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Recombinant Production and One-Pot Purification for Enhancing Activity of Haloacid Dehalogenase from *Bacillus cereus* IndB1

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Abstract

In recent years we have witnessed the emergence of organohalogen utilization in various chemical-based industries, particularly polymer-based, agricultural, and pharmaceutical sectors. Despite this, organohalogen compounds are actually very dangerous to the environment, as they are difficult to be naturally degraded and generally toxic to organisms. A green and biocompatible method to overcome this issue is by employing enzymes that could convert organohalogens into non-toxic compounds, such as the class of enzymes known as haloacid dehalogenases. To enhance the activity of haloacid dehalogenase isolated from local strains of Bacillus cereus IndB1, we have developed a recombinant expression system using pET-bcfd1 plasmid in E. coli BL21 (DE3) host cells. Following enzyme production, we also demonstrated a one-pot purification system for the expressed dehalogenase, harnessing the presence of His-tag in the recombinant clones. Purification was carried out using Ni-NTA affinity column chromatography, using imidazole eluent with a concentration gradient of 10 mM to 500 mM. The enzyme activity was tested against the monochloroacetic acid (MCA) substrate according to the Bergmann and Sanik method, and the protein content in the solution was measured using the Bradford method. The purity of the enzyme after one-pot purification was confirmed by SDS-PAGE analyses, showing a single band of 40 kDa in size. Remarkably, the purified haloacid dehalogenase specific activity was increased by 12-fold compared to its crude enzyme extract. Therefore, the expression and purification system developed in this study allow further exploration of dehalogenases from local strains as an efficient catalyst for MCA biodegradation.

Keywords: *recombinant expression, haloacid dehalogenase, monochloroacetic acid, enzyme purification*

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INTRODUCTION

Organohalogens are a group of organic compounds containing one or more halogen atoms, which are widely used in polymer industry, agriculture, and pharmaceutics (Fetzner and Lingens 1994). Despite their benefit, exposure to organohalogen compounds can cause great damage to organisms and ecosystem, as they are neurotoxic,

immunotoxic, and possibly carcinogenic (Rose *et al.*, 2009). As organohalogen compounds are difficult to be naturally degraded in the environment, the risk of exposure to these compounds is relatively high (Rose *et al.*, 2009). Monochloroacetic acid (MCA) is an example of organohalogens that would survive in the environment for a long time (European Commision, 2005). The chemical hydrolysis of MCA is slow and photolysis of MCA in air or in water does not occur because this compound does not absorb UV rays. Due to its persistence in the environment and its use in industries, the risk of exposure to MCA is significantly high (European Commision, 2005).

To address this environmental issue, accelerating MCA degradation by employing microbes and green plants is an attractive option, owing to their green and biocompatible nature (Megharaj *et al.*, 2011). Specifically, a group of enzymes obtained from microorganisms can be employed for this purpose, as they are capable of altering the chemical structures of organohalogen pollutants into non-toxic compounds. This class of enzymes is known as the haloacid dehalogenases that catalyze the carbon-halogen bond-breaking reaction in halogenated aliphatic acid compounds (Fetzner and Lingens 1994). We have previously reported a local strain of *Bacillus cereus* IndB1 that expressed a haloacid dehalogenase (Ratnaningsih and Idris, 2018). However, as the yield of the enzyme obtained from *Bacillus cereus* IndB1 wild type was relatively low, it was not quite sufficient for an efficient MCA biodegradation. A recombinant expression system is generally very effective approach to improve enzyme production (Ratnaningsih and Idris, 2018). Furthermore, in order to enhance the activity of such enzyme, a purification method of the recombinant expressed enzyme is generally required.

In this study, we aimed to produce the recombinant haloacid dehalogenase from Bacillus cereus IndB1 local strains in its recombinant pET-*bcfd1* clone using *E. coli* BL21 (DE3) as host cells and improve the activity of the expressed enzyme by means of purification. Efficiency of the purification was expected to be high since the recombinant clone contains the His-tag. Therefore, a one-pot purification system for this dehalogenase utilizing Ni-NTA affinity column chromatography, could be developed (Bornhorst and Falke, 2000; Mahmoodi *et al.*, 2019*)* The enzyme purity was evaluated using SDS-PAGE. To further confirm that the purification would result in the expected enhancement of specific activity, we compared the pure enzyme specific activity to the crude enzyme extract expressed under identical environments.

