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A Promising Novel Rhizobacteria Isolate UBCR_12 as Antifungal for *Colletotrichum gloeosporioides.*

Elly Syafriani[1], Femi Riwany[1], Rahmi Kamelia[2], Istino Ferita[1], Fatchiyah Fatchiyah[3], and Jamsari Jamsari[1]*.

[1]Plant Breeding Department, Faculty of Agriculture, Andalas University, Padang, West Sumatra, Indonesia.
[2]Chemistry Department, Faculty of Mathematics and Science, Andalas University, Padang, West Sumatra, Indonesia.
[3]Biology Department, Faculty of Mathematics and Natural Science, Brawijaya University, Malang, Jl Veteran, Malang, 65145, East Java, Indonesia.

ABSTRACT

Anthracnose is a serious fungal disease for many horticultural crops which is attended by a significant decrease in crop yield. One of these disease-causing pathogens is *Colletotrichum gloeosporioides*. Synthetic fungicides are currently used by farmers to control this fungus. However, excessive application of synthetic fungicides has caused some negative impacts on human health and environment. Since it could be shown that some bacteria of the plant rhizosphere act as natural suppressors of fungal diseases, we screened 101 isolates of rhizobacteria and analysed their capability to control *C. gloeosporioides* in-vitro. One isolate designated as UBCR_12 showed high antagonistic activity. Further analysis of UBCR_12 indicated that this effect could be due to protease and chitinase activity. Using a sequencing-based approach we identified *Serratia plymuthica* to be the inhibitor of *C. gloeosporioides*. Our data support the idea that rhizobacteria can be used as natural alternatives to biocidal chemical compounds. In addition we showed that UBCR_12 is a promising candidate for controlling *C. gloeosporioides*.

Keywords: *Colletotrichum gloeosporioides*, rhizobacteria, *Serratia plymuthica*, antifungal, and biofungicide.

Corresponding author

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INTRODUCTION

As a tropical country, Indonesia faces an extensive pathogen attack, especially for horticultural crops [1]. One of the notorious plant pathogens are fungi. This situation is due to the climatic condition characterized by high humidity, that makes it very favorable for fungal growth and development [2]. Small and large monocultures provide optimal conditions for spreading of plant diseases. One of the fungal pathogens which has been frequently reported in many horticultural crops is *Colletotrichum gloeosporioides* [3]. The disease caused by *C. gloeosporioides* is commonly known as anthracnose [4]. Anthracnose disease is widely spread in many countries over the world. As a consequence, significant decline in crop production is frequently reported [5].

A traditional approach for managing anthracnose is the application of synthetic fungicides. However, with the increase of environmental issues, such synthetic fungicide application has to be reconsidered, since such approach has brought serious impact on human health as consumers, other organisms, and many environmental elements [6,7]. Recently, several studies are designed in an effort to develop some biocontrol agents which are regarded to be more environmental friendly [8]. Such biofungicide products will be an ideal solution to answer the drawback of synthetic fungicide application in agricultural practices.

With its great biodiversity, Indonesia has advantages as habitat for many valuable biological materials, so there is a great opportunity to acquire some biocontrol agents for *C. gloeosporiodes*. Especially the plant rhizosphere layer is well known as a favorable habitat for many indigenous bacteria that could have an antagonistic ability against fungal pathogens. Several rhizobacteria have been described to have such ability and practically have been applied to control many fungal pathogens [9,10]. Unfortunately, current studies analyzing domestic rhizobacteria adapted to the Indonesia agroclimate, for their biofungicidal properties especially against *C. gloeosporioides*, have not been widely reported so far.

In this study, we successfully identified 101 rhizobacteria isolates that showed significant antagonistic effect to *C. gloeosporioides*. Started from this point we further analyzed our promising isolates. Here we describe our selection results and performing species identification by applying 16S rRNA gene sequencing. Furthermore, we analysed the isolates for enzymatic capabilities in hydrolytic systems, particularly their protease and chitinase activity.

