Research Article

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# 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone in biphasic ionic liquid/water system by *Aspergillus ochraceus*

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## Abstract

**BACKGROUND: The improved efficiency of steroid biotransformation using the biphasic system is generally attributed to the positive effect on the solubility of substrate in aqueous media. A promising alternative for the application of organic solvents in biphasic systems is the use of ionic liquids (ILs). This study aims to investigate the applicability of the biphasic ILs/water system for 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone (HEP) by *Aspergillus ochraceus*.**

**RESULTS: Of the seven ILs tested, [$C_3$mim][$PF_6$] exhibited the best biocompatibility, with markedly improved biotransformation efficiency. In the [$C_3$mim][$PF_6$]-based biphasic system, substrate conversion reached 90% under the condition in which buffer pH, volume ratio of buffer to ILs, cell concentration, and substrate concentration were 4.8, 10/1, 165 g $L^{-1}$ and 20 g $L^{-1}$, respectively. This is more efficient than that of the monophasic aqueous system. The effects of the cations and anions of these ILs on the 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone (HEP) by *A. ochraceus* is also discussed.**

**CONCLUSION: The above results showed that IL/water biphasic system improved the efficiency of 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone (HEP) by *A. ochraceus,* thus suggesting the potential industrial application of ILs-based biphasic systems for steroid biotransformation.**


*Supporting information may be found in the online version of this article.*

**Keywords:** 11$\alpha$ hydroxylation; *Aspergillus ochraceus*; 16$\alpha$, 17-epoxyprogesterone; byphasic system; ionic liquids

## INTRODUCTION

Microbial hydroxylation of steroid at position C11 plays an essential role in the pharmaceutical intermediate production of corticosteroids and progestogens.[1–8] For steroid 11$\alpha$ hydroxylation via biotransformation, the typical problems arise from the poor solubility of substrate and product in water, as well as their inhibitory/toxic effects on the biocatalyst.[9] These seriously limit the biotransformation of steroid production. To improve the solubility of substrate, hydrophilic co-solvents and surface-active agents are used.[10] Subsequently, the biphasic approach was developed using water-immiscible organic solvent as the second phase for a substrate reservoir and *in situ* product recovery.[11–14] However, commonly used conventional organic solvents often damage the microbial cells, and they are a threat to operator health due to their volatile and environmentally hazardous nature.[15,16]

Ionic liquids (ILs), a new promising class of solvents with non-volatility, non-flammability and high thermal and chemical stability, were recommended as attractive alternatives to organic solvents to construct biphasic systems.[17,18] Moreover, ILs dissolved a wide range of chemical compounds. In several encouraging results, hydrophobic ILs were biocompatible solvents especially for those containing hexafluorophosphate and bis(trifluoromethylsulfonyl)imide cations.[18–20] They also reduce the inhibitory/toxic effects of substrate and product on the biocatalyst.[18–20] Therefore, ILs were considered most effective for biotransformation. In fact, several studies have already confirmed the improved whole-cell biotransformation efficiency in the biphasic ILs/water system in comparison with biocatalysis in organic solvents or those performed in pure aqueous systems.[20–25] Some bioprocesses of commercial interest have already been developed to process scale. However, to the best of our knowledge, microorganisms used in ILs/water biphasic system were restricted to single-celled microbes,[21–27] although hydroxylation in steroid drugs was mainly catalyzed by filamentous fungi. Moreover, most of the reported biotransformation so far are asymmetric reduc-

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tions of ketones,[22,23,25] and only a few attempts have been made to carry out steroid bioconversion in an IL-based biphasic system.[8,26]

