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Molecular Cloning and Expression of Haloacid Dehalogenase Gene from a Local *Pseudomonas aeruginosa* ITB1 Strain and Tertiary Structure Prediction of the Produced Enzyme

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Abstract

Organohalogens are widely utilized as pesticides, herbicides, solvents, and for many other industrial purposes. However, the use of these compounds caused some negative impacts to the environment due to their toxicity and persistency. In the light of this, some microbes have been identified and employed to perform dehalogenation, converting halogenated organic compounds to non-toxic materials. In this research, we successfully cloned and sequenced the haloacid dehalogenase gene from a local *Pseudomonas aeruginosa* ITB1 strain, which is involved in the degradation of monochloroacetate. First, the haloacid dehalogenase gene was amplified by PCR using a pair of primers designed from the same gene sequences of other *P. aeruginosa* strains available in the GenBank. The cloned gene in pGEM-T in *E. coli* TOP10 was sequenced, analyzed, and then sub-cloned into pET-30a(+) for expression in *E. coli* BL21 (DE3). To facilitate direct sub-cloning, restriction sequences of *Eco*RI (G/AATTC) and *Hin*dIII (A/AGCTT) were added to the forward and reversed primers, respectively. The expressed protein in *E. coli* BL21 (DE3) appeared as a 26-kDa protein in SDS-PAGE analysis, which is in good agreement with the size predicted by ExPASy Protparam. We obtained that the best expression in LB liquid medium was achieved with 0.01 mM IPTG induction at 30°C incubation for 3 hours. We also found that the enzyme is more concentrated in the pellet cells as inclusion bodies. Furthermore, the in-silico analysis revealed that this enzyme consists of 233 amino acid residues. This enzyme's predicted tertiary structure shows six β-sheets flanked by α-helixes and thus belongs to Group II haloacid dehalogenase. Based on the structural prediction, amino acid residues of Asp7, Ser121, and Asn122 are present in the active site and might play essential roles in catalysis. The presented study laid the foundation for recombinant haloacid dehalogenase production from *P. aeruginosa* local strains. It provided an insight into the utilization of recombinant local strains to remediate environmental problems caused by organohalogens.

1. Introduction

Halogenated organic substances and their derivatives are widely used as herbicides, pesticides, and other industrial purposes. Despite their usefulness, halogenated organic compounds are considered xenobiotics due to their toxicity to many organisms, though some microorganisms are known to have the ability to degrade such compounds. Some organisms are known to produce dehalogenases, which refers to an

enzyme that catalyzes the cleavage of carbon-halogen bond with seven possible mechanisms, which are reductive, oxygenolitic, hydrolytic, and thiolytic dehalogenation, intra-molecular nucleophilic displacement, dehydrohalogenation, and hydration [1]. Therefore, microorganisms producing dehalogenases, particularly bacteria, are potentially used in bioremediation, as they could facilitate the conversion of organohalogen xenobiotics at a contaminated site into non-harmful compounds [2, 3]. The general feature of bacterial dehalogenase, including its structural properties, mechanism of its catalysis, and improvement of its applications, have been thoroughly studied [1, 4]. Despite the rare natural function of this enzyme in metabolic pathways, the use of enzymatic dehalogenation is best known for removing halogen atoms from artificial organohalogen compounds with various mechanisms [5].

Based on its catalytic mechanism and substrate specificity, hydrolytic haloacid dehalogenase is classified into two different groups, known as Group I (DehI) and Group II (DehII) [6]. Molecular phylogenetic studies suggest that these two groups are not evolutionary closely related, as indicated by fundamental differences in their catalytic strategies. However, both groups are active towards α-halogenated carboxylic acid with low molecular weight, undergo SN2 substitution catalysis, and produce substrate inversion products [7]. In terms of its stereo-specificity, dehalogenase of Group I catalyzes the dehalogenation on D- and L-haloacid, whereas dehalogenase of Group II is only active on L-haloacid [8, 9]. This characteristic has opened a possibility for haloacid dehalogenase for fine chemical synthesis [10].

Among bacterial dehalogenases, 2-haloacid dehalogenases are best studied. It is well known that most haloacid dehalogenase utilizes aspartate residue as the catalytic nucleophile [11]. Haloacid dehalogenases of Group I directly use aspartate activated water molecules to attack the substrate's alpha-carbon and displace the halogen atom. In comparison, haloacid dehalogenase of Group II starts the catalysis by nucleophilic attack of carboxylate group of catalytic aspartate residue to the alpha-carbon of the substrate to form an ester intermediate, which then hydrolyzed by activated water molecule to produce the hydroxy acid [9, 12, 13]. In addition, the difference between Group I and Group II haloacid dehalogenase could also be recognized from its tertiary structure. The tertiary structure of Group I haloacid dehalogenase possesses only an alpha-helical structure, whereas Group II haloacid dehalogenase consists of alpha-helices and some beta-sheets [8, 14].

