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Degradation of 3-Chloropropionic Acid by *Escherichia coli* JM109 Expressing Dehalogenase (*deh*) Gene used as Selection Marker

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Abstract: 3-Chloropropionic acid (3CP) in its carboxylate ionic form is a synthetic compound found in herbicide. The biodegradability of 3CP is not well documented but a microbe that has the ability to utilise 3CP as sole carbon and energy source has been isolated. The dehalogenase gene (*deh*) cloned from *Rhodococcus* sp. HJ1 could be used as a selection marker gene for vector in *E. coli*. Halogenated compound, especially 3CP inhibit the growth of some microorganisms. In current investigation, a 4 kb *Eco*R1 fragment of genomic DNA from *Rhodococcus* sp. HJ1 was cloned into pUC18 plasmid and transformed into an *E. coli* JM109 conferred 3CP resistance on them. Therefore, *E. coli* transformed with vector marked with *deh* could be easily selected on plates containing 3CP. The *E. coli* JM109 transformed with pTY096 (*deh*$^+$) weakly expressed the *deh* gene as shown from its slow growth with cells doubling time of 22 h with minimal amount of chloride ion released in the growth medium.

Key words: Dehalogenase, *deh* gene, 3CP resistance, dehalogenase gene

INTRODUCTION

The application of chemically synthesised herbicide in agricultural areas has shown a remarkable success in doubling the yield, but at the same time can cause environmental issues such as contamination of ground water and rivers. The biological methods involve the use of microbial or enzymic biocatalysts as a process of bioremediation. Various microorganisms are able to utilise halogenated alkanoic acids as a sole carbon source and therefore, are assumed to have a significant role in their natural detoxification (Chaudhry and Chapalamadugu, 1991; Janssen *et al*., 2001). Dehalogenation is used for degradation of chlorinated aliphatic acids for example degradation of α-chloro substituted haloalkanoates, 2,2-dichloropropionate (2,2-DCP), D,L-2chloropropionate (D,L-2CP) and Monochloroacetate (Kerr and Marchesi, 2006; Sui-Yi *et al*., 2007). Mineralisation of these compounds is attributed to a group of enzymes which are called dehalogenases (Hardman, 1991; Janssen *et al*., 1994; Jing and Huyop, 2007; Jing *et al*., 2008; Thasif *et al*., 2009) and these enzymes have great potential of being applied in the emerging bioremediation technologies. Currently, the interest of the subject was apparent when dehalogenase enzymes from α-chloroalkanoate (Fig. 1a) degrading bacteria were unable to dechlorinate the β-substituted haloalkanoate for example 3CP (Fig. 1b).

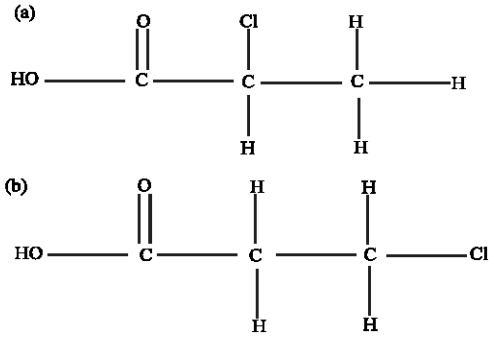

Fig. 1: Molecular structures, (a) D,L-2-chloropropionic acid (D,L-2CP) and (b) 3-chloropropionic acid (3CP)

A species from genera *Rhodococcus* sp., HJ1 has been isolated from soil, which showed the ability to utilise 3CP as the sole source of carbon (Jing and Huyop, 2007). A dehalogenase gene responsible for 3CP-utilisation is located in the chromosomal DNA as there was no plasmid has been detected. The 3CP is toxic to many organisms and inhibit their growth. Dehalogenase from the isolated bacteria is assumed to play two main roles: the detoxification of harmful 3CP and the utilisation of them as carbon source.

In order to study the properties of dehalogenase that can degrade 3CP, cloning and expression of this gene is

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necessary. As a first step, we demonstrate that *E. coli* acquiring the β-substituted haloalkanoate dehalogenase gene became resistant to 3CP and therefore this gene could be used as genetic markers of vectors to be easily selected. This study also presents an alternative way of molecular cloning and expression of the gene encoding 3CP dehalogenase enzyme in *E. coli*.

MATERIALS AND METHODS

Bacteria, plasmids and growth conditions: Plasmid pTY096 (*deh*$^+$, *Amp*^{r}) was constructed by inserting a 4.0 kb DNA fragment containing the *deh* gene into pUC18. The plasmid was maintained and propagated in *E. coli* JM109 (PROMEGA). An *E. coli* strain was grown aerobically at 37°C for 1 h prior to plating in LB medium (Maniatis *et al*., 1982) consisting of 10 g tryptone, 5 g yeast extract, 5 g NaCl L^{-1}, pH 7.3, when required Ampicillin (final concentration 100 μg mL^{-1}) and isopropyl thio-β-D-galactoside (IPTG, final concentration 0.05 mM) were incorporated.

