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ORIGINAL ARTICLE

Multi Facilitated Activation of Endophytic Bacteria Isolated from Macroalgae

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ABSTRACT
In this study macroalgae were collected from Veraval Sea coast and Okha sea coast, Gujarat, India and identified as Ulva sp. and Tribophyta sp. respectively. The endophytic bacteria were isolated from collected macroalgae and different morphology, biochemical studies and growth pattern study was performed. A total of 18 endophytic bacteria were isolated and they all were gram's positive in nature. Isolates were capable of tolerating 11% of salt concentration. All endophytic bacteria were tested for plant growth-promoting activities like phosphate solubilization, ammonia production, and indole acetic acid production. Based on this study three bacteria SUN 3, SUL 24 and OTY 36 were selected for analyses of various antimicrobial potentials like antibacterial activity, antifungal activity, and antibiotic activity. These three endophytic bacteria were identified by 16s RNA method as the potential of phosphate solubilization, ammonia production and indole acetic acid production, antibiotic activity and tree topology were evaluated.
Keywords: *Macroalgae, endophytic bacteria, Plant Growth Promoting activity, Antimicrobial potential*

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INTRODUCTION

Bacteria that live in the internal tissue of the plants either symbiotically or in a mutuality relationship called endophytic bacteria [2]. They can be isolated after surface sterilization bacteria and fungi are found from any plant tissues are known as endophytes and they may start from the seed, the roots of surrounding soil or the aerial portions of plants. All plants in nature have various types of endophytic bacteria which can completely affect host plant growth [3].

Endophytic bacteria have beneficiary effect for degrading the different types of metal like copper, iron etc [8]. Endophytic bacteria are the symbionts that present within the plant, and by properties they occurs mutualisms with the plant and bacteria, both take a benefit of each other presence and increases the stress tolerance like drought stress, salt stress, metal stress or plant growth promotion [4]. The endophytic bacteria have to colonize the plant endosphere. The colonization process traits are involved such as motility, connection, plant-polymer degradation, and illusion of plant defences. The diversity of endophytic bacteria depends on several things such as plant and environment specific factors. Some endophytic bacteria are broad host range and used as bio inoculants in developing agriculture system that enhance the plant growth promotion, enhanced plant mineral uptake and yield and as well as reduction of oxidative stress responses [7]. Thus endophytic bacteria establish a double condition attribute with the host where in both get synergistically benefitted [9]. Endophytic bacteria are also caused an enhancement in water retention and in biomass accomplishment by a waiting in flowering and leaf senescence, that fix a large amount of carbon within the host [6, 7]. In terms of antioxidant status, endophytic bacteria inoculated plants expressed a high level of antioxidant production that exposed to abiotic or biotic stress. An augmented antioxidant activity directly correlated to an increase in host biomass and root length [10, 27]. Endophytic bacteria garner the attention of sustainable agriculture and

also use as the alternative to rhizospheric bacteria, their benefits are that the endophytes get a good competitor for plant growth promoting rhizobia (PGPR). This research work focus on isolation of endophytes from macroalgae formed on sea coast and study of their plant growth promoting properties like phosphate solubilization, ammonia production and indole acetic acid production phytohormones and in saline stress.

MATERIAL AND METHODS

Macroalgae Sample Collections

Fresh macro algae sample were collected from Veraval sea in Arabian sea Western Sea coast of Gujarat (20° 54' 21.1896'' N and 70° 23' 15.0180'' E.), Sea cost of Okha, in Dwarka, Gujarat (22° 15' N, 69° 1' E).The samples were collected in a sufficient quantity in non-reactive bag to easily transport for the analysis and taken care that there were no chemical or physical reactions that occurred in relevant components.

Satellite map of sample collection

Satellite map of macroalgae collection from Veraval sea coast and Okha sea coast from

Figure 1

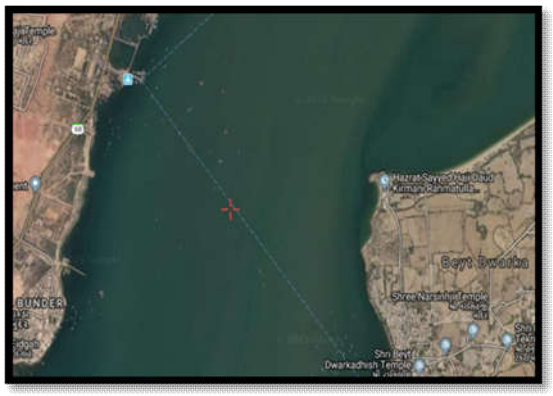


Figure 2

Figure 1 is satellite map of Veraval Sea, Arabian Sea Western Sea coast of Gujarat, and **Figure 2** is Sea cost of Okha, in dwarka, Gujarat

