XYLANOLYTIC ENZYMES FROM MARINE BACTERIA AND THEIR APPLICATION IN PAPER AND PULP INDUSTRY

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Goa University

BY

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DECLARATION/STATEMENT

As required under the University Ordinance OA-19A, I hereby declare that the present thesis entitled **"Xylanolytic enzymes from marine bacteria and their application in paper and pulp industry"** is my original work carried out in the National Institute of Oceanography, Dona Paula, Goa and the same has not been submitted in part or full elsewhere for any other degree or diploma to any institute or University.

The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgements have been made wherever facilities and suggestions has been availed of.

There were no corrections/modifications suggested by the honorable examiner (s).

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CERTIFICATE

This is to certify that **Mr. Pankaj Parab** has duly completed the thesis entitled **'Xylanolytic enzymes from marine bacteria and their application in paper and pulp industry'** under my supervision for the award of the degree of Doctor of Philosophy.

This thesis being submitted to the Goa University, Taleigao Plateau, Goa for the award of the degree of Doctor of Philosophy in Microbiology is based on original studies carried out by him.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institutions.

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।। गुरुर्ब्रह्मा गुरुर्विष्णुः गुरुर्देवो महेश्वरः ।।

।। गुरुरेव परं ब्रम्ह तस्मै श्रीगुरवे नमः ।।

Since the eternal time, our tradition has given the supreme place to our Guru in our life. The Guru is the dispeller of Darkness; HE is Brahma, the creator of Knowledge, HE is Vishnu, the protector and sustainer of our acquired knowledge, and HE is Maheshwara, the destroyer of our Ignorance. The Guru is Verily the Para-Brahman, the Supreme Being with ultimate consciousness and therefore I express my deep sense of gratitude and offer my salutation to my Guru, Dr. Rakhee Khandeparker, Principal Scientist, National Institute of Oceanography, Goa. I am very fortunate that she took me under her wings and gave her continuous guidance, useful advice and invaluable support throughout my research work. Irrespective of whether it was my personal or professional life battlefront she stood behind me with unwavering support. Her unflinching convictions will always inspire me, and I hope to work with her noble thoughts. I earnestly thank her for inculcating in me scientific temperament and appreciable work ethics which helped me to achieve this goal.

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Pankaj Parab

Dedicated to my Beloved

Family

Table of Contents

Chapter 1

General Introduction

1.1 Introduction

The gravity of modern biotechnological applications has been increased noticeably over the past few decades, and it escalated rapidly from the beginning of the twentieth century. The applications underscored their significance in various industrial processes and products, which ultimately improved the living standards. The application of enzymes in multiple processes is one of the premier contributions of modern biotechnology. The benefits of enzymes are well known from ancient times but modern biotechnology proved its potential in current industrial processes as well. An enzyme is a bio-molecule, almost always a protein, having a catalytic domain and prevailed in all living forms to perform complex metabolic pathways for their survival. Microorganisms are well known for their ability to produce enzymes. The type and characteristics of the enzymes produced by microbes vary based on metabolic requirements of the microbe and physio-chemical conditions of the surrounding environment. In general, enzymes enhances any biochemical reaction rate by lowering the activation energy of the reaction and thus also known as bio-catalysts. Unlike processes carried out using chemical catalysts, the processes involving these bio-catalysts are less energy-intensive. These molecules are highly unambiguous and carry out a narrow range of reactions with specific substrates.

1.2 Enzyme

The usage of enzymes is known from the era of the earliest human civilizations. By the early 18^{th} century the processes like meat digestion by stomach secretions and starch conversion to sugars were known to mankind but the actual mechanisms in the back of these processes were still unknown. It was 1833 when a French chemist Anselme Payen discovered first enzyme, diastase. After a few decades while studying sugar fermentation to alcohol using yeast Louis Pasteur inferred that the process is the result of some vital force within living cells. In 1877, Wilhelm Kuhne for the first time coined the term enzyme. The remarkable "Lock and Key model" was demonstrated by Emil Fischer in the year 1894. Eduard Buchner in the year 1897 submitted report in which the use of cell-free extracts in the fermentation process was described. These were some the milestone works carried out in the field of enzymology which gave an early kick start to this subject area. The entire 19^{th} century witnessed some of the crucial breakthroughs in enzymology subject which unfurled

various applications of enzymes, and ultimately paved path for some of the recent developments.

