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BIOCATALYSIS

Self-sufficient redox biotransformation of lignin-related benzoic acids with *Aspergillus flavus*

Martín A. Palazzolo[1] · María L. Mascotti[1,2] · Elizabeth S. Lewkowicz[3] · Marcela Kurina-Sanz[1]

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Abstract Aromatic carboxylic acids are readily obtained from lignin in biomass processing facilities. However, efficient technologies for lignin valorization are missing. In this work, a microbial screening was conducted to find versatile biocatalysts capable of transforming several benzoic acids structurally related to lignin, employing vanillic acid as model substrate. The wild-type *Aspergillus flavus* growing cells exhibited exquisite selectivity towards the oxidative decarboxylation product, 2-methoxybenzene-1,4-diol. Interestingly, when assaying a set of structurally related substrates, the biocatalyst displayed the oxidative removal of the carboxyl moiety or its reduction to the primary alcohol whether electron withdrawing or donating groups were present in the aromatic ring, respectively. Additionally, *A. flavus* proved to be highly tolerant to vanillic acid increasing concentrations (up to 8 g/L), demonstrating its potential application in chemical synthesis. *A. flavus* growing cells were found to be efficient biotechnological tools to perform self-sufficient, structure-dependent redox reactions. To the best of our knowledge, this is the first report of a biocatalyst exhibiting opposite redox transformations of the carboxylic acid moiety in benzoic acid derivatives, namely oxidative decarboxylation and carboxyl reduction, in a structure-dependent fashion.

Keywords Benzoic acids · Redox biotransformation · *Aspergillus flavus* · Carboxyl reduction · Oxidative decarboxylation

Abbreviations

$CDCl_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
d_6-DMSO	Deuterated dimethyl sulfoxide
GC-FID	Gas chromatography-flame ionization detector
NMR	Nuclear magnetic resonance
TLC	Thin-layer chromatography
UV	Ultra-violet

Introduction

Lignin is formed by aromatic molecules such as *p*-coumaryl, coniferyl and sinapyl alcohols (usually referred to as monolignols), linked predominantly by β-O-4 bonds. This macrostructure acts as a barrier to preserve cellulose and hemicellulose in living plants and it is the second most abundant polymer in nature [1, 2]. Under the 'biorefinery concept', the lignocellulosic biomass is not often fully converted to biofuels and platform chemicals since methods for the valorization of the lignin fraction are generally scarce—despite lignin incineration to generate process heat—as thoroughly stated by several authors [2, 3]. For the sake of bio-based economy, it is necessary to develop robust technologies to produce valuable chemicals out

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of lignin, which could be used as antioxidants in the food industry [4], as mediators for laccase-catalyzed reactions [5] or as ingredients in bioresins [6].

Nature offers an immeasurable arsenal of biotechnological tools to transform a plethora of chemical compounds. In particular, the biotransformation of aromatic carboxylic acids is at the forefront [7, 8]. Goodwin has compiled extensive data concerning the biotransformation of these molecules [9]. Schoemaker [10] and Hatakka [11] described lignin biodegradation leading to these compounds. The study of the metabolism of aromatic carboxylic acids by microorganisms was pioneered by Higuchi [12] and Raman [13]. Later, Arfmann and Abraham reported the transformation of some benzoic, cinnamic and phenylacetic acid derivatives using mainly fungal growing cells as biocatalysts [14]. Although they showed that it was possible to reduce the carboxylic group to their corresponding alcohol, no structural pattern could be established for the substrates to be transformed. The use of the carboxylic acid reductases from the bacteria *Nocardia asterodies* and the archea *Pyrococcus furiosus* with biosynthetic purposes was performed by Chen and Rosazza [15] and van den Ban et al. [16], respectively. Recently, these enzymes were successfully applied to the biohydrogenation of some aromatic carboxylic acids [17, 18], as well as to the production of biofuels and bio-based chemicals [19]. The biotechnological valorization of aromatic carboxylic acids from agricultural wastes has also been explored. The most remarkable processes might be the ones producing vanillin from bio-based ferulic acid using wild-type species of *Aspergillus niger* and *Pycnoporus cinnabarinus* [20, 21].

