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ORIGINAL ARTICLE **OPEN ACCESS**

Molecular Characterization of Indole Acetic Acid Producing Endosymbiotic Bacteria Isolated from *Gracillaria corticata* (J. Agardh) of Visakhapatnam Coast

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ABSTRACT
Six types of endosymbiotic bacteria were isolated from Gracillariacorticata, of Visakhapatnam coast and screened for Indole Acetic Acid production by spectrophotometric method. Isolates were subjected to taxanomical characterization and identified as Vibrio (KX8227220), Enterobacter (KX822723) and Bacillus, (KX822724) and quantities of IAA production, 109.43±0.08 µg/ml, 102 µg/ml and 89.4±0.17 µg/ml respectively.
Keywords:*Gracillaria, Seaweeds, Indole Acetic Acid, Endosymbiotic bacteria, Vibrio, Enterobacter, Bacillus.*

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INTRODUCTION

Marine macro algae play a significant role as primary producers and provides substratum to the number of marine organisms. Seaweeds grow in shallow waters up to 180m [1] depth in intertidal regions [2] and subjected to biotic and abiotic stresses. They are known to produce structurally unique, pharmacologically active and novel secondary metabolites also. The marine bacteria associated with macro algae influence the life cycle of seaweeds by producing different enzymes [3], Plant growth promoting hormones such as auxins (Indole acetic acid) and cytokines (adenine and kinetin) which play a crucial role in the development and morphogenesis of macroalgae [4, 5, 6].
Seaweeds in turn provide substratum and nutrients for survival of the marine bacteria. IAA enhances the plant growth by acting as a signalling molecule which coordinates the cell division and cell elongation. Fries [7] reported that symbiotic bacteria of *Enteromorpha spp*., converts tryptophan to IAA. Studies on molecular mechanism of IAA production by marine symbiotic bacteria is not well established. Many beneficial bacteria found to synthesize IAA predominantly using tryptophan dependent pathway [8]. As the symbiotic bacteria of the seaweeds which had the trait of IAA production were also salt endurable can be beneficial to improve the agricultural productivity in arid and semiarid regions [9, 10, 11, 8 & 13].
Plant growth promoting bacteria (PGPB) are being preferred nowadays as inoculants for influencing crop via multiple direct or indirect mechanisms but isolation of effective PGPB strain is important.

MATERIAL AND METHODS

Study area

Samples were collected from Visakhapatnam coast, located on the East coast of India between the latitudes 17^0 14^1 30^{11} and 17^0 45^1 N and the longitudes 83^0 16^1 25^{11} and 83^0 21^1 30^{11} E. The red alga *Gracilariacorticata*were collected at intertidal regions of Rushikonda, Mangamaripeta and Thotlakonda beaches of Visakhapatnam (Fig: 1).

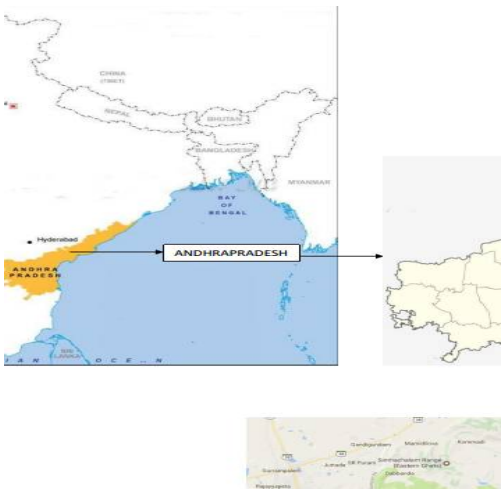

Figure 1. Map showing sample collection areas

Sample collection

Whole plants of *Gracilariacorticata* were collected at the intertidal regions and carefully scrapped by using a sterile knife. Collected samples (100 g) were transferred into ice packed zip-lock bags, brought to the laboratory and identified based on standard taxonomical keys [14, 15 & 16]. The algal surface was cleaned with a disinfectant solution containing 2% Sodium hypochlorite and 0.1% Tween 20. Samples were thoroughly washed with sterile Milli-Q water followed by seawater.

Isolation of endosymbiotic bacteria

The samples were ground in sterile mortar and pestle. The ground suspension was serially diluted, spread plated over Zobell's marine Agar medium (Himedia, India) and incubated at 29^0C for 48 hours. Morphologically different colonies obtained were restreaked onto the same medium to obtain pure cultures [17].

