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Organic Matter and Anaerobic Cellulolytic Activity in Sediments of Ashtamudi Estuary, Kerala, India

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Abstract
Estuarine sediments are best suited for bioprospecting cellulose-degrading microorganisms because of continuous input of cellulosic carbon from rivers and terrestrial runoff, and such sediments act as a substrate for decomposition by microbes. Sediment samples were collected from thirteen stations of Ashtamudi estuary, a tropical Ramsar site during April 2016 and January 2017 and analyzed for environmental variables such as temperature, pH, electrical conductivity, oxidation-reduction potential, sulphate, total organic carbon (C_{org}), carbohydrate, protein, lipid and labile organic matter. Microcosm experiments were conducted in the sediment samples to compare native and substrate-induced cellulase enzyme activities in mesophilic and thermophilic conditions added with crystalline cellulose and cellobiose as substrates. Abundance of cellulolytic anaerobes in the roll tubes was higher with cellobiose (3.15 in April 2016 and 3.38 in January 2017) than crystalline cellulose (2.84 in April 2016 and 3.92 in January 2017). Substrate induced enzyme activity was more than native enzyme activity [0.0012 ± 0.0001–0.004 ± 0.002 (April 2016) and 0.004 ± 0.001–0.161 ± 0.002 mg glucose h^{-1} (January 2017) in the sediment samples and cellulolytic activity was more pronounced in thermophilic conditions during April 2016. Redundancy Analysis indicated that salinity was the highest determining factor for explaining variations among bacterial abundance and activity during April 2016 and sediment lipid content during January 2017. The study reveals that estuarine sediments can act as a potential source of thermophilic cellulase enzyme producing bacteria, which needs to be further explored owing to their vast industrial applications.

Keywords Anaerobic sediments · Cellulolytic activity · Environmental variables · Labile organic matter

Introduction

Estuaries are considered to be an important link between land and the sea and function as natural sinks of organic matter that come from marine, terrestrial and anthropogenic sources (Zwolsman and Van Eck 1999). Most of the organic matter in sediments is dominated by cellulose due to the plant derived sources (Cowie and Hedges 1984; Bacic et al. 2012).The organic carbon rich sediments of estuaries harbour abundant cellulolytic bacteria which have considerable influence on the carbon cycle in both aerobic and anaerobic environments. The structure and function of microbial communities in the estuarine sediment depend upon the human input of soluble and insoluble particulate form of organic matter (Babu et al. 2010). The soluble form of organic matter is contributed by carbohydrate, protein, lipid and other biological compounds which can be easily metabolized (He et al. 2010). Studies indicate that carbohydrate accounts for ~3–10% of total sedimentary organic matter (Skoog and Benner 1997; Bergamaschi et al. 1999; Burdige et al. 2000; Kerhervé et al. 2002) whereas, protein is an important biochemical compound which can be easily utilized by bacteria than other biochemical compounds (Newell and Field 1983).

Deposition of labile and refractory organic matter from the water column provides energy and nutrients for microbes inhabiting coastal sediments (Middelburg and Levin 2009). A major part of the organic matter consists of plant structural polymers referred as lignocelluloses and these polymers need to be hydrolyzed by extracellular enzymes before

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it is taken by microbes (Billen 1982; King 1986; Gunny et al. 2015). These enzymes play an important role in organic matter degradation in natural ecosystems (Boschker and Cappenberg 2006). In anaerobic environment, cellulolytic bacteria hydrolyze organic polymers to monomers through fermentation; the monomers are further degraded by secondary fermenters such as methanogens (Zinder 1993).

In the global carbon cycle, enzymatic degradation of cellulose by microorganism is a key process (Malhi 2002; Wilson 2011; Sorokin et al. 2014). Especially, extracellular enzymes such as cellulase have an important role in organic matter decomposition in the sediments, which depends upon the quantity and quality of organic matter (Meyer-Reil 1987; Boetius and Lochte 1996). Enzymatic conversion of lignocellulosic material is important because of their insolubility in water (Behera et al. 2017). The cellulose degradation occurs by the hydrolysis of β 1–4 glycosidic bonds, which needs the concerted action of three enzymes such as endoglucanase, exoglucanase and β- glucosidase (Jørgensen et al. 2007). These enzymes acts synergistically: endoglucanases hydrolyze the exposed cellulose chains of the cellulose polymer; exoglucanases (cellobiohydrolases) act to release cellobiose from the reducing and nonreducing ends, and *β*-glucosidases help to cleave the cellobiose and short-chain cello-oligosaccharide into glucose (Chandra and Madakka 2018). However, in anaerobic cellulolytic bacteria a "cellulosome" consisting of cellulose binding proteins and hydrolytic enzymes are responsible for cellulolysis (Doi et al. 1994). A high molecular weight protein (>2 MDa) was reported in cellobiose grown anaerobic cellulolytic bacteria such as *Clostridium* sp., *Acetivibrio* sp. and *Bacteroides* sp. that could bind to filter paper cellulose (Vincent and Ramasamy 2001). Ultimately, cellulolytic microorganisms degrade cellulose into simple sugar derivatives (Tengerdy and Szakacs 2003). Because of the complexity and high molecular weight of cellulose, it needs the action of various enzymes for degradation which is associated with various environmental conditions such as temperature, pressure and salinity (Taketani et al. 2010).

