HMFCA (0.1-3 mM) and FFCA (0.2-5  
481 mM). Kinetic parameters were obtained by fitting the data to the Michaelis-  
482 Menten equation using R statistical software (R Core Team, Vienna Austria).

483

### 484 **HPLC Analysis of Reaction Products**

485 All oxidation reactions were performed at 30 °C under agitation at 800 rpm in  
486 Eppendorf ThermoMixer tubes (Eppendorf, Montesson, France) over 24 hours  
487 period in 100 mM tartrate buffer, pH6. To follow the reaction of the three  
488 *Pci*GLOX isoenzymes on HMF, DFF, HMFCA and FFCA over time, reaction  
489 mixtures containing *Pci*GLOX (20 µg), HRP (8 Units), ABTS (0.1 mM) and the  
490 substrate (3 mM) were prepared. Samples were taken after 0, 0.5, 1, 1.5, 2, 3,  
491 4, 5, 6, 8 and 24 hours and analyzed. Reactions containing the same  
492 constituents except *Pci*GLOX were followed for the same period of time and  
493 used as controls. All measurements were performed in duplicates.

494 Reaction mixtures were separated on Aminex HPX-87H column (300 x 7.8  
495 mm) (BioRad) at 45 °C, with 2.5 mM sulfuric acid as the mobile phase with a  
496 flow rate of 0.6 mL/min. Eluted compounds were detected using a diode array  
497 detector at 280 nm. The reactions were stopped by incubating the mixture at  
498 90 °C for 10 min and centrifuging at 15000 xg for 15 minutes. The samples  
499 were then filtered using 0.45µm polyvinylidene difluoride syringe filters  
500 (Restek, Lisses, France) before injection in the column. Peak areas from the



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

501 obtained chromatograms were converted to molar concentration using  
502 calibration curves of pure substrates and products standards.

503 The percentage conversion of the used substrate was determined according  
504 to the following equation:

$$505 \quad \% \text{Conversion} = \left( 1 - \frac{\text{Concentration of substrate}}{\text{Initial concentration of substrate}} \right) \times 100$$

506 The percentage yield of the reaction products was determined according to  
507 the following equation:

$$508 \quad \% \text{Yield} = \left( \frac{\text{Concentration of product}}{\text{Initial concentration of substrate}} \right) \times 100$$

509

### 510 **Effect of H<sub>2</sub>O<sub>2</sub> on HMF Oxidation**

511 The stability of the three *Pci*GLOX isoenzymes in the presence of H<sub>2</sub>O<sub>2</sub> was  
512 assessed by measuring the residual activity of the enzymes after pre-  
513 incubation with different concentrations of H<sub>2</sub>O<sub>2</sub> over 24 hours. The H<sub>2</sub>O<sub>2</sub> was  
514 removed before adding the enzyme to the reaction mixture by washing the  
515 samples with buffer in an Amicon ultrafiltration unit with a 10-kDa-molecular-  
516 mass-cut-off membrane (Merck Millipore). All measurements were performed  
517 in duplicate.

518 To determine the effect of hydrogen peroxide on the oxidation of HMF and its  
519 derivatives by *Pci*GLOX, the standard reaction was performed in the presence  
520 of 10 µg catalase (2000 – 5000 Units/mg protein). The reaction was followed  
521 over time as described above. A control reaction containing all the  
522 components except *Pci*GLOX was analyzed to determine the effect of  
523 catalase on HMF oxidation.

524

## 525 ***PciGLOX* and *UmaAAO* Cascade Reactions**

526 The oxidation of HMF by *UmaAAO* in the presence and absence of 10 µg  
527 catalase was followed. The reaction mixture contained *UmaAAO* (20 µg) and  
528 HMF (3 mM) in 100 mM tartrate buffer, pH6. Samples were taken after 0, 0.5,  
529 1, 1.5, 2, 3, 4, 5, 6, 8 and 24 hours and analyzed on HPLC. All measurements  
530 were performed in duplicates.

531 *UmaAAO* was then used in a cascade reaction with *PciGLOX3* for the  
532 oxidation of HMF and its derivatives. The reaction was performed by reacting  
533 *UmaAAO* with HMF for 2 or 24 hours before adding *PciGLOX3*. Following the  
534 addition of *PciGLOX3*, the samples were further incubated for 4 or 24 hours  
535 and then analyzed. The reactions were again performed in the presence and  
536 absence of catalase.

537

## 538 **List of abbreviations**

539 HMF, 5-hydroxymethylfurfural; FDCA, 2, 5-Furandicarboxylic Acid; DFF,  
540 diformylfuran; HMFCA, 5-hydroxymethyl-2-furancarboxylic acid; GAO,  
541 galactose oxidase; AAO, aryl alcohol oxidase; UPO, unspecific peroxigenase;  
542 HMFO, HMF oxidase; GLOX, glyoxal oxidase; FFCA, 5-formyl-2-furan  
543 carboxylic acid; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid;  
544 GA, glyoxylic acid; HRP, horseradish peroxidase

545

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

546 **Declarations**

547 ***Ethics approval and consent to participate***

548 Not applicable.

549 ***Consent for publication***

550 Not applicable.

551 ***Availability of data and materials***

552 The datasets used and/or analyzed during the current study are available

553 from the corresponding author on reasonable request.

554 ***Competing interests***

555 The authors declare that they have no competing interests.

556 ***Funding***

557 This project has received funding from the Bio Based Industries Joint

558 Undertaking under the European Union's Horizon 2020 research and

559 innovation program under grant agreement No. 720303 (project Zelcor), and

560 the FP7 program within the INDOX project (KBBE-2013-7-613549). We would

561 also gratefully acknowledge the research federation ECCOREV (ECosystème

562 COntinentaux et Risques EnVironnementaux) for providing a Masters grant to

563 BY, and to the French Embassy in Thailand for a Junior Research Fellowship

564 for SW.

