Steroids 77 (2012) 85–90

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Entrapment of mycelial fragments in calcium alginate: A general technique for the use of immobilized filamentous fungi in biocatalysis

Patrice C. Peart [a], Avril R.M. Chen [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, Canada M5S 3H6

ARTICLE INFO

Article history:
Received 22 August 2011
Received in revised form 24 September 2011
Accepted 19 October 2011
Available online 28 October 2011

Keywords:
Filamentous fungus
Calcium alginate
Immobilization
Hydroxylation
Biotransformation

ABSTRACT

Transformation reactions on 3β,17β-dihydroxyandrost-5-ene using free fungal cells were compared with those carried out by macerated mycelia, immobilized in calcium alginate beads. Six fungi were utilized in this study, namely *Rhizopus oryzae* ATCC 11145, *Mucor plumbeus* ATCC 4740, *Cunninghamella echinulata* var. *elegans* ATCC 8688a, *Aspergillus niger* ATCC 9142, *Phanerochaete chrysosporium* ATCC 24725 and *Whetzelinia sclerotiorum* ATCC 18687. The results show, for the first time, that encapsulated mycelial fragments essentially carry out the same bioconversions as those observed with growing cells. As the immobilized cells were "resting", the products formed were free of contamination by natural products, and this greatly aided the purification of the metabolites. Conditions for bead preparation were optimized. Furthermore, it was noted that the beads could be reused, once they had been subjected to a rejuvenation process.

1. Introduction

There is increasing interest in the immobilization of whole cells of microorganisms. This thrust is partly fuelled by the desire to replace the usage of isolated enzymes in industrial and laboratory processes. Unfortunately, most of the work on cell entrapment has been performed using bacteria and yeasts. These microorganisms are unicellular which makes immobilization of their whole cells relatively simple. However, the majority of fungi form filamentous strands and, therefore, are not so easily immobilized using conventional methods. Nevertheless, there have been many modifications of the different methods to make them applicable to the filamentous fungi [1].

Entrapment is one such technique that is widely used. Polyacrylamide and calcium alginate gels have been employed for this technique. In the first method cells are entrapped by the polymerization of an aqueous solution of acrylamide monomers in which the fungal cells were suspended, followed by subdivision into pellets. The process can result in loss of cell activity. Studies have shown that calcium alginate gives a higher retention of cell activity when compared with polyacrylamide [1]. Fungi are trapped by dripping a suspension of fungal spores in aqueous sodium alginate into a stirred solution of chilled calcium chloride. The monovalent sodium ion is replaced by the divalent calcium ion, which cross-links the polysaccharide, resulting in spontaneous polymerization to form spherical pellets. These mild conditions allow for higher cell viability. Furthermore, these pellets are well suited for usage in commercial reactors [2].

Yeast cells and the spores of several fungi have been entrapped in alginate. Using this method Baker's yeast, *Saccharomyces cerevisiae*, has been employed in the asymmetric reduction of ketones [3–9]. In some cases the fermentation was carried out in the presence of organic solvents, for example, hexane [5,10]. More recently immobilized cells of *S. cerevisiae* have been used in the production of ethanol from sucrose [11]. Spores of the filamentous fungi *Rhizopus stolonifer* [12] and *Curvularia lunata* [13], trapped in a calcium alginate matrix, were used in the bioconversion of progesterone. Freshly germinated spores of *Penicillium digitatum*, another filamentous fungus, enmeshed in alginate, have found utility in the transformation of limonene to α-terpineol [14]. In a rare case *Mortierella isabellina* mycelium has also been immobilized in calcium alginate and used to transform dehydroabietic acid [15]. Normally the fungus is grown for few days and the spores are harvested and trapped in the alginate gel (and possibly allowed to germinate *in situ*) [16,17]. In some cases the gel network affected the growth of the mycelia.

There is only one previous report where homogenization was used in the preparation of entrapped cells in alginate [18]. It was unclear whether this was done using spores or mycelia. Additionally, the immobilized cells were dried and used for soil treatment. This is the first report where macerated fungal cells are immobilized and used for fermentation purposes.

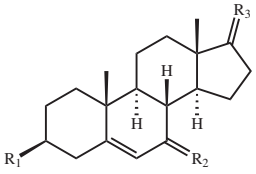
* Corresponding author. Tel.: +1 876 927 1910; fax: +1 876 977 1835.
E-mail address: paul.reese@uwimona.edu.jm (P.B. Reese).

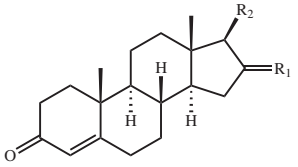
0039-128X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2011.10.008

2. Experimental

2.1. General procedure

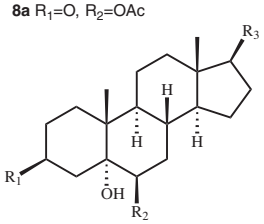
Melting points were obtained using a Reichert Hot Stage melting point apparatus and are uncorrected. Infrared data was acquired using a Perkin Elmer Fourier transform infrared spectrophotometer 1000 using sodium chloride disks. NMR spectra were obtained on Bruker Avance 200, Bruker Avance 500 and Varian Unity 500 spectrometers. Compounds were analyzed using $CDCl_3$, unless otherwise stated, with tetramethylsilane as the internal standard. Optical rotations were performed using a Perkin Elmer 241 MC polarimeter and solutions were prepared in 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 carried out 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 (dehydro*epi*androsterone, DHEA) (**1**) was obtained from Productos Químicos Naturales, S.A. de C.V. (Orizaba, Mexico). 3β,17β-Dihydroxyandrost-5-ene (**2**) was prepared from **1** in methanol using sodium borohydride. The fungi used for these experiments were obtained from the American Type Culture Collection, Rockville, MD, USA.