The cellulolytic microbes and their enzymes receive much attention due to their diverse applications in several industries such as textile, food, paper and pulp, beer and wine brewing, fuel and chemical industries (Gao et al. 2008; Behera et al. 2016). A study conducted by Odisi et al. (Odisi et al. 2012) on five different strains of bacteria revealed the potential of cellulase enzyme activity in both mesophilic and thermophilic conditions. Isolation of cellulose degrading bacterial strains were well documented from various coastal habitats such as salt marshes on Sapelo island, Ga (Benner et al. 1984), Sundarbans mangrove of West Bengal, India (Ramanathan et al. 2008), mangrove soil of Bhitarkanka, Odisha, India (Thatoi et al. 2012), Philippine's mangrove (Tabao and Monsalud 2010), Uppanar estuary, India (Kalaiselvi et al. 2013) and mangrove soil of Mahanadi river delta, Odisha, India (Behera et al. 2014). Tropical estuaries receives multiple input of organic matter from allochthonous and autochthonous source. The increased microbial activities on organic matter mineralization leads to the depletion of oxygen and cause the prevalence of anoxic conditions and further leads to anaerobic metabolic activities. In Ashtamudi Estuary, the predominant anaerobic microbial activity was previously related to sulphate reduction, although denitrification and methanogenesis also occurred in the sediments (Vincent et al. 2017). Hence, this study was done to explore the hydrolytic activity of Ashtamudi estuarine sediment by analysing the abundance and activity of anaerobic cellulolytic bacteria and also to investigate the environmental factors influencing the spatio-temporal variations in native and substrate-induced cellulolytic activity.

Materials and methods

Study Area and Relevance

Ashtamudi estuary, located between 76°32' and 76°41'E longitude and 8°52' and 9°2' N latitude (Fig. 1) is the second-largest estuarine system having a surface area of 32 km^2 and gains international importance as a Ramsar site. It is a palm shaped estuarine system and opens into Neendakara, which is one of the most important fishing harbours of India. Kollam city is located in the southern side of the estuary, and it receives freshwater input from Kallada river (on the eastern side) with a length of 120 km with basin area of 1,699 km^2 and an annual average discharge of 3,375 Mm^3. Ashtamudi estuary receives organic matter from non-point and point sources such as urban and agricultural runoff, tourism, waste and sewage disposal, discharge from coconut husk industries, clay factory and fish processing industries(Babu et al. 2010) and also a huge amount of organic matter is transported by Kallada river (Jennerjahn et al. 2008). Based on the geography, the entire Ashtamudi estuary can be subdivided into three zones- river zone in the northeast part where Kallada River joins the estuary Kallada zone (S1-S5), open estuary and the marine influenced zone (S6-S10) and the Kollam city zone (S11- S13) (Jennerjahn et al. 2008). The anthropogenic pressure on the sampling stations were: S1- influence of clay factory, S4- confluence of Kallada River and also owing pressure of direct sewage disposal from the population inhabiting in the river catchments, S8- The sediments dredged for widening or national waterways were dumped, S10- Fishing harbour and hydrocarbon discharges from the fishing boats, S11- Solid waste

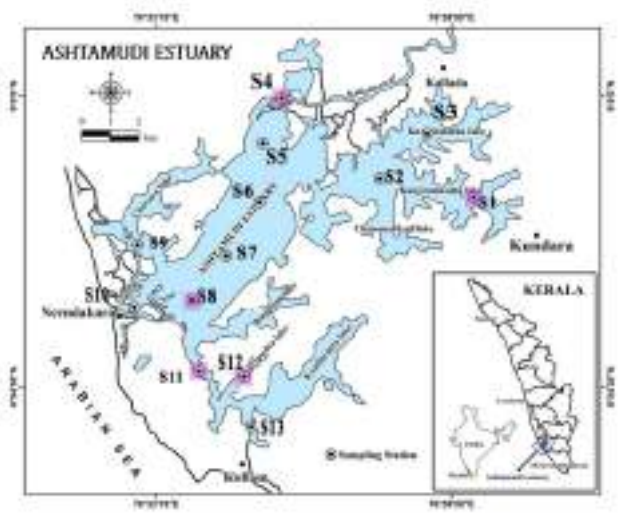


Fig. 1

plant of Kollam city, S12 – Presence of coir retting industries (Reshmi et al. 2015).

Sample Collection and Preparation

Sediment samples were collected from thirteen stations of Ashtamudi estuary using Van Veen's grab sampler during April 2016 and January 2017. The depth of the estuary varied from 1.0 to 7.3 m in the sampling stations. The samples for microbial analysis were transferred to sterile airtight bottles and brought to lab and kept under 4 °C. The remaining sediment samples were collected in polythene bags for physicochemical analysis.

Environmental Variables of Sediment

The environmental variables of estuarine sediments [temperature, pH, electrical conductivity (EC), oxidation- reduction potential (ORP), sulphate, total organic carbon (C_{org}), carbohydrate, protein, lipid and labile organic matter (LOM)] were analysed using standard procedures(Trivedy et al. 1998; Grasshoff et al. 1999; Danovaro 2009) Lipid, carbohydrate and protein were converted into carbon equivalents using 0.75, 0.40 and 0.49 $\mu gC \mu g^{-1}$ conversion factors, respectively (Fabiano and Pusceddu 1998). The biopolymeric carbon fraction (BPC) was calculated by taking the sum of lipid, carbohydrate and protein (Pusceddu et al. 2000). The protein: carbohydrate and lipid: carbohydrate ratios were calculated to determine the quality of sedimentary organic matter (Pusceddu et al. 2003).

Abundance of Cellulolytic Anaerobes

The sediment samples were enriched in pre-reduced Hungate's mineral broth containing (gL^{-1}) Potassium dihydrogen phosphate (0.2); Di-potassium hydrogen phosphate (0.3); Magnesium sulphate (0.1); Calcium chloride (0.1); Sodium chloride (1.0); Ammonium sulphate (1.0); Cysteine HCl (0.2); Sodium bicarbonate (0.2); Resazurin (0.001). Vitamin solution (Wolin et al. 1963) and trace element solution (Ferguson and Mah 1983) were added to final concentration of 1%(v/v). Crystalline cellulose (0.2%) and cellobiose (0.2%) were the two substrates used in this study as insoluble and soluble substrate respectively. Cellulolytic anaerobes were enumerated by the roll tube method (modified method of Hungate (Ramasamy et al. 1992). Growth of cellulolytic anaerobes observed as cleared zones or colonies in the roll tube, were counted and expressed as colony forming units per gram of sediments ($CFUg^{-1}$).