In these systems, the evaluation of ILs is crucial for the design of biphasic ILs/water biotransformation. Based on the physical properties, some ILs were excluded, then ILs were commonly ranked according to ILs' biocompatibility and distribution coefficients.[19,20,23,27] As a matter of fact, the catalytic performance displayed by microorganisms, which were affected by different structure of ILs, varied widely in the ILs-based biphasic system.[28] Given various structures of ILs, Luis *et al*.[29] thought that the effect of ILs on the bioconversion came from three main groups: the cation, the anion and the substitutions (carbon chains linking to the cation). Considering the charged hydrophilic head group and hydrophobic tail in ILs, the long-chain imidazolium and pyridinium based ILs would display the inherent amphiphlic nature. And it can be further anticipated that with elongation of the side chain of cations, the lipophilic property of these ILs increased, which impaired the membrane integrity more significantly.[30] Recent results showed the close correlation between the lengths of the alkyl chain linking ILs' cation and the biological behavior.[31,32] The minor effect of the anion on the toxicity of pyridinium- and imidazolium-based ILs to the microorganism was found,[33] which suggested that toxicity was largely driven by the cation. However, the opposite result, which suggested the toxicity of ILs mainly coming from the anion, was also reported.[34] Therefore, research is necessary to analyze the effect of cation and anion of ILs on biocatalysis, and to explore the relationship between their structure and biological activity, as well as their effect on biotransformation.

Hydroxylation of epoxyprogesterone at the position C11 is well-known and often chosen as a model system when introducing a hydroxyl group into a steroidal ring system by the filamentous fungus.[8] In this research, the utility of ILs in the biphasic system for 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone (EP) by *A. ochraceus*, an effective hydroxylator of epoxyprogesterone at the position C11, was evaluated, and the effect of cation and anion of ILs on the biotransformation was discussed. This study aims to provide an improved understanding of the structural parameters of the long-chain ILs affecting biological activity. The research is expected to contribute to the industrial application of ILs in biphasic systems for steroid biotransformation.

## EXPERIMENTAL

### Material

All ILs were purchased from Lanzhou Greenchem ILS (LICP, CAS, China). The names of each IL are given in Table 1. 16$\alpha$ 17-epoxyprogesterone (EP) and 11$\alpha$-hydroxy-16$\alpha$ 17-epoxyprogesterone (HEP) of 99% purity, and other reagents were of chromatographic quality or analytical grade and available commercially.

### Methods

*Microorganisms and growth condition*

*A. ochraceus* (TCCC 41 060) was maintained in a slant medium containing 20 g $L^{-1}$ sucrose, 1 g $L^{-1}$ yeast extracts, 200 g $L^{-1}$ potato juice and 20 g $L^{-1}$ agar at 28 °C. After cultivation for 5–6 days, The harvested spore suspensions were inoculated into 50 mL fermentation medium in a 250 mL flask, with a final concentration of $5 \times 10^6$ spores $mL^{-1}$. The fermentation medium containing 20 g $L^{-1}$ glucose, 20 g $L^{-1}$ yeast extracts, 20 g $L^{-1}$ peptone, pH was adjusted to 5.8 ± 0.2. Incubation was performed on a rotary shaker (180 rpm) at 28 ± 2 °C. After 24 h cultivation, mycelia were collected and washed with physiological saline. Then mycelia were prepared for the biotransformation.

*Distribution coefficients*

In order to determine the distribution coefficients of substrates and products between ILs and water (log D), 20 mg substrate and 20 mg product were dissolved in 20 mL saturated IL/water system. After vibration by a mixer mill at 180 rpm for 24 h at 28 °C, the system stood for 30 min. Aqueous phase was extracted with ethyl acetate and ILs phase was diluted by acetonitrile of chromatographic grade, and subsequently the substrate and product concentration were determined by HPLC. Log D was calculated according to:

$$\log D = \log \frac{C_{ILs}}{C_{water}}$$

*Biocompability of ILs on A. ochraceus*

The biocompatibility of ILs on *A. ochraceus* was assayed in various biphasic systems consisting of 6 mL ILs and 24 mL glucose buffer medium (NaAc-HAc, pH = 4.5), without substrate and with 15 mg substrate, respectively. The control flask contained no ILs. After cultivation for 12 h, the mycelia were removed by centrifugation (5000 rpm, 5 min), and 100 µL supernatant was diluted 10-fold, and the glucose concentrations were measured at 540 nm using the dinitrosalicylic acid (DNS) assay and a decrease in aqueous glucose concentration was taken as an indicator of biocompatibility.

