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Large-scale Production of Genetically Engineered CYP3A4 in E. coli: Application of a Jarfermenter

Yoichi Kanamori1, Ken-ichi Fujita1, Kazuo Nakayama1, Hideki Kawai2 and Tetsuya Kamataki1

1Laboratory of Drug Metabolism, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan
2Bio Laboratory, Nihon Nosan Kogyo K.K., Tsukuba, Japan

Summary: To produce a large amount of CYP3A4, we applied a jarfermenter (ABLE, BMJ-PI or BMS-PI) to culture the genetically engineered E. coli cells harboring CYP3A4 along with NADPH-cytochrome P450 reductase (OR). The jarfermenter is a stirred bacterial culture vessel in which the pH, the dissolved oxygen (DO) and the temperature of a culture medium can be controlled. The expression of CYP3A4 in E. coli cells in the 500 mL of culture medium contained in the BMJ-PI (1 L vessel) (JFM-1) was examined by altering the parameters mentioned above. The highest expression of CYP3A4 in E. coli cells was attained when cultured at pH 6.0, at 30°C under the DO of 0.1 ppm. The incubation was performed 18 hr after the addition of 1.5 mM isopropyl β-D-(-)-thiogalactopyranoside. The expression levels of CYP3A4 and the OR in the membrane fraction of E. coli cells were 267 nmol/L culture and 552 units/L culture, respectively. The CYP3A4 level was about three times higher than that obtained by incubation in a 500 mL flask (100 mL of medium) (84 nmol/L culture). The testosterone 6β-hydroxylase activity of CYP3A4 expressed in the membrane fraction of E. coli obtained with the JFM-1 was examined. The apparent Kₘ and Vₘₐₓ values were 66.4 μM and 57.8 nmol/min/nmol CYP, respectively.

Expecting the mass production of the CYP3A4 by a culture of E. coli, the possibility of a scale up of the culture with the BMS-PI (10 L vessel) (JFM-10) was examined. The optimal culture condition to achieve the highest expression of CYP3A4 with JFM-1 was employed. The expression levels of CYP3A4 and the OR obtained with JFM-1 and JFM-10 were almost equal. The total level of CYP3A4 obtained by using JFM-10 (5 L of medium) was calculated to be about 1.4 μmol.

Based on these results, we confirm that the jarfermenter is a useful tool to produce large amounts of CYP3A4.

Key words: heterologous expression; drug metabolism; pH; dissolved oxygen; high throughput screening

Introduction

Cytochrome P450 (CYP) is a heme-containing enzyme which catalyzes the oxidation of a wide variety of endogenous and exogenous compounds including drugs, carcinogens, and other xenobiotic chemicals.1-3) Since most clinically used drugs are metabolized by CYP, metabolism by CYP has been regarded as a necessary matter to be examined for the development of new drugs. Thus, the metabolism of a drug candidate by CYP is examined during the pre-clinical trials. This examination is usually performed after the screening of chemicals for pharmacological effects as indices.

However, in some cases, the drug candidates thus screened are known to show pharmacokinetic problems, which prevent further development of chemicals as drugs. The other is a drug-drug interaction caused by the inhibition of the metabolism of a certain drug by other drugs. There are many chemicals which are metabolized by CYP3As.4) Thus, the drug interaction is expected to occur if more than two drugs are applied to a patient, which are metabolized mainly by CYP3A4. In fact, azole antifungal agents have been known to inhibit the metabolism of other drugs to cause undesired effects of other drugs metabolized mainly by the same enzyme, CYP3As, because the drugs given in combination with the antifungal agents cannot be metabolized by the CYP3As.5,6)

To predict the problems mentioned above, pharmaceutical companies have performed research to clarify the metabolism by CYP in the pre-clinical stages. One of the most important matters in this stage of research is
to identify a toxic metabolite(s) of a candidate drug to avoid a possible toxicity seen only in humans.

