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Antidiabetic screening of some Indonesian marine cyanobacteria collection

SRI PRIATNI[1,♥], THELMA A. BUDIWATI[1], DIAH RATNANINGRUM[1], WAWAN KOSASIH[1], RINA ANDRYANI[1], HANI SUSANTI[2], DWI SUSILANINGSIH[2]

[1]Research Unit for Clean Technology, Indonesian Institute of Sciences. Jl. Sangkuriang, Bandung 40135, West Java, Indonesia.
[2]Research Centre for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Jakarta-Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia.
Tel.: +62-22-2503051 Fax.: +62-22-2503240, ♥email: sripriatni@yahoo.com

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Abstract. *Priatni S, Budiwati TA, Ratnaningrum D, Kosasih W, Andryani R, Susanti H, Susilaningsih D. 2016. Antidiabetic screening of some Indonesian marine cyanobacteria collection. Biodiversitas 17: 642-646.* Cyanobacteria have been known as a potential extracellular-polysaccharide (EPS) producer. The objective of this study was to screen the marine cyanobacteria as potential antidiabetic agents. The present investigation was designed to determine the antidiabetic activity of EPS, intracellular-polysaccharide (IPS) and biomass extracts from marine cyanobacteria isolates. 10 cyanobacteria isolates were cultivated in IMK medium, at 25^{o}C for 21 days. The morphology of cells was identified by a light microscope. EPS and IPS were separated by ethanol precipitation method and their antidiabetic activity was analyzed by the inhibition of α-glucosidase activity method. Results of morphology identification of 10 cyanobacteria isolates consist of *Oscillatoria limnetica, Oscillatoria* sp., *Leptolyngbya* sp., *Pseudanabaena* sp., *Lyngbya* sp. and *Phormidium* sp., *Coelastrella sp., Aphanothece* sp. and *Synechococcus* sp., and *Chroococcus* sp. Almost all of EPS from marine cyanobacteria isolates were potential as inhibitor of α-glucosidase, except for *Oscillatoria limnetica* and *Phormidium* sp. isolates. The highest activity in α-glucosidase inhibition was detected in *Pseudanabaena* sp. (14.02%) and *Chroococcus* sp. (13.0%) isolates.

Keywords: antidiabetic, cyanobacteria, extracellular-polysaccharide, screening

INTRODUCTION

Diabetes mellitus is a common metabolic disease in which the concentration of glucose in the blood is above the standard level. This is due to insulin deficiency or functional disturbance of the receptors, which causes blood glucose to rise and induce disorders in the metabolism of fat and proteins (Yang et al. 2012). Diabetes is a silent disease and generally detected after chronic symptom. Unhealthy lifestyle has an impact on weight gain that can trigger diabetes. Another factor that triggers diabetes is urbanization and lack of activity. Consumption of suitable food for diabetics is indispensable so that the blood sugar levels can be controlled. Nutrition intake for people with diabetes must be maintained through the consumption of functional food product. In Indonesia, in 2013 8.5 million people were diabetic and in 2030 they are expected to be 21.3 million. This will put Indonesia on the fourth rank highest diabetes prevalence in the world (Mulyanti et al. 2010).

A sudden increase in blood glucose levels, which causes hyperglycemia in type 2 diabetes patients, occurs as the result of the hydrolysis of starch by pancreatic α-amylase and glucose uptake due to intestinal α-glucosidase. An effective strategy for the management of type 2 diabetes patients involved the profound inhibition of intestinal α-glucosidase and the mild inhibition of pancreatic α-amylase. Several natural resources have been evaluated for their ability to suppress the production of glucose from carbohydrates in the gut or glucose absorption from the intestine (Lee and Jeon 2013). The study on anti-diabetic compound was conducted with intense interest using a mangrove species *Sonneratia alba* because its close relative terrestrial plant, *Lagerstroemia speciosa* had previously shown many anti-diabetic properties. When subjected to anti-diabetic bioassay using standard Glucometer, data showed that it has significantly high attenuating activity for blood glucose because it reduced blood sugar level by 19.2% during the first 6 hours and reduced further to 66.9% after 12 hours (Morada et al. 2011).