*Biotransformation runs*

In the ILs–aqueous biphasic system with the volume ratio of aqueous buffer (NaAc-HAc, pH = 4.5) to ILs 4:1 (NaAc-HAc, pH = 4.5), 10 g $L^{-1}$ substrate (based on the volume of the whole bioconversion medium) was dissolved in IL followed by the addition of the aqueous phase with mycelia. In the control experiment, the same substrate was added to the aqueous system. After completion of biotransformation, 20 µL samples of IL phase were diluted by 50-fold methanol of chromatographic grade, and the conversion results analyzed by HPLC.[25] The samples in the control bioconversion were extracted by ethyl acetate, and supernatant was dried and dissolved in acetonitrile of chromatographic grade and analyzed by HPLC.[25] The percentage conversion was defined as the ratio of converted substrate to total amount of initial substrate ×100. (see supporting information Fig.S1, Fig.S2 and Fig.S3)

*Analytical methods*

The samples were analyzed by HPLC (Agilent) with a UV detector at 240 nm. The column used was 4.6 mm × 250 mm × 10 µm Hypersil ODS C18 (Thermo, American). The mobile phase was composed of 60% acetonitrile and 40% water, and the mobile phase flow rate was 1.2 mL $min^{-1}$. 10 µL samples were injected with a total elution time of 10 min.

## RESULTS AND DISCUSSION

### Biocompability of ILs

Based on the toxic damage to the whole cell, ILs were rated according to their biocompatibility for the biotransformation. Cell viability was usually assayed by Methylene Blue and the LIVE/DEAD *Bac*light test kit in order to evaluate the effects of ILs on cells.[20–23,25] However, LIVE/DEAD *Bac*light assay for determining cell viability seemed to be unsuitable for assaying filamentous fungus. Vrionis



  

**Table 1.** Log D data for the substrate and the product in the different solvents

| | $[C_3mim][NTf_2]$ | $[C_4mim][NTf_2]$ | $[C_6mim][NTf_2]$ | $[Amim][NTf_2]$ | $[C_3mim][PF_6]$ | $[C_4mim][PF_6]$ | $[C_6mim][PF_6]$ |
|---|---|---|---|---|---|---|---|
| Substrate | 2.12 | 2.16 | 2.20 | 2.25 | 2.15 | 2.17 | 2.25 |
| Product | 2.34 | 3.20 | 3.45 | 3.60 | 3.31 | 3.46 | 3.70 |

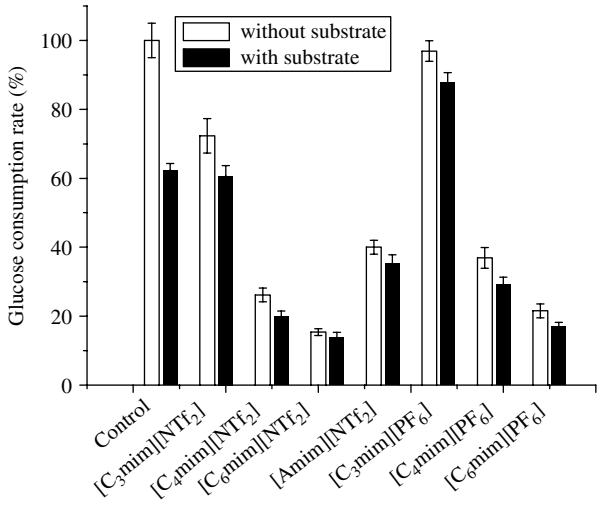


**Figure 1.** Effect of 20% ILs on the glucose consumption of *A. Ochraceus* compared with pure buffer system with and without substrate.

