

Phylogenetic diversity of carbohydrate degrading culturable bacteria from Mandovi and Zuari estuaries, Goa, west coast of India

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Abstract

Coastal and estuarine waters are highly productive and dynamic ecosystems. The complex carbohydrate composition of the ecosystem would lead to colonisation of microbial communities with abilities to produce an array of complex carbohydrate degrading enzymes. We have examined the abundance and phylogenetic diversity of culturable bacteria with abilities to produce complex carbohydrate degrading enzymes in the Mandovi and Zuari estuaries. It was interesting to note that 65% of isolated bacteria could produce complex carbohydrate degrading enzymes. A majority of these bacteria belonged to *Bacillus* genera followed by *Vibrio*, *Marinobacter*, *Exiguobacterium*, *Alteromonas*, *Enterobacter* and *Aeromonas*. Most abundant bacterial genus to degrade hemicellulose and cellulose were *Bacillus* and *Vibrio* respectively. Most abundant bacterial genus to degrade hemicellulose and cellulose were *Bacillus* and *Vibrio* respectively. It was seen that 46 % of *Bacillus* had ability to degrade both the substrate while only 14 % of *Vibrio* had bifunctionality.

Keywords: Hemicellulose, Cellulose, Bacteria, Carbohydrates, Phylogeny

1. Introduction

The rich estuarine ecosystem offers an example of dynamics of river with contribution from activities along the coast. Total soluble carbon is an important measure of richness and bioactivity of this ecosystem. However understanding the metabolic fluxes would require not only the composition of organic and inorganic components but also the contribution of microbes to these cycles. In aquatic systems the dissolved carbohydrates, especially the polymeric substrates, are the most abundant molecules in nature and are used by most organisms as structural and/or storage compounds. Among the carbohydrates the most abundant are cellulose, starch and xylans, which also represent the largest renewable source of energy known (Rosado and Govind, 2003).

Studies of lignocellulose degradation under saline conditions are important in terms of understanding carbon flux within marine environments. (Pointing and Hyde, 2000). Carbohydrates are one of the major forms of organic substrates in the aquatic environment contributing up to 80% of the dissolved and 5–25% of the particulate organic carbon (Decho, 1990, Benner et al. 1992, Biersmith and Benner, 1998). Bacteria play an important role in the regulation of rate of organic matter mineralization, nutrient cycling, and energy transfer in aquatic environments (Lennon 2007, Azam and Warden, 2004). The remineralization and the turnover of carbohydrates has been used to evaluate the efficiency of the microbial community and the lability of the organic carbon in coastal waters. The ocean environment is complex, consisting of extreme variations in pressure, salinity, temperature, and biological habitats. The biota of marine microorganisms has developed unique metabolic and physiological functions that not only ensure survival in extreme habitats but also offer a potential for the production of novel enzymes and bioactive metabolites for potential exploitation (Ninawe and kuhad, 2006, Beleneva et al., 2010).

Carbohydrases are enzymes that break down carbohydrates into simple sugars. They are important in life processes and find a number of industrial applications. Acting in a singular or synergistic manner they permit the use of low value biomass for the industrial production of paper, textiles, chemicals, feed stuff, bio-fuels and offer alternatives for currently used toxic substances (Gilbert and Hazlewood, 1991 Rosado and Govind, 2003, Maki et al. 2009).

In view of the important role played by microbes in recycling of carbon and bioremediation, we have isolated marine bacteria which are producers of carbohydrate degrading enzymes and characterised their phylogenetic diversity.

2. Materials and Methods

2.1. Study area

The coastal region selected for our study was the Mandovi–Zuari riverine area. The Mandovi river originates from the Western Ghats (mountain pass) of the Indian peninsula and flows from east to west over a length of N 75 km through the rocky terrain of Goa, draining into the Arabian Sea, along the west coast of India. Fig. 1 shows the sampling area in the Mandovi river estuarine system. The

estuarine banks of Mandovi and Zuari have mangroves, which harbour sediments rich in organic matter. They support several flora and fauna that are either resident or migratory and act as nursery grounds and nutrient sources for a variety of economically and ecologically important organisms, including fishes, prawns and many other invertebrates. Also these estuaries are used for transport of iron and manganese ores.

Fig.1. Mandovi and Zuari estuary.

2.2. Sample collection and isolation of bacterial cultures

Soil and water samples (2 samples per site) were collected in sterile 50 ml polypropylene tubes. These were immediately transported to the laboratory. 1% soil suspension with sterile distilled water or sterile sea water was prepared and 0.1 ml of the sample suspension was plated out on solid rich nutrient medium Luria-Bertani Agar or Zobell Marine Agar. Water samples were plated out with different dilutions on the plates. Individual colonies were isolated from these plates and streaked separately on Zobell marine agar plates to get isolated pure colonies. From here they were transferred on Zobell marine agar slants and preserved at 4°C.

