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Instrumental use of Marine Bacteria to Stimulate Growth in Seaweed

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ABSTRACT
Edible seaweed - *Caulerpa lentillifera* is being cultivated along the coast of Khanh Hoa province, Vietnam, and makes a relatively large contribution to the economic development of this region. Bacterial strains originating from marine sources such as those associated with seaweed and hard coral were screened for properties of promote plant growth with the capacity of indole-3-acetic acid (IAA) - a phytohormone belonging to auxin group, the phosphate solubilization ability and antibacterial activity of IAA-producing strains were also performed in this study. Robust strains were identified by morphological methods with biochemical tests and analysis of 16s RNA sequences. Isolate RN06 produced high amounts of IAA, utilized inorganic phosphate, and inhibited *Bacillus subtilis* ATCC6633, *Escherichia coli* 0157, and *Serratia marcescens* PDL100. The IAA producer HRA5 isolated from hard coral demonstrated the ability to solubilize phosphate and exhibited antibacterial activity against *B. subtilis*. Morphological analysis and 16sRNA sequencing showed that isolate RN06 was the closest strain to *Bacillus amyloliquefaciens* and HRA5 was linked to *Pseudomonas* sp. This is the first report of isolated bacteria from seaweed and corals from the Vietnamese sea served as potential strains for further research of the application of biological inoculants specifically for seaweed farming.

Keywords: Indole-3-acetic acid-producing bacteria, Phosphate solubilizing bacteria, Antimicrobial activity, Marine potential bacteria

1. Introduction

Seaweeds are divided into three groups based on their pigment content: Green (Chlorophyta), brown (Phaeophyta), and red (Rhodophyta). They are a group of marine plant-like organisms distributed geographically, from tropical to polar regions, with ecoregions ranging from intertidal to submerged zones that are still exposed to sunlight (Tirtawijaya et al. 2022). The diversity of seaweed-associated bacteria, the role and the relationship between bacteria and their host as well as their production of bioactive compounds have been investigated for a long time. However, the research on the edible seaweed *Caulerpa lentillifera* was focused on the nutrient requirement, optimum cultivational conditions, and production of bioactive substances such as antioxidants, antimicrobials, antitumor compounds as well as pigments. Not much is known about the diversity of microorganisms associated with the sea grape *C. lentillifera*. Among phytohormones, indole-3-acetic acid (IAA) increased polysaccharide content in edible seaweed rather than other normal functions of phytohormones which induce the growth such as 6-benzyl aminopurine and gibberellin (Tao et al. 2017). It was worth that Polysaccharides exhibited anticoagulant, and immunomodulatory effects and prevented cancer activity (Chen et al. 2019). Auxin is a phytohormone that controls almost every aspect of growth and development in plants. They are the basic compounds that regulate the growth and development of plants. One of the most important natural auxins is IAA produced by plants, bacteria, fungi, and brown algae (Ali et al. 2009; Leyser 2010; Sunarpi et al. 2021). So far, auxin has also been found in the marine sediment (Maruyama et al. 1989), and IAA-producing marine microorganisms affected the growth of thallus of coenocytic alga *Caulerpa sertularioides* (Mishra & Kefford 1969). Indole-3-acetic acid was detectable in Dictyota dichotoma germlings and mature tissue and auxin play a role during the apical-basal patterning of the embryo of brown algae (Bogaert et al. 2019). Indole-3-acetic acid (IAA) was produced by a brown algae *Ectocarpus siliculosus* so far and affected the growth of its host (Le Bail et al. 2010). However, another paper showed that an un-culturable microbe associated with the alga might be the true producer of IAA instead of alga (Dittami et al. 2014). The plant hormone IAA from plant growth-promoting *Pseudomonas putida* UB1 was reported to play a major role in the development of the host plant root system (Bharucha et al. 2013). A *Sulfitobacter* isolate associated with the diatom *Pseudonitzschia* could promote diatom cell division via the secretion of indole-3-acetic acid. The hormone indole-3-acetic acid was synthesized by this bacterium using both diatom-secreted and endogenous tryptophan. Both IAA and tryptophan acted as biological signaling molecules in their interaction for nutrient exchanges (Amin et al. 2015). It was illustrated that an *Enterobacter* strain that overproduces IAA acts as a bio-herbicide (Park et al. 2015). In another paper, the marine bacterium *Ruegeria* sp. R11 was thought to be responsible for algal blooms (hosts) and produced IAA, but the role of IAA in the interaction between bacteria and algae

had not been clearly established (Mayers et al. 2016). Without a doubt IAA is an important substrate in the communication between algae and marine bacteria (Lin et al. 2022).

Although, phosphorus is one of the most important and major components of seawater. It is normally found in the form of both inorganic and organic phosphates. Microorganisms play a vital role in the phosphorus cycle both in terrestrial and aquatic ecosystems. Thus, many kinds of phosphate-solubilizing bacteria like *Bacillus*, *Pseudomonas*, *Nitrosomonas*, *Erwinia*, *Serratia*, *Rhizobium*, *Xanthomonas*, *Enterobacter*, and *Micrococcus* have been isolated from various habitats compassing of coastal, offshore, and mangrove (Rawat et al. 2021) *Pseudomonas aeruginosa* KUPSB12 isolated from the river Ganga west Bengal India was effective in phosphate solubilization with Pikovskaya's agar method and also was a good candidate for the production of antimicrobial substances. Crude extract from *P.aeruginosa* inhibited the growth of both Gram-negative and Gram-positive test strains (Paul & Sinha 2017). *Enterobacter* and *Serratia* strains isolated from *Mimosa pudica* root nodules have plant growth-promoting characters compassing auxin production, phosphate solubilization, and enzymatic activities. Moreover, *Serratia* strains had antimicrobial activity against plant pathogenic fungi *Fusarium* sp. and *Alternaria solani*. Other strains of *Enterobacteria* sp. strain NOD1 and NOD10 were very efficient in promoting shoot height and increasing the size of the legumes *Phaseolus vulgaris* and *Mimosa pudica* in vitro (Sánchez-Cruz et al. 2019). Recently the diversity and functional traits of actinobacteria from the coastal salt marsh soil from Jiangsun province have been investigated. The results from that paper showed that 2.8-43.0% of actinobacterial sequences belonged to rhizosphere bacterial communities by high throughput sequencing methods. Among all isolates, two strains were identified as plant growth-promoting and were inoculated into wheat seed under salt-stress conditions. Both strains promoted seed germination and significantly enhanced plant growth. They were identified as Streptomyces sp. KLBMP S0051 and Micromonospora sp. KLBMP S0019 (Gong et al. 2018). The diversity of seaweed-associated bacteria, the role and the relationship between bacteria and their host as well as their production of bioactive compounds have been investigated for a long time. Previously, we found that *Micrococcus* sp. strain A-2-28 associated with the soft coral *Alcyonium digitatum* showed antimicrobial activity and metabolite analysis showed the presence of IAA in the crude extract of this bacteria (Pham 2014). On the other hand, *Micrococcus* was the most common bacterium among the isolates from the soft corals, the natural compounds from this bacterium have also been reported so far (Palomo et al. 2013). In addition, we coincidentally detected that the most abundant *Enterobacter* and *Pseudomonas* from *Acropora* the majority species of building coral reefs in the center of Vietnam when PO_4 concentration in ambient seawater was lowest compared to those in three other sampling times (Pham et al. 2019).