Macroalgae identification

Macroalgae were identified by their morphological classification within 24 hr of sample collection. Firstly macroalgae were identified by Colour and morphological differences in genera/species and taxonomic characteristics. Taxonomic identification key should be followed to identify the seaweed specimen. The taxonomic description of the specimen and anatomical characteristic of the specimen to be identified should be referred from the identification books. Macroalgae Identification was also confirmed by the Botany Department, Gujarat University [49, 50].

Physiochemical analysis of collected Sea water sample

Sea water samples were analyzed for Physico-chemical characteristics as per standard methods for analysis of water and wastewater [12].

Morphological study of Endophytic Bacteria

Primarily the collected macro algae were washed with tap water and other physical particles were removed by distilled water wash. After washing surface sterilization was carried out with 70 % ethanol, $HgCl_2$ and1 % NaCl [4]. After surface sterilization macroalgae were turned into pest by mortar and pestle and 0.1 ml was inoculated in nutrient agar medium and incubated the plate for 48 hr at 25˚ to 30˚C temperature. After the growth colony was picked for pure isolation and incubate at 25˚ to 30˚C temperature for growth. Isolated bacteria were preserved on Nutrient agar slant at 4˚ C temperature.

Biochemical characterization of endophytic bacteria

Phenotypic and morphological characters were carried out for the entire identification test like grams' reaction, KOH reaction, Sodium chloride tolerance (1%, 3%, 5%, 7%, 9%, 11%, 13%, 15%, 17%, 19%, and 21%) study.

Growth pattern study

All isolated endophytic bacteria were studied for their growth pattern. Bacteria were inoculated in nutrient broth and check their growth interval of 24 hr up to 96 hr and note down the results [11].

Plant Growth Promoting Activity of endophytic Bacteria

Plant growth promoting activities were checked for all isolated endophytic bacteria by the phosphate solubilization on Pikovskaya's agar medium [39], ammonia production using peptone water in liquid media [40]. IAA production by Salkowsky's reagent method [41].

Pesticides sensitivity assay of endophytic Bacteria

Pesticides test was evaluated by Agar well diffusion method that was widely used to the plants or microbial extracts the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100mL) of the 3ppb pesticide like Alpha BHC, Gamma BHC, Beta BHC Aldrin, Dieldrin, 4 4DDT, Alpha Endosulphon, Beta Gamma chlordane, Alpha chlordane, 4 4 DDE, Endrin, 4 4 DDD, Endrinaldehyde, Endosulfun sulphate, Endrin ketone) was introduced in to the well. Then, agar plates are incubated under 37˚C tempera for 48 to 72 hr. The pesticide solution diffuses in the agar medium and sensitive inhibits the growth of the microbial strain tested [17].

Antimicrobial Potential of selected endophytic bacteria from macroalgae

This activity was carried out for selected isolates from growth pattern and plant growth promoting study. Anti bacterial, antifungal, antipesticides activities were checked in secondary metabolite activity. Antibacterial potential of selected three isolates SUN 3, SUL 24 and OTY 36 were checked against the pathogenic bacteria such as *P. aeruginosa, E. coli, S. aureus* and *B. subtilis* using overlay methods [13]. *Sclerotinum sclerotiorum and Macrophomina* was used to test the antifungal activity of three selected isolates [15]. By the disc diffusion method antibiotic study was conducted and chloramphenicol (25mcg), erythromycin (5mcg), fusidic acid (10 mcg), methicillin (10mcg), novobiocin (5mcg), streptomycin (10 mcg) tetracycline (25 mcg) and penicillin-G (1 unit) antibiotic were used for the screening of isolates [15, 29].

Cross-streak test:

To screen for antagonism test of microorganisms Cross streak method is used. The microbial strain of interest is put on single streak in the centre of the agar plate and the perpendicular streaking of tested microorganisms. After streaking plates were put for incubation, the antimicrobial interactions are analyzed by observing the inhibition zone size [16]. All three endophytic bacteria were checked for the Cross-streak test against each other on Nutrient agar plate. One loop full of all three bacterial broths were streaked on Nutrient agar plat and incubate at 25˚ to 30˚ C for 48hr [17].

