

## **Biosynthesis of Drug Metabolites Using Microbes in Hollow Fiber Cartridge Reactors: Case Study of Diclofenac Metabolism by *Actinoplanes sp.***

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Abbreviations: DIC, diclofenac; 4'-OH-DIC, 4'-hydroxy diclofenac; 5-OH-DIC, 5-hydroxy diclofenac; 4',5-diOH-DIC, 4',5-dihydroxy diclofenac; HFC, hollow fiber cartridge; ECS, extra-capillary space; CID, collision induced dissociation; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multi-bond coherence; ATCC, American type culture collection.

## Abstract

Fungal and bacterial microbes are known to mimic mammalian cytochrome P450 metabolism. Traditionally, microbial biotransformation screening and small scale-ups (< 1L) are performed in shake-flask reactors. An alternative approach is the use of hollow fiber cartridge (HFC) reactors. The performance of HFC reactors is compared to shake-flask reactors using diclofenac as a model substrate. *Actinoplanes sp.* (ATCC-53771) in a shake-flask reactor hydroxylated diclofenac (50  $\mu$ M) with 100% turnover in less than 5 hours. A scaled-up production resulted in the formation of 4'-hydroxy (169 mg, 54% yield), 5-hydroxy (42 mg, 13 % yield) and 4',5-dihydroxy (25 mg, 7.7% yield) metabolites. HFC reactors with Teflon, poly-sulfone, and cellulose membranes were screened for non-specific binding of diclofenac. Concentration-time profiles for turnover of 50-2000  $\mu$ M diclofenac by *Actinoplanes sp.* were then determined at 22°C and 30 °C in an HFC reactor. Cellulose-based HFC reactors exhibited the lowest non-specific binding (87% of 50  $\mu$ M diclofenac remaining after 5 hr) and offered the best conditions for its biotransformation (100% conversion; < 5hr at 30 °C at 50  $\mu$ M; 25 hr at 500  $\mu$ M). The time profile for substrate turnover was equivalent in both a cellulose membrane HFC reactor and shake-flask reactor. Two cellulose membrane HFC reactors were also tested to evaluate the reusability of the cartridges for diclofenac metabolism (50  $\mu$ M, 22 °C, 15 hr; 500  $\mu$ M, 30 °C, 36 hr). Up to seven reaction cycles with intermediate wash cycles were tested. At least 98 % conversion was observed in each reaction cycle at both diclofenac concentrations.

## Introduction

There are several fungal and bacterial microbial systems known to mimic mammalian metabolism of xenobiotics in which the biotransformations are carried out by the constituent cytochrome P450(s). The term “Microbial Models of Mammalian Metabolism” was coined in the mid-70s by Smith and Rosazza (1974) to describe these systems. Since then many examples have been published where milligram amounts of mammalian metabolites, sufficient for biological and other evaluations, have been produced by microbial fermentation methods (recent examples, Zmijewski et al. 2006; Zhang et al. 2006).

Microbial fermentation methods typically utilize a two-stage fermentation protocol (Goodhue CT, 1982). A common approach (Hilton MD, 1999) is to add substrate to a stage II culture in a sterile shake flask, incubate on an orbital shaker for a suitable period of time, quench, centrifuge or filter, and then extract supernatants to obtain the bio-transformed products. This method has been successfully used to screen for microbes that make the metabolite of interest as well as perform small scale (< 1 L) biosynthesis with the identified microbe. One shortcoming of this configuration, however, is the destruction of the microbial bio-catalyst when the reaction is quenched, which limits each preparation to a single reaction.

A hollow fiber cartridge (HFC) reactor (described in the experimental section) provides a configuration that isolates a relatively small volume of microbial culture from a larger reservoir of culture medium via a semi-permeable membrane (Knazek et al. 1972). The membrane permits exchange of nutrients, substrate, and metabolites, and provides two potential advantages of an HFC reactor over a shake-flask system. The excretory products that over time might otherwise inhibit the viability of the microorganism diffuse away from the culture and are diluted by the larger volume of medium in the reservoir. Also, because the metabolites also diffuse through the capillaries into the circulating medium, they can be harvested without disturbing the bio-catalyst. The bio-catalyst may then be potentially reused many times over.

Diclofenac sodium (Voltaren) is a non-steroidal anti-inflammatory drug widely prescribed anti-inflammatory and anti-pyretic analgesic. Its principal phase-I metabolic

products formed in vivo and in vitro in rats, dogs, baboons, and humans, are 4'-hydroxy and 5-hydroxy metabolites (Stierlin et al 1979, Shen et al. 1999, Leemann et al. 1993). Minor *in vivo* oxidative metabolites include 3'-OH and 4',5-dihydroxy diclofenac (Stierlin et al. 1979).

In this study, we report (1) the efficient turn over of diclofenac to its hydroxylated metabolites by the bacterium *Actinoplanes sp.* in a shake-flask incubation; (2) use of a shake-flask system to scale-up production of the 4'-hydroxy, 5-hydroxy, and 4',5-dihydroxy metabolites of diclofenac; (3) the efficient use of HFC reactors to produce turnover rates comparable to a shake-flask configuration; and (4) demonstration that an HFC reactor can be reused for many ( $n > 5$  over 10 days) cycles.

## Experimental Methods

### Solvents and reagents

Diclofenac (sodium salt) was obtained from Sigma Chemicals (St. Louis, MO) and its stock solutions were prepared in a 1:1 mixture of DMSO/H<sub>2</sub>O. All solvents were of analytical grade or higher. Sterile super-optimal medium C (SOC), prepared in house, consisted of 2 % tryptone, 0.5 % yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 20 mM glucose, 10 mM each of magnesium sulfate and magnesium chloride. Sterile terrific broth complete (TBC) medium, obtained from TEKnova (Hollister, CA), consisted of 1.2 % tryptone, 2.4 % yeast extract, 0.4 % glycerol, 17 mM monobasic potassium phosphate, and 72 mM dibasic potassium phosphate. Microbial cultures were obtained from American Type Culture Collection (ATCC, Manassas, VA).

