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ORIGINAL PAPER

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Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in *Escherichia coli*

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Abstract Three human cytochrome P450s, 3A4, 2C9 and 1A2, were each co-expressed with NADPH-P450 reductase in *Escherichia coli* and used in the preparative synthesis of drug metabolites. Low dissolved oxygen (DO) concentration ($< 1\%$) during expression was found to be critical for producing active P450s. Control of temperature, pH and glycerol supplementation in 10-L fermentations enhanced enzyme expression 31–86%. Additional improvements were obtained by altering media formulations, resulting in bicistronic expression levels of 890, 1,800 and 1,010 nmol/L for 3A4, 2C9 and 1A2, respectively. The P450 titers achieved in fermentors exceeded those in flask fermentations by 3- to 6-fold in this study and up to 10-fold when compared with previously reported literature [FEBS Lett (1996) 397:210–214, Arch Biochem Biophys (1996) 327:254–259, Biochem Pharmacol (1998) 55:1315–1325, Drug Metab Pharmacokinet (2003) 18:42–47, Nat Biotechnol (1997) 15:784–788; Metab Eng (2000) 2:115–125]. Intact cells and isolated membranes obtained from 10-L fermentations were used to establish an efficient bioconversion system for the generation of metabolites. To demonstrate the utility of this approach, known metabolites of the anabolic steroid testosterone, the anti-inflammatory agent diclofenac and the analgesic agent phenacetin, were generated using 3A4, 2C9 and 1A2, respectively. The reaction conditions were optimized for pH, temperature, DO concentration, use of co-solvent and glucose supplementation. Conversion yields of 29–93% were obtained from 1-L reactions, enabling isolation of 59 mg 6β-hydroxytestosterone, 110 mg 4′-hydroxydiclofenac and 88 mg acetaminophen.

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Introduction

Cytochrome P450s belong to a family of heme-containing proteins found in practically all living organisms ranging from bacteria to mammals [19, 22]. They play a critical role in oxidation of xenobiotics including drugs and environmental pollutants and therefore represent a primary focus of toxicology and drug metabolism studies [5, 7, 16]. This group of enzymes (EC 1.14.14.1), estimated to contain about 4,000 members (http://drnelson.utmem.edu/P450.stats.all.htm), is classified into 17 families based on amino acid sequence similarity. The so called ''drug metabolizing P450s'' are membrane-bound enzymes belonging to families 1 through 4 and are found in highest concentration in the liver [11, 19]. During the catalytic cycle, the P450 functions as a terminal oxidase that utilizes molecular oxygen, whereby one oxygen atom is introduced into the substrate while the other is incorporated into water [6]. The reducing electrons are supplied by oxidation of NADPH, catalyzed by NADPH-P450 reductase (NPR), a 78-kDa flavoprotein closely associated with the P450s in the endoplasmic reticulum [21]. In addition to hydroxylation, dealkylation (N and O), and epoxidation, P450s are capable of catalyzing a remarkably wide range of reactions, including desaturation, oxidative ester and ether cleavage, dehydration, and various couplings and rearrangements [6, 7]. Since the majority of these transformations proceed in a highly regio- and stereoselective manner, synthesizing these metabolites by purely chemical means can present difficult technical challenges. Thus it would be highly desirable to be able to selectively generate metabolites directly from drug candidates using P450s.

Hepatic microsomes provide a direct source of a multitude of different P450 activities but are available in only limited supply and enzyme expression levels are often dependent on prior induction and exposure to xenobiotics. These limitations severely restrict their practical application for preparative synthesis of metabolites. Unlike hepatic microsomes, recombinant fermentations provide the opportunity for a readily renewable source of large quantities of individual P450s. Several groups have been successful in co-expressing P450s in bacteria with a number of ancillary proteins resulting in fully functional drug metabolizing systems [1, 2, 12, 15, 23]. Surprisingly, despite numerous reports of successful expression of P450s in flask fermentations, application of engineered bacteria toward preparative synthesis of drug metabolites has been limited [15, 25].

