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Original Article

SEAWATER DEPENDENCY OF MARINE ACTINOMYCETES FOR ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

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ABSTRACT

Objective: This study was done to examine the seawater dependency of marine actinomycetes in the production of its bioactive secondary metabolites.

Methods: A total of 100 marine actinomycetes strains were isolated from marine sponges collected within the conserved sea area of Sipadan Island. All of the strains were fermented in mannitol-peptone broth with the presence and absence of seawater prior to the antimicrobial and DPPH assay.

Results: The data from DPPH assay showed that 22 strains have antioxidant properties (IC50 in the range of 56.3µg/ml to 99.1µg/ml) in the presence of seawater and 15 strains have antioxidant properties (IC50 in the range of 82.6µg/ml to 99.4µg/ml) in the absence of seawater. On the other hand, the presence of seawater during fermentation has induced the production of antimicrobial compounds of 31 strains with inhibition zone ranging from 8mm to 19mm. In contrast, without seawater, only 22 strains were able to exhibit antimicrobial properties with inhibition zone ranging from 8mm to 11mm.

Conclusion: The data from both assays showed that seawater indeed played an important role in the production of the antioxidant and antimicrobial compounds from marine actinomycetes.

Keywords-Antimicrobial, Antioxidant, Marine actinomycetes, Sipadan Island

INTRODUCTION

Oceans are highly complex environment with extreme variations in pressure, salinity and temperature which are habitat of diverse assemblage of microbes[1]. In these harsh environments, these microbes are able to survive due to their specialized adaptation, which may include the production of various chemicals that enable them to sustain their life. Although the ocean have biodiversity that are richer compared to terrestrial rainforests, most part of it are still underexplored [2-3]and the exploitation the microbes from these environments might bring great value to the pharmaceutical field.

From all marine microorganisms that live in the ocean, marine actinomycetes are regarded as the one that has greatest potential to be exploited as new sources of bioactive compounds. The adaptation of marine actinomycetes towards the harsh sea environment resulted that most of these microbes show the requirement of seawater for growth, a physiological trait that contrast to the terrestrial actinomycetes[4]. Based on this unique adaptation to the high salinity in the sea, it is assumed that marine actinomycetes might be able to produce unique secondary metabolites for their successful survivability. In the ocean, marine actinomycetes are widely dispersed where it can be found in seawater, sediments and also in association with many marine organisms. In this study, marine sponges were used as the source for actinomycetes isolation due to its well-known symbiotic interaction with marine microbes[5]. In addition to that, actinomycetes are the common microbes that can be successfully isolated from sponges[6].

The aim of this research was to investigate the effect of seawater towards the production of secondary metabolites from marine actinomycetes for their antioxidant and antimicrobial properties. 100 strains of marine actinomycetes were fermented in broth with the presence and absence of seawater, and the resulting secondary metabolite extracts were tested for its potential antimicrobial and antioxidant activities by using disc diffusion methods and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, respectively.

Antimicrobial and antioxidant properties of the actinomycetes from this unique environment were examined due to its enormous benefits to the pharmaceutical field because bacterial infection and oxidative stress-related diseases possess major threat to the human health worldwide. The usages of currently available antibiotics drugs in the treatment of some bacterial infections are nearly impossible as these pathogens are able to develop resistance towards these antibiotics[7]. Aside from bacterial infections, oxidative stress related diseases such as liver damage, nephrotoxicity, pathogenesis of diabetes, inflammation, cancer, cardiovascular disorder, neurological disorders and also the acceleration of aging process[8] are also the major threat to the human population. These diseases are caused by the oxidative stress due to the accumulation of free radicals as the result of the cellular metabolisms in the body, and these free radicals have damaging effect on cellular components such as lipids, proteins and DNA[9]. Besides that, the exposure to the exocellular factors such as ultraviolet light, cigarettes smoke and atmospheric pollutants can also contribute to the formation of free radicals in the body[10-14].