### LC-MS/MS and NMR

The progress of biotransformations and purities of isolated metabolite were determined by LC-MS/MS analysis. Reverse-phase HPLC separations were carried out on a Agilent 1100 system (Agilent Technologies, Delaware, DE) consisting of a temperature controlled auto-sampler, a binary pump, and a photo-diode array detector in-line with a LCQ-DecaXP<sup>Plus</sup> (ThermoFinnigan, San Jose, CA) ion-trap mass spectrometer. The mobile-phase consisted of 10 mM ammonium formate in water at pH 5.0 (solvent A) and 10 mM ammonium formate in 95% MeOH / 5% water (solvent B) with the following

linear gradient system: flow rate of 0.3 mL/min; 0 to 2.5 min, 60% A; 2.5 to 9.5 min, 60 to 5% A; 9.5 to 11.5 min, 5% A; 11.5 to 12 min, 60% A. Separation was achieved on a C18 (Symmetry, 2.1 x 100 mm, 5  $\mu$ m; Waters, Milford, MA) column maintained at 40 °C. An electrospray ionization source operated in negative ion mode was used to acquire LC-MS/MS data. Diclofenac turnover (measured by its depletion) and formation of its metabolites was approximated by measuring peak areas in extracted ion chromatograms for the pseudo-molecular ions of each component ( $m/z$  294: diclofenac;  $m/z$  310: 4'-hydroxy/5-hydroxy;  $m/z$  326: 4',5-dihydroxy metabolites).

NMR spectra were acquired on a 600 MHz spectrometer equipped with a 5-mm cryoprobe (Bruker Instruments, Billerica, MA). Isolated metabolites were dissolved in 160  $\mu$ L of methanol- $d_4$  and transferred to 3-mm tubes. Complete structural assignment was carried out by analyzing the 1D  $^1\text{H}$ ; 2D  $^1\text{H}/^1\text{H}$  TOCSY; 2D  $^1\text{H}/^{13}\text{C}$  HSQC and HMBC data sets.

### Microorganism Screening

Microbial cultures (shipped lyophilized or frozen) were reconstituted according to ATCC instructions and preserved in either nutrient or potato dextrose agar slants at 4-6 °C. The following bacteria were screened for diclofenac (50  $\mu$ M) turnover: *Rhodococcus erythropolis* (ATCC-4277), *Streptomyces griseus* (ATCC-13273), *Nocardia corallina* (ATCC-31338), *Pseudonocardia autotrophica* (ATCC-55293), *Saccharopolyspora hirsuta* (ATCC-20501), *Pseudomonas sp.* (ATCC-17483), and *Actinoplanes sp.* (ATCC-53771). For each bacteria, spores from slant/frozen stock were used to inoculate 25 mL SOC medium in a 125 mL Erlenmeyer flask and this stage I culture was incubated at 30 °C for three days on an orbital shaker (250 rpm, Max<sup>Q</sup> 4000, Barnstead, Dubuque, IA). A stage II culture was initiated by adding approximately 2 mL of stage I culture into 25 mL fresh SOC medium in a 125 mL Erlenmeyer flask. The stage II culture was incubated at 30 °C for 24 hr, followed by addition of a stock solution of substrate. At selected time points, 1mL aliquots were placed in polypropylene tubes with 0.5 mL acetonitrile, vortex mixed, centrifuged at  $16000 \times g$  for 10 min, and supernatants were subjected to LC-MS/MS analysis.

The following fungi were also screened: *Aspergillus ochraceus* (ATCC-1008), *Cunninghamella elegans* (ATCC-9245), *C. elegans PA-1* (ATCC-36112), *Beauveria bassiana* (ATCC-7159), *Absidia blakesleeana* (ATCC-10148b), *Cunninghamella echinulata* (ATCC-9244), *C. echinulata* (ATCC-11585b), and *Rhizopus oryzae* (ATCC-11145). The methods used for the bacterial incubations were also employed for the fungal cultures except that all incubations were carried out in TBC medium at ambient temperature (~22 °C).

### Concentration tolerance and conversion time profiles

Experiments were performed to determine the tolerance of stage II cultures of *Actinoplanes sp.* to various concentrations of diclofenac, and to determine the progression of metabolism. The culture methods were identical to those used for screening of bacterial metabolism. Aliquots of substrate stock solution were added to stage II cultures to achieve final concentrations of 50 µM, 100 µM, and 500 µM using a 50 mM stock; and 1, 2, 5, and 10 mM using a 500 mM stock. 1 mL aliquots were sampled at indicated time points and analyzed by LC-MS/MS as previously described.

The effect of using a diluted *Actinoplanes sp.* culture for the conversion of diclofenac was also studied at ambient (22 °C) and 30 °C incubation temperatures. 15 mL of culture medium obtained 24-hours after inoculation of the stage II culture was mixed with 135 mL of fresh SOC in 500 mL Erlenmeyer flasks. Appropriate amounts of substrate stock (400 mM) were added to achieve target final concentrations of 50 µM, 500 µM, 1 mM, and 2 mM. The time profile of the reaction was monitored via LC-MS/MS as described above.

### Preparative-Scale Incubation and Chromatography

The scale-up procedure was similar to the microbial screening. Metabolites were produced in a 200 mL stage II *Actinoplanes sp.* culture (2 × 100 mL in 500 mL Erlenmeyer flasks, 200 rpm). Diclofenac sodium (1.25 mL of 400 mM stock added per flask; 5 mM final concentration) was added 24 hr post stage II inoculation and incubated for 20 hr. At the end of the incubation, the fermentation broth was adjusted to pH 5 with 3N HCl and then extracted with ethyl acetate (8 × 50 mL). The pooled organic phases

were dried over anhydrous  $\text{MgSO}_4$ , and solvent was removed on a Rota-vap<sup>®</sup> to yield a light-yellow oil. The crude oil was purified via flash column chromatography (CombiFlash system, Teledyne ISCO, Lincoln, NE) on a 40g Redi-sep silica cartridge with an isocratic mobile phase (hexanes/ethyl acetate; 6:4) to yield three metabolites. The isolated metabolites were characterized by LC-MS/MS and NMR.

### **Hollow Fiber Cartridge (HFC) Reactor Procedures**

The composition of an HFC reactor and its operation in a closed-cycle loop is depicted in Figure 1. An HFC consists of a bundle of capillaries composed of a semi-permeable membrane (molecular cutoff varies with the membrane material) and an extra-capillary space (ECS), all enclosed in a poly-carbonate cylinder. The semi-permeable membrane permits exchange of  $\text{O}_2$ , nutrients, substrate, metabolites, and cellular excretory products (with molecular weights below the cutoff) while retaining the microorganism in the ECS. The capillary bundle is potted on both ends to poly-urethane adapters resulting in a common flow path when connected to an external reservoir. The ECS, between the capillary membranes and inner-walls of the cartridge, is loaded with a microbe culture. A medium (containing  $\text{O}_2$ , nutrients, and the substrate) is then continuously circulated at a constant flow rate using a peristaltic pump.

