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Expression of Mesophilic-Alkali-Stable Catalase Hydroperoxidase II from *Staphylococcus equorum* in *Escherichia coli* BL21(DE3)

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Abstract

Introduction: Catalase is a widely used enzyme with numerous advantages in industrial, diagnostic, and therapeutic applications. This study elucidates the characteristics of recombinant catalase hydroperoxidase II (rHPIISeq) from *Staphylococcus equorum*.

Materials and Methods: A synthetic gene of catalase (*hpII*) was expressed in *Escherichia coli* BL21(DE3). The gene was codon-optimized and cloned commercially into vector pET-15b. Gene expression was performed under 0.5 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction for 24 hours at 25 °C. The soluble recombinant catalase, rHPIISeq, was partially purified using ammonium sulfate precipitation followed by dialysis. Its activity was measured at 440 nm using a UV-visible spectrophotometer. Additionally, the effects of pH and temperature on the enzyme activity and stability were evaluated by incubating the enzyme across various pH levels and temperatures.

Results: The pET-15b_HPIISeq recombinant plasmid was successfully constructed. The optimized gene of *hpII* consisted of 1,989 bp and encoded the rHPIISeq protein with a size of 75.22 kDa. The yield of soluble rHPIISeq was 9.73 mg in 1 L culture with 1.5–1.6 g of wet weight bacterial mass. Notably, approximately 90% of the produced protein formed inclusion bodies (IBs). Following partial purification, a 7-fold increase in the purification of soluble rHPIISeq was achieved using 40% ammonium sulfate precipitation, resulting in a purity level of about 60% with a yield of 93.7%. The enzyme exhibits optimal activity at a pH of 7 and a temperature of 40 °C, and it remains stable within a pH range of 6 to 10 at temperatures between 20 °C and 50 °C.

Conclusions: This recombinant catalase is proposed as a mesophilic-alkali-stable enzyme, which is potentially beneficial for industrial applications, particularly in processes under alkaline conditions and a wide range of temperatures.

Keywords: Mesophilic-Alkali-Stable, Monofunctional Catalase, rHPIISeq, Synthetic Gene

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Introduction

Catalases (EC 1.11.1.6) are peroxidase enzymes produced by both prokaryotic and eukaryotic organisms. These antioxidant enzymes play a crucial role in the cell's defense system against oxidative stress by decomposing hydrogen peroxide (H_2O_2) into water and oxygen molecules.[1,2] Catalases are classified into four types: mono-functional heme catalase, bi-functional peroxidase catalase, non-heme catalase, and minor catalase.[3] This study focuses on monofunctional catalase, which is the largest and most extensively explored cluster of catalases.[4,5]

Monofunctional catalase consists of four subunits, which are classified based on size: small subunits (<60 kDa) and large subunits (>75 kDa). Each subunit is associated with heme *d* and heme *b*, respectively.[6] The larger subunits have been identified with NADP(H) bounds and are significantly more stable towards high temperature and proteolysis.[5,6] The thermal stability data reported that catalase activity slightly increased above 60 °C and that activity began to decrease only above 80 °C with a Tm of 83 °C. Furthermore, larger subunits of catalase will lose activity due to secondary structure changes, but the dimer association will only be dissociated after boiling in a buffer or heating to 65 °C in 5.6 M urea.[7]

Catalases are widely used in industrial applications, including food processing preservation and bleaching in the textile industry.[8] In the pharmaceutical field, catalase is used to remove hydrogen peroxide from contact lens disinfectants. Catalase and superoxide dismutase (SOD) are used in cosmetics as anti-aging ingredients and antioxidants.[9] In wastewater treatment, catalase provides an alternative way of bioremediation that is more eco-friendly than chemical decomposition methods.[10] The widespread

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applications of catalase in various industrial and health fields, alone or combined, lead to the high demand for these enzymes. The global catalase market size was worth around \$387.4 million in 2022 and was predicted to grow to around \$416.6 million by 2030 with a compound annual growth rate (CAGR) of roughly 3.2% between 2023 and 2030.[9] The utilization of catalases in industry requires several characteristics, including stability at high temperatures (above 60 °C) and a wide pH range (above 9).[11] However, most commercial catalases, such as bovine liver catalase, have optimal activity at 20-50 °C and pH 7-10,[12] which makes the enzyme unable to withstand the harsh conditions in some industrial processes.[13] Therefore, it is essential to find an alternative catalase with suitable criteria for industry requirements.

Many studies have focused on exploring marine microbes as a source of novel enzymes with unique properties and diverse applications.[1,14,15] Molecular analysis of monofunctional catalase gene diversity in the Yellow Sea surface water in China was predominantly represented by clade 3 of monofunctional catalase.[14] Several studies have reported that catalases derived from marine bacteria are thermostable, with an optimum temperature of 60 ºC, and classified as alkaline enzymes with an optimal reaction pH of 11,[1,14] making them potential candidates for commercial industrial enzymes. Interestingly, enzymes from marine bacteria exhibit unique properties due to their high catalytic activity under various stressful and fluctuating conditions, such as temperature, pH, and salinity, which are common during several industrial processes.[15] However, research related to enzymes from marine bacteria in Indonesia is still very limited.[16]

In our previous study, the exploration of Indonesian marine diversity resulted in *Staphylococcus equorum* isolates that produce high concentrations of SODs.[17] Since SODs and catalases are categorized in the same cluster of antioxidant enzymes, it is promising to investigate catalase from *S. equorum*, which has not been reported yet. This study aims to provide a catalase profile from *S. equorum* that has the potential to be combined with its SOD as an antioxidant substance in the future for various applications.

In this work, the ORF encoding recombinant HPIISeq (rHPIISeq) was genetically optimized for high-level expression in *E. coli* BL21(DE3). We reported the production of rHPIISeq from S. equorum in *E. coli* BL21(DE3) and elaborated on its characteristics, as well as the optimum temperature and pH stability for the first time, to our knowledge.

Materials and Methods

Bacterial Strains, Plasmids and Cultivation

E. coli BL21(DE3) was used for gene expression. Recombinant plasmids pET-15bHPIISeq were synthesized and cloned by GenScript (USA), based on the results of codon optimization in this study. Bacteria harboring the recombinant plasmid were cultivated in Luria Bertani (LB) medium (1% NaCl, 1% tryptone, 0.5% yeast extract, 1.5% bacto agar) with the addition of 100 µg/ml of ampicillin (Bernofarm) and 0.5 mM of isopropyl β-D-1-thiogalacto pyranoside (IPTG) (Sigma, USA) for induction and characterized by SDS-PAGE.[18]

Codon Optimization and Synthesis of The Gene

The codon usage of the *hpII* gene (GenBank accession no. CP013114.1) from *S. equorum* was analyzed and optimized by replacing the codons predicted to be less frequently used in *E. coli* with the frequently used ones (https://sg.idtdna.com/CodonOpt). The optimized gene (*hpII* gene-opt) was synthesized by GenScript, USA. A translation tool was used to deduce amino acids from *hpII* gene-opt (https://web.expasy.org/translate/). The results were compared with the HPIISeq sequence from BLAST (http://www.ncbi.nlm.nih.gov/blast) and multiple sequence alignment of the catalase amino acid sequences was performed using Clustal Omega.