Indonesian as a tropical country has rich of biodiversities, primarily from marine resources. Tropical marine microalgae is an interesting subject for research because its potency as producer of unsaturated fatty acids, carotenoids and polysaccharide. A great diversity in the chemical composition of these organisms, and therefore, this makes them extremely attractive for bio-prospecting and potential exploitation as commercial sources of a wide range of biomolecules. The potential of microalgae as new sources of valuable chemicals and other products recently has regained wide interest (Ko et al. 2000; Borowitzka 2013). Microalgae have the advantage because they can be cultured in a small area, they are very efficient in light capture and their cultivation varies between 7 and 10 days (Kabinawa 2001; Huang et al. 2010). Microalgae can be used to produce a wide range of metabolites such as proteins, lipids, carbohydrates, carotenoids or vitamins for

health, food and feed additives, cosmetics and for energy production (Priyadarshani and Rath 2012). The range of polysaccharides produced by microalgae is large and the polysaccharides of unicellular red algae such as *Porphyridium* and *Rhodella*, as well as many cyanobacteria have long been studied for their properties and potential applications. However, as yet, cyanobacteria have not been significantly accepted in the market, mainly for the existence of cheaper alternatives than macroalgae and higher plants. However, few microalgal polysaccharides have found niche markets, mainly in the area of cosmetics (Borowitzka 2013). Cyanobacteria are a rich source of potentially useful natural products. Some research has been focused on adapting cyanobacterial collections and cyanobacterial-derived compounds for screening the new bioactive compounds (Burja et al. 2001). Extracellular polysaccharide (EPS) from marine bacterial contain the new combination that very important in pharmaceutical industries (Satpute et al. 2010). Cyanobacteria have been known since long as a potential EPS producer. The presence of proteins, uronic acids, pyruvic acid, and *O*-methyl, *O*-acetyl and sulfate groups emphasizes the complex nature of cyanobacterial EPS (Parikh and Madamwar 2006). The information about antidiabetic activity from marine cyanobacteria is still not clear. Therefore, the objective of this study was to screen marine cyanobacter which potential as α-glucosidase inhibitory, so that potential for nutraceutical and functional food. The screening of α-glucosidase inhibitory from marine cyanobacteria was carried out to the extracellular polysaccharide, intracellular polysaccharide and the total extract of biomass.

MATERIALS AND METHODS

Materials

Ten marine cyanobacteria isolates was obtained from culture collection of InaCC Indonesian Institute of Sciences (LIPI). The IMK medium was sea water which enriched with the following additives (per liter): $NaNO_3$ 0.2 g, $Na_2HPO_4.H_2O$ 14 mg, K_2HPO_4 50 mg, NH_4Cl 268 mg, Na-EDTA 372 mg, $ZnSO_4.7H_2O$ 23 µg, $CoSO_4.7H_2O$ 140 µg, $Na_2MoO_4.H_2O$ 7.3 µg, $CuSO_4.5H_2O$ 25 µg, $MnCl_2.4H_2O$ 180 µg, $FeCl_3.6H_2O$ 315 µg. The medium was sterilized (120°C, 20 min) prior to use. Vitamin B_{12} 15 µg, thiamin HCl 2 mg and biotin 15 µg (per liter) was added to the sterile medium.

Methods

Cyanobacteria cultivation

The cultivation of marine cyanobacteria was carried by some steps cultivation as follow: (i) 1 ml stock culture was cultivated in first 5 ml of medium, continued to 10 ml and 20 ml of medium. The cultivation was carried out in incubator at 28°C for 3 days. (ii) The cultivation of marine cyanobacteria was increased to 100 ml of medium. Cyanobacteria in these experiments was cultivated in bottles which connected to an aeration pump (pump output: 70 L per minutes) exposed to 2x10 W white lamp equal to 500-2000 lux at 25°C. The experiments were carried out for 21 days.

