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SHORT COMMUNICATION

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Production of drug metabolites by immobilised *Cunninghamella elegans*: from screening to scale up

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Abstract *Cunninghamella elegans* is a fungus that has been used extensively as a microbial model of mammalian drug metabolism, whilst its potential as a biocatalyst for the preparative production of human drug metabolites has been often proposed, little effort has been made to enable this. Here, we describe a workflow for the application of *C. elegans* for the production of drug metabolites, starting from well-plate screening assays leading to the preparative production of drug metabolites using fungus immobilised either in alginate or as a biofilm. Using 12- and 96-well plates, the simultaneous screening of several drug biotransformations was achieved. To scale up the biotransformation, both modes of immobilisation enabled semi-continuous production of hydroxylated drug metabolites through repeated addition of drug and rejuvenation of the fungus. It was possible to improve the productivity in the biofilm culture for the production of 4′-hydroxydiclofenac from 1 mg/l h to over 4 mg/l h by reducing the incubation time for biotransformation and the number of rejuvenation steps.

Keywords Biotransformation · Biocatalysis · Biofilm · Immobilisation

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Introduction

Drug metabolites that are required for toxicity testing are classically prepared via organic synthesis. However, it is possible to use microorganisms, which are able to transform drugs in an analogous fashion to mammals owing to the presence of cytochromes P450 (CYP). Furthermore, the biological approach is attractive since the conditions are mild, with less waste and fewer side products being generated. Fungi, in particular the zygomycete *Cunninghamella elegans*, have been extensively studied in relation to their ability to biotransform drugs to the same metabolites that are found in mammals [4]. The typical approach to date has been to study a single drug incubated with the fungus in a shake flask and extract sufficient metabolite (usually <10 mg) to permit structural characterisation [2]. However, little effort has been made either to scale down the screening to enable the biotransformation of numerous drugs simultaneously or to scale up the biotransformation so that preparative quantities of valuable drug metabolites can be generated.

Most reported microbial drug transformations involve suspended cells, whereas immobilisation of cells can improve metabolite production. For example, *Actinoplanes* sp. trapped in a hollow-fibre cartridge produced hydoxylated diclofenac more efficiently than suspended cultures [15]. In the case of *C. elegans*, we have recently shown that the fungus can form a biofilm, and that the transformation of the non-steroidal anti-inflammatory drug flurbiprofen can be catalysed over an extended timeframe compared to the typical suspended culture [1]. Immobilisation of cells in calcium alginate is technically straightforward and has proven effective in numerous biocatalytic applications [5], including biotransformation reactions catalysed by *C. elegans* protoplasts [8]. In this paper, we have attempted

to address the screening and scale up issues by developing a well-plate assay for convenient assessment of drug biotransformation, and application of immobilised fungus to the semi-continuous production of drug metabolites.

Materials and methods

Fungal cultivation

Previously developed methods were employed for the growth of the fungus in shake flasks [1, 2]. *Cunninghamella elegans* DSM1908 was grown on a Sabouraud dextrose agar plate for 5 days at 28 °C and then aseptically homogenised into 100 ml of sterile saline solution using an ATO MSE course mix blender. For planktonic growth, the homogenate (10 % v/v) was used directly to inoculate 250 ml Erlenmeyer flasks containing 45 ml of Sabouraud dextrose broth. To cultivate the fungus as a biofilm, the homogenate (2 % v/v) was used to inoculate 49 ml of Sabouraud dextrose broth in 250 ml Erlenmeyer flasks in which stainless steel compression springs (1.2 mm T316 wire; Shannon Coiled Springs, Ireland) were inserted. To entrap the fungus in alginate, the homogenate (4.4 ml) was mixed with cooled sterile sodium alginate (2.25 % v/v, final volume = 20 ml) and added drop wise to a sterile calcium chloride solution (0.15 M). After approximately 20 min of incubation at room temperature, calcium alginate beads were rinsed thoroughly with sterile H_2O and finally transferred into 45 ml Sabouraud dextrose broth in a 250 ml Erlenmeyer flask. All the cultures were incubated at 28 °C with shaking at 150 rpm for 72 h.