2.3. Screening marine bacteria for production of cellulases, hemicellulases, and amylases

To screen the isolates degrading hemicellulose all the isolates were spotted on xylan-agar plates (Zobell marine agar/ Luria agar containing 1% xylan) and incubated at room temperature for 24 h and the zone of clearance was observed by flooding the plate with 1% Congo-red and then washing the agar with 1.5 M NaCl (Sa-Pereira et al. 2002). Similarly for the bacteria degrading cellulose all the isolates were spotted on CMC-agar plates (Zobell marine agar/ Luria-Bertini agar containing 1% carboxymethyl cellulose), and the zone of clearance was observed as described above for xylan plates. The amylase producers were isolated on starch agar plates.

2.4. Bacterial DNA extraction

Bacterial genomic DNA was isolated using a modification of existing techniques (Ausubel et al. 1995). Briefly the technique entailed the following: 5ml of mid- to late log phase bacteria were collected by centrifugation and resuspended in a small volume of lysis buffer (0.05M Tris HCL pH 8.0, 0.01M EDTA). SDS was added to this solution at a final concentration of 0.5%, mixed gently and incubated for five minutes at room temperature. The solution was sequentially extracted with equal volumes of TE (pH 8.0) saturated phenol, phenol-chloroform-isoamyl alcohol and chloroform. DNA was precipitated by adding 1/10 volume of 1M NaCl and 2.2 volumes of 100% ethanol and incubated for one hr. at 20°C. The precipitated DNA, after centrifugation was rinsed three times with 70% ethanol, dried and resuspended in sterile distilled water.

2.5. Polymerase chain reaction

The genomic DNA of individual bacterial samples was used for PCR amplification. The 16S rDNA gene fragments were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-30) (Weisburg et al., 1991). The amplification was carried out in 25 µl of reaction mixture containing DNA template, PCR buffer, dNTP (10 mM), MgCl₂ (25 mM), the primers 27F (10 ppm) and 1492R (10 ppm) respectively, Taq polymerase (Sigma) (2U) and autoclaved Milli-Q water (Millipore). The amplification was carried out for 35 cycles of 94 °C, for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72 °C for 7 min with initial 05-min denaturation at 94 °C using a PCR-Express thermal cycler (Hybaid). The DNA sequences of the PCR products were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer) using the protocol recommended by the supplier. Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems). Databases (GenBank) were searched for sequences similarity analysis of the 16S rDNA sequence obtained. The sequences obtained were aligned with clustal x and the tree was built by using MEGA 4.

3. Results

3.1. Screening of carbohydrate degrading bacteria

Screening of marine bacteria from Mondovi and Zuari estuaries resulted in the isolation of 536 bacterial isolates. The isolates were maintained and subcultured on Luria Agar or Zobell Marine Agar. Each bacterial isolate was then screened for the production of amylase, xylanase and cellulase by plate assay as described under the materials and methods (Fig. 2a & 2b). It was seen that almost 66% of total bacteria isolated from this area had carbohydrate degrading abilities (Fig. 3a). In order to investigate the influence of salinity with abundance of carbohydrate degrading bacteria regression analysis was carried out (Fig. 3b) which showed that carbohydrate degrading bacteria are negatively correlated with salinity ($p \leq 0.001$) (Fig. 3b).

Fig. 2a. Detection of Amylase production as a clear white zone using Gram's-iodine in starch agar plates

Fig. 2b. Detection of xylanase/cellulase activity in xylan/CMC agar plates using Congo red

Fig. 3a. Percentage of carbohydrate degrading bacteria in Mandovi and Zuari estuary

Fig. 3b. Analysis of regression on carbohydrate degrading bacteria and salinity of different stations

The percentage of total carbohydrate degrading bacteria in Mandovi estuary was 60% as compared to Zuari estuary which was 77%. Amongst the carbohydrate degrading bacteria, amylase degrading bacteria were most dominant in Mandovi estuary (i.e. 83%) than in Zuari estuary (75 %), followed by xylanase degraders (10%) and (13%) while cellulose degraders were (6.7%) and (12%) in Mandovi and Zuari estuaries respectively (Fig. 4a, 4b and Fig. 5a,5b).

Fig. 4a. Total carbohydrate degrading bacteria in Mandovi estuary

Fig. 4b. Percentage of xylanase, cellulase and amylase degrading bacteria in Mandovi estuary

Fig. 5a. Total carbohydrate degrading bacteria in Zuary estuary

Fig. 5b. Percentage of xylanase, cellulase and amylase degrading bacteria in Zuary estuary

3.2. *Distribution of carbohydrate degrading bacteria*

The estuarine system of mandovi and Zuari estuaries are amongst one of the well studied systems and have been investigated from hydrological, tidal, biological viewpoints (Shetye et al., 2007). The results obtained in our study showed interesting trend of bacterial distribution at each station. In all the stations of Mandovi and Zuari, amylase degrading bacteria were the most dominant. It was seen that Donapaula station had only starch degraders while amylase producers were 10-20 times more than cellulose and hemicellulose degraders at Akkada, Usgao and Cortalim, while at Savordem and Sanguem they were just two times higher (Fig. 6). Observed differences in carbohydrate degrading bacteria at different stations is highly significant as evident from one-way ANOVA (Table 1).

