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ORIGINAL RESEARCH PAPER

# 15α-Hydroxylation of a steroid (13-ethyl-gon-4-en-3,17-dione) by *Penicillium raistrickii* in an ionic liquid/aqueous biphasic system

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**Abstract** Biphasic processes are used in whole-cell biotransformation to overcome the low water solubility of substrates and products as well as their inhibitory effects on the biocatalyst. Commercially available [$NTf_2$]- and [$PF_6$]-based ionic liquids (ILs) were used in a biphasic system for the 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione by *Penicillium raistrickii*. With the substrate at 5 g $l^{-1}$ and a volume ratio of IL to buffer, buffer pH and cell density at, 1:9, 6.5, 16.8 $g_{DW}$ $l^{-1}$, respectively, the 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione was achieved with a yield of 70 % after 72 h using [BMIm][$NTf_2$] in a 50 ml biphasic system. This is compared to a 30 % yield in a monophasic aqueous system. This suggests the potential industrial application of ILs-based biphasic systems for steroid biotransformation.

**Keywords** Biphasic system · 15α-Hydroxylation · Ionic liquid · *Penicillium raistrickii* · Steroid biotransformation

## Introduction

The low solubility of steroids in aqueous media considerably limits their biotransformations.

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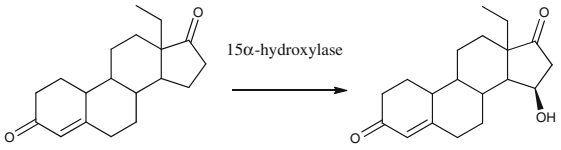


**Fig. 1** The 15α-hydroxylation reaction of 13-ethyl-gon-4-en-3,17-dione

A biphasic process, which consists of a cell-containing phase and a water-immiscible organic phase as a substrate reservoir and an in situ extractant for the product, has been established to overcome this problem (Angelova et al. 2005). However, most organic solvents used as the second phase frequently have negative effects on catalytic activity of microbial cells (Heipieper et al. 2007). Furthermore, these solvents are volatile and environmentally hazardous.

Ionic liquids (ILs) are attractive alternatives to organic solvents for biocatalysis in a biphasic process (Weuster-Botz 2007; Oppermann et al. 2011). The beneficial properties, such as non-flammability and non-volatility, make ILs greener, cleaner and safer than organic solvents (Gangu et al. 2009; Oppermann et al. 2011). Moreover, whole-cell biotransformation processes are more efficient in biphasic IL/aqueous systems compared to biphasic organic/aqueous systems or in pure aqueous systems (Pfrunder et al. 2006; Wang et al. 2009; Gangu et al. 2009). However, the microorganism used in a whole-cell ILs biphasic system is mainly restricted to bacteria and yeasts (Brautigam et al. 2007; Wang et al. 2009). There are few reports about the application of filamentous fungi-based steroid bioconversions in ILs (Wu et al. 2011), although most of the documented microbial biocatalysts for steroid hydroxylation are filamentous fungi.

15α-Hydroxylation of 13-ethyl-gon-4-en-3,17-dione (Fig. 1), an important intermediate in the production of gestodene which is an exogenous female sex steroid with potent oral contraceptive properties and few side effects, can be produced by *Penicillium raistrickii*-mediated biotransformation (Schlosser et al. 1993). As an extension of our research on the biphasic systems for the whole-cell bioconversion, this study has investigated the biocompatibility of four ILs on *P. raistrickii* and has evaluated the applicability of the biphasic IL/aqueous system for 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione by *P. raistrickii*.

## Materials and methods

### Chemicals

Four ILs ([BMIm][$PF_6$], [HMIm][$PF_6$], [BMIm][$NTf_2$] and [HMIm][$NTf_2$]) were purchased from Lanzhou Greenchem ILS (LICP, CAS, China). 13-Ethyl-gon-4-en-3,17-dione, 15α-hydroxy-13-ethyl-gon-4-en-3,17-dione, 99 % purity, and all other chemicals were obtained from commercial sources.

### Cell growth

*Penicillium raistrickii* (ATCC 10490) was grown at 28 °C with shaking in 250 ml flasks supplemented with 50 ml defined medium containing (g $l^{-1}$): glucose 30, corn syrup 10, $NaNO_3$ 2, $K_2HPO_4{\cdot}3H_2O$ 2, $KH_2PO_4$ 1, $FeSO_4{\cdot}7H_2O$ 0.02, KCl 0.5, $MgSO_4{\cdot}7H_2O$, 0.5; pH 7.3 ± 0.2. After 30 h, mycelia were harvested by filtration and washed twice with sterile water.