Sterile hollow fiber cartridges containing different semi-permeable membranes (Teflon, C2025; polysulfone, C2011; cellulose, C3008) and the peristaltic pump were obtained from FiberCell systems (Frederick, MD). After assembly of the cartridges, the non-specific binding of diclofenac to the cellulose and poly-sulfone membranes was first determined. The ECS in each cartridge and the external-reservoir was filled with 15 and 135 mL SOC medium, respectively. The medium was circulated for 10 min at 10 mL/min and diclofenac stock solution was added to the reservoir to achieve a final concentration of 50  $\mu\text{M}$ . Aliquots were sampled after 15 hours and analyzed by LC-MS/MS. The experiment for the Teflon membrane HFC was the same except the ECS and the reservoir was filled with 2 mL and 98 mL of SOC broth, respectively.

The ECS in each HFC was then loaded with the microbial culture and time-profiles for diclofenac turnover were determined. Two sterile 50 mL syringes, one empty and another loaded with 40 mL of stage II *Actinoplanes* sp. culture were attached to the two ECS



ports. The microbial suspension was flushed slowly through the ECS space to the other syringe, back and forth, for 2 min. The cartridge ports were wiped with 70% ethanol and closed. The external reservoir volumes remained the same as described above. The system was equilibrated by starting the flow of medium at 10 mL/min for 5 min, and then diclofenac stock solution (50  $\mu$ M final concentration in the cellulose and Teflon HFC reactors; 2 mM in the poly-sulfone HFC reactor) was added to the external reservoir. Aliquots (1 mL) were sampled at different times and analyzed by LC-MS/MS. The same experiment was also conducted with cellulose membrane HFC reactor at 2 mM diclofenac concentration.

Experiments to evaluate the reusability of the HFC reactors were performed at two substrate concentrations (50 and 500  $\mu$ M) in cellulose membrane cartridges. The microbial suspensions were loaded and the reactors were equilibrated as described above. 19  $\mu$ L of 400 mM diclofenac stock (50  $\mu$ M final concentration) was then spiked into the reservoir. The reactions proceeded for 15 hr at ambient temperature (22  $^{\circ}$ C) after which the contents of the external reservoir were removed and analyzed by LC-MS/MS. The reactors were washed by circulating 100 mL of fresh SOC broth for one hour. Then 135 mL of fresh SOC broth was placed in each reservoir. Medium was recirculated for 5 min and then aliquots were collected and analyzed to ensure minimal carry over from the preceding reactions. The medium was then amended with diclofenac stock and formation of the reaction products was monitored. This cycle was repeated a total of 7 times. The experimental procedure to evaluate the reusability at 500  $\mu$ M concentrations of diclofenac was slightly modified. The external reservoir containing SOC medium was placed on a hot-plate stirrer with its temperature set to 34  $^{\circ}$ C so that the temperature of the medium (measured at the reservoir with the pump set at a flow-rate of 10 mL/min) was 30  $^{\circ}$ C. 188  $\mu$ L of 400 mM diclofenac stock was added and the reaction proceeded for 36 hours. The reaction was repeated four more times for a total of five cycles with two wash cycles between each reaction.

## Results

### Microorganism screening

Nine of the fifteen microbes screened turned over < 1% of 50  $\mu$ M diclofenac. A time course of diclofenac consumption from the seven microorganisms that did catalyze turnover is summarized in Table 1. In the fungi family, the majority of turnover catalyzed by *C. elegans*, *C. echinulata* (ATCC -11585a) and *B. bassiana* occurred after 27 hours of incubation and was complete by 120 hours. The other strain of *C. echinulata* (ATCC-9244) was less efficient in catalyzing turnover of diclofenac. Of the several bacterial strains tested, *S. griseus* showed a small amount of turnover while the actinomycete *Actinoplanes sp.* was very efficient in the catalysis and turned over 100% of the diclofenac within 5 hours. In all cases, diclofenac was primarily mono-hydroxylated at the 4' and 5 positions (labels shown in Table 2). *Actinoplanes sp.* was the microbe chosen for all subsequent experiments.

### Shake-flask concentration tolerance and time profiles

Time-profiles of diclofenac turnover when incubated at various concentrations (50  $\mu$ M – 5 mM) with *Actinoplanes sp.* at 30 °C in a shake-flask are shown in Figure 2. At concentrations of up to 1 mM, substrate turnover was rapid and complete within 5 hr. The turnover at 2 and 5 mM was complete in 10 and 24 hr, respectively. LC-MS/MS analysis indicated the reaction products predominantly consisted of 4'-OH ( $R_t$  10.6 min) and 5-OH ( $R_t$  10.8 min) diclofenac in ratios ranging from 8:2 to 7:3 for all of the tested concentrations. At later time points 4',5-dihydroxy diclofenac ( $R_t$  8.2 min) was also formed. Turnover at a substrate concentration of 10 mM was dramatically diminished (0.5% over 48 hr, data not shown) and substantial cell-death was observed.

### Scale up of metabolite production and definitive metabolite identification

Three hydroxylated metabolites were isolated from a preparative fermentation of diclofenac (5 mM; 318 mg) with *Actinoplanes sp.* and characterized by MS and NMR. The results are summarized in Table 2. 169.5 mg of 4'-hydroxy (54% yield) and 42.1 mg of 5-hydroxy (13.4% yield) diclofenac were isolated as solid white powders. Both mono-hydroxylated metabolites formed  $[M-H]^-$  ions at  $m/z$  310, 312 and 314 in a typical two-

chlorine isotopic ratios of ~1:0.65:0.1. Collision induced fragmentation of the  $m/z$  310 ion resulted in a major ion at  $m/z$  266 (-44, loss of  $\text{CO}_2$ ); and minor ions at  $m/z$  230 (loss of  $\text{CO}_2$  and  $\text{HCl}$ ) and 194 (loss of  $\text{CO}_2$  and  $2\text{HCl}$ ).  $\text{MS}^3$  analysis of the  $m/z$  266 ion resulted in secondary ions at  $m/z$  230 and 194. The proton NMR spectra of the 4'-OH and 5-OH diclofenac metabolites matched well with previously published data (Shen et al. 1999, Stierlin et al 1979, Tang et al. 1999). A complete listing of identifiable proton and carbon chemical shifts are listed in Table 2.

The bis monooxygenated metabolite 4',5-dihydroxy diclofenac (25.3 mg; 7.7 % yield) was isolated as a beige solid and it formed  $[\text{M}-\text{H}]^-$  ions at  $m/z$  326, 328, and 330. MS/MS analysis of the  $m/z$  326 ion resulted in a major fragment at  $m/z$  282 (-44, loss of  $\text{CO}_2$ ) and a minor fragment at  $m/z$  246 (loss of  $\text{CO}_2$  and  $\text{HCl}$ ).  $\text{MS}^3$  analysis of the  $m/z$  282 ion resulted in peaks at  $m/z$  246 and 210 (successive loss of two  $\text{HCl}$ ). The NMR data, listed in Table 2, confirmed bis-hydroxylation had occurred at the 4' and 5 positions.

