World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:6, No:5, 2012

Enhance Halorespiration in *Rhodopseudomonas palustris* with Cytochrome P450cam System from *Pseudomonas putida*

Shou-Chen Lo, Chia-Ching Lin, Chieh-Chen Huang

***Abstract*—**To decompose organochlorides by bioremediation, co-culture biohydrogen producer and dehalogenation microorganisms is a useful method. In this study, we combined these two characteristics from a biohydrogen producer, *Rhodopseudomonas palustris*, and a dehalogenation microorganism, *Pseudomonas putida*, to enchance halorespiration in *R. palustris*. The genes encoding cytochrome P450cam system (camC, camA, and camB) from *P. putida* were expressed in *R. palustris* with designated expression plasmid. All tested strains were cultured to log phase then presented pentachloroethane (PCA) in media. The vector control strain could degrade PCA about 78% after 16 hours, however, the cytochrome P450cam system expressed strain, CGA-camCAB, could completely degrade PCA in 12 hours. While taking chlorinated aromatic, 3-chlorobenzoate, as sole carbon source or present benzoate as co-substrate, CGA-camCAB presented faster growth rate than vector control strain.

***Keywords*—**cytochrome P450, halorespiration, nitrogen fixation, *Rhodopseudomonas palustris* CGA009

I. Introduction

ORGANOCHLORIDE pollution made by industry factory is harmfull to enviroment and human health. Some microoganisum could dehalogenate those materials and futher assimilate them as carbon source by halorespiration. Halorespiration is an anaerobic respiration constituted by an electron donor, an electron carrier, and an electron acceptor. In halorespiration, organochloride are the final electron acceptor; organochloride accept electron then release chloride ion and become a new organic compound [1]. As for electron donor, hydrogen was considered a main factor in dehalogenation. In previous reports, hydrogen had been taken as electron donor to be added in or supported by anaerobic bacterium for halorespiration [2]-[5]. The intermedium, electron carrier is also an important factor in halorespiration. An electron carrier would transfer electrons between metabolites, like cytochrome, iron-sulfur cluster, flavins, quinones, hemes, and cobalamin [6], [7].

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To focus on study halorespiration of organochloride, an *in vivo* activity assay should be complete with the factors; electron donor, electron carrier, and electron accepter. Due to halorespiration reaction need hydrogen as electron donor and proper electron carrier, we use a photoheterotrophic biohydrogen producer, *Rhodopseudomonas palustris*, and cytochrome P450cam from *Pseudomonas putida* to construct an enhanced halorespiration reaction. *R. palustris* is a purple nonsulfur bacterium with versatile metabolic pathway [8]. It could generate hydrogen under photoheterotrophic and nitrogen fixation condition [9]. Under nitrogen fixation condition, the electrons would be released as hydrogen for redox balance in bacteria [10]. The excessed electron amount could be used in halorespiraion. According to the transcriptome data of Rey's report at 2007 [11], more 10 to 40 fold of electron transfer protein genes would be expressed in nitrogen fixation conditon than in nonnitrogen fixation condition. This high expression level of electron proteins makes electron could be easily transfered to cytochrome P450 and organochloride for halorespiration. Even under nonnitrogen fixation condition, in previous reports, *R. palustris* WS17 and CGA009 strains could utilize 3-chlorobenzoate as carbon source with benzoate as co-substrate to grow [12], [13]. Cytochrome P450 is a heme-thiolate protein superfamily and exsist in broad range of species, like bacteria, plants, and animals, including human. Cytochrome P450 would be stimulated by many enviromental factors, like exposed under lead, cadmium, and mercury pollutants [14]. There is a heme iron in the activity cite of cytochrome P450; when this iron had been reduced and combined with carbon monoxide, an optimal absorbs light at wavelengh 450 nm could be detected [15]. Cytochrome P450cam belongs to class I of cytochrome P450 superfamily, and is composed of P450 monooxygenase (*camC*), putidaredoxin reductase (*camA*), and putidaredoxin (*camB*). Cytochrome P450cam of *P. putida* could catalyze camphor to 5-exo-hydroxycamphor in aerobic condition [16], and dehalogenate pentachloroethane (PCA) to trichloroethylene in anaerobic condition [17], [18]. The X-ray protein structure of cytochrome P450cam shows that its activity cites is composed of several hydrophobic amino acids. Therefore the hydrophobic organochloride could easily be dehalogenated in its activity cite [19]. According to the genomic data of *R. palustris* CGA009 [8], there are seven cytochrome P450 genes and two associated ferredoxin genes. Three of these cytochrome P450 proteins have been proved that they could bind with aromatic compounds. Futhermore, CYP199A2, one of these three cytochrome P450 proteins, had been purified and tested activity reconstitution with putidaredoxin reductase [20]. Generally, halorespiraion in bacteria cultures need hydrogen as an electron donor to provide energy [2], [5], [21], and proper cytochrome P450 enzyme system. For performance of an enhanced halorespiraion reaction, *R. palustris* CGA009 was transferred cytochrome P450cam genes from *P. putida* PpG1 plasmid by

