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► 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-02622737>

Submitted on 26 May 2020

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Fungal Biology and Biotechnology

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

--Manuscript Draft--

Manuscript Number:									
Full Title:	Pycnoporus cinnabarinus glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives								
Article Type:	Research								
Funding Information:	<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
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Abstract:	<p>Background</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>Results</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>Conclusions</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>								
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	Marco Fraaije Rijksuniversiteit Groningen
	Competitor in this area
Additional Information:	
Question	Response
Is this study a clinical trial?<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>

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

4
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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>

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.

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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.

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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

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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].

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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 (H₂O₂) [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 H₂O₂-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

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.

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

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

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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 H₂O₂**

186 The stability of the three *PciGLOX* isoenzymes was investigated in the
187 presence of varying concentrations of H₂O₂. *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 H₂O₂ for 24 hours (**Figure 3**). Although more
190 stable than *PciGLOX3*, *PciGLOX2* significantly lost activity with increasing
191 concentrations of H₂O₂ and lost more than 95% of its activity after incubation
192 in the presence of 8 mM H₂O₂. *PciGLOX3* on the other hand lost 60% of its
193 activity with only 2 mM H₂O₂ and was completely inactive with 10 mM H₂O₂
194 after 24 hours of incubation.

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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

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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⁻¹ M⁻¹
220 and $K_m = 0.2$ mM; $K_{cat}/K_m = 7267$ s⁻¹ M⁻¹, respectively) were significantly
221 better than that of *PciGLOX1* ($K_m = 4.3$ mM; $K_{cat}/K_m = 124.3$ s⁻¹ M⁻¹). 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 HMFCA 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 HMFCA,
228 with *PciGLOX3* having the best conversion rate (2%; **Figure 5b**). The extent
229 of HMFCA 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**).

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234 **GLOX and AAO Cascade Reactions**

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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

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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

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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 H₂O₂ over time, the three
288 isoenzymes again showed differences in stability. The inhibition of *PchGLOX*
289 by exogenous H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 ¹). 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 ($K_{cat}/K_m = 787 \text{ s}^{-1} \text{ M}^{-1}$, $K_m = 0.02 \text{ M}^{-1}$)

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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 HMFCFA. 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 HMFCFA.
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 μM
329 of this enzyme, HMFCFA 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 HMFCFA obtained with *Pci*GLOX2 after 24
332 hours of reaction was significantly lower. However, a two folds increase in

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333 HMFCA yields was observed when catalase was added to *PciGLOX2*
334 reaction. This was in agreement with the results showing that *PciGLOX2* was
335 very sensitive to accumulating H₂O₂ concentrations. Catalase converts H₂O₂
336 to O₂, protecting the enzymes from oxidative damage and supplying O₂. This
337 enzyme was previously used to eliminate the accumulating H₂O₂ 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 *PciGLOX1*. This result shows that *PciGLOX* 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 *MtGLOX*; 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 *PciGLOX*. However, the substrate specificity and catalytic
351 efficiencies of *PciGLOX2* and *PciGLOX3* 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]. *PciGLOX2* and *PciGLOX3* were also more efficient than
356 *PciGLOX1* in oxidizing DFF which explains the presence of constant
357 concentrations of DFF with *PciGLOX1*. On the other hand, in the cases of

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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.

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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 H₂O₂ 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 H₂O₂
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 H₂O₂ 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

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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 H₂O₂, 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].

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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.

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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 μ 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 μ 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.

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454 ***PciGLOX3* Characterization**

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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.

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477 **Kinetic Studies**

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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

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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 H₂O₂ on HMF Oxidation**

511 The stability of the three *Pci*GLOX isoenzymes in the presence of H₂O₂ was
512 assessed by measuring the residual activity of the enzymes after pre-
513 incubation with different concentrations of H₂O₂ over 24 hours. The H₂O₂ 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

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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***

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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.

