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Bioprospecting of Halotolerant Bacteria from the Rhizosphere of a Halophyte for the Production of Industrial Enzymes

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ABSTRACT

Halophytes are the plants that thrive in hypersaline environments such as solar salterns. Their rhizosphere may have beneficial microorganisms which are not studied adequately. A total number of 8 rhizosphere soil samples were collected during a monsoon season from Suaeda maritima from the solar salterns along Kelambakkam and Thaiyur, Tamil Nadu, India from which 73 rhizobacteria were isolated in pure culture. When they were grown on different salt concentrations, it has been found that they grow well in nutrient media having salt concentrations ranging from 0-20% of NaCl. They were further characterized for their alkaline tolerance also. In an attempt to study the bioprospecting of these HTRB, they were subjected to qualitative screening for various environmental, agricultural, medical and industrial beneficial bioactivities. They were not able to decolorize the five dyes tested. All of them were also not able to produce plant growth promotion activities such as production of IAA and solubilization of tri-calcium phosphate. Surprisingly none of the strains could exhibit any antibacterial and antifungal activity. However, they were found to produce some economically important industrial enzymes such as amylase, lipase and protease extracellularly. Few strains alone could to produce proteolytic activity event at 10% of slat concentrates. While our previous studies in which we collected samples from the similar locations during summer has yielded many beneficial organisms, the present study concludes that though the HTRB from the halophytes of Kelambakkam salterns collected during monsoon season were not showing any plant growth promoting or environmental beneficial applications. However, they were efficient in producing salt active hydrolytic enzymes. So, these HTRB can further be characterized for their extracellular enzymes which may be exploited commercially also.

Key words: *Halophytes, rhizosphere, solar salterns, rhizobacteria, industrial enzymes, salinity*

INTRODUCTION

Halotolerant or halophilic microorganisms, able to live in saline environments, offer a multitude of actual or potential applications in various fields of Biotechnology. As a result of adaptation to their environment, many extremophilic microorganisms have evolved unique properties of considerable Biotechnological and, therefore, commercial significance[12]. In recent years, halophilic microorganisms have been explored

for various Biotechnological applications in different area of fields. Our earlier studies have proved that the rhizosphere of halophytes from the salterns could be able to produce plant growth hormones, decolorize the textile dyes and also could do bioremediation of industrial wastes[9,10,11]. These studies have also concluded that the rhizobacteria of halophytes can be potentially exploited for many bioprospecting aspects. Hence, studies on bioprospecting of rhizobacteria from the halophytes of solar salterns are becoming momentum in recent years.

Rhizosphere, the soil area around the root system has been considered as the most active component for the biological and chemical characteristics in soil and hence receives considerable attention always. Various factors such as difference in root exudates, root uptake, microorganism activity and water relationship, the chemical and physical characters of the rhizosphere are significantly different from those of the bulk soil (non-rhizosphere) and hence the rhizophere region always maintains good biological activity[13]. Dominant halophytes of salterns and other hypersaline marine environments such as *Salicornia brachiata*, *Aeluropus lagopoides* and *Suaeda maritima* play significant role in carbon sequestration, nutrient mineralization, nutrient cycling and improving micro-environment. There are scanty reports on the rhizosphere microbial communities of halophytes. Whether the rhizosphere of halophytes harbors unique microbial communities or has species specificity is yet to be established[4].

