BIODIVERSITY OF GRAM POSITIVE BACTERIA IN MARINE WATER BODIES AND THEIR SEASONAL VARIATION

P.Swapna, A. Bindu Madhavi and Prof .V.V.Lakshmi

Abstract: The marine realm is one of the major habitats of the biosphere and covers around 70% of the earth's surface. The knowledge of the oceanic biodiversity, as a whole is limited, in spite of the advances in sampling techniques and use of insitu methods to study natural communities. The biodiversity of the Indian subcontinent is one of the richest in the world owing to its vast geographic area, varied topography and climate. There is significant variation in the spatial and temporal distribution of microbial diversity. A complete knowledge on the diversity physiology of the marine microflora is essential to enable optimum explotation of the marine environment. The diversity of gram positive bacteria was studied in the coastal environment of Andhra Pradesh. The microbial counts of gram positive organisms was high though the diversity in terms of the generae isolated was low.Among the gram positive bacilli, Bacillus and Lactobacillus sp. were predominant forms whereas Micrococcus and Planococcus were predominant among cocci. There was a two-three fold quantitative variation in the microbial counts with higher in winter as compared to summer

Index terms: Biodiversity Marine microflora, Gram positive bacteria, Seasonal variation

1 INTRODUCTION

Marine environment covering around 70% of the earth's surface has eighty to ninety percent of all life forms of earth are present only in the oceans. The microbial diversity is enormous in marine habitat. The diverse structure, and metabolism enables microorganisms to survive in this extreme environment. It has been estimated that though more than half of the earth's biomass is microbial, though not obviously conspicuous in natural environment. World renewed interest in biodiversity of microbial marine ecosystems is relatively recent[1]. The knowledge of the oceanic biodiversity, as a whole is limited, in spite of the advances in sampling techniques and use of insitu methods to study natural communities.

India is included among 12 mega biodiversity countries. The subcontinent has sea on three sides and is bestowed with a long coastline of ~ 8000 Kms. The biodiversity of the Indian subcontinent is one of the richest in the world owing to its vast geographic area, varied topography and climate. There is significant variation in the spatial and temporal distribution of microbial diversity.

A complete knowledge on the diversity physiology of the marine microflora is essential to enable optimum explotation of the marine environment. The present study reports the biodiversity and seasonal variation of gram positive bacteria studies from selected coastal areas of Andhra Pradesh studied over a period of three years.

2 PROCEDURE

Marine samples were collected from the coastal areas of Bay of Bengal around Nellore and Vishakapatnam (17 40′ N, 83 10′ E) in Andhra Pradesh. The sample collection was carried out twice a year during high tides times at the specified locations in periods between 2009-2012. The Samples were collected from depths upto 10-20km by using a specially fabricated sampling device. The slides were carefully removed from the devise and placed in sterile Artificial Sea Water (ASW) to mimic the marine environment.

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For primary isolation of bacteria marine water and scrapings samples, were inoculated onto GP/10 media. Scraping samples were also inoculated into selective liquid media and incubated at 27°C and 37°C for 3-5 days. After incubation the samples were plated by streaking as well as by dilution plating on both the selective agar plates to obtain isolated colonies. Initial identification of isolates were carried out by studying morphological characters. The colony morphology, pigment production and its diffusibility were observed and morphologically distinct colonies were selected for staining. The staining properties and bacterial morphology was studied using modified Leifson's gram staining method[2]. The cultural characters of the selected isolates were further studied by determining growth at different temperatures, tolerance to pH and salinity concentrations, oxygen requirement for growth, motility and spore production. Further, characterization of the isolates were carried out by performing various biochemical tests adopting standard protocols[3], unless otherwise specially mentioned. The procedure in all the cases was suitably modified by using GP/10 medium or supplementing the required media with 1.2% NaCl to facilitate the growth of halophilic bacteria. Known positive and negative organisms for the various biochemical tests performed were included as controls.

