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RESEARCH ARTICLE

DIVERSITY OF CULTIVABLE BACTERIA BY STRATEGIC ENRICHMENT ISOLATED FROM FARMED EDIBLE RED SEAWEED, *Gracilaria sp*.

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ABSTRACT

Research on bacteria associated with various red seaweed species are emerging due to the interest to understand bacteria-seaweed interaction and functional roles of bacteria in a seaweed environment. Edible red seaweed, *Gracilaria* sp. is farmed primarily in China, followed by Indonesia and Malaysia but little is known on its associated bacteria and potential functions. This study aimed to isolate and identify cultivable bacteria from extracts of seaweed samples collected from a seaweed cultivation farm in Kedah, Malaysia. The results are hoped to provide insights into beneficial bacteria that exist in the farmed seaweed environment. Through cultivation on strategic enrichment media, 18 isolates OTUs were identified from bacterial phyla Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. These phyla are ubiquitous in seawater and some isolates were homologous to bacteria in marine host cluster. Further investigation on these isolates is hoped to reveal how the isolated bacteria can be beneficial in a seaweed environment or for other biodiscoveries.

KEYWORDS

Seaweed-associated bacteria, Halide salts, KBr, Inorganic nitrate, NH_4Cl, $NaNO_3$.

1. INTRODUCTION

Red seaweeds are generally farmed to be consumed as edible food source, or to harvest its hydrocolloids such as agar, carrageenan, and alginate. Farming of red seaweeds are dominant in Asian countries such as China, which contributes towards 62.8% of the global production, followed by Indonesia and Philippines at 13.7% and 10.6%, respectively. The annual global harvest is estimated at 26 million tonnes for a revenue of about US$ 6 billion (Food and Agriculture Organization of the United Nations (FAO, 2017). Red seaweeds such as *Porphyra* and *Gracilaria* are edible and excellent sources of fibre, proteins, carbohydrates, vitamins, micronutrients, and omega-3 fatty acids. Hence, consuming seaweeds can help sustain a balanced nutrition intake.

However, bacterial community studies related to red seaweed are limited, despite the red seaweeds position as agarophytes with high economical values. Due to its economic importance, most studies regarding red seaweeds were focused on seaweed cultivation and optimisation of agar or carrageenan yield (Charrier et al., 2015; Hurtado et al., 2014; Wenno et al., 2018). Only recently studies on bacteria associated with seaweeds emerge as researchers seek to understand the interaction between the resident bacteria and its host seaweed in a symbiosis relationship where both parties benefit mutually (Hollants et al., 2011). Seaweed exchange organic carbon with dissolved inorganic carbon to its symbiont bacteria, which is important for nutrient cycling in marine ecosystem (Ramanan et al., 2016).

Moreover, different bacteria classes contribute to different holobiont niche functions such as host health, nitrogen fixation, growth development, defense against pathogen, and zoospore settlement and are dominant to different seaweed host, making the bacterial community exists based on a functional profile structure (Egan et al., 2013; Hollants et al., 2013; Burke et al., 2011). Classification of the bacteria in seaweed farm may help to identify beneficial bacteria against disease-inducing bacteria and this knowledge is important for infection management in seaweed farming. Infection by epiphytism is one of the major biological problems in *Gracilaria* farms due to the high density of individuals maintained under single species or monoculture conditions (Fletcher, 1995).

Hence, this study aimed to investigate the bacterial communities associated with the edible red seaweed species, *Gracilaria* sp. cultivated in a seaweed farm in Peninsular Malaysia though culture-dependent approach as little is known to date. Previous research on bacteria from farmed seaweed were mostly related to pathogens or disease, and not on beneficial bacteria in seaweed farming. Furthermore, strategic enrichment culture is employed by supplementing halide salts (KBr, KI, NaCl) and inorganic nitrate ($NaNO_3$, NH_4Cl) to encourage growth of diverse bacteria that can utilise halogen ions and nitrogen which are abundant in the seaweed environment and used by seaweeds for metabolic processes. As several metabolites have been extracted from *Gracilaria* spp. with antioxidant and cytotoxic potentials, it will be interesting to observe bacteria isolates with functional profile that can benefit the seaweed host environment (de Almeida et al., 2011; Abdullah et al., 2013). Therefore,

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knowledge gained from this study is hoped to enlighten on the bacteria that exist in a seaweed farm and the relationship of the bacteria to seaweed in farmed conditions.

