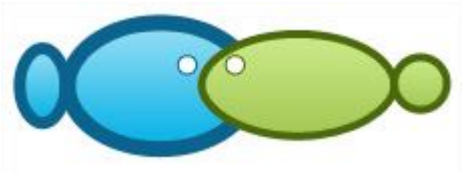

Polysaccharides-producing bacteria isolated from marine sponge, *Theonella* sp. and their bioactivities

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Abstract. Marine bacteria associated with marine sponge, *Theonella* sp. is regarded as promising source of biologically-active compounds. It has a huge potential as a primary drug-producer due to its unique structure, which is rare relative to other organisms of terrestrial region. Thus, this study aimed to identify polysaccharide-producing bacteria isolated from *Theonella* sp., and to screen their polysaccharides for anti-bacterial and toxicity tests. Four out of seven types of bacteria isolated from Bidong Island in Terengganu was determined as polysaccharide-producing bacteria, with a high yield of polysaccharide being recorded by *Shewanella putrefaciens* and *Capnocytophaga* sp. at 239.7 and 215.8 mg L^{-1}, respectively. Two other bacteria were *Brevundimonas diminuta* and *Burkholderia cepacia*, which produced polysaccharides at a low yield (179.0 and 167.6 mg L^{-1}, respectively). In reference to Disc Diffusion Test, growth inhibition was occurred against *Salmonella typhimurium* (15.67±1.15 mm), *Klebsiella pneumoniae* (13.67±0.58 mm) and *Bacillus subtilis* (7.33±0.58 mm). Polysaccharide produced by *S. putrefaciens* showed a strong growth inhibition against *B. subtilis* with Minimum Inhibitory Concentration (MIC) value recorded at 100 µg mL^{-1}. In a meantime, the growth of *S. putrefaciens* was also inhibited by both *S. typhimurium* and *K. pneumoniae*, in which MIC value was pronounced at of 50 µg mL^{-1}. Additionally, toxicity activity as measured by LC_{50} value indicated polysaccharide secreted by *S. putrefaciens* tested in brine shrimp, *Artemia salina* was at 0.52 mg mL^{-1}. In future, further study is needed to highlight the potential pharmaceutical value of the compounds from polysaccharide-producing bacteria.

Key Words: *Artemia salina*, anti-bacterial activity, *Theonella* sp., polysaccharides-producing bacteria, toxicity test.

Introduction. Nature plays a crucial role for an acquisition of medicinal sources. To date, a large number of natural resources used in medical practice, were originated from traditional preparation of medicinal extracts of animals and plants (Ji et al 2016). In relation to this, most of the marine natural products that are currently in clinical-trial were produced by marine invertebrates. Due to its unique-characteristics such as soft-bodied, slow-moving and lack of morphological defense structure, these special features attributed it as a subject for an intensive exploration of the chemical components to elucidate its biological-functions (Giubergia et al 2016). As an effective defensive-strategy, marine invertebrates accumulate toxic natural products in order to repel predation threats or to compete for a space (Giubergia et al 2016; Berne et al 2016). Such toxicants are known as secondary metabolites with a diverse structure and stereo-chemically complex eliciting a specific biological-activity (Wakimoto et al 2016). Unlike terrestrial sources, many marine-derived chemical compounds are structurally novel due to the multiple interactions between genetic-drifts and ecological-factors. Up to this extend, marine compounds are insufficiently configured, but it is significant to indicate that the marine environment will be an important source of novel compounds in the near future (Cragg & Newman 2013).

Microorganisms are distributed abundantly in nature. Most of the bioactive compounds secreted by microorganisms gained a very important focus in today's

pharmaceutical products for instance the use of antibiotics (Penicillin and Aminoglycosides) and anti-cancer drugs (Anthracyclins and Bleomycin) (Sathishkumar et al 2018). In another context, polysaccharides secreted by marine bacteria are receiving a significant interest nowadays because it is thought to play a role to withstand an extreme environmental-stresses, in particular, alterations of normal pattern of pressure, pH, temperature and salinity, nutrient depletion and heavy-metal contamination (Manivasagan & Kim 2014). Hence, the objective of this study is to identify bacteria producing-polysaccharides from marine sponge, *Theonella* sp. and to screen their anti-bacterial and toxicity activities.