Assay of Native and Substrate induced enzyme activities

For native enzyme assay, 2.0 g sediment was incubated with 0.7% carboxy methyl cellulose in acetate buffer (pH-5.5) at 50°C for 24 h. For substrate induced enzyme assay, 10 mL of pre-reduced Hungate's mineral broth was dispensed to screw capped vials (40 mL) sealed with butyl rubber stopper and aluminium cap assembly. Crystalline cellulose (1.0%) was added as the substrate and to maintain anaerobic conditions, the vials were flushed with high purity nitrogen gas. 2.0 g sediment samples were transferred to the vials and incubated for 10 days. Two sets of the vials for the sediment samples were prepared and each set were maintained in mesophilic and thermophilic conditions separately. After the incubation period, 1.0 mL of supernatant was incubated with 0.7% carboxy methyl cellulose in acetate buffer (pH-5.5) at 50°C for 24 h. The amount of reducing sugar was analyzed by the dinitrosalicyclic acid method (Miller 1959). Enzyme activity was expressed in terms of milligram of glucose released per hour (Schinner and Von Mersi 1990).

Statistical analysis

Two-way analysis of variance (two-way ANOVA) and principal component analysis (PCA) were conducted to test for significant spatial and temporal differences in the variables and also study the influence of environmental parameters on the activity of cellulolytic anaerobes. Redundancy Analysis (RDA) analysis is a method to extract and summarize the variation in a set of response variables that can be explained by a set of explanatory variables. RDA reveals the relationship between environmental parameters and activity of

enzyme and bacterial abundance. All statistical analyses were performed using SPSS 20.0 and R studio.

Results and Discussion

Spatio- Temporal Variation of Environmental Variables

The changes in environmental variables of Ashtamudi estuarine sediments is summarized in Table 1. The overall sediment temperature was higher during April 2016 (28.61^0 C) than January 2017 (28.2^0 C). The spatial variation of sediment temperature was statistically insignificant along the estuary whereas the temporal variation was significant ($p = 0.001$) (Table 2). Previous study by Reshmi et al. (Reshmi et al. 2015) in Ashtamudi estuarine sediment also points out similar results. During both sampling periods, Ashtamudi estuary exhibited brackish to marine conditions with regard to pH and salinity values (Jennerjahn et al. 2008). Alkaline pH was observed all over the estuary during the sampling seasons with an average value of 7.97 and 8.26. However, the spatial variation of salinity was statistically significant ($p < 0.001$) and temporal variation was insignificant. Ashtamudi estuary opens to the Arabian sea through a wide opening which obviously shows a marine influence all over the estuary (Vincent et al. 2017). Maximum electrical conductivity was observed in the Kollam city part of the estuary during April 2016 and Kallada River part of the estuary during January 2017, which indicates the influence of dissolved nutrient load from the Kallada River (Jennerjahn et al. 2008) during the post-monsoon season.

Spatio- Temporal Variation and Source of Organic Matter

The quality and quantity of organic matter can regulate the composition and activity of microbial communities in aquatic sediments (Torres et al. 2011; Cawley et al. 2012). Particularly, the lignocellulosic biomass forms the substrate for cellulolytic anaerobes in the anaerobic sediment. Hence, it is important to analyze the quality and quantity of organic matter in the sediment. Significant spatial variations ($p < 0.001$) in the C_{org} values were observed, whereas the temporal variations were statistically insignificant in the Ashtamudi estuary. In both seasons, average C_{org} values was higher in Kollam city part as compared to the marine and river influenced part of the estuary. The sampling station S12 in the Kollam city region showed higher values during both seasons. Previous studies (Jennerjahn et al. 2008; Vincent et al. 2017) also showed the dominance of C_{org} in the Kollam city region. Lowest C_{org} values were observed in the open estuary portion particularly S9 and S10. That is related to the flushing of sea water to the open estuary during the tidal cycles and high rate of microbial degradative activities (Jonathan et al. 2004; Hussain et al. 2020). Stable isotopic δ13 C_{org} study by Jennerjahn et al. (Jennerjahn et al. 2008) points out the different source and diagenesis of organic matter, in which open estuary had highest value of δ13 C_{org} indicating the presence of marine derived organic matter (Thimdee et al. 2003) and lowest value of δ13 C_{org} in the upper part, which is attributed to the presence of river derived organic matter. LOM and BPC values showed significant spatio- temporal variations ($p < 0.001$). The higher LOM and BPC values were observed in Kallada River region during April 2016 and in Kollam city region of the estuary during January 2017. The LOM to TOM value can be used as an index of organic matter lability (Gonsalves et al. 2011). In Ashtamudi estuarine sediments, higher LOM of TOM values were observed during January 2017 (21.82%) than during April 2016 (13.22%), which indicates that the organic matter was more labile during January 2017. LOM was dominated by protein (4.51 mg/g and 1.81 mg/g for April 2016 and January 2017 respectively) followed by lipid(0.57 mg/g and 0.79 mg/g for April 2016 and January 2017 respectively) and carbohydrate (0.42 mg/g and 0.41 mg/g for April 2016 and January 2017 respectively) for both seasons. Similar result was observed in Kerala coast (Nair and Sujatha 2012), Galician coast (Cividanes et al. 2002) and Ross sea, Antarctica (Fabiano et al. 1995). Low carbohydrate values were also observed in cochin estuary (Joseph et al. 2008) of Kerala. High protein concentration in the sediment is due to allochthonous inputs (Danovaro 1996) of organic matter. Usually, proteins can be readily utilized by the bacteria than carbohydrate (Newell and Field 1983) and the dominance of protein concentration indicates the presence of fresh organic matter. Contrastingly, high values of lipid were reported from decayed organism (Danovaro et al. 1993). Nevertheless, carbohydrates are important fraction of C_{org} contributed by living organisms (Børsheim et al. 1999; Burdige et al. 2000; Bacic et al. 2012) and low carbohydrate values show the refractory nature of organic matter (Danovaro et al. 1993). Protein: carbohydrate ratio was observed to be higher in Kallada River region during April 2016 and in Kollam region during January 2017. The high protein to carbohydrate ratio also indicates that the organic matter is fresh and recently generated (Danovaro et al. 1993; Fabiano and Pusceddu 1998; Joseph et al. 2008; Joy et al. 2019), whereas higher lipid carbohydrate ratio was observed in the open estuary during both seasons.