565 ***Authors' contributions***

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

566 MD, CBF and ER designed the experiments. MD and SW performed the  
567 production and biochemical characterization of *PciGLOX3*. MD and BY  
568 performed the reactions and HMF and the HPLC analysis. MD, EB, CBF and  
569 ER analyzed and interpreted the data and results. MD was the major  
570 contributor in writing the manuscript. CBF, EB, FD and ER provided critical  
571 feedback and helped in writing the manuscript. All authors read and approved  
572 the final manuscript.

573 ***Acknowledgements***

574 Not applicable.

575

576

577

578

579

580

581

582

583

584

585

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

586

587

588 **References**

589 [1] Röper H. Renewable raw materials in Europe—industrial utilisation of  
590 starch and sugar [1]. *Starch-Stärke*. 2002;54:89-99.

591 [2] Li H, Yang S, Riisager A, Pandey A, Sangwan RS, Saravanamurugan S,  
592 Luque R. Zeolite and zeotype-catalysed transformations of biofuranic  
593 compounds. *Green Chem*. 2016;18:5701-5735.

594 [3] Chheda JN, Roman-leshkov Y, Dumesic JA. Production of 5-  
595 hydroxymethylfurfural and furfural by dehydration of biomass-derived mono-  
596 and poly-saccharides. *Green Chem*. 2007;9:342-350.

597 [4] Conner AH, Bhuiyan MSH. Wood : adhesives. Reference module in  
598 materials science and materials engineering1. 2017; doi:10.1016/b978-0-12-  
599 803581-8.01932-9

600 [5] Gandini A, Silvestre AJ, Neto CP, Sousa AF, Gomes M. The furan  
601 counterpart of poly (ethylene terephthalate): An alternative material based on  
602 renewable resources. *J Poly Sci A*. 2009;47:295-298.

603 [6] Rosatella AA, Simeonov SP, Frade RF, Afonso CA. 5-  
604 Hydroxymethylfurfural (HMF) as a building block platform: Biological  
605 properties, synthesis and synthetic applications. *Green Chem*. 2011;13:754-  
606 793.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 607 [7] Hopkins KT, Wilson WD, Bender BC, Mccurdy DR, Hall JE, Tidwell RR,  
608 Kumar A, Bajic M, Boykin DW. Extended Aromatic Furan Amidino Derivatives  
609 as Anti-*Pneumocystis carinii* Agents. J Med Chem. 1998;41:3872-3878.
- 610 [8] Del Poeta M, Schell WA, Dykstra CC, Jones S, Tidwell RR, Czarny A,  
611 Bajic M, Bajic M, Kumar A, Boykin D, Perfect JR. Structure-In vitro activity  
612 relationships of pentamidine analogues and dication-substituted bis-  
613 benzimidazoles as new antifungal Agents. Antimicrob Agents Chemother.  
614 1998;42:2495-2502.
- 615 [9] Benahmed-Gasmi A, Frere P, Jubault M, Gorgues A, Cousseau J,  
616 Garrigues B. 2, 5-Bis (1, 4-dithiafulven-6-yl) substituted furans, thiophenes  
617 and N-methyl pyrroles as precursors for organic metals. Synth Met.  
618 1993;56:1751-1755.
- 619 [10] Amarasekara AS, Green D, Williams LD.. Renewable resources based  
620 polymers: Synthesis and characterization of 2,5-diformylfuran–urea resin. Eur  
621 Polym J. 2009;45:595-598.
- 622 [11] Hirai H. Oligomers from Hydroxymethylfuran carboxylic Acid. J Macromol  
623 Sci A Chem. 1984;21:1165-1179.
- 624 [12] Braisted AC, Oslob JD, Delano WL, Hyde J, Mcdowell RS, Waal N, Yu C,  
625 Arkin MR, Raimundo BC. Discovery of a potent small molecule IL-2 inhibitor  
626 through fragment assembly. J Am Chem Soc. 2003;125:3714-3715.
- 627 [13] Munekata M & Tamura G. Antitumor Activity of 5-Hydroxy-methyl-2-furoic  
628 Acid. Agric Biol Chem. 1981;45:2149-2150.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 629 [14] Werpy T, Petersen G, Aden A, Bozell J, Holladay J, White J, Manheim A,  
630 Eliot D, Lasure L, Jones S. Top value added chemicals from biomass. Volume  
631 1-Results of screening for potential candidates from sugars and synthesis  
632 gas. 2004. DTIC Document.
- 633 [15] Zhang Z, Deng K. Recent Advances in the Catalytic Synthesis of 2,5-  
634 Furandicarboxylic Acid and Its Derivatives. ACS Catal. 2015;5:6529-6544.
- 635 [16] Qin YZ, Li YM, Zong MH, Wu H, Li N. Enzyme-catalyzed selective  
636 oxidation of 5-hydroxymethylfurfural (HMF) and separation of HMF and 2,5-  
637 diformylfuran using deep eutectic solvents. Green Chem. 2015;17:3718-3722.
- 638 [17] Zhang Z, Liu B, Lv K, Sun J, Deng K. Aerobic oxidation of biomass  
639 derived 5-hydroxymethylfurfural into 5-hydroxymethyl-2-furancarboxylic acid  
640 catalyzed by a montmorillonite K-10 clay immobilized molybdenum  
641 acetylacetonate complex. Green Chem. 2014;16:2762-2770.
- 642 [18] Carro J, Ferreira P, Rodriguez L, Prieto A, Serrano A, Balcells, B, Arda A,  
643 Jimenez-Barbero J, Gutierrez A, Ullrich R, Hofrichter M, Martinez AT. 5-  
644 hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and  
645 unspecific peroxygenase. FEBS J. 2015;282:3218-29.
- 646 [19] Dijkman WP, Groothuis DE, Fraaije MW. Enzyme-Catalyzed Oxidation of  
647 5-Hydroxymethylfurfural to Furan-2, 5-dicarboxylic Acid. Angew Chem Int Ed.  
648 2014;53:6515-6518.
- 649 [20] Dijkman WP, Binda C, Fraaije MW, Mattevi A. Structure-based enzyme  
650 tailoring of 5-hydroxymethylfurfural oxidase. ACS Catal. 2015;5:1833-1839.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 651 [21] Whittaker MM, Kersten PJ, Cullen D, Whittaker JW. Identification of  
652 catalytic residues in glyoxal oxidase by targeted mutagenesis. J Biol  
653 Chem. 1999;274:36226-36232.
- 654 [22] Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat  
655 B. The Carbohydrate-Active EnZymes database (CAZy): an expert resource  
656 for Glycogenomics. Nucleic Acids Res. 2009;37:D233-238 [PMID :  
657 18838391].
- 658 [23] Levasseur A, Drula E, Lombard V, Coutinho P, Henrissat B. Expansion of  
659 the enzymatic repertoire of the CAZy database to integrate auxiliary redox  
660 enzymes. Biotechnol Biofuels. 2013;6:41.
- 661 [24] Kersten P. Glyoxal oxidase of *Phanerochaete chrysosporium*: its  
662 characterization and activation by lignin peroxidase. Proc Natl Acad Sci USA.  
663 1990;87:2936–2940.
- 664 [25] Daou M, Piumi F, Cullen D, Record E, Faulds CB. Heterologous  
665 production and characterization of two glyoxal oxidases from *Pycnoporus*  
666 *cinnabarinus*. Appl Environ Microbiol. 2016;82:4867-4875
- 667 [26] Kersten PJ, Kirk TK. Involvement of a new enzyme, glyoxal oxidase, in  
668 extracellular H<sub>2</sub>O<sub>2</sub> production by *Phanerochaete chrysosporium*. Journal of  
669 Bacteriology. 1987;169:2195-201.
- 670 [27] Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J,  
671 Helfenbein KG, Ramaiya P, Dette JC, Larimer F, Coutinho PM. Genome  
672 sequence of the lignocellulose degrading fungus *Phanerochaete*  
673 *chrysosporium* strain RP78. Nature biotechnol. 2004;22:695.



- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 674 [28] Levasseur A, Lomascolo A, Chabrol O, Ruiz-Dueñas FJ, Boukhris-Uzan  
675 E, Piumi F, Kües U, Ram AF, Murat C, Haon M. The genome of the white-rot  
676 fungus *Pycnoporus cinnabarinus*: a basidiomycete model with a versatile  
677 arsenal for lignocellulosic biomass breakdown. *BMC Genomics*. 2014;15:486.
- 678 [29] Roncal T, Munoz C, Lorenzo L, Maestro B, Diaz De Guerenú MDM. Two-  
679 step oxidation of glycerol to glyceric acid catalyzed by the *Phanerochaete*  
680 *chrysosporium* glyoxal oxidase. *Enzyme Microb Technol*. 2012;50:143–150.
- 681 [30] Kurek B, Kersten P. Physiological regulation of glyoxal oxidase from  
682 *Phanerochaete chrysosporium* by peroxidase systems. *Enzyme Microb*  
683 *Technol*. 1995;17:751–756.
- 684 [31] Hernandez K, Berenguer-Murcia A, C Rodrigues R, Fernandezlafuente R.  
685 Hydrogen peroxide in biocatalysis: A dangerous liaison. *Curr Org Chem*.  
686 2012;16:2652-2672.
- 687 [32] Kadowaki M, Godoy M, Kumagai P, Costa-Filho A, Mort A, Prade R,  
688 Polikarpov I. Characterization of a New Glyoxal Oxidase from the  
689 Thermophilic Fungus *Myceliophthora thermophila* M77: Hydrogen Peroxide  
690 Production Retained in 5-Hydroxymethylfurfural Oxidation. *Catalysts*. 2018;  
691 19:476.
- 692 [33] Karich A, Kleeberg SB, Ullrich R, Hofrichter M. Enzymatic Preparation of  
693 2, 5-Furandicarboxylic Acid (FDCA)—A Substitute of Terephthalic Acid—By  
694 the Joined Action of Three Fungal Enzymes. *Microorganisms*. 2018;6:5.
- 695 [34] Hanke PD, Archer Daniels Midland Company. Enzymatic oxidation of  
696 hydroxymethylfurfural. 2012. U.S. Patent 8,183,020.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 697 [35] Mckenna SM, Leimkühler S, Herter S, Turner NJ, Carnell AJ. Enzyme  
698 cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic  
699 acids using oxidases in tandem. *Green Chem.* 2015;17:3271-3275.
- 700 [36] Couturier M, Mathieu Y, Li A, Navarro D, Drula E, Haon M, Grisel S,  
701 Ludwig R, Berrin JG. Characterization of a new aryl-alcohol oxidase secreted  
702 by the phytopathogenic fungus *Ustilago maydis*. *Appl microbiol*  
703 *biotechnol.* 2016;100:697-706.
- 704 [37] Whittaker MM, Kersten PJ, Nakamura N, Sanders-Loehr J, Schweizer  
705 ES, Whittaker JW. Glyoxal oxidase from *Phanerochaete chrysosporium* is a  
706 new radical-copper oxidase. *J Biol Chem.*1996;271:681-687.
- 707 [38] Leatherbarrow, RJ. GraFit version 4.0. Erithacus Software Ltd.,  
708 Staines, 1998. United Kingdom.
- 709  
710  
711  
712  
713  
714  
715  
716  
717

718

719

720

721 **Table 1.** Substrate specificity of *PciGLOX3* compared to previously  
 722 characterized *PciGLOX1* and *PciGLOX2* (Daou et al. 2016).

Substrate (10 mM)	Activity (nkat/mg)		
	<i>PciGLOX1</i>	<i>PciGLOX2</i>	<i>PciGLOX3</i>
Methyl glyoxal	814	291	90
Glyoxal	392	163	68
Glyoxylic acid	1562	384	59
3-phenylpropionaldehyde	10	135	51
Formaldehyde	50	202	133
DL-glyceraldehyde	11	66	26
Dihydroxyacetone	108	157	64
Glycerol	2	61	88
2,4-dimethoxybenzaldehyde	ND	2	ND
Veratraldehyde	ND	ND	ND
4-hydroxybenzaldehyde	ND	ND	ND
Phenyl glyoxilic acid	ND	ND	ND
Formic acid	ND	ND	ND
D-Glucose	ND	ND	ND
D-Galactose	ND	ND	ND
D-Xylose	ND	ND	ND
Methanol	ND	ND	ND

723 ND, activity not detected under these assay conditions

724

725

726

727 **Table 2.** Kinetic constants of *PciGLOX3* on Methylglyoxal, glyoxal, glyoxylic  
 728 acid and glycerol compared to *PciGLOX1* and *PciGLOX2* (Daou et al. 2016).

