

16SrRNA and enzymatic diversity of culturable bacteria from the sediments of Oxygen Minimum Zone in the Arabian Sea

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Abstract

Sediment underlying the oxygen minimum zone of the eastern Arabian Sea is rich in organic matter. Bacteria in this sediment-water interface are of great ecological importance as they are responsible in decomposing, mineralizing and subsequent recycling of organic matter. This study has for the first time addressed the phylogenetic and functional description of culturable bacteria of this region. Genotypic characterization of the isolates using amplified rDNA restriction analysis (ARDRA) followed by 16SrRNA sequencing grouped them into various phylogenetic groups like Firmicutes, Gammaproteobacteria, Low G+C Gram positive bacteria, Actinobacteria and unaffiliated bacteria. Among the enzyme activities, phosphatase was predominant (52%) and was associated with all the phylotypes followed by amylase (37%) and gelatinase (33%). These hydrolytic enzymes exhibited wide range of temperature and pH. Firmicutes expressed most of the hydrolytic activities and thus signify its role in degradation of organic matter. Multiple enzyme expression (>3) was exhibited by Actinobacteria (100%), followed by unaffiliated group (62.5%) and Firmicutes (61.5%). Besides hydrolytic enzymes, the phylotypes also elaborated functional enzymes such as nitrate reductase and catalase (58 and 81% of the isolates, respectively). In the oxygen minimum zone, the diversity was high with 28 phylotypes. Culturable bacterial assemblages encountered were *Bacillus* sp, *Halobacillus* sp, *Virgibacillus* sp, *Paenibacillus* sp, *Marinilactibacillus* sp, *Kytococcus* sp, *Micrococcus* sp, *Halomonas* sp and *Alteromonas* sp. The high diversity and high percentage of extracellular hydrolytic enzyme activities of the culturable bacteria reflect their important ecological role in biogeochemical cycling of organic matter in the oxygen minimum zone.

Keywords: Bacteria, 16SrRNA, Extracellular Hydrolytic enzymes, Oxygen minimum zone, Sediment, Arabian Sea

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Introduction

Oxygen Minimum Zones (OMZ) are unique ecosystems in the most productive coastal areas and open oceans. The high oxygen demand along with flux of labile organic matter and limited intermediate water ventilation lead to an intense, basin wide mid depth layer of oxygen depleted waters ($<0.5 \text{ ml.l}^{-1}$ dissolved oxygen concentration), which impinges on the continental slope, extending from water depths of 150 to 1500 m (Wyrski 1962; Kamykowski and Zentara 1990; Helly and Levin 2004; Cowie 2005). Naqvi et al. (2006) have shown an increase in OMZ as oceans are losing dissolved oxygen at high rates in intermediate water depths. Arabian Sea (AS) which covers 59% of oxygen minimum area of the world oceans (Helly and Levin 2004) is known for high primary productivity and vertical decrease in the dissolved oxygen concentrations in the water column from 0.5 ml l^{-1} to non-detectable levels. Sediment underlying the OMZ is rich in organic matter and a primary site of sedimentary organic carbon burial with high sedimentation rates (Hedges and Keil, 1995). The organic matter in the sediments below the OMZs is contributed by the detritus exported from the overlying water column along with considerable fluvial and aeolian inputs (Cowie, 2005). By virtue, organic particles get modified by bacterioplankton during sinking to deeper depths so these settled organic matters do not contain any of its original components. The aerobic heterotrophic bacteria are capable of producing certain hydrolytic enzymes depending on the supply and nature of organic matter (Kristensen et al. 1995). Being a zone of high organic matter, we hypothesize that the continental margin sediments underlying the OMZ harbour numerous bacteria with hydrolytic enzymes responsible for transformation of old, refractory and structurally complex organic matter that settles on the ocean floor. Very little effort has been made to understand this microbial community and its function. In this study we have made an attempt to understand the diversity and extracellular hydrolytic enzyme activities of the aerobic culturable heterotrophic bacteria inhabiting the OMZ sediment of the eastern Arabian Sea (200, 500 and 1000 m contours) along the western continental margin of India. Though 16SrRNA based techniques are widely used and provide taxonomic classification in a culture-independent approach (Woese et al. 1985; Pace et al., 1986; Amann et al., 1995) they do not elaborate their metabolic activities in biogeochemical cycles. In this study we have made an attempt to understand the diversity using 16SrRNA and extracellular hydrolytic enzyme activities of the aerobic culturable heterotrophic bacteria inhabiting the OMZ sediment of the eastern Arabian Sea (200, 500 and 1000 m contours) along the western continental margin of India.

Materials and methods

Collection of sediment samples

Sampling was carried out during an inter-disciplinary cruise of FORV Sagar Sampada (#253). The sampling stations were in the western continental margin of India (Fig. 1). Samples were collected along a transect off Goa at 200, 500 and 1000 m depth contours using a box corer. The collected sediment samples were sub-sampled using sterile syringes and stored at 4-5°C until analysis.

Isolation of bacteria

Bacteria were isolated after serial dilution of sediment samples and plating on standard nutrient medium (Hi Media, India) prepared in aged sea water. The composition of the medium was peptic digest and sodium chloride -5g each; beef and yeast extract -1.5g each and 15g of agar per litre of seawater. The plates were monitored at 24-hour interval for a period of 1 week in order to isolate even the slow growing bacteria. Morphologically different isolates (150) from the 3 depth zones were isolated and purified for genotypic and enzymatic studies.

Genotypic characterization of isolates

Genomic DNA was extracted and purified from bacterial cultures grown in nutrient broth (Hi Media, India) using standard protocol (Ausbel et al. 2003). 16SrRNA genes were amplified using the primers 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-CGTTACCTTGTTACGACTT-3') (Lane et al. 1991), using thermal cycler (Eppendorf, Germany). Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed to group the isolates into different phlotypes. 16SrRNA gene was digested using the four base-cutting restriction enzyme Hae III (1U) at 37°C for one hour. The restricted products were electrophoresed in 1.2% agarose gel and viewed on Gel Imaging System (Kodak Gel Logic). The patterns in the gels were compared using Bionumerics Software Version 4.6 (Applied Maths, Belgium). Representative phlotypes were sequenced and compared with sequences in NCBI database using BLAST software (www.ncbi.nih.nlm.gov). The sequences were submitted in the GenBank (GenBank accession numbers: GQ900674-GQ900701).

