Expression induction of *Serratia plymuthica* UBCF_13 metalloprotease gene with various types of metal ions

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**Abstract.** Bacteria as biocontrol agents can produce hydrolytic enzymes, one of them is a protease which plays an important role to degrade cell membranes or plasmalemma in fungi composed of protein. Metalloproteases are proteases that contain one or two metal ions on their active sites. The role of metal ions in metalloproteases is to activate water molecules, which act as nucleophiles in the catalysis process. The metalloprotease function of *Serratia plymuthica* UBCF_13 is unknown, therefore this study aimed to determine the effective metal ion to induce the expression of the UBCF_13 metalloprotease gene. The metalloprotease gene from *Serratia plymuthica* UBCF_13 was ligated into a pGEM T-easy plasmid. The recombinant plasmid was transformed into *Escherichia coli* BL21 by the heat shock method. The metalloprotease gene expression was induced by IPTG and a combination of several metal ions namely Zn²⁺, Mn²⁺, Fe²⁺, and Ca²⁺. The highest gene expression was characterized by the thickest protein band based on the result of SDS-PAGE visualization with a molecular weight of 27 kDa after being induced by Fe²⁺ metal ions. Proteolytic activity of metalloprotease UBCF_13 was tested using solid LB media containing 2% skim milk. The longest clear zone diameter was obtained up to 0.83 cm after being induced by IPTG combined with Ca²⁺ metal ion. These results can be used as a reference for the expression of the UBCF_13 metalloprotease gene. However, further enzyme purification is to be able the enzyme as an antifungal compound.

**Keywords:** clear zone, *Escherichia coli* BL21, gene expression, *Serratia plymuthica* UBCF_13
1. Introduction
Bacteria as biocontrol agents can produce hydrolytic enzymes which function as inhibitors of plant pathogenic fungi growth through the mechanism of antibiosis [3]. Protease is one of the effective hydrolytic enzymes to be used as bio-fungicides for controlling plant diseases. Protease enzymes have the function to degrade cell membranes in fungi that are composed of protein molecules [8]. Metalloproteases are proteases that contain one or two metal ions on their active site. Most metalloproteases contain Zn$^{2+}$, while some metalloproteases contain Mg$^{2+}$, Ni$^{2+}$, or Cu$^{2+}$. The role of metal ions in metalloproteases is to activate water molecules, which act as nucleophiles in the catalysis process [17].

Based on [1], UBCF_13 phylloplane bacterial isolate identified as Serratia plymuthica showed high fungal suppression activity against Colletotrichum gloeosporioides compared to Fusarium oxysporum and Sclerotium rolfsii. Antifungal effectiveness of the S. plymuthica bacterium extracellular compound UBCF_13 was tested by adding five types of metal ions [Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Ca$^{2+}$, and Zn$^{2+}$] with a concentration of 1 mM. The results of testing the antifungal activity of the bacterium Serratia plymuthica UBCF_13 conducted by [2] showed a different effect. The growth of these three fungal species can be inhibited by the addition of metal ions, including Colletotrichum gloeosporioides [Mn$^{2+}$], Sclerotium rolfsii [Fe$^{2+}$], and Fusarium oxysporum [Ca$^{2+}$].

[16] succeeded in isolating the metalloprotease gene from the bacterium S. plymuthica UBCF_13 with a nucleotide length of 1,059 bp. The metalloprotease gene from the Serratia plymuthica UBCF_13 bacterium was successfully transformed into E. coli DH5α by using the pGEM T-easy plasmid. The next step is testing the expression of that gene. This test serves to synthesize the protein from the target gene, so the protein can be used for further testing such as protein identification and activity.

2. Methods

2.1 Transformation of Recombinant Plasmids
The starter culture of E. coli BL21 was grown using LB media with a pH of 7 for 18 hours with 160 rpm. The method for transformation was heat shock based on [6]. Culture of 50 μL was transferred to selective media [LB with 100 μg/ml ampicillin]. The single colony was used as a template for colony PCR and T7/SP6 as the primer.

2.2 Isolation of Recombinant Plasmids and Sequencing
Isolation of recombinant plasmids was using the Plasmid Mini Kit [ATP Biotech Inc.] method. The results were used as the samples for sequencing. The sequence was edited and contigs using sequence editor software. Then, it was analyzed by VecScreen and ORF on NCBI, the molecular weight was measured using the ExPASY website [https://www.expasy.org].

2.3 Induction of UBCF_13 Metalloprotease Gene Expression
The protocol was adapted from [20], which was modified by the addition of ampicillin 100 μg/mL. Expression of E. coli BL21 transformant was induced by 10 different treatments, namely without induction, induction with 1 mM IPTG, induction with different types of metal ions [Mn$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, and Fe$^{2+}$] with a concentration of 1 mM then induction with IPTG and all types of metal ions.