Substrate	<i>PciGLOX1</i>		<i>PciGLOX2</i>		<i>PciGLOX3</i>	
	$K_m$ (mM)	$K_{cat}/K_m$ ( $s^{-1}$ mM)	$K_m$ (mM)	$K_{cat}/K_m$ ( $s^{-1}$ mM)	$K_m$ (mM)	$K_{cat}/K_m$ ( $s^{-1}$ mM)
Methylglyoxal	1.3	58.4	0.2	7	0.1	75.8
Glyoxal	13.1	6.3	2.2	0.6	0.7	11.2
Glyoxylic acid	0.08	2136.3	0.1	17	0.5	15.3
Glycerol	660.5	0.04	9.4	0.06	5.5	1.4

729

730

731

732

733

734

735

736

737

738

739

740

741 **Table 3.** Kinetic parameters for the oxidation of HMF, DFF, and FFCA by the  
 742 three *PciGLOX* isoenzymes.

Substrate		<i>PciGLOX1</i>	<i>PciGLOX2</i>	<i>PciGLOX3</i>
HMF	$K_m$ (mM)	15.66	5.872	6.353
	$K_{cat}$ (s <sup>-1</sup> )	2.59	0.56	0.75
	$K_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> ) 1)	101.66	96.04	118.35
DFF	$K_m$ (mM)	4.38	0.205	0.176
	$K_{cat}$ (s <sup>-1</sup> )	0.54	4.80	1.28
	$K_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> ) 1)	124.39	23403.66	7267.72
FFCA	$K_m$ (mM)	0.85	1.404	0.608
	$K_{cat}$ (s <sup>-1</sup> )	0.03	2.02	0.04
	$K_{cat}/K_m$ (s <sup>-1</sup> M <sup>-1</sup> ) 1)	38.55	1435.45	72.03

743 **Table 4.** Percentage content of HMF, DFF, FFCA and FDCA in the cascade  
 744 reactions of *UmaAAO* and *PciGLOX3* following condition (1) that consisted of  
 745 reacting *UmaAAO* with HMF for 2 hours before adding *PciGLOX3* and  
 746 condition (2) in which *PciGLOX3* was added after 24 hours of the reaction of  
 747 *UmaAAO* with HMF. Reactions were performed in the presence (C) and  
 748 absence of catalase (NC) for both conditions. *PciGLOX3* was reacted for 2  
 749 or 24 hours before analyzing the reaction products.

	Condition (1)				Condition (2)			
	After 2 hours		After 24 hours		After 2 hours		After 24 hours	
	NC	C	NC	C	NC	C	NC	C
HMF	ND	ND*	ND	ND	ND	ND	ND	ND
DFF	17%	ND	1%	ND	ND	ND	ND	ND
FFCA	83%	96%	98%	95%	91%	88%	87%	84%
FDCA	ND	4%	1%	5%	9%	12%	13%	16%

751 \*ND, values below 1 %

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

752 **List of Schemes**

753 **Scheme 1.** The oxidation reaction pathways of HMF to FDCA (Carro et al.  
754 2015)

755 **List of Figures**

756 **Figure 1.** Custal W alignment of *P. cinnabarinus* GLOX proteins. Identical  
757 residues are highlighted in red. Arrows indicate residues essential for  
758 catalysis

759 **Figure 2.** Residual activity of the three *Pci*GLOX after incubation at 30°C and  
760 800 rpm shaking in 50 mM tartrate buffer pH6 in the absence (a) and  
761 presence (b) of 3 mM HMF, and in the presence of 3 mM HMFCa (c). The  
762 residual activity was calculated as a percentage of the activity before  
763 incubation.

764 **Figure 3.** Stability of the three *Pci*GLOX in increasing concentrations of  
765 hydrogen peroxide after an incubation period of 24 hours.

766 **Figure 4.** HMF oxidation reaction follow-up over time in the absence (left) and  
767 presence (right) of catalase by (a) *Pci*GLOX1, (b) *Pci*GLOX2 and (c)  
768 *Pci*GLOX3.

769 **Figure 5.** Follow-up of the reactions of *Pci*GLOX1, *Pci*GLOX2 and *Pci*GLOX3  
770 on (a) DFF, (b) HMFCa and (c) FFCA as initial substrates.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

771 **Figure 6.** Oxidation of HMF overtime by UmaAAO in the presence **(A)** and  
772 absence **(B)** of catalase.

773

774 **Additional files**

775

776 **Additional file 1.** Residual activity of HRP on ABTS after incubation for  
777 different time periods in tartrate buffer pH 6 at 30°C and 800 rpm in the  
778 presence and absence of HMF or HMFCA.

779 **Additional file 2.** HPLC chromatogram of the reactions of **(A)** the three  
780 *Pci*GLOX enzymes on HMF after 24 hours of incubation and of **(B)** the  
781 reaction of *Uma*AAO on HMF overtime.