Recently, the use of CYP preparations expressed in heterologous expression systems has become more popular for examining human drug metabolism, partly because the preparations possessing the same properties can be supplied constantly. Among the heterologous expression systems, *E. coli* expression systems have advantages compared to other expression systems in terms of low cost to maintain, ease of use, and the high yield of protein with a relatively short period of incubation. Thus, we established eleven strains of *E. coli* each co-expressing a form of human CYP (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) along with the human NADPH-cytochrome P450 reductase (OR). The metabolic pathways of various chemicals have been successfully predicted with the established *E. coli* strains. We have also succeeded in evaluating the inhibition of the metabolism of a certain drug by human CYP by other chemicals, which indicated that the established genetically engineered *E. coli* strains were applicable to predict drug-drug interactions in humans. The large amount of production of human CYPs with the bacterial expression systems may allow us to screen a wide variety of candidates on the metabolism by CYP in a relatively short period, namely high throughput screening.

So far, we have cultured the bacteria in 100 mL of medium in a 500 mL flask to express human CYP in *E. coli* cells. This amount of culture is not enough to apply to the high throughput screening for drug-drug interaction and to the identification of the metabolites of the drug candidates.

To yield large amounts of CYP3A4 by the *E. coli* expression system, we employed a jarfermenter with a stirred bacterial culture vessel (1 L) (JFM-1), where the pH, dissolved oxygen (DO) and temperature of culture medium could be controlled. In the present study, we examined the expression of CYP3A4 in *E. coli* cells with the JFM-1 by altering the parameters mentioned above, and compared the result with that obtained by using a 500 mL flask.

In addition, considering the mass production of the CYP3A4 by a culture of *E. coli*, the possibility of a scale-up of the culture with a jarfermenter, in which vessel volume was 10 L (JFM-10), was examined. We tested whether or not the optimal culture condition determined by using the JFM-1 could be applicable to the JFM-10.

**Materials and Methods**

**Chemicals and apparatus:** Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ were obtained from Oriental Yeast (Tokyo, Japan), and testosterone was from Sigma (St. Louis, MO). 6β-Hydroxytestosterone was purchased from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available. Jarfermenters, BMJ-PI (JFM-1) and BMS-PI (JFM-10) were purchased from ABLE (Tokyo, Japan).

**Culture conditions for the expression of CYP and OR in *E. coli* DH5α cells:** A strain of *E. coli* DH5α expressing human CYP3A4 together with the OR was used. The strain of *E. coli* was established by Iwata *et al.*

CYP and the OR were expressed in a culture containing the genetically engineered *E. coli* cells according to the method by Iwata *et al.* with minor modifications. Briefly, twenty μL of bacterial stock solution was inoculated into 50 mL of a Luria-Bertantni medium supplemented with ampicillin (100 μg/mL). Five or 50 mL of the culture was inoculated into 500 mL (JFM-1, Fig. 1(A)) or 5 L (JFM-10, Fig. 1(B)) of modified Terrific Broth and grown with shaking at 30°C for 8 hr prior to induction by addition of 1.5 mM isopropyl β-D(-)-thiogalactopyranoside (IPTG), according to the protocol of the manufacture (ABLE, Tokyo, Japan). The culture conditions such as pH, DO and temperature were changed to obtain the maximal expression of CYP3A4 as follows; pH of the medium was 5, 6 or 7, DO was 0.01, 0.1 or 1 ppm and temperature
Optimal conditions to express CYP3A4 in E. coli cells by using JFM-1: The culture conditions to achieve the highest expression of CYP3A4 in E. coli cells were examined by using JFM-1. The effects of pH and DO of the culture medium on the expression of CYP3A4 are shown in Fig. 2. The temperature of the culture medium was 30°C. The time course of the expression of CYP3A4 after the addition of IPTG to the culture medium is shown. When pH of a culture medium was adjusted to 6.0, the expression level of CYP3A4 was the highest. The expression of CYP3A4 was low with DO at 1.0 ppm. CYP3A4 was not expressed when pH of the culture medium was 7.0. Thus, the high level of expression of CYP3A4 was achieved under conditions at relatively low DO at pH lower than 7.0.