Material and Method

Sample preparation. Marine sponge, *Theonella* sp. samples were collected along the sea-bed of Bidong Island, Terengganu, Malaysia at a latitude 05°37.271' and longitude 103°03.366'. All samples were collected by underwater scuba-diving at a depth of 10 to 16 m.

Bacteria identification. *In-situ* sampling of bacteria from sponges was aseptically performed on boat according to Webster & Hill (2001) with some modifications. After bacteria-sampling, all plates were incubated at 27°C for one to three days in the incubator-shaker at the Microbiology Laboratory, Universiti Malaysia Terengganu (UMT). The viscous colony grown on the medium was picked and re-streaked onto agar plate for isolation purpose. Single colony of bacteria (isolate) was randomly picked and purified by repeated-plating on Sucrose Sea Water (SSW) agar plates and maintained on SSW agar slant (Shamsuddin 2000; Webster & Hill 2001). Bacterial isolates obtained from *Theonella* sp. were classified in reference to Bergey's Manual of Determinative Bacteriology (Bergey et al 1994). The characteristics of bacteria from conventional test were further used in subsequent step for identification purpose following protocol described by Maloney et al (2014) using Bacterial Diagnostic Systems {RapID NF Plus System, RapID ONE System and RapID ANA System (Remel, USA)}.

Isolation of polysaccharides-producing bacteria. Screening for polysaccharide-producing bacteria was performed using a protocol described by Kambourova et al (2009) with some modifications. Bacteria isolates were screened for polysaccharide production using SSW broth consisting of peptone (5 g L^{-1}), yeast (1 g L^{-1}), sucrose (30 g L^{-1}) and sea water (1 L). Sterile sea water was used as a basal solution. Crude polysaccharides were isolated following protocol reported by Shamsuddin (2000).

Disc Diffusion Test (DDT) and Minimum Inhibitory Concentration (MIC). Eight bacteria test strains were used namely *Bacillus cereus (*ATCC 11778), *Staphylococcus aureus (*ATCC BAA-1026), *Bacillus subtilis (*ATCC 6051), *Enterococcus faecalis (*ATCC 51299), *Escherichia coli (*ATCC 4157), *Salmonella typhimurium* (ATCC 29629) and *Klebsiella pneumoniae* (ATCC 700603). Protocol of DDT was performed according to Kirby-Bauer method (Bauer et al 1966) with some modifications. First, 6-mm sterile Whatman paper-discs were impregnated into 30 µL of sterile-filtered of crude polysaccharides produced by each bacterium isolate, which was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 µg mL^{-1}. Next, the wet paper-disc was dried in air for 30 min inside a safety cabinet, before it was placed onto the surface of Muller-Hinton agar plates (Merck, USA) with swabs of bacteria test strains. Then, the plates were incubated at 37°C for overnight. Gentamicin and DMSO were used as positive and negative controls, respectively. Anti-bacterial activity is evaluated by measuring the diameter zone of growth inhibition against the bacteria test strains which was expressed as millimeter (mm). MIC test for selected bacterial polysaccharide, *Shewanella putrefaciens* was determined using the same method employed for DDT. For MIC test, polysaccharide was diluted with two-fold dilution at a respective concentration of 100, 50, 25, 12.5, 6.25 and 3.125 µg mL^{-1} (Pfaller et al 2010).

Toxicity test using brine shrimp, Artemia salina. In general, the procedure for toxicity test using brine shrimp, *A. salina* was applied according to the method developed

by Carballo et al (2002) with some modifications. For evaporation purpose, the selected crude polysaccharides were dissolved in DMSO at a respective final concentration of 1000, 500, 250, 125, 62.5, 31.3 and 15.6 µg mL^{-1} in 96-wells microtiter plate. After complete evaporation, the dried-crude polysaccharides were dissolved in 100 µL of sea water. Next, 100 µL sea water containing 10 to 20 individuals of *A. salina* were added into each well to produce a total volume of 200 µL. After 12-hours approximate duration for hatching the phototropic nauplii, the brine shrimps were collected from the lighted-side and concentrated into a small vial. A total of 20 individuals of brine shrimps were transferred into each well. Each test consisted of exposing 20 individuals of *A. salina* at 12 hours of age to various concentrations of toxic compound. The toxicity of nauplii at instar stages I and II was determined after 12 hours of exposure (Carballo et al 2002). All assays were conducted at 28°C under continuous light exposure. The numbers of survival were counted and the percentage of mortality was calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. Lethality dose curve for crude polysaccharides which expressed as LC_{50} was determined using Probit Analysis on Graph-Pad Prism Software version 5.00.