2. Methodology

The materials and experimental procedures are described below.

2.1. Materials

The local *P. aeruginosa* ITB1 strain was obtained from the School of Life Science, Institut Teknologi Bandung (SITH ITB), Indonesia. Classification of this bacterium had been carried out with 16s ribosomal DNA typing (unpublished data). The pGEM-T Easy Kit and pET-30a(+) were purchased from Fermentas. The *E. coli* TOP10 and *E. coli* BL21 (BE3) were available in the Biochemistry Laboratory of Institut Teknologi Bandung.

Yeast extract, tryptone, and bacto agar were purchased from Bio Basic. Chromosomal DNA Purification Kit was obtained from Promega. GeneJET Plasmid Miniprep Kit was purchased from Thermoscientific. Restriction enzyme and ligase were purchased from Promega. Kappa Taq Ready Mix PCR was obtained from Kappa Biosystem. The 1 kb DNA ladder as a DNA size marker and the protein molecular weight marker were purchased from Promega. Antibiotics were purchased from pharmacy stores in Bandung, Indonesia, and other chemicals were purchased from Merck, Aldrich, or Sigma.

2.2. DNA Isolation

Chromosomal DNA isolation was performed according to the protocol of DNA Purification Kit from Promega, which was carried out on 1 mL overnight LB liquid fresh culture of a local *P. aeruginosa* ITB1 strain. Plasmid DNA isolation was performed using GeneJET Plasmid Miniprep Kit from Thermoscientific for mini preparation, utilizing 1 mL overnight fresh liquid culture. Large-scale plasmid DNA isolation, particularly for restriction analysis and sequencing, was carried out by alkaline lysis method [15], using 10 mL of overnight liquid fresh culture. All DNA precipitation was performed using isopropanol.

2.3. Primer Design

Pair of primers used to fish out the haloacid dehalogenase gene from the local *P. aeruginosa* ITB1 strain was designed by utilizing the identified haloacid dehalogenase gene's conserved region in addition to the *P. aeruginosa* strains available in the GenBank. As there are many haloacids dehalogenase genes in the NCBI database, nucleotide sequence alignment was only performed utilizing nine genes with the highest homology. GenBank accession number for these 9 aligned haloacid dehalogenase genes were CP000438.1; CP000744.1; NZ-ALBW.01000008; NZ-ALIO01000121.1; NZ-AEVW02000298.1; NZ-AKZF01000393.1; NZ-AKZG01000127.1; NZ-AKZH01000152.1; and NZ-ABKZ01000456.1. After verifying the isolated gene in the pGEM-T vector, these primers were again used for further sub-cloning into the pET-30a(+) expression vector with the addition of *Eco*RI (5'-G/AATTC-3') and *Hin*dIII (5'-A/AGCTT-3') restriction sites on the respective forward and reverse primers.

2.4. PCR, Cloning, and Sub-Cloning

All PCR was performed utilizing the Kappa Taq Ready Mix PCR from Kappa Biosystem with standard composition. The gene was first fished out from the isolated chromosomal DNA of *P. aeruginosa* ITB1 with the initial primers (termed as F and R primers) for 30 PCR cycles at 48°C annealing temperature. The obtained amplicon was termed as *paed-d*. This amplicon was analyzed through gel electrophoresis, cloned into the pGEM-T Easy vector, and then used to transform the *E.*

coli TOP10. Blue-white screening on LB agar medium containing 100 µg/mL ampicillin, colonies re-PCR, and plasmid size analysis gave pGEM/*paed-d(i)* recombinant clone, which was then confirmed by sequencing.

For sub-cloning into the pET-30a(+) expression vector, the second PCR was performed using pair of primers that contain *Eco*RI and *Hin*dIII sites on its ends (termed as Fs and Rs primers). The PCR was performed using both chromosomal DNA of *P. aeruginosa* ITB1 and the pGEM/*paed-d(i)* DNA as templates, carried out with a standard composition mixture for 34 cycles at 58.4°C annealing temperature. The obtained amplicon was analyzed by agarose gel electrophoresis, cloned again into pGEM-T vector, and used to transform *E. coli* TOP10 for selection by blue-white screening and plasmid size. The obtained clone was named pGEM/*paed-d*. Further confirmation was done by restriction analysis.

The *paed-d* fragment was then isolated from this confirmed pGEM/*paed-d* clone by *Eco*RI and *Hin*dIII double digest, inserted into pET-30a(+) that had been linearized with the same enzymes and used to transform *E. coli* BL21 (DE3). Selection for pET/*paed-d* recombinant clone was carried out on LB agar medium containing 50 µg/mL of kanamycin. The obtained recombinant plasmid was further reconfirmed by plasmid size, re-PCR, and restriction analysis using *Eco*RI and *Hin*dIII. The *paed-d* fragment in the confirmed pET/*paed-d* recombinant clone was then sequenced.