Vector Construction

The synthetic gene was digested by *NdeI* and *BamHI* and then ligated into pET-15b, forming pET-15bHPIISeq by GenScript, USA. The recombinant plasmids were checked by DNA sequencing at Macrogen (Seoul, Korea) using a flanking primer. The constructed recombinant plasmids pET-15bHPIISeq were transformed into *E. coli* BL21 (DE3) using the heat shock method.[19]

Gene Expression in E. coli BL21(DE3)

Optimization of overproduction was achieved by inducing IPTG at a final concentration range of 0.1 to 1 mM at 25 and 37 °C, 150 rpm for 5 and 24 hours. A single colony of *E. coli* BL21(DE3) harboring pET-15bHPIISeq was inoculated into 10 ml of LB broth and cultivated overnight at 37 °C with shaking at 150 rpm. Then, 500 µl of the overnight culture was then inoculated into fresh LB broth and incubated under the same conditions until the OD_{600} reached 0.7-0.8. Subsequently, a final concentration of 0.5 mM IPTG was added, and the cells were incubated for the next 24 hours at 25 and 37 °C. The cells were harvested by centrifugation (9500 g for 15 minutes at 4 °C). The media was removed, and the cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.0) and lysed by sonication. The lysates were then centrifuged at 9500 g for 15 minutes at 4 °C. The supernatant (SN) and insoluble fraction (inclusion bodies, IB) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel at 90 V for 90 minutes. The IB was directly mixed with a loading buffer for SDS-PAGE analysis.

Partial Purification of rHPIISeq

Partial purification of rHPIISeq was performed using ammonium sulfate (AS) precipitation and dialysis. AS was slowly added to 30 ml of SN (saturation range at 20%, 40%, 60%, and 80%) with stirring for 24 hours at 4 ºC. The precipitate was isolated by centrifugation for 30 minutes at 10,000 g at 4 ºC and dissolved at a 1:5 ratio in 50 mM sodium phosphate buffer (pH 7.0), then dialyzed using a 20 kDa cutoff dialysis membrane (Thermo Fisher Scientific) for 24 hours at 4 ºC. The partially purified rHPIISeq was analyzed by the SDS-PAGE method and compared with the SN fraction. Total protein concentrations were determined using the Bradford method[20] with bovine serum albumin (BSA) as a standard. AS precipitation was also performed on the SN of *E. coli* BL21(DE3) that does not carry the recombinant plasmid as a control.

Catalase Activity Assay

The activity of catalase was measured using a UV-visible spectrophotometer at 440 nm. All assay reagents and enzymes were allowed to reach room temperature before being used. The test tube was prepared by mixing 15 µl of samples with 185 µl of 50 mM phosphate buffer (pH 7.0) and 400 µl of 10 mM hydrogen peroxide as a substrate, then incubated for 2 min at 37 ºC. The enzymatic reaction was stopped by the addition of a working solution (2.03% cobalt (II), 1% Graham salt, and 9% sodium bicarbonate solution) in the dark for 10 min at room temperature (RT). The decomposition of hydrogen peroxide was identified by the decrease in absorbance value at 440 nm. As a standard, 400 µl of 10 mM hydrogen peroxide in 200 µl ultrapure water was used. One unit of catalase was defined as the amount of catalase that decomposes 1 µmol of hydrogen peroxide to oxygen and water per minute at 37 ºC. The rate constant of a first-order reaction (k) equation was used to determine catalase activity using Eq. 1[11]:

(Eq. 1) $k(U) = 2.303*/t \times \log(S°/S)$

where U: unit of catalase, t: time, S°: the absorbance of the standard tube, and S: absorbance of the test tube.

Effect of pH on Catalase Activity and Stability

The effect of pH on the activity and stability of rHPIISeq catalase was investigated using the catalase activity assay. To determine the optimum pH, samples were mixed with buffer solutions at pH 4.0, 7.0, and 10.0. To assess the effect of pH on the stability of rHPIISeq, samples were pretreated with a buffer solution at pH 4.0–10.0 for 1 and 24 hours at room temperature. Residual activities were then determined using the catalase activity assay.

Effect of Temperature on Catalase Activity and Stability

The optimum temperature was determined by incubating the assay mixture (as described in the catalase activity assay) at 20, 37, and 50 °C in a buffer solution with a pH of 7. The effect of temperature on stability was assessed by pre-treating samples at temperatures ranging from 10 to 60 °C for 30-minute and 1-hour intervals. Residual activities were then determined using the catalase activity assay.

Statistical Analysis

All of the experiments to determine catalase activity and stability were carried out three times. Statistical analysis was performed using one-way ANOVA in Minitab® Statistical Software (minitab.com).

Results and Discussion

Codon Optimization and Analysis of Sequence

To optimize the expression of the *hpII* gene in *E. coli* BL21(DE3), 121 out of the original 663 codons were modified. Optimization using the OligoAnalyzerTM Tool (idtdna.com) increased the codon adaptation index (CAI) from 0.650 to 0.813 and the GC content from 37.6% to 51.4%. These conditions met the requirements of the expression system in *E. coli* B, the strain from which *E. coli* BL21(DE3) originates, for CAI (0.7 – 0.9) and GC content (51.06%).[21] The total length of the *hpII* synthetic gene was 1992 bp, encoding a putative rHPIISeq with 663 amino acids (Figure 1). This open reading frame (ORF) was inserted into a pET-15b vector using *NdeI* and *BamHI* restriction enzymes. As a result, rHPIISeq includes the initial methionine, three extra amino acids, a 6x histidine tag for purification, three additional amino acids, a six-amino-acid thrombin cleavage site, one more extra amino acid from the *NdeI* site (originating from the plasmid), followed by the sequence of HPIISeq from N to C-terminus, respectively. Consequently, rHPIISeq contains an additional 20 amino acids apart from the catalase and excluding its signal peptides. The putative rHPIISeq protein experimentally has a molecular weight of 75.22 kDa. Based on its size, the subunit was classified as a clade 2 monofunctional heme catalase large subunit (75 to 84 kDa) with associated heme d.[22,23]

The conserved features of this catalase are similar to *E. coli* catalase HPII, which binds heme and consists of a heme-binding pocket, forming tetramers.[18] However, it is quite different in terms of molecular weight, as catalase HPII of *E. coli* is the largest catalase characterized so far, existing as a homotetramer of 84 kDa subunits.[24] The heme prosthetic group at the catalytic center is located between the internal walls of the beta-barrel and several helices and plays an important role in catalase enzymatic activity.[19,20]

