Identification of marine bacteria HPP.4A and HPP.T13 and its anticancer activity against P388 murine leukaemia cell

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Abstract. Sea cucumber has been widely studied as a source of bioactive compounds with various biological activities such as antibacterial, antifungal, antioxidant, and anticancer. However, there are a few studies have reported on the biological activity of its associated bacteria. The purpose of this study were to determine the potential of sea cucumber associated bacteria from Panjang Island, Jepara, Central Java, Indonesia as a natural source of anticancer compounds and identify the prospective isolates through DNA barcoding. Bacteria HPP.4A and HPP.T13 were isolated from the gut of sea cucumber Holoturia atra. The bacteria were cultivated in three different media (A3, A11, and A16) then extracted using 1-butanol with maceration method. Cytotoxic assay of each extract was conducted against P388 murine leukaemia cell. Bacteria HPP.4A and HPP.T13 were identified through molecular approach as Sallinicoccus roseus and Sphingobium yanoikuyae with 99.73% similarity. The strongest anticancer activity was showed by Sallinicoccus roseus extract which cultivated in A11 medium while Sphingobium yanoikuyae extract in A3 medium.

1. Introduction

Indonesia as a tropical country is known through its high diversity of marine resources with diverse benefits. One of Indonesia's marine resources with promising potential is marine natural products. Research on marine natural products from marine organisms has been carried out over the past decade and has produced highly prospective results as a source of new drugs for various diseases, such as cancer [1-5]. Investigation to find chemotherapeutic agents has become the main focus in cancer treatment research, interestingly marine natural products shows great potential to solve this issue [6]. Unfortunately, from the total of Indonesian marine natural products reported, only 4.0% of their biological activity is anticancer [2,4]. For this reason, further research is needed regarding the isolation of anticancer compounds from Indonesian marine organisms. According to previous studies, one of the marine organisms that have the potential to be a natural source of anticancer compounds is sea cucumber [3,12-14].
Various anticancer compounds such as stichoposide C, philinopside A and frondoside A have been reported successfully isolated from various species of sea cucumbers \[3,12,13\]. Unfortunately, isolation of anticancer compounds directly from sea cucumbers in the long term is not environmentally friendly because it requires large amounts of sea cucumbers which will cause a reduction in the number of sea cucumbers in nature. In this point of view, another source of anticancer compounds that can be utilized is sea cucumber association bacteria. Further, associated bacteria have been known to possibly produce compounds that are similar to or similar to their host \[18\]. However, research on anticancer compounds from sea cucumber association bacteria has not been widely reported \[2\]. Therefore, the purpose of this study is to identify \textit{Holoturia atra} associated bacteria species from Panjang Island, Indonesia through molecular approach and to understand the anticancer activities of \textit{Holoturia atra} associated bacteria crude extract.

2. Materials and methods

2.1. Preparation of bacterial culture

Sea cucumber sample was obtained from Panjang Island, Jepara, Indonesia. Bacteria HPP.4A and HPP.T13 were isolated from sea cucumber intestinal. The bacteria then were cultured using streak method on marine agar (Difco\textsuperscript{TM} Marine Agar 2216) and incubated for 3 days at room temperature (27\textdegree C).

2.2. Salinity experiment

Salinity test was carried out to determine the obligation of bacteria HPP.4A and HPP.T13. The isolates were cultivated on seawater and freshwater agar medium. The bacteria that able to growth only on seawater indicated that the isolate is marine obligate bacteria.

2.3. Metabolite production and extraction

Metabolite production is carried out in 3 medium namely A3 medium (0.3% yeast extract, 0.5% glucose, 2.0% glycerol, 2.0% soluble starch, 1.5% Pharmamedia, and 1% Diaion HP-20), A11 (0.5% yeast extract, 2.5% soluble starch, 2% glucose, 0.5% polypeptone, 0.5% CaCO3, 0.5% NZ-amine, and 1% Diaion HP-20), and A16 (glucose 2%, CaCO3, and 0.5% pharmamedia). HPP.4A and HPP.T13 was cultivated in seed culture for 3 days and production culture for 7 days. The isolate was cultivated by shaking at 200 r.p.m and temperature of 30\textdegree C. Extraction is done by adding 1-butanol to the cultivation medium with a ratio of 1: 1. The medium was then macerated by shaking at 200 r.p.m for 1 hour and then centrifuged at 10,000 r.p.m and 4\textdegree C temperature. The supernatant was then evaporated to obtain crude extract \[7,8,11\].

2.4. Anticancer assay

Anticancer test of bacteria HPP.4A and HPP.T13 were evaluated to inhibit P388 murine leukemia cells according to Sibero et al. \[15\].

2.5. Molecular identification

Chelex method was carried out to extract the DNA of the bacteria according to Walsh et al. \[16\] with several modifications that are explained by Sibero et al. \[17\].

