Stability assay of antagonistic activity of *Serratia plymuthica* strain UBCR_12 under various environmental factors

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Abstract. *Serratia plymuthica* strain UBCR_12 exhibited promising antifungal activity against *Colletotrichum gloeosporioides*. The development of this strain for field use requires a certain level of stability towards various environmental exposures that might occur in the natural condition. This study was aimed to evaluate the stability level of antifungal activity produced by the culture supernatants of *S. plymuthica* strain UBCR_12 under several environmental factors. Antifungal assay of this strain was performed through the application of culture supernatants under several parameters and was performed in five technical replications. Data were confirmed statistically using one-way and two-way ANOVA and significance among treatments were verified through DNMRT with a p<0.05. The antagonistic effect of this strain varied in each environmental parameter tested. The highest suppression was recorded at pH 8.0 [30.32%] after eight days of application indicating that the alkaline condition might trigger its defense mechanism. Other parameters showed quite similar inhibition efficacy, such as 24.06% [100°C for 45 minutes] and 21.15% [CaCl₂ addition]. Compared to control, this strain could suppress anthracnose-causing fungi up to 24.06% suggesting that its antifungal activity was considered as quite stable over the presence of heat and calcium.

1. Introduction

Microorganisms can secrete a variety of metabolite compounds that can interfere with or inhibit the growth and or activity of pathogenic organisms, one of which is a hydrolytic enzyme. This enzyme group functions in the process of hydrolysis of various polymer compounds, such as chitin, protein, cellulose, and hemicellulose. The ability of hydrolysis of these polymers is very useful, especially in controlling phytopathogenic fungi through the activity of damaging or destroying the structure of fungal cell walls [1].

Previous studies conducted by Syafriani *et al.* [2] successfully isolated the antagonistic rhizobacteria identified as a species of *Serratia plymuthica* strain UBCR_12. These bacteria were reported to have the ability to suppress the growth of the fungi *Colletotrichum gloeosporioides* through its protease activity [33.48%] and chitinase [26.82%] [2]. Besides, Aisyah *et al.* [3] also successfully tested the antifungal activity of the UBCR_12 bacterial extracellular compound against the same pathogenic species with an inhibition value of 30.66% [3].
The development of this strain for field use requires a certain level of stability towards various environmental exposures which might occur in the natural condition. This study was aimed to evaluate the stability level of antifungal activity produced by the culture supernatants of *S. plymuthica* strain UBCR_12 under several environmental factors.

2. Materials and methods

2.1. Origins and culture conditions of bacterial and fungi isolates

The *Serratia plymuthica* strain UBCR_12 and *Colletotrichum gloeosporioides* [isolated from chili pepper] were obtained from the internal collection of the Biotechnology Laboratory of Andalas University. *Serratia plymuthica* strain UBCR_12 was isolated from a healthy onion plant [*Allium cepa*] rhizosphere. The bacteria were grown on Nutrient Agar [NA] medium for 24 h at 30°C in darkness. Fungal mycelium was grown on Potato Dextrose Agar [PDA] medium for 7 days at room temperature in darkness. Luria Bertani [LB] broth was used as seed liquid culture medium and Potato Dextrose Broth [PDB] medium was modified by adding 1% [w/v] peptone was used for extracellular compounds production.

2.2. Stability assays

In this assay, *S. plymuthica* strain UBCR_12 was cultured in 50 mL LB broth medium at pH 7. The culture was incubated for 16 h at 80 rpm and 29°C until the optical density [OD600 nm] had reached a level 1.0. Cells were harvested by centrifugation at 14,000 rpm and 4°C for 15 min. The pellet is equalized by the standard solution Mc. Farland 1.0 [1x10^8 cell/mL suspension] using sterile aqua dest. Furthermore, 5 mL bacteria suspension were cultured in PDB modified medium at pH 7 and grown for 24 h under shaking condition at 100 rpm and 29°C. The supernatant culture was collected by centrifugation [14,000 rpm for 15 min at 4°C] and then filtered using a syringe filter with a pore size of 0.02 μm to obtain cell-free extracellular compounds. Subsequently, this filtrate was treated at several high-temperature levels [50, 60, 70, 80, 90 and 100°C] over the time ranging from 15, 30, 45 and 60 min.