MATERIALS AND METHODS

Sample Collection

Marine sponges were collected around Semporna coastal area (4°06'52.92"N and 118°38'04"E) as shown in Fig 1. This area is a conserved region that is located of a close distance from Sipadan Island. The samples were taken by scuba diver at depth between 10-15 m. All samples were kept in a sterilized slip bag and stored in -40°C freezer upon arrival to the laboratory.

Actinomycetes Isolation

Approximately 3 cm of tissue from mesophyll part of sponge was excised and homogenised in 10 ml seawater using sterile mortar. Homogenate serially diluted and plated on starch casein agar (SCA) prepared by using seawater (nalidixic and cycloheximide supplemented). Plates of isolation media were incubated for 3 weeks

in the incubator at 28°C. The resulting colonies on HV agar were further sub-cultured on oatmeal (OA) agar with pH 7.2.

Fig. 1: Sabah sea area (Red circle shows the sampling area).

Determination of Actinomycetes by non-staining KOH method

A drop of 3% aqueous KOH were placed on a slide. Using a sterile loop, a visible amount of bacterial growth is transferred from the agar culture to the drop of KOH. The cells and KOH were mixed thoroughly on the slide, constantly stirring over an area about 1.5 cm in diameter. If the bacterium-KOH suspension becomes markedly viscid or gels within 5 to 60 seconds, the isolate is Gram negative. If no gelling is observed, the isolate is Gram positive. The best way to test the viscosity is to raise the loop about 1 cm from the slide. If an obvious stringiness is present, then the culture was gram negative. The actinomycetes can be identified by the sporulation on OA media and also non gelling formation on slides.

Fermentation and Extraction

Production of microbial secondary metabolites were from actinomycetes grown in 2% mannitol + 2% peptone + 1% glucose liquid medium at 28°C for 5 days. The liquid medium was prepared in the presence or absence of seawater. The 25 ml culture in conical flask (250 ml) was shaken at 220 rpm in shake flask incubator. The secondary metabolites from actinomycetes were extracted by using equal volume of acetone to culture medium.

Antioxidant Test

Different concentration (100, 50, 25, 12.5 & 6.25 µg/ml) for each sample were dissolved in water and taken in tubes separately. Ascorbic acid was also prepared in different concentrations (100, 50, 25, 12.5 & 6.25 µg/ml) and this was used as a reference standard. DPPH (1, 1, Diphenyl-2-Picryl hydrazyl) 0.002% was freshly prepared in ethanol. DPPH (2.7 ml) was added to each tube containing different concentrations of extracts (0.3 ml) and of standard solution (0.3 ml). It was shaken vigorously with a vortex. They were then allowed to stand for 60 minutes for room temperature in dark place. The control was prepared without any extracts (0.3ml water added with 2.7ml of 0.002% DPPH). Ethanol was used for base line corrections in absorbance (OD) of sample measured at 517 nm. The below formula was used to interpret the value of the sample.

% DPPH Scavenging Activity = [(control O.D – sample O.D) / control O.D] x 100

The working concentration of antioxidant was expressed as IC50 value, indicating the concentration of each extracts that are able to scavenge 50% of DPPH radicals.

Antimicrobial Assay

Antibacterial activities were assayed by using well diffusion method against the eight standard test organisms namely *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella spp., Streptococcus pneumonia* and *Pseudomonas aeruginosa*. The test organisms were grown in sterile nutrient broth at 37°C for 12 hours and 200µl of broth containing the test organism is added into 100ml of molten nutrient agar. Sterile filter paper discs 6 mm in diameter were impregnated with 20 µl of each extract (30µg/ml) which were prepared using the same solvents employed to dissolve the extracts, and then sterilized, and placed on the agar surface containing each test organism. Standard 6-mm discs containing chloramphenicol were used as positive controls. Negative controls were made using paper discs loaded with the solvents. The plates were incubated overnight at 37 °C for 18-24 hours. At the end of the incubation period antimicrobial activity was evaluated by measuring the zones of inhibition.

