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Characterization and Identification of Secondary Metabolite Compounds from Bacteria in Symbiont with *Ascidia gemmate*

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Abstract: Isolation of secondary metabolites from bacteria that have a symbiont with Ascidians has been shown to have enormous potential. This study aims to identify the types of secondary metabolites in *Bacillus sp.* which has a symbiont with *A. gemmata*. The research was carried out using laboratory experimental methods by means of phytochemical screening. After testing, the results of the characterization of bacteria that lead to the genus Bacillus and the secondary metabolites contained are alkaloid compounds.

Keywords: *A. gemmate*; Alkaloids; *Bacillus*

Introduction

Ascidians are marine biota that belong to the phylum chordata, subphylum urochordata (Hotta et al., 2020). Marine biota with a unique position between vertebrates and invertebrates is classified into the Chordata phylum because when they are pre-adults they are tadpoles, but they become filter animals for the invertebrate group when they are adults (Abitua et al., 2012). Ascidians belong to the Ascidiacea class with approximately 3000 species, which is why the Ascidiacea class is the largest marine sessile community (Sahuma et al., 2021). Ascidian habitat depends on three environmental factors, namely temperature, saltiness of seawater, and diversity of hard substrates (Maciver et al., 2017). Some Ascidians live in colonies and some are solitary. According to Shenkar et al. (2017), in the Ascidian World Database that the ascidians of the order Aplousobranchia have a colonial way of life, the orders Phlebobranchia and Stolidobranchia have a colony and solitary way of life.

Ascidians began to be the object of research since 1847, this organism is thought to be a living place for bacterial symbionts which have pharmacological potential (Chen et al., 2019). The potential for Ascidians is very promising, but large-scale marine biota extraction activities conflict with marine conservation (Bara et al., 2015). In connection with this conflict, other alternatives are needed without wrong cultivation to isolate secondary metabolite compounds from Ascidian. Another alternative that can be used is to isolate secondary metabolites from the symbiont bacteria. Symbion bacteria are bacteria that have symbiosis with the host organism. Bacteria that are symbiotic with ascidians can be a potential source of secondary metabolites.

Secondary metabolite compounds are biosynthetically derived compounds from primary metabolites (Sumilat et al., 2018), some examples of secondary metabolite compounds are alkaloids, flavonoids, saponins and tannins. Secondary metabolite compounds in Ascidian symbiont bacteria have various biological activities. Ascidians produce antibacterial

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compounds in the form of secondary metabolites to fight all the threats they face (Opa et al., 2018).

Ascidian symbiont bacteria have the ability to synthesize secondary metabolite compounds like their host organisms (Hellio et al., 2015). Newman (2016), explained that bacteria that have symbionts with ascidians can be a source of producing secondary metabolite compounds. Several bacteria that have symbionts with ascidians are proteobacteria and cyanobacteria (Sathishkumar et al., 2018). Many references show that secondary metabolite compounds previously isolated from ascidians are very similar to secondary metabolite compounds in their bacterial symbionts. The similarity of compounds in ascidians to symbiotic bacteria is due to the presence of a single molecule that regulates biosynthesis in symbiotic bacteria (Morita & Schmidt, 2018).

Secondary metabolite compounds isolated from bacteria that are symbiotic with ascidians have been shown to have enormous potential (Asolkar et al., 2010; Olguin-Uribe et al., 1997; Socha et al., 2006). Some examples of compounds isolated from ascidian symbiont bacteria are a) Ecteinascidin 743 from ascidian symbiont bacteria of the species Ecteinascidia turbinata (Rinehart et al., 1990); Didemnin B compounds from the bacteria Tistrella mobilis and Tistrella bauzanensis which are symbionts with ascidians are very useful for the pharmaceutical world (Rinehart et al., 1981; Tsukimoto et al., 2011; Xu et al., 2012). Secondary metabolite compounds produced by bacteria that have a symbiont with ascidians have various benefits such as antibacterial (Mogea, 2015).

This research focuses on what types of secondary metabolites are contained in ascidian symbiont bacteria. The type of ascidian used in this research is Ascidia gemmata. Many studies have been carried out on the topic of the potential of ascidian symbiont bacteria as antibacterials, but until now references to the potential of secondary metabolite compounds from bacteria that have symbionts with Ascidia gemmata have never been presented.

Method

This research uses laboratory experimental methods. The research was carried out using a sequence of procedures in the form of: a) Rejuvenation of bacterial isolates; b) Characterization of bacteria; c) Identification of secondary metabolites. Identification of secondary metabolite compounds was carried out using the phytochemical screening method. The secondary metabolite compound groups tested were alkaloids, flavonoids, saponins and tannins.