3 RESULTS

Most of the marine isolates being slow growers, formed discrete colonies only after incubating for 3-5 days. Morphologically distinguishable colonies were selected and were subjected to morphological characterization. Among the 35 gram positive bacilli isolated 24 isolates were able to grow between 4°C – 37°C. 6 isolates (MB 38, 54, 55, 56, 87 and 90) grew between 27°C – 45°C, 5 isolates showed growth between 27-37°C only. All the isolates tolerated upto 5% salinity and 17 isolates tolerated upto 10%. 14 of the total 35 isolates had higher salt tolerance upto 15% salinity. None of the isolates tolerated 20% salt concentration. In terms of pH all the isolates were able to grow between pH 6.5-7.5. Four isolates exhibited growth over the entire pH range tested in between 4.5-10.5. Other isolates showed varied preference of pH for growth

(Table -1). 25 isolates were facultative in nature and other 10 isolates were aerobic. Among the 35 gram positive bacilli, 10 were sporulating and the rest being non-sporulating. The same 10 tested isolates were also motile and the rest were non-motile.The cultural characters of the 57 gram positive cocci isolates showed that 15 isolates were able to grow in the temperature range of 4-45°C, 16 between 4-37°C and 13 between 27-45°C and another 13 between 27-37°C only. Of these 57 isolates, 41 were aerobic and the remaining were facultative in nature. All the isolates were non-spore formers of which 8 were motile. Initial characterizations of the 135 isolates were carried out by conducting sugar utilization tests, IMViC and other biochemical tests. Based on the morphological and biochemical characters attempts were made to categorize the isolates upto genus level. Further characterization of isolates to species level was carried out using Probabilistic Identification of Bacteria for Windows PIBWin (1.9.2 version) software designed by Bryant, 1995, in addition to comparing with Bergeys' Manual of Determinative Bacteriology Bergeys' Manual of Determinative Bacteriology[4]

ISOLATES WITH GRAM POSITIVE BACILLI MORPHOLOGY: The biochemical characters of 35 gram positive bacilli showed that 10 isolates among these (MB 33, 38, 54, 55, 56, 62, 87, 90, 130 and 131) were gram positive, aerobic, sporulating rods. These isolates were positive for catalase, and positive for H2S production. Based on the morphological and biochemical characters, these isolates could be characterized to be belong to the genus Bacillus. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc. The other 25 isolates were gram positive facultative forms which are non-motile, non-sporulating, oxidase and catalase negative rods. Based on these biochemical characters the isolates were characterized to belong to the genus Lactobacillus. Further identification considered halotolerance and utilization of sugars (Table-1).

ISOLATES WITH GRAM POSITIVE COCCI MORPHOLOGY: The biochemical characters of 57 gram positive cocci showed that 33 isolates were aerobic, non-capsulated, non-motile, non-sporulating cocci arranged in clusters or tetrads which were catalase positive and indole negative. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Micrococcus. Further, the cultural, biochemical and enzymatic characters were considered to differentiate these isolates upto species level. Another 8 isolates were observed to be motile, aerobic non-capsulated irregular clustered cocci that were catalase and oxidase positive. Based on these morphological, biochemical characters and halotolerance these isolates were categorized under the genus Planococcus. The isolates were negative for urease, indole, H2S production, VP, citrate and reduction of nitrate. The other 16 isolates were microaerophilic / facultative, non-motile cocci arranged in tetrads / pairs. The isolates were negative for catalase, oxidase, indole and positive for MR and reduction of nitrates. These morphological and biochemical characters suggest that these isolates belong to the genus Pedicoccus (Table-2).

IDENTIFICATION OF GRAM POSITIVE BACILLI- Among the 10 isolates belonging to Bacillus species characterized above, four isolates (MB 33, 62, 130 and 131) utilized glucose, fructose, mannitol, xylose, glycerol and galactose. Further these isolates, which were VP and citrate positive, were grouped with B pumulis species using PIBWin. Another four isolates (MB 38, 54, 55 and 56) were identified to belong to B. subtilis based on the utilization of sucrose, maltose, glucose, fructose, mannitol, xylose and starch. These isolates did not utilize lactose, glycerol and galactose as carbon sources and were VP, citrate, NO3 positive and oxidase, urease negative. MB 87 and 90 isolates were grouped to belong to B. firmis depending on the utilization of glucose, mannitol and starch and non-utilization of sucrose, lactose, fructose, maltose, xylose, glycerol and galactose as sole carbon source. These isolates were NO3 positive and oxidase negative. Among the 25 gram positive facultative rods identified as Lactobacillus, 12 isolates utilized glucose, fructose, maltose, starch and did not utilize other sugars. These twelve isolates were grouped under Lactobacillus halotolerance with high ID scores. The other 13 isolates were grouped under Lactobacillus brevis based on non-utilization of maltose, starch and other biochemical characters with ID score >0.999. Members of each species were further differentiated into 2 distinct phena based on numerical taxonomy parameters.

