MULTIMETABIOPROD

Reconstitution of a human metabolic pathway in yeast for the bioproduction of chemical entities - CP2D 2007

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Introduction

The study of human metabolism of exogenous molecules (as active ingredient) (Fig. 1) is a key-step in the study and the use of these molecules (pharmacokinetic, metabolism, toxicology). We developed an alternative system to the synthesis and production by a chemical way of the corresponding human metabolites. This system involves the heterologous expression of human hepatic proteins in micro-organisms and reconstitutes the phase I of the metabolism (CYP450, NADPH CYP450 reductase and cytochrome b5) of drugs or toxic molecules in the yeast S. cerevisiae. The formation of human metabolites was demonstrated mainly analytically in incubations with microsomes or in bioconversion with whole yeast cells. In order to develop a production use (metabolites and new chemical entities) an optimisation of the expression system is required. This optimisation constitutes one of the two part of this project, the other is the development of yeast strains allowing the phase II of drug metabolisation (UGT, GSTs,...). The main goal of this project is to reconstitute a multistep human system allowing the bioproduction of chemical molecules by an alive organism.

Figure 1. Human hepatic metabolisation pathway of xenobiotics

Material and Methods

Molecular biology: We amplify and clone all the yeast and human genes using classical methods of molecular biology (PCR, restriction/ligation, transformation, amplification in *E.coli* and purification of nucleic acids)
Plasmids and strains: pYeDP60 is a plasmid developed by Dr D. Pompon for the inducible expression of genes. It comprises an expression module (a polycloning sequence, an inducible galactose promoter, a yeast sequence terminator), two selection markers (gene URA3 and ADE2) used in ura- and ade- yeast strains. It includes also a β-galactosidase gene bearing the ampicilline resistance for selection in *E.coli*.
Microbiology: the wild type and recombinant yeast and *E.coli* strains were grown in complete and selective culture media. *E.coli* strains were grown in Luria-Bertani media with or without Ampicilline for selection of plasmid bearing strains and yeast strains were grown in YPGA (complete media) and SW10 and S-X, selective synthetic media for selection of plasmid bearing auxothrophic strains.
Bioconversion: The cultures for bioconversion were performed in YPGE (for ethanol) media. A preculture is used for seeding a culture of YPGE, after a growth step, the expression of the genes is induced (and then production of the human proteins) by addition of galactose. In the same time the substrate to be metabolise is added in the culture media. The enzymatic activity of the CYP450s and of the UGTs is followed in samples taken at different times during incubation. The analysis of the bioconversion products is performed by RP-HPLC-UV/MS/MS.
Biochemistry: The microsomal fractions containing the recombinant proteins are prepared in the same conditions excepted for the addition of substrate. The cells are disrupted and after several centrifugation steps, the intracellular membranes corresponding to reticulum endoplasmic (microsomes) are purified and used for protein dosage and enzymatic activity assays.

Optimisation of the phase I system

In order to enhance the bioconversion rate, we have modified, in a first step, three parameters of the phase I recombinant system, the surrounding cloning sequences, the co-expression of the partners and the yeast strain.

The composition of the **upstream and downstream DNA sequences** is important for a good heterologous expression. We have changed these sequences for several CYP isoforms (Fig. 2) and we have observed an increased level of human protein expression in yeast for several isoforms (Fig. 3)

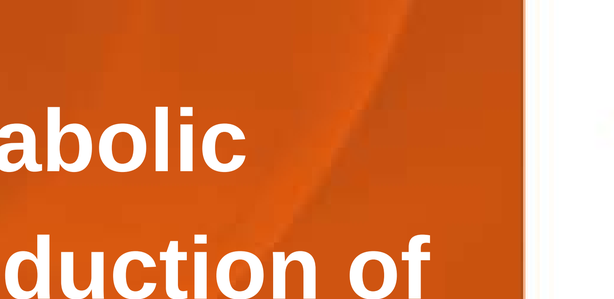


Figure 2. Structure of the surrounding DNA sequences

Figure 3. Increase of protein level in yeast membranes for the re-cloned isoforms

Certain CYP450 isoforms, as CYP3A4, require the presence of the Cytochrome b5 for an optimum enzymatic activity in vivo. We have constructed **co-expression plasmids** allowing the expression in the same cells of the CYP450 and the human cytochrome b5 (Fig. 4). We observe a 4 to 20 fold increase of the enzymatic activity for two CYP450 isoforms (6 β OH testostérone hydroxylase for CYP3A4 and 7 coumarin hydroxylase for CYP2A6). In parallel different isoforms of cytochrome b5 were tested in association with the human CYP3A4, in order to enhance the activity. The cytochrome b5 of a plant (vernicia fordii) was selected for further activity tests in vivo.