Of the human P450 genes identified [18], Five, including 3A4, 2C9 and 1A2, represent a major portion of the total P450 content in human liver [9] and hence are the most frequently encountered in the metabolism of clinically important drugs in humans [11]. In this study, we report the successful high level bicistronic expression of human P450s 3A4, 2C9 and 1A2 together with NPR in *Escherichia coli* and their use in metabolite generation. Intact cells and isolated membranes derived from batch fermentations were used to establish reaction conditions for generating metabolites on a 100-mg scale employing the well-characterized model substrates testosterone, diclofenac and phenacetin [23, 25].

Materials and methods

Materials

Terrific broth, yeast extract, peptone, chromatography grade solvents and isopropyl-β-D thiogalactopyranoside (IPTG: dioxane free) were obtained from Fisher Scientific (Springfield, NJ). Tastone 154 (yeast extract) and casamino acids were obtained from Sensient (Juneau, WI) and Difco/Becton Dickinson (Sparks, MD). Testosterone, 6β-hydroxytestosterone, 4-acetamidophenol, diclofenac, δ-aminolevulinic acid, glucose-6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were obtained from Sigma (St. Louis, MO). Phenacetin and 4′-hydroxydiclofenac were obtained from Fluka (Steinheim, Switzerland) and BD Biosciences (Bedford, MA).

E. coli DH5-α was obtained from Invitrogen (Carlsbad, CA) and plasmids containing the P450 and NPR genes were provided by F. Guengerich (Vanderbilt University).

P450 expression and culture growth conditions

Each P450 used in this study was modified and truncated (at the 5′ end) for optimal expression in *E. coli* as described by Parikh et al. [23]. Co-expression of human P450s with NPR was achieved using a bicistronic expression vector with a double *tac* promoter and selecting for ampicillin resistance. For all *E. coli* propagations, plasmid was maintained by supplementing all growth media with 100 μg/ml ampicillin. Frozen inoculum stocks were prepared from single colonies of *E. coli* DH5-α cells transformed with plasmid DNA. Colonies were grown on Luria-Bertani (LB) solid medium followed by propagation at 37°C in LB broth (25 ml/125-ml flask) for ~6 h (OD_{600}~1.0) with agitation (200 rpm). The culture was then transferred into vials (in 20% glycerol) and stored at −80°C.

Flask fermentations

Expression of P450s and NPR in flasks was initiated by inoculating 100 μl frozen stock into LB-ampicillin broth (100 ml/300-ml flask) followed by growth at 37°C with agitation (200 rpm) for 16–20 h. A portion of this primary seed culture (5 ml) was transferred into LB-ampicillin broth (100 ml/300-ml flask) and grown at 37°C with agitation (200 rpm). When the culture achieved $OD_{600} = 2–3$, a 10 ml portion of this secondary seed culture was used to inoculate 100 ml Terrific broth medium (24 g/l yeast extract, 14 g/l tryptone, 12.5 g/l dibasic potassium phosphate, 2.3 g/l monobasic potassium phosphate) with glycerol (5.0 ml/L) in a 300 ml Erlenmeyer flask supplemented with ampicillin (100 mg/l), trace minerals, 1 mM thiamine, 1 mM IPTG and 0.5 mM δ-aminolevulinic acid [18]. Cultures were grown at 28°C with agitation (150 rpm) for 48–72 h.

P450 expression in fermentors

A 40-ml portion of the primary seed culture (described above) was transferred into LB-ampicillin broth (1 L/2.8 L Fernbach flask) and grown at 37°C with agitation (200 rpm). When the desired culture density ($OD_{600} = 2.0–3.0$) was reached (~5 h), the entire content of this secondary seed culture was transferred into the fermentor (Bioflow 4500 with Rushton impellers; New Brunswick Scientific, Edison, NJ) containing 10-L Terrific broth (prepackaged), YECA 1 or YECA 2 medium with glycerol (5.0 ml/L) and supplemented as described for flask fermentations and grown at 28°C. YECA 1 medium contains 24 g/l Fisher yeast extract, 14 g/l casamino acids, 12.5 g/l dibasic potassium phosphate and 2.3 g/l monobasic potassium phosphate. YECA 2 medium contains all components of YECA 1 medium except that Tastone 154 yeast extract is used instead of Fisher yeast extract. Dissolved oxygen (DO) levels (expressed as a percent of saturation) were controlled by automatically adjusting the agitation rate between 150 and 450 rpm in the presence of fixed aeration (5 Lpm) and backpressure [5 psi (34.5 kPa)]. Fermentation pH was not controlled until cellular metabolism caused a drop in the medium pH to the desired value (where indicated), at which point pH was controlled through automatic

addition of 1 *N* sodium hydroxide or 1 *N* sulfuric acid. At this time, feeding of a 10% glycerol solution (1 L per 10-L fermentation) was initiated at a rate of 1 ml/min (where indicated).

