THE POTENCY OF CHITOSAN AS AN ELICITOR ON ANTIBACTERIAL ACTIVITY
OF \textit{Streptomyces} sp. GMR-22 AGAINST HISTAMINE-PRODUCING BACTERIA

Mohamad Aji Ikrami\textsuperscript{1}, Jaka Widada\textsuperscript{2}, Indun Dewi Puspita\textsuperscript{1} and Masagus Muhammad Prima Putra\textsuperscript{1*}

\textsuperscript{1}Fish Product Technology, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora gedung A4, Bulaksumur Yogyakarta 55281
\textsuperscript{2}Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora gedung A4, Bulaksumur Yogyakarta 55281

\textsuperscript{*}Corresponding author, e-mail: primaputra@ugm.ac.id

ABSTRACT

\textit{Streptomyces} is a Gram-positive bacteria that produces the largest secondary metabolite compounds. The results of whole-genome sequence analysis showed that \textit{Streptomyces} can carry more than 30 Biosynthetic Gene Clusters (BGC) encoding secondary metabolites that have the potential to be explored in the exploration for new bioactive compounds. However, not all BGC can be expressed in the laboratory scale and requires a specific activation method. This study aims to explore the potential of chitosan as an elicitor compound to activate and or increase the antibacterial activity of \textit{Streptomyces} sp. GMR-22 was tested against histamine-producing bacteria (HPB) \textit{Morganella morganii} TK7 and \textit{Citrobacter freundii} CK1. Chitosan was added to the fermentation medium with the final concentration of 250, 500, and 750 µg/ml while without the addition of chitosan used as control. Fermentation was carried out for 10 days at room temperature, with constant agitation 200 rpm. The supernatant was separated by centrifugation at 3500 rpm for 15 minutes, then fractionation with ethyl acetate, concentrated by vacuum rotary evaporator, and freeze-dried. The test for antibacterial activity was carried out by the microdilution method with an extract concentration of 100 mg/ml. The test results of the microdilution method showed that the addition of chitosan successfully increases the antibacterial activity with the highest activity shown by the water fraction of 250 µg/ml addition of chitosan which effective in inhibiting the growth of \textit{Morganella morganii} TK7 and \textit{Citrobacter freundii} CK1 by 97.29% and 97.92% respectively.

Key words: antibacterial, histamine-forming bacteria, elicitor, chitosan, \textit{Streptomyces} sp. GMR-22
INTRODUCTION

Streptomyces is a group of filamentous Gram-positive bacteria belonging to the Actinomycetes phylum. These bacteria can be found in nature in various types of habitats such as the sea, soil, and in symbiosis with insects, plants and marine sponges (Cheng et al., 2015). Streptomyces is one type of potential bacteria that has produced nearly two-thirds of the antibiotic compounds that have been discovered and used to date (Procópioa et al., 2012). Several types of secondary metabolite compounds produced by Streptomyces include antifungal, anticancer, antiviral, and other antibiotics (Azerang & Soroush, 2017). One of the Streptomyces species that has a large potential for bioactive compounds production is Streptomyces sp. GMR-22 which was isolated from soil samples in Wanagama Forest, Gunungkidul, Yogyakarta (Nurjasmi et al., 2009). Several studies have been conducted to detect the presence of bioactive compounds produced by Streptomyces sp. GMR-22 includes antifungal activity (Alimuddin et al., 2011) and antiviral (Mentari et al., 2019).

