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Isolation, Screening And Identification of Marine Bacteria for the Plant Growth Promoting Activities

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ABSTRACT

The variability of chemical composition and physical conditions in the oceans play a major role in the development of a great diversity of microbes.These distinctive characteristics have attracted many researchers to explore in depth since there is the potential of marine microorganisms used in industry. Plant growth promoting rhizobacteria (PGPR) is beneficial bacteria that colonize plant roots and enhance plant growth by wide variety of mechanism like phosphate solubilisation, etc. The present research work was designed to isolate and characterize the PGPR activity of marine bacteria. For this purpose bacterial isolates were isolated from the marine water and sediment samples and the efficient bacterial strain was screened for various parameters of plant growth promotion activities. Because of there PGP activities, bacteria were explored as bio-fertilizers to improve the soil fertility and plant growth and offers attractive way to replace chemical fertilizers, pesticides and other growth supplements.

KEYWORDS : Marine bacteria, Plant Growth Promoting Activities

INTRODUCTION

Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses. Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep-sea water. These distinctive characteristics have attracted many researchers to explore in depth since there is the potential of marine microorganisms used in industry. (K AshaDevi et al,2011) .Marine microorganisms that provided biotechnological applications in enzymes industry and pharmaceutical products and also provided an overview of the challenge faced by researchers in order to explore and exploit the marine reservoir (Debnath M et al, 2007)

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can stimulating plant growth and production and nutrition or site competition , protection of minor and major pathogen, increasing crop yields has evolved over the past several years.(Chaihan et al,2008) PGPR can affect plant growth by different direct and indirect mechanisms. Some examples of these mechanisms, which can probably be active simultaneously or sequentially at different stages of plant growth, are (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soilborne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants. By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length. The bacteria presenting one or more of these characteristics are known as plant growth promoting rhizobacteria – PGPR.(Ibiene et al, 2010)

MATERIALS AND METHOD

Isolation of marine bacteria:

Marine water and sediment samples were collected from Thiruvananthapuram coastal area (50km from the sea shore) at the depth of 100 meter with the help of local sampling facilities, during March 2015. The marine water and sediment samples were subjected to SPC method for the isolation of marine bacteria on Zobell marine agar and incubated at 30ºC for 24 hrs. After the incubation morphologically distinct bacteria from zobell marine agar medium were subjected to purity on the respective culture media.

Invitro Screening for plant growth promoting activities of the marine bacterial isolates

Phosphate solubilization assay: The solubilization of phosphate was tested using Pikovskaya agar medium. The marine bacterial isolates were spot inoculated on the Pikovskaya agar medium. All the plates were incubated for the 4 days at 28±2°C. The halo zone around the colony was measured and considered as phosphate solubilizing bacteria.(T. Karpagam et al, 2014)

Detection of Organic acid production: Bacterial isolates that produced organic acids were identified by their ability to produce change in color of methyl red pH indicator (added at a concentration of 0.03%), from yellow (pH8.0) to red (pH 5 or below) on Pikovskaya agar plates.(Brahim Bouizgarne et al 2013)

***Production of Indole acetic acid*:** Indole acetic acid produced by bacteria was determined as described by Brick et al. Bacterial cultures were grown in NB amended with tryptophan (100 µg/ml) at 30°C for 48 h on shaker (120 rpm). The cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2 ml) was mixed with two drops of *o*-phosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution). Development of pink color indicated IAA production.(Barriaso J et al, 2008)

Nitrogen fixation: Bacterial isolates were cultured on nitrogen free Jensen's media and incubated for 2 or 3 days at 30ºC. If the organism grown on the media , it indicates that the organism is capable to fix nitrogen from the atmosphere.(Rokhzadi A et al, 2008)

***Production of siderophore*:** All the marine bacterial isolates were subjected for the production of siderophore by using Chrome Azurol Sulfonate (CAS) assay. The tertiary complex Crome Azurol S (ACS)/ Fe+3 / hexadecyltrimethyl ammonium bromide served as an indicater. Spot inoculation of bacterial isolates is done on CAS agar and-incubated at 30°C for 48–72 h. Development of yellow–orange halo around the growth is considered as positive for siderophore production.(Amar Jyoti et al, 2013)

***Potassium solubilization assay*:**All the isolates were spot inoculated on to the modified Aleksandrov medium. All the plates were incubated for 3 days at 30°C. The halo zone around the colony was measured and considered as potassium solubilizing bacteria(Mshra D.J et al, 2013).