2.4 Proteolytic Activity Test
The proteolytic activity was tested using recombinant E. coli BL21 cell culture after induction. This test was carried out using LB agar media containing 2% skim milk. The 5 μL of cell culture was applied to the application point in media. The applied plate was incubated at 37 °C for 3 days. Clear zone diameters were calculated using the formula from [12].
2.5 Total Protein Extraction
Total protein extracellular and intracellular extraction were carried out based on the method of [21]. Total protein concentration was measured using the method of [5]. Determination of the protein concentration was using BSA. SDS-PAGE visualization was done using SDS-PAGE gel with 12% separating gel.

2.6 Data analysis
Obtained data from the results of proteolytic activity testing were processed statistically using the one-way ANOVA test [10]. The results were continued to be analyzed using DMRT at a 5% significance level [7].

3. Results and Discussion

3.1 Transformation of Recombinant Plasmids
The quality of the recombinant plasmid was still good, as seen from the thickness of the target fragment [Figure 1]. The quantity was conformable with the estimated nucleotide length, 1.059 bp [16] and 1.200 bp.

![Figure 1](image1.png)

Figure 1. Results of recombinant plasmid; 1 = metalloprotease F/R primer, 1 kb = size marker [Thermoscientific-USA], 2 = T7/SP6 primer.

![Figure 2](image2.png)

Figure 2. [a] Recombinant E. coli BL21 colonies that has been transformed; 1 kb = size marker [Thermoscientific-USA], 1, 2, 3 = single colony of plates 1, 2, 3. [b] PCR results of 5 single colonies of recombinant E. coli BL21 from 1 plate.
Based on the results, the obtained fragment had a length of 1.200 bp [Figure 2a], the result was conformable with the estimated length. The efficiency of recombinant plasmid transformation into E. coli BL21 in this study was 100% [Figure 2b].

Figure 3. [a] Isolation of recombinant plasmid [b] Amplification of recombinant plasmid; 1kb = size marker [Thermoscientific-USA].

Based on the visualization results, there were 2 fragments [Figure 3a], one of them was the genome of E. coli BL21 which was isolated with a size of ± 10,000 bp. The next fragment was a recombinant plasmid, the size of the target fragment should be 4074 bp. The recombinant plasmid was circular, therefore the size of the fragment did not match the size of the ladder that should be used to measure the linear DNA. Circular DNA moves slower because of the mobility of them [9].

The amplification result of the recombinant plasmid [Figure 3b] showed the presence of 3 fragments, the target fragment was 1.200 bp. The result of the sequencing was the electropherogram [Figure 4] and amino acid sequence [Figure 5]. The results were conformable with the previous studies by [16]. Based on the nucleotide BLAST result [Figure 6], it shows the gene of metalloprotease UBCF_13 has 100% similarity with metalloprotease UBCF_01 with accession MK524937.1 and UBCF_13 with accession MK524938.1.

Figure 4. Electropherogram of UBCF_13 metalloprotease gene.
**Metalloprotease-F** Primer : ATGCCAAATGAGAGCGAGTT

| ATGCCAAATGAGAGCGAGTT |
|-----------------------|
| TCACAACTAATTCAGCTGGA |
| CCACTCTCCCTGGATAGT   |
| CACATAGTCCTGCGGCAAGAT |
| TCCAAAATTCAATCAAGTTCG |
| GCTCCATCCCTGTTAACATT |
| AGGACGAGATTACGACGAC |
| TCTGGGCGCGGAGGCGAGT |
| TGGCTGACCTGGGCTGCTG |
| GTGATTAGGTGCTCTGTT |
| GATGAGGAATGTCGCGTTTC |
| CACGCTTGACTCGTGTTT |
| AGCAGGGTGCTGCGCTT |
| ATCGAGGCGTCAGTATGAT |
| CCGCTTCCGCCCA |

**Metalloprotease-R** Primer : ATAGTTAATCGCTTCCGCGCA

| ATAGTTAATCGCTTCCGCGCA |

Figure 5. Nucleotide sequences of UBCF_13 metalloprotease gene.