In this work, we conducted the search of microorganisms capable of transforming vanillic acid, and we further tested a range of other lignin-related benzoic acid derivatives as substrates. The metabolic pathways displayed under the experimental conditions were also explored.

Materials and methods

Chemicals and microorganisms

All the chemicals were purchased in analytical grade from Sigma-Aldrich, Argentina and were used without further purification. The bacterial strains belong to the Colección Española de Cultivos Tipo (CECT) from Universidad de Valencia (Spain) and the fungal species were acquired from the collections of Universidad de Buenos Aires (UBA) and Universidad Nacional de Rio Cuarto (UNRC) (Argentina).

Culture conditions and microbial screening

All the microorganisms were cultured under their optimal conditions as defined by the supplier (see Supplementary Information). To assay their biocatalytic performances, bacterial and fungal strains were employed both as resting and growing cells, following similar procedures as described by Mascotti et al. [22, 23]. Standard biotransformation conditions were set as follows. Biocatalysts were prepared using 3- or 5-day old, 500 mL cultures of bacteria or fungi, respectively, inoculated from agar plate stocks. After the proper time, microbial cells were centrifuged for 20 min and 5000 rpm, washed with one volume of distilled water and centrifuged again with the same parameters. Then, to run bacterial-catalyzed transformations, 50 mg of wet cells was resuspended in 4 mL of 50 mM triethanolamine-HCl buffer pH 7.5 (when preparing resting cells) or fresh culture media (for biotransformations using growing cells) and transferred to 20 mL erlenmeyer flasks. Alternatively, when running biotransformations catalyzed by fungal strains, 950 mg of wet cells was resuspended in 20 mL of 50 mM potassium phosphate buffer pH 6.0 (for resting cells) or fresh culture media (for biotransformations using growing cells) and transferred to 50 mL erlenmeyer flasks. Bacterial biotransformations were initiated by adding 1 mL of a warm 1 g/L vanillic acid stock solution in 50 mM triethanolamine-HCl buffer pH 7.5 or fresh culture media (for biotransformations using resting or growing cells, respectively) to 4 mL of biocatalyst suspension (0.2 g/L substrate final concentration). Similarly, biotransformations carried out with fungal cells were started by adding 200 μL of a 50 g/L vanillic acid stock solution in DMSO (used as cosolvent, 1 % (*v*/*v*) final concentration) to 20 mL of biocatalyst suspension in batch, reaching 0.5 g/L final concentration for the substrate (standard conditions). All the batches were incubated at 28 °C with orbital shaking at 180 rpm. DMSO was omitted in bacterial biotransformations, since these cells were not viable in the presence of this organic solvent (data not shown). The biotransformations with growing cells as biocatalysts were performed under sterile conditions.

A total of five batches *per* strain were prepared to independently evaluate the time-dependent consumption of vanillic acid during 0, 24, 48, 72 and 96 h. Control batches without biocatalyst and without substrate were also run. When biotransformations were catalyzed by bacteria, cell separation from the reaction mixture was done by centrifuging the batch at 5000 rpm for 20 min. In the case of fungal-catalyzed reactions, cells were removed by filtration. In every case, the supernatant was acidified by the addition of 100 μL of HCl 6 N and it was then extracted twice with one volume of ethyl acetate. Organic layers were combined, dried with sodium sulfate and spotted on silica gel TLC plates to qualitatively assess the substrate consumption and the appearance of biotransformation products. The chromatographic analysis was done using the mixture *n*-hexane: ethyl acetate (6: 4) as mobile phase and plates

were revealed under UV light and by immersion in a supersaturated potassium permanganate solution in water.

Biotransformation of benzoic acids

As a result from the microbial screening, the growing cells of *A. flavus* were selected as the biocatalyst to quantitatively assay the transformation of other 7 benzoic acid derivatives (**1–3a** and **5–8a**) besides **4a**. To carry out these experiments, 50 g/L stock solutions of these compounds were prepared in DMSO. Biotransformations were initiated by adding 200 μL of substrate stock solution to a 50 mL erlenmeyer flask containing 20 mL of fresh culture medium and 950 mg of wet cells (37 mg on a dry-weight basis) obtained as described above, and they were placed in an orbital shaker at 28 °C and 180 rpm. Several batches *per* substrate were prepared to monitor its transformation during 0, 6, 24, 48, 72 and 96 h. Additionally, control batches without biocatalyst and without substrate were included. Samples were taken as described for the microbial screening. Experiments were done in duplicate.