Identification of isolated strain

Molecular classifications of the strains were identified by 16S rRNA gene sequencing, which had closed homology with Lysinibacillus and bacillus strain. The 16S rRNA sequence of three strain were submitted into GenBank with a unique accession number were. Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using the MEGA 6.06 software [51].

RESULTS

Macroalgae sample collection and identification

Collected macroalgae from Veraval Sea and sea coast Okha named as Sample 1 and Sample 2 respectively. By the Morphological classification of macroalgae sample1 was achieved as Ulva sp. and sample 2 achieved as Tribophyta sp.

Physiochemical analysis of collected Sea water sample

Physicochemical analysis was done of sea water in laboratory. At the time of sample collection temperature was 25˚C to30˚C for sample 1 and 25˚C to 28˚C for sample 2.The pH of sea water Sample 1 and 2 was slightly acidic to neutral and turbidity was between 6 to 7 NTU. Dissolve oxygen was around 5 to 5.6 ppm for sample 1 and 5.2 to 5.9 ppm for sample 2. Average value of Biological oxygen demand was around 60 to 65 ppm and alkalinity was 88 to 100ppm (Table 1).

Table 1: Physiochemical analysis of collected Sea water sample

NO	Physiochemical analysis	Sample 1	Sample 2
1	pH	7-8	7-8
2	Turbidity	6.1-6.9NTU	6.05-6.8 NTU
3	Temperature	25-30C	25-28C
4	EC	34-41mS	36-43mS
5	TDS	25-30	28-31
6	DO	5.0-5.6ppm	5.2-5.9ppm
7	BOD	0.5-0.60ppm	0.5-0.65ppm
8	Total Alkalinity	90-98ppm	93-100ppm

Sample1-Veraval sea water and Sample 2 –Okha sea water

Morphological study Bacterial strain

A total of 18 endophytic bacteria were isolated in the morphological study mostly bacterial colonies observed medium and big in size, round and irregular in shape, even in margin, Vesicular and smooth in surface, moist in Consistency and translucent or white in optical observation (Table 2).

Table 2: Colony characteristic of isolates

NO	Culture	Size	Shape	Margin	Elevation	Surface	Consistency	Odour	Opacity
1.	SUN 3	Medium	Irregular	Entire	Raised	Smooth	Moist	No	Opaque
2.	SUN 8	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque
3.	SUN 12	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
4.	SUK 17	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
5.	SUK 18	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque
6.	SUK 19	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
7.	SUL 24	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
8.	SUL33	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
9.	SUL 34	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque
10.	SUL 40	Medium	Round	Entire	Raised	Smooth	Moist	No	Translucent
11.	OTK 15	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
12.	OTK 16	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
13.	OTL 27	Medium	Round	Entire	Raised	Smooth	Moist	No	Translucent
14.	OTL28	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
15.	OTL 30	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
16.	OTY 34	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque
17.	OTY 35	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque
18.	OTY 36	Medium	Round	Entire	Raised	Smooth	Moist	No	Translucent

Biochemical characterization of Endophytic Bacteria

In the Phenotypic identification of isolates Grams staining, KOH reaction and NaCl concentration was checked. Isolated all 18 bacteria were Grams positive in nature and they were big in size. In microscopy they were arranged in singular, chain or cluster form. KOH test was performed and it did not adhere with the bacteria so all endophytic isolates were positive in nature (Table 3).

Table 3: Grams staining and KOH reaction

No	Culture	Gram's staining	Bacterial size	Arrangements	KOH Reaction
1.	SUN 3	Positive	Big	Singular, Chain	Negative
2.	SUN 8	Positive	Big	Singular, Chain	Negative
3.	SUN 12	Positive	Big	Singular, Cluster	Negative
4.	SUK 17	Positive	Big	Singular, Chain	Negative
5.	SUK 18	Positive	Big	Singular, Cluster	Negative
6.	SUK 19	Positive	Big	Singular, Cluster	Negative
7.	SUL 24	Positive	Big	Singular, Cluster	Negative
8.	SUL33	Positive	Big	Singular, Cluster	Negative
9.	SUL 34	Positive	Big	Singular, Chain	Negative
10.	SUL 40	Positive	Big	Singular, Cluster	Negative21
11.	OTK 15	Positive	Big	Singular, Chain	Negative
12.	OTK 16	Positive	Big	Singular, Chain	Negative
13.	OTL 27	Positive	Big	Singular, Chain	Negative
14.	OTL28	Positive	Big	Singular, Cluster	Negative
15.	OTL 30	Positive	Big	Singular, Cluster	Negative
16.	OTY 34	Positive	Big	Singular, Chain	Negative
17.	OTY 35	Positive	Big	Singular, Chain	Negative
18.	OTY 36	Positive	Big	Singular, Cluster	Negative

