Optimizing the Expression of Polyethylene Terephtalate Hydrolase-Encoding Synthetic Gene in Escherichia coli Arctic Express (DE3)

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Optimizing the Expression of Polyethylene Terephthalate Hydrolase-Encoding Synthetic Gene in Escherichia coli Arctic Express (DE3)

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Abstract

The waste of polyethylene terephthalate (PET) plastic waste in Indonesia is a pressing concern due to its slow degradation and potential environmental damage. One promising solution is to utilize polyethylene terephthalate hydrolase from Ideonella sakaiensis (IsPETase), an enzyme that specifically degrades PET. However, inducing the expression of IsPETase synthetic gene in Escherichia coli BL21 (DE3) has been challenging because much of it remains insoluble. This study aimed to express IsPETase in E. coli Arctic Express (DE3) and optimize the conditions to enhance its production. First, pET22b(+)pelB-IsPETase was inserted into E. coli Arctic Express (DE3). The recombinant E. coli Arctic Express (DE3) was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 10 °C. The fraction expressing soluble IsPETase was determined in different culture media, IPTG concentrations, induction times, and sonication durations. Parameters were optimized using a one-factor-at-a-time approach and then evaluated based on esterase specific activity and SDS-PAGE analysis. Results showed that IsPETase can be expressed in extracellular, periplasmic, and cytoplasmic soluble fractions. However, the extracellular fraction should be concentrated. Subsequent optimization focused only on the cytoplasmic fraction under optimal conditions, achieving a threefold increase in PETase specific activity compared with that under uninduced IPTG conditions. The reaction of PETase enzyme with PET and PCL was proven by weight loss, scanning electron microscope (SEM), and Fourier transform infrared spectroscopy (FTIR). Although successful IsPETase expression and production optimization have been achieved, the specific activity remains low, prompting the need for ongoing expression optimization.

Keywords: E. coli Arctic Express, IsPETase, polyethylene terephthalate

Introduction

Polyethylene terephthalate (PET) is a plastic used for industrial purposes, such as products, foods, and beverages packaging [1]. According to the Indonesian Plastic Industry Association and the Central Statistics Agency, Indonesia produces 64 million tons of plastic waste per year [2]. Owing to its slow decomposition and environmentally unfriendly management techniques, the management of PET trash has become a severe issue [1, 3]. A solution to this plastic waste problem is Ideonella sakaiensis polyethylene terephthalate hydrolase (IsPETase). This enzyme degrades PET into mono-hydroxyethyl terephthalate, which is then degraded by mono-2-hydroxyethyl terephthalate hydrolase into terephthalic acid and ethylene glycol [4].

Escherichia coli is a commonly used expression host cell. The problem with gene expression in E. coli is that the lack of post-translational modification proteins can trigger protein misfolding and form inclusion bodies, which are detected in the insoluble fraction [5]. E. coli BL21 (DE3) is commonly used to express PETase. A study constructed recombinant plasmid pET22b(+) pelB-IsPETase containing mutant IsPETase with pelB as a signal peptide to obtain extracellular PETase [6]. However, when this recombinant plasmid was expressed in E. coli BL21 (DE3) host, PETase was predominantly detected in insoluble fractions when PETase gene expression was examined via Western blot analysis, indicating that the results were less than ideal [6]. The utilization of E. coli Arctic Express (DE3) (E. coli AE (DE3) may provide a solution to the issue of insoluble PETase protein [6]. Owing to its ability to be cultivated at temperatures between 10 °C and 13 °C and the
presence of the chaperones Cpn10 and Cpn60 that assist in protein folding at low temperatures and enhance refolding activity by up to 30%. *E. coli* AE (DE3) can be applied to increase the solubility of expressed proteins [7, 8]. A previous study expressed the *IsPETase* gene using the same host cell, *E. coli* AE (DE3), and focused on the effects of salt concentration, pH, and organic solvents on enzyme activity [9]. However, they used different plasmid constructions and few mutations. In the present study, we employed pelB as a signal peptide and mutated *IsPETase* and focused on optimizing PETase expression through the induction of different isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentrations. Previous reports used *E. coli* AE (DE3) and conducted partial optimization of a parameter, such as IPTG concentration [10, 18].

This study aimed to evaluate whether switching the host cells to *E. coli* AE (DE3) can improve PETase gene expression and to identify the optimal parameters that can increase esterase specific activity. The PETase gene expression was analyzed by SDS-PAGE and evaluating the PETase activity of all three fractions: extracellular, periplasmic, and intracellular. Gene expression was assayed quantitatively using esterase specific activity values and qualitatively using SDS-PAGE. The highest gene expression was then subjected to the one-factor-at-a-time (OFAT) method of expression condition optimization, with variables such as various growth media, IPTG concentrations, IPTG induction times, and sonication times for the cytoplasmic fraction [6, 7].