Rhodococcus sp. HJ1 (Accession Number AM231909) was isolated from soil taken from University Technology Malaysia plantation, was grown in 100 mL culture of minimal medium supplied with 10 mM 3CP and 0.05% yeast extract. The biomass concentration was measured at A_{600nm}. Minimal medium containing 0.05% yeast extract only, with inoculum was incubated in the same condition and served as a control.

The minimal medium was prepared as 10x concentrated containing K_2HPO_4. $3H_2O$ (42.5 g L^{-1}), NaH_2PO_4. $2H_2O$ (10.0 g L^{-1}) and $(NH_4)_2SO_4$ (25.0 g L^{-1}). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g L^{-1}), $MgSO_4$ (2.0 g L^{-1}), $FeSO_4$. $7H_2O$ (120.0 mg L^{-1}), $MnSO_4.4H_2O$ (30.0 mg L^{-1}), $ZnSO_4$. H_2O (30 mg L^{-1}) and $CoCl_2$ (10.0 mg L^{-1}) in distilled water (Hareland *et al*., 1975). Minimal media for growing bacteria contained 10 mL of 10×basal salts and 10 mL of 10×trace metal salts per 100 mL of distilled water and were autoclaved (121°C, for 15 min).

DNA manipulations: Plasmid preparations and DNA ligations were carried out by standard procedures. Restriction digests of plasmid DNA were carried out using 0.5-1 μg DNA with 5-10 U restriction enzyme for 1 h at 37°C in the supplied buffers. Restriction digest of chromosomal DNA was carried out overnight at 37°C. Restriction fragments were separated by electrophoresis in 0.8% (mass vol^{-1}) agarose and an approximately 4 kb DNA in size was extracted from gel using a QIAquick gel extraction kit (Qiagen). Transformations were performed using the Mops/RbCl method (Kushner, 1978). All molecular and microbiological works were carried out in the Microbiology Laboratory, University Technology Malaysia.

Production of pTY096 (*deh*$^+$): Plasmid pUC18 was cut using *Eco*R1 (1U) and dephosphorylated using 1-20 picomoles of DNA termini with 1 U of calf intestine alkaline phosphatase for 30 min at 37°C. The dephosphorylated plasmid was purified using a DNA extraction kit (Fermentas). The digested chromosomal DNA was run on 1.0 % (w/v) agarose gel electrophoresis. The 4 kb DNA fragment was extracted from the agarose gel and purified. The purified fragments were then ligated into the dephosphorylated pUC18. Ligation reaction was carried out by standard procedure (Maniatis *et al*., 1982). Plasmid preparation from transformants were carried out using Qia-Miniprep kit (Qiagen) as described by the manufacturers.

Chemicals: The 3-Chloropropionic acid (3CP) was from SIGMA and neutralized using 10 M NaOH before filter-sterilised (Acrodisc). All other chemicals were of analytical grade.

RESULTS

Transformants were selected on a gene library screened for clones able to grow in solid minimal medium contained 10 mM 3CP as a carbon source, IPTG (0.05 mM) and ampicillin. After 1-week incubation at 37°C, 1 potentially colony was observed due to the fact that *E. coli* with *deh* gene could grow on 3CP, while the cells without *deh* gene could not suggested the usage of *deh* gene as a selection marker of vectors. The plasmid DNA was prepared from the colony and subjected to *Eco*R1 digest to check for the presence 4 kb insert fragment. Clone contains an approximately 4 kb insert DNA was then further investigated and was designated as pTY096.

The pTY096 was transformed into *E. coli* JM109 but failed to grow in liquid minimal medium plus 10 mM 3CP and IPTG (0.05 mM). However, growth was seen only when 0.05% yeast extract was added into the same minimal medium. Cell doubling time was calculated to be 22 h with maximum growth A_{600} 0.58 (Fig. 2). In control experiment, minimal medium containing 0.05% yeast extract only, yielded A_{600} 0.28 with doubling time 1 h. A_{600} 0.30 may contributed possibly by 3CP. *E. coli* JM109 without transformed plasmid did not show any growth in liquid minimal medium plus 10 mM 3CP and IPTG (0.05 mM), suggesting 3CP toxic to the *E. coli*.