1 R_1=OH, R_2=H_2, R_3=O
2 R_1=OH, R_2=H_2, R_3=βOH,αH
3 R_1=OH, R_2=αOH,βH, R_3=βOH,αH
3a R_1=OAc, R_2=αOAc,βH, R_3=βOAc,αH
4 R_1=OH, R_2=βOH,αH, R_3=βOH,αH
4a R_1=OAc, R_2=βOAc,αH, R_3=βOAc,αH
5 R_1=OH, R_2=βOH,αH, R_3=O
5a R_1=OAc, R_2=βOAc,αH, R_3=O
6 R_1=OH, R_2=αOH,βH, R_3=O
6a R_1=OAc, R_2=αOAc,βH, R_3=O

7 R_1=H_2, R_2=OH
7a R_1=H_2, R_2=OAc
8 R_1=O, R_2=OH
8a R_1=O, R_2=OAc



9 R_1=R_2=R_3=OH
9a R_1=R_2=R_3=OAc

2.2. Free cell fermentation conditions

One slant was used to inoculate four 500 mL Erlenmeyer flasks, each containing 125 mL of liquid culture medium. The substrate (**2**) (1 g) in ethanol (20 mL) was distributed among the flasks, in the following way. A solution of 10% of the total mass of the substrate was fed 24 h after inoculation. Then 20%, 30% and 40% of the substrate in ethanol were fed at 36, 48 and 60 h after inoculation respectively. The fermentation was allowed to proceed for an additional 5 d after the final feed. The mycelia were filtered from the broth. The broth was divided into two portions. Each portion was extracted with ethyl acetate (2 × 500 mL). The mycelia were placed in ethyl acetate (500 mL) and homogenized, warmed, and then filtered. The organic extracts were dried using sodium sulfate. The solvent was removed *in vacuo*, and the residues were analyzed by thin layer chromatography. The extracts were combined and absorbed on silica gel and purified by flash column chromatography or by preparative thin layer chromatography. After isolation of the untransformed substrate (**2**), the mixture of products of biotransformation was acetylated (acetic anhydride-pyridine) as an aid to purification.

2.3. Preparation of immobilized fungal cells

One slant was used to inoculate four Erlenmeyer flasks each containing 125 mL liquid culture medium. The fungus was allowed to grow for 3 d. At the end of the incubation period the cells were harvested by filtration. The cells were suspended in water (40 mL) and 3% aqueous sodium alginate solution (140 mL) was added. The cells were macerated for 1 min at 8000 rpm using an IKA Ultra-Turrax T25 homogenizer. The cell-alginate suspension was then added dropwise to stirred chilled aqueous 0.1 M calcium chloride (200 mL). The alginate beads formed were allowed to harden for 30 min in the calcium chloride solution. The calcium chloride solution was decanted, the beads were rinsed with water and were stored under water at 4° until used.

2.4. Immobilized cell fermentation conditions

The alginate beads derived above were divided into equal portions and placed into four 500 mL Erlenmeyer flasks, each containing water (125 mL). The substrate (200 mg) in ethanol (5 mL) was distributed among the flasks. The flasks were shaken at 180 rpm for 5 d. After the fermentation was complete the aqueous solution was decanted from the beads and the former was extracted using ethyl acetate (2 × 300 mL). The organic solution was dried using sodium sulfate, filtered, and the solvent was removed *in vacuo*. The residue was analyzed by TLC and purified by column chromatography. After isolation of the untransformed substrate (**2**), the mixture of products of biotransformation was acetylated (acetic anhydride-pyridine) as an aid to purification. The used beads were covered with distilled water and stored at 4°.

2.5. Rhizopus oryzae ATCC 11145

This fungus was maintained on malt agar slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks, each containing 125 mL liquid culture. The medium was prepared from glucose (20 g/L), peptone (5 g/L), sodium chloride (5 g/L) and yeast extract (5 g/L) [19]. The flasks were shaken at 250 rpm.

2.5.1. Free cell fermentation

The combined extract (2.08 g) was purified using column chromatography. Elution with ethyl acetate/petrol (1:19 v/v) yielded fed substrate **2** (28 mg). Further elution using ethyl acetate/petrol (1:9 v/v) afforded 3β,7α,17β-trihydroxyandrost-5-ene (**3**). This compound was characterized as the triacetate (**3a**) (77 mg), Rf = 0.89, acetone/dichloromethane (1:19 v/v), which crystallized from acetone/methanol as plates, m.p. 142–144°, $[\alpha]_D$ −176.8° (c 0.22), lit. [20] 156–158°, $[\alpha]_D$ −152°; IR: ν_{max} 1747, 1723, 1246 cm^{-1}; ^1H NMR: δ 0.80 (3H, s, H-18), 1.03 (3H, s, H-19), 2.04 (9H, s, 3 × CH_3CO_2), 4.68 (1H, m, w/2 = 12 Hz, H-17α), 4.69 (1H, dd, J = 9.8 Hz, H-3α), 4.98 (1H, t, J = 4.5 Hz, H-7β), 5.59 (1H, d, J = 5 Hz, H-6).