Biotransformations with increasing concentrations of vanillic acid

To assess the vanillic acid tolerance of *Aspergillus flavus* under the standard biotransformation conditions, substrate 100 × stock solutions in DMSO were prepared so as to yield 0.5, 1, 2, 4, 6 and 8 g/L final concentration in the batches but keeping constant the solvent concentration (1 % v/v). The batches were prepared by adding 200 μL of the corresponding stock solution to 50 mL erlenmeyer flasks containing 20 mL of suspended biocatalyst in a fresh culture medium as described before for standard conditions. Biotransformations were initiated by the addition of the substrate and incubated at 28 °C and 180 rpm. Several batches were prepared so as to sample at 0, 10, 24, 29, 33, 48, 72 and 96 h. Samples were treated as described above. All the experiments and analyses were done in duplicate.

Analytical methods

Biotransformation samples were analyzed by GC-FID using a Perkin Elmer-Clarus 500 instrument. Conversion was determined using a 007 methyl 5 % phenyl silicone column and calculated using relative areas (see Supplementary Information for further details). When needed, product purification was done by column chromatography on silica gel, employing mixtures of *n*-hexane: ethyl acetate as mobile phases. The identity of the isolated compounds was determined by ^{1}H and ^{13}C NMR spectra recorded at 200 and 50.2 MHz, respectively, on a Bruker AC-200 spectrometer using $CDCl_3$ as solvent, or alternatively in d_6-DMSO, both with TMS as internal standard (see Supplementary Information).

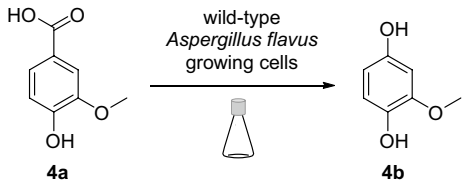


Fig. 1 Biotransformation of vanillic acid (**4a**) as model of lignin-related carboxylic acid by *A. flavus* growing cells

Results and discussion

Microbial screening

Based on previous literature reports, a screening to detect microorganisms capable of transforming benzoic acids was run. Thirty-one wild-type bacterial and fungal species from the genera *Erwinia*, *Nocardia*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, *Pycnoporus*, *Rhizopus*, *Rhodotorula* and *Saccharomyces* [24–26] were qualitatively tested using both growing and resting cell cultures with vanillic acid (**4a**)—a bulk, readily available chemical from the degradation of biomass-derived lignin [27, 28]—as model substrate (Table S1). Only *Aspergillus flavus* transformed vanillic acid significantly, yielding 2-methoxybenzene-1,4-diol (**4b**) (Fig. 1). This result is the first report of *A. flavus*—a fungal strain with probed capability to biotransfom natural products [29, 30]—catalyzing an oxidative decarboxylation reaction, which is in agreement with early studies made by Buswell et al. for several lignin-degrading white-rot and brown-rot fungi [31, 32]. Since *A. flavus* transformed **4a** with growing cultures but not with resting cells, it could be assumed that the oxidative decarboxylation pathway is triggered only under primary metabolism conditions.

Substrate scope

With the aim of establishing the substrate scope of the biocatalyst, seven lignin-related benzoic acid derivatives, including other monolignols as well as compounds that can be obtained from them through classic chemical reactions [2, 27], were also chosen as biotransformation substrates in analytical scale (Table 1). When benzoic acid (**1a**), (4-nitrophenyl)methanol (**6a**) and 3-chlorobenzoic acid (**7a**) were employed, the corresponding alcohols **1b**, **6b** and **7b** were obtained with very good and excellent conversions (c = 87 %, c = 93 % and c = 100 %, respectively). These alcohols were detected as the only biotransformation products, thereby showing the exquisite selectivity of the