electroporation. In this study, by appliying different characteristic of *R. palustris* and *P. putida*, we demonstrated that halorespiration could be enhanced in *R. palustris*.

II. Materials and Methods

To express cytochrome P450cam genes in *R. palustris*, the promoter we chose is base on Inui's report at 1999 [22]. The promoter of pckA expressed in log phase with different carbon sources in aerobic or anaerobic condition. The promoter DNA fragment was amplified by pckA-F/R primer pair from genomic DNA of a local *R. palustis* 636 (BCRC 16636) from Bioresource Collection and Research Center, Taiwan. For translation of cytochrome P450cam mRNAs, a ribosome binding site (AGGAGG) was designed within each forward primer to add in the upstream of cytochrome P450cam genes by polymerase chain reaction (PCR). According to Peterson's report at 1990, exchange start codon of camA by ATG woud increase protein expression level about 18 times [23]. Therefore, we made the start codon of cytochrome P450cam genes are ATG. The PCR was performed with TaKaRa Ex Taq™ (TakaRa Shuzo Co., Ltd.) and TaKaRa PCR Thermal Cycler Dice™ (TP600). To discover more about heterologous expressed cytochrome P450cam in *R. palustris*. We constructed a plasmid pMG-camC to exrepss only camC (monooxygenase) in *R. palustris*.To construct two plasmids, pMG-camCAB and pMG-camC, for expression of a serial cytochrome P450cam system from *P. putida* and single monooxygenase, respectively, DNA fragments were ligated by designated restriction enzyme sites. Then RNA expression was checked by reverse transcriptase polymerase chain reaction (RT-PCR). To perform RT-PCR, RNeasy mini kit (Qiagen) was used to extract total RNA of *R. palustris* then AccessQuick RT-PCR system (Promega Co.) was used to ampilify target mRNA.

A. Bacterial strains, plasmids, and primers

All bacterial strains, plasmids, and primers used in this study are listed in Table I.

B. Culture conditions

R. palustris was cultured in modified *Rhodospirillaceae* medium [24]. During photoheterotrophic growth, sodium acetate (1 g/L) was added as a carbon source. To test halorespiraion, acetate was exchanged by 5 mM of 3-chlorobenzoate (ACROS) (dissolved in methanol), or with 5 mM of benzoate (Riedel-de Haën). Glutamate was added as nitrogen source into the culture media and adjusting the initial pH of the medium to 7.0 with 5 N NaOH. The space of culture tubes top were filled with argon gas for anaerobic condition. *R. palustris* was cultured at 32°C under illumination with a tungsten filament lamp at 50 W/m^2 light. Bacteria growth was determined by measuring the optical density at 650 nm (GeneQuant 1300) then calculate dry cell weight (DCW). To select transgenic strains, *R. palustris* strains were grown in the presence of 200 μg/mL kanamycin (Km). *E. coli* strains were grown in the presence of 50 μg/mL Km or ampicillin (Ap).

C. DNA manipulation and construction of recombinant strains

The Plasmid Miniprep Kit II (GeneMark) was used to extract plasmids. Cytochrome P450cam genes, *camC*, *camA*, and *camB* from *P. putida* PpG1, were amplified with the primer pairs camC-F/R, camA-F/R, and camB-F/R to obtain PCR products of 1282 bp, 1314 bp, and 362 bp, respectively. The PCR fragments were cloned into the yT&A cloning vector (Yeastern Biotech Co., Ltd, Taiwan), and yT&A-camC, yT&A-camA, and yT&A-camB were cloned and sequenced (Genomic BioSci & Tech, Taiwan).

