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Invitro Screening of Siderophore Producing Bacteria from Arabian Sea off the Coast of Thiruvananthapuram

KEYWORDS: marine bacteria, siderophore, chrome azurol sulfonate

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ABSTRACT Adaptation of marine bacteria to the harsh environment has lead to a rich biological and genetic diversity. These bacteria can be a potential sources of new bioactive compounds for industrial, environmental, medicinal, pharmaceutical and agricultural uses. The present paper reveals the qualitative and quantitative evaluation of siderophore production by marine bacteria. It was achieved by Chrome Azurol Sulfonate (CAS) assay, a universal siderophore detection method. Formation of orange halos in blue agar plates confirmed the CAS assay. Siderophore and their derivative have large application in agriculture as to increase soil fertility and used as bio-control agents against fungal pathogens. Thus the siderophore producing bacterias 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.

INTRODUCTION

Marine ecosystem represent 95% biosphere and coastal regions are particularly promising, because of the rightly adapted species found in these harsh environment. The marine environment is a rich source of both biological and chemical diversity (Nidhi et al, 2012). This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals and agrochemicals. The ocean represents virtually untapped resource from discovery of even more novel compounds with useful activity (Mousumi et ai, 2007). Siderophores are relatively low molecular weight, ferric ion specific chelating agents produced by bacteria and fungi growing under low iron stress. These compounds scavenge iron from the environment and make the mineral, available to the microbial cell. Siderophores have been related to virulence mechanisms in microorganisms pathogenic to both animals and plants. In addition, they have clinical applications and are possibly important in agriculture. Siderophores not only improve rhizosphere colonization of producer strain but also play an important role in iron nutrition of plant and antagonism against phytopathogens.

The Chrome Azurol Sulfonate (CAS) assay has become widely used since it is comprehensive, exceptionally responsive, and more convenient than microbiological assays which, although sensitive, may be rigidly specific. The CAS assay may be applied on agar surfaces . It is based on the color change that accompanies transfer of the ferric ion from its intense blue complex to the siderophore (Louden et al, 2011).

MATERIALS AND METHOD

Isolation of marine bacteria: Marine sediment samples were collected from the coastal areas of Arabian sea (50km from the sea shore) at the depth of 100 meter with the help of local sampling facilities, during March 2014. The dried samples were then subjected to standard plate count method for the isolation of marine bacteria on Zobell marine agar and incubated at 30°C for 24 hrs. After the incubation morphologically distinct bacteria were subjected to purity on the respective culture media and used for further screening purposes.

Screening of marine bacteria for the production of siderophore

Qualitative production of siderophore

All the marine bacterial isolates were subjected for the production of siderophore by using Chrome Azurol Sulfonate (CAS) assay (Payne et al, 1994). The tertiary complexChromeAzurolS(ACS)/Fe+3/hexadecyltrimethyl ammonim bromide served as an indicater. To prepare blue or green agar 60.5mg of CAS was dissolved in 50ml of distilled water and mixed with 10ml of Iron(III) solution(1Mm FeCl.6H2O in 10Mm HCl). While constantly stirring, this solution was slowly added to 72.9 mg of hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40ml of water. The resultant dark blue/green liquid was added in nutrient agar to make Chrome Azurol S (CAS). Spot inoculation of bacterial isolates was done on CAS agar and incubated at 30°C for 48–72 h. Development of yellow–orange halo around the growth was considered as positive for siderophore production(Guan et al, 2011).

Quantitative production of siderophore

The quantitative estimation of siderophore was done by CAS-shuttle assay (Yamamoto et al 1994). In which the strains are grown on succinate medium and incubated for 24-30 hrs at 28°C with constant shaking at 120 rpm on shaking

incubator separately. During incubation, in every 20 min 5 ml broth was centrifuged at 10,000 rpm at 4°C in cooling centrifuge for 10 minute and 0.5ml cell free supernatant was mixed with 0.5 ml CAS solution. The color intensity was measured using UV spectrophotometer at 630 nm with reference containing 0.5ml uninoculated succinate medium and 0.5 ml CAS solution (Schwyn et al,1987).

Identification of marine isolates

The potent isolates producing siderophores 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

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VITEK cards containing 64 wells to ensure accurse (Table no.2).

RESULTS AND DISCUSSION

Fourteen isolates were obtained when the sample were grown in Zobell marine agar medium and they were denoted as MB1, MB2,MB3,……,MB14. These isolates were then sub- cultured for isolation in pure culture form.

Qualitative production of siderophore

The isolates produce orange halo around the colony considered as positive for the production of siderophore shown in the figure -1, and the diameter of the orange halo shown in the table no.1

TABLENO.1ABOUT HERE

Isolates	CAS assay(halo formation in blue agar)	Diameter of halo(in mm)
MB1	+	10
MB2	+	8
MB3	+	8
MB4	+	9
MB5	+	8
MB6	+	12
MB7	-	-
MB8	-	-
MB9	+	9
MB10	+	10
MB11	+	8
MB12	-	-
MB13	+	12
MB14	+	8

Table:1 Study of siderophore production by marine bacteria by using CAS assay. (+) means CAS assay was positive and orange halo were formed on the blue agar plates while (-) means no halo formation, also showing the diameter of the halo.

FIGURE NO1: ABOUT HERE

Figure 1. control plate (left) and formation of orange halo zone around the bacterial isolate (right).