MATERIALS AND METHODS

Preparation of Rhizobacteria and C. gloeosporioides

Bacterial isolates were collected from eight different plant rhizospheres using standard protocols [11]. In total, 101 of rhizobacteria isolates were obtained from this study after growing them on Nutrient Agar (NA), pH 7. The isolates were incubated at room temperature overnight and stored at 4 ^{0}C for working stock and part of them were stored at -80 ^{0}C for main stock. The *C. gloeosporioides* isolate was prepared from our laboratory stock, and its authenticity was verified with specific primer by PCR analysis.

In-Vitro Inhibition Assay

Antagonistic assay was performed by agar diffusion method using a bacterial colony assay as well as an extracellular supernatant assay [12]. The colony based inhibition assay was performed as follows. Mycelia of *C. gloeosporioides* were cut in 0.6 cm x 0.6 cm in size, and laid down in the center of Potato Dextrose Agar (PDA) medium. After three days-incubation period, a single colony of bacteria was picked by sterile toothpick and put on 3 cm distant from the center of fungi. The co-culture was then incubated at room temperature for seven days. Diameter of *C. gloeosporioides* was compared with the control. Antagonistic activity was determined by a formula [12]:

$$\frac{Dc - Dt}{Dc} x\ 100\%$$

Where:
Dc = diameter of *C. gloeosporioides* from control
Dt = diameter of *C. gloeosporioides* co-cultured with bacteria

A similar formula was used also for extracellular supernatant assay. For this, we performed assay as follows. A single bacteria colony from each isolate was grown in 20 ml Tetrazolium Chloride (TZC) medium and shaken at 150 rpm at room temperature for 48 hours. Two milliliters bacteria culture was centrifuged at 4 ^{0}C for 15 minutes, at 14.000 rpm. The extraction step was repeated three times. 100 µL of extract supernatant were pippeted into a cork borer hole prepared three cm away from the center of fungal mycelia which previously grown for three days. The assay was then incubated at room temperature for seven days. Antagonistic activity was determined as inhibition percentage and calculated with the above formula.

Species Identification Using 16S rRNA Gene Sequence

Chromosomal DNA was extracted from bacterial cells by harvesting 10 mL of overnight-cultured bacteria, using Wizard® Genomic DNA Purification Kit according to Promega's protocol (Promega, USA). After quality control of the isolated DNA, in-vitro amplification was proceed by using PCR technique. The 16S rRNA gene was amplified with 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') primers [13]. PCR reaction was performed in 25 µL of volume, containing 15 ng / 3 µL of DNA, 1.5 µL of each primer (10 pmol/µL), and filled until 25 µL with PCR-grade H_2O. PCR reaction was run using 35 cycles contained of 94 ^{0}C (60 s), 55 ^{0}C (60 s), and 72 ^{0}C (90 s) for each cycle. Initial denaturation was done at 94 ^{0}C for three minutes and followed by a final extension at 72 ^{0}C for five minutes. The amplicon which was expected as a single fragment of 1500 bp in size was ligated to pGem-T easy vector. The ligation was transformed into *Escherichia coli* DH5α as host by heat shock method. Enrichment of transformants was done by incubating for 20 minutes in 250 µL Luria Bertani (LB) medium under shaking condition at 37 ^{0}C for 150 rpm. Transformation suspension was grown on LB selective solid medium, completed with 100 mM IPTG, 3% X-Gal, and 100 mg/mL ampicilin prior incubation at 37 ^{0}C, for overnight.

The recombinant DNA plasmid was further purified using Wizard® Plus SV Minipreps DNA Purification System according to Promega's protocol (Promega, USA). The amplicon was sent to 1st BASE for sequencing. Sequencing was done from both T7 and SP6 termini. Nucleotide sequences were compared for their homology using BLASTn tool available at the NCBI website (http://blast.ncbi.nlm..nih.gov). Multi-alignment were performed with ClustalW2 available at EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Furthermore, the alignment results were used in Mega6 [14] to design the phylogenetic tree.