Well-plate screening of drug metabolism

Broth containing homogenate (10 % v/v) was transferred to either 12- or 96-well plates (5 ml or 150 µl, respectively) immediately after inoculation and incubated for 72 h at 28 °C. The well plates were agitated on a Luckham R100/TW rotatest shaker at 100 rpm. Drug dissolved in dimethylformamide (0.1 mg/ml) was added to the cultures and incubated for 24 h under the same conditions. The supernatant was removed with a pipette and extracted with ethyl acetate for analysis by gas chromatography–mass spectrometry (GC–MS).

Shake flask transformation conditions

After cultivation, drug (0.1 mg/ml) dissolved in dimethylformamide was either added directly to the culture, or the spent medium was replaced with the same volume of water containing the drug. For routine transformation experiments, the fungus was incubated for a further 3–7 days at 28 °C with shaking at 150 rpm. Incubations with biofilm varied as detailed in the Results section. Supernatant from the biotransformation experiments was extracted with ethyl acetate; the solvent was removed under reduced pressure and the residue redissolved in 1 ml ethyl acetate.

The biomass of planktonic and biofilm cultures was suspended in 30 ml water and sonicated (Fischer Scientific Sonic Dismembrator, 70 % amplitude, 1 s pulse for 10–30 min) prior to extraction. The alginate beads were softened using a method similar to that described by Maguire et al. [14] by incubation at 55 °C in 20 mM MOPS buffer (pH 7) containing 200 mM sodium citrate and 54 mM NaCl, for 2 h to soften the beads. The buffer was decanted, and the 30 ml of water was added to the beads which were then sonicated (50 % amplitude, 1 s pulse, 5 min). The sonicate was extracted with ethyl acetate.

Analysis of metabolites

Gas chromatography–mass spectrometry analysis was routinely conducted on per-trimethylsilylated extracts. Silylation was performed by adding 100 µl *N*-methyl-*N*-(trimethyl-silyl)trifluoroacetamide (MSTFA, Sigma) to a dried aliquot of the extracted supernatant, and heating at 100 °C for 1 h. Derivatised samples (1 µl) were injected in the splitless mode onto a HP-5MS column (30 m × 0.25 mm × 0.25 µm) and the oven temperature held at 120 °C for 2 min, and then raised to 300 °C at 10 °C/min. The mass spectrometer was operated in the scan mode.

Organic extracts from shake flasks were analysed by reversed-phase high-performance liquid chromatography (HPLC) with a varian prostar HPLC system equipped with Zorbax SB-C18 5 µm 4.6 × 150 mm column (Agilent technologies) and a UV–Vis detector monitoring at 250 nm. Compounds were eluted with a gradient of acetonitrile/water (20–80 % acetonitrile) over 35 min at a flow rate of 1 ml/min.

Scaling the transformation for preparative scale production of metabolites

Multiple Erlenmeyer flasks containing alginate-immobilised fungus were prepared as described and incubated for 72 h. The spent medium was removed and replaced with water supplemented with diclofenac (0.1 mg/ml) and incubated for 48 h. The liquid fraction was removed, and the cells were incubated in sabouraud dextrose broth (SDB) without any drug for 24 h to allow them to recover, before the cycle was repeated with water and drug. The aqueous fractions containing the drug metabolites were extracted with ethyl acetate; the organic solvent was removed under reduced pressure and the solid redissolved in methanol. The