Screening of Enzymes

Enzymatic activities were determined by agar diffusion method as per the protocols given in manual of general bacteriology (Gerhardt et al. 1981). Enzyme activities were tested in nutrient agar medium of pH 7.5 in sea water amended with respective substrates [amylase - 0.2% soluble starch; gelatinase - 0.4% gelatin; nitrate reductase -0.1% potassium nitrate; citrate

synthase -0.2% sodium citrate]. The cultures that showed growth or clearing zones when tested with respective reagents were considered as positive cultures. DNase activity was determined by the growth of blue colonies on DNase test medium while phosphatase activity was determined by the development of pink colouration on phenolphthalein agar when subjected to ammonia fumes. Urease activity was detected by pink colouration of urease agar medium. Deposition of the calcium chloride-lipid complex in degradative zones detected in medium amended with 1% Tween 80 and Tween 20 showed esterase and lipase activity, respectively. Cytochrome oxidase and catalase activities were determined with N; N; N; N-tetramethyl-p-phenyl-diaminedihydrochloride and hydrogen peroxide solutions, respectively. Utilization of amino acids by the isolates was assessed by their ability to grow in 1% of ornithine, arginine, lysine and tryptophan. The isolates were also tested for enzyme activity at pH 6 and 7 and temperature 4, 20 and 28°C. Data from the 3 stations were pooled for a homogenous representation of the OMZ region.

Results and Discussion

Phylotypes distribution

The sediment-water interface is a zone of high microbial abundance and processes/fluxes occurring are of great biogeochemical importance especially in the continental margin in the AS (Walsh, 1991) creating unique sedimentary environment. Though earlier study in Pacific Ocean has reported low species richness (Levin 2003), cultivation-independent studies of the sediments in OMZs of Pacific and Atlantic oceans have shown enormous bacterial diversity (Schippers and Neretin 2006; Liu et al. 2003 a, b). Further, this technique has facilitated the detection of uncultured bacteria which at times constitute 99% of the total bacteria (Olsen 1986; Schloss and Handelsman 2004). Notwithstanding the prospective of cultivation-independent techniques to provide taxonomic identification; in this study we have adopted pure culture technique to link bacterial species with their functions. In the present study, ARDRA of the culturable bacterial isolates categorized them into various phylotypes which were then sequenced for identification. ARDRA analysis showed 28 phylotypes, affiliated to Firmicutes, Proteobacteria, Low G+C Gram positive bacteria and Actinobacteria (Table 1). Being the first report from OMZ, the results are being discussed with those obtained using different methods of direct analysis of 16SrDNA of culturable bacteria of sediments from other ecosystems such as Shimokita peninsula, Southern Okinawa trough and mangroves (Kobayashi et al. 2008; Dang et al. 2009, Dias et al. 2009). Unlike the deep sea sediments of Pacific Ocean where the dominant groups were Gammaproteobacteria and Alphaproteobacteria (Kato et al. 1996; DeLong et al. 1997; Wang et al. 2004), the OMZ sediment in the AS was dominated by Firmicutes. Interestingly, most of the sequences had high similarity (>98%) with earlier reported culturable bacterial strains of the

deep sea sediment (Dang et al. 2009). However, few sequences showed lesser similarity and these were categorized as unaffiliated bacteria and the possibility of new species endemic to this region cannot be ruled out. The genera obtained from the OMZ sediments were *Bacillus* sp, *Halobacillus* sp, *Virgibacillus* sp, *Paenibacillus* sp, *Marinilactibacillus* sp, *Kytococcus* sp, *Micrococcus* sp, *Halomonas* sp and *Alteromonas* sp (Table 1). *Bacillus* was the dominant genus in the OMZ sediment unlike in Okinawa Trough and southwestern Okhotsk Sea where *Halomonas* was the major group (Dang et al. 2009; Inagaki et al. 2003). In addition, Low G+C Gram positives and unidentified groups were also obtained. High bacterial diversity was reported for OMZ waters of the AS by culture independent method (Fuchs et al. 2005). However in the sediment, most of cultivable bacteria were restricted to Firmicutes and a few representatives of other phyla, in spite of the fact that the sediment was rich in organic matter. This may be due to the competition for the variable and limited labile substrates that reach the bottom of the sea from the overlying euphotic zone especially in the slope of the AS. The sediment carbohydrate content gives the best idea of concentration of readily assimilable organic matter which ranged from 1.33-17.99 mg g⁻¹ of dried sediment with higher concentration in the middle region of the shelf and the least at the base of the slope of the AS (Bhosle et al 1978). Occurrences of a few dominant species have been reported from different areas, in spite of different methodologies used for typing the isolates (Giuliano et al. 1999; Uphoff et al. 2001). The occurrence of a few dominant phylotypes may be habitat adaptations enabling them to survive in the OMZ environment.

Extracellular enzymatic activity and its ecological Implications

Studies have revealed that the oxic and anoxic bacteria are able to degrade organic matter at similar rates (Kristensen et al. 1995; Hall et al. 1998). However, aerobic heterotrophic bacteria are capable of producing certain hydrolytic end products of their metabolism that are effective in initiating degradative pathways of old, refractory and structurally complex organic matter (Mayer et al.1994; Hedges and Keil 1995; Kristensen et al. 1995; Hall et al. 1998) compared to anaerobic bacteria and thus play an important role in nutrient cycling. The present study covers the continental slope of the eastern AS which is an excellent site of deposition and preservation of organic carbon (>4%) (Paropkari et al. 1993). The unsuitability of the sedimentary organic matter for direct assimilation by the bacteria leads to the production of several extracellular enzymes that makes it more assimilatory (Hoppe et al 1991; Meyer-Reil et al. 1991).

