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Enantioselective hydrolase type bioconversions of exogenous substrates using cell suspension cultures of bryophytes

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Abstract—We have prepared green cell suspension cultures of *Marchantia polymorpha*, *Marchantia plicata*, *Riccia fluitans* and *Asterella blumeana*. Whole cell biotransformations of the hydrolase type were studied using different exogenous substrates resulting in a small to large-scale production of useful homochiral compounds.

1. Introduction

Plant cell suspension cultures can serve as tools for the in vivo production of secondary metabolites[1,2] as well as for the biotransformation of foreign substrates.[3,4] Higher plants such as *Nicotiana tabacum*, *Glycine max* and *Catharanthus roseus* have been studied extensively in this area.[5,6] Green cell suspension cultures obtained from bryophytes were prepared for growth analyses, biogenetic studies and for the production of metabolites.[7] Only few reports are known on hydrolase-type bioconversions of exogenous substrates using such cell cultures and these involve *Marchantia polymorpha* exclusively: Hydrolysis of 2-methylcyclohexyl acetates,[8] 1,2- or 1,3-diacetoxy cyclohexanes or norbornanes[9,10] and enol acetates.[11,12]

In the course of our studies concerning the 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.[13] Reaction rates and selectivities were studied and the reactions scaled up from the typical 10 mg to an efficient gram scale.

2. Results and discussion

Green (photomixotrophic[14]) cell suspension cultures can be obtained from the spores or decontaminated gametophytes of liverworts by a routine but uncertain procedure described by Katoh.[15] For bryophytes, only suspension cultures of *M. polymorpha* have been tested in bioconversion reactions to date. We started our study with gametophytes of sterile agar cultures from *M. polymorpha*, *Marchantia plicata*, *Riccia fluitans* and *Asterella blumeana* (the last one could not be studied in detail because of culture decay). A static 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 fresh phosphate buffer.

The enantioselective hydrolysis of acetates with biocatalysts containing hydrolase-type enzymes[16] is a useful process for the preparation of chiral alcohols.[17] We tested the general capability of the bryophyte cell suspension cultures in the hydrolysis of acetates, attempting a large scale hydrolysis of benzyl acetate **1** to benzyl alcohol **2**. It was found that the hydrolysis could be performed with all suspension cultures in a 0.5–1.5 mmol batch in 5 days with 80–90% yields (Table 1 and Scheme 1, entry 1).

For an enantiodifferentiating hydrolysis of *sec*-acetates (kinetic resolution) we tested (±)-(1-phenylethyl)acetate **3** as substrate.[18] Enantiopure (*R*)- or (*S*)-1-phenylethanol can be used as chiral reagents for the determination of the enantiomeric purity and the resolution of carboxylic acids[19] or as chiral synthons.[20] All cell cultures hydrolyse the (*R*)-acetate to the (*R*)-alcohol **4** preferentially, leaving the enantiomerically enriched (*S*)-acetate **5**. This is in agreement with

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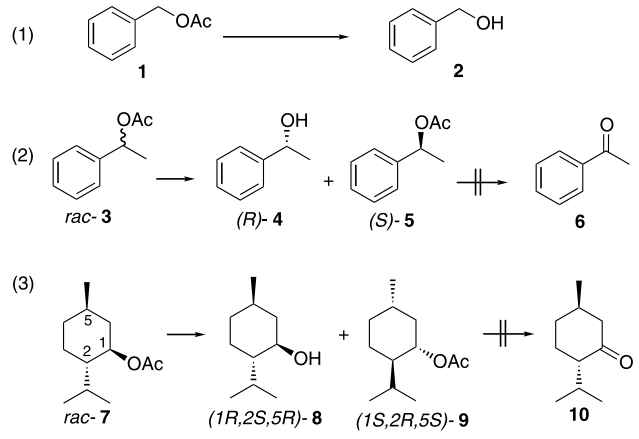
Table 1. Biotransformations of the hydrolase type: enantiomer-differentiating hydrolysis (kinetic resolution)

