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Filamentous fungal biofilm for production of human drug metabolites

Jessica Amadio,¹ Eoin Casey² and Cormac D. Murphy^{1*}

1. School of Biomolecular and Biomedical Science and Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland
2. School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland

*Corresponding author Fax: +353 (0)1 716 1183, Telephone: +353 (0)1 716 1311, email: Cormac.d.murphy@ucd.ie

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Abstract

In drug development access to drug metabolites is essential for assessment of toxicity and pharmacokinetic studies. Metabolites are usually acquired via chemical synthesis, although biological production is potentially more efficient with fewer waste management issues. A significant problem with the biological approach is the effective half-life of the biocatalyst, which can be resolved by immobilisation. The fungus *Cunninghamella elegans* is well established as a model of mammalian metabolism, although it has not yet been used to produce metabolites on a large scale. Here we describe immobilisation of *C. elegans* as a biofilm, which can transform drugs to important human metabolites. The biofilm was cultivated on hydrophilic microtiter plates and in shake flasks containing a steel spring in contact with the glass. Fluorescence and confocal scanning laser microscopy revealed that the biofilm was composed of a dense network of hyphae, and biochemical analysis demonstrated that the matrix was predominantly polysaccharide. The medium composition

was crucial for both biofilm formation and biotransformation of flurbiprofen. In shake flasks the biofilm transformed 86 % of the flurbiprofen added to hydroxylated metabolites within 24 h, which was slightly more than planktonic cultures (76 %). The biofilm had a longer effective lifetime than the planktonic cells, which underwent lysis after 2×72 h cycles, and diluting the Sabouraud dextrose broth enabled the thickness of the biofilm to be controlled while retaining transformation efficiency. Thus, *C. elegans* biofilm has the potential to be applied as a robust biocatalyst for the production of human drug metabolites required for drug development.

Introduction

The production of metabolites, required for the safety assessment of drugs, traditionally involves the use of animals or chemical synthesis. There are disadvantages to both approaches, e.g. ethical concerns, environmental and waste management issues, which can be avoided by employing biocatalysis. In this study we aimed to develop an immobilised microbial biocatalyst for the production of human drug metabolites.

The key enzyme class involved in phase I (oxidative) metabolism of drugs is the cytochromes P450 (CYP). These enzymes are also present in microorganisms and can be exploited for biotransformation reactions. The relative ease of scalability of microorganisms means that they can be used in the production of key drug metabolites (Dragan et al. 2011). Bacterial CYPs are soluble, whereas eukaryotic CYPs are membrane-bound, and the bacterial enzymes have narrow substrate specificities, thus are not good models of mammalian drug transformation (Jezequel, 1998). In contrast, the fungus *Cunninghamella elegans* has been shown to transform a broad range of drugs and xenobiotics to the same metabolites as humans (Asha and Vidyavathi 2009; Amadio et al. 2010; Amadio and Murphy 2011). Although the majority of experimental protocols for drug transformation refer to planktonic cells cultivated in liquid cultures in small scale vessels, immobilizing cells can improve metabolite production, for example, Osorio-Lozada et al. (2008) employed *Actinoplanes* sp. trapped in a hollow-fibre cartridge to generate hydroxylated metabolites of diclofenac.

Biofilms are surface-associated communities of microorganisms encased in extracellular polymeric substances (EPS) and are widely studied in the fouling of surfaces and medical device infections (Flemming 2002; Hall-Stoodley et al. 2004). Interestingly, the same characteristics that make biofilms problematic in some areas, such as resistance to toxic compounds and long-term stability, are beneficial for biocatalysis, and reports are now emerging of the application of biofilms in this area. Gross et al. (2007) demonstrated that a

mutant strain of the styrene degrader *Pseudomonas* sp. VLB120, that contained a knock-out mutation of the isomerase involved in the styrene degradation pathway, could transform styrene to (*S*)-styrene oxide in a tubular biofilm reactor, which was stable for 55 days. A biocatalyst biofilm of recombinant *E. coli* expressing tryptophan synthase from *Salmonella enterica* sv Typhimurium was generated by spin coating poly-L-lysine covered slides and used to produce halotryptophans from serine and 5-haloindole (Tsoligkas et al. 2011). Recently, Singh et al. (2011) reported the first observations of zygomycete fungi growing as biofilms, by studying the morphology and matrix characteristics of the medically relevant fungi *Rhizopus oryzae*, *Lichtheimia corymbifera* and *Rhizomucor pusillus*. However, as far as we are aware, no work has been reported of a filamentous fungus growing as a biofilm being applied as a biocatalyst for fine chemicals production.

We now report for the first time the cultivation of the zygomycete model of mammalian metabolism, *C. elegans*, as a biofilm and the application of the surface-attached fungal cells as biocatalysts for the production of hydroxylated metabolites of the fluorinated anti-inflammatory drug flurbiprofen.