MATERIALS AND METHODS

Source of the recombinant microorganism

The *E. coli* BL21 (DE3) harboring pET-*bcfd1* recombinant clone carrying the haloacid dehalogenase gene from *Bacillus cereus* IndB1 local strain was obtained from our previous research (Ratnaningsih and Idris, 2018). The haloacid dehalogenase gene, namely *bcfd1*, has been sequenced and the sequence has already been deposited in GenBank (accession number KU49803). This gene was initially cloned into pGEM-T for analysis and subsequently sub-cloned into pET-30(a) expression vector under the control of the T7 promoter. The clone has been stored as a stock culture at −80ºC in 20% (v/v) glycerol-LB medium.

Refreshing the *E. coli* BL21 (DE3)/pET-*bcfd1* culture

To refresh the *E. coli* BL21 (DE3) carrying pET-*bcfd1* recombinant plasmid, the culture from glycerol stock was inoculated into selective LB broth medium without NaCl (contains 5 g/L yeast extract and 10 g/L tryptone, and 50 µg/mL kanamycin). The culture was shaken overnight at 37ºC rotary shaker. Single colony for further experiments was obtained by streaking this fresh culture onto a solid LB medium containing 50 µg/mL kanamycin, followed by overnight incubation at 37ºC. Confirmation of the recombinant plasmid was conducted by resriction analysis as well as by PCR employing the primers used for initial gene isolation.

Recombinant expression of haloacid dehalogenase from *E. coli* BL21 (DE3)/pET-*bcfd1*

The crude enzyme was prepared by inoculating 500 µL of overnight fresh culture of *E.coli* BL21 (DE3)/pET-*bcfd1* into 100 mL LB broth medium without NaCl, containing 50 µg/mL kanamycin. The culture was incubated at 37ºC with shaking until the OD_{600} reached 0.6. Then, IPTG was added into the culture with 10 µM final concentration. The culture was further incubated at 30ºC for 2 hours with shaking, moved into ice for 10 minutes, and the cells were harvested by centrifugation. The supernatant was collected, and the cells pellet was re-suspended into 10 mL of 50 mM Tris-acetate buffer pH 7.5. The cells were lysed by intermittent ultrasonication for 20 minutes, 30 seconds on and 30 seconds off with 40% amplifier level at 4ºC. The lysate was then centrifuged at 4ºC and the supernatant (which is the crude enzyme extract) was transferred into a new tube and store at 4ºC for further analysis.

Enzyme purification

Purification of the crude enzyme was carried out using batch method on Ni-NTA affinity column, chromatography employing the His-tag in the recombinant enzyme (Bornhorst and Falke, 2000; Mahmoodi *et al.*, 2019). One milliliter of Ni-NTA resin suspension was transferred into a 15 mL centrifuge tube. The alcohol in the resin mixture was decanted and the resin was washed several times with MiliQ water until the alcohol odor disappears. Into the tube, 2 mL of phosphate buffer binding solution was added, and the mixture was vigorously shaken at 10ºC for about 10 minutes. The supernatant was then discharged by means of centrifugation. Then, 3 mL of cold crude enzyme extract was added into the resin in the tube, and the mixture was incubated overnight on rotary shaker at 10ºC. The supernatant was collected

into a new tube as flowthrough and stored at −20ºC for further analysis. The resin was subsequently washed 5 times with 2 mL each of washing buffer 1 (10 mM imidazole), and the supernatants were pooled into a new sterile tube. The washing process was repeated using washing buffer 2 (50 mM imidazole) under identical procedure.

After completing the washing process, 600 µL of elution buffer 1 (100 mM imidazole) was used to take up the enzyme from the resin, which were carried out 3 times, and each resulted eluates were placed in separate new sterile tube. The elution process was repeated using elution buffer 2 (250 mM imidazole) and elution buffer 3 (500 mM imidazole) with the same procedure. All imidazole solution was prepared in 50 mM Tris-Cl pH 7.5. Overall, this elution process resulted in 9 fractions, namely fraction 1 of eluate 1, fraction 2 of eluate 1, fraction 3 of eluate 1, fraction 1 of eluate 2, fraction 2 of eluate 2, fraction 3 of eluate 2, fraction 1 of eluate 3, fraction 2 of eluate 3, and fraction 3 of eluate 3. Enzyme purity of each fraction was evaluated by SDS-PAGE and its protein content was quantified by Bradford method (Bradford, 1976; Kruger, 2009; Aminian *et al.*, 2013), using BSA standard curve. All fractions were subsequently subjected to haloacid dehalogenase activity assay, where fractions from the same eluates were pooled.