IDENTIFICATION OF GRAM POSITIVE COCCI: Among the isolates grouped under Micrococcus, 13 isolates (MB 1, 6, 7, 8, 9, 12, 16, 17, 63, 100, 102, 108 and 117) were non- pigmented that utilized glucose, sucrose, fructose, maltose, mannitol and glycerol as carbon source and produced acid. These isolates were citrate, oxidase positive and negative for urease, H2S production and nitrate reduction. Based on the characters they were grouped with M. halobius. Seven isolates (MB 18, 19, 20, 83, 93, 96 and 97) were categorized to belong to the species M.sedentarius with good reliability depending on the utilization of only glucose, sucrose, maltose and being citrate and oxidase negative. MB 31, 35, 53, 119, 121, 123 and 124 utilized glucose, sucrose, lactose, fructose, maltose and galactose as the sole carbon source. These isolates were positive for VP, citrate, urease, NO3 reduction and catalase hence were grouped as M.varians. Further 6 isolates (MB 28, 29, 30, 72, 79 and 82) were categorized to belong to the species M. luteus depending upon the utilization of only glucose, maltose and being positive for citrate, oxidase and NO3 reduction using the PIBWin package. Based on pigmentation, halotolerance and sugar utilization the isolates belonging to Planococcus were further grouped into two species. P.citreus isolates were lemon yellow pigmented which utilized glucose, fructose, maltose, mannitol, glycerol, galactose and starch whereas others grouped under P.halophilus were non-pigmented and did not utilize mannitol and starch. The further characterization of Pedicoccus isolates showed that all of them belonged to the species P.halophilus depending on similar characters of utilization of glucose, sucrose, fructose, maltose and glycerol as the carbon source and halotolerance to above 20% salt concentration. The isolates were not able to utilize xylose, lactose, galactose,

starch, mannose as carbon source.

TABLE 1: DIFFERENTIAL CHARACTERISTICS OF GRAM POSITIVE BACILLI

Test			Lb1	Lb2	Lh1	Lh2	Bp	Bs	Bf
Morphology	Microscopic observation		Rods	Rods	St.rods	St.rods	Rods	St.rods	Rods
	Colony pigmentation		-	-	-	-	-	-	-
	Spore formation		-	-	-	-	+	+	+
	Motility		-	-	-	-	+	+	+
Cultural characters	Growth(°C)	4°C	+	+	+	-	+	-	-
		27°C	+	+	+	+	+	+	+
		37°C	+	+	+	+	+	+	+
		45°C	-	-	-	-	-	+	+
	Growth at different pH	4.5	+	-	+	-	+	-	-
		5.5	+	-	+	-	+	+	-
		6.5	+	+	+	+	+	+	+
		7.5	+	+	+	+	+	+	+
		8.5	-	-	+	+	+	+	+
		9.5	-	-	+	+	+	+	-
		10.5	-	-	-	-	+	-	-
	Salt tolerance(%)	1.2%	+	+	+	+	+	+	+
		2.4%	+	+	+	+	+	+	+
		5%	+	+	+	+	+	+	+
		10%	+	+	+	+	-	+	+
		15%	-	-	+	-	-	-	+
		20%	-	-	-	-	-	-	-
	Oxygen requirement	F	+	+	+	+	-	-	-
		A	-	-	-	-	+	+	+
Biochemical tests	Indole production		-	-	-	-	-	-	-
	Methyl Red test		-	-	-	-	-	-	+
	Voges Proskaeur test		+	+	+	+	+	+	-
	Citrate		+	+	+	+	+	+	-
	Oxidase		-	-	-	-	-	-	-
	Urease		+	+	+	+	-	-	-
	H2S production		-	-	-	-	+	+	+
	NO3 reduction		-	-	-	-	-	+	+
	Catalase		-	-	-	-	+	+	+
Sugar utilization	Glucose		+	+	+	+	+	+	+
	Sucrose		-	-	-	-	-	+	-
	Lactose		-	-	-	-	-	-	-
	Fructose		+	+	+	+	+	+	-
	Maltose		-	-	+	+	-	+	-
	Mannitol		-	-	-	-	+	+	+
	Xylose		-	-	-	-	+	+	-
	Glycerol		-	-	-	-	+	-	-
	Galactose		-	-	-	-	+	-	-
	Starch		-	-	+	+	-	+	+
Production of	Caseinase		-	-	-	-	+	+	+
	Amylase		-	-	+	+	-	+	+
	Gelatinase		-	-	-	-	+	+	+

St.rods-Straight rods,
Phenon 1 Lf1 – *Lactobacillus brevis1* -(MB 14, 32, 34); Phenon 2 Lf2 - *Lactobacillus brevis2* - (MB13, 21, 24, 36, 46); Phenon 3 Lh1 – *Lactobacillus halotolerance*1 -(MB 40, 48,); Phenon 4 Lh2 - *Lactobacillus halotolerance2* - (MB 39, 41, 42); Phenon 5 Bp – *Bacillus pumilis* -(MB 33); Phenon 6 Bs – *Bacillus subtils*- (MB 38); Phenon 7 Bf – *Bacillus firmis.*