Results. All isolated bacteria were categorized as Gram-negative bacteria based on morphological characteristics of Gram's staining and their response to biochemical test (Table 1). The bacteria isolates were identified as *Shewanella putrefaciens* (4 isolates), *Brevundimonas diminuta* (3 isolates), *Alcaligenes faecalis* (1 isolate), *Burkholderia cepacia* (1 isolate), *Fusobacterium varium* (1 isolate), *Vibrio damsela* (1 isolate), *Capnocytophaga* sp. (1 isolate). Each bacteria isolate exhibited a varied result for each biochemical test (Table 1). All of the bacteria isolates respond positively to catalase test, but for oxidase test only *Capnocytophaga* sp. was seen unresponsive. Most of the bacteria isolates were yellowish in colour and some of them appeared swarming on the surface of agar. The bacteria colonies appeared to be mucoid after a few days of sub-cultured on SSW agar. Following this step, the bacteria isolates were picked randomly in order to determine a suitable candidate for polysaccharide-producing bacteria. Weights of polysaccharides produced were determined after isolation procedure of the crude polysaccharide as shown in Figure 1. Four bacteria isolates (*S. putrefaciens*, *Capnocytophaga* sp., *B. diminuta* and *B. cepacia*) were selected for biological screening tests because they produced a high yield of polysaccharide (239.7, 215.8, 179.0, 167.6 mg L^{-1}, respectively) than any other polysaccharides-producing bacteria (*V. damsela*, *F. varium* and *A. faecalis*) ranging from 15.1 to 26.7 mg L^{-1}.

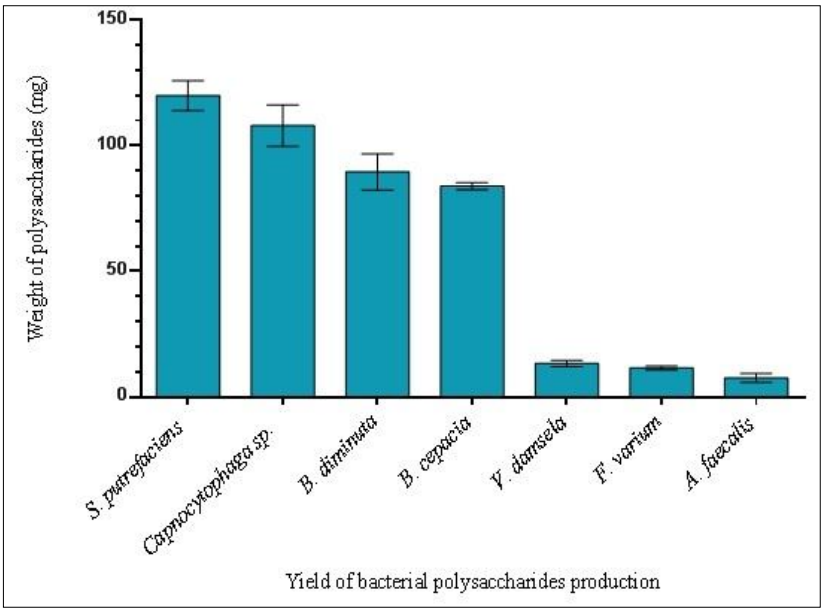

Figure 1. Production yield of polysaccharide selected from bacterial isolates of marine sponge, *Theonella* sp.

DDT assay was applied to screen growth-inhibition effect against selected pathogenic bacteria. As shown in Table 2, only polysaccharide of *S. putrefaciens* exhibited a positive growth inhibition against *S. typhimurium* (15.67±1.15 mm), *K. pneumoniae* (13.67±0.58 mm) and *B. subtilis* (7.33±0.58 mm). As for MIC test, polysaccharide secreted by *S. putrefaciens* showed a positive growth inhibition against *B. subtilis* at 100 µg mL^{-1}. Whereas, MIC value of *S. typhimurium* and *K. pneumoniae* were at 50 µg mL^{-1} (Table 3).