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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

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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

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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^{-1})	2.59	0.56	0.75
	K_{cat}/K_m ($s^{-1} M^{-1}$) 1)	101.66	96.04	118.35
DFF	K_m (mM)	4.38	0.205	0.176
	K_{cat} (s^{-1})	0.54	4.80	1.28
	K_{cat}/K_m ($s^{-1} M^{-1}$) 1)	124.39	23403.66	7267.72
FFCA	K_m (mM)	0.85	1.404	0.608
	K_{cat} (s^{-1})	0.03	2.02	0.04
	K_{cat}/K_m ($s^{-1} M^{-1}$) 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 %

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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.

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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-DDFLQINNHSAWGALANSET 58
 PciGLOX2 APSTPTLWQFNLKAERNGIVALESVVSPTDLWFFDPAF-NDFLQINNHSAWGALANSET 59
 PciGLOX3 -APSAFQMSFDLAKETNGIVALESAVVSPTDLWFFDPAF-DDFLQINNHSAWGALANSES 58

Cys70

PciGLOX1 STVRFNPLNDFCRAGALLNSNTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 118
 PciGLOX2 STVRFNPLNDFCRAGALLNSNTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 119
 PciGLOX3 STVRFNPLNDFCRAGALLNSNTMASVVDQDFVFNPNPAINPNCALNIPFCASITD 118

Tyr135

PciGLOX1 OCTLFEDQVLLLEKDWYSSVRIIFDQSLLVQDTWNEVFNLHANSFEFFPPSSS 178
 PciGLOX2 OCTLFEDQVLLLEKDWYSSVRIIFDQSLLVQDMSEETPTVNTDVALSFEFFPPSSS 179
 PciGLOX3 OCTLFEDQVLLLEKDWYSSARIFDQSLLVQDMSEATPTVNTDVALSFEFFPPSSS 178

PciGLOX1 VVPSALESLPANLFFVIFALPDKKQVMVANNOSITYDIEANTERELIPIINWAVTNI 238
 PciGLOX2 VVPSALESLPANLFFVIFALPDKKQVMVANNOSITYDIEANTERELIPIINWAVTNI 239
 PciGLOX3 VVPSALESLPANLFFVIFALPDKKQVMVANNOSITYDIEAKTERELIPIINWAVTNI 238

PciGLOX1 IDGSAITLQPTVPIYIENVLVCSTSTPLDLSLISSTPATTQCSMRDEEIIAKGW 297
 PciGLOX2 IDGSAITLQPTVPIFVENVLVCSTQTUTIDLSLISSTPASSQCSMRDEEIIARSH 298
 PciGLOX3 MDGSAITLQPTVPIFVENVLVCSTQTUTIDLSLISSTPASSQCSMRDEEIIAKGW 297

PciGLOX1 QVWVLEPITVPIWVPIGIVLAVGARGVAVIKQVQDIIINNSRAVIVPIVPI 357
 PciGLOX2 EVWVLEPITVPIWVPIGIVLAVGARGVAVIASVSDVWVGNASRAVIVPIVPI 358
 PciGLOX3 EVWVLEPITVPIWVPIGIVLAVGARGVAVIASVSEVWVGNASRAVIVPIVPI 357