PciGLOX1 -APSAFQWRFDLKAERNGIVALESVVSPTDLWFDPAS-DDFLQINNHSAWGAALNSET 58  
 PciGLOX2 APSTPTLWQFNLKAERNGIVALESVVSPTDLWFFDPAF-NDFLQINNHSAWGAALNSET 59  
 PciGLOX3 -APSAFQMSFDLAKETNGIVALESAVVSPTDLWFFDPAF-DDFLQINNHSAWGAALNSES 58

Cys70

PciGLOX1 STVRFNPLNNECAREGALLSNSTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 118  
 PciGLOX2 STVRFNPLNNECAREGALLSNSTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 119  
 PciGLOX3 STVRFNPLNNECAREGALLSNSTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 118

Tyr135

PciGLOX1 OCTLFEDQVLLLEKDWYSSVRIIFDQSLIVGDTWNEFYNLHANSFEFFPPASS 178  
 PciGLOX2 OCTLFEDQVLLLEKDWYSSVRIIFDQSLIVGDMEEETPTVTDVALSFEFFPPASS 179  
 PciGLOX3 OCTLFEDQVLLLEKDWYSSARIFDQSLIVGDMEEATPTVTDVALSFEFFPPASS 178

PciGLOX1 WPFSEFLNSELNANLFFVVALPDKKQVMWANNOSITYDIEANTERELIPIINWAVTNI 238  
 PciGLOX2 WPFSEFLNSELNANLFFVVALPDKKQVMWANNOSITYDIEANTERELIPIINWAVTNI 239  
 PciGLOX3 WPFSEFLNSELNANLFFVVALPDKKQVMWANNOSITYDIEAKTERELIPIINWAVTNI 238

PciGLOX1 IDGSAITLIPSTWYIENLVGDSSTPLDLSLISSTPATTQCSMRDEEIIAKGW 297  
 PciGLOX2 IDGSAITLIPSTWYFVENVLVDSTQTLTIDLSLISSTPASTQCSMRDEEIIARSH 298  
 PciGLOX3 MDGSAITLIPSTWYFVENVLVDSTQTLTIDLSLISSTPASTQCSMRDEEIIAKGW 297

PciGLOX1 QVWMLPITPFWIPIGIIANGARGFPAIKQDIIINNSRAWVWPIVPI 357  
 PciGLOX2 EVWMLPITPFWIPIGIIANGARGFPAIASDVIWGNASRAWVWVPLVPI 358  
 PciGLOX3 EVWMLPITPFWIPIGIIANGARGFPAIASSEWVWGNASRAWVWVPLVPI 357

Tyr377      His378

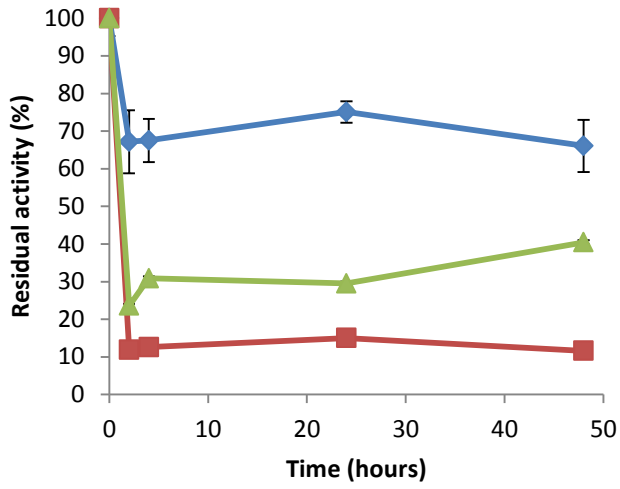
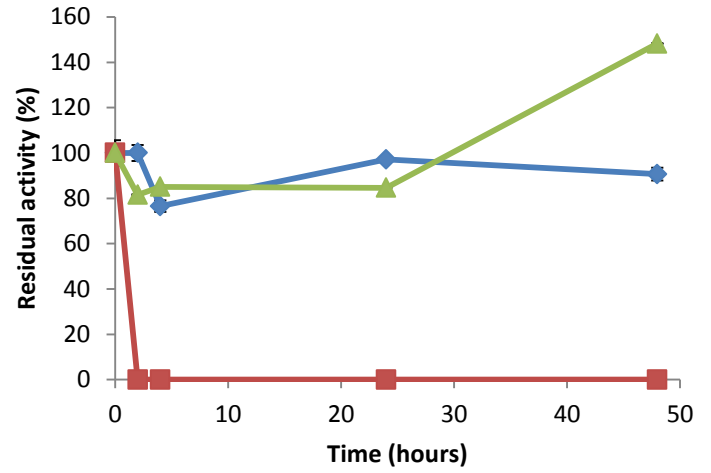
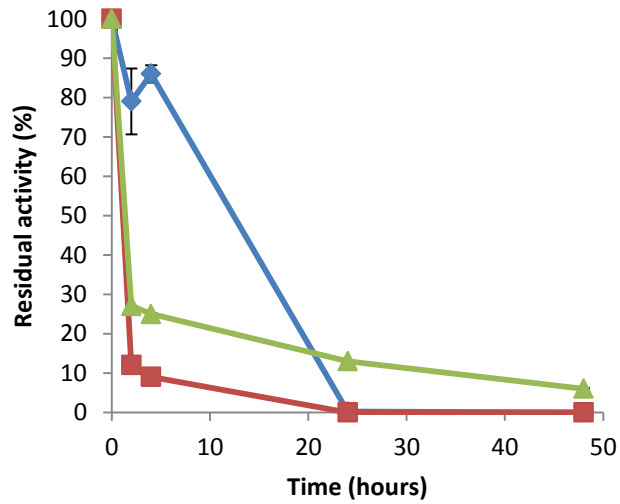
PciGLOX1 AFLQRTISSAEMSSGAVVYHSSIFLPQGNLIASWNNNTT--VGPVITPSEFV 415  
 PciGLOX2 AFLQRTISSVLLSSGAVVYHSSIFLPQGNLIASWNNSS--VTAVITPSEFV 416  
 PciGLOX3 AFLQRTISSVLLSSGAVVYHSSVFLPQGNLIASWNNRTT--VGPVITPSEFV 415

His471

PciGLOX1 QLEDTTFVVEEKIINTIEAANKTFVYSVSTIASPGAQVVSMLQGFSSHAF 475  
 PciGLOX2 QLEDTTFVVEEKISMKKLAAGKSFVYSVSTIAHPGAQVVSMLQGFSSHAF 476  
 PciGLOX3 QLEDTTFVVEEKINMKKLAGKSFVYSVSTIARPGAQVVISMLQGFSSHAF 475