To construct pMG105P, the promoter P*pckA1* was amplified with the pckA-F/R primer pair from genomic DNA from *R. palustris* 636, a local PNSB strain in Taiwan, and then inserted into the *Eco*RI and *Bam*HI sites of pMG105 [23] (a generous gift from Hideaki Yukawa, Research Institute of Innovative Technology for the Earth, Japan). The sequence of the conserved promoter regions -35 (TCGCCC) and -10 (TATATT) in P*pckA1* were identical to the P*pckA* sequence of *R. palustris* NO.7 in Inui's report [22].

To construct pMG-camC, *camC* DNA fragment was digested and ligated to pMG105P at *Bgl*II restriction enzyme site. As for pMG-camCAB, *camC*, *camA*, and *camB* DNA fragments were first digested and ligated together in yT&A-camCAB then put into *Bgl*II/*Xba*I restriction enzyme site of pMG105P.

All restriction enzymes and the T4 ligase were obtained from TAKARA. *E. coli* and *R. palustris* strains were transformed using the $CaCl_2$ method [26] and electroporation [27], respectively.

D. Pentachloroethane degradation test

Different concentrations of PCA (Tokyo Kasei Kogyo Co., Ltd.) were desolved in n-hexane (ECHO Chemical Co., Ltd.) and storage in brown bottles with Teflon seal for making standard concentration curve. To consider about nonbiodegradation of PCA, a blank control was made with autoclaved (121°C, 1.2 kg/cm^2) *R. palustris*. Tested *R. palustris* strains were cultrued under nitrogen fixation condition until cell concentration up to OD650 nm 0.7-0.8, 50 μM PCA desolved in methanol (ECHO Chemical Co., Ltd.) was added to cultrue by injection then determined PCA concentration in next 16 hours. Samples were added into n-hexane (1:1) and extracted with ultrasonic cleaner, then injected 1 μL into gas chromatography (Agilent 6890 GC) equipped HP-5ms column (Agilent) and flame ionization detector by 5 μL microsyringe (HAMILTON) to analysis PCA concentration changes. Carrier gas was nitrogen, make up gas was air and hydrogen (20:1), the flow of gas was 16 mL/min.

Column temperature was started at 40°C for 5 min, then increased to 300°C by the rate of 50°C/min. The temperature of injector and detector was 200°C and 300°C respectively.

TABLE I
BACTERIA STRAINS, PLASMIDS, AND PRIMERS USED IN THIS STUDY

Strains, plasmids, or primers	Genotype or sequence of primer (5' to 3')	origin
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1*Δ*(lac-proAB)/F'*[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Takara[a]
R. palustris CGA009 (ATCC BAA-98)	*hupV* mutant; spontaneous frame-shift (4-bp deletion)	ATCC[b]
yT&A	Ap^r; α-*lac*/multicloning site, M13 *ori* (T vector)	YB[c]
pMG105	Km^r; α-*lac*/multicloning site, pHSG299 with a 3.0-kb *Sal*I-*Xho*I fragment of pMG101	RITE[d]
pMG105P	Km^r; pMG105 with the promoter of *pckA1* fragment inserted into *Eco*RI and *Bam*HI site	this study
yT&A-camA	Ap^r; 1314 bp fragment containing the camA gene of *Pseudomonas putida* PpG1	this study
yT&A-camB	Ap^r; 362 bp fragment containing the camB gene of *Pseudomonas putida* PpG1	this study
yT&A-camC	Ap^r; 1282 bp fragment containing the camC gene of *Pseudomonas putida* PpG1	this study
yT&A-camAB	Ap^r; 1676 bp fragment containing the camA and camB genes of *Pseudomonas putida* PpG1	this study
yT&A-camCAB	Ap^r; 2958 bp fragment containing the camC, camA, and camB genes of *Pseudomonas putida* PpG1	this study
pMG-camC	Km^r; pMG105P with a 1.2 kb *Bgl*II fragment of yT&A-camC containing the camC gene of *Pseudomonas putida* PpG1	this study
pMG-camCAB	Km^r; pMG105P with a 2.8 kb *Bgl*II/*Xba*I fragment of yT&A-camCAB containing the camC, camA, and camB genes of *Pseudomonas putida* PpG1	this study
pckA-F	GAATTCCATATGCCCGGGGCCGAGGGCTACAT	this study
pckA-R	GGATCCAGATCTCGAATCCGCGTTGTCTCGAT	this study
camA-F	GCTAGCGCGGCCGCAGGAGGTGCGTGCTAAATGAACGCA	this study
camA-R	GAATTCCCATGGTCAGGCACTACTCAGTTCAG	this study
camB-F	CCATGGCACGTGAGGAGGATAAACAGATGTCTAAA	this study
camB-R	GAATTCTCTAGATTACCATTGCCTATCGGGAA	this study
camC-F	AGATCTACTAGTAGGAGGAACAACAACAATGACGACT	this study
camC-R	GCTAGCTTATACCGCTTTGGTAGTCGCCGG	this study

