Biochemical and Pathogenic Potential

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Original Research Article

Biochemical and pathogenic potential characterization of *Serratia plymuthica* UBCR_12 as promising biological agents for controlling *Colletotrichum gloeosporioides*

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Abstract

In effort to develop bacterial based biofungicide, a rhizobacterium *Serratia plymuthica* UBCR_12 showing antifungal activity against phytopathogen *Colletotrichum gloeosporioides* was successfully isolated. Prior to its mass application its safety aspect to the human being and environmental factors has to be characterized in detail as well as its efficacy. For this purpose, biochemical characterizations of the isolate involving chitinolytic activity assay based on colloidal chitin degradation, siderophore production assay using CAS medium, and hemolytic test to identify its pathogenicity potential was performed. Its antifungal activity based on its capability to inhibit mycelial growth of fungus finally was determined. The results showed that optimal chitinolytic activity (1.63 ± 0.15) of the isolate was at pH 7.0, temperature 30 °C. The isolate showed a typical response to substrate concentration 2% (w/v) of colloidal chitin. Furthermore, the isolate showed a positive reaction with CAS medium and could lyse red blood cell on the blood agar. This result will imply to mass production and future application of *S. plymuthica* UBCR_12 as a biocontrol agent for *C. gloeosporioides*. Hence precaution to the human health and environmental safety has to be taken into consideration for its application.

Keywords: *S. plymuthica* UBCR_12, Chitinase, Hemolysis, *C. gloeosporioides*

Introduction

Rhizosphere is soil-plant root interphase and thus in practice consists of many rhizobacterial populations. The significance of some rhizobacteria competence as biocontrol agent has been widely considered. Our previous study showed that a rhizobacterium species designated as *Serratia plymuthica* UBCR_12 could be a promising agent in controlling *Colletotrichum gloesporioides*, an anthrachnose causing agent in chilli pepper cultivation (Syafriani et al., 2016). Further analysis indicated that the *Serratia plymuthica* UBCR_12 also showed high suppression activity against *Sclerotium rolfsii* and *Fusarium oxysporum* isolated from soybean (Aisyah et al., 2016).

One of the interesting characters from our above isolate is its chitinolytic activity. Chitinolytic activity is connected with capability in degrading cell wall of wide range phytopathogenic fungal and therefore is attracted to be considered as a biocontrol agent.

Furthermore, the need of microbial chitinase production currently has increased, and it serves two purposes; reduce environmental hazards and increase production of industrially important value-added products (Chaitanya et al., 2014).

Many chitinase-producing organisms involving actinobacteria, firmicute, and proteobacteria including the genus of Serratia have been used so far in agricultural practices as an effective biocontrol agent against a number of phytopathogenic fungi (Bhattacharya et al., 2007; Veliz et al., 2017; El Khaldi et al., 2015). Two species of the genus Serratia ie. *S. marcescens* and *S. plymuthica* have been studied extensively and offer an attractive alternative to overcome some diseases caused by a broad spectrum of phytopathogenic fungi (Iyozumi et al., 1996; Someya et al., 2000; Syafriani et al., 2016). The most recent report on *S. plymuthica* regarding its potential as a biocontrol agent against anthracnose causing agent *C. gloesporioides,* in chili pepper cultivation, was reported by Syafriani et al., (2016) and Aisyah et al., (2016).

However further analysis, particularly concerning its impact in case of direct living cell form application on health and ecological aspects has to be detailed investigated. This is of most important since many spesies of *Serratia* especially *S. marcescens* have been reported as harmful to the human being due to its hemolysis activity (Hertle and Schwarz, 2004; Krithika and Chellaram, 2016; Mahlen, 2011). The first report of the pathogenic role of *S. plymuthica* was reported by Horowitz et al., (1987) in a case of sepsis while in a case of chronic osteomyelitis was reported by (Zbinden and Blass, 1988). Furthermore, Carrero et al (1994) have reported six cases of *S. plymuthica* infection from the patients with lymphoblastic leukemia, lymphoma, and stroke.

Here biochemical characterization of *S. plymuthica* UBCR_12 claimed as promising isolate for biocontrol agent of *C. gloeosporioides* in chilli pepper is reported. This result will be very important in consideration of future application of *S. plymuthica* UBCR_12 in agricultural practices.

