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RESEARCH ARTICLE

DIVERSITY IN THE CULTURAL CHARACTERISTICS OF RHIZOBACTERIA FROM COASTAL SAND DUNE PLANTS AND BIOPROSPECTING THEM FOR THE PRODUCTION OF EXTRACELLULAR ENZYMES

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ABSTRACT

Microorganisms have become increasingly important as producer of industrial enzymes and antibiotics. Due to their diversity in their biochemcial properties and growth characteristics, they are much suitable for environmental applications and genetic manipulations. Hence, attempts are being made to isolate and characterize bacteria from less explored ecosystems so as to have diverse cultural and biochemical characteristics. Coastal sand dunes are one such relatively unexplored marine ecosystem. The plants surviving in these harsh environmental systems may harbor diverse and new beneficial bacteria in their rhizosphere. Hence, in the present study, a total of 40 different bacteria were isolated from seven different rhizosphere samples of coastal sand dune plants along the East Coast of Tamil Nadu, in Bay of Bengal India. Among the samples maximum numbers of bacterial colonies were observed in the rhizosphere of *Spinifex* sp. that were collected from Kanathur, Chennai. The bacterial colonies were found to be in various shapes such as rhizoidal, irregular, and circular. Some colonies were found be mucoidal in nature. The results for the biochemical characterization showed that all the bacteria were rod shaped and most of them were gram positive. Out of 40, 35 strains produced catalase and 17 produced oxidase positive results. Majority of them were actively motile bacteria. A total of 13 strains were found to be fluorescent pseudomonads as they exhibited fluorescence in King's B Medium. The screening for their useful bioactivities showed that the bacterial strains were efficient for producing different enzymes such as the tannase, lipase, amylase and protease which can be used for industrial applications. Hence, the study is significant in finding morphologically, culturally and biochemically diverse rhizobacteria capable of producing industrially important enzymes from a less explored marine ecosystem.

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INTRODUCTION

Coastal sand dunes (CSD) are one of the least explored marine ecosystems for beneficial microorganisms. They are distributed throughout the world except in Antarctica; they serve as an ecological niche between marine and terrestrial systems (Padmavathy and Anbarashan, 2011; Jayaprakashvel *et al.,* 2010; Jayaprakashvel *et al.,* 2014a). Coastal sand dunes (CSD) are important marine ecosystems but received less attention. India has a vast coast line of a coastline of over 7000 km and this area has been concentrated on various marine and marine related researches. The coastal sand dune plants of west coast of India are studied for their diversity, biology and bioprospecting adequately. However, the coastal sand dunes of East Coast of India along the Bay of Bengal received less attention only (Jayaprakashvel *et al.,* 2014b). Bacteria are very useful microorganisms because of their fast growth rate, easy adaptability to the new environments and easy amicability with genetic modifications. Hence, bacteria from the coastal sand dune ecosystem may also harbor potential uses. Microorganisms, especially bacteria, isolated from rare or relatively unexplored ecosystems such as coastal sand dunes have always produced many beneficial bioactivities such as production of industrial enzymes and promotion of plant growth (Jayaprakashvel *et al.,* 2014 a & b; Muthezhilan *et al.,*

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2012). Plant Growth Promoting Rhizobacteria (PGPR) are beneficial bacteria that colonize the plant root and enhance the plant growth. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements (Muthezhilan *et al.,* 2012). Fewer studies have been concentrated on isolation and bioprospecting of rhizobacteria from coastal sand dune plants. From the West Coast of India along Goa, Godinho *et al.* (2010) have isolated a total of 400 rhizobacteria from coastal sand dunes and characterized for their plant growth promotion bioactivities such as phosphate solubilization, ACC deaminase activity and production of IAA, siderophores and HCN. They have selected four potential strains from the total of 400 rhizobacteria and demonstrated their plant growth promotion potentials on egg plant. Jayaprakashvel *et al.,* (2010) have isolated several rhziobacteria from the coastal sand dune plants in East Coast of India along the beaches of Chennai and demonstrated the hydrogen cyanide mediated biocontrol potential of *Pseudomonas* sp. AMET1055 isolated from the rhizosphere of coastal sand dune vegetation. Siderophore producing bacteria were isolated from a sand dune ecosystem and the effect of sodium benzoate on siderophore production by a potential isolate was characterized (Gaonkar *et al.,* 2012).