3. Results and discussion

The sampling of sea cucumber samples was done by collecting the \textit{Holoturia atra} on Panjang Island, Jepara, Indonesia. The sea cucumber \textit{H. atra} was identified by its morphological identification, which is entirely black color, smooth skin, and the existence of the sand on their body \[19\]. The previous study showed that sea cucumber crude extract has potential as the source of various bioactive compounds. Many of study have tried to find \textit{H. atra} bioactive compounds and their potential \[6,9\]. There still a lack of studies about the diversity of \textit{H. atra} associated bacteria and its potential. Based on the previous study, there were two reported species isolated from \textit{H. atra} intestinal. They were
*Vibrio algynolyticus* and *Bacillus paramycoides* which is considered as pathogenic bacteria without any potential of anticancer nor antibacterial [10]. In this regard, a longer period of incubation time during the isolation step was performed in order to get more unique slow-growing bacteria from *H. atra* intestinal.

**Figure 1.** Sample’s intestine (A) and *Holothuria atra* from Panjang island (B)

The associated bacteria were isolated from *H. atra* intestinal segments. They were isolated on different medium to find the diversity of *H. atra* associated bacteria. There were twenty bacteria were cultivated on the medium with different morphological and two of them were taken to the next identification by DNA barcoding.

**Figure 2.** HPP.4A (left) and HPP.T13 (right) cultured on marine agar

Sample HPP.4A (Figure 2.a) was cultivated on marine agar and it showed the orange to rose pigment perfectly. The pigment was produced as a intracellular pigment. Bacteria pigment is a reaction to survive in different places so it might be produced more secondary metabolites. In another case, Sample HPP.T13 (Figure 2.b) did not produce any pigment.

**Figure 3.** HPP.4A (left) and HPP.T13 (right) cultured on sea water and fresh water medium

Isolates HPP.4A and HPP.T13 were identified by DNA barcoding then analysed using Neighbour Joining (NJ) to reconstruct the phylogenetic tree. Based on the NCBI BLAST homology, HPP.4A was identified as *Salinicoccus roseus* with 99.73% similarity to the *Salinicoccus roseus* 16S ribosomal RNA gene partial sequence (AF237976) (Figure 4). On the other hand, isolate HPP.T13 was identified
as *Sphingobium yanoikuyae* with 99.58% similarity to *Sphingobium yanoikuyae* strain ANT3D 16S ribosomal RNA gene partial sequence (MG686734) (Figure 4).

![Figure 4. The Phylogenetic analysis by neighbour joining tree of HPP.A4 and HPP.T13](image)

Referring to the previous reports, *Salinococcus roseus* was reported to produce orange pigment. *S. roseus* pigment production depends on the medium. Isolate *S. roseus* HPP.A4 was identified as a marine obligate bacterium since it was grown on sea water medium but, it did not survive on freshwater medium. In contrast to HPP.4A, *Sphingobium yanoikuyae* HPP.T13 did not produce any pigmentation. In addition, *S. yanoikuyae* HPP.T13 was able to survive on both of sea water and freshwater agar media so it was suggested that this bacterium was a facultative strain.

In order to understand the anticancer potential of these bacteria, the percentage of cell viability after addition of the bacterial crude extracts were measured. The results of the test were presented in Table 1.

Table 1. P388 murine leukaemia cell viability after addition of bacterial crude extracts

| Medium | HPP.T13 | HPP.A4 |
|--------|---------|--------|
| A3     | 27.93   | 60.12  |
| A11    | 69.34   | 29.63  |
| A16    | 70.01   | 87.55  |

The anticancer assay was identified by cell viability screening against P388 murine leukaemia cell. Cell viability is a measurement to provide an indication of the number of healthy cells on the population. *S. roseus* HPP. A4 and *S. yanoikuyae* HPP.T13 were cultivated in A3, A11, and A16 medium to find the best nutrient for the bacteria to produce anticancer compounds. Cell viability percentage showed the anticancer potential by comparing all of the results. The lowest percentage result of the cell viability showed the best anticancer activity. The result indicated that *S. yanoikuyae* HPP.T13 showed the best result on A3 medium with 27.93% cell on the population. In another hand, *S. roseus* HPP.A4 gave the best result on medium A11 with 29.63% cell on the population. The
growth and development of microorganisms depend on the nutritional composition of the medium. Every single species needs different nutrition to produce its primary and secondary metabolites. The composition of A3 medium gives S. yanoikuyae HPP.T13 a better nutrition to produce more anticancer potency. Meanwhile, S. roseus HPP.A4 gave a better potency for anticancer after cultivation in A11 medium.

4. Conclusion
Isolates HPP.4A and HPP.T13 were isolated as Holothuria atra-associated bacteria. Based on the salinity experiment, HPP.4A was identified as a marine obligate bacterium while HPP.T13 was a marine facultative bacterium. Further, 16S rRNA genes analysis discovered that isolate HPP.4A was similar to Salinicoccus roseus with 99.73% similarity while isolate HPP.T13 was closely related to Sphingobium yanoikuyae with 99.58% similarity. In addition, A-11 medium was suggested as the best medium to produce anticancer agent for S. roseus HPP.4A, while A-3 medium was suggested to S. yanoikuyae HPP.T13.

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