2.5. Expression Analysis

Expression of the putative haloacid dehalogenase gene in pET/*paed-d* recombinant clone in *E. coli* BL21 (DE3) was carried out with 0.01 mM or 0.1 mM IPTG induction followed by 4°C or 30°C incubation for 3 hours with shaking. The inducer was added when the OD_{600} of the liquid LB culture had reached 0.6 at 37°C. The cells were harvested by centrifugation, which was then sonicated to obtain enzyme crude lysate. The obtained lysate and the cell debris were analyzed by SDS-PAGE using methylene blue staining, and the size of the protein was estimated using protein molecular weight standard (Promega). The high intensity of the blue band on the gel at around 26 kDa would indicate haloacid dehalogenase.

2.6. Bioinformatics Analysis

The obtained *paed-d* gene sequence was compared to other similar sequences using BioEdit Sequence Alignment Editor. Protparam predicted the deduced amino acid sequence and the biophysical characteristics of the protein on the Expasy website. In addition, amino acid sequence homology was also analyzed using PSI-BLAST on the NCBI database. Using the ESPript 3.0, the secondary structure was predicted using the Swiss model, Expasy, and VMD program. The tertiary structure prediction was made. Enzyme catalytic residues were predicted through SAS (Sequence Annotated by Structure) and EXIA Server.

3. Results and Discussion

3.1. Primer design, amplification, and initial cloning of the paed-d gene

The initial primers to fish out the gene from the chromosomal DNA of a local *P. aeruginosa* ITB1 strain were designed according to the conserved region of haloacid dehalogenase genes from 9 different *P. aeruginosa* strains available in the GenBank, which was squeezed from 23 strains. GenBank accession numbers for these nine aligned haloacid dehalogenase genes were presented in Materials and Methods. The haloacid dehalogenase genes in these strains appeared to have 100% homology on its flanking region. Hence the primers could be designed by including the start and the stop codons. The constructed primers were 5'-ATGCGCGCGATCCTGTTCGA-3' as a forward primer (F) and 5'-TCAGGCCGAGGCCGCCAGTT-3' as a reverse primer (R).

The performed PCR utilizing the isolated chromosomal DNA of *P. aeruginosa* ITB1 as a template (shown in Figure 1a) resulted in an obvious strong single band amplicon at ~700 bp (Figure 1b). This amplicon was cloned into a pGEM-T Easy vector which was then used to transform *E. coli* TOP10. The pGEM-T Easy linearized vector contains 3'-T overhangs at the insertion site, hence facilitated the cloning of the PCR products generated by *Taq* DNA polymerase, which mainly contains 3'-A fragments. The recombinant plasmid was termed as pGEM/*paed-d(i)*. Subsequently, the gene in this clone was sequenced, and the obtained sequence was presented in Figure 2. Utilizing the Blast program (http://blast.ncbi.nlm.nih.gov/), this gene sequence was then compared to the haloacids dehalogenase gene from other *P. aeruginosa* strains available in the GenBank. Remarkably, this sequence showed 100% homology to haloacid dehalogenase genes from four *P. aeruginosa* strains, namely *P. aeruginosa* PAO1H2O (Accession number: CP008749.1), *P. aeruginosa* YL84 (Accession number: CP007147.1), *P. aeruginosa* SCV20265 (Accession number: CP006931.1), and *P. aeruginosa* M18 (Accession number: CP002496.1).

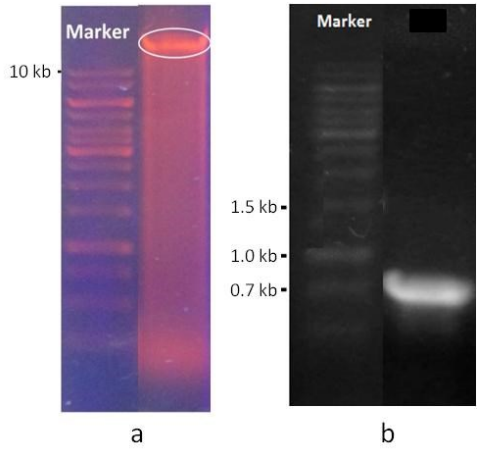

Figure 1. (a) Isolated chromosomal DNA of *P. aeruginosa* ITB1 and (b) the amplicon obtained utilizing F and R primers using *P. aeruginosa* ITB1 chromosomal DNA as a template. M = 1 kb DNA ladder (Promega)