Further elution with ethyl acetate/petrol (1:9 v/v) gave 3β,7β,17β-trihydroxyandrost-5-ene (**4**). This compound was characterized as the triacetate **4a** (38 mg), Rf = 0.88, acetone/dichloromethane (1:19 v/v), which crystallized as needles from

acetone-methanol, m.p. 178–179°, $[\alpha]_D$ +42.9° (c 0.15), lit. [21] m.p. 210–211°, $[\alpha]_D$ +52°; IR: ν_{max} 2947, 1739, 1733, 1370, 1238 cm^{-1}; ^1H NMR: δ 0.79 (3H, s, H-18), 1.05 (3H, s, H-19), 2.10 (9H, s, 3 × CH_3CO_2), 4.60 (2H, d, J = 8.1 Hz, H-3α, H-17α), 5.08 (1H, d, J = 8.2 Hz, H-7β), 5.37 (1H, s, H-6).

2.5.2. Immobilized cell fermentation

The extract (147.5 mg) was partially purified by column chromatography to give the fed substrate (**2**) (12.3 mg). Further purification using ethyl acetate/petrol (1:9 v/v) afforded 3β,7α,17β-trihydroxyandrost-5-ene (**3**), which was characterized as the triacetate (**3a**) (36.9 mg).

Further elution with ethyl acetate/petrol (1:9 v/v) gave 3β,7β,17β-trihydroxyandrost-5-ene (**4**) which was characterized as the triacetate **4a** (65.7 mg).

Elution with ethyl acetate/petrol (3:17 v/v) afforded a third metabolite, 3β,7β-dihydroxyandrost-5-en-17-one (**5**) (10.3 mg), which was characterized as the diacetate **5a**, and which did not crystallize, Rf = 0.76, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +200° (c 0.09), lit. [22] m.p. 164–165°, $[\alpha]_D$ +105°; IR: ν_{max} 2947, 1736, 1373, 1241, 1032 cm^{-1}; ^1H NMR: δ 0.91 (3H, s, H-18), 1.12 (3H, s, H-19), 2.04 (3H, s, CH_3CO_2-3), 2.06 (3H, s, CH_3CO_2-7), 4.61 (1H, m, w/2 = 17.5 Hz, H-3α), 5.17 (1H, dt, J = 8.7, 2.1 Hz, H-7α), 5.29 (1H, t, J = 2 Hz, H-6).

2.6. Mucor plumbeus ATCC 4740

This fungus was maintained on potato dextrose agar slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks, each containing 125 mL liquid culture medium. The medium was prepared using glucose (30 g/L), potassium chloride (0.5 g/L), corn steep solids (5 g/L), sodium nitrate (2 g/L), magnesium sulfate heptahydrate (0.5 g/L) and iron(II) sulfate heptahydrate (0.02 g/L) [23]. The flasks were shaken at 250 rpm.

2.6.1. Free cell fermentation

The extracts were purified separately using column chromatography. Purification of the mycelial extract (746 mg) gave only the fed steroid (**2**) (109.6 mg), which was collected in the fractions eluted with ethyl acetate/petrol (1:19 v/v). The broth extract (626 mg) afforded two transformed products. Elution with ethyl acetate/petrol (1:9 v/v) gave 3β,7α,17β-trihydroxyandrost-5-ene (**3**), which was characterized as the triacetate **3a** (148 mg).

Further elution with ethyl acetate/petrol (3:17 v/v) yielded a second metabolite, 3β,7α-dihydroxyandrost-5-en-17-one (**6**). This was characterized as the diacetate **6a** (52 mg), Rf = 0.81, acetone/dichloromethane (1:19 v/v), which resisted crystallization, $[\alpha]_D$ −228° (c 0.21), lit. [22] m.p. 168–170°, $[\alpha]_D$ −178°; IR: ν_{max} 1722, 1622, 1228 cm^{-1}; ^1H NMR: δ 0.88 (3H, s, H-18), 1.05 (3H, s, H-19), 2.05 (6H, s, 2 × CH_3CO_2), 4.68 (1H, m, w/2 = 16.5 Hz, H-3α), 5.12 (1H, dt, J = 8.7, 2.1 Hz, H-7β), 5.62 (1H, dd, J = 5.3, 1.5 Hz, H-6).

2.6.2. Immobilized cell fermentation

Partial purification of the broth extract (172 mg) allowed for the recovery of the fed substrate (**2**) (30.5 mg). Further percolation using ethyl acetate/petrol (1:9 v/v) gave 3β,7α,17β-trihydroxyandrost-5-ene (**3**) (37.4 mg), which was characterized as the triacetate **3a**. Further elution with ethyl acetate/petrol (3:17 v/v) yielded 3β,7α-dihydroxyandrost-5-en-17-one (**6**), characterized as the diacetate **6a** (53.8 mg).