Screening of bacterial isolates for IAA production

Test for production of Indole acetic acid

*In-vitro*auxinproduction by isolated marine bacterial isolates were detected by following Brick et al. [18]. Isolates were grown in 250 ml conical flasks in 100 ml Yeast Malt Dextrose broth (Himedia, India) and supplemented with tryptophan (1000 µg/ml). The flasks were inoculated with 100 µl of bacterial cell suspension adjusted to an optical density of 10^{-8}CFU/ml. All inoculated flasks (in triplicate) were incubated at 37°C for 72 h in a shaker at 120 rev/min. After incubation, cells were centrifuged at 2300 × g for 15 min. Two ml of supernatant was taken and mixed with 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) to determine auxin production in bacterial culture supernatant [19].

Quantitative assay of IAA production

Cultures were grown for 72 hours in Yeast Malt Dextrose broth and centrifuged at 10,000 rpm for 10 minutes and collected the supernatant. The amount of IAA produced by the bacterial isolates were quantified by using Gordon & Weber method [19]. To the 2 ml of supernatant, 4 ml of Salkowski reagent were added and incubated in dark for 30 minutes. A blank containing Salkowski reagent (50 ml, 35% perchloric acid, 1ml 0.5 N $FeCl_3$solution) and water (4+2 ml) were used for calibration in UV-Vis Spectrophotometer (SL Elico 159). The test samples were read at 530 nm for absorbance (OD). OD values were compared with a standard graph made from authentic IAA [20] to calculate the actual quantity of IAA and expressed in µg/ml.

Identification of IAA producing bacteria associated with *G. corticata* and their biochemical characterization

Biochemical characterization of the selected bacterial isolates was done by observing colony morphology, microscopic examination and by performing different biochemical characterization tests [21].

Molecular characterization of endosymbiotic bacteria of *G. corticata*

Bacterial DNA was extracted by simple Phenol chloroform method [22]. The isolated DNA concentration was measured by diluting 2 µl of DNA in 198 µl of TE (1:100 dilution) and measured the absorbance at 260nm and 280nm.

Concentration of original DNA solution in µg/ml = OD@ 260nm x 50 x dilution factor. (Here dilution factor = 100). Gel check on 0.9% Agarose.

PCR amplification

PCR amplification of 16S rRNA gene sequences of the isolates were performed by using universal forward 27F (5'- AGAGTTTGATCCTGGCTCAG 3') and reverse 907R (5´CCGTCAATTCMTTTRAGTT 3') primers [22]. The PCR was set up with Initial denaturation at 95°C for 5 mins: 40 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C followed by a final extension step consisting of 7 min at 72°C. Amplified PCR products were run on 1.5% agarose gel electrophoresis simultaneously 1 kB DNA ladder added as a reference to estimate the size of the unknown DNA. Experiment was conducted at 0.7^{-1} V ml^{-1}. The size and purity of the DNA and PCR products were checked by 260/280 ratio and size of the DNA bands were estimated by comparison to the DNA ladder.

16S rRNA gene sequence analysis

Pure Link™ PCR Purification Kit was used to purify the PCR products. Gel fragments were solubilized and loaded on a Pure Link™ spin column and DNA was isolated using a simple elute procedure. DNA fragments were recovered in TE buffer and sequence analysis was performed by using an ABI PRISM 3100 Genetic Analyser (Bioserve technologies India). The sequences were submitted to the NCBI facility (www.ncbi.nlm.nih.gov/BLAST) and recorded the nearest matches by using UPGMA, MEGA SOFTWARE version .7.0.

Confirmation of the presence of IAA in culture filtrate by Thin layer Chromatography

Culture filtrate was taken by centrifuging the mass culture content at 10,000 rpm for 10 minutes. The culture filtrate was mixed with equal volume of ethyl alcohol, performing the solvent separation to concentrate solvent layer. Thin layer chromatography was performed with the extracted ethanol fraction of crude compounds using pre-coated silica gel. TLC plates of grade Silica gel GF 254, thickness 0.25 mm, (Merck, Germany) were used to detect IAA produced by the bacterial isolates [24].

The crude extract was spotted with the capillary tube and solvent front was allowed to run for approximately 80% of the plate. The crude was eluted with butanone-ethyl acetate-ethanol-water (3:5:1:1) solvent system [25, 26]. Spots with R_f values which coincided with that of authentic IAA were identified under UV light (254 nm) after the movement of the solvent up to the solvent front, the bands in the TLC were identified after soaking in iodine[27].

Retention factor was calculated according to the standard formulae as follows:

Retention factor = Distance travelled by Solute/Distance travelled by the Solvent.