The application of pH treatment is done by adjusting the pH of the modified PDB medium starting from pH 5, 6, 7, 8, and 9 through the addition of a solution of HCl [to decrease pH] and NaOH [to increase pH]. Meanwhile, metal ions Fe^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+} in the form of FeSO_4, MgSO_4, CaCl_2, and MnSO_4 compounds were added to the modified PDB medium as much as 1 mM before the medium was used for bacterial culture UBCR_12. The treatment of adding metal ions was applied using the optimum pH conditions that have been obtained previously.

2.3. Antagonistic assays

The antagonistic assay was performed by the agar diffusion method using a cell-free culture supernatant [4]. Mycelia of *C. gloeosporioides* [0.5 cm x 0.5 cm] were cultured on PDA medium at pH 7 for two days at room temperature. After two days incubation period, each 50 mL cell-free supernatant filtrate was applied to four agar well plates with 3 cm away from the center of fungal growth. The plate was subsequently incubated at room temperature for eight days and observed as routinely. The fungal culture without cell-free extracellular compounds application was used as a control. Each treatment was performed in five replicates.

2.4. Data analysis

The observation was performed in this study is the inhibition percentage [%]. Inhibition was measured daily using this following formula [5]:

\[
\text{Inhibition (\%)} = \left(\frac{DC - DT}{DC}\right) \times 100
\]
Where:
DC is the diameter of untreated fungi spot
DT is the diameter of fungi spot treated with extracellular compounds.

Data were confirmed statistically using one-way and two-way ANOVA and significance among treatments were verified through DNMRT with a p<0.05 [6]. The experimental design used was a factorial completely randomized design. Significant differences from each treatment group were further processed using Duncan’s New Multiple Range Test with a p<0.05.

3. Results and discussion

3.1. Interaction between temperature factor and heat duration factor on extracellular compound antagonist activity UBCR_12

Referring to the interaction of each factor, there are significant differences in the inhibition effect both from temperature and duration factor. However, at the same temperature level, the highly significant differences in suppression effectiveness in each heating duration are only seen at 90 and 100°C [Table 1]. Meanwhile, significant differences inhibition showed by other temperature levels is not so far in terms of values obtained. Even at 60°C, differences inhibition in each heating duration was not significantly different [Table 1]. Conversely, in terms of the heating duration factor, the effectiveness of the resulting suppression at each temperature level shows highly significant differences [Table 1].

Table 1. Inhibition of extracellular compounds of UBCR_12 at 8 DAT post-heating at several temperatures level and heating duration. The letter notation was obtained based on the two-way ANOVA result followed by the post hoc test using Duncan’s New Multiple Range Test [DNMRT] with a p<0.05.

| Temp [°C] | Heating Duration [minute] |
|-----------|---------------------------|
|           | 15 | 30 | 45 | 60 |
| 50°C      | 19.83 ± 6.05 b | 0.83 ± 1.76 a | 2.44 ± 4.87 a | 15.66 ± 7.3 b |
|           | D  | A  | A  | B  |
| 60°C      | 8.00 ± 3.71a   | 11.59 ± 3.44 a | 12.24 ± 3.19 a | 11.40 ± 4.68 a |
|           | B  | B  | C  | B  |
| 70°C      | 9.91 ± 2.51b   | 3.65 ± 4.97 a  | 6.60 ± 3.47 ab | 15.30 ± 1.58 c |
|           | BC | A  | AB | B  |
| 80°C      | 0.00 ± 0.00 a  | 4.81 ± 0.96 b  | 7.21 ± 2.47 b  | 1.57 ± 2.64 a  |
|           | A  | A  | B  | A  |
| 90°C      | 0.00 ± 0.00 a  | 18.89 ± 3.78 b | 18.54 ± 3.52 b | 15.04 ± 6.07 b |
|           | A  | C  | D  | B  |
| 100°C     | 12.86 ± 1.74 a | 17.33 ± 3.25 b | 24.06 ± 0.53 c | 22.84 ± 1.99 c |
|           | C  | C  | E  | C  |

Numbers followed by the same small letter on the same rows are not significantly different. Numbers followed by the same capital letters in the same columns are not significantly different.