2.7. Cunninghamella echinulata var. elegans ATCC 8688a

This fungus was maintained on maltose-peptone slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks, each containing 125 mL of liquid culture. The medium was prepared using glucose (20 g/L), yeast extract (5 g/L), soya meal (5 g/L), sodium chloride (5 g/L) and di-potassium hydrogen phosphate (5 g/L) [24]. The flasks were shaken at 180 rpm.

2.7.1. Free cell fermentation

The mycelia and broth extracts were combined (2.04 g) and partially purified by column chromatography to give the fed compound **2** (469 mg). Acetylation and further purification gave two products, **3a** (18 mg) and **4a** (12 mg).

2.7.2. Immobilized cell fermentation

The broth extract (170 mg) was partially purified by column chromatography to give the fed compound **2** (24.5 mg). Acetylation and further purifications yielded two metabolites, **3a** (23.6 mg) and **4a** (16.9 mg).

2.8. Aspergillus niger ATCC 9142

This fungus was maintained on potato dextrose agar slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks, each containing 125 mL of liquid culture. The medium was prepared using glucose (20 g/L), yeast extract (5 g/L), soya meal (5 g/L), sodium chloride (5 g/L) and di-potassium hydrogen phosphate (5 g/L) [24]. The flasks were shaken at 180 rpm.

2.8.1. Free cell fermentation

The combined extracts (1.22 g) were purified using column chromatography. Elution with ethyl acetate/petrol (1:19 v/v) afforded 254 mg of the fed compound (**2**). After acetylation, further purification gave three transformed products. Compound **4a** (10 mg) was identified by comparison of its spectral data with that of an authentic sample.

Elution with ethyl acetate/petrol (1:4 v/v) afforded a second metabolite, 17β-hydroxyandrost-4-en-3-one (**7**), which was characterized as the acetate **7a** (17 mg), Rf = 0.14, acetone/dichloromethane (1:19 v/v), which crystallized from acetone as needles, m.p. 130–132°, $[\alpha]_D$ +92° (c 0.01), lit. [25] m.p. 141–142°, $[\alpha]_D$ +96.2°; IR: ν_{max} 2932, 1737, 1675, 1247 cm^{-1}; ^1H NMR: δ 0.84 (3H, s, H-18), 1.20 (3H, s, H-19), 2.05 (3H, s, CH_3CO_2-17), 4.60 (1H, dd, J = 7.7, 8.8 Hz, H-17α), 5.74 (1H, s, H-4).

Further elution with ethyl acetate/petrol (3:7 v/v) gave a third compound, 17β-hydroxyandrost-4-ene-3,16-dione (**8**), which was characterized as the monoacetate **8a** (104 mg), Rf = 0.62, acetone/dichloromethane (1:19 v/v), which crystallized from acetone-ethanol as plates, m.p. 202–204°, $[\alpha]_D$ −43.4° (c 0.15), lit. [26] m.p. 200–201.5°, $[\alpha]_D$ −48°; IR: ν_{max} 2939, 1742, 1672, 1234 cm^{-1}; ^1H NMR: δ 0.88 (3H, s, H-18), 1.22 (3H, s, H-19), 2.18 (3H, s, CH_3CO_2-17), 5.02 (1H, s, H-17α), 5.76 (1H, bs, H-4).

2.8.2. Immobilized cell fermentation

The broth extract (161.8 mg) was purified using column chromatography. Elution with ethyl acetate/petrol (1:19 v/v) afforded 133 mg of the fed compound (**2**). Acetylation and further purification gave three transformed products, namely, **4a** (5 mg), **7a** (3 mg), and **8a** (4 mg).

2.9. Phanerochaete chrysosporium ATCC 24725

This fungus was maintained on potato dextrose agar slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks each containing 125 mL of liquid culture. The medium was made using potassium nitrate (10 g/L), magnesium sulfate heptahydrate (1.5 g/L), potassium di-hydrogen phosphate (2.5 g/L), glucose (40 g/L) and yeast extract (2 g/L) [27]. The flasks were shaken at 180 rpm.

The fermentation of 3β,17β-dihydroxyandrost-5-ene (**2**) by *P. chrysosporium* produced several metabolites, however, the quantities were insufficient to allow for their isolation and characterization.

2.10. Whetzelinia sclerotiorum ATCC 18687

This fungus was maintained on potato dextrose agar slants at 28°. Five slants were used to inoculate twenty 500 mL Erlenmeyer flasks, each containing 125 mL of liquid culture. The medium was made using potassium nitrate (10 g/L), magnesium sulfate heptahydrate (1.5 g/L), potassium dihydrogen phosphate (2.5 g/L), glucose (0.5 g/L), yeast extract (0.5 g/L) and cellulose (10 g/L) [28]. The flasks were shaken at 180 rpm.