### Hollow fiber cartridge reactions

The non-specific binding of diclofenac to HFC reactors with three different semi-permeable membrane materials was evaluated in absence of microorganism in ECS. Non-specific binding to a poly-sulfone membrane HFC reactor was rapid and high with only about 18 % of nominal concentrations of diclofenac unabsorbed at 1 hr and available for biotransformation. The non-specific binding to Teflon and cellulose membrane HFC reactors were substantially lower: 98.5 % and > 85 % diclofenac remaining in both at 1 hr and 5 hr, respectively.

Diclofenac was turned over in all three HFC reactors at ambient temperature to its 4'-OH and 5-OH metabolites, and the results are summarized in Table 3. The time for complete substrate depletion, however, differed widely. *Actinoplanes sp.* in a cellulose membrane HFC turned over 50  $\mu\text{M}$  diclofenac rapidly: ~ 95 % conversion in 15 hr and 100 % in 24 hr. Diclofenac was metabolized at the slowest rate in the Teflon HFC reactor, probably because its ECS can hold the smallest volume (2 mL) of microorganism culture. In the poly-sulfone HFC, 95% of substrate (at 2 mM nominal concentration) had disappeared in 96 hr. However, there was a proportionally lower amount of metabolites formed. This is a result of combination of irreversible binding to the poly-sulfone membrane and

metabolic consumption. Diclofenac at a 2 mM substrate concentration in a cellulose HFC reactor was not turned over probably because of lethality to cells as observed in the shake-flask experiment.

Incubations were conducted in shake flasks using the amounts of stage II culture that were used in the cellulose-membrane HFC reactors. The results are presented in Figure 3A. The amounts of stage II culture were matched in the way to provide a more direct comparison of the two techniques. The cellulose HFC reactor holds 15 mL of stage II bacterial culture in a total reaction volume of 150mL, which is an approximately 10-fold lower microbial density than was used in the shake-flask cultures used to determine the time course of turnover shown in Figure 2. At ambient temperature, 50  $\mu$ M diclofenac was completely consumed in 24 hr and 500  $\mu$ M diclofenac required 72 hr. Figure 3B shows results from the same incubations conducted under identical conditions except temperature was 30 °C. The reaction rate was approximately 3 to 4 fold higher at higher temperature. At 1 mM or higher concentrations, the substrate turnover was dramatically lower with < 1% turnover observed at 72 hr. The 4'-OH and 5-OH metabolites were observed approximately in the same ratio as in earlier incubations.

Time profiles of 50  $\mu$ M diclofenac conversion in a shake-flask and a cellulose-membrane HFC reactor under identical conditions (same amount of stage II culture, 22 °C) is shown in Figure 4. The progression of turnover appears to be very similar for both techniques.

### **Reuse of biocatalyst in HFC reactors**

Plots of diclofenac turnover versus number of reutilization cycles at 50  $\mu$ M (at 22 °C, 15 hr, 7 cycles) and 500  $\mu$ M (at 30 °C, 36 hr, 5 cycles) concentrations are displayed in Figure 5. At 50  $\mu$ M concentration, the conversion was > 98% in 15 hr for each reaction. The ratio of 4'-OH to 5-OH, the two mono-hydroxylated metabolites, changed from 8:2 in cycle number 1 to 6:4 in cycle number 7. The 4',5-dihydroxy metabolite accounted for < 3% of the metabolite products. Carry over, monitored by analyzing the wash medium, was < 1%. At a 500  $\mu$ M diclofenac concentration, the reaction time to completion (> 98% turnover) was 36 hr and required two wash cycles to reduce the carryover to < 1%. The ratio of 4'-OH to 5-OH metabolite was 7:3 in all cycles and the 4',5-dihydroxy

metabolite formed was < 2% of all products. In both settings, the cartridges could be reused at least 5 times over 10 days.

## Discussion

*Actinoplanes sp.* (ATCC-53771), a bacterium from the actinomyces family, was demonstrated to efficiently turn over diclofenac. It tolerated substrate concentrations of up to 5 mM under the shake-flask conditions. Previous examples of metabolites generated by *Actinoplanes sp.* were all reported to be hydroxylations on either an allylic (Kuhnt et al. 1996) or alkyl carbon (Haag et al. 1998; Chen et al. 1993; Hsu et al. 2002; So et al. 1995); no aromatic hydroxylations have been previously reported.

A 200 mL scale-up of a shake-flask incubation and isolation of the reaction products yielded three metabolites: 4'-hydroxy, 5-hydroxy, and 4',5-dihydroxy diclofenac. 169 mg of 4'-hydroxy diclofenac was obtained, and a 54 % yield was significantly higher than previously reported bio-reactor scale-ups. Previously obtained yields were 15 % from *Epicoccum nigrum* (Webster et al. 1998), 26 % from human CYP450 2C9 expressed in Sf21 insect cells (Rushmore et al. 2000), and 35 % from human CYP450 2C9 expressed in an *Escherichia coli* system (Vail et al. 2005). 5-Hydroxy diclofenac has been previously reported to be formed in fungal incubations (Webster et. al. 1998) but was never isolated. It has been isolated from human urine (Stierlin et al. 1979) as well as synthesized (Kenney et al. 2004, Tang et al. 1999, Bort et al. 1996). 4',5-dihydroxy diclofenac has also been isolated from human urine (Stierlin et al. 1979); however, a microbial or synthetic route has not been previously reported.

The tolerance of *Actinoplanes sp.* to high concentrations of diclofenac (up to 5 mM, Figure 2) in a shake flask nominally appeared to be higher than in the HFC reactor (only up to 500  $\mu$ M, Figure 3). The microbial content was, however, approximately 10 times higher in the shake flask than in the HFC reactor. When the lower microbial content in the HFC reactor was compensated, the rate of 50  $\mu$ M diclofenac conversion was observed to be similar in both the shake flask and cellulose HFC reactor (Figure 4).

Hollow fiber cartridge reactors were demonstrated to turn over substrate and produce metabolites with similar efficiency to shake-flask reactors. They also offer several

potential advantages compared to shake-flask configurations. As was demonstrated, the bio-catalyst in an HFC reactor can be re-used many times over. In contrast, the bio-catalyst in a shake flask can be used only once because the quenching process kills the microbes. The purification of metabolites biosynthesized in an HFC reactor is also theoretically easier because the products can be harvested from the external medium which is devoid of the cellular matrix.