The gene sequence showed in Figure 2 also showed 99% homology to haloacid dehalogenase genes from other 25 *P. aeruginosa* strains available in the GenBank, notably *P. aeruginosa* c7447m (Accession number: CP006728), *P. aeruginosa* PA38182 (Accession number: HG530068.1), *P. aeruginosa* PA96 (Accession number: CP007224.1), *P. aeruginosa* MTB-1 (Accession number: CP006853.1), *P. aeruginosa* DK2 (Accession number: CP003149.1), *P. aeruginosa* VRFPA04 (Accession number: CP008739.1), *P. aeruginosa* LESlike4 (Accession number: CP006985.1), *P. aeruginosa* LESlike1 (Accession number: CP006984.1), *P. aeruginosa* LESB65 (Accession number: CP006983.1), *P. aeruginosa*LES400 (Accession number: CP006982.1), *P. aeruginosa* LESlike7 (Accession number: CP006981.1), *P. aeruginosa* LESlike5 (Accession number: CP006980.1), *P. aeruginosa* LES431 (Accession number: CP006937.1); *P. aeruginosa* PA1R (Accession number: CP004055.1), *P. aeruginosa* PA1 (Accession number: CP004054.1), *P. aeruginosa* PAO1-VE13 (Accession number: CP006832.1), *P. aeruginosa* PAO1-VE2 (Accession number: CP006831.1), *P. aeruginosa* PAO581 (Accession number: CP006705.1), *P. aeruginosa* RP73 (Accession number: CP00006245.1), *P. aeruginosa* NCGM2.S1 DNA (Accession number: AP012280.1), *P. aeruginosa* LESB58 (Accession number: FM209186.1), *P. aeruginosa* PAO1 (Accession number: AE004091.2), *P. aeruginosa* UCBPP-PA14 (Accession number: CP000438.1), *P. aeruginosa* B136-33 (Accession number: CP004061.1), and *P. aeruginosa* PA7 (Accession number: CP000744.1).