According to BLAST analysis, rHPIISeq showed a high percentage of similarity with other catalases from the *Staphylococcus* genus (>87%), but only shared 38% homology

```
   1 M   S   N   K   K   K   D   Q   L   N   E   V   E   K   N   N   D   D   K   A
   1 atgagcaacaaaaagaaagatcaattaaatgaagtagaaaaaaacaatgacgataaagca
  21 M   T   T   N   N   G   V   K   V   S   E   D   E   N   T   L   T   V   G   E
  61 atgacgactaataatggtgtcaaagtaagtgaagatgaaaacacgttaactgttggtgaa
  41 R   G   P   S   L   L   E   D   F   H   F   R   E   K   I   M   H   F   D   H
 121 cgtggtcctagcttattagaagatttccatttcagagaaaaaatcatgcacttcgatcat
  61 E   R   I   P   E   R   V   V   H   A   R   G   F   G   A   H   G   E   F   Q
 181 gaacgcattccagaacgtgttgtccatgcccgtggctttggtgcacatggcgaatttcaa
  81 V   Y   E   D   L   S   K   Y   T   S   A   D   F   L   T   H   P   E   K   T
 241 gtttatgaagatttatcgaaatatacttcagccgacttttttaactcaccccgagaaaaca
 101 T   P   V   F   V   R   F   S   T   V   Q   G   S   K   G   S   P   D   T   V
 301 acgccagtatttgttagatttccacagttcaaggttctaaaggctctcctgatacagtt
 121 R   D   V   R   G   F   A   T   K   F   Y   T   D   E   G   I   F   D   L   V
 361 agagatgtccgtggctttgcgacaaaatttatacagatgaaggtattttttgacctagta
 141 G   N   D   I   P   V   F   F   I   Q   D   A   I   K   F   P   D   L   I   H
 421 ggtaacgatataccagtatttttcatacaagatgcaatcaaattcccagatttaattcac
 161 A   V   K   P   E   P   H   N   E   M   P   Q   G   G   T   A   H   D   T   F
 481 gcagttaaacctgaaccacataacgaaatgcctcaaggtggtacagcacatgatacattt
 181 W   D   F   F   A   Q   N   P   E   S   T   H   T   A   V   W   A   M   S   D
 541 tgggatttctttgctcaaaacccagaatctacacataccgctgtatgggctatgagtgat
 201 R   G   I   P   K   D   F   R   Q   V   E   G   F   G   V   H   T   F   R   L
 601 cgcggtatacctaaagatttagacaagtcgaaggtttcggtgtgcatacattccgcctt
 221 V   N   N   E   G   Q   S   Y   F   V   K   F   H   W   K   P   L   Q   G   L
 661 gttaataacgagggacagtcttattttgttaaattccattggaaaccacttcaagggcta
 241 E   S   L   V   W   D   E   A   Q   M   L   H   G   K   D   V   D   F   H   R
 721 gagtctctcgtatgggatgaagcacaaatgctacatggtaaagacgtcgactttcatcgt
 261 K   D   L   Y   E   S   I   E   K   G   D   Y   P   E   W   E   L   G   L   Q
 781 aaagatctttatgaatctatcgaaaaaggcgattatcctgagtgggaattaggcctacaa
 281 I   I   R   P   E   Q   E   F   D   F   D   F   D   I   L   D   P   T   K   I
 841 attattcgtcctgaacaagaatttgactttgattttgatattttagaccctacgaagatt
 301 W   P   E   D   D   V   P   V   Q   R   V   G   K   M   T   L   N   Q   N   V
 901 tggcctgaagatgatgttccagtacaaagagttggtaaaatgacactaaatcaaaatgtt
 321 T   N   V   F   D   E   T   E   Q   A   A   F   H   P   G   H   I   V   P   G
 961 acgaatgtgtttgatgaaacagagcaagccgcattccatccaggacatattgtaccaggt
 341 I   D   F   S   N   D   P   L   L   Q   G   R   L   F   S   Y   T   D   T   Q
1021 atcgacttttctaatgatccattattacaaggacgtttattctcctatacagatacacaa
 361 I   T   R   L   G   G   P   N   F   N   Q   I   P   I   N   R   P   V   N   E
1081 atcacgagattgggcggtccaaacttcaatcaaattccaattaatcgtcctgtaaatgaa
 381 V   H   N   N   Q   R   E   G   M   H   Q   T   S   V   N   K   G   Q   V   A
1141 gtacacaacaaccaaagagaaggcatgcatcaaacaagtgtaaacaagggacaagttgcg
 401 Y   H   K   N   A   L   N   N   N   D   P   H   T   T   P   K   E   E   G   G
1201 tatcataaaaatgcattgaataataatgatcctcatacaacaccgaaagaagaaggtggt
 421 Y   E   H   Y   Q   E   K   V   E   G   R   K   I   Q   K   R   S   E   S   F
1261 tatgagcactaccaagaaaaagttgaaggccgcaagattcaaaaacgcagtgaaagtttc
 441 K   D   Y   Y   S   Q   A   K   L   Y   L   N   S   L   T   Q   P   E   F   D
1321 aaagattattatagccaagccaaactttatttaaacagtttgactcagcctgaatttgat
 461 H   T   V   D   G   F   S   F   E   I   G   M   C   K   S   V   M   V   K   Q
1381 catacagtagatggcttttcatttgaaatcggtatgtgtaaatcggttatggttaaacaa
 481 N   A   V   N   Q   L   N   K   V   D   R   T   L   A   E   R   V   A   K   N
1441 aatgctgttaatcaattgaacaaagttgaccgtacattagctgaacgtgtagctaaaaat
 501 V   G   V   E   V   P   A   E   N   E   E   V   Q   S   D   A   K   D   S   K
1501 gtaggtgtggaagtacccgcagaaaatgaagaagtccaatcagatgcaaaagatagtaaa
 521 L   T   M   E   K   F   D   I   P   L   A   G   H   S   V   A   V   A   V   N
1561 ctgacaatggaaaaattcgatatcccattagctggacattctgtagctgttgcagtaaat
 541 G   D   I   S   A   D   T   L   K   S   Y   A   K   T   F   T   E   N   K   L
1621 ggcgatattagtgctgacacgctaaaatcatatgctaaaacgttcactgaaaacaaattg
 561 N   Y   A   F   V   G   Q   H   P   K   D   I   S   E   D   Y   G   I   T   E
1681 aactacgcatttgtgggacaacatcctaaagatatctcagaagactacggtatcactgaa
 581 T   F   D   T   A   H   P   T   L   F   D   S   L   I   V   L   S   D   G   S
1741 acatttgatactgctcatccaactttatttgatagcttaattgtactttcagatggtagc
 601 D   I   L   P   P   V   E   E   F   A   E   L   I   Y   K   H   N   K   P   L
1801 gatatactcccacctgtcgaggagtttgcagaactcatttataaacacaataaaccatta
 621 I   V   N   Q   T   A   A   T   N   L   A   D   A   K   L   N   L   A   A   P
1861 atcgttaaccaaacagctgcgacaaatttagcagatgccaaattgaatctagcagcacct
 641 G   V   F   V   S   D   D   P   N   T   I   V   Q   A   F   D   R   A   R   Y
1921 ggtgttttcgtatcggatgaccctaatacaattgtgcaagcttttgatagagcaagatac
 661 W   D   R   *
1981 tgggacagataa
```

Figure 1. Deduced Amino Acid Sequences of rHPIISEq. The nucleotide base sequences are shown in small letters with start codons (atg) and stop codons (taa) in bold. Bold capital letters show the highly conserved residues of the heme-binding pocket domain in all heme-catalases.

with the catalase of *Staphylococcus warneri* (Figure 2A). The native catalase of *S. warneri* belongs to the KatA monofunctional group, which is the only catalase from Staphylococcus that had been previously studied.[26] HPII monofunctional enzymes that have been elaborated are from *E. coli* K-12 and *Gluconobacter oxydans*, and HPIISeq shared only 54% and 45% homology with them, respectively.