Tyr377 His378

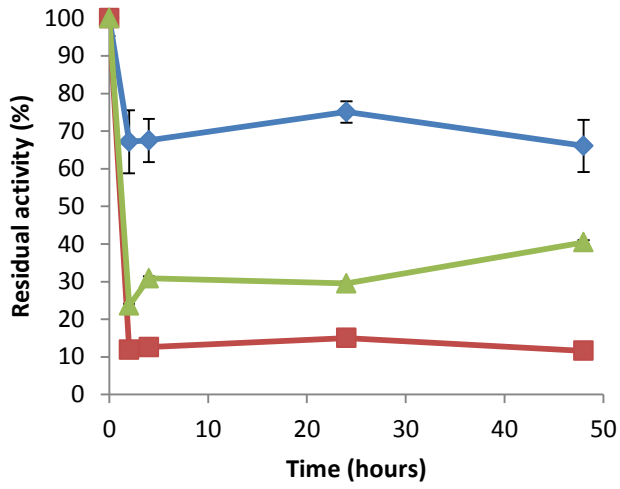
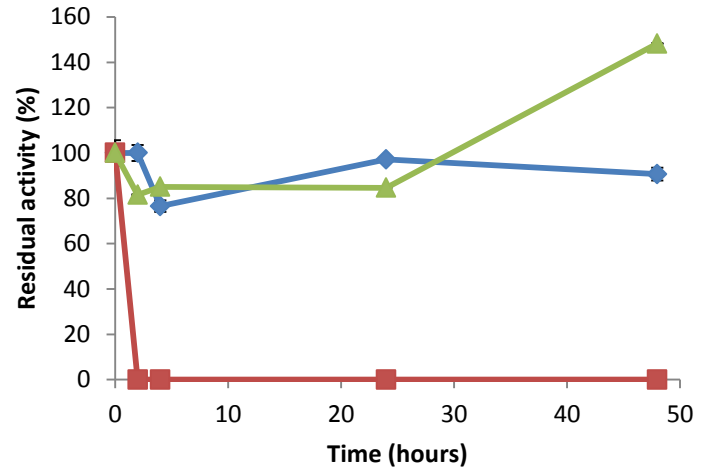
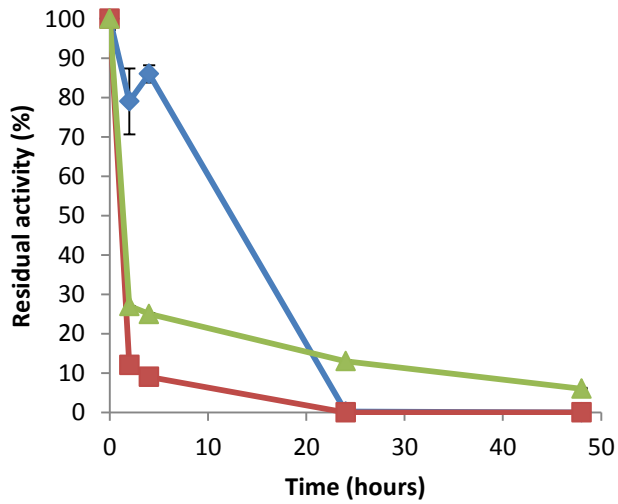
PciGLOX1 AFLQRTISSAEMSSGAVVYHSSIFLPQGNLIASNNGNTT--VGPVITPSEFFV 415
 PciGLOX2 AFLQRTISSVQLSSGAVVYHSSIFLPQGNLIASNNGNNS--VTAVITPSEFFV 416
 PciGLOX3 AFLQRTISSVQLSSGAVVLYHSSIFLPQGNLIASNNGNRTT--VGPVITPSEFFV 415

His471

PciGLOX1 QVLDPTTFVVEEKIINTVIAANKTFVYVSVVETIASPGAQVVSQVLDGFSHAF 475
 PciGLOX2 QVLDPTTFVVEEKISMKKIAAGKSFVYVAVVETIAHPGAQVVSQVLDGFSHAF 476
 PciGLOX3 QVLDPTTFVVEEKINMKKIAAGKSFVYVSVVETIARPGAQVVISQVLDGFSHAF 475

PciGLOX1 ESARLVFMDATIERRRRLTFTTFVNGPVFNGPATVFLITIDVTEAWDQNGSQSP 535
 PciGLOX2 ESARLVFMNAKISQVSKSLTFTTFVNGPVYNGPATVFLITIDVTEAWDQNGSQSP 536
 PciGLOX3 ESARLVFMNGKISQVSKSLTFTTFVNGRMYNGPATVFLITIDVTEAWDQNGSQSP 535

