Biodegradation of 4-chloroaniline using toluene dioxygenase-based biocatalyst

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Abstract

Chloroaniline is a group of toxic chemicals which is widely used in the industrial production of pesticides, polyurethanes, pharmaceutical products and dye. 4-chloroaniline (4CA) is also an intermediate compound of natural degradation of herbicides and pesticides. Due to its toxicity and contamination in both industrial and agriculture environment, bioremediation which is an effective technique that relies on biocatalyst activity has been used to remove the pollutants. This study showed the oxidation of 4CA by toluene dioxygenase (TodC1C2BA) of *Pseudomonas putida* T57 that was heterologously expressed in *Escherichia coli*. This study showed that the recombinant host and strength of the promoter that controlled *tod* cluster expression affected the remediation efficiency. The recombinant strain with high-strength promoter controlled *tod* (pHKTodC1C2BA) could degrade 4CA to more than 98% within 48 hours of incubation, while it was at 46% degradation by the recombinant strain with moderate-strength promoter controlled *tod* (pBKTodC1C2BA). When the remediation was based solely upon enzyme catalyst, it was found that the enzyme produced from pHKTodC1C2BA-harboring strain could completely oxidize 4CA, while the performance of the enzyme from pBKTodC1C2BA-harboring strain was comparatively at 50%. According to the degradation pathways of 4CA previously reported, it can be inferred that the oxidation of 4CA leads to non-toxic product. The results demonstrate the potential application of enzyme bioremediation.

Keywords: 4-chloroaniline, biodegradation, enzyme bioremediation, toluene dioxygenase, *Pseudomonas putida* T57

Introduction

A 4-chloroaniline is an industrial chemical, of which the application involves the production of pesticides, polymers, dyes, as well as other industrial products. In addition, it has been reported to moderately to highly accumulate in agricultural soil and water due to heavily used pesticides and herbicides in agricultural activities. Due to its persistence in the environment and toxicity to human and living organisms, 4CA is classified as one of important environmental pollutants, and thus the treatment of 4CA is essential (Scheunert, 1981). Bioremediation is one of effective techniques, which involves in the ability of microorganisms to utilize or biotransform the toxic pollutant to non-toxic form(s). Biodegradation of 4CA has been reported by several bacteria such as *Pseudomonas* sp. strain CA16 (Vangnai and Petchkroh, 2007), *Acinetobacter baylyi* strain GFJ2 (Hongsawat and Vangnai, 2011) etc., but the information of gene(s) and enzyme(s) involved in this degradation pathway is scarce. Nevertheless, recently toluene dioxygenase gene cluster from *Pseudomonas putida* strain T57 was heterologously expressed in *E. coli* DH5α generating the recombinant strain that could degrade 4CA Nitisakulkan et al., (2014). This study investigated the recombinant host and strength of the promoter that controlled *tod* cluster

expression could influence the remediation efficiency. The results suggested the potential application of the enzyme-based bioremediation of 4-chloroaniline.

Methodology

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for plasmid construction and DNA manipulation. *E. coli* recombinant strains were cultivated in Luria-Bertani (LB) medium supplemented with an appropriate antibiotic at 37°C with shaking condition at 200 rpm. For solid medium, 1.5% agar was added to LB medium. Alternatively, for degradation test, cells were grown in minimal salt basal (MSB) medium consisted of the following components; 4.3 g of K_2HPO_4, 3.4 g of KH_2PO4, 2.0 g of $(NH_4)_2SO_4$, 0.34 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $MnCl_2 \cdot 4H_2O$, 0.006 g of $FeSO_4 \cdot 7H_2O$, 0.026 g of $CaCl_2 \cdot 2H_2O$, 0.02mg of $Na_2MoO_4 \cdot 2H_2O$, 0.01mg of $ZnCl_2 \cdot 7H_2O$, 0.01mg of $CoCl_2 \cdot 6H_2O$, 0.01mg of $CuSO_4$, 0.001 mg of $NiSO_4 \cdot 6H_2O$, 0.001 mg of Na_2SeO_4. When it was supplemented with yeast extract (0.01% w/v), it was designated as MSBY.