Among the studied enzymes, 52% of the OMZ isolates expressed phosphatase (Fig 2a). Phosphatase is known to be prevalent in marine bacteria (Hoppe, 2003) and these bacteria have been reported in sediments of east coast of India (Ayyakkannu and Chandramohan, 1971). An increased level of phosphate concentration in the pore waters of suboxic continental margins is

due various factors such as microbial degradation of organic matter (Froelich et al., 1988; Ruttensburg and Berner 1993), desorption from iron oxides (Sundby et al., 1992; Slomberg et al., 1998) and dissolution of fish debris (Suess, 1981). It has been reported that benthic phosphorous regeneration and consequent dissolution of phosphate in the sediment-water interface are observed under oxygen-depleted conditions (Ingall and Jahnke 1994). The occurrence of DNase among the OMZ isolates provides a source of phosphorous by degrading the extracellular DNA in the sediment. Dell'Anno and Corinaldesi (2004) have demonstrated that the extracellular DNA concentration as well as the degradation of DNA in sediments was higher than that in water. Also they have suggested that preservation of DNA in deeper sediment layers may be favoured in the benthic systems with higher sedimentation rates. It is not only a source of phosphorous but also a suitable carbon and nitrogen source for prokaryotic metabolism (Jorgensen et al, 1993, Dell'Anno and Danovaro 2003). Therefore phosphatase and Dnase are the two enzymes that play an important role not only in phosphorous cycling but also in the carbon and nitrogen cycling of OMZ sediments.

Carbohydrates and proteins generally form about <90% of the biopolymeric carbon in marine sediments (Poremba, 1995; Azam 1998). In the Chilean margin, it has been reported that most of the photosynthetically produced particulate protein in the photic zone gets degraded in the upper water column up to 300m and only 2% reaches the sediment-water interface (Pantoja et al., 2004). Studies by Bhosle et al., (1989) in shallower sediments of Arabian Sea in the western continental margin, have demonstrated that the total carbohydrate in the sediments was high depending on the season. Furthermore, Alagarsamy (2003) has reported that the contribution of hydrolysable carbohydrates and aminoacids to the total organic carbon in Oman margin sediments is only 10% and the remaining being recalcitrant in nature. Proteolytic and starch hydrolyzing microflora are well documented in non -OMZ sediments of India (Nair et al. 2000, Raghukumar et al. 2006). In the OMZ sediments amylase and gelatinase were expressed by 37 and 33% of the isolates, respectively reflecting the presence of these biopolymers. These biopolymeric compounds are degraded by the benthic bacteria extracellularly and their oligomeric products become available as substrates for the microbial uptake. Studies have shown that marine heterotrophic bacteria are capable of synthesizing extracellular proteases which breakdown proteins into smaller units that can act as immediate precursors for protein synthesis and other microbial cell metabolism (Pantoja and Lee, 1994; Mudryk and Podgorska, 2006). Studies have also shown that the lipolytic bacteria are of great significance as they aid in the processes of modification and transformation of lipid compounds (Wakeham, 1995). *In situ* lipase activity experiments conducted by Boetius and Lotche (2000) in the North East Atlantic demonstrated that the lipids which are the nutritional compounds of the deep sea microorganisms, get rapidly consumed during sedimentation in the water column. However, in

the AS, bacterial and terrestrial fatty acids are enriched in the sediments within the oxygen minimum zone (Schulte et al., 2000). Thus the production of lipase enzyme by the bacterial isolates reflects the high lipase activity in the sediments (Middelboe et al, 1995). The aerobic isolates also expressed hydrolytic enzymes at wide range of pH and temperature (Table 2). Maximum amylase activity was at 28°C both at neutral (44%) and acidic (30%) conditions. Interestingly, about 21 and 48% of the isolates were able to produce amylase enzyme at wide range of temperature and pH, respectively. However, the highest DNase activity was observed with 52% positive isolates at 20°C in neutral conditions, while high acidic DNase activity was recorded at 4°C and 28°C (12% each), respectively. Other enzymes such as arginine decarboxylase, lysine decarboxylase, ornithine decarboxylase, tryptophanase (lyases) and the oxidoreductases such as catalase, oxidase and nitrate reductase were also produced by most of the OMZ isolates (Fig 2b). About 32, 33 and 32% of OMZ isolates expressed arginine, lysine and ornithine decarboxylases, respectively and 58% of the isolates were able to reduce nitrate. Nitrate is an abundant electron acceptor in the Arabian Sea (Neretin 2006). It has also been demonstrated that denitrification is the major mode of respiration in the sub-oxic zones of the Arabian Sea (Codispoti et al. 2001; Naqvi 1994). Inagaki et al. (2003) and Biddle et al. (2005) found that aerobic bacteria from the deep sea sediments of Peru margin and Okhotsk Sea formed a major part of heterotrophs and can be facultative in nature. Oxidase enzymes are generally present in bacteria which possess an aerobic type of metabolism for the regeneration of NAD in the respiratory system (Nishiyama et al. 1997). Oxidase was present in more than 50% of the OMZ isolates. Later studies on these enzymes revealed the presence of NADH oxidases belonging to peroxiredoxin oxidoreductases that are not only involved in the regeneration of NAD, but also in scavenging of peroxides (Poole et al. 2000). Thus, the anaerobic or facultative anaerobic bacteria without respiratory systems or peroxide scavenging systems are able to survive in the aerobic conditions with the help of these enzymes (Niimura et al. 1990). About 81% of the OMZ isolates were able to express catalase enzyme. Rocha and Smith (1995) have demonstrated that this enzyme helps in the detoxification of oxygen byproducts even in anaerobic conditions. Such diverse and abundant hydrolytic enzyme producers have been reported from organic and methane rich deep sub seafloor of off Shimokita Peninsula (Kobayashi et al. 2008). These enzyme activities signify the role of culturable bacteria in the degradation and mineralization of organic matter and the production and activity depend primarily on the availability, distribution, type and concentration of the organic substrates (Boetius, 1995). These complex organic matters may be relatively fresh or partially degraded organic carbon (Paropkari et al. 1992).