PciGLOX1 FLR 538
 PciGLOX2 FLR 539
 PciGLOX3 FLR 538

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

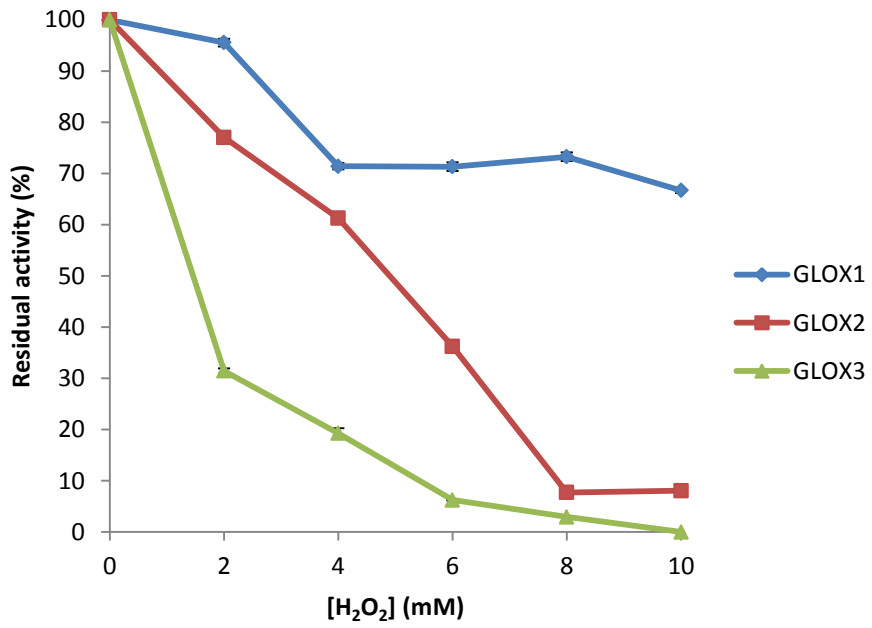


Figure 4