Haloacid dehalogenase activity assay

The enzymatic reactions were performed in 1.0 mL solutions containing 50 µL of enzyme crude extract or wash buffer, or 5 µL of enzyme fractions from Ni-NTA column (diluted to 50 µL with buffer), 850 µL of 50 mM glycine-NaOH buffer pH 10, and 100 µL of 5 mM MCA to make 0.5 mM final concentration. The control was prepared with the same composition except that the enzyme was replaced with buffer solution. The reaction mixture was homogenized by inverting the tube several times, followed by incubation at 37ºC water bath for 10 minutes. The reaction was stopped by placing the tube on ice for about 5 minutes and the amount of chloride ion released into the medium was quantified by spectrophotometer according to Bergmann and Sanik method (Bergmann and Sanik, 1957).

Chloride ion determination

Bergmann and Sanik method (Bergmann and Sanik, 1957) for chloride ion determination was performed as follows. One milliliter of the enzymatic reaction sample was mixed with 100 µL of 0.1% (w/v) $Hg(SCN)_2$, mixed well by inverting the tube several times, and incubated for 5 min at room temperature. Then, 100 µL of 0.25 M $FeNH_4(SO_4)_2$ in 9 M HNO_3 was added into the mixture, mixed well by inverting the tube, and further incubated for 5 min at room temperature. Absorbance of the resulted color solution was measured at 460 nm, and the chloride ion concentration was determined using NaCl standard curve.

RESULTS AND DISCUSSION

Culture of *E. coli* BL21 (DE3)/pET-bcdf1

The gene encoding for haloacid dehalogenase from *Bacillus cereus* IndB1 local strain was referred as *bcfd1* gene. This gene has been inserted to a pET-30a(+) expression vector, resulting in pET-*bcfd1* recombinant clone. The *E. coli* BL21 (DE3) harboring pET-*bcfd1* recombinant clone carrying the haloacid dehalogenase gene from *Bacillus cereus* IndB1 was named as *E. coli* BL21 (DE3)/pET-*bcdf1*, which was obtained from our previous research (Ratnaningsih and Idris, 2018). In this study, these recombinant clones were cultured in a selective LB medium containing 50 g/mL kanamycin. In this medium, only the bacteria that carrying the recombinant plasmid would grow as the *kanR* gene responsible for resistance to kanamycin was carried by the recombinant pET-*bcfd1* plasmid. Using serial streaking technique on selective agar plates, followed by overnight incubation at 37ºC, we obtained a single colony of *E. coli* BL21 (DE3)/pET-*bcfd1* for further experiments (Figure 1). A single colony that healthily grew on the medium and well separated from other colonies was assumed to be derived from a single cell. Such a single colony was required as to ensure that the cultures were genetically homogeneous.

Figure 1. Culture of recombinant *E. coli* BL21(DE3)/pET-*bcfd1* on selective solid LB medium

Isolation and analyses of pET-*bcfd1* recombinant plasmid

To confirm that *E. coli* BL21 (DE3) was carrying the correct pET-*bcfd1* recombinant plasmid, we isolated the plasmid from the single colony (Figure 1) derived culture by alkaline lysis method (Birnboim and Doly, 1979). The isolated recombinant plasmid was then subjected to agarose gel electrophoresis using 1% (w/v) agarose gel in TA buffer and run at 55 V for 60 min. The electrophoregram revealed that the size of pET-*bcfd1* from the single colony (line S) was larger compared to the empty pET-30a(+) (line E) (Figure 2a). The larger size of the recombinant plasmid was indicated by its smaller migration distance in comparison to empty pET-30a(+). Such an increase in size confirmed that the single colony

cultivated in this study indeed contained a recombinant plasmid.

To ensure that the inserted gene in the plasmid was indeed the *bcfd1*gene, restriction analysis was performed using the *Eco*RI and *Hind*III restriction enzymes (Ratnaningsih and Idris, 2018). Following double-digestion with these restriction enzymes, we analyzed the samples by agarose gel electrophoresis using 1% (w/v) agarose gel in TA buffer and run at 55 V for 60 minutes. The electrophoregram is presented in Figure 2(b), revealed that the double-digested pET-*bcfd1* (line S) produced two DNA fragments with the size of ~5500 bp and ~850 bp. The heavier fragment was indicated to be the pET-30a(+) whose original size was 5422 bp. On the other hand, the lighter fragment of ~850 bp confirmed the presence of the *bcfd1*gene as this particular size is in agreement with the estimated 852 bp of *bcfd1* gene suggested by previous *in silico* analyses (Ratnaningsih and Idris, 2018). Further confirmation was performed by amplifying the *bcfd1* gene using pair of primers utilized in initial gene isolation, which also resulting the same size of strong single band amplicon (Figure 2(c)).