Table 1: Bacterial strains and plasmids were used in this study

Strain or plasmid	Characteristics	Reference
Strain		
Pseudomonas putida T57	The strain containing *todC1C2BA* gene	(Faizal et al., 2005)
Recombinant strains		
E. coli (pHKTod)	*E. coli* JM109 harboring pHKTodC1C2BA	This study
E. coli (pBKTod)	*E. coli* DH5α harboring pBKTodC1C2BA	This study
Plasmid		
pHKTodC1C2BA	pHK1 containing kan promoter-*todC1C2BA*; Km^r	(Nitisakulkan et al., 2014)
pBKTodC1C2BA	pBluescript II sk(-) containing *todC1C2BA*; Amp^r	This study

Amp, amplicilin; Km, kanamycin

Whole-cell biodegradation activity test

The starting culture (5 ml) of *E. coli* (pHKTod) and *E. coli* (pBKTod) were cultivated overnight by shaking at 200 rpm at 37°C in LB medium with kanamycin and ampicillin, respectively, and used as an inoculum (1% v/v) for a 200-ml culture cultivation. For *E. coli* (pBKTod), isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to this stage to the final concentration of 0.5 mM for an induction. Cells were grown for 3 hrs to the optical density (OD_{600}) of 0.5 before cell harvesting, washing twice with sterile saline solution, and resuspending to 10 ml suspension using MSBY for *E. coli* (pHKTod), and MSBY-IPTG for *E. coli* (pBKTod). This concentrated cell suspension was used for 4CA biodegradation test later on. Five ml of the concentrated cells was aliquoted into a 30-ml glass vial. The biodegradation test was started by adding 1 mM 4CA and incubated at 30°C. At time interval, the sample was taken to analyze the remaining 4CA using high performance liquid chromatography (HPLC) as described below.

Preparation of cell-free extract and enzymatic-based biodegradation test
Cell suspension of *E. coli* (pHKTod) and *E. coli* (pBKTod) was prepared as described above. After cell harvesting, cells were washed once with 25 mM potassium phosphate buffer pH 7.2, and resuspended with the same buffer in the presence of 1 mM dithiothreitol (DTT). Cells were then disrupted by two-cycles through a chilled French press cell. The broken cells were centrifuged at 13,000 x *g* at 4°C for 10 min to remove cell debris and the supernatant was used in enzymatic-based biodegradation assay. Protein concentration of the crude cell-free extract was determined.
4CA biodegradation test by the action of toluene dioxygenase activity was determined using a modified method of Jenkins and Dalton (1985) (Jenkins and Dalton, 1985). In brief, the reaction mixture (2.5 ml) comprised 50-µM NADH, 50-µM FAD, 25-mM phosphate buffer, pH 7.2 and crude cell-free extract of either *E. coli* (pHKTod) or *E. coli* (pBKTod). The reaction was started by adding 4CA to the final concentration of 0.1 mM. At time interval, the sample was taken to analyze the remaining 4CA using high performance liquid chromatography (HPLC) as described below.

Protein quantification
Protein concentrations were determined by the method of Lowry (Lowry et al., 1951), with bovine serum albumin as a standard protein. SDS-PAGE is prepared according to Bollag (1996) (Daniel M. Bollag 1996).

Analysis of 4CA
4CA concentration was determined using a reverse phase HPLC (LC-10AD, Shimadzu, Japan). The separation was performed at 30 °C on C18 column (5 µm, 250 x 4.6 mm; Hyperclone, Phenomenex, USA) using acetonitrile: water mixture (70:30, v/v) as a mobile phase at a flow rate of 1 ml min^{-1} and the detection wavelength of 240 nm. The retention time of 4CA under this condition was 3.45 min.

