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Efficient biodegradation of dihalogenated benzonitrile herbicides by recombinant *Escherichia coli* harboring nitrile hydratase-amidase pathway

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ABSTRACT

Worldwide use of benzonitrile herbicides has caused a contamination hazard for groundwater, and their removal has attracted increasing attention. Microbial degradation has been considered as a major route of removing toxic nitriles from the environment. However, the process is very inefficient for degrading benzonitrile herbicides by natural microbes. In this study, a recombinant microbial cell was constructed to degrade benzonitrile herbicides by co-expression of nitrile hydratase and amidase in *Escherichia coli*. Both enzymes were functionally over-expressed in the cytoplasm of *E. coli*. The NHase activities of the cell reactor on dichlobenil and ioxynil were 15.4 and 21.3 U/mg dry cell weigh (DCW), respectively. And the amidase activities on 2,6-dichlorobenzamide and 3,5-iodo-4-hydroxybenzamide were 8.3 and 13.6 U/mg DCW, respectively. Furthermore, the degradation of dichlobenil and ioxynil was investigated using the recombinant cell reactor. The degradation process suggested that dichlobenil and ioxynil were degraded to corresponding carboxylic acids via nitrile hydratase-amidase pathway, and the intermediate amides (2,6-dichlorobenzamide and 3,5-iodo-4-hydroxybenzamide) did not accumulate in the reaction mixture. The degradation rates of dichlobenil and ioxynil were 43 and 185 mg/g DCW/h, respectively. The recombinant *E. coli* cell reactor was observed to be promising catalysts for the bioremediation of wastewater containing benzonitrile herbicides.

1. Introduction

The occurrence of herbicides and their residues in the environment has caused great concern worldwide [1,2]. Dichlobenil (2,6-dichlorobenzonitile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) are a group of benzonitrile herbicides containing a cyano-group and a dihalogenated phenyl [3]. Dichlobenil is a broad-spectrum contact herbicide, and used mostly in gardens, fruit orchards and plant nurseries. Bromoxynil and ioxynil are used for post-emergence control of broad-leaved weeds. According to statistics of Danish Environmental Protection Agency and United States Environmental Protection Agency, 556 tons of dichlohbenil, 106 tons of bromoxynil and 96 tons of ioxynil were sold between 2009 and 2011 in Denmark, and 68–102 tons of dichlohbenil was used between 1993 and 1995 in the United States [4,5]. These herbicides have caused a contamination hazard for soil and groundwater, and may threaten human health [6,7]. Particularly, the risk associated with the persistent metabolites of benzonitrile herbicides has recently been increasingly investigated because of their mobility and toxicity [8,9]. Dichlobenil can be slowly degraded to 2,6-dichlorobenzamide (BAM) by microorganisms in soil [10,11]. The solubility of BAM in water (1830 mg/L) is significantly higher than dichlobenil (21.2 mg/L) (data from Pesticide Properties DataBase, PPDB) [12]. BAM is recalcitrant to further degradation and easily leaches through the soil profile to contaminate groundwater. Therefore, dichlobenil was detected in only 0.9% of Danish drinking water abstraction wells, but BAM was found in 20.5% of wells in the period from 1992 to 2002 [13]. The metabolite was also detected in groundwater in the Netherlands, Germany, and Italy [14]. 3,5-dibromo-4-hydroxybenzamide (BrAM) and 3,5-iodo-4-hydroxybenzamide (IAM) from bromoxynil and ioxynil are also biodegraded by soil microbes and detected in groundwater [15,16]. Dichlobenil exhibits lower acute toxicity than bromoxynil and ioxynil. The toxicity of BAM is decreased compared to the parental nitrile, while

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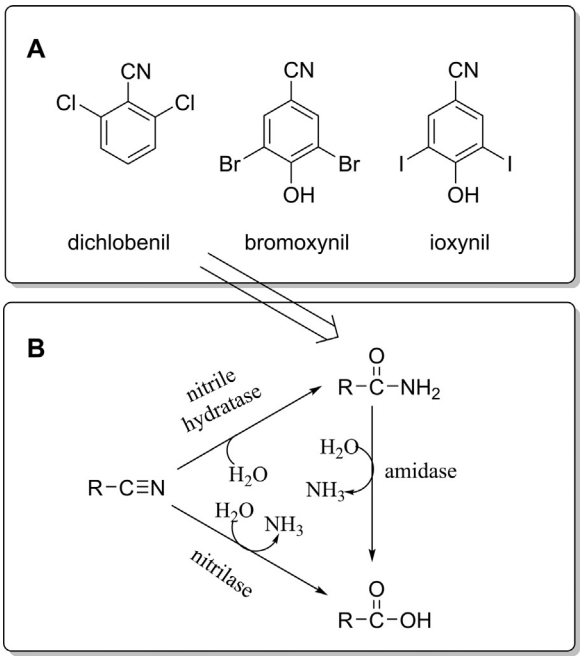

Fig. 1. Three dihalogenated benzonitrile herbicides and hydrolysis by nitrile-degrading enzymes.

BrAM and IAM all exhibit a higher toxicity than the corresponding nitriles [17]. To decrease the toxicity of benzonitrile herbicides, it is necessary to further degrade these amides to corresponding acids. Therefore, how to efficiently remove nitrile herbicides and their metabolites from the environment remains an important research subject.