The marine animals including corals, sponges, and muscles from Vietnamese seawater become interesting for investigation of bioactive compounds. Along the coast of ca 3000 km from the north to the south are many activities of aquaculture in open water. In addition, many coastal regions now are farming edible sea grapes for commercial trades, especially in Khanh Hoa province, which is the best place to grow green seaweed in Vietnam, developing sustainable farming both in soil and water is a challenge for Vietnamese, we need more and more basic research and application technology.

This present study is first searching for indigenous bacterial strains with benefit features such as the production of phytohormone IAA, solubilization of phosphate, and inhibition of pathogenic bacteria to use for further use as biological inoculants in seaweed farming.

2. Material and Methods

The living coral *Acropora hyacinthus* was collected from the Center of Vietnam by a diver with SCUBA at the station in Hang Rai (109°18'28" E and 11°67'71" N), Ninh Hai, Ninh Thuan province, Viet Nam. The living edible seaweed *C. lentillifera* cultivated in natural seawater in the VAST Key Lab for Food and Environment Safety in Center Vietnam was used to isolate bacteria for this study. After collecting, the samples were preserved in a sterile dark polyethylene bag, stored in an ice box, and transferred to the laboratory as soon as possible for further study. In the laboratory, samples were immersed in ethanol 70% for 30 seconds and then washed three times with sterile filtered seawater. At the laboratory, samples were immersed in the ethanol 70% for 30 seconds and then washed three times with sterile filtered seawater. The wet weight of 2 g of each sample was homogenized in 18 mL of sterile 0.45 µm (Whatman) filtered seawater collected at the sampling sites and diluted samples were used to isolate until a pure strain (Pham et al. 2018). The pure isolates were long time preserved in TM medium (Tryptone: 1 g/L, Yeast extract: 1 g/L, Agar: 15 g/L, NaCl: 5 g/L) plus 50% glycerol at room temperature (approx. 30 °C) at Marine Ecology Department, Institute of Oceanography, VAST.

2.1. Screening of IAA producer strains

Total of 50 pure microorganisms derived from corals and edible seaweed were screened for IAA production in modified LB broth medium (LBM g/L) consisting of KH_2PO_4: 1, Na_2HPO_4: 1, $MgSO_4 \cdot 7H_2O$: 0.2, pH= 7.0, glucose:10; yeast extract: 5, NaCl: 5, Tryptone: 5, L-tryptophan: 0.5 at 30 ± 1 °C on a rotary shaker at 120 rpm. After 3 incubated days, broth cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was collected for testing of IAA production by using Salkowski's reagent after keeping for 30 min at 30 °C in the dark, the development of pink to reddish color was noted as slight pink (+), pink (++) and dark pink (+++) for increment IAA producing levels. The positive control of pink color development was added by using 1 µg/mL of authentic IAA (Sigma) and negative control was performed with distilled water instead of cultured broth in the same manner (Gupta et al. 2012).

2.2. Quantitative of IAA

All IAA production strains were inoculated in LB-modified broth medium and incubated at 30±1 °C for 10 days on a rotary shaker at 120 rpm. Each 2 mL broth culture was harvested and centrifuged at 10,000 rpm for 15 min. The cell pellets were dried at 70 °C in a hot oven to get dry weight for biomass values of triplicate samples. Then 1 mL of supernatant sample of day 4, day 7, and day 10 was taken and mixed with 2 mL of Salkowski's reagent and kept for 30 min to develop color. Color intensity was assayed with a spectrophotometer at 530 nm by the colorimetric method (Gordon & Paleg 1957) and calculated based on the curve of standard IAA (Sigma) with a series of concentrations of 0, 5, 10, 20, 50, and 100 µg/mL. Two-time distilled water was used as a blank sample.

2.3. Detection of phosphate solubilizing activity of IAA-producing strains

The IAA-producing bacterium was screened on Pikovskaya's agar (PKV) medium for phosphate solubilization by the Agar Spot method. PKV consisted of 2.5 g/L calcium triphosphate $Ca_3(PO_4)_2$ as phosphate source, glucose 10 g/L, $(NH_4)_2$ SO_4 0.5 g/L, $MgSO_4$. $7H_2O$ 0.1 g/L, KCl 0.2 g/L, NaCl 2 g/L, $FeSO_4$ 0.002 g/L, Yeast extract 0.5 g/L, $MnSO_4$ 0.002 g/L, Agar 15 g/L. The pH was adjusted to 7.0 ± 0.2 before sterilization. The cultured plates were incubated at 30 ± 1 °C for 5 days and colonies with a clear halo marked positive for phosphates solubilization (Gupta et al. 2012). The Agar Spot method yielded the results in terms of solubilization index (SI). The phosphate solubilization index was calculated by the ratio of the sum of colony diameter and halo zone diameter divided by colony diameter or the ratio of total zone over colony zone (Paul & Sinha, 2017) as the equation (1). PKV broth medium was used for pH drop check daily for 10 incubation days at 30 ± 1°C on a rotary shaker at 120 rpm with a glass electrode (Ahmad et al. 2013).

$$SI = \frac{CD+HZD}{CD} \quad (1)$$

Where; SI: Solubilization index, CD: Colony diameter, and HZD: Halo zone diameter.

