Bioactivities Screening of Indonesian Marine Bacteria Isolated from Sponges

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Abstract

Currently marine bacteria are considered as important source of natural products for drug discovery. The objective of this study is to conduct an *in vitro* bioactivities (antidiabetic, antioxidant and antibacterial) screening of 9 Indonesian marine bacteria isolated from sponges that belongs to the Research Center for Oceanography, Indonesian Institute of Sciences collections. The marine bacteria were cultured for 2 days in liquid medium containing yeast, peptone and sea salt under shaking condition and extracted with ethyl acetate. Antidiabetic was measured using inhibition of α-glucosidase inhibitory activity method; antioxidant was measured using DPPH free radical scavenging activity method; antibacterial was tested using disc diffusion method.Screening results show that at sample concentration of 200 µg/mL, there was significant α-glucosidase inhibitory activity detected in the extracts of strain Sp 7.9 (84% inhibition) and Sp 8.10 (75% inhibition), however the antioxidant activities of these two strains were low only around 30% inhibition, antioxidant activities of other strains were very low. Screening for antibacterial activities using 10 µL samples show that extract of strain Sp 8.5 was best for *Staphylococcus aureus* (14 mm inhibition); Sp 7.9, and Sp 8.5 for *Bacillus subtilis* (18 mm inhibition); Sp 8.10 for *Escherichia coli* (10 mm inhibition); Sp 8.9 and Sp 8.10 (10 mm inhibition) for *Pseudomonas aeuriginosa*. Based on these results marine bacteria strain Sp 7.9 and Sp 8.10 were selected to be used for further studies in the isolation of bioactive that has potential as antidiabetic and antibacterial. Results of molecular identification conducted by InaCC show that identity of both strains based on BLAST Homology using NCBI database were *Bacillus thuringiensis* strain Ou2.

Keywords: marine bacteria, sponges, anti diabetes, antioxidant, anti bacterial

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Introduction

Indonesia is an archipelago that has the ocean area of 5.8 million km^2. Extensive ocean areas caused Indonesia has a rich of marine biodiversity, both in the form of natural resources that can be renewed such as fisheries, coral reefs, mangrove forests, sea grass and biotechnology products, as well as unrenewable natural resources such as petroleum. Some of marine species live in extreme environmental conditions such as cold, low light and high pressure causing the high diversity of species (Debbab *et al*., 2010).The uniqueness of the sea ecosystemcould lead to a lot of potential as a source of raw material for medicine and cosmetics with unique molecular structure and mechanism which can be used for development of new drugs. Metabolites from sponges, algae and marine endophytic microbe extracts are reported to have antidiabetic, antiobesity and antioxidants activities as shown in Table 1 (Debbab *et al*., 2010; Wang *et al*., 2014a). Research of bioactive compounds from marine organisms have developed rapidly and discover a wide variety of new compounds that have the potential to be developed into drugs in the future.

Currently marine bacteria are considered as important source of natural products for drug discovery.The objective of this study is to conduct an *in vitro* bioactivities (antidiabetic, antioxidant, and antibacterial) screening of several Indonesian marine bacteria, isolated from sponges that belongs to the Research

Center for Oceanography, Indonesian Institute of Sciences collections.

Materials and Methods

Marine Microorganisms and Culture Conditions. Ninesponge endophytic bacterial strains, Sp 4.3, Sp 5.8, Sp 6.3, Sp 7.4, Sp 7.5, Sp 7.9, Sp 8.5, Sp 8.9, and Sp 8.10, from Research Center Oceanography, LIPI collection were used. The isolates were maintained in solid media containing 5 g/L yeast,1 g/L peptone, 33 g/L sea salt,and 15 g/L agar. For screening experiment, bacterial strains were transferred into fresh solid medium, which has the same compositions as the maintenance media and grew overnight prior to cultures in liquid media for 2 days under shaking condition at room temperature.