Materials and methods

Standardization of biofilm formation on microtiter plates.

Spores of *Cunninghamella elegans* strain DSM 1908 (DSMZ, Germany), grown on Sabouraud dextrose agar plates for 5 days at 28 °C, were collected by washing with 10 ml phosphate buffer saline (PBS) pH 7.2 and were counted using a haemocytometer cell counting chamber. Spore suspensions were prepared in Roswell Park Memorial Institute Medium (RPMI) 1640, Sabouraud Dextrose Broth (SDB), or RPMI and SDB (1:1). All media were purchased from Sigma, Ireland. The inoculum (1×10^4 - 1×10^7 spores/ml) was transferred to standard 96-well plates (Sarstedt, Co. Wexford, Ireland) with a final volume of

200 μ l, and incubated at 28 °C for 24 h. The biofilms were washed twice with PBS, fixed with 95 % ethanol at 37 °C for 15 min and stained with safranin for 5 min. Biofilm formation was observed under a microscope (Nikon, Japan) and was quantified by measuring the absorbance of the bound safranin at 490 nm using a microtiter plate reader (Spectra Max 340, Molecular Devices).

Preliminary characterization of biofilm matrix.

C. elegans 1908 spores in RPMI (1×10^5 spores/ml) were incubated in 96-well plates in static conditions from 24 to 72 h at 28 °C. Biofilms were washed twice with PBS and treated separately with 200 μ l of sodium (meta)periodate (40 mM), proteinase K from *Engyodontium album* (0.5-1 mg/ml) and deoxyribonuclease I from bovine pancreas (0.5-5 μ g/ml) (all from Sigma). After incubation for 24 h at 37 °C the biofilms were washed twice with PBS, fixed and stained; biofilm was quantified by measuring the absorbance of the bound safranin at 490 nm.

Fluorescence and confocal laser scanning microscopy of biofilm matrix.

C. elegans 1908 was inoculated in 96-well plates (Cornig® CellBind®, Costar) as described. Biofilms were washed twice with PBS and stained with 200 μ l of 10 μ g/ml wheat germ agglutinin (WGA) Oregon green® 488 conjugate (Invitrogen) for 10 minutes at 37 °C. Biofilms were washed twice with PBS and analysed by fluorescence (Axiovert 200 M, Carl Zeiss) and confocal laser scanning microscopy (LSM510Meta, Carl Zeiss). The constrained, iterative deconvolution was performed by the Autoquant X2.2.0 software based on theoretical PSF and the volume rendering using Imaris 7.2.3 software.

Biotransformation of flurbiprofen on biofilm on microtiter plate.

C. elegans 1908 in 2 ml RPMI (1×10^5 spores/ml) was inoculated in 12-well plates (Cornig® CellBind®, Costar) in static conditions for 72 hours at 28 °C. Biofilms were washed twice with PBS and 2 ml of RPMI, a mixture of RPMI and SDB (1:1) or SDB was added. Flurbiprofen dissolved in dimethylformamide was added to the cultures at final concentration 0.1 mg/ml. After up to three days incubation at 28 °C the biofilms and supernatants were extracted with 2 ml of ethyl acetate; the organic layer was removed and evaporated to dryness.

Biotransformation of flurbiprofen by biofilms on stainless steel spring.

C. elegans homogenate (2 % v/v) was used to inoculate 50 ml of either SDB or RPMI medium in 250 ml Erlenmeyer flasks with or without stainless steel compression springs (1.2 mm T316 wire, Shannon Coiled Springs, Ireland). Cultures were incubated at 28 °C with shaking at 150 rpm for 72 hours. Flurbiprofen (0.1 mg/ml) was added and cultures incubated for up to three days. Control experiments were conducted in either the absence of flurbiprofen or fungi. Biomass and supernatants were separated either by centrifugation (suspended cells) or by decanting (biofilm), and extracted with 50 ml of ethyl acetate, and the organic layer evaporated to dryness. Organic extracts were finally dissolved in 1 ml of methanol.

Biofilm was quantified *in situ* by decanting the liquid from the flask, drying the flask and contents at 80 °C overnight and weighing. The dry weights of suspended cells were measured after harvesting the cultures by centrifugation and drying at 80 °C overnight.

Analysis of fluorometabolites.

Gas chromatography-mass spectrometry (GC-MS) analysis was routinely conducted on *per*-trimethylsilylated extracts. Silylation was performed by adding 100 µl *N*-methyl-*N*-

(trimethyl-silyl)trifluoroacetamide (MSTFA, Sigma) and heating at 100 °C for 1 hour. 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 then raised to 300 °C at 10 °C min⁻¹. The mass spectrometer was operated in the scan mode. The degree of biotransformation was determined from peak areas of flurbiprofen and the hydroxylated metabolites.

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 5µm 4.6x150mm 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 30 minutes at a flow rate of 1 ml/min. Flurbiprofen from 0.1 to 1 mg/ml final concentration dissolved in methanol was used to construct a calibration curve.