**Materials and Methods**

Transformation of a plasmid containing a gene encoding PETase in *E. coli* Arctic Express (DE3) and the expression. The recombinant plasmid pET22b (+) pelB-*IsPETase* was constructed as previously described [6]. Competent cell of *E. coli* AE (DE3) was prepared using calcium chloride, and the recombinant plasmid was transformed into the competent cell using heat shock method [16] to create recombinant *E. coli* AE (DE3) containing pET22b (+) pelB-*IsPETase*. The recombinant *E. coli* AE (DE3) was grown overnight with a 1% starter until OD₆₀₀ 0.7–0.8, induced with a specific concentration of IPTG, and incubated at 10 °C for a specified duration. These steps were performed to induce PETase gene expression. The distribution of PETase enzyme was observed in extracellular, periplasmic, and intracellular fractions. The intracellular fraction was extracted from the harvested cells using sonication, and the periplasmic fraction was harvested in accordance with previous reports [6, 7, 13, 14, 17]. The fraction exhibiting the highest PETase specific activity (represented by esterase activity) was selected for further optimization of gene expression using the OFAT method with two repetitions. The results were compared with the data of Aji et al. [6]. The data were then processed and presented through bar and line graphs and SDS-PAGE documentation [17].

Optimization of PETase expression in *E. coli* AE (DE3). The fraction exhibiting the highest PETase specific activity was selected to further optimize the gene expression using the OFAT method (Table 1) with two repetitions [23]. Optimization was carried out in stages, starting from the medium (Luria–Bertani (LB) or terrific broth (TB)). The same media not induced with IPTG was used for the negative control [10]. The next step was to study the effect of IPTG concentration (0 as negative control, 0.1, 0.5, and 1.0 mM) [10], IPTG induction time (0, 3, 8, 21, and 24 hours; the prepared samples that were not induced with IPTG were used as the negative control) [10], and sonication duration (5, 10, 15, and 20 minutes; a sample that was not induced with IPTG was prepared as a negative control) [18]. The results were compared with the data of Aji et al. [6]. The qualitative and quantitative data were processed and presented through bar and line graphs.

**Table 1.** Gradual Condition Optimization Design using the One Factor at the Time (OFAT) Method

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Luria Bertani IPTG (Uninduced Condition)</td>
<td>0 mM</td>
<td>0 h</td>
<td>5 minutes</td>
<td></td>
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<tr>
<td>Luria Bertani IPTG (Induced Condition)</td>
<td>0.1 mM</td>
<td>3 h</td>
<td>10 minutes</td>
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<tr>
<td>Luria Bertani IPTG (Uninduced Condition)</td>
<td>0.5 mM</td>
<td>8 h</td>
<td>15 minutes</td>
<td></td>
</tr>
<tr>
<td>Terrific Broth IPTG (Induced Condition)</td>
<td>1.0 mM</td>
<td>21 h</td>
<td>20 minutes</td>
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PETase specific activity represented by esterase was measured by dividing the volumetric activity of PETase (U/mL) by the protein concentration (mg/mL) [24]. Protein concentration was measured using the Bradford assay [25, 26], and PETase volumetric activity was measured using esterase assays. PETase is belong to esterase that can degrade PET. Therefore, activity testing can also be performed using esterase assay with p-nitrophenyl acetate (4-pNP). The assay requires 0.02 M sodium phosphate buffer pH 8, 10 mM 4-pNP dissolved with sodium phosphate buffer, and 10 μL of PETase enzyme sample. The samples were prepared in triplicate and incubated at 37 °C for 5 minutes. Esterase activity was measured using an A280 spectrophotometer [17, 27].

**Determination rate of biodegradation.** Polycaprolactone (PCL) was prepared by dissolving 1.5 g of PCL beads into 60 mL of acetone and heating at 60 °C for 30 minutes. The solution was poured into a Petri dish, and the diluent was removed [28]. Plastic samples were prepared by cutting into a square form with dimensions of 6x 6 mm. PET was prepared by cutting into a circular form of 6 mm diameter, and was sterilized with alcohol and SDS 1%. PCL and PET were soaked in 750 μL of glycine/NaOH, pH 9.0, and 250 μL of PETase enzyme. The reaction was incubated at 37 °C for 7 days. The biodegradation was terminated, and the samples were washed with alcohol and SDS 1%. The samples were subjected to Fourier transform infrared spectroscopic (FTIR) analysis using the services provided by the Laboratory of Advanced Chemistry, BRIN (Serpong, Bandung), and scanning electron microscopy (SEM) analysis using the services provided by the Laboratory of Mineral and Mining, BRIN (Lampung) through E-Layan Sains BRIN, Indonesia. The percentage of weight loss was calculated using the formula (initial weight−final weight)/initial weight [29, 30].

