

EXPLORATION OF SEA CUCUMBER INTESTINAL SYMBIONT MICROBE AS PROBIOTIC MICROBE CANDIDATE IN HEALTHCARE PRODUCTS

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Abstract

Natural ingredients from animals and plants generally have potential in pharmaceutical applications. However, there still relatively little exploration made on the active ingredients from marine life. Sea cucumbers inhabit seabed and are filter feeders, consuming all microorganisms within their vicinity, which opens the prospect of medicinal applications of sea cucumber intestinal symbiont microbes. This study aims to obtain sea cucumber intestinal symbiont microbes with a potential pharmaceutical applications as probiotic microbe candidates. The study was carried out by collecting samples, microbe isolation, antimicrobial screening, identification of potential probiotic microbe candidates. Characteristics of the microbe were studied by biochemical screening and molecular identification. The study found 21 microbial isolates from *Holothuria atra* and 30 microbial isolates from *Holothuria leucospilota*. Antimicrobial activity screening results against pathogens of *Bacillus cereus* dan *Pseudomonas aeruginosa* showed that 13 microbial isolates with positive activity. Four species were identified by molecular identification, namely *Bacillus aquimaris*, *Bacillus maritimus*, *Bacillus toyonensis*, and *Virgibacillus chiguensis*, whereas the other nine were identified by biochemical screening. Four microbe genus, namely *Rothia* sp., *Listeria* sp., *Micrococcus* sp., and *Staphylococcus* sp, were found to be the most viable candidate for probiotics. It was concluded that *Bacillus* sp., *Rothia* sp., *Micrococcus* sp., and *Staphylococcus* sp. exhibited the most potential as probiotic microbes.

Keywords: Sea Cucumber, Symbiont Bacteria, Probiotic

INTRODUCTION

Human health is one of important thing in human life. One effort for that is food supplement consumption. Probiotic product known to maintain human health especially for digestive health stability. According to [1] There is increasing evidence in favour of the claims of beneficial effects attributed to probiotics, including improvement of intestinal health, enhancement of the immune response, reduction of serum cholesterol, and cancer prevention.. Probiotic as consortium of microorganism will effect human health. This was since combination of microorganism in probiotic will improve intestinal microflora. Other than intestinal probiotic microorganism also will give positive effect for immunity, urogenital, and allergic effect [2]. The ability of probiotic microorganism is inhibiting virus

or pathogenic microorganism as well as other microorganisms that produce lactic acid included as probiotic.

Lactic acid bacteria will change carbohydrates and sugar to become lactic acid. Probiotic had been widely used in food industry. Most of food product using probiotic will have sour taste because of fermentation process [2].

Exploration of probiotic bacteria can be isolated from the symbiotic organism in the intestinal organism of *Holothuria atra* and *Holothuria leucospilota*. Research of [3] had isolated probiotic bacteria from rumen of cow used for human health. A study of [4], showed that probiotic bacteria from the intestine of catfish can be used in fermentation. This can be supporting evidence that probiotic bacteria isolation of any organism can be applied for human health. Sea cucumber as marine

invertebrate known as a filter feeder which means that sea cucumber will only feed on suspended particles in the seawater [5]. So that can be assumed that symbiotic bacteria in sea cucumber intestine can be potential for probiotic.

Based on that, the aim of this study is to explore the bacterial species in sea cucumber intestine as potential candidate for probiotic based on isolation, bacterial screening and biochemical test.

MATERIAL AND METHOD

Sample of symbiotic bacteria was collected from the two species of sea cucumber *H. atra* and *H. leucospilota* from Bandengan Beach, Jepara. Bacterial isolation was conducted in Tropical Marine Biotechnology Laboratory, Faculty of Fisheries and Marine Science, Diponegoro University.

Isolation and purification of symbiotic bacteria

One milliliter macerated sea cucumber intestine suspended with 5 ml sterile seawater to obtain a serial dilution from 10^{-1} to 10^{-5} dilution. Diluted bacteria will be isolated with pourplate technique on Zobell agar medium and incubated for 48 hours [4]. Characteristics of the bacterial colony would be observed to identify form, elevation, margin, size, and colour. Each colony will be purified and incubated for 2x24 hours to obtained single colony. [6].

Screening of antibacterial activity

Screening of bacterial activity using the overlay method. Bacterial isolate inoculated in Zobell agar medium and incubated for 48 hours at 38 °C temperature. Pathogenic bacteria of *Pseudomonas aeruginosa* and *Bacillus cereus* growth on 10 ml liquid medium for 24 hours. One milliliter culture of pathogenic bacterial added to soft Zobell agar medium and poured on a petri dish with bacterial inoculate and incubated for 48 hours [7].

Antibacterial activity test

Antibacterial activity was tested using diffusion technique on a paper disk (Kirby-bauer test). Result of bacterial isolate screening would be extracted using centrifuge to get supernatant. Culture of pathogenic bacteria was added into Zobell agar medium using swap method. Sterile paper disc was dipped into bacterial supernatant and medium content was put on medium with pathogenic bacteria. Petri dish incubated for 2x24 hours and observed inhibitory zone around isolate [8].