### Biocompatibility of ILs with *P. raistrickii*

The toxicity of ILs to *P. raistrickii* was investigated by the glucose uptake assay. 0.1 $g_{DW}$ mycelia was suspended in 5 ml glucose (50 g $l^{-1}$), followed by addition of 5 ml different ILs. The blank control contained no ILs. After incubating at 28 °C for 10 h, the mycelia were removed by centrifugation (8,000×*g*, 10 min), and glucose in aqueous phase was measured at 540 nm using the dinitrosalicylic acid (DNS) assay. A decrease (if any) in aqueous glucose concentration was used as an indicator of ILs biocompatibility.

### Biotransformation runs and analysis

The substrate was dissolved in 4 ml IL, followed by addition of 16 ml aqueous phase (0.05 M phosphate buffer, pH 6.5) with a certain amount of mycelia. The biotransformation was carried out in a 30 ml shake-flask at 28 °C for 72 h. 500 µl samples were withdrawn from the aqueous and IL phase separately, and the substrate and product concentrations were determined by HPLC (Schlosser et al. 1993).

**Table 1** Effect of 20 % ILs on the glucose uptake of *P. raistrickii* in ILs/aqueous biphasic system compared to aqueous system

| Solvent | Glucose uptake[a,b] (%) |
|---|---|
| Water | 80 ± 2 |
| [BMIm][$PF_6$] | 52 ± 2 |
| [HMIm][$PF_6$] | 20 ± 1 |
| [BMIm][$NTf_2$] | 80 ± 2 |
| [HMIm][$NTf_2$] | 32 ± 1 |

Data are reported as mean ± standard deviation of triplicate

[a] The % glucose uptake = (initial concentration) − (final concentration)/initial concentration × 100, and the glucose concentrations were measured at 540 nm using the dinitrosalicylic acid (DNS) assay

[b] The initial glucose concentration (50 g $l^{-1}$) was taken to calculate % glucose uptake

## Results and discussion

### Biocompatibility of ILs with *P. raistrickii*

Biocompatibility of ILs is an essential criterion for their applicability in whole-cell biotransformation. Table 1 illustrates the glucose uptake in a biphasic system and in a pure aqueous system. Glucose uptake was maximum in the presence of [BMIm][$NTf_2$], indicating that it had little inhibitory effect on the growth of *P. raistrickii*.

The toxicity of the cation and anion of ILs on *P. raistrickii* cells was also investigated. ILs with hexyl-cation possessed lower biocompatibility than ILs with butyl-chain cation (Table 1). This different effect could be explained by the increasing surfactant characteristics of ILs with elongation of the alkyl cation, which tends to cause more damage on the cell membrane (Couling et al. 2006; Luis et al. 2010). On the other hand, ILs with [$NTf_2$]-anion were less toxic to *P. raistrickii* cells than the ILs with [$PF_6$]-anion (Table 1). However, opposite results were found using these ILs for *Rhodotorula* sp. AS 2.2241 and *R. nigricans* cells (Wang et al. 2009; Wu et al. 2011). Further work is needed to establish the relationship between the structures of ILs and biocompatibility, as well as their effect on biotransformation.

### Whole-cell biotransformation on 20 ml scale

The substrate conversion in the [BMIm][$NTf_2$]/buffer biphasic system was higher than that in the [BMIm][$PF_6$]/buffer biphasic system (Table 2), which was ascribed to the higher distribution coefficient of substrate and product in [BMIm][$NTf_2$]. Many studies have shown that the most suitable ILs for a biphasic reaction system exhibit higher product- and substrate-related distribution coefficients (Brautigam et al. 2007; Roosen et al. 2008). On the contrary, our results showed that the substrate conversion was much higher in [BMIm]-based ILs/buffer biphasic systems than that in [HMIm]-based ILs/buffer biphasic systems (Tables 2 and 3) although the former systems have lower distribution coefficients. The lower viscosity of ILs with shorter alkyl cationic chain could account for our observations to some extent (Lou et al. 2009).