Cell and membrane isolation

E. coli cells were isolated from fermentation broth using a Pellicon ultra filtration apparatus (Millipore, Bedford, MA) equipped with 0.2 μm Durapore PVDF membranes (2×0.5 m^2). Cells were concentrated approximately 20-fold and then sequentially washed (5×1.5 volumes) with 100 mM potassium phosphate buffer pH 6.0, followed by centrifugation (10,000 *g*) for 15 min to pellet the cell mass. Cell pellets were stored at −80°C.

Bacterial membranes were prepared by sonicating cell pellets suspended in lysis buffer (100 mg/ml) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and aprotinin (10 units/ml) [13].

Carbon monoxide binding spectra and NADPH reductase assays

The P450 content of cells and membranes was determined by CO binding spectral analysis according to the method of Omura and Sato [20] using a dual beam spectrophotometer (Shimadzu UV 2401). NPR content of bacterial membranes was determined by measuring the reduction of cytochrome *c* based on the method of Yasukochi and Masters [26].

Cell-catalyzed bioconversion of testosterone

Washed cells (100 g) were added to a round bottom jacketed vessel (5 L) equipped with a half moon Teflon impeller (10 cm) containing 1 L 100 mM potassium phosphate buffer pH 7.4 and 10.0 g glucose. Bioconversion was initiated by addition of 25 ml of a 40 mM solution of testosterone in ethanol. The reaction pH was controlled at 7.4 by the automatic addition of 0.5 M NaOH. To ensure that NADPH depletion did not limit the course of hydroxylation, 10 g glucose was added 20 and 44 h following initiation of bioconversion. After 48 h incubation at 20°C with agitation (400 rpm), the reaction was terminated by pelleting cells by centrifugation (10,000 *g* for 15 min). The clarified supernatant was then loaded (at 20 ml/min) onto a 25×250 mm steel column (Phenomenex, Torrance, CA) packed with Amberchrome CG161m resin (Rohm & Haas, Philadelphia, PA) and equilibrated with water/acetonitrile (9/1, buffer A). The column was washed with five volumes of buffer A and eluted at a flow rate of 20 ml/min with a linear gradient of acetonitrile in water (10–90% over 30 min). The peak fractions were collected, the acetonitrile removed under vacuum, and the aqueous solution lyophilized. The product, 6*β*-hydroxytestosterone, was purified further on a preparative 10 μm× 21.2×250 mm Luna C18 (2) column (Phenomenex, Torrance, CA) using the above gradient.

Cell-catalyzed bioconversion of diclofenac and phenacetin

Intact cell bioconversions of diclofenac and phenacetin were carried out in a manner similar to that described above, with the following exceptions: the bioconversions were carried out at 28°C, diclofenac was added as a solution in acetonitrile/water (1/1), and phenacetin was added as a solution in acetonitrile. In the case of the diclofenac conversion, the cell pellet was washed (2×500 ml) with acetonitrile:water (3:1) to recover 4′-hydroxydiclofenac associated with cellular material. Acetonitrile was removed from the wash under vacuum and the resultant aqueous slurry was combined with the clarified supernatant prior to Amberchrome CG161m chromatography (as above). For the phenacetin conversion, the reverse-phase chromatography was substituted with a silica gel column chromatography as a final purification step of acetaminophen. The column was eluted with methylene chloride/methanol (9/1).