Heckmann and Paradisi, (2020) described in their article some of the major developments that occurred in the area of enzymology (**Figure 1.1**).

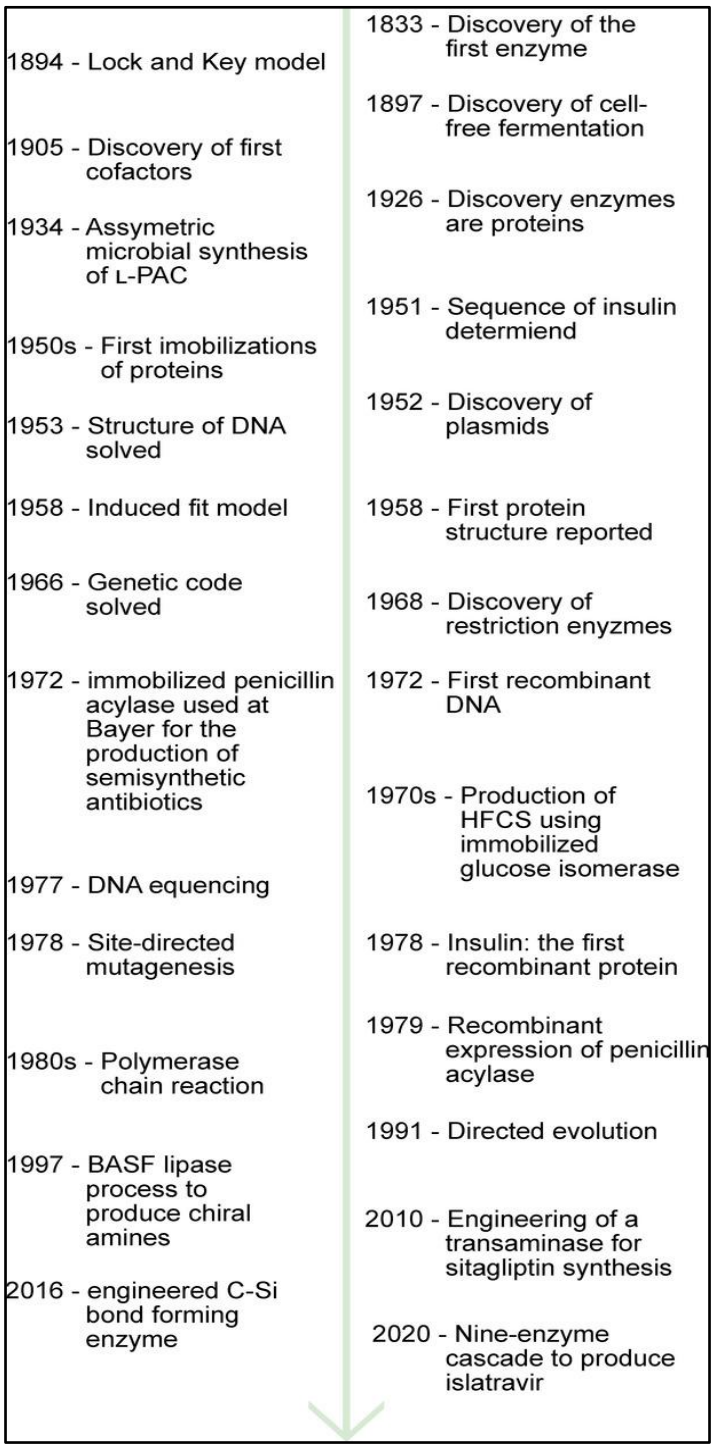

Figure 1.1 Timeline of major developments in enzymology, molecular biology, and biocatalysis (Heckmann and Paradisi, 2020)

1.3 Xylan

The broad classification of plant cell wall leads us to celluloses, hemicelluloses, pectin and at some extent proteins (Hao and Mohnen, 2014) (**Figure 1.2**). The overall proportion of these constituent components varies in plant cell wall based on geographical location and botanical origin. Hemicelluloses are the components which act as cementing agent for celluloses and other biopolymers. These are the ones which exhibit rigidity to plant along with other components such as lignin. Some of the major terrestrial wood hemicelluloses include xylan, xyloglucans, mannans, glucomannans, arabinoxylan, galactan, (Scheller and Ulvskov, 2010; Polizeli et al., 2005). Some of these hemicelluloses form a complex structure made of more than one polymer. The level of complexity of any hemicellulose composition describes its susceptibility to enzymatic damage. More the complex hemicellulose polymer, it requires an equally complex enzyme system to de-polymerize it, effectively and efficiently.