Table 1. Result of one-way ANOVA comparing the carbohydrate degrading bacteria and different stations.

When only cellulose and hemicellulose degrading bacteria were studied it was seen that Donapaula station didn't show any cellulose or hemicellulose degrading bacteria. Choraao was mainly dominated by hemicellulase degraders. Akkada had both cellulose and hemicellulose degrading bacteria with almost equal proportion. Usgao and St. Cruze stations had bacteria with equal proportion of hemicellulose degraders and bacteria which can degarade both cellulose and hemicellulose together. Chikalim and Savordem stations showed all three types of bacteria, while Cortalim had only hemicellulase degrading bacteria. Loutlim was the first station which was dominated by cellulose degrading bacteria, while Sanguem was dominated by bacteria having multiple substrate degrading capabilities (Fig. 7)

Fig. 6. Distribution of Cellulose and Hemicellulose and Amylose degrading bacteria in Mandovi and Zuary estuary

Fig. 7. Distribution of Cellulose and Hemicellulose degrading bacteria in Mandovi and Zuary estuary

3.3. *Phylogenetic diversity*

Molecular phylogeny of Xylanase and cellulase producing isolates was carried out by employing 16s rDNA sequencing. Comparison of these sequences with those in gene bank database (accession numbers: HM486447-HM486493) indicated that the majority of cellulose and hemicellulose degrading isolates from Mandovi and Zuary estuary belong to *Bacillus* genera

followed by *Vibrio*, *Marinobacter*, *Exiguobacterium*, *Alteromonas*, *Enterobacter* and *Aeromonas* (Table 2).

Table 2. Relative abundance (%) of cellulose and hemicellulose degrading marine bacteria.

Most abundant bacterial genus to degrade hemicellulose and cellulose were *Bacillus* and *Vibrio* respectively. It was seen that 46 % of *Bacillus* isolates had ability to degrade both the substrate while only 14 % of *Vibrio* showed had bifunctionality. It was seen that 39 % *Bacillus* degraded hemicellulose but only 15 % *Bacillus* sp could degrade cellulose exclusively (Fig. 8).

Fig. 8. Degradation pattern of *Bacillus* sp (a). and *Vibrio* sp. (b) with respect to hemicellulose and cellulose

Fig. 9 shows a phylogenetic tree based on 16S r DNA sequences of 47 culturable isolates degrading cellulose and hemicellulose found in and Zuari estuarine region. The *Bacillus* and *Vibrio* isolates although very similar in rDNA sequences show variations in enzyme production and appear to colonise the diverse ecological niches.

Fig. 9. Phylogenetic relationship based on 16S rDNA sequences of representative isolate and related species degrading carbohydrate found in Mandovi and Zuary estuary-Goa. This tree was generated using the program ClustalX. It can be clearly seen that all the *Bacillus* species show clustering and several *Bacillus pumilis* isolates clearly differ in the rDNA sequence compositions. Although similar trend is seen in *Vibrio* isolates there is a lot of diversity amongst the various bacillus isolates in terms of their abilities to produce enzymes and rDNA sequence homologies.

4. Discussion

Marine microorganisms possess a wide range of enzymes which metabolise carbohydrate and are different from those of terrestrial microbes. Detection of the enzymes that hydrolyze natural compounds in the ocean and their further investigation is scientifically and practically significant, making it possible to elucidate the mechanisms of the transformation of organic substances in marine ecosystems and to fulfil the requirements of modern biotechnology (Beleneva et al., 2010). Thus, this research is oriented to the isolation of carbohydrate degrading bacterial enzymes in marine environment with special emphasis on celluloses and hemicelluloses.

Most of the samples collected from Mandovi and Zuary estuary had high densities of culturable bacteria (10^3 to 10^6). The number of carbohydrate degraders isolated amongst them was quite large (66%). The isolation of large number of carbohydrate degraders belonging to different groups highlights the important role of these micro organisms in carbohydrate turnover in Mandovi and Zuary estuary. It has been reported by Beleneva et al. (2010) that the strains isolated from temperate climatic zone possess higher enzymatic activity than those isolated from the tropical zone. Extracellular enzyme activity of these organisms is a very important feature in the ecology of

water because it is responsible for that part of detritus which is introduced into the food chain via bacterial heterotrophic activity.

If we compare Mandovi and Zuary estuary, the carbohydrate degraders were more in Zuary compared to Mandovi. The Amylase producers dominated at both the estuaries. Dereková et al. (2008) also reported that amylose was the most preferable substrate, and about half of the isolates from Bulgarian hot springs degraded it. The observed high frequency of amylolytic activity between the isolated strains could be explained by ubiquitous occurrence of starch in nature and its comparatively easy degradation.

