



## Isolation and identification of IAA producing endosymbiotic bacteria from *Gracilaria corticata* (J. Agardh)

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**Abstract:** Indole acetic acid is a natural phytohormone which influence the root and shoot growth of the plants. Six (GM1-GM6) endosymbiotic bacteria are isolated from *Gracilaria corticata* and screened for the production of IAA out of six, three bacterial strains GM3, GM5 and GM6 produced significant amount of IAA 102.4 µg/ml 89.40 µg/ml 109.43 µg/ml respectively. Presence of IAA in culture filtrate of the above strains is further analyzed and confirmed by TLC. As these bacterial strains, able to tolerate the high salinity these can be effectively used as PGR to increase the crop yield in saline soils.

**Key words:** IAA- Indole acetic acid; PGR- plant growth Regulators; TLC- Thin layer chromatography

### Introduction

The phytohormone indole-3-acetic acid (IAA) is the most commonly occurring naturally produced and extensively studied plant growth regulator (Klopper and Schroth 1978) it is a product of L-tryptophan metabolism of microorganisms. 80% of rhizospheric bacteria produces IAA (C. L. Patten and B. R. Glick, 1996). IAA directly influences several physiological processes in plant growth and development including the induction and regulation of cell division, root extension, vascularization, apical dominance, seed dormancy, fruit ripening and abscission. (P.J. Davies, 2004, J.K. Vessey, 2003, Aloni *et al.*, 2006, M. Kelen, 2004) Present study focus on isolation, identification of IAA producing endosymbiotic bacteria from marine macro algae *Gracilaria corticata*. These bacterial strains can be exploited as biofertilizers to improve the crop productivity in saline soils and also we can reduce the use of chemical fertilizers.

Seaweed associated bacteria were initially identified only based on morphological and biochemical tests (Berland *et al.*, 1972; Laycock, 1974; Shiba & Taga, 1980). Johannes Reinke (1903) for the first time demonstrated a true symbiotic association between seaweed and bacteria Johannes Reinke (1903) for the first time demonstrated a true symbiotic association between seaweed and bacteria. IAA is the most abundant member of the auxin family of phytohormone (Bartel, 1997). Fries (1975) reported that bacteria living on *Enteromorpha spp.* showed capacity to convert tryptophan into IAA.

Roseobacter group associated with red seaweed caused a gall formation in *Prionitis lanceolata* due to over production of IAA in comparison to other thallus (Ashen & Goff, 2000). Molecular mechanism of IAA production from seaweed associated bacteria is not well understood. But, it can predict a molecular mechanism of IAA production from seaweed associated bacteria, based on the reports from those bacteria which are associated to higher plants.

### Material and Methods

#### Study area:

Visakhapatnam located on the east coast of India between the latitudes 17° 14' 30" N and 17° 0' 45" N and the longitudes 83° 16' 25" E and 83° 21' 30" E. The red alga *Gracilaria corticata* (A.V.S.S.Sambamurthy, 2005) were collected at intertidal regions of the Mangamma vari peta beach and Thotla konda beach where anthropogenic activity is less. Sample collection was done during the lowest tide time according to the tide time table provided by the NIO, Visakhapatnam. Sample collection was done one time in November, 2014.

#### Sample collection:

The whole plant of *Gracilaria corticata* (A.V.S.S.Sambamurthy, 2005) was collected at the intertidal regions the samples were carefully scrapped from substratum by using a sterile knife. Collected samples (100 g) were transferred in ice packed zip-lock bags. Fresh healthy plant with no

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sign of injury were washed with sterile seawater and processed 24 hours for isolation of associated bacteria. The algal surface sterilized with disinfectant solution containing 2% Sodium hypochlorite and 0.1 % Tween 20. Samples were rinsed thoroughly with sterile Milli-Q water to remove disinfectant.