Unlike other salterns studied the Tamil Nadu salterns are fed by hypersaline spring water mixed with seawater and led to the ponds from bore wells. In addition, prokaryotic community development is restricted as salterns operate only during the arid part of the year[16]. In this context, the presently reported study has been undertaken to isolate rhizobacteria from rhizosphere of a common and dominant halophyte *Suaeda maritima*. The plant could be seen in all seasons in various solar salterns along the coastal area of Tamil Nadu, India. In most of our previous studies, we have collected rhizosphere soil samples from the solar salterns during summer while the present study concentrate on the microbiology of rhizophere of *Suaeda maritima* during a monsoon season. We would like to ascertain whether the beneficial activities of rhizopabteria are prominent. This study reports the isolation of halotolerant rhizobacteria from the rhizoshere of *Suaeda maritima*, characterize them based on their cultural characteristics and study their tolerance towards salinity and alkalinity. Further this study also reports the bioprospecting of these HTRB for their ability to produce industrially important enzymes which shows activity even at high salt concentrations. Thus, this study assumes much significance in reporting salt active industrial enzymes from the HTRB.

MATERIALS AND METHODS

Collection of rhizosphere samples

A total of 8 sediment samples were collected from the rhizosphere of *Suaeda maritima* from Kelambakkam and Thaiyur salterns, Tamil Nadu, India. The samples were collected using sterile spatula and aseptically transferred into sterile polythene bags. The soil samples were processed on the same day for the isolation of rhizobacteria.

Isolation of rhizobacteria from the halophytes

Ten grams of each rhizosphere sediment sample was suspended in 90 ml of sterile distilled water blank and shaken vigorously for 2 mins. It was then serially diluted up to 10^{-6}. Then 1.0 ml of sample was taken from the dilution 10^{-4}, 10^{-5} and 10^{-6} and pour plated in sterile Halophilic Agar Medium. The halophilic agar medium containing (gm/L) Peptone - 5, Yeast extract - 3, $CaCl_2$ - 0.1, Potassium chloride 5, $MgSO_4$ - 6, NaCl- 30, Agar - 20. The Plates were incubated at 37ºC ± 2 for 7 days. After incubation period, the distinct bacterial colonies were isolated and pure cultured and were given a unique accession number with a prefix AMET followed by numbers ranging from 7001-7073 and preserved as sea water stock in eppendorf tubes at 4°C[25].

Characterizing HTRB for salt tolerance

The isolated HTRB strains were characterized for their salt tolerance, by streaking them in Halophilic Agar medium with NaCl concentrations ranging from 0 - 40% w/v. The plates were kept in room temperature for 48 hrs and the growth was observed and recorded.

Screening of halotolerant rhizobacteria (HTRB) for alkaline tolerance

All the isolated 73 HTRB were streaked on nutrient agar medium prepared in Glycine NaOH buffer (0.2 M; pH 9 & 11) and KCl NaOH buffer (0.2 M; pH 13) respectively. The plates were kept in room temperature for 48 hrs and the growth of all the 73 HTRB in these media was compared.

Screening of HTRB for environmental, agricultural and industrial applications

a. Screening for decolorization of dyes

All the isolates were screened for their potential to decolorize the dyes, in solid medium. For screening the potential to degrade, five different dyes such as Congo red, Malachite green, Methylene blue, Amido black, and Crystal violet were used. The dyes were amended in the medium at 100 µg/mL concentration in the Halophilic agar medium and sterilized. All the 73 HTRB were streaked on these media and were observed for halo-clear zone of dye degradation around the bacterial colonies after an incubation period of 3 days at room temperature.

b. *In vitro* screening of bacterial isolates for IAA production

The halotolerant strains were tested for IAA production by the method, described by Brick et al.[2]. The halotolerant strains were inoculated in sterilized Nutrient broth supplemented with tryptophan (10 µg/mL) and incubated at 37^{o}c for 3 days in shaking conditions. After incubation period, fully grown bacterial cultures were centrifuged at 10,000 rpm for 10 minutes. To the supernatant (2 ml), two drops of ortho phosphoric acid was added and incubated at room temperature for 10minutes, followed by addition of 4 ml of Salkowski reagent (50 ml, of 35% sulphuric acid, 1 ml of 0.5 M $FeCl_3$). Development of pink colour indicates the positive result for IAA production and no color change indicates the negative result for IAA production.