Expression and one-pot purification of haloacid dehalogenase

The recombinant expression of the *bcfd1* gene in *E. coli* BL21 (DE3) hosts occurred upon the addition of the inducer to the growth medium (Rosano and Ceccarelli, 2014; Rosano, *et al.*, 2019). In this study, we employed isopropyl-β-D-thiogalactopyranoside (IPTG) as the inducer, which is an analogue compound of lactose (Ma *et al.*, 2020; Schein, *et al.*, 1988; Winograd, *et al.*, 1993). Following after induction incubation at 30ºC for 2 h with shaking, the resulting cultures were harvested by centrifugation and the cells were lysed by intermittent ultrasonication.

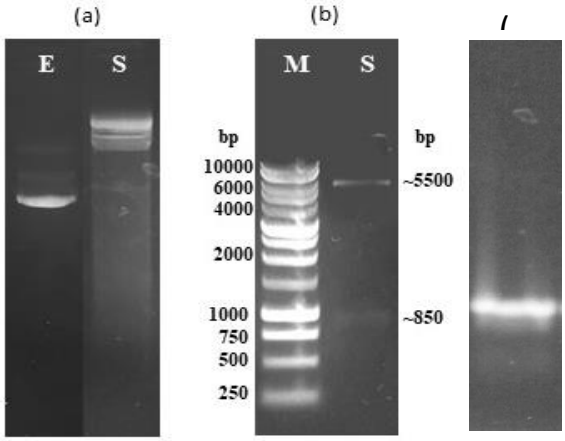

Figure 2. Electrophoregram of (a) empty pET-30a(+) plasmid (line E) and pET-*bcfd1* recombinant plasmid isolated from single colony (line S), (b) double-digested pET-*bcfd1* recombinant plasmid isolated from single colony (line S). M = marker, I kb DNA Ladder (Promega), and (c) amplicon obtained from pET-*bcfd1* by PCR

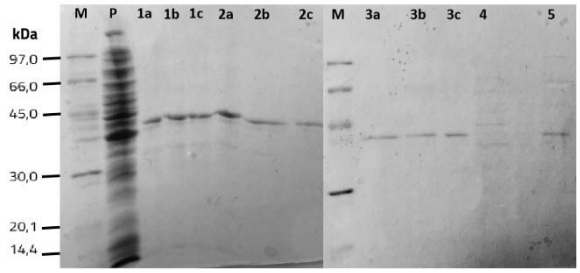

Figure 3. SDS-PAGE electrophoregram of protein fractions from *E. coli* BL21 (DE3)/pET-*bcfd1*. M = marker, P = flowthrough, 1a = fraction 1 of eluate 1, 1b = fraction 2 of eluate 1, 1c = fraction 3 of eluate 1, 2a = fraction 1 of eluate 2, 2b = fraction 2 of eluate 2, 2c = fraction 3 of eluate 2, 3a = fraction 1 of eluate 3, 3b = fraction 2 of eluate 3, 3c = fraction 3 of eluate 3, 4 = wash 1, and 5 = wash 2

Upon centrifugation, the lysate was transferred into a new sterile tube and used as the crude enzyme extract.

To improve the purity of the enzyme, and thus enhancing its specific activity, we set to purify the enzyme using a Ni-NTA affinity column. The recombinant haloacid dehalogenase gene in *E.coli* BL21 (DE3)/pET-*bcfd1* contains 6 x His-tag in its N-terminal sequence, which facilitated the separation of this enzyme from other cellular proteins using this column (Hengen, 1995). At certain pH, histidine residue in His-tag would specifically bind to nickel ions immobilized with nitrilo triacetic acid in the resin matrices (Ni-NTA) (Hengen, 1995). Once all other proteins were washed off from the resin, the target protein could be eluted with improved gradient concentration of imidazole. This approach allowed a one-pot purification system for the expressed enzyme.

