Indonesian Journal of Biotechnology

VOLUME 22(2), 2017, 55-60 | RESEARCH ARTICLE

Cloning and expression of haloacid dehalogenase gene from *Bacillus cereus* IndB1

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ABSTRACT Organohalogen compounds, widely used as pesticides in agriculture and solvents in the industrial sector, cause environmental pollution and health problems due to their toxicity and persistence. Numerous studies have been conducted on the biodegradation of organohalogen compounds, with many focusing on the use of dehalogenase from bacteria. Haloacid dehalogenase is a group of enzymes that cleaves the carbon-halogen bond in halogenated aliphatic acids. In a previous study, the *bcfd1* gene encoded haloacid dehalogenase from *Bacillus cereus* IndB1 was successfully isolated and characterized. This research aimed to create an expression system of the *bcfd1* gene by subcloning this gene into pET expression vector and to overexpress the gene in *Escherichia coli* BL21 (DE3). In addition, the recombinant protein was characterized to gain a better understanding of the catalytic action of this enzyme. A high expression of *bcfd1* was obtained by inducing the culture at OD_{550} 0.8–1.0 using 0.01 mM IPTG as determined by SDS-PAGE. Zymogram analysis proved that the recombinant protein possessed dehalogenase activity. Bcfd1 activity toward monochloroacetic acid (MCA) showed specific activity of 37 U/mg at 30°C, pH 9. The predicted tertiary structure of Bcfd1 was estimated has conserved α/ß hydrolase folding motif for haloacid dehalogenase superfamily.

KEYWORDS *Bacillus cereus* IndB1; *bcfd1* gene; heterologous expression; haloacid dehalogenase; organohalogen

1. Introduction

Organohalogen compounds are organic compounds that are widely used as pesticides, plasticizers, precursors, and solvents in several industries. These compounds are xenobiotic and can cause environmental pollution and health problems because of their toxicity, persistence, and ability to transform into other hazardous metabolites (Huyop et al. 2004).

Organohalogen compounds are one of the biggest pollutants in the hydrosphere (Römpp et al. 2001). Biodegradation using microorganisms has been considered an environmentally friendly and economical method of handling organohalogen pollutants as these microorganisms can metabolize and transform organohalogen compounds to make them non-toxic (Maier et al. 2009). To this end, a number of microorganisms with dehalogenase activity have been isolated and characterized, such as *Moraxella* sp. (Kawasaki et al. 1992), *Pseudomonas* sp. (Liu et al. 1994), *Bulchordia* sp. (Tsang and Sam 1999), *Rhizobium* sp. (Huyop et al. 2004), *Bacillus* sp. (Olaniran et al. 2004), *Xanthobacter* sp. (Torz and Beschkov 2005), and *Rhodobacteraceae* sp. (Novak et al. 2013).

Haloacid dehalogenases are a group of hydrolase class enzymes that break the carbon-halogen bond in halogenated aliphatic acids (Hardman and Slater 1981), and are classified into two major classes, DehI and DehII (Hill et al. 1999). DehI is a L-haloacid dehalogenase that only works at L-enantiomer substrate, whereas DehII consists of two kinds of enzymes, D-haloacid dehalogenase which only works at D-enantiomer substrate, and DL-haloacid dehalogenase which can work at both enantiomer forms.

The development of recombinant DNA technology allows enzyme production in large quantities with similar properties to the native form (Cohen et al. 1973; Choi et al. 2006). A previous study successfully isolated and characterized the *bcfd1* gene that encoded dehalogenase enzyme from *Bacillus cereus* IndB1 obtained from the Indonesian Agriculture Research and Development Association (Arif 2013). This research aimed to create an expression system of the haloacid dehalogenase gene from *B. cereus* IndB1 by subcloning the gene into the pET expression vector in *Escherichia coli* BL21 (DE3). Further research in characterizing its protein activity will also be performed through *in silico* and *in vitro* analysis to gain a better understanding of the catalytic activity of this enzyme.