An HFC reactor potentially offers a readily available system to generate metabolites for multiple compounds over time because there is no theoretical reason to believe that the substrate would have to be the same in each reaction cycle. The recirculation of medium (containing the substrate) in a closed-loop system allows for multiple passes for the substrate to be turned over. The cartridges employed in this report had an ECS volume between 2 and 15 mL and were sufficient to achieve a 100 % turnover of diclofenac (up to 500  $\mu$ M) in a closed-loop system. Larger volume cartridges are also commercially available (cellulose HFC with a 70 mL ECS, FiberCell Systems) to facilitate scaled up reactions.

The one drawback observed with an HFC reactor is the potential for non-specific binding of substrate. Non-specific binding could occur to any of the components in the reactor. The three cartridges used in this study all had the same length of silicone tubing and the same cartridge shell. The only differences were in the material that comprised the semi-permeable membrane. Differences in the membrane material were probably responsible for the differences observed in non-specific binding. Binding to Teflon and cellulose membranes was substantially less than to the poly-sulfone membrane. The extent to which other substrates (e.g. hydrophobic or basic compounds) might non-specifically bind to these materials was not determined.

To our knowledge, this work represents the first example of an HFC reactor used to produce mammalian CYP450 metabolites using microbial cultures. Previously published examples employing HFC reactors for biochemical reactions include conversions of glucose to ethanol using entrapped *Saccharomyces cerevisiae* (Inloes et al. 1983); L-histidine to urocanic acid by *Pseudomonas fluorescens* (Kan and Shuler, 1978); and 2-

methyl-1,3-propane diol to (*R*)- $\beta$ -hydroxyisobutyric acid by *Acetobacter* sp. (Leon et al. 2001).

## Conclusion

It was demonstrated that the actinomyce bacterial strain, *Actinoplanes* sp. (ATCC-53771), turned over diclofenac very efficiently to its hydroxylated metabolites. Scale-up productions and characterization of three metabolites: 4'-hydroxy, 5'-hydroxy, and 4',5-dihydroxy diclofenac were accomplished. It was also shown using diclofenac as a model substrate that microorganisms in hollow fiber cartridge reactors can be reused many times over ( $n > 5$  over 10 days) and they performed similarly to conventional shake-flask reactors.

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## Footnotes

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## Figure Captions.

**Figure 1.** Hollow fiber cartridge reactor set up. (A) Cross sectional view of an HFC. The fiber bundle on either end is potted onto poly-urethane adapters such that there is a common fluid path through the central compartments. The microorganism is loaded onto the ECS. (B) HFC operation in a closed loop cycle. The medium containing oxygen, nutrients, and substrate is continuously cycled at a flow rate of 10 mL/min.

**Figure 2.** Concentration tolerance and time profile of diclofenac turnover by *Actinoplanes sp.* in a shake-flask setup (25 mL stage II culture in SOC at 30 °C).

**Figure 3.** Effect of employing 10-fold diluted *Actinoplanes sp.* stage II culture on diclofenac turnover in a shake-flask setup at two temperatures. (A) 22 °C; and (B) 30 °C.

**Figure 4.** Time profile of diclofenac (50 µM, 22 °C) turnover in shake-flask and cellulose membrane HFC setups. Other reaction conditions were identical in both reactors.

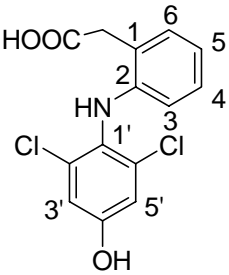
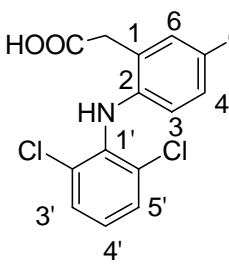
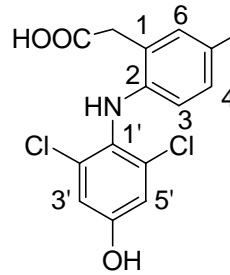
**Figure 5.** Diclofenac turnover on reutilization of *Actinoplanes sp.* in cellulose membrane HFC. (A) 50 µM diclofenac, 22 °C, and 15 hr reaction time/cycle. (B) 500 µM diclofenac, 30°C, and 36 hr reaction time/cycle.

Tables.

**Table 1.** Microorganism metabolism of diclofenac. Substrate was incubated at 50  $\mu$ M in a 25 mL stage II microbial culture. Other details are as listed in experimental methods.

	Time (hr)	5	20	27	72	120
<b>Bacteria</b>		Turnover (%)				
<i>Streptomyces griseus</i> (ATCC-13273)		0	0	0	0	17
<i>Actinoplanes sp.</i> (ATCC-53771)		100	-	-	-	-
<b>Fungi</b>						
<i>Cunninghamella elegans PA-1</i> (ATCC-36112)		0.2	1.1	4.2	90	100
<i>Beauveria bassiana</i> (ATCC-7159)		0	2.1	2.2	59	100
<i>Cunninghamella echinulata</i> (ATCC-9244)		0	2.5	4.2	31	54
<i>Cunninghamella echinulata</i> (ATCC-11585a)		0	1.1	1.2	37	100

**Table 2.** Summary of scale-up reaction and structural characterization of diclofenac metabolites. 318 mg of diclofenac sodium was incubated with *Actinoplanes sp.* and the reaction products were isolated and characterized by MS and NMR.

	4'-OH-DIC	5-OH-DIC	4',5-di-OH-DIC
			
$R_f^a$	0.47	0.28	0.30
Yield	169.5 mg (54.5 %)	42.1 mg (13.4 %)	25.3 mg (7.7 %)
MS, $m/z$	310	310	326
MS/MS, $m/z^b$	266, 230, 195	266, 230, 195	282, 246, 210
NMR <sup>c</sup>	$\delta_H$ ppm (multiplicity, J-coupling in Hz)/ $\delta_C$ ppm		
COOH	174.4	174.4	174.6
CH <sub>2</sub>	3.72 (s) / 37.8	3.71 (s) / 37.8	3.68 (s) / 38.1
1	123.0	127.9	126.0
2	143.8	134.9	136.4
3	6.28 (dd, 8.2,0.9)/114.9	6.38 (d, 8.5) / 120.4	6.22 (d, 8.6) / 117.9
4	7.03 (ddd, 8.3, 7.7, 1.5)/127.4	6.56 (dd, 8.5,2.8) / 113.8	6.52 (dd, 8.6, 2.8) / 113.8
5	6.82 (ddd, 8.3, 7.5, 1.1)/119.8	152.6	151.4
6	7.18 (dd, 7.5, 1.3) / 130.3	6.72 (d,7.8) / 116.9	6.69 (d, 2.8) / 117.0
1'	132.1	138.9	130.4
2',6'	128.9	127.6	NI
3',5'	6.89 (s) / 115.3	7.34 (d, 8.0) / 128.5	6.85 (s) / 115.4
4'	154.7	6.96 (dd, 8.7, 8.0) / 122.6	153.7

a. TLC Retention factor ( $R_f$ ): 4' and 5-OH DIC in 1:1 hexanes-ethyl acetate; 4',5'-di-OH DIC in 4:6 hexanes-ethyl acetate.