Production of ammonia: Bacterial isolates were grown in peptone water. 1% inoculum was added to 5 ml of peptone water in each

tube and incubated for 72 h at 30°C. Nessler s reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production(Radziah et al, 2014)

***Production of HCN*:** Isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck .Briefly, NA was amended with glycine (4.4 g/l) and bacteria were streaked on modified agar plates. Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid was placed at the inner surface of the lid of the Petri plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of orange to red color indicated HCN production.(Sunil T.Pawar et al, 2013)

***Zinc solubilization assay*:** All the isolates were inoculated on to the modified Pikovskaya medium containing 1% insoluble zinc compound (ZNO). All the plates were incubated for 48 h at 28°C. The halo zone around the colony was measured and considered as zinc solubilizing bacteria.(Fauziay Hafeez et al, 2006)

Identification of marine isolates:The potent isolates showing high plant growth promoting activity were identified using VITEK 2 compact-Biomerieus, France automatic system. VITEK-2 system provides an automated, computer based method of species identifications, relies on advanced colorimetry technology, the measurement of light attenuation associated with each biochemical reactions in VITEK cards containing 64 wells to ensure accurse (Table no.2).

Table No.1 Biochemical reactions in the VITEK -2 system

No.	Test	No.	Test
1	Ala-Phe-Pro-ARYLAMIDASE(APPA)	15	D-GLUCOSE(dGLU)
2	ADONITOL(ADO)	16	GAMMA-GLUTAMYL TRANSFERASE(GGT)
3	L-Pyrrolydonyl-ARYLAMIDASE(PryA)	17	FERMENTATION/GLUCOSE(OFF)
4	L-ARABITOL(IARL)	18	BETA GLUCOSIDASE(BGLU)
5	D-CELLOBIOSE(dCEL)	19	D-MALTOSE(dMAL)
6	BETA-GALACTOSIDASE(BGAL)	20	D-MANNITOL(dMAN)
7	H2S PRODUCTION(H2S)	21	D-MANNOSE(dMNE)
8	BETA-N-ACETYL-GLUCOSAMINIDASE(BNAG)	22	BETA-XYLOSIDASE(BXYL)
9	Glutamyl Arylamidase Pna(AGLTp)	23	BETA-Alanine arylamidase pNA(BAlap)
10	L-Proline ARYLAMIDASE(ProA)	24	L-LACTATEalkalinization(ILATk)
11	LIPASE(LIP)	25	ALPHA-GLUCOSIDASE(AGLU)
12	PALANTINOSE()PLE	26	PHOSPHATASE(PHOS)
13	Tyrosine ARYLAMIDASE(TyrA)	27	Glycine ARYLAMIDASE(GlyA)
14	UREASE(URE)	28	ORNITHINE DECARBOXYLASE(ODC)
29	D-SORBITOL(dSOR)	38	LYSINE DECARBOXYLASE(LDC)
30	SACCAROSE/SUCROSE(SAC)	39	DECARBOXYLASE BASE(0DEC)
31	D-TAGATOSE(dTAG)	40	L-HISTIDINE assimilation(IHISa)
32	D-TREHAE(dTRE)	41	COURMARATE(CMT)
33	CITRATE(SODIUM)(CIT)	42	BETA-GLUCURONIDASE(BGUR)
34	MALONATE(MNT)	43	O/129 RESISTANCE(comp. vibrio.)
35	5-KETO-D-GLUCONATE(5KG)	44	Glu-Gly-Arg-ARYLAMIDASE(GGAA)
36	L-MALATEassimilation(IMILTa)	45	ELLMAN(ELLM)
37	L-LACTATE assimilation(ILATa)		

RESULTS AND DISCUSSION

The marine environment is the largest habitat on Earth, representing more than 70 % of the surface of our planet. Oceans include the greatest extremes of temperature light and pressure encountered by life (Munn, 2004). Adaptation of marine to the harsh environment leads to a rich biological and genetic diversity. Marine bacteria are attracting attention as new biotechnological resources. Total 25 isolates were obtained when the sample were grown in Zobell marine agar medium. These isolates were then sub- cultured for isolation in pure culture form and they were denoted as MB1, MB2,MB3,…… ….,MB25.