2. Materials and methods

2.1. Bacterial strains and chemicals

B. cereus IndB1 obtained from the Agriculture Research and Development Association of Indonesia was used as a chromosomal DNA source. *E. coli* TOP 10 was used as a host cell in the cloning process and *E. coli* BL21(DE3) was used as a host cell in gene expression. Two kinds of plasmids were used in this study, namely pGEM-T Easy (Promega, United States) and pET-30a(+). pGEM-T Easy was used as a cloning vector and pET-30a(+) was used as an expression vector. The genomic DNA of *B. cereus* IndB1 was isolated using a DNA extraction kit from Qiagen (Germany). Restriction endonucleases (*Eco*RI and *Hind*III), T4 DNA ligase and DNA ladder 1 kb were purchased from Promega. Oligonucleotide primers for amplification of haloacid dehalogenase gene were designed manually and ordered from 1stBASE (Malaysia). Monochloro acetic acid (MCA) was purchased from Merck (United States). Isopropyl-this-ß-D-galactoside (IPTG) was used to induce the gene expression under control of *lac* promoter. All the chemicals used in this work were analytical grade.

2.2. Construction of dehalogenase gene expression

A pair of primers was designed based on nucleotide sequences of the haloacid dehalogenase gene from *B. cereus* IndB1 named *bcfd1* (Arif 2013). This gene has been submitted to GenBank with Accession Number KU498039. The designed primers were 5'-GCAGAATTCATGGATGGAACACTACTATC-3' as the forward primer and 5'-GCGAAGCTTTTATTTACTAGATGAAGTTTG-3' as the reverse primer. The underline sequences indicate the *Eco*RI and *Hind*III recognition sites, respectively.

DNA amplification was performed using the KAPA Taq ReadyMix PCR kit (Kapa Biosystems) with the genomic DNA of *B. cereus* strain IndB1 serving as a template. The PCR reaction of 20 µL was prepared using KAPA Taq ReadyMix in accordance with the KAPA Taq PCR protocol. PCR reaction was initiated with predenaturation at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. The reaction was completed by a final extension at 72°C for 5 min. The obtained amplicon was confirmed by electrophoresis on a 1% w/v agarose gel.

The amplicon was first cloned into pGEM-T Easy vector then subcloned into pET-30a(+) expression vector. Recombinant pGEM-*bcfd1* was digested with *Eco*RI and *Hind*III, ligated into linear pET-30a(+), transformed into *E. coli* BL21 (DE3) and grown on kanamycin selection medium. The pET-*bcfd1* recombinant clone was isolated and confirmed by the size screening , re-PCR, restriction analysis and sequencing.

2.3. Expression of recombinant protein

E. coli BL21 (DE3) carrying pET-*bcfd1* was grown in liquid LB media supplemented with 50 µg/ml kanamycin at 37°C to reached 0.8–1.0 of OD_{550} and induced with 0.01 mM IPTG for protein expression and further incubated at 30°C for 2–4 h. The crude extract recombinant protein was prepared from the cells taken every hour from the culture. The cells were harvested by centrifugation, washed with 50 mM Tris-acetate (pH 7.5), lysed by sonication for 20 min, and the crude extract was separated from the cell debris by centrifugation.

2.4. Preparation of crude extract protein

The crude extract was prepared by collecting cells from a culture that was induced for 2 h by centrifugation at 9820x*g* for 20 min at 4°C. The supernatant was disposed and the cell pellet was washed with 50 mM Tris-acetate, pH 7.5 buffer twice. The cell pellet was resuspended in the same buffer and lysed by sonicating on ice for 20 min with intervals 30 s on/ 30 s off. The cell debris was separated from the supernatant by centrifugation at 6000x textitg for 30 min, 4 °C. The supernatant is crude extract recombinant protein and immediately used for activity assay.