Table 1 Benzoic acid derivatives used as substrates for biotransformations catalyzed by *A. flavus* growing cells and the obtained products

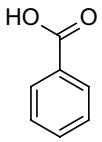
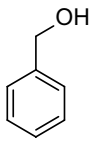
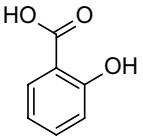
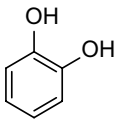
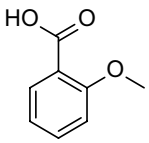
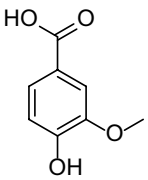
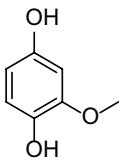
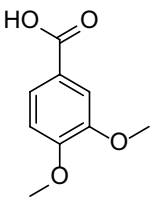
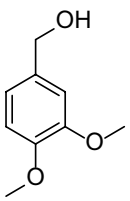
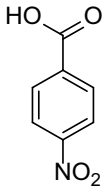
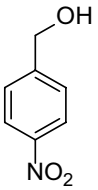
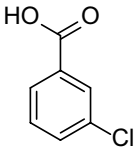
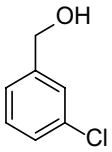
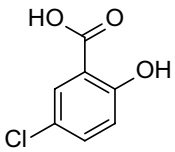
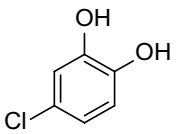
Entry	Substrate	Product	Conversion (%)[a]	Time (h)
1	 Benzoic acid **1a**	 Benzyl alcohol **1b**	87	72
2	 2-Hydroxybenzoic acid **2a**	 Pyrocatechol **2b**	100	48
3	 2-Methoxybenzoic acid **3a**	-	0	-
4	 4-Hydroxy-3-methoxybenzoic acid **4a**	 2-Methoxybenzene-1,4-diol **4b**	100	48
5	 3,4-Dimethoxybenzoic acid **5a**	 (3,4-Dimethoxyphenyl)methanol[b] **5c**	78	96

Table 1 continued

Entry	Substrate	Product	Conversion (%)[a]	Time (h)
6	 4-Nitrobenzoic acid **6a**	 (4-Nitrophenyl)methanol **6b**	93	96
7	 3-Chlorobenzoic acid **7a**	 (3-Chlorophenyl)methanol **7b**	100	48
8	 5-Chloro-2-hydroxybenzoic acid **8a**	 4-Chlorobenzene-1,2-diol **8b**	93	72

[a] Determined by GC-FID (See "Materials and methods")

[b] Main product

biocatalyst. Interestingly, the substitution of **1a** with a weak deactivating group like –Cl in *meta* (**7a**) or with a strong deactivating one as $–NO_2$ in *para* (**6a**) position seems to enhance the carboxylic acid reduction. To the best of our knowledge, this is the first report of the reduction of carboxylic acids **1a**, **6a** and **7a** to their alcohols with *A. flavus* as catalyst.

When attempting to transform 2-methoxybenzoic acid (**3a**), no conversion was evidenced. This result is in agreement with the observations made by Milstein et al. [33] for *Aspergillus japonicus*. This can be explained considering that the methoxy substitution in *ortho* may sterically prevent the carboxyl group to interact with the biocatalyst. Furthermore, apart from vanillic acid (**4a**), the compounds 2-hydroxybenzoic acid (**2a**) and 5-chloro-2-hydroxybenzoic acid (**8a**) were also selectively transformed to the corresponding oxidative decarboxylation products with

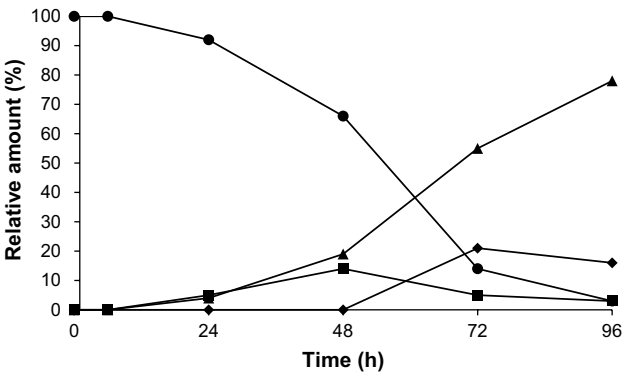


Fig. 2 Biotransformation profile of 3,4-dimethoxybenzoic acid (**5a**) (*circle*) with growing cultures of *A. flavus*, yielding 3,4-dimethoxybenzaldehyde (**5b**) (*square*), (3,4-dimethoxyphenyl)methanol (**5c**) (*triangle*) and 2-methoxybenzene-1,4-diol (**4b**) (*diamond*)

