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RESEARCH PAPER

Effect of Culture Conditions on the Whole Cell Activity of Recombinant *Escherichia coli* Expressing Periplasmic Organophosphorus Hydrolase and Cytosolic GroEL/ES Chaperone

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Abstract Specific whole cell activity strongly affects sensitivity and detection limit of whole cell-based biosensors. Previously, we developed recombinant *Escherichia coli* co-expressing periplasmic organophosphorus hydrolase (OPH) and cytosolic chaperone GroEL-GroES (GroEL/ES). In present work, we investigated the effect of culture conditions on whole cell OPH activity. Especially, the whole cell OPH activity was significantly affected by the concentration of tetracycline that is an inducer for chaperone GroEL/ES. When cultured at 20°C for 31 h in M9 medium containing 1 mM IPTG, 50 ng/mL tetracycline, and 500 μM $CoCl_2$, the recombinant *E. coli* exhibited a specific whole cell OPH activity (U/OD_{600}) of ~0.55, which is 2.6-fold higher than that of recombinant *E. coli* cultured as previously described conditions. In addition, recombinant cells showed adequate storage stability for 1 week with 100% of original response. Finally, the improved activity and adequate stability in the whole cell biocatalyst will contribute to sensitivity, detection time, and stability of a whole cell-based biosensor for the detection of toxic organophosphates.

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1. Introduction

Organophosphates (OPs) are widely used as pesticides and insecticides in agriculture and homes due to their wide-spectrum activities and low cost. However, the compounds have also been considered a risk element because they act as acetylcholinesterase (AChE) inhibitors, and can have serious effects in man due to acetylcholine accumulation [1,2]. Because of these effects, some OPs are used as chemical warfare agents. Thus, the threats posed by OPs necessitate the development of sensitive, reliable, and cost-effective analytical tools for their detection, and in this context, organophosphorus hydrolase (OPH)-based biocatalysts have been developed for the sensitive and effective detection of OPs by biosensors [3-6].

Organophosphorus hydrolase (OPH) is a homodimeric organophosphotriesterase from *Pseudomonas diminuta* or *Flavobacterium* sp. with a broad spectrum of substrate specificity and high turnover rates for various phosphorus-ester bonds of toxic OPs [7-9]. Although these properties make OPH useful as a biocatalyst for the detection, its low stability and high purification cost of the enzyme remain major challenges. Recombinant *Escherichia coli* expressing OPH has attracted considerable attention because it addresses both of these issues [7,10,11]. However, the use of whole-cell biocatalysts present some problems in OPs detecting biosensors, such as limited mass transfer through the cell membrane, which reduces catalytic efficiency [12]. To overcome this limitation, several strategies have been used,

for example, the specific cellular localization of the enzyme and enhancer co-expression [3,4,13-15]. In previous study, we developed recombinant *E. coli* co-expressing periplasmic OPH and cytosolic chaperone GroEL-GroES (GroEL/ES) and identified the function of GroEL/ES on the folding and translocation of OPH [13]. In addition, we confirmed the feasibility of recombinant *E. coli* as biocatalyst in biosensor for the detection of toxic OPs [16,17]. However, activity and stability of whole cell biocatalyst are considered to be of important issues in whole cell-based biosensors since they significantly affects the sensitivities, detection limits, reusability, and long-term stability of biosensors [5]. In this work, we investigated the effect of culture conditions on whole cell OPH activity, including the concentrations of isopropyl β-D-thiogalactoside (IPTG), tetracycline, and $CoCl_2$, and culture temperature and time. Furthermore, we examined storage stability of the recombinant *E. coli* culture medium at 4°C