2.5. Protein expression analysis with SDS-PAGE

The crude extract recombinant protein was loaded on 12% SDS-PAGE to confirm the protein expression. The protein was visualized by staining the gel in a Coomassie brilliant blue solution while a protein ladder was used as the size standard.

2.6. Qualitative assay of haloacid dehalogenase activity

Haloacid dehalogenase activity was qualitatively analyzed through native-polyacrylamide gel electrophoresis (Hardman and Slater 1981). The gel was incubated at 30°C in 100 mM Tris-acetate buffer pH 9 containing 50 mM monochloroacetic acid (MCA) for 30 min. Dehalogenase activity was detected after further incubation in 0.1 M $AgNO_3$ solution. The white precipitate of AgCl on the gel around protein band indicated positive dehalogenase activity.

2.7. Quantitative assay of haloacid dehalogenase activity

Haloacid dehalogenase activity was quantitatively determined using colorimetry method by measuring released chloride ion concentration (Bergmann and Sanik 1957). The enzymatic reaction was performed in 1 mL solution containing 50 µL of crude extract and 5 mM MCA. The reaction mixture was incubated at various pH and temperature for 10 min. The pH ranged from 6 to 11 and the temperature ranged from 20 to 70 °C. The buffers used were K_2HPO_4-KH_2PO_4 (pH 6.0), Tris-acetate (pH 7.0–8.0) and Glycine-NaOH (pH 9.0–11.0). The chloride ion

was measured spectrophotometrically at 460 nm from the absorbance of colored ferric thiocyanate complex. One unit activity was defined as the amount of enzyme that was required to release 1 µmol chloride ion per minute. The protein concentration of crude extract was determined using Bradford method and bovine serum albumin (BSA) as a reference (Bradford 1976).

2.8. Bioinformatic analysis

Three-dimensional structure prediction of protein was carried out using I-TASSER program (Zhang 2008). MolProbity program was used to validate of the stereochemistry of the structure (Chen et al. 2010).

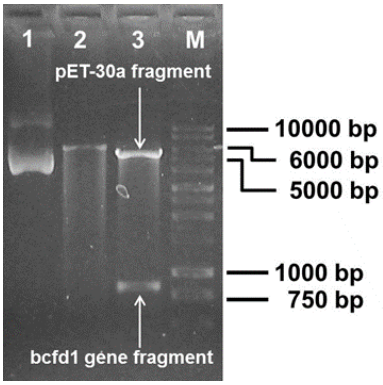

FIGURE 1 Confirmation of the *bcfd1* gene subcloning into pET-30a(+) by restriction analysis (M: 1 kb DNA Ladder, 1: non-digest pET-*bcfd1*, 2: *Hind*III digested pET-*bcfd1*, 3: *Hind*III and *Eco*RI digested pET-*bcfd1*).

3. Results and discussion

3.1. Construction of pET-bcfd1 expression system

The recombinant pET-*bcfd1* obtained from pGEM-*bcfd1* were size screened and digested with *Eco*RI and *Hind*III (Figure 1), confirmed by re-PCR and sequenced. Sequences analysis showed that *bcfd1* gene was integrated into expression vector pET-30a with the correct orientation and in frame with plasmid sequence (Figure 2). These analyses confirmed that the *bcfd1* gene has been successfully subcloned into pET-30a(+) expression vector.

3.2. Expression of recombinant pET-bcfd1

The SDS-PAGE analysis confirmed that recombinant Bcfd1 protein has been successfully expressed by pET-*bcfd1* in *E. coli* BL21(DE3). The expression of the recombinant protein presence as prominent protein band with Mr about 37 kD (Figure 3a). This size is greater than the molecular weight of Bcfd1 actually is but it is consistent with prior *in silico* prediction. Prior *in silico* analysis informed that the expressed recombinant protein has protein fusion on its N terminus, that is, 6xHis-tag and S-tag. This fusion made the molecular weight of protein increase than before, from 31.5 kD to 37.1 kD. The overexpression of *bcfd1* gene still observable though IPTG concentration was lowered to 0,01 mM (Figure 3b). This is of fortunate as low IPTG will increase soluble protein production (Pacheco et al. 2012), as indicated by the large amount of recombinant Bcfd1 in the cell lysate (Figure 3b).