2.4. Antibacterial testing

The isolated strains were streaked in Marine Agar (Himedia) plates for 3-5 days and then inoculated into 300 mL Erlenmeyer flasks containing 100 mL BM medium (yeast extract: 1 g/L, beef extract: 1 g/L, tryptone 2 g/L, glucose 10 g/L, 1000 mL filtered seawater). After 72 h of incubation at 30 °C with shaking at 120 rpm, the bacterial cells and the supernatants were homogenized by using of Ultrasonic processor for breaking the cells in 30 s. The homogenized broth was extracted by ethyl acetate 1/1 (v/v). Crude extracts were dried and re-suspended in 1 mL of methanol (Merck). The methanolic extracts were applied for antimicrobial activities by agar well diffusion method on Mueller Hinton Agar (MHA-Himedia, India) according to Bauer et al. (1966). A total of 30 µL methanolic extract of each isolated bacteria was inoculated into four available wells on MHA-containing indicator bacteria. The same amount of methanol without extract was used as a negative control. All the plates tested with *Bacillus subtilis* ATCC6633, *Salmonella typhimurium* ATCC6994, and *Escherichia coli* 0157 were incubated at 37 °C for 24 h. The plates tested with *Serratia marcescens* PDL100 were incubated at 25 °C for 24 h. The zone of inhibition was measured and expressed in the mean of the four wells excluding the well diameter.

2.5. Identification of potential marine bacteria

The potent plant growth-promoting strains were identified by traditional methods with biochemical tests and commercial identification compassing of API 20E, API-ZYM, and API 50CH (BioMérieux, France) as well. Individual tests were performed according to West and Colwell (1984), and Cowan and Steel (1970), as cited in (Buller 2014), Gram determination was decided based on the result of the KOH reaction (Halebian et al. 1981). Some antibiotic-producing strains were identified by the 16sRNA gene analysis according to Pham (2014).

2.6. Statistical analysis

One-way ANOVA statistical analysis of the results was carried out by using the Statgraphics Centurion XV software. Multiple Range Least Significant Difference was used to measure specific differences between pairs of means. Significant differences between the two treatments were tested using t-test: Paired Two Sample for Means with a significance level of $P \leq 0.05$ and compared t Stat and t Critical one-tail, t Stat and t Critical two-tail.

3. Results

3.1. Screening of IAA producer and phosphate solubilization strains

Out of 50 tested strains, six cultures isolated from edible algae *C. lentillifera* cultured at the Key Lab for Food and Environment Safety in Center Vietnam, and five other strains isolated from *Acropora* sp. from a coral reef in the center of Viet Nam produced IAA into screening broth medium. The phosphate solubilization screening and IAA-producing marine isolates are listed in Table 1.

Table 1- Microorganisms producing IAA and solubilizing phosphate

No	*Isolates*	*Sources of isolation*	*Development of pink color*	*Phosphates solubilization*
RN02	Gram-positive, rod-shape	*C. lentillifera*	+++	Positive
RN04	Gram-positive rod shape	*C. lentillifera*	++	Negative
RN05	Gram-positive rod shape	*C. lentillifera*	+	Positive
RN06	Gram-positive rod shape	*C. lentillifera*	+++	Positive
RN07	Gram-negative, coccus	*C. lentillifera*	++	Negative
RN08	Gram-positive rod shape	*C. lentillifera*	+	Negative
HRA1	Gram-negative rod shape	*Acropora* sp.	+++	Positive
HRA2	Gram-negative rod shape	*Acropora* sp.	+++	Positive
HRA3	Gram-negative rod shape	*Acropora* sp.	+++	Negative
HRA4	Gram-negative rod shape	*Acropora* sp.	+++	Negative
HRA5	Gram-negative rod shape	*Acropora* sp.	+++	Positive

+: Slight pink color development, ++: pink color development, +++ : dark pink color

The strains derived from edible algae showed dark pink color development including RN02, and RN06, other strains RN04, RN07 possessed pink color development (++), whereas two other strains RN05, RN08 produced IAA slightly (+). All cultures from hard corals (HRA1, HRA2, HRA3, HRA4, HRA5) were considered as potential IAA producers with dark pink color development.

3.2. Production of IAA

The IAA production of isolates is shown in Table 2 and biostatistics of signification for means of treatment with the range of different letters. Most of the isolates revealed the highest IAA production on day 7 of cultivation. Strains RN02 and RN06 revealed dark pink color development (Table 1) and also yielded high levels of IAA production (Table 2). In the same manner, two isolates from the edible seaweed strain RN05 and RN08 produced very low amounts of IAA. The biostatistics analysis showed that those values were not significantly different. It inferred the two mentioned strains produced the same amount of IAA with the same trend.

Table 2- Biostatistics analysis of the significantly different treatment of IAA producers from marine-derived bacteria. The values are the mean of triplicate samples. The significantly different values are identified with different letters (P<0.05)

STRAINS	*IAA (µg/mL)*		
	Day 4	*Day 7*	*Day 10*
RN02	17.80^{fg}	12.15^{ghi}	14.70^{gh}
RN04	9.63^{hijk}	12.82^{gh}	4.99^{lmnop}
RN05	1.12^{pq}	6.08^{jklmn}	1.62^{opq}
RN06	8.75^{ijkl}	25.75^{ab}	12.82^{gh}
RN07	7.71^{1jklm}	11.39^{ghij}	3.56^{nopq}
RN08	0.63^{q}	1.24^{opq}	1.31^{opq}
HRA1	5.23^{lmno}	18.93^{dfg}	18.37^{defg}
HRA2	4.08^{mnopq}	27.42^{a}	15.00^{gh}
HRA3	4.38^{mnopq}	23.73^{abc}	5.99^{klmn}
HRA4	21.82^{bcde}	21.36^{cdef}	1.88^{opq}
HRA5	2.79^{nopq}	18.00^{efg}	23.00^{bc}

Three strains HRA1, HRA2, and HRA3 from hard corals were good IAA producing (+++) in the screening step and also produced a high amount of IAA after 7 days of cultivation with 18.93 µg/mL 27.42 µg/mL and 23.73 µg/mL, respectively. Particularly, the strain HRA2 showed the highest amount of IAA on day 7 with 27.42 µg/mL. The best producer seaweed derived strain RN06 also yielded 25.75 µg/mL IAA on day 7. Those values of IAA produced from HRA2 and RN06 were significantly different from IAA from day 4 and day 10 for both strains.