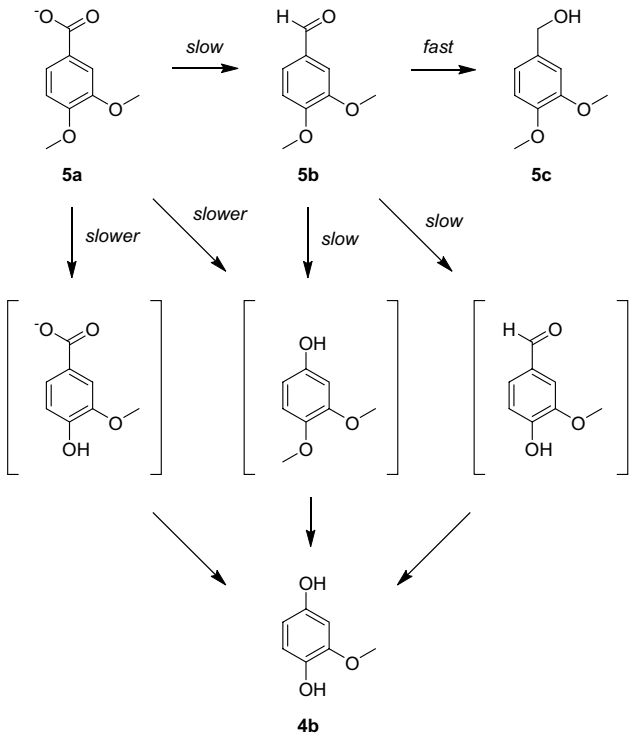


Fig. 3 Suggested metabolic pathway for the biotransformation of 3,4-dimethoxybenzoic acid (**5a**) by *A. flavus*. Reaction velocities are inferred on the basis of the observed kinetic profile. Compounds between brackets were not isolated

excellent rates ($c = 100$ % and $c = 93$ %, respectively). Remarkably, there is no previous report of *A. flavus* performing the oxidative decarboxylation of these compounds. As in substrate **4a**, they bear a strong electron donating group like –OH but in *ortho*. This suggests that the electronic density in the π system might necessarily be reinforced by substitution (with reference to benzoic acid) to facilitate the oxidative removal of the carboxyl group. Additionally, when a halogen atom is present—as in **8a**-, it may not produce enough inductive effect so as to prevent decarboxylation.

An interesting metabolic sequence leading mainly but not solely to the carboxyl reduction final product was observed when 3,4-dimethoxybenzoic acid (**5a**) was used as substrate. Although the alcohol **5c** was obtained as the major product ($c = 78$ %) after 96 h, 2-methoxybenzene-1,4-diol (**4b**) was also produced ($c = 16$ %). Moreover, very small amounts of the corresponding aldehyde **5b** ($c = 3$ %) were detected. Figure 2 depicts the time-dependent biotransformation profile of substrate **5a**. The biotransformation route displayed by *A. flavus* in the presence of substrate **5a**, which resembles the one described for *Pycnoporus cinnabarinus* by Hatakka [34], is shown in Fig. 3. By extending the electronic considerations stated above, it is worth to notice that the exchange of a strong electron donating group like –OH for a moderate one as –OCH_3 in *para* position-like in substrates **4a** and **5a**- might be enough so as to trigger the mechanisms leading to the carboxyl reduction in parallel to the oxidative decarboxylation route. Furthermore, it is interesting to note that substrates **1a**, **6a** and **7a** could also follow the same metabolic routes as described for **5a**, but the enzyme(s) responsible for their reduction might act considerably faster on those compounds than on **5a**. Despite the electronic effects, the