Protease and Chitinase Assay

The bacteria were grown on 50 mL LB medium for 24 hours in shaking condition. Bacterial cell density was checked in a spectrophotometer at 600 nm of wavelength. Measurement was performed every two hours. Analysis of enzymatic activity was performed as previously described by Queiroga *et al*. [15] for protease, and Mubarik *et al*. [16] for chitinase. Briefly, at first a reaction that consisted of 2 mL colloidal chitin (2% (w/v)) and 1 mL enzyme solution was incubated at 30 ^{0}C for two hours. The reaction was stopped by heating in boiling water for 20 minutes, then centrifuged immediately at 14.000 rpm for 10 minutes. The supernatant was used for reduced sugar analysis using Dinitrocalysilic (DNS) method. The cell-free supernatants were assayed for protease activity using a colorimetric determination of the casein (1% (w/v)) breakdown extent at 660 nm of wavelength. In-vitro inhibition assay using extracellular protease and chitinase on *C. gloeosporioides* was performed as follows. Protease producing was obtained after 18 hours of UBCR_12 cultured in LB medium induced with casein, while the chitinase producing was obtained after six day of UBCR_12 cultured in LB medium induced with colloidal chitin. 100 µL extracellular protease were pippeted into the hole of medium prepared 3 cm away from the center of fungal mycelia which previously grown for three days. The assay was then incubated at room temperature for seven days. Antagonistic activity was determined as inhibition percentage and calculated with the same formula as before. This procedure was also done for extracellular chitinase.

RESULTS AND DISCUSSION

In-Vitro Inhibition Assay

In this study, we tested 101 rhizobacteria isolates by using them for in-vitro inhibition assays. Seventeen isolates showed antagonistic effects against *C. gloeosporioides* **(Table 1)**, while the others either

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showed no inhibition effect or failed to grow. Three out of 17 isolates showed high percentage of inhibition activity. They are designated as UBCR_12, UBCR_36, and UBCR_39 **(Figure 1)**.

Table 1. Percent of inhibition of the 17 positively tested isolates to *C. gloeosporioides*

No.	Isolate ID	Percent of inhibition (%) based on single colony assay
1.	UBCR_05	13.3
2.	UBCR_07	15.8
3.	UBCR_12	43.3
4.	UBCR_19	10.8
5.	UBCR_36	43.3
6.	UBCR_39	40.0
7.	UBCR_52	16.7
8.	UBCR_57	14.2
9.	UBCR_59	16.7
10.	UBCR_61	21.7
11.	UBCR_64	18.3
12.	UBCR_71	20.0
13.	UBCR_72	14.2
14.	UBCR_74	19.2
15.	UBCR_76	7.5
16.	UBCR_86	23.3
17.	UBCR_94	24.2