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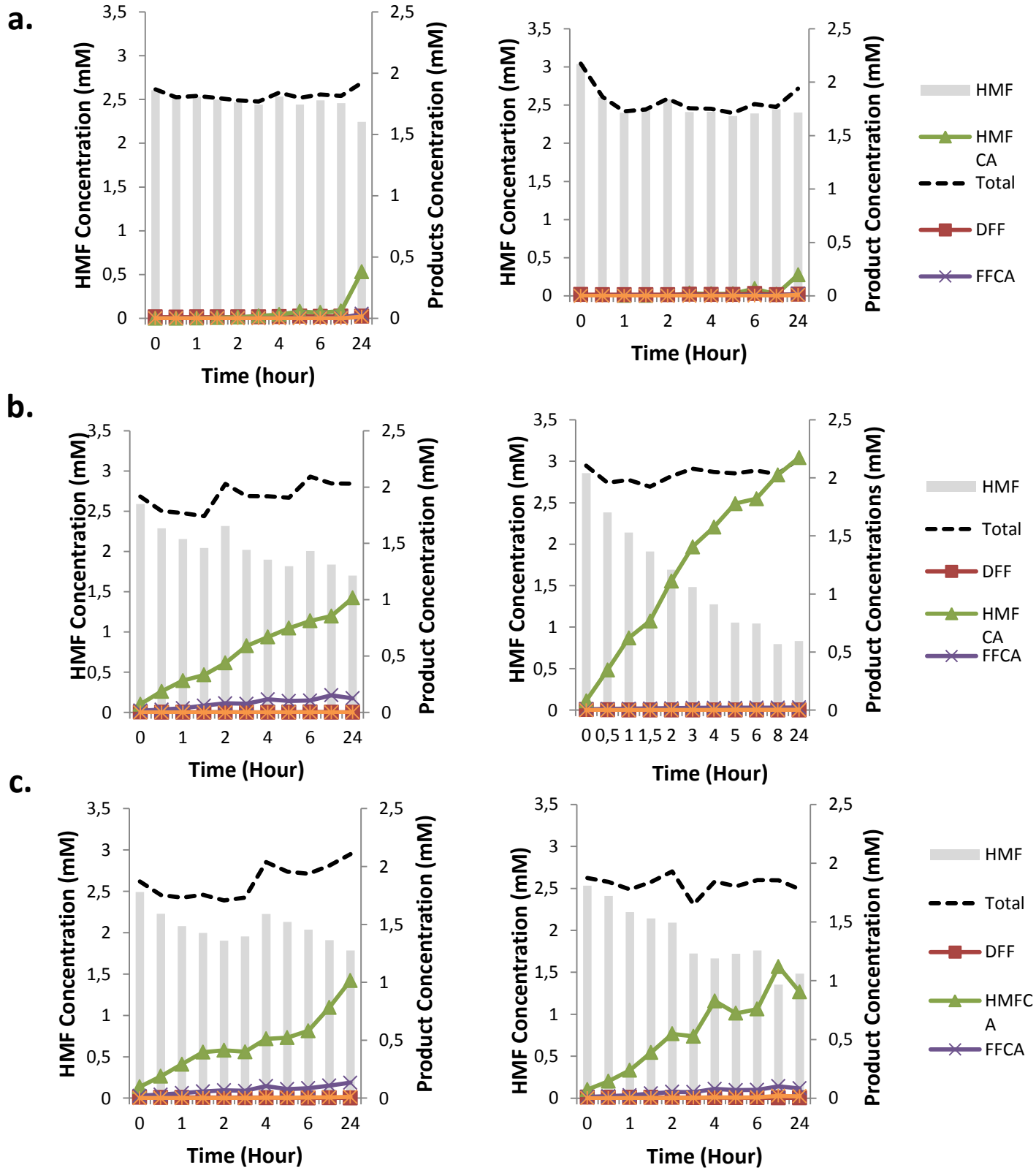
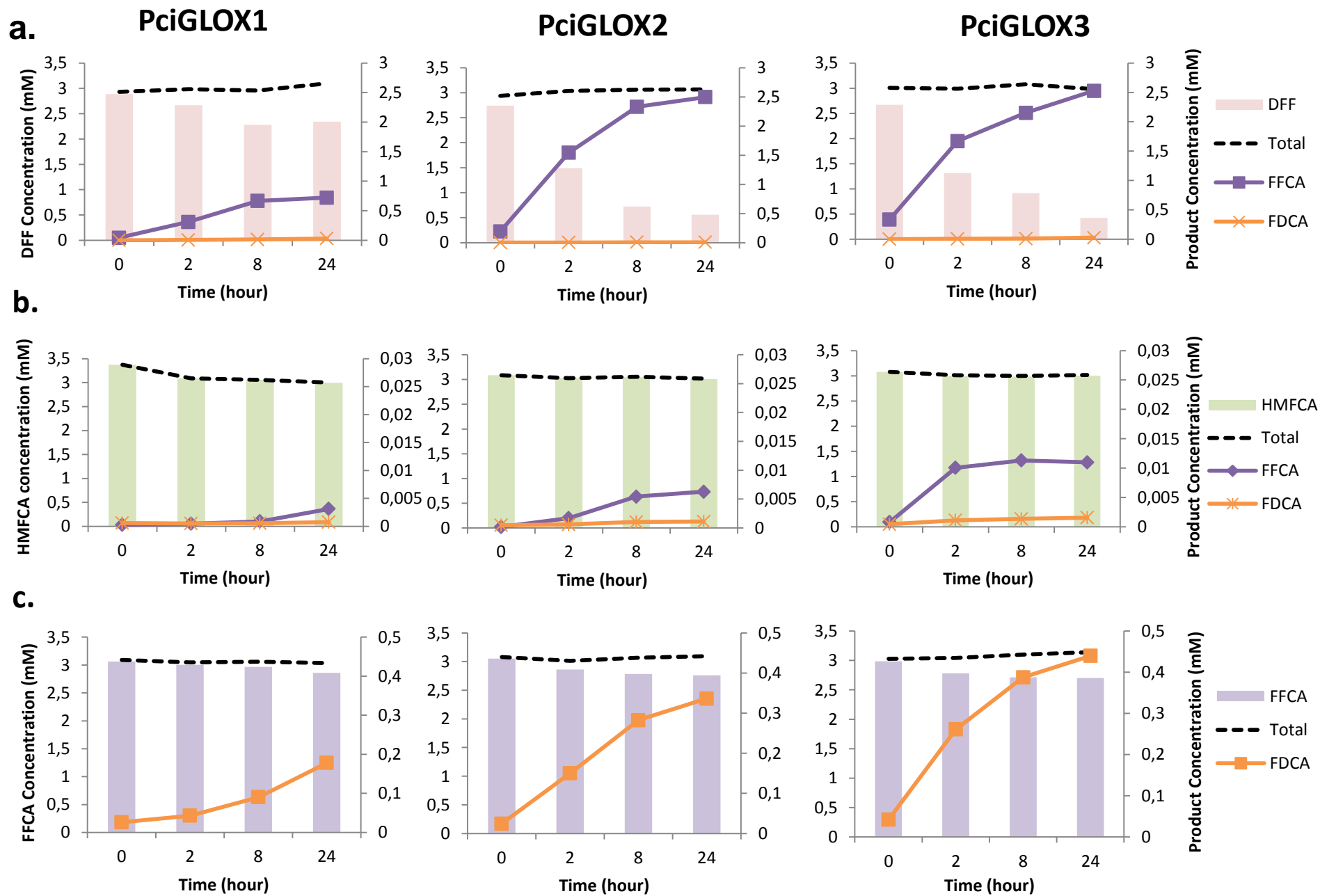
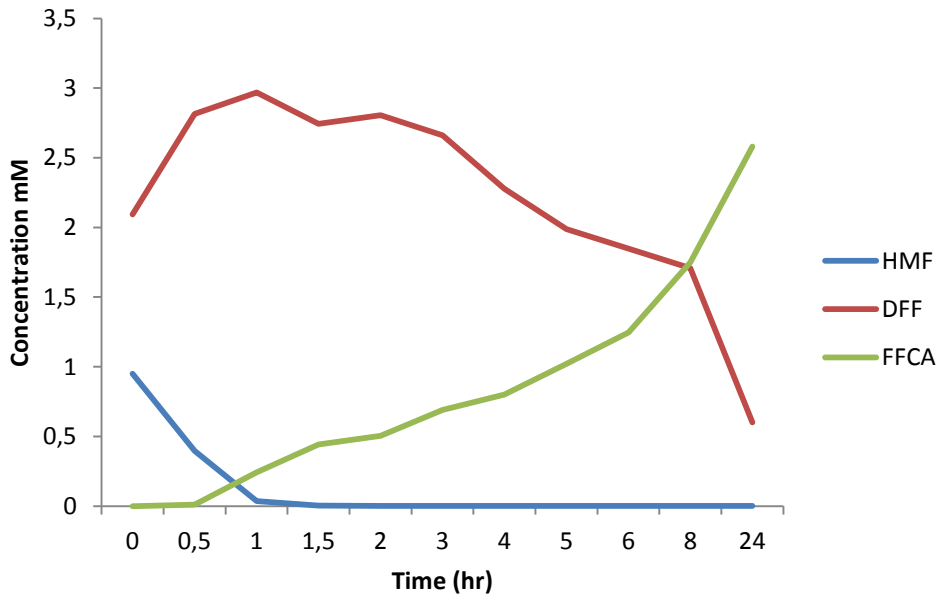