Multiple sequence alignment analysis showed a heme-binding pocket active site in the form of seven amino acids: His69, Ser108, Asn142, Phe147, Phe155, Arg352, and Tyr356 (Figure 2B), which are conserved in all heme catalases and play an important role in the chemical binding site.[27] play an

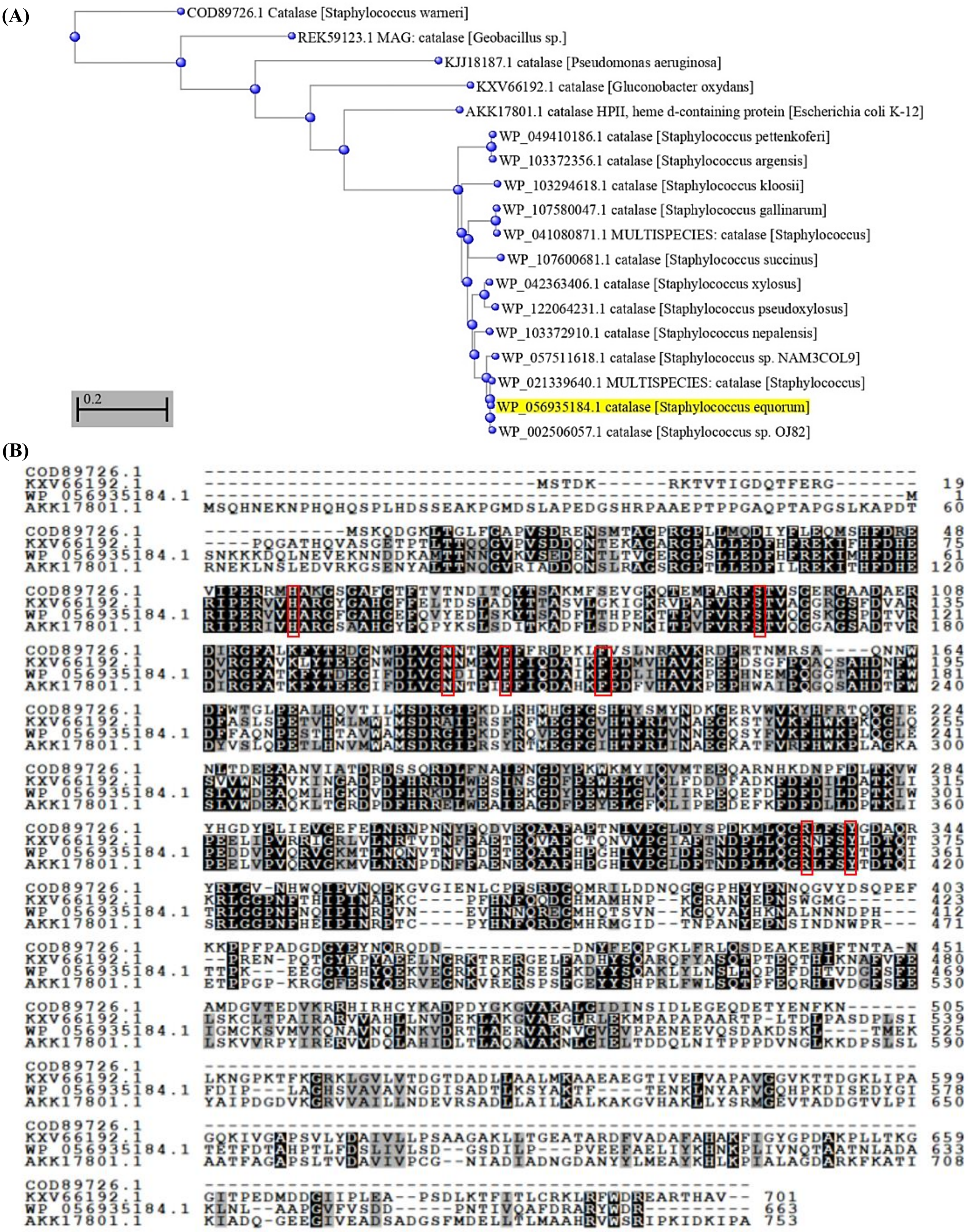


Figure 2. Multiple Sequence Alignment and Phylogenetic Tree of the Amino Acid Sequences of rHPIISeq with other Monofunctional Bacterial Catalases from the NCBI Database. (A) Phylogenetic tree of rHPIISeq showing the relationship with other strains using BLAST pairwise alignment with the Neighborhood-Joining method. The most related strains with more than 99% similarity were chosen from the NCBI database to construct the phylogenetic tree. rHPIISeq in this study is shaded in yellow, and the studied catalases are marked by an asterisk. (B) Multiple sequence alignment of rHPIISeq with another studied catalase. The sequences are from *S. warneri* (COD89726.1), *G. oxydans* (KXV66192.1), *S. equorum* (this study, WP_056935184.1), and *E. coli* K-12 (AKK17801.1). Conserved residues are shaded in gray. The red rectangle shows the highly conserved residue of the heme-binding pocket domain in all heme-catalases.

important role in the chemical binding site.[27] Domain analysis results showed that rHPIISEq contained three catalase-typical domains and conserved residues: Kat E domain (Ser2-Arg663), heme-binding domain (Asp59-Val501), and GAT_1 domain (Gly532-Phe655).[16] The position of the heme group in all catalases is buried deep inside the core homotetramer structure,[16,21] and the closest distance is about 20 Å from the surface.[28,29] Three residues are believed to be essential for catalysis: tyrosine on the proximal side of the heme (Tyr415), histidine, and asparagine on the distal side (His128 and Asn201).[23]

Different from the HPII catalase of *E. coli*, Tyr415 in the structure of rHPIISEq was replaced by Pro415. This amino acid has exceptional rigidity and is commonly found in the formation of beta turns in protein secondary structure.[31] The histidine and asparagine residues on the distal side of the heme create a strongly hydrophobic environment.[23]

The sequence of the C-terminus domain of rHPIISeq is also similar to those of proteins belonging to the type-I glutamine amidotransferase superfamily.[32] However, as with HPII catalase from *E. coli*, the catalytic triad (Cys-His-Asp/Glu) essential for glutamine amidotransferase activity is not conserved in the rHPIISeq sequence, similar to catalase from *G. oxydans*.[25] Therefore, the presence of the GAT_1 domain at the C-terminal of the deduced amino acid sequence suggests that the addition of a 6xHis-tag at the N-terminal in this study will not interfere with the active site of rHPIISeq.

Vector Construction

The *hpII* gene was inserted upstream of the T7 promoter, RBS, and polyhistidine coding sequence and constructed at the multiple cloning sites (MCS) of the pET-15b vector using *NdeI* and *BamHI* restriction sites (Figure 3A). Migration analysis on a 1% agarose gel electrophoresis showed that pET-15b_HPIISeq (7692 bp) had two conformations and migrated slower than pRTase (7000 bp) as a control, due to its larger size (Figure 3B, Lanes 1 & 2). Double digestion analysis using *NdeI* and *BamHI* restriction enzymes produced two DNA bands approximately at 6000 and 2000 bp (Figure 3B, Lanes 3 & 4). These results matched the expected backbone and inserted DNA sizes theoretically, at 5708 and 1989 bp, respectively. Recombinant plasmid characterization revealed that the *hpII* gene was cloned appropriately into the pET-15b expression vector without any mutations. Consistent with the migration and restriction results, sequencing analysis of the recombinant plasmid (data not shown) confirmed that the *hpII* gene encoding rHPIISEq had successfully transformed into *E. coli* BL21(DE3) cells.