Among the various groups, Firmicutes was the major enzyme producing group expressing all the enzymes tested and was found to be metabolically versatile (Fig.3). Though

Actinomycetales are known to be a producer of numerous important enzymes (Nakai 1987), in the present study they were restricted to the production of only six out of the eight hydrolytic enzymes tested. Interestingly, 61.5% of Firmicutes exhibited multiple enzyme expression (>3) (Fig 4). The unidentified group also formed a major producer of all the enzymes tested and exhibited multiple expressions (62.5%) with only marginal difference with the Firmicutes. Though Actinomycetales produced only six out of the eight hydrolytic enzymes tested, highest multiple expressions of enzymes were shown by this group with all the representatives expressing 3 or more enzymes. Proteobacteria showed the highest phosphatase activity (100%) followed by unaffiliated bacteria (81%). Phosphatase enzyme production has been reported in heterotrophic bacteria falling in Proteobacteria and Firmicutes (Chrost and Overbeck, 1987). The phylogenetic group with the highest DNase activity was shown by Low G+C Gram positive group (42.8%). Amylase activity was observed in all phylogenetic groups with the highest in the Actinobacteria (100%) followed by Low G + C gram positives (57%). A similar trend was observed in gelatinase activity. Though esterase activity was observed in four phylogenetic groups lipase activity were restricted to only 3 phylogenetic groups. Interestingly, Actinobacteria formed the dominant group showing esterase and lipase activity.

The significance of metabolically active aerobic culturable bacteria in cycling of organic matter has been reiterated in our present study. The OMZ sediments of the AS harbour diverse bacteria which have enormous catabolic efficiencies. The supply of organic material to the OMZ sediment is a key factor in stimulating the production of extracellular hydrolytic enzymes. These hydrolytic enzymes are responsible for organic matter mineralization and thereby play important role *in situ* biogeochemical processes.

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References

- Alagarsamy R (2003) Organic matter composition in sediments of the Oman margin. *Chem Ecol* 19: 419–429
- Amann RI, Ludwig W, Schleifer K (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*.59:143-169.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhls K (eds) (2003) *Current protocols in Molecular Biology*. John Wiley and Sons Inc., USA
- Ayyakkannu K, Chandramohan D (1971) Occurrence and distribution of phosphate solubilizing bacteria and phosphatase in marine sediments at Port Novo. *Mar Biol* 11: 201-205
- Azam F (1998) Microbial control of oceanic carbon flux: the plot thickens. *Science* 280:694-696
- Bhosle NB, Dhargalkar VK, Braganca AM (1978). Distribution of biochemical compounds in the sediments of the shelf and slope regions of the west coast of India. *Ind J Mar Sci*. 7:155-158.
- Bhosle NB, Sawant SS, Sankaran PD, Wagh AB. 1989. Sedimentation of particulate material in stratified and non stratified water columns in the Bombay High area of the Arabian Sea. *Mar Ecol Progress Ser*,57:225-236
- Biddle JF, House CH, Brencely JE (2005) Microbial stratification in deeply buried marine sediment reflects changes in sulfate/methane profiles. *Geobiol* 3:287-295
- Boetius, A and Lochte, K. 2000. Regional variation of total microbial biomass in sediments of the deep Arabian Sea. *Deep Sea Research II*, 7: 149-168
- Boetius A (1995). Microbial hydrolytic enzyme activities in deep-sea sediments. *Helgoländer Meeresuntersuchungen* 49, 177-178.
- Codispoti LA, Brandes JA, Christensen JP, Devol AH, Naqvi SWA, Pearl HW, Yoshinari T (2001) The oceanic fixed nitrogen and nitrous oxide budgets: moving targets as we enter the anthropocene? *Sci Mar* 65:85-105
- Cowie G (2005) The biogeochemistry of the Arabian Sea surficial sediments: A review of recent studies. *Prog Oceanograph* 65: 260-289
- Dang H, Zhu H, Wang J, Li T (2009) Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. *World J Microbiol Biotech* 25:71-79
- Dell'Anno A, Corinaldesi C (2004) Degradation and Turnover of Extracellular DNA in Marine Sediments: Ecological and Methodological Considerations *Appl Environ Microbiol* 70:4384-4386
- Dell'Anno A, Danovaro R (2003) Extracellular DNA plays a key role in deep –sea ecosystem functioning. *Science* 309:2179
- DeLong EF, Franks DG, Yayanos AA (1997) Evolutionary relationships of cultivated psychrophilic and barophilic deep sea bacteria. *Appl Environ Microbiol* 63:2105-2108