2. Materials and Methods

2.1 Sample Collection and Processing

Specimens of the seaweed were collected from a seaweed farm in Kedah (5.6195748N, 100.2949879E) and physical-chemical parameters of the sampling site were recorded (salinity: 16 ppt, temperature: 32.6 °C, pH: 7.68). Seaweed samples collected were rinsed with sterilised seawater to remove dirt, epiphytes and other contaminants before the seaweed were ground by mortar and pestle until slurry.

2.2 Bacterial Isolation

The seaweed slurry was then spread on 8 agar plate culture media which included commercial type media: Marine Agar Zobell 2216 (HiMedia, USA) and Actinomycetes Isolation Agar (HiMedia, USA); and strategic enrichment media containing halide salts (KBr, KI, NaCl; 10mmol/L) or inorganic nitrate (NH_4Cl, $NaNO_3$; 10mmol/L) (Joint et al., 2010; Yuan et al., 2014). The plates were incubated at 30 °C for 2 weeks. Culture colonies were counted and sub-cultured to achieve single colony culture plates where bacteria isolates were obtained.

2.3 Colony PCR and Sequencing

Isolates from agar plates were picked using sterilised toothpicks and dissolved in 20 µL ddH_2O. in a 200-µL PCR tube. Tubes were then treated to heat-boiling at 98 °C for 10 min. Aliquots (1.5 µL) of these samples was used to amplify the 16S rRNA gene in a 25-µL PCR mixture with 10 µM universal bacterial primers 27F (5'-AGRG TTTG ATCM TGGC TCAG-3') and 1492R (5'-GGTT ACCT TGTT ACGA CTT-3'), and 2X MyTaq™ Mix (Bioline, Applied Biosystems, USA) PCR buffer containing Taq DNA polymerase, dNTPs, and magnesium chloride, $MgCl_2$. The PCR program was as follows: initial denaturation for 5 min at 95 °C; 30 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 55 °C, and elongation for 1.5 min at 72 °C; and a final extension step for 5 min at 72 °C. Amplicons were visualised on a 1% agarose gel and amplicons with the correct expected size (~1500 bp) were sequenced with the 27F primer.

2.4 Sequencing and Analysis

For the 16S rRNA sequences obtained from cultivable bacteria, the sequence chromatograms were manually inspected and curated to remove unambiguous regions using BioEdit software V7.1.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All sequences were converted to FASTA format by EBI online sequence conversion tool (https://www.ebi.ac.uk/Tools/sfc/emboss_seqret/), and the compiled sequences were checked for possible chimeric origins using DECIPHER software (Wright et al., 2012) available online (http://decipher.cee.wisc.edu/FindChimeras.html). Sequences with similarities of >97% were considered as 1 OTU (Operational Taxonomic Units) and were clustered by CD-HITs to obtain number of OTUs (http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi). Sequence homology searches were performed against prokaryotic 16S rRNA gene sequences maintained in NCBI genbank database using a Blastn algorithm to determine nearest neighbours, and sequences obtained were deposited at the NCBI GenBank database with the accession numbers MN396133–MN396175.

3. Results

A total of 214 growing colonies were counted from eight different media. From that total colony count, 36 isolates were selected based on different media source and morphologies for species identification based on 16S rRNA gene analysis. Total number of visible colony, PCR amplicons, and OTUs based on media is presented in Table 1.

Table 1: Number of visible colony, PCR amplicons, and OTUs based on media.