RESULTS

Isolation

A total number of 100 marine actinomycetes strains were successfully isolated from sponges by using starch casein agar (SCA) supplemented with seawater. All the isolated strains were examined by using non-staining KOH method to determine either it was gram positive or gram negative. In this test, all the strains were identified as gram-positive bacteria given that there were no gelling formations in the KOH droplets. Besides the morphology of strains on the purification media, the results of non-staining KOH method were further confirmed that all the isolated strains were actinomycetes.

Antioxidant Assay

In this study, out of 100 marine actinomycetes strains tested, 33 strains showed to have antioxidant activities with or without seawater as shown in TABLE 1. The antioxidant activity of each strain was different in fermentation media with or without the presence of seawater.From 33 strains mentioned above, 22 strains have antioxidant activity when fermented in the presence of seawater (IC50 in the range of 56.3µg/ml to 99.1µg/ml) whilst only 15 strains have antioxidant activity when fermented without seawater (IC50 in the range of 82.6µg/ml to 99.4µg/ml). From these bioactive strains, it is interesting to note that there were four strains such as MSA07, MSA22, MSA34 and MSA47 showed antioxidant activity on both fermentation media with or without seawater. The highest antioxidant activity was observed on strain MSA07 with IC50 value of 56.3µg/ml in the present of seawater. On the other hand, MSA52 exhibited the lowest antioxidant activity with the IC50 value of 99.4µg/ml in the absence of seawater. These data clearly revealed that the antioxidant potential of the secondary metabolites produced are much stronger if fermentation is done in the presence of sea water. Besides, these marine sponges-associated actinomycetes isolated from conserved sea area of Sipadan Island exhibited remarkable potential of antioxidant properties as the IC50 values and working condition of samples are in the range of µg/ml instead of mg/ml.

Antimicrobial Assay

The data in this assay revealed that a total of 43 strainsfrom the 100 actinomycetes strains isolated did possess antimicrobial activities towards the tested pathogens at 30mg/ml as shown in TABLE 2. The inhibition zones obtained ranging from 8mm to 19mm, and the antimicrobial activities were different when fermented in the presence or absence of seawater.

Antimicrobial assay showed that 31 strains exhibited activity on test pathogens in the presence of seawater (inhibition zone within the range of 8mm to 19mm). On the other hand, only 22 strains have antimicrobial activity in the absence of seawater (inhibition zone within the range of 8mm to 11mm). From this data, it is impressive to find that 10 strains of marine actinomycetes such as MSA03, MSA07, MSA23, MSA34, MSA44, MSA62, MSA69, MSA77, MSA84 and MSA98 possess antimicrobial activity when fermented by using both mannitol-peptone broth prepared with distilled water or seawater. However, it is also important to note that the antimicrobial strength of these 10 strains were different in both conditions. In addition, the number of the tested pathogens successfully inhibited by these actinomycetes was also different.

For example, the extract from MSA62 was able to inhibit only four pathogens (*Streptococcus pyogenes, Staphyloccoccus aureus, Streptococcus pneumonia and Pseudomonas aeruginosa*) in the absence of seawater, but the range of activity was increased to five

pathogens (*Streptococcus pyogenes, Staphyloccoccus aureus, Bacillus cereus, Bacillus subtilis* and *Streptococcus pneumonia)* in the presence of seawater. The presence or absence of seawater in the fermentation broth also affects the strength of antimicrobial activity in the crude extracts. For example, in the presence of seawater, the antimicrobial activity of MSA77 and MSA84 towards *Streptococcus pneumonia* and *Pseudomonas aeruginosa* in increased from 8mm to 12mm and 10mm to 12mm, respectively.