[a] Takara, Takara Bio Inc.; [b] ATCC, the American Type Culture Collection; [c] YB, Yeastern Biotech. Co., Ltd.; [d] RITE, Research Institute of Innovative Technology for the Earth

E. Hydrogen gas analysis

The total gas generation was determined by releasing generated gas in to syringes with graduation. To analysis hydrogen concentration, 250 μL syringes (SUPELCO, Pressure-Lok Series A-2 Syringe 250 μL) were used to collect 200 μL samples and inject into gas chromatography (Shimadzu, GC-14A) equipped with a thermal conductivity detector (TCD) and column (molecular sieve, 5A 60/80, 3MX 1/8"). Nitrogen gas was used to be carrier gas. The working temperature of column, injectior, detector, and TCD was 60°C, 100°C, 100°C, and 100 ° C, respectively. The hydrogen concentration of samples was determined by comparing with pure hydrogen gas.

III. RESULTS AND DISSCUSION

A. Construction of transgenic R. palustris strains

The plasmid, pMG105P, was constructed by inserting P*pckA1* fragment into *Eco*RI/*Bam*HI site of pMG105 and confirmed by *Eco*RI and *Bam*HI restriction enzyme digest experiments (Fig. 1). The *camC*, *camA*, and *camB* expression plasmid, pMG-camCAB, was confirmed by *Hind*III, *Eco*RI, and *Xba*I restriction enzyme sites (Fig. 2). The *camC* expression plasmid, pMG-camC, was confirmed by *Eco*RI, *Sal*I, and *Bgl*II restriction enzyme sites (Fig. 3). Each constructed plasmids were transfered into *R. palsutris* by electroporation and confirmed by PCR with specific primer pairs. The total DNA of wild-type and transgenic strains were extracted and checked the specific of primer pairs (Fig. 4 (a)) and transgenic gene containing (Fig. 4 (b)). The results indicate that the transgenic strain, *R. palustris* CGA-camCAB, containing *camC*, *camA*, and *camB* genes.

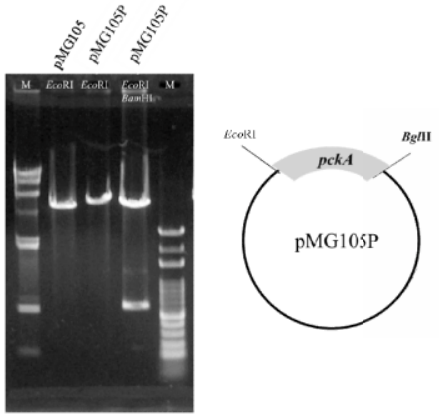

Fig. 1 Restriction enzyme digestion of plasmid pMG105P to confirm vector construction

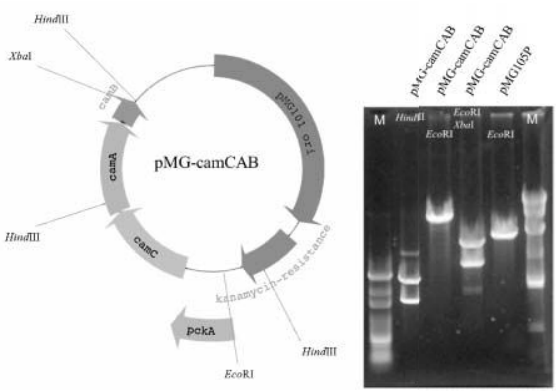

Fig. 2 Restriction enzyme digestion of plasmid pMG-camCAB to confirm vector construction