As shown in Table 3, the edible seaweed isolates RN02, RN05, RN08, and HRA3 reached the highest biomass on day 7 and the lowest biomass on day 10, the differences in biomass values between days 4 and 7, also days 7 and 10 were statistically significant. However, differences in biomass on days 4 and 10 were not statistically significant (Table 3). Both edible seaweed isolates RN06 and RN07 reached the maxima biomass on day 7 and the lowest on day 10. Biomass of strain RN06 on day 7 (14.22 mg/mL) had a statistically significant difference with biomass on day 4 (5.92 mg/mL) but there was no significant difference with biomass from day 10 (11.85 mg/mL). In contrast, the sea grape-associated isolate RN07 reached the highest

amount of biomass on day 7 with 10.67 mg/mL and there was a significant difference in biomass on day 10 with 6.89 mg/mL while there was no significant difference in biomass on day 4 (7.63 mg/mL).

Table 3- Biostatistics analysis of the significantly different treatment of biomass producers from marine-derived bacteria. The significantly different values are identified with different letters (*P*= 0.05).

STRAINS	*BIOMASS (mg/mL)*		
	Day 4	*Day 7*	*Day 10*
RN02	$10.81^{abcdefg}$	14.96^{a}	$9.04^{bcdefghi}$
RN04	7.29^{ghij}	$8.17^{defghij}$	7.67^{fghij}
RN05	7.85^{efghij}	11.63^{abcde}	5.26^{j}
RN06	5.93^{ij}	14.22^{ab}	11.85^{abcd}
RN07	7.63^{ghij}	$10.67^{abcdefg}$	6.89^{hij}
RN08	7.78^{fghij}	11.41^{abcdef}	5.93^{ij}
HRA1	11.48^{abcde}	$10.15^{bcdefgh}$	12.37^{abc}
HRA2	11.85^{abcd}	11.26^{abcfef}	7.78^{fghij}
HRA3	$8.52^{cdefghij}$	12.15^{abc}	$8.15^{defghij}$
HRA4	11.26^{abcdef}	12.15^{abc}	10.52^{bcdefg}
HRA5	$8.29^{cdefghij}$	$9.55^{bcdefgh}$	11.85^{abcd}

Most strains reached maxima of biomass on day 7, except HRA1, HRA2, and HRA5 isolates (Table 3). The isolates HAR1 and HRA5 had the highest biomass on day 10, but there were no statistically significant differences. The coral associate isolates HRA2 reached the highest biomass on day 4 with 11.85 mg/mL and slightly dropped a non-significant difference on day 7 with 11.26 mg/mL. The biostatistics analysis showed that only biomass yielded on day 4 (11.85 mg/mL) and day 10 (7.78 mg/mL) was a statistically significant difference (Table 3). Comparing of single strain, among isolates from seaweed RN02 produced the highest biomass and the total biomass produced from this strain was significantly different from other edible algae isolates except RN06. The results from the IAA screening step (Table 1) and quantitative IAA production looked like proof that RN04 and RN07 should be grouped in the moderate IAA producer strains whereas the strains RN05, and RN08 revealed rather weak IAA producer group and two other strains RN02 and RN06 showed as good IAA producers. Other coral-associated strains compassing HRA1, HRA2, HRA3, HRA4, and HRA5 should be placed in the last group for IAA producer.

3.3. Qualitative estimation of phosphate solubilization

Three out of six isolates from edible algae strains RN02, RN05, and RN06, and other three from coral strains HR1, HR2, and HR5 revealed phosphate soluble activities (Table 1, Figure1). Five strains consisting of RN02, RN06, HRA1, HRA2, and HRA5 could be considered as potential strains for both IAA producing and phosphate solubilization.