Table 1 Environmental variables of Ashtamudi estuary (April 2016 and January 2017)

April 2016													
Environmental variables	**S 1**	**S 2**	**S 3**	**S 4**	**S 5**	**S 6**	**S 7**	**S 8**	**S 9**	**S 10**	**S 11**	**S 12**	**S 13**
Temperature (°C)	29 ± 0.53	29 ± 0.58	29 ± 0.40	29 ± 0.74	29 ± 0.61	28 ± 0.79	28 ± 0.36	28 ± 0.36	29 ± 0.25	28 ± 0.31	28 ± 0.36	29 ± 0.40	29 ± 0.31
pH	8.00 ± 0.31	8.15 ± 0.03	7.95 ± 0.10	8.11 ± 0.07	7.91 ± 0.03	7.95 ± 0.04	7.97 ± 0.03	7.8 ± 0.20	7.85 ± 0.02	7.99 ± 0.31	7.88 ± 0.08	8.13 ± 0.01	7.96 ± 0.03
EC (µs/cm)	2.32 ± 0.02	2.23 ± 0.03	1.75 ± 0.01	2.00 ± 0.11	1.03 ± 0.04	2.47 ± 0.04	1.93 ± 0.03	2.33 ± 0.03	1.34 ± 0.03	1.50 ± 0.46	1.93 ± 0.04	1.93 ± 0.13	2.24 ± 0.02
Salinity (ppt)	24.2 ± 0.30	27.00 ± 0.44	25.70 ± 0.25	21 ± 0.35	22.5 ± 0.40	25.8 ± 0.26	26.4 ± 0.10	25.9 ± 0.47	26.3 ± 0.15	25.6 ± 0.32	27.3 ± 0.08	29.6 ± 0.36	27.1 ± 0.38
Redox potential (mv)	-128 ± 2.31	-188 ± 4.16	-269 ± 3.51	-251 ± 4.93	-313 ± 5.03	-223 ± 2.52	-298 ± 3.51	-303 ± 3.06	-204 ± 4.04	-288 ± 8.33	-300 ± 8.54	-415 ± 3.06	-277 ± 5.29
Sulphate (mg/g)	63.61 ± 0.05	63.05 ± 0.02	40.89 ± 0.04	22.77 ± 0.03	23.44 ± 0.03	27.38 ± 0.04	28.05 ± 0.04	37.94 ± 0.04	23.33 ± 0.01	16.27 ± 0.032	44.05 ± 0.03	70.11 ± 0.14	63 ± 2.64
C_{org} (%)	6.21 ± 0.02	5.08 ± 0.05	4.26 ± 0.03	4.5 ± 0.15	0.92 ± 0.03	3.88 ± 0.03	3.12 ± 0.02	1.89 ± 0.03	0.89 ± 0.03	0.89 ± 0.03	5.55 ± 0.01	7.54 ± 0.02	4.17 ± 0.03
TOM (%)	10.7 ± 0.04	8.76 ± 0.09	7.35 ± 0.05	7.75 ± 0.26	1.6 ± 0.06	6.7 ± 0.06	5.39 ± 0.04	3.26 ± 0.05	1.54 ± 0.06	1.54 ± 0.06	9.57 ± 0.02	13.01 ± 0.04	7.2 ± 0.07
Carbohydrate (mg/g)	0.23 ± 0.02	0.19 ± 0.03	0.84 ± 0.02	0.68 ± 0.02	0.24 ± 0.03	0.57 ± 0.02	0.43 ± 0.02	0.24 ± 0.02	0.6 ± 0.25	0.03 ± 0.03	0.79 ± 0.04	0.25 ± 0.02	0.36 ± 0.02
Protein (mg/g)	7.36 ± 0.03	8.4 ± 0.34	3.2 ± 0.20	3.97 ± 0.02	4.43 ± 0.03	4.19 ± 0.04	2.57 ± o.02	3.91 ± 0.01	2.37 ± 0.01	3.66 ± 0.01	5.83 ± 0.01	3.15 ± 0.02	6.01 ± 0.09
Lipid (mg/g)	1.02 ± 0.02	0.33 ± 0.01	0.6 ± 0.15	1.22 ± 0.01	1.37 ± 0.02	0.03 ± 0.02	0.12 ± 0.04	0.51 ± 0.01	0.03 ± 0.02	0.02 ± 0.02	0.56 ± 0.02	0.78 ± 0.02	0.92 ± 0.02
LOM (%)	0.86 ± 0.42	0.89 ± 0.44	0.4 ± 0.2	0.54 ± 0.26	0.6 ± 0.28	0.44 ± 0.23	0.29 ± 0.15	0.46 ± 0.22	0.26 ± 0.13	0.39 ± 0.19	0.66 ± 0.34	0.41 ± 0.20	0.72 ± 0.35
LOM of TOM (%)	8.05 ± 0.56	10.24 ± 0.55	5.49 ± 0.20	6.99 ± 0.38	37.76 ± 4.90	6.65 ± 0.23	5.43 ± 0.05	14.29 ± 0.88	17.08 ± 0.99	25.38 ± 1.10	6.92 ± 0.01	3.2 ± 0.32	9.99 ± 0.66
BPC ($mgCg^{1}$)	4.46 ± 0.04	4.44 ± 0.04	2.35 ± 0.03	3.13 ± 0.02	3.29 ± 0.06	2.3 ± 0.06	1.52 ± 0.04	2.39 ± 0.02	1.42 ± 0.05	1.82 ± 0.04	3.59 ± 0.02	2.23 ± 0.02	3.78 ± 0.03
Protein:Carbohydrate	32 ± 2.67	44.21 ± 5.23	3.81 ± 0.15	5.84 ± 0.14	18.46 ± 2.19	7.35 ± 0.25	5.98 ± 0.29	16.29 ± 1.30	3.95 ± 1.63	122 ± 163	7.38 ± 0.44	12.6 ± 1.57	16.69 ± 0.66
Lipid:Carbohydrate	4.43 ± 0.44	1.74 ± 0.19	0.71 ± 0.16	1.79 ± 0.03	5.71 ± 0.61	0.05 ± 0.04	0.28 ± 0.07	2.13 ± 0.11	0.05 ± 0.02	0.67 ± 0.69	0.71 ± 0.02	3.12 ± 0.31	2.56 ± 0.07