| No. | Description | Total score | E-value | Query coverage | Identity | Accession |
|-----|-------------|-------------|---------|---------------|----------|-----------|
| 1.  | *Serratia plymuthica* strain UBCF_13 metalloprotease gene, complete cds | 1956 | 0,0 | 100 % | 100,00 % | MK524938.1 |
| 2.  | *Serratia plymuthica* strain UBCF_01 metalloprotease gene, complete cds | 1956 | 0,0 | 100 % | 100,00 % | MK524937.1 |
| 3.  | *Serratia plymuthica* strain UBCR_12 metalloprotease gene, complete cds | 1917 | 0,0 | 100 % | 99,34 % | MK524936.1 |
| 4.  | *Serratia plymuthica* strain NCTC8900 genome assembly, chromosome: 1 | 1480 | 0,0 | 100 % | 95,00 % | LR134151.1 |
| 5.  | *Serratia plymuthica* strain NCTC8015 genome assembly, chromosome: 1 | 1480 | 0,0 | 100 % | 95,00 % | LR134478.1 |

Figure 6. Nucleotide BLAST result.
3.2 Proteolytic Activity Test

The qualitative proteolytic activity testing was carried out using skim milk as a substrate. Skim milk contains casein, which is a milk protein consisting of calcium that is bound to phosphate protein to form arsenic calcium [19]. The clear zone formed after 24 hours of bacterial application on the test media shows that the casein in the media has been broken down into amino acids [14].

The diameter of the clear zone produced by the metalloprotease UBCF_13 [Figures 7 and 8] for 3 days after the application had been measured using the ruler. On the 1st day, the highest value of the clear zone diameter was in treatment B with 0.58 cm. Then, on observations of the 2nd and 3rd day, treatment J had the longest clear zone diameters of 0.72 cm and 0.83 cm. Based on the results, recombinant *E. coli* BL21 culture induced by IPTG plus Ca²⁺ was the best treatment in hydrolyzing casein.

![Figure 7](image)

**Figure 7.** The data shown is an average of 3 biological replications. Standard deviation is marked with an error bar. The notation shows a significant difference based on the DMRT test at the 5% level.

The diameter of the clear zone produced in treatment K was smaller on the 1st to 3rd day. The clear zone formed in the K treatment was caused by the presence of proteases in the *E. coli* BL21. [4] states that the *E. coli* BL21 lacks two proteases encoded by the lon [cytoplasmic] and ompT [periplasmic] genes, so this statement proves that it has proteases even though in small amounts.

Metal ions as cofactors in the work of enzymes can regulate enzyme activity but metal inhibitors can reduce the hydrolysis of the substrate by proteolytic enzymes [15]. Based on the research of [11], the addition of Ca²⁺ and Co²⁺ with a concentration of 1 mM can increase the proteolytic activity of *Serratia proteamaculans* HY-3.
3.3 Total Protein Extraction

Based on the results of the ORF analysis [Figure 9], the longest was ORF 7 with a size of 753 bp starting from the 162nd to the 914th base and estimated to be able to encode 250 amino acids. The molecular weight of protein with an amino acid number of 250 was 27 kDa.

Protein profile gives the pattern pictures of protein change, there are proteins whose expression decreases [down-regulated] or increases [up-regulated]. The results of total protein extracellular extraction can be seen from the visualization of SDS-PAGE [Figure 10a]. The protein band was conformable with the estimated molecular weight of UBCF_13, 27 kDa. The thickest protein band was seen in treatment E, meaning the expression was higher in that treatment. In treatment A the protein band with a size of 27 kDa looks very thin as well as in other treatments without IPTG addition.
Figure 10. SDS-PAGE visualization of [a] total protein extracellular [b] total protein intracellular; A [without induction], B [IPTG], C [Zn^{2+}], D [Mn^{2+}], E [Fe^{2+}], F [Ca^{2+}], G [IPTG and Zn^{2+}] H [IPTG and Mn^{2+}] I [IPTG and Fe^{2+}], J [IPTG and Ca^{2+}], BL21 [non-recombinant bacteria].

Based on the results of total protein intracellular extraction [Figure 10b], the protein target was not present in the visualization. This shows that the metalloprotease of UBCF_13 was found outside the cell membrane. Based on [16], the UBCF_13 metalloprotease gene has an M48C loiP-like domain, loiP is a membrane in the outer layer of lipoprotein that can secrete proteins into the periplasm [13]. So, it can be ascertained that UBCF_13 metalloprotease is an exoprotease. Besides, E. coli BL21 without recombinant plasmids did not show the presence of a protein target. The protein band with size 27 kDa could be ascertained as a metalloprotease from S. plymuthica UBCF_13.

4. Conclusion
Metalloprotease activity of UBCF_13 had the largest clear zone which is inducted by IPTG plus Ca^{2+} and the highest expression gene was inducted by Fe^{2+}. The result of sequence gene close to metalloprotease gene from UBCF_13 and UBCF_01.

5. Acknowledgment
This research is funded by Directorate General of Higher Education-Ministry of Research and Technology via PMDSU research grant fiscal year 2019, contract No. T/15/UN.16.17/PT.01.03/PP/2019.

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