Induction of gene expression and chitinolytic activities of putative chitinase gene bacteria *Serratia plymuthica* UBCF_13

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**Abstract.** The most dominant disease that causes low production of chili in Indonesia is anthracnose. Anthracnose disease is caused by the pathogenic fungi called *Colletotrichum* sp. The control of anthracnose disease can be done by using bio fungicides which can be developed from the class of enzymes that degrade pathogenic fungal cell walls. These enzymes are known as hydrolase enzymes, one of which is *Chitinase*. *Chitinase* is an enzyme that catalyzes the breakdown of chitin polymer compounds in β-1,4 glycosidic bonds. Chitin is a polymer compound that makes up the fungal cell wall. Chitinolytic bacteria can produce chitinase enzymes. *S. plymuthica* bacteria produce chitinase enzyme, one of which is putative chitinase [Chi_Put]. The purpose of this study was to express the Chi_Put gene from *S. plymuthica* UBCF_13 in pGEM-T Easy vector using *Escherichia coli* BL21 and to test the chitinolytic activity of Chi_Put in degrading chitin in-vitro. Chi_Put gene expression was induced by IPTG and in combination with Mg²⁺ and Cu²⁺ metal ions. The highest gene expression was marked by the thickest protein band on the SDS-PAGE visualization with a molecular weight of 47 kDa, namely in the treatment of expression induction with IPTG plus Mg²⁺ metal ion. The chitinolytic activity of Chi_Put UBCF_13 was tested using solid LB media containing 3% colloidal chitin. The longest clear zone diameter on the last day of observation was the expression induction treatment with Mg²⁺ with a length of 2.90 cm.

**Keywords:** Chi_Put, gene expression, *Serratia plymuthica*

1. **Introduction**

Chitinase is an enzyme that catalyzes the breakdown of chitin polymer compounds in β-1,4 glycoside bonds. Chitin is a polymer compound that makes up the cell walls of fungi. The chitinase enzyme can be produced by bacteria, fungi, plants, and animals [1].

Yani [2] successfully identified UBCF_01 and UBCF_13 [UBCF: Unand Bacterial Collection Filoplan] isolates as *Serratia plymuthica* bacteria. Syafriani et al. [3] reported that the *Serratia plymuthica* bacteria strain UBCF_13 was proven in vitro to produce proteases and chitinases which were able to inhibit the growth of the fungus *Colletotrichum gloeosporioides*. Syafriani et al. [4] have isolated...
several chitinase genes from *S. plymuthica* strain UBCF_13, one of which is a putative chitinase gene [Chi_Put] with a size of 1,281 bp. Syafriani et al.[4] further reported that the putative chitinase gene from *S. plymuthica* showed 93% similarity with the chitinase gene from several other *S. plymuthica* bacteria that had been registered in the gene bank. Based on its phylogenetic, the putative chitinase gene is thought to be related to the chitinase A [ChiA] gene.

The chitinase structure is known as a multi-domain structure because it has more than one domain, namely the catalytic domain [accelerates the work of enzymes], the peptide signal domain [directs gene secretion inside the cell to the outside of the cell], and the fibronectin III domain [substrate binding and substrate hydrolysis efficiency] [5]. Syafriani et al. [4] stated that Chi_Put only has one domain of the three proper domains, namely the catalytic domain, while the peptide signal domains and FN-III-Like were not found. Even so, interestingly Chi_Put is still able to degrade chitin compounds, so further research is carried out on its expression and characteristics, which can provide an overview of the opportunities for using the Chi_Put gene in the development of a bio-fungicide compound.

2. Methods

2.1 Transformation of the Recombinant Plasmid into *E. coli* BL21

*E. coli* were prepared by growing them in liquid LB media [pH 7]. Plasmid transformation using the heat shock method [6]. 50 μL bacterial cultures were grown on selective solid LB media [containing 100 μg/mL ampicillin] Single colonies resulting from plating were used as templates for PCR colony. PCR mixture contains 1.5 μL T7/SP6 primer 1.5 μL, 9.5 μL nuclease-free water of, and 12.5 μL My Taq Green Mix. Then analyzed by VecScreen on NCBI to find out what base sequence the original Chi_Put sequence was. Then a BLAST analysis was carried out to determine the similarity of the target gene nucleotide sequences with the nucleotide sequences of other genes in the database. The determination of the ORF (Open Reading Frame) region and protein sequence is carried out on the NCBI website, which can later be used to estimate the molecular weight of proteins using the ExPasy website.

2.2 Induction of Putative Chitinase Gene Expression UBCF_13

The protocol used was adapted from Waschkowitz et al [7] with the addition of ampicillin. After that, 1 mM IPTG and 1 mM metal ion were added with 6 treatments, without IPTG and Metal ion, IPTG, metal ion Cu²⁺, metal ion Mg²⁺, IPTG+Cu²⁺, IPTG+Mg²⁺, which is used as a source of metal ions, namely compounds MgSO₄.7H₂O and CuSO₄.5H₂O in 4 replications. Furthermore, it was centrifuged to separate the precipitate and the culture supernatant.