**Results and Discussion**

**Expression of the gene encoding PETase in various fractions of E. coli AE (DE3).** According to SDS-PAGE and esterase specific activity results, the PETase gene expression in E. coli AE (DE3) was observed in all fractions: extracellular (concentrated), periplasmic (concentrated sucrose solution), and soluble cytoplasmic. We examined all the possible fractions to determine whether the signal peptide is responsible for the extracellular expression. Given that we found the extracellular expression in our experiments, the use of PelB signal peptide was effective (Figure 1). For further optimization, only the cytoplasmic (soluble) fraction was observed. The extracellular and periplasmic fractions (sucrose solution) showed high esterase specific activity only when they were concentrated. Therefore, only the cytoplasmic (soluble) fraction was subjected to further optimization using the OFAT method [21, 22, 23].

The PETase enzyme was expressed in the extracellular and periplasmic (sucrose solution) fractions due to the construction of the plasmid pET22b(+) pelB-IsPETase, which was fused with a mutant pelB signal peptide. This fusion allowed PETase to be expressed to a less extent in the periplasmic space and abundantly translocated to the extracellular space [4, 14]. The expression of PET-ase protein in the BL21 (DE3) strain of E. coli was also observed in the extracellular, periplasmic, and cytoplasmic fractions. However, the intensity band of PETase expression in AE (DE3) was thicker than that in BL21, which was aligned with the research goal but was not yet optimal for further scale-up. PETase was expressed in a soluble form in E. coli AE (DE3) because the protein solubility was enhanced by the presence of chaperones Cpn 10 and Cpn 60, which increased the folding activity of recombinant proteins at low temperatures [8, 29].

**Optimization of expression conditions for the recombinant PETase.** The tested experimental conditions included the type of growth media, concentration of IPTG, induction time, and duration of sonication to express the soluble fraction. The use of LB medium resulted in the highest PETase-specific activity (0.15 ± 0.00 U/mg) (Figure 2). Employing 1.0 mM IPTG resulted in the highest PETase-specific activity (0.08 ± 0.01 U/mg) (Figure 3). An 8-hour induction time yielded the highest PETase-specific activity (0.33 ± 0.03 U/mg) (Figure 4 and 5). A sonication time of 10 minutes produced the highest PETase specific activity (0.26 ± 0.00 U/mg) as confirmed by the SDS-PAGE data (Figure 6 and 7). All four optimized parameters were validated, resulting in a threefold increase in PETase specific activity compared with that under non-optimized conditions (Figure 8). The optimal growth medium was LB due to its lower total amino acid content (0.9%) than TB (3.4%) [13]. This result is consistent with the findings of Silaban et al. [10], who expressed prethrombin 2 using E. coli AE (DE3) in LB medium. The reduced amino acid content maintained a decreased cytoplasmic redox environment, leading to slow E. coli growth but increased folding activity for PETase, a protein featuring disulfide bonds in its structure. This process enhanced PETase specific activity [13, 28, 31]. The second optimization result was IPTG concentration at 1.0 mM, which is in line with the study by Jhamb and Sahoo [32], and this concentration increased the expression of the target protein in the cytoplasm (soluble), such as PETase, which contains disulfide bonds [29].

The third optimization indicated that an 8-hour induction time was the most effective. Prolonged induction with high IPTG concentrations could reduce PETase specific activity. This result does not correlate with the study of Silaban et al. [10], who reported the requirement of a long expression time (18-hour induction time) likely due to their use of a low IPTG concentration (0.1 mM) [10]. This effect was attributed to increased bacterial
metabolism resulting from the expression of foreign proteins [33]. The final optimized sonication time was 10 minutes, which is consistent with the findings of Shrestha et al. [18], who also used 10 minutes to achieve high enzyme activity [18]. Too long sonication duration, such as 15–20 minutes, would generate excess heat, potentially degrading the extracted protein [34].