Biochemical test

Identification including macroscopic identification that are gram bacterial colour, spore test, motility test, and anaero/b test. Biochemical test including of catalase test, oxidase test, and glucose test [9].

RESULT AND DISCUSSION

Isolation and bacterial colony characteristic

Isolation result from sea cucumber intestine produce 51 isolate with 21 isolates from *Holothuria atra* and 30 isolates from *Holothuria leucospilota*. Each colony was characterized by its form, elevation, margin, size, and colour. Types of colonies form were circular, irregular, spindel, filamentous, and rhizoid. Types of elevations were flat, raised, convex, and umbonate. Types of margins were entire, lobate, serrate, filamentous, and undulate. Colonies size were punctiform, small, moderate and large. Colours of the colony were creamy white, white, creamy yellow, yellow, orange, pink, and also transparant. The form of bacterial colony was effected of growing and enviromental factor as biotic and abiotic, nutrition and temperature [10]. The colour if this natural pigment can be use for many other used in human health such as anti-tumor, anti-inflammation, anti-obesity and increase brain ability and antioxidant.

Table 1. Characteristic identification of bacterial colony

	Morphology	Bacterial Code
		TB TH
Form	Circular	12
		13
	Irregular	16
		6
	Spindel	-
		1
	Filamentous	1
		1
	Rhizoid	1
		-
Elevation	Flat	17
		14
	Raised	4
		1
	Convex	8
		5
	Umbonate	1
		1
Margin	Entire	12
		12
	Lobate	11
		6
	Serrate	3
		2
	Filamentous	1
		1
	Undulate	3
		-
Size	Punctiform	5
		5
	Small	12
		10
	Moderate	7
		3
	Large	6
		3
Colour	Creamy white	11
		10
	White	1
		4
	Creamy yellow	5
		3
	Yellow	6
		1
	Orange	2
		1
	Pink	1
		-
	Transparant	4
		2

Antibacterial Activity Test

Antibacterial test become one important step in bacterial exploration for probiotic candidate for pathogenic inhibition. Thirteen isolates resulted for pathogenic inhibition are P.a B.c where P.a known as bacteri caution for urinary infection and digestive infection [11]. While B.c produce toxin which will cause toxicity [12]. Test result showed that 13 bacteria had capable of inhibiting pathogens.

Table 2. Antibacterial activity test

Code	<i>P. aeruginosa</i>	<i>B. cereus</i>
TH 12	-	+
TH 18	+	+
TH 19.1	-	+
TH 20.1	-	+
TB 3.2	-	+
TB 16	+	+
TB 18.2	-	+
TB 23	-	+
TB 29	-	+
TB 12	-	+
TH 15	-	+
TB 7	-	+
TH 20	-	+

Biochemical Test

All bacteria that can pass through the antibacterial test will be identified. Four isolates from two samples of sea cucumber were identified using molecular method [13] are *Bacillus aquimaris* TF-12 (TB 12), *Bacillus maritimus* KS 16-9 (TH 15), *Bacillus toyonensis* BCT-7112, dan *Virgibacillus chiguensis* NTU-101 (TH 20). Another 9 bacterial such as TH 18, TB 3.2, TB 16, TB 18.2, TH 20.2, TH 12, TH 19.1, TB 23, and TB 29 had been identified using biochemical test to genus level.

Bacterial gram test indicates 9 isolates as gram positive bacteria. According to [14] that gram positive bacteria can be classified as probiotic. According to [15], characters of gram positive bacteria are they have thick cell wall and do not have the outer membran. In contrast, gram negative bacteria have thin cell

wall in between two layers of their cell membrane. Gram positive bacteria will have purple colour because of peptidoglycan bind the crystal violet and gram negative will showed red colour because of safranin [16].

In aerobic respiration, the oxidative enzyme will role in the process of electron transfer [16]. The negative result on bacteria TH 18, TB 3.2, TB 16, and TB 18.2 assumed that these bacteria don't have an oxidative enzyme. Bacterial with the positive result which can produce oxidative enzymes are TH 20.1, TH 12, TH 19.1, TB 23, dan TB 29. Bacterial with negative oxidase belongs to anaerob bacteria while positive oxidase as aerobic bacteria. Oxidation reaction was depend on the presence of peroxide and peroxidase. The presence of enzyme as catalysis of cytochrome oxidase will be reduced by molecular oxygen in electron

transfer process. Most of aerobic and anaerobic facultative bacteria will have cytochrome system in aerobic respiration [17].

Nine bacterial candidates have negative spore that showed not producing spore. Results of 9 probiotic candidate bacteria in the category of lactic acid bacteria that are gram positive, non spore, and have coccus and bacil form [18]. Result of motility test showed that bacteria with code TH 18, TH 20.1, TH 12, TB 3.2, TB 16, and TB 18.2 have negative motility while bacteria of 19.1, TB 23, and TB 29 have positive motility. The presence of positive motility can be assumed that the bacteria have flagel. Flagela in bacteria function as moving organ as structure outside the cell and made from unit of protein. Bacterial with non motility will have limited biosyntethic ability [19].