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TABLE 2: DIFFERENTIAL CHARACTERISTICS OF THE PHENA IDENTIFIED AS GRAM POSITIVE COCCI

Test		Ph1 Mh	Ph2 Ms	Ph3 Mv	Ph4 Ml	Ph5 Ph	Ph6 Plc	Ph7 Plh
Morphology	Microscopic observation	T,C	T,Tc	T,Ic	T,Ic	Tc	T	T,Ic
	Colony pigmentation	-	-	+	+	-	+	-
	Spore formation	-	-	-	-	-	-	-
	Motility	-	-	-	-	-	+	+
Cultural characters	Growth(°C)	4°C -	-	+	-	+	+	+
		27°C +	+	+	+	+	+	+
		37°C +	+	+	+	+	+	+
		45°C -	+	+	+	-	+	+
	Growth at different pH	4.5 -	-	-	-	+	+	+
		5.5 -	-	-	-	+	+	+
		6.5 +	+	+	+	+	+	+
		7.5 +	+	+	+	+	+	+
		8.5 +	+	+	+	+	-	-
		9.5 -	-	-	-	+	-	-
		10.5 -	-	-	-	-	-	-
	Salt tolerance(%)	1.2% +	+	+	+	+	+	+
		2.4% +	+	+	+	+	+	+
		5% +	+	+	+	+	+	+
		10% -	+	+	+	+	+	+
		15% -	-	+	-	+	-	-
		20% -	-	-	-	+	-	-
	Oxygen requirement	F -	-	-	-	+	-	-
		A +	+	+	+	-	+	+
Biochemical tests	Indole production	-	-	-	-	-	-	-
	Methyl Red test	-	+	-	-	+	+	+
	Voges Proskaeur test	-	-	+	+	-	-	-
	Citrate	+	-	+	+	-	-	-
	Oxidase	+	-	-	+	-	+	+
	Urease	-	-	+	-	-	-	-
	H2S production	-	-	-	-	-	-	-
	NO3 reduction	-	-	+	+	+	-	-
	Catalase	+	+	+	+	-	+	+
Sugar utilization	Glucose	+	+	+	+	+	+	+
	Sucrose	+	+	+	-	+	-	-
	Lactose	-	-	+	-	-	-	-
	Fructose	+	-	+	-	+	+	+
	Maltose	+	+	+	+	+	+	+
	Mannitol	+	-	-	-	-	+	-
	Xylose	-	-	-	-	-	-	-
	Glycerol	+	-	-	-	+	+	+
	Galactose	-	-	+	-	-	+	+
	Starch	-	-	-	-	-	+	-
Production of	Caseinase	-	-	-	-	-	-	-
	Amylase	-	-	-	-	-	+	-
	Gelatinase	+	+	+	+	+	+	+

T-Tetrads, Ic-Irregular clusters, Tc-Tetrads in cubical patches, C-Clusters Ph- Phenon,

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Phenon 1 Mh – *Micrococcus halobius*: (MB 1, 6, 7, 8, 9, 12, 16, 17); Phenon 2 Ms - *Micrococcus sedentarius* - (MB 18, 19, 20); Phenon 3 Mv – *Micrococcus varians*: (MB 31, 35,); Phenon 4 Ml - *Micrococcus luteus*-(MB 28, 29, 30,); Phenon 5 Ph – *Pedicoccus halophilus*:(MB 5, 10, 37, 44) Phenon 6 Plc – *Planococcus citreus*- (MB 50); Phenon 7 Plh – *Planococcus halophilus* (MB22,25)

GROUPING OF THE MB ISOLATES INTO PHENA: The clustering of the isolates into Phena was done using Numerical Taxonomy and Multivariant analysis system NTSYS package (version 2.0) to understand the biodiversity of the marine bacteria. 45 physiological, morphological and biochemical characters were considered for computing similarity data. The multivariate data matrix of the isolates were created using NT edit program using codes of 0 for negative and 1 for positive results and 9 for non-comparable or missing values. Similarity matrices were calculated using Simple matching coefficient (Ssm) and Jaccard's coefficient (Sj) followed by clustered phena based on Ssm/UPGMA results. Tests showing variance higher than 0.1 were deleted from the data set matrices as suggested in the Manual[5]. Sm coefficient with r values of ≥0.8 or 80% indicating high reliability where as ~ 60% was used as cut off for identification of isolates [6]. Cophenetic correlations showed the best fit and are indicated in the respective dendrograms. (Fig. - 1). The Bacillus isolates were characterized into 3 phena and the Lactobacillus were characterized into 4 phena based on the characters. The phena were designed with a Ssm value of ≥80 % for most of the isolates indicating good reliability. The Micrococcus isolates were characterized into 4 phena, Pedicoccus into one phena and Planococcus isolates into 2 phena based on all the characters considered. The Ssm coefficient for gram positive cocci was ≥60% in all the cases.