- Dias ACF, Andreote FD, Dini-Andreote F, Lacava PT, Sa ALB, Melo IS, Azevedo JL, Araujo WL (2009) Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. *World J Microbiol Biotech* 25:1305-1311
- Froelich PN, Kim KH, Burnett R, Deakin WC, Hensley V, Jahnke R L, Kaul L, Kim KH, Soutar K, Vathakanon C, 1988. Early diagenesis of organic matter in Peru continental margin sediments : Phosphorite precipitation. *Mar Geol*, 80:309-343.
- Fuchs BM, Woebken D, Zubkov MV, Burkill P, Amman R (2005) Molecular Identification of picoplankton populations in contrasting waters of Arabian Sea. *Aquat Microb Ecol* 39: 145-157
- Gerhardt P, Murray RGE, Costilov RN, Nester EW, Wood WA, Kreig NR, Philips GB (1981) Manual methods for general bacteriology. American Society for Microbiology, Washington DC. pp: 409-443
- Giuliano L, De Domenico M, De Domenico E, Hofle MG, Yakimov MM (1999) Identification of Culturable Oligotrophic Bacteria within Naturally Occurring Bacterioplankton Communities of the Ligurian Sea by 16SrRNA Sequencing and Probing. *Microb Ecol* 37:77-85
- Hall POJ, Hulth S, Hulthe G, Landen A, Roos P and Tenberg A (1998) Recycling and burial of organic carbon in continental margin sediments of the Skagerrak (Northeastern North Sea). *Cont Shelf Res* 23: 1787-1809
- Hedges JL, Keil RG (1995) Sedimentary organic-matter preservation: an assessment and speculative synthesis. *Mar Chem* 49: 81–115
- Helly JJ, Levin LA (2004) Global distribution of naturally occurring marine hypoxia on continental margin. *Deep Sea Res I* 51:1159-1168
- Hoppe HG (1991) Microbial extracellular enzyme activity: a new key parameter in aquatic ecology. In: R.J. Chróst, Editor, *Microbial enzymes in aquatic environments*, Springer, New York. pp60–83.
- Hoopes HG (2003) Phosphatase activity in the sea. *Hydrobiol* 493:187-200
- Inagaki F, Suzuki M, Takai K, Oida H, Sakamoto T, Aoki T, Neilson KH, Horikoshi K (2003) Microbial communities associated with geological horizons in coastal seafloor sediments from the sea of Okhotsk. *Appl Environ Microbiol* 69:7224-7235
- Ingall E, Jahnke R (1994) Evidence for enhanced phosphorus regeneration from marine sediments overlain by oxygen depleted waters. *Geochim Cosmochim Acta* 58:2571–2575
- Jorgensen NOG, Kroer N, Coffin RB, Lee C (1993) Dissolved free amino acids, combined amino acids, and DNA as source of carbon and nitrogen to marine bacteria. *Mar Ecol Progress Ser* 98: 135-148
- Kato C, Inoue A, Horikoshi K (1996) Isolating and characterizing deep-sea marine microorganisms. *Trends Biotechnol* 14:6-12
- Kamykowski DZ, Zentara SJ (1990) Hypoxia in the world ocean as recorded in the historical data set. *Deep Sea Res* 37:1861-1874
- Kobayashi T, Koide O, Mori K, Shimamura S, Matsuura T, Miura T, Takaki Y, Morono Y, Nunoura T, Imachi H, Inagaki F, Takai K, Horikoshi K (2008) Phylogenetic and enzymatic

- diversity of deep seafloor aerobic microorganisms in organics and methane rich sediments off Shimokita Peninsula. *Extremophiles* 12:519-527
- Kristensen E (1995) Aerobic and anaerobic decomposition of organic matter in marine sediment: Which is fastest? *Limnol Oceanograph* 40:1430-1437
- Lane DJ (1991) 16S/23S rRNA sequencing in nucleic acid techniques. *Bacterial Systematics* (Stackebrandt E & Goodfellow M eds) John Wiley and sons, New York. pp 115-148
- Levin LA (2003) Oxygen Minimum Zone Benthos: Adaptation and Community Response to Hypoxia. *Oceanography and Marine Biology: An Annual Review* 41: 1-45
- Liu X, Bagwell CE, Wu L, Devol AH, Zhou J (2003a) Molecular diversity of sulfate reducing bacteria from two different continental margin habitats. *Appl Environ Microbiol* 69:6073-6081
- Liu X, Tiquia SM, Holguin G, Wu L, Nold SC, Devol AH, Luo K, Palumbo AV, Tiedje JM, Zhou J (2003b) Molecular diversity of denitrifying genes in continental margin sediments within the oxygen deficient zone off the Pacific coast of Mexico. *Appl Environ Microbiol* 69: 3549-3560
- Mayer LM (1994) Surface area control of organic carbon accumulation in the continental shelf sediments. *Geochim. Cosmochim Acta* 58:1271-1284
- Meyer –Reil LA (1991) Ecological aspects of enzymatic activity in marine sediments. In RJ Chrost (Eds) *Microbial enzymes in aquatic environments*. Springer Verlag, New York, 84-95.
- Middelboe M, Borch NH, Kirchman DL (1995) Bacterial utilization of dissolved free amino acids, dissolved combined amino acids and ammonium in the Delaware Bay estuary: effects of carbon and nitrogen limitation. *Mar Ecol Prog Ser* 128:109–120
- Mudryk ZJ, Podgorska B (2006) Enzymatic activity of bacterial strains isolated from marine beach sediments. *Pol J Environ St.* 15: 441-448.
- Nair S, Mohandas C, Loka Barathi PA, Sheelu G., Raghukumar C (2000) Microscale response of sediment variables to benthic disturbance in the Central Indian Ocean Basin. *Mar Geores Geotechnol* 18:273-283
- Naqvi SWA (1994) Denitrification processes in the Arabian Sea. In: Lal, D. (Ed.) *Biogeochemistry of the Arabian Sea* Indian Academy of Sciences, Bangalore, India, pp. 181–202
- Naqvi SWA, Naik H, Jayakumar DA, Shailaja MS, Narvekar PV (2006) Seasonal oxygen deficiency over the western continental shelf of India. In: Neretin (Ed.), *Past and present water column anoxia*. In Springer, pp. 195–224
- Niimura Y, Yanagida F, Suzuki K, Komagata K, Kozaki M (1990) *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone, and catalase. *Int J Syst Bacteriol* 40:297–301
- Nishiyama Y, Massey V, Anzai Y, Watanabe T, Miyaji T, Uchimura T, Calzi ML (2000) AhpF and other NADH : peroxiredoxin oxidoreductases of *Sporolactobacillus inulinus* NADH oxidase and its physiological role in aerobic metabolism of the bacterium. *J Ferment Bioeng* 84:22–27
- Olsen GJ, Lane DL, Giovannoni SJ, Pace NR (1986) Microbial ecology and evolution: a ribosomal RNA approach. *Ann Rev Microbiol* 40: 337-365