Optimal conditions to express CYP3A4 in E. coli cells by using JFM-1: The culture conditions to achieve the highest expression of CYP3A4 in E. coli cells were examined by using JFM-1. The effects of pH and DO of the culture medium on the expression of CYP3A4 are shown in Fig. 2. The temperature of the culture medium was 30°C. The time course of the expression of CYP3A4 after the addition of IPTG to the culture medium is shown. When pH of a culture medium was adjusted to 6.0, the expression level of CYP3A4 was the highest. The expression of CYP3A4 was low with DO at 1.0 ppm. CYP3A4 was not expressed when pH of the culture medium was 7.0. Thus, the high level of expression of CYP3A4 was achieved under conditions at relatively low DO at pH lower than 7.0.

Next, the effects of temperature and DO of culture medium on the expression levels of CYP3A4 were examined. In these experiments, pH of the culture medium was fixed at 6.0. The results are shown in Fig. 3. When the culture temperature was 30°C, the expression of CYP3A4 clearly depended on the DO. The expression level of CYP3A4 was found to be higher under 0.01 or 0.1 ppm DO than 1.0 ppm DO. On the other hand, the expression level of CYP3A4 was not dependent on the
Fig. 3. The effects of pH and DO of the culture medium on the time course of the expression level of CYP3A4 in E. coli cells by using JFM-1. The volume and pH of the culture medium were 500 mL and 6.0, respectively. The temperature of the culture was 25°C (A), 30°C (B) or 37°C (C). (∆), DO 1.0 ppm; (○), DO 0.1 ppm; (●), DO 0.01 ppm. Each point represents the mean (n = 3).

Table 1. Expression levels of CYP3A4 and OR

<table>
<thead>
<tr>
<th>Source</th>
<th>Culture/Vessel</th>
<th>CYP3A4 (nmol/L culture)</th>
<th>Total CYP3A4 (nmol)</th>
<th>OR (units/L culture)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>500 mL/JFM-1</td>
<td>267 ± 5a</td>
<td>134</td>
<td>552 ± 88a</td>
<td>Present Study</td>
</tr>
<tr>
<td>E. coli</td>
<td>100 mL/500 mL flask</td>
<td>84 ± 54a</td>
<td>8.4</td>
<td>436 ± 18a</td>
<td>15</td>
</tr>
<tr>
<td>Yeast</td>
<td>80 L/110 L fermenter</td>
<td>7–11</td>
<td>7–11</td>
<td>—</td>
<td>22</td>
</tr>
<tr>
<td>Yeast</td>
<td>1 L/2 L flask</td>
<td>2</td>
<td>160</td>
<td>—</td>
<td>22</td>
</tr>
</tbody>
</table>

a Values are presented as mean ± SD (N = 3).

DO when the culture temperature was 37°C. The highest expression of CYP3A4 in E. coli was observed with a culture condition of pH 6.0, 0.01 ppm DO, the temperature at 30°C and the period of the incubation after the addition of IPTG for 18 hr (267 nmol/L culture). The expression level of OR under the culture condition was 552 units/L culture.

The expression levels of CYP3A4 and the OR by using JFM-1 were compared with those obtained by using a 500 mL flask (Table 1). The expression levels of CYP3A4 and the OR achieved with the flask were 84 nmol/L cultures and 436 units/L culture, respectively.15) Thus, the content of CYP3A4 prepared with JFM-1 was three times higher than that obtained with the flask. The level of the OR seen with JFM-1 was 1.27 times higher than that observed with the flask. The total amount of CYP3A4 obtained with JFM-1 was 134 nmol, whereas that obtained with the flask was 8.4 nmol.