The results of statistical analysis, temperature factor and the duration of heating proved to have an interaction that further affected the antagonistic activity of the UBCR_12 extracellular compound. As shown in Figure 1, it appeared that the interaction between the two factors can be either positive or negative. Positive interaction will show a synergistic effect that encouraged an increase in the
antagonistic ability of the extracellular compound. Conversely, negative interaction will result in inhibition of even the loss of antagonistic activity of the extracellular compound.

The synergistic interactions in this study are evident at temperatures of 60, 80, 90 and 100°C where the increase of heating duration increased the effectiveness of suppression of the extracellular compound [Figure 1]. However, the antagonistic activity of these extracellular compounds tends to decrease after heating for 60 minutes. Meanwhile, two other temperature levels [50 and 70°C] show an uncooperative interaction because the extension of the incubation period caused decreasing in antagonistic activity [Figure 1].

Reviewed from the magnitude of inhibition value, the resulting suppressed is still low despite the interaction between the two factors showing a synergistic effect. This indicated that these bacterial extracellular compounds tend to be less stable at certain temperature levels and incubation periods. The low stability may be due to the characteristics of the compounds contained in the extracellular compound. Since the applied extracellular compound is still a mixture of various compounds, it is difficult to determine which specific compounds act as compounds directly related to the bacterial antagonistic activity UBCR_12.

Figure 1. Illustration interaction between time factor and incubation temperature [a] and temperature factor with incubation time [b] on the effectiveness of fungal suppression C. gloeosporioides by S. plymuthica strain UBCR_12. Graphs were obtained based on statistical analysis results using SPSS version 16.0.

The thermal stability tested was performed on the compound mixture as well as this study has been reported by Le lay et al [7] that tested the antifungal activity of the lactic acid bacteria Leuconostoc citreum L123 supernatant. The supernatant antifungal activity is known can be conserved post-heating at 90°C for 20 minutes. In another study, the antifungal compound extracted from B. subtilis strain B154 was also reported to be stable at 90°C [8]. Korenblum et al. [9] also suggested the stability of antimicrobial compounds of B. licheniformis T6-5 at 100°C for 1 hour and even after autoclaved with 121°C for 15 minutes. Besides, the antifungal activity of cell-free cultures of Lysobacter enzymogenes OH11 bacteria is also retained to temperatures of 100°C and 121°C [autoclaved] so that it is still able to degrade Fusarium graminearum PH1 hyphae [10].

In contrast to this study, thermal stability testing in several studies is generally was performed post-stage purification of specific compounds. It is intended that the information obtained is specific to one type of compound and minimizes the risk of interference from other compounds having different stability levels. In a study, purified chitinase enzymes were purified from Bacillus sp. R2 can maintain its antifungal activity up to 30% after being heated at 60°C. However, the optimal activity of this enzyme is obtained at a temperature of 40°C which reached 100% and is relatively stable after one
hour of heating [11]. Meanwhile, other studies also reported that the thermal stability of pure chitinase enzymes derived from the same bacterial species can last for 5 hours at 70°C and one hour at 80°C with 100% optimal activity at 65°C. This capability is related to this bacterial habitat was isolated from the Tompaso Hot Spring in North Sulawesi [12]. In addition to chitinase, protease enzymes from some bacterial species such as *Staphylococcus sp.* and *Streptomyces flavogriseus* are also relatively stable in activity at temperature range 40-60°C [13] [14].

The scientific behavior and habitat of the producing microorganisms of a compound are many factors that also affect the thermal stability characteristics of the compound. Some microorganisms originating from hot springs are known to survive at high temperatures as described previously [12]. Another example, chitinase produced by the mushroom *Microbispora sp.* V2 from 51°C hot spring in Mumbai, India. The activity of chitinase is optimal [100%] at 60°C. The enzyme is also tolerant of a highly acidic state, where its optimum pH at 3 pH [15].

Other studies have also reported on chitinase that are tolerant of high temperatures where activity reaches 100% at 70°C. Besides, that enzyme is also highly tolerant of high salt levels. This is because the bacterium *Planococcus rifitoensis* producer of thermophilic chitinase is isolated from the saltwater lake [16]. Based on these explanations, it can be concluded that the sensitivity of UBCR_12 bacterial extracellular compounds to exposure to high temperatures due to the habitat of the producing bacteria is not from a high-temperature environment. Bacteria *S. plymuthica* strain UBCR_12 was isolated from the rhizosphere onion [*Allium cepa*] [1]. Rhizosphere is a relatively rare ecosystem to get high-temperature exposure. Therefore, the bacteria living in these ecosystems tend to have no resistance to high temperatures [17].