- Pace NR, Stahl DA, Lane DJ, Olsen GJ (1985) Analysing natural microbial populations by rRNA sequences. *ASM News*, 51: 4-12
- Pantoja S, Lee C (1994) Cell surface oxidation of amino acids in sea water. *Limnology and Oceanography*. 39:1718-1725
- Pantoja S, Sepulveda J, Gonzalez HE (2004) Decomposition of sinking proteinaceous material during the fall in the oxygen minimum zone off northern Chile. *Deep Sea Res Part I* 51: 55-70
- Paropkari AL, Babu CP, Mascareahas A (1992) A critical evaluation of depositional parameters controlling the variability of organic carbon in the Arabian Sea sediments. *Mar Geol* 107:213-226
- Poole LB, Reynolds CM, Wood ZA, Karplus PA, Ellis HR, Calzi ML (2000) AhpF and other NADH: peroxiredoxin oxidoreductases, homologues of low Mrthioredoxin reductase. *Eur J Biochem* 267:6126–6133
- Poremba K (1995) Hydrolytic enzymatic activity in deep sea sediments. *FEMS Microbiol Ecol* 16: 213-222
- Raghukumar C, Nath NB, Sharma R, Bharathi PAL, Dalal SG (2006) Long term changes in microbial and biochemical parameters in the Central Indian Basin. *Deep Sea Res I*, 53: 1695-1717
- Rocha ER and Smith CJ (1997) Regulation of *Bacteriodes fragilis* katB mRNA by oxidative stress and carbon limitations. *J Bacteriol* 179:7033-7039
- Rottenberg KC and Berner R ,1993. Authigenic apatite formation and burial in sediments from non-upwelling, continental margin environments. *Geochim Cosmochim Acta*, 57, 991-1007
- Schippers A, Neretin LN (2006) Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. *Environ Microbiol* 8:1251-1260
- Schloss PD, Handelsman J (2004) Status of the microbial censuses. *Microbiol Mol Biol Rev* 68:686-691
- Schulte, S., Mangelsdorf, K., & Rullkoetter, J. (2000). Organic matter preservation on the Pakistan continental margin as revealed by biomarker geochemistry. *Organic Geochemistry*, 31, 1005–1022.
- Slombe CP, Malschaert JFP, Van Raaphorst W (1998) The role of adsorption in sediments-water exchange of phosphate in North Sea continental margin sediments. *Limnol Oceanography* 43: 832-846.
- Suess E (1981) Phosphate regeneration from sediments of the Peru continental margin by dissolution of fish debris. *Geochim Cosmochim Acta* 45: 577-588
- Sundby B, Gobeil C, Silverberg N and Mucci A (1992) The phosphorus cycle in coastal marine sediments. *Limnol Oceanography* 37:1129-1145

- Uphoff HA, Felske A, Fehr W, Wagner-Dobler I (2001) The microbial diversity in picoplankton enrichment cultures: a molecular screening of marine isolates FEMS Microbiol Ecol 35: 249-258
- Wakeham, SG (1995) Lipid biomarkers for heterotrophic alteration of suspended particulate organicmatter in oxygenated and anoxic water columns of the ocean, Deep-Sea Res.I, 42:1749–1771
- Walsh JJ (1991) Importance of continental margins in the marine biogeochemical cycling of carbon and nitrogen. Nature 350: 53-55
- Wang F, Wang P, Chen M, Xiao X (2004) Isolation of extremophiles with the detection and retrieval of *Shewanella* strains in deep-sea sediments from the west Pacific. Extremophiles 21: 165-168
- Woese CR, Stackebrandt E, Macke TJ, Fox GE (1985) A phylogenetic definition of the major eubacterial taxa. Syst Appl Microbiol 6: 143-151
- Wyrтки K (1962) The oxygen minima in relation to ocean circulation. Deep Sea Res 9: 11-23.

Table 1: Nearest neighbours of the phylotype representatives in the GenBank.

| Phylotype representative | Phylogenetic group | Accession numbers | Nearest neighbour | Similarity (%) |
|--------------------------|--------------------------|-------------------|---|----------------|
| G12-15A | Firmicutes | GQ 900675 | Halobacillus (FJ237396) | 97 |
| G12-10A | Firmicutes | GQ 900678 | Bacillus licheniformis (FN298317) | 99 |
| G12-11B | Firmicutes | GQ 900681 | Bacillus sp.(DQ078995) | 96 |
| G12-8A | Firmicutes | GQ 900685 | Halobacillus mangrovi strain (DQ888316) | 99 |
| G9-1A | Firmicutes | GQ 900686 | Halobacillus sp (AB305183) | 99 |
| G9-2 | Firmicutes | GQ 900687 | Bacillus sp (FJ977613) | 89 |
| G9-6 | Firmicutes | GQ 900688 | Bacillus sp. (DQ868675) | 99 |
| G9-15A | Firmicutes | GQ 900689 | Virgibacillus sp (FJ477404) | 97 |
| G9-18B | Firmicutes | GQ 900690 | Halobacillus sp (AB305183) | 99 |
| G9-12 | Firmicutes | GQ 900693 | Paenibacillus sp.(DQ854976) | 99 |
| G6-4 | Firmicutes | GQ 900694 | Uncultured Firmicutes (EU753617) | 98 |
| G6-13A | Firmicutes | GQ 900696 | Halobacillus sp. (AB198765) | 96 |
| G6-10 | Firmicutes | GQ 900698 | Marinilactibacillus sp (DQ344859) | 96 |
| G12-23A | Actinobacteria | GQ 900676 | Kytococcus sedentarius (AY881239) | 98 |
| G12-22B | Actinobacteria | GQ 900677 | Micrococcus sp.(EF491952) | 97 |
| G12-14A | Low G + C Gram positives | GQ 900683 | Low G+C gram positive organism (AB002642) | 92 |
| G12-12A | Low G + C Gram positives | GQ 900684 | Low G+C gram positive organism (AB002643) | 94 |
| G6-15 | Low G + C Gram positives | GQ 900695 | Low G+C Gram-positive bacterium (AF348730) | 99 |
| G6-16 | Proteobacteria | GQ 900700 | Halomonas sp (EU781513) | 98 |
| G6-9 | Proteobacteria | GQ 900701 | Alteromonas hispanica (EU529840) | 99 |
| G12-11A | Unidentified bacteria | GQ 900674 | Glacial ice bacterium (AF479338) | 99 |
| G12-21B | Unidentified bacteria | GQ 900679 | Uncultured bacterium (FJ479457) | 99 |
| G12-21A | Unidentified bacteria | GQ 900680 | Uncultured compost bacterium clone (DQ346631) | 99 |
| G12-1A | Unidentified bacteria | GQ 900682 | marine sediment bacterium (AY936960) | 95 |
| G9-15B | Unidentified bacteria | GQ 900691 | Uncultured compost bacterium (DQ346631) | 97 |
| G9-5 | Unidentified bacteria | GQ 900692 | Marine sediment bacterium (AY911022) | 94 |
| G6-8 | Unidentified bacteria | GQ 900697 | Uncultured bacterium (AM183073) | 93 |
| G6-14 | Unidentified bacteria | GQ 900699 | Uncultured compost bacterium (DQ346631) | 97 |