Media	AIA	MA	SA	KB	KI	NC	NH	NN
Visible colony (n)	33	20	28	35	16	14	29	39
No. of PCR amplicons	6	5	5	4	3	3	6	4
No. of OTU(s)	2	1	2	3	3	0	5	2

Media abbreviations: AIA: Actinomycetes Isolation Agar (DifcoTM); MA: Marine Agar 2216 (DifcoTM), SA: 1 L seawater + 1.5% agar, KB: 1 L seawater + 1.5% agar + 10mmol/L KBr; KI: 1 L seawater + 1.5% agar + 10mmol/L KI, NC: 1 L seawater + 1.5% agar + 10mmol/L NaCl; NH: 1 L seawater + 1.5% agar + 10mmol/L NH_4Cl; NN: 1 L seawater + 1.5% agar + 10mmol/L $NaNO_3$.

From the isolates selected, 16S rRNA gene amplicons were successfully amplified and sequence. A total of 18 OTUs were clustered at 97% sequence similarity. Interestingly, out of 18 OTUs, there are 10 OTUs with percentage of similarity less than 97%, indicating possible novel isolates (Table 2). Comparison of isolates with its nearest neighbours from NCBI database showed various isolation sources which can be grouped into 3 clusters: (1) seawater; (2) sediment and soil; and (3) seaweed and other marine hosts such as sponges and coral.

Table 2: Isolates BLAST Table

Isolate	Nearest neighbour description [Accession number]	Identity (%)	Isolation source	Phylum
AI112	*Bacillus* sp. strain 70049, 16S ribosomal RNA gene, partial sequence [MF045085]	97	Sediment	Firmicutes
AIA114	*Bacillus firmus* strain BSCS11, 16S ribosomal RNA gene, partial sequence [HQ397584]	89	Sediment	Firmicutes
SA117	*Labrenzia alba* isolate M2B125, partial 16S rRNA gene [LN812985]	98	*Cotylorhiza tuberculate*	α-Proteobacteria
SA119	*Labrenzia alba* isolate CMS163, partial 16S rRNA gene [FR750958]	95	Seawater	α-Proteobacteria
KB120	*Microbulbifer variabilis* strain Ni-2088, 16S ribosomal RNA gene, partial sequence [NR_041021]	94	Marine macroalgae	γ-Proteobacteria
KB122	*Microbulbifer variabilis* strain HNS025, 16S ribosomal RNA gene, partial sequence [JN128259]	98	Marine sponge	γ-Proteobacteria
KB123	*Virgibacillus salarius* gene for 16S ribosomal RNA, partial sequence, strain: BAFBB5 [LC259996]	96	Coral	Firmicutes

KI124	*Alcanivorax gelatiniphagus* strain MEBiC08158, 16S ribosomal RNA, partial sequence [NR_136483]	94	Seawater	γ-Proteobacteria
KI125	*Labrenzia aggregata* strain 0194, 16S ribosomal RNA gene, partial sequence [KP236323]	92	Sediment	α-Proteobacteria
KI126	*Stappia* sp. SCS5m-7 16S ribosomal RNA gene, partial sequence [JX533670]	95	Seawater	α-Proteobacteria
NH130	*Bacillus aquimaris* strain NIOT-Cu-5, 16S ribosomal RNA gene, partial sequence [KJ575054]	97	Sediment	Firmicutes
NH132	*Alcanivorax* sp. CBF L53 gene for 16S rRNA, partial sequence [AB166953]	94	Sediment	γ-Proteobacteria
NH133	*Flavobacterium* sp. strain Bacter-13 16S ribosomal RNA gene, partial sequence [MH671385]	89	Soil	Bacteroidetes
NH134	*Solibacillus silvestris* strain Am1 16S ribosomal RNA gene, partial sequence [GU226320]	96	Sediment	Firmicutes
NH135	*Microbulbifer* sp. HB09007, 16S ribosomal RNA gene, partial sequence [FJ796077]	97	Seawater	γ-Proteobacteria
NN138	Uncultured *Sphingobacterium* sp. clone DQ20 16S ribosomal RNA gene, partial sequence [KT427395]	94	Soil	Bacteroidetes
NN139	*Streptomyces* sp. LCB 0297 16S ribosomal RNA gene, partial sequence [JF922881]	99	Host: *Podocarpus macrophyllus*	Actinobacteria
MA141	*Erythrobacter* sp. 1LE25 gene for 16S rRNA, partial sequence [AB435653]	96	Seawater	α-Proteobacteria