Results

Standardization of biofilm formation on microtiter plate.

It was recently reported that selected zygomycete strains showed biofilm-forming capacity on flat-bottomed polystyrene microtiter plates (Singh et al. 2011). The starting inoculum concentration, presence of nutrients and charged surfaces may strongly influence biofilm formation and adhesion (Djordjevic et al. 2002; Cerca et al. 2005; Pierce et al. 2008), thus these factors were investigated in relation to *C. elegans*.

To investigate the importance of spore density on biofilm formation, *C. elegans* spores, in densities ranging from 1x10⁷ to 1x10⁴ spores/ml, were inoculated in RPMI or SDB medium in 96-well plates, and the biofilm growth observed over 72 hours. Fungal spores inoculated in the nutrient-rich SDB grew at the liquid-air interface showing aerial hyphae and

submerged mycelium, the morphological growth typical of that on solid media such as agar. There was no attachment at the bottom of the wells for any spore concentration. In contrast, fungal spores in RPMI medium attached to the plastic surface forming biofilm to varying degrees depending on the initial spore density (Fig 1A). After 24 hours inocula with 1×10^6 and 1×10^5 spores/ml showed the highest and most uniform biofilm formation (Fig 1B), whereas 1×10^4 spores/ml resulted in substantially reduced hyphal density. The wells inoculated with 1×10^7 spores/ml had biofilm growth around the circumference of the wells, but there was no attachment in the centre, resulting in a very low absorbance measurement. These conditions are very similar to those that resulted in optimum biofilm growth of other zygomycete fungi (Singh et al., 2011).

Biofilm matrix characterization.

The presence of polysaccharides, proteins and DNA, which are the major components of the matrix of bacterial biofilms (Flemming and Wingender 2010), was investigated in the *C. elegans* biofilm. Biochemical characterization of the biofilm matrix was investigated by the addition of sodium meta-periodate, proteinase K and DNase I, which are reagents used to determine the composition of biofilm matrices (Tetz et al. 2009; Gjermansen et al. 2010). The amount of biofilm attached to the surface after treatment was quantified by measuring the absorbance at 490 nm of bound safranin (Fig 2). Addition of sodium meta-periodate, which specifically destroys sugars containing unsubstituted dihydroxyl groups, to biofilm cultivated in 96-well plates resulted in complete disintegration of the biofilm matrix after 2 h. Biofilms in presence of both proteinase K and DNase I were incubated up to 24 h, but no release of adherent cells was observed at any tested concentration. These data strongly suggest that *C. elegans* biofilm matrix consists mainly of polysaccharides while the presence of proteins and DNA is scarce. The results are in accordance with the previous finding

reporting that EPS of selected zygomycetes was primarily composed of amino sugars with proteins present in small quantities (Singh et al. 2011).

The presence of polysaccharides within the matrix was visually identified by staining *C. elegans* biofilms with wheat germ agglutinin (WGA) conjugated to Oregon green, a fluorescent lectin that selectively binds to *N*-acetylglucosamine residues. Fig 3 shows both the elongated hyphae and the surrounding hazy area that forms the polysaccharide part of the matrix. Confocal laser scanning microscopy revealed that the thickness of the biofilm cultivated in the wells increased from approx. 106 μ m after 24 h to 166 μ m after 48 h, and the hyphae were less densely packed with more gaps after 48 h (supplemental information, Video S1 and S2).

Biotransformation of flurbiprofen on microtiter plate.

C. elegans DSM 1908 is very well known to be a model for mammalian drug metabolism (Asha and Vidyavathi 2009), and we have shown previously that the suspended mycelia transform the drug flurbiprofen to mono- and di-hydroxylated metabolites (Amadio et al. 2010). This drug was used to study the capability of the biofilm for biotransformation. *C. elegans* was grown in RPMI in 12-well plates for 72 hours, the original medium removed, biofilms washed and fresh medium (RPMI, SDB or a 1:1 mixture of RPMI + SDB) with flurbiprofen (0.1 mg/ml) was added. GC-MS analysis of silylated extracts of the supernatant and the biofilm revealed that flurbiprofen was degraded over 3 days to varying degrees depending on the medium used (Fig 4). In RPMI almost no flurbiprofen was metabolized, but when the medium was replaced with a mixture of RPMI and SDB (1:1), 22% of flurbiprofen was converted into 4'-hydroxyflurbiprofen. When the original RPMI medium was removed and replaced with SDB, flurbiprofen was completely transformed to hydroxylated metabolites.