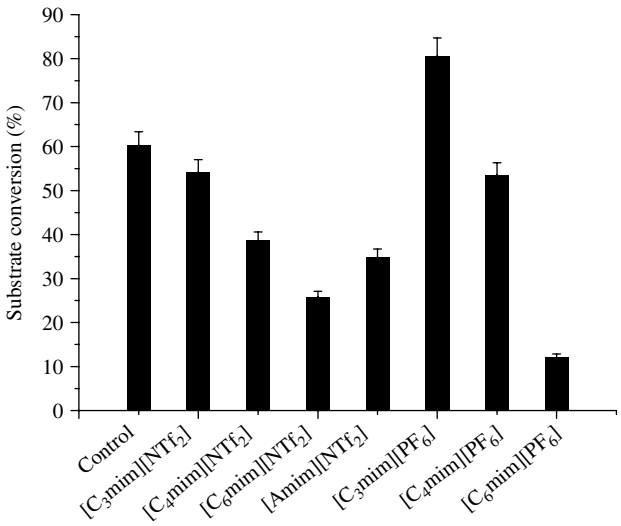


**Figure 2.** Effect of ILs on the efficiency of whole cell biotransformation in $[C_3mim][PF_6]$/buffer biphasic systems. Phase ratio of aqueous/ionic liquid is 4 : 1, EP concentration is 10 g $L^{-1}$. In the control, substrate was dispersed into aqueous solution.

*et al*.[35] noted that both substrate uptake and cell growth were essentially interchangeable as a measure of biocompatibility. Here the reduced glucose consumption relative to the positive control (no ILs) was taken as an indicator of toxicity.

As shown in Fig. 1, in the absence of substrate, the glucose consumption was lower in all tested ILs-based biphasic systems compared with the aqueous monophasic system. This suggested that ILs were toxic to *A. ochraceus*. It was also noted that in the water-immiscible ILs tested, $[C_3mim][PF_6]$ exhibited less toxic effects on *A. ochraceus*. Furthermore, compared with the $[C_3mim][NTf_2]$-containing system, $[Amim][NTf_2]$ was more toxic to fungus, suggesting that ILs containing unsaturated alkyl side chains could increase the toxicity.

Fig. 1 further indicated that cell viability decreased with elongation of the alkyl side chain of ILs containing the same anions, probably due to the more lipophilic property of longer alkyl chains. These results were in accordance with a number of other toxicity studies.[32,36,37] Additionally, ILs used in the biphasic system possessed similar structures to cationic surfactant, especially for the imidazolinium compounds. And it was well-known that cationic surfactant displayed narcotic effects due to the increased membrane permeability with elongation of the alkyl chain.[30,38,39]

It should be noted that in the presence of the same cation, the $[PF_6]$-based ILs displayed higher cell viability than those containing $[NTf_2]$. This suggested that the anion also has a major effect on ILs toxicity which most probably was related to the presence of fluoride atom.[40]

Finally, to further elucidate toxic and/or inhibitory effects of substrate on the fungus in the ILs-based biphasic system, cell viability was explored in the presence of 5 g $L^{-1}$ substrates. As can be seen in Fig. 1, glucose consumption decreased in the presence of substrate compared with its absence in all reaction systems, implying that the substrate exerted toxic effects on the fungus. In the biphasic system containing $[C_3mim][PF_6]$, cell viability clearly increased compared with that in the aqueous monophasic system, suggesting that ILs effectively decreased the toxic substrate concentration in the aqueous phase.

## Distribution coefficients

Distribution coefficients, another important criterion for rating the suitability of ILs for biotransformation, were investigated in the biphasic whole-cell biotransformation. As shown in Table 1, it was found that $[PF_6]$-based ILs afforded higher distribution coefficients for the substrate and the product, compared with $[NTf_2]$-based ILs under the same conditions. This implied that substrate and product in the $[PF_6]$-based ILs biphasic systems may exert less toxicity to *A. ochraceus*. The cationic effects were also investigated. Higher distribution coefficients were obtained with increasing alkyl chain length, due to the increasing surfactant characteristics of ILs. However, Wang found that partition coefficients of MOAP between ILs and buffer were slightly reduced with elongation of the alkyl chains.[39] Until recently, there was insufficient data to allow a detailed understanding of the influence of ILs on distribution coefficients of the specific compounds. So approaches should be applied to describe the thermodynamic behavior of ILs and the solubility of selected compounds, and this may accelerate the industrial application of ILs in steroid biotransformation.