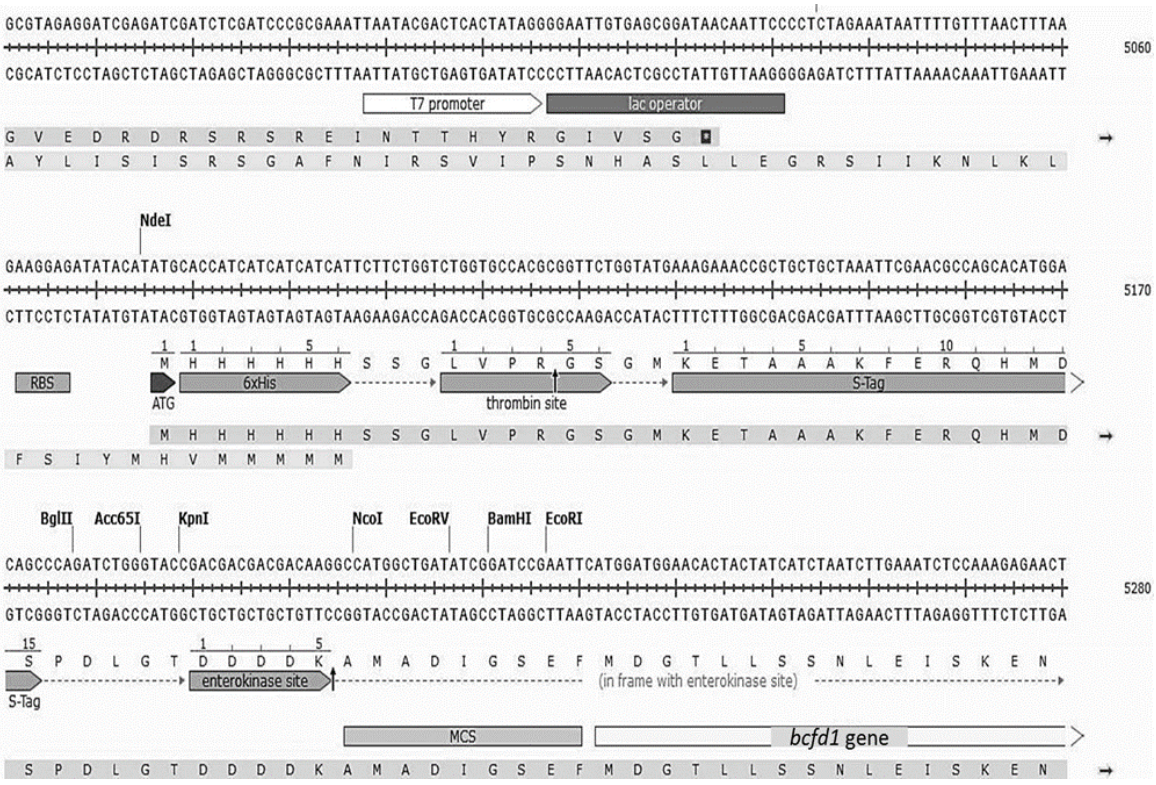

FIGURE 2 The orientation of the *bcfd1* gene in pET-*bcfd1* expression system.