Materials and Methods

Chitinolytic Activity

The isolate *S. plymuthica* UBCR_12 was collected from rhizosphere of onion plant. Detail isolation procedure and characterization of the isolate has been described by Syafriani et al (2016). Chitinase activity of the isolate was determined according to the method described by Ahmed et al (2014). The isolate was inoculated on colloidal chitin agar medium pH 7.0 and incubated for 1-14 days at 30 ˚C. The screening was carried out based on chitinolytic index (ratio of clear zone and colony diameter). The colonies showing clear zones were considered as chitinase–producing bacteria.

Production of Chitinase

Fifty milliliter of LB medium, containing 2% of colloidal chitin (w/v), were inoculated with 0.5 mL suspension of isolate UBCR_12 and incubated in a rotary incubator at 160 rpm and 30 ˚C for 7 days. The culture broths were centrifuged at 14000 rpm for 30 min. The cell free supernatant was used for chitinase assay using dinitrosalicylic (DNS) acid method as described by Miller (1959) and antifungal activity test for inhibition assay to *C. gloeosporioides*.

Preparation of Colloidal Chitin

Colloidal chitin was prepared from the commercialized chitin with modification as described by Liu et al., (2015). In total of 4 g chitin powder, was slowly added with 60 mL of concentrated HCl and kept for 2 hours at 4 ˚C with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 250 mL of ethanol 50% at 4 ˚C. The suspension was collected by centrifugation at 14.000 rpm for 30 min and washed until pH of the suspension was 6.5. After the above treatment, the colloidal chitin was used as a substrate.

Siderophore Production Assay

The medium for siderophore production originally was prepared by using method described by Schwyn and Nelland (1987) and modified as described by Anand et al., (2000). For 1 L of medium, 100 mL CAS-FE-HDTMA dye was mixed with 900 mL of growth medium.

Pathogenicity Test (Hemolytic Test)

Medium for pathogenic test consisted of LB agar medium containing 5 % sheep blood. The medium was inoculated with the isolate of bacteria for 48 h at 30 ˚C. The colonies showing colorless zones were considered as hemolytic (Islam et al., 2012).

Antifungal Activity

Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of

actively growing bacteria (Nochure et al., 1993). The data were expressed by percentage of difference between the fungal growth without crude extract application as a control and fungal growth with addition of crude extract of chitinase extracellular from the isolate *S. plymuthica* UBCR_12.

Results and Discussion

Chitinase Activity

The effect of incubation time on chitinase production are shown in Fig.1. The isolate *S. plymuthica* UBCR_12 produced highest halo zone index at 10 days after incubation. Enzyme production gradually decreased after 10 days. One of the reasons for such decreased production may be the lack of nutrients or production of toxic chemicals in the medium resulting in the inactivation of secretory machinery of the enzymes (Velzahan et al., 1990).Started from 1 day after incubation until 14 days after incubation colonies of bacterium showing clear zone on chitin agar plates were regarded as chitinase-producing isolates. Clear zone surrounding the colony indicated chitinase activity that break down chitin compound in medium. The highest chitinolytic index was reached up to 1.63 ± 0.15 by the 10 days incubation. Mode of action of the antagonistic organism against various soil-borne plant pathogenic fungi and bacteria, including biosynthesis of antibiotic, production of hydrolytic enzymes (chitinase, glucanase, protease) which are responsible for the lysis and cause antifungal (Matzanke et al., 1991) and antibacterial (Syukur et al., 2017). Thus, in our study, the chitinolytic activity was determined as an important trait.

