ORIGINAL PAPER

원저

Bioremediation을 위한 유기인계 화합물의 분자생물공학적 제거연구

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Study on Molecular Biotechnplogical Removal of Organophosphate Compunds for Bioremediation

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1. Introduction

Organophosphorus compounds are widely used in many pesticides (Paraoxon, Parathion, Coumaphos, and Diazinon) and chemical nerve agents (Sarin and Soman). Organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* or *Flavobacterium sp.* is a homodimeric organophosphotriesterase that can degrade a broad spectrum of toxic organophosphates. This enzyme can hydrolyse various phosphorus-ester bonds including P-O, P-F, P-CN, and P-S bonds. The hydrolytic mechanism involves the addition of an activated water molecule at the phosphorus center. The application of OPH for bioremediation is of great interest due to its high turnover rate.

Recombinant *Escherichia coli* expressing OPH can degrade a variety of organophosphates. The ability of *E. coli* to grow to much higher densities than *P. diminuta* and *Flavobacterium* enables the development of large-scale detoxification processes. However, recombinant *E. coli* has a very low production yield of OPH due to its low solubility. Therefore, several techniques have been attempted to enhance OPH production yield or bioconversion efficiency such as insertion of multiple gene fusions, fusion with a soluble partner to increase solubility, and display on a cell surface.

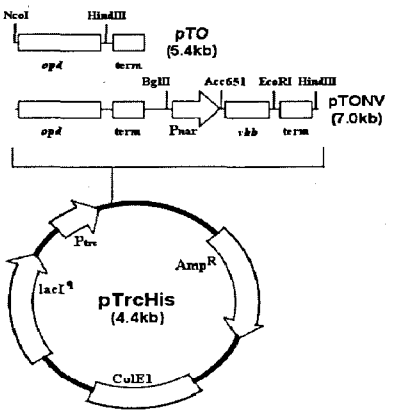
Vitreoscilla hemoglobin (VHb) has been introduced into a variety of microorganisms to improve the productivity of a target metabolite, especially a foreign protein, under microaerobic conditions. VHb increases the rate of O2 usage, especially when dissolved O2 (DO) is less than 5% of air saturation. Recently, VHb-expression was applied to degrade toxic chemicals using whole bacterial cells.

In the present work, we have constructed a recombinant, whole cell bioconversion system that co-expresses OPH and VHb to increase the OPH expression yield. The O2 dependent *nar* promoter was introduced for *self-tuning* of VHb expression by cellular O2 environment instead of original vhb promoter that is subjected to severe catabolite repression by glucose.

2. Materials and Methods

E. coli TOP10 [*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*] (Invitrogen, USA) was used for constructing recombinant plasmid containing the opd and vhb genes. *E. coli* BL21 [*F ompT hsdSB (rB- mB-) gal dcm*] (Novagen, USA) was used for expressing the OPH and VHb. Recombinant *E. coli* strains were grown at 37℃ in 50ml of LB (Luria broth) medium (5g yeast extract, 10g tryptone, and 10g NaCl in 1liter distilled water) containing ampicillin 50mg/ml using a 250ml flask. These cultures were inoculated (2% v/v) from 37℃ overnight cultures in the same medium. When cell concentration reached about 0.3mg/ml, *E. coli* strains were induced by the addition of 1mM IPTG to express the OPH.

Plasmid pTO (see [**Fig. 1**]) was constructed as an OPH control vector by inserting the polymerase chain reaction (PCR) amplified (DNA Thermal Cycler; Eppendorf Scientific, USA) opd gene (primers: ggccatgggatcgatcggcacaggcg and ggaagctttcatgacgcccgcaaggtcg) from the plasmid DNA of *Flavobacterium sp.* (ATCC 27551) based on the GenBank sequence (M22863; http://www3.ncbi.nlm.nih.gov/Entrez) into the *NcoI-HindIII* sites of pTrcHis C vector (Invitrogen). The *HindIII* digested fragment of nar promoter andvhb gene from the plasmid pTGNV was subcloned in the pTO plasmid. This plasmid was denoted pTONV (see [**Fig. 1**]).



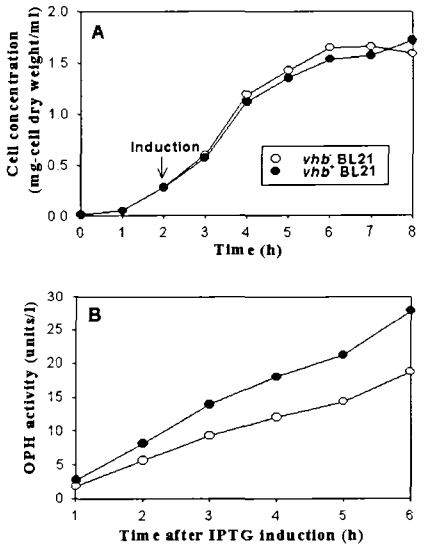
[Fig. 1] Gene maps of recombinant plasmid pTO and pTONV. Abbreviations: Ptrc, *trc* promoter; AmpR, ampicillin resistance gene; lacIq, overexpressed *Lac* repressor; ColE1, replication origin; Pnar, *nar* promoter; term, termination sequence

Cell growth was monitored by measuring cell concentration (mg-cell dry weight/ml). Whole cell OPH activity was measured by following the increase in absorbancy of p-nitrophenol from the hydrolysis of substrate (0.1mM Paraoxon) at 400nm (ε400 = 17,000 M-1 cm-1). Samples of culture (1ml) were centrifuged at 10,000g and 4℃ for 5min. The cells were washed, resuspended with distilled water, and 100μℓ was added to an assay mixture containing 400μℓ 250mM CHES [2-(N-cyclohexylamino)ethane-sulfonic acid] buffer, pH 9.0, 100μℓ 1mM Paraoxon, and 400μℓ distilled water. One unit of OPH activity was defined as mmoles Paraoxon hydrolyzed per min.