RESULTS

IAA producing endosymbiotic bacteria from *Gracilariacorticata*(J. Agardh)

The total number of bacterial colonies obtained by spread plate of *Gracilariacorticata*(J. Agardh)sample collected from Rushikonda, Mangamaripeta beach and Thotlakonda beach were 25, 30 and 35 CFU/g at 10^{-7} dilution Six isolates of endosymbiotic bacteria were and designated as GM1, GM2, GM3, GM4, GM5 and GM6 (Fig: 2).

Figure 2. Growth of pure bacterial isolates on Zobell's Marine agar

Screening of the endosymbiotic bacteria for IAA production

Preliminary screening

To confirm the production of Indole acetic acid by the bacterial isolates, the culture filtrate was treated with salwoski reagent. Change of the colour from yellow to pink confirmed the presence of IAA. All the 6 isolates (GM1-GM6) produced pink to light red color with salwoski reagent indicating that all they are capable to produce Indole acetic acid (Fig: 3). No color change was observed in control. The bacterial isolates GM3, GM5 and GM6 produced red color indicating that they were potent IAA producers (Fig: 3).

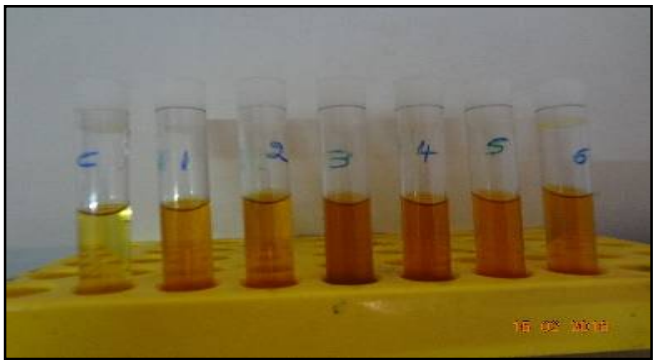

Figure 3. IAA production by bacterial isolates

Quantification of IAA by Spectrophotometric method

Amounts of Indole acetic acid produced by the isolates were depicted in the graph (Fig: 4). Bacterial strains GM3, GM5 and GM6 produced good amount of IAA. The highest amount (109.43±0.08 µg/ml) was produced by GM6 and the least amount (17.16±0.18µg/ml) by GM2. GM3 (102µg/ml) and GM5 (89.4±0.17µg/ml) also produced significant amounts of IAA. GM1 and GM4 produced 35.35±0.41µg/ml and 38.4 ±0.36 µg/ml (Fig-4).

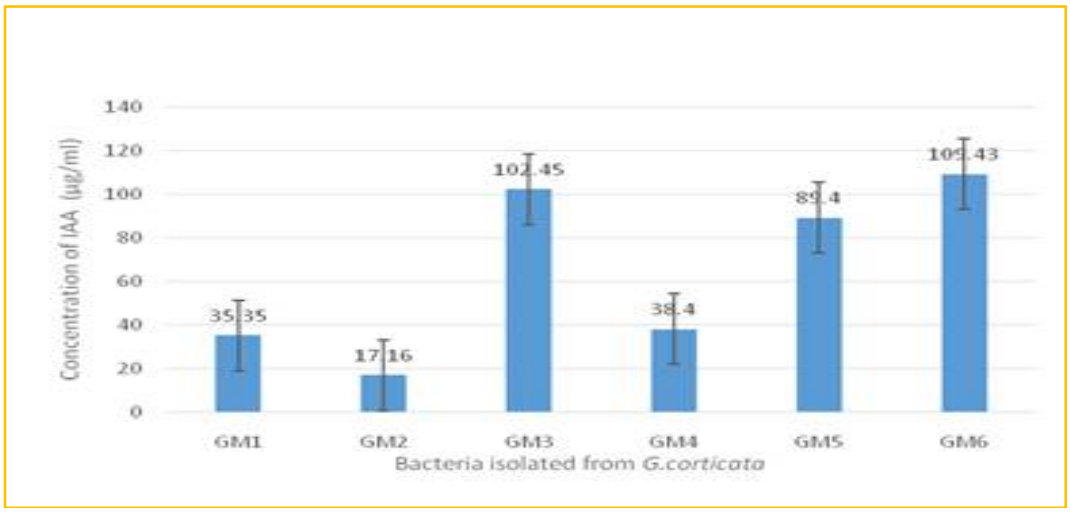

Figure 4. Graph showing Quantity of IAA produced by different isolates obtained from *G. corticata*

Conformation of IAA by Thin layer chromatography

It was identified that GM3, GM5 and GM6 produced significant amounts of IAA (Fig-5).