Testosterone 6β-hydroxylase activity of CYP3A4 produced by JFM-1: The testosterone 6β-hydroxylase activity of CYP3A4 expressed in E. coli cells obtained with JFM-1 was examined by using the membrane fraction of the E. coli cells. The apparent $K_m$ and $V_{max}$ values were 66.4 μM and 57.8 nmol/min/nmol CYP, respectively. These values were almost equal to those obtained with whole cells of E. coli expressing CYP3A4 ($K_m$, 70.8 μM; $V_{max}$, 59.7 nmol/min/nmol CYP).15)

Scale-up of the culture to express CYP3A4 from JFM-1 to JFM-10: The possibility of the scale-up of the culture with the jarfermenter was tested. We examined whether or not the optimal culture condition determined by using the JFM-1 was applicable to the culture with JFM-10. The optimal culture condition to achieve the highest expression of CYP3A4 with JFM-1 was applied to the culture of E. coli cells by using JFM-10. Figure 4 shows the time course of the expression of CYP3A4 after the addition of IPTG to the culture medium. The expression levels of CYP3A4 and the OR in the E. coli cells cultured with JFM-1 and JFM-10 were nearly the same. The total amount of CYP3A4 expressed in E. coli cells obtained by using JFM-10 with 5 L of medium was calculated to be about 1.4 μmol.
Fig. 4. Comparison of JFM-1 and JFM-10 for the expression of CYP3A4 and OR in E. coli cells. The pH, temperature and DO were 6.0, 30°C and 0.1 ppm, respectively. The volumes of the culture were 0.5 L for JFM-1 and 5 L for JFM-10. (○), The expression level of CYP3A4 (JFM-1); (●), The level of OR (JFM-1); (△), The expression level of CYP3A4 (JFM-10); (▲), The level of OR (JFM-10). Each point represents the mean ± SD (n = 3).

Discussion

We applied the jarfermenter to culture the genetically engineered E. coli cells to produce a large amount of CYP3A4. The highest expression of CYP3A4 in E. coli (267 nmol/L of culture) was seen with the culture conditions of pH 6.0, 0.1 ppm DO and 30°C of temperature, when the incubation was performed 18 hr after the addition of IPTG (Fig. 1 and Fig. 2). The level of CYP3A4 expressed in one liter of a culture of E. coli cells corresponded to that expressed in 61.8 g of the human liver, assuming that 1 g of the human adult liver contained about 4.32 nmol of CYP3A4 protein, as reported by Shimada et al.22)

In previous studies, the cultures of E. coli cells for the heterologous expression of human CYP were performed by using glass flask. The culture conditions such as DO and pH changed according to the growth of E. coli cells, when the flasks were employed. Therefore, DO and pH could not be maintained as optimal for the production of CYP3A4 protein. On the other hand, applying the jarfermenter, the culture conditions of E. coli cells have been optimally controlled during the culture for the synthesis of CYP3A4 protein by E. coli cells, allowing us to obtain the large amount of CYP3A4 protein.

The possibility of the scale-up of the culture with the jarfermenter was tested. As shown in Fig. 4, time course of the expression of CYP3A4 seen with JFM-10 was almost the same as that seen with JFM-1, when cultured under the same culture condition, probably suggesting that the culture condition could be applied to a jarfermenter with a volume larger than 10 L.

The total level of CYP3A4 expressed in E. coli cells obtained by using JFM-10 with 5 L of medium was calculated to be about 1.4 μmol. Guengerich et al.22) have shown that the total expression level of CYP3A4 in Yeast cells was about 0.16 μmol even though they used a 110 L fermenter containing 80 L of medium (Table 1), indicating that the efficiency of the expression of CYP3A4 in E. coli cells seen with JFM-10 was extremely higher than that in Yeast cells. The large amount of the production of CYP3A4 by JFM-10 may allow us to examine the possibility of drug-drug interaction caused by the inhibition of the metabolism of a therapeutic drug by CYP3A4 for many drug candidates.

The production of a large amount of genetically engineered CYPs in E. coli cells may realize the application of the enzymes to the high throughput screening for pre-clinical drug metabolism studies.

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