Expression *ChiPut*-II gene from *Serratia plymuthica* UBCR_12

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**Abstract.** Putative chitinase II gene (*ChiPut*-II) was isolated from rhizosphere bacteria strain UBCR_12 [NCBI accession number KX863673]. The *ChiPut*-II gene encodes chitinase enzyme which has the ability as a *bio fungicide*. Expression of the gene could be identified in the extracellular as well as in the intracellular of the host cell. The chitinolytic ability of the *ChiPut*-II enzyme effectively works on 3% colloidal chitin exhibiting specific activity up to 1.235 U/μg. The inhibition capacity of the *ChiPut*-II enzyme extracted from intracellular against *Colletotrichum gloeosporioides* is 15.59%. It’s chitinolytic and inhibition activity indicated the bacteria to be developed as a *bio fungicide*.

Keywords: intracellular, extracellular, putative chitinase, *Serratia plymuthica*

1. Introduction

Antibiotic control of pathogens by bacteria through antifungal compounds such as chitinase enzymes will be active when interacting with pathogenic fungi [1]. Chitinase enzyme has an antibiosis mechanism by degrading the cell wall of pathogenic fungi formed from chitin. The fungal cell wall degraded by chitinase is then damaged and inhibits the growth of pathogenic fungi [2]. Chitinase is an enzyme that can degrade chitin from the polymer β-1,4-N-acetylglucosamine (GlcNAC) to its monomers and oligomers [3]. Chitin is the second-largest polymer found in nature and construct cells of various organisms such as fungal cell walls, insects, crustaceans and some higher plants [4]. Classification of chitinase enzymes based on the catalytic site of the domain is divided into chitinase A [ChiA], chitinase B [ChiB] and chitinase C [ChiC] [5].

Bacteria isolate *Serratia sp.* UBCR_12 has been reported to have antifungal activity by producing chitinase. The ability to inhibit pathogenic fungi is reported can reach up to 43%. Identification using 16S rRNA sequence data exhibited that the UBCR_12 isolate is *S. plymuthica* [6]. The UBCR_12 isolate has also been tested for its extracellular product which also shows an ability to inhibit *C. gloeosporioides* by 30.66% [7].

One of the putative chitinase genes that has been isolated and sequenced from UBCR_12 is the *ChiPut*-II gene. The *ChiPut*-II gene sequence was cloned into pGem-T Easy vector plasmid using *E. coli* strain DH5α [8] as host. The expression of *ChiPut*-II was identified from extracellular as well as in the intracellular of its host cell.
2. Materials and Method

2.1. Cells and plasmid
The plasmids used were recombinant pGem-T Easy containing inserted ChiPut-II gene, *E. coli* strain DH5α, *E. coli* strain BL21 and pathogenic fungi *C. gloeosporioides*.

2.2. Transformation and verification of plasmid recombinant
Recombinant plasmid verification was performed using specific primers F-[5'TAACATGGCATTACCCCGTA 3'] R-[5'ATCGCCCTGTNGGTATTAGCG3'] and T7SP6 primer for pGem T-Easy T7-[5'AATACGACTCAGTATAG3'] SP6- [5'ATTTAGGTGACACTATAG3']. The competent cell for transformation was prepared using 0.1 M CaCl$_2$, while transformation was done using a heat shock method in a water-bath using 42°C for 90 seconds. The transformation results were added 250 μL of liquid LB media and incubated for 2 hours on a shaking incubator at 37°C and a speed of 160 rpm. The bacterial suspension was pipetted as much as 100 µL and plated on selective solid LB media containing ampicillin [30 μg/mL]. Incubation was done in an oven at 37°C overnight.

2.3. Isolation and profile analysis of crude protein containing ChiPut-II gene
Induction of gene expression was carried out by adding 1 mM isopropyl β-D-1-thiogalactoside [IPTG] when OD$_{600}$ [600] reached 0.6 and then incubated again for 3 hours. Isolation of extracellular proteins from recombinant bacterial cultures was transferred in a 50 ml tube and centrifuged at 4°C at 10,000 rpm for 5 minutes to obtain bacterial pellets and their supernatant. The supernatant was separated using centrifugation at 4°C at 10,000 rpm for 15 minutes. The protein obtained was then dried at room temperature and then added with 10 mL of pH 7 phosphate buffer.