Bioconversion catalyzed by isolated cell membranes

Membrane-catalyzed reactions (20 ml) were conducted in 125-ml Erlenmeyer flasks containing 100 mM potassium phosphate buffer pH 7.4, NADPH regeneration system (50 mM glucose-6-phosphate, 30 units glucose-6-phosphate dehydrogenase and 10 mM NADPH), 1 mM substrate and 2.5% of the appropriate co-solvent (as described above). The reaction was initiated by the addition of the respective P450 (1.5 μM), followed by incubation at 28°C with agitation (250 rpm). Conversion of testosterone and diclofenac was determined using HPLC (Waters, Milford, MA) by monitoring absorbance at 242 and 270 nm, respectively, employing a 3 μm×4.6×75 mm Luna C18 (2) column (Phenomenex, Torrance, CA) and a linear 10–90% gradient of acetonitrile in water at 1 ml/min (0.1% trifluoroacetic acid was added to the mobile phase to improve separation of diclofenac). Conversion of phenacetin was measured by monitoring absorbance at 248 nm using a 3 μm×4.6×100 mm Discovery HS F5 column (Supelco, Bellafonte, PA) and a linear 10–90% gradient (starting at 2 min) of 10 mM aqueous ammonium acetate-acetonitrile at 1 ml/min for 10 min. LCMS analysis was conducted with a ZQ mass spectrometer (Waters) using electrospray positive conditions, with capillary voltages of 3.0–3.5 kV and cone voltages of 20–35 V.

Results and discussion

The ability to generate metabolites of drug candidates in a timely manner is essential for expediting toxicity and biological activity testing. The timing of this assessment is highly critical as the outcome of these studies often determines whether a drug candidate is selected for development. Typically, metabolites are generated using multi-step chemical syntheses, requiring considerable allocation of time and resources. To circumvent this limitation we decided to investigate an alternative biological approach based on recombinant expression of human P450s. This approach has been successfully demonstrated with suspension cultures of insect cells infected with recombinant baculovirus expressing various individual P450 enzymes with NPR [25]. Some of these enzymes are commercially available and commonly used in various drug metabolism related studies. Although the baculovirus-based expression system proved useful in generating metabolites on a 1–2 mg scale [25], low expression levels and high cost limit the utility of this system in larger synthetic applications.

In contrast, it has been shown that bacteria can express high levels of heterologous P450s and NPR, and their fermentations are readily amenable to scale-up [3, 4, 24]. Recombinant expression in *E. coli* has emerged as one of the most versatile systems for expressing active P450s, primarily due to its ease of propagation, well-established genetics, and the absence of any interfering native P450 genes [3, 4, 8]. By concomitantly expressing P450 and NPR genes using a bicistronic vector, adequate levels of each enzyme can be obtained [12, 23], enabling the direct use of recombinant *E. coli* cells (or membranes) for generation of metabolites. To develop this recombinant microbial approach we focused on the three most prominent hepatic P450 enzymes associated with human drug metabolism, namely 3A4, 2C9 and 1A2 [9, 10].

Fermentation development

Initial fermentation development to examine the impact of temperature, medium supplementation, inoculum age and volume, as well as induction of enzyme expression with IPTG, was conducted in flasks. Under optimal flask fermentation conditions (see Materials and methods) maximum titers of 250, 300 and 260 nmol P450/L were obtained for expression of 3A4, 2C9 and 1A2, respectively. Further optimization of P450 expression was carried out in fermentors.

Expression levels of active P450s in 10-L fermentations were strongly dependent on the DO level (Table 1). Although the final cell densities attained in high and low DO fermentations were similar, a 3- to 10-fold increase in overall P450 expression levels was observed in fermentations conducted at low DO. This result was in agreement with recent findings for the dual expression of 3A4 and NPR in jar fermentors as reported by Kanamori et al. [15]. In general, under low DO conditions, P450 expression and cell density increased proportionately. In contrast, under high DO conditions (>10%), the initial growth rate of the culture rose significantly, up to 3-fold, and only modest amounts of active P450 were produced. Moreover, high growth rate was accompanied by a dramatic increase in absorbance at 420 nm, most likely due to incorrect folding of newly synthesized P450.

Glycerol feeding and pH control during early stationary phase also resulted in improved titers, the impact of which was enzyme specific. For example, 3A4 expression levels increased 31% in glycerol-fed batches (under pH control), compared to an 86% increase in the

Table 1 Effect of dissolved oxygen (DO) concentration, pH and glycerol on expression of human P450s by *E. coli*

P450	Conditions[a]	Titer (nmol/l)[b]
3A4	High DO (>10%)	70 ± 20
	Low DO (≤ 1%)	320 ± 40
	Low DO, glycerol feed, pH 6.0	420 ± 20
2C9	High DO (>10%)	44
	Low DO (≤ 1%)	430 ± 20
	Low DO, glycerol feed, pH 6.0	800 ± 80
1A2	High DO (>10%)	220
	Low DO (≤ 1%)	670 ± 20
	Low DO, pH 5.8	1,010 ± 110