Table 1. Bioactive compounds from marine biota and marine endophytic microbes

Bioactive compound	Source	Activity
Fucoxanthin	Brown seaweed	Antidiabetic, antiobesity, antioxidant
Astaxanthin	Marine algae	Antidiabetic, antiobesity, antioxidant
Kolagen peptide	Deep sea fish	Antidiabetic
Dieckol	*Ecklonia cava*	Antidiabetic, antihyperlipidemic
Fucoidan	Brown algae	Antidiabetic, antiobessity
Hyrtiosal	Marine sponge	Inhibitor of protein tyrosin phosphatase-1B (PTP-1B)
Sargaquinoic (SQA) and sargahydroquinoic (SHQA)	*Sargassum yezoense*	Reduce insulin resistance
Aquastatin	*Cosmospora* sp	Inhibitor of protein tyrosin phosphatase-1B (PTP-1B)
Fucofureeckol A	*Eisenia bicyclis*	Inhibitor of pancreatic lipase
7-phloroeckol		
Bromophenol	Marine algae	Inhibitor of protein tyrosin phosphatase-1B (PTP-1B)and inhibitor of α-glucosidase
2,4,6-tribromophenol dan 2,4-dibromophenol	Red alga *Grateloupia elliptica*	Inhibitor of α-glukosidase
Aspergillusol A	*Aspergillus aculeatus* CR1323-04	Inhibitor α-glucosidase
Carotenoid	Marine algae	Antioxidant

Extraction. The culture broth of 2 days' culture bacteria was extracted with ethyl acetate (EtOAc). The extract was filtered and concentrated with a rotary evaporator under vacuum at 40 ºC.Subsequently, these extracts were used for bioactivity screening of antidiabetic, antioxidant, and antibacterial activities.

Antidiabetic Assay. The antidiabetic activity was measured as the inhibitory activity for α-glucosidase using the method reported by Kim *et al*.(2004) with minor modifications. *p*-Nitrophenyl-α-d-glucopyranoside (3 mM) was used as substrate and yeast α-glucosidase (0.065 units/mL) was used as the enzyme. Screening of α-glucosidase inhibitory activity was conducted at sample concentration of 200 µg/mL. The inhibitory effect on α-glucosidase activity was determined by measuring the amount of *p*-nitrophenol released at λ 400 nm. All the tests were run in duplicate.

Antioxidant Assay. Antioxidant activity was measured as 1,1-diphenyl-2-picryl-hydrazyl DPPH free radical scavenging assay using the method reported by Yen and Chen (1995) with minor modification. Sample at concentration of 200 µg/mL was mixed with 1 mL of a methanolic solution containing DPPH radicals at 1 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm.The ability to scavenge the DPPH radical was calculated using the following equation:
DPPH scavenging effect (%) = [(A0-A1)/A0x100] where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample. The assays were carried out in duplicate.

Antibacterial assay. Antibacterial assay was conducted using disc diffusion method against *Staphylococcusaureus*, *Bacillus subtilis*,

Escherichia coli, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Prior to the assay, each bacterium was grown in nutrient agar and incubated at 37°C for 24 hours. For antibacterial assay, each bacterium was placed in the Petri dish, mixed with 15-20 mL of nutrient agar and allowed to solidify at room temperature, and then filter paper disc (6 mm diameter) was placed on the agar surface. The 10 µL of each sample dissolved in DMSO at concentration of 40 mg/mL was placed on the disc. The plates were then incubated at 37°C for 24 hours. All plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters. The assays were carried out in duplicate.

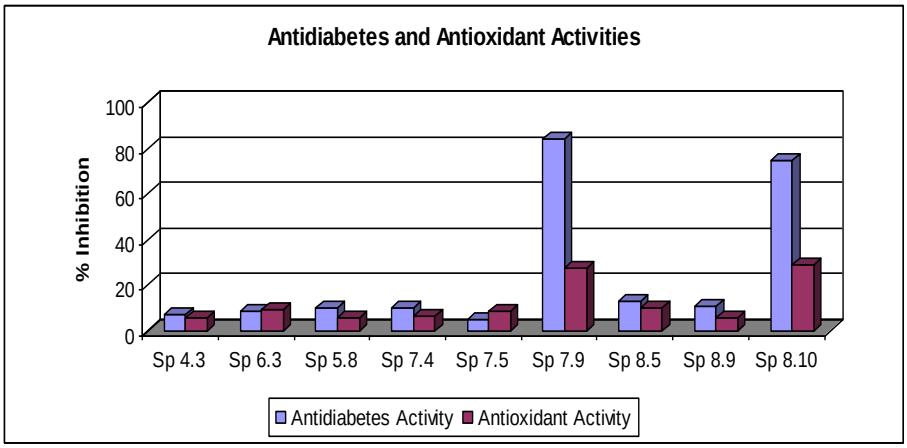
Chemical screening analysis. Preliminary analysis of marine bacteria chemical constituents were conducted by qualitative analysis of alkaloids, flavonoids, phenols, tannins and terpenoids according to Harbourne (1973). Quantitative analysis of total phenolic content as Phloroglucinoldihydrate equivalent using Follin-Ciocalteu method (Singleton & Rossi, 1965) and total flavonoid content as quercetin equivalent using $AlCl_3$ method(Sultana *et al*., 2009) were also conducted.