January 2017													
Environmental variables	**S 1**	**S 2**	**S 3**	**S 4**	**S 5**	**S 6**	**S 7**	**S 8**	**S 9**	**S 10**	**S 11**	**S 12**	**S 13**
Temperature (°C)	28 ± 0.30	29 ± 0.30	29 ± 0.20	26 ± 0.51	27 ± 0.41	28 ± 0.55	30 ± 0.52	29 ± 0.40	28 ± 0.30	28 ± 0.43	28 ± 0.17	29 ± 0.34	28 ± 0.37
pH	8.62 ± 0.03	8.37 ± 0.03	8.2 ± 0.12	8.13 ± 0.04	7.77 ± 0.02	8.39 ± 0.04	8.37 ± 0.02	8.18 ± 0.03	8.49 ± 0.03	8.07 ± 0.04	8.21 ± 0.01	8.41 ± 0.03	8.42 ± 0.01
EC (µs/cm)	6.25 ± 0.03	6.3 ± 0.29	8.04 ± 0.02	7.8 ± 0.32	6.48 ± 0.01	6.38 ± 0.02	6.27 ± 0.01	6.21 ± 0.04	6.34 ± 0.02	6.25 ± 0.02	6.34 ± 0.01	6.27 ± 0.03	6.14 ± 0.03
Salinity (ppt)	25.8 ± 0.2	26.3 ± 0.15	26 ± 0.25	19.1 ± 0.32	24 ± 0.35	26.3 ± 0.30	27 ± 0.25	28.3 ± 0.20	27 ± 0.37	26.7 ± 0.25	28.6 ± 0.26	29.1 ± 0.39	26.4 ± 0.1
Redox potential (mv)	-393 ± 3.05	-291 ± 5.00	-234 ± 3.51	-301 ± 2.00	-262 ± 2.51	-209 ± 2.08	-277 ± 3.60	-302 ± 2.51	-229 ± 4.16	-234 ± 1.52	-289 ± 2.51	-175 ± 2.51	-391 ± 2.51
Sulphate (mg/g)	58.27 ± 0.02	37.72 ± 0.02	14 ± 0.20	15.66 ± 0.02	19.27 ± 0.04	33.72 ± 0.02	20.05 ± 0.03	25.55 ± 0.03	8.55 ± 0.03	17.77 ± 0.02	34.16 ± 0.03	4.44 ± 0.02	38.05 ± 0.02
C_{org} (%)	3.37 ± 0.02	0.9 ± 0.05	0.52 ± 0.03	1.35 ± 0.03	0.37 ± 0.01	0.3 ± 0.17	0.07 ± 0.02	0.6 ± 0.15	0.3 ± 0.11	0.97 ± 0.02	2.17 ± 0.03	5.02 ± 0.03	2.85 ± 0.02
TOM (%)	5.82 ± 0.03	1.55 ± 0.09	0.9 ± 0.06	2.33 ± 0.06	0.64 ± 0.02	0.52 ± 0.29	0.12 ± 0.03	1.03 ± 0.36	0.52 ± 0.20	1.67 ± 0.03	3.75 ± 0.05	8.66 ± 0.05	4.91 ± 0.04
Carbohydrate (mg/g)	0.18 ± 0.01	0.15 ± 0.03	0.94 ± 0.01	0.05 ± 0.01	0.71 ± 0.03	0.73 ± 0.03	0.57 ± 0.02	0.92 ± 0.03	0.23 ± 0.02	0.01 ± 0.01	0.37 ± 0.02	0.17 ± 0.02	0.35 ± 0.02
Protein (mg/g)	3.07 ± 0.02	1.47 ± 0.03	1.24 ± 0.02	1.38 ± 0.01	0.77 ± 0.01	1.44 ± 0.02	0.88 ± 0.02	1.05 ± 0.02	1.32 ± 0.03	0.31 ± 0.02	2.44 ± 0.02	4.51 ± 0.02	3.64 ± 0.03
Lipid (mg/g)	0.96 ± 0.02	1.31 ± 0.02	0.74 ± 0.02	1.08 ± 0.02	1.23 ± 0.02	0.45 ± 0.02	0.27 ± 0.02	0.56 ± 0.03	0.51 ± 0.01	0.71 ± 0.02	0.15 ± 0.02	0.91 ± 0.03	1.43 ± 0.02
LOM (%)	0.42 ± 0.05	0.29 ± 0.07	0.29 ± 0.04	0.25 ± 0.06	0.27 ± 0.07	0.26 ± 0.02	0.17 ± 0.01	0.25 ± 0.03	0.2 ± 0.03	0.1 ± 0.04	0.29 ± 0.01	0.55 ± 0.05	0.54 ± 0.08
LOM of TOM (%)	7.24 ± 0.94	18.97 ± 4.4	32.46 ± 3.61	10.87 ± 2.56	42.03 ± 10.42	50.49 ± 12.48	13.42 ± 76.25	24.51 ± 2.98	39.4 ± 7.46	6.02 ± 2.43	7.92 ± 0.16	6.45 ± 0.60	11.05 ± 1.67
BPC ($mgCg^{1}$)	2.3 ± 0.3	1.76 ± 0.03	1.54 ± 0.02	1.51 ± 0.01	1.58 ± 0.03	1.34 ± 0.02	0.86 ± 0.02	1.3 ± 0.25	1.12 ± 0.01	0.69 ± 0.01	1.46 ± 0.02	2.96 ± 0.02	3 ± 0.01
Protein:Carbohydrate	17.06 ± 1.44	9.8 ± 2.20	1.32 ± 0.001	27.6 ± 7.08	1.08 ± 0.03	1.97 ± 0.04	1.54 ± 0.02	1.14 ± 0.01	5.74 ± 0.44	31 ± 10.78	6.59 ± 0.30	26.53 ± 4.17	10.4 ± 0.67
Lipid:Carbohydrate	5.33 ± 0.35	8.73 ± 2.04	0.79 ± 0.01	21.6 ± 5.35	1.73 ± 0.05	0.62 ± 0.01	0.47 ± 0.01	0.61 ± 0.01	2.22 ± 0.16	71 ± 26.46	0.41 ± 0.04	5.35 ± 0.70	4.09 ± 0.23