2.10.1. Free cell fermentation

The both extracts were combined (782 mg) and partially purified by column chromatography to give the fed substrate (**2**) (468 mg). Acetylation and further purification gave **3a** (133 mg), **4a** (134 mg) and **5a** (4.9 mg). Elution with acetone/petrol (1:9 v/v) afforded 3β,6β,17β-triacetoxy-5α-hydroxyandrostane (**9a**) (36.2 mg), Rf = 0.43, acetone in dichloromethane (1:19 v/v), which resisted crystallization, $[\alpha]_D$ −97.1° (c 0.21); IR: ν_{max} 3420, 2943, 1735, 1373, 1247 cm^{-1}; HREIMS: *m/z*: 473.2533, MNa^+ ($C_{25}H_{38}O_7Na$ requires 473.2509); ^1H NMR: δ 0.81 (3H, s, H-18), 1.16 (3H, s, H-19), 2.02 (3H, s, CH_3CO_2-3), 2.04 (3H, s, CH_3CO_2-17), 2.08 (3H, s, CH_3CO_2-6), 4.59 (1H, dd, J = 7.5, 8.8 Hz, H-17α), 4.69 (1H, t, J = 2.5 Hz, H-6α), 5.14 (1H, tt, J = 12, 6 Hz, H-3α). ^{13}C NMR: δ 12.3 (CH_3-18), 16.4 (CH_3-19), 20.5 (CH_2-11), 21.2 ($\underline{C}H_3CO_2$-17), 21.4 ($\underline{C}H_3CO_2$-3), 21.5 ($\underline{C}H_3CO_2$-6), 23.4 (CH_2-15), 26.6 (CH_2-2), 27.5 (CH_2-16), 30.6 (CH-8), 31.0 (CH_2-7), 31.8 (CH_2-1), 36.8 (CH_2-12), 36.9 (CH_2-4), 38.6 (C-10), 42.8 (C-13), 45.0 (CH-9), 50.1 (CH-14), 70.5 (CH-3), 74.9 (C-5), 76.0 (CH-6), 82.7 (CH-17), 170.1 ($CH_3\underline{C}O_2$-6), 170.6 ($CH_3\underline{C}O_2$-3), 171.2 ($CH_3\underline{C}O_2$-17).

2.10.2. Immobilized cell fermentation

The broth extract (182.6 mg) was partially purified by column chromatography to give the fed substrate (**2**) (57.9 mg). Acetylation and further purification gave **3a** (28.9 mg), **4a** (26.7 mg) and **9a** (8 mg).

3. Results

A summary of the products of transformation obtained is found in Table 1.

3.1. Biotransformations using R. oryzae

3.1.1. Free cell fermentation

The ^{13}C NMR spectrum for the first acetylated metabolite (**3a**) showed the occurrence of a new methine at δ 67.6. COSY data indicated that the corresponding proton showed correlations to protons at H-6 and H-8, and as such the position of hydroxylation was determined to be C-7. The proton at C-7 showed T-ROESY correlations to protons at H-8 and H-16β. The transformed product was, therefore, determined to be 3β,7α,17β-trihydroxyandrost-5-ene (**3**), which was characterized as the triacetate (**3a**) [19]. The NMR data of the triacetate of the second metabolite (**4a**) was quite similar to that of **3a**. Loss of the methylene at δ 31.4, C-7, was accompanied by the appearance of a new methine at δ 75.3. The metabolite was determined to be 3β,7β,17β-trihydroxyandrost-5-ene (**4**), which was characterized as the triacetate (**4a**) [21].

Table 1
Products of transformation under different fermentation conditions.

Fungi	Fermentation method	Products
R. oryzae	Free cell	3, 4
R. oryzae	Immobilized cell	3, 4, 5
M. plumbeus	Free cell	3, 6
M. plumbeus	Immobilized cell	3, 6
C. echinulata var. *elegans*	Free cell	3, 4
C. echinulata var. *elegans*	Immobilized cell	3, 4
A. niger	Free cell	4, 7, 8
A. niger	Immobilized cell	4, 7, 8
W. sclerotiorum	Free cell	3, 4, 5, 9
W. sclerotiorum	Immobilized cell	3, 4, 9

3.1.2. Immobilized cell fermentation

This fermentation afforded three metabolites. 3β,7α,17β-Trihydroxyandrost-5-ene (**3**) and 3β,7β,17β-trihydroxyandrost-5-ene (**4**) were characterized as their respective acetates (**3a** and **4a**), both found in the free cell fermentation. The ^{13}C NMR spectrum of the diacetate (**5a**) of the third metabolite showed the appearance of a new peak at δ 220.3. The spectrum also revealed the loss of the methine at δ 83.1, C-17. There was a new methine signal at δ 74.9 which was coupled to a proton resonance at δ 5.17. This was accompanied by the disappearance of the methylene at δ 31.8, indicating that hydroxylation had occurred at C-7. The COSY data showed correlations between the H-6 and H-8 with the proton at δ 5.17. The stereochemistry of the hydroxyl group was determined as β, as there were correlations between H-7 and H-9, H-14, H-15α. Compound **5** was determined to be 3β,7β-dihydroxyandrost-5-en-17-one, characterized as the diacetate **5a** [22].

3.2. Biotransformations using M. plumbeus

3.2.1. Free cell fermentation

The NMR data confirmed that the first metabolite, after acetylation, was **3a**. The ^{13}C NMR spectrum of the second metabolite showed the loss of the methine at δ 83.1, C-17, along with the loss of the signal at δ 4.56 in the ^1H NMR. There was a new peak in the ^{13}C NMR spectrum at δ 220.3, indicating the presence of an unconjugated ketone moiety. The HMBC data showed that H-12, 16, and 18 were coupled to the carbon at δ 220.3. A new methine was observed at δ 67.0, which was attached to a proton resonating at δ 5.12. The COSY data showed that there was correlation of this new proton with H-6 and H-8. This confirmed that hydroxylation had occurred at C-7. T-ROESY data showed that H-7 was coupled to H-8. The acetylated compound **6a** was determined to be 3β,7α-diacetoxyandrost-5-en-17-one (**6a**) [22], derived from the metabolite 3β,7α-dihydroxyandrost-5-en-17-one (**6**).