2. Materials and Methods

2.1. Bacterial strains, plasmids, and cell culture

The plasmid pETO [3] and a commercial vector pG-KJE6 (TakaRa, Shiga, Japan) were used to express OPH in periplasm and chaperone GroEL/ES in cytosol, respectively. Plasmids were transformed into *E. coli* strain BL21 (DE3) (Novagen, Darmstadt, Germany) to facilitate OPH and chaperone GroEL/ES co-expression [13]. Recombinant cells were grown to an optical density at 600 nm (OD_{600}) of 0.6 ~ 0.8 at 37°C in shake flasks containing 50 mL of Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptophan, and 1% NaCl) containing 50 μg/mL ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and 25 μg/mL chloramphenicol (Sigma-Aldrich). OPH expression was induced by adding IPTG (isopropyl β-D-thiogalactoside) (Sigma-Aldrich) with 0.5 mM $CoCl_2$ (Sigma-Aldrich) to final concentrations ranging from 0.05 to 1 mM. To induce the expression of chaperone GroEL/ES, tetracycline (Sigma-Aldrich) was also added at final concentrations ranging from 1 to 50 μg/mL. Recombinant *E. coli* were also grown at different temperatures (20, 25, and 37°C) and in M9 minimal medium containing 0.5% (w/v) glucose and 50 μg/mL ampicillin to investigate the effect of $CoCl_2$ concentration (0.005 ~ 5 mM) on OPH expression.

2.2. Measurement of whole-cell activity

Cell density (OD_{600}) was measured at 600 nm using an UV/VIS spectrometer (Beckman Coulter, Brea, CA, USA). Paraoxon solution was added to 100 mM 2-(N-cyclohexylamino) ethane-sulfonic acid (CHES) buffer (pH 9.0; Sigma-Aldrich) containing the recombinant *E. coli*. The hydrolysis reaction of paraoxon to *p*-nitrophenol (PNP) and diethyl phosphoric acid was allowed to proceed for 1 min at room temperature. The final product PNP was detected by measuring absorbance at 410 nm using an UV/VIS spectrometer. One unit of whole cell activity was defined as the hydrolysis of 1 micromole of paraoxon per min [18].

2.3. Western blot analysis

For Western blot analysis, each sample was mixed with Tris-HCl buffer (pH 6.8) containing 10% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue and 50% (v/v) glycerol, and boiled for 15 min. Proteins were resolved by 15% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane (GE Healthcare, Giles, Buckinghamshire, UK) pre-equilibrated with transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 20% methanol; pH 9.2) using a Trans-Blot SD Cell (Bio-Rad, Hercules, CA, USA). After blocking with TBS (Tris-buffred saline) buffer (20 mM Tris-HCl and 500 mM NaCl; pH 7.5) containing 5% (w/v) non-fat dry milk, membranes were sequentially incubated with monoclonal anti-His_6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich) in TBS containing 0.05% (v/v) Tween 20 (TTBS) and 1% (w/v) non-fat milk) for 1 h at room temperature. After successive washing with TTBS and TBS, membranes were treated with 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine salt/nitroblue tetrazolium chloride color development solution (Roche, Welwyn Garden City, UK) to detect proteins of interest. The reaction was quenched with distilled water.

3. Results and Discussion

3.1. Effect of IPTG concentration for whole cell OPH activity

Previously, we found that a chaperone GroEL/ES assists proper folding of OPH and enhances the translocation of active OPH into the periplasmic space of *E. coli*, which leads to markedly increases whole cell OPH activity [13]. Enhancing whole cell activity of the recombinant *E. coli* plays a vital factor in microbial biosensor for detection of toxic OPs because the activity dictates sensitivity and detection limit of the biosensor [16,19,20]. The whole cell activity of recombinant *E. coli* co-expressing periplasmic OPH and cytosolic GroEL/ES can be affected by several factors, such as, the concentrations of IPTG, tetracycline, and $CoCl_2$, culture temperature, and culture time. Initially, we investigated the effect of IPTG concentration on the whole cell OPH activity of recombinant *E. coli*. When the