Table 2 Two-way ANOVA on environmental variables of Ashtamudi estuary

	Season		Station		Season * Station	
Variables	**F ratio**	**P value**	**F ratio**	**P value**	**F ratio**	**P value**
Carbohydrate(mg/g)	0.013	0.910	12.953**	0.000	7.545**	0.000
Protein(mg/g)	1307.239**	0.000	83.884**	0.000	48.461**	0.000
Lipid(mg/g)	14.492**	0.000	15.233**	0.000	3.638*	0.001
LOM(%)	310.832**	0.000	29.960**	0.000	12.722**	0.000
$C_{org(\%)}$	90.779**	0.000	15.958**	0.000	2.679*	0.007
TOM(%)	90.779**	0.000	15.958**	0.000	2.679*	0.007
LOM of TOM(%)	200.676**	0.000	69.189**	0.000	71.196**	0.000
Sulphate(mg/g)	1333.328**	0.000	697.103**	0.000	84.337**	0.000
pH	9.769*	0.003	0.499	0.906	0.413	0.952
Temperature (°C)	4.318*	0.043	2.268*	0.021	3.733**	0.000
Conductivity (µs/cm)	1245.138**	0.000	3.085	0.002	5.463**	0.000
ORP	4.832*	0.032	9.204**	0.000	30.056**	0.000
salinity	15.091**	0.000	0.050	1.000	0.045	1.000
Substrate induced enzyme activity (Mesophilic)	1.836	0.181	1.966*	0.047	2.178*	0.027
Substrate induced enzyme activity (Thermophilic)	0.762	0.387	2.165*	0.028	1.034	0.434
Native enzyme activity	165.349**	0.000	31.944**	0.000	32.167**	0.000
BPC($mgCg^{1}$)	67764.499**	0.000	8799.901**	0.000	3014.457**	0.000
Protein: carbohydrate	27.555**	0.000	16.801**	0.000	9.381**	0.000
Lipid: Carbohydrate	8.232*	0.006	3.759**	0.000	4.064**	0.000

** $P < 0.001$; *$P < 0.05$

Abundance and Distribution of Cellulolytic Anaerobes

The abundance of cellulolytic anaerobes was comparatively higher during January 2017 than April 2016 (Table 3). During both seasons, maximum cellulolytic population was observed with cellobiose and limited or slow growth with crystalline cellulose as substrate, which is attributed to the insolubility nature of crystalline cellulose (Leschine 1995). In S7 (open estuary) maximum growth was observed in April 2016 and S8 (open estuary) in January 2017 for cellobiose substrate. In case of crystalline cellulose substrate, S12 (Kollam city region) showed maximum growth in April 2016 and S9 (open estuary) in January 2017. The cellulolytic population of cellulolytic anaerobes were very low in Ashtamudi estuary as compared to the populations of other group of obligate anaerobes like methanogens and sulphate reducing bacteria (Reshmi et al. 2015).

Cellulolytic Enzyme Activity

In natural lake sediments, most of the cellulose degradation occurs aerobically and only 5–10% is available for anaerobic degradation (Leschine 1995). During April 2016, native enzyme activity (NEA) was predominant in the open estuary (Fig. 2). Whereas, during January 2017, NEA was more pronounced in the Kallada River region, that might be due the confluence of Kallada river and the availability of fresh organic load from the river as native substrate. Transport of terrestrial organic matter by river includes plant residues, agricultural residues, eroded top soils and became fresh substrate for inhabitant microbial communities (Meybeck 1993). In this study, the cumulative substrate induced enzyme activity (SIEA) (Figs. 3 and 4) was more than NEA. This reveals the potential of added substrates to induce cellulosic activity. Previous studies in Lake Gooimeer also showed increased activity of β –glucosidase by addition of cellulose (Boschker and Cappenberg 2006). During April 2016, thermophilic activity was predominant in the Kallada River region, whereas mesophilic enzyme activity was higher in the Kallada River and Kollam city region of the estuary. During January 2017, thermophilic cellulase activity predominated in the open estuary and mesophilic enzyme activity in the Kollam city and Kallada River regions. Overall cellulolytic activity was higher towards Kallada River region of the estuary. Biogeochemical analysis of Ashtamudi estuarine sediment by Jennerjahn et al. (Jennerjahn et al. 2008) revealed that, most of the Kallada River load is deposited in the upper part of the estuary and middle and lower parts are subjected to strong marine influence. So, it is evident that the riverine dissolved organic load contains sufficient amount of labile fraction, that can be easily consumed by the microbial communities (Abril et al. 2002).

