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Journal of Molecular Catalysis B: Enzymatic 22 (2003) 71–77
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Stereoselective oxidoreductase type bioconversions of exogenous substrates by cell suspension cultures of bryophytes

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Received 25 September 2002; accepted 8 January 2003

Abstract

We established green cell suspension cultures of *Marchantia polymorpha*, *Marchantia plicata*, *Riccia fluitans* and *Asterella blumeana*. Whole cell biotransformations of the oxidoreductase type were studied using different exogenous substrates. The results show that cell cultures of liverworts can serve as effective tools in bioconversion reactions for the enantio- or diastereoselective reduction of simple ketones, β-ketoesters and α,β-unsaturated carbonyl compounds. The reduction of ketones and β-ketoesters resulted in products according to Prelog's rule.

Keywords: Bioconversion; Cell cultures; Bryophytes; Oxidoreductase; Enoate reductase

1. Introduction

Chemo-, regio- and stereoselective oxidations and reductions are basic chemical transformations which can be performed by well established chemical methods as well as by enzyme systems of the *oxidoreductase* type. These enzymes demand cofactors like $NAD(P)^+$/NAD(P)H which make bioconversions using isolated enzymes expensive without an effective cofactor regeneration. Whole cell systems like yeasts and plant cell cultures overcome this disadvantage though large biomasses or an uncontrolled metabolism must be taken in account. *Alcohol dehydrogenases* (ADH) are the mainly applied subtype of this enzyme class which catalyze the transfer of hydrogen and so oxidize alcohols to carbonyl compounds and vice versa. Applied in reductions they may distinguish between enantiotopic and diastereotopic sides of prochiral carbonyl compounds and so allow the preparation of non racemic alcohols. *Horse liver alcohol dehydrogenase* (HLADH) and *lactate dehydrogenase* (LDH) are the most established isolated dehydrogenases [1,2] whereas baker's yeast (*Saccharomyces cerevisae*) is by far the most widely used whole cell system with a broad substrate and functional group tolerance [3] and which can be optimized by recombinant engineering to "designer yeasts" [4,5].

Plant cell suspension cultures can serve as tools for the in vivo production of secondary metabolites [6,7] as well as for the biotransformation of foreign substrates [8,9]. Mainly higher plants like *Nicotiana tabacum*, *Glycine max* and *Catharanthus roseus* were extensively studied in this area [10,11]. Green cell suspension cultures obtained from bryophytes were preferentially established for growth analyses and biogenetic studies or for the production of metabolites [12]. Only few reports were given on oxidoreductase type bioconversions of exogenous substrates using

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1381-1177/03/$ – see front matter
doi:10.1016/S1381-1177(03)00008-0

such cell cultures and exclusively for *Marchantia polymorpha*: reduction of ethyl 2-methyl-3-oxobutanoate [13], reduction of 4-androstene-3,17-dione [14], reduction of 1,4-androstadiene-3,17-dion and andrenosterone [15] as well as oxidation of epitestosterone [16].

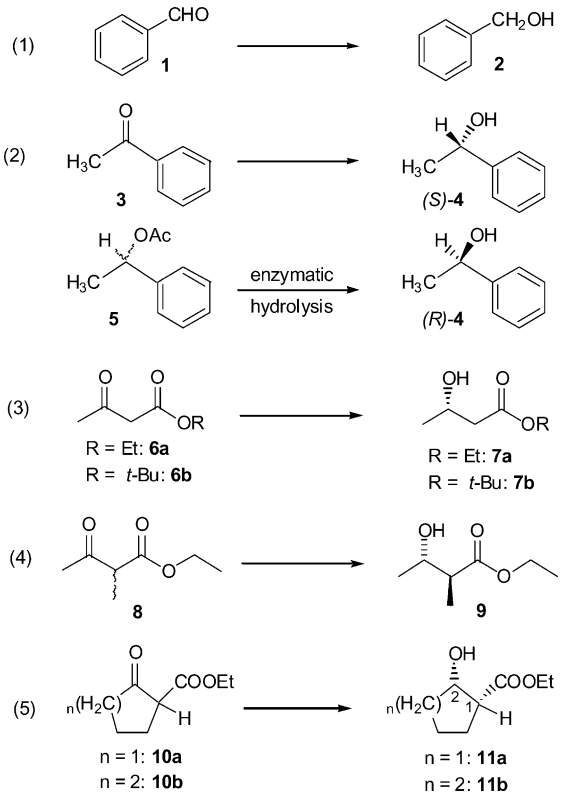
2. Results and discussion

In the course of our studies concerning chemistry and biology of bryophytes we established green cell suspension cultures of different liverworts (growing and resting cells) which were checked in bioconversion studies with different substrates [17]. Reaction rates and selectivities were studied and the reactions were scaled up from the typical 10 mg to an efficient g scale.