b. MS/MS lists major fragment ions from the MS2 and MS3 fragmentations.

c. NMR acquired in CD<sub>3</sub>OD, solvent signal appears at  $\delta_H$  3.32 ppm /  $\delta_C$  47.9 ppm. s, singlet; d, doublet; NI = not identified.

**Table 3.** Diclofenac conversion by *Actinoplanes sp.* in HFC reactors at ambient temperature (22 °C). Abbreviations: ECS, extra capillary space; MWCO, 50% molecular weight cutoff.

Membrane	MWCO (kDa)	ECS (mL)	[DIC] $\mu$ M	Time (hr) <sup>a</sup>	4'-OH/5-OH
Teflon	nd <sup>b</sup>	2	50	> 144	8:2
cellulose	5 kDa	15	50	15	8:2
poly-sulfone	20 kDa	15	2000 <sup>c</sup>	96	6:4

<sup>a</sup> 87% turnover at 144 hr in Teflon HFC; > 95% turnover in the cellulose and poly-sulfone HFCs.

<sup>b</sup> 0.1  $\mu$ m pore size; MWCO not determined (nd) but expected to be > 100 kD.

<sup>c</sup> Higher substrate concentration employed to offset non-specific binding.

(A)

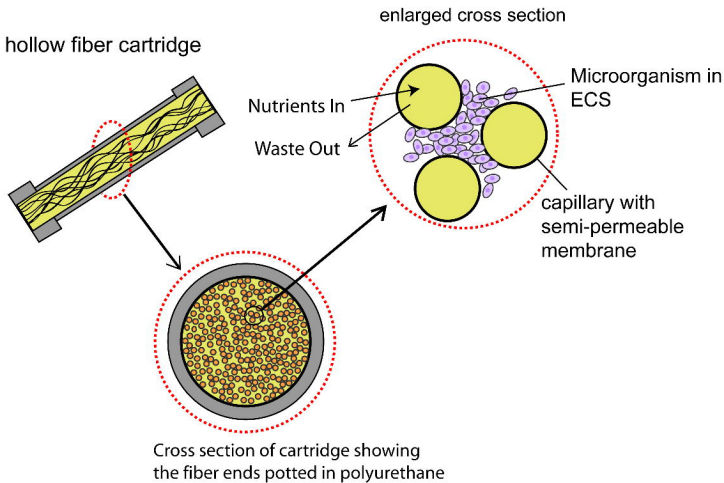


Figure 1A



(B)