2.3 Total Protein Extraction

In the next stage, total protein extraction was carried out using the method of Zhang et al [8], measurement of total protein concentration using the Bradford method, while for protein concentration determination using the standard BSA curve.

2.5 Chi_Put Chitinolytic Activity Test of *E. coli* BL21 Transformant on Chitin Colloidal Media

The chitinolytic activity test was carried out using post-induction recombinant E. coli cell culture, then as much as 5 μL of suspension was applied to the center of the chitin colloidal medium. After application, the plates were incubated at 37 °C. Chitinolytic activity is obtained by measuring the clear zone formed around the hole which indicates that the bacterial suspension can hydrolyze colloidal chitin.

**Clear zone diameter = Total Diameter - Coloni Diameter**

2.6 Data analysis

The data obtained from the test results of chitinolytic activity in each type of culture were statistically processed using the one-way ANOVA test. Some treatments that showed significant differences were
continued to be analyzed using the advanced test of Duncan's New Multiple Test Range at the 5% real level [9].

3. Results and Discussion

3.1. Transformation of the Recombinant Plasmid into E. coli BL21

The total length of the PCR product of the recombinant plasmid that was amplified with \textit{Chi\_Put} specific primer is 1.281 bp. The T7/SP6 primer is the promoter and terminator area found in the T-easy pGEM plasmid [10].

![Figure 1](image1.png)

\textbf{Figure 1.} Verification of recombinant plasmids [pGEM T-easy + Chi\_Put] using PCR; A = primer T7/SP6, M: size marker 1 kb ladder [Thermo-scientific], B = primer Chi\_Put F/R

Verification of the success of the transformation can be seen from the number of bacterial colonies growing on selective media containing ampicillin antibiotics. The verification of the success of the transformation was then carried out using the colony PCR technique. Bacterial colonies growing on selective LB media were randomly selected.

![Figure 2](image2.png)

\textbf{Figure 2.} PCR results of recombinant \textit{E. coli} BL21 bacterial colonies that had been transformed with recombinant plasmids [pGEM and \textit{Chi\_Put}] using primer T7/SP6. M = size marker 1 kb ladder [Thermo scientific], 1, 2 = single colony of plates 1, 2.

The fragments of each sample according to the estimate of 1,422 bp. These data prove that the colonies tested were transformant \textit{E. coli} BL21 containing \textit{Chi\_Put} gene fragments. Colony PCR amplicon is then used for the sequencing stage, to determine the identity and function of genes by comparing the sequences obtained with other known DNA sequences, a database available online [11].

The sequencing result of the colony PCR amplicon in the form of the contig sequence chromatogram. The quality of the resulting sequence data is quite good with a total sequence length of 1,410 bp. The
VecScreen result showing the position of the original *Chi_Put* sequence and its vector position. The original length of the *Chi_Put* sequence is 1,299 bp, while the forward vector positions of bases 1-47 and reverse are from bases 1.347-1.409.

**Figure 3.** Chromatography resulting from the sequencing of the *Chi_Put* UBCF_13 gene.

### 3.2. Effect of Metal Ion Induction and IPTG on Expression of the *Chi_Put* Gene

SDS-PAGE visualization was carried out by equalizing the concentration of each sample to 100 ng / μl with a volume of 10 μl so that can be seen the difference in the thickness of the protein bands for each treatment given.

The size of the putative chitinase molecular weight is estimated at 47 kDa. The results of this prediction were obtained from the calculation of the amino acid composition that makes up *Chi_Put* using ExPASY. The visualization in Figure 3 shows that the target protein from *Chi_Put* gene expression has been successfully obtained. This protein was seen in all treatments, including non-transformant *E. coli* BL21 treatment. The band produced by non-transformant bacteria at a size of 47 kDa is probably another type of protein, namely phosphodiesterase [12] which has the same molecular weight as *Chi_Put*.

Bacterial induction treatment on extracellular fraction, thick protein band in C treatment [Mg^{2+} induction], D treatment [IPTG induction] and F treatment [IPTG induction and Mg^{2+}] where fragments with a size of about 47 kDa were estimated to be *Chi_Put* protein has an intensity three times thicker than the other induction treatments. However, the thickest of the three treatments was treatment F [induction of IPTG and Mg^{2+}].

**Figure 4.** Visualization of the separation of *E. coli* BL21 transformant proteins using SDS-PAGE. A = extracellular fraction, B = intracellular fraction, BL21 = non-transformant bacteria, A [transformant BL21 [without induction]], B [Cu^{2+} induction], C [Mg^{2+} induction], D [IPTG induction], E [Induction of IPTG and Cu^{2+}], F [Induction of IPTG and Mg^{2+}].
In the intracellular fraction, D treatment [IPTG induction] did not produce a thick protein band like in the extracellular fraction. Visualization of SDS-PAGE on the intracellular fraction looks thicker when compared to the extracellular fraction, this is probably because the peptide signal is not there so that the secretion out of the cell from the cell is not as much as the intracellular fraction.