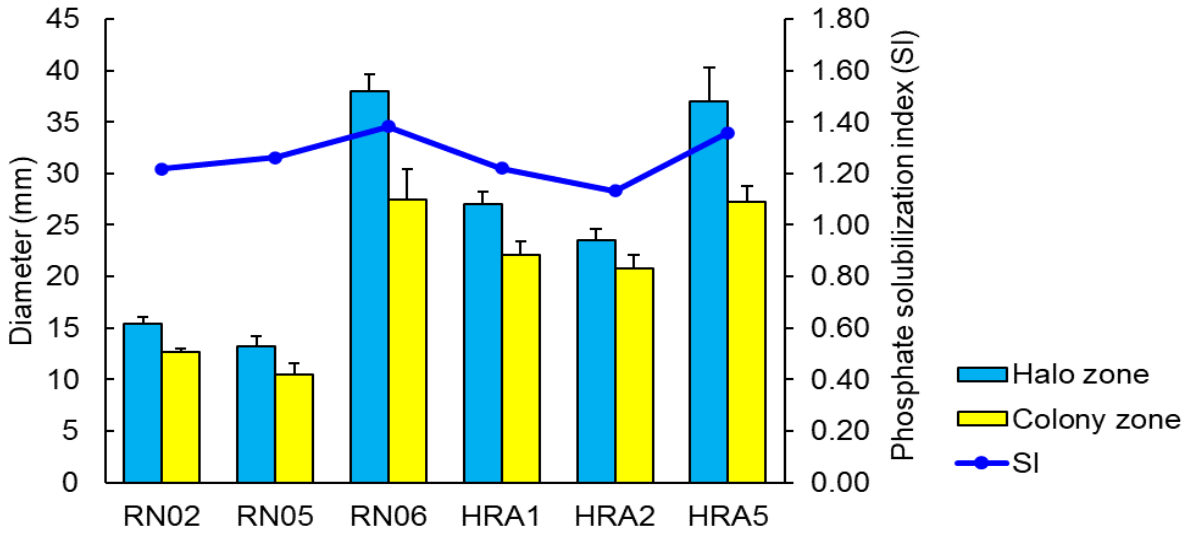

Figure 1- Phosphate solubilization of potential strains

The results of the Agar spot and pH drop methods are shown in Table 1 and Figure2. From these results of the comparison of the two methods revealed that isolates HRA1 showed a maximum of SI in the agar spot whereas minimum in the pH drop method, it showed a significant drop of pH in the incubation medium, it referred that HRA1 has the highest phosphate solubilizing potential. The pH drop was usually company with the phosphate solubilization, the pH was slightly dropped in the first days of incubation and reached the lowest point of 3.4 on day 5 and then remained stable. This trend of pH in broth medium for all strains in this present study.

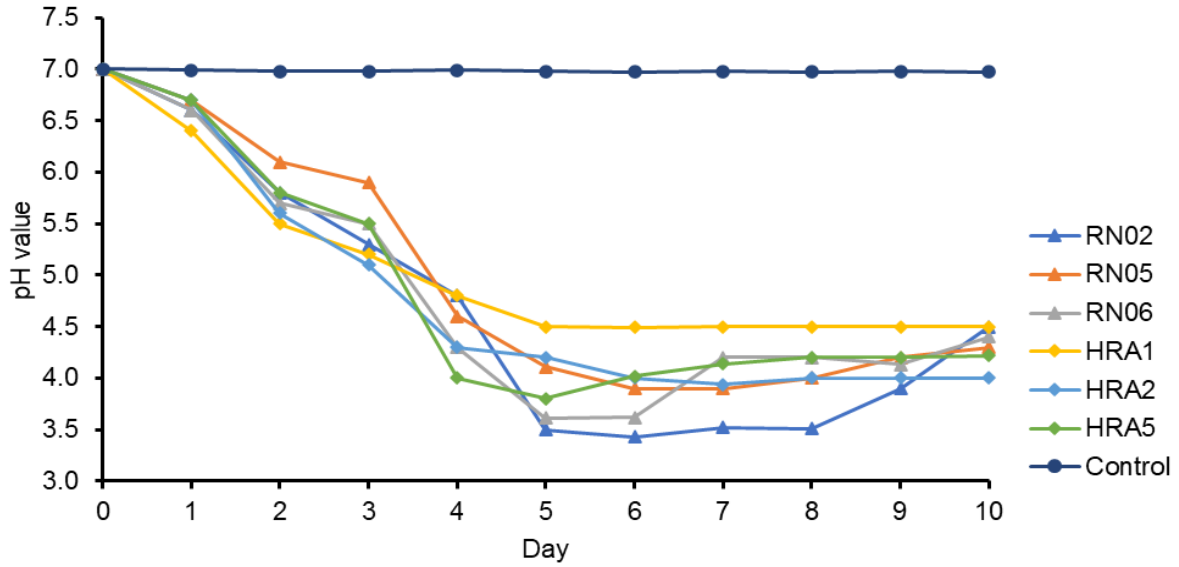

Figure 2- The pH change over cultivation time

As can be seen from Figure1, the most effective phosphate solubilization isolates are RN06 and HRA5 with SI equal to 1.38 and 1.35, respectively, both strains showed a pH drop from 7.00 to 3.61 and 3.80 in sequences on day 5 of incubation then the pH increased slightly up to ca. 5 on day 10. The pH drop in liquid media was thought to be a company with the solubilization of inorganic phosphate.

3.4. Antimicrobial activities

Antimicrobial activities of the IAA producer strains are shown in Figure3 with the mean and standard deviation. There were four strains RN04, RN06, RN07, and HRA5 showed inhibition of Gram-positive *B. subtilis*, the strain HRA5 had the strongest activity with a diameter of 7.85 mm and RN06 showed the lowest antibacterial activity with an average inhibition zone of 4.70 mm in diameter. The strain RN06 showed active against both two Gram-negative tested *E. coli* and coral pathogenic *S. marcescens* PDL100, whereas the strain RN04 had no inhibition of *E. coli*

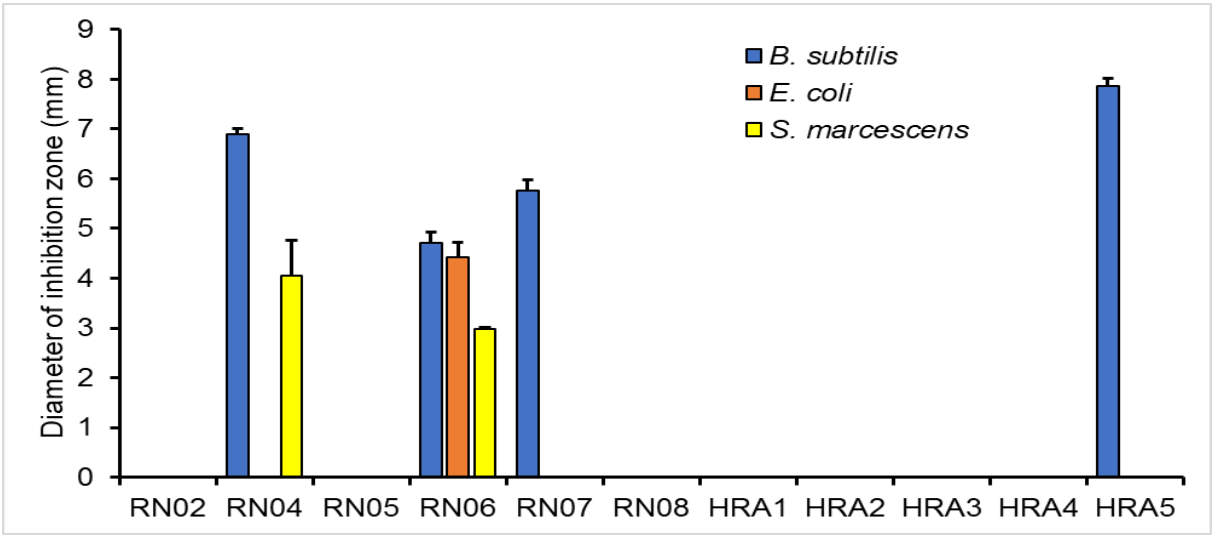

Figure 3- Antimicrobial activity of IAA producer strains

3.5. Identification of RN06 and HRA5

The potential strains RN06 and HRA5 from this study were closest to *Bacillus* and *Pseudomonas* respectively. The results of morphological and chemical tests are shown in Table 4 and the results of 16sRNA sequencing analysis are shown in Table 5, and Figure 4.