Hence, we have been routinely isolating rhizobacteria from the coastal area of East Coast in Tamil Nadu along Bay of Bengal, India. In the present study, the differential population density of rhizosphere of 3 commmon coastal sand dune plants is analyzed and rhizobacteria were also isolated in pure culture from the samples. These coastal sand dune rhizobacteria were characterized for their morphological and biochemical characteristics. Further, screening for their useful bioactivities showed that the bacterial strains were efficient for producing different industrially important extracellular enzymes such as the tannase, lipase, amylase and proteases.

MATERIALS AND METHODS

Collection of rhizosphere samples

Rhizoshpere samples of coastal sand dune plants of three commonly occurring species namely *Canavalia rosea*, *Spinifex* sp. *and Ipomoea* sp. were collected from seven different locations of Chennai coastal area for isolating Rhizobacteria. The plants were uprooted and loosely adhering soil aggregates along with roots were collected in sterile polypropylene bags and brought to lab, where the isolation of rhizobacteria was carried out on the same day. The details of sampling locations and samples are provided in Table 1.

Isolation of rhizobacteria

10 grams of each soil sample was suspended in 90 ml of sterile distilled water blank in a 250 mL conical flask and incubated at shaking condition in an environmental shaker at 150 rpm for 15 minutes at 35˚C. It was then serially diluted up to 10^{-6}. Then 0.1 ml of sample was taken from each dilution and spread plated in King's B Agar (KBA) medium (King *et al.,* 1954). The Plates were incubated at 35ºC ± 2 for 24 hours. Distinct colonies appeared within 48 hours were isolated and subcultured to purity on KBA.

Morphological diversity of the isolated rhizobacteria

The morphological nature of the bacterial colonies was characterized by observing the colonies in pure culture form on nutrient agar growth medium. Morphological diversity was recorded on the basis of visual observation of characteristics such as colony size (Tiny, small, medium, large), shape (raised, Flat, domed, irregular, rhizoid, serrated, umbonate etc), colony texture (dry, rough, mucoid, slimy etc) and pigmentation.

Biochemcial characteristics of the isolated rhizobacteria

Standard biochemical tests such as gram staining, simple staining, endospore staining, motility test, indole test, MR-VP tests, catalase and oxidase tests, citrate utilization test and fluorescene tests were done using standard protocols as suggested by Cappuccino and Sherman (2004).

Screening for the production of extracellular enzymes

The pure cultures of rhizobacteria isolated from the coastal sand dune plats were screened for the potential to produce industrially important extracellular enzymes such as lipase, protease, amylase, pectinase and tannase. Nutrient agar medium was prepared and supplemented with substrates for lytic enzymes such as tween 80, gelatin, starch, pectin and tannic acid for the assay of extracellular enzymes viz., lipase, protease, amylase, pectinase and tannase, respectively at a concentration of 1% using a qualitative assay as described by Vijayan *et al.* (2012) and Vinothini *et al.* (2014). Lipase activity was visualized as a zone of opalescence around the bacterial growth. Gelatin activity was visualized as a zone of clearance around bacterial colony when the plates were flooded with saturated ammonium sulphate prepared in 1 N HCl. Amylase activity was visualized as a zone of clearance around the bacterial plates when the plates were flooded with Grams Iodine. Pectinase activity was also visualized as a zone of clearance against a red background when plates were flooded with cango red (3% aqueous solution for 15 mins) and destained for 15 mins with 1 N NaCl.

Table 1. Sampling location details for the collection of rhizosphere soil samples from coastal sand dunes of East Coast along Bay of Bengal India

Sl. No.	Sample code	Plant species	Location along Bay of Bengal	GPS details (from Google Maps)
1	KNI	*Ipomoea pes caprae*	Kanathur, Chennai	12.850273, 80.248561
2	KPI	*Ipomoea pes caprae*	Meiyur, Kalpakkam	12.507868, 80.161652
3	SDI	*Ipomoea pes caprae*	Sadras, Kalpakkam	12.526604, 80.165998
4	TCC	*Canavalia rosea*	Tiger cave, Mahabalipuram	12.655462, 80.209993
5	KPC	*Canavalia rosea*	Meiyur, Kalpakkam	12.507868, 80.161652
6	TCS	*Spinifex* sp.	Tiger cave, Mahabalipuram	12.655462, 80.209993
7	KNS	*Spinifex* sp.	Kanathur, Chennai	12.850273, 80.248561