It is likely that some detachment also occurred during the experiment; therefore, it was important to determine if the detached cells were partially or totally responsible for the biotransformation observed. Supernatants were collected from biofilm-containing wells after 24 and 48 hours incubation with flurbiprofen in SDB medium, and transferred to empty clean wells for a further 48 and 24 hours, respectively (thus, the total incubation time with the drug was 72 hours). The metabolism of flurbiprofen in these wells was then compared with that from biofilm wells. GC-MS analysis showed that 39 and 52% of flurbiprofen from supernatants removed at 24 and 48 hours, respectively, was converted into 4-hydroxyflurbiprofen compared with complete transformation when the drug remained in contact with biofilm. Approximately 10% of flurbiprofen was transformed in the biofilm wells after 24 h, thus some further metabolism occurred when the supernatant containing any suspended cells was transferred to fresh wells. Nevertheless, these data suggest that cells embedded in biofilm play the predominant role in drug metabolism.

Shake flask biofilm transformation.

The application of immobilized, viable microbial cells for biotransformation and production of useful metabolites has gained increasing attention in recent years (Illanes et al. 2012). Several studies have been reported on immobilization techniques applied to filamentous fungi (Pakula and Freeman 1996; Dlugonski et al. 1997) but little is known about the potential of biofilms (Harding et al. 2009). Pakula and Freeman (1996) showed that a stainless steel mesh was required to catch mycelium fragments and start a gradual biofilm development in a continuous biofilm bioreactor for an oil-degrading filamentous fungus, *Tyromyces sambuceus*. Based on this experimental procedure, stainless steel supports of different sizes and shapes inserted into petri dishes or conical flasks were tested for *C. elegans* biofilm development in SDB medium (supplemental information, Fig S1). Under almost all

conditions biofilm growth was poor, or aerial hyphae were observed; the only support that showed good biofilm growth was with a stainless steel spring when in contact with the inside of the flask, and when the culture was shaken. After 6 h all the mycelium fragments had adhered onto the metallic spring leaving the medium completely clear. The surface-attached cells were washed thoroughly and vigorously several times during the incubation period of 6 days and the cells were persistently and strongly attached to the spring (Fig 5A-C). It was observed that the length and position of the spring was crucial; loose springs not in contact with the flask wall resulted in non-uniform or no biofilm adhesion (Fig 5D). Flasks containing springs, but incubated without shaking, resulted in growth as aerial mycelia at the air-liquid interface only (Fig 5E), which is similar to the morphology observed in well-plates with SDB. Dry weight measurement indicated that biomass in flasks peaked at 0.55 g on day 3 and declined to 0.4 g by day 6.

The next step was to investigate biotransformation performance of *C. elegans* biofilm and compare it with suspended cells. After 72 hours of fungal growth 5 mg of flurbiprofen was added and HPLC analysis of extracts showed that the amount of flurbiprofen transformed over 24 h was 4.28 mg (86%) for surface-attached cells and 3.6 mg (72%) for planktonic cells, which equated to a specific transformation of $16 \mu\text{g} \cdot \text{mg}^{-1} \cdot \text{day}^{-1}$ in biofilm and $13 \mu\text{g} \cdot \text{mg}^{-1} \cdot \text{day}^{-1}$ in planktonic cells. The main metabolite detected in both cases was 4'-hydroxyflurbiprofen but other hydroxylated metabolites were present. Most of the product was detected in the supernatant, but some was extracted from the biomass (17% from biofilm and 9% from suspended cells). Thus, transformation in the biofilm is at least as efficient as planktonic cultures.

It was further investigated if RPMI was the optimum medium for the formation of biofilm by filamentous fungal cells on springs as it was for microtiter plates. After 72 h growth of both planktonic cells and biofilm showed a drastically reduced biomass compared

with cultures grown in SDB, although this was expected since SDB is a much richer medium. Biotransformation of flurbiprofen was carried out to test if the RPMI medium would allow the production of CYP enzymes responsible for the transformation of the drug. GC-MS data showed that only 3% of flurbiprofen was biotransformed into 4'-hydroxyflurbiprofen by suspended cells and 23% in the biofilm flasks. Thus, for transformation in shake flasks the optimal medium for *C.elegans* biofilm formation and biotransformation was SDB. It was interesting to note that although the growth was not optimal in RPMI, fungal cells immobilised as biofilm were able to convert the drug more efficiently than non-attached filamentous hyphae, suggesting increased CYP activity in biofilm.

Recycling of biofilm in shake flask.

One of the greatest advantages of using immobilized cells is their application in continuous processes for greater productivity (Illanes et al. 2012). A preliminary study on the potential of reusing filamentous fungi as biofilms compared to suspended cells was conducted. Fig 6A shows GC-MS analysis of two cycles (72 h) of biotransformation of flurbiprofen conducted over 6 days in SDB (replaced after each cycle). Biotransformation was complete in biofilm growing cells after the first and second cycle and 4'-hydroxy- and 3',4'-dihydroxy-metabolites were extracted from the supernatants. In comparison, planktonic cells converted about 80% of flurbiprofen in the first cycle and completely in the second, which yielded only 4'-hydroxyflurbiprofen. One additional cycle of 3 days incubation with drug was performed but after 24 hours there was complete lysis of the cells in suspended culture. In contrast, cells growing as biofilm retained their structural integrity. This might be explained since cells in the biofilm have the advantage of being protected from mechanical stresses caused by continuous shaking and repeated centrifugation cycles applied to non-immobilized cells. The third cycle of biotransformation for biofilm growing cells showed that flurbiprofen was

completely converted into the main monohydroxy (82%) and dihydroxy (18%) fluorometabolites. It can be concluded that biofilm is a more robust system and easier to handle for repeated processes than planktonic cells.