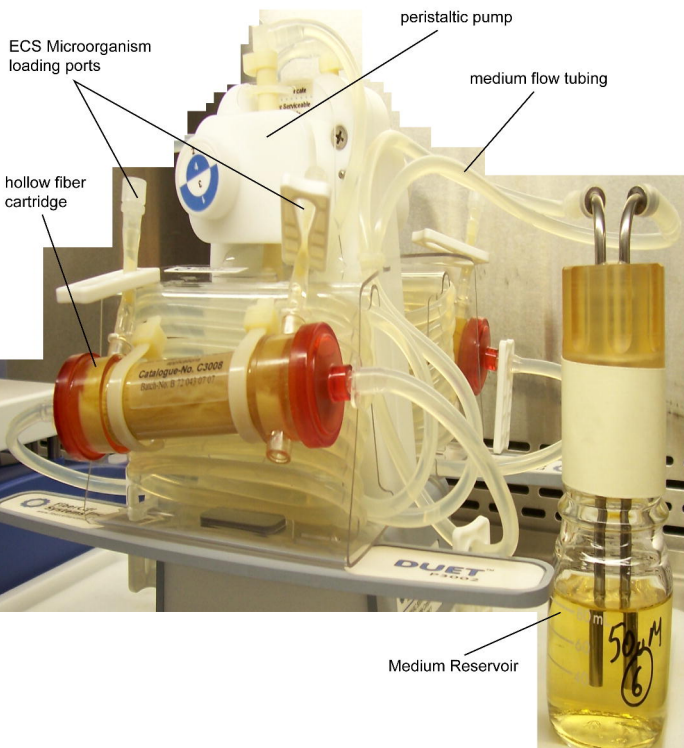


Figure 1B

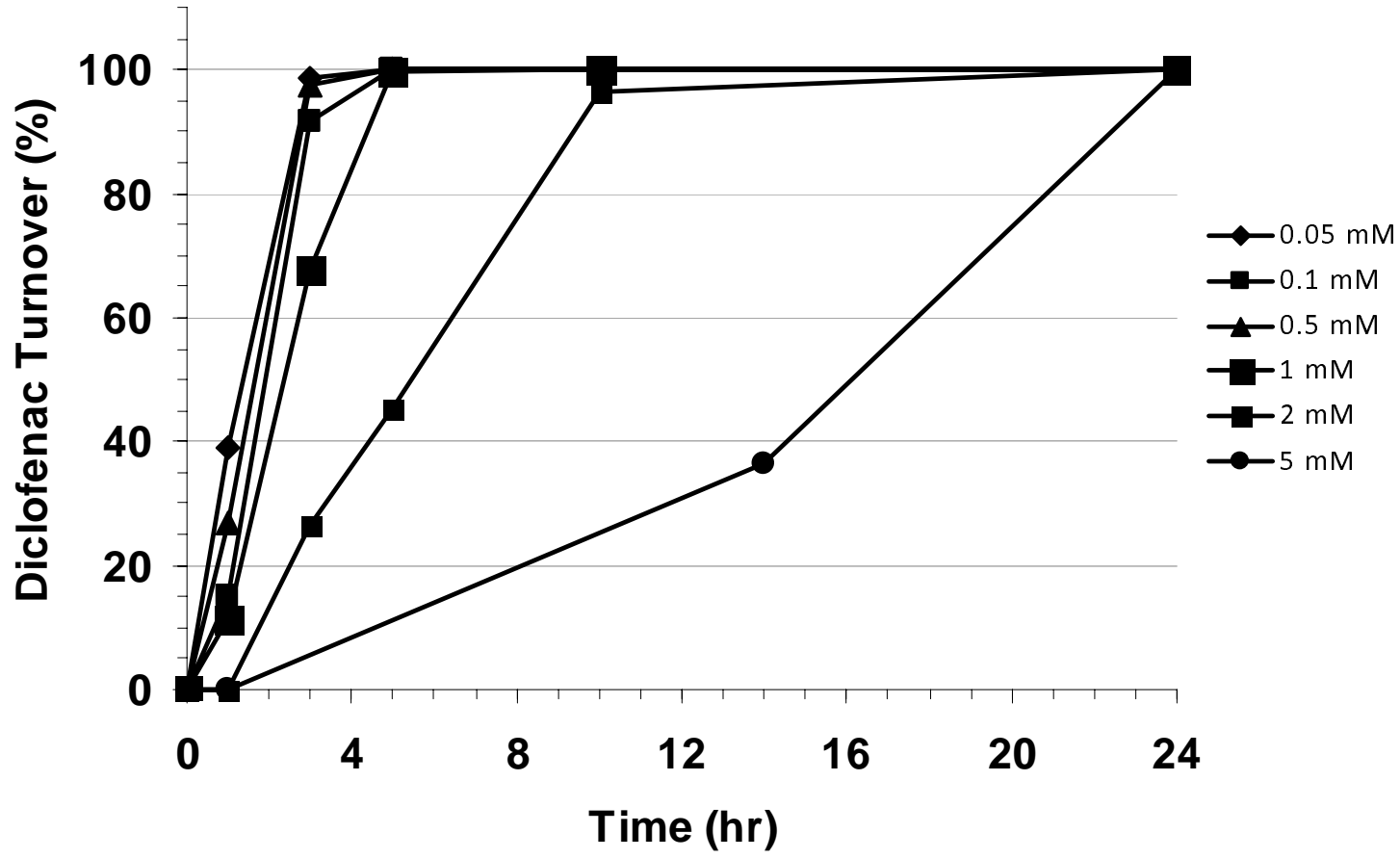


Figure 2

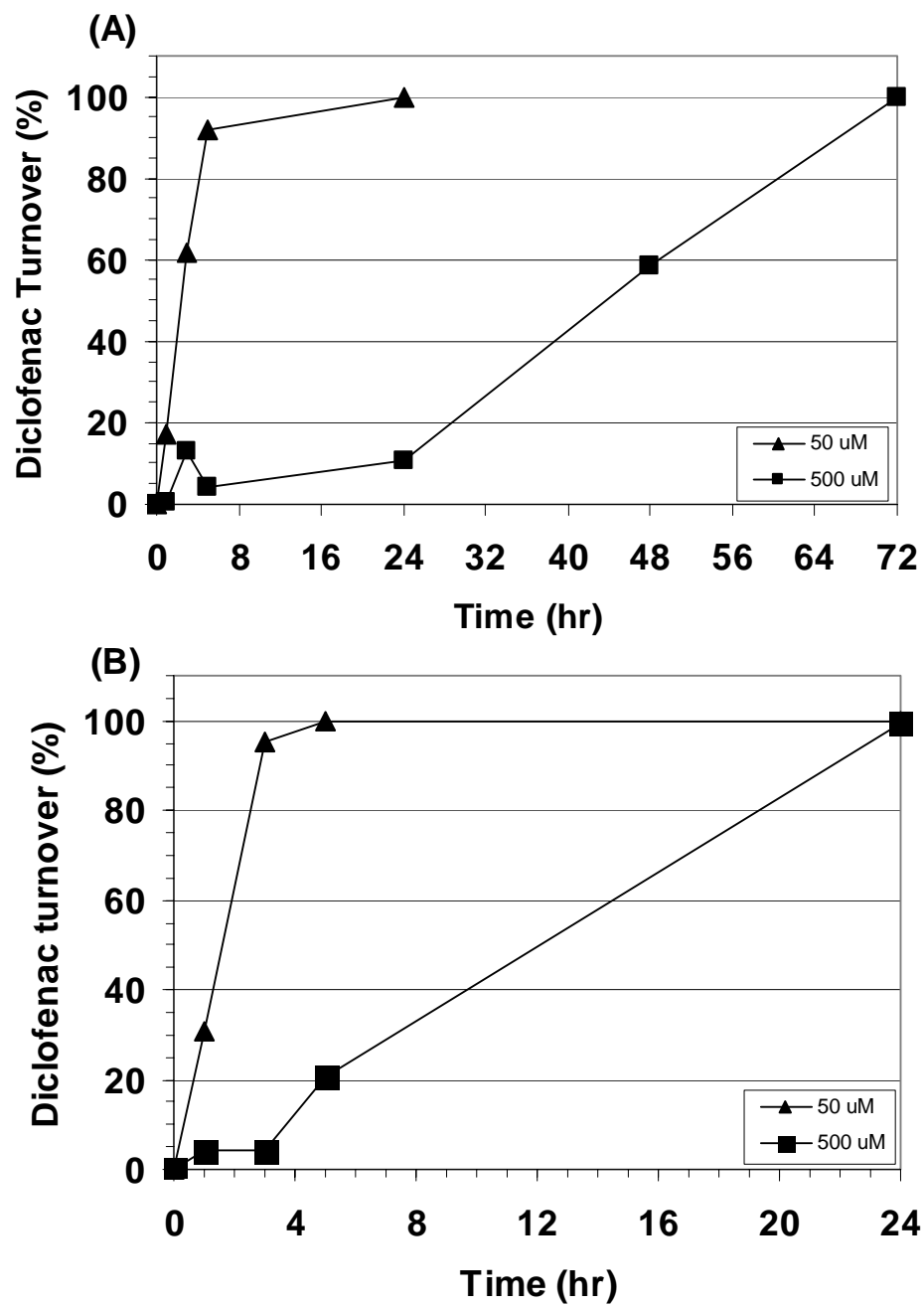