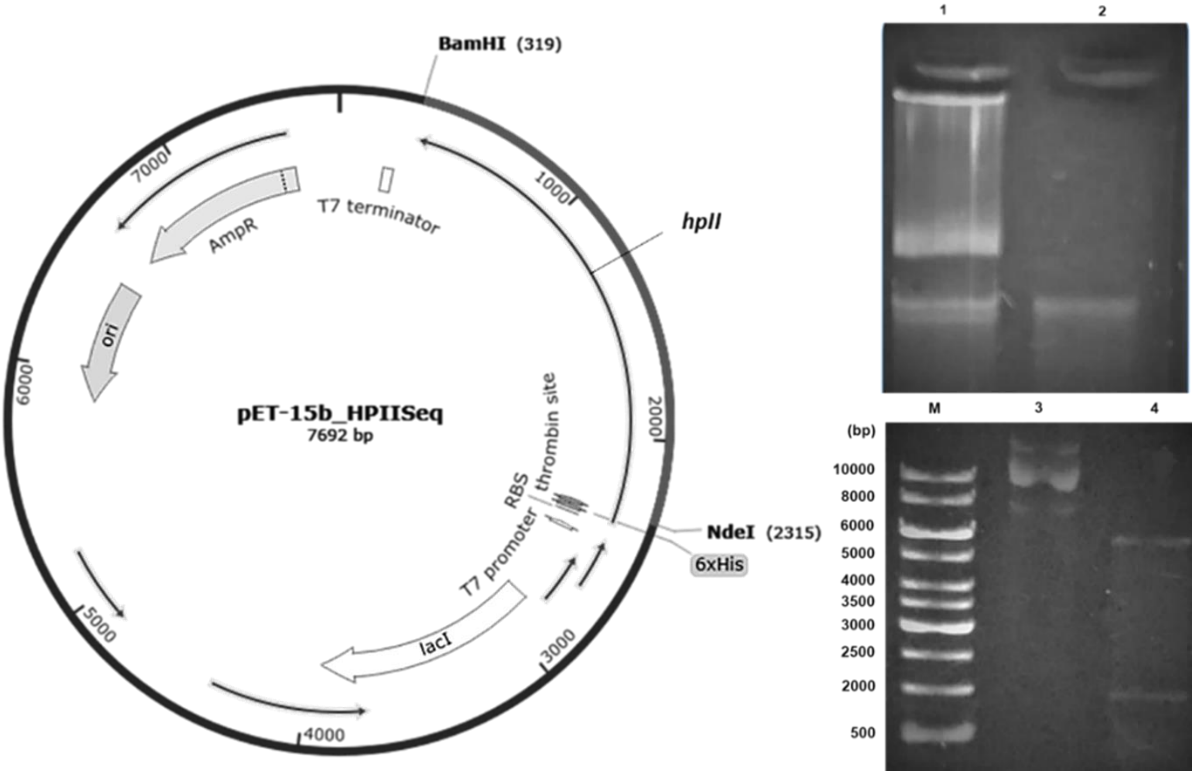


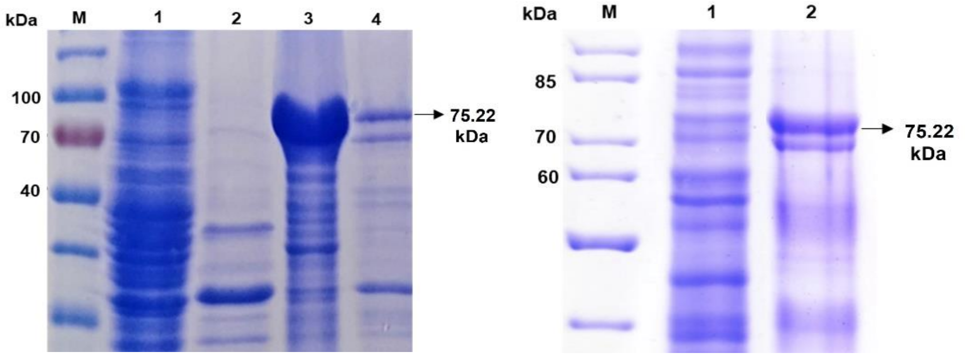
Figure 3. Recombinant Plasmid Characterizations. (A). Construction of recombinant plasmid pET-15b_HPIISeq. The *hpII* gene encoding rHPIISeq catalase was inserted into an expression vector with an upstream T7 promoter, RBS, and 6×His tag (Illustrated by SnapGene). (B). Agarose gel electrophoresis analysis of pET-15b_HPIISeq. Lane 1: pET-15b_HPIISeq (7692 bp); Lane 2: pRTase as plasmid standard (7000 bp); Lane M: DNA ladder 1 kb; Lane 3: Circular plasmid pET-15bHPIISeq; Lane 4: Double digestion of plasmid pET-15bHPIISeq (*NdeI*/*BamHI*).

Expression and Partial Purification of rHPIISeq in E. coli BL21 (DE3)

The optimal condition for soluble HPIISeq overproduction was achieved by inducing 0.5 mM IPTG at 25 °C for 24 hours (Figure 4A). SDS-PAGE analysis revealed a protein band with a molecular weight of 75.22 kDa, which is close to the theoretical size of rHPIISeq (77.5 kDa) (Figure 4A). Based on protein band intensity measurements using ImageJ software,[33] rHPIISeq was expressed with a low soluble fraction, accounting for only about 2% of the total protein, and was predominantly insoluble as inclusion bodies (IBs), making up around 90% of the total *E. coli* BL21(DE3) protein (Figure 4A, Lanes 3 & 4). The soluble fraction and IBs were approximately 0.67% and more than 90%, respectively, when expressed at 37 °C (data not shown). Several recombinant bacterial catalases that were overexpressed in *E. coli* have been reported to be dominated by IB formation.[26,27] The formation of aggregates as IBs suggests a probable mismatch between the rate of synthesis of the recombinant protein and the molecular capacity of the cells to fold the newly synthesized protein into its native state. The formation of IBs mainly depends on the kinetic competition between protein-specific folding and aggregation rates associated with the synthesis rate.[34]

It was reported that low temperatures for protein induction improve the solubility of proteins.[35] By lowering the temperature to 18-25 ºC, the translation rate can decrease, providing sufficient time for proteins to fold correctly and avoid the formation of inclusion bodies (IBs).[28,29] The expression rate and correct folding of heterologous proteins are determined by the level of gene induction, codon bias, and mRNA stability.[36] Furthermore, soluble proteins are mostly produced at a low specific synthesis rate and require a long cultivation time after induction, typically ranging from 6 to 24 hours.[31,32] In our study, lowering the cultivation temperature did not significantly decrease the formation of IBs. The amount of IBs at 18 ºC showed a similar thickness of protein band compared to 25 and 37 ºC (data not shown).

rHPIISeq was partially purified from a crude cell extract by performing AS precipitation and dialysis. This partially purified enzyme was analyzed by SDS-PAGE, exhibiting a molecular mass of 75.22 kDa (Figure 4B, Lane 2). The highest activity fraction was obtained from the precipitation at 40% saturation of AS and was then subjected to a dialysis process to remove AS, resulting in a purification fold of 15 and a total protein concentration of 515 µg/ml. The amount of rHPIISeq in this fraction is approximately 41% of the total protein, which is 211 µg/ml. As a control, SN protein from *E. coli* BL21(DE3) was also precipitated with AS under the same conditions. The total protein concentration for dialyzed 40% AS saturation of this *E. coli* strain was 450 µg/ml.