3.2.2. Immobilized cell fermentation

The two products of transformation obtained from this incubation were the same as those that were found in the free cell fermentation, namely, 3β,7α,17β-trihydroxyandrost-5-ene (**3**) and 3β,7α-dihydroxyandrost-5-en-17-one (**6**), which were characterized as **3a** and **6a**.

3.3. Biotransformations using C. echinulata var. elegans

3.3.1. Free cell fermentation

Two metabolites were isolated from this incubation, compounds **3** and **4**. Both were characterized as their respective acetate derivatives, **3a** and **4a**.

3.3.2. Immobilized cell fermentation

The results of this biotransformation paralleled those of the free cell fermentation. Two metabolites were obtained, 3β,7α,17β-trihydroxyandrost-5-ene (**3**) and 3β,7β,17β-trihydroxyandrost-5-ene (**4**), characterized as **3a** and **4a** respectively.

3.4. Biotransformations using A. niger

3.4.1. Free cell fermentation

The first metabolite **4** was characterized as the acetate **4a**. The ^{13}C NMR spectrum of the acetate (**7a**) of second metabolite showed that the methine at δ 74.2 had disappeared, and this was accompanied by the appearance of a new peak at δ 199.8. The position of this resonance indicated that the ketone was conjugated. This was corroborated by the changes in the signals for the unsaturated carbons. Therefore, there had been oxidation of the 3-hydroxyl, followed by migration of the carbon-carbon double bond from C-5,6 to C-4,5. Compound **7a** was determined to be 17β-acetoxyandrost-4-en-3-one [25], which was derived from 17β-hydroxyandrost-4-en-3-one (**7**). The final transformed product (**8**) was acetylated to yield **8a**. The ^{13}C NMR spectrum showed the disappearance of the peak at δ 74.2, along with the appearance of a new nonprotonated carbon at δ 199.2. HMBC data revealed that the protons at C-1 were coupled to this conjugated ketone, establishing that this moiety was located in ring A. The hydroxyl group at C-3 had been oxidized to a ketone, and this was followed by a migration of the double bond from C-5,6 to C-4,5. Additionally, there was the loss of a methylene at δ 27.9, C-16, accompanied by the appearance of a new signal at δ 210.3. The HMBC spectrum showed that there were correlations between H-15 and H-17 to the carbon at δ 210.3. This confirmed that the group was present in ring D. The final metabolite was 17β-hydroxyandrost-4-ene-3,16-dione (**8**), which was characterized as the acetate **8a** [26].

3.4.2. Immobilized cell fermentation

The biotransformed compounds isolated from this fermentation were the same as those from the free cell fermentation: 3β,7β,17β-trihydroxyandrost-5-ene (**4**), 17β-hydroxyandrost-4-en-3-one (**7**) and 17β-hydroxyandrost-4-ene-3,16-dione (**8**). These metabolites were converted to their acetates (**4a**, **7a** and **8a**) for characterization.

3.5. Biotransformations using P. chrysosporium

3.5.1. Free cell fermentation

The fermentation produced several metabolites, however, their quantities were not significant enough to allow for their isolation and characterization.

3.5.2. Immobilized cell fermentation

As with the free cell fermentation, the immobilized cells of *P. chrysosporium* produced several metabolites in poor yields, and as such it was not deemed practical to isolate and characterize these products.

3.6. Biotransformations using W. sclerotiorum

3.6.1. Free cell fermentation

The first three metabolites were identified as compounds **3**, **4** and **5** by examination of the NMR data of their respective acetates **3a**, **4a** and **5a**. The fourth product of biotransformation, compound **9**, was converted to the triacetate **9a**. The ^{13}C NMR spectrum revealed the loss of signals representing the carbon-carbon double bond. There was a new methine resonating at δ 75.9 as well as a new nonprotonated carbon at δ 74.9. This showed that there was a hydroxyl group at C-5. For these reasons it was deduced that epoxidation of the carbon-carbon double bond had taken place, and this was followed by hydrolysis. This compound was determined to be 3β,6β,17β-triacetoxy-5α-hydroxyandrostane (**9a**), which was derived from metabolite **9**. The latter is completely characterized here for the first time.

3.6.2. Immobilized cell fermentation

This incubation afforded three transformed products: 3β,7α,17β-trihydroxyandrost-5-ene (**3**), 3β,7β,17β-trihydroxyandrost-5-ene (**4**) and 3β,5α,6β,17β-tetrahydroxyandrostane (**9**). These were the same as those obtained from the free cell incubation with the exception that the free cell fermentation also produced 3β,7β-dihydroxyandrost-5-en-17-one (**5**).

3.7. Optimization of immobilized cell fermentations

3.7.1. Mycelial fragmentation time

It was envisaged that the size of the mycelial fragments would affect the viability of the fungal cells within the alginate beads. This is because the smaller the hyphae fragments, the lower the likelihood that intact cells remained. It was observed that mycelium macerated for periods longer than 3 min retained very little or no enzymatic activity (Table 2). Yields were determined from the masses of transformed metabolites separated from the organic extract of the fermentation.