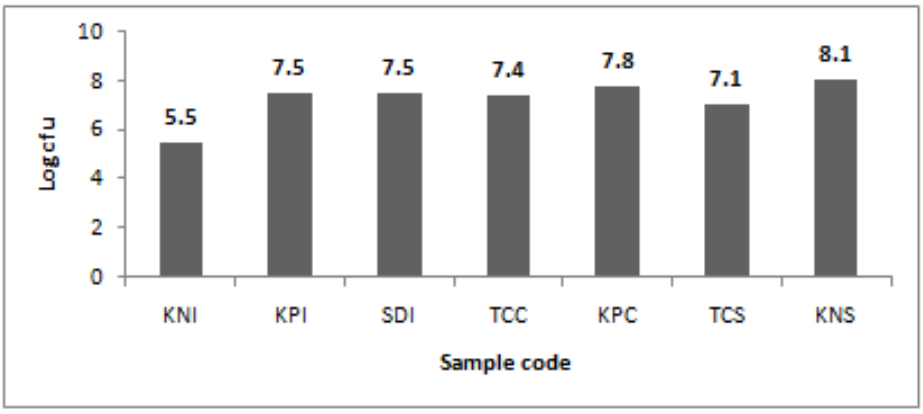

Figure 1. Bacterial population of rhizosphere of commonly occurring coastal sand dune plants along Bay of Bengal, Tamil Nadu, India