Invitro Screening For The Plant Growth Promoting Activity Of Marine Bacterial Isolates

The presence of bacteria in rhizosphere is based on the concentration of nutrient available. Due to constant delivery of nutrients from plant roots, soil microbes are found to dominate the niche and their by helps in plant growth promotion under various mechanisms. PGPR are mainly used as inoculants for enhancing the growth and yield of agriculture crops, however screening for the efficient PGPR strains selection needs to be very critical(N. K Asha Devi et al, 2011). This study mainly focuses on the screening for a potential PGPR strains on the basis of direct plant growth promoting traits *viz.,* Solubilization of Phosphate, Indole acetic acid production, Biological Nitrogen fixation, Siderophore production, Potassium solubilization, Ammonia production, HCN production and Zinc solubilization. The phosphate solubilization is based on the production of low molecular weight organic acid. The production of gluconic acid, acetic acid, formic acids during the solubilization of insoluble tricalcium(Dehnath M et al, 2007). Out of 25 marine bacterial isolates 21 isolates shown the phosphate solubilization on pikovskaya's agar medium(Fig:1.A). Phytohormone IAA works as a signal molecule in the regulation of plant development. Plant system uses auxin like hormones for their optimal growth. In our study 14 isolates were produce IAA(Fig:1.C) in the presence of L-Tryptophane. Nitrogen and potassium are major essential macronutrients for plant growth and development(Farah Ahmad et al, 2008). The organisms were isolated using N free media and 20 isolates able to fix atmospheric nitrogen(Fig:1.D), it can be identified by the growth of isolates on the medium. 17 isolates were able to solubilizing insoluble potassium compound, from which stem endophytes isolates were able to solubilizing potassium on modified Aleksandrov medium. The siderophore production was performed using these different strains by using Chrome Azurol Sulfonate (CAS) assay given by Schwyn and Neilands (1987), a universal siderophore detection method.The isolates produce orange halo around the colony considered as positive for the production of siderophore(Kannahi M et al, 2014). Out of 25 marine bacterial isolates 17 were shown the siderophore production(Fig:1.E). Ability for hydrogen cyanide synthesis was observed for selected isolates of 15(Fig:1.F) ,the hydrogen cyanide have role in the defense mechanism of plant against the major and minor pathogens. The production of ammonia observed in all the 17 isolates(Fig:1.G). The ammonia is useful for plant as directly or indirectly. Ammonia production by the plant growth promoting bacteria helps influence plant growth indirectly(Deshwal et al, 2013).Zinc is on of the macro nutrient essential for the plant growth , out of the 25 marine bacterial isolates only nine isolates shown the solubilisation of insoluble ZO in the medium(Fig:1.H).

Identification of potent isolates: The most potent Plant Growth Promoting marine bacterial isolate MB5 was identified as *Acinectobacter lwoffii*(Fig.2 [a]), using vitek 2 automatic compact system. The biochemical reactions of bacterial isolate MB5 was shown in the Table No. 3.

CONCLUSSION

It can be concluded from the above discussion that Plant growth promoting rhizobacteria are increasingly used for crop improvement and protection. In the same context, present study was focused for the isolation and characterization of PGPR from marine water and marine sediment samples collected from Thiruvananthapuram costal area. Phosphate solubilization, Indole acetic acid production, Nitrogen fixation ,Siderophore production, Potassium solubilization ,Hydrogen cyanide production, Ammonia production and zinc solubilization were considered for the present study. From the screening tests marine bacterial isolate MB5 was shown high plant growth promoting activities. These isolate was identified as *Acinectobacter* lwoffii(MB5) on the basis of biochemical analysis by using the VITEK-2 system. And the isolates were consider as a good plant growth promoting bacterias and it can be further explored as potential biofertilizer for the sustainable agriculture. The results are promising for design of potentially active plant growth promoting PGPR strain based formulation which would be beneficial for crop improvement and crop protection. The potential of this strain could be investigated in detail and field application shall be studied for its bio-control potential.