Dominant phylum isolated by the culture dependent approach was Proteobacteria (56%), with only Alphaproteobacteria (28%) and Gammaproteobacteria (286%) groups. Other phyla isolated were Firmicutes (28%), Bacteroidetes (11%), and Actinobacteria (5%) (Figure 1).

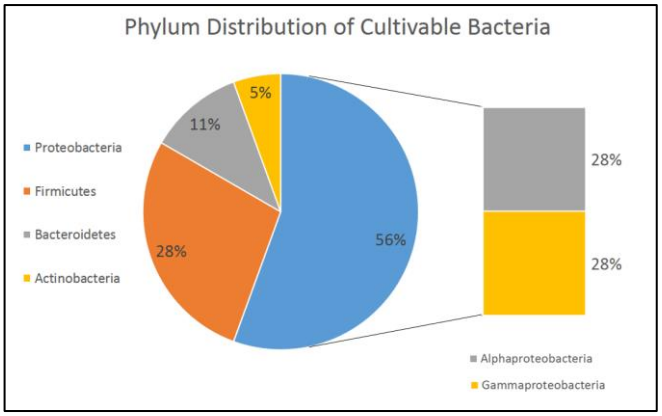

Figure 1: Distribution of cultivable bacteria isolated from *Gracilaria* sp. by phylum.

These bacterial isolates can be classified into 5 genera for Proteobacteria: *Alcanivorax* sp., *Erythrobacter* sp., *Labrenzia* sp., *Microbulbifer* sp., *Stappia* sp.; 3 genera for Firmicutes: *Bacillus* sp., *Solibacillus* sp., *Virgibacillus* sp.; 2 genera for Bacteroidetes: *Sphingobacterium* sp., *Flavobacterium* sp.; and 1 genus for Actinobacteria: *Streptomyces* sp.

4. Discussion

This is the first reporting on cultivable bacteria isolated from edible red seaweed, *Gracilaria* sp. in a farmed condition. Previous research on bacteria associated to seaweeds in farmed conditions are limited to pathogens and diseases (Azizi et al., 2018). The bacterial diversity observed in this study is similar to other environmental bacterial studies which revealed large numbers of Proteobacteria isolates due to the members of the phylum being oligotrophs that can exist in various habitat around the world and in association with hosts such as seaweeds, sponges, and corals (Carlos et al., 2013; Hollants et al., 2013; Naim et al., 2014). Alphaproteobacteria and Gammaproteobacteria are predominant in seawater, in marine macroorganism hosts, and in organic-rich coastal sediments due to their role in nutrient cycling, such as *Alcanivorax* sp. (KI124 and NH132) and *Erythrobacter* sp. (MA141). Other phyla identified were Firmicutes, Actinobacteria, and Bacteroidetes which are also commonly isolated from seaweeds or other marine environments (Hollants et al., 2011; Joint et al., 2010).

The strategic enrichment media supplemented with halide salts (KBr, KI) and inorganic nitrate (NN, NH) allowed growth of seaweed-associated bacteria and improved cultivable bacteria diversity. Commercial nutrient-rich media (AIA) resulted in an overgrowth of fast-growing *Bacillus* sp. (AIA112, AIA114). Colonies of fast-growing bacteria often hinders the cultivability of slow-growing bacteria (Naim et al., 2014). Proteobacteria were present on most culture media types experimented because of the phyla predominance in the natural marine environment and the members have diverse bacterial characteristics which can grow on enrichment media that strategically replicate essential aspects of their environment in terms of nutrients, pH, osmotic conditions, and temperature (Singh and Reddy, 2016). Bacteroidetes and Actinobacteria isolates grew well on NH and NN media where nitrogen in the form of ammonium and nitrate was supplemented.