There has been considerable interest in microbial and enzymatic conversion of cellulosic and hemicellulosic substrates, such as waste plant cell wall material. Its successful utilization as renewable raw material depends on the development of economically feasible technologies for the hydrolysis into low molecular weight products and for the conversion of these hydrolysis products into commercial products such as liquid fuel, food materials, and chemicals (Risna and Suhirman, 2002). In this context, cellulose and hemicellulose degrading bacteria from this region were studied more closely. It was seen that Donapaula station didn't show any cellulose and hemicellulose degrading bacteria, while some stations such as Cortalim and Loutlim were completely dominated by xylanase and cellulase degraders respectively. Most interestingly some stations such as Sanguem and Savordem showed the dominance of bifunctional bacteria which could degrade both hemicellulose and cellulose. Sanguem and Savordem is a riverine end of Mandovi estuary with fresh water, while Donapaula is a mouth of estuary with salinity of 35ppt. It is been reported that the growth rate of lignocellulose-degrading populations (50%) is inhibited by NaCl (Liu and Boone, 1991). Also according to Park et al. 2006 the high salinity seems to significantly reduce ectoenzyme activities. Thus it is possible that the type of bacterial population found at particular station can also be influenced by salinity changes. It has also been reported that in most of the aquatic environments there is a significant relationship between extracellular enzyme activities, their corresponding substrates (polymers) and their hydrolysis products (monomers) (Munster et al., 1992). Thus the presence of cellulolytic and hemicellulolytic bacteria in particular region while absence of these bacteria in other region indicates the presence of wide-ranging organic matter in each region. Previous research has also put forth a widely accepted concept that hydrolytic enzymes are induced by the presence of polymeric substrates (Chrost, 1991; Vetter and Deming, 1999).

It was observed that the genus *Bacillus* and *Vibrio* were the dominant hemicellulase and cellulase producers in both the estuaries. According to Ruger, (1989) the genus *Bacillus* is comprised of a phylogenetically and phenotypically diverse species, which are ubiquitous in terrestrial and fresh water habitat and are also widely distributed in sea water. While the family *Vibrionaceae* is one of the most important bacterial groups in marine environments. Members of this family often predominate in the bacterial flora of seawater, plankton, and fish. In a survey carried out

in the West Pacific Ocean, *Vibrios* accounted for nearly 80% of the bacterial population in surface seawater (Shimidu et al., 1980). Phylogenetic diversity of the culturable bacterial species from Bulgarian hot springs has been described along with their carbohydrate utilising abilities (Derekova et al., 2008)

It was seen that organisms belonging to the bacterial genus *Bacillus* could efficiently degrade hemicelluloses and also showed effective bifunctionality. While only few *Bacillus* isolates could degrade cellulose exclusively. *Bacillus* species are known to produce a number of extracellular polysaccharide hydrolysing enzymes (Priest, 1977) and several *Bacillus* spp. are known to produce xylanases (Nakamura et al., 1993, Morales et al., 1993, Ratanakhanokchai et al., 1999, Yin et al., 2010). It has also been reported that bacilli lack the complete cellulase system, the main activity being that of carboxymethylcellulase (endoglucanase) which does not hydrolyse crystalline cellulose (Robson and Chambliss, 1987, Dhillon et al., 1985, Au and Chan, 1987, Okoshi et al., 1990, Ozaki and Ito, 1991, Aa et al., 1994). This may be the reason behind reduced efficiency of *Bacillus* in degrading cellulose. On the other hand, *Vibrio* was seen to degrade cellulose very efficiently. Although a few *Vibrio* isolates demonstrated bifunctionality, very few had ability to degrade hemicellulose. While all cellulases cleave a single type of bond in a chemically simplistic substrate, the extensive intermolecular bonding pattern of cellulose generates a fascinating crystalline substrate that is particularly resistant to microbial degradation. Multiple enzyme systems are thus required to efficiently degrade cellulose. Such systems comprise either a collection of free cellulases and/or multicomponent complexes thereof, called cellulosomes. It is likely that marine *Vibrios* may possess cellulosomes which help them to degrade cellulose while *Bacillus* does not. It is reported by Demain, (2006) that along with other bacteria such as some of *Clostridium* sp. and *Ruminococcus* sp., *Vibrio* sp. also possess cellulosomes.

Different strains of *Bacillus subtilis* and *Bacillus pumilis* were found all over the Mandovi and Zuari estuary involved in cellulose and hemicellulose degradation. Miranda et al., (2008) have described the identification of 23 marine *Bacillus* isolates by rDNA sequencing. In our molecular phylogenetic analysis it can be seen that amongst the *Bacillus* species described, these isolates show variation in rDNA sequence and also differ in their abilities to produce glucanases. Thus *Bacillus* seems to adapt to changing salinity and differential availability of complex carbohydrates by colonising different specialised niches

It is estimated that the 50% of the primary production is cycled as dissolved organic carbon (DOC) through the microbial loop to higher trophic levels (Azam, 1998). Carbohydrates are one of the major forms of organic substrates in the aquatic environment, contributing up to 80% of the dissolved and 5–25% of the particulate organic carbon. Thus, Detection of the enzymes that hydrolyze carbohydrates in the ocean and their further investigation is scientifically and practically significant, making it possible to elucidate the mechanisms of the transformation of organic substances in marine ecosystems and to fulfil the requirements of modern biotechnology.