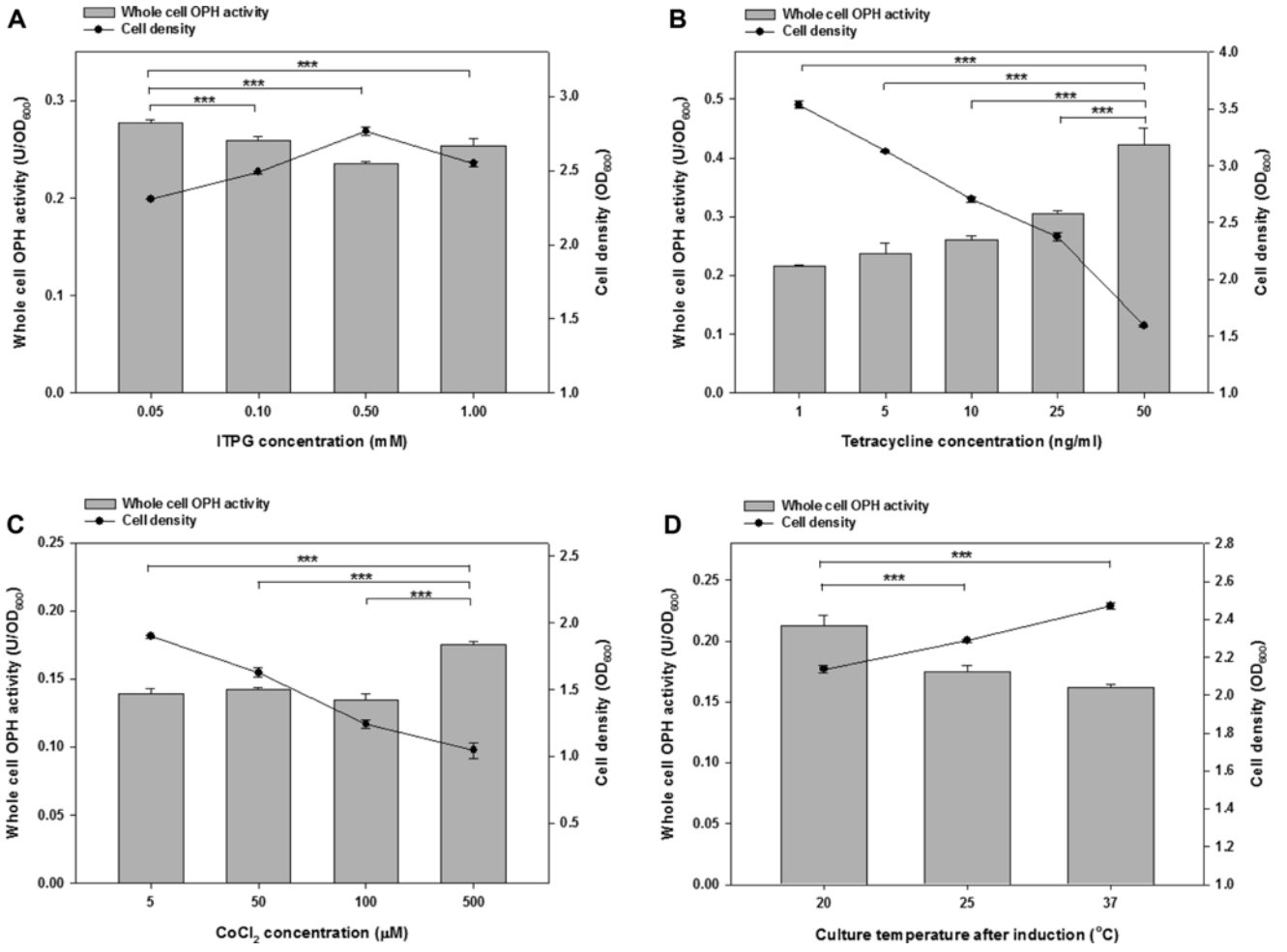

Fig. 1. Effects of (A) IPTG, (B) tetracycline, and (C) $CoCl_2$ concentrations, and (D) culture temperature on specific whole cell OPH activity. Whole cell OPH activity was determined in 100 mM CHES buffer (pH 9.0) containing 1 mM paraoxon by measuring the absorbance at the λmax of 410 nm. Values and error bars represent the means of four independent experiments and their standard deviations with statistical significance (*$p < 0.05$, **$P < 0.01$, ***$P < 0.005$, unpaired t-test).

OD_{600} reached 0.6 ~ 0.8, four different IPTG concentrations (0.05, 0.1, 0.5, and 1 mM) were added to cultures, which were then incubated at 37°C for 6 h. The specific whole cell OPH activity (U/OD_{600}) was not found to be dependent on IPTG concentration in the range of 0.5 ~ 1 mM, (Fig. 1A), possibly because IPTG can enter cells *via* lactose permease-dependent and/or the permease-independent pathway [21].