The secondary metabolite compounds that can be produced by Streptomyces are encoded by various types of protein coding genes that are located close to each other. These gene groups are called gene clusters and are located on chromosomes in the cell nucleus. Based on the analysis of genome mining using the AntiSMASH 3.0 program conducted by Herdini et al., (2017), Streptomyces sp. GMR-22 was reported to have around 63 types of biosynthetic gene clusters. However, Abdelmohsen et al. (2015) explained that the major gene clusters encoding secondary metabolites of this bacterial group were a little difficult to express under laboratory conditions. Streptomyces coelicolor A3 (2) was reported to have 29 types of BGC (Bentley et al., 2003), but only 6 types of BGC were expressed on a laboratory scale (Rutledge & Challis, 2015). The other gene clusters that are not expressed named as silent or cryptic. Several strategies can be done to activate the silent gene cluster includes co-culture (Onaka, 2017), heterologous expression (Matsuda et al., 2017), ribosome engineering (Thong et al., 2018) and elicitation (Tyurin et al., 2018). Elicitation is one of the silent gene cluster activation methods by adding elicitor compounds to influence the expression of secondary metabolites. Elicitor is a compound that has the ability to stimulate changes in the production of secondary metabolites in cells (Abdelmohsen et al.,
Several elicitor compounds reported have the potential to stimulate the expression of secondary metabolites by bacteria, namely ethanol; dimethylsulfoxide (DMSO) (Pettit, 2011); N-acetylglucosamine (NAG) (Rigali et al., 2008; Nazari et al., 2013); and chitin (Nazari et al., 2013).

Nazari et al. (2013) reported that the addition of chitin and N-acetylglucosamine to the Streptomyces coelicolor A3 (2) culture resulted in an increase in the expression of antibiotics encoded by the Act, Red, Cpk cluster genes. Moreover, Rigali et al. (2008) also reported an increase in the expression of the production of the antibiotics undecylprodigiosin (Red) and actinohordin (Act) in the culture of Streptomyces coelicolor A3 (2) supplemented with N-acetylglucosamine. However, research related to the application of chitosan as an elicitor compound to influence the expression of BGC in the production of Streptomyces secondary metabolites has not been reported. Chitosan is a chitin derivative compound that has undergone a deacetylation reaction.

Despite of the already reported bioactive compound produced by Streptomyces sp. GMR-22, information regarding the antibacterial activity has not been done yet. This study aim to observe the potency of chitosan as an elicitor compound to increase and or activate Streptomyces antibacterial compounds. Antibacterial test is carried out on histamine-producing bacteria (HPB) which is one of the pathogenic bacteria in fishery products. HPB produce histamine compounds that is toxic at certain concentrations. Apart from being able to trigger the formation of histamine, this group of bacteria is also reported to have various types of virulence factors that can be dangerous. Citrobacter freundii as one of HPB is also reported to cause food poisoning and diarrhea and can produce several toxic compounds such as Shiga-like toxins and heat-stable toxins (Liu et al., 2020).

MATERIAL AND METHODS

Material

Autoclave (Hirayama HVE-50), oven (Eyela NDO-451SD), orbital shaker (Ohaus), showcase chiller (Sanyo SR-D180F), freezer (Sanyo MDF-U3333), vortex mixer (Ohaus), single beam spectrophotometer (Hanon 13 UV / Vis), hot plate stirrer (Labnet PC-420D; Thermo Scientific SP88857107), micropipette (Ohaus), centrifuge (Corning 6766-HS), rotary evaporator, analytical balance (Shimidzu BX-320D) micropipet, microtube, magnetic stirrer, petridish, inoculation
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Methods

Isolate refreshment

Isolate *Streptomyces* sp. GMR-22 from glycerol stock stored in frozen conditions at -23°C was inoculated on yeast extract malt extract agar medium (yeast extract 0.4%; malt extract 1%; dextrose 0.4%; and agar 2%) and incubated at room temperature for ± 5 days. The histamine-forming bacteria (BPH) used were *Citrobacter freundii* CK1, and *Morganella morganii* TK7 from glycerol stock stored in frozen conditions at -23°C inoculated on tryptone soy agar (TSA) medium then incubated at 37°C for ± 24 hours.

Preparation of chitosan solution

Preparation of chitosan stock solution was carried out by dissolving 0.5 g of commercial chitosan powder into 100 ml of glacial acetic acid solution (1% (v/v)), then the solution was homogenized for ± 1 hour. The chitosan solution was then sterilized using an autoclave at a temperature of 121°C for ± 10 minutes. The chitosan is then stored in the refrigerator at 4°C.

