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The generation of a steroid library using filamentous fungi immobilized in calcium alginate

Patrice C. Peart[a], William F. Reynolds[b], Paul B. Reese[a,*]

[a] Department of Chemistry, University of the West Indies, Mona, Kingston 7, Jamaica
[b] Department of Chemistry, University of Toronto, Toronto, Ontario M5S 3H6, Canada

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Dedicated to the memory of Professor Sir John W. Cornforth, University of Sussex (1917–2013).

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ABSTRACT

Four fungi, namely, *Rhizopus oryzae* ATCC 11145, *Mucor plumbeus* ATCC 4740, *Cunninghamella echinulata* var. *elegans* ATCC 8688a, and *Whetzelinia sclerotiorum* ATCC 18687, were subjected to entrapment in calcium alginate, and the beads derived were used in the biotransformation of the steroids 3β,17β-dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**). Incubations performed utilized beads from two different encapsulated fungi to explore their potential for the production of metabolites other than those derived from the individual fungi. The investigation showed that steroids from both single and crossover transformations were typically produced, some of which were hitherto unreported. The results indicated that this general technique can be exploited for the production of small libraries of compounds.

1. Introduction

The concept of using a number of microorganisms in a single fermentation vessel for the bioconversion of a single substrate to multiple products is not new. Unfortunately, such an environment would lead to inhibitory competition, where either one microorganism prevails whilst the other dies, or the growth of both suffers. Presumably even if the fermentation were successful, the conditions would not be highly reproducible. Additionally, as each microorganism has different nutritional requirements, it could be difficult to find a suitable medium to perform the fermentation [1]. Employing immobilized mycelial cells could avert these problems. With each microorganism trapped in its own sphere, the possibility of negative competition would be reduced or eliminated.

Most of the applications of cell immobilization have been concentrated on monoculture bioconversions. It is, therefore, of immense interest to explore the potential of mixed cultures. There have been previous reports of co-immobilized biocatalysts [1]. Typically, the microorganisms employed for such an objective tend to be symbiotic. For example, one might be oxygen consuming while the other is oxygen producing, as in the case of *Cephalosporium acremonium* (an oxygen consuming fungus) and *Chlorella pyrenoidosa* (an oxygen producing alga). Both were co-immobilized and used for the production of cephalosporin C [2]. A similar mixed culture system was previously used for the production of β-lactam antibiotics [3]. Another study, with entrapped cells of *Chlorella vulgaris* and *Provedencia* sp., as well as *Aspergillus nidulans* and *Provedencia* sp., were used produce α-keto acids from amino acids [4]. A mixed culture-entrapped preparation of *Chlorella pyrenoidosa* and *Glucanobacter oxydans* was employed in the bioconversion of glycerol to dihydroxyacetone, in an effort to study the effects that co-immobilization with oxygen-producing algae had on the transformation yields [5].

It has been shown that immobilized cell bioconversions parallel those of the free cell fermentations to some extent [6,7]. A progression from this would involve the mixing of beads of immobilized fungus A in water with those of mould B in a single vessel, followed by feeding of the appropriate substrate. The expectation was the isolation of additional metabolites that were different from those obtained from the individual single immobilized cell fermentations. Each of these new metabolites would be the product of "crossover"; that is, a compound formed when the transformed

* Corresponding author. Fax: +876 977 1835.
E-mail address: paul.reese@uwimona.edu.jm (P.B. Reese).

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Table 1
^{13}C NMR data (δ) for compounds **2a–9a** determined in $CDCl_3$.