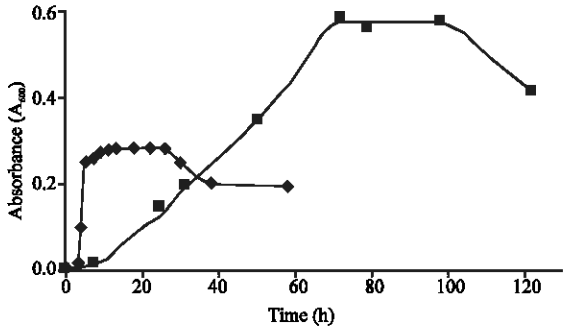

Fig. 2: Growth curve of *E. coli* JM109::pTY096. (■) Minimal medium supplied with 10 mM 3CP+ 0.05% yeast extract + 0.05 mM IPTG; (◆) Minimal medium + 0.05% yeast extract + IPTG

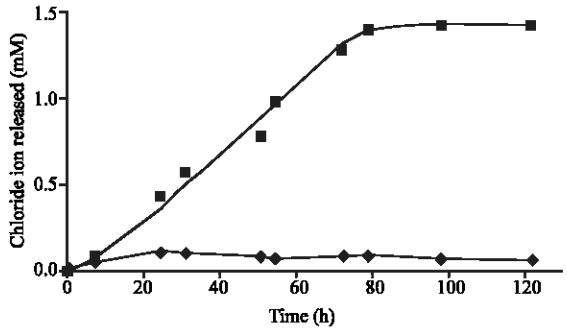

Fig. 3: Chloride ion released in the growth medium: (■) Amount of chloride ion released in the growth medium supplied with 10 mM 3CP+0.05% yeast extract + 0.05 mM IPTG in the presence of *E. coli* JM109::pTY096 (*deh*$^+$); (◆) Amount of chloride ion released in the growth medium supplied with 10 mM 3CP+0.05% yeast extract + 0.05 mM IPTG without *E. coli* JM109::pTY096

Further investigation was carried out to detect chloride ions in the growth medium. The presence of chloride ions indicated the utilisation of 3CP in the growth medium. The maximum amount of chloride ion released was 1.43 mM (14%), whereas 3CP in control flask remains stable suggesting no auto degradation of 3CP in the growth medium (Fig. 3).

DISCUSSION

Current investigation has suggested that it was possible to isolate the dehalogenase gene using shot gun cloning method by expressing the gene in *E. coli* that resist to 3CP as a selection marker in vector system. Other cloning strategy may include specific amplification of dehalogenase gene by Polymerase Chain Reaction (PCR) (Kocabiyik *et al*., 1995) or using a specific gene probe designed from N or C terminal of the dehalogenase protein to probe gene of interest by Southern Blotting analysis.

Growth of *E. coli* JM109 harbouring pTY096 plasmid in 3CP minimal medium was due to the fact that the presence of *deh* gene, while the cells without pTY096 (*deh*$^+$) could not grow on 3CP plates, suggested the usage of *deh* gene as a selection marker of vector. The *E. coli* JM109 was transformed with pTY096 (*deh*$^+$) and transformants were selected on 3CP and ampicillin. Prior to plating the transformed cells on the selection plates, they were incubated in fresh LB medium at 37°C for 1 h to express the *deh* and *amp*^{r} genes and to acquire the resistance to 3CP and ampicillin, respectively. Since 3CP resistance phenotype conferred by *deh* was easily selected, the gene was usable as genetic marker for vectors.

Slow growth and little chloride released were observed (Fig. 2, 3). These phenomena may due to the creating of an environment that may not suitable for further growth of cells. The 3CP dehalogenase enzyme is an intracellular protein and the uptake of the substrate into the cell is the main factor that affects the amount of the substrate to enter the cells resulted in slow chloride released in the growth medium. The uptake of 3CP into *E. coli* is not well understood. If the gene coding for 3CP symport protein is included in the construct DNA, then the uptake of 3CP will be efficient.

Expression of *deh* in pTY096 was induced by IPTG using *lac* promoter system. However, if there was an intrinsic promoter on the 4 kb 3CP dehalogenase gene from *Rhodococcus* sp., it will regulate the dehalogenase expression without IPTG present provided that the host polymerase recognise the 3CP dehalogenase promoter gene. Low gene expression could happen as well, if there were a significant gap between the dehalogenase gene and the promoter (Ohkouchi *et al*., 2000). If the dehalogenase gene expressed in *E. coli* was subjected to protease attack, or form inclusion bodies, or the enzyme that has low affinity to the substrate (high K_m values), the 3CP degradation will be less, as seen by the phenomena of low chloride released in the growth medium.

In conclusions, the gene encoding for 3CP-dehalogenase was successfully isolated using selection of transformants on 3CP plates. The poor growth of transformant (*E. coli* JM109::pTY096) in 3CP minimal medium and the low amount of chloride ion released possibly due to inefficient dehalogenase gene expression and substrate uptake into the cells. In future this gene is potentially useful if the gene is cloned and well expressed. Therefore, it can be used as a plant selectable marker in plant transformation studies and for the production of herbicide resistant plants.

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