B. RNA expression of transgenic camC, camA, and camB

By agarose gel electrophoresis results of specific primer PCR, we confirmed that expression plasmids are transferred into *R. palustris*. The tranfered gene expressions were futher checked by RT-PCR with the specific primer pairs. Fig. 5 (a) with 1.2 kb fragment shows that camC gene is successful expressed in CGA-camC strain. As for CGA-camCAB, we can only detect *camB* gene expression from total RNA (Fig. 5 (b)). There is also a band occurred in the *camB* without reverse transcriptase control, it indicates that there are some DNA exist with our extracted RNA. However, the amount of DNA is less than that of RNA, so the RT-PCR results still could be told that *camB* is expressed. According to Fraga's article [28], the suitable size of RT-PCR ampilcon shoud be around 300 bp, otherwise if the extracted RNA quailty was defective, results would be unstable. It should be the reason why the expression of *camC* was detected in CGA-camC but not in CGA-camCAB and why only the expression of *camB* (~0.3 kb) was detected in CGA-camCAB. Nevertheless, according to the previous report [22], the promoter would express downstream gene in log phase and together with PCA degradation results, it could be told that the transgenic cytochrome P450cam genes are expressed.

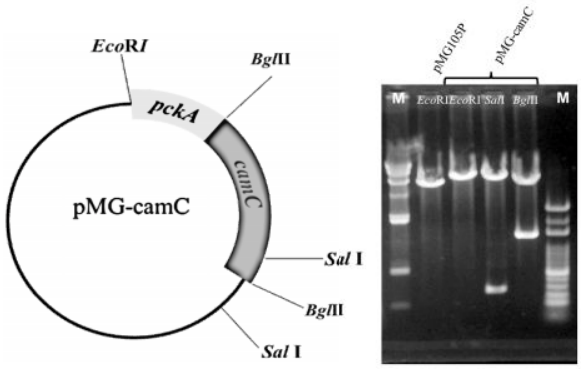

Fig. 3 Restriction enzyme digestion of plasmid pMG-camC to confirm vector construction

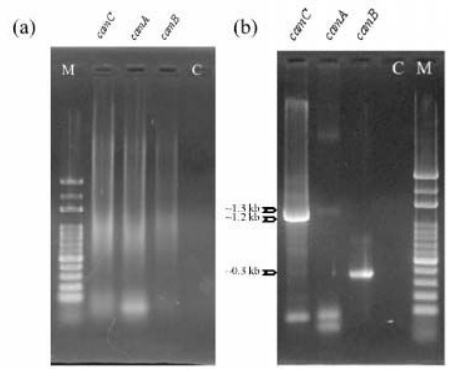

Fig. 4 Polymerase chain reaction results with camC-F/R, camA-F/R, and camB-F/R as primer pairs, genomic DNA of *R. palustris* CGA009 (a) or CGA-camCAB (b) were as templates

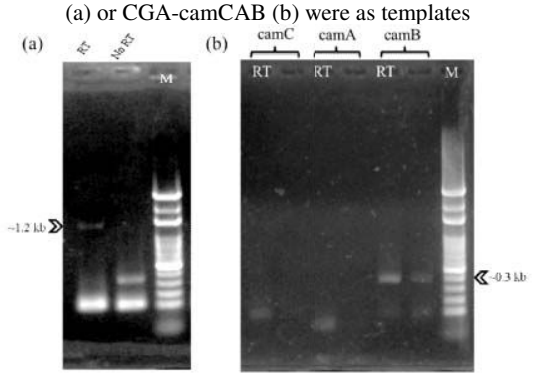

Fig. 5 RT-PCR results of camC expression in CGA-camC strain (a), and that of camC, camA, and camB expression in CGA-camCAB strain (b)

C. Pentachloroethane degradation test of transgenic R. palustris strains

Each transgenic strain was cultured to log phase with monitoring their hydrogen production then added PCA for final concentration of 50 μM and keep on cultrue. About twelve hours later, there was no PCA been detected in CGA-camCAB culture media. After four more hours later, the PCA concentration in CGA-camC culture media was degraded about 89% and that in CGApMG was degraded about 78% (Fig. 6). On the other hand, the hydrogen generation from CGA-camC is less than CGApMG. These results indicate that the two redox reactions, dehalorespiration and hydrogen generation, are competing the same source of electrons.