Results and Discussion

Collection of endophytic bacteria from sponge collected from ocean surrounding Barrang Lompo island, Makassar, on June 2009 (Murniasih&Rasyid, 2010) was conducted by direct plating method describedin Murniasih *et al*.(2013). Isolation of bacteria was based on different morphology (shape, color) of colonies, further purification was conducted to get a single bacterial colony (Murniasih *et al*., 2013).

Screening results show that at sample concentration of 200 µg/mL (Figure 1), it was considered that % inhibition >70% = strong, 50-70% = medium, < 50% = weak activities.There was significant α-glucosidase inhibitory activity, detected in the extracts of strain Sp 7.9 (84% inhibition) and Sp 8.10 (75% inhibition). However the antioxidant activity of these two strains were weak, only around 30% inhibition. Antidiabetic and antioxidant activities of other strains were very weak.

Screening for antibacterial activities using 10 µL samples at concentration 40 mg/mL were presented in Table 2, it was considered that inhibition zone>20mm = strong, 10 - 20 mm = medium, <10mm = weak activities. Observation on minimum inhibition concentration (MIC) was also conducted as shown in Table 3. The results show that extract of strain Sp 8.5 was best for *S. aureus* (14 mm inhibition with MIC 2.5 mg/mL), Sp 7.9 and Sp 8.5 for *B.subtilis* (18 mm inhibition with MIC 2.5 mg/mL), Sp 8.10 for *E. coli* (10 mm inhibition with MIC 5.0 mg/mL); Sp 8.9 and Sp 8.10 for *P. aeuriginosa* (10 mm inhibition with MIC 10.0 mg/mL). Based on these results, marine bacterial strain Sp 7.9 (isolated from sponge *Lisoclinum* sp.) and Sp 8.10 (isolated from sponge *Clathria* sp.) were selected for further studies in the isolation of bioactive that has potential as antidiabetic and antibacterial.



Note: % inhibition >70% = strong, 50-70% = medium, < 50% = weak

Figure 1. Antidiabetic and antioxidant activities of marine bacteria

Table 2. Antibacterial activity of marine bacteria extracts at sample concentration 40 mg/mL

Sample	Inhibition Zone (mm)			
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Sp 7.9	7.5	18	8	9
Sp 8.5	14	18	9	8
Sp 8.9	8.5	14	9	10
Sp 8.10	11	13	10	10

Notes: inhibition zone > 20 mm= strong, 10-20 mm= medium; <10 mm = weak

Table 3. Minimum Inhibition Concentration (MIC) of marine bacteria extracts

Sample	MIC (mg/mL)			
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Sp 7.9	5.0	2.5	10.0	10.0
Sp 8.5	2.5	2.5	10.0	10.0
Sp 8.9	5.0	5.0	10.0	10.0
Sp 8.10	5.0	5.0	5.0	10.0

Table 5. Total flavonoid content and total phenolic content of endophytic marine bacteria extracts

Bacterial Strain	Total Flavonoid Content mg quercetin equivalent/g extract	Total Phenolic Content mg phloroglucinol dihydrate equivalent/g extract
Sp 7.9	22.4	11.8
Sp 8.5	29.8	6.8
Sp 8.9	16.6	14.4
Sp8.10	6.6	5.0

Preliminary analysis of possible chemical constituent in endophytic marine bacterial is shown in Table 4. The results show that all extracts have positive content of alkaloids, flavonoids, phenols and saponins. Quantitative analysis results are shown in Table 5. These preliminary results will be used as a guide for further studies on the isolation of bioactive compounds.

