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High throughput screening of heterologous P450 whole cell activity

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Abstract

Cytochrome P450s are a superfamily of monooxygenases that catalyze numerous stereo- and regio-specific oxygenation reactions that are potentially valuable biotransformations. In particular, plant P450s typically have higher substrate specificity than mammalian P450s. Since most eukaryotic P450s are membrane-bound proteins, require the cofactor NAD(P)H and cytochrome P450 reductase (CPR), a whole-cell system is preferred for application in biosynthesis. Cinnamate 4-hydroxylase (C4H) is a P450 monooxygenase from the phenylpropanoid pathway, which catalyzes the hydroxylation of cinnamic acid with high specificity. In this study, recombinant *Saccharomyces cerevisiae* co-expressing C4H and CPR from *Arabidopsis thaliana* was selected as a system to develop an optimal medium for the heterologous expression of plant P450s. A high throughput screening (HTS) method based on the natural substrate was developed to examine factors important for whole cell C4H activity, which included the ratio of inducer (galactose) to glucose and concentration of casein hydrolysate. A single-stage procedure that combined cell growth and induction was optimized through factorial design to simplify cell culture and enzyme expression.

Keywords: Media optimization; Full factorial design; Yeast; Biocatalysis

1. Introduction

Cytochrome P450s are one of the largest superfamilies of enzymes found in almost all living organisms. In particular, there are more than 1000 P450 sequences known from all plant species [1]. Because the reactions catalyzed by P450s are at ambient conditions with molecular oxygen as the oxygen source, P450s have an enormous potential for application as biocatalysts [2]. Furthermore, due to the specificity of enzymes, multiple protection and de-protection steps typical of organic synthesis can be avoided. Typically, plant P450s, which catalyze most of the oxidation steps in secondary metabolism, have been shown to have a higher specificity than the mammalian P450s [3,4]. However, there are considerable challenges which must be overcome before the practical application of P450s as biocatalysts. These challenges include low activity towards non-natural substrates, poor stability under industrial process conditions, and the requirement of associated proteins for electron transfer [5].

Directed evolution is a very efficient and practical strategy to improve the performance of P450s, which mimics natural evolution but at a much shorter timescale [6]. The key to success of directed evolution is an effective high throughput screening method. The commonly used high throughput screening assays of P450 activity are based on the detection of fluorescent substrate surrogates, coupling products with fluorescence generating enzymes, or the degradation product of cofactors, all of which are not direct assays of enzyme activity towards natural substrate [7–9].

To develop a high throughput screening method of P450s, we chose *Arabidopsis thaliana* cinnamate-4-hydroxylase (C4H; EC 1.14.13.11), which catalyzes the *para*-hydroxylation of *trans*-cinnamic acid to produce *p*-coumaric acid (Fig. 1). C4H was the first plant P450 to be cloned and has been extensively studied due to its key involvement in the plant phenylpropanoid pathway [1,4,10]. In this study, we developed an effective high throughput screening method of heterologous C4H activity with its natural substrate, which includes a single-stage procedure of C4H expression and a whole cell activity assay via a microplate reader.

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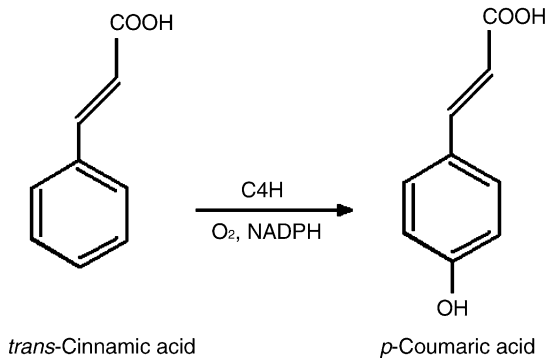

Fig. 1. Reaction catalyzed by cinnamate 4-hydroxylase (C4H).

2. Materials and methods

2.1. Chemicals

Glucose, galactose, casein enzymatic hydrolysate (EHC), tryptophan, methanol, acetonitrile, dimethyl sulfoxide (DMSO), agar, cinnamic and *p*-coumaric acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). Yeast nitrogen base without amino acids and ammonium sulfate (YNB-AA/AS) was purchased from Becton Dickinson Company (Sparks, MD).