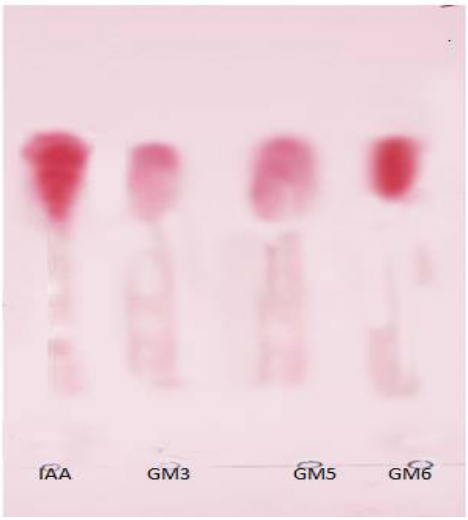

Figure 5. TLC picture of the bacterial isolates GM3, GM5 and GM6

The R_f value of the standard IAA is 0.58 cm. The R_f values of the GM3, GM5 and GM6 were 0.55 cm, 0.56 cm and 0.58 cm respectively.

Biochemical characterization tests

Based on biochemical characterization tests, the three isolates GM3, GM5 and GM6 have been identified as *Vibrio spp, Enterobactersp* and *Bacillus sp*

Molecular characterization by using 16S rRNAGenesequence analysis

The PCR amplified products of the isolates (GM3, 5 and 6) were electrophoresed in 1.5% Agarose gel and were presented in Fig-6. It is estimated that amplicon corresponds to 900bp.

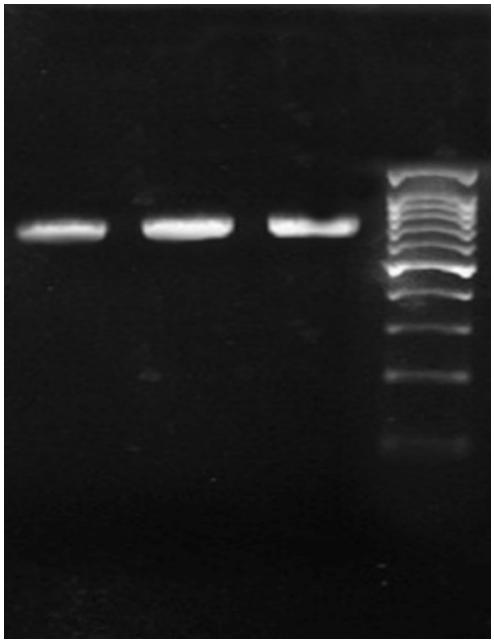

Figure 6. Gel electrophoresis of amplified 16S rRNA Gene of the GM3, GM5 and GM6

16s r RNA Gene based identification

The identification of bacterial strains based on 16S rRNA gene sequences is presented in the table. BLAST nucleotide search of the three bacterial isolates GM3, GM5 and GM6 showed 99% homology with genus vibrio (*Vibrio alginolyticus* strain NBRC 15630 (Accession No. NR 122050), *Enterobacter (Enterobacteraerogenes* strain NCTC10006 (Accession No. NR 114737) and *Bacillus (Bacillus licheniformis*strain*I*SM 13 (Accession No.NR 118996). The nucleotide sequences were submitted to Gen Bank and accession numbers were tabulated (Table-1).

Table 1. Molecular identification of GM3, GM5 and GM6

Assigned code	Sequence length (bp)	Similarity (%)	BLAST Results	NCBI's Accession
GM-3	776	99%	*Vibrio alginolyticus*	KX822722
GM-5	962	99%	*Enterobacteraerogenes*	KX822723
GM-6	794	99%	*Bacillus licheniformis*	KX822724

Phylogenetic tree

Phylogenetic tree derived from maximum parsimony analysis of the 16S r RNA gene from marine bacteria *Vibrio alginolyticus, Enterobacteraerogenes* and *Bacillus licheniformis* (GenBank accession no.s KX822722, KX822723, KX822724). The Gram-negative bacterium *Enterobacteraerogenes* was used as the out group. This tree is the most parsimonious generated by the UPGMA software.