Table 4- Basic biochemical tests of potent strains RN06 and HRA5

Characteristics		*RN06*	*HRA5*
Morphological and supplementary tests			
1	Gram Staining	Positive	Negative
2	Cell width	0.8	0.2
3	Shape	Rods	Rods
4	Motility	Motile	Motile
5	Spore	Sporing	Non-Sporing
6	Catalase	Positive	Positive
7	Oxidase	Positive	Positive
8	MCA	-	+
9	TCBS	w	+
10	NaCl (%)	0/6	0/6
11	Methyl Red	+	-
12	V-P (Acetoin production)	+	-
13	Indole production	-	-
14	Citrate utilization	+	+
15	Nitrate reduction	+	+
16	H_2S production	-	-
17	Acetate utilization	+	-
18	Gelatin hydrolysis	+	-
Fermentation			
19	GLU (Glucose)	+	-
20	MAN (Mannitol)	+	w
21	INO (Inositol)	+	-
22	SOR (Sorbitol)	+	-
23	RHA (L-rhamnose)	-	-
24	SAC (Sucrose)	+	-
25	MEL (Melibiose)	w	+
26	AMY (Amygdalin)	+	-
27	ARA (Arabinose)	+	-
28	SAL (Salicin)	+	-
29	Starch	+	-
30	LAC (Lactose)	w	-
31	MAL (Maltose)	+	-
Enzymatic Reactions			
32	ONPG (Galactosidase)	+	-
33	ADH (Arginine decarboxylase)	-	+
34	LDC (Lysine decarboxylase)	-	-
35	ODC (Ornithine decarboxylase)	-	-
36	TDA (Tryptophane deaminase)	-	w
37	Urease	-	w

Vp: Voges–Proskaüer, MCA: Growth on MacConkey, w: weak, +: positive reaction,-: negative, TCBS: thiosulfate–citrate–bile–sucrose agar, NaCl%: Growth in 0% and 6% salt in LBM medium.

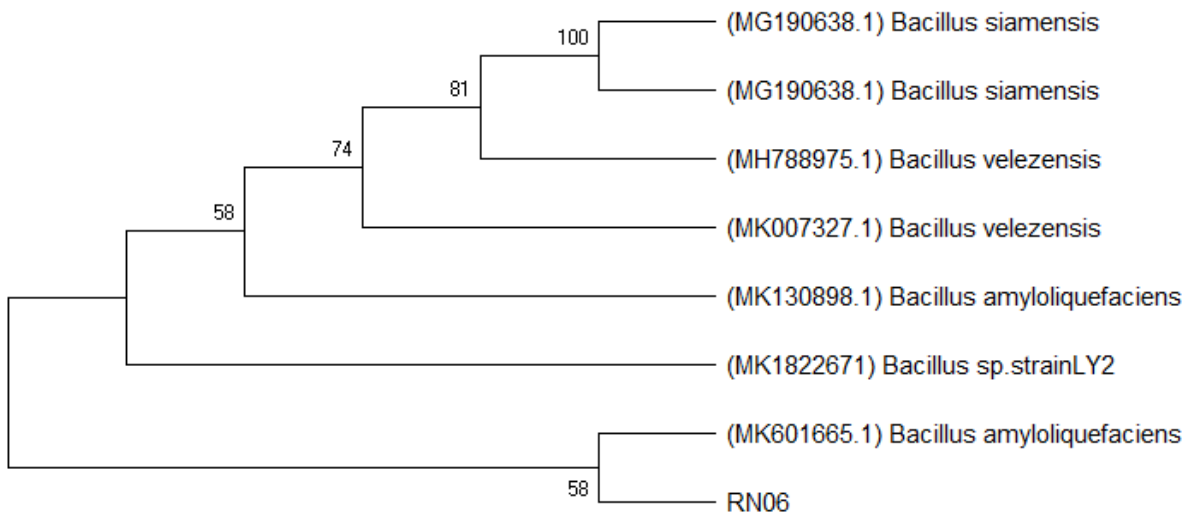

Figure 4- Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences. Evolutionary relationships of taxa: The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. There were a total of 1342 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

It was clear that the strain RN06 belonged to the genera *Bacillus*, however, the results from the analysis of 16s rRNA have been given some different results. Compared to other results for the identification of *Bacillus* with conventional biochemical tests with API kit (Logan & Berkeley 1984). The results from the biochemical test strongly suggested that this strain could be affiliated with *Bacillus amyloliquefaciens* rather than other *Bacillus* species (Table 5). In addition, the phylogenetic analysis showed that the strain RN06 was the closest to *Bacillus amyloliquefaciens* strain CIFTMFBB7 (Figure 4).

Both two potent strains from this study can grow at no salt concentration and 6% NaCl as well (Table 4). It could be due to the sources of isolation, the strain RN06 and HRA5 were associated with sea grapes and hard coral respectively and the salinity of seawater in the ambient sampling site was less than 35‰. However, this test was done here for getting a basic chemical test (up to 6% NaCl only) rather than a salt tolerance test, so all potential strains in this study were suggested to further experiment including salt tolerance, other plant-hormone producing or antifungals test with plant pathogens.