Results

Recombinant plasmid construction
A full-length *todC1C2BA* gene cluster was amplified from *P. putida* T57 genomic DNA using the following PCR primer set: C1-F (5' ATGAATCAGACCGACACATCAC 3'), and A-R
(5' TCACGTTAGGTCTCCTTCATTC 3'), cloned into pGEM-T Easy® vector, and then subcloned with *Apa*I-*Sac*I sites into pBluescript II sk(-) to obtain the plasmid pBKTodC1C2BA, in which *todC1C2BA* gene cluster was under control of *lac* promoter (P_{lac}). The other plasmid, pHKTodC1C2BA, in which *todC1C2BA* gene cluster was under control of kanamycin promoter (P_{kan}) was previously constructed by our group. Each plasmid was transformed into *E. coli* hosts generating *E. coli* (pHKTod) and *E. coli* (pBKTod).

Analysis of 4CA degradation using recombinant *E. coli* strains harboring toluene dioxygenase gene
The degradation pathways of 4CA in *E. coli* (pHKTod) have been proposed by Nitisakulkan et al. (2014) (Nitisakulkan et al., 2014). In this study, ability of two recombinant strains, *E. coli* (pHKTod) and *E. coli* (pBKTod), were investigated and compared. Although the test was carried out within the same test period (48 hrs) using similar amount and density of cell suspension (OD_{600} at 0.6), *E. coli* (pHKTod) was able to degrade 4CA up to 98%, while it was only at 50% by *E. coli* (pBKTod) (Figure 1). This was probably due to differences of promoter activity and host activity.

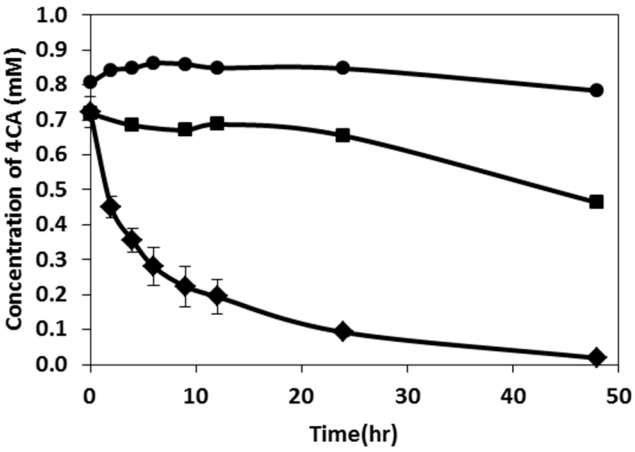

Figure 1: 4CA degradation by *E. coli* recombinant strains. The test was conducted as a growth-independent resting cell test using *E. coli* (pHKTod) (♦), *E. coli* (pBKTod) (■), and *E. coli* DH5α with an empty vector of pBluescript (a control) (●).

The expression of *todC1C2BA* in *E. coli*

The result showed that *todC1C2BA* gene could be expressed in *E. coli* hosts, but with different expression levels in *E. coli* (pHKTod) and *E. coli* (pBKTod) (Figure 2A and 2B, respectively). In both hosts, expression of each protein component of TodC1C2BA cluster was indicated by the overexpressed protein at expected molecular weight shown in the SDS-PAGE analysis and as follows: TodC1 (alpha subunit of toluene dioxygenase) at 52.5-kDa, TodC2 (beta subunit of toluene dioxygenase) at 20.8-kDa, TodB (ferredoxin) at 15.3-kDa and TodA (reductase) at 46.0-kDa, respectively (Finette et al., 1984).