Additionally, the bacterial isolates observed reflect the nature of bacteria that tolerate the polyculture conditions of the seaweed farm in Kedah. Salinity recorded (16 ppt) was lower than coastal waters (in the range of 28 to 34 ppt) but temperature and pH value were in the range of temperate climate seawater. In environmental samples, salinity had the strongest effect on bacterial community structure compared to other environmental parameters such as pH and temperature because salinity can cause stress to the bacterial cells by having to defend against the salinity gradient and osmotic pressure (Yang et al., 2016). Hence, low salinity such as observed in the seaweed farm is optimum for seaweed culture and its associated beneficial bacteria.

Furthermore, isolates such as SA117, KB120, KB122, KB123, and NN139 from enrichment media showed homologs or nearest neighbours isolated from marine hosts such as marine macroalgae, sponge, coral. Reported

bacterial communities associated with marine hosts suggest their beneficial role in the host environment in a mutualistic relationship, where the bacteria help the host in metabolic processes by chemical cues whilst the host provide shelter to the symbiont bacteria (Singh and Reddy, 2016). The symbiont bacteria are also believed to be beneficially active bacteria as the chemical cues can be elucidated as bioactive compounds. Therefore, marine environment is widely considered as promising source of identifying new bacteria and their novel metabolites.

Nevertheless, cultivation attempts could be further elaborated and optimised in the future to promote growth of other symbiotic bacteria, especially from other marine bacterial phyla such as Cyanobacteria and Planctomycetes. For example, the natural environment of the seaweed host could be mimicked even more by adding other metabolites or seaweed extracts to the culture media to favour the growth of nutrient-dependent symbiotic bacteria. As some isolates are related to up to now uncultivated clones (such as NN138) and possibly represent new bacterial species, they may offer great opportunities for future identification of beneficial bacteria for seaweed environment or other biodiscovery research.

5. Conclusion

The bacterial OTUs isolated from farmed edible red seaweed, *Gracilaria* sp. provided an interesting niche to identify novel beneficial bacteria species among the isolates obtained as none of the OTUs indicated seaweed diseases-related bacteria. Strategic enrichment media improved bacteria cultivability and isolates diversity. Additionally, isolates with nearneighbours associated with marine hosts suggest the bacterial beneficial role in the host environment. Hence, it is strongly recommended that future research increase nutrient variables for strategic enrichment so that more beneficial bacteria from other marine bacterial phyla can be isolated.

References

Abdullah, N.S., Muhamad, S., Omar, I.C., Abdullah, H., 2013. Determination of Antioxidant and Cytotoxic Activities of Red Seaweed, (Gracilaria manilaensis) against Different Cancer Cell Lines. Journal of Food Science and Engineering, 3, 616–624.

Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., Thomas, T., 2011. Bacterial community assembly based on functional genes rather than species. Proceedings of the National Academy of Sciences of the United States of America, 108 (34), 14288–14293. https://doi.org/10.1073/pnas.1101591108

Carlos, C., Torres, T.T., Ottoboni, L.M.M., 2013. Bacterial communities and species-specific associations with the mucus of Brazilian coral species. Scientific Reports, 3 (April), 1–7. https://doi.org/10.1038/srep01624

Charrier, B., Rolland, E., Gupta, V., Reddy, C.R.K., 2015. Production of genetically and developmentally modified seaweeds: exploiting the potential of artificial selection techniques. Frontiers in Plant Science, 6, 127. https://doi.org/10.3389/fpls.2015.00127

de Almeida, C.L.F., Falcão, H. de S., Lima, G. R. de M., Montenegro, C. de A., Lira, N. S., de Athayde-Filho, P. F., Petrônio, F., Rodrigues, L.C., De Souza, M.D.F.V., Batista, L.M., 2011. Bioactivities from marine algae of the genus Gracilaria. International Journal of Molecular Sciences, 12 (7), 4550–4573. https://doi.org/10.3390/ijms12074550