Preliminary research

The preliminary research was done to observe the minimum inhibitor concentration (MIC) of chitosan to *Streptomyces* sp. GMR-
The concentration of chitosan used in this preliminary study refers to the research of Ghinet et al. (2010) which has been modified in chitosan concentrations of 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml, and without chitosan as control. The inoculum concentration used was 1% (v/v) with the final volume of the medium around ± 5 ml. The medium was then incubated using an orbital shaker at room temperature with an agitation of 200 rpm for ± 1 day. Optical Density (OD) of each treatment replication was observed using a spectrophotometer with a wavelength of 600 nm.

Production and isolation of secondary metabolite

Single colony of *Streptomyces* sp. GMR-22 was pre-cultured on tryptic soy broth (TSB) medium with a volume of ± 7 ml and incubated at room temperature for ± 2 days in an orbital shaker with an agitation speed of 200 rpm. Fermentation process was done by inoculating 1% (v/v) pre-cultured inoculum in 250 ml flasks containing 100 ml of starch nitrate broth (SNB) medium. Each treatment was added with a sterile chitosan solution with varying final concentrations of chitosan 250 µg/ml, 500 µg/ml, 750 µg/ml, and 0 as a control (E250, E500, E750 and E0).

Kanamycin (50 mg/ml) was used as positive control, while sterile TSB medium used as negative control. The medium was then incubated using an orbital shaker at room temperature with an agitation speed of 200 rpm for 10 days. The extraction of secondary metabolites from the fermentation process was carried out by transferring the culture into a 50 ml falcon tube. The sample was then centrifuged at 3500 rpm for 15 minutes to separate the pellets and supernatant. The supernatant was then transferred to a separating funnel, then extracted using ethyl acetate solvent with a ratio of 1: 1 (v/v), then shaker for ± 25 minutes. The extract was then separated into two types of fractions, water fraction and ethyl acetate. The sample extract was put into a rotary evaporator, the water temperature in the water bath was set at 40°C with a speed of 100 rpm. The sample was allowed to reach a volume of 1-2 ml, then transferred to a 5 ml vial tube. The extract will then be dried using a freeze dryer.

Antibacterial activity assay

Microdilution assay was conducted according to Balouiri et al. (2016). HPB isolate was inoculated into TSB medium with a volume of ± 7 ml incubated at 37°C for ± 24 hours. Extract the water fraction of the metabolite *Streptomyces* sp. GMR-22 was
dissolved in sterile distilled water and mixed into TSB medium with a final concentration of 100 mg / ml with a final volume of ± 1 ml. The inoculum concentration used for each BPH was 1% (v/v). The medium was then incubated at 37°C for ± 24 hours. Optical Density (OD) was observed using a spectrophotometer with a wavelength of 600 nm (OD$_{600}$) to determine the effectiveness of antibacterial activity. The antimicrobial activity was described in terms of the percentage of antibacterial effectiveness in the growth of histamine-producing bacteria between negative control and treatment.

RESULTS AND DISCUSSION

*Streptomyces* sp. GMR-22 was isolated from cajuput plant rhizosphere soils in Wanagama I Forest UGM Yogyakarta (Nurjasmi *et al.*, 2009). The strain GMR-22 was identified afterward by Alimuddin *et al.* (2011) by using 16S rRNA primer pairs 27f (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492r (5’-GGTTACCTTGTAGCGACTT-3’) and reported as the isolate which possessed the strongest antifungal activity. In this study, we aims to continue the exploration of bioactive compounds produced by *Streptomyces* sp. GMR-22 and focused on the antibacterial activity against histamine-producing bacteria (HPB). In the same time, we also want to explore the potency of chitosan (chitin derivate) as elicitor compounds.