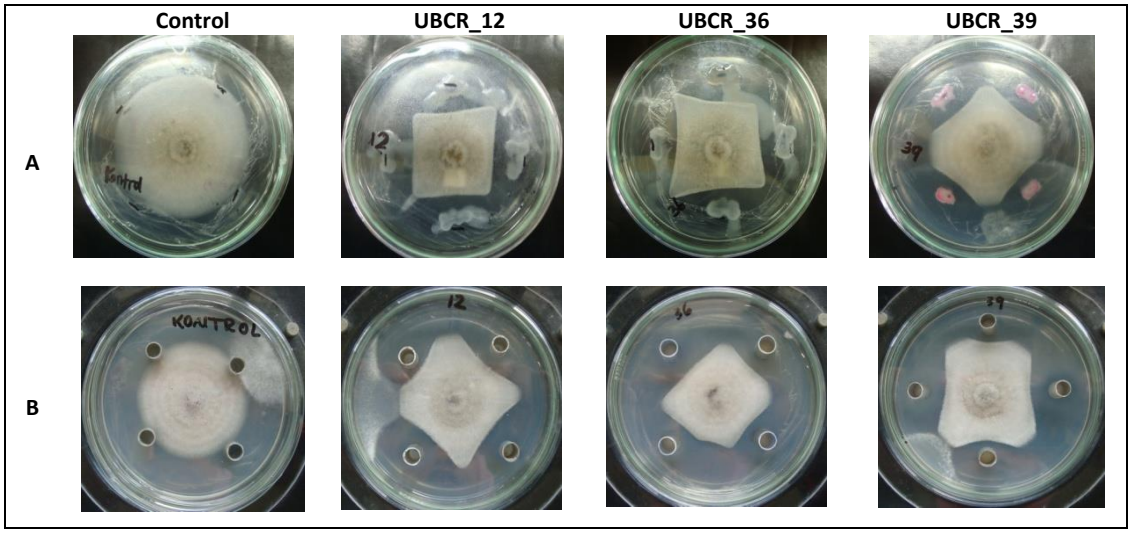

Figure 1. In-vitro inhibition assay of the best three isolates. A: single colony assay; B: extracellular supernatant assay.

Based on single colony assay, UBCR_12 and UBCR_36 showed similar levels of inhibition activity (43.3%), while UBCR_39 showed 40%. Further analysis by an extracellular assay showed that the UBCR_12 had the highest activity (data not shown). Based on these results we selected UBCR_12 for further detailed analysis.

Compared to our results, some previous studies reported that several rhizobacteria showed a variety of inhibition activity on *C. gloeosporioides*. Ann [17] reported six different species of rhizobacteria which showed an inhibition 40.4 to 52.4% by dual culture assay measurements. Lee *et al.* [18] reported that one of their best rhizobacteria isolate against *C. gloeosporioides*, TRL2-3 bacterial strain, has a percentage of 58.6%. A bit lower inhibition, Ashwini and Srividya [19] reported *Bacillus subtilis* from chili rhizosphere soil has 57%. Otherwise, Jamal *et al*. [20] reported their soil bacterial isolate, *B. amyloliquefaciens*, has a variety of percentage inhibition to *C. gloeosporioides* depending on its bacterial culture concentration. Based on these, we concluded that inhibition activity of our isolates are still acceptable.

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Identification of Bacterial Isolates Based on 16S rRNA Gene Sequence

The 16S rRNA gene is commonly used for bacteria species identification [21]. Two directional sequencing analysis exhibited a 1534 bp sequence corresponding to 16S rRNA gene from the UBCR_12. The sequence has been deposited at NCBI database with accession number KU299959. Nucleotide BLAST homology search showed that the UBCR_12 isolate has highest (16S rRNA gene) homology (99.61%) with *Serratia* sp. AS12 (CP002774) that is known as *S. plymuthica* AS12 [22]. Based on this result, we designated the UBCR_12 isolate as *S. plymuthica*.

We have chosen several other accessions having high similarity with UBCR_12, then performed multi-alignment analysis using ClustalW2. Multi-alignment showed that insertion-deletion and base substitution events occurred in the 16S rRNA gene sequence of UBCR_12 compared with the others. Data entry of multi-alignment analysis was used to build a phylogenetic tree among some closely selected accessions **(Figure 2)**.