3.2. Sub-Cloning of *paed-d* Gene into pET-30a(+) Expression vector

To facilitate the sub-cloning of the *paed-d* gene into the pET-30a(+) expression vector, a second PCR was performed using pair of primers that contain *Eco*RI and *Hin*dIII sites. These two restriction sites were required to ensure the correct orientation of the *paed-d* gene for expression. These two sites are absent in the *paed-d* gene sequence, hence would facilitate the sub-cloning of the entire gene. The forward primer (termed as Fs) was 5'-GAATTCATGCGCGCGATCCTGTTCGA-3', and the reverse primer (termed as Rs) was 5'-AAGCTTTCAGGCCGAGGCCGCCAGTT-3'. The PCR was carried out using both chromosomal DNA of *P. aeruginosa* ITB1 and the pGEM/*paed-d(i)* DNA as the templates, separately. As expected, both templates gave the same size of the amplicon, which is approximately 700 bp, as corroborated by gel electrophoresis (Figure 3 line 1 and 2), which was of the same size as those obtained from chromosomal DNA of *P. aeruginosa* ITB1 as for initial cloning (Figure 1).

```
5'    ATGCGCGCGA TCCTGTTCGA TGTGTTCGGT ACCCTGGTGG ACTGGCGTTC CAGCCTGATC GAGCAGTTCC AGGCGTTGGA GCGCGAACTC      90
3'    TACGCGCGCT AGGACAAGCT ACACAAGCCA TGGGACCACC TGACCGCAAG GTCGGACTAG CTCGTCAAGG TCCGCAACCT CGCGCTTGAG

      GGCGGGACCC TGCCCTGCGT GGAACTGACC GACCGCTGGC GCCAGCAATA CAAGCCGGCG ATGGACCGGG TACGCAACGG CCAGGCGCCC     180
      CCGCCCTGGG ACGGGACGCA CCTTGACTGG CTGGCGACCG CGGTCGTTAT GTTCGGCCGC TACCTGGCCC ATGCGTTGCC GGTCCGCGGG

      TGGCAGCACC TCGACCAGTT GCACCGGCAG AGTCTCGAGG CCCTGGCCGG AGAGTTCGGC CTGGCGCTGG ACGAGGCCCT GCTGCAACGT     270
      ACCGTCGTGG AGCTGGTCAA CGTGGCCGTC TCAGAGCTCC GGGACCGGCC TCTCAAGCCG GACCGCGACC TGCTCCGGGA CGACGTTGCA

      ATCACTGGCT TCTGGCACCG CCTGCGGCCG TGGCCGGACA CGCTCGCCGG GATGCATGCG CTGAAGGCCG ACTACTGGCT CGCCGCGCTG     360
      TAGTGACCGA AGACCGTGGC GGACGCCGGC ACCGGCCTGT GCGAGCGGCC CTACGTACGC GACTTCCGGC TGATGACCGA GCGGCGCGAC

      AGCAACGGCA ACACCGCGCT GATGCTCGAC GTCGCGCGGC ACGCCGGGCT GCCCTGGGAC ATGCTGCTGT GCGCCGACCT GTTCGGCCAC     450
      TCGTTGCCGT TGTGGCGCGA CTACGAGCTG CAGCGCGCCG TGCGGCCCGA CGGGACCCTG TACGACGACA CGCGGCTGGA CAAGCCGGTG

      TACAAGCCCG ACCCGCAGGT CTACCTCGGC GCCTGCCGCC TGCTCGACCT GCCGCCGCAG GAGGTGATGC TCTGCGCGGC GCACAACTAC     540
      ATGTTCGGGC TGGGCGTCCA GATGGAGCCG CGGACGGCGG ACGAGCTGGA CGGCGGCGTC CTCCACTACG AGACGCGCCG CGTGTTGATG

      GACCTCAAGG CCGCGCGCGC CCTCGGCCTG AAGACCGCGT TCATCGCCCG GCCGTTGGAA TACGGCCCCG GCCAGTCCCA GGACCTCGCC     630
      CTGGAGTTCC GGCGCGCGCG GGAGCCGGAC TTCTGGCGCA AGTAGCGGGC CGGCAACCTT ATGCCGGGGC CGGTCAGGGT CCTGGAGCGG

      GCCGAGCAGG ACTGGGACCT GATCGCCAGC GACCTGCTGG ACCTGCACCG GCAACTGGCG GCCTCGGCCT GA    3'
      CGGCTCGTCC TGACCCTGGA CTAGCGGTCG CTGGACGACC TGGACGTGGC CGTTGACCGC CGGAGCCGGA CT    5'   702
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Figure 2. Sequence of the *paed-d* gene in pGEM/*paed-d(i)* recombinant clone

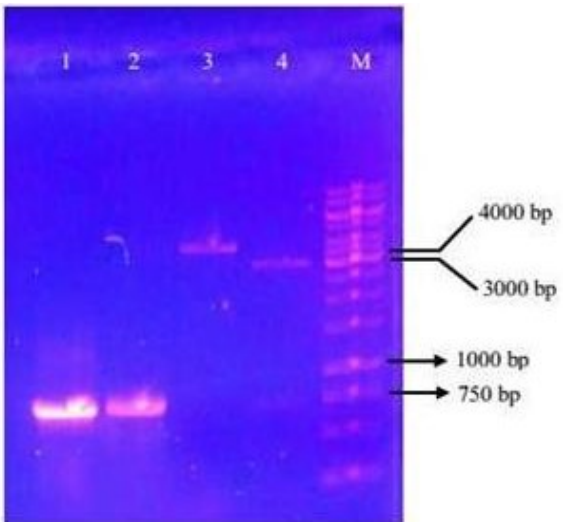

