Utilizing a Novel Biocatalytic Human P450 System for the Multi-Cycle Production of Metabolites

Enrique Martinez², Shuvendu Das¹, Denis Callewaert² and Mani Subramanian¹
(1) Department of Chemical & Biochemical Engineering, The University of Iowa, Coralville, IA,
(2) Oxford Biomedical Research, Inc., Rochester Hills, MI

ABSTRACT

The large-scale preparation of drug metabolites is typically a lengthy and problematic step in pharmaceutical development. We have characterized a robust P450 Catalytic System (PCS), which contains unmodified recombinant human cytochrome P450 (CYP) and oxidoreductase enzymes. The engineered inclusion of cofactors and antioxidants within its semipermeable structure greatly stabilizes the PCS and increases its usable lifetime. The present study focused on evaluating the ability of PCS expressing different isoforms to absorb and sequester substrate and generate additional product in multiple reaction cycles. After incubation at 30°C with 500 μM substrate for an initial 4 hour reaction cycle, the PCS was pelleted via low speed centrifugation and resuspended in fresh buffer containing glucose-δ-phosphate but no additional substrate, and incubated for an additional 4 hours (cycle 2). The substrate and concentrations in the reaction supernatant and that sequestered within the PCS system after each reaction cycle, were determined by reversed phase HPLC. The second cycle produced significant amounts of additional product.

INTRODUCTION

CypExpress™ exhibits several characteristics for improved P450 metabolite production, including:
- The ability to absorb and retain hydrophobic substrates from the reaction mixture.
- Retention of catalytic activity for several reaction cycles, thereby increasing overall yield.
- Easily removed from the reaction mixture via low speed centrifugation for metabolite analysis.
- A higher total product yield for multi-cycles vs. a longer single cycle reaction.
- Room-temperature handling and freeze/thaw stability.

METHODOLOGY

Testosterone/Dextromethorphan multi-cycle runs: 20 μL of CypExpress™ was pre-washed with 50 mM potassium phosphate buffer pH 7.5, centrifugation at 3,000 g for 5 min, then re-suspended in the same buffer containing 12 mM Na-Glucose and 500 or 1,000 μM of substrate in buffered flasks. The reaction mixtures were shaken at 30°C and 225 rpm in rotary orbital shaker for the specified times. On the second day, the pellet was removed from 4°C storage and re-suspended in fresh reaction buffer containing only G6P and incubated for an additional five hours. Aliquots were taken at the indicated intervals for HPLC analysis. CypExpress™ multi-cycle runs: 44 mg of CypExpress™ was added to 5.0 mL of 100 mM potassium phosphate buffer pH 7.4 containing 1.0 M G6P and 1.5 mM NADPH with 1.0 mM dextromethorphan, diiodom salt. After the first five hour cycle, the suspension was pelleted via centrifugation at 25,100 g for 10 minutes and the pellet re-suspended in the same buffer. The second, third, and fourth cycles were run for 38, 5 and 18 hours, respectively.

TESTOSTERONE MULTI-CYCLE EXPERIMENT

To distinguish between these two possibilities, a 400 μL reaction was run in which 500 μM testosterone was incubated in 150 mM phosphate buffer with CypExpress™ for 2 cycles at 30°C. At the specified time intervals aliquots of the suspension were removed, extracted and analyzed by HPLC. After 4 hours, the reaction mixture was centrifuged at 16,000 g, and the pellet stored overnight.

DICYClOFEVAC MULTI-CYCLE EXPERIMENT

Diclofenac, an excellent P450-2C9 substrate, was converted to 4'-hydroxydiclofenac in high yield with a CypExpress™ 2C9 loading of 100 mg/mL. However, to examine whether lower levels of CypExpress™ could efficiently produce large quantities of product, diclofenac was incubated with 20 mg/mL of CypExpress™ 2C9 for a total of four reaction cycles. A cost-comparable quantity of rat liver microsomes were used to compare total yield for both systems, shown below in Figure 5.

OPTIMIZATION

As each substrate is unique, the user should determine the following before scaling-up the reaction:
- Amount of retained substrate and metabolite after the first cycle.
- Difference in the product yield obtained by adding additional substrate to each reaction cycle using the substrate retained in the CypExpress™.
- Conversion rate for each substrate based on its solubility or ionic charge.
- Washing of CypExpress™ can be skipped if the metabolites are well separated in the HPLC profile.

SUMMARY

- CypExpress™ retains catalytic activity over multiple cycles and can be used to prepare P450 metabolites in high yield.
- Certain substrates, such as testosterone, accumulate in CypExpress™ and allow for additional cycles to be run with only the addition of cofactors.
- High turnover substrates such as diclofenac can be efficiently metabolized using lower CypExpress™ 2C9 loadings.
- Dextromethorphan at 1.0 mM required only one cycle to produce a significant quantity of dextromethan, with the second cycle yielding significantly less product and being unnecessary.

For more information contact: martinez@oxfordbiomed.com