c. Screening for phosphate solubilization

All the isolates were screened for phosphate solubilization by streaking in Pikovskaya's agar medium as described by Chung et al.[5]. All the 73 HTRB were spot inoculated in sterile Pikovskaya's medium (Hi Media, Mumbai) and incubated for five days at room temperature. After incubation period, the plates were observed for the solubilization of phosphates. Halo zone around the colonies indicates the positive result for solubilization of phosphates by bacteria.

d. Screening for antibacterial activity

The ability of the bacterial cultures to inhibit the growth of the infectious pathogens is determined by screening for antibacterial activity. The isolates were tested for antibacterial activity by agar well diffusion assay against *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus* and *Pseudomonas aeruginosa.* All the test bacteria were grown in nutrient broth medium for two days at room temperature. The cell free culture filtrate was obtained by centrifugation at 8000 rpm. The bacterial pathogens were inoculated on NA as a lawn culture and wells were made on the agar plate. The cell free culture filtrate at 100 µl concentration was placed in each well and antibacterial as a zone of inhibition was recorded if any after two days of incubation at room temperature.

e. Screening for anti fungal activity

The antifungal activity of the halotolerant bacteria was tested *in vitro* by Dual plate assay against a phytopathogenic fungi *Rhizoctonia solani.* The fungal discs measuring 8 mm were cut out from 4 days old culture of *R. solani* on Potato Dextrose Agar (PDA) and inoculated at the center of a fresh PDA plate. The bacterial cultures are placed on the medium in the form of circular patches. Efficient antagonists were identified by their ability to produce a zone of inhibition of the pathogen after three days of incubation.

f. Screening for the production of extracellular enzymes

The Halotolerant strains were screened for the potential to produce Extracellular enzymes such as Protease, Amylase and Lipase. Nutrient agar medium and Halophilic agar medium was prepared supplemented with substrate such as Gelatin, Starch and Tween 80 for the assay of extracellular enzymes viz., protease, amylase and lipase, respectively at a concentration of 1% using a qualitative assay as described by Vijayan et al.[24].

Effect of salinity on the production of protease

Five strains that have shown maximum protease activity were selected and they were screened for their activity in different salt concentration. For screening the protease activity of the selected halotolerant bacteria, three different substrates such as Gelatin, Casein and Skimmed milk in three different concentrations were used. Nutrient agar medium with different salt concentration of 10 %, 20 %, 30 %, supplemented with three different substrates at a concentration of 0.1%, 0.5%, 1.0% was prepared. The selected halotolerant bacteria were spotted on substrate-amended media and kept incubation for two days after which subjected to visualize enzyme activity. Protease activity was visualized as a clear zone around bacterial patches and it is visualized by flooding with saturated ammonium sulfate in 0.1N HCl[24,25].

RESULTS AND DISCUSSION

Isolation of Halotolerant Rhizobacteria

Halophytes are frequently used as a source of the isolation of halotolerant rhizobacteria. Several studies were undertaken to isolate halotolerant rhizobacteria from different halophytes. Recently, the rhizospheres of *Salicornia* plants and bulk soils were collected from hypersaline ecosystems in Tunisia and a large collection of 475 halophilic and halotolerant bacteria was established from *Salicornia* rhizosphere and the surrounding bulk soil, and the bacteria were characterized for the resistance to temperature, osmotic and saline stresses, and plant growth promotion (PGP) features. It was ultimately concluded that these halophilic/halotolerant strains could be exploited in biofertilizer formulates to sustain crop production in degraded and arid lands[17]. In the present study, a total of 73 morphologically diverse bacteria were isolated from 8 rhizosphere soil samples of *Suaeda maritima* from Kelambakkam Salterns and Thaiyur, Tamil Nadu. These bacterial strains were represented by giving the code number ranging from AMET7001 to AMET 7073. These 73 bacterial cultures are selected for characterization and for analysing various beneficial bioactivities.