Figure 3. Electropherogram of amplicons obtained through PCR using Fs and Rs primers: 1: amplicon obtained from chromosomal DNA of *P. aeruginosa* ITB; 2: amplicon obtained from pGEM/*paed-d(i)* recombinant plasmid; 3: *Hin*dIII digest of pGEM/*paed-d*; 4: *Hin*dIII and *Eco*RI double digest of pGEM/*paed-d*; M: 1 kb DNA ladder (Promega)

The obtained amplicon was cloned again into the pGEM-T Easy vector and used to transform *E. coli* TOP10 for selection by blue-white screening. The obtained recombinant clone was referred to as pGEM/*paed-d*. Restriction analysis to pGEM/*paed-d* recombinant clones confirmed *Hin*dIII and *Eco*RI sites (Figure 3 lines 3 and 4). Further confirmation was performed by sequencing, which indicated that the *paed-d* sequence in pGEM/*paed-d* (Figure 5) is identical to those in pGEM/*paed-d(i)* (Figure 2).

The *paed-d* fragment from pGEM/*paed-d* clone was then isolated using *Eco*RI and *Hin*dIII double digest and then inserted into linearized pET-30a(+) expression vector, which was further used to transformed *E. coli* BL21 (DE3). The selection was performed on LB medium with kanamycin, which was then screened by plasmid size. As the size of pET-30a(+) was 5,422 bp, the recombinant clones were expected to be 6,112 bp in size. The suspected pET/*paed-d* recombinant clones were isolated from *E. coli* BL21 (DE3) and further confirmed by re-PCR and restriction analysis. Comparison of the amplicon size obtained from (1) chromosomal DNA of *P. aeruginosa* ITB1, (2) pGEM/*paed-d*, and (3) pET/*paed-d* recombinant clones is shown in Figure 4. This analysis indicated that the subcloning was successfully performed, as the amplicon size obtained from pET/*paed-d* is the same as those from chromosomal DNA and pGEM/*paed-d* clones (Figure 4). We then proceeded with DNA sequencing for further confirmation, in which we obtained that the *paed-d* fragment from all our clones (Figure 5) is 100% identical.

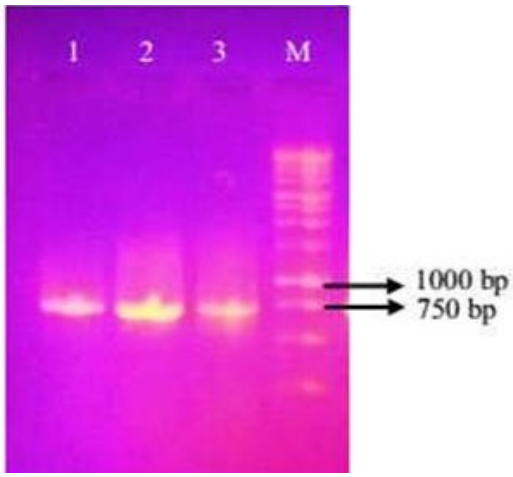

Figure 4. Electropherogram of amplicon from re-PCR of the *paed-d* gene. Line 1: amplicon obtained from chromosomal DNA of *P.aeruginosa* ITB1; Line 2: amplicon obtained from pGEM/*paed-d* recombinant plasmid; Line 3: amplicon obtained from pET/*paed-d* recombinant plasmid; M: 1 kb DNA ladder (Promega)

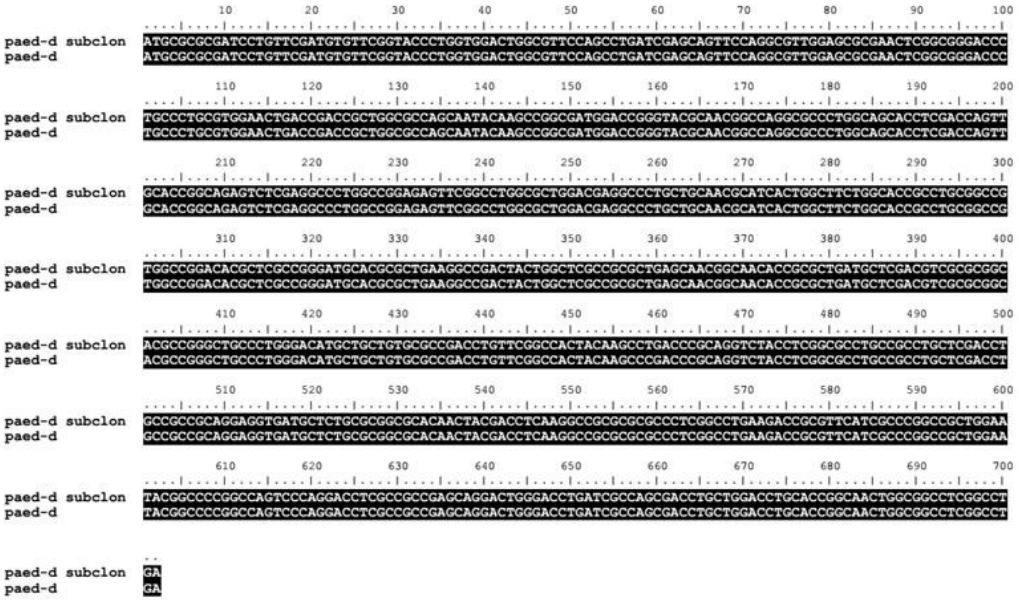

Figure 5. Comparison of a *paed-d* gene sequence in pET/*paed-d* (marked as *paed-d* subclone) and in pGEM/*paed-d* (marked as *paed-d*). The pET/*paed-d* recombinant plasmid was isolated from *E. coli* BL21 (DE3), whereas pGEM/*paed-d* was isolated from *E. coli* TOP10

3.3. Expression Analysis

Expression of the *paed-d* gene in *E. coli* Bl21 (DE3)/pET/*paed-d* recombinant clone was studied by varying the IPTG concentration as the inducer (0.01 and 0.10 mM) and the induction incubation temperature (4°C and 30°C) for 3 hours. Among the variation studied in this experiment, we observed a similar result where a 26-kDa protein was concentrated in the cell pellet (Figure 6). This finding suggests that the protein was predominantly present in the form of inclusion bodies in