## Whole cell biotransformation of 30 mL scale

To better understand the effect of ILs on biotransformation, 11α hydroxylation of 10 g $l^{-1}$ EP was performed in a biphasic whole-cell



  

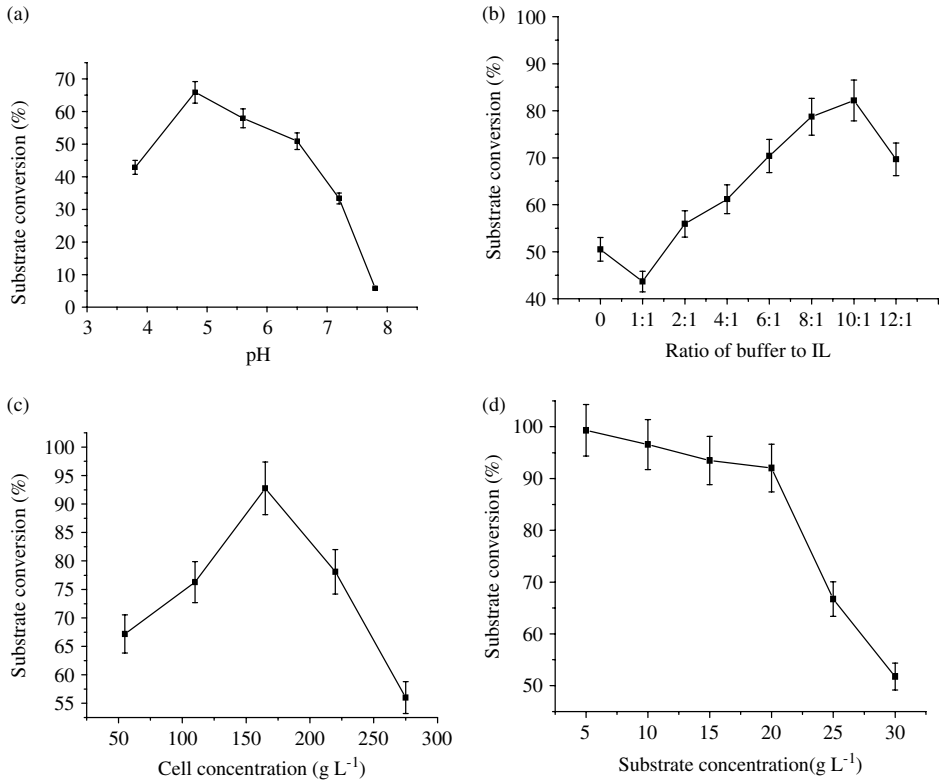


**Figure 3.** 11$\alpha$ Hydroxylation of 16, 17-epoxyprogesterone mediated by *A. Ochraceus* in the [$C_3$mim][$PF_6$]/buffer biphasic system. (a) effect of buffer pH (buffer/IL volume ratio 4 : 1, cell concentration 100 g $L^{-1}$, substrate 10 g $L^{-1}$); (b) effect of volume ratio of IL to buffer (buffer pH 4.8, cell concentration 100 g $L^{-1}$, substrate 10 g $L^{-1}$); (c) effect of cell concentration (buffer pH 4.8, buffer/IL volume ratio 10 : 1, substrate 10 g $L^{-1}$); (d) effect of substrate concentration (buffer pH 4.8, buffer/IL volume ratio 10 : 1, cell concentration 165 g $L^{-1}$).