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Figure captions

Fig.1. Mandovi and Zuari estuary

Fig. 2a. Detection of Amylase production as a clear white zone using Gram's-iodine in starch agar plates

Fig. 2b. Detection of xylanase/cellulase activity in xylan/CMC agar plates using Congo red

Fig. 3. Percentage of carbohydrate degrading bacteria in Mandovi and Zuari estuary

Fig. 4a. Total carbohydrate degrading bacteria in Mandovi estuary

Fig. 4b. Percentage of xylanase, cellulase and amylase degrading bacteria in Mandovi estuary

Fig. 5a. Total carbohydrate degrading bacteria in Zuari estuary

Fig. 5b. Percentage of xylanase, cellulase and amylase degrading bacteria in Zuari estuary

Fig. 6. Distribution of Cellulose and Hemicellulose and Amylose degrading bacteria in Mandovi And Zuari estuary

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Fig. 8. Degradation pattern of *Bacillus* sp. (a). and *Vibrio* sp. (b) with respect to hemicellulose and cellulose

Fig. 9. Phylogenetic relationship based on 16S rDNA sequences of representative isolate and related species degrading carbohydrate found in Mandovi and Zuari estuary-Goa. This tree was generated using the program Clustal X

Table 1

Result of one-way ANOVA comparing the carbohydrate degrading bacteria and different stations.

Source of Variation	df	SS	MS	F	P-value
<i>% Carbohydrate degrading</i>					
<i>bacteria</i>					
Between Groups	9	2186.8	242.9778	121.4889	5.09E-09
Within groups	10	20	2		
Total	19	2206.8			

Table 2

Relative abundance (%) of cellulose and hemicellulose degrading marine bacteria.

Bacterial Genera	(%)
<i>Bacillus</i>	70
<i>Vibrio sp</i>	17
<i>Marinobacter</i>	4
<i>Exiguobacterium auranticum</i>	2
<i>Alteromonas</i>	2
<i>Enterobacter oryzae/</i>	2
<i>Aeromonas</i>	2

Fig.1. Mandovi and Zuari estuary.

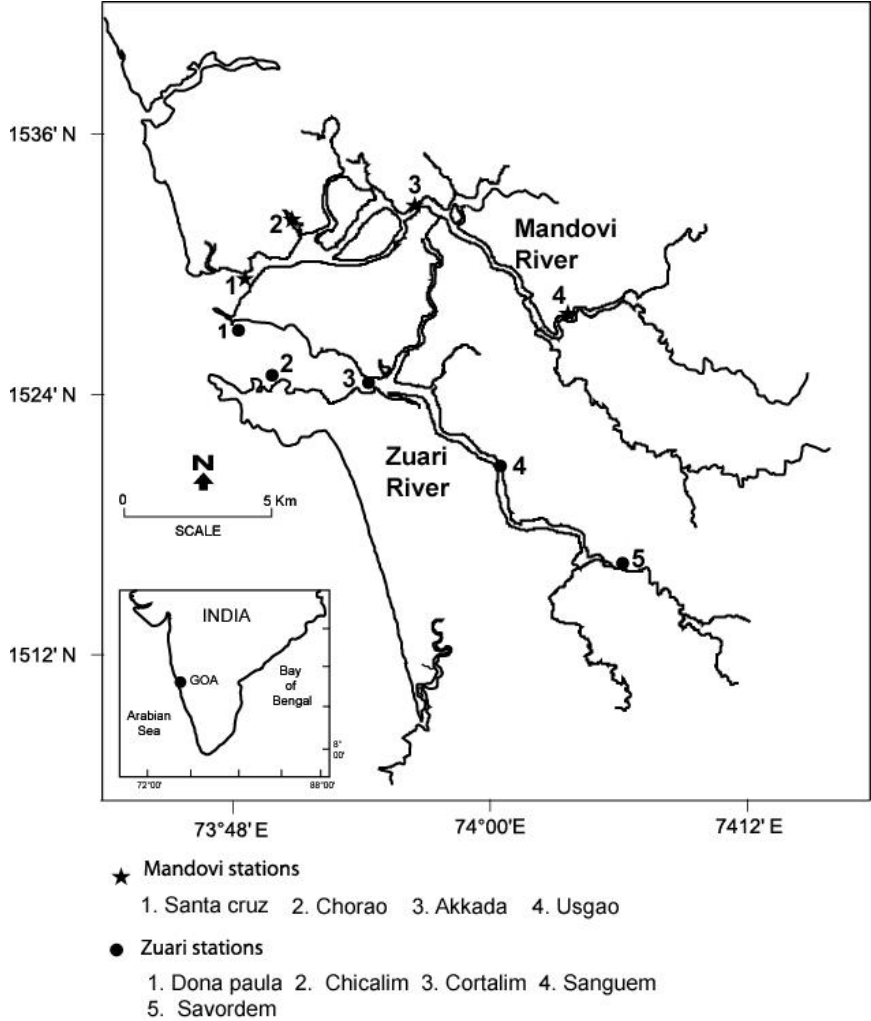


Fig. 2a. Detection of Amylase production as a clear white zone using Gram's-iodine in starch agar plates.