(A)

(B)

Figure 4. SDS-PAGE Analysis of Soluble and Insoluble Fractions of rHPIISeq in *E. coli* BL21(DE3). (A) Production with and without IPTG induction at 25 °C for 24 h. Lane M: Protein marker (PageRuler™ Prestained protein ladder); Lane 1 & 2: Insoluble and soluble protein (without IPTG); Lane 3 & 4: Insoluble and soluble protein (with 0.5 mM IPTG). (B) Fractions obtained by partial purification of rHPIISeq using ammonium sulfate precipitation at 40%. Lane M: Protein marker (PageRuler™ unstained protein ladder). Lane 1: Crude cell extract; Lane 2: Partially purified rHPIISeq. The arrow marks rHPIISeq with a molecular weight of approximately 75.22 kDa.

Catalase Activity Assay

Catalase activity was measured spectrophotometrically at 440 nm, based on the rapid formation of a stable and colored carbonato-cobaltate (III) complex.[33] Catalase activity is directly proportional to the rate of dissociation of hydrogen peroxide, which oxidizes cobalt (II) to cobalt (III) in the presence of bicarbonate ions. This process results in the production of a carbonato-cobaltate (III) complex ($[Co(CO_3)_3]Co$), identified by its intense olive green color. Various sample volumes were used to assess catalase activity (Figure 5A), with larger sample volumes showing a decrease in color intensity, indicating higher catalase activity. These results are consistent with previous findings.[11]

As shown in Figure 5B, positive catalase activity was confirmed by the slide catalase test,[34] as evidenced by the rapid formation of oxygen bubbles produced by hydrogen

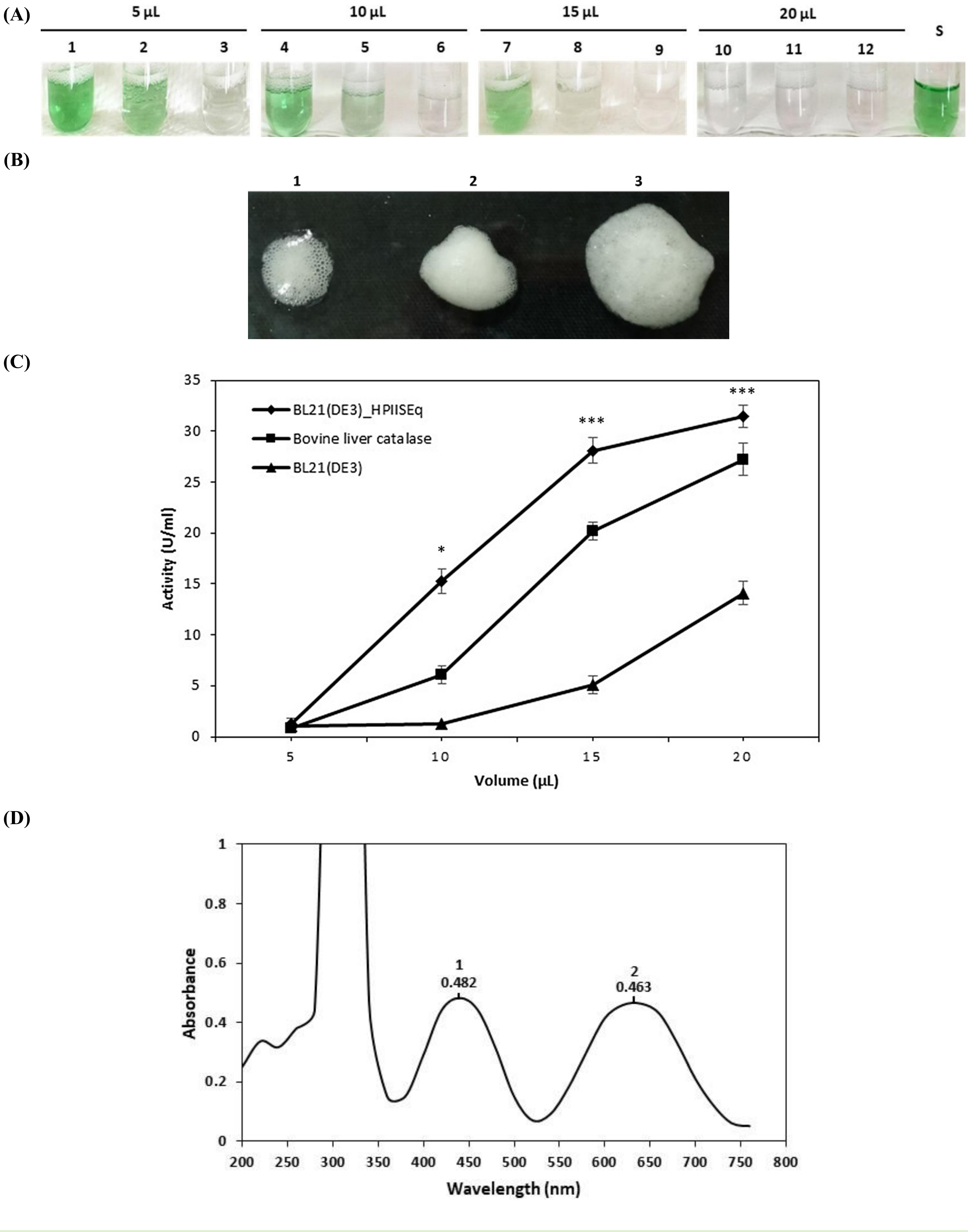


Figure 5. The Spectrophotometric Characteristic of the Carbonato-Cobaltate (III) Complex Correlates with the Activity of the Catalase Enzyme. (A) An increase in catalase activity is associated with a decrease in color intensity. Lanes 1, 4, 7, and 10 show partially purified crude extract of *E. coli* BL21(DE3) harboring pET-15b; Lanes 2, 5, 8, and 11 show bovine liver catalase; Lanes 3, 6, 9, and 12 show partially purified crude extract of *E. coli* BL21(DE3) harboring pET-15b_HPIISeq; Lane S shows hydrogen peroxide as a standard. (B) Bubbles develop following the reaction of catalase with hydrogen peroxide. Lane 1 shows partially purified crude extract of *E. coli* BL21(DE3) harboring pET-15b; Lane 2 shows monofunctional bovine liver catalase 1.4 x 10^6 Units, Lane 3 shows partially purified crude extract of *E. coli* BL21(DE3) harboring pET-15b_HPIISeq. (C) Catalase activity measured by the carbonato-cobaltate (III) complex method. N = 3, *$p < 0.05$, ***$p < 0.001$. (D) UV-Vis spectra of rHPIISeq show two maximum absorbance peaks at 440 nm and 640 nm.