cell pellets. We attribute the formation of such inclusion bodies to the overexpression occurring in *E. coli*, which might have led to the aggregation of produced proteins [16]. Media composition was also stated as the most significant effect on the expression of soluble or insoluble recombinant protein [17]. To obtain a soluble enzyme, further actions such as solubilization, refolding, fine-tune on induction conditions, and purification needs to be performed. Nevertheless, the high intensity of Coomassie-stained bands at 26 kDa (Figure 6) is a strong indication that the cloned *paed-d* gene was expressed at both temperatures. Based on band intensity, the best expression in LB liquid medium was achieved with a 0.01 mM IPTG induction followed by incubation at 30°C. Our result was in good agreement with the previous report that stated lower IPTG concentrations were advantageous at elevated temperatures, as this would not put such a metabolic burden on the host cells [18].

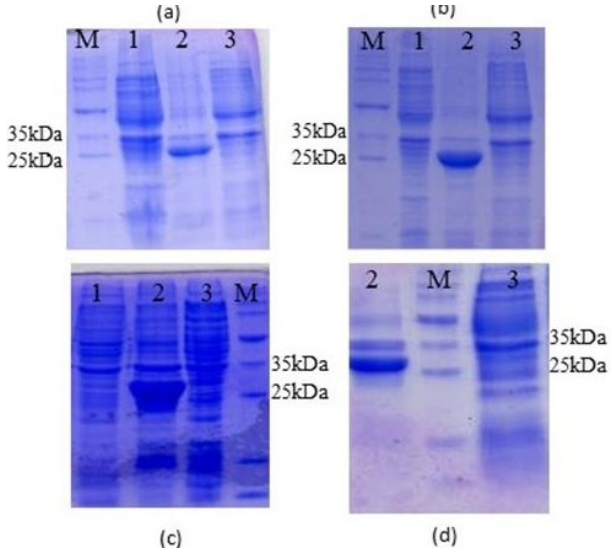

Figure 6. Expression of *paed-d* gene in pET/*paed-d* in *E. coli* BL21 (DE3) with induction of (a) 0.1 mM IPTG at 30°C, (b) 0.01 mM IPTG at 30°C, (c) 0.1 mM IPTG at 4°C, and (d) 0.01 mM IPTG at 4°C, 1: negative control; 2: fraction from cell pellet; 3: fraction from crude lysate; M: Protein molecular weight marker (Promega)

3.4. Bioinformatics Analysis and Tertiary Structure Prediction

ProtParam analysis showed that the *paed-d* gene would be translated to 233 amino acids with a pI of 5.42 and a molecular weight of 26.251 kDa (Figure 7). The latter is in good agreement with gel analyses conducted in this study (Figure 6). The predicted secondary structure of the deduced Paed-d protein using ESPript 3.0 is presented in Figure 7a. The presence of β-sheets in this protein suggests that the enzyme belongs to Group II haloacid dehalogenase [8, 14]. Furthermore, upon inspection, the translated sequence showed a 98% similarity to L-2-haloacid dehalogenase from *P. aeruginosa* (Accession number: 3UMC_A). The pdb file of this enzyme is available in NCBI. Therefore, this protein is further used as a reference to conduct tertiary structure prediction of our deduced enzyme. Using the Swiss model Expacy, we revealed that the tertiary structure of haloacid dehalogenase from a local *P. aeruginosa* ITB1 strain consists of five β-sheets surrounded by α-helixes (Figure 7 and 8a). A previous report [19] has also indicated that haloacid dehalogenase has a signature core domain resembling a Rossmann-fold, where five α-helixes flanked six parallel β-sheets. Notably, more than five α-helixes are surrounding only five β-sheets in our predicted model (Figures 7 and 8a). Nevertheless, the presence of β-sheets in this tertiary structure model (Figure 8a) also confirmed that our enzyme belongs to Group II haloacid dehalogenase (8, 14).

3.5. Prediction of Catalytic Residues