Carbon	2a	3a	4a	5a	6a	7a	8a	9a
1	36.1	36.5	36.6	36.5	35.8	35.5	36.5	35.5
2	35.8	27.5	27.5	27.4	33.9	33.9	27.7	33.9
3	199.9	73.0	73.1	72.9	199.4	198.7	72.9	198.8
4	124.4	37.8	37.5	37.8	124.1	125.6	37.5	126.5
5	171.4	146.7	144.2	146.9	170.2	165.5	144.4	166.3
6	33.2	120.6	122.0	120.4	32.5	38.1	121.9	37.3
7	31.9	67.6	75.3	67.1	26.2	75.5	74.9	70.3
8	34.3	35.6	36.3	35.5	38.6	39.5	36.2	38.2
9	54.1	43.1	47.9	43.3	46.7	50.5	48.1	46.4
10	39.0	37.3	36.3	37.5	38.7	37.9	36.7	38.4
11	20.9	20.2	20.5	19.9	19.6	20.5	20.4	20.4
12	37.0	36.1	36.5	30.9	28.9	36.3	31.1	36.1
13	42.9	42.2	42.7	47.3	46.8	43.1	47.7	42.5
14	50.7	43.7	49.8	44.7	82.9	49.2	50.6	44.8
15	23.9	23.3	24.5	21.8	32.8	25.3	23.1	22.9
16	27.9	27.4	27.5	35.7	26.9	27.6	35.7	27.3
17	82.9	82.5	82.2	220.3	80.9	81.9	220.3	82.2
18	12.4	11.5	11.8	13.1	15.9	12.0	13.6	11.8
19	17.8	18.2	18.9	18.2	17.3	17.2	19.0	17.1
OAc	21.6	21.2	21.1	21.3	21.3	21.2	21.4	21.1
OAc	171.6	21.3	21.3	21.4	171.2	21.6	21.6	21.2
OAc	–	21.4	21.5	170.4	–	170.2	170.3	170.5
OAc	–	170.4	170.3	170.6	–	171.1	170.9	171.2
OAc	–	170.7	170.9	–	–	–	–	–
OAc	–	171.3	171.1	–	–	–	–	–

Table 2
^{13}C NMR data (δ) for compounds **10a**–**16a** and **6** determined in $CDCl_3$.

Carbon	10a	11a	12a	13a	14a	15a	16a	6
1	35.9	26.0	31.8	33.0	31.9	31.9	32.0	35.7
2	27.4	24.4	26.6	27.0	26.6	26.6	26.6	33.9
3	72.2	70.6	69.9	70.4	70.1	70.3	70.4	201.0
4	38.0	31.2	35.9	37.0	37.2	37.0	36.9	123.6
5	166.0	76.7	75.8	75.7	75.9	75.9	74.9	172.8
6	126.7	72.7	73.0	76.0	74.9	76.0	76.2	32.8
7	201.8	71.4	71.6	31.3	73.0	30.1	25.8	26.2
8	48.8	36.6	34.2	30.2	35.6	26.7	34.0	38.7
9	44.4	37.0	39.4	48.0	44.2	45.1	38.2	46.8
10	38.6	41.0	39.1	40.3	38.2	38.9	38.9	39.0
11	20.0	20.9	20.4	70.3	20.8	20.4	19.5	19.0
12	27.9	36.1	36.3	43.5	36.8	38.0	29.4	28.6
13	46.9	42.6	42.8	42.9	43.5	42.7	47.1	47.0
14	80.4	44.7	44.3	48.9	49.1	53.0	83.0	83.3
15	35.2	23.1	23.1	24.4	25.3	71.7	32.3	32.3
16	27.2	27.3	27.3	27.4	27.7	38.3	26.9	29.2
17	80.5	82.3	82.2	81.6	82.4	81.2	81.0	78.3
18	16.1	11.7	11.9	12.8	12.3	14.1	16.1	15.0
19	17.2	16.9	16.5	16.4	17.2	16.4	16.3	17.3
OAc	21.2	21.1	20.9	21.2	21.2	20.8	21.1	–
OAc	21.4	21.1	21.0	21.4	21.3	21.1	21.3	–
OAc	170.2	21.4	21.1	21.5	21.4	21.3	21.4	–
OAc	171.2	169.3	21.4	21.9	21.5	21.3	170.0	–
OAc	–	170.9	168.7	170.1	170.2	169.7	170.7	–
OAc	–	171.4	168.8	170.5	170.3	170.3	171.4	–
OAc	–	–	170.5	170.6	170.8	170.7	–	–
OAc	–	–	171.4	171.2	171.2	171.0	–	–

Table 3
^{13}C NMR data (δ) for compounds **7**, **9** and **17**–**22** determined in $CDCl_3$.