Sodium chloride Tolerance

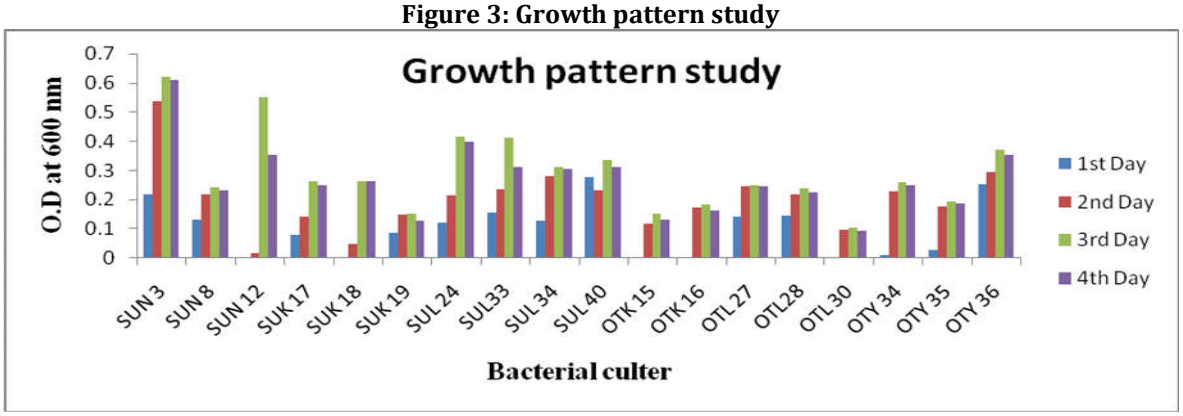
Growth as range of sodium chloride concentrations were checked on nutrient agar media and broth by adding the NaCl. Bacterial strain could grow up to 11% in Nutrient agar plate but bacterial strain could not grow 13% to 21% of NaCl concentration. Optimum growth has observed on 11% of sodium chloride

concentration when this concentration were increasing up to 21% bacterial growth were decrease and SUN 3, SUL 24 and OTY 36 were tolerate Sodium chloride concentration at higher growth rate .

Growth pattern study of endophytic bacteria

This Study was carried out up to 96hr and results were taken after 24hr of interval. Maximum bacterial growths were observed at 48 hr and 72 hr at 3% of sodium chloride concentration after that bacterial growth were decreased. Bacterial growths were study to know their multiplication time their survival time and bacterial cell devastation time (Figure 3).

Figure 3: Growth pattern study



Plant Growth Promoting activity of endophytic bacteria

Isolated all 18 endophytic bacteria were studied for the various plant growth promoting activities like phosphate solubilization, ammonia production, IAA production. Isolates were shows zone of solubilization after 48 hr of incubation and all bacteria were solubilise the maximum amount of phosphate. Ammonia production was also maximum for SUN 3, SUL 24 and OTY 36 isolates. Plant growth hormone IAA was also produced in maximum amount by the isolates SUN 3, SUL 24 and OTY 36 from macroalgae. These isolates were giving the beneficiary effect to the plant growth and other plant activities such as nutrient uptake. After all the PGPR activity SUN3 (0.236 µg/ml) give the higher phosphate solubilization (Figure 3) followed by SUL 24(0.2310.236 µg/ml) and OTY 36(0.1890.236 µg/ml). 11% 9% and 36% ammonia (Figure 4) and 1.531 µg/ml, 1.689 µg/ml and 1.896 µg/ml IAA (Figure 5) was produced by SUN 3, SUL 24 and OTY 36 respectively. These three bacteria were show maximum activity of PGPR.