**Table 2** Effect of ILs on biotransformation of 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione mediated by *P. raistrickii* in biphasic systems

| IL/buffer | Bioconversion[a,b] (%) |
|---|---|
| Buffer | 35 ± 1 |
| [BMIm][$PF_6$]/buffer | 42 ± 3 |
| [HMIm][$PF_6$]/buffer | 2 ± 1 |
| [BMIm][$NTf_2$]/buffer | 77 ± 3 |
| [HMIm][$NTf_2$]/buffer | 40 ± 1 |

The reaction was carried out at 28 °C for 72 h in 20 ml biphasic system in which the IL/phosphate buffer ratio, buffer pH, cell density and substrate concentration in IL are 1:5, 6.5, 5 $g_{DW}$ $l^{-1}$ and 3 g $l^{-1}$, respectively. In control, substrate was dispersed into aqueous solution. Data are reported as mean ± standard deviation of triplicate

[a] The percent conversion was defined as the percentage of the converted substrate to the initial substrate

[b] The initial substrate concentration was taken to calculate the % substrate conversion

**Table 3** Data of log D[a] of the substrate and the product in the different solvents

| Solvent | log D of the substrate | log D of the product |
|---|---|---|
| [BMIm][$PF_6$] | 0.79 | 0.82 |
| [HMIm][$PF_6$] | 0.73 | 0.75 |
| [BMIm][$NTf_2$] | 1.63 | 1.69 |
| [HMIm][$NTf_2$] | 1.05 | 1.18 |

ILs containing 0.05 mM of substrate and product were combined with an equal volume of water in a gas-tight vial and vigorously shaken in a mixer mill for 4 h, and the distribution coefficient was determined via mass balance, and the substrate (product) concentration was determined by HPLC

[a] log D = log($C_{IL}/C_{water}$)

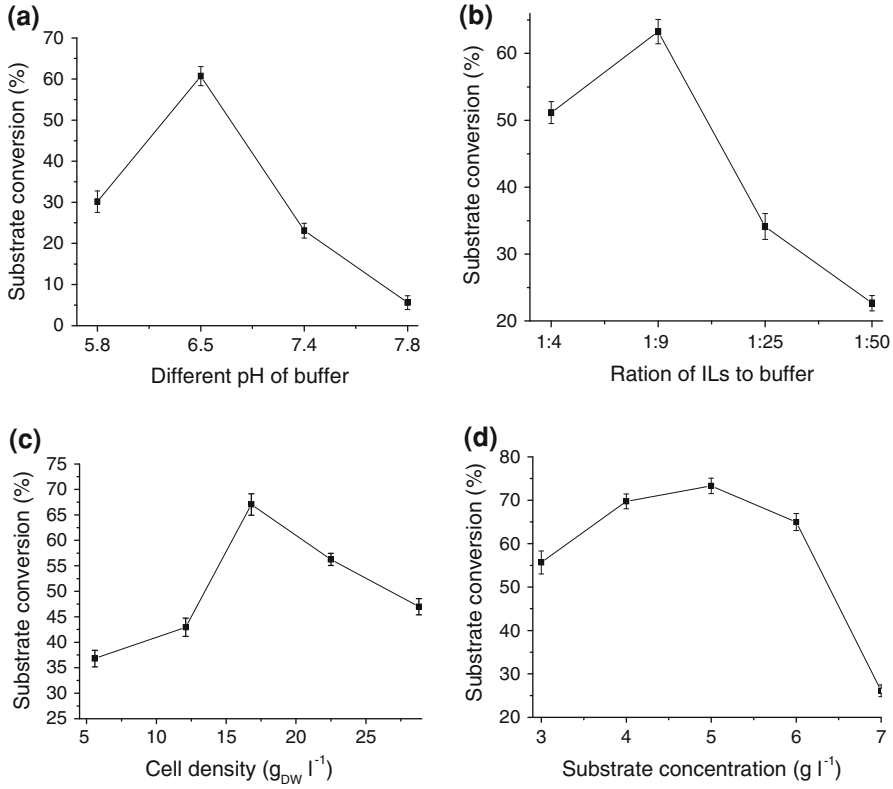


**Fig. 2** 15α-Hydroxylation of 13-ethyl-gon-4-en-3,17-dione mediated by *P. raistrickii* in a 20 ml [BMIm][NTf2]/phosphate buffer biphasic system, the reaction was carried out at 28 °C for 72 h. **a** Effect of buffer pH (IL/buffer volume ratio: 1:4, substrate 3 g $l^{-1}$, cell density 5 $g_{DW}$ $l^{-1}$); **b** effect of volume ratio of IL to buffer (pH 6.5, substrate 3 g $l^{-1}$, cell density 5 $g_{DW}$ $l^{-1}$); **c** effect of cell density (pH 6.5, IL/buffer volume ratio 1:9, substrate 3 g $l^{-1}$); **d** effect of substrate concentration (pH 6.5, IL/buffer volume ratio 1:9, cell density 16.8 $g_{DW}$ $l^{-1}$)

In conclusion, for the four ILs tested, [BMIm][$NTf_2$] markedly enhanced the whole-cell biotransformation efficiency, and was consequently chosen as the second phase in an ILs/buffer biphasic system for further investigation.