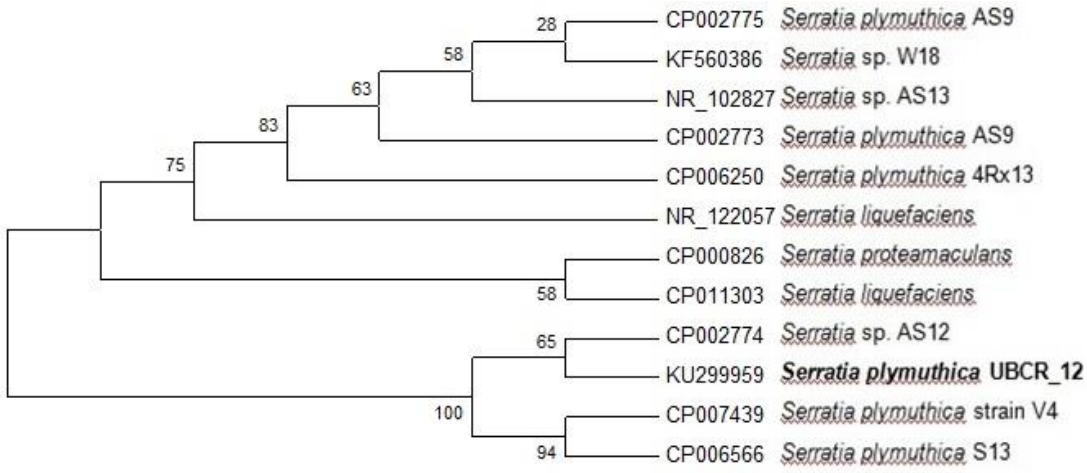

Figure 2. Phylogenetic tree based on the 16S rRNA gene sequence of UBCR_12 among 11 closely relative selected accessions. The tree was designed by Kimura 2-model parameter method embedded in Mega6 by bootstrap value with 1000 replication.

S. plymuthica is well known as an antagonistic bacteria to some fungal pathogens, e.g. *Rhizoctonia solani* [23] and *Botrys cinera* by producing chitinase [24]. The fungicidal effect seemed to be based on the potential to produce chitinase enzymes. Furthermore, Vleesschauwer and Hofte [25] reviewed 10 strains of *S. plymuthica* as a biocontrol agent to several plant pathogens. But so far, *S. plymuthica* was not reported as biocontrol agent for *C. gloeosporioides*. Based on this, it is the first time that *S. plymuthica* (UBCR_12) showed antifungal activity against *C. gloeosporioides* and could be proposed as a promising novel antifungal agent for controlling anthracnose disease. However, *S. plymuthica* is known as a pathogen to human being [26], therefore the utilization of this bacteria in a living cell form has to be taken with caution. Safe applications could therefore only be done by using extracts of intra or extracellular products.

Protease and Chitinase Activity of UBCR_12 Isolate

Sharma and Tiwari [27] described that the extracellular product of *Serratia* spp. could contain several types of enzymes such as: amylase, protease, lipase, and chitinase. The antifungal function of these enzymes is to destruct fungal pathogen cell wall which is generally composed of a complex network of proteins and polysaccharides such as glucan, mannan, chitin, and cellulose [28-31]. The specific cell wall constituent are responsible for the particular functions of this structural layer. Crystalline polysaccharides (chitin and β-glucan) for example, are responsible for the rigidity and mechanical strength of the cell wall [31-33]. Although the percentage of chitin as a constituent of fungal cell wall is not as much as β-glucan, chitin is essential for cell viability for many types of fungi [34]. Since the fungal cell wall is full of proteins, protease is involved in hydrolysis of proteins in fungal cell wall into amino acids or smaller peptides as nutrients for bacterial propagation [35]. Therefore, inhibiting the growth of fungal pathogen through protease- or chitinase-based approaches would be rational. For that reason we further investigated protease and chitinase activity of UBCR_12.

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Growth condition was measured during 24 hours in order to figure out its dynamical growth phase. Our isolate achieved its lag phase after four hours, after that the log phase was achieved until 20 hours and continued with the stationary phase. The protease activity assay was performed after 18 hours during the culture (end of log phase and beginning of stationary phase). At this point, we assumed that the bacteria produce several enzymes with the highest activity such as protease. Data of protease and chitinase activity of UBCR_12 and their percent of inhibition against *C. gloeosporioides* is shown in **Table 2**.

Table 2. Protease and chitinase activities of UBCR_12.