When cytochrome P450 was expressed, many electrons that supplied to generate hydrogen were transferred to cytochrome P450. These results also indicate that the transgenic *camC*, *camA*, and *camB* genes could enhance the degradation of PCA in *R. palustris*. Halorespiraion of PCA by cytochrome P450cam would become trichloroethylene [17]. The hydrogen persentage in total gas generation data indicate that there are other gases exsit besides hydrogen (Table II). They might be volatile organochloride gases, like trichloroethylene, catalyzed from PCA with camC protein. By coexpression of putidaredoxin reductase and putidaredoxin, PCA woud be degraded faster and those nonhydrogen gases would be generated more. These results indicate that the completely degradation of PCA in *R. palustris* would generate hydrogen and other gases.

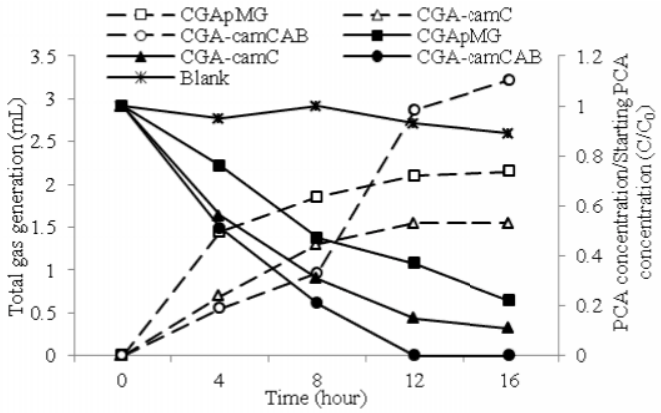

Fig. 6 Total gas generation (open symbols) and PCA degradation (closed symbols) of transgenic *R. palustris* strains

TABLE II
HYDROGEN AND OTHER GASES GENERATION IN 132 HOURS AFTER ADDING PENTACHLOROETHANE

Strains	Hydrogen (μmole)	Other gases (μmole)
CGApMG	162.47	38.42
CGA-camC	26.01	119.07
CGA-camCAB	233.11	339.21

Before adding PCA, the hydrogen production of CGA-camCAB was faster than two other strains, but three strains produced almost the same amount of hydrogen in 108 hours (Fig. 7). According to Ducat's report [29], the expression of electron transfer protein would help hydrogen production by transportation of electrons. As we coexpressed putidaredoxin reductase and putidaredoxin in *R. palustris,* it might help the electron transportation between with metabolites and nitrogenase to generate hydrogen faster. In Fig. 6, the gas production of CGA-camCAB increased when PCA was completely undetectable. While PCA was completely degraded, putidaredoxin reductase and putidaredoxin still transfer electrons between metabolites; it might be the reason why CGA-camCAB could generate more hydrogen than other transgenic strains in this experiment (Table II).

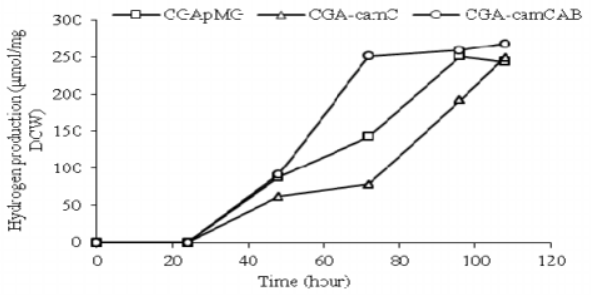

Fig. 7 Hydrogen production of transgenic *R. palustris* strains before adding PCA

D.The growth of transgenic R. palustris strains with 3-chlorobenzoate

According to Egland's report [13], *R. palustris* CGA009 could grow with 3-chlorobenzoate while presenting benzoate in cultrue media. Therefore, the halorespiration of aromatic organochloride had been tested with the transgenic *R. palustris* strains in this study. Fig. 8 shows the growth curve of CGApMG and CGA-camCAB with 3-chlorobenzoate, or 3-chlorobenzoate and benzoate, as carbon sources. Under these conditions, CGA-camCAB was growth faster than CGApMG. These results indicate that transgenic cytochrome P450, putidaredoxin reductase, and putidaredoxin would enhance the ability to utlize aromatic organochloride as carbon sources.