Table 5- Identification of RN06 and HRA5

Strains	Most closed relative strains from GenBank (NCBI)	Query cover (%)	Identify (%)	Possible genus
RN06	*Bacillus amyloliquefaciens* (MK130898.1)	99	99.93	*Bacillus* sp. strain RN06
	Bacillus siamensis (MG190638.1)	99	99.93	
	Bacillus sp. LY2 (MK182267.1)	99	99.93	
	Bacillus velezensis (MH788975.1)	99	99.93	
	Bacillus amyloliquefaciens (MH788971.1)	99	99.93	
	Bacillus amyloliquefaciens (MK601665.1)	99	99.93	
	Bacillus velezensis (MK007327.1)	99	99.93	
HRA5	*Pseudomonas fluorescens* (CP032618.1)	97	100	*Pseudomonas* sp. strain HRA5
	Pseudomonas sp. LG1D9 (CP026881.1)	97	100	
	Pseudomonas chlororaphis (CP011020.1)	97	99.93	
	Pseudomonas fluorescens (CP008896.1)	97	99.93	
	Pseudomonas fluorescens (GU198103.1)	97	100	
	Pseudomonas gessardii (NR_024928.1)	97	99.86	
	Pseudomonas synxantha (LR590482.1)	97	99.86	

4. Discussion & Conclusions

4.1. IAA producers

Compared to other results the marine-derived bacteria from this present study produced rather low amounts of IAA (maxima ca 30 µg/mL). These results are likely to be the same as those of Jeon et al. (2003) when using L-tryptophan as a precursor with a

concentration of 500 mg/L at initial cultivation, the *Pseudomonas fluorescens* strain M45 produced 17.7 µg/mL IAA, and *P. fluorescens* strains MCO7 yielded 22.7 µg/mL IAA. The results from this study reflected the reliability of those methods which were applied successfully in other studies (Gutierrez et al. 2009). They also revealed that when the strain was strongly acted with Salkowski's reagent, it inferred that they could be a good IAA-producing strain. This study was the first report of a good source of IAA produced from marine bacteria isolated from edible algae and hard coral from the Vietnamese sea. All isolates showed good growth in modified LB medium and cultivation conditions. This medium was desired for marine strains with some marine element components such as marine salts. However, the concentration of IAA was not always parallel to the amount of high biomass. The results from this study were similar to those of results from other studies of screening estuary *Vibrio* strains for IAA production. It was shown that the bacterial cells in the cultured broth of the highest IAA (21.43 µg/mL) producing strain J-S2-8 were similar to quite low IAA (5.52 µg/mL) producing strain J-S2-26 with $3.64x10^9$ cell/mL and $3.42x10^9$ cell/mL, respectively (Gutierrez et al. 2009). The other study of screening IAA producers from bacteria associated with sponges from the Gulf of Mannar, southeast coast of India showed that many associated bacteria could produce IAA in rather low concentration with a maximum of 0.41 µg/mL in LB plus tryptophan medium. The most abundant IAA-producer bacteria belonged to *Pseudomas fluorescens, P. aeruginosa, P. putida, B. subtilis, B. licheniformis, and B. megaterium* (Vasanthabharathi & Jayalakshmi 2017). It is worth noting that the IAA amount produced from isolates in this study is in low concentration. Indeed, the high concentration of auxin could be toxic to plants, when IAA is the principal natural auxin in higher plants and overdose effects of IAA involve the use of herbicides for weed control (Grossmann 2003). On the other hand, the IAA produced from bacteria should be lower in vivo if they are considered biofertilizers. This phytohormone also is induced from the host plants and tryptophan was not usually found in vast amounts from soils.

4.2. Phosphate solubilization strains

The pH drop-in liquid media was thought to be a company with the solubilization of inorganic phosphate. The phosphate-soluble bacteria produced organic acids in the media and induced the pH drops to dissolve phosphorus-containing minerals or insoluble phosphates (Cooper 1959). The pH drop on the first five days of bacterial incubation was significantly correlated with phosphate solubilization. The group of very good phosphate solubilization bacteria was all strains with a significant drop in pH at the first five days; the group bacteria were decided as good phosphate solubilization when the pH dropped to 4 and remained for the whole last time of incubation, whereas other bacteria group showed the pH still a decrease of the pH up to 3 along with the experimental time were poor phosphate solubilization (Sánchez-Cruz et al. 2019). However, all isolates from this present study showed a pH trend like the group of very good phosphate solubilization bacteria (Sánchez-Cruz et al. 2019). The results of phosphate solubilization with low SI values inferred that all tested strains possessed weak phosphate solubilization capacity in comparison with *P. aeruginosa* strain KUPSB12 with phosphate solubilization index of 2.85 in screening Pikovskaya's agar and a high amount of phosphate production of 219.64±0.330 mg/mL in liquid medium as well (Paul & Sinha 2017).

The pH values and phosphate solubilization of all isolates were not found to be strictly correlated, thus the pH drop in the liquid medium was effective by other aspects such as the type of microorganisms and the cultivation media. It was shown in the study of phosphate solubilization by fungal isolates from the Brazilian soils (de Oliveira Mendes et al. 2014), that result showed that *Aspergillus niger* FS1 was able to solubilize 71, 36, 100, and 14% of the phosphate from $AlPO_4$, $FePO_4$, $Ca_3(PO_4)_2$, and RPs, respectively. The phosphate solubilization was effective by medium acidification, particularly for $Ca_3(PO_4)_2$.

The microorganisms associated with corals in this study were decided as the most dominant bacteria in Acropora muricata isolated in August 2016 (Pham et al. 2019). The pH value and PO4+ in ambient seawater were measured with 7.61±0.03 and 4.77±1.29 µgP/L, respectively, at the same time of collecting sample. While those parameters measured in May 2016 were 8.10±0.00 and 15.89±2.30 µgP/L, respectively, it could be seen at the time of sample collection, the concentration of PO4 + in seawater was rather low. The data also revealed that the number of phosphate solubilization bacteria was strongly correlated with the pH values in ambient seawater (Pham et al. 2019). In some cases, phosphate deficiency has accelerated bacteria to dissolve insoluble phosphate (Gyaneshwar et al. 1999). One of the most effective inorganic phosphorus solubilizers was the genera of Enterobacter and Pseudomonas (Yazdani et al. 2009).