Siderophore Production

Siderophore assay of *S. plymuthica* UBCR_12 on CAS medium agar showed positive reaction, indicated with forming of large orange halo surroundings colonies (Fig. 3). Visually, this result is distinct in terms of halo formation, because there was a contrast of orange halos against the green medium. This process can be explained by the reaction between the CAS-Fe-HDTMA that contained in the medium reacting with the siderophore produced by *S. plymuthica* UBCR_12. Siderophore bonded with Fe (III) and released the dye agent to form the orange halo in the medium. The presence of HDTMA, CAS is competitive in chelating the metal below neutral pH, while ferric hydroxide seems to have a higher stability at pH values above 7.0 (Islam et al., 2012). Microorganism produces a wide range of siderophore. Most of siderophores produced by bacteria are cathecholates and some carboxylates and hydrozamates (Cornelis et al., 2010). However, there are also certain types of bacterial siderophores that contain a mix of the main functional groups (Radzki et al., 2013). Siderophore production play a direct role in promoting plant growth due to their ability to acqusition iron uptake, solubilize inorganics nutrients and responsible for iron availability. Several recent studies reported the importance of bacteria-produced siderophore in active interaction between plants and beneficial bacteria (Koo and Cho, 2009). A rhizobacterium *Serratia* sp. strain SY5 has been reported to produce siderophore and has been attributed to the plant-growth promoting effect (Jenifer et al., 2013). Siderophore enable bacteria to take up iron under conditions of limited availability of the element in the environment (Schenk et al., 2013).Siderophore also have been suggested to be an environmental friendly alternative to hazardous pesticides (Gamalero and Glick, 2011). It has been known for more than three decades that different *Pseudomonas* species can improve plant growth by producing siderophore and protecting them from pathogen (Yu et al., 2011).Some other bacteria species producing siderophores were reported by some authors. For instance *Bacillus subtilis* that also producing siderophore has been used as biocontrol agents for *F. oxyporum*, a pathogen causing the Fusarium wilt of pepper (Yu et al., 2011). Ahmed et al., (2014) also reported that *Bacillus thuringiensis* strain C110 has been used to control *Rhizoctonia solani* 21E. They reported also that the produced shiderophore had high affinity to chelat Fe (III) from soil and thereby cause negative effect to the growth of fungal pathogen.

Hemolysis Assay

Incubation of isolate *S. plymuthica* UBCR_12 on LB agar medium containing 5 % sheep blood for 48 h produced halo colorless zone (Fig. 4). The formed colorless zone related to capability of isolate to lyse the erythrocytes of sheep blood supplied in the medium (Shimuta et al., 2009). The destruction is indicated by appearing of colorless zones around the colonies.Certain bacteria species produce extracellular enzymes that lyses red blood cells in the blood agar (hemolysis). The hemolysis process move outwards from the colony causing complete or partial lysis of the red cells (RBC) in the medium and complete denaturation of hemoglobin within the cells to

colorless products (Shimuta et al., 2009; Sowemimo-Coker, 2002).Some authors reported that most of genus Serratia have a hemolytic capability and is related to cause some problem in human health. Krithika and Chellaram (2016) reported that *S. marcescens, S liquefaciens, S. ficaria, S. fonticola, S. grimessi, S. odorifera, S. quinivorans, S rubidaea and also S plymuthica* have been known can caused nosocomical infection in human. Petersen and Tisa (2013) reported that *S. marcescens* has been identified as the causing agent of various infections, such as bacteremia, pneumonia, keratis, endocarditis, urinary tract infection, meningitis and necrotizing fasciitis. Hemolytic activity of *S. marcescens* has also been reported in Hertle and Schwarz (2004) and Mahlen (2011) as a major cause of cytotoxicity against a diverse number of mammalian cell lines, including epithelia; cells and fibroblast and is a major virulence factor against both *C. elegans* and human.Recent studies reported novel proteases from *S. grimessi* and *S. proteamaculans* and these proteases play a significant role in the invasion of human larynx carcinoma Hep-2 cells. These proteases appear to disrupt the integrity of the targeted cell (Bozhokina et al., 2008; Bozhokina et al., 2011; Tsaplina et al., 2009). One characteristic of most of Serratia sp. is secretion of potent lytic enzyme, and these enzymes have been postulated play important roles in immune resisten, invasiness and destruction of competitors (Petersen and Tisa, 2013).Overall, the wide range of Serratia proteases activity enables these bacteria to escape host immune defense and also destroy certain cell types to survive and proliferate in the human host (Petersen and Tisa, 2013). The production of hemolysins plays a critical role in the selection of bacteria that applied as biocontrol agent. Due to safety and human health consideration, utilization of *S. plymuthica* UBCR_12 as a biocontrol agent should be applied not in a form of living cell. Application by using the metabolites products is recommended.