It was observed during the course of the recycling experiments that the biofilm became very thick, reaching a final wet weight of 56 g. Furthermore, after the third cycle, over half the original volume of liquid added (50 ml) was retained by the biofilm. The extensive growth can be attributed to the use of fresh SDB in each cycle, thus in an effort to control the biofilm thickness and improve product recovery, the medium used in the recycling experiments was modified. Thus the biofilm was initially grown in SDB for 48 h then replaced with either diluted SDB (half-strength) or water containing flurbiprofen. The biofilm thickness over the same timeframe, in which the medium was replaced every 72 h, was reduced to 43 g and 16 g when the biofilm was incubated with dilute SDB and water, respectively, and the volumes of liquid recovered were 70 % (diluted SDB) and 100 % (water) of the original volume.

To assess whether the biofilm could transform flurbiprofen in dilute media, the original medium was replaced after 72 h growth with either diluted SDB (half strength and quarter strength) or water, plus flurbiprofen. After 24 h the supernatant was decanted and extracted and fresh dilute SDB or water (plus drug) was added and the experiment repeated. Fig 6B shows the GC-MS analysis of the extracted products for up to 4 cycles. The drug was completely transformed to the main hydroxy metabolites after 24 hours in half-strength SDB with an increase of the proportion of di-hydroxy- metabolite from cycle one to four. After the first cycle both cultures in quarter-strength SDB or water showed unmetabolized flurbiprofen (36% and 72% respectively), but after the second cycle all the flurbiprofen was converted into the hydroxylated metabolites.

Discussion

We have shown for the first time that the filamentous fungus *C. elegans* can form a robust biofilm, which can transform a drug to generate valuable human metabolites. The capacity of *C. elegans* in forming biofilms was firstly confirmed on flat bottom microtiter plates, which enabled investigation of some characteristics of the biofilm, and opened the possibility of miniaturized screening of drug biotransformation. Medium, inoculum concentration and surface type were the parameters that impacted on biofilm formation. RPMI, a basal medium (pH 7.0-7.4), strongly promoted biofilm formation compared with nutrient rich SDB (pH 5.6). The pH and reduced nutrient load, together with an optimum spore density, might be triggers for switching on genes necessary for biofilm formation on this particular surface.

The matrix consists of extracellular polymeric substances (EPS) secreted by biofilm cells and it provides the microenvironment needed to sustain microbial life taking part in each of the fundamental steps of biofilm development (Flemming and Wingender 2010). Biochemical assays conducted on *C. elegans* matrix revealed that the main component was polysaccharide, and microscopy images showed how hyphae were enfolded by this matrix, which appeared as a haze (Fig 3). Singh et al. (2011) determined that the main monomeric constituent of the biofilm matrix in other zygomycete fungi was glucosamine and concluded that the matrix was mainly chitosan. It is likely that the matrix of *C. elegans* has a similar composition, since chitosan is a major cell wall component of this fungus (Stamford et al. 2007).

Recent studies suggested the application of biofilms as robust, self-immobilized and self-regenerating catalysts, thus have the potential to be effective tools for the industrial production of chemicals (Li et al. 2007; Winn et al. 2012) but relatively little interest has been shown in filamentous fungi. Having demonstrated that *C. elegans* biofilms in well plates could transform flurbirpofen, we then investigated biotransformation in biofilm

growing on stainless steel supports in conical flasks. Measurements of microbial adhesion and biofilm formation on stainless steel surfaces have been extensively studied in the past especially in relation to biofouling and biocorrosion (Kielemoes et al. 2000). It was noticed in our experiments that *C. elegans* fragmented hyphae or spores inoculated in liquid medium strongly attached to a stainless steel spring coiled inside a flask and gradually formed a biofilm. The localized hydrodynamic turbulence adjacent to the spring surface most likely enhanced the adhesion and accumulation of biofilm. Furthermore, it was observed in this study that in static well-plates and flasks, with SDB as the medium, the fungus formed a mat with aerial hyphae at the liquid air interface, strongly suggesting that oxygen availability plays an important role in the formation of biofilm.

Immobilized cells may have a more efficient rate of biotransformation over a longer time period compared with planktonic cells, owing to the protective microenvironment given by the immobilized structure (Illanes et al. 2012). A comparison of flurbiprofen biotransformation in planktonic and biofilm *C. elegans* cultures demonstrated that in a small scale fermentation the conversion of flurbiprofen was similar among the two systems, but upon recycling of the biocatalyst the planktonic cells underwent lysis and could not be reused, whereas the biofilm maintained its integrity and had a longer effective lifetime.