Table 1: It shows theIC50 values of crude extracts

Strains	IC50	
	MP with distilled water	MP with Seawater
MSA01	-	+ (98.2µg/ml)
MSA03	-	+ (95.4µg/ml)
MSA05	+(98.2µg/ml)	-
*MSA07	+(82.6µg/ml)	+ (56.3µg/ml)
MSA10	+(97.0µg/ml)	-
MSA12	+(98.4µg/ml)	-
MSA14	+(88.4µg/ml)	-
MSA18	-	+ (97.1µg/ml)
*MSA22	+(96.5µg/ml)	+(89.3µg/ml)
MSA24	-	+(98.6µg/ml)
MSA27	-	+(98.0µg/ml)
MSA29	-	+ (97.2µg/ml)
MSA30	+(90.1µg/ml)	-
MSA31	-	+(97.9µg/ml)
*MSA34	+(97.2µg/ml)	+(79.5µg/ml)
MSA37	+(92.4µg/ml)	-
MSA42	-	+(91.4µg/ml)
MSA45	-	+(90.1µg/ml)
*MSA47	+(98.5µg/ml)	+(85.0µg/ml)
MSA48	-	+(98.5µg/ml)
MSA50	-	+(99.1µg/ml)
MSA52	+(99.4µg/ml)	-
MSA55	+(90.6µg/ml)	-
MSA57	-	+(78.5µg/ml)
MSA62	+(95.8µg/ml)	-
MSA69	+(98.4µg/ml)	-
MSA73	+(92.6µg/ml)	-
MSA74	-	+(92.1µg/ml)
MSA76	-	+(85.5µg/ml)
MSA79	-	+(96.4µg/ml)
MSA82	-	+(56.8µg/ml)
MSA84	-	+(91.4µg/ml)
MSA92	-	+(95.2µg/ml)

*Strains with antioxidant activity both in the presence and absence of seawater.(MP: Mannitol-peptone).

Table 2: It shows the antimicrobial activity of crude extracts from MSA01 until MSA40. (MSA=Marine Sponge Actinomycetes)

Strains		Antimicrobial (mm)							
		Staph A	Strep P	Kleb	B.C	B.S	S.P	E.C	P.A
MSA01	MP	-	-	-	-	-	-	-	-
	MP+ ASW	9±0.0	-	-	10±0.0	-	-	-	-
*MSA03	MP	8±0.0	9±0.0	-	8±0.0	8±0.0	-	-	-
	MP+ ASW	12±0.0	11±0.0	-	-	-	-	-	-
*MSA07	MP	-	-	9±0.0	-	9±0.0	-	-	9±0.0
	MP+ ASW	9±0.0	-	10±0.0	-	8±0.0	-	-	12±0.0
MSA09	MP	-	-	-	-	-	-	-	-
	MP+ ASW	8±0.0	-	-	9±0.0	12.3±0.6	-	-	-
MSA12	MP	9±0.0	8±0.0	-	-	-	-	10±0.0	-
	MP+ ASW	-	-	-	-	-	-	-	-
MSA15	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	12.3±0.6	10±0.0	-	-	10±0.0
MSA18	MP	-	-	-	-	-	-	-	-
	MP+ ASW	8±0.0	8±0.0	-	8±0.0	8±0.0	-	-	8+0.0
MSA20	MP	8±0.0	8±0.0	9±0.0	-	8±0.0	-	-	9±0.0
	MP+ ASW	-	-	-	-	-	-	-	-

MSA22	MP	-	-	-	-	-	-	-	-
	MP+ ASW	10±0.0	19±0.0	-	-	9±0.0	-	-	-
*MSA23	MP	9±0.0	8±0.0				9±0.0		-
	MP+ ASW	9±0.0	11±0.0-	8±0.0	8±0.0	10±0.0	-	-	-
MSA26	MP	-	-	-	-	-	-	-	-
	MP+ ASW	10±0.0	10±0.0	-	10±0.0	10±0.0	10±0.0	-	-
*MSA34	MP	9±0.0	8±0.0	-	-	-	-	9±0.0	
	MP+ ASW	8±0.0	-	-	11±0.0	-	-	9±0.0	8±0.0
MSA36	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	10.3±0.6	11±0.0	8+0.0	9±0.0	-
MSA37	MP	-	-	-	-	-	-	-	-
	MP+ ASW	9.3±0.6	-	9±0.0	-	-	-	10±0.0	-
MSA40	MP	9±0.0	9±0.0	-	-	-	-	-	9±0.0
	MP+ ASW	-	-	-	-	-	-	-	-

Values present three replicates. * Strains with antimicrobial properties both in the presence or absence of seawater. (MP: Mannitol-peptone without seawater, MP + ASW: Mannitol-peptone with seawater).