Antifungal Assay

Compared with the control, addition of crude extract of extracellular chitinase inhibited about 31.33 ± 6.12 % (Fig 4). The activity of chitinase in the crude extract of extracellular was 4.95 ± 0.042 ($U\mu g^{-1}$). Degradation of chitin as the major component of fungal cell wall by chitinolitic enzyme causes inhibition of fungal cell wall development that in turn will decrease significantly the fungal cell's life. The inhibitory activity of chitinolytic enzime against fungal pathogen is affected by proportion of chitin compounds in the cell wall. Fungal cell wall should be arranged in a scale-shape material that allow chitinase easily contact in the surface of cell wall. The higher the rate of chitin hydrolysis would come out when the chitinase contact its substrate in the fungal cell wall (Yan et al., 2008; Arlorio, 1992; Feofilova, 2001). There was a positive correlation between the inhibitory activity of chitinase against fungi and the extent of exposure of the chitin fiber bundles on the surface of the fungal cell wall (Yan et al., 2008). The activity of chitinase that degrade the polymers in fungal cell wall may has been hypothesized that they are involved as part of the plant defence mechanism against fungal pathogen (Collinge et al., 1993).Chitinase from *Pichia pastoris* also showed different antifungal activity against four fungal pathogens (Yan et al., 2008). Chitinase from *P. pastoris* could efficiently inhibited the growth of *Rhizopus stolonifer* and *Botrytis squamosa*, but no significant inhibitory effect on *Aspergilus niger* and *Pythium aphanidermatum*. Scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) observation showed that the four fungal pathogens have different surface microstructure and proportion of chitin in the fungal cell wall.

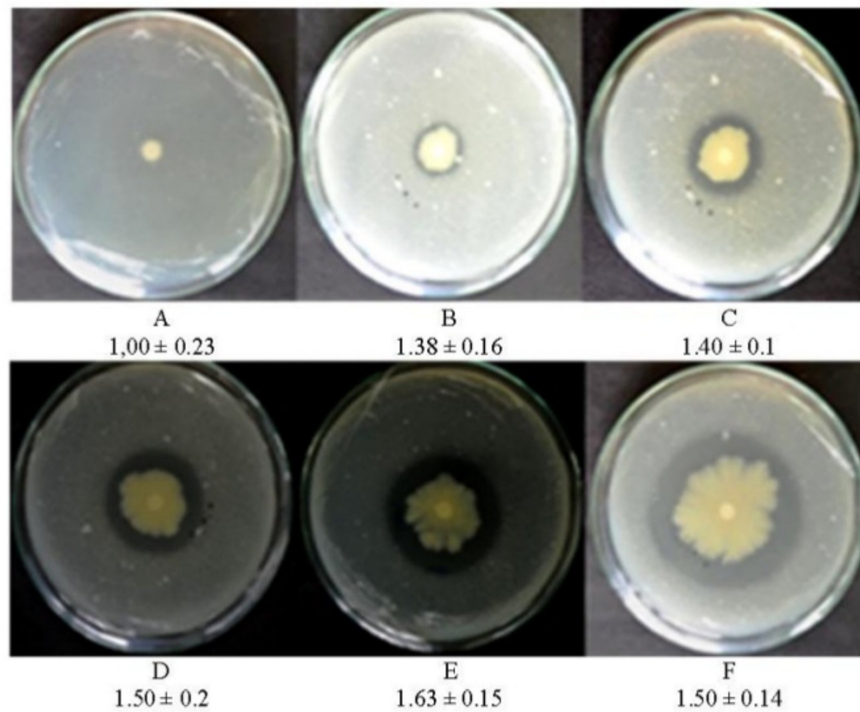

Fig.1 :Bacterial colony of *S. plymuthica* UBCR_12 showing clear hydrolysis zone on colloidal chitin, incubation 1 day (A), 3 days (B), 5 days (C), 7 days (D), 10 days (E) and 14 days (F).