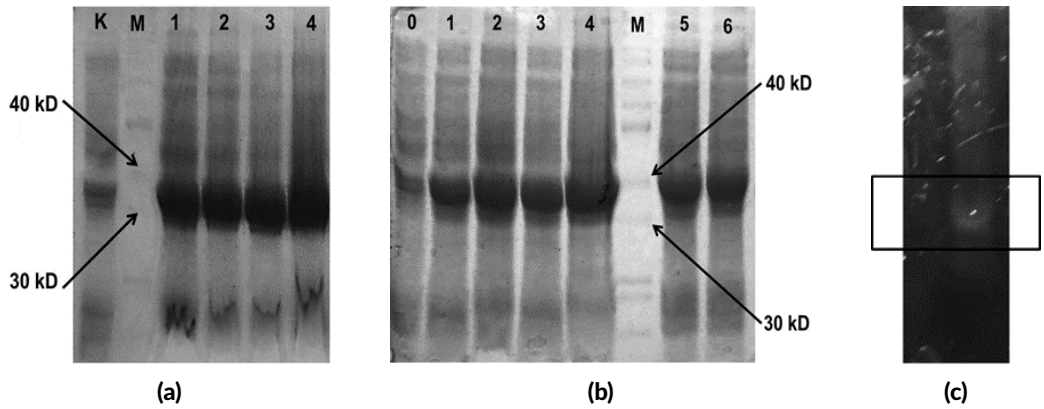

FIGURE 3 SDS-PAGE and *native* PAGE of the recombinant Bcfd1 expressed by pET-*bcfd1*. (a) Total protein cell analysis (M:protein ladder; K: *E. coli* BL21; 1–4: recombinant Bcfd1 expressed by pET-*bcfd1* on 1–4 h after IPTG induction at 30°C); (b) Total protein cell and crude extract analysis (M: protein ladder; 0–2: total protein cell on 0–2 h induced by 0.01 mM IPTG; 3–4: total protein cell after 1–2 h on 0.1 mM IPTG induction; 5–6: cell lysate on 2 h with 0.01 mM and 0.1 mM IPTG induction at 30°C); (c) Qualitative dehalogenase assay of the recombinant Bcfd1 protein on *native* PAGE.

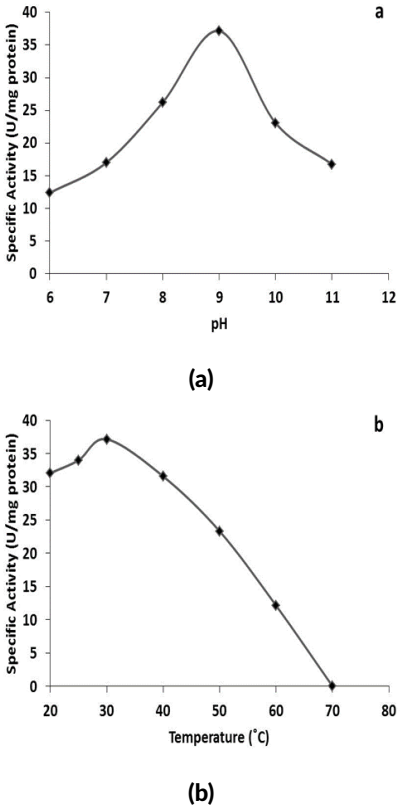

FIGURE 4 Dehalogenase activity of Bcfd1 at various pH (a) and temperature (b)

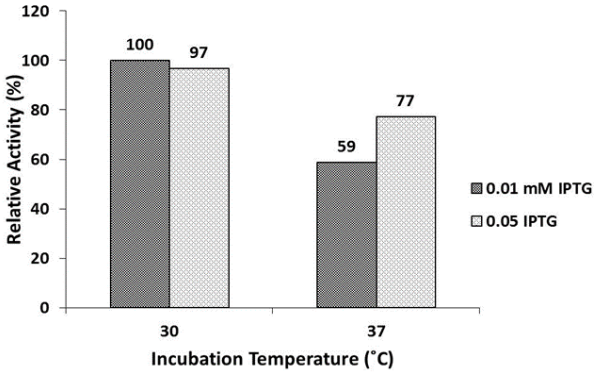

FIGURE 5 Effect of induction temperature and inducer concentration on recombinant Bcfd1 activity.