[a]Fermentations (10 L) were conducted with Terrific broth where DO (% saturation) and pH were allowed to decrease to the indicated set points prior to onset of automatic control of agitation and addition of sodium hydroxide or sulfuric acid. Glycerol feeding was initiated when the fermentation reached the pH set point as indicated (see Materials and methods)

[b]Titers of replicate fermentations are expressed as average value ± standard deviation

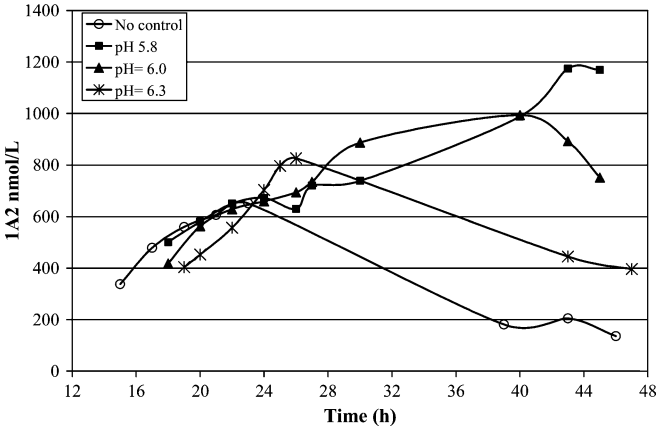

Fig. 1 Effect of fermentation pH on expression of active human 1A2 in *E. coli*. The culture was propagated in fermentors containing Terrific broth (10 L) at 28°C. Agitation and pH were automatically controlled after the growing culture achieved the set point values of dissolved oxygen (DO) (<1%) and the pH indicated (see Materials and methods)

Table 2 Effect of medium on expression of human P450s by *E. coli*

P450	Conditions[a]	Titer (nmol/l)[bc]
3A4	Terrific broth	410 ± 30
	YECA 1 medium	440 ± 60
	YECA 2 medium	890 ± 20
2C9	Terrific broth	680 ± 170
	YECA 1 medium	950 ± 60
	YECA 2 medium	1,800 ± 200
1A2	Terrific broth	840 ± 210
	YECA 1 medium	900
	YECA 2 medium	620

[a]Conditions providing maximum P450 expression indicated in Table 1 were used to evaluate various production medium formulations in 10-L fermentations (see Materials and methods)
[b]The value reported is the average titer ± standard deviation of all fermentations using three lots of Terrific broth (prepackaged medium containing yeast extract and peptone)
[c]Titers reported for YECA medium represent single or multiple fermentations conducted with two sources of yeast extract (1 = Fisher brand, 2 = Tastone 154) with casamino acids

case of 2C9. The elevated P450 titers observed in glycerol-fed batches could not be attributed to enhanced growth, which was determined to be unaffected, but were most likely the result of improved expression and/or protein folding, as proposed by Kagawa and Cao for 4A14 [14]. Without glycerol feeding and pH control, the pH of the fermentation medium in early stationary phase rose significantly, eventually reaching over pH 8. This rise was accompanied by a major loss in absorbance at 450 nm in the CO-binding assay, and in the concomitant appearance of a large peak at 420 nm. Upon further incubation, the 420 nm peak also disappeared, suggesting that both degradation and misfolding contributed to low P450 titers. The influence of pH on enzyme expression and accumulation was best exemplified with 1A2, where glycerol feeding did not provide any enhancement in expression but pH control was found to be crucial. As depicted in Fig. 1, continuous accumulation of active 1A2 was observed at pH 5.8 for as long as 48 h (reaching > 1,000 nmol/l), whereas maintaining the fermentation pH at 6.3 resulted in lower expression and a rapid degradation of synthesized protein with no influence on culture growth.

Further titer improvements were attempted by supplementing the fermentations with a sub-lethal addition of chloramphenicol (1 mg/l), which has been reported to increase protein synthesis by slowing culture growth, thereby promoting folding of active P450 enzymes [10, 17]. However, in our study, chloramphenicol supplementation not only reduced growth (24–27%) but decreased P450 titers (24–39%). Using fermentors with precise control of operating conditions, P450 titers between 420 and 1,010 nmol/l were obtained on a 10-L scale (Table 1).