Green (photomixotrophic [18]) cell suspension cultures can be obtained from spores or decontaminated gametophytes of liverworts by a routine but uncertain procedure described by Katoh [19]. Up to now for bryophytes only suspension cultures of *M. polymorpha* were tested in bioconversion reactions. In our hands, we started with gametophytes of sterile agar cultures from *M. polymorpha*, *Marchantia plicata*, *Riccia fluitans* and *Asterella blumeana*. A stationary phase of the cell growth in the liquid medium (with a factor of ∼5 in biomass) was observed after 2–3 weeks. Bioconversion reactions could be performed in the original cell culture medium as well as in a suspension of resting cells in a phosphate buffer.

We confirmed the general capability of the bryophyte cell cultures for alcohol dehydrogenase reactions in a reduction of benzaldehyde (**1**) to benzyl alcohol (**2**) (1–2 mmol, see Table 1 and Scheme 1, entry 1). Acetophenone (**3**) is a simple prostereogenic ketone for testing a stereoselective reduction [20]. Cell cultures of *M. polymorpha*, *M. plicata* and *R. fluitans* reduced **3** with 34–74% e.e. to (*S*)-1-phenylethanol (**4**), the product according to Prelog's rule [21]. The conversion ratio is maximum 74% in a 0.8–3.2 mmol batch. So, this bioconversion is only moderate effective, though the enantiomeric alcohol to the hydrolysis product from (1-phenylethyl)acetate (**5**) according to the Kazlauskas rule [22] can be obtained.

β-Ketoesters like alkyl 3-oxobutanoates are standard objects for stereoselective chemical [23] or enzymatic reductions [24]. The baker's yeast reduction



Scheme 1. Alcohol dehydrogenase reactions with bryophyte cell cultures.

was studied in detail [25]. Plant cell cultures from *N. tabacum* were used for this biotransformation resulting in 60–98% e.e. [26]. In our studies we subjected ethyl and *t*-butyl acetoacetate (**6a** and **6b**) to a bioconversion using bryophyte cell cultures which reduced these substrates to (*S*)-(+)-ethyl and (*S*)-(+)-*t*-butyl 3-hydroxybutanoate (**7a** and **7b**), respectively (entry 3). Cultures from *M. polymorpha* and *M. plicata* gave moderate e.e. values (40–76%), cultures from *R. fluitans* and *A. blumeana* however resulted in high e.e. values (90 to >95%). These results were even obtained in 6.0 mmol batches within 1–2 days reaction time and in high yields. Being in accordance with Prelog's rule the *tert*-butyl ester **6b** gave better stereoselectivities than **6a** because of the bulkier ester moiety.

The enantio- and diastereoselectivity in the reduction of ethyl 2-methyl-3-oxobutanoate (**8**) (generally leading to valuable 2-alkyl-3-hydroxyalkanoates [27])