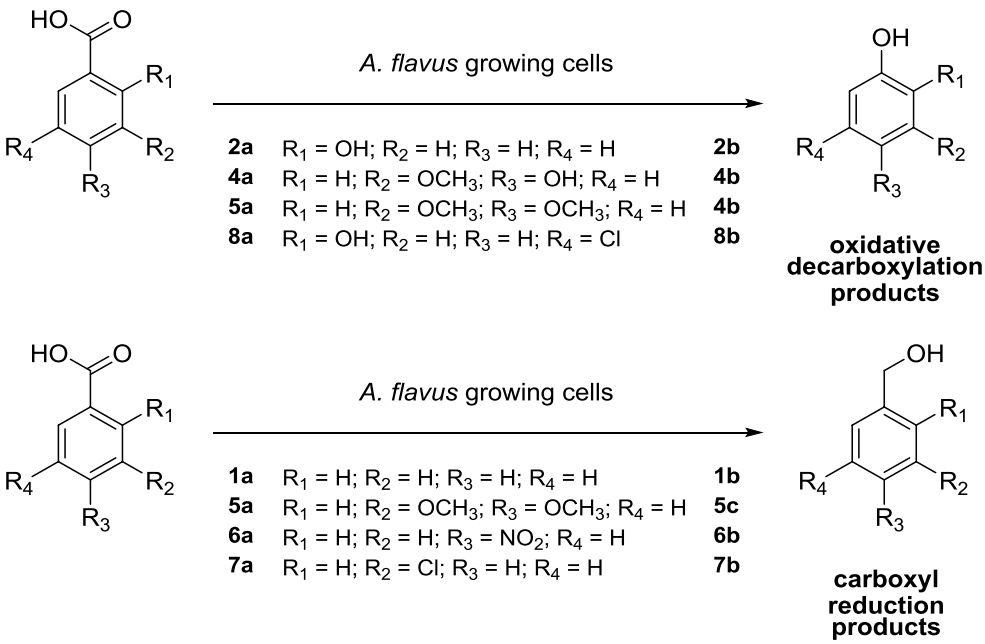


Fig. 4 Structure-dependent, self-sufficient transformation of lignin-related benzoic acid derivatives by *A. flavus*

presence of two bulky substitutions on the aromatic ring in this molecule could also be a reason for such differences. The structure-dependent, self-sufficient redox biotransformation outcome observed for the first time for *A. flavus* is depicted in Fig. 4.

Vanillic acid tolerance

To determine how much vanillic acid could be transformed by the selected biocatalyst, several experiments using substrate loadings from 0.5 g/L (standard condition) up to 10 g/L as final concentrations were conducted. Figure 5 illustrates that *A. flavus* was active when vanillic acid was supplied in the range of 0.5–8 g/L. From these results, it was evident that there is a strong dependence of the decarboxylation rate on the substrate concentration. Using 0.5–2 g/L of vanillic acid, almost complete conversion is reached after 48 h (Fig. 5a–c). When the substrate is supplied at 4 g/L, the system needs twice as much time (96 h) to reach 100 % conversion (Fig. 5d) and, at this stage, culture density becomes high enough to complicate orbital shaking (data not shown). Therefore, mass transfer might be low enough to ensure competitive rates when vanillic acid loading is 6 or 8 g/L, as can be observed in Fig. 5e, f, respectively. Considering these observations, and since the substrate is fairly soluble at 10 g/L in the reaction conditions, this experiment was discarded (data not shown). Finally, it should be highlighted that with vanillic acid concentration ranging from 1 to 8 g/L, the biocatalyst retains the complete selectivity exhibited under standard conditions, since it solely yielded the product **4b**, 2-methoxybenzene-1,4-diol. These results may not only envision larger scale biosynthesis of **4b** or similar derivatives, but they may also ease