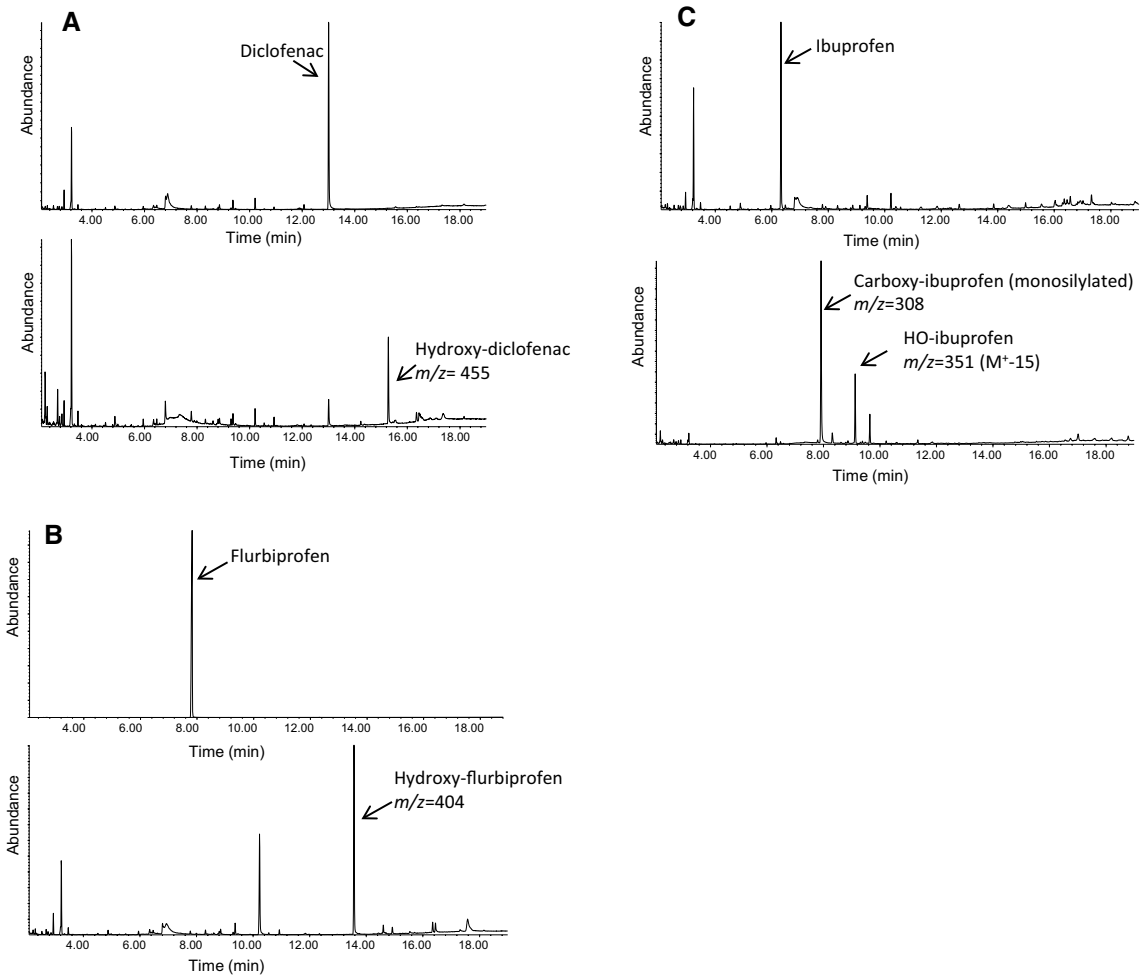


Fig. 1 GC–MS analysis of extracts from 12-well plates before (*upper chromatograms*) and after (*lower chromatograms*) incubation of *C. elegans* with diclofenac (**a**), flurbiprofen (**b**) and ibuprofen (**c**). Similar results were observed in the 96-well plates

metabolite was purified by semi-preparative HPLC with an Ascentis C18 column (15 cm × 10 mm, Supelco), eluted with a gradient of acetonitrile–water (20–80 % acetonitrile) over 40 min at a flow rate of 4 ml/min. Fractions containing hydroxylated metabolite were pooled, dried and weighed. Confirmation of structure was afforded by ^{1}H NMR, which was recorded on a Varian Inova 400 MHz spectrometer, after dissolving the compound in d_4-methanol.

