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Biocatalysed reduction of carboxylic acids to primary alcohols in aqueous medium: A novel synthetic capability of the zygomycete fungus *Syncephalastrum racemosum*

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ABSTRACT

The zygomycete fungus *Syncephalastrum racemosum* shows the remarkable capability of reducing carboxylic acids to primary alcohols in water medium, at ambient temperature and pressure. The reaction does not require molecular hydrogen, and in most cases affords quantitative transformations. The results herein reported highlight the basic molecular scaffold that can be accepted by the fungus, the effects due to substituents, and also the possibility that carboxylic acids can be generated in the reaction medium by enzymatic hydrolysis of the corresponding methyl esters. This biocatalysed reduction implements the scarcely supplied enzymatic toolbox for the conversion of carboxylic groups into primary alcohols, which can be exploited for the optimisation of sustainable synthetic procedures.

1. Introduction

The reduction of esters and carboxylic acids to primary alcohols is a key reaction of organic chemistry [1], which has widespread application in the manufacturing processes of fine and bulk chemicals. This transformation still relies on the stoichiometric use of metal hydride reagents and their derivatives [2], often with the requirement of extensive heating, and anhydrous conditions. The highly exothermic hydrolytic work up is generally troublesome, and yields voluminous precipitates. Recently, transition metal catalysed hydrogenation reactions [3] employing molecular hydrogen as a reducing agent have been investigated in order to address the waste challenge connected with hydride promoted reductions. However, only a limited number of these new procedures work at room temperature and low hydrogen pressure. Other chemocatalytic methods, such as the metal-catalysed hydrosilylation of esters, have received considerable interest as a tool for carboxylic group reduction to primary alcohols [4]. Nevertheless, the development of a cost-effective, and highly selective catalyst for this transformation is still desirable because most of the known procedures either require expensive silanes or have limited functional group tolerance.

As for biocatalysed methods, this is a very challenging task, because the reduction of acids is characterised by a very low redox potential, and it has been described up to now only for a very limited number of microorganisms and cultured plant cells. The two classes of enzymes which have been reported so far as biocatalysts of this reaction are [5]: carboxylic acid reductases (CARs, E.C.1.2.1.30) from aerobic sources, such as bacteria, fungi and plants, and aldehyde ferredoxin oxidoreductases (AORs, E.C. 1.2.99.6) from anaerobic bacteria and archaea.

In 1969 Gross et al. isolated a CAR [6] from *Neurospora crassa* and performed preliminary studies on the reaction mechanism. The reduction was found to be adenosine triphosphate (ATP), Mg^{2+} and NADPH dependent [6c]. An homologous enzyme was isolated and purified by Rosazza and co-workers from *Nocardia* species NRRL 5646 in 1997 [7]. This CAR enzyme was overexpressed, employed successfully in biocatalytic reductions [7a,c], and its reaction mechanism was fully elucidated [7d]. Recently [8], the conversion of 3,4-dihydroxyphenylacetic acid into 3-hydroxytyrosol has been

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performed in whole *Escherichia coli* BL21(DE3) cells overexpressing CAR from *Nocardia* and phosphopantetheinyl transferase from *E. coli*, with the concomitant action of an endogenous *E. coli* aldehyde reducing activity. In 2013 [9], Turner et al. described the conversion of fatty acids into the corresponding alcohols, by coexpression of a CAR from *Mycobacterium marinum* and an aldehyde reductase from *Synechocystis* species.

Sodium carboxylates C_6–C_{10} were transformed into the corresponding alcohols by growing cultures of the fungus *Colletotrichum gloeosporioides*, but the method lacks general applicability to different substrates [10]. Some examples of aerobic reductions of aromatic carboxylic acids by fungi have been described, especially in connection with the conversion of ferulic acid into vanillin [5].

Resting cells of *Clostridium thermoaceticum* and *Clostridium formicoaceticum* were described to reduce [11] carboxylic acids and carboxylates to alcohols at the expense of carbon monoxide or, less efficaciously, in the presence of formate. The enzymes responsible for the carboxylic acid reduction in these two organisms were isolated and characterised to be highly oxygen sensitive tungsten enzymes (W-AORs). In spite of the efficiency of the reaction, the requirement for strictly anaerobic conditions makes this alternative poorly appealing for preparative applications.

In 1999, it was first reported [12a] that the hyperthermophilic archaea *Pyrococcus furiosus* reduced carboxylic acids with starch as carbon and energy source at 90 °C. Recently, the use of this microorganism has been reconsidered [12b], because it contains suitable enzymes to couple the oxidation of molecular hydrogen (catalysed by hydrogenases) to the reduction of carboxylic acids to aldehydes (mediated by an AOR). The presence of endogenous alcohol dehydrogenases promotes the final conversion of aldehydes into primary alcohols. A biocatalytic hydrogenation procedure has been optimised for the reduction of carboxylic acids at 40 °C under 5 bar molecular hydrogen pressure. The same authors have also described the possibility to employ syngas instead of hydrogen to promote this *P. furiosus*-mediated reduction, thus hypothesising the presence in this microorganism of enzymes capable of coupling CO oxidation to acid reduction [12c].