Figurer 4: Phosphate Solubilization by endophytic bacteria

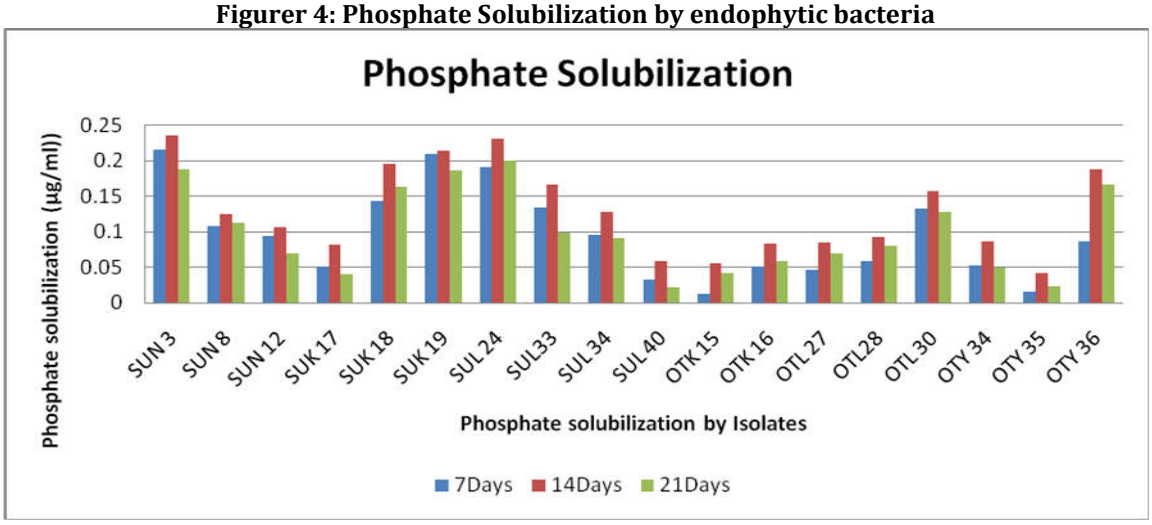


Figure 5: Ammonia Production by endophytic bacteria

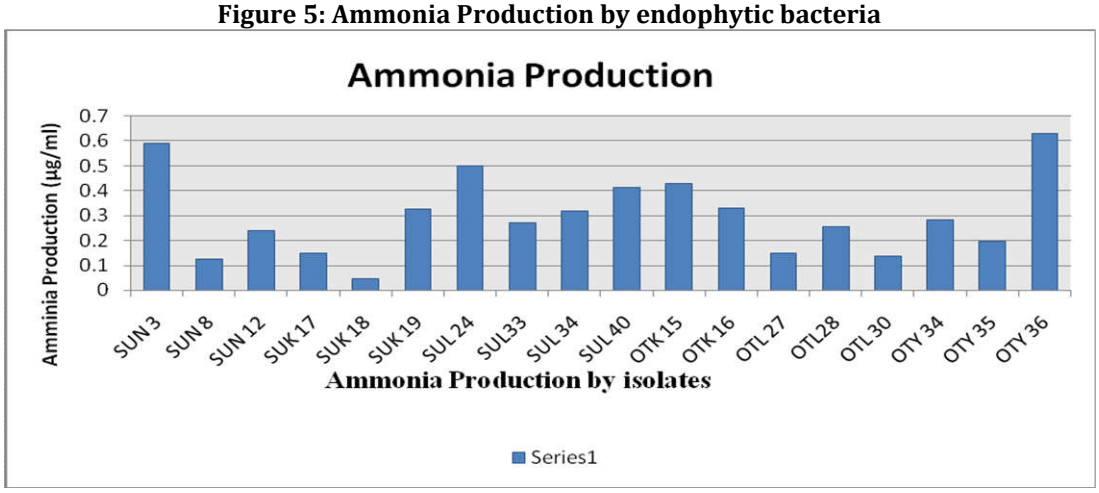
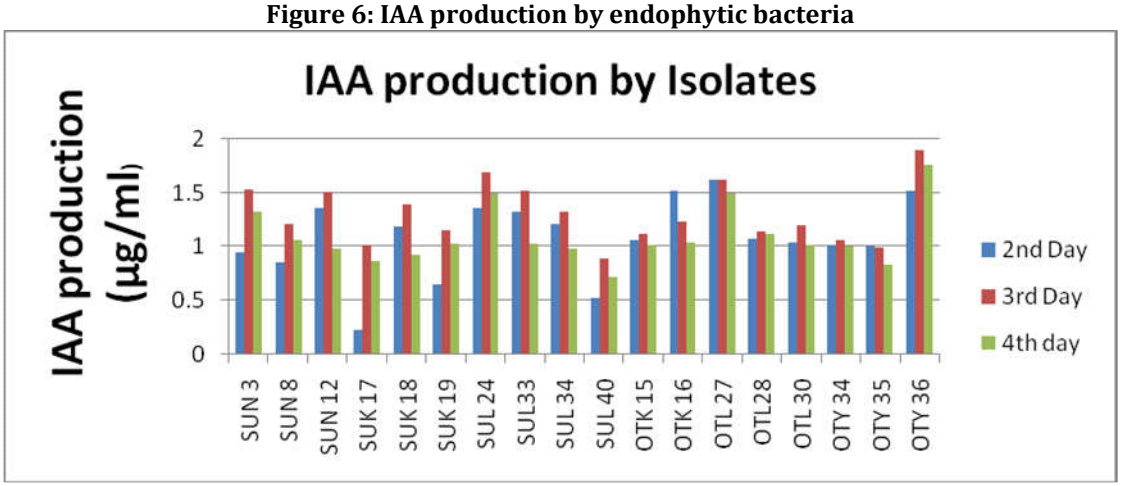