Analysis of viable counts of water samples for free living organisms showed an approximate 2-3-fold variation between the seasons. The bacterial counts were higher in winter (1.7-4.9 X106 CFU/ml) as compared to summer (0.6-1.9X106 CFU/ml) indicating that there was a significant seasonal variation in the number of bacteria in the marine environment. The trend was consistent for the samples analyzed in all the three years. The earlier studies of,[7] [8} also found that direct viable count of living bacteria in summer were between 1.5-39.8% of the total content with mean percentage of 11.2%, whereas the percentage was several folds higher in winter at Taiwan coast. Seasonal variations in marine biotype have been observed in several other studies also[8],[9},[10}. Gauthier, 1975 [11} observed that diversity both in the form and number of bacterial types was higher in the second half of the year with maximum densities reaching between September and January. It was observed that heterotrophic bacteria appeared in good part of winter and could form upto 5% of cultured microorganisms. Marine diversity was found to be higher in benthic rather than pelagic system and in coast than in open ocean [8].Similar observations of seasonal influence were also made on marine biodiversity along the coast of India[9],[12]. The diversity remained fairly high during the southwest monsoon and winter. It was shown that nutrient levels are important determinants of marine biodiversity as they influence the process of competition and community structure.

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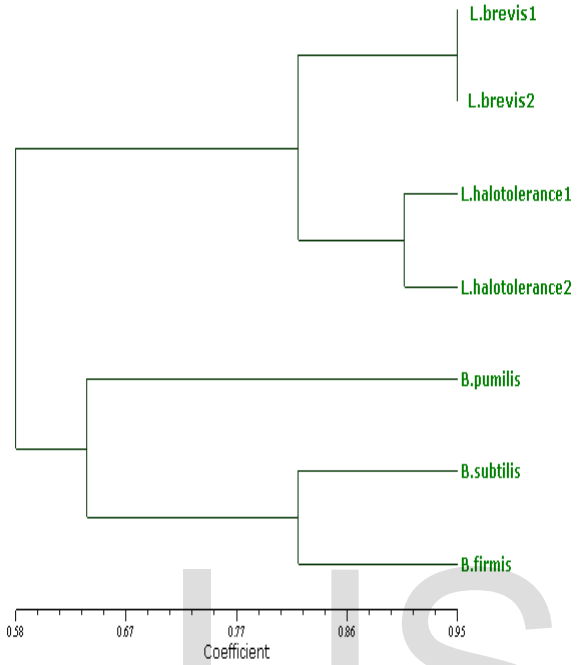
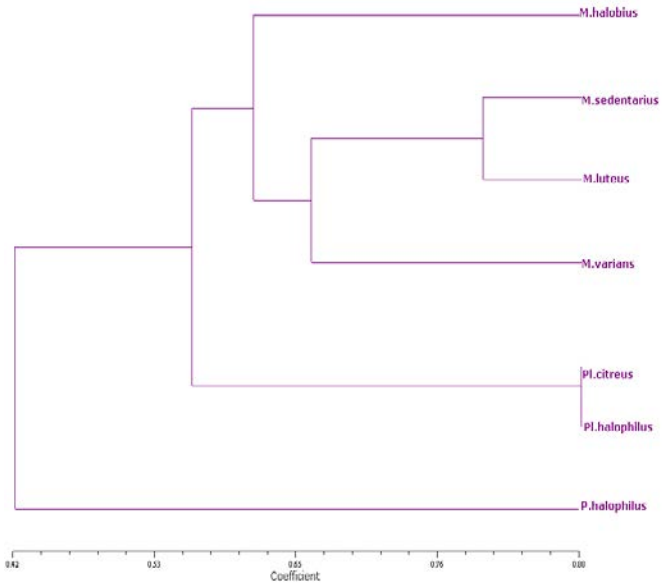


(A)



(B)

FIG.1 : DENDROGRAM OF GRAM POSITIVE(A) BACILLI AND (B) COCCI

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