Table 3 Factor loadings for various environmental variables

	Rotated Component Matrix[a]			
		Components		
		PC1	PC2	PC3
April 2016	Temperature(°C)	0.181	0.739	−0.069
	pH	0.511	0.203	0.182
	EC(µs/cm)	0.754	−0.241	0.172
	Salinity	0.462	−0.546	−0.031
	Redoxpotential	0.111	0.072	0.408
	Sulphate(mg/g)	0.828	0.230	0.213
	C_{org}(%)	0.938	0.178	−0.097
	TOM(%)	0.938	0.177	−0.098
	Carbohydrate(mg/g)	0.161	−0.005	−0.757
	Protein(mg/g)	0.520	0.325	0.708
	Lipid(mg/g)	0.091	0.941	−0.056
	LOM(%)	0.493	0.514	0.633
	BPC($mgCg^{1}$)	0.501	0.600	0.525
	Protein:carbohydrate	−0.292	−0.273	0.743
	Lipid:carbohydrate	−0.025	0.829	0.299
	Eigen value	5.125	3.525	2.829
	% of Variance	32.029	22.034	17.682
	Cumulative %	32.029	54.063	71.744
January 2017	Temperature(°C)	−0.050	−0.217	0.811
	pH	0.458	0.070	0.500
	EC(µs/cm)	−0.144	−0.098	−0.591
	Salinity	0.056	−0.071	0.938
	Redoxpotential	−0.544	0.057	0.278
	Sulphate(mg/g)	0.490	−0.149	−0.016
	C_{org}(%)	0.750	0.554	0.239
	TOM(%)	0.750	0.554	0.239
	Carbohydrate(mg/g)	−0.121	−0.859	0.102
	Protein(mg/g)	0.870	0.283	0.301
	Lipid(mg/g)	0.534	0.167	−0.569
	LOM(%)	0.948	0.099	0.123
	BPC($mgCg^{1}$)	0.946	0.156	0.034
	Protein:Carbohydrate	0.068	0.962	−0.183
	Lipid:Carbohydrate	−0.478	0.740	−0.190
	Eigen value	5.060	3.615	2.862
	% of Variance	31.627	22.592	17.890
	Cumulative %	31.627	54.219	72.109

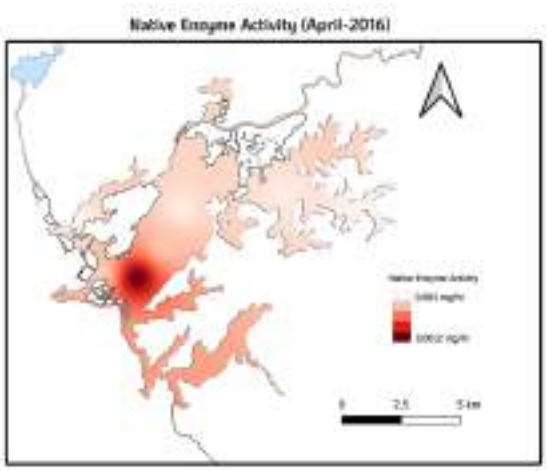
Extraction Method: Principal Component Analysis

Rotation Method: Varimax with Kaiser Normalization

Environmental Controls on Abundance and Activity of Cellulolytic Anaerobe

The spatial factors of the habitat are more important for shaping the structure of sedimentary microorganisms (Yang et al., 2016). The production of cellulolytic enzymes and their activity mainly depends upon the size of inoculum, pH, temperature, various activators, additives and inhibitors of the environment (Immanuel et al. 2006). The relationship between environmental variables and cellulolytic activity were analysed using principal component analysis (PCA) and redundancy analysis (RDA). The PCA generated three principal components (Table 3; Fig. 5). During April 2016, the first PC accounted for 32.02% of total variance. Whereas PC2 and PC3 explained 22.03% and 17.68% of total variance respectively and also explained a cumulative variance of 71.74%. In the first PC, EC, sulphate C_{org} and TOM showed strong positive loadings and the factors were collectively referred as conductivity- nutrient factor. In PC 2 lipid:carbohydrate ratio showed strong positive loading and in PC3 carbohydrate showed strong negative loadings.

During January 2017, the first PC accounted for 31.62% of total variance. Whereas PC2 and PC3 explained 22.59



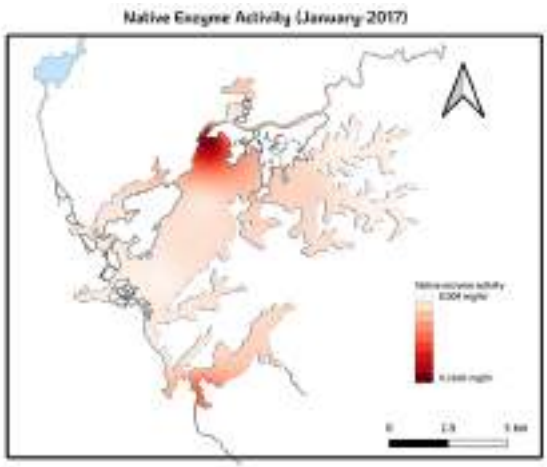
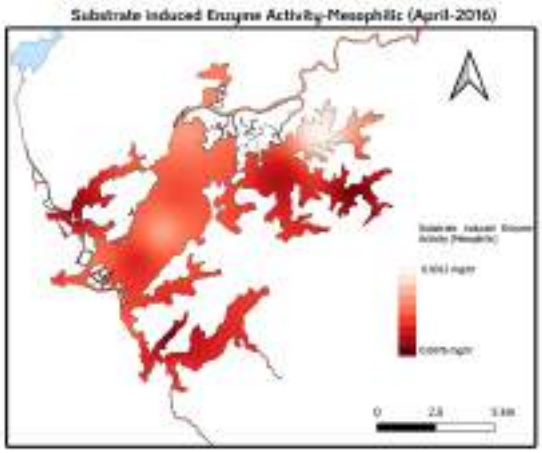


Fig. 2



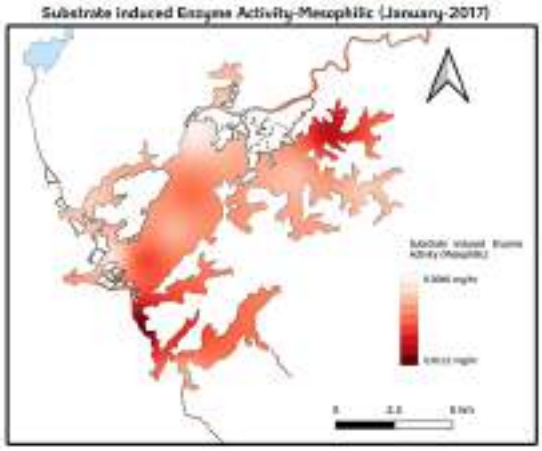


Fig. 3

and 17.89 of total variance respectively and also showed a cumulative variance of 71.74%. In the first PC, C_{org}, TOM, protein, LOM and BPC showed strong positive loadings and was referred as nutrient factor. The composition of organic matter can influence extra cellular enzyme activities in lake sediments (Boschker and Cappenberg 2006). The second PC exhibited strong positive loading for protein:carbohydrate ratio and had a negative loading for carbohydrate. The third PC showed high positive loading on temperature and salinity. Hence, the factor was named as temperature- salinity factor. Comparing PCA results of the two seasons it is evident that the organic matter turned more labile during January 2017. For monsoonal estuaries, complex fractions of organic matter derived from multiple sources undergoes microbial degradation and became labile (Gawade et al. 2018).