#### Isolation of endosymbiotic bacteria:

The *Gracilaria corticata* sample was prepared by grinding the seaweed in sterile mortar and pestle. Isolation of endosymbiotic bacteria was done by serial dilution method. 0.1 ml ground seaweed suspension of each dilution was spread over Zobell marine Agar Medium (HiMedia, India, Peptic digest of animal tissue 5g/l, Yeast extract 1g/l, Ferric citrate 0.1g/l, Sodium chloride 9.45g/l, Magnesium chloride 8.8g/l, Sodium sulphate 3.24g/l, Calcium chloride 1.8g/l, Potassium chloride 0.55g/l, Sodium bicarbonate 0.16g/l, Potassium bromide 0.08g/l, Strontium chloride 0.034g/l, Boric acid 0.022g/l, Sodium silicate 0.004g/l, Sodium fluoride 0.0024g/l, Ammonium nitrate 0.0016g/l, Disodium phosphate 0.008g/l, Agar 15g/l) Plates were incubated at 29°C for 1 to 6 weeks. Pure cultures were obtained by streaking morphologically different colonies on the Zobell marine agar medium. For further analysis cultures were preserved in Zobell Marine Agar broth with 20% glycerol and stored at -20°C. To know the validity of surface sterilization tissue which were surface sterilized soaked in 5 ml sterile Milli Q and kept for 1 min and then Milli Q (500 µl) distilled water was spread onto Zobell Marine Agar and incubated for 7 days at 29°C. No bacterial growth was observed on the plate which is an indicative of effective surface sterilization. (Mirshiba *et al.*, 2010, Polpass rose *et al.*, 2013).

#### Hydrographic Parameters:

Immediately after the collection of the sample Temperature and pH of the sea water is checked. Salinity of the sea water is identified by Knudsen (Barnes, 1959) method. Dissolved Oxygen is estimated by Winkler's method (APHA, 1971).

#### Identification of isolated bacteria

Selected bacterial isolates were identified by colony morphology, microscopic examination and biochemical characterization was done by following Bergey's Manual of Determinative Bacteriology (Bergey, 2009) by performing tests such as catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, starch hydrolysis, carbohydrate fermentation i.e. glucose, sucrose, mannitol and lactose etc., and urea utilization

#### Preliminary screening of bacterial isolates for IAA production:

**Test for production of Indole acetic acid:** *In vitro* auxin production by isolated marine bacterial strains were determined in the presence of

precursor L-tryptophan (Brick *et al.*, 1991) Strains were grown in 100 ml Yeast malt Dextrose broth (HiMedia, India) (Peptic digest of animal tissue 5g/l, Yeast extract 3g/l, Malt extract 3g/l, Dextrose 10g/l) each 250 ml Erlenmeyer flask are supplemented with L-tryptophan (1000 µg ml<sup>-1</sup>). The flasks were inoculated with 100 µl of bacterial cell suspension adjusted to an optical density of 10<sup>7</sup> CFU/ml. All inoculated flasks (in triplicate) were incubated at 30 ± 2 °C for 72 h at 120 rev/min. After incubation, centrifugation was done at 2300 × g for 15 min to remove cells and prepare a cell free extract. 2 ml of Cell free extract was mixed with 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M FeCl<sub>3</sub> solution) to determine auxin production in bacterial culture supernatant (Ali *et al.*, 2009a).

**Quantitative assay of IAA production:** Cultures were grown for 3 days in YMD broth, the 72 hours old cultures were centrifuged at 10,000 rpm for 10 minutes and cell free extract was collected. 2 ml of Cell free extract was mixed with 4 ml of Salkowski's reagent and kept in dark for 30 minutes. Simultaneously a Blank was setup with Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 N FeCl<sub>3</sub> solution) (Brick *et al.*, 1991) and water (4+2 ml). For calibration ELICO UV Vis Spectrophotometer is used. The test samples were read at 530 nm for absorbance (OD). To calculate the actual quantity of IAA OD values were compared with a standard graph made from standard IAA and concentration of IAA produced was expressed in µg/ml.