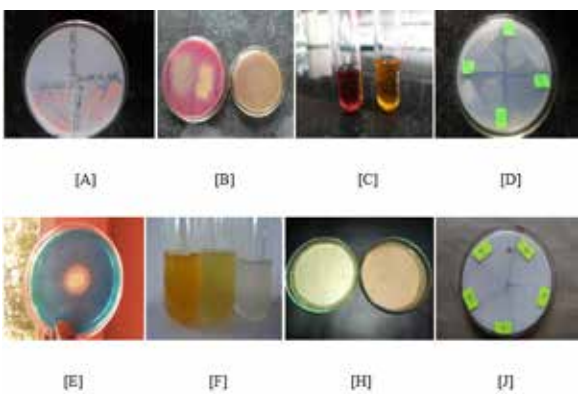

Figure No.1: [A] Phosphate solubilization(clear zone around the colony); [B] Organic acid production (left: +ve; pink colour of medium, right:control); [C] IAA Production (left: +ve; pink colour, right:-ve yellow colour); [D] Nitrogen fixation(bacterial growth on the media);[E] Siderophore production(orange halo zone around the colony); [F] Ammonia production(left: +ve, middle: -ve, right: control); [G] HCN Production(left: -ve, right: +ve); [H] Zinc solubilization(clear halo zone around the colony).

Table No.2 Plant Growth Promoting Traits of Marine Bacterial Isolates

No.	Isolates	Plant growth promoting traits							
		PO_4	IAA	N_2	Siderophore	K	NH3	HCN	ZN
1	MB1	+	+++	+++	+	+	+	-	-
2	MB2	+	+++	+++	+	+	+	+	-
3	MB3	+	-	+++	+	-	-	+	-
4	MB4	+	-	+++	+	+	+	-	-
5	MB5	+++	+	+++	+	+	+	+	+
6	MB6	-	-	+	+	-	+	+	-
7	MB7	+	++	-	+	+	-	+	+
8	MB8	+++	-	+++	+	+	+	-	-
9	MB9	+++	-	+++	-	+	-	+	-
10	MB10	+++	+++	-	-	-	+	+	+
11	MB11	-	-	-	-	-	-	-	-
12	MB12	+	++	+	+	+	+	-	+
13	MB13	-	-	-	-	-	+	+	-
14	MB14	+	++	+	+	+	+	-	-
15	MB15	+++	++	+++	+	+	+	+	-
16	MB16	+	+	+++	-	-	-	+	-
17	MB17	+	++	+++	+	+	-	+	+
18	MB18	+	-	+	+	+	+	+	-
19	MB19	+	++	-	-	-	+	-	+
20	MB20	++	++	+++	+	+	-	+	+
21	MB21	+++	+++	+	+	+	+	+	+
22	MB22 - -			+	-	+	+	+	-
23	MB23	+	-	+	+	+	+	-	-
24	MB24	+++	+	+++	+	-	-	-	-
25	MB25	+	-	+	-	+	+	-	+
TOTAL		21	14	20	17	17	17	15	9

Table No.3. Biochemical Reactions of isolate MB12- *Acinectobacter lwoffii*

2	APPA	-	21	BXYL	-	42	SUCT	-
3	ADO	-	22	BAlap	-	43	NAGA	-
4	PyrA	-	23	ProA	-	44	AGAL	-
5	IARL	-	26	LIP	-	45	PHOS	-
7	dCEL	-	27	PLE	-	46	GlyA	-
9	BGAL	-	29	TyrA	(-)	47	ODC	-
10	H2S	-	31	URE	-	48	LDC	-
11	BNAG	-	32	dSOR	-	53	IHISa	-
12	AGLTp	-	33	SAC	-	56	CMT	-
13	dGLU	-	34	dTAG	-	57	BGUR	-
14	GGT	-	35	dTRE	-	58	O129R	-
15	OFF	-	36	CIT	-	59	GGAA	-
17	BGLU	-	37	MNT	-	61	IMLTa	-
18	dMAL	-	39	5KG	-	62	ELLM	-
19	dMAN	-	40	ILATk	-	64	ILATa	-
20	dMNE	-	41	AGLU	+			

Figure No.2 Biochemically identified marine bacterial isolates- *: [a] Acinectobacter lwoffii,*

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