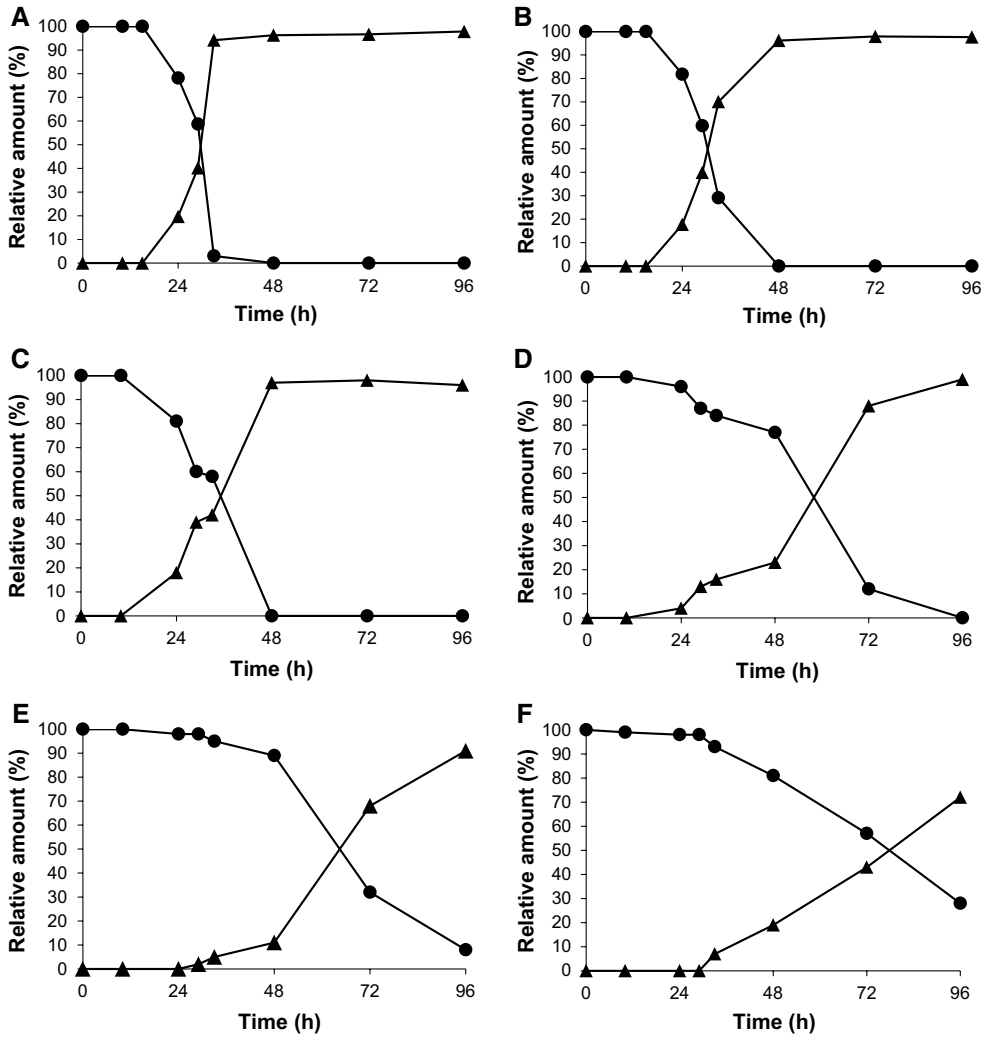


Fig. 5 Time-course biotransformation profiles with increasing concentrations of vanillic acid (**4a**). **a**–**f** depict **4a** consumption (*circle*) and product **4b** appearance (*triangle*), when the carboxylic acid load was 0.5, 1, 2, 4, 6 and 8 g/L, respectively

the design of a greener and more efficient alternative for the traditional chemical processes [35] leading to these molecules.

Conclusion

This is the first report of *Aspergillus flavus* performing the structure-dependent, self-sufficient reduction of the carboxyl group and the oxidative decarboxylation of selected lignin-related benzoic acids. These compounds are cheap bulk chemicals or readily available in vast amounts as by-products from the treatment of lignocellulosic biomass. *A. flavus*—a wild-type strain grown in a defined medium under simple culture conditions—exhibited not only good to excellent biotransformation rates, but also showed high tolerance to large substrate concentrations. For these reasons, the biocatalyst could be used as a biotechnological toolbox for chemical synthesis, and especially for lignin valorization in biomass processing.

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