All protein fractions from Ni-NTA column were subjected to SDS-PAGE and the observed electrophoregram is presented in Figure 3. In our one-pot purification system, the resin was initially washed 5 times with 2 mL each of washing buffer 1 (10 mM imidazole) and subsequently with washing buffer 2 (50 mM imidazole). After the completion of resin washing, elution buffer 1 (100 mM imidazole) was used to elute the enzyme out of the resin. The elution process was repeated using higher concentrations of imidazole in elution buffer 2 (250 mM imidazole) and then elution buffer 3 (500 mM imidazole). Particularly at the highest imidazole concentration (elution buffer 3), all fractions from imidazole elution gave only a single band at around 40 kDa, indicating that the recombinant haloacid dehalogenase was successfully purified using this method. Our previous research had identified the size of haloacid dehalogenase from *Bacillus cereus* IndB1 as 37 kDa (Ratnaningsih and Idris, 2018).

Enhanced activity of haloacid dehalogenase upon one-pot purification

The activity of haloacid dehalogenase *bcfd1* was quantitatively analyzed by measuring the amount of

free chloride ions into the medium as a result of MCA degradation by haloacid dehalogenase (Adamu, *et al*., 2020; Torz and Beschkov, 2005; Ang, *et al.,* 2018). The measurement of chloride ions in solution was carried out using the Bergmann and Sanik method (Bergmann and Sanik, 1957). In this method, the chloride ions from MCA degradation would bind the Hg^{2+} ions from $Hg(SCN)_2$, releasing thiocyanate ion (SCN^-) into the solution. As iron(III) ions were also present in the solution, the colored complex of $Fe(SCN)^{2+}$was accordingly formed and monitored at the wavelength of 460 nm (A_{460}). In this method, the concentration of the colored complex was directly proportional to the chloride ions released by MCA degradation (Bergmann and Sanik, 1957). To obtain the extinction coefficient required for spectrophotometric concentration determination, we prepared standard solutions of NaCl and separately carried out the Bergmann and Sanik reactions under identical procedure.

Specific activities of all fractions obtained from this purification are presented in Table 1. Fractions of the same eluates were pooled, because they all contained the same single band in SDS-PAGE. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of chloride ion into the medium in a 1-minute enzymatic reaction using 0.5 mM of MCA as the substrate. Accordingly, unit of specific activity was concurrently defined as unit activity per microgram protein in the sample in a 1-minute enzymatic reaction.

Table 1. Specific activity of haloacid dehalogenase in each fraction from purification steps

Sample	[Cl^- (mM)]	Activity (U/mL)	[Total protein] (µg/ml)	Specific activity (U/µg protein)
Medium	0.065	0.130	11.11	0.011
Crude extract	0.329	0.658	95.21	0.007
Wash 1	0.238	0.476	15.38	0.030
Wash 2	0.197	0.394	6.20	0.064
Elute 1	0.155	0.100	8.98	0.345
Elute 2	0.179	3.580	11.11	0.322
Elute 3	0.249	4.980	5.94	0.838

Notes: Unit activity was calculated from observed chloride ion concentration divided by reaction time (10 minutes) and enzyme volume used in the reaction. The volumes of medium, crude extract, wash1, and wash 2 solutions were 50 µLeach in 1 mL of total reaction volume, whereas the volume of elution 1, elution 2, and elution 3 were 5 µL each, in 1 mL of total reaction volume.

Table 1 clearly indicated that the specific activity of the enzyme was significantly increased with the purification steps. Best specific activity was obtained in eluates 3, where 500 mM imidazole was used as an eluent. Specific activity in this fraction was 0.838 U/µg protein, which was 12-fold higher compared to the crude enzyme extract (0.007 U/µg protein). Gradual increase of imidazole concentration in the eluent apparently did not give any significant effect on this haloacid dehalogenase purification.

CONCLUSIONS

Through genetic engineering, we have producedhaloacid dehalogenase from local strain as an intracellularly expressed active enzyme for MCA degradation, employing *E. coli* BL21 (DE3) as the host cells. Double-digestion and gel analyses of the recombinant pET-*bcfd1* isolated from *E. coli* BL21 (DE3) single colony confirmed that the gene inserted to the pET-30a(+) was indeed the *bcfd1 from Bacillus cereus* IndB1 encoding for haloacid dehalogenase. Moreover, IPTG-induced expression of the enzyme resulted in a ~40 kDa protein on SDS-PAGE analyses, which was in agreement with the predicted size of haloacid dehalogenase from *Bacillus cereus* IndB1. Importantly, a one-pot purification set-up using Ni-NTA affinity column chromatography had successfully increased the specific activity of the purified enzyme by 12-fold compared to its crude enzyme extract. Such an increase in activity was obtained particularly at higher concentration of imidazole as the eluent. The reported findings are useful to further utilize the dehalogenase from local strain as an efficient catalyst for MCA biodegradation as well as for fine chemical syntesis.

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