Toxicity test was performed using 12-hour age of brine shrimp, *A. salina* nauplii at instar stages I and II. The exposure at the lowest concentration did not show any mortality for each crude polysaccharide used. Mortality was started by *S. putrefaciens* polysaccharide at a concentration of 31.25 µgmL^{-1}, followed by polysaccharides produced by *B. cepacia*, *B. diminuta* and *Capnocytophaga* sp. which were ranged from 125 to 250 µg mL^{-1}. At 50% mortality, lethality dose curve (Figure 2) showed a minimum value of LC_{50} possessed by *S. putrefaciens* polysaccharide (0.52 mg mL^{-1}) as compared to *Capnocytophaga* sp. polysaccharide which recorded a maximum LC_{50} value at 25.08 mg mL^{-1}. On the other hand, *B. cepacia* and *B. diminuta* polysaccharides gave a LC_{50} values at 2.08 and 3.62 mg mL^{-1}, respectively.

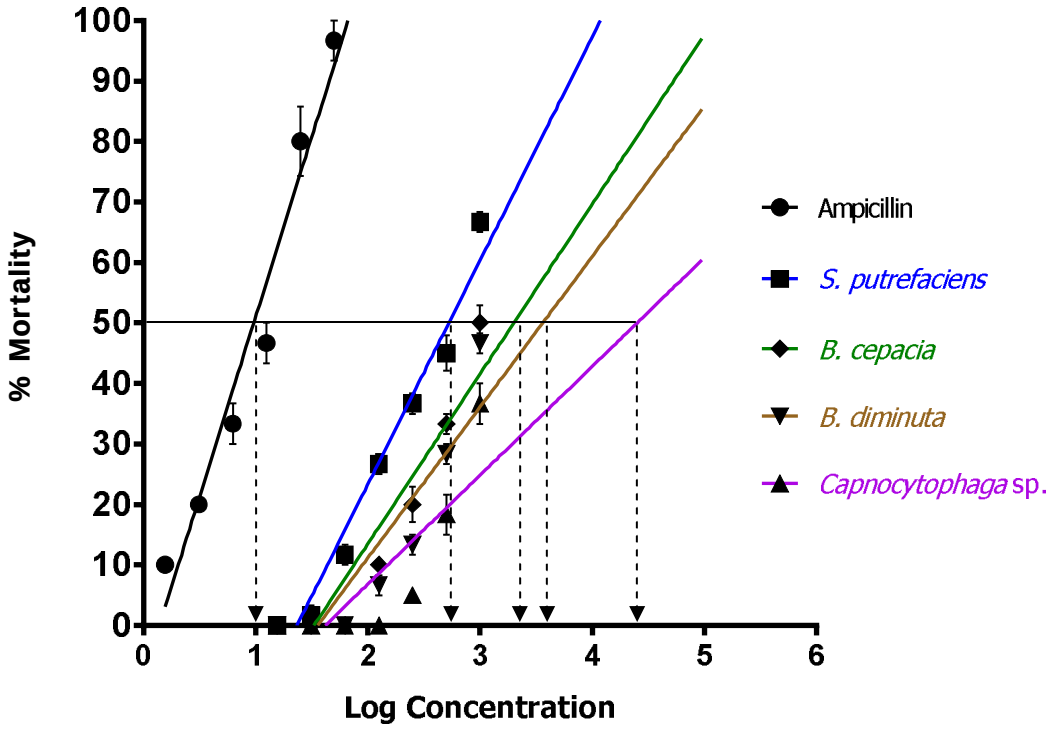

Figure 2. Determination of toxicity activity recorded at 50% mortality (LC_{50}) in selected bacterial polysaccharides at a dosage of 15.63 to 1000 µg mL^{-1} upon exposure to 12 hour of age brine shrimp, *A. salina* nauplii. Ampicillin was used as a positive control at a concentration of 1.56 to 50 µg mL^{-1} and DMSO acts as negative control.