3.2. Effect of tetracycline concentration for whole cell OPH activity

The overexpression of molecular chaperones in recombinant *E. coli* is commonly used to produce target proteins in a properly folded form [22-24]. We examined whole cell OPH activity at different concentrations of tetracycline (1 ~ 50 μg/mL), an inducer of GroEL/ES [25]. Whole cell OPH activity increased with increasing tetracycline concentration, and at 50 ng/mL tetracycline exhibited twice the specific whole cell OPH activity (U/OD_{600}) of tetracycline at 1 ng/mL (Fig. 1B). As was expected, the growth of recombinant *E. coli* was reduced by increasing tetracycline concentration, which could have been influence by the ATP-dependent GroEL/ES-mediated folding of OPH [26]. We investigated whether the expression level of chaperone GroEL/ES was dependent on the concentration of tetracycline by Western blotting (Fig. 2). The expression level of GroEL/ES was increased by tetracycline (Fig. 2A), and this seemed to affect the expression level of premature and mature OPH (Figs. 2B and S1). By facilitating properly OPH folding, GroEL/ES appeared to prevent the proteolytic degradation of unfolded or inappropriately folded OPH, and subsequently, the level of premature OPH increased on increasing GroEL/ES level; the expression level of premature OPH at tetracycline concentration of 50 ng/mL was ~300% of that at 1 ng/mL tetracycline (Fig. S1). This may be due to the

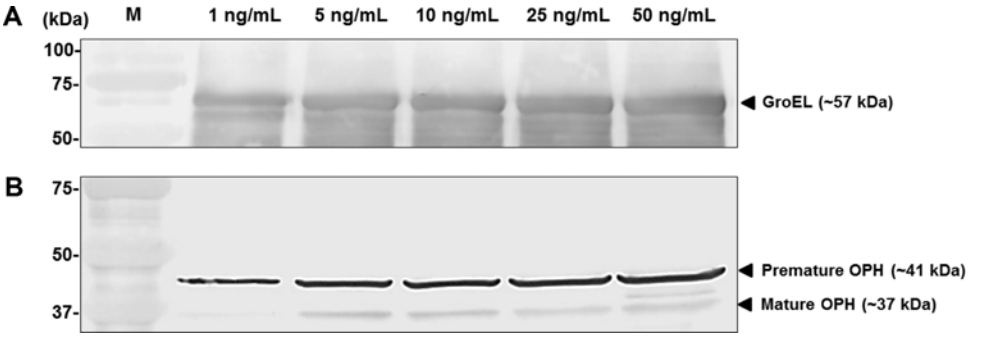

Fig. 2. Effects of tetracycline concentration on (A) the expression levels of premature and mature OPH, and (B) on expression level of GroEL/ES as determined by Western blotting.

slow protein folding rate of GroEL/ES chaperone, for example, GroEL/ES showed turnover number of 0.015 ~ 0.018/sec for malate synthase G [27]. The high levels of properly folded premature OPH might lead to an increase in the level of mature OPH (Figs. 2Band S1); the expression level of mature OPH at tetracycline concentration of 50 ng/mL was ~9.6-fold higher than that at 1 ng/mL tetracycline (Supplementary data, Fig. S1). This seems to be possibly because the twin-arginine (Tat) translocation machinery can only act on properly folded protein [28] with a certain translocation efficiency; the periplasmic translocation efficiencies were almost steady (~6%) except for 1 ng/mL of tetracycline concentration (Fig. S1). In short, high tetracycline concentration induces a large amount of GroEL/ES, resulting in increasing amounts of appropriately folded premature OPH. This leads to high periplasmic translocation yield of OPH and, in turn, enhanced whole cell OPH activity. Thus, the expression level of the GroEL/ES appears to play an important role in folding and periplasmic translocation of OPH, which are important contributors to whole cell OPH activity.

3.3. Effect of $CoCl_2$ concentration and culture temperature with respect to whole cell OPH activity

The effect of $CoCl_2$ concentration was investigated for active OPH expression in *E. coli* because the cobalt ion plays an important role in its folding and activity [29,30]. Cells showed similar whole cell OPH activities at $CoCl_2$ concentrations of 5 ~ 100 μM, but at higher concentration (5,000 μM), cells agglomerated to form large clusters (data not shown). The recombinant *E. coli* showed an optimal specific whole cell OPH activity (U/OD_{600}) when incubated in culture media supplemented with 500 μM $CoCl_2$ (Fig. 1C).