Table 3. Biochemical identification of bacterial isolate

No	Biochemical Test	Bacterial Code								
		TH 18	TH 20.1	TH 12	TH 19.1	TB 3.2	TB 16	TB 23	TB 18.2	TB 29
1	Gram	+	+	+	+	+	+	+	+	+
2	Form	C	B	B	B	C	C	B	C	B
3	Catalase	+	+	+	+	+	+	+	+	+
4	Oxidase	-	+	+	+	-	-	+	-	+
5	Spore	-	-	-	-	-	-	-	-	-
6	Glukosa carb	Fer	-	-	-	-	-	-	-	-
7	Glukosa acid	+	+	+	+	+	+	+	+	+
8	Anaerob	+	-	-	-	+	+	-	+	-
9	Motility	-	-	-	+	-	-	+	-	+
		<i>Staphylococcus</i> sp.	<i>Rothia</i> sp.	<i>Rothia</i> sp.	<i>Listeria</i> sp.	<i>Micrococcus</i> sp.	<i>Micrococcus</i> sp.	<i>Listeria</i> sp.	<i>Micrococcus</i> sp.	<i>Listeria</i> sp.

Note:

C : Coccus, B : Bacil, Fer : Fermentation

Biochemical test for identification indicate for genera of bacteria that are *Staphylococcus* sp. (TH 18), *Micrococcus* sp. (TB 3.2 dan TB 16), *Rothia* sp. (TH 20.1), and *Listeria* sp. (TH 19.1, TB 23, dan TB 29). Although having pathogenic inhibitory as probiotic should be non pathogenic for human being and give benefit for the host organism. Benefit of bacteria will be different from one to another but sometime will have similar clinical benefit [20]. According to [21], that probiotic candidate from intestinal target will support the growth of probiotic in the intestine.

Staphylococcus sp. as gram-positive bacteria can cause pathogens. However, research of [22], prove that *Staphylococcus* sp. with negative coagulase (CNS) is known as a non-pathogenic bacterium and acts as a good probiotic. The negative coagulase *Staphylococcus* can be used as antitubercular. *Staphylococcus hominis* is one type of the *Staphylococcus* genus capable of being a probiotic candidate because it has the ability as antimicrobial activity [23].

Micrococcus sp. can be easily found in the environment. This bacteria known as normal skin microflora and have antibacterial activity against several pathogens [24]. Not much research has been done to explore this bacteria. Potential of *Micrococcus* sp. besides the ability to be antibacterial, it also has the potential as an antioxidant. One type of this bacteria namely *Micrococcus luteus* is able to produce exopolysaccharides. This bioactive ability can be utilized as an antioxidant [25].

Rothia sp. is a gram positive bacterium that belongs to phylum of Actinobacteria. These bacteria can be found in the oral cavity, respiratory tract, and duodenum. *Rothia* sp. has the ability to degrade physiologically dangerous gluten peptides. Colonization of the duodenum with these bacteria can reduce gluten level due to *Rothia* sp. capable of producing the gluten degrading enzymes [26]. *Listeria* sp. classified into facultative bacteria and included in gram-positive bacteria. The bacteria live in optimal temperatures of 24-37 °C and have size range of 0.4-2.0 µm [6]. The

bacteria can be motile at temperature of 28 °C and non-motile at 37 °C [26]. The genera has 6 species, namely *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi*. Three types of *Listeria* sp. group are known to be pathogenic that infectious to human, namely *L. ivanovii*, *L. seeligeri*, and *L. monocytogenes* which are currently better known in the world of health [28]. Even though it is found in the intestine, this bacterium is thought not to qualify as a candidate for probiotics because it has a greater potential for pathogenic properties.

Bacillus sp. is a facultative bacterium and belongs to the category of gram-positive. The biochemical properties of these bacteria include positive catalase and oxidase and have spores [6]. Some strains of *Bacillus* sp. have potential function as probiotics. *Bacillus* sp. has higher acid tolerance and better stability during heat processing and low temperature storage. Exploration of *Bacillus* sp. as probiotics show the ability to produce antimicrobial peptides and small extracellular effector molecules [22].

Genus *Virgibacillus* sp. is a gram positive bacterium, motile, and has spores. [29]. The results of species identification showed that TH 20 isolate had an identical character to *Virgibacillus chiguensis* NTU-101. So far, there is still a lack of information about the use or application of biological activity from *Virgibacillus chiguensis* [30].

CONCLUSION

Based on the results of identification of symbiotic bacteria from sea cucumber, not all genera are able to become candidates for probiotics that are good for human health. The sea cucumber intestinal bacteria symbionts that can be a candidate for probiotics for health are *Staphylococcus* sp., *Micrococcus* sp., *Rothia* sp., and *Bacillus* sp.

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