2.2. Strain and plasmids

A *Saccharomyces cerevisiae* strain WAT11U, derived from the W303-B strain (*MAT a*; *ade*2-1; *his*3-11, -15; *leu*2-3, -112; *ura*3-1; *can*^{R}; *cyr*$^{+}$) with chromosomal integration of an *A. thaliana* NADPH-cytochrome P450 reductase gene was used as the host strain in this study [11]. The *S. cerevisiae* expression vector pYeDP60 that contains C4H gene whose expression was driven by a *GAL10-CYC1* hybrid promoter was constructed by Pompon et al [11].

2.3. Culture media

The starting medium composition was based on a previously optimized medium for ferulate 5-hydroxylase expression in the WAT11U strain (modified synthetic medium SGI) [12]. The compositions are: 20 g/L glucose or mixture of glucose and galactose (for yeast growth or enzyme expression induction, respectively), 5 g/L casein enzyme hydrolysate, 40 mg/L tryptophan and 3.4 g/L YNB-AA/AS. In this study, a statistical analysis was used to find the optimum medium composition.

2.4. Single-stage cell cultivation procedures

A single yeast colony from a plate containing modified SGI medium was transferred to 25 mL of modified SGI medium in a 125 mL flask and cultivated at 30 °C with shaking at 300 RPM for 24–28 h until OD_{600} reached 1.0–2.0. The cells were then subcultured to a 96-well standard microplate with 200 μL induction medium in each well and diluted to an initial OD_{600} of 0.02. The microplate was covered with a Breathe-Easy™ membrane (Diversified Biotech, Boston, MA) to keep cells aerated and contamination free. The microplate was incubated at 30 °C for 18–24 h with a shaking speed of 800 RPM on a microplate shaker (National Labnet Co., Woodbridge, NJ). Higher liquid volume or lower shaking speed did not provide good mixing as judged by comparison to growth curves from identical cultures in shake flasks (data not shown).

2.5. Whole cell in vivo enzyme assay

2.5.1. Spectrophotometric assay

Different concentrations of *trans*-cinnamic acid and *p*-coumaric acid were dissolved in either DMSO, supernatant of yeast (WAT11U) cell culture or well-mixed yeast (WAT11U) cell culture, with the total molar concentration of cinnamate and coumarate kept at the same level. The absorbance at 314 and 320 nm was recorded with a DU® Series 500 spectrophotometer (Beckman Instruments Inc., CA). The supernatant assay and whole cell assay methods were applied in parallel with the HPLC assay to follow the formation of *p*-coumaric acid in the whole cell reaction according to the standard curve above.

2.5.2. HPLC assay

After induction, a stock solution of substrate (250 mM *trans*-cinnamic acid dissolved in DMSO) was added to the cell culture to a final concentration of 0.5 mM. Initial reaction rates were calculated based on the first 20 min of reaction to ensure that less than 10% of the substrate was converted to product. Samples were taken every 5 min and mixed with an equal volume of methanol to quench the reaction. The mixture was centrifuged at 18,000 × *g* for 2 min and the supernatant was analyzed by HPLC on an Agilent SB-C18 column (4.6 mm × 75 mm) with temperature maintained at 30 °C. Solvent A was 1.5% (v/v) acetic acid in water and solvent B was 100% acetonitrile. The flow rate was 0.9 mL/min and the injection volume was 10 μL. Initially, solvent B was maintained at 5% for 4 min, increased to 45% over 8.5 min, and held for 1 min before returning to 5% solvent B. Cinnamic acid was quantified at 254 nm with a retention time of 12.1 min, and *p*-coumaric acid was quantified at 314 nm with a retention time of 9.1 min.

2.5.3. Microplate reader assay

After induction for 18–21 h, 100 μL of cells from each well of the microplate were transferred to a 96-well UV-transparent microplate (Corning Incorporated, NY). The substrate was added to each well to a final concentration of 0.5 mM. The absorbance was read in the kinetic mode of a SpectraMax190 microplate reader (Molecular Devices Corporation, CA). The absorbance of the products at 320 and 600 nm were recorded every 2 min and the absorbance at