To evaluate the effect of culture temperature on whole cell OPH activity, cultures were incubated at 20, 25, and 37°C for 6 h after adding IPTG, tetracycline and $CoCl_2$. Specific whole cell OPH activity increased on lowering culture temperature (20 ~ 37°C), and at 20°C, cells exhibited 1.3-fold higher specific whole cell activity (U/OD_{600}) that cells cultured at 37°C (Fig. 1D). A low incubation temperature, which is commonly used to control of protein synthesis rate [31], seems to provide sufficient time to allow the proper folding of recombinant proteins at lower OPH production rate [32].

3.4. Effect of culture time with respect for whole cell OPH activity

Recombinant *E. coli* was cultured at 20°C in M9 minimal medium containing 1 mM IPTG, 50 ng/mL tetracycline, and 500 μM $CoCl_2$. After induction with IPTG and tetracycline at ~0.7 OD_{600}, cells grew to ca. 0.9 OD_{600} within 2 h. Cell densities were maintained over 49 h of culture without any significant reduction (Fig. 3A). The whole cell OPH activity increased with culture time, which may have been due to an increase in the periplasmic translocation of OPH. This could be supported by the expression level of mature OPH according to culture time (Fig. S2). The recombinant *E. coli* exhibited a specific whole cell OPH activity (U/OD_{600}) of ~0.55 when cultured for 31 h after induction (Fig. 3A). The whole cell OPH activity in current culture condition was approximately 2.6-fold higher compared to that (a specific whole cell activity (U/OD_{600}) of ~0.21) of recombinant *E. coli* cultured as previously described condition [13] (Fig. 3B). Its activity also exceeds those for recombinant *E. coli* expressing OPH on the surface *via* lipoprotein-transmembrane domain of outer membrane protein A fusion-based or an ice nucleation protein-based surface display system [14,33].

3.5. Storage stability of recombinant *E. coli*

To investigate the effect of GroEL/ES on the storage stabilities of cell, we periodically measured specific whole cell OPH activity and cell density of suspended cultures over a 2-weeks period. As shown in Fig. 4, specific whole cell OPH activity was maintained at essentially the original level for 1 week, whereas recombinant *E. coli* cell expressing