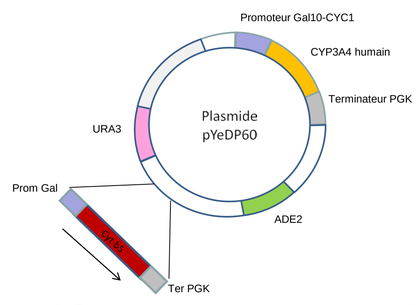


Figure 4. Structure of the co-expression plasmid for the CYP3A4

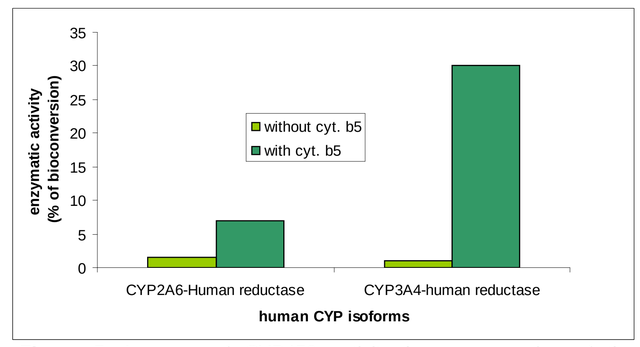
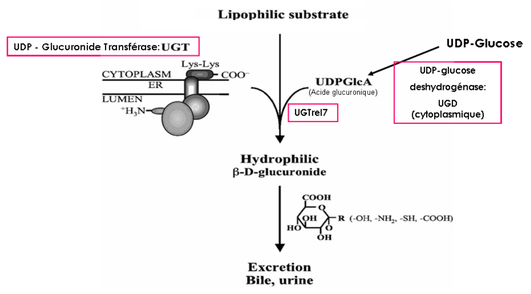


Figure 5. Increase of CYP450 activity by co-expression of the cytochrome b5.

In an other part of the work we have tested several genetic backgrounds for the host yeast strain. FY, BY and W303 (current strain) have similar genetic markers and were tested for the growth and the expression of heterologous genes. Regarding these two parameters FY seems to be the best strain (biomass yield and CYP1A1 production). We have also re-integrated a wild type reductase gene in order to recover a good health for the recombinant strains.

Reconstitution of the phase II system

For the reconstitution of the glucuronidation pathway in yeast we have cloned and expressed in yeast, the human gene coding for the enzyme (UDPglucose deshydrogenase, UGD) catalyzing the formation of the UDP-glucuronic acid (absent in yeast) starting from UDP-glucose (pool present in yeast) and the genes coding for the different human isoforms of UDP-glucuronyl transferases (UGTs). In order to express thes genes in yeast we have developed a set of expression plasmids replicative (model of pYeDP 60, above) and integrative with different selection markers



The human UGD and several isoforms of UGTs (1A6, 1A9, 1A10 and 2B7) have been cloned and transferred in yeast. We have observed in bioconversions, the formation of glucuronidated metabolites with the UGT1A6 and UGT1A9 (wild type genes) but a very low level (10% and 1% respectively) and not with all substrates. No bioconversion was observed with the UGT2B7. We decided to optimise the coding DNA sequences of these genes to obtain a higher level of expression in yeast. The tests are in progress.

Conclusions - Outlooks

Regarding the objectives of the project, for the phase I we have obtain several fold of increase for the expression and activity of the CYP450 and their partners, as the cytochrome b5. The optimisations for the genes, the plasmids and the strains will be combined to obtain new strains for each CYP isoform (almost 13) allowing an enhanced production of oxidized metabolites (scale range from mg to hundreds grams).
Regarding the second goal of the project, we have reconstituted the glucuronidation pathway in yeast as we observed the fomation of metabolites. However the level of bioconversion is too low for the expected level of production. The analysis of the bioconversion level of the optimised UGT genes in yeast is still under process and we expect an increase for the formation of glucuronides.
For the last objectives, the combination of the two phases in vivo, we will test the bioconversion with the optimised strains and genes.

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