The isolation of intracellular proteins was performed using 12.5 mL lysis buffer containing 50 mM HEPES pH 7.4, 150 mM KCl and 1 mM EDTA pH 8. Lysis was done twice using the freeze-thawing method. Lysis incubation was performed at a temperature of -80°C and 95°C for 5 minutes and repeated 3 times. After the freeze-thawing step, the pellet was centrifuged at 10,000 rpm at 4°C for 5 minutes. The supernatant was transferred to a new falcon tube and added with a solution of ammonium acetate methanol with a ratio of 1:1 to the supernatants and incubated at -15°C overnight. Protein was then dried at room temperature and added 1 mL of pH 7 buffer phosphate. The intracellular protein is stored at 4°C and then is measured for protein concentration and protein profile analysis.

2.4. Antagonist test of crude protein.
Functional analysis of crude proteins containing ChiPut-II enzymes from extracellular and intracellular proteins was tested against pathogenic fungi *C. gloeosporioides*. The fungi were grown on PDA media and incubated at room temperature for 7 days. The *C. gloeosporioides* fungi were located in the center of the petri dish.

The application of crude protein containing the ChiPut-II enzyme was carried out on the 2nd day. The crude enzyme [20 ng/μL] was applied with a volume of 20 μL. The observation was started from the first day after the application until the 7th day. Percentage of inhibition was calculated by the following formula:

\[
\text{Inhibition}(\%) = \frac{D_c - D_t}{D_c} \times 100\%
\]

Where:

- $D_c$ is the diameter of the untreated fungi
- $D_t$ is the diameter of the treated fungi sample

Each treatment was performed in three replicates

2.5. Chitinolytic assay of crude protein
The chitinolytic test of the ChiPut-II enzyme was based on the ability of the enzyme to hydrolyze glycoside bonds on the choline substrate. The sample solution was prepared with 1 mL phosphate
buffer [20 mM pH 7] composing 5 different concentrations [1%, 2%, 3%, 4%, and 5%] of colloidal chitin mixed with 500 μL crude protein containing the enzyme chitinase. Sample solutions were also prepared 3 replications. The sample solution was incubated at 30°C for 2 hours, then the reaction was stopped by incubating at 95°C for 10 minutes. The solution is vortexed until homogeneous and then incubated in ice for 30 minutes. Blank solution and sample solution were then centrifuged with a speed of 10,000 rpm for 10 minutes. The supernatant of 1 mL solution was transferred into a 2 ml Eppendorf tube and then mixed with 500 μL of DNS reagent and incubated at 95°C for 5 minutes. The solution is cooled then the absorbance is measured at λ 540 nm using a spectrophotometer.

3. Results and discussion

3.1. Transformation and verification
Recombinant pGem-T Easy-ChiPut-II plasmid was verified by PCR using a specific primer [Figure 1a]. The T7SP6 primer produced a single 1,422 bp fragment while a specific primer for ChiPut-II exhibited a 1281 bp fragment [Figure 1b]. The two PCR products were under the estimated size of the ChiPut-II gene. This data concluded that pGem-T Easy-ChiPut-II contains the ChiPut-II gene insert. The ChiPut-II gene has been cloned and its sequence data are deposited at the NCBI gene bank with accession number KX863673.

Cloning of some Chitinase genes has been carried out in several species of Serratia sp. They are for instance S. marcescens [9], S. proteamaculans [10] and S. plymuthica strain UBCR 12 [6]. The size of each chitinase gene is almost similar even though they isolated from different species. The ChiPut genes isolated from S. marcescens, S. proteamaculans, and S. plymuthica have 1,280 bp in size, while the ChiB and ChiC gene of those three species also have a similar size.

3.2. Crude protein profile
Induction of ChiPut-II gene expression using 1 mM IPTG was performed when OD culture reached 0.6 [9]. The isolated protein was profiled using SDS-PAGE to separate protein molecules from other types of proteins or other molecules based on size, solubility, charge, and affinity of bonds [11] [Figure 2].