**Preliminary test**

Determination of the concentration of chitosan that will be used as a treatment for the fermentation of *Streptomyces* sp. GMR-22 was done through a preliminary test. Based on Table 1, it can be observed that *Streptomyces* sp. GMR-22 grown on TSB medium with the addition of chitosan treatment 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, and control gave OD$_{600}$ values in the range of 2.414 - 2.523. The OD$_{600}$ value tends to be high and indicates that the environmental conditions of the medium in the treatment of adding chitosan 10 µg/ml to 250 µg/ml still provide optimal environmental conditions for the growth of *Streptomyces* sp. GMR-22. Based on Table 1, the addition of chitosan which is first dissolved using 1% glacial acetic acid still provides an optimal pH conditions for the growth of *Streptomyces*. This results was in accordance with Kontro *et al.* (2005) which reported that several types of *Streptomyces* grown on various types of medium able to grow at pH conditions range from 4 - 11.5.
In addition, it is also assumed that there has been a change in pH during the mixing process of chitosan solution with TSB medium which affects the protonation mechanism of chitosan, which results in weakening of the antibacterial activity produced by chitosan. Chitosan has a pKa value of around 6.2 - 7.0 which causes chitosan to dissolve in dilute organic acids (Zargar et al., 2015). In this condition, the chitosan amine group will undergo protonation so that it is positively charged to become NH₃⁺ ions which will affect the antibacterial activity of chitosan (Zargar et al., 2015). Increasing the pH value due to mixing chitosan with TSB medium resulted in chitosan not being protonated, so that the antibacterial activity of chitosan did not tend to affect the growth of *Streptomyces* sp. GMR-22. *Streptomyces* is also reported to have the ability to utilize chitin and its derivatives as a nutrient source by producing chitinase and chitosanase. *Streptomyces* is able to produce the chitosanase which has a role to hydrolyze the long chain of chitosan into its oligomers so that it can be transported into cells and is useful as a source of carbon and nitrogen (Ghinet et al., 2010).

Based on Table 1 the growth of *Streptomyces* sp. GMR-22 was a dramatically decrease in the addition of 500 µg / ml chitosan (OD₆₀₀ 1.129); 750 µg/ml (OD₆₀₀ 0.030); and 1000 µg/ml (OD₆₀₀ 0.072). This condition indicates that *Streptomyces* sp. GMR-22 slowly cannot withstand the antibacterial activity of chitosan which causes its growth to be inhibited. The mixing process of chitosan and TSB medium still provides a pH condition in the pKa value range of chitosan (6.2 - 7.0) which allows chitosan to undergo protonation so that it still has the ability to be antibacterial.
Table 1. Effect of chitosan concentration on *Streptomyces* sp. GMR-22 growth

| Treatment          | pH  | OD<sub>600</sub> |
|--------------------|-----|------------------|
| Control            | 7.4 | 2.523            |
| Chitosan 10 µg/ml  | 7.3 | 2.458            |
| Chitosan 25 µg/ml  | 7.3 | 2.436            |
| Chitosan 50 µg/ml  | 7.3 | 2.436            |
| Chitosan 100 µg/ml | 7.2 | 2.414            |
| Chitosan 250 µg/ml | 7.1 | 2.441            |
| Chitosan 500 µg/ml | 6.9 | 1.129            |
| Chitosan 750 µg/ml | 6.7 | 0.03             |
| Chitosan 1000 µg/ml| 6.5 | 0.072            |

Based on these conditions, the selection of the concentration of chitosan was carried out by referring to the OD<sub>600</sub> value which is considered to provide environmental conditions that can stimulate changes in the production of secondary metabolite compounds *Streptomyces* sp. GMR-22. Secondary metabolites are compounds produced by microorganisms under certain conditions in response to survival. The chitosan treatment that will be used for the main research stage is assumed to create environmental conditions that can threaten the growth of *Streptomyces* sp. GMR-22. Based on this results, chitosan concentration of 250 µg/ml, 500 µg/ml, and 750 µg/ml were further used as treatment.