Entry	Substrate	Culture	Scale (mmol)	Reaction time/% conversion	% yield	Product/config./% ee		
1	**1**	*M. polymorpha*	0.5–1.5	4–8 days/100	85		**2**	
1	**1**	*M. plicata*	1.5	6 days/100	90		**2**	
1	**1**	*R. fluitans*	0.6	4 days/100	83		**2**	
1	**1**	*A. blumeana*	0.6	4 days/100	81		**2**	
						(*R*)-(+)-**4**	(*S*)-(−)-**5**	*E*-value
2	**3**	*M. polymorpha*	0.6–1.2	1–2 days/45		76–88	82–96	20–30
			1.2–6.0	3–7 days/14–68		54–88	40–96	5–30
2	**3**	*M. plicata*	0.6–1.2	6 h/49–52		70–80	66–85	11–21
			1.2–6.0	3–7 days/26–89		28–80	22–85	5–21
2	**3**	*R. fluitans*	0.6–1.2	6 h/50–55		78–80	52–65	19–24
			1.2–6.0	3–7 days/32–99		26–80	36–65	8–24
2	**3**	*A. blumeana*	0.6–1.2	6 h/41–54		58–64	34–48	6
			1.2–6.0	3–7 days/29–99		52–64	20–48	4–6
						(1*R*)-(−)-**8**	(1*S*)-(+)-**9**	*E*-value
3	**7**	*M. polymorpha*	0.5–1.5	6–10 days/36–45	65–90	>96	35–60	95
3	**7**	*M. plicata*	0.5–1.5	6–10 days/32–45	68–90	>96	35–80	95
3	**7**	*R. fluitans*	0.5–1.5	6–10 days/26–31	40–60	>96	30–60	95



Scheme 1. Simple hydrolysis and kinetic resolution.

Kazlauskas' rule.[21] Subsequent oxidation to acetophenone **6** was not observed (entry 2).

The enantiomeric ratio (*E* value) describes the relationship between the conversion rate and the enantiomeric purity of the starting material and the product, and so the efficiency of a kinetic resolution.[22] Therefore, we performed detailed kinetic studies concerning substrate charge, conversion rate and the resulting enantioselectivities. The results are summarized in Table 1, entry 2. In the first series the bryophyte suspension cultures gave low (<20) to moderate (*E* 20–<50) *E*-values with 0.6 to 1.2 mmol charges. Scaling the reaction up to 1.2–6.0 mmol resulted in a slight decrease in the efficiency of the reaction. In an optimized reaction using *M. polymorpha*, at nearly 50% conversion the (*R*)-(+)-alcohol **4** could be obtained with 88% ee, leaving the (*S*)-(−)-acetate with 96% ee (see Fig. 1).

We also performed an enzymatic resolution of DL-menthyl acetate **7**.[21] Resolution using plant tissue cultures have been described for orchid species,[23] but occur in low enantioselectivities. The bryophyte cell cultures hydrolyze—in agreement with Kazlauskas' rule—the (1*R*)-acetate to the (1*R*)-alcohol (L-(−)-menthol) **8** preferentially, leaving the (1*S*)-acetate **9** unreacted (entry 3). Subsequent oxidation of **8** to menthone **10** was not observed. Detailed charge and kinetic studies showed that the conversion rate did not exceed 45% in a 0.5 to 1.5 mmol scale so that L-(−)-menthol **8** was obtained with >96% ee [(+)-acetate **9**: ≤80% ee, *E* ~95].

In contrast to kinetic resolution, prostereogenic molecules can theoretically be transformed into one enantiomer in 100% yield. *meso*-Diols or *meso*-diacetates are suitable compounds which can be transformed by an enantiotopos differentiating hydrolase reaction to complementary *mono*-acetates ('*meso*-trick').[24] The enantioselectivity in the hydrolysis of *cis*-1,2-diacetoxycyclohexane **11** by the cultured cells of *M. polymorpha* was already investigated[9] for a 0.1 mmol charge and resulted in 63% yield and 91% ee for the (1*S*,2*R*)-monoacetate **12**. Less than 2% of the undesired *meso*-diol **13** were obtained within 12 h. Our more detailed conversion studies with 1.0–2.0 mmol charges showed the formation of the monoacetate in 78–95% ee leaving 1–4% of the unreacted diacetate and producing 15–31% of the undesired diol within 24–60 h (see Table 2 and Scheme 2).