In both well plates and shake flasks, the medium composition was crucial to biotransformation of flurbiprofen. Biofilm formed when *C. elegans* was grown in RPMI, in both the well plates and shake flasks, but little or no transformation was observed in this medium. Substantial biotransformation by biofilm only occurred in the presence of SDB, which is a more nutritious medium. The extent of biotransformation is related to the amount of biomass and the induction of cytochrome P450 (CYP). In shake flasks there was clearly more biomass when SDB was used as the culture medium, which contributed to the more efficient biotransformation. However, in well plates, where the biofilm was initially cultivated

in RPMI, which was then replaced with SDB to promote biotransformation, the induction of CYP was likely to be the most important factor. While induction of CYP in microorganisms has not been extensively investigated, there are some studies that illustrate the importance of carbohydrate concentration in biotransformation. For example, Dai et al. (2007) demonstrated that sucrose played a key role in the CYP-catalysed transformation of the insecticide imidacloprid by the bacterium *Stenotrophomonas maltophilia*, and suggested that the addition of sucrose might be linked to the regeneration of NADH, which is required for CYP.

In the shake flask biofilm became very thick when fresh SDB medium was continually used. CYP incorporates oxygen from O₂ into substrates and excessive biofilm thickness might reduce specific biocatalytic efficiency owing to a reduction in oxygen transfer (Misiak et al. 2011). By employing diluted medium it was possible to control thickness and shorten the timeframe required for biotransformation. For large scale application it is important to reduce the production costs, such as those associated with media, without interfering with the performance. Furthermore, separation of the biocatalyst from the product was very straightforward (decanting), which adds to the potential of *C. elegans* biofilms as industrial biocatalysts. Clearly the potential of these biofilms requires further investigation and optimisation, as does the underlying mechanisms of their formation.

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Figure legends

Fig. 1 *C. elegans* biofilm formation on 96-well plate after 24 hours using an inoculum of varying spore density. The histogram (A) shows the absorbance at 490 nm of safranin-stained biofilm in RPMI medium from 1×10^7 to 1×10^4 spore/ml starting inoculum concentration; a well to which no inoculum had been added is shown as a control. Fig B shows images of microtiter plate wells with *C. elegans* biofilm at concentration from 1×10^7 to 1×10^4 taken with a camera mounted on the microscope (magnification $\times 25$). Experiments were conducted in quintuplicate.

Fig. 2 *C. elegans* biofilm matrix characterization. The figure shows the measure of absorbance at 490 nm of safranin bound to biofilm after 24 hours treatment with sodium meta-periodate (SMP), proteinase K (PK) and DNase I (DNase) (blank: empty wells; control: stained empty wells, buffer: biofilm in buffer). Experiments were conducted in quintuplicate.

Fig. 3 Fluorescence micrograph of *C. elegans* biofilm at 24 hours growth in RPMI. Biofilm was stained with WGA that selectively binds to *N*-acetylglucosamine residues of hyphae and matrix.

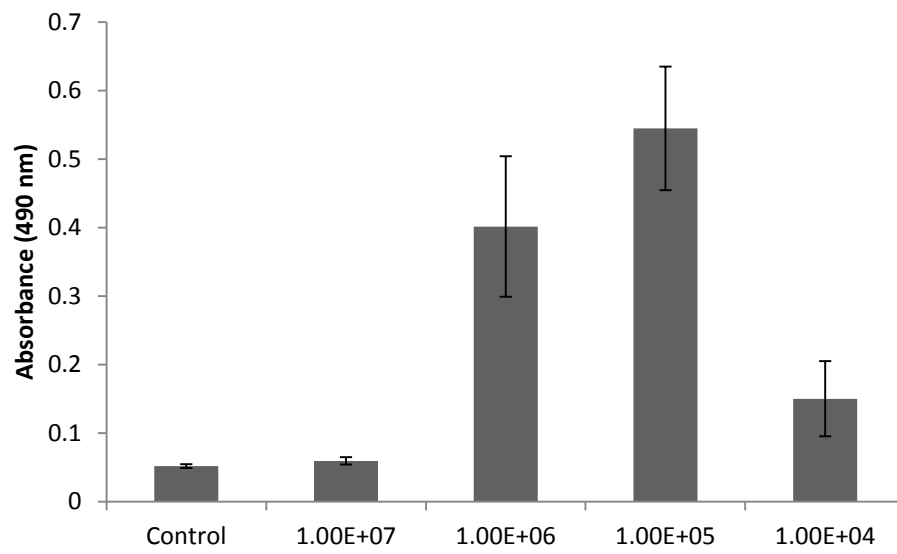
Fig. 4 Biotransformation of flurbiprofen to 4'-hydroxy- and 3',4'-dihydroxy-flurbiprofen by *C. elegans* biofilm in different media. Experiments were conducted in duplicate; the data are presented as a mean of the replicates, based on the relative areas from the total ion chromatogram. The difference between the replicates was less than 5 %.