Figure 6: IAA production by endophytic bacteria



Pesticides sensitivity assay of endophytic Bacteria

Three selected bacteria from Growth pattern study and PGP activity were SUN 3, SUL 24 and OTY 36. They were tested against 3ppb concentration of pesticides such as Alpha BHC, Gamma BHC, Beta BHCAldrin,Dieldrin,4 4DDT, Alpha Endosulphon, Beta Gamma chlordane, Alpha chlordane, 4 4 DDE, Endrin, 4 4 DDD, Endrinaldehyde, Endosulfun sulphate, Endrin ketone.

Table 4: Pesticides sensitivity assay of endophytic Bacteria

No	Culture No	Alpha BHC	GammBHC	Beta BHC Aldrin	Dieldrin	4 4DDT	Alpha Endosulphon	Gammachlord	Alpha chlordane	4 4 DDE	Endrin	4,4 DDD	Endrinaldehyde	Endosulfun sulphate	Endrin ketone
1	**SUN 3**	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	**SUL 24**	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	**OTY36**	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Positive, - Negative

Antimicrobial Potential of selected endophytic bacteria from macroalgae

Antimicrobial potential was carried out with selected isolates Bactria SUN 3, SUL 24 and OTY 36. In antibacterial potential bacteria give the zone of inhibition against *E.coli, S. aureus and B.subtilis* and their zone measurements were as shown in table 5. In antibiotic study all three bacteria were sensitive against seven antibiotics like chloramphenicol (25mcg), erythromycin (5mcg), fusidic acid (10 mcg), methicillin (10mcg), novobiocin (5mcg), streptomycin (10 mcg) and tetracycline (25 mcg) and only resistant with penicillin-G (1 unit).

Table 5: Antimicrobial Potential of selected endophytic bacteria from macroalgae

No	Culture No	Anti Bacterial				Anti Fungal	
		P.aeruginosa	*E. coli*	*S. aureus*	*B.subtilis*	SS	MP
1	SUN 3	-	21 ±0.5 cm	21.67 ±0.5 cm	19.33±0.5 cm	+	+
2	SUL 24	16 ±0.5 cm	14 ±0.5 cm	-	27 ±0.5 cm	+	+
3	OTY 36	19 ±0.5 cm	18.33 ±0.5 cm	-	20 ±0.5 cm	+	+

-, negative**; +,** positive; **SS-***Sclerotinum sclerotiorum***, MP:** *Macrophomina,*

Cross-streak test of endophytic bacteria:

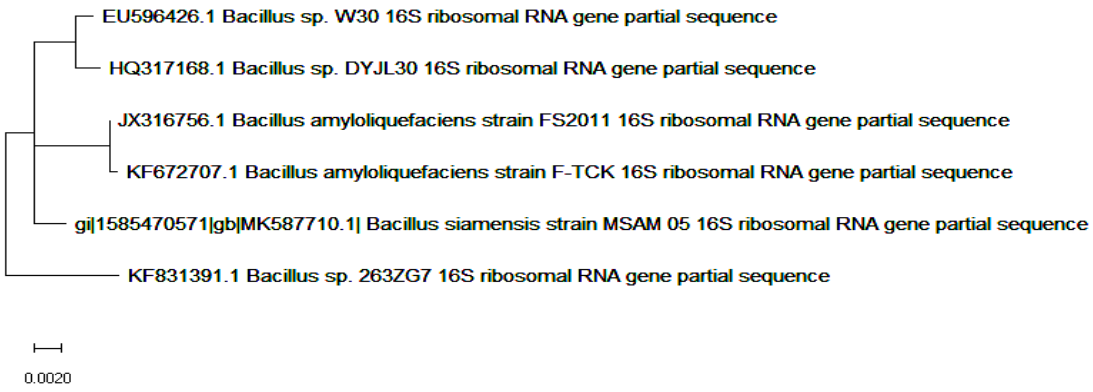
Cross-streak test between co-inoculated strains were tested. This test was reflected by the co-culture of all three strains on the same plate with no trace of growth inhibition at the centre where the three strains crossed each other. Each of the co-inoculated strains was streaked perpendicularly. The compatible combination of SUN 3, SUL 24 and OTY 36 were showed the co-existence of all strains.