Characterization of Bacteria

The characterization carried out is in physiology and biochemistry. The physiological characteristics observed were colony color, colony diameter, colony shape, colony edge, Gram reaction, cell shape, spore formation, elevation and consistency. Biochemical characteristics observed were oxidase, motility, nitrate, lysine, ornithin, H2S, glucose, mannitol, xylose, ONPG, indole, urease, V-P, citrate, TDA, gelatin, malonate, inositol, rhamnose, sucrose, lactose, arabinose, adonitol , raffinosa, salicin, arginine, catalase, coagulase, hemolysis, novoniosin sensitive test, and casein hydrolysis. Biochemical characterization was tested using the Microbact 12A and 12B Kits. The results of the characterization of the symbiont bacterial isolates will be presented in the form of Tables 1 and 2.

Identification of Secondary Metabolites

Identification of secondary metabolite compounds aims to identify groups of compounds in bacterial isolates that have symbionts with A. gemmata. The identification treatment used experimental methods, by means of phytochemical screening consisting of testing alkaloids, flavonoids, saponins and tannins.

Alkaloid Identification

Alkaloid testing was carried out using Dragendorf, Meyer, and Wagner reagents. Samples of the symbiont bacteria A. gemmata were put into four test tubes, each tube filled with 3 ml of sample. Three tubes were filled with Dragendorf, Meyer, and Wagner reagents, while one tube was left for comparison. If there is a precipitate in the three reactants, then it is positive for alkaloids.

Identification of Flavonoids

Flavonoid testing was carried out by inserting 1 ml of bacterial isolate into 3 test tubes. The first tube was added with 3 drops of concentrated H2SO4, the second tube was added with 0.5 mL of concentrated HCL and magnesium powder, and the third tube was used as a blank. If an orange color forms in the first tube, and a red to purple, green or light blue color forms in the second tube, then it is positive for flavonoids.

Saponin Identification

The symbiont bacterial isolates were added with 1 mL of distilled water and then shaken. If there is stable foam for 30 minutes, then the sample is positive for saponins.

Identify Tannins

A total of 3 ml of isolate was added with 5 drops of 10% NaCl. The sample is then divided into two test tubes. The first tube was used as a blank and the second tube was added with 1% $FeCl_3$. If there is precipitate in

the second tube, then the positive isolate contains tannin compounds.

Result and Discussion

Characterization of Bacteria

Characterization aims to identify the characteristics of bacterial isolates that have symbionts with A. gemmata. The characterization was carried out in the form of physiology and biochemistry using the microbact 12A and 12B kits. The results of physiological characterization can be seen in Table 1.

Table 1. Results of Physiological Characterization of A.gemmata Symbiont Bacterial Isolates

Characterization Parameters	Results
Colony Color	Beige
Colony Diameter	3.02mm
Colony Form	Ovals
Colony Edge	Uneven
Gram's reaction	Positive
Cell Shape	Basil
Spore Formation	+
Colony Elevation	Flat
Colony Consistency	Dry
Oxidase	Positive
Motility	Non Motile

Note: (+) = Yes

The results of the physiological characterization in Table 1 show that the bacterial isolate that is a symbiont with A. gemmata has physiological characteristics in the form of a cream colony color, a diameter of 3.02, an oval colony shape, Gram positive, has a bacillus cell shape, forms spores, and has a dry consistency. The physiological characters obtained show similarities to bacteria of the genus Bacillus (Turnbull, 1996).

The isolates also showed characteristics of the genus Bacillus because they were positively identified in tests for nitrate, glucose, mannitol, xylose, Voges Phroskauer, Arabinosa, catalase, casein hydrolysis, and starch hydrolysis which can be seen in Table 2. The study entitled antagonistic activity test of several isolates of Bacillus spp . against bacterial wilt (Ralstonia solanacearum) on several tomato varieties and their identification, wrote that 6 species of Bacillus namely 4 species of Bacillus spp., Bacillus subtilis, and Bacillus licheniformis were positive for nitrate, glucose, mannitol, xylosa, Voges Phroskauer, Arabinosa, catalase, casein hydrolysis, and starch hydrolysis (Saputra et al., 2015).

Table 2. Results of Biochemical Characterization of A.gemmata Symbion Bacterial Isolate

Parameter	Results
Nitrate	+
Lysine	-
Ornithin	-
H2S	-
Glucose	+
mannitol	+
Xylose	+
ONPG	+
Indole	-
Urease	-
Voges Praskauer	+
Citric	-
TDAs	-
gelatin	-
Malonat	-
Inositol	-
Rhamnosa	-
sucrose	-
Lactose	-
Arabinosa	+
Adonitol	-
Raffinosa	-
Salicin	-
Arginine	-
Catalase	+
Coagulase	-
Hemolysis	Beta
Novoniosin Sensitive Test	TD
Casein hydrolysis	+
Starch Hydrolysis	+

Note: (+) = Yes; (-) = None; (TD)= No Identification carried out.

The biochemical characteristics of the bacteria are Gram positive with positive catalase, rod-shaped, positive for spores, lysine, glucose, xylose, ONPG, indole, V-P, gelatin, catalase, beta hemolysis, starch hydrolysis, and casein hydrolysis predicted as Bacillus subtilis bacteria (Kurniawan et al., 2017; Mulyasari et al., 2015).