Table 2: Percentage of bacteria producing enzyme at different temperature and pH

| Enzymes | Temperature (4, 10, 28°C) | pH (6, 7, 8) |
|----------------|--------------------------------------|-------------------------|
| Amylase | 21 | 48 |
| Lipase | 3 | 24 |
| Gelatinase | 3 | 15 |
| DNase | 9 | 21 |

Legends to Figures

Figure 1: Location of sampling stations

Figure 2 a: Percentage of bacteria producing hydrolytic enzymes in OMZ

Figure 2 b: Percentage of bacteria producing other enzymes in OMZ

Figure 3: Multiple hydrolytic enzymes (≥ 3) by phylogenetic groups

Figure 4: Extracellular hydrolytic enzyme production by different
phylogenetic groups

Figure 5: Percentage of the phylogenetic groups exhibiting other enzymes

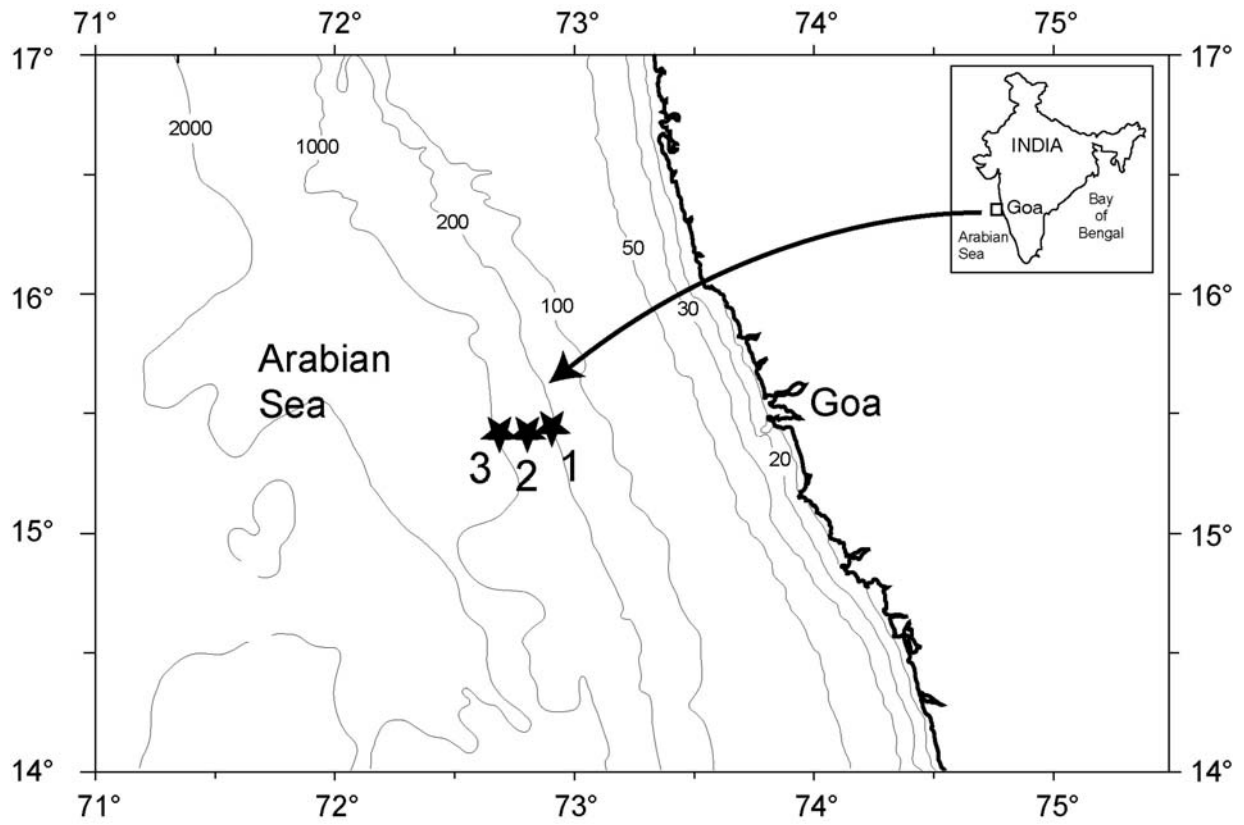


Figure. 1

Fig 2a

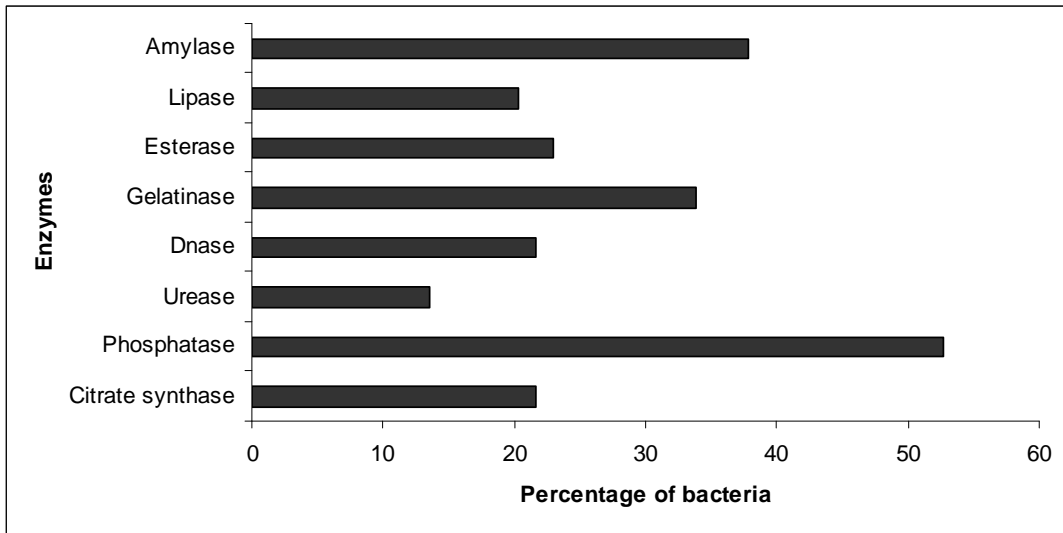


Fig 2b

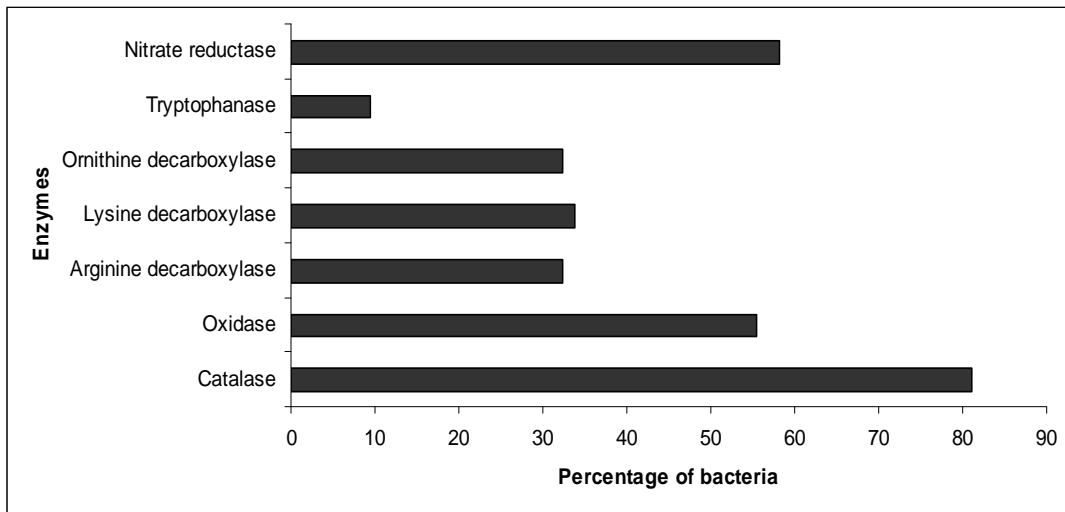


Fig 3

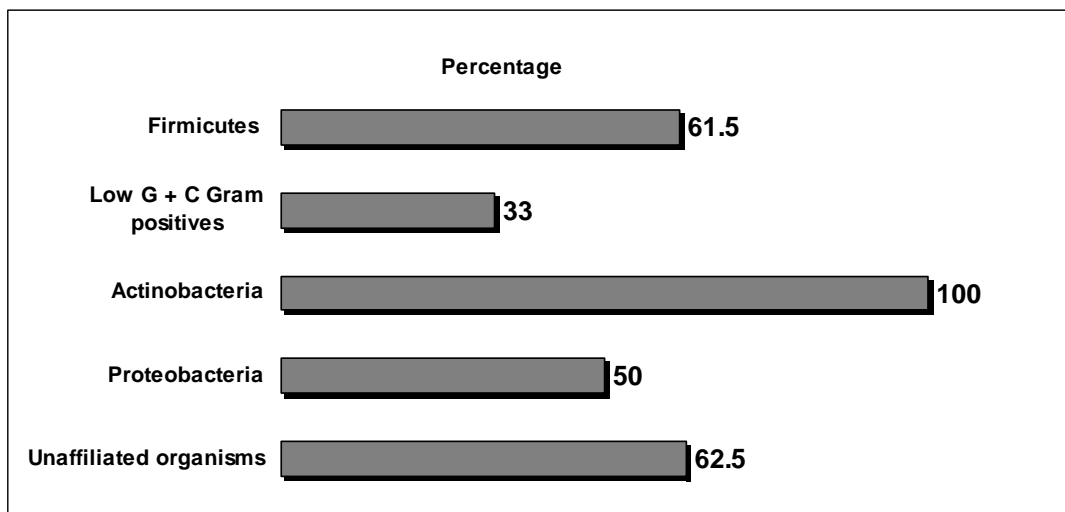


Fig 4

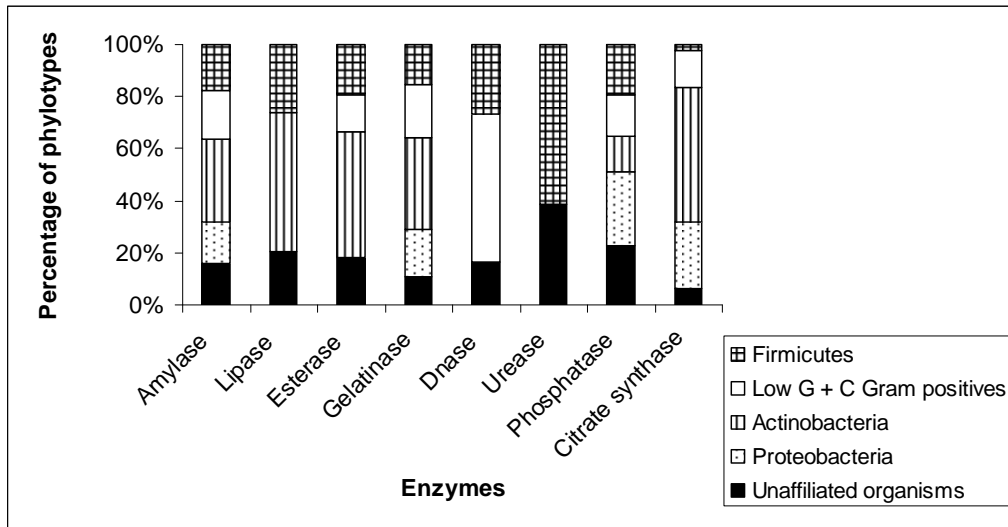


Fig 5

