Optimization of recombinant Cytochrome P450 2C9 protein production in *Escherichia coli* DH5α by statistically-based experimental design

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**Abstract** Cytochrome P450 (CYP) 2C9 is of major importance in drug metabolism. However, the low yield of recombinant CYP2C9 protein in *E. coli* strains prevents its extensive use in the study of *in vitro* drug metabolism. In the present study, Taguchi design and desirability function were first used to investigate the effect of medium components (glycerol, δ-ALA, IPTG, ampicillin, chloramphenicol, inoculum density, peptone, thiamine, trace elements, NH₄Cl, and MgSO₄) on recombinant human CYP2C9 production by *E. coli* DH5α. An L₁₂(2¹¹) orthogonal array was used to design the experiments to screen out the most influential factors. The CYP concentration and the specific content of CYP were considered as two product quality variables. A desirability function was applied to combine these two qualities as a single objective function. Optimization via central composite design (CCD) was then undertaken to yield the best performance. The confirmation experiments indicated that the expression performance under the optimized conditions was better than those obtained under other conditions. A compromise between conflicting goals, such as achievement of good yield of recombinant CYP2C9 and facility of the following purification, was found by means of the desirability function *D*. This is the first report that combined Taguchi design and CCD, and performed experiments in a multiresponse framework to optimize the production of human CYP in a recombinant *E. coli* strain.

**Keywords** Cytochrome P450 2C9 · Heterologous expression · Optimization · Taguchi orthogonal design · Desirability function · Central composite design

**Introduction**

Cytochrome P450 (CYP, P450) 2C9 is known to be one of the most important members of the drug-metabolizing CYP isoforms in the human liver. Some of these drugs have narrow therapeutic indices, such as the anticoagulant warfarin and the anticonvulsant phenytoin (Delozier et al. 2005). Other drugs metabolized principally by CYP2C9 include the antidiabetic drugs tolbutamide and glipizide, the diuretic torsemide, the antihypertensive drug losartan and numerous nonsteroidal anti-inflammatories including flurbiprofen, ibuprofen and diclofenac.

Heterologously expressed CYP2C9 is indispensable in the *in vitro* study of relevant drug metabolism (Boye et al. 2004). Recombinant CYP2C9 production is influenced by various factors, especially the medium components. However, it is not an easy task to explore all the main nutritional factors and try to obtain their optimum levels in a single process. Published experimental results concerning medium composition are often conflicting. The traditional method of optimization involves varying one factor at a time, while keeping the others constant. This strategy requires a relatively large number of experiments and fre-
frequently fails to anticipate the optimal conditions (Montgomery 2004). This essential shortcoming is due to the inability of the approach to consider the effects of possible interactions between factors. The deficiency can be overcome by applying more efficient, statistically based experimental design. In this respect, Taguchi orthogonal design (Taguchi et al. 2004), central composite design (CCD), and desirability function (Derringer and Suich 1980) are important tools to determine the optimal process conditions. The advantages of using the Taguchi method and CCD are that many more factors can be screened and optimized simultaneously and much quantitative information can be extracted by only a few experimental trials. Therefore, these methods have been extensively applied in parameter optimization and process control (Ambati and Ayyanna 2001; Escamilla-Silva et al. 2001; Fontani et al. 2003; Ratnam et al. 2003, 2005).

Most of Taguchi’s and CCD applications aim to optimize single-response problems. However, due to the complex process of recombinant CYP expression and purification, at least two quality characteristics need to be considered simultaneously to enhance the product quality. Moreover, correlations among multiple responses always exist and these correlations may generate conflicts in determining optimal parameter settings. Hence, simultaneously optimizing a multi-response problem is a relevant issue.

This work attempts to increase the CYP concentration and the CYP specific content by optimizing the production conditions using the Taguchi orthogonal design and CCD. The model CYP production studied was human CYP2C9 expressed in E. coli DH5α that is commonly used in heterologous expression of human CYPs. This work presents an effective procedure which utilizes the desirability function (Paterakis et al. 2002; Safa and Hajmohammadi 2005) to optimize multi-response problems.

Materials and methods

Recombinant strain, media, and culture conditions

Escherichia coli DH5α, genotype: supE44, ΔlacU169 (φ80 lacZAM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1, harboring plasmid pCW2C9dH has been used throughout this study. The purified human CYP2C9 produced from this expression plasmid was used in X-ray crystallography to determine the structure of CYP2C9 complexed with flurbiprofen (Wester et al. 2004). In this plasmid, the expression of CYP 2C9 is under the control of tac promoter.

δ-Aminolevulinic acid (δ-ALA), thiamine, ampicillin (Amp), chloramphenicol (Cm), isopropyl-β-d-thiogalactopyranoside (IPTG), and glycerol were from Sigma. NH4Cl and MgSO4·7H2O were prepared and sterilized as stock solutions of 2.5 M and 0.5 M, respectively. Terrific broth (TB) and Luria-Bertani (LB) media were prepared as described in pET system manual (Novagen). Tryptone, peptone, agar, and standard salt-free yeast extract were from Oxoid.

Competent DH5α cells, prepared using a standard calcium chloride method, were transformed with pCW2C9dH. Colonies transformed with pCW2C9dH were selected on LB agar plates supplemented with 100 μg/ml Amp. A single colony was inoculated into 5 ml LB medium supplemented with 100 μg/ml Amp and incubated with shaking overnight at 37°C. On the following day, 25 ml of TB medium in a 250 ml flask conditionally supplemented with Amp, Cm, δ-ALA, thiamine, peptone, glycerol, and trace elements (composition: 27 g FeCl3·6H2O, 2.0 g ZnCl2·4H2O, 2.0 g CoCl2·6H2O, 2.0 g Na2MoO4, 1.0 g CaCl2·2H2O, 1.0 g CuCl2, 0.5 g H3BO3, and 100 ml concentrated HCl (li- ter⁻¹) was inoculated with 0.25 ml or 0.5 ml pCW2C9dH (DH5α) overnight culture. The cultures were grown at 30°C in an incubator shaker with shaking at 200 rev/min. When the OD600 nm reached 0.6 (usually 3–4 h after inoculation), CYP2C9 expression was induced by addition of IPTG. NH4Cl and MgSO4·7H2O were added simultaneously when necessary. The cultures were grown for another 48 h under the same conditions. The culture volume was increased to 100 ml in the central composite design.

Spectral analysis and determination of protein content

The reduced CO difference spectrum was obtained as described previously (Omura and Sato 1964). Cells were solubilized in 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 1 mM EDTA, and the insoluble fraction was removed by centrifugation at 10,000 × g for 10 min at 4°C. The soluble fraction was then divided into reference and sample cuvettes, CO was gently bubbled into the sample cuvette for 30 s, and sodium dithionite (Sigma) was dissolved in both cuvettes. CO difference spectra were recorded using a JASCO V-530 spectrophotometer. CYP concentration was calculated according to the difference of absorbance at 450 nm and 490 nm, and an extinction coefficient of 91 cm⁻¹ M⁻¹ for P450 was used as described previously (Omura and Sato 1964). The same spectrophotometer was used to measure protein concentration of the whole cell lysate via the Bradford assay using bovine serum albumin as the standard. The CYP specific content (sc, nmol/mg protein)=CYP concentration (conc, nmol/ml) / protein concentration (mg/ml).

Taguchi design and data analysis

Table 1 displays the eleven control factors selected in the optimization study. A standard orthogonal array L12 (211)