PciGLOX1 ESARLVFMDATIERRRRLTFTTFPNGPVFNGPATVFLTIDTDEEAWDQNGSQSP 535  
 PciGLOX2 ESARLVFMNAKISQGGKSLTFTTFPNGPVYNGPATVFLTIDTDEEAWDQNGSQSP 536  
 PciGLOX3 ESARLVFMNGKISQGGKSLTFTTFPNGRMYNGPATVFLTIDTDEEAWDQNGSQSP 535

PciGLOX1 FLR 538  
 PciGLOX2 FLR 539  
 PciGLOX3 FLR 538

**a.****b.****c.**

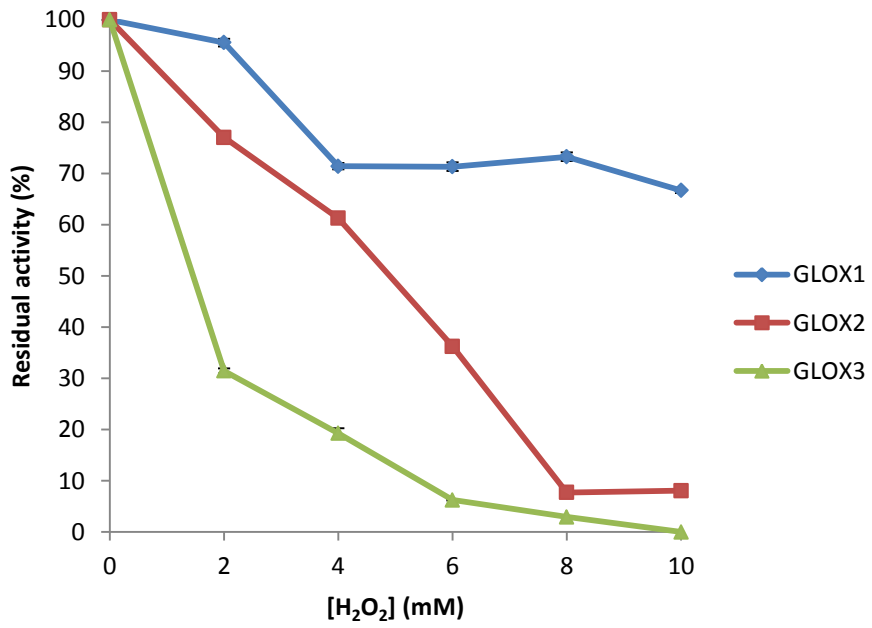


Figure 4

[Click here to access/download;Figure;Figure 4.pdf](#)