Microscopic observation

Morphological observation of cyanobacteria cells was done with the light microscope Olympus BX 53 (x 1000) and image analysis was done with the Sklanlt RE for various Flash 2.4.3 program.

Extracellular polysaccharides (EPS) separation

Extracellular polysaccharide (EPS) was separated by Velea et al. (2011) method. After separation of cyanobacteria biomass by centrifugation, the aqueous solution containing EPS and the remaining dissolved salts in the nutrient medium are concentrates through evaporation, at 50°C up till 25% from the initial volume. The resulting creamy white product was then mixed with ethanol (1:2 v/v) and let in 4°C for overnight. The precipitate was centrifuge at 2500 rpm for 20 minutes. The precipitate was then rinsed and dried at 100°C for 2 hours.

Intracellular polysaccharides (IPS) separation

Intracellular polysaccharide (IPS) was separated by El-Sheekh et al. (2012) method. IPS was separated by homogenizing the biomass in distilled water (50 mL). The homogenates were then heated in water bath at 95°C for 6 hours. The extracts were filtrated through Whatman No.2 filter paper, then precipitated with four volumes of 95% ethanol, stirred vigorously and left overnight at 4°C. The precipitated IPS was recovered by centrifugation at 8.000 rpm for 15 min and the supernatant was discarded.

Biomass extraction

Extraction of cyanobacteria biomass was carried out by modification of Akah et al. (2011) method, using methanol with ratio 1:20, at 60°C, 100 rpm for 2 hours. Methanol extract was separated by centrifuge at 4°C, 10.000 rpm for 10 minutes and the extract was then concentrated.

Analysis of total carbohydrate content

The total carbohydrate content was analyzed by Carbazole method (Frazier et al. 2008) with modification. The anhydrous glucose (Merck) was used as standard. 1 ml of EPS was mixed with 0.5 ml of 1.5% cystein solution and 6 ml of 70% sulfuric acid. The sample solution was then mixed with 0.2 ml of 0.12% carbazole in ethanol 95%. The sample solution was incubated at 60oC for 10 minutes and cooled to room temperature. Total sugar content of sample was measured on a Spectrophotometer UV Vis at 560 nm.

Analysis of inhibition of α-glucosidase activity

In vitro, the method used to analyze the inhibition of α-glucosidase activity was that of Yang et al. (2012) using ρNPG as a substrate and modifying the dosage. The assay mixture contained 0.25 mL of 0.1 M phosphate buffer (pH 7.0), 0.25 mL of substrate solution (2.5 mM ρNPG in 0.1 M phosphate buffer) and 0.1 mL of sample solution in DMSO. The mixture of solution was incubated at 37 ◦C for 5 minutes, 0.25 mL of enzyme solution (0.2 U/mL α-

glucosidase in 0.01 M phosphate buffer containing 0.2% BSA) was added, and the reaction mixture was incubated for 15 min at 37 ◦C. The reaction was stopped by adding 0.1 mL of 0.2 M Na_2CO_3. The amount of ρNP released was measured on a Spectrophotometer UV Vis at 400 nm. The results were expressed as % inhibition calculated using the formula:

$$\%\,inhibition = \frac{\text{Abs (Control)} - \text{Abs (Sample)}}{\text{Abs (Control)}} \times 100$$

RESULTS AND DISCUSSION

Cultivation of marine cyanobacteria has been carried out to 10 isolates using IMK medium in processed of sea water. On the preliminary study, the cultivation was carried out in a shaker incubator (28^{o}C, 150 rpm) and in bottles connected to an aeration pump. The growth of cyanobacteria was monitored for 10-21 days. Results shown that the cultivation in shaker incubator was much slower than cultivation in bottles with aeration. The simple design of small scale cyanobacteria cultivation was shown on Figure 1. Aeration pump system can improved the mixing of microalgae during its growth. Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (*e.g.* in outdoor cultures) and to improve gas exchange between the culture medium and the air. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, erlenmeyers), aerating (bags, tanks), or using paddle wheels and jet pumps (Lavens and Sorgeloos 1996). The light intensity also is important in microalgae culture growth. Doubling the irradiance level has led to 25% higher yields in algal mass. Also, the irradiance proves to be a determining factor in the biosynthesis and accumulation of EPS (Velea et al. 2011).