Characterizing the HTRB for salt and alkaline tolerance

Salinity is one major limiting factor to plant growth and crop productivity. Recent advance in soil salinity alleviation is to alleviate salt stress by inoculating crop seeds and seedlings with plant growth promoting bacteria (PGPB). Looking into the perspectives of crop production losses due to the severity of abiotic stresses, especially salinity, tolerance to stress provided by microbial inoculants becomes more important[6,20]. In the past, several studies were undertaken to isolate holotolerant and alkalotolerant rhizobacteria from various saline and alkaline environments. Haloalkaliphiles are polyextremophiles adapted to grow at high salt concentrations and alkaline pH values. El Hidri et al.[7] have isolated 122 haloalkaliphilic bacteria upon enrichments of 23 samples from 5 distinct saline systems of southern Tunisia, growing optimally in media with 10% salt and at pH 10. Liu et al.[15] have taken up a study to isolate promising halotolerant and alkalotolerant plant growth-promoting rhizobacteria and to study their effects on the growth of tall fescue and phytodegradation efficiency in a petroleum-contaminated saline-alkaline soil. A total of 115 PGPR strains were isolated from the rhizosphere of tall fescue grown in petroleum-contaminated saline-alkaline soils.

In the present study, all the 73 pure cultures of HTRB isolated from the rhizosphere of halophytes were further characterized for their salt tolerance. Surprisingly, all the strains showed growth in 0%, 10% and

20%. However, only 16 strains have grown up to 30% of NaCl. However, none of the strains grew at 40% salt concentration. An organism that grows well only at higher salt concentration *i.e.*, above 10% has been considered as halophilic. If the growth was observed in both low (0%) and high (20%) NaCl concentration, it is said to be a halotolerant. However, if a strain grows only at low NaCl concentrations below 10%, it has been considered as mesophilic organisms with no salt tolerance ability. Hence, all these 73 HTRB can be categorized as Halotolerant strains. Invariably, all the bacterial strains have exhibited luxuriant growth in alkaline medium also. Since these bacteria were isolated at near neutral pH and can tolerate a pH of 11 for their growth, they were characterized as alkaline tolerant strains. Less growth was also exhibited by all the strains in the pH of 13 excepting three strains such as AMET7069, AMET7070, AMET7072 showing no growth. Thus this experiment concludes that these strains are both alkaline and salt tolerant.

Similar to our study, other studies have also concluded that halotolerant bacteria grew well up to 20% of salinity. Eighty four halotolerant bacterial strains were isolated from the saline habitats and screened for growth at different NaCl concentrations. All grew well at 5% NaCl, but only 25% isolates showed growth at 20% NaCl concentration. Five strains SL3, SL32, SL35, J8W and PU62 growing well in 20% NaCl concentrations[20]. We have isolated halotolerant bacteria from solar salterns having alakaline tolerance also. Similar to our study, a *Halobacterium* species isolated from solar evaporation ponds and sodium sesquicarbonate deposits at Lake Magadi, Kenya, which is obligately alkalophilic with a pH optimum between 9.0 and 10.0, and having a Mg2+ requirement of between 0.1 and 2.0 mM for optimum growth.

Screening of HTRB for environmental, agricultural, medical and industrial applications

Characterization of halotolerant bacteria for agricultural applications includes many screening techniques. In another study, 140 halotolerant bacterial strains were isolated from both the soil of barren fields and the rhizosphere of six naturally growing halophytic plants in Republic of Korea. All of these strains were characterized for multiple plant growth promoting traits, such as the production of indole acetic acid (IAA), nitrogen fixation, phosphorus (P) and zinc (Zn) solubilization, thiosulfate (S_2O_3) oxidation and the production of ammonia (NH_3)[22]. In a study by Ramadoss et al.[20] eighty four halotolerant bacterial strains were isolated from the saline habitats and screened for multiple plant growth promoting traits such as indole −3- acetic acid (IAA) production, HCN and siderophore production, ACC deaminase activity and P-solubilization. From that study it was concluded that halotolerant bacteria isolated from saline environments have potential to enhance plant growth under saline stress through direct or indirect mechanisms and would be most appropriate as bioinoculants under such conditions. Thus, to characterize halotolreant bacteria for multiple biotechnological applications require several screening tests.