Results and discussion

Screening of fungal drug metabolism

Numerous studies have been conducted employing *Cunninghamella* spp. in the biotransformation of drugs, and typically these experiments involve the fungus being cultivated in an Erlenmeyer flask. Whilst this is suitable for the transformation of a single drug, it is not practical for the screening of numerous compounds to determine whether the fungus can transform them to mammalian metabolites. Here, we employed well plates to cultivate *C. elegans* and incubate the grown fungus with diclofenac, flurbiprofen, ibuprofen and flumequine. After 24 h incubation, the supernatants in the wells were extracted with ethyl acetate, dried and *per*-trimethylsilylated for analysis by GC–MS. Hydroxylated metabolites were detected from all the drugs in both 12-well and 96-well plates, with the exception of flumequine (Fig. 1). However, the biotransformation of this drug was previously shown to occur over 7 days in shake flasks [20], and thus the shorter incubation period employed here is probably the reason for the absence of detectable metabolites. Nevertheless, the experiment demonstrates that employing well plates is a convenient and inexpensive method for screening several drugs at once.

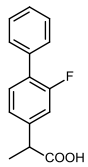
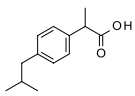
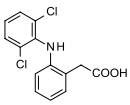
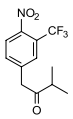
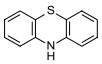
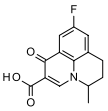
Earlier work by Griffiths et al. [10] used smaller culture volumes to screen a range of micro organisms,

including *C. elegans*, but employed 25-ml culture bottles rather than well plates. Corcoran et al. [6] used 24-well plates to screen microbial drug biotransformations, but no experiment was conducted with *C.elegans*. Interestingly, Shanmugam et al. [17] previously used 6-well plates to cultivate *C. elegans* in the presence of solid supports such as glass beads and florisil. In this case, the fungal morphology changed depending on the solid support used, and the cultures were used to transform the drug amoxapine over 14 days. The authors of this study were concerned with the effect of solid supports on the metabolism of the drug and did not indicate if the method could be used for a broader screening of drug metabolism. The morphology of the *C. elegans* in our study was similar to that of planktonic cultures in Erlenmeyer flasks, whereas in the previous work, the fungus aggregated as a pellet when grown in the absence of solid support, which may have influenced the authors' view on the general applicability of their method.

Transformation of drugs by alginate-immobilised fungus

Immobilisation of fungal mycelia in alginate for biocatalytic production of hydroxylated steroids was previously demonstrated by Peart et al. [16]; here, we used *C. elegans* spores that were allowed to grow entrapped in alginate. To evaluate the applicability of alginate-immobilised fungus in the production of drug metabolites, a comparative study was undertaken in which the immobilised and planktonic fungus was incubated with a range of drugs previously known to be transformed by suspended cultures of *C. elegans*. Analysis of the products by GC–MS revealed that the production of phase I metabolites by the immobilised

Table 1 Comparison of metabolites produced by alginate-immobilised and suspended (planktonic) *C. elegans* upon incubation with a range of drugs

Drug	Known metabolites[a]	Alginate	Planktonic	Reference[a]
 Flurbiprofen	4′-Hydroxy flurbiprofen	+++	+++	[2]
	3′,4′-Dihydroxy flurbiprofen	++	+	
	3′-Hydroxy-4′-methoxy-flurbiprofen	N.D.	+	
 Ibuprofen	Hydroxyibuprofen	+++	+++	[7]
	Carboxyibuprofen	N.D.	++	
 Diclofenac	4′-Hydroxydiclofenac	+++	+++	[13]
	3,4′-Dihydroxydiclofenac	+	++	
 Flutamide	2-Hydroxyflutamide	+++	+++	[3]
	4-Nitro-3(trifluoromethyl) analine	++	++	
	Hydroxylated flutamide	+	+	
 Phenothiazine	Phenothiazine sulphoxide	+++	+++	[18]
	3-Hydroxyphenothiazine sulphoxide	++	++	
	Phenothiazine-3-one	N.D.	N.D.	
	3-Hydroxyphenothiazine	N.D.	N.D.	
 Flumequine	7-Hydroxyflumequine (1)	+++	+++	[20]
	7-Hydroxyflumequine (2)	+++	+++	
	7-Oxoflumequine	N.D.	N.D.	