The ChiPut-II protein is estimated to be 47 kDa. Based on that estimation position of the ChiPut-II protein fragment is be determined in the SDS-PAGE visualization [Figure 2]. Figure 2 indicates that the target fragment can be obviously in intracellular lysis 2. The extracellular fraction shows also similar fragment, but it seems not so clear like its counterpart. This could be caused by an incomplete lysis process, particularly during the first lysis. The intracellular proteins in recombinant E. coli mostly tend to be inseparable. Therefore, the protein profile in the first lysis in ChiPut-II could not be
visualized. The putative II chitinase protein has smaller molecular weight compared to ChiA, ChiB, and ChiC. The ChiA proteins, for instance, have protein size in the range of 57-58 kDa [12]. Another study described that chitinase protein in S. marcescens has a molecular weight of ChiA, ChiB, and ChiC of 57, 55, and 51 kDa respectively [9].

![Figure 2](image.png)

**Figure 2.** Profile of the intracellular and extracellular proteins of the ChiP-ll. M = Protein ladder, A = intracellular fraction lysis 1, B = intracellular fraction lysis 2, C = extracellular protein

3.3. Antagonistic test of ChiP-ll crude Protein

The inhibitory or antagonistic test of crude extracellular and intracellular ChiP-ll crude Protein was performed using the agar diffusion method [13]. The results are presented in Figure 2. The inhibitory ability of intracellular 2 fractions showed up to 15.59% after 7th days of treatment, while extracellular fraction reached 10.20% on 6th and intracellular 1 reached only 11.27% on day 7 [Figure 3].

![Figure 3](image.png)

**Figure 3.** Antifungal activity of extracellular and intracellular fraction lysis 1-2 in 7 days.

The inhibition capability of chitinase against pathogenic fungi showed varied results. Such ability is related to the type and domain structure of the chitinase enzyme. The intracellular fraction of the ChiA enzyme isolated from S. plymuthica strain UBCF_01 expressed in E coli containing pGem T-
Easy could inhibit \textit{C. gloeosporioides} up to 27.05\%. While it’s extracellular fraction can inhibit up to 20.40\%. The chitinases [A, B, and C] isolated from \textit{S. marcescens CFFSUR-B2} showed inhibition activity ranged from 40 to 80\% against \textit{Mycosphaerella fijiensis}. The study also indicated that optimal inhibition could be more effective if the three chitinases were working together simultaneously [14].

3.4. \textit{The chitinolytic activity of ChiPut-II crude protein}

The chitinolytic test of the intracellular and extracellular fraction of \textit{ChiPut-II} was performed using N-acetyl-D-glucosamine. Releasing 1 μmol of N-acetyl-D-glucosamine is measured as 1 activity unit of the \textit{ChiPut-II} enzyme and termed as specific activity [Figure 4]. Figure 4 shows that all three fractions have the highest specific activity on 3\% colloidal chitin. The intracellular elution 2 revealed high activities in 1 and 2\% colloidal chitin compared to extracellular and intracellular elution 1.

![Figure 4. The specific activity of the crude \textit{ChiPut-II} enzyme](image)

The specific activity of the crude \textit{ChiPut-II} enzyme in colloidal chitin 1\% to 5\% showed a dynamic pattern. The maximum specific activity of the three fractions reached 3\% of colloidal chitin. Their specific activity decreased at concentrations of 4\% and 5\%. In the two last concentrations probably the enzyme has reached its saturation point, causing the decrease of enzyme activity rate [15].

4. Conclusion

The cloned \textit{ChiPut-II} gene is successfully expressed in the \textit{E. coli} BL21. Inhibitory test of expressed \textit{ChiPut-II} protein against \textit{C. gloeosporioides} exhibited inhibitory ability up to 15.59\% obtained from the second intracellular protein lysis fraction. The fraction showed specific activity up to 1.235 U/μg on 3\% colloidal chitin. This result indicates that the expressed \textit{ChiPut-II} protein has a potential activity to be developed as a candidate for \textit{biofungicide}.

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