**Characteristics of secondary metabolite compounds of *Streptomyces* sp. GMR-22**

The fermentation process was carried out using 3 treatments of chitosan concentration 250 µg/ml, 500 µg/ml, and 750 µg/ml which were obtained from the preliminary test and 1 control. This process was carried out for 10 days using an orbital shaker with an agitation speed of 200 rpm and incubated at room temperature. During the fermentation process, there was a color change in the SNB medium in each treatment which can be seen in Table 2. In the control
treatment without the addition of chitosan, the color changes in the medium from initially white to brownish orange. Whereas in the chitosan treatment of 250 µg/ml, 500 µg/ml, and 750 µg/ml in general, the color characteristics were not different from the respective treatments which caused the medium to turn yellowish. The change in the color of the medium is caused by the presence of secondary metabolites produced by *Streptomyces* sp. GMR-22. This color change is also caused by several factors such as temperature, pH, agitation, aeration, and fermentation time during the fermentation process.

The fractionation of secondary metabolites from *Streptomyces* sp. GMR-22 was carried out using two solvent which were water and ethyl acetate. The ethyl acetate fraction was done with a ratio of 1:1 (v/v) in constant shaking to dissolve the secondary metabolite compound which is semi-polar. The insoluble portion of ethyl acetate hereinafter referred to water fraction. The extract was then concentrated using a rotary evaporator using a temperature of 40°C in each of the water and ethyl acetate extracts. Furthermore, the extract is freeze dried to get the dry extract.

Based on Table 2, it can be seen that the appearance of ethyl acetate extract is generally similar in the whole treatment which will produce yellow to orange crystals after freeze dried with a production capacity of 0.10 - 0.33 g/l. In water extract, the resulting compound is more yellowish to brownish in color. The treatment of chitosan administration has an effect on changes in the brighter color of fermented products. This may be due to changes in environmental conditions in the SNB medium which affect the response of *Streptomyces* sp. GMR-22 utilizes available nutrients which also have an impact on the metabolites produced. The production capacity of the compounds obtained from aqueous extracts tends to be greater when compared to ethyl acetate extracts. Based on Table 2 it was shown that the water extract production capacity of the entire treatment was in the range of 12.54 - 15.27 g/l.
Table 2. The characteristics of the secondary metabolite extracts of *Streptomyces* sp. GMR-22

| Fraction   | Treatment | Weight (g/l) | Appearance          | Color                |
|------------|-----------|--------------|---------------------|----------------------|
|            | Control   | 14.97        | Brown crystalline powder | Brownish orange     |
| Water      | 250 µg/ml | 15.27        | White fine powder   | Yellowish clear      |
|            | 500 µg/ml | 12.54        | White fine powder   | Yellowish cloudy     |
|            | 750 µg/ml | 14.44        | White fine powder   | Yellowish cloudy     |
| Ethyl Acetate | Control | 0.32         | Orange crystals     | Clear                |
|            | 250 µg/ml | 0.10         | Yellow crystal      | Clear                |
|            | 500 µg/ml | 0.29         | Yellow crystal      | Clear                |
|            | 750 µg/ml | 0.33         | Yellow crystal      | Clear                |

**Antibacterial activity against histamine-producing bacteria**

The antibacterial activity of the secondary metabolite extracts of *Streptomyces* sp. GMR-22 was performed using microdilution assay with extract concentration 100 mg/ml. Antibacterial activity produced by *Streptomyces* sp. GMR-22 is shown in Table 3.

Table 3. Antibacterial activity of *Streptomyces* sp. GMR-22 on histamine-producing bacteria

| Tested strain         | Percent inhibition (%) |
|-----------------------|------------------------|
|                       | Kanamycin | Negative | E0     | E250   | E500   | E750   |
| *Morganella morganii* TK7 | 99.45a     | 0        | 75.69c | 97.29a | 87.91b | 79.61c |
| *Citrobacter freundii* CK1 | 98.01a     | 0        | 97.78b | 97.92a | 99.69a | 58.29b |