The detection of metabolites was by GC-MS or HPLC (supplemental information)

+++ = Highly abundant (as determined by peak area); ++ = Intermediate abundance; + = Trace amounts detected; N.D. = not detected

[a] The known metabolites listed refer to those reported in the literature

fungus was broadly similar to that of suspended cells when grown in shake flasks and incubated with drug (Table 1). There were differences in the minor metabolites produced from some drugs, for example, carboxyibuprofen is produced by planktonic cells, but was not detected in the alginate-immobilised culture. Also, whereas Williams et al. [20] observed only partial transformation of flumequine with suspended *C. elegans*, the immobilised cells completely transformed the substrate over the same incubation period (7 days) forming both diastereomers of 7-hydroxyflumequin. Furthermore, in the previous study, 7-oxoflumequin was also detected, but was formed neither by the suspended cells nor the immobilised cells in this study.

Preparative scale production of 4′-hydroxydiclofenac by alginate-immobilised fungus

One of the key reasons for employing an immobilised biocatalyst is that it can be recycled and used over an extended time period, which cannot be done with suspended cells. Thus a considerable effort was made to identify a feeding regime that would prolong the biocatalyst lifetime and enable up-scaling of drug metabolite production. The immobilised fungus was incubated for 72 h, and the spent medium removed and replaced with water supplemented with drug (5 mg per flask) and incubated for 48 h. The liquid fraction was removed, and the cells were incubated in Sabouraud dextrose broth without any drug for 24 h to allow them to recover, before the cycle was repeated with water and drug. By employing this protocol, 20 flasks of immobilised beads were established and incubated with a total of 400 mg of diclofenac over a period of 12 days. The products were extracted and purified by semi-preparative HPLC, yielding 171 mg of hydroxydiclofenac. The structure of the metabolite was confirmed as 4′-hydroxydiclofenac by ^{1}H NMR analysis (supplemental information), which matched the previously published data [9], and is the main human metabolite of the drug.

The yield of 4′-hydroxydiclofenac from the alginate-immobilised fungus was 43 %. This is much more efficient than the production of 4′-hydroxydiclofenac by *Epicoccum nigrum*, which was cultivated in 20 l and yielded 0.3 g metabolite from 2 g of drug (15 % yield) [19]. The recovery from the present method also compares reasonably well to that of Dragan et al. [9] who employed the fission yeast *Schizosaccaromyces pombe* that was engineered to express CYP2C9 and CYP reductase. From a total culture volume of 30 l, 2.8 g of 4′-hydroxydiclofenac was recovered, which is 93 mg/l; in our protocol, including the re-generation steps, the total volume was 3 l, and thus the recovery was 57 mg/l. However, with the immobilised *C. elegans*, no genetic manipulation was involved, no centrifugation to remove biomass was required as almost all of the metabolite is in the supernatant and the transformation can be conducted with the biocatalyst in water.