In the present study, all the HTRB tested were exhibited negative response when they are screened for environmental, medical and agricultural applications. None of them have produced IAA in their culture filtrates. Similarly no tested HTRB could produce halo zones after 7 days in Pikovskaya's medium which indicates that none of the strains have the ability to solubilize tri calcium phosphate. The screening for the antibacterial activity of the halotolerant bacterial strains against the pathogenic bacteria also showed that these halotolerant bacteria were not producing antibacterial substances in their culture filtrates. Similarly, none among the 73 HTRB could inhibit the plant pathogenic fungi *R. solani* in agar plates suggesting that they are also unable to produce any antifungal substances in their culture filtrates. All the isolates were screened for their potential to decolorize the dyes, in solid medium. However, none of the strains could were able to decolorize any of the dyes tested.

It is quite surprising from the present study that none of the 73 HTRB could exhibit any of the tested beneficial bioactivities such as IAA production, phosphate solubilization, dye decolorization and antibacterial and antifungal activities. This shall be due to the fact that these strains were isolated during a

monsoon season where isolates present that period might be of less beneficial. However, in our earlier studies we could able to record maximum number of beneficial bioactivities from the same sampling area but the samples were collected during summer[25,9,10].

Screening for the production of extracellular enzymes

Hydrolases constitute a class of enzymes widely distributed in nature from bacteria to higher eukaryotes. In recent years, different screening programs have been performed in saline habitats in order to isolate and characterize novel enzymatic activities with different properties to those of conventional enzymes. Several halophilic hydrolases have been described, including amylases, lipases and proteases, and then used for biotechnological applications[18]. Halotolerant bacteria form a versatile group; adapted to life at the lower range of salinities, with the possibility of rapid adjustment to changes in the external salt concentration. This property of halotolerant bacteria makes them better candidates for bio-prospecting than their halophilic counterparts. While most of the reports on halotolerant bacteria focus on molecular phylogeny, only a few reports are available on designing bioprocesses that improve growth conditions, and then positively influence the productivity of biomass, enzymes or metabolites[14,21]. 184 halophilic bacterial strains were isolated from coastal regions of Karnataka and were characterized that these isolates required atleast 10% of NaCl for their growth and were screened for the production of extracellular hydrolytic enzymes such as amylases,lipases, proteases, inulinases and β -galactosidases. Many of the isolates with combined hydrolytic activities belonged to the moderate halophilic bacteria, which were fascinating results, as moderate halophiles have potential biotechnological applications with respect to their ability to produce different variety hydrolases[8].

Hence, we have made a comparative study to screen the extracellular production of industrially important enzymes. Surprisingly, all the 73 HTRB could produce protease and lipase activity in both neutral and high salt concentrations. Protease and lipase activity was produced by all the 73 HTRB in both halophilic agar and nutrient agar added with respective substrates. A total of 51 strains produced amylase activity in halophilic agar amended with starch and a total of 64 HTRB strains could produce amylase activity in starch amended nutrient agar medium. The results of enzyme screening are quite encouraging and interesting. Almost all the strains could produce all the three enzymes. This could be due to the induced survivability adaptation in hypersaline environments where the complex nutrients are less available to these microorganisms and hence they might produce all hydrolytic enzymes to degrade complex nutrients for their growth.

While these 73 halotolerant bacteria were not producing positive results for the screening of environmental, agricultural, medical and industrial applications, surprisingly almost all of them have produced the industrial enzymes tested. The halotolerance of many enzymes derived from halophilic bacteria can be exploited wherever enzymatic transformations are required to function under physical and chemical conditions, such as in the presence of organic solvents and extremes in temperature and salt content[18].