Table 1

Biochemical characteristics of bacteria isolated from marine sponge, *Theonella* sp.

Bacteria isolates	*Number of bacteria tested*	*Number of positive results (%)*						
		Oxidase	*Catalase*	*Indole*	*Methyl Red*	*Voges-Proskauer*	*Citrate*	*Hydrogen sulphide production*
S. putrefaciens	4	100	100	0	0	0	0	100
B. diminuta	3	100	100	100	100	0	0	0
A. faecalis	1	100	100	0	0	0	0	0
B. cepacia	1	100	100	0	0	0	0	0
F. varium	1	100	100	0	0	0	0	0
V. damsela	1	100	100	0	100	100	0	0
Capnocytophaga sp.	1	0	100	0	100	100	0	0

Table 2

Screening of anti-bacterial activity of crude polysaccharides produced by bacterial isolates using DDT assay

Bacteria isolates	*Crude polysaccharides concentration (μgmL^{-1})*	*Diameter of growth inhibition zone (mm); mean±S.D.*					
		B. subtilis	*E. faecalis*	*E. coli*	*K. pneumoniae*	*S. typhimurium*	*S. aureus*
S. putrefaciens	100	7.33±0.58	-	-	15.67±1.15	13.67±0.58	-
B. cepacia	100	-	-	-	-	-	-
B. diminuta	100	-	-	-	-	-	-
Capnocytophaga sp.	100	-	-	-	-	-	-
Gentamicin (Positive control)	0.5	17.33±0.58	21.67±0.58	21.67±0.58	18.67±0.58	27.33±0.58	24.68±1.53
DMSO (Negative control)	1%	-	-	-	-	-	-

*Note: "-" signs indicate no growth inhibition.

Table 3

Screening of anti-bacterial activity of crude polysaccharide produced by *S. putrefaciens* using MIC test

Crude polysaccharides concentration (µg mL^{-1})	*Dilution factor*	*Diameter of growth inhibition zone (mm); mean±S.D.*		
		B. subtilis	*K. pneumoniae*	*S. typhimurium*
100	2°	7.33±0.58	15.67±1.15	13.67±0.58
50	2^{-1}	-	8.33±0.58	7.33±0.58
25	2^{-2}	-	-	-
12.5	2^{-3}	-	-	-
6.25	2^{-4}	-	-	-
3.125	2^{-5}	-	-	-

Discussion. Marine bacteria have become increasingly popular and novel sources of polysaccharides. Many marine bacteria are able to produce polysaccharides, but it has not been utilized extensively, particularly to aid a new development of medical application. A few of the medical products derived from bacteria is currently in market are anti-biofilm (Junter et al 2016), wound-healing, surgical-dressing, drug-delivery system, tissue-engineered blood vessels (Di Donato et al 2016). Despite of the limited usage, a search of polysaccharides that might have innovative applications is still of potential interest (Squillaci et al 2016). In this study, a total of seven bacteria were isolated from *Theonella* sp. namely *S. putrefaciens*, *Capnocytophaga* sp., *B. diminuta*, *B. cepacia*, *V. damsela*, *F. varium* and *A. faecalis*. They exhibit a mucoidal morphology on SSW agar, and their capacity to produce polysaccharide was screened. The highest yield of polysaccharide was produced by *S. putrefaciens* at a concentration of 239.7 mg L^{-1} suggested that the initial mucoidal observation consistent with the yield of polysaccharide. This finding was corroborated with Kambourova et al (2016). Furthermore, they observed that the outer-layer of bacteria cells secrete mucous and appeared to be bacterial-polysaccharides when preceded for polysaccharide production (Kambourova et al 2016).

Bacterial-polysaccharides exhibited a self-biological activity. It is interesting to note that *S. putrefaciens* polysaccharide indicated a broad-spectrum activity against both Gram-positive and negative bacteria. The reason behind sensitivity and specificity response to pathogenic bacteria is related to the differences in external morphology characteristics, biochemical composition and structure of the cell wall in both types of bacteria (Mingeot-Leclercq & Décout 2016). Gram-negative bacteria contain outer membranes carrying-lipopolysaccharide components, which makes the cell wall impermeable to lipophilic solution. In contrast, Gram-positive bacteria have only an outer peptidoglycan layer, which is not as effective permeable barrier as Gram-positive bacteria (Silhavy 2015).