Xylan is the second most abundant biopolymer and one of the major hemicellulosic polysaccharide present in the plant cell wall. The structure of xylan is variable, fluctuating from linear polysaccharide made up of D-xylosyl units connected by β-1,4- glycosidic bonds to highly branched heteropolysaccharides. The 'hetero' prefix indicates a combination of other substituent sugar moieties other than xylose attached to the main backbone chain and side branches. The complexity increases with an increase in number and types of sugar residues attached which is shown in **Figure 1.3**. It's side chains are composed of various moieties which leads to the formation of its various derivatives which are as follows; Arabinoxylan (L-arabinofuranose moiety attached to xylose by α-1,2 or α-1,3 glycosidic linkages), in certain cases even ferulic acid esters from lignin are attached with arabinofuranose sugars at *O*-5 position. Glucuronoxylan (4-*O*-methyl-D-glucuronic acid linked to xylose backbone by the α-1,2-glycosidic bond), Acetylated xylan (Acetic acid esterifies xylose units at *O*-2 or *O*-3 positions) (Shallom and Shoham, 2003; Madeira Jr et al., 2017). Xylan holds the major percentage in hardwood plants as compared to softwood plants and its overall content varies in wood from 15-30% to 7-12% respectively (Richards and Whistler, 1970). Since it is present in abundance, it becomes one of the major renewable energy source and industrially important raw material.