#### Standard IAA graph (Gordon and Weber 1951)

Standard graph of IAA was prepared with different IAA concentrations in aqueous solution of IAA ranging from 3.125 µg/ml to 100 µg/ml. To each 2 ml of the standard, 4 ml of 2% 0.5 M FeCl<sub>3</sub> in 35% perchloric acid i.e. Salkowski reagent is added and incubated for 30 minutes. After 30 minutes readings were read at 530 nm by UV-Visible spectrophotometer SL Elico 159. Standard graph is prepared by plotting concentration of IAA in µg/ml Vs Optical Density at 530 nm.

#### Identification of IAA by Thin layer chromatography:

To identify the IAA in culture filtrate cell free filtrate was prepared by centrifuging the 72 hours aged mass culture at 10,000 rpm for 10 minutes. The culture filtrate was mixed with equal volume of ethyl acetate, and then solvent separation was done in separating funnel the solvent layer was concentrated in a rotavapor. TLC was performed with the extracted ethyl acetate fraction of crude compounds using pre-coated silica gel TLC plates of grade Silica gel GF 254, thickness 0.25 mm, (Merck, Germany) to detect IAA produced by the bacterial isolates. The crude extract was spotted with 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 (Ahmad *et al.*, (2005), Hariharan Harikrishnan *et al.*, 2014). Spots with R<sub>f</sub> values coincide 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. (M. Jayaprakashvel *et al.*, 2014). Retention factor was calculated according to the standard formulae as follows,  
Retention factor = Distance travelled by Solute/Distance travelled by the Solvent.

**Results and Discussion**

*Gracilaria corticata*, (Rhodophyta) is a marine red seaweed profusely grow at the intertidal regions of the sea. Seaweeds provide substratum for colonization of bacteria which are beneficial to complete their life cycle and for the morphogenesis of seaweeds (Armstrong *et al.*, 2000 Delbridge *et al.*, 2004; Burke *et al.*, 2011a; Singh *et al.*, 2011a, b, c). In the present study six endosymbiotic bacteria are isolated from the *G.corticata* on Zobell marine agar (Fig.1). Morphology, biochemical characters of six bacterial strains GM1, GM2, GM3, GM4, GM5 and GM6 (Table-1) are studied out of six bacteria five are gram-ve rod shaped bacteria, GM6 is gram +ve bacilli. Fifty-three bacterial isolates were isolated by R.P.Singh *et al.*, from naturally collected thalli of *Ulva fasciata*, *U. taeniata*, *U. lactuca*, *Gracilaria*

*corticata*, *G.dura*, and *G. salicornia* from the coast of Veraval out of which 18 from *Gracilaria spp.* Out of 18 five from *G.corticata*. Production of IAA is the trait of the rhizospheric bacteria but the studies revealed that seaweed associated marine bacteria produce plant growth regulators (PGRs) (Maruyama *et al.*, 1986, 1988, 1990; Mooney and Van,1986), indol-3-acetic acid (IAA; Provasoli & Pintner,1953; Singh *et al.*, 2011b) and other PGRs and vitamins(Provasoli & Carlucci, 1974) which are effective in inducing the morphogenesis in *Ulva spp.* (Fries & Aberg, 1978; Bradley, 1991; Spoerner *et al.*, 2012) Nayomi John & M Thangavel, 2015 isolated 25 marine bacteria in that 14 strains producing IAA in presence of L-tryptophan. Phytohormone IAA works as a signal molecule in the regulation of plant development. Plant system uses auxin like hormones for their optimal growth.