The present study found a varied result for growth inhibition between bacteria isolates. This was probably caused by specificity of polysaccharide and the choice of technique use as in agreement with Cappuccino & Sherman (2001). In the bacterial cell, there are four major sites that mediate an effective clinical-drug action. They are specifically known as cell wall, ribosome, nucleic acids and cell membranes. Up to now, the occurrence of bacteria drugs resistance is a worse-case scenario in the medical field. Therefore, understanding of mechanism governing bacterial drugs resistance is utmost important. Shamsuddin et al (2010) proposed four mechanisms underlying bacterial drugs resistance: first, bacteria produce enzymes to inactivate the drug; second, bacteria synthesize modified targets resulting negligent effects on drugs; third, bacteria decrease their cell wall's permeability causing an effective intracellular concentration of the drug is not achieved; and fourth, bacteria actively export drugs using a 'multi-drug resistances pump' (MDR pump). These mechanisms distinguished the activities for different treatments, which were used on each of specific bacteria. The differences in susceptibility among bacteria also may be explained by the different cell wall composition and the

inherited-genes on plasmids of the anti-microbial compounds, which can be easily transmitted among bacteria strains tested (Shamsuddin et al 2010).

While there is abundant evidence reported by Blunt et al (2015) and Shamsuddin et al (2010), who found that crude organic extracts of marine invertebrates exhibit anti-bacterial activity against medically important bacteria. The invertebrates are potentially vulnerable to microbial-infection, which may have led to the evolution of chemical defense. In marine invertebrate, several kinds of immune-related humoral activities have been reported by Qian et al (2016), but it is species-specific. The presence of anti-bacterial effects of certain polysaccharide suggested that it could be due to the different composition of polysaccharides carrying by each of them (Billings et al 2013). This could attribute to physical environmental and biological factors, extremes temperature, nutrient deficiency, perpetual threat from predator, microbial-pathogen and competition for limited resources. Potential roles of these chemical defenses include protection against invasion, settlement by other invertebrates and predation (Singh & Thakur 2016).

The toxicity assay using brine shrimp is an excellent method for preliminary investigations on how several bioactive molecules from various sources affect cellular functions in live organism (Wu 2014). Alam et al (2016) reported that, the toxic effect of their sample used was positively correlated between the lethality to brine shrimp and the corresponding oral lethal dose in mice. Our results have revealed that bacterial-polysaccharides used had toxic effect against the brine shrimp, *A. salina*. In the brine shrimp lethality test, *S. putrefaciens* had most toxic effect against *A. salina* followed by other bacterial polysaccharides used. Mortality was observed during the incubation period at various concentrations of polysaccharides. The degree of lethality was found to be directly proportional to the concentration of the polysaccharides. Maximum mortalities took place at a concentration of 1 mg mL^{-1} whereas lower mortality was observed at 0.031 mg mL^{-1}. The results showed significant differences in percentage mortalities between different concentrations within the polysaccharide samples indicating brine shrimp lethality compared to that of control. The LC_{50} values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts and the best-fit line was obtained from the data by means of regression analysis. This significant lethality of several bacterial polysaccharides to brine shrimp is an indicative of the presence of potent toxic components which warrants further investigation (Waliullah et al 2016).

Conclusions. To the best of our knowledge, the present study is the first report in its kind on production of polysaccharide by marine bacteria from marine sponge, *Theonella* sp. We have identified seven types of bacteria isolates which are *Shewanella putrefaciens*, *Brevundimonas diminuta*, *Alcaligenes faecalis*, *Burkholderia cepacia*, *Fusobacterium varium*, *Vibrio damsela* and *Capnocytophaga* sp. and four of them producing polysaccharide that are *S. putrefaciens, Capnocytophaga* sp*., B. diminuta* and *B. cepacia.* Only *S. putrefaciens* crude polysaccharide showed moderate activity in anti-bacterial and toxicity tests. Hence, crude polysaccharide from *S. putrefaciens* is recommended for the medicinal preparation for anti-bacterial and toxic properties.

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