At this juncture, we had not anticipated any further pursuit of titer improvements. Terrific broth, commonly reported for use with *E. coli* to express recombinant P450s [1, 4, 12–15, 23] had provided high P450 titers. Unfortunately, we discovered that use of different lots of this prepackaged complex medium resulted in up to 50% variation in enzyme synthesis while having little impact on culture growth. Therefore, a new medium providing improved culture productivity was sought. Two new media, YECA1 and YECA2, were compared to Terrific broth as summarized in Table 2. YECA 1 medium

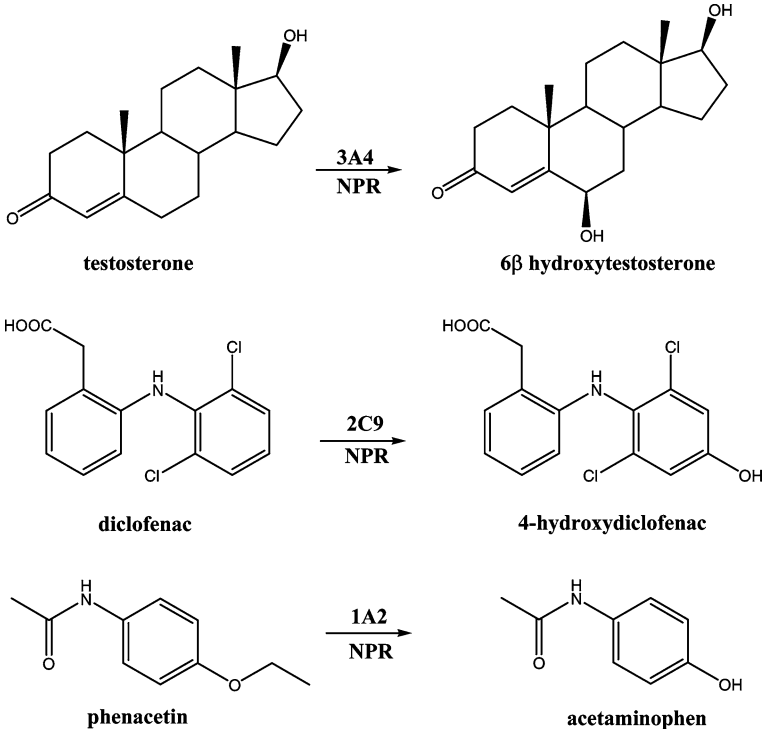

Fig. 2 Model reactions for metabolite generation by human P450s expressed in *E. coli*

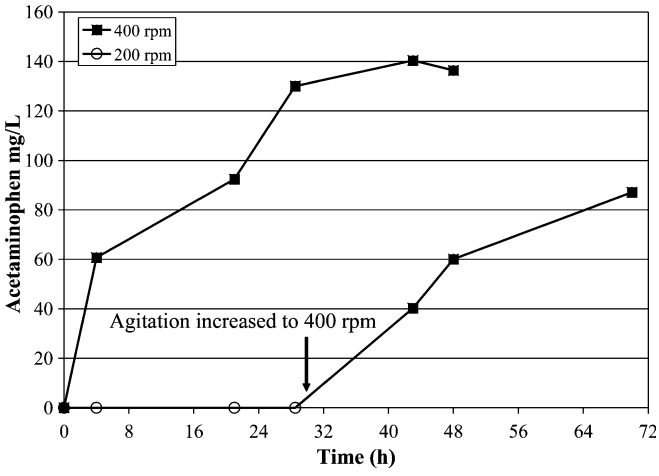

Fig. 3 Effect of agitation on phenacetin deethylation by *E. coli* cells expressing 1A2/NPR. Washed cells (100 g) were added to a round bottom jacketed vessel equipped with an overhead stirrer, containing 1 L 100 mM potassium phosphate buffer pH 7.4, glucose and phenacetin, and incubated at 28°C with agitation as indicated (see Materials and methods)

provided comparable productivity to Terrific broth for expression of 3A4 and 1A2, whereas 2C9 titers improved ~40%. YECA 2 medium provided over a 2-fold improvement in the expression of 3A4 and 2C9, but decreased expression of 1A2. Under optimal medium and fermentation conditions, titers of 890, 1,800 and 1,010 nmol/l of 3A4, 2C9 and 1A2, respectively, were achieved in 10-l fermentations. These expression levels represent a 3- to 6-fold increase compared to flask fermentations in this study, and up to a 10-fold increase in titer compared with prior literature reports [1, 2, 12, 15, 23, 25].