Out of 14 marine bacterial isolates 11 isolates shown siderophore production in the CAS blue agar medium. Results were visually distinct in terms of halo formation, because there was a contrast of orange halos against the blue medium. Out of this 11 marine bacterial isolates MB6 and MB13 shows highest diameter of orange halo.

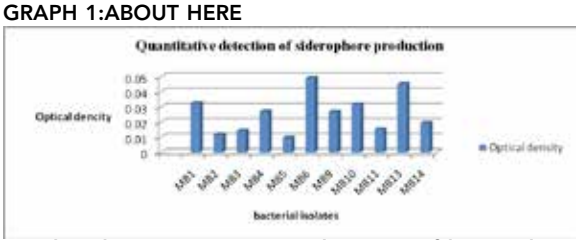
Quantitative production of siderophore

The optical density of siderophore positive bacterial isolates were shown in graph no. 1. The bacterial isolates MB6 and MB13 shows the highest rate of siderophore production compared to other bacterial isolates

Identification of potent isolates

The most potent siderophore producing marine isolates MB6 and MB13 were identified as *Stenotrophomonas maltophilia* (Fig.2 left) and *Pseudomonas alcaligenes* (Fig.2 right) respectively using vitek 2 automatic compact system. The biochemical reactions of bacterial isolates MB6 and MB13were shown in the Table No. 3 and Table No.4 respectively.

GRAPH 1:ABOUT HERE



Graph:1 showing quantitative detection of bacterial isolates

TABLE NO.2 ABOUT HERE

No.	Test	No.	Test
1	Ala-Phe-Pro-ARYLAMIDASE(APPA)	8	D-GLUCOSE(dGLU)
2	ADONITOL(ADO)	9	GAMMA-GLUTAMYL TRANSFERASE(GGT)
3	L-Pyrrolydonyl-ARYLAMIDASE(PryA)	10	FERMENTATION/ GLUCOSE(OFF)
4	L-ARABITOL(IARL)	11	BETA GLUCOSIDASE(BGLU)
5	D-CELLOBIOSE(dCEL)	12	D-MALTOSE(dMAL)
6	BETA-GALACTOSIDASE(BGAL)	13	D-MANNITOL(dMAN)
7	H2S PRODUCTION(H2S)	14	D-MANNOSE(dMNE)
15	BETA-N-ACETYL-GLUCOSAMINIDASE(BNAG)	31	BETA-XYLOSIDASE(BXYL)
16	Glutamyl Arylamidase Pna(AGLTp)	32	BETA-Alanine arylamidase pNA(BAlap)
17	L-Proline ARYLAMIDASE(ProA)	33	L-LACTATEalkalinization(ILATk)
18	LIPASE(LIP)	34	ALPHA-GLUCOSIDASE(AGLU)
19	PALANTINOSE()PLE	35	PHOSPHATASE(PHOS)
20	Tyrosine ARYLAMIDASE(TyrA)	36	Glycine ARYLAMIDASE(GlyA)
21	UREASE(URE)	37	ORNITHINE DECARBOXYLASE(ODC)
22	D-SORBITOL(dSOR)	38	LYSINE DECARBOXYLASE(LDC)

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23	SACCAROSE/ SUCROSE(SAC)	39	DECARBOXYLASE BASE(0DEC)
24	D-TAGATOSE(dTAG)	40	L-HISTIDINE assimilation(IHISa)
25	D-TREHAE(dTRE)	41	COURMARATE(CMT)
26	CITRATE(SODIUM)(CIT)	42	BETA-GLUCURONIDASE(BGUR)
27	MALONATE(MNT)	43	O/129 RESISTANCE(comp. vibrio.)
28	5-KETO-D-GLUCONATE(5KG)	44	Glu-Gly-Arg-ARYLAMIDASE(GGAA)
29	L-MALATEassimilation(IMILTa)	45	ELLMAN(ELLM)
30	L-LACTATE assimilation(ILATa)		

Biochemical reactions of Gram negative and Gram positive organisms in the Vitek -2 system

TABLENO.3. ABOUTHERE

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	+			

Table No.2 Biochemical Reactions of isolate MB6-*Stenotrophomonas maltophilia*

FIGURE NO.2: PASTE HERE

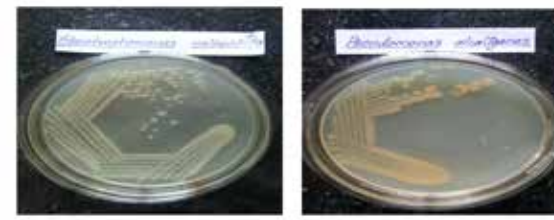

Figure 2. *Stenotrophomonas maltophilia* (left) and *Pseudomonas alcaligenes* (right)

TABLENO.4: PASTEHERE

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	-			

Table No.3 Biochemical Reactions of isolate MB6-*Pseudomonas alcaligenes*

CONCLUSION

The present study deals with the collection of marine sediment samples from Thiruvananthapuram costal areas of Arabian sea for the isolation and identification of marine bacteria and used to study the qualitative and quantitative detection of siderophore production. The present study concludes that the marine bacterial isolates producing siderophores in large amount. These siderophores bind to the available form of iron in the rhizosphere soil thus making it unavailable to the phytopathogens and protecting the plant health. Therefore it needs further studies to understand the mechanisms and characteristics of microbial siderophores. Moreover the Siderophore producing microorganisms on plant growth under field conditions is also important and necessary to be studied and explored as potential biofertilizers.

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