3.2. Variation of the stability level of antagonistic activity of extracellular compounds of UBCR_12 at high-temperature levels

The extracellular compound of UBCR_12 bacterial exhibited varying levels of stability of antagonistic activity at each high-temperature level and incubation periods [Figure 4]. The tendency is also shown patterns that are not in line between one treatment with other treatments. At the same temperature level, the compressive activity generated from each incubation period tends to fluctuate. Some temperature levels show an increase in drag in longer incubation periods, whereas, in a shorter incubation period, the inhibition is lower.

Referring to Figure 2, the highest inhibition is generated from the incubation treatment at 100°C for 45 minutes at 24.06%. Meanwhile, these extracellular bacterial compounds lose their antifungal ability at lower temperature levels, i.e., 80 and 90°C after being heated for 15 minutes [Figure 2]. However, in longer incubation periods at these temperatures, extracellular compounds still exhibited antifungal activity. At a temperature of 90°C for example, the antagonistic activity produced after high-temperature applications during longer incubation periods [30-60 minutes] is capable of reaching a range of values between 15 and 19% [Figure 2].

Different activity dynamics are also seen in temperature treatments of 50 and 70°C. These two temperature levels have the opposite pattern when compared to other temperature levels. The antagonistic activity generated at the 50°C temperature level was quite high when incubated for 15 minutes, but decreased dramatically in the incubation period of 30 and 45 minutes [Figure 2]. However, its antagonistic activity again increased when heated for 60 minutes. The same is true at the 70°C temperature level where the inhibition increase in the 60 minute incubation period is capable of exceeding the post-incubation inhibition of 15 minutes [Figure 2].

Based on the results obtained, it is suspected that the antifungal compounds contained in the extracellular compound of UBCR_12 bacteria belong to a compound that is not heat resistant. Nevertheless, the highest inhibition generated from this study is still higher when compared with Fuaddi’s [18] research results. The highest inhibition effect of the UBCR_12 extracellular compound which was not treated with heat only reached 20.78% [18], while the highest inhibition in this study was 24.06%. However, the compressive effectiveness of these bacterial extracellular compounds was able to achieve a figure of 30.66% in unmodified media and without treatment of high-temperature
applications [19] as was done in this study [18] [19]. Referring to these results, the high-temperature applications provided in this study have not been able to increase the effectiveness of suppression of extracellular compounds of UBCR_12.

**Figure 2.** Variations in stability levels of antibacterial activity of UBCR_12 bacterial extracellular compounds at various temperature levels and incubation periods after 8 days post-application. The data shown are the average of five replications \([n = 5]\). Error bars represent standard deviation values. The significant difference between treatments according to DNMRT analysis at a 5% real level is indicated by a *.

The thermal resistance characteristics of an antifungal compound should be known. The ability to tolerate the temperature factor in the natural environment is very important for the antifungal compound as the active compound used in agriculture. It is useful in commercial applications, where the compound is expected to be thermostable [20]. Also, the thermal stability of an enzyme is needed in industrial processes, it is because they use enzymes in more extreme environmental conditions is often desirable [21], [22]. The high temperature in the production process is beneficial to factors such as substrate solubility and product, viscosity, processing velocity and percentage of microbial contamination [21].

The thermal stability of a compound is influenced by the type or composition of the compound itself. Microorganisms can synthesize various compounds as secondary metabolite products such as proteins, enzymes, biosurfactants, antibiotics, and others [1]. Proteins and enzymes are less stable molecules. Therefore, the molecule is easily denatured by various environmental factors such as temperature, pressure and pH change. At high temperatures, many enzymes become inactive, which is because the protein molecules in the enzyme are partially unfolded or do not form folds so the enzyme is unable to perform the desired task [1]. However, some additives are known to stabilize proteins from stress to environmental factors. Proteins binding to compounds such as glycerol, sugars, amino acids, and certain salts are reported to be retained to denaturation of environmental factors [23].