Drug metabolite production in biofilm

Whilst relatively straightforward, the preparation of alginate-immobilised cells is labour and time intensive. Therefore, we employed cells cultivated as biofilm to produce drug metabolites in a semi-continuous manner, which did not require an immobilisation step or additional reagents beyond culture medium. Biofilms are microbial cells and communities attached to a solid surface and encased in extracellular polymeric substances. They are stable for prolonged periods and demonstrate resistance to antibiotics and xenobiotics compared to planktonic cultures. These characteristics make biofilms attractive for biocatalysis [21]. We have recently shown that the fungus can form a biofilm, and that the transformation of the non-steroidal anti-inflammatory drug flurbiprofen can be catalysed over an extended timeframe compared to the typical suspended culture [1]. However, this initial work was qualitative and did not measure the concentration of the metabolites produced. Here, we quantitatively compared the production of the main phase 1 drug metabolites from three drugs, flurbiprofen, diclofenac and flutamide, by planktonic (shake flask), alginate-immobilised and biofilm cultures of *C. elegans* (Table 2). Flurbiprofen and diclofenac were undetectable in the cultures after 48 h, whereas most of the flutamide was still present after 7 days. Approximately, the same amount of 4′-hydroxydiclofenac (2 mg) was extracted from the planktonic and biofilm cultures after 24 h incubation; however, after 72 h, more 4′-hydroxydiclofenac was extracted from the planktonically grown cells (3.4 mg) than from either biofilm or alginate-immobilised cultures (2.49 and 2.28 mg, respectively). It was noticeable that more of the metabolite was detected in the biomass of the biofilm cultures that in the planktonic cells. Indeed, after 24 h incubation, the 4′-hydroxydiclofenac was predominantly present in the biomass of the biofilm cultures, and as the incubation time increased, the metabolite slowly diffused into the bulk liquid.

After 24 h incubation of *C. elegans* with flurbiprofen, 4′-hydroxyflurbiprofen was generated in approximately equal amounts (~2.5 mg) in the planktonic and biofilm cultures. After 72 h, the amount of 4′-hydroxyflurbiprofen recovered from the biofilm cultures fell dramatically in comparison to the planktonic cultures. The reason for this is not immediately clear, although it may be related to further transformation to other phase I and phase II metabolites, as 0.81 mg of 3′,4′-dihydroxyflurbiprofen was recovered from the biofilm cultures after 72 h incubation. The amount of 4′-hydroxyflurbiprofen recovered from the

Table 2 Metabolite production upon incubation of *C. elegans* planktonic, alginate-immobilised and biofilm cultures with 5 mg diclofenac, flurbiprofen and flutamide

Metabolite	Incubation time (h)	Amount (mg)					
		Planktonic		Alginate-immobilised		Biofilm	
		SN	BM	SN	BM	SN	BM
4′-OH diclofenac	24	1.92	0.70	2.08[a]	N.D.[b]	0.35	2.05
	72	2.86	0.54	2.49	N.D.	1.3	0.98
4′-OH flurbiprofen	24	1.91	0.64	1.01	0.10	1.55	1.03
	72	2.1	1.19	1.84	0.08	0.41	0.19
2-OH flutamide	168	0.34	0.16	0.51	0.04	0.35	0.15

Metabolites were extracted from culture supernatant (*SN*) and biomass (*BM*) of duplicate flasks and the values reported are the mean amounts recovered

[a] Incubation time = 48 h

[b] N.D. = not detected

Table 3 Metabolite productivity in extended biotransformation experiments with alginate-immobilised cells and biofilm

Immobilisation method	Productivity (mg/l h)			
	4′-OH diclofenac		4′-OH flurbiprofen	
	Max	Average	Max	Average
Alginate	1.16	0.71	1.40	1.03
Biofilm I	1.02	0.46	0.77	0.60
Biofilm II	1.63	1.13	0.83	0.69
Biofilm III	1.80	0.99	1.26	0.75
Biofilm IV	4.12	2.66	2.58	0.58

The alginate-immobilised cells and biofilm I were rejuvenated with Sabouraud dextrose broth for 24 h after each biotransformation experiment, biofilm II followed the same regime except that diluted medium was employed, Biofilm III was rejuvenated only once in the course of the experiment and Biofilm IV was not rejuvenated. Full details are in the text

alginate-immobilised fungus (1.94 mg after 72 h) was less than that from the planktonic culture and probably reflects the production of more dihydroxylated metabolite (Table 1).