Table 4. Chemical constituent analysis of endophytic marine bacteria extracts

Chemical Constituent	Sp 7.9	Sp 8.5	Sp 8.9	Sp 8.10
Alkaloids	+	+	+	+
Flavonids	+	+	+	+
Phenols	+	+	+	+
Tannins	-	-	-	-
Triterpenes	-	-	-	-

Results of molecular identification conducted by Indonesian Culture Collection (InaCC) confirm that identity of both strain based on BLAST Homology using NCBI database are *Bacillus thuringiensis* strain Ou2. Previous study by Qian *et al*. (2006) also reported isolation of *B. thuringiensis* from sponge *Callyspongia* sp., hence this bacterium species seems to be common found as sponge endophyte.

Although most studies on *B. thuringiensis* were on bioinsecticide and genetic engineering of Bt plants, there are some reports on other potensial of *B. thuringiensis*. Theability of *B. thuringiensis* 2e2 to generate (2S,3R,4S)-4-hydroxyisoleucine an amino acid that has potential as insulinotropic and anti-obesity from metabolism of L-isoleucine a potential amino acid was reported byOgawa and colleagues(2011). Our studies show that marine bacteria *B. thuringiensis* Ou2 Sp 7.9 and Sp 8.10 have α-glucosidaseinhibitory activity, so far that we aware, there is no previous report on α-glucosidase inhibitor from *B. thuringiensis*. However, there are reports of other *Bacillus* species produced α-glucosidase inhibitor such as *Bacillus* sp. that showed produced 1-deoxynojirimycin a known α-glucosidase inhibitor (Kim *et al*., 2011) and *Bacillus lentimorbus* B-6 that showed 72% inhibition to α-glucosidase at pH 8 as optimum pH (Kim *et al*., 2002).

Compare to antidiabetic as α-glucosidase inhibitor activity, *B. thuringiensis* Ou2 Sp 7.9 and Sp 8.10 show relatively much lower antioxidant activity as DPPH free radical

scavenging activity.We could not found report on antioxidant activity from *B. thuringiensis*, however other *Bacillus* species are reported to have antioxidant activity such as *B. subtilis* B38 (Tabbene*et al*., 2009),*B. subtilis*NRC1aza (Abdel-Fatah *et al*., 2012)and *Bacillus mojavensis* A21 (Ayed*et al*., 2015). Antioxidant activity of *B. thuringiensis* Ou2 Sp 7.9 and Sp 7.8 found to be lower than those studies such as compare to levan from B. substilis NRC1aza that has 50% inhibition at concentration < 10 µg/mL(Abdel-Fatah *et al*., 2012).In our studies, it was observed that*B. thuringiensis* Ou2 Sp7.9 and Sp 8.10 also show inhibition on *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* growth although much weaker if it is compared to antibiotic streptomycin that have inhibition zone > 20 mm at concentration 10 mg/mL. There are some reports about antibacterial from *B. thuringiensis* and other *Bacillus* species. *B. thuringiensis* subsp. *israelensis* was reported to produce delta-endotoxin protein(Cyt1Aa) that has activities as bactericidal for *E. coli* and bacteriostatic for *S. aureus* (Cahan*et al*., 2008). Other *Bacillus* species such as *B. subtilis* B38 (Tabbene*et al*., 2009), *B. cereus* (Wang *et al*., 2014b), *Bacillus amyloliquefaciens* AP 183 (Ravu*et al*., 2015) also produced antibacterial activity.

Conclusions

Based on screening results, marine bacteria strain Sp 7.9 (isolated from sponge *Lisoclinum* sp.) and Sp 8.10 (isolated from sponge *Clathria* sp.) have potential for further studies in the isolation of bioactives as antidiabetic and antibacterial. These bacteria were identified as *Bacillus thuringiensis* strain Ou2. Marine bacteria showed potential as a source of bioactive compounds for drug development.

Acknowledgements

This work has been supported by *Riset Mandiri* project for Research Center for Chemistry Indonesian Institute of Sciences 2015.

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