Egan, S., Harder, T., Burke, C., Steinberg, P., Kjelleberg, S., Thomas, T., 2013. The seaweed holobiont: Understanding seaweed-bacteria interactions. FEMS Microbiology Reviews, 37(3), 462–476. https://doi.org/10.1111/1574-6976.12011

FAO (Food and Agriculture Organization of the United Nations), 2017. The state of world fisheries and aquaculture, Available from: http://www.fao.org/fishery/en. Accessed Jan 23, 2017.

Fletcher, RL. 1995. Epiphytism and fouling in Gracilaria cultivation: an overview. Journal of Applied Phycology, 7, 325–333.

Hollants, J., Leliaert, F., De Clerck, O., Willems, A., 2013. What we can learn from sushi: a review on seaweed-bacterial associations. FEMS Microbiology Ecology, 83 (1), 1–16. https://doi.org/10.1111/j.1574-6941.2012.01446.x

Hollants, J., Leroux, O., Leliaert, F., Decleyre, H., De Clerck, O., Willems, A., 2011. Who Is in There? Exploration of Endophytic Bacteria within the Siphonous Green Seaweed Bryopsis (Bryopsidales, Chlorophyta). PLoS ONE, 6(10), e26458. https://doi.org/10.1371/journal.pone.0026458

Hurtado, A.Q., Gerung, G.S., Yasir, S., Critchley, A.T., 2014. Cultivation of tropical red seaweeds in the BIMP-EAGA region. Journal of Applied Phycology, 26, 707–718. https://doi.org/10.1007/s10811-013-0116-2

Joint, I., Mühling, M., Querellou, J., 2010. Minireview Culturing marine bacteria – an essential prerequisite for biodiscovery. Microbial Biotechnology, 3(5), 564–575. https://doi.org/10.1111/j.1751-7915.2010.00188.x

Kim, K.M., Park, J.H., Bhattacharya, D., Yoon, H.S., 2014. Applications of next-generation sequencing to unravelling the evolutionary history of algae. International Journal of Systematic and Evolutionary Microbiology, 64 (PART 2), 333–345. https://doi.org/10.1099/ijs.0.054221-0

Naim, M.A., Morillo, J.A., Sørensen, S.J., Waleed, A.A.S., Smidt, H., Sipkema, D., 2014. Host-specific microbial communities in three sympatric North Sea sponges. FEMS Microbiology Ecology. https://doi.org/10.1111/1574-6941.12400

Ramanan, R., Kim, B.H., Cho, D.H., Oh, H.M., Kim, H.S., 2016. Algae-bacteria interactions: Evolution, ecology and emerging applications. Biotechnology Advances, 34 (1), 14–29. https://doi.org/10.1016/j.biotechadv.2015.12.003

Singh, R.P., Reddy, C.R.K., 2016. Unraveling the Functions of the Macroalgal Microbiome. Frontiers in Microbiology, 6, 1488. https://doi.org/10.3389/fmicb.2015.01488

Wenno, P.A., Latumahina, M.C.A., Loupatty, S.R., Soumokil, A.W., 2018. Growth, Biomass Production, and Carrageenan Yield of Kappaphycus alvarezii (Doty) Cultivated in Deep Seawater of Saparua Bay in Central Maluku. Aquacultura Indonesiana, 19 (1), 28–32.

Wright, E.S., Yilmaz, L.S., Noguera, D.R., 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Applied and Environmental Microbiology, 78(3). https://doi.org/10.1128/AEM.06516-11

Yang, J., Ma, L., Jiang, H., Wu, G., Dong, H., 2016. Salinity shapes microbial diversity and community structure in surface sediments of the Qinghai-Tibetan Lakes. Scientific Reports, 6 (April), 6–11. https://doi.org/10.1038/srep25078.