## [BMIm][$NTf_2$]/aqueous biphasic systems for whole-cell biotransformation on 50 ml scale

In addition to the effects of ILs on specific conversion systems, pH, $V_{IL}/V_{Aq}$, substrate concentration and cell density also play essential roles in steroid biotransformation. The substrate conversion was enhanced with increasing pH value of the reaction buffer from 4.5 to 6.5. However, a further increase in buffer pH resulted in a decreased substrate bioconversion (Fig. 2a). $V_{IL}/V_{Aq}$ also had an effect on substrate conversion (Fig. 2b). Since active cells are commonly inactivated by direct contact with the interface between the aqueous phase and non-aqueous phase (Lou et al. 2009), the enhancement in final substrate conversion with the decrease of $V_{IL}/V_{Aq}$ from 1/4 to 1/9 can be easily understood because of the reduced interface area as $V_{IL}/V_{Aq}$ decreased. A further decrease of $V_{IL}/V_{Aq}$ resulted in declined substrate conversion, most likely owing to the toxicity of substrate and product in the aqueous phase. Moreover, an increase in the amount of *P. raistrickii* cells led to

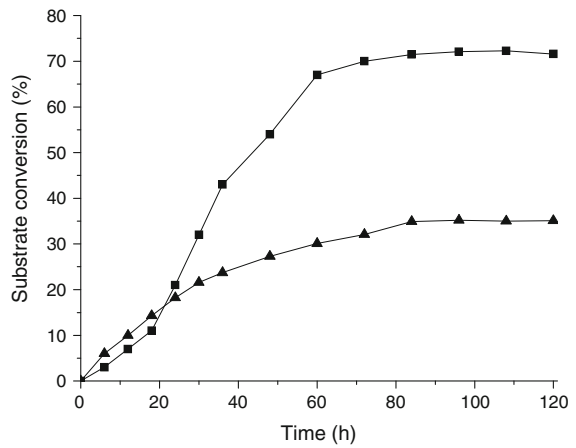


**Fig. 3** Time-course profiles of substrate conversion of 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione with *P. raistrickii* in the [BMIm][$NTf_2$]-containing biphasic system (*filled square*) and in a pure aqueous system (*filled triangle*). The initial substrate concentration was taken to calculate the % substrate conversion

an apparent improvement of the bioconversion yields (Fig. 2c): the maximum yield was with 16.8 $g_{DW}$ $l^{-1}$ in the biphasic system. Addition of more *P. raistrickii* cells decreased the substrate conversion. In addition, the conversion yields increased with increasing substrate concentrations (<5 g $l^{-1}$) and decreased when substrate concentration was above 5 g $l^{-1}$ (Fig. 2d), indicating that substrate toxicity occurred even if the applied substrate concentration in an aqueous phase was limited (Wang et al. 2009).

Figure 3 showed that the initial reaction rate was lower in the [BMIm][$NTf_2$]/buffer biphasic system compared with that in the corresponding aqueous monophasic system, most likely due to the much lower substrate concentration in the aqueous phase caused by in situ extraction of substrate into IL-containing phase (Wang et al. 2009). This also lowered toxicity of substrate and product in the aqueous environment, resulting in a significant increase of substrate conversion in the [BMIm][$NTf_2$]-based biphasic system. The maximum substrate conversion of 70 % was obtained at 72 h in the biphasic system (Fig. 3), as opposed to 30 % in the aqueous monophase.

## Conclusions

Our investigations of the effect of ILs on *P. raistrickii* broaden the applicability of biphasic IL/water systems for whole-cell biocatalysis. [BMIm][$NTf_2$] has the potential for promoting the 15α-hydroxylation of 13-ethyl-gon-4-en-3,17-dione as mediated by *P. raistrickii* in a biphasic system. Furthermore, considering the current cost of the ILs, research for the efficient recovery and reuse of ILs for the process development in such biphasic reaction systems is warranted.

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