Fig. 5 *C. elegans* biofilm formation and growth on stainless steel spring after 0 (A), 1 (B) and 6 (C) days. When the spring was not completely in contact with the flask inner wall there was poor and non-uniform biofilm formation (D). If the flask was not shaken during incubation, the fungus grew only at the air-liquid interface (E) forming aerial mycelia.

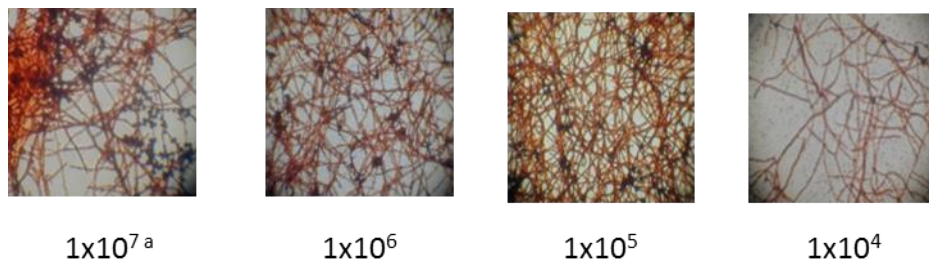
Fig. 6 Repeated cycles of biotransformation of flurbiprofen by *C. elegans* biofilms. (A) Cycles (1-3) of 72 hours each of biotransformation in biofilm and non-immobilized free cells in SDB medium. (B) Cycles (1-4) of 24 hours each of cell-immobilized biofilm in SDB:water 1:1 (a), SDB:water 1:4 (b) and water (c). Vertical axes report the percentage of metabolites converted and horizontal axis the number of cycles.

Fig 1. Amadio et al.

A



B



- a. This image was taken from the edge of the well as there was no biofilm in the middle.

Fig 2. Amadio et al.

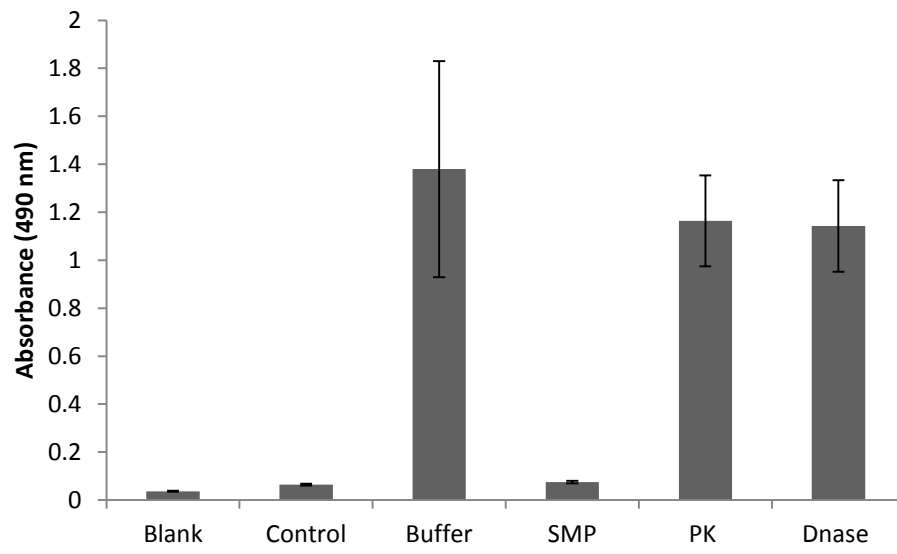


Fig 3. Amadio et al.

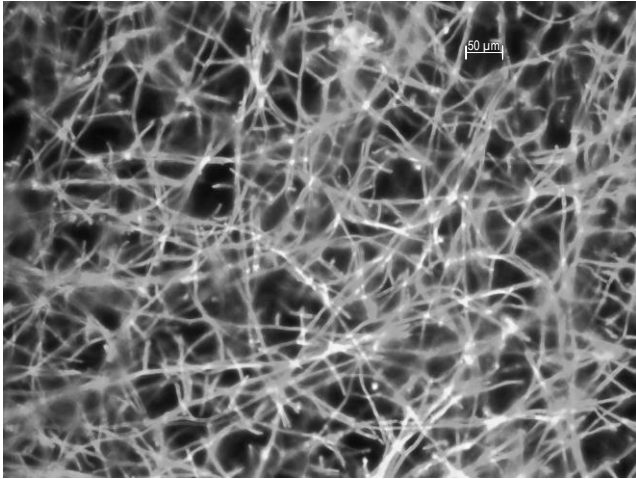


Fig 4. Amadio et al.