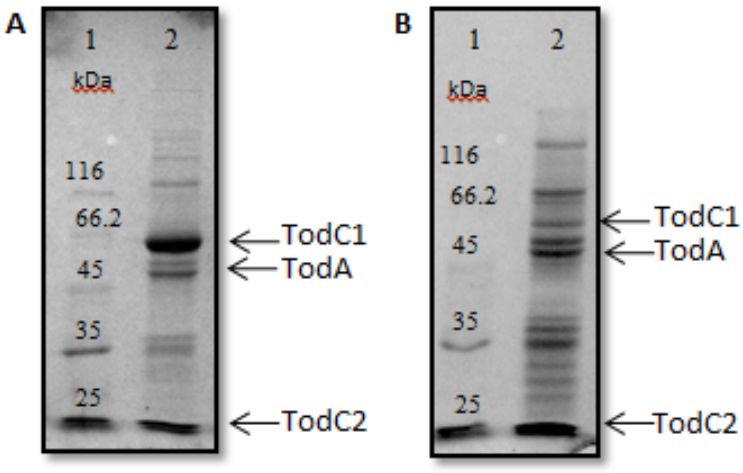

Figure 2: SDS-PAGE analysis of the overexpressed TodC1C2BA proteins in *E. coli* recombinants. (A) crude cell-free extract of *E. coli* (pHKTod) (Lane 2) and (B) crude cell-free extract of *E. coli* (pBKTod) (Lane 2, in order of IPTG concentration). Protein was loaded at approximately 3 mg ml^{-1} and stained with Coomassie brilliant blue R250. Lane 1 was unstained protein molecular weight marker.

Analysis of 4CA biodegradation using crude cell-free extract

The toluene dioxygenase-based 4CA biotransformation test was conducted as described above. The result showed that without the crude enzyme, there was no abiotic biotransformation within the test period, and that the enzyme expressed in both *E. coli* hosts could biotransformed 4CA, although with different rates and extents. At the same protein content in the reaction mixture at approximately 3.2 mg, 4CA was transformed to a near complete transformation within 24-hr of incubation with the crude enzyme from *E. coli* (pBKTod), while the transformation occurred at 50% with that of *E. coli* (pBKTod). Increase of protein content in the reaction mixture to 6.3 mg confirmed the positive transformation as the rate of the transformation increased approximately twice as much (Figure 3). The results indicated that unlike other unstable oxygenases reported previously, toluene dioxygenase from *P. putida* T57 has sufficient stability to carry out the degradation or biotransformation of 4CA.

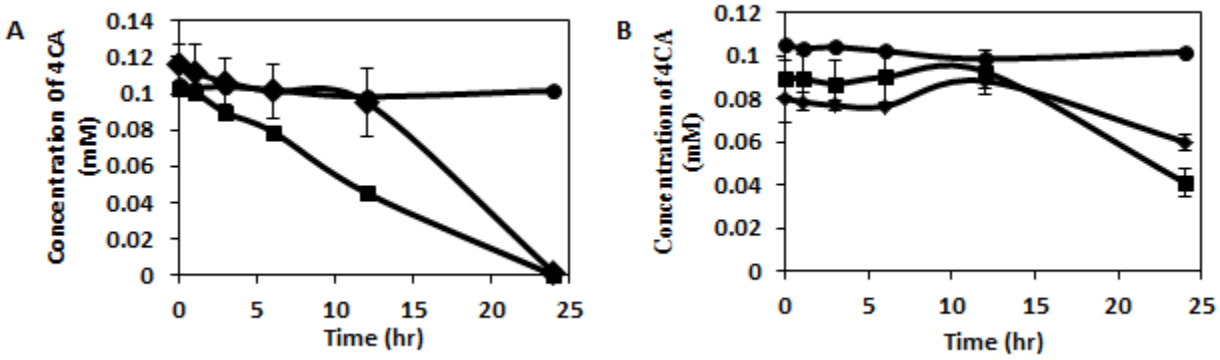

Figure 3: 4CA biotransformation by crude enzyme from (A) *E. coli* (pHKTod), and (B) *E. coli* (pBKTod). The 4CA biotransformation reaction was catalyzed using different protein contents at: 3.2 mg (♦), 6.3 mg (■), a non-enzyme reaction control (●).

Discussion and conclusion

This work investigated the biotransformation of 4-chloroaniline using recombinant strains of *E. coli* harboring toluene dioxygenase gene from *P. putida* strain T57. The results suggested that differences of promoter strength (strong promoter P_{kan}, and moderate-strength promoter P_{lac}) and host (*E. coli* strain DH5α and *E. coli* strain JM109) influence the transformation efficiency. Nevertheless, the result positively indicated that unlike other unstable oxygenases reported previously, toluene dioxygenase from *P. putida* T57 has sufficient stability to carry out the degradation or biotransformation of 4CA. This result demonstrated the potential use of enzyme-based bioremediation.

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