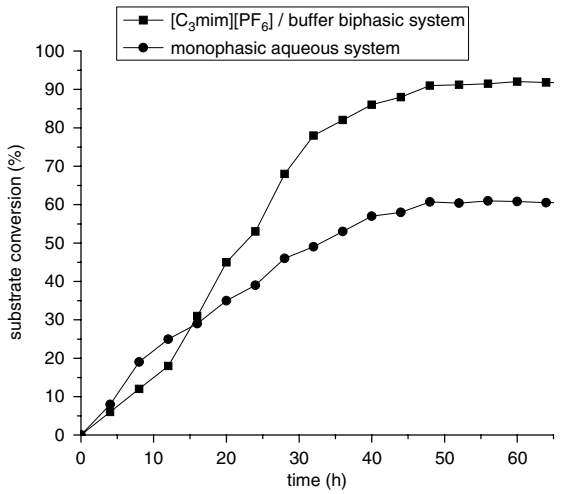


**Figure 4.** Time-course profile of substrate conversion of 11$\alpha$ hydroxylation of 16$\alpha$, 17-epoxyprogesterone by *A. ochraceus* in the [$C_3$mim][$PF_6$]-containing biphasic system (buffer pH 4.8, volume ratio of buffer to ILs 10/1, cell concentration 165 g $L^{-1}$, and substrate concentration 20 g $L^{-1}$) and in a pure aqueous system (substrate concentration 20 g $L^{-1}$).

biotransformation system. Furthermore, simple and parallel batch hydroxylation was carried out on a 30 mL scale. As shown in Fig. 2, [$C_3$mim][$NTf_2$] produced the highest product yield, suggesting its suitability for the biphasic biotransformation. While the remaining six ILs showed lower bioconversion yields in comparison with the aqueous system, and the poor performance of the biocatalysis in these IL-containing systems is probably due to the markedly toxic effect of the ILs on the biocatalyst.

The results from the ILs-based biphasic whole-cell biotransformation showed that the substrate conversion went down slightly with elongation of the alkyl chain of the ILs' cation, possibly resulting from the increase in ILs' viscosity with elongation of the alkyl chain,[30] which may lead to a decrease in mass transfer between the two phases. Furthermore, the above observation could be explained by the lower biocompatibility of ILs with *A. ochraceus* (Fig. 1) with the elongation of alkyl chain. In addition, in the case of [$NTf_2$]-based ILs, the changing profiles of substrate conversion with elongation of the alkyl chain are similar to those observed for [$PF_6$]-based ILs.

Generally, distribution coefficients were used as criteria for estimation of ILs in the ILs-based biphasic biotransformation. It was expected that ILs with higher distribution coefficients had higher product yields, due to the lower toxicity of substrate and product to cells in the aqueous phase. Published work seems to confirm this assumption. However, the distribution coefficient cannot be the only measure for an increased or decreased yield in biotransformation. In this study, as shown in Table 1 and Fig. 2, lower substrate conversions were obtained for both [$NTF_2$]- and [$PF_6$]-based ILs with higher distribution coefficients under the same conditions. This observation may be related to lower biocompatibility and higher viscosity of ILs with longer alkyl chains.

It was noteworthy that the variation in the structure of ILs also afforded a substantial impact on the bioconversion. Interestingly, the minor change in structure from [$C_3$mim][$PF_6$] to [Amim][$PF_6$]



  

also led to apparently worse catalyst performance, which coincided with the decline in cell viability (Fig. 1).

Additionally, it can be seen from Table 1 and Fig. 2 that substrate conversions were much higher in $[PF_6]$-based ILs systems than those in $[NTf_2]$, indicating that the nature of the anion in the ILs also has an important effect on the biotransformation. Both the slightly lower partition coefficients of EP and HEP between ILs and buffer (Table 1) and the lower biocompatibility of the ILs with *A. ochraceus* (Fig. 1) could explain this observation.

Of the seven water-immiscible ILs tested, $[C_3mim][PF_6]$ afforded the highest substrate bioconversion, it was consequently chosen as the second phase in the ILs/buffer biphasic system for further investigation.