Identification of Secondary Metabolites

The results of testing alkaloid, flavonoid, saponin and tannin compounds from bacterial isolates that have symbionts with A. gemmata are presented in Table 3.

Table 3. Identification Results of Secondary Metabolites of Symbion Bacteria A. gemmate

Testing	Observation result
Alkaloids	+
Flavonoids	-
Saponins	-
Tannin	-

Note: (+++) = presence of strong compounds; (-) = no compounds

The test results in Table 1 show that the bacteria which are symbiotic with A. gemmata contain secondary metabolites in the form of alkaloid compounds, and are negative or do not contain flavonoids, saponins, and tannins. Identification of alkaloid compounds was carried out using three reactants, namely Dragendorf, Meyer and Wagner reactants. The Dragendorf reactant shows a positive result when a brownish-white precipitate forms (Raal et al., 2020), Mayer's reactant states positive when it forms a red precipitate (Goldstein & Mayer, 1958), and the Wagner reactant states positive for alkaloids when it produces a brownish red precipitate (Furr & Mahlberg, 1981). In the alkaloid test, the only reactant that causes precipitate formation is the Dragendorf reactant which is characterized by the formation of a brownish white precipitate. The use of the Dragendorf reactant showed positive results which were indicated by the presence of a brown precipitate which was a potassium alkaloid (Suteja et al., 2020). Alkaloid compounds contain nitrogen atoms which are paired with free electrons, thus forming metal ion covalent bonds (Marliana & Suyono, 2005).

Flavonoid testing was carried out using concentrated H_2SO_4, concentrated HCL, and magnesium powder. If an orange color forms in the first tube, and a red to purple, green or light blue color forms in the second tube, then it is positive for flavonoids. Flavonoid compounds are one of the active ingredients with the ability to inhibit disease-causing bacteria (Zulkifli et al., 2018). Other compounds tested were saponins and tannins. Saponins are active compounds that have a detergent-like surface, this surface causes damage to the permeability of the microbial plasma membrane (Noer et al., 2023). Meanwhile, tannins are bioactive compounds that belong to the type of phenolic compounds, with characteristics that dissolve in polar solvents and water (Muthmainnah, 2017).

After testing, the results obtained in both tubes were that there was no color change. The same case occurred in the identification of saponins and tannins. Saponin testing was carried out by adding distilled water to the sample and then shaking the sample. After testing, there is no stable foam in the sample. In the tannin test, the solution used was 10% NaCl and 1% $FeCl_3$. $FeCl_3$ is used because it will form a complex compound that is blue to black in color. After the test, there is no precipitate in the test tube. After testing for flavonoids, saponins and tannins, it was concluded that samples of bacteria that had a symbiont with Ascidia gemmata tested negative for flavonoids, saponins and tannins.

Secondary metabolite compounds are components of natural materials with low molecular mass. Secondary metabolite compounds function as compounds for protection and self-defense, resistance to disease, and act as hormones (Nugroho, 2017). According to Saifudin (2014), secondary metabolite compounds have the following characteristics: a) Not involved in the processes of growth, development and reproduction; b) secondary metabolites are only distributed in certain species; c) the absence of secondary metabolite compounds in the short term will not have any impact on living creatures, while the absence in the long term will result in a lack of protection and self-defense; d) organic compounds known as micro molecules; e) the main groups are alkaloids, polyketides, phenylpropanoids, and terpenoids; f) can be used as ingredients for cosmetics, food, and medicine by humans. Secondary metabolites are produced by almost all living things for self-defense. Based on the compound framework, secondary metabolite compounds are classified into alkaloids, flavonoids, saponins and tannins (Saidi et al., 2018).

Bacteria that have a symbiont with Ascidia gemmata showed positive results for alkaloids, this discovery shows its potential for the pharmaceutical world. Alkaloids are widely used in the treatment of cancer, pain, dementia, and various other diseases (Debnath et al., 2018). Alkaloids are a very potential source of medicinal ingredients because they have quite large therapeutic abilities (Amirkia & Heinrich, 2014). Lichman (2021), explained that alkaloids consist of true alkaloids, protoalkaloids and pseudoalkaloids. True alkaloids are complex alkaloids where the nitrogen atom comes from amino acids and are heterocyclic. Protoalkaloids or simple alkaloids are amino acid derivatives but are not heterocyclic. Pseudoalkaloids are compounds containing nitrogen introduced at a final stage through enzymatic processes such as transamination.

Conclusion

Based on the results of the study, it was concluded that the bacteria which have a symbiont with A. gemmata were identified as having a kinship with bacteria of the genus Bacillus and producing alkaloid compounds.

Author Contributions

The contributions of the three authors are as follows: N.R., contributed to data collection, data analysis, and data interpretation; R.A.M, preparing research concepts and designs; M. M., contributed to the review and editing of the manuscript draft.

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Conflicts of Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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