Figure 3

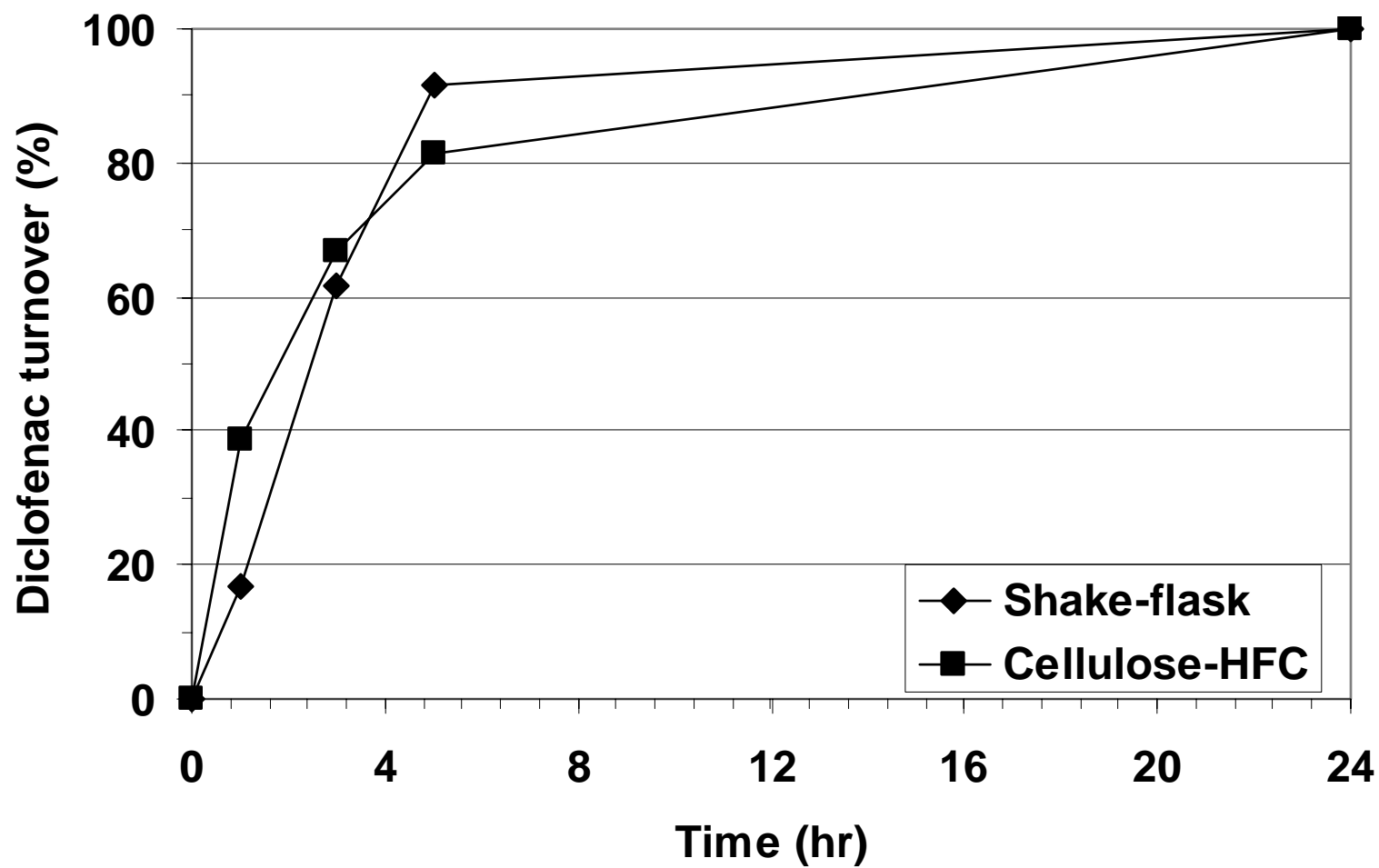
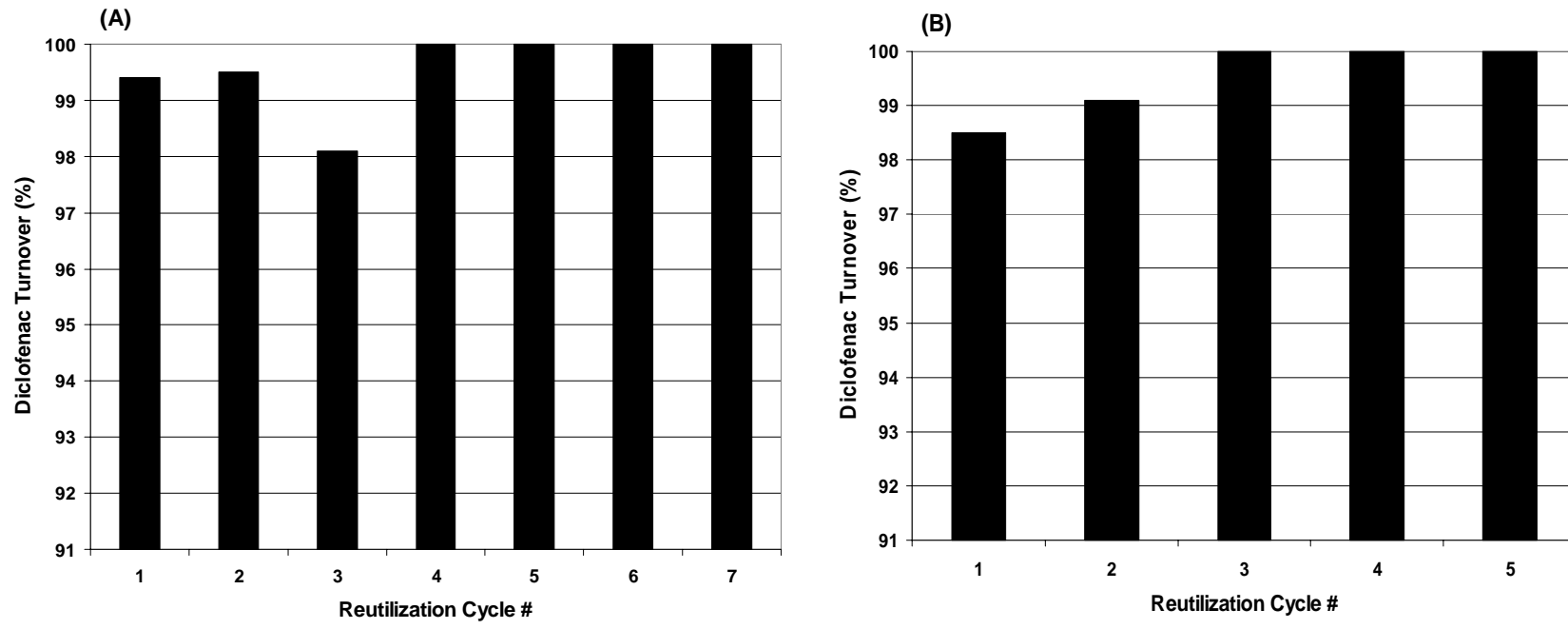


Figure 4



**Figure 5**