As for eukaryotic cells, nine different plant cell cultures have also been successfully employed on several carboxylic acids, but only in modest yields and with long pre-culture times and reaction times [13].

We now wish to report on the possibility of preparing primary alcohols by reduction of carboxylic acids in a very mild and efficient way, using the whole cells of the zygomycete fungus *Syncephalastrum racemosum* in aqueous medium, at room temperature and pressure. In some cases the carboxylic acids can be generated in the reaction medium by enzymatic hydrolysis of the corresponding methyl esters. This peculiar reducing activity was discovered during a functional screening of the enzymatic capabilities of a selection of filamentous fungi of the *Mycotheca Universitatis Taurinensis* (MUT), performed on model substrates in the aim of finding new biocatalysts for synthetic applications.

2. Experimental

2.1. Materials and equipment

The carboxylic acids employed for the work were commercial products purchased from Sigma–Aldrich. The corresponding methyl and ethyl esters were either commercial products from Sigma–Aldrich or were prepared by Fischer esterification. Stock solutions (500 mM) of each substrate in DMSO were employed for the biotransformations.

GC/MS analyses were performed on an Agilent HP 6890 gascromatograph equipped with a 5973 mass detector and an HP-5-MS column (30 m × 0.25 mm × 0.25 μm, Agilent), employing the following temperature program: 60 °C (1 min)/6 °C min^{-1}/150 °C (1 min)/12 °C min^{-1}/280 °C (5 min). The identity of the reduction products was established by comparison with reference samples (either commercially available or prepared by lithium aluminium hydride reduction of the corresponding methyl esters).

2.2. Fungal strains

The strain of the zygomycete fungus *S. racemosum* MUT 2770 employed for this work is preserved at *Mycotheca Universitatis Taurinensis* (MUT, Department of Life Sciences and Systems Biology, University of Turin).

2.3. Biotransformation experiments

Fungal strains were pre-grown in Petri dishes containing malt extract solid medium (MEA: 20 g L^{-1} glucose, 20 g L^{-1} malt extract, 20 g L^{-1} agar, 2 g L^{-1} peptone) from which the inoculum for liquid cultures was set up. The fungus was inoculated as conidia suspension (1×10^6 conidia/mL) in 50 mL flasks containing 40 mL of malt extract liquid medium. Flasks were incubated at 25 °C and were maintained under agitation (110 rpm).

After 2 days of pre-growth, a 500 mM solution of the substrate in DMSO was added, to a starting substrate concentration (c_0) of 1–5 mM. For each substrate, three biological replicates were run. The experiment was run for 3 days after the addition of the substrates, during which time 1 mL samples were taken, at specified intervals (usually 24, 48, and 72 h). Each sample was extracted with EtOAc (500 μL), the organic phase was dried over anhydrous Na_2SO_4 and analysed by means of GC/MS. In some cases (see Section 2.4) the isolation of the reduced product has been carried out.

For each set of biotransformations, one flask was used to measure the initial biomass and pH before the addition of the substrate. These parameters were also evaluated at the end of the experiment for all the flasks. The liquid media was separated from the biomass by filtration and was used for pH measurement while the mycelia were dried at 60 °C for 24 h to measure the biomass dry weight.

2.4. Isolation procedure of biotransformation products

In the case of benzoic acid, methyl benzoate, phenylacetic acid, methyl phenylacetate, 3-phenylpropanoic acid, phenoxyacetic acid, methyl phenoxyacetate, 2-furoic acid, 2-(thiophen-2-yl)acetic acid, methyl 2-(thiophen-2-yl)acetate, 2,4-hexadienoic acid, methyl 2,4-hexadienoate, and methyl octanoate the isolation of the final products was carried out. For benzoic acid, 2-(thiophen-2-yl)acetic acid and 2,4-hexadienoic acid c_0 = 1 mM was employed, while for all the other substrates c_0 = 5 mM was used (in both cases the reaction total volume was 40 mL). After 72 h, the reaction mixture was filtered to remove the biomass. The filtrate was extracted with EtOAc (3 × 20 mL), dried on Na_2SO_4, and concentrated under reduced pressure. When the conversion was not complete (GC/MS analysis) the residue was purified either by treatment with a saturated $NaHCO_3$ solution or by column chromatography (silica gel, elution with hexane and increasing amount of EtOAc).

Benzyl alcohol: from benzoic acid (3.8 mg, 89%) and from methyl benzoate (19.9 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.35–7.25 (m, 5H, aromatic hydrogens), 4.67 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ = 141.2, 128.3, 127.3, 126.9, 65.0. GC/MS: t_R = 6.46 min, m/z 108 (M^+, 95), 91 (20), 79 (100).

1-Phenylethanol: from phenylacetic acid (9.0 mg, 37%) and from methyl phenylacetate (6.3 mg, 26%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.35–7.15 (m, 5H, aromatic hydrogens), 3.85 (t, 2H, J = 6.6 Hz, $PhCH_2CH_2OH$), 3.85 (t, 2H, J = 6.6 Hz, $PhCH_2CH_2OH$). ^{13}C