Identification of isolated endophytic Bacteria

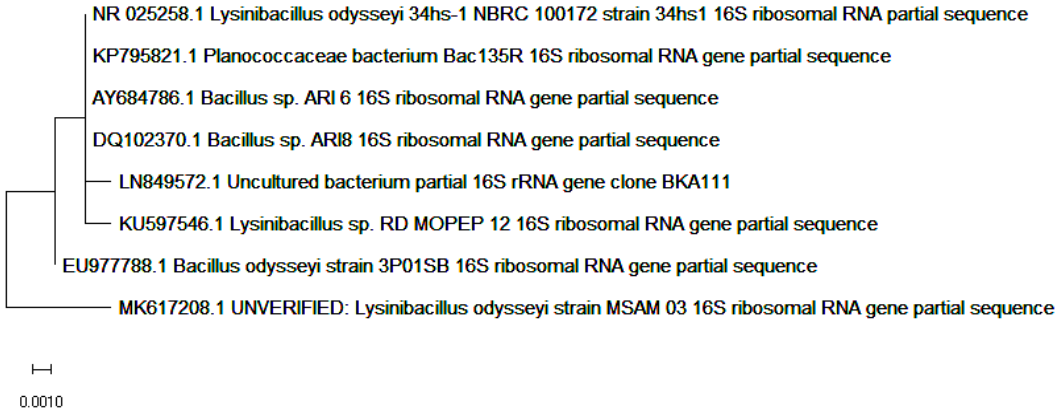
Molecular classifications of the strains were identified by 16S rRNA gene sequencing, which had closed homology with Lysinibacillus and bacillus strain. SUN 3 was identified as *Bacillus siamensis*, SUL 24 was identified as *Lysinibacillus odyssey* and **OTY** 36 was identified as *Lysinibacillus RD MOPEP*. The 16S rRNA sequence of three strain were submitted into GenBank with a unique accession number were MK587710, MK617208, and MK589719respectively. Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using the MEGA 6.06 software.

Tree Topologies of Isolated Endophytic Bacteria

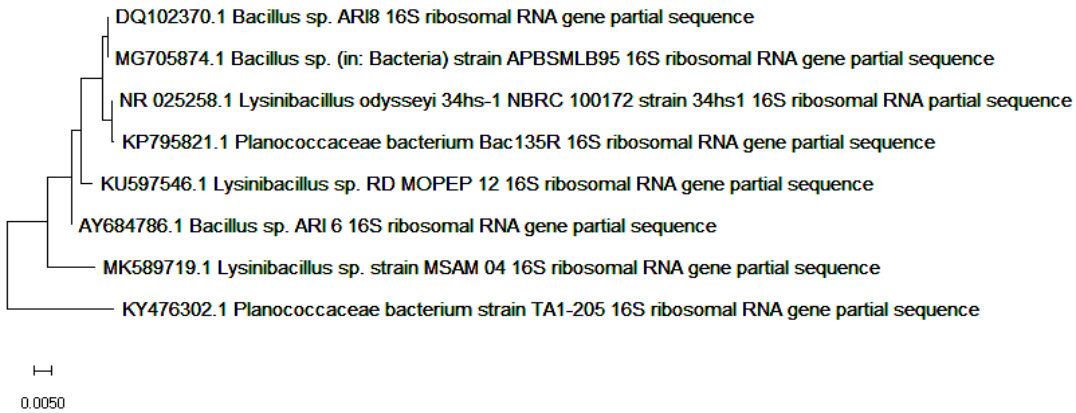
(A) SUN 3-Bacillus siamensis(MSAM 05)



(B) SUL 24-Lysinibacillus odyssey (MSAM 03)



(C) OTY 36-Lysinibacillus rdmopep(MSAM04)



DISCUSSION

In our experiments, macroalgae were collected from Veraval sea coast and Okha sea coast. Then this macroalgae were identified by their morphology. Then endophytic 18 isolate were obtained from collected macroalgae. These strains were Gram-positive, aerobic, rod shaped and spore-forming bacterium. Endophytic isolates were identified as *Bacillus* and *Lysinibacillus strains* by 16 s RNA sequencing. This endophytic isolates were give their maximum activity against antimicrobial activity.