peroxide decomposition.[37,38] Consistent with the spectrophotometry assay results using the carbonato-cobaltate (III) method, the activity of rHPIISeq was significantly higher than the control (dialyzed 40% AS saturation of *E. coli* BL21(DE3) without recombinant plasmid and bovine liver catalase) (Figure 5B, Lanes 1 & 2) ($p < 0.05$). An equal volume of samples was used in the assay with concentrations of 450 µg/ml total protein for *E. coli* BL21_DE3 and 515 µg/ml total protein for *E. coli* BL21_DE3_HPIISeq. The unit activity of 20 ul rHPIISeq (equal to 4.22 µg) was calculated as 1.56×10^6 U/ml, based on the known activity of bovine liver catalase (1.36×10^6 U/ml) (EC 1.11.1.6) (Figure 5C). This enzyme was used as a standard for monofunctional catalase, and until recently, there has been no standard catalase derived from bacteria commercially available. The UV–Vis spectrum of purified rHPIISeq is shown in Figure 5D. The spectrum of the colored end product [carbonato-cobaltate (III) complex ($[Co(CO_3)_3]Co$)] was scanned from 200 to 700 nm and showed peaks at 440 and 640 nm. The 440-nm absorbance was used for measuring catalase activity.[11]

Effect of pH on rHPIISeq Activity and Stability

As much as 20 µl of partially purified rHPIISeq (equal to 4.22 µg) was assayed at 37 ºC in 50 mM phosphate buffer with various pH values to investigate its activity and stability. The enzyme demonstrated catalase activity in a pH range of 4-10, with the optimum reaction observed at pH 7 with an activity of 5 U/ml (Figure 6A). The high activity was found over a wide range of pH (7–10); however, the activity rapidly decreased below pH 7, with only a little activity remaining at pH 4 ($p < 0.05$). These characteristics were similar to the catalase of *Pseudomonas aeruginosa*[39]

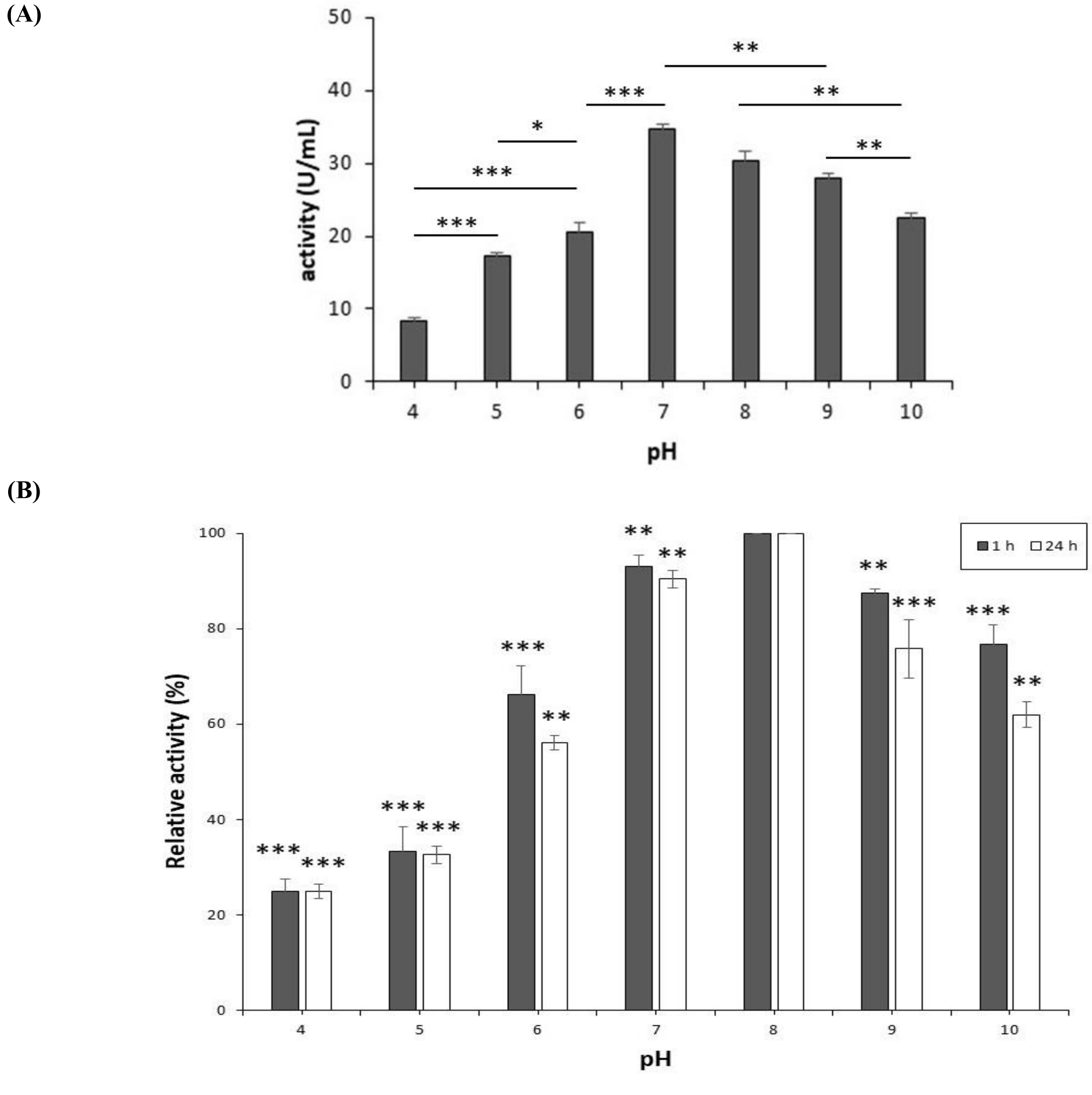


Figure 6. Effect of pH on rHPIISeq Activity. (A) Optimum activity of the enzyme (under standard assay conditions at 37 °C and different pH values). (B) pH stability of the enzyme at pH 4-10 for 1 and 24 h. Relative activity was compared with the highest activity at pH 8. N = 3, **$p < 0.01$, ***$p < 0.001$.

and *Serratia marcescens* SYBC08,[37] which had optimum activity between pH 7 and 9, and *Rhodospirillum rubrum* S1 at pH 7 and 8.[37] Some bacteria have higher pH activity of catalase, such as *S. marcescens* FZSF01, which exhibits maximum activity between pH 10 and 11. This saprophytic bacterium was isolated from the rice field soil of an industrial farm in Fuzhou, China.[37]

Below pH 7, acid denaturation of recombinant catalase might take place. As the pH drops into the acidic range, the enzyme tends to gain hydrogen ions from the solution. This condition will change the ionization state of amino acids at the enzyme's active sites, which impacts the enzyme's overall 3-D structure and could result in reduced substrate binding. At extreme pH levels, the protein might become so altered that it loses its functionality and can no longer recognize the substrate.[12,39]

The optimum pH of catalase is also influenced by the composition of its constituent amino acids, which are classified based on their side chains, whether they have an even distribution of electrons (nonpolar) or an uneven distribution of electrons (polar). Polar basic amino acids contain an amine group (which may be neutral or charged) in the side chain (R group), while polar acidic amino acids contain a carboxylic acid (or carboxylate) group.[43] The composition of amino acids will also represent the pI value of a protein. The calculated pI value of *S. marcescens* FZSF01's catalase (GenBank Acc. Nr KU350659) using https://web.expasy.org/compute_pi/ was higher (6.15) than rHPPIISeq's (5.28). This could explain why the catalase from *S. marcescens* FZSF01 has a higher pH optimum than our catalase.