The transformation of flutamide by planktonic *C. elegans* cultures was previously shown to be very poor [3], most likely owing to the toxicity of the compound and its metabolites. Immobilised cells of other microbes, whether trapped in alginate or cultivated in biofilm, have been shown to have increased resistance to toxic xenobiotics; however, in this case, immobilisation did not improve the production of metabolites relative to planktonically grown *C. elegans*. It was observed that most of the flutamide in the biofilm cultures was situated in the biomass, and the thickness of the biofilm noticeably decreased over the course of the experiment (results not shown). Therefore, in this instance, the presence of extracellular polymeric substance may have encouraged inhibition rather than prevented it possibly by concentrating the toxic compounds in the biomass.

Potential of biofilm for up-scaled production of drug metabolites

To further develop the observations of Amadio et al. [1], the productivity of the main phase 1 metabolites by repeated additions of diclofenac and flurbiprofen to biofilm was measured. Biofilm was cultivated for 72 h before the spent medium decanted and replaced with water supplemented with drug (5 mg). Several different rejuvenation regimes were employed: Biofilm I followed the same regime as for the alginate-immobilised cells, i.e. after 48 h incubation with drug, the supernatant was decanted and fresh Sabouraud dextrose broth was added; the biofilm was incubated for 24 h before the cycle was repeated. Biofilm II followed the same protocol except a 1:1 dilution of Sabouraud dextrose broth and water was used for rejuvenation. Biofilm III employed a single rejuvenation step with Sabouraud dextrose broth after the second drug incubation, and biofilm IV did not have a rejuvenation step, but the incubation period with drug was reduced from 48 to 24 h. For each experiment, a total of four drug biotransformation cycles was conducted by adding 5 mg drug to the culture flask at the start of each cycle. The metabolites were extracted from the combined aqueous fractions and the concentration determined by HPLC (Table 3). Compared with alginate-immobilised cells, the average production of 4′-hydroxydiclofenac was greater in biofilm, but lower for 4′-hydroxyflurbiprofen. Biofilm IV, which did not have any rejuvenation step and a shorter incubation time, was the most productive. There was a substantial variation between the maximum and average production of 4′-hydroxydiclofenac (4.12 and 2.66 mg/l h, respectively), which can

be accounted for by the observation that in the first addition of drug, the amount of 4′-hydroxydiclofenac detected in the supernatant was much lower compared with that in subsequent additions. This observation reflects the earlier measurement of substantial metabolite detected in the biomass (Table 1) and it is likely that the lag observed is a result of the establishment of a diffusion gradient across the extracellular polymeric substance. This lag period is not as pronounced in the other biofilm experiments as the incubation period for each biotransformation was 48 h. In a further experiment with biofilm IV, to determine if the lifetime of the biocatalyst could be extended, fresh medium was added after the fourth transformation of diclofenac was completed. After 24 h, the medium was decanted and the sequence of drug additions was repeated as before. The productivity did not change substantially (2.06 mg/l h), and thus the biofilm can be used for an extended period with minimal rejuvenation.

Conclusion

Although there are many reports of microorganisms transforming drugs into mammalian metabolites, a relatively small number of studies have focussed on the up-scaling of metabolite production. The US Food and Drug Administration's MIST (Metabolites In Safety Testing) guidelines [11] has created an additional need for drug metabolites, underscoring the relevance of improving microbial production. In this study, we report a workflow for the small-scale screening of drug biotransformation using well plates and application of alginate- and biofilm-immobilised *Cunninghamella elegans* for the scaled up production of hydroxylated drug metabolites. It was possible to cultivate *C. elegans* in 12- and 96-well plates and use the cultures directly for drug biotransformation, which is much more convenient than the commonly employed shake flask method. Alginate-immobilised fungus suspended in water catalysed the biotransformation of drugs to the same metabolites as the suspended cultures, and could be used to produce preparative amounts of 4′hydroxydiclofenac upon repeated additions of the parent drug. The biofilm biocatalyst was more efficient than the alginate-immobilised fungus, in both the preparation phase, requiring no immobilisation step, and in relation to productivity of hydroxylated metabolites. Crucially, the volumetric productivity of the biofilm exceeded the minimum process requirements for pharmaceuticals of 1 mg/l h [12], though further optimisation and bioreactor development is necessary to improve productivity.

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