Table 1
Biotransformations of the alcohol dehydrogenase type

Entry	Substrate	Culture	Scale[a] (mmol)	Reaction time in days (%conversion)	%Yield	Product/configuration/%e.e.			
1	**1**	*M. polymorpha*	1.0–2.0	5 (100)	70–90	Benzyl alcohol (**2**)			
1	**1**	*M. plicata*	2.0	5 (100)	70	Benzyl alcohol (**2**)			
1	**1**	*R. fluitans*	1.0	5 (100)	81	Benzyl alcohol (**2**)			
1	**1**	*A. blumeana*	2.0	5 (100)	90	Benzyl alcohol (**2**)			
						(S)-(−)-1-Phenylethanol (**4**)			
2	**3**	*M. polymorpha*	0.8–1.6	10 (30–45)		70–74			
2	**3**	*M. plicata*	0.8–3.2	10 (28–70)		34–44			
2	**3**	*R. fluitans*	0.8	10 (60)		74			
						(*S*)-(+)-3-Ethyl hydroxybutanoate (**7a**)	(*S*)-(+)-3-*t*-Butyl hydroxybutanoate (**7b**)		
3	**6a/6b**	*M. polymorpha*	0.75–7.5	2–7 (100)	70–90	40	56–66		
3	**6a/6b**	*M. plicata*	0.75–7.5	2–7 (100)	70–90	70	64–76		
3	**6a/6b**	*R. fluitans*	0.6–6.0	1–2 (100)	70–90	>95	>95		
3	**6a/6b**	*A. blumeana*	1.0–4.0	3–7 (100)	70–90	90–93	93–95		
						anti-(2*S*,3*S*)-**9** (d.e.)	anti-(2*S*,3*S*)-**9** (e.e.)		
4 [28]	**8**	*M. polymorpha*	0.1	1	88	92	99		
4	**8**	*M. polymorpha*	1.4–7.0	5–9 (100)	84–86	92–96	64–86		
4	**8**	*M. plicata*	1.4–7.0	5–6 (100)	86–92	96– >98	92–96		
4	**8**	*R. fluitans*	1.4–7.0	5–9 (100)	82–85	98	86–86		
4	**8**	*A. blumeana*	1.4–7.0	5–9 (100)	83–90	>97	54–68		
						(1*R*,2*S*)-(+)-**11a** (d.e.)	(1*R*,2*S*)-(+)-**11a** (e.e.)	(1*R*,2*S*)-(+)-**11b** (d.e.)	(1*R*,2*S*)-(+)-**11b** (e.e.)
5	**10**	*M. polymorpha*	1.2–3.2	7–10	50–90	78	0	90–94	68–90
5	**10**	*M. plicata*	1.2–3.2	7–10	50–65	80–88	14–42	28–52	5–36
5	**10**	*R. fluitans*	1.2–3.2	7–10	50–90	52–64	0	90–94	32–58

[a] Total amount in 200–400 ml cell culture medium.

was already studied with cell cultures of *M. polymorpha* and some other plants [28]. *M. polymorpha* yielded anti-(2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid ethyl ester (**9**) with 92% d.e. and 99% e.e. in 0.1 mmol batches (entry 4). In contrast, *G. max* resulted in the formation of the syn-(2*R*,3*S*)-product with 84% d.e. and 97% e.e. The formation of preferably one of the four possible products is possible because of the diastereoselective reduction with concomitant dynamic resolution (rapid in situ racemization of substrate via enolization).

In our experiments (1.4–7.0 mmol batches) all cell cultures reduced **8** preferably to the anti-(2*S*,3*S*) product **9**. Only moderate selectivities (92 to >98% d.e., 54–86% e.e.) were obtained with *M. polymorpha*, *R. fluitans* and *A. blumeana* but better results with cultures of *M. plicata* (86–92% yield, 96 to >98% d.e., 92–96% e.e.). So, the latter bioconversion proved to be an effective process.

Reductions of cyclic β-ketoesters using baker's yeast are well studied [29] whereas plant cell cultures were yet not used for this purpose. For a reduction with bryophyte cell cultures we tested ethyl cyclopentanone and hexanone-2-carboxylates **10a** and **10b** which, due to the cyclic structure, were reduced with 30% d.e. (65:35) to the corresponding *cis*-hydroxyesters **11a** and **11b** even by simple chemical reducing agents [30]. The results obtained with the cell cultures of bryophytes proved to be moderate compared with acyclic ketoesters and baker's yeast reductions and better for cyclohexanone than the cyclopentanone carboxylic esters (entry 5). The best results were obtained with *M. polymorpha* yielding ethyl *cis*-(1*R*,2*S*)-2-hydroxycyclohexane carboxylate (**11b**) with 90–94% d.e. and up to 90% e.e.