The effect of pH on rHPIISeq showed that the enzyme was stable at neutral and basic conditions (Figure 6B). After 1 hour of incubation, about 90% of catalase activity was retained between pH 7-9, and 80% at pH 10, indicating that rHPIISeq is a characteristically alkaline enzyme. Relative activity was higher than 90% with the highest activity at pH 7 to 8, which remained constant after 24 hours and slightly decreased at pH 9-10, respectively. At pH 6, 5, and 4, this enzyme still retained more than 60%, 30%, and 25% activity, respectively. This data was similar to monofunctional catalase from the marine bacterium *Acinetobacter* sp. YS0810, which had stability at pH 5-12[1] and *Oceanobacillus oncorhynchi* subsp. *Incaldaniensis*, which was stable in the pH range of 5 to 10 at 40 ºC for 1 hour. Alkaline catalases are usually suitable for textile and paper processing industries as these processes are performed under alkaline conditions.[40]

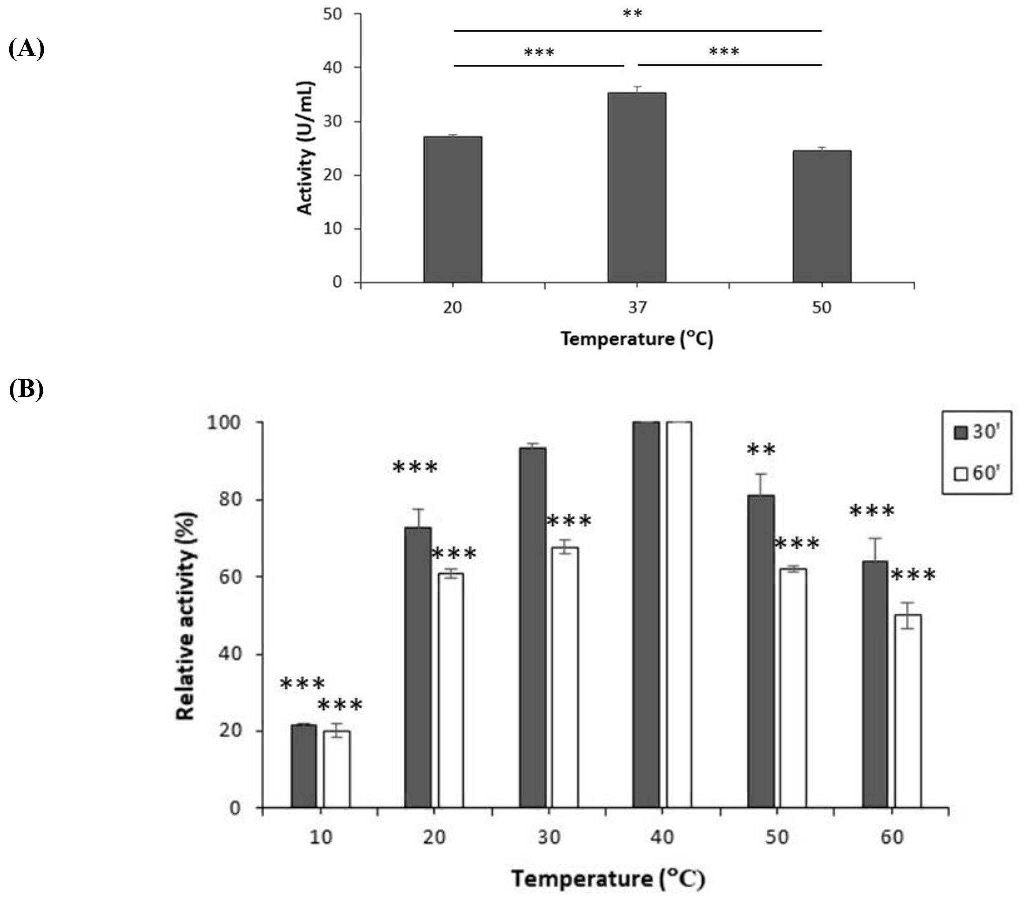


Figure 7. Effect of Temperature on rHPIISeq Activity. (A) Optimum activity of the enzyme (under standard assay conditions at different temperatures). (B) Temperature stability of the enzyme. Relative activity of the recombinant enzyme by incubation at 10-60 °C for 30 and 60 min. N = 3, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effect of Temperature on rHPIISeq Activity and Stability

To determine the effect of temperature on rHPIISeq activity, the assay was performed at pH 7 at various temperatures: 20, 37, and 50 °C. Optimum activity was observed at 37 °C (Figure 7A). The thermostability of rHPIISeq activity was assessed over the temperature range of 50–80 °C and is shown in Figure 7B. The enzyme remained stable at temperatures between 20-60 °C after a 30-minute treatment ($p < 0.05$). Overall, the relative activity decreased by approximately 20% after 60 minutes of incubation, except at 60 °C where the activity retained half of its maximum activity. This recombinant enzyme showed no significant activity at low temperatures (20 °C), with only about 20% remaining after 30 and 60 minutes, classifying rHPIISeq as a mesophilic enzyme ($p < 0.05$).

The thermostability of rHPIISeq is superior to that of *P. aeruginosa*, which displayed an 83% reduction in activity after being treated at 55 °C for 1 hour and completely lost activity after being incubated for 1 hour at 60 °C. This stability is presumed to be related to the structure of rHPIISeq as a large subunit catalase, as larger subunits of catalase are known to have good stability against high temperatures and proteolysis. This comprehensive evaluation highlights the potential utility of rHPIISeq in various industrial and biotechnological processes, particularly in high-temperature processes under alkaline conditions.

Conclusion

In this study, the gene for catalase hydroperoxidase II (hpII) from *S. equorum* was successfully expressed in *E. coli* BL21(DE3) with induction of 0.5 mM IPTG at 25 ºC for 24 hours. The recombinant HPIISeq has an experimental molecular weight of 75.22 kDa. Partially purified enzyme maintained stability and exhibited high activity over a wide temperature range of 20 to 60 ºC and a pH range of 6 to 10. These enzyme characteristics are consistent with the native alkali environment of *S. equorum*, which was isolated from the sea of Indonesia and identified as a mesophilic-alkali-stable enzyme. The stability of rHPIISeq at a wide range of pH and reaction temperatures makes it suitable for industrial applications, particularly in textile, paper production, and other industries.

Authors' Contributions

RZ: Concept, design, resources, materials, data collection and/or processing, data analysis and/or interpretation, literature search, writing; CR: Concept, design, supervision, data analysis and/or interpretation, literature search, writing, critical reviews; RAU: Concept, supervision, literature search, writing, critical reviews; DM: Supervision, literature search, writing, critical reviews.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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