3.3 Chitinolytic Activity Test of Chi _ Put Protein on Chitin Colloidal Media
The formation of a clear zone around the bacterial colony proves that these bacteria are capable of carrying out the chitinolytic activity. This is a result of the activity of the chitinase enzyme which results in the degradation of chitin in media containing chitin [Figure 4].

Degradation and utilization by bacteria make the media appear clear [clear], especially around bacterial colonies. The results of chitinase activity testing on LB media containing colloidal chitin showed clear zones in all treatments. However, for non-transformant bacteria, there is no colloidal chitin that is degraded so that no clear zone is formed.

Figure 5. Testing of chitinase activity of transformant and non-transformant BL21 bacteria on LB media containing 3% colloidal chitin.

Figure 6. The diameter of the clear zone on LB agar media containing 3% colloidal chitin in various types and inducer conditions. The data displayed is the average value of 4 replications. The error bar represents the standard deviation [SD]. Different lowercase letters above each bar indicate a significant difference based on the DNMRT follow-up test at the 5% level.
Induction treatment with the addition of metal ions either alone or in combination with IPTG began to show a clear zone on day 2 after treatment application. Figure 5 shows that there is a real difference of treatment inducers given to the activity of the gene expression product chitinolytic *Chi_Put*. The treatment without inducer [transformant bacteria only] was used as control compared to other treatments. The clear zone was formed after the 2nd day after application, namely by giving an inducer with Mg\(^{2+}\) and Cu\(^{2+}\) ions. On observations on days 2, 3, and 4, the combination of the addition of Mg\(^{2+}\)+IPTG metal ions resulted in the largest clear zone diameter compared to other treatments with diameters of 0.73, 1.36, and 2 cm, respectively. On the 5th day after application, the addition of Mg\(^{2+}\) metal ion, the clear zone diameter was 2.90 cm. Likewise, the addition of Mg\(^{2+}\) metal ions combined with IPTG was able to produce a diameter of 2.82 cm. The addition of Cu\(^{2+}\) metal ions either singly or in combination with IPTG resulted in a clear zone diameter that was not much different [not significant] compared to the controls. Thus the addition of metal ions Mg\(^{2+}\) and IPTG has the potential to increase chitinolytic activity against colloidal chitin. However, this assumption must be tested empirically on real fungal cells.

4. Conclusion
Gene *Chi_Put* from strain UBCF_13 was successfully expressed both extracellularly and intracellularly in *E. coli* strain BL21 cells. The Cu\(^{2+}\) inducer for the expression level and chitinolytic activity had no significant effect compared to that without the inducer. The Mg\(^{2+}\) inducer was able to significantly increase the expression level and chitinolytic activity of the *Chi_Put* gene expression product compared to the transformant BL21 [without induction] with a diameter of 2.90 cm and 2.82 cm.

Reference
[1] Herdyastuti, N., TJ Raharjo, M. Mudasir, and S. Matsjeh. 2009. Chitinase and chitinolytic microorganism: isolation, characterization, and potential. *Indonesian Journal of Chemistry* 9 37-47.
[2] Yani, RH 2012. Selection of antagonistic bacteria from mustard plants *Brassica juncea* L. as biofungicides against *Colletotrichum gloeosporioides* causes Anthracnose disease in chili *Capsicum annuum* L. [Essay]. Padang. Faculty of Agriculture. Andalus University. 41 p
[3] Syafriani, E., FF Syafirzayanti, and A. Jamsari. 2017. Isolation of Putative Chitinase II Gene fragment from *Serratia plymuthica* Strain UBCR_12. *Der pharmacia Lette* 9 26-37.
[4] Malecki, PH, JE Raczynska, CE Vorgias, and W. Rypniewski. 2013. Structure of a complete fourdomain Chitinase from *Moritella marina*, a marine psychrophilic bacterium. *Acta Crystallographica Section D* 69 821-829.
[5] Sambrook, J., EF Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual No. Ed. 2. *Cold Spring Harbor Laboratory Press*.
[6] Waschkowitz, T., Rockstorn, S, and Daniel. R. 2009. Isolation and characterization domain structure by construction and screening metagenic. *Libraries Applied and Environmental Microbiology* 75 2506-2516.
[7] Zhang, CX, X. Zhao, F. Han, and MF Yang. 2009. Comparative proteome analysis of two antagonists of *Bacillus subtilis* strains. *J Microbial Biotechnology* 19 351-357.
[8] Duncan, D. B. 1995 Multiple range and multiple F Test. *Biometrics* 11 1-42
[9] Zhou, L., X. Wang, Q. Liu, S. Xu, H. Zhao, M. Han, and J. Li. 2019. Visualization of Turbot *Scophthalmus maximus* primordial germ cells in vivo using fluorescent protein mediated by the 3 untranslated region of nanos3 or vasa gene. *Marine Biotechnology* 1-12.
[10] Klug, WS, MR Cummings, CA Spencer, MA Palladino, and SM Ward. 2009. Conscepts of genetics. Vol. 9: *Peraron*.
[11] Rajagopal. 2014. Antifungal activity against plant pathogenic fungi of *chaetoviridins* isolated from *Chaetomium Globosum*. *FEMS Microbiology Letters* 252 309-313.