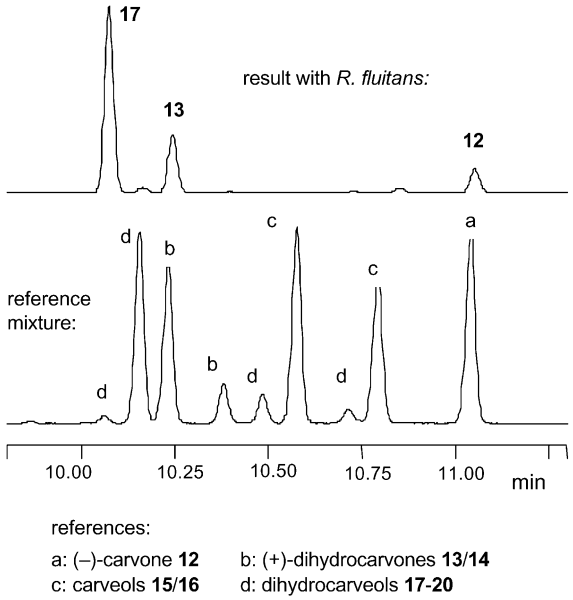


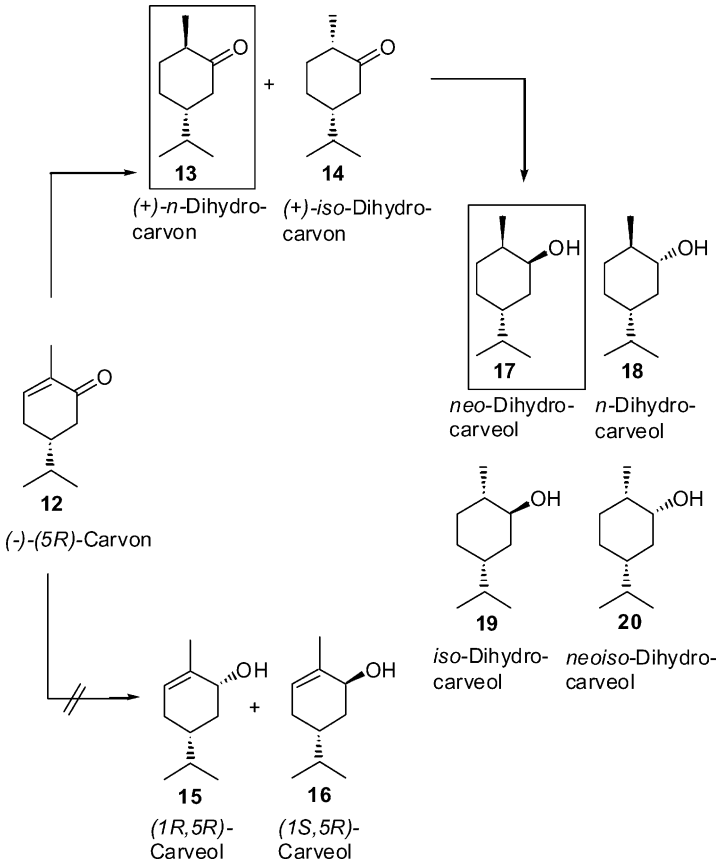
Fig. 1. GC analysis of carvones, dihydrocarveols, carveols and dihydrocarvones (Column: OV-1, 30 m).

The diastereo- or enantioselective reduction of C=C bonds of α,β-unsaturated carbonyl compounds is a difficult chemical transformation [31], which however was successfully performed using whole cell systems possessing NADH dependent enoate reductase activity [32]. We tested (−)-(5*R*)-carvone (**12**) as substrate for this bioconversion. The different steps [33] and the stereochemical outcome of the enzymatic enoate reduction have been elucidated and is normally made up of a starting *trans*-addition of hydrogen across the C=C bond [34] (yielding e.g. dihydrocarvones **13**/**14**) followed by an alcohol dehydrogenase reduction of the saturated carbonyl compounds (yielding e.g.

Table 2
Reduction of (−)-(5*R*)-carvone **12** with plant cell cultures

Culture	Scale (mmol)	Culture (ml)	Time (days)	Product distribution (%)						
				12	**13**	**15**	**17**	**18**	**19**	**20**
N. tabacum [35]	0.065	200	10	86.6	1.6	<0.01	10.7	1.1	<0.01	<0.01
M. sativa [36]	3.2[a]	1600	3	21	27	<0.5	52	<0.5	<0.5	<0.5
M. polymorpha	0.7[a]	300	9	40	28	<0.5	32	<0.5	<0.5	<0.5
M. plicata	0.7[a]	300	9	52	16	<0.5	26	<0.5	<0.5	<0.5
R. fluitans	0.7[a]	300	9	5	23	<0.5	72	<0.5	<0.5	<0.5

[a] Total amount in 200 ml cell culture medium.