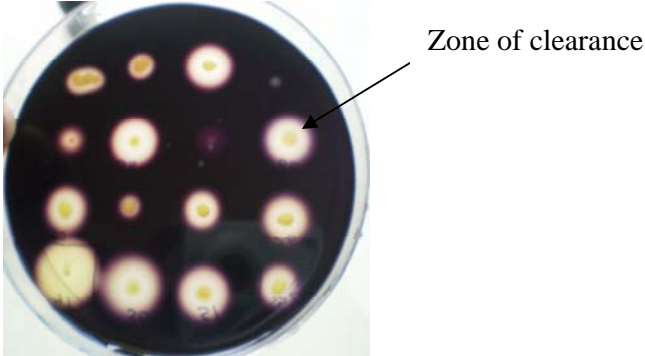


Fig. 2b. Detection of xylanase/cellulase activity in xylan/CMC agar plates using Congo red.

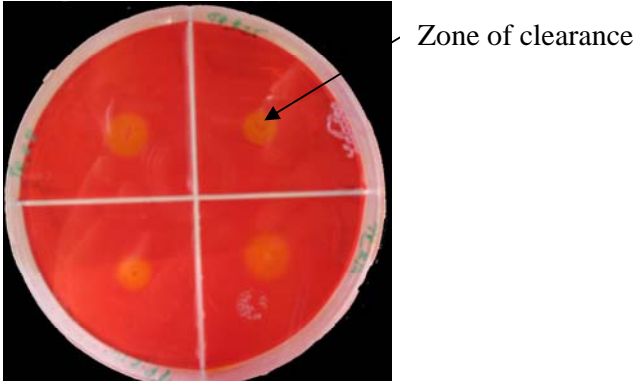


Fig. 3. Percentage of carbohydrate degrading bacteria in Mandovi and Zuari estuary.

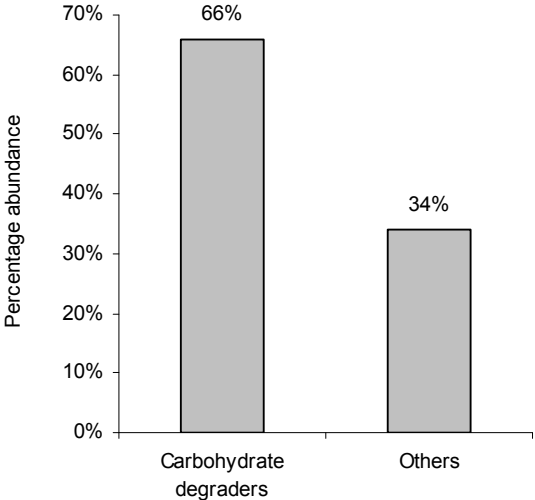


Fig. 3b. Analysis of Regression on carbohydrate degrading bacteria and salinity of different stations

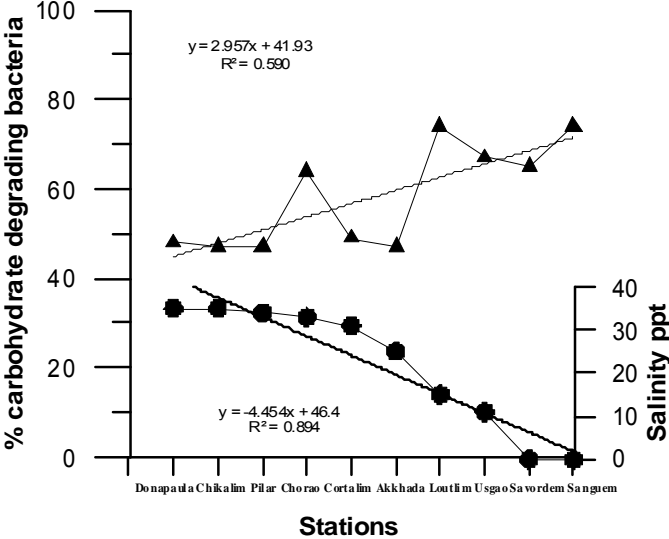


Fig. 4a. Total carbohydrate degrading bacteria in Mandovi estuary

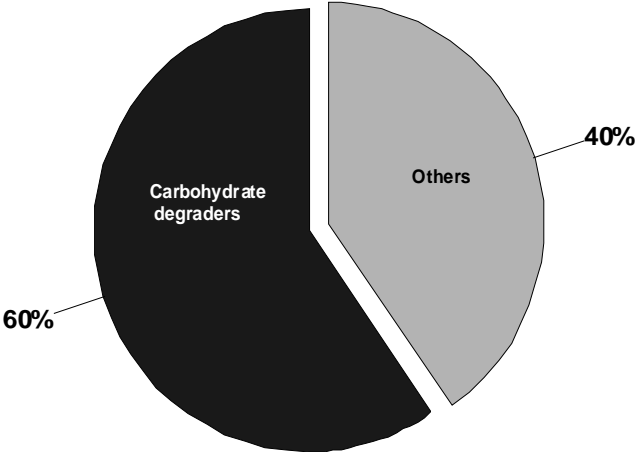


Fig. 4b. Percentage of xylanase, cellulase and amylase degrading bacteria in Mandovi estuary