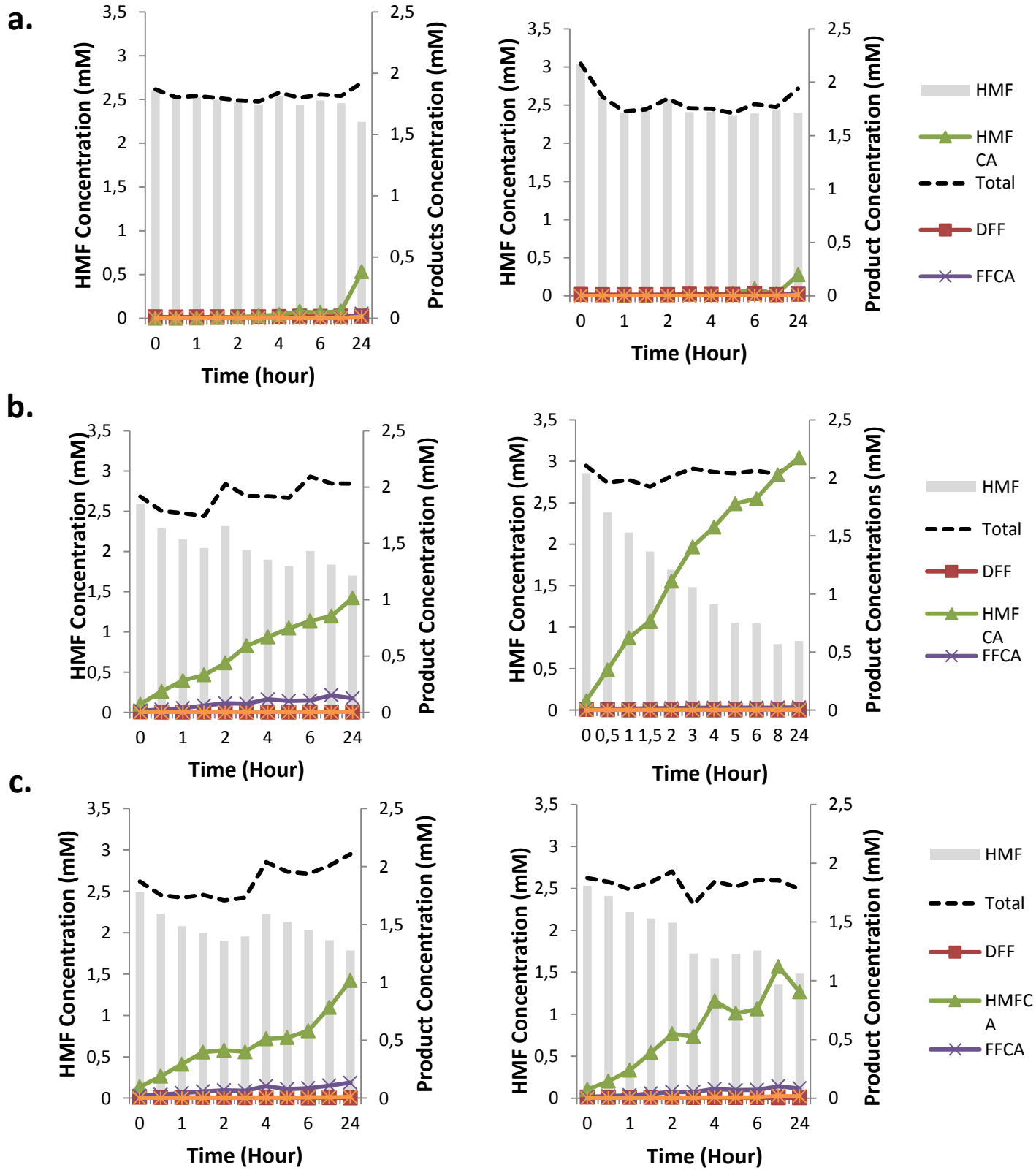
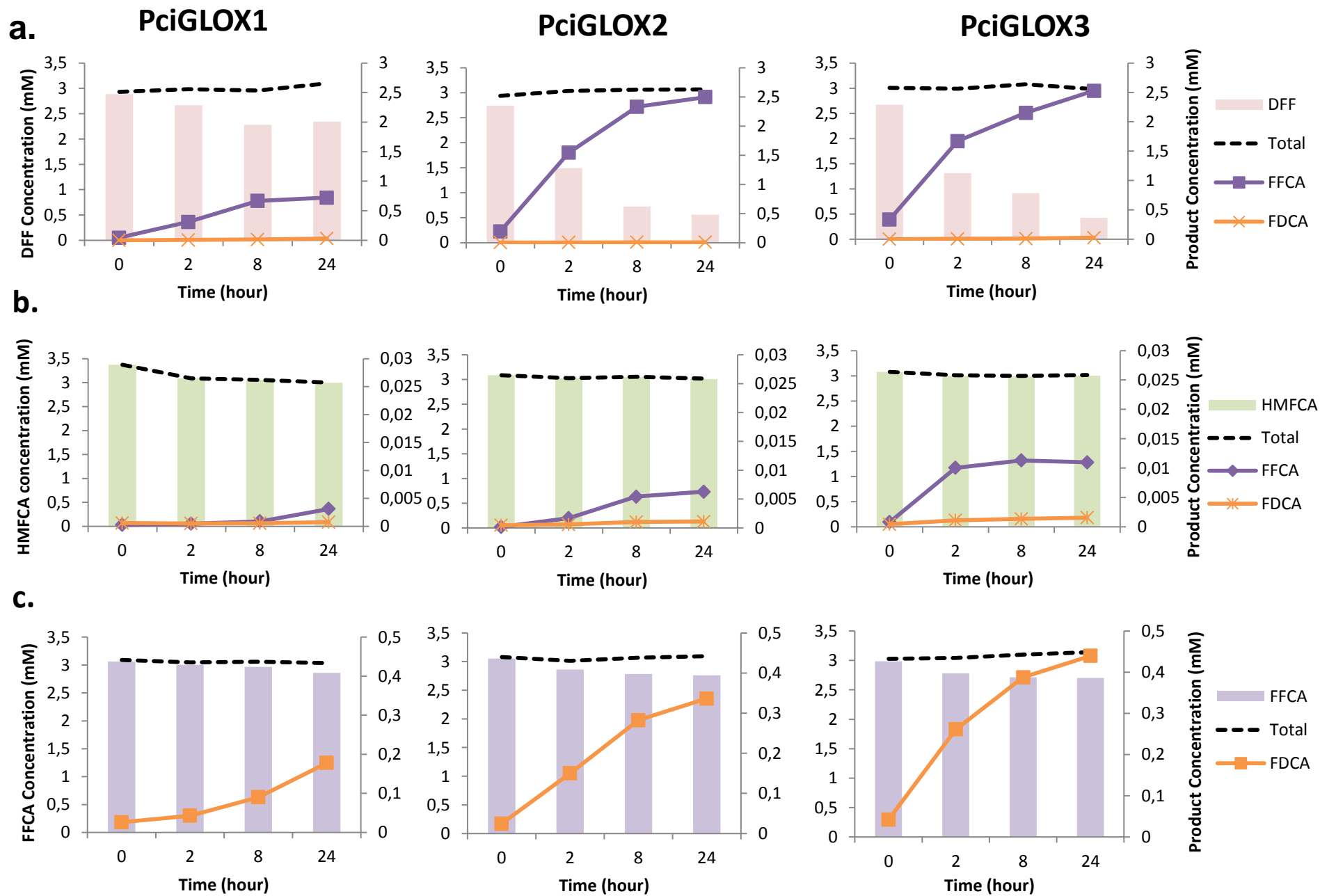
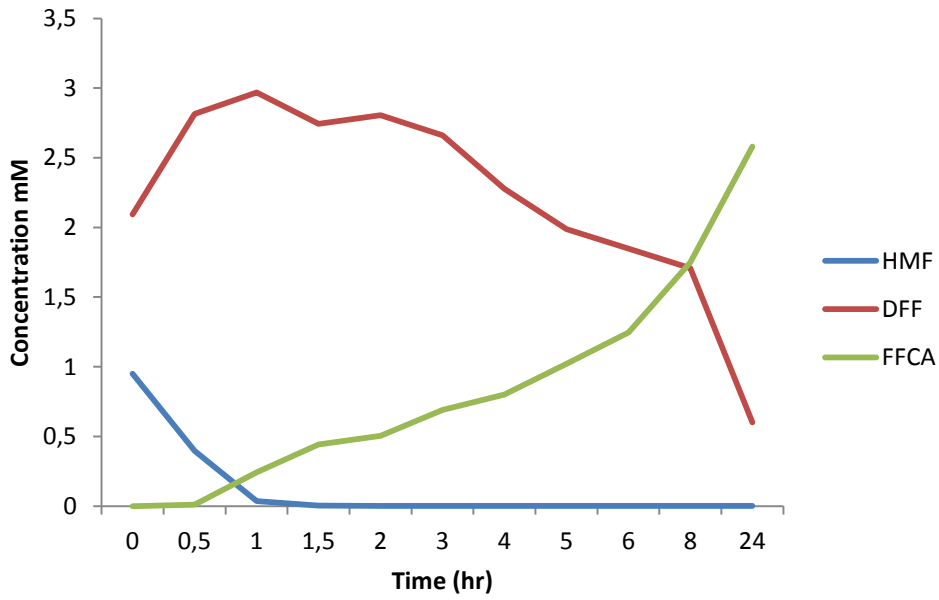
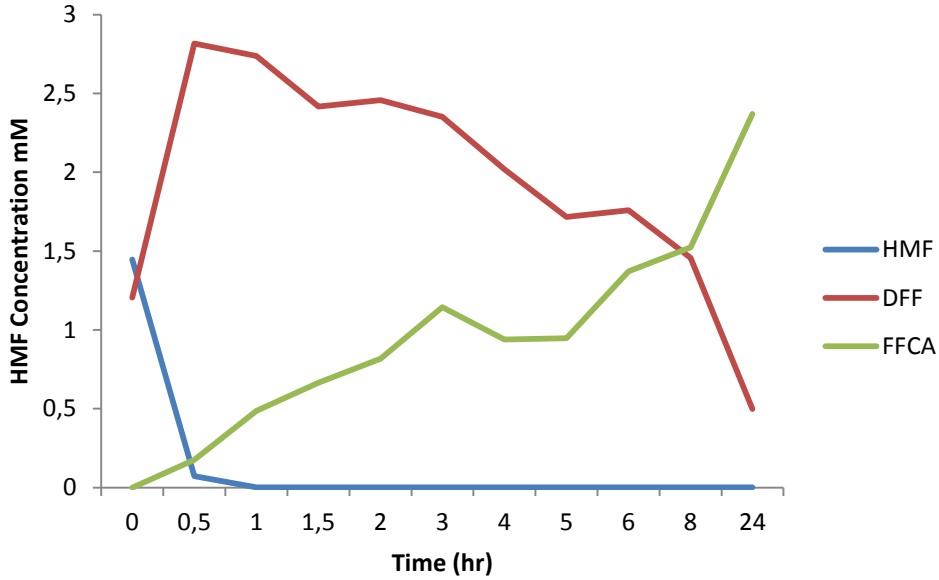
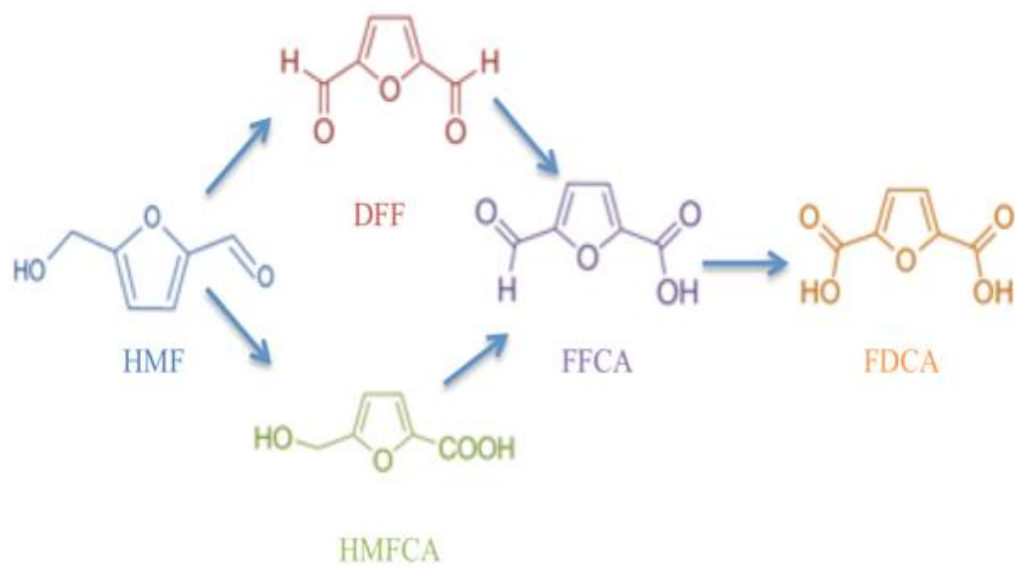
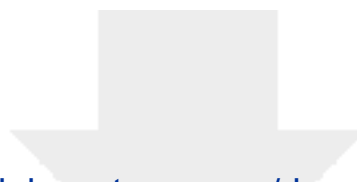