Microbial degradation has been demonstrated to be an efficient and environmentally friendly method for removing toxic contaminants [18]. Microbial degradation of nitriles to corresponding carboxylic acids generally occurs via the hydrolytic route, which consists of two enzymatic systems [19,20]. Nitrile hydratase (NHase, EC 4.2.1.84) catalyzes nitriles to amides, which are subsequently converted to carboxylic acids and ammonia by amidase (EC 3.5.1.4). Alternatively, nitrilase (EC 3.5.5.1) directly converts nitrile compounds into corresponding carboxylic acid and ammonia (Fig. 1). These nitrile-degrading enzymes have received increasing general attention for enzymatic production of chemicals, biosynthesis of plant hormones, and bioremediation of toxic nitriles [21,22]. For the NHase/amidase system, NHase and amidase generally exist in a complex gene cluster. Some elements in gene cluster precisely control the biosynthesis of NHases, particularly activator gene [23]. The activator gene is adjacent to α- or β-subunit gene of NHase, and is necessary for the functional expression of NHase and amidase *in vivo*. The characteristic gene cluster means that it is difficult to efficiently express NHase and amidase in native microbes because the two enzymes were generally induced by different inducers [23]. Recently, the degradation of BAM, BrAM and IAM was reported by *Rhodococcus erythropolis* A4 and *Aminobacter* sp. MSH1, but the degradation efficiency was very low [11,17]. Therefore, it is necessary to reconstruct novel microbes for efficiently degrading benzonitrile herbicides from the environment.

With the development of gene recombination techniques, it is an alternative strategy to construct a cell reactor harboring NHase and amidase for degradation of benzonitrile herbicides. Given the convenience to express NHase and amidase in the host cell, the removal of environmental pollutants must be more efficient than wild microbes. In our previous studies, various NHase genes and amidase genes were discovered by genome mining and further functionally expressed in *Escherichia coli* [24,25]. In this study, NHases and amidases were investigated for degrading dichlobenil and ioxynil and their metabolites (BAM and IAM). Furthermore, some recombinant cell reactors were constructed by the co-expression of NHase and amidases in *E. coli*. Then the cell reactors were investigated in terms of degradation efficiency of dichlobenil and ioxynil. The strategy highlights the potential of recombinant *E. coli* cell reactors in the removal of toxic nitrile pollutions from the environment.

2. Methods

2.1. Chemicals, bacterial strain and plasmids

Chemicals used in this study were purchased from Sinopharm Chemical Reagent (Shanghai, China) unless otherwise specified. Dichlobenil (DCB), 2,6-dichlorobenzamide (BAM) and 2,6-dichlorobenzoic acid (DCBA) were purchased from Sigma-Aldrich. Ioxynil (DIHB), 3,5-iodo-4-hydroxybenzamide (IAM) and 3,5-iodo-4-hydroxybenzoic acid (DIHBA) were purchased from Energy Chemical (Shanghai, China). Acetonitrile and methanol for HPLC analysis were purchased from Merck (Darmstadt, Germany). Yeast extract and tryptone were obtained from OXOID (Basingstoke, England). *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and BL21 (DE3) were served as cloning and recombinant protein expression hosts, respectively. Plasmids pET24a and pCDFDuet-1 were employed to construct recombinant vector for recombinant expression of NHase and amidase.

2.2. Medium and culture for enzyme expression

Luria–Bertani (LB) medium was used for the growth of recombinant *E. coli*. LB media were supplemented with appropriate antibiotic (100 μg/mL ampicillin, 50 μg/mL kanamycin or 50 μg/mL streptomycin) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 μM) as required. Recombinant *E. coli* harboring different plasmids were cultivated in 100 mL LB media at 37 °C until the OD_{600} reached 0.8. Then, IPTG was added to a final concentration of 0.1 mM to induce the expression of recombinant enzymes at 18 °C for 12 h.

2.3. Construction of recombinant cell bioreactors

The recombinant plasmids containing NHase and amidase gene had been constructed and preserved in our laboratory. To achieve the co-expression of NHase and amidase in *E. coli*, two strategies were designed: two genes in two compatible plasmids and two genes in a plasmid (Fig. 2). Primers used in this study are described in Table 2. In the first scenario, the NHase gene was cloned into plasmid pET24a, and the amidase gene was cloned into plasmid pCDFDuet-1. The amidase gene from *Rhodococcus erythropolis* was amplified with plasmid pEAm02 as a template through polymerase chain reaction (PCR) using primers Rho_Am-F and Rho_Am-R, and the amidase gene from *Agrobacterium tumfaciens* D3 was amplified with plasmid pEAm03 as a template using primers Agro_Am-F and Agro_Am-R. PCRs were conducted with PrimeSTAR™ Max DNA polymerase [Takara (Dalian) Biotech, China]. PCRs were carried out as follows: a preliminary denaturation was conducted at 95 °C for 2 min, followed by 30 cycles of 10 s denaturation at 95 °C, 15 s annealing at 58 °C, and 15 s extension at 72 °C. Final extension was conducted at 72 °C for 5 min. The PCR products were recovered using a DNA gel extraction kit (Axygen, New York, USA). A fragment of approximately 1.6 kb was digested by *Nde*I/*Xho*I or *Nde*I/*Eco*RV and ligated into pCDFDuet-1 with the same restriction digestion to obtain recombinant plasmids pCAm02 and pCAm03, respectively (Table 2). Recombinant plasmid pENh11 containing NHase gene from *Bradyrhizobium japonicum* USDA 110 was co-transformed into