Bicistronic expression of NPR with P450s was confirmed by measuring the reduction of cytochrome *c* by isolated membranes using a standard procedure [11, 26]. Membrane preparations contained 1,344–4,719 nmol NPR/L having a specific activity of 0.3–1.7 μmol min^{-1} $(mg\ protein)^{-1}$. This level of NPR activity exceeded that of all three P450 enzyme activities by at least 50-fold, ensuring that the rate of metabolite generation was not likely to be limited by flow of the reducing equivalents.

Metabolite generation

The significant improvement in expression levels obtained through fermentation development provided a consistent means to produce ample quantities of both the P450 enzymes and NPR. Intact cells and membranes containing P450 and NPR were evaluated as catalysts for preparative metabolite generation using a series of well-characterized P450 substrates (Fig. 2). To our surprise, when used directly to catalyze the bioconversion of testosterone, cells expressing 3A4 recovered from fermentation broth by centrifugation

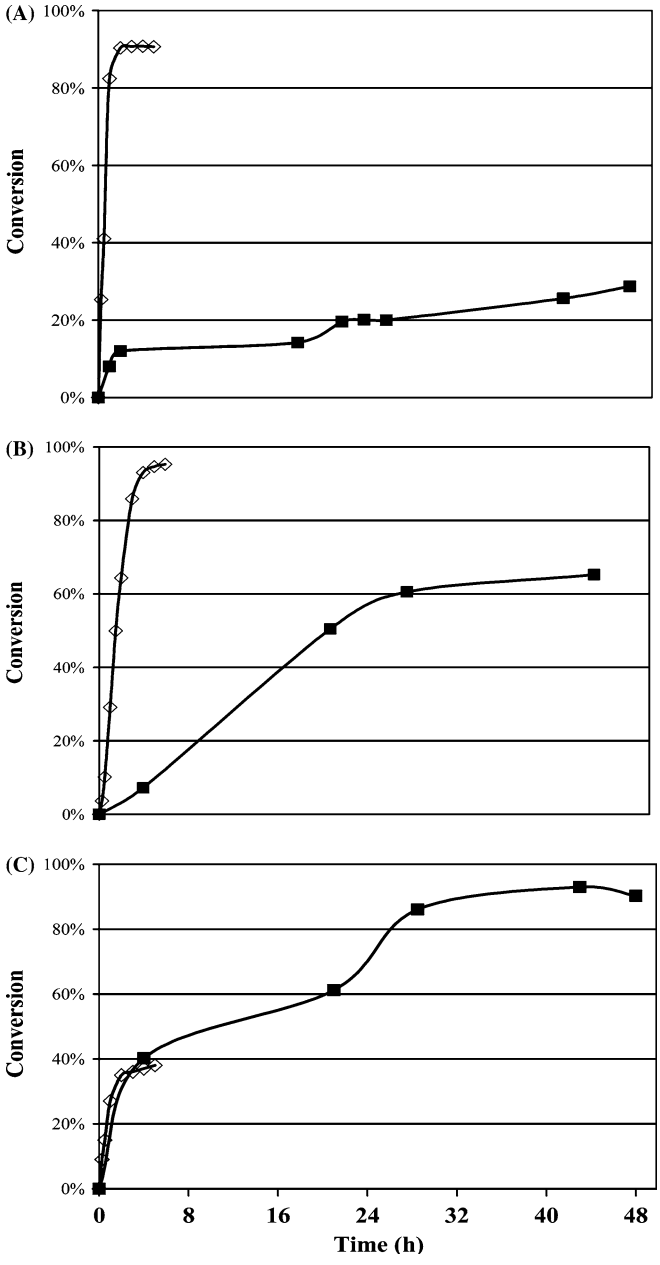

Fig. 4 Profile of model bioconversions by recombinant *E. coli* cells (■) and membranes (◇). **a** Testosterone hydroxylation to 6β-hydroxytestosterone by 3A4/NPR, **b** diclofenac hydroxylation to 4′-hydroxydiclofenac by 2C9/NPR, **c** phenacetin deethylation to acetaminophen by 1A2/NPR. Metabolites were generated using intact cells in 1-L reactions containing phosphate buffer and glucose, or with membranes in 20-ml reactions containing phosphate buffer with a NADPH regeneration system (see Materials and methods)