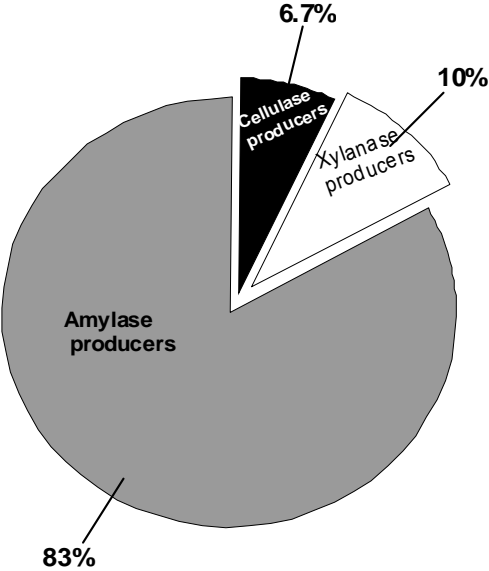


Fig. 5a. Total carbohydrate degrading bacteria in Zuary estuary.

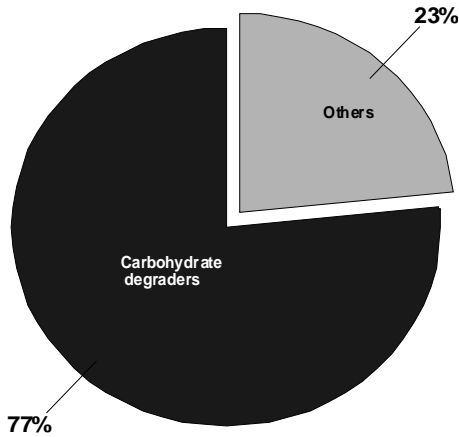


Fig. 5b. Percentage of xylanase, cellulase and amylase degrading bacteria in Zuary estuary

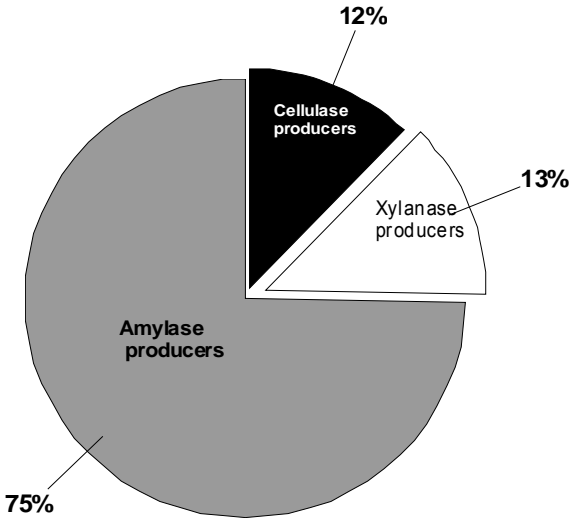


Fig. 6. Distribution of Cellulose and Hemicellulose and Amylose degrading bacteria in Mandovi And Zuary estuary.

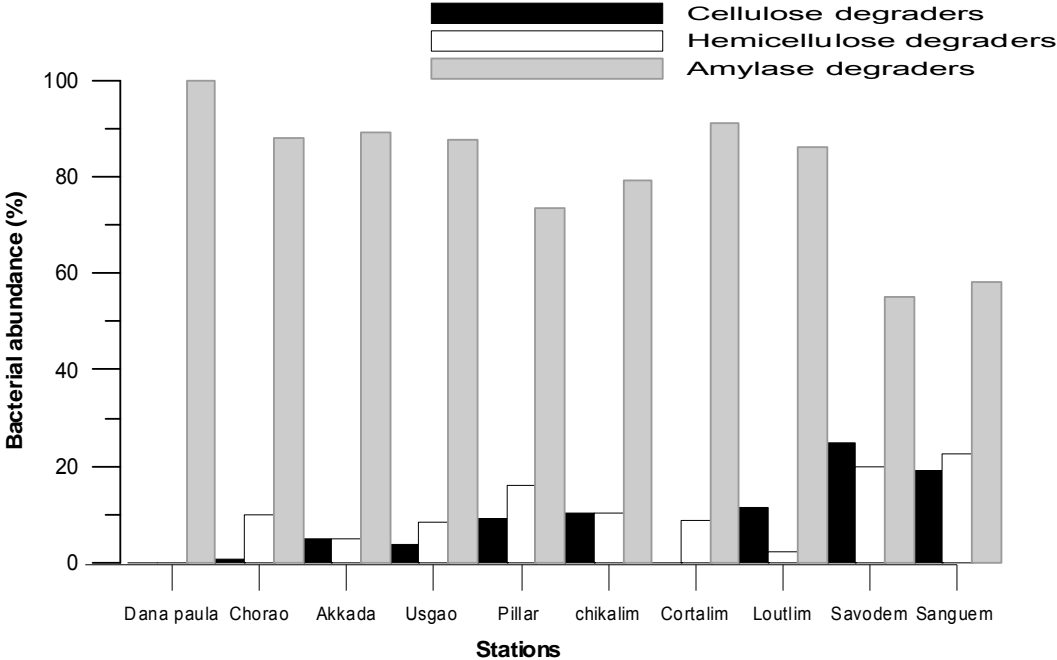


Fig. 7. Distribution of Cellulose and Hemicellulose degrading bacteria in Mandovi And Zuary estuary.