3.7.2. Alginate bead diameter

In any fermentation the rate at which xenobiotes in the culture medium diffuse into the mycelia will affect the yield of transformed products. This same is true for incubations using immobilized cells. The substrate has to diffuse through not one, but two semi-permeable barriers (gel matrix and cell membrane). The surface area:volume ratio is a function of the diameter of the alginate beads. With this in mind the search for an alginate bead size that would give the highest transformation yields was investigated. This was executed by varying the diameter of the bore of the dropping tube. These results indicated that with larger alginate beads the transformation yield was reduced. The optimal diameter was found to be 3 mm (Table 3). Yields were determined from the masses of transformed metabolites separated from the organic extract of the fermentation.

3.7.3. Rejuvenation of used immobilized cells

Some enzymes must be associated with other compounds in order to carry out their reactions. Exhausted cofactors have to be replenished before further reactions can occur. Sonomoto showed that incubation of immobilized cells in a nutrient medium could rejuvenate these systems [12]. Replenishment of the cofactor NADPH is crucial for the success of cytochrome P450 hydroxylations. In this regard four possible media were investigated: water, 1% glucose solution, potato broth (PB), and potato dextrose broth (PDB). The exhausted immobilized cells were incubated in each medium with shaking for 12 h. The beads were then washed with water and reincubated with the steroid. Rejuvenation with PDB

Table 2
Effect of maceration time on transformation yields (%) using different fungi.

Fungi	Maceration time @ 8000 rpm			
	1 min	3 min	5 min	7 min
M. plumbeus (% transformation)	10	31	8	2
A. niger (% transformation)	4	7	1	1
R. oryzae (% transformation)	9	12	8	1

Table 3
Effect of bead diameter on transformation yields (%) using different fungi.

Fungi	Alginate bead diameter/mm		
	1	3	5
M. plumbeus (% transformation)	10	31	7
A. niger (% transformation)	3	5	1
R. oryzae (% transformation)	6	12	8

Table 4
Effect of different media (for rejuvenation) on transformation yields (%) using different fungi

Fungi	Media for rejuvenation			
	H_2O	Glucose	PB	PDB
M. plumbeus (% transformation)	19	23	20	49
A. niger (% transformation)	1	3	1	5
R. oryzae (% transformation)	3	7	7	11

gave the best results overall (Table 4). Yields were determined from the masses of transformed metabolites separated from the organic extract of the fermentation. Immobilized cells rejuvenated in the glucose solution exhibited outgrowth of mycelia from the alginate matrix. This led to the collapse of the beads. It was concluded from the data that the cells needed glucose and inorganic salts for cofactor regeneration.

4. Discussion

Our investigations have led to the development of a general technique for the immobilization of filamentous fungi. This was accomplished by growing the microorganism in a suitable medium, after which the mycelium was harvested. The latter was subjected to a fragmentation process and this was followed by entrapment in the alginate matrix. Incubation of the alginate beads in water with **2** led to the preparation of transformed analogues. This study showed that, for the first time, transformation reactions by immobilized cells paralleled those from the growing cells, in that, to a large extent, they produced the same metabolites with most of the fungi that were used. The reason that they are not exactly the same is probably due to the fact that, in the free versus immobilized cell fermentations, there must be some small differences in fungal metabolism. Additionally, in a majority of the entrapped cell incubations there was an improvement in yields over those from the free cells. Purification of the transformed products was significantly facilitated (and reduced solvent volumes were utilized in chromatography), since cells in the matrix were not producing secondary metabolites. The procedure is straightforward, and the fact that the immobilized cells can be reused makes the technique effective and efficient. An additional advantage of this new method over previous ones is its applicability in cases where the mould does not produce spores, for example, as is the situation with many endophytic fungi.

Acknowledgments

The authors wish to thank the University of the West Indies, Mona/Inter-American Development Bank (UWI/IDB) Programme for partial funding. PCP and ARMC are grateful to Tanaud International B.V. (Jamaica) and the University of the West Indies, respectively, for the granting of postgraduate scholarships. PCP and ARMC also acknowledge the University of the West Indies for providing Tutorial Assistantships.