(22–39 g/l) showed very little activity, even though they contained significant levels of P450 and NPR as determined by carbon monoxide binding and cytochrome *c* reduction assays. We hypothesized that the lack of activity could be attributed to an inhibitor present in the fermentation broth. Indeed, cells washed extensively with buffer prior to use exhibited a 3-fold increase in activity when compared to unwashed cells.

Moreover, fermentation broth added to the reaction mixture inhibited 3A4 activity in a concentration-dependent manner. Interestingly, inhibition was not observed with fresh growth medium, indicating that the inhibitor was likely a fermentation by-product. Due to the complexity of fermentation broth and the fact that inhibition could be eliminated by washing cells with buffer, no attempt was made to identify the nature of the inhibitor.

To improve the aqueous solubility of the substrates, and potentially the efficiency of catalysis, a series of water miscible solvents (1–5% v/v) of varying polarity were screened as additives. As anticipated, the effect of co-solvent was enzyme- and substrate-specific. For example, acetonitrile (2.5% v/v) provided the highest improvement in diclofenac conversion by 2C9 and phenacetin conversion by 1A2, while ethanol (2.5% v/v) was the superior co-solvent for enhancing conversion of testosterone by 3A4. Conversions conducted at temperatures between 20 and 28°C resulted on average in a 30–60% higher product yield when compared to reactions conducted at higher temperatures (37–45°C), presumably due to higher stability of the P450s and possibly increased oxygen solubility at lower temperatures.

To provide sufficient NADPH generation during prolonged reactions, intact cell-catalyzed transformations were supplemented with glucose. Glucose catabolism dramatically decreased the pH of the reaction and depleted the DO level, prompting the need for pH control (7.0–7.5) and high agitation (400 rpm). The impact of these factors was best exemplified by the conversion of phenacetin by cells expressing 1A2. As illustrated in Fig. 3, little if any conversion was observed at an agitation rate of 200 rpm corresponding to a DO saturation level of $<1\%$. Increasing the agitation rate to 400 rpm resulted in a DO level $>60\%$ saturation, which dramatically increased the conversion rate. High agitation combined with sequential glucose supplementation afforded $>90\%$ deethylation of phenacetin to acetaminophen within 40 h. Similar but less pronounced trends were observed with conversion of testosterone and diclofenac.

Conversion profiles of testosterone, diclofenac and phenacetin by both intact cells and membranes are depicted in Fig. 4 (panels a–c). Isolated membranes provided higher specific conversion activity for testosterone and diclofenac when compared with cellular conversions, which were likely limited by substrate diffusion across the cell membrane. In contrast, the highly water-soluble compound, phenacetin, was efficiently metabolized by both intact cells and membranes. Despite the higher conversion of testosterone and diclofenac afforded by isolated membranes, intact cells were chosen as catalyst for all preparative level syntheses to avoid the need to prepare large quantities of membranes and supply an exogenous NADPH regeneration system. Preparative bioconversions of the model compounds on a 1-L scale with intact cells provided conversion yields of 29, 65 and 93% for testosterone, diclofenac and phenacetin, respectively. Metabolite products 6β-hydroxytestosterone, 4′-hydroxydiclofenac and acetaminophen were isolated using solid phase extraction, followed by chromatographic separation (see Materials and methods) with an overall recovery of 53–71%. The identity of each bioconversion product was confirmed by LC MS analysis. The optimized bioconversions conducted on a 1-L scale yielded 59 mg 6β-hydroxytestosterone, 110 mg 4′-hydroxydiclofenac and 88 mg acetaminophen.

In summary, we have demonstrated both the efficient expression and preparative use of recombinant human P450s to generate metabolites of drugs in amounts sufficient to support toxicity and biological activity assessment. The utility of this approach can be easily expanded to the drug discovery area to support the synthesis of new drugs [8, 25].

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