Protease[a)]		Chitinase[b)]	
Activity (U/mL)	Percent of inhibition (%)	Activity (U/mL)	Percent of inhibition (%)
3.88±0.09	33.48±4.24	0.67±0.06	26.82±5.01

Data was obtained from 5 samples as replication for protease and 4 samples as replication for chitinase. In vitro inhibition data was taken after 7 days of treatment.

a. One unit of protease is defined as the enzyme capability to hydrolyze casein and produce 1 µmol tyrosine / minute at pH 8 (37 ^{0}C).
b. One unit of chitinase is defined as a number of units was needed to produce 1 mg N-acetylglucosamine from chitin colloidal for one hour at pH 7 (30 ^{0}C).

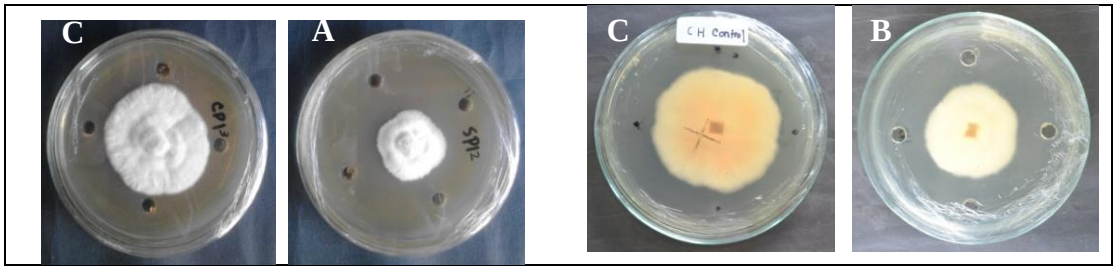

Figure 3. In-vitro inhibition assay of extracellular crude protease and crude chitinase from UBCR_12 against *C. gloeosporioides*. C: control; A: in-vitro inhibition assay by protease; and B: in-vitro inhibition assay by chitinase.

In summary, data in Table 2 and Figure 3 proved that protease activity of extracellular crude extract from *S. plymuthica* (UBCR_12) is more pronounced than chitinase activity. The data of this study describe that the growth inhibition of *C. gloeosporioides* by protease of *S. plymuthica* is more effective compared to inhibition by chitinase. In general, bacterial proteases are important enzymes in pathogenesis which attack the plasmalemma after the degradation of the cell wall [36]. Plasmalemma or plasma membrane is a fragile and thin layer inside the cell wall composed of different proteins and lipids. This layer is important because several cell wall synthesis enzymes likes chitin synthase, polysaccharide synthase, and mannosyltransferases are located here [31]. Destructing of this plasmalemma could inhibit the cell wall growth since the cell wall is critical for protection and survival of the cell in the diverse environments of fungal live [37]. Therefore, degradation of the plasmalemma will significantly inhibit the growth of the fungal pathogens as we can see in Figure 4. However, the kinetic of protease activity showed a delay in *C. gloeosporioides* inhibition for the initial 3 days. It is likely that this time is required for primary degradation of the cell wall. From that point, protease in-vitro assay indicated a clear inhibition effect on fungal growth.

Meanwhile, the inhibition effect of chitinase in this study showed a unique pattern (Figure 4). In the first day of application, its inhibitory effect was high but significantly decreasing for the second and third day. Antifungal effect was re-increasing at the fourth and fifth day. It even started to inhibit slowly at day six, but increased at day seven again. These data indicated that the inhibition effect of chitinase to *C. gloeosporioides* depends on the time of cell wall degrading. The first day application is the effective time for chitinase to degrade chitin in the cell wall since its amount is high. In the second and the third day, the fungi started to build up cell wall again as a defense mechanism, which resulted in a decrease of inhibition. Cell wall degradation by chitinase action led to a re-increasing of *C. gloeosporioides* inhibition at day four and five. This pattern was repeated at the sixth and seven day. As we know, the fungal wall is a complex structure that is typically composed of chitin, 1,3-β- and 1,6-β-glucan, mannan and proteins [38]. Chitinases activity will degrade chitin into disaccharides and larger oligometric saccharides by hydrolysis of the β-(1,4)-linkages [39]. Anita and Rabeeth [40] concluded that chitinases are able to lyse the chitin of the cell wall and provide a

carbon source, indicating that these substrates can also act as inducers of lytic enzyme synthesis. It is also responsible for distrupting the fungal cell wall and/or prevention of hyphal growth [41,42]. This enzyme may contribute to breakage and re-forming of bonds within and between polymers, leading to re-modelling of the cell wall during growth and morphogenesis [38].