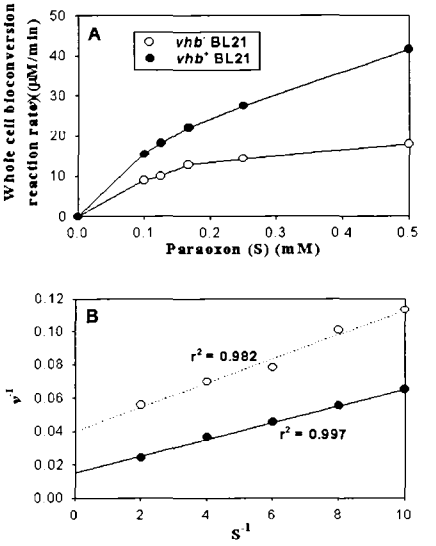
The values for kinetic parameters, Vmax and Km, were determined by analyzing Lineweaver-Burk plots over the range 0.1 to 0.5mM Paraoxon in 100mM CHES buffer, pH 9.0.

3. Results and Discussion

To evaluate the effect of VHb co-expression on cell growth and OPH expression, we performed flask cultures (see [**Fig. 2**]). Although we expected a positive effect of *vhb* co-expression on cell growth as previous reports, we observed no notable advantages on cell growth from the *vhb* co-expressing (*vhb+*) BL21 compared to the *vhb* non-expressing (*vhb-*) strain (see [**Fig. 2A**]). This agreed with our previous result using green fluorescent protein (GFP) as a foreign protein model. While the effect on cell growth was negligible, the presence of VHb had a significant



(Fig. 2) Time profiles of cell growth (A) and organophosphorus hydrolase (OPH) expression (B) in *vhb-* and *vhb+ E. coli* BL21. Recombinant *E. coli* strains were grown at 37℃ in 50ml of LB medium containing ampicillin 50㎍/ml using a 250ml flask. Arrow indicates the point of induction with 1mM IPTG



(Fig. 3) Whole cell bioconversion reactions (A) and Lineweaver-Burk plot analysis (B) with *vhb-* and *vhb+ E. coli* BL21. Bioconversion reactions were performed in resting cell condition. All data were based on unit cell concentration (1mg-cell dry weight/ml)

impact on OPH expression and therefore, on OPH activity. As shown in (Fig. 2B), whole cell OPH activity of the *vhb+ E.* coli was higher than that of the *vhb-* strain from an initial induction point. Difference of whole cell OPH activity between two strains increased with culture time. Finally, the *vhb+* strain showed 48% enhanced whole cell OPH activity compared to the *vhb-* strain. We obtained similar result of OPH activity enhancement by VHb co-expression in other *E. coli* strain W3110 (a derivative of *E. coli* K12) that has different metabolic characteristics with BL21 (a derivative of B) strain. From these results, we found that VHb co-expression successfully worked to enhance OPH production yield and could be applied to all types of *E. coli* cells.

We performed whole cell bioconversion reaction with various substrate (Paraoxon) concentrations. Whole cell reaction rates (v) were plotted against substrate concentrations (S) as depicted in (Fig. 3A). All data shown here were based on unit whole cell concentration (1mg-cell dry weight/ml). Both reactions showed Michael-Menten kinetic patterns and the *vhb+* strain exhibited higher bioconversion rates in all ranges of Paraoxon concentrations. Interestingly, the biodegradation rate in the *vhb+* strain was much increased with substrate concentrations compared to that in the *vhb-* strain and showed a 2.3-fold higher conversion rate with 0.5mM Paraoxon. These results demonstrated that the developed whole cell bioconversion system could more efficiently degrade organophosphates, especially high concentration of organophophate. From the linear Lineweaver-Burk graphs (see (Fig. 3B)), kinetic parameters Vmax and Km were determined for both strains. The *vhb+* whole cells with 15.4units/g-cell dry weight showed 2.7-fold higher maximum bioconversion rate, Vmax (0.067mM/min per unit cell concentration), than that (0.025mM/min) of the *vhb-* strain with 8.9

units/g-cell dry weight. However, the *vhb+* strain had higher Michaelis-Menten constant; implying that substrate affinity of the *vhb+* whole cell biocatalyst was lower. When we calculated overall reaction efficiency, Vmax/Km, the *vhb+* strain exhibited highly enhanced bioconversion efficiency by 44%. Therefore, from these results, we confirmed that VHb co-expression could be successfully employed to develop a whole cell biocatalysis system with notable enhanced bioconversion efficiency and capability for organophosphate, Paraoxon.

4. Acknowledgement

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