The characteristic growth of marine cyanobacterial isolates was evaluated by monitoring the biomass color, and cultivation time when harvested. Based on the data on Table 1, it is shown that almost all marine cyanobacteria isolates have green color except the isolate of *Chroococcus* sp. The growth of marine cyanobacteria was monitored until 21 days. *Oscillatoria limnetica, Coelastrella* sp. and *Leptolyngbya* sp. were shown grew faster compared to other isolates. These cultures grown well and can harvested after 10 days. The dark green color of these cultures is probably due to its high chlorophyll content. Cyanobacteria contain chlorophyll *a* as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments that harvest light in the green, yellow and orange part of the spectrum (500-650 nm), which is hardly used by other phytoplankton species (Luuc et al. 1999). Meanwhile, *Chroococcus* sp., *Pseudanabaena* sp., *Phormidium* sp. and *Synechococcus* sp. grew very slowly at the selected experimental conditions. Presumably, the salinity values and the presence of some trace elements in medium influenced the growth of these strains. The growth of cyanobacteria is influenced by a number of factors, therefore it is necessary to evaluate the optimum condition for their mass culture. Due to their complicated requirements for salts, pH, light, temperature, vitamins, organic carbon, nitrogen and trace elements, marine cyanobacteria are difficult to isolate and culture. These requirements may differ from species to species. The successful cultivation and growth of cyanobacteria require modification in the media composition as well as other physico-chemicals parameters (Bano and Siddiqui 2004). The growth rate of cyanobacteria is usually much lower than that of many alga species. Slow growth rates require long water retention time to enable cyanobacteria to bloom. Therefore cyanobacteria do not bloom in water with short retention times (Luuc et al. 1999).

The morphology cell of marine cyanobacteria culture was identified by a light microscope (Olympus BX 53). Cell images of these isolates are shown in Figure 2. Some isolates are filamentous cyanobacteria such as *Oscillatoria limnetica, Oscillatoria* sp., *Leptolyngbya* sp., *Pseudanabaena* sp., *Lyngbya* sp. and *Phormidium* sp. Species in the order Oscillatoriales, with unseriated and unbranched trichomes, are composed of essentially identical cells (Luuc et al. 1999). Other isolates such as *Coelastrella* sp., *Aphanothece* sp. and *Synechococcus* sp. are conidial forms. *Chroococcus* sp. has a unique morphology with its unicellular forms. The unicellular cells may aggregate in irregular colonies, being held together by the slimy matrix secreted during the growth of the colony. By means of a more or less regular series of cell division, combined with sheath secretions, more ordered colonies may be produced (Luuc et al. 1999).

Figure 1. Small scale cultivation of marine cyanobacteria in bottles with aeration

Table 1. Characteristic growth of marine cyanobacteria culture

Isolates	Biomass color	Cultivation time (day)
Oscillatoria limnetica	Dark green	10
Coelastrella sp.	Dark green	10
Oscillatoria sp.	Green	16
Chroococcus sp.	Black	21
Leptolyngbya sp.	Dark green	10
Pseudanabaena sp.	Green	21
Lyngbya sp.	Green	16
Aphanothece sp.	Green	16
Phormidium sp.	Dark green	21
Synechococcus sp.	Green	21