Scheme 2. Enoate reductase/alcohol dehydrogenase reaction with (−)-(5*R*)-carvone (**12**).

dihydrocarveols **17**–**20**). Only in rare cases the ketone is reduced first which would lead to the intermediates like the carveols **15**/**16** (Scheme 2).

Our investigations with bryophyte cell cultures in the reduction of carvone were compared with the results obtained with *N. tabacum* [35] and *Medicago sativa* [36]. The enoate reductase step of (−)-(5*R*)-carvone (**12**) occurs with high diastereoselectivity to (+)-*n*-dihydrocarvone (**13**). The following alcohol dehydrogenase step yielding *neo*-dihydrocarveol (**17**) is also highly diastereoselective. In summary (see Table 2), conversion rates and selectivities are equal or even better than with *N. tabacum* and *M. sativa* especially for *R. fluitans* (an example for GC analysis is given in Fig. 1). No side reactions like formation of carveols (**15**/**16**) or oxygenase reactions were observed.

3. Conclusion

These results show that the green cell suspension cultures of different liverworts can serve as tools in bioconversion reactions (0.6–7.0 mmol batches with 100–400 ml tissue cultures) for the enantio- or diastereoselective reduction of ketones, β-ketoesters and α,β-unsaturated carbonyl compounds. The reduction of ketones and β-ketoesters resulted in products according to Prelog's rule.

4. Experimental

The sterile liverwort agar cultures were provided by Prof. Dr. H. Becker and Dr. K.P. Adam, Institut für Pharmakognosie und Analytische Phytochemie,

University of the Saarland, D-66041 Saarbrücken. All liverwort cell cultures were handled in laminar flow boxes under sterile conditions.

4.1. Preparation of cell suspension cultures

In the first step, the callus induction was performed according to procedure described by Katoh [19] starting with gametophytes of sterile agar cultures from *M. polymorpha*, *M. plicata*, *R. fluitans* and *A. blumeana* using a solid MSK 2.2 agar medium [37] containing 4% of glucose and 1 ppm 2,4-dichlorophenoxy acetic acid (2,4-D). Suspension cultures were obtained transferring rapidly growing callus to a modified Gamborg B5 liquid medium (100–400 ml) [38]. All culture flasks were agitated on a rotary shaker at 100 rpm at 20 °C while illuminating up to 3000 lux and were subcultured every 4 weeks. A stationary phase of the cell growth was observed after 2–3 weeks. Resting suspension cultures were obtained by filtration under sterile conditions and suspending in a 0.05 M phosphate buffer (pH 6.0). They proved to be stable for more than 3 weeks and could be used alternatively.

4.2. Carrying out and monitoring bioconversion reactions

Biotransformations were started at the stationary phase by adding the substrate (typically 50–1000 mg or 0.6–7.5 mmol, see Table 1) in a minimum amount of ethanol to culture flasks containing 200–400 ml of liquid medium (dry cell mass after workup: 3–8 g). The bioconversion was monitored up to 10 days (GC analyses of batch probes after filtration through a Chem Elut® cartridge) and worked up by filtration from the biomass, saturating the aqueous layer with $(NH_4)_2SO_4$ or NaCl and extraction with diethylether. The products were separated and purified by LC and HPLC (silica gel/EtOAc–*n*-hexane) and analyzed by NMR as well as by achiral and chiral GC using the corresponding reference probes. GC analyses were performed on Varian GC 3400 and 3380: Achiral column: fused silica type OV-1 (30 m × 0.25 mm × 0.25 μm); chiral columns: CP-chirasil-Dex CB and CP-cyclodextrin-β-2,3,6-M-19, fused silica (25 m × 0.25 mm × 0.25 μm) from Chrompack.

Acknowledgements

We are very grateful to Prof. Dr. H. Becker and Dr. K.P. Adam, Institut für Pharmakognosie und Analytische Phytochemie, University of the Saarland, D-66041 Saarbrücken for providing the sterile bryophyte cultures and the guidance in our tissue culture work.

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