4.3. Antimicrobial activities

The inhibition level of bacteria in this study is similar to those of isolates from the hard coral (Nguyen et al. 2016) when the clear zones received in both studies were quite weak. However, the results of other studies reported that many coral-derived bacteria had strong antimicrobial activities against the growth of several tested strains (de Castro et al. 2010; Zhang et al. 2012). Three strains P9, P11, and P20 belonged to *Pseudomonas* and showed inhibition against nine tested strains. Those strains were most effective against the test strains *S. aureus, S. typhymurium*, and *Micrococcus* sp. However, there was no inhibition against *Candida albicans*. The isolate P8 identified as *Bacillus pumilus* had antimicrobial activity against 12 tested bacteria (*Aeromonas salmonicida, A. hydrophila, Enterobacter xiangfangensis, Enterococcus faecium, E. coli, Micrococcus* sp., *S. typhimurium, Staphylococcus aureus, Streptococcus* sp., *Vibrio alginoliticus, V. proteolyticus, V. vulnificus*) and the yeast *C. albicans*. The results emphasized the potential use of epiphytic bacteria isolated from brown algae *Padina pavonica* (Peacocks tail) from the northern coast of Tunisia as producers of novel antibacterial compounds (Ismail et al. 2016). *Bacillus licheniformis* strain A2

isolated from the halotolerant plant *Suaeda fruticosa* from the saline desert, Gujarat (India) was reported to solubilize phosphate, produce IAA, and inhibit pathogenic fungus against *Fusarium oxysporum* (Goswami et al. 2014).

In addition, the potential strain RN06 isolated from sea grape possessed antimicrobial capacity against human pathogen *E. coli* 0157. It is inferred that this strain is very powerful in use for aquaculture or agriculture when it is applied in the field, it is very effective in preventing microbial contamination from human activities. The results from the basic chemical test and analysis of the 16S rRNA gene showed that the strain RN06 was most relative to *Bacillus* sp. and isolates HRA5 were identified as *Pseudomonas* sp. It could be seen that both genera were good candidates for PGP so far. A strain of *Bacillus subtilis* LK14 isolated from a medical plant *Moringa peregrina* growing in the arid region of Arabia has shown significant prospects in phosphate solubilization after 5 days of cultivation, *B. subtilis* LK14 produced IAA with the highest concentration of 8.7 µM after 14 days of growth. This strain was applied to *Solanum lycopersicum* and it significantly increased the shoot and root biomass as well as chlorophyll contents as compared to control plants (Khan et al. 2016). On the other hand, the estuary bacteria *Pseudomonas aeruginosa* strain KUPSB12 was illustrated as a potential strain for antimicrobial capacity against Gram-negative tested strains including *E. coli*, *Shigella flexneri*, *V. cholerae*, and three Gram-positive tested strains *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* (Paul & Sinha 2017). None of the total of 11 strains which were IAA producer strains in this study were actinomycetes according to the morphological results. However, actinomyces was thought to be a good candidate for plant growth promotion. Both strains produced a bit low amount of IAA with 3.04±0.16 µg/mL from *Streptomyces* and 4.24±0.21 µg/mL from *Micromonospora*. The actinobacteria isolated from salt marsh soil have been shown to have tolerance of NaCl up to 5 and 11%, respectively, the ability of nitrogen-fixing, produce some hydrolytic enzymes, and could able to serve as plant growth promoting with proof in application in wheat plants under salt stress (Gong et al. 2018).

Macroalgae were reported as good sources of marine microorganisms, the microorganisms are beneficial and harmful partners as well. The *Bacillus* spp. and *Pseudomonas* spp. were the most commonly associated bacteria which were confirmed from algae (del Olmo et al. 2018; Karthick & Mohanraju 2018). The strain RN06 was an IAA producer, phosphate solubilization also possessed antimicrobial activity against three out of four tested strains including of human pathogenic strain *E. coli* 0157. It is worth noting that this bacterium was isolated from edible seaweed so that it could be a potent strain for use as a biological inoculant in commercial aquaculture of seaweed when the demand for seaweed as a healthy food has been increasing recently, especially in Southeast Asia (Chen et al. 2019). The results from the basic chemical test and analysis of the 16S rRNA gene showed that the strain RN06 was most relative to *Bacillus* sp., and isolate HRA5 was identified as *Pseudomonas* sp. It could be seen that both genera were good candidates for plant growth promotion so far (Comeau et al. 2021). Noteworthy, the strain *S. marcescens* strain PDL100 known to cause a severe disease named "white spot" on reef-building coral *Acropora palmate* in the Florida Keys, United States was used as a tested strain (Gyaneshwar et al. 1999).

The estuary bacteria *P. aeruginosa* strain KUPSB12 illustrated as a potential strain for plant growth-promoting when it showed high effectiveness with phosphate solubilization and possessed antimicrobial capacity against *E. coli*, *S. flexneri*, *Vibrio cholerae*, *B. subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* (Paul & Sinha 2017).

Both two potent strains from this study can grow at no salt concentration and 6% NaCl as well (Table 2). It could be due to the sources of isolation, the strain RN06 and HRA5 were associated with sea grapes and hard coral respectively and the salinity of water in the ambient sampling site was less than 35‰. However, this test was done here for getting of basic chemical test (up to 6% NaCl only) rather than a salt tolerance test, so all potential strains in this study were suggested to further experiment including salt tolerance, other plant hormone-producing or antifungals test with plant pathogens.

Seaweed-associated bacteria *Bacillus amyloliquefaciens* strain RN06 produced high amounts of IAA, solubilized inorganic phosphate, and produced antibiotics including antimicrobial agent against human pathogen *E. coli* 0157, and coral pathogen *S. marcescens* PDL100. The derived hard coral bacterium HRA5 was identified as *Pseudomonas* genera. Both two marine potent isolates from this study were identified as *Bacillus* and *Pseudomonas* at the general level. Interestingly, they have been well-known plant growth-promoting bacteria in soils so far. Although the results from this work are still limited, it can clearly pave the way for research on the application of biological inoculants of original marine isolates to seaweed farming as the demand for this food has been increasing rapidly in recent years.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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