In previous reports about *R. palustris* strains that could dehalogenate 3-chlorobenzoate are all isolated from enrichment culture about 1 to 3 months [12], [13], [30]. However, the host strain CGA009 in this study is not reported that it was isolated from 3-chlorobenzoate and benzoate enrichment culture. According to Egland's report [13], *R. palustris* CGA009 would not grow with 3-chlorobenzoate as sole carbon source. But the result in this experiment, the vector control without additional expressing cytochrome P450 which should be negative control did grow. The experiment bacteria culture condition was followed Egland's report at 2001 [13], except methanol dissolved carbon sources and nitrogen source; according to the genomic sequence report [8], *R. palustris* CGA009 could assimilate methanol. It indicates that methanol would be utilized by *R. palustris* CGA009. On the other hand, in Egland's report, ammonium sulfate was used as nitrogen source, but glutamate was used in this study. When all ammonium compounds in the media were replaced by glutamate, the ammonium ions would be minimized and make *R. palustris* grow under nitrogen fixation condition [24]. In this condition, nitrogenase would be expressed to absorb protons and electrons for generating ammonium and hydrogen. According to the transcriptome data in previous report [11], electron transfer proteins would be expressed 10 to 40 times more for transfering electrons to nitrogenase under nitrogen fixation condition. It indicates that electrons are also easier to transfer between metabolites, inculding 3-chlorobenzoate in this case. Therefore, 3-chlorobenzoate is able to accept more electrons to be dehalogenated then assimilated within *R. palustris*. These might be the reason why the negative control *R. palustris* strain in this experiment could grow.

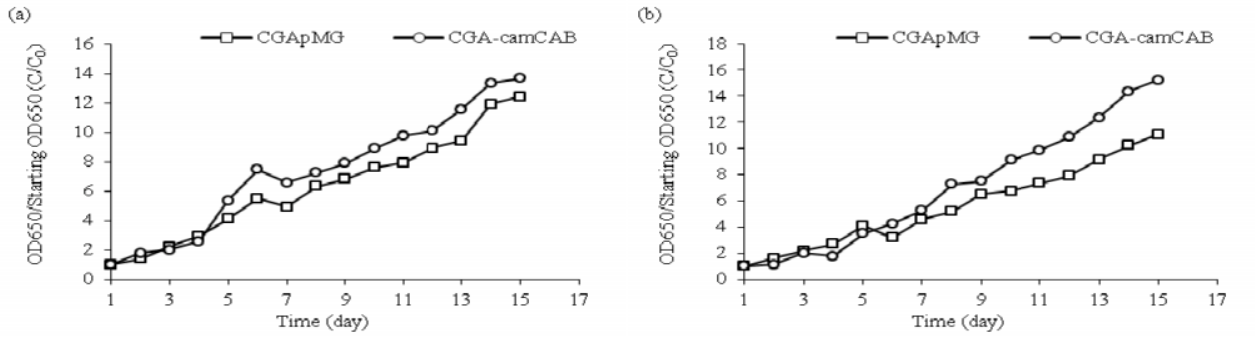

Fig. 8 The growth curve of CGApMG and CGA-camCAB with 3-chlorobenzoate (a), or 3-chlorobenzoate and benzoate (b), as carbon sources

IV. Conclusion

In this study, we enhanced halorespiration reaction in *R. palustris* by expressing cytochrome P450, putidaredoxin reductase, and putidaredoxin form *P. putida*. This result could be able to apply to any *R. palustris* strains to enhance halorespiration.

Acknowledgment

We thank Dr. Hideaki Yukawa from The Research Institute of Innovative Technology for the Earth in Japan for supplying the shuttle vector pMG105 and Yang-hsin Shih who was with Department of Soil and Environmental Sciences, National Chung Hsing University, now is with Department of Agricultural Chemistry, National Taiwan University, Taiwan, for helping PCA degradation experiment.

This research is financial supported by National Science Council; project number is NSC 95-2221-E-005-074-MY3.

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