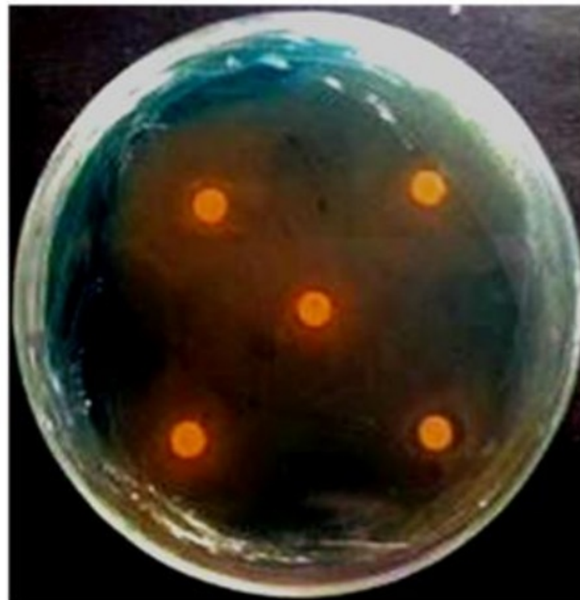

Fig. 2: Siderophore assay of *S. plymuthica* UBCR_12 on CAS medium agar

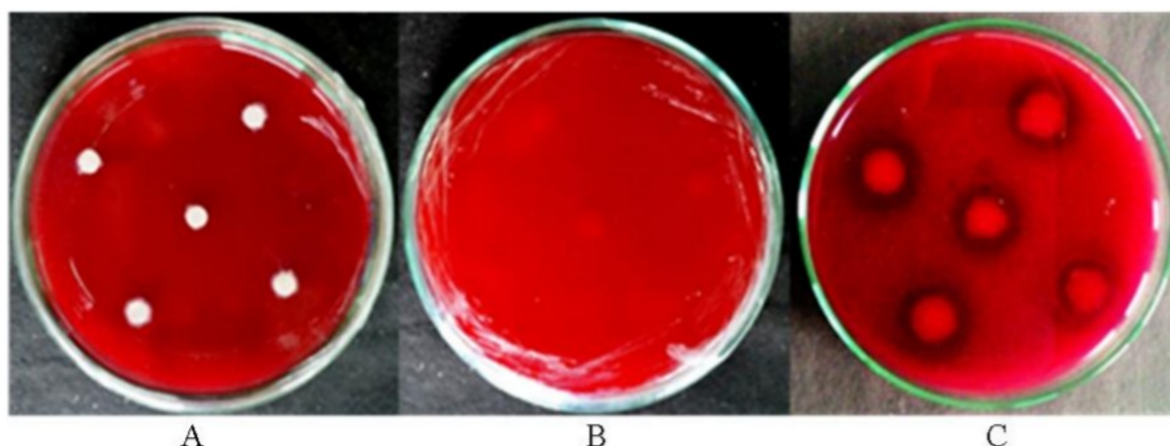

Fig. 3: **Hemolysis assay of *S. plymuthica* UBCR_12 on blood agar, A, B: 24 h incubation and C: 48 h incubation**

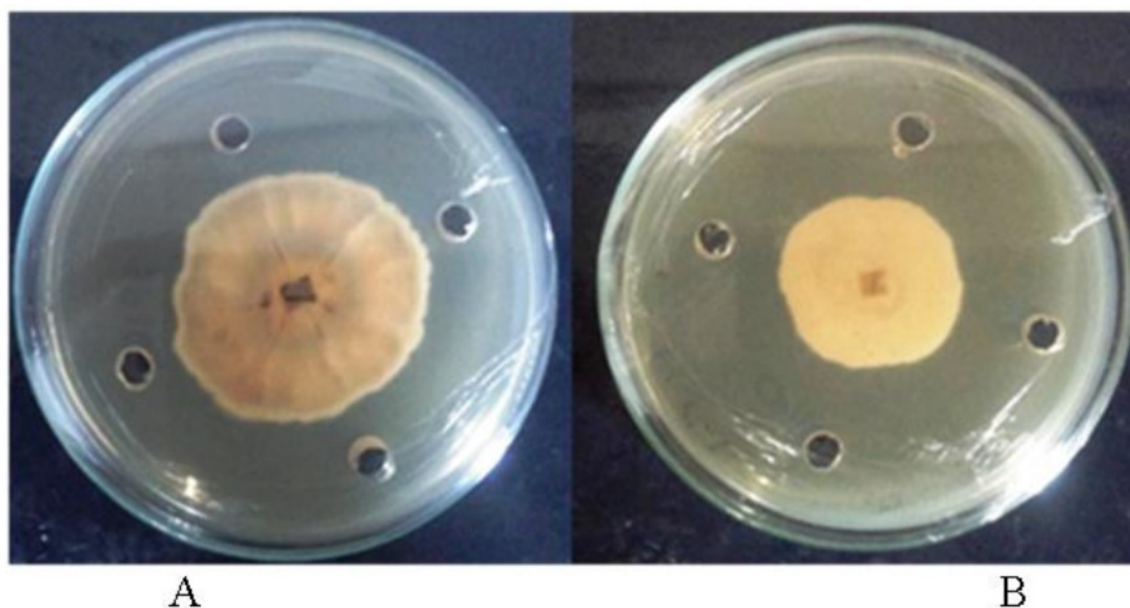

Fig. 4: **In-vitro inhibition assay of crude extracellular of chitinase from *S.plymuthica* UBCR-12 against *C. gloeosporioides*. A: Control , B: In- vitro inhibition assay by chitinase**

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