Screening for effect of salinity on the production of protease

Enzyme-substrate interaction under the presence of high concentration of salts is of great interest for biotechnology applications and basic enzymology[19]. Hence in the present study, five halotolerant rhizobacterial strains namely AMET7009, AMET 7012, AMET 7021, AMET 7022, AMET 7036 were randomly selected to study the effect of salinity on the production of protease based on the result obtained in both medium. Three different concentrations of three substrates were used to screen the proteolytic effect in different saline concentrations. Strain AMET 7009 and AMET 7036 showed high proteolytic activity up to 10% salinity using 0.1% gelatin as substrate (Tables 1, 2 & 3). In 20% salinity, strains AMET7009, AMET7012, AMET7021 and AMET7022 have produced considerable protease activity when gelatin was used as substrate and strains AMET7021 and AMET7022 have also produced proteolytic activity when 0.5% skimmed milk was used as substrate. However, none of the strains could

produce proteolytic activity at 30% salt concentration irrespective of the substrates and their concentrates (Tables 1, 2 & 3). From this study it has been concluded that gelatin at 0.1% (w/v) concentration at10% of NaCl in the medium optimally enhance the production of halotolerant protease from the haloalkalotolreant rhizobacteria used in the present study.

Table 1. Effect of 0% salinity on the production of protease by selected strains

Strain no	Gelatin Zone of lysis (cm)			Caesin Zone of lysis (cm)			Skimmed Milk Zone of lysis (cm)		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
AMET7009	0.5	0.7	0.6	-	-	-	0.8	-	-
AMET7012	0.5	0.4	0.5	-	-	-	0.1	-	-
AMET7021	0.4	0.5	0.3	0.3	0.3	0.1	0.4	0.5	0.4
AMET7022	0.4	0.3	0.3	0.1	-	-	0.2	0.1	0.2
AMET7036	0.6	0.9	0.3	0.5	0.7	0.3	1	0.6	0.3

Table 2. Effect of 10% salinity on the production of protease by selected strains

Strain no	Gelatin Zone of lysis (cm)			Caesin Zone of lysis (cm)			Skimmed Milk Zone of lysis (cm)		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
AMET7009	0.9	0.4	0.5	0.8	0.5	0.2	-	-	-
AMET7012	0.5	0.7	0.2	0.2	0.3	-	-	0.2	-
AMET7021	0.7	0.4	0.5	0.8	0.8	0.1	-	-	-
AMET7022	0.4	0.5	0.2	0.5	0.5	-	-	-	-
AMET7036	0.7	0.5	0.2	0.3	0.3	0.3	-	0.4	-

Table 3. Effect of 20% salinity on the production of protease by selected strains

Strain no	Gelatin Zone of lysis (cm)			Caesin Zone of lysis (cm)			Skimmed Milk Zone of lysis (cm)		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
AMET7009	0.3	0.2	0.3	-	-	-	-	-	-
AMET7012	0.3	0.2	0.2	-	-	-	-	-	-
AMET7021	0.4	0.3	0.3	-	-	-	-	0.3	-
AMET7022	0.3	0.4	0.1	-	-	-	-	0.2	-
AMET7036	-	-	-	-	-	-	-	-	-

CONCLUSION

The rhizobacteia isolated from the halophyte *Suaeda maritima* from the solar salterns along Kelambakkam and Thaiyur, Tamil Nadu, India were having both halotolerant and alkaline tolerant ability. Though they could not exhibit any of the tested beneficial bioactivities such as IAA production, phosphate solubilization, dye decolorization and antibacterial and antifungal activities, they were found to be excellent producers of industrially important hydrolytic enzyemes such as protease, lipase and amylase. This study enhances the scope for exploiting the scope of producing commercially viable hydrolases from these bacteria to use them at physiologically challenged environmental conditions.

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