Table 2 (cont): It shows the antimicrobial activity of crude extracts from MSA41 until MSA71. (MSA=Marine Sponge Actinomycetes)

Strains		**Antimicrobial (mm)**							
		Staph A	**Strep P**	**Kleb**	**B.C**	**B.S**	**S.P**	**E.C**	**P.A**
MSA41	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	11±0.0	-	-	9±0.0	8±0.0	-
*MSA44	MP	9±0.0	8±0.0	-	-	-	10±0.0	-	-
	MP+ ASW	9±0.0	9±0.0	-	-	-	-	-	-
MSA46	MP	-	-	-	-	-	-	-	-
	MP+ ASW	10±0.0	9±0.0	11±0.0	-	-	-	-	-
MSA48	MP	-	-	-	8±0.0	9±0.0	9+0.0	8±0.0	
	MP+ ASW	-	-	-	-	-	-	-	-
MSA49	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	14±0.0	10±0.0	10±0.0	-	-
MSA52	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	-	11±0.0	13±0.0
MSA54	MP	9±0.0	10±0.0	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-
MSA57	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	10±0.0	-	-
MSA59	MP	-	-	-	9±0.0	10±0.0	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-
MSA60	MP	-	-	-	-	-	-	-	-
	MP+ ASW	10±0.0	10±0.0	-	-	-	13±0.0	-	-
*MSA62	MP	9±0.0	9±0.0	-	-	-	8±0.0	-	9±0.0
	MP+ ASW	9±0.0	10±0.0	-	14±0.0	10±0.0	10±0.0	-	-
MSA65	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	11±0.0	13±0.0	8±0.0
MSA68	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	-	11±0.0	-	-	-	-	-
*MSA69	MP	8±0.0	8±0.0	-	-	-	-	-	10±0.0
	MP+ ASW	9±0.0	10±0.0	11±0.0	-	-	-	-	-
MSA71	MP	8±0.0	8±0.0	-	9±0.0	10±0.0	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-

Values present three replicates. * Strains with antimicrobial properties both in the presence or absence of seawater. (MP: Mannitol-peptone without seawater, MP + ASW: Mannitol-peptone with seawater).

DISCUSSION

The most significant finding in this study is the differential potential of both antioxidant and antimicrobial activities of marine actinomycetes when fermented by using mannitol-peptone broth with the presence or absence of seawater. This shows that the presence of seawater does affect the production of secondary metabolites by marine actinomycetes strains. Microbes adapt to changes in their surroundings, which ultimately affects the type of secondary metabolites they produced [15]. The presence of seawater in fermentation media will affect the growth of marine actinomycetes as different strains required the presence or absence of seawater for them to grow in the media. In favourable condition, marine actinomycetes will grow faster, and they will eventually manage to produce secondary metabolites during the seven days fermentation period. Microorganisms produce secondary metabolites during the stationary phase of the growth cycle. The process is believed to be triggered by fermentation conditions such

as the depletion of nutrients, the biosynthesis of an inducer or a decrease in growth rate. In response to these conditions the microorganism generates signals, which trigger a cascade of regulatory event resulting in chemical differentiation (secondary metabolism) and morphological differentiation (morphogenesis)[16]. The growth rate difference in the presence or absence of seawater may influence the production of secondary metabolites based on the ability of marine actinomycetes strains to reach its stationary phase during the fermentation period. The data on both antimicrobial and antioxidant assays demonstrated that marine actinomycetes strains could produce different set of compounds in the presence or absence of seawater. This can be observed by the bioactive activities in both assays. Seawater requirements are really essential for the growth of marine actinomycetes as marine organisms usually have specific requirement for NaCl for growth. There are generally about 3% NaCl plus a small amount of any other minerals and elements in seawater[15].