References

[1] Anderson JG. Immobilized cell and film reactor systems for filamentous fungi. In: The Filamentous Fungi. In: Smith JE, Berry DR, Kristiansen B, editors. Fungal Technology, IV. London, UK: Arnold; 1983. p. 145–9.
[2] Smidsrød O, Skjåk-Bræk G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71–8.
[3] Nakamura K, Higaki M, Ushio K, Oka S, Ohno A. Stereochemical control of microbial reduction. 2. Reduction of β-keto esters by immobilized bakers' yeast. Tetrahedron Lett 1985;26:4213–6.
[4] Sakai T, Nakamura T, Fukuda K, Amano E, Utaka M, Takeda A. Enantioselective reduction of ethyl 2-acyloxy-3-oxobutanoate with immobilized baker's yeast. Bull Chem Soc Jpn 1986;59:3185–8.
[5] Griffin DR, Yang F, Carta G, Gainer J. Asymmetric reduction of acetophenone with calcium-alginate-entrapped baker's yeast in organic solvent. Biotechnol Prog 1998;14:588–93.
[6] Naoshima Y, Maeda J, Munakata Y, Nishiyama T, Kamezawa M, Tachibana H. Bioreduction with immobilized bakers' yeast in hexane using alcohols as an energy source. J Chem Soc Chem Commun 1990:964–5.
[7] Naoshima Y, Nakamura A, Munakata Y. Synthesis of (5Z, 13S)-5-tetradecen-13-olide, aggregation pheromone of the Cryptolestes grain beetle, employing asymmetric bioreduction with immobilized bakers' yeast entrapped in calcium alginate beads. Bull Chem Soc Jpn 1990;63:1263–5.
[8] Fadnavis NW, Deshpande A, Chauhan S, Bhalerao UT. Peptide synthesis mediated by immobilized and viable baker's yeast in reverse micelles: synthesis of leucine enkephalin analogues. J Chem Soc Chem Commun 1990:1548–50.
[9] Bhalerao T, Chanderaprakash Y, Babu RL, Fadnavis NW. β-Ketoester reduction by baker's yeast immobilized in calcium alginate: an examination of pH effects on enantiospecificity. Synth Commun 1993;23:1201–8.
[10] Griffin DR, Gainer JL, Carta G. Asymmetric ketone reduction with immobilized yeast in hexane: biocatalyst deactivation and regeneration. Biotechnol Prog 2001;17:304–10.
[11] Najafpour G, Younesi H, Tsmail KSK. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. Bioresource Technol 2004;92:250–1.
[12] Sonomoto K, Nomura K, Tanaka A, Fukui S. 11α-Hydroxylation of progesterone by gel-entrapped living *Rhizopus stolonifer* mycelia. Eur J Appl Microbiol Biotechnol 1982;16:57–62.
[13] Tan Q, Day DF. Bioconversion of limonene to α-terpineol by immobilized Penicillium digitatum. Appl Microbiol Biotechnol 1998;49:96–101.
[14] Mazumder TK, Sonomoto K, Tanaka A, Fukui S. Sequential conversion of cortexolone to prednisolone by immobilized mycelia of *Curvularia lunata* and immobilized cells of *Arthrobacter simplex*. Appl Microbiol Biotechnol 1985;21:154–61.
[15] Kutney JP, Choi LSL, Hewitt GM, Salisbury PJ, Singh M. Biotransformation of dehydroabietic acid with resting cell suspensions and calcium alginate-immobilized cells of *Mortierella isabellina*. Appl Environ Microbiol 1985;49:96–100.
[16] Ohlson S, Flygare S, Larsson PO, Mosbach K. Steroid hydroxylation using immobilized spores of *Curvularia lunata* germinated *in situ*. Eur J Appl Microbiol Biotechnol 1980;10:1–9.
[17] Sonomoto K, Hoq MM, Tanaka A, Fukui S. 11β-Hydroxylation of cortexolone (Reichstein compound S) to hydrocortisone by *Curvularia lunata* entrapped in photo-cross-linked resin gels. Appl Environ Microbiol 1983;45:436–43.
[18] Walker HL, Connick WJ, Quimby PC (inventors). Method for mycoherbicide-containing pellets. The United States of America (assignee). US Patent 4,718,935. January 12, 1988.
[19] Hufford CD, Badria FA, Abou-Karram M, Shier WT, Rogers RD. Preparation, characterization, and antiviral activity of microbial metabolites of stemodin. J Nat Prod 1991;54:1543–52.
[20] Wilson MR, Gallimore WA, Reese PB. Steroid transformations with *Fusarium oxysporum* var. *Cubense* and *Colletotrichum musae*. Steroids 1999;64:834–43.
[21] Defaye G, Luche MJ, Chambaz EM. Microbiological 7- and 15-hydroxylations of C-19 steroids. J Steroid Biochem 1978;9:331–6.
[22] Dodson RM, Nicholson RT, Muir RD. Microbiological transformations. IV. The oxidation of dehydroisoandrosterone at C-7. J Am Chem Soc 1959;83:6295–7.
[23] Lamm AS, Chen ARM, Reese PB. Steroid hydroxylation by *Mucor plumbeus*, *Whetzelinia sclerotiorum* and *Phanerochaete chrysosporium*. Steroids 2007;72:713–22.
[24] Belan A, Bolte J, Fauvre A, Gourcy JG, Veschambre H. Use of biological systems for the preparation of chiral molecules. 3. Application in pheromone synthesis: preparation of sulcatol enantiomers. J Org Chem 1987;52:256–60.
[25] Tada H, Sawa YK. Preparation of A-ring conjugated enones and the corresponding.alpha.,.beta.-epoxy ketones of 17.beta.-acetoxy-5.alpha.-androstane. J Org Chem 1968;33:3347–9.
[26] Dodson RM, Mizuba S. Microbiological transformations. VIII. The oxidation of androstenedione at C-16. J Org Chem 1962;27:698–700.
[27] Bhutani KK, Thakur RN. The microbiological transformation of parthenin by *Beauveria bassiana* and *Sporotrichum pulverulentum*. Phytochemistry 1991;30:3599–600.
[28] Chen ARM, Ruddock PLD, Lamm AS, Reynolds WF, Reese PB. Stemodane and stemarane diterpenoid hydroxylation by *Mucor plumbeus* and *Whetzelinia sclerotiorum*. Phytochemistry 2005;66:1898–902.