Carbon	7	9	17	18	19	20	21	22	23
1	35.7	35.5	37.1	37.4	37.2	37.4	33.9	35.6	35.4
2	33.9	34.0	34.2	34.2	34.3	34.2	33.8	34.0	33.9
3	199.3	200.7	200.3	199.9	200.4	202.1	199.7	199.2	198.5
4	124.6	126.3	126.5	124.8	126.4	124.2	123.7	126.4	127.2
5	167.5	170.7	167.9	170.1	168.3	173.9	163.7	166.7	166.4
6	42.3	41.2	72.8	33.4	73.0	34.0	128.0	37.4	41.0
7	74.9	67.5	37.2	30.3	38.1	31.4	140.4	70.5	67.1
8	43.1	40.0	29.6	34.6	29.8	35.6	37.6	38.6	39.3
9	50.7	45.4	53.7	59.2	53.8	59.1	50.7	45.5	45.4
10	38.0	38.8	38.1	40.0	38.1	40.4	36.1	38.4	38.5
11	20.6	20.6	20.3	68.7	20.6	69.5	20.3	20.5	20.1
12	36.3	36.2	31.3	43.0	36.4	48.0	36.3	36.0	30.9
13	43.5	42.9	47.7	48.0	42.9	43.6	43.8	42.9	47.3
14	49.9	45.1	50.9	50.1	50.6	50.0	48.2	45.0	45.6
15	26.3	22.8	21.7	21.7	23.3	23.4	23.0	22.7	21.3
16	30.7	29.9	35.8	35.7	30.5	29.9	30.4	30.4	35.6
17	81.1	81.3	220.5	218.4	81.7	80.8	81.3	81.4	220.2
18	11.1	11.0	13.8	14.7	11.1	12.3	11.0	10.9	13.5
19	17.3	17.2	19.6	18.4	19.6	18.4	16.3	17.2	17.0
OAc	–	–	–	–	–	–	–	21.2	–
OAc	–	–	–	–	–	–	–	170.6	–

congener from one fungus would be used as the substrate by the second microorganism. This would lead to the production of multiple compounds. The results of our experiments show this to be the case. In previous work [6,7]. we reported on the use of six encapsulated fungi (*Rhizopus oryzae*, *Mucor plumbeus*, *Cunninghamella echinulata* var. *elegans*, *Aspergillus niger*, *Phanerochaete chrysosporium* and *Whetzelinia sclerotiorum* in the transformation of two steroidal substrates, 3β,17β-dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**). In general, it was observed that the transformations of **1** and **2** by encapsulated *A. niger* and *P. chrysosporium* gave modest results. Therefore, only *R. oryzae*, *M. plumbeus*, *C. echinulata* var. *elegans* and *W. sclerotiorum* were chosen for the crossover experiments. 3β,17β-Dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**) were used as substrates, as the current work is an extension of earlier studies [6,7]. With four fungi available for crossover, there were six possible permutations with each substrate, giving a total of 12 possible mixed bead fermentations. Once it was determined that one could generate new compounds from **1** using four combinations of fungi, only two mixed bead experiments using **2** as substrate were attempted. Products of transformation derived from **1** were acetylated. This aided in their purification. It also generated compounds that were readily soluble in $CDCl_3$. Furthermore, comparison of NMR spectra (for structure elucidation purposes) was facilitated when the biotransformation products from **1** had been derivitized in this way.

2. Experimental

2.1. General procedure

Melting points were obtained using a Reichert Hot Stage melting point apparatus and are uncorrected. Infrared data was acquired using a PerkinElmer Fourier transform infrared spectrophotometer 1000 using sodium chloride disks. NMR spectra were obtained on Bruker Avance 200, Bruker Avance 500 and Varian Unity 500 spectrometers. Compounds were analyzed using $CDCl_3$, unless otherwise stated, with tetramethylsilane as an internal standard. ^{13}C NMR data for all compounds are reported in Tables 1–3 . 1D and 2D NMR data for new compounds are reported in Tables S1–S7 (Supplementary data). 1D and 2D NMR data for compounds that were not previously fully characterized are in Tables S8–S11 (Supplementary data). Optical rotations were performed using a PerkinElmer 241 MC polarimeter and solutions were prepared using CH_2Cl_2 unless otherwise stated. Purifications were done by column chromatography using silica gel (230–400 mesh) as the stationary phase. Additionally, preparative thin layer chromatography (PLC) glass backed plates with silica gel (60 Å, 250 μm and 1000 μm thicknesses) were used. Thin layer chromatographic (TLC) analyses were done using polyester backed plates. Both the TLC and PLC plates were visualized under ultraviolet light or by spraying with ammonium molybdate-sulfuric acid or methanol-sulfuric acid reagents. After spraying the plates were warmed for colour development using a heat gun. Petrol refers to the petroleum fraction boiling between 60 and 80°. 3β-Hydroxyandrost-5-en-17-one (dehydroepiandrosterone, DHEA) was obtained from Productos Químicos Naturales,