During April 2016, RDA output showed that lipid followed by salinity, protein and C_{org} (explanatory variables) were the highest determining factors for explaining most of the variation in the abundance and activity of cellulolytic anaerobes (response variables) (Fig. 6). During January 2017, salinity followed by sulphate and organic matter were the highest determining factors. Energetic requirements of bacteria are associated with higher salinity and that can affect the abundance and activity of microbial groups (Gu et al. 2012).

Conclusion

Estuarine ecosystems are an untapped resource for cellulosic biomass which provide opportunity for identifying novel microbial species that carry cellulase enzyme with novel biotechnological potential. Microbial cellulases are now commercially produced by several industries globally and are being widely used in food, animal feed, fuel, paper industry, textile industry and also various chemical industries. In the present work, even though, cellulolytic

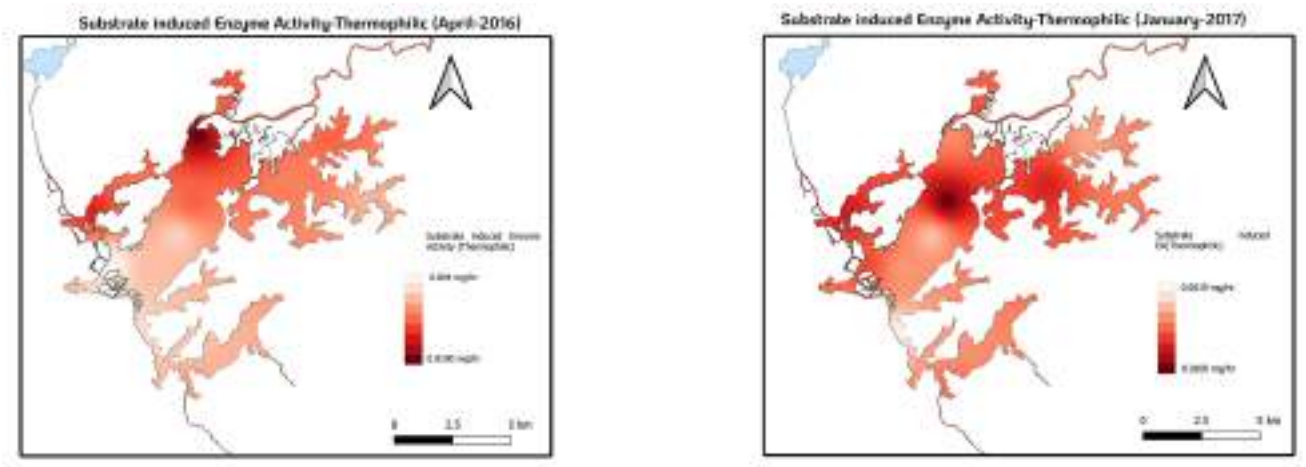


Fig. 4

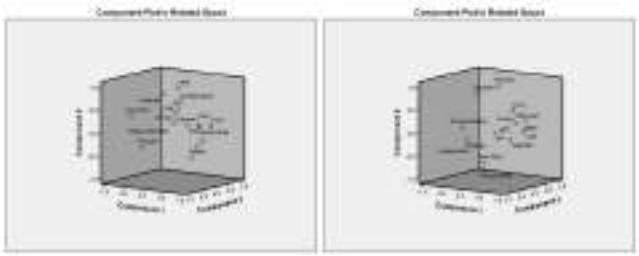


Fig. 5

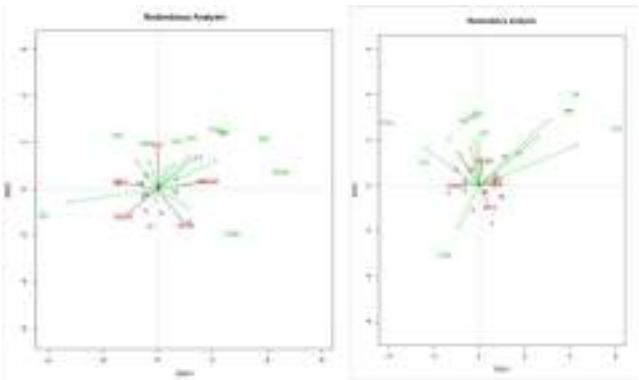


Fig. 6

anaerobic abundance was found to be very low in Ashtamudi estuarine sediments, higher enzyme activity was observed in thermophilic conditions than mesophilic conditions. This clearly updates the evidence of tapping the enormous potential of thermophilic anaerobic cellulolytic bacteria for commercial and industrial applications. The substrate induced enzyme activity was more than native activity, which shows that addition of substrates induced the growth of cellulolytic anaerobe and enhanced their enzyme activities in the sediments. RDA revealed the importance of salinity and lipid with enzyme activity. To meet the growing demand for cellulases and to realize their full potential in biotechnology and research, continued research on bioprospecting cellulolytic microbes from potential sources such as estuarine sediments are vital. The development of rapid and reliable methods for the screening of cellulases from microorganisms within inhospitable environments like estuaries will allow a greater number of novel bacterial cellulases to be isolated with purpose for various applications in future.

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Data Availability Most of the data produced from this study are provided in this manuscript and the remaining datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Code Availability Not Applicable.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval Not Applicable for this study.

Consent to Participate Not Applicable.

Consent for Publication Not Applicable.

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