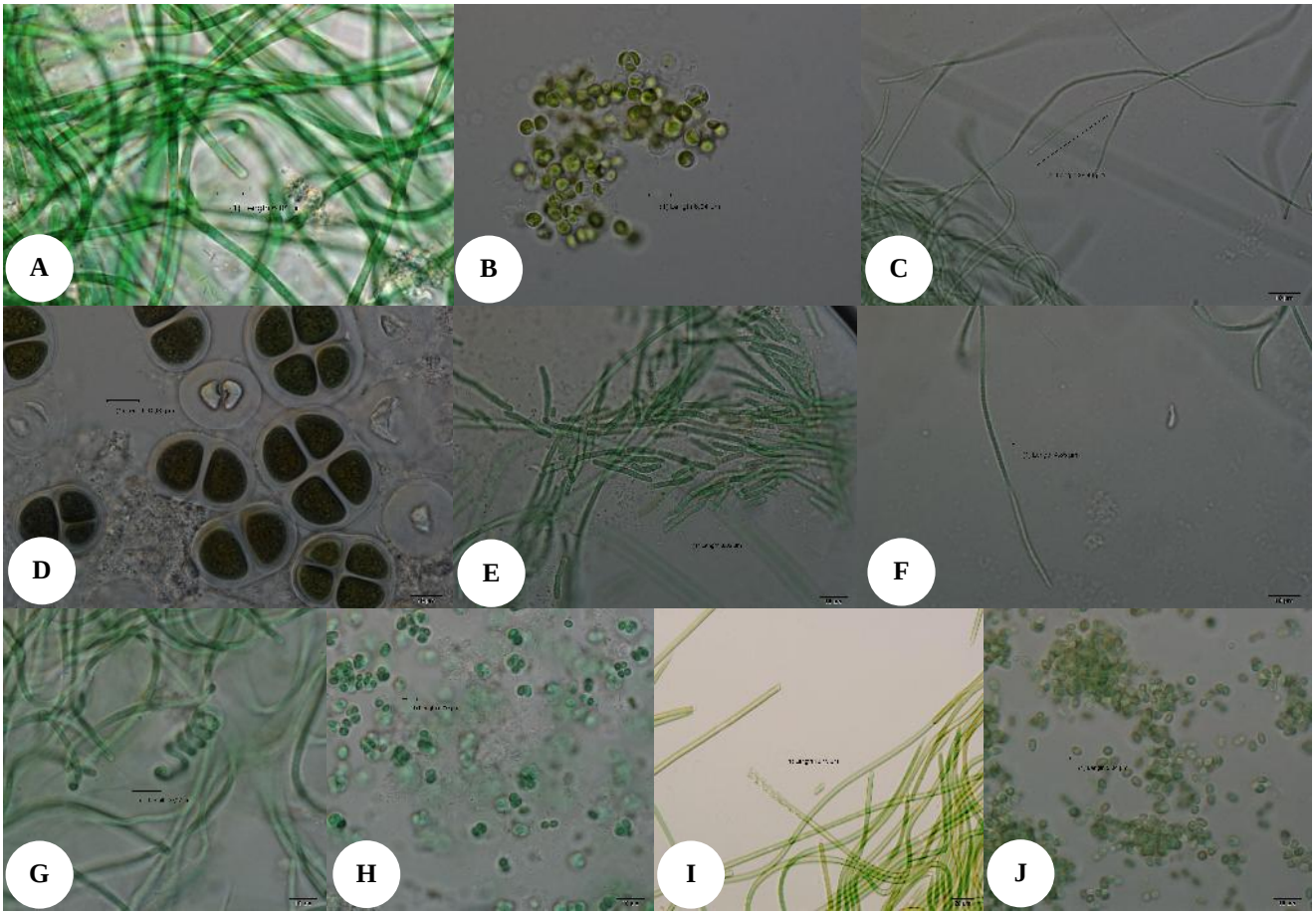

Figure 2. Cell images of marine cyanobacteria isolates; A. *Oscillatoria limnetica*, B. *Coelastrella* sp., C. *Oscillatoria* sp., D. *Chroococcus* sp., E. *Leptolyngbya* sp., F. *Pseudanabaena* sp., G. *Lyngbya* sp., H. *Aphanothece* sp., I. *Phormidium* sp., J. *Synechococcus* sp.