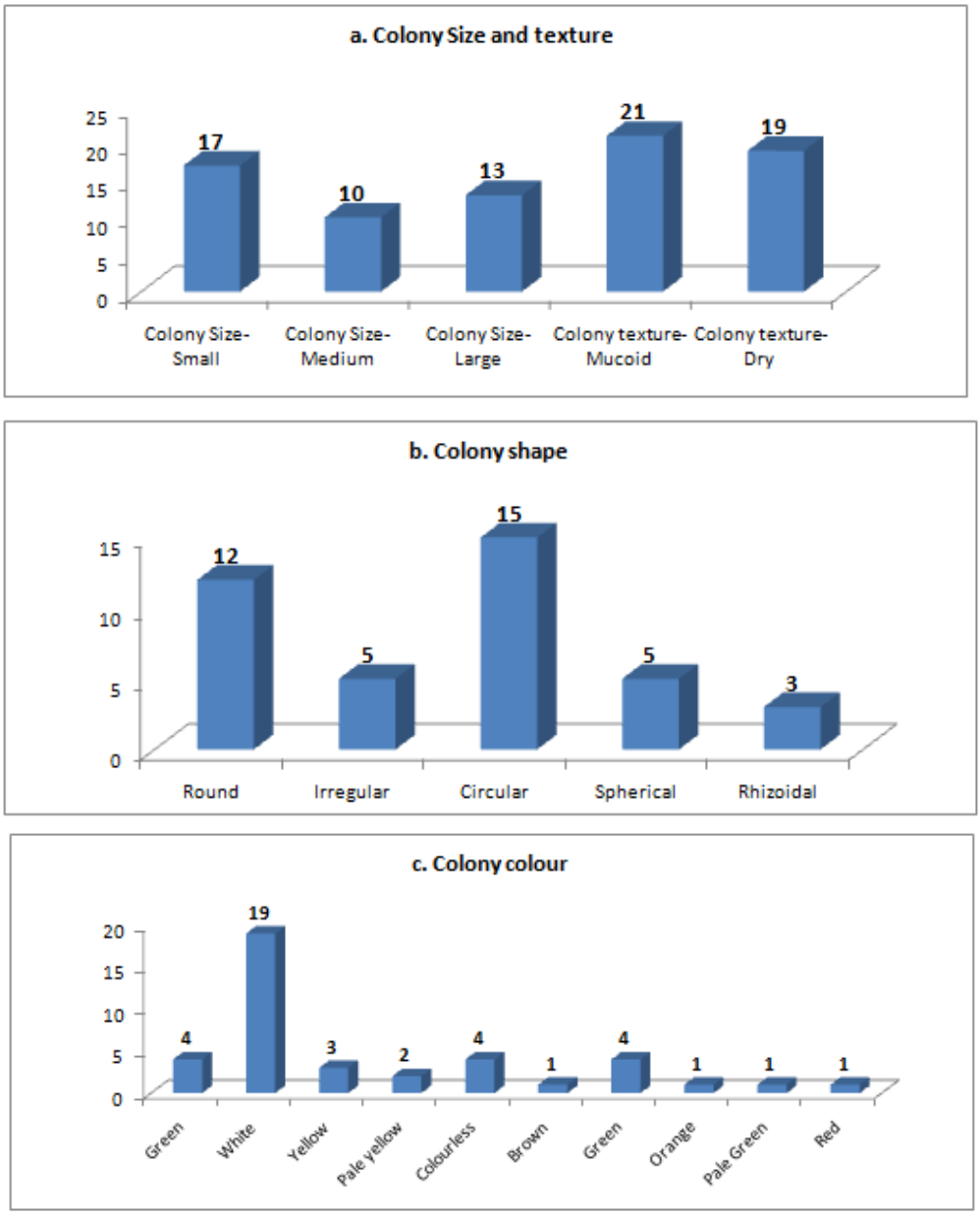

Figure 2. Morphological diversity of Rhizobacteria isolated from the coastal sand dune plants

RESULTS AND DISCUSSION

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions. Vast research across the globe has concluded that there are two main drivers of soil microbial community structure, *i.e.,* plant type and soil type, are thought to exert their function in a complex manner (Garbeva *et al.,* 2004). Coastal sand dunes are a hostile environment for forests. Little is known about the composition and diversity of the bacterial community in forest soils established on sand dunes. Hence, Lin *et al*. (2014) have studied the soil bacterial community in the upland and lowland *Casuarina* and lowland *Hibiscus* successional forests in a coastal sand dune ecosystem. In the present study, attempt has been made to estimate the bacterial population in the rhizosphere of three majorly occurring coastal sand dune plants along the coastal area of Bay of Bengal in Tamil Nadu India. Three plants viz., *Ipomoea pes caprae, Spinifex* sp. and *Canavalia rosea* were found predominant in the sampling locations (Table 1). The rhizosphere soil samples collected from the mentioned sampling locations were processed in the lab to enumerate total heterotrophic bacteria.

It has been found there has been not much difference in the total population of heterotrophic bacteria in these locations. However, comparatively the rhizosphere of *Spinifex* sp. was found to harbor more bacterial load. Among the locations, the rhizosphere of *Spinifex* sp. from Kanathur was found to have more bacterial density (8.1 log cfu). Assessments of morphological, physiological and functional attributes of bacterial communities are very important aspects for the appropriate bioprospecting and understanding. Hence, the diversity of morphological features in the isolated pure cultures of rhizobacteria from coastal sand dune plants is studied. Features such as colony size, shape texture and pigmentation were observed visually. It has been found that, majority of the bacterial strains produced small sized (17 strains), mucoidal (21 strains) and circular shaped (15 strains) colonies. And most of them have also produced white pigmentation (21 strains). The detailed results are presented in Figure 2 (a, b & c). Routine biochemical tests were done to characterize their diversity in physiological functions. It has been found that only 7 (17.5% of strains) out of 40 strains were gram positive and remaining 33 are gram negative (82.5% of strains). 35 out of 40 strains produced catalase positive (87.5% of strains) and 10 strains produced oxidase positive (47.5 % of strains). Among 40 strains, 28 were actively motile in nature (70% of strains).