### 11α Hydroxylation of 16α, 17-Epoxyprogesterone by *Aspergillus ochraceus* in whole cell biotransformation in $[C_3mim][PF_6]$/aqueous system on 50 mL scale

Besides the effect of ILs on the specific bioconversion system, other important factors, such as pH, the volume ratio between two phases, cell concentration and substrate concentration also played vital roles in the whole-cell biotransformation. First, Fig. 3(a) illustrates the effects of pH on the 11α hydroxylation of EP in the $[C_3mim][PF_6]$/buffer system. Substrate conversion rate increased when pH increased from 3.8 to 4.8, and decreased with further increase in the buffer pH. This might be ascribed to the inactivation of cells after being incubated for a long period of time at an unsuitable buffer pH. Subsequently, the volume ratio of the aqueous phase to the IL phase ($V_{aq}/V_{IL}$, mL $mL^{-1}$) was explored. As depicted in Fig. 3(b), the product yield enhanced with the increase of $V_{aq}/V_{IL}$ from 1/1 to 10/1 and declined with further increase of $V_{aq}/V_{IL}$. Previous works indicated that enzymes and active cells are often inactivated by direct contact with the interface between the aqueous and non-aqueous phases,[30] thus the increased conversion could be due to a decreasing interface area between the aqueous and IL phases. Therefore, 10/1 ($V_{aq}/V_{IL}$) was regarded as the most suitable volume ratio for the bioconversion. Moreover, as shown in Fig. 3(c), the gradual increment of *A. ochraceus* resulted in apparent improvement of product yields, and the yield reached the maximum as the concentration of 165 g $L^{-1}$ mycelia in the biphasic system. Further addition of *A. ochraceus* cells led to decreased substrate conversion owing to the increased viscosity because of excessive *A. ochraceus* cells. Thus, 165 g $L^{-1}$ *A. ochraceus* were the selected cell concentration for EP bioconversion. Finally, the effect of substrate concentration in ILs on the 11α hydroxylation of EP was investigated. Fig. 3(d) showed that substrate conversion maintained at a higher level (>90%) with increased substrate feeding (<20 g $L^{-1}$) and declined with further addition of substrate (>20 g $L^{-1}$) owing to the toxic effect of substrate.

Fig. 4 depicts the time-course profile of substrate conversion of 11α hydroxylation of 16α, 17-epoxyprogesterone by *A. ochraceus* in the $[C_3mim][PF_6]$-containing biphasic system and in the pure aqueous system. As shown in Fig. 4, in comparison with the monophasic aqueous system, higher yield was obtained in the $[C_3mim][PF_6]$/buffer biphasic system, maybe due to the ability to decrease the concentrations of toxic substrates and products in the biocatalyst's aqueous environment. However, at the beginning, the reaction rate was lower in the $[C_3mim][PF_6]$/buffer biphasic system than in the aqueous monophasic system. This might be in accordance with the much lower substrate concentration in the aqueous phase, which was caused by the mass transfer limitation with the IL-containing biphasic system.

It should be noted that this study has only evaluated the applicability of ILs in the biphasic system for 11α hydroxylation of 16α, 17-epoxyprogesterone (HEP) by *A. ochraceus*. This study does indicate the potential application of IL/aqueous biphasic systems for steroid biocatalysis by filamentous fungi. Furthermore, the comparison of substrate bioconversion between ILs/aqueous and organic solvents/aqueous biphasic system, as well as the reuse of ILs will be reported in due course.

## CONCLUSION

11α Hydroxylation of 16α, 17-epoxyprogesterone (HEP) can be successfully carried out using *A. ochraceus* in water-immiscible ILs-based biphasic systems. Various cations and anions of ILs have significant but different effects on the biotransformation. Of all the ILs examined $[C_3mim][PF_6]$/aqueous biphasic system was the most effective for substrate conversion which increased to above 90% with a substrate concentration of 20 g $l^{-1}$ under the selected conditions. These results indicate the potential industrial application of ILs-based biphasic systems for steroid biotransformation.

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### Supporting information

Supporting information may be found in the online version of this article.

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