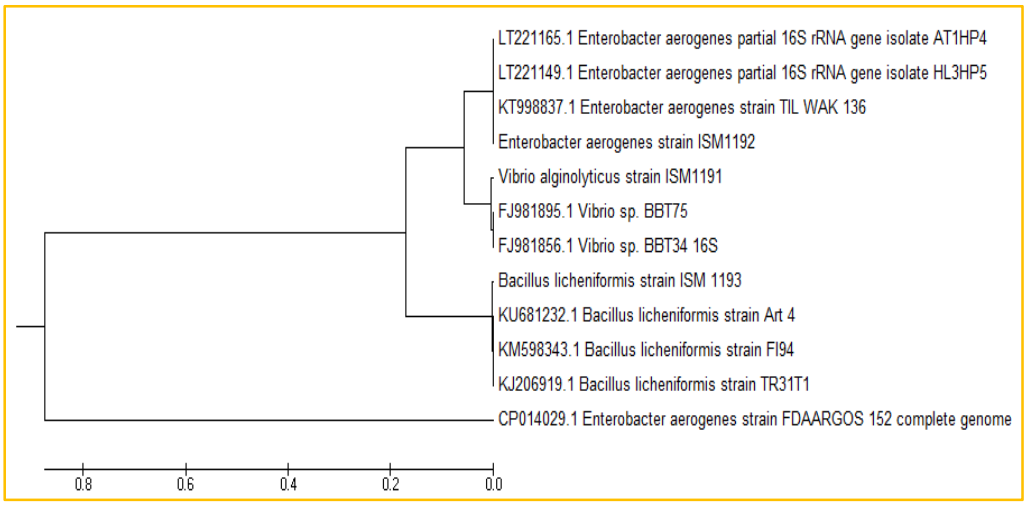

Figure 7. Phylogenetic tree analysis of IAA producing endosymbiosis bacteria of *G. corticata*

DISCUSSION

In the present study a total of six, IAA producing endosymbiotic bacteria GM1-GM6 were isolated from the *Gracilariacorticata.* All the bacterial isolates showed clear growth on Zobell's marine agar. Preliminary screening was done to know the amount of IAA produced [20]. Maximum amount of IAA was produced by the isolate GM6 i.e., (109.43 ± 0.08) followed by GM3 (102.45 ± 0.39**)**, GM5 (89.40 ±0.17), GM1 (35.35 µg/ml), GM4 (38.40 µg/ml) and GM2 (17.16 ± 0.18) respectively. Molecular characterization was done to only these endosymbiotic bacteria which produce significant amounts of IAA. Earlier studies of Singh *et al.* [28] also revealed that symbiotic bacteria of macroalgae had the IAA producing trait, they isolated 39 symbiotic bacteria from various *Gracilaria spp.,* from (*G. corticata* isolated 10 bacterial strains). From those isolates, three species *B. pumilus, B. licheniformis* and *E. homiense* produced IAA and the amount of IAA produced was 445.5, 335 and 184.1 $\mu g.ml^{-1}$ IAA in the culture filtrates respectively.

Present study with the interpretation of the results obtained from the preliminary screening for IAA production *Vibrio alginolyticus, Enterobacteraerogenes* and *Bacillus licheniformis* were identified as potential IAA producers. Earlier works showed that IAA producing organisms were Gram negative [28] [29]. Gram positive strains belonging *Bacillus* strain were also known to produce IAA [30]. In the present work also GM3 and GM5 were Gram negative rod shaped bacteria except GM6. *Exiguo bacterium homiense* and *Bacillus spp*. were shown to have the trait to produce IAA that determined the morphogenesis in *G. dura* [31]. It was also reported that green, brown and red seaweeds have the ability to produce plant growth hormones. In *Ectocarpussiliculosus* [32, 33], *Kappaphycusalvarezii* [34] and *Ulvaspp* [35] phytohormones determined morphogenesis and [36] showed a relationship with bacterial auxin. It has been reported that production of IAA by bacteria varies with species and strains, and also depends on culture condition, growth stage and substrate availability [37, 38]. In the present study all the six isolated bacterial strains produced IAA but only three produced a significant amounts of IAA. A better understanding of seaweed-bacterial interaction would be possible with the development and improvement of molecular techniques [39]. Hence further identification of bacteria was done at molecular level using 16S rDNA sequencing. The homologies of 16S rDNA sequences were obtained by blasting the sequences against the NCBI data base. The taxonomic positions of the isolates were shown in the phylogenetic tree. Most reliable method to check and confirm the presence of IAA in culture filtrate was Thin layer chromatography [10, 40, 41]. The $R_f$values of the IAA produced by culture filtrates compared with the standard IAA R_f value [25, 42]. The chromatogram of the culture filtrates of the isolates of GM3, GM5 & GM6 showed the near with the Standard IAA $R_f$value (0.58 cm). The results support and confirm the production of IAA. Our TLC findings were in agreement with reports by other scientists [43 & 44].

CONCLUSION

IAA produced by the marine symbiotic bacteria can be used as bio-fertilizers. Use of these bacteria as bio-inoculants profoundly increase the crop yield and reduce the use of chemical fertilizers. As these bacteria are halo tolerant they can efficiently produce IAA even in the saline soils and can be used as bio inoculants to improve the crop.

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