Table 2 (cont): It shows the antimicrobial activity of crude extracts from MSA73 until MSA99. (MSA=Marine Sponge Actinomycetes)

Strains		Antimicrobial (mm)							
		Staph A	**Strep P**	**Kleb**	**B.C**	**B.S**	**S.P**	**E.C**	**P.A**
MSA73	MP	-	-	-	-	-	-	-	-
	MP+ ASW	8±0.0	11±0.0	-	10±0.0	13±0.0	-	-	-
MSA76	MP	9±0.0	8±0.0	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-
*MSA77	MP	8±0.0	9±0.0	-	-	-	8±0.0	-	-
	MP+ ASW	-	-	9±0.0	-	-	12±0.0	-	15±0.0
MSA79	MP	-	-	-	-	-	-	-	-
	MP+ ASW	13±0.0	13±0.0	13±0.0	-	-	-	-	14±0.0
MSA80	MP	-	-	8±0.0	9±0.0	8±0.0	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-
MSA82	MP	-	-	-	-	-	-	-	-
	MP+ ASW	-	10±0.0	-	11±0.0	14±0.0	-	-	-
*MSA84	MP	-	-	-	9±0.0	10±0.0	-	-	10±0.0
	MP+ ASW	-	-	13±0.0	-	-	-	10±0.0	12±0.0
MSA86	MP	9±0.0	11±0.0	-	-	-	-	-	-
	MP+ ASW	-	-	-	-	-	-	-	-
MSA93	MP	8±0.0	8±0.0	-	-	-	-	9±0.0	10±0.0
	MP+ ASW	-	-	-	-	-	-	-	-
MSA95	MP	-	-	-	-	-	-	-	-
	MP+ ASW	12±0.0	13±0.0	12±0.0	-	-	-	-	8±0.0
MSA97	MP	-	-	10±0.0	-	-	-	9±0.0	11±0.0
	MP+ ASW	-	-		-	-	-	-	-
*MSA98	MP	-	-	8±0.0	-	-	11±0.0	-	-
	MP+ ASW	-	-	-	13±0.0	-	-	9±0.0	9±0.0
MSA99	MP	-	-	-	-	-	-	-	-
	MP+ ASW	12±0.0	15±0.0	-	-	-	-	8±0.0	-

Values present three replicates. * Strains with antimicrobial properties both in the presence or absence of seawater. (MP: Mannitol-peptone without seawater, MP + ASW: Mannitol-peptone with seawater).

This study also revealed the unusual characteristics of some marine actinomycetes strains, in which these strains can only produce bioactive secondary metabolites in the absence of seawater. However, this finding did come to the agreement with the previous researches which stated that marine actinomycetes strains that showed poor growth in seawater indeed exist despite their high adaptation in the marine environment[17-18]. Some strains showed activity in both fermentation media either with the presence or absence of seawater. This might be because of these actinomycetes do not have absolute requirement of seawater for growth. Genus *Micromonospora,* for example, does not require the presence of NaCl or seawater for growth, although it can tolerate concentration of NaCl up to 4%[19]. Some obligate marine actinomycetes can still grow well in both media with or without seawater[20].

CONCLUSION

This study proved the dependency of secondary metabolites production from marine actinomycetes with the presence of seawater during fermentation. This is clearly observed from the differential antioxidant and antimicrobial activities from all of the 100 strains tested. The fact that more actinomycetes strains exhibited antioxidant and antimicrobial properties with stronger activity when fermentation is done in the presence of seawater, indicating that sea water is essential for the production of therapeutic bioactive compounds. Nonetheless, it is worth noting that the production of bioactive secondary metabolites from certain marine actinomycetes isolated from Sipadan Island may not require the presence of seawater, these marine actinomycetes may produce a complete different set of bioactive compounds. The knowledge

obtained from this research is crucial for the further optimization of secondary metabolites production from marine actinomycetes.

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