### 3.3. Effect of pH and addition of metal ion to the antagonistic activity of UBCR_12

Differences in pH levels applied in the bacterium culture of UBCR_12 show various effects on the effectiveness of extracellular compounds in suppressing the growth of the fungus *C. gloeosporioides*. Figure 3 shows the dynamics variation of antagonistic activity produced at different pH levels. The highest inhibition was obtained from the pH 8 treatment of 30.32%, while the lowest was obtained from the pH 7 of 13.62%. These results indicate that the extracellular compound has a high level of antagonistic activity under alkaline pH conditions. These results are consistent with previous studies conducted by Kamelia [24] that *S. plymuthica* strain UBCR_12 has an optimum pH of 8 in extracellular chitinolytic production.
Figure 3. Inhibition activity of UBCR_12 bacterial extracellular produced at different levels of pH culture medium against *C. gloeosporioides*.

The importance of proper pH conditions when producing antifungal compounds produced by bacteria is very important, especially during the bacterial culture process. This is consistent with the statement of Joshi *et al.*, [25] who reported that the conditions of the bacterial growth environment such as during culture and the length of the incubation process greatly influenced the antimicrobial activity produced by the bacteria. The results of his study showed that the pH conditions and the length of incubation time resulted in different levels of emphasis on fungal growth.

Research conducted by Zhao *et al*. [26] reported that the activity of the protease K enzyme produced by the *Bacillus* strain BH071 was able to be stable at pH 5-9 at 100°C compared to a combination of pH and several other temperatures during the production process of antifungal compounds.

In the treatment of the addition of metal ions, the optimum pH conditions are used at pH 8. Figure 4 shows variations in the dynamics of antagonistic activity resulting in the addition of several different types of metal ions. All metal ion treatment shows that the inhibitory power generated continues to increase from 6 days after application to 8 days after application. The highest inhibitory yield was resulted from the treatment with the addition of CaCl₂ of 21.15%, while the lowest was the addition of MgSO₄ which was equal to 13.01%.

Referring to the lower inhibition percentage of other metal ion treatments compared to CaCl₂ treatment, it can be said that CaCl₂ acts as an activator while MnSO₄, FeSO₄ and MgSO₄ act as inhibitors. Metal ions with a concentration of 1 mM can act as activators or enzyme inhibitors. Activators are chemical compounds that function to activate substrate enzyme complexes [27]. Metal ions are needed in the form of monovalent and divalent cations as activators to increase activity on certain enzymes. But these ions can also act as inhibitors at certain concentrations. Suryadi *et al.*, [28] described that Ca²⁺ and Mg²⁺ ions can increase chitinase activity. Ions K⁺, Na⁺, Mn²⁺, Zn²⁺, Cu²⁺, Fe²⁺ and EDTA with concentrations of 10 mM respectively reduce the chitinase activity of *B. cereus 11 UJ*.

Metal ions greatly influence the antagonistic activity produced by extracellular compounds from *S. plymuthica* strain UBCR_12 in suppressing the growth of the fungus *C. gloeosporioides*. This is due to the hydrolysis reaction that can occur in degrading chitin compounds found in bacterial walls. Like the research conducted by Hamer *et al*. [29] who reported that metabolite compounds produced from extracellular bacteria such as the chitinase enzyme are enzymes that catalyze the hydrolysis reaction of chitin polymers.
In the hydrolysis reaction of chitin metal ions Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) form a coordination bond with amino acid residues of chitinolytic enzymes and acts as an electron acceptor [Lewis acid] so that they can interact with bases namely OH groups of water molecules. Metal ions will polarize the carbonyl substrate group and facilitate the deproteination of water molecules making it easier for hydrolysis reactions.

![Figure 4](image)

**Figure 4.** Inhibition activity of UBCR_12 bacterial extracellular produced from culture medium with the addition of various types of metal ions against *C. gloeosporioides*.

4. Conclusions
The antagonistic effect of UBCR_12 varied in each environmental parameter tested. The highest suppression was recorded at pH 8 [30.32\%] after eight days of application indicating that the alkaline condition might trigger its defense mechanism. Other parameters showed quite similar inhibition efficacy, such as 24.06\% [100 C for 45 minutes] and 21.15\% [CaCl\(_2\) addition]. Compared to control, this strain could suppress anthracnose-causing fungi up to 24.06\% suggesting that its antifungal activity was considered as quite stable over the presence of heat and calcium.

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