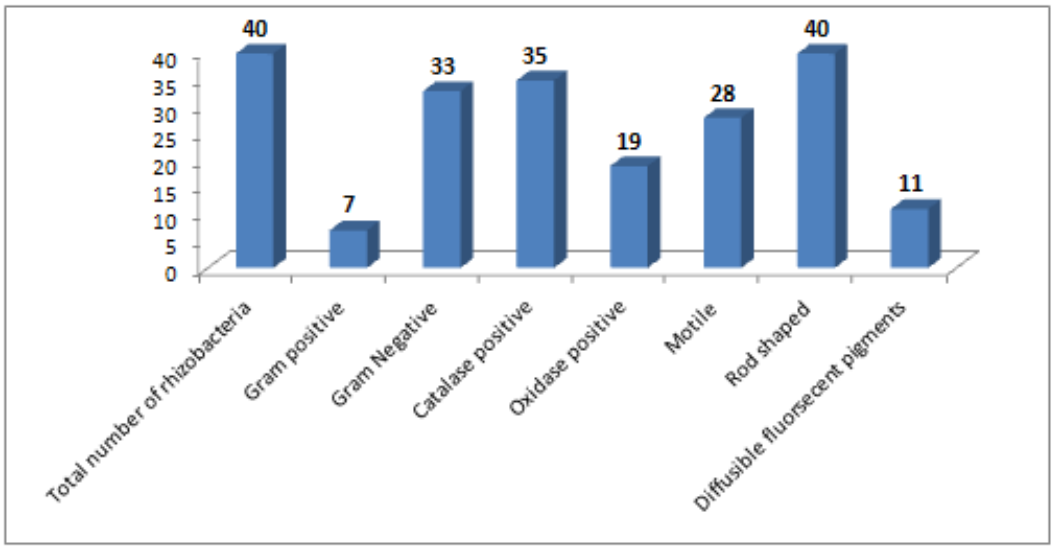

Figure 3. Diversity in biochemical characteristics of rhizobacteria isolated from the coastal sand dune plants

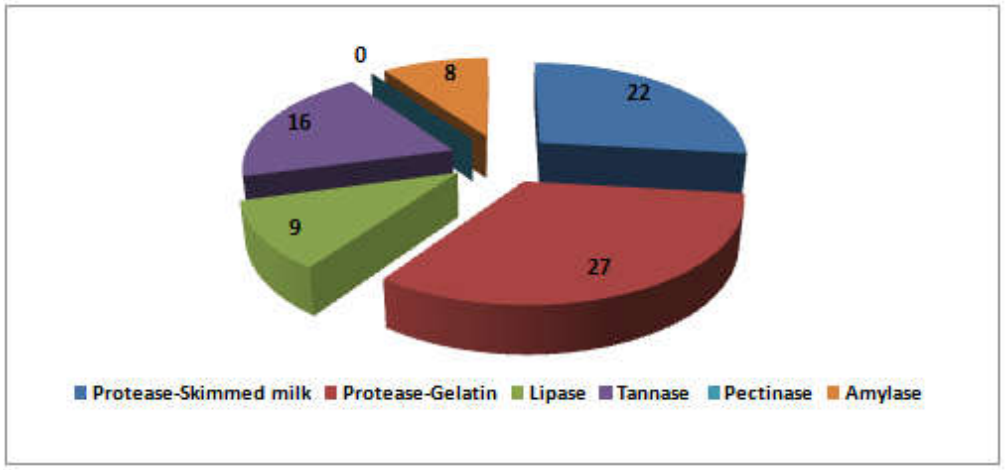

Figure 4. Summary of qualitative screening of rhizobacteria for the production of extracellular enzymes

All the strains were found to be rod in their cell shape. Only 11 strains (27.5% of the strains) were found to produce diffusible fluorescent pigments in KBA medium indicating that they are the members of fluorescent pseudomonads. Pseudomonadaceae is a very large and important family of gram-negative bacteria. They are chemoheterotrophs with versatile functions and are predominantly present in the soil. Fluorescent pseudmonads are important soil microbial communities known to be involved in the control of plant diseases through production of secondary metabolites and are able to solubilize insoluble inorganic phosphates which have made them a promising group of plant growth promoting rhizobacteria (Kloepper *et al.,* 1980; Megha *et al.,* 2007). Organisms associated with marine environments have greater potential to produce hydrolyzing enzymes such as amylase, lipase, protease, chitinase etc. and hence the bio-prospecting for these compounds is of greater importance. Various screening approaches could be developed to increase the ease with which the microbial products can be retrieved

(Jayadev and Navami, 2014). Hence, in the present study, qualitative plate assay was done to screen the rhizobacteria of coastal sand dunes for the production of extracellular enzymes. Among 40, 22 strains (55%) produced protease activity by using skimmed milk as substrate while 27 strains (67.5%) produced protease by utilizing gelatin as substrate. A total of 16 strains (40%) produced tannase activity; 9 strains (22.5%) have produced lipase activity and only 8 strains have produced amylase activity. However, none of the strains have produced pectinase activity in the present study (Figure 4). The marine realm represents 70% of the surface of the biosphere and contains a rich variety of organisms. The number of marine species used by humans is growing at unprecedented rates. Till date 18,000 natural products and 4,900 patents associated with genes of marine organisms were developed (Arrieta *et al.,* 2010). Hence, bioprospecting of marine microorganisms is gaining momentum in recent years and this study has been one of preliminary reports on the bioprospecting of marine bacteria for the production of industrially important enzymes.

Conclusion

The present study is significant in finding morphologically, culturally and biochemically diverse rhizobacteria capable of producing industrially important enzymes from a less explored marine ecosystem.

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