Based on the results, it can be seen that the response of each histamine-producing bacteria used as the test bacteria is different, however, all bacterial tested grew normally in negative control. Treatment E250 has the highest antibacterial
activity in every antibacterial activity test carried out on the three types of histamine-forming bacteria. E250 extract was effective in inhibiting the growth of *Morganella morganii* TK7 and *Citrobacter freundii* CK1 with inhibition percentage values of 97.29% and 97.92%, respectively and no significant different with positive control. The antibacterial activity of E250 was slightly higher compared to E0. This shows that the addition of 250 µg/ml chitosan might be stimulate *Streptomyces* sp. GMR-22 to increase the expression of genes that play a role in increasing the antibacterial activity. Based on the preliminary test conducted previously, it can be seen that the addition of 250 µg/ml of chitosan has an OD600 value of 1.129. This absorbance value indicates that the addition of 250 µg/ml of chitosan creates a stress-response condition that can be used by *Streptomyces* sp. GMR-22 to be able to grow even with stressful conditions that trigger the expression of genes that affect certain metabolite pathways.

Changes in growth environmental conditions can affect the morphology of each Streptomyces life cycle and can affect the production of secondary metabolites. The addition of chitosan dissolved using acetic acid has the potential to affect the pH conditions of the fermentation medium, besides the antibacterial activity of chitosan can cause a stress response from *Streptomyces* sp. GMR-22 which might be resulted in the response changes in gene expression produced on producing secondary metabolites during the fermentation process. Ghinet *et al.* (2010) reported that the addition of different chitosan concentrations could affect morphological changes in *Streptomyces lividans* TK24 grown on agar medium. Morphological changes in Streptomyces can affect the process of the production of secondary metabolites produced during its life cycle. Viollier *et al.* (2001) also reported that different pH conditions affect the growth and development of *Streptomyces coelicolor*. Chitosan and chitin have the same constituent components, it's just that chitin is dominated by N-acetylglucosamine, while chitosan tends to be composed of glucosamine monomers (Zerger *et al*., 2015).

To confirm that the antibacterial activity of the extract produced during the fermentation process in the addition of chitosan treatment did not come from the activity of chitosan, an antibacterial activity test of chitosan was also carried out which can be seen in Table 4.
Table 4. Antibacterial activity of chitosan on histamine-producing bacteria

| Tested strain               | Inhibitory Percentage (%) | K250 | K500 | K750 |
|-----------------------------|---------------------------|------|------|------|
| Morganella morganii TK7     | 10.54^a                   | 32.95^b | 97.95^c |
| Citrobacter freundii CK1    | 74.76^a                   | 77.18^a | 93.89^a |

When compared with the results of the antibacterial activity described in Table 3, it can be seen that the antibacterial activity produced between extracts of secondary metabolites and chitosan is not the same. This shows that the antibacterial activity of the secondary metabolite extract is not derived from chitosan, thus indicating that the addition of chitosan during the fermentation process can affect the response of *Streptomyces* sp. GMR-22 in producing secondary metabolite compounds.

CONCLUSIONS AND SUGGESTIONS

Conclusions

*Streptomyces* sp. GMR-22 has the potential to produce antibacterial compounds that can inhibit the growth of histamine-producing bacteria. The addition of chitosan had an effect on the color change and the characteristics of the extract of secondary metabolite compounds. The addition of chitosan has an effect on the antibacterial activity of secondary metabolites of *Streptomyces* sp. GMR-22 with the most effective antibacterial activity was found in E250 treatment which inhibited the growth of *Morganella morganii* TK7 and *Citrobacter freundii* CK1 with inhibition percentage values of 97.29% and 97.92%. This shows that chitosan has the potential as an elicitor compound which can influenced the metabolites produced by *Streptomyces* sp. GMR-22.

Suggestions

In this research, it is still necessary to test the characterization of the secondary metabolite extracts of *Streptomyces* sp. GMR-22 to determine the differences in the compound components of each extract produced. In addition, the volume of production of secondary metabolites of *Streptomyces* sp. GMR-22 needs to be increased so that the optimal amount of extract is obtained for activity testing using a higher concentration. The extraction process of secondary metabolites can use various
types of solvents to confirm the secondary metabolites of *Streptomyces* sp. GMR-22 which has antibacterial activity.

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