Figure 1: Isolated cultures

Table 2: Biochemical identification

Parameter	MG-1	MG-2	MG-3	MG-4	MG-5	MG-6
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Dull
Size	Small	Small	Small	Small	Small	Medium
Color	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy	Cloudy
Form	Circular	Circular	circular	Circular	Circular	Irregular
Texture	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Dry
Elevation	Raised	Raised	Raised	Convex	Convex	Umbonate
Mrgin	Entire	Entire	Entire	Entire	Entire	Undulate
Gram stain	-ve	-ve	-ve	-ve	-ve	+ve
Shape	rod shape	rod shape	rod shape	rod shape	rod shape	bacilli
Salt 0 %	-	-	-	-	-	-
Salt 3 %	+	+	+	+	+	++
Salt 6%	++	+	+	++	+	++
Salt 8%	+	+	+	+	+	+
Salt 10%	+	+	+	+	+	+
Salt 12%	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	-	+	-	+
Urease	-	-	-	-	-	+
Indole	-	+	-	+	-	+
Methyl red	+	+	+	+	+	+
Voges prosecure	-	-	-	-	-	-
Citrate	-	-	-	-	-	+
Gelatin	++	++	++	++	++	++
Tween 80	+	+	+	+	+	+
Iron	+	+	+	+	+	+
Starch	++	++	++	++	++	++
<b>Amino acids utilization tests</b>						
IAA	+	+	+	+	+	++
Ornithine	+	+	-	-	-	-
Arginine	+	-	+	+	+	-
Lysine	-	-	+	-	-	-
<b>Carbohydrate utilization tests</b>						
Raffinose	-	-	-	-	-	-

Sucrose	+	-	-	-	-	+
Lactose	+	+	-	+	+	-
Maltose	+	+	+	+	+	-
Trehalose	+	+	+	+	++	-
Cellobiose	+	+	+	+	+	-
Melibiose	-	+	-	-	-	+
Ribose	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-
Xylose	+	+	-	-	-	-
Rhamnose	-	-	-	+	+	-
Mannose	+	-	+	-	+	+
Galactose	+	-	-	-	+	-
<b>Sugar alcohol utilization tests</b>						
Fructose	+	+	-	-	+	+
Adonitol	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+
Sorbitol	-	-	+	+	+	-
Dulcitol	-	-	-	-	-	+
<b>TCBS</b>		Milky Yellow color growth	Milky Yellow color growth	Milky Yellow color growth	Milky Yellow color growth	Milky Yellow color growth

+ Positive, - Negative

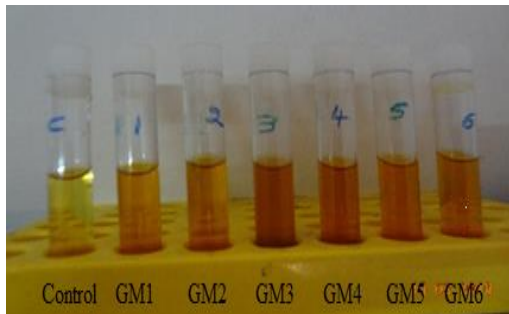
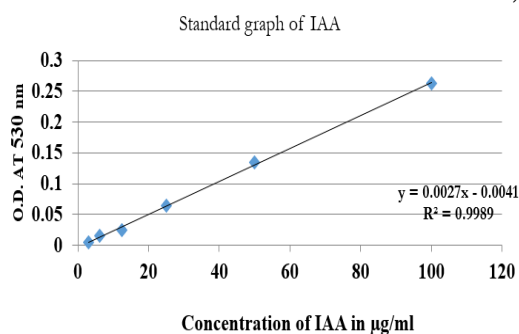


Fig.2: IAA production by GM1, GM2, GM3, GM4, GM5 and GM6

Table 3: O.D values of standard IAA at 530nm

Concentration of IAA(µg/ml)	Optical density at 530nm
100	0.262
50	0.133
25	0.063
12.5	0.024
6.25	0.015
3.125	0.004

Fig.3: Standard graph of IAA (concentration of IAA on X-axis vs O.D value at 530nm on Y- axis)



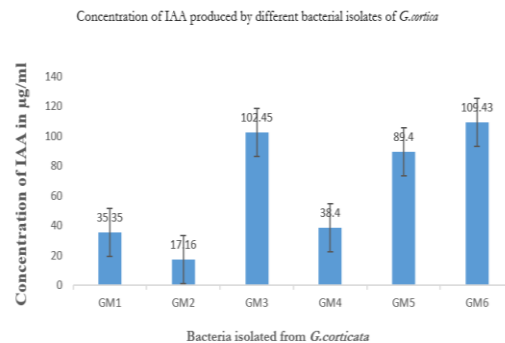
By plotting the concentration of IAA against O.D. a straight line was constructed the straight-line equation was represented on the graph.