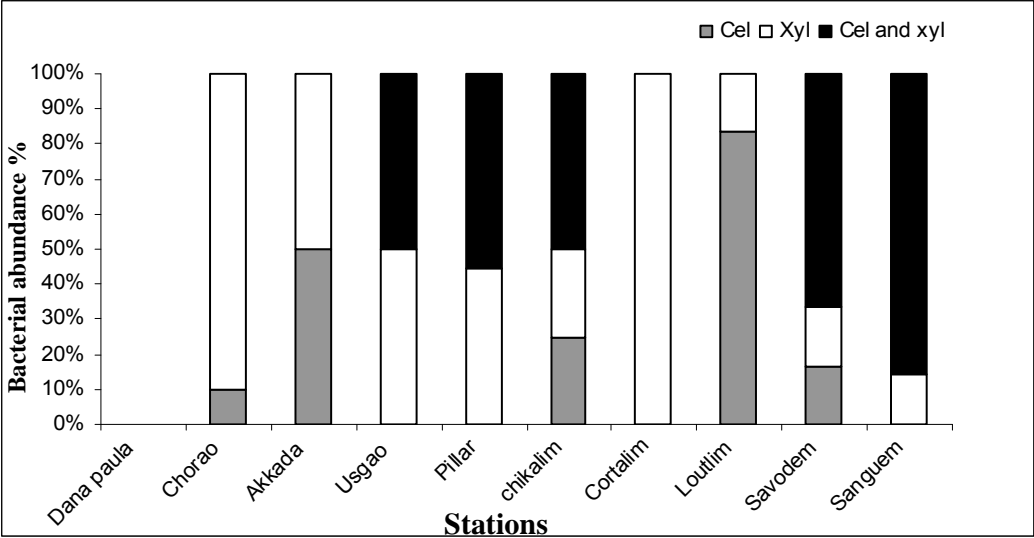
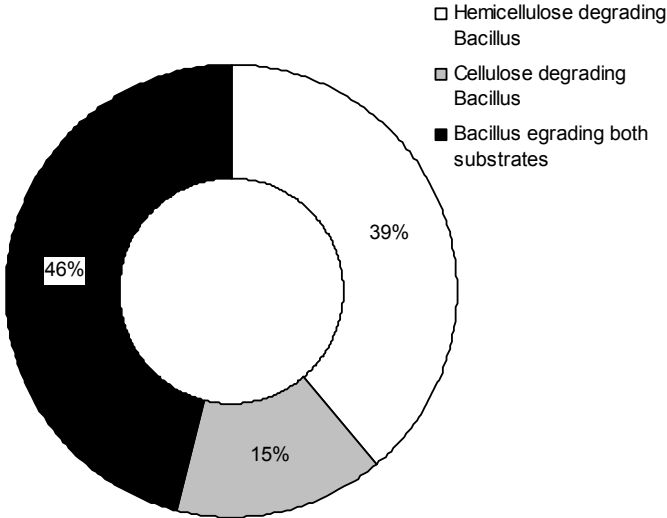


Fig. 8. Degradation pattern of *Bacillus* sp (a). and *Vibrio* sp. (b) with respect to hemicellulose and cellulose.

a



b

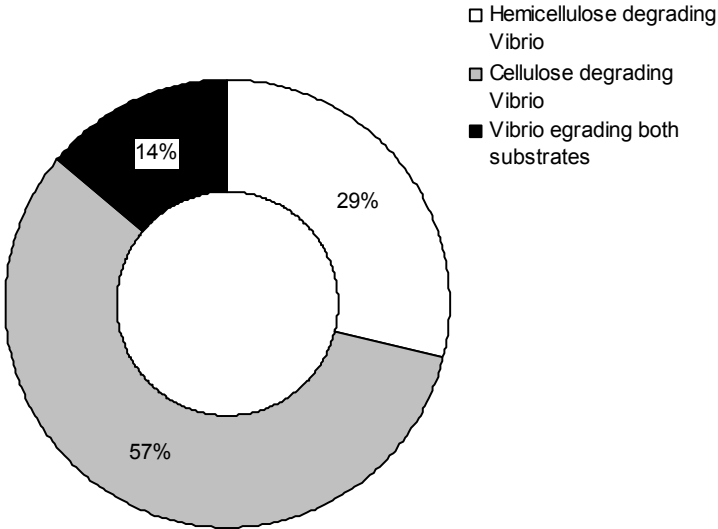


Fig. 9. Phylogenetic relationship based on 16S rDNA sequences of representative isolate and related species degrading carbohydrate found in Mandovi and Zuary estuary-Goa. This tree was generated by using the program ClustalX