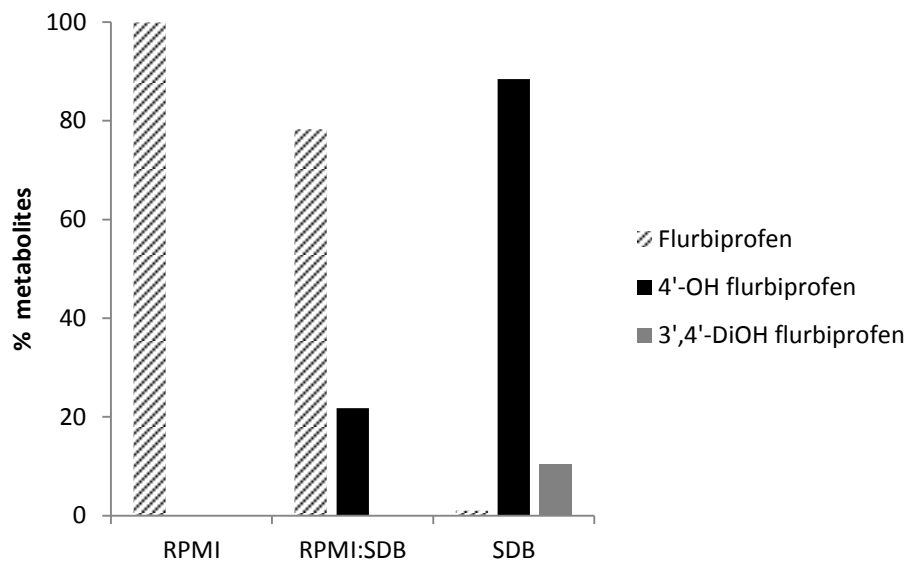


Fig 5. Amadio et al.

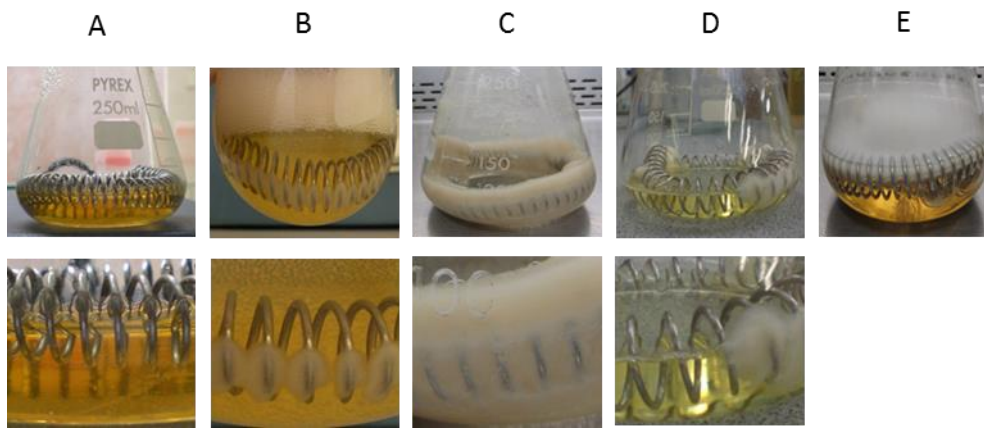
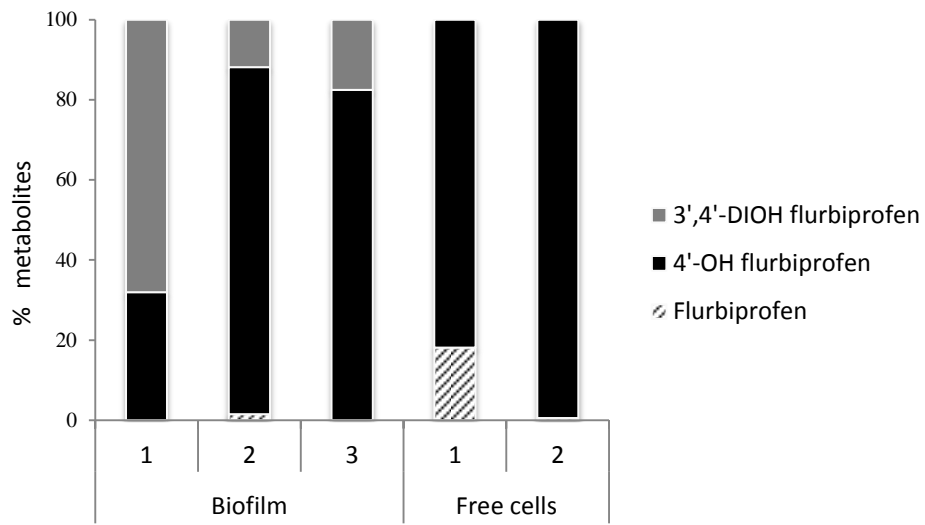


Fig 6. Amadio et al.

A



B