We used Sequence Annotated by Structure (SAS) and EXIA server to predict the catalytic residues of the expressed enzyme. SAS analyses indicated that Asp7, Arg44, Ser121, Asn122, Lys152, His178, and Asp181 were present at the active site and strong candidates for the catalytic site (Figure 8b). Meanwhile, EXIA analyses suggested that Asp7, Ser121, Thr11, Asn122, and Trp15 as essential amino acids in the catalytic site. Taken together, we could predict that Asp7, Ser121, and Asn122 might play essential roles in the catalysis of the expressed enzyme. Our findings are in good agreement with previous reports, such as haloacid dehalogenase from *Pseudomonas* sp. YL listed Asp, Thr, Arg, Ser, Lys, Tyr, and Asn on its active site [14] and haloacid dehalogenase from *S. tokodaii* that identified Asp, Thr, Arg, Lys, Tyr, and Ser as its essential catalytic residues [20]. It is well known that Asp is the key catalytic residue that is conserved in most L-2-haloacid dehalogenase [21, 22, 23] as this residue plays an essential role in nucleophilic attack to the substrate.

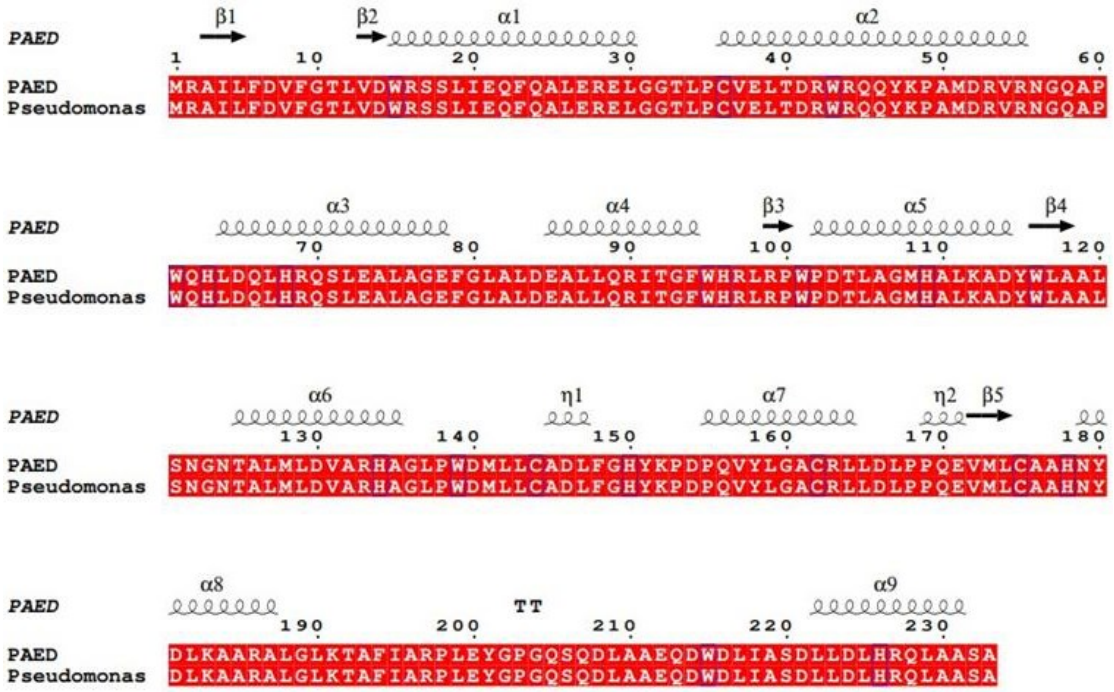

Figure 7. Deduced amino acid sequence and secondary structure of haloacid dehalogenase from a local *P. aeruginosa* ITB1 strain. Coil indicates α-helices, and arrow indicates β-sheets

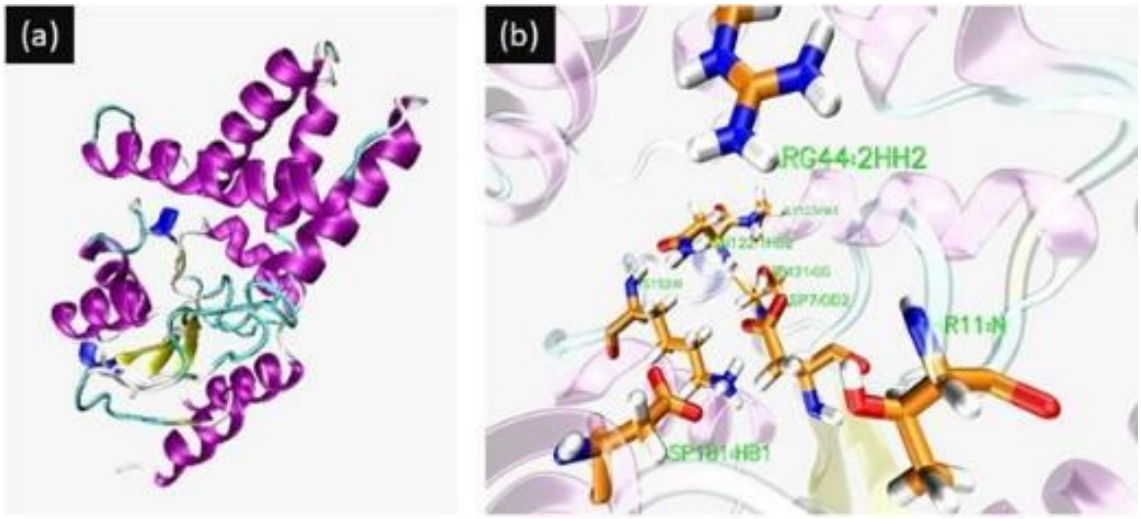

Figure 8. Predicted (a) tertiary structure and (b) catalytic residues of haloacid dehalogenase from a local *P. aeruginosa* ITB1 strain

4. Conclusion

We have demonstrated the construction of a recombinant expression system of the haloacid dehalogenase gene from a local *P. aeruginosa* ITB1 strain using *E. coli* BL21 (DE3) as the host cells. Gel analysis showed that the cloned gene was successfully expressed as a 26-kDa protein, despite being predominantly obtained as inclusion bodies. We concluded that the best gene expression in LB liquid medium was achieved with 0.01 mM IPTG at 30°C of 3 hours of induction incubation upon variation of temperature and inducer concentration. Further studies are required to solubilize the expressed enzyme and examine the catalytic activity. We have also predicted the sequence, pI, and tertiary structure of the expressed enzyme, which indicated a characteristic pattern of β-sheets flanked by α-helixes commonly observed for Group II haloacid dehalogenases. Structural prediction also hinted that Asp7, Ser121, and Asn122 are likely to be the catalytic site of the expressed enzyme. The expression system presented in this study could be employed as a starting point for the recombinant protein production and characterization of haloacid dehalogenase from the *Pseudomonas aeruginosa* local strain.

5. Further studies

Using this expression system, the produced recombinant protein could be further purified by affinity column chromatography utilizing the incorporated His-taq in the protein. Moreover, a more soluble recombinant protein might also be obtained by moving the recombinant plasmid into other possible hosts harboring chaperones plasmids that help correct folding of the produced recombinant protein.

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