Table: 4 Concentration of IAA produced by bacteria GM1-GM6 isolated from *G.carticata*

Bacterial isolate	Concentration of IAA (µg/ml)
GM1	35.35 ± 0.41
GM2	17.16 ± 0.18
GM3	102.45 ± 0.39
GM4	38.40 ± 0.36
GM5	89.40 ± 0.17
GM6	109.43 ± 0.08

Values are tabulated as means ± the standard error of the mean (SEM) and N=3.

Fig.4: Standard error bar diagram representing amount of IAA produced by the bacteria GM1-GM6 isolated from *G.carticata*



The above figure depicts that all the bacterial isolates are producing IAA but the Strains GM3, GM5 and GM6 are producing more amount of IAA in these GM6 is producing significant amount of IAA.

**Fig.5:** IAA Thin layer chromatography of the bacterial isolates GM3, GM5 and GM6



$R_f$  values of the GM3, GM5 and GM6 were calculated by using the formula.

$R_f$  value = the distance travelled by solute/the distance travelled by the solvent.

The  $R_f$  values of the GM3, GM5 and GM6 are compared with the standard IAA,  $R_f$  value.

Same findings are observed in the recent study that six bacteria (GM1...GM6) which are isolated has formed pink to red color with salkowski reagent which indicates that they are capable of IAA production. (Fig.2). The amount of IAA produced is estimated from standard graph (fig.3). Mary Franceena *et al.*, 2016 screened marine bacteria for the production of IAA the amount of IAA produced is quantified in which strain Tb3 showed maximum production of IAA (14.35 $\mu$ g/ml) of all the strains Bb2 showed minimum IAA (6.05  $\mu$ g/ml) in our study the maximum amount is produced by the GM6 (109  $\mu$ g/ml) and minimum amount by GM2 (17.16  $\mu$ g/ml). GM3 and GM5 also produced significant amount of IAA 102  $\mu$ g/ml and 89.40  $\mu$ g/ml. GM1& GM4 produced 35.35  $\mu$ g/ml and 38.40  $\mu$ g/ml which is an indicative that these bacteria are more potential to produce the IAA. The studies of Singh R.P. *et al.*, 2011 showed that three strains isolated from *G.corticata* the *B.pumilus*, *B.licheniformis* and *E.homiense* produced 445.5, 335 and 184.1  $\mu$ g /ml-IAA which supports our study that symbiotic bacteria are best IAA producers. Bacteria can synthesize IAA through six different pathways namely, indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TAM), indole-3-acetonitrile (IAN), tryptophan side-chain oxidase (TSO) and tryptophan independent pathways, a high degree of similarity between biosynthesis of IAA in plants and bacteria has been reported (Spaepen S. *et al.*, (2007), Patten, C. L. and Glick, B. R. 1996) an amino acid, tryptophan, is identified as the main precursor for IAA biosynthesis in bacteria. The identification of IAA can be done by TLC by comparing the  $R_f$  values of the IAA produced by culture filtrates with standard IAA  $R_f$  value (2014, Ahmad *et al.*, 2005, Hariharan Hari Krishnan *et al.*, 2014 Sutthinan Khamna *et al.*) The chromatogram of the GM3, GM5 & GM6 culture filtrate showed the near  $R_f$  values with the

Standard IAA value (0.58cm) (fig.5). The results support and confirms the production of IAA by the above bacterial strains.

## Conclusion

Study of these bacterial strains for the production of PGR can be an effective tool to improve the crop productivity these can be used as bio fertilizers in saline soils and also minimize the use of chemical fertilizers. To increase of agriculture, yield in sustainable and eco-friendly manner is possible with the intervention of IAA producing endosymbiotic bacteria from *Gracilaria corticata*.

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