3.3. Qualitative dehalogenase assay of recombinant Bcfd1

The zymogram analysis suggested that recombinant Bcfd1 shows dehalogenase activity as indicated by AgCl formation on *native* PAGE (Figure 3c). Degradation of MCA by haloacid dehalogenase release chloride ion. The free chloride ion will react with a silver ion from $AgNO_3$ solution to produce AgCl. The formation of AgCl was indicated by the presence of white precipitation on the gel.

3.4. Optimum pH and temperature of dehalogenase activity of recombinant Bcfd1

Variation of pH and temperature indicated that recombinant Bcfd1 has its optimum activity at pH 9 at 30°C (Figure 4), consistent with bacterial L-haloacid dehalogenase (Kurihara et al. 2000). At this pH, Asp8 (corresponds to L-haloacid dehalogenase from *Xhantobacterium autotropicus*, DhlB) available in negative charges and attacks the substrate to release chloride ion (Ridder et al. 1997). It could be seen from Table 1 that the recombinant Bcfd1 dehalogenase has 37.14 U/mg specific activity that degraded 1.7% of 5 mM MCA in 10 min. This activity higher compared to wildtype dehalogenase obtained from *B. cereus* IndB1, which was only12 U/mg.

The optimum induced condition was obtained by 0.01 mM IPTG at 30°C (Figure 5). This result suggests that lower-temperature may form correct folding to produce an active enzyme (Schein and Noteborn 1988; Pacheco et al. 2012). As an addition, the low temperature also restricts activity some protease that can degrade target protein (Sahdev et al. 2007).

TABLE 1 Activity of recombinant Bcfd1 dehalogenase against MCA.

pH	Temp (°C)	Free Cl^- release (μmol)	Protein concentration (mg/ml)	Activity (U/ml)	Specific activity (U/mg)	Percent degradation (%)
9	30	85	4.58	170	37.14	1.70

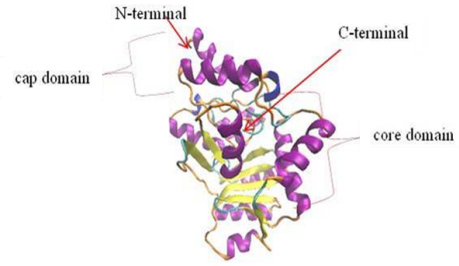

FIGURE 6 Tertiary structure prediction model of Bcfd1. Alpha helix: purple, beta sheet: yellow, turn: cyan, and coil: brown. Tertiary structure prediction carried out I-TASSER server, structure validation using MolProbity and structure visualization using VMD.

3.5. Tertiary structure prediction of Bcfd1

The structure prediction was based on the sequence of the full-length construct of the Bcfd1. The predicted tertiary structure of Bcfd1 was estimated has conserved α/ß hydrolase folding motif for haloacid dehalogenase superfamily (Figure 6). This folding motif consists of two domains, named by cap domain and core domains. The active site was predicted located at the interface of two domains and composed by catalytic residues and binding residues (Novak et al. 2013).

4. Conclusions

Haloacid dehalogenase *bcfd1* gene from *B. cereus* IndB1 was successfully cloned into pET-30a(+) in *E. coli* BL21(DE3). Overexpression of Bcfd1 was obtained by 0.01 mM IPTG induction on culture OD_{550}≈1.0 for 2 h at 30°C. On 5 mM MCA, Bcfd1 has specific activity of 37.14 U/mg at 30°C, pH 9, with 1.70% degradation for 10 min. The tertiary structure of Bcfd1 predicts the presence of conserved α/ß hydrolase folding motif, indicated that the Bcfd1 is closely related to haloacid dehalogenase superfamily.

Acknowledgments

The authors are grateful to Chemistry Department, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Indonesia, and The Asahi Glass Foundation, Japan. This research was (partially) supported by the Asahi Glass Foundation.

Authors' contributions

ER designed the study. I carried out the laboratory work, analyzed the data and wrote the manuscript. Both authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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