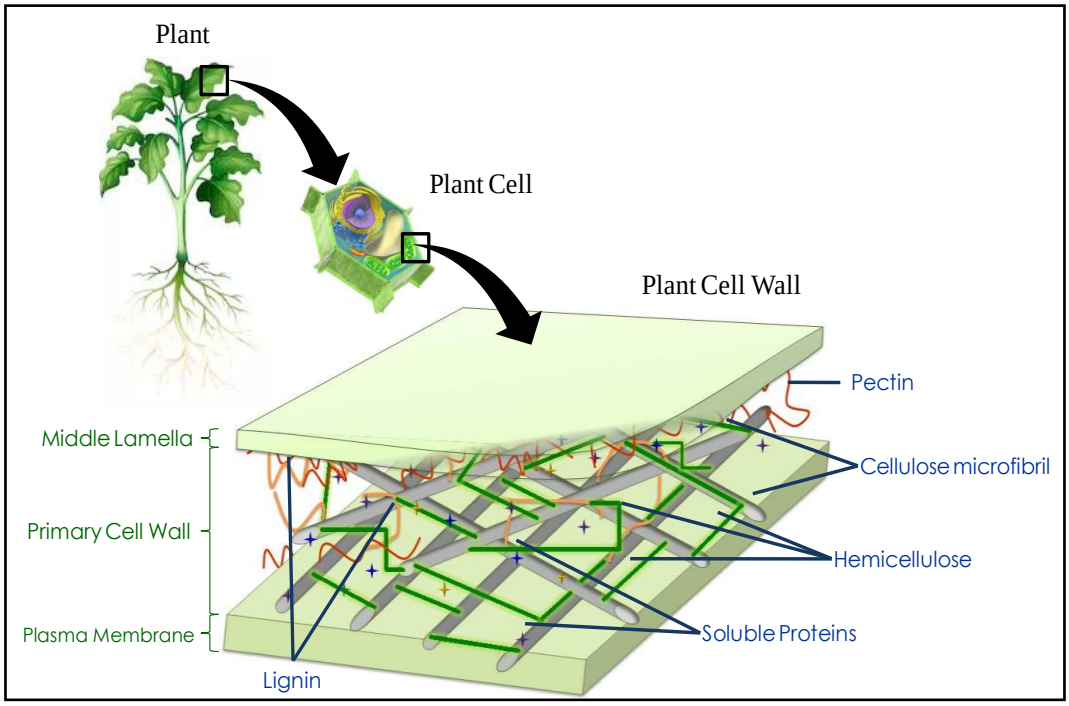

Figure 1.2 Components and structure of plant cell wall

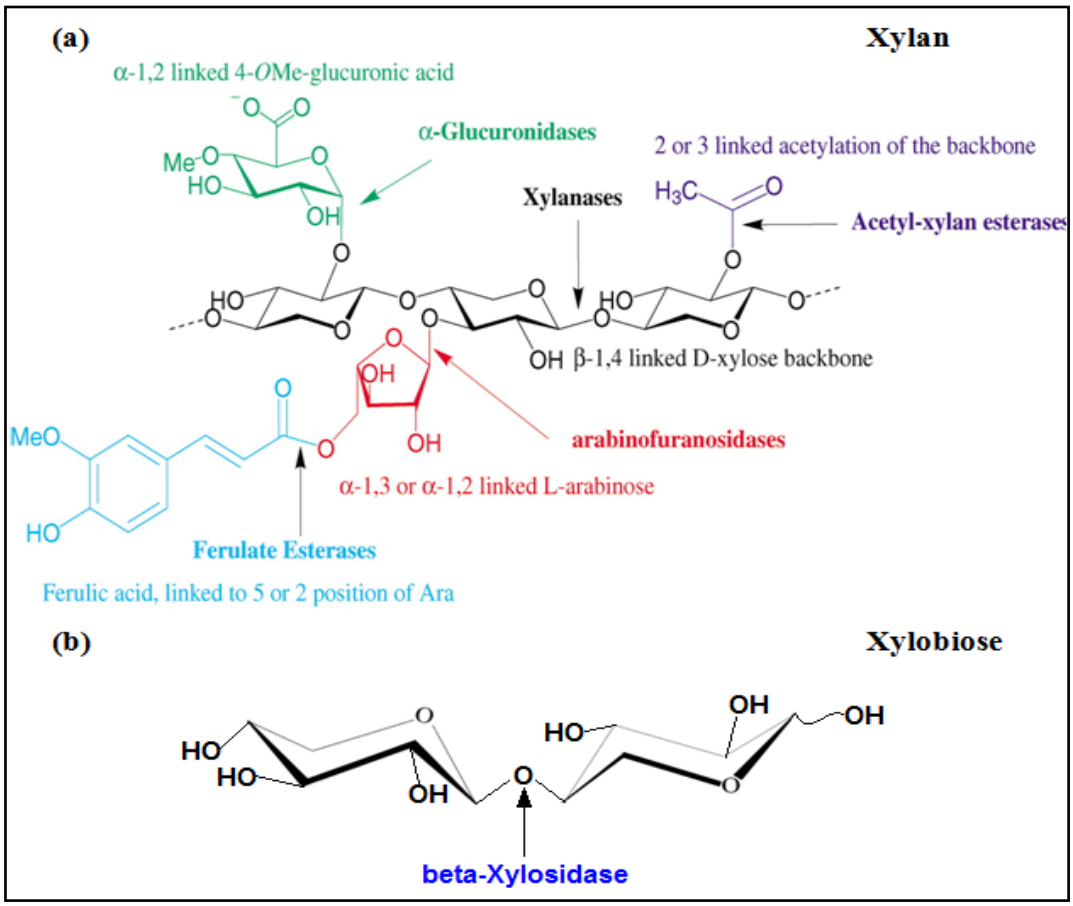

Figure 1.3 Structure of xylan and catalytic sites of xylanolytic enzymes

1.4 Xylanolytic enzymes

A set of enzymes responsible for catabolic hydrolysis of xylan is collectively known as xylanolytic enzyme consortia. This consortium is comprised of enzymes which are operational at the distinct site of action on xylan molecule (**Table 1.1**). The degree of de-polymerization or hydrolysis by a xylanolytic enzyme is inversely proportional to the degree of hetero-polymerization of xylan. The degree of hetero-polymerization and unambiguous nature of enzymes with substrate specificity are the primary reasons for multiple enzymes requirement for complete hydrolysis of complex xylan molecule. Based on the site of cleavage these xylanolytic enzymes categorized as primary and accessory xylanolytic enzymes. Endo-xylanase (EC. 3.2.1.8) and β-xylosidase (EC. 3.2.1.37) are considered to be primary xylanolytic enzymes, wherein prior one randomly cleaves glycosidic linkages of linear xylan backbone and latter hydrolyzes glycosidic linkages of intact and fragmented xylan oligomers (including xylobiose) strictly from reducing end. Enzymes such as α-L-arabinofuranosidase (EC.3.2.1.55) which hydrolyzes non-reducing α-L-arabinofuranoside residues, α-glucuronidase (EC.3.2.1.139) which cleaves α-D-glucuronoside and acetyl xylan esterase (EC.3.1.1.72) which catalyzes the hydrolysis of acetyl groups linked to linear xylan chain are known to be accessory xylanolytic enzymes. Unless the catalytic sites for primary xylanolytic enzymes are not freely available for hydrolysis on xylan linear chain, these enzymes may not work efficiently. Hence for better and effective xylan hydrolysis results, all the xylanolytic enzymes should work synergistically together rather than an individual. With this regard, a brief description of these enzymes is given below.

Table 1.1 Xylanolytic enzymes, their substrate and family classes

Name of the enzyme	Substrate	EC	GH/CE family
Endo-1,4-β xylanase	β-1,4-xylan	3.2.1.8	GH5, 8, 10, 11, 43
β-xylosidase	β-1,4-xylooligomers Xylobiose	3.2.1.37	GH3, 39, 43, 52, 54