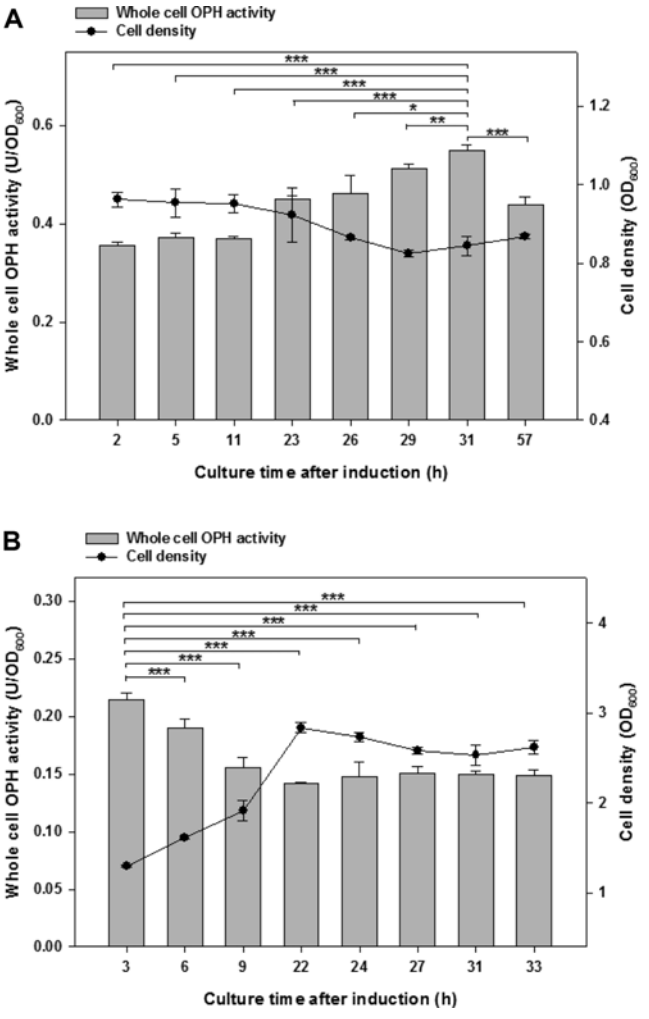

Fig. 3. Time profiles of cell density and specific whole cell OPH activities of recombinant *E. coli* expressing periplasmic OPH and cytosolic GroEL/ES (A) when cultured at 20°C in M9 minimal medium containing 50 μg/mL ampicillin, 25 μg/mL chloramphenicol, 1 mM IPTG, 50 ng/mL tetracycline, and 500 μM $CoCl_2$, and (B) when cultured at 37°C in LB medium containing 50 μg/mL ampicillin, 25 μg/mL chloramphenicol, 1 mM IPTG, 10 ng/mL tetracycline, and 500 μM $CoCl_2$. Whole cell OPH activities were determined in 100 mM CHES buffer (pH 9.0) with 1 mM paraoxon by measuring absorbance at a λmax of 410 nm. Values and error bars represent the means of three independent experiments and their standard deviations with statistical significance (*$p < 0.05$, **$P < 0.01$, ***$P < 0.005$, unpaired *t*-test).

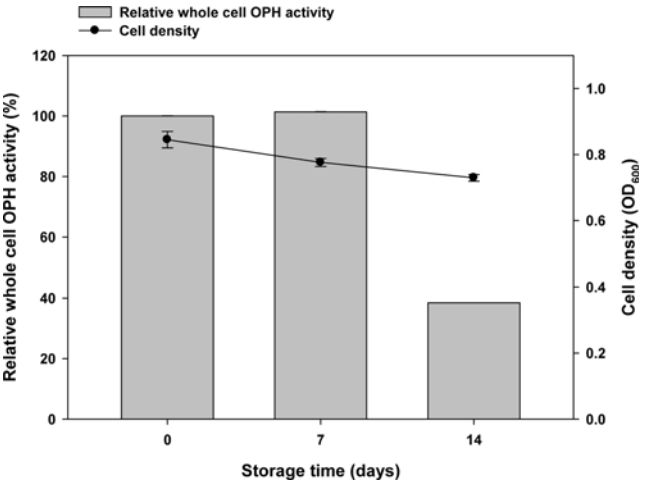

Fig. 4. Storage stability of recombinant *E. coli* expressing periplasmic OPH and cytosolic chaperone GroEL/ES stored at 4°C. Whole cell OPH activities were determined in 100 mM CHES buffer (pH 9.0) with 1 mM paraoxon by measuring absorbance at a λmax of 410 nm. Values and error bars represent the means of three independent experiments and their standard deviations.

only periplasmic OPH exhibited gradually decreased OPH activity with 80% of initial activity after 5 day [3]. This enhanced stability seems to be because GroEL/ES prevent thermal denaturation and aggregation of OPH [34]. The enhancement might contribute to stabilities of the whole cell-based biosensor. However, activity dramatically decreased to 40% of the original at 2 weeks, although the cell density was maintained. This decreased activity, which is similar to that described in other studies [12,16,35], may have been due to weakened transport machinery in periplasmic OPH and cytosolic GroEL/ES-expressing recombinant *E. coli* cells.

4. Conclusion

In the present work, we investigated the effect of culture condition on whole cell OPH activity of recombinant *E. coli* expressing periplasmic OPH and cytosolic GroEL/ES. Unlike IPTG concentration, tetracycline concentration significantly affected specific whole cell OPH activity; OPH activity increased with increasing tetracycline concentration. In addition, $CoCl_2$ concentration and culture temperature and time after induction were found to influence on OPH activity of recombinant *E. coli*. When cultured in M9 medium containing 1 mM IPTG, 50 ng/mL tetracycline, 500 μM $CoCl_2$ at induction temperature of 20°C for 31 h after induction, recombinant *E. coli* exhibited a specific whole cell OPH activity of ~0.55 U/OD_{600} and storage stability for 1 week with complete retention of activity. It is hoped that the enhanced specific activity of whole cell biocatalyst will result in enhancement of sensitivity and detection limits of recombinant *E. coli*-based whole cell biosensors designed to detect toxic OPs.

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