3. Conclusion

These results show that the green cell suspension cultures of different liverworts can serve as effective tools in bioconversion reactions for the small to medium large-scale production of useful chiral compounds. The

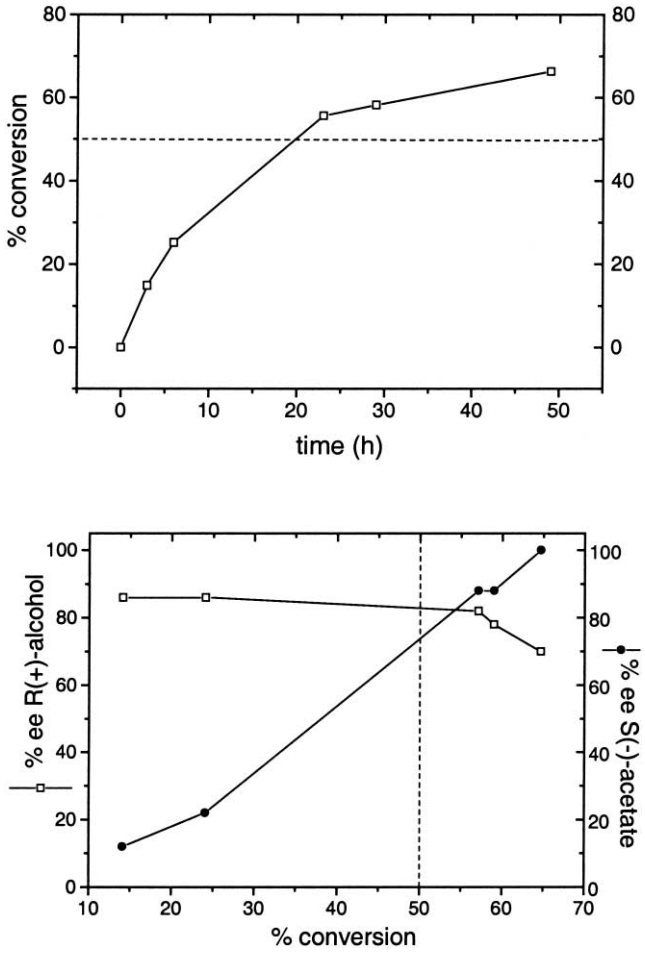
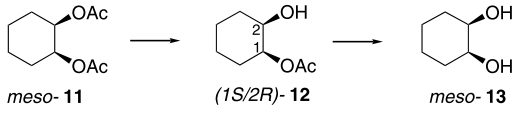


Figure 1. Kinetic studies on the hydrolysis of (±)-**3**.

kinetic resolution of secondary acetates induced preferential hydrolyses of the (*R*)-acetate to the (*R*)-alcohol leaving enantiomerically enriched (*S*)-acetates which is in agreement with Kazlauskas' rule. An enantiotopos differentiating hydrolysis of *meso*-diacetates yields enantiomerically enriched *mono*-acetates.

4. Experimental

The sterile liverwort agar cultures were provided by Professor 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.



Scheme 2. Enantiotopos-differentiating hydrolysis.

4.1. Preparation of cell suspension cultures

In the first step the callus induction was performed according to procedure described by Katoh[15] 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[25] 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).[26] 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 (50–1000 mg) in a minimum amount of ethanol to culture flasks containing 200–400 ml of liquid medium (dry cell mass after work-up: 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 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.

Table 2. Biotransformations of the hydrolase type: enantiotopos differentiating hydrolysis

Entry	Substrate	Culture	Scale (mmol)	Reaction time/conversion[a]	% yield	Product/config./% ee
						(1*S*/2*R*)-Monoacetate **12**
1[9]	**11**	*M. polymorpha*	0.1	12 h	63	91
1	**11**	*M. polymorpha*	1.0–2.0	60 h/1:68:31[a]	68[b]	93
1	**11**	*M. plicata*	1.0–2.0	24 h/2:66:32[a]	66[b]	>95
1	**11**	*R. fluitans*	1.0–2.0	60 h/4:81:15[a]	81[b]	82

[a] Diacetate:monoacetate:diol.
[b] Detected in the reaction mixture.

Control experiments were performed in all cases to exclude non-enzymatic hydrolysis of the product.

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