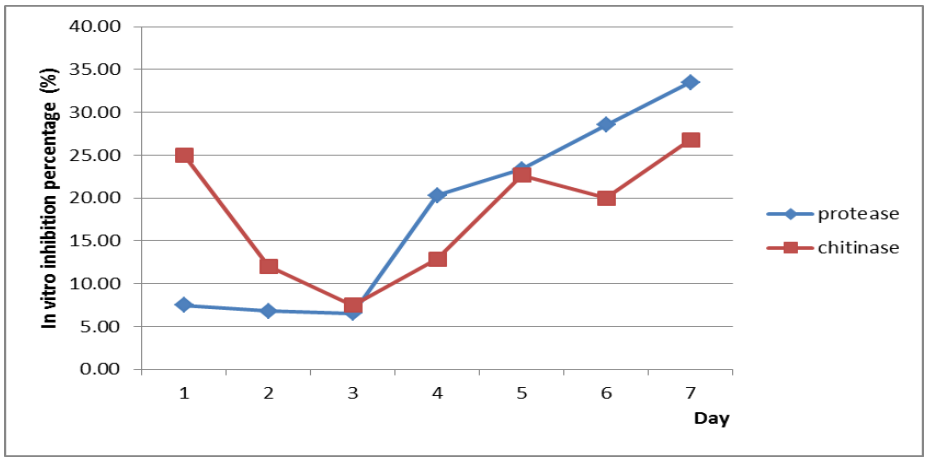

Figure 4. Graphic of in-vitro inhibition percentage of protease and chitinase from UBCR_12 to *C. gloeosporioides* for 7 days of observation.

Our study identified a highly antifungal rhizobacteria isolate and revealed *S. plymuthica* as the biocontrolling agent. We suppose that inhibition of *C. gloeosporioides* may occur due to bacterial protease and chitinase activity. In order to obtained the maximum results for in-vitro inhibition analysis, further research related to optimize media conditions like pH, nitrogen and carbon sources, and also incubation temperature for both enzymes is needed. Beside that, protease and chitinase activities of *S. plymuthica* (UBCR_12) could also describe that the growth inhibition of *C. gloeosporioides* may occur through antibiosis mechanism. Antibiosis mechanism is a inhibition of pathogen growth by metabolic compounds that are produced by biocontrol agent [43]. Hydrolitic enzymes such as protease and chitinase are two of kind metabolic compounds by antagonistic bacteria. Both of these enzymes, protease [44] and chitinase [45] functions were well known as effective enzymes against plant pathogens. Overall, it is still possible that other enzymes, antibiotics, and also other antifungal compounds were included in the extracellular supernatant. A further investigation will be needed to characterize putative additional compounds. It is useful to understand the comprehensively antibiosis mechanism that occurs between *S. plymuthica* UBCR_12 to *C. gloeosporioides*.

CONCLUSION

In this present study, it has been found that *Serratia plymuthica* (UBCR_12) is useful as biocontrol agent for *Colletotrichum gloeosporioides*. It produces protease and chitinase that well known as hydrolytic enzymes with their each activities are 3.88 U/mL and 0.67 U/mL respectively. The percent inhibition of protease to *C. gloeosporioides* (33.48) is higher than the chitinase (26.82), indicating that the protease in this study is more effective than the chitinase. Based on the hydrolytic enzymes assay, we concluded that the growth inhibition of *C. gloeosporioides* by *S. plymuthica* UBCR_12 may occur through antibiosis mechanism.

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