Figure 5

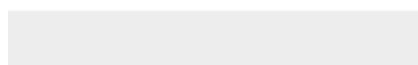
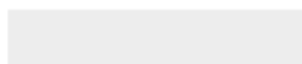


**A.****B.**






Click here to access/download  
**Supplementary Material**  
Daou et al COVER\_LETTER.pdf



Comment citer ce document :

Faulds, C., Daou, M. (Co-premier auteur), Yassine, B., Wikee, S., Record, E., Duprat, F. (Collaborateur), Bertrand, E. (Collaborateur) (2019). *Pycnoporus cinnabarinus* glyoxal oxidases display differential catalytic efficiencies on 5hydroxymethylfurfural and its oxidized derivatives. *Fungal Biology and Biotechnology*. 6. 4. . DOI : <https://doi.org/10.1186/s40694-019-0067-8>






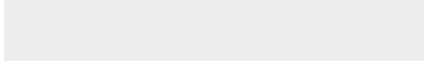
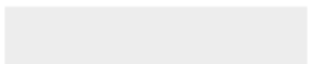
Click here to access/download  
**Supplementary Material**  
Additional file 1.pdf

Comment citer ce document :

Faulds, C., Daou, M. (Co-premier auteur), Yassine, B., Wikee, S., Record, E., Duprat, F. (Collaborateur), Bertrand, E. (Collaborateur) (2019). *Pycnoporus cinnabarinus* glyoxal oxidases display differential catalytic efficiencies on 5hydroxymethylfurfural and its oxidized derivatives. *Fungal Biology and Biotechnology*. 6. 4. . DOI : <https://doi.org/10.1186/s40694-019-0067-8>



Click here to access/download  
**Supplementary Material**  
Additional file 2.pdf



Comment citer ce document :

Faulds, C., Daou, M. (Co-premier auteur), Yassine, B., Wikee, S., Record, E., Duprat, F. (Collaborateur), Bertrand, E. (Collaborateur) (2019). *Pycnoporus cinnabarinus* glyoxal oxidases display differential catalytic efficiencies on 5hydroxymethylfurfural and its oxidized derivatives. *Fungal Biology and Biotechnology*. 6. 4. . DOI : <https://doi.org/10.1186/s40694-019-0067-8>