The genus *Lysinibacillus* has 18 species [18]. Singh *et al*, [19] reported that bacteria can help the growth and other metabolism of plant or macro algae. Macroalgae associated bacteria were also isolated and studied by Armstrong [20]. Marine bacteria are important resources of new biotechnological activity [21]. Bacterial growth can start after some hour of inoculation in medium then bacterial cell can grow and multiply in number and at some stages when nutrient requirement enough to utilize then cell number is high and nutrient were utilized more so bacterial cell number also decreases. In present study bacterial growth were higher at 48 hr and 72 hr after that bacterial were go through their decline phase and decreases their cell number.

The plant and Plant Growth Promoting bacteria play a major role by promoting the growth and the health of widely diverse plants. Phosphate-solubilising bacteria (PSB) have the ability to convert insoluble to soluble forms of phosphorus for maximum plant yields [48]. Phosphate-solubilising bacteria release more available form of phosphorus and water soluble source of phosphorus [44]. The endophytes produce IAA, is the valuable characteristic that help in the influences of plant growth directly. It has been reported that many endophytes bacteria produced IAA which stimulated plant growth. For plant growth promoting attributes ammonia production is another source which has a signalling role between plant and bacterial interactions. Ammonia can be used by plants as a source of nitrogen [2]. Bacilli strains that can produce IAA that promotes the growth of different plants. Bacteria secreted relatively maximum amount of IAA [45]. It has been studied that ammonia production induces the plant growth, plant growth promotion and found the maximum Ammonia production in the isolates [46] .

Macro algae have produces good bioactive product which can also use for antimicrobial activity. Gram positive bacteria were more efficient for anti microbial activity then gram negative bacteria (Abdel et al 2017). Antibacterial activity macroalgae and its bacteria show broader and higher against pathogen [36]. Some reports say that macroalgae are efficient for bioactive compound and in antibiotics analysis they are good. Pesticides sensitive activity was also recover to be high in macroalgae isolates and extracts. Isolated microbes were able to inhibit the pathogens of plant and help to growth of plant and increase the nutrient uptake. Macroalgae was inhibiting the fungal pathogens and also inhibit the seed borne pathogens [37]. Some studies also have that macroalgae extract with solvent give good results with respect to disease control. Ethyl extract is good solvent for macroalgae extract and give higher activity against pathogen fungus [25]. Marine algae have large number of compounds like terpenes, polyphenols, phlorotannins, and polysaccharides shown several biological activities, such as anticancer activity.

CONCLUSION

Macroalgae were collected from the marine environment of Veraval and Okha endophytic bacteria were isolated. Bacteria were able to grow up to 11% of NaCl concentration. In the growth phase study most bacteria were able to show optimum growth after 24 hr of incubation and reached in decline phase after 72 hr of inoculation. Plant growth promoting activity was also study against the all isolates and SUN3,

SUL 24 and OTY 36 which solubilised the higher amount of phosphate, and shows the higher production of Ammonia. The maximum activities were observed in IAA production. From the bacterial growth pattern study and Plant growth promoting activity three bacteria were selected for antimicrobial potential SUN 3 only gave negative response against *P.aeruginosa* and positive response against *E.coli* (21,14and 19 cm followed by SUN3,SUL24 and OTY 36), *S. aureus* (21.67cm for SUN 3) *and B. subtilis* (19.33,27.0 and 20.0 cm by SUN3,SUL24 and OTY 36). SUL 24 and OTY 36 gave positive response against all pathogenic bacteria. Antifungal and pesticide activity were positive for all three isolates. All bacteria remain live while streaking against each other in Cross-streak test. All three bacteria were then identified with16 s DNA method and SUN 3 was identified as *Bacillus siamensis*, SUL 24 was identified as *Lysinibacillus odyssey* and OTY 36 was identified as *Lysinibacillus RD MOPEP*. Macroalgae isolates can also use in agricultural purpose, pesticide sensitivity and especially in saline areas they are able to tolerate up to 11% salt.

FUTURE ASPECT

Macroalgae has a divers use in the world. It can use pharmaceutical for medicines, for making cosmetic products also use as a biofertilizer purpose in agriculture. In the Future this macroalgae and its bacteria will use for agriculture to enhance the fertility of soil to increase the plant health and also it will use for remediation of metal.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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