Figure 1. SDS-PAGE Analysis and Specific Activity of PETase Expressed in All Fractions of E. coli AE (DE3); (1) Extracellular (50X Concentrated); (2) Cytoplasmic (Soluble); (3) Cytoplasmic (Insoluble); (4) Periplasmic (25X Concentrated MgSO₄ Solution); (5) Periplasmic (25X Concentrated Sucrose Solution)

Figure 2. SDS PAGE Analysis and Specific Activity of PETase Expressed in the Cytoplasmic Fraction in Different Media and with/without IPTG Induction; (1) Luria Bertani Medium (Uninduced Condition); (2) Luria–Bertani Medium (IPTG Induction); (3) Terrific Broth Medium (Uninduced Condition); (4) Terrific Broth Medium (IPTG Induction)

Figure 3. SDS PAGE Analysis and Specific Activity of PETase Expressed in Soluble Cytoplasmic Fractions with Different IPTG Concentrations: (1) 0; (2) 0.1; (3) 0.5; and (4) 1.0 mM
Figure 4. Specific Activity of PETase on 1 mM IPTG Induction (1) After 0 h IPTG (Uninduced Condition); (2) After 0 h IPTG Induction; (3) After 3 h (Uninduced Condition); (4) After 3 h IPTG Induction; (5) After 8 h IPTG (Uninduced Condition); (6) After 8 h IPTG Induction; (7) After 21 h (Uninduced Condition); (8) After 21 h IPTG Induction; (9) 24 h (Uninduced Condition); (10) After 24 h IPTG Induction

Figure 5. SDS PAGE Analysis of PETase on 1 mM IPTG Induction (1) After 0 h (Uninduced Condition); (2) After 0 h IPTG Induction-1; (3) After 0 h IPTG Induction-2; (4) After 3 h (Uninduced Condition); (5) After 3 h IPTG Induction-1; (6) After 3 h IPTG Induction-2; (7) After 8 h (Uninduced Condition); (8) After 8 h IPTG Induction-1; (9) After 8 h IPTG Induction-2; (10) After 21 h (Uninduced Condition); (11) After 21 h IPTG Induction-1; (12) After 21 h IPTG Induction-2; (13) After 24 h (Uninduced Condition); (14) After 24 h IPTG Induction-1; (15) After 24 h IPTG Induction-2

Figure 6. Specific Activity of PETase Expressed in Soluble Fractions using Different Sonication Time with 1 mM IPTG; (1) 5 Min (Uninduced Condition); (2) 5 Min (IPTG Induction); (3) 10 Min (Uninduced Condition); (4) 10 Min (IPTG Induction); (5) 15 Min (Uninduced Condition); (6) 15 Min (IPTG Induction); (7) 20 Min (Uninduced Condition); (8) 20 Min (IPTG Induction)
Figure 7. SDS PAGE Analysis of PETase Expressed in Soluble Fractions using Different Sonication Time with 1 mM IPTG; (1) 5 Min (Uninduced Condition); (2) 5 Min (IPTG Induction 1); (3) 5 Min (IPTG Induction 2); (4) 10 Min (Uninduced Condition); (5) 10 Min (IPTG Induction 1); (6) 10 Min (IPTG Induction 2); (7) 15 Min (Uninduced Condition); (8) 15 Min (IPTG Induction 1); (9) 15 Min (IPTG Induction 2); (10) 20 Min (Uninduced Condition); (11) 20 Min (IPTG Induction 1); (12) 20 Min (IPTG induction 2)

Figure 8. SDS PAGE Analysis of PETase Expressed in Optimal Condition (Grown in LB Medium, 8 h Induction with 1 mM IPTG, and Extracted by 10 Min Sonication (2); Compared to Negative Control (IPTG Uninduced Sample) (1)

Table 2. Assay Summary of PETase Expressed in Optimal Condition (Grown in LB Medium, 8 h Induction with 1 mM IPTG, and Extracted by 10 Min Sonication (2); Compared to Negative Control (IPTG Uninduced Sample) (1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Protein Concentration (mg/mL)</th>
<th>Protein Total (mg)</th>
<th>Volumetric Enzyme Activity (U/mL)</th>
<th>Specific Enzyme Activity (U/mg)</th>
<th>Total Enzyme Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninduced by IPTG (1)</td>
<td>1</td>
<td>26.71 ± 0.13</td>
<td>20.63</td>
<td>0.16 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.16</td>
</tr>
<tr>
<td>Induced by IPTG (2)</td>
<td>1</td>
<td>23.92 ± 0.11</td>
<td>23.92</td>
<td>1.35 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>1.35</td>
</tr>
</tbody>
</table>