Table 2. The yield data of biomass, EPS and total carbohydrate content of EPS from 100 ml culture of marine cyanobacteria

Isolates	Biomass (g)	EPS (g)	Total carbohydrate (mg/mL)
Oscillatoria limnetica	1.4010	0.3737	0.0651
Coelastrella sp.	0.9825	0.3798	0.0976
Oscillatoria sp.	1.0272	0.3706	0.0849
Chroococcus sp.	0.3971	0.6318	0.5332
Leptolyngbya sp.	1.1241	0.1690	0.0665
Pseudanabaena sp.	0.2132	0.6175	0.0523
Lyngbya sp.	0.4246	0.3842	0.0863
Aphanothece sp.	2.1796	0.0462	0.0651
Phormidium sp.	1.2266	0.3096	0.0778
Synechococcus sp.	0.3806	0.6015	0.0693

Table 3. Antidiabetic activity of extracellular polysaccharide (EPS), intracellular polysaccharide (IPS) and methanol extract of marine cyanobacterial strains

Isolates	Inhibition of α-glucosidase (%)		
	EPS	IPS	methanol extract
Oscillatoria limnetica	-	-	-
Coelastrella sp.	10.93	3.91	-
Oscillatoria sp.	9.27	1.90	-
Chroococcus sp.	13.0	-	-
Leptolyngbya sp.	2.13	-	-
Pseudanabaena sp.	14.02	-	-
Lyngbya sp.	9.19	-	5.48
Aphanothece sp.	8.08	-	-
Phormidium sp.	-	-	-
Synechococcus sp.	3.13	-	-

Note:-= negative

Microalgae enable in the production of polysaccharides or whichever other compounds with similar properties, either chemical or physical. Polysaccharides and sulphated exopolysaccharides are released by many species of microalgae (de Jesus Raposo et al. 2013). EPS are high molecular weight carbohydrate polymers. Many marine microorganisms produce extracellular polymers which form a layer surrounding the cells that helps them to withstand or resist adverse and extreme environmental conditions industries (Satpute et al. 2010). On this study, EPS was separated from concentrated of culture media by precipitated method with ethanol. The yield data of

biomass and EPS, and also total carbohydrate content of EPS was presented in Table 2.

Data on Table 2 shown that *Chroococcus* sp. and *Pseudanabaena* sp. produced higher yields of EPS compared to other marine cyanobacteria culture, although its biomass yields were very low. This indicated the marine cyanobacteria growth rate was not influenced by the production of EPS. The algal cells can release extra-cellular polysaccharides EPS into the environment. The production of polysaccharide by green algal species indicated the involvement of this polysaccharide in protecting the algal cells against toxic species. The increasing in polysaccharides contents in crude microcystins treated cultures in algae indicated that these polysaccharides may be involved in certain defense mechanisms in response to toxin stress (El-Sheekh et al. 2012). We assumed that the production of EPS by *Chroococcus* sp. and *Pseudanabaena* sp. is the response to toxin that contained in culture medium.

EPS and IPS from marine cyanobacter culture were screened for antidiabetic activity by analysis its inhibition to the α-glucosidase activity, the results are shown in Table 3. The data shown that almost all of EPS from marine cyanobacteria culture were potential as inhibitor of α-glucosidase, except *Oscillatoria limnetica* and *Phormidium* sp. isolates were negative or no inhibition to the α-glucosidase activity. The highest activity in inhibition of α-glucosidase was *Pseudanabaena* sp. (14.02%) and *Chroococcus* sp. (13.0%) isolates. This data shown has correlated with EPS production (Table 2), in which these cultures were produced high yields of EPS.

The distribution of *Pseudanabaena* is widespread, although their actual abundance is still to be determined (Acinas et al. 2009). C-phycoerythrin was isolated and purified from marine *Pseudanabaena* sp. to evaluate its fluorescence properties for future applications in biochemical and biomedical research (Mishra et al. 2011). The antidiabetic activity of EPS from *Pseudanabaena* sp. is the first report and potential for further research.

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