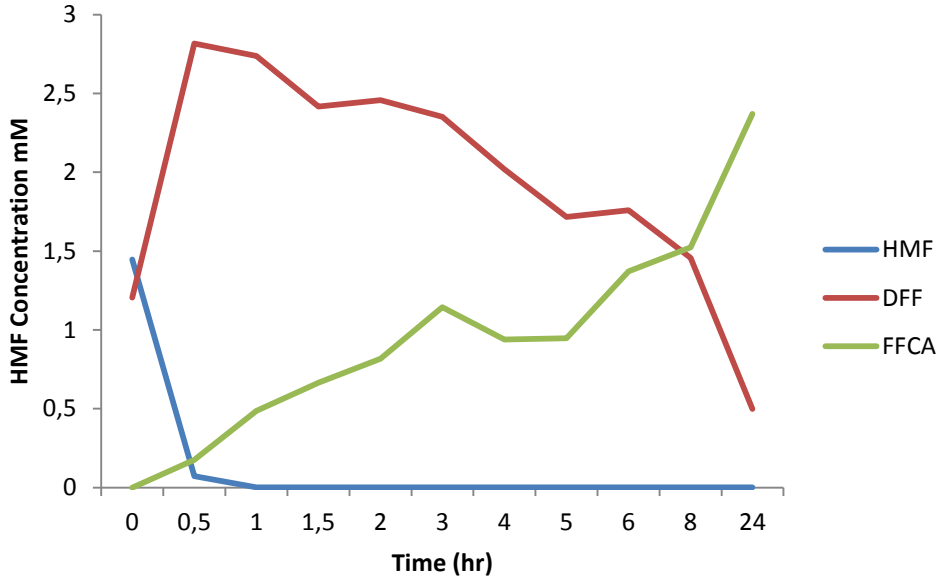
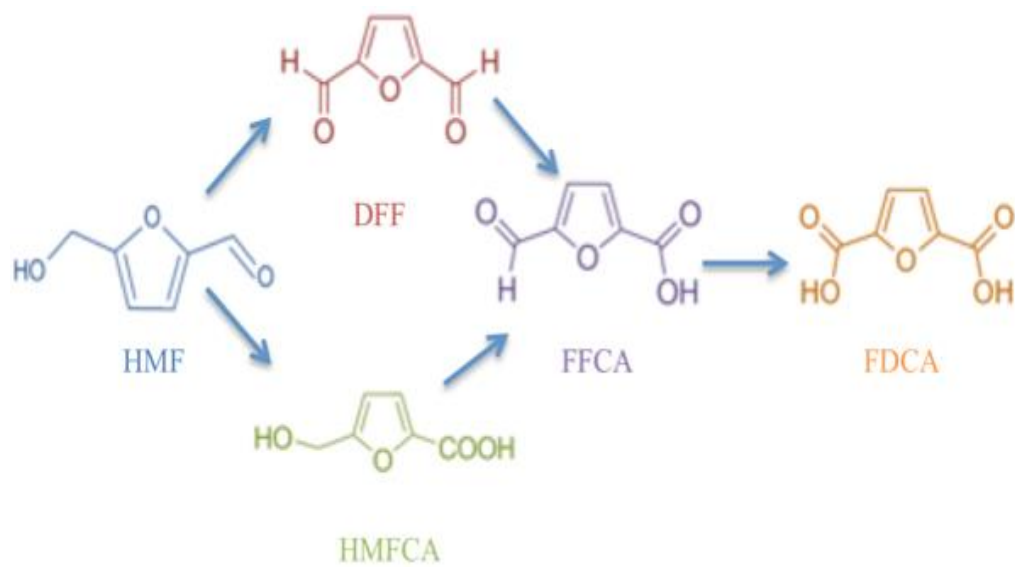
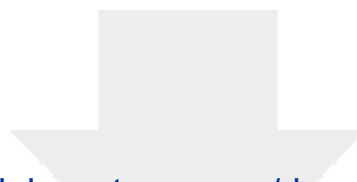


Figure 5



A.**B.**






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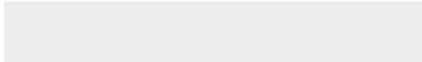

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