Figure 9. Percentage Weight Loss of PET and PCL after Treatment with PETase Enzyme; (1) PET Dissolved in Buffer Sodium Phosphate as Negative Control; (2) PET Dissolved in PETase Enzyme; (3) PCL Dissolved in Buffer Sodium Phosphate as Negative Control; and (4) PET Dissolved in PETase Enzyme
Figure 10. SEM Analysis of PET and PCL Treated by PETase Extract; (1) PET (Negative Control, Treated with Buffer Na-trium Phosphate Only); (2) PET Treated by Enzyme Extract Showed Many Holes; (3) PCL (Negative Control, Treated with Buffer Natrium Phosphate Only; (4) PCL Treated by PETase Enzyme Showed Large Holes

Figure 11. FTIR Analysis PET (A) and PCL (B) Treated with PETase Enzyme (Black Line); Negative Control PET and PCL Treated with Buffer Natrium Phosphate (Red Line)
The optimal parameters to express PETase in *E. coli* AE (DE3) were as follows: LB medium, 1 mM IPTG, 8-hour induction, and 10 minute sonication. To validate these parameters, we induced PETase gene expression using selected parameters and discovered higher specific activity and higher band intensity in SDS-PAGE compared with the uninduced gene expression (Table 2 and Figure 8).

**Determination Rate of Biodegradation**

**Weight loss method.** The weight loss values for the degraded PET and PCL samples are shown in Figure 9. The PETase-treated PET degraded by 2.58% ± 0.06%, which was three times higher than the untreated sample’s weight loss in 7 days. Meanwhile, the PETase-treated PCL degraded by 57.58% ± 0.08%, which was two times higher than the value for the untreated sample.

**SEM.** SEM analysis revealed the PETase enzyme degradation activity. The surface structure of the PETase-treated PET showed more and larger holes compared with the negative control (Figure 10). Hence, the SEM images confirmed the biodegradation.

PETase degrades PCL faster than PET due to the effect of molecular weight, rigidity, and different flexibility. Although both plastics are semicrystalline, the molecular weight of PET (16,000–20,000 g/mol) is greater than that of PCL (14,000 g/mol). PCL is a semicrystalline linear aliphatic polyester that is ring-opening polymerized by ε-caprolactone [33]. In addition, plastics with aliphatic forms of polyester, such as PCL, are more susceptible to degradation compared with plastics containing aromatic ring chains, such as PET. Aromatic rings maintain rigidity and reduce flexibility [35].

**FTIR spectroscopy.** PET samples treated with or without (negative control) PETase enzyme were analyzed with FTIR (Figure 11). Characteristic peaks (1713, 1372, 1241, 1093, 1042, 872, and 725) were observed and aligned with literature [36]. Compared with the untreated PET (red line), the PETase-treated PET (black line) had higher transmittance in 1042 and 872, which indicated C-O stretching and C-H meta stretching. A new peak detected in 598 showed C-I stretch [37]. PCL samples treated with or without (negative control) PETase enzyme were also analyzed with FTIR (Figure 11). Characteristic peaks (2939, 2863, 1722, 1239, and 1161) were observed and aligned with literature [36]. Compared with the untreated PCL (red line), the PETase-treated PCL (black line) had lower transmittance in 2939 and 2863, which were assigned to C-H asymmetric and hydroxyl stretching. In addition, the PETase-treated PCL (black line) had higher transmittance in 1161, which was assigned to C-O-C symmetric stretching [37], and showed a new peak at 527, which indicated C-I stretch [38, 39].

**Conclusion**

The expression of PETase in *E. coli* Arctic Express (DE3) was observed in the extracellular (concentrated), periplasmic (concentrated), and cytoplasmic (soluble) fractions. The optimal expression of PETase in the cytoplasmic fraction (soluble) was obtained under the conditions of LB medium induced with 1.0 mM IPTG for 8 hours. Cell sonication for 10 minutes resulted in PETase (represented by esterase) specific activity of 0.07 ± 0.00 U/mg and a total enzyme activity of 1.35 U. The optimal parameters led to a threefold increase in PETase specific activity compared with that under uninduced IPTG conditions. The reaction of PETase enzyme with PET and PCL was proven by weight loss, SEM, and FTIR.

**Acknowledgements**

The expression of PETase in *E. coli* Arctic Express (DE3) was observed in the extracellular (concentrated), periplasmic (concentrated), and cytoplasmic (soluble) fractions. The optimal expression of PETase in the cytoplasmic fraction (soluble) was obtained under the conditions of LB medium induced with 1.0 mM IPTG for 8 hours. Cell sonication for 10 minutes resulted in PETase (represented by esterase) specific activity of 0.07 ± 0.00 U/mg and a total enzyme activity of 1.35 U. The optimal parameters led to a threefold increase in PETase specific activity compared with that under uninduced IPTG conditions. The reaction of PETase enzyme with PET and PCL was proven by weight loss, SEM, and FTIR.

**References**


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