α-L-arabinofuranosidase	α-arabinofuranosyl (1→2) or (1→3)xylooligomers α-1,5-arabinan	3.2.1.55	GH3, 43, 51, 54, 62
α-glucuronidase	4-O-methyl-α-glucuronic acid(1→2) xylooligomers	3.2.1.139	GH67
Acetyl xylan esterase	2- or 3-O-acetyl xylan	3.1.1.72	CE 1, 2, 3, 4, 5, 6, 7

1.4.1 Endo-1,4-β xylanase

Endo-1,4-β xylanase (EC-3.2.1.8) also know by other synonyms such as 1,4-β-xylan xylanohydrolase and β-xylanase or simply xylanase. It is specialized to cleave glycosidic linkages of xylan backbone (**Figure 1.3a**). Xylan molecule is converted to small oligomers by xylanase (Khandeparkar and Bhosle, 2006a) and ultimately gets converted to mono, biose and triose units (Khandeparker et al., 2017). The efficiency of the xylanase enzyme depends on the nature of the substrate molecule along with physiological parameters. Nature of substrate includes chain length, degree of branching and type of substituent residues attached to xylose linear chain (Reilly, 1981; Li et al., 2000).

1.4.2 β-Xylosidase

β-Xylosidase (EC. 3.2.1.37) is another group member of the xylanolytic enzyme system which is also known with other synonyms such as; xylan 1,4-β-xylosidase, β-D-xylopyranosidase. These are specific to act on glycosidic linkages of D-xylose residues at reducing end of xylan backbone as well as smaller xylan oligomers, including xylobiose (**Figure 1.3b**). Due to its mode of action on xylan molecule, it is also known with another synonym exo-1,4-xylosidase. During initial depolymerization of xylan, it may not have much role to play, since xylan is not its specific substrate, but once main xylan linear chain is de-polymerized into xylooligosaccharides by endo-xylanase it works cooperatively along with endo-xylanase. The accumulation of xylo-oligomers may create hindrance for endo-xylanase enzyme action, but due to beta-xylosidase the hydrolysis of xylan can be more efficiently carried out (de Vargas Andrade et al., 2004).

1.4.3 α-L-Arabinofuranosidase

α-L-Arabinofuranosidase knew with other synonyms such as arabinosidase, α-arabinosidase, α-L-arabinosidase and α-L-arabinofuranoside hydrolase. This enzyme is usually known to be auxiliary or accessory enzyme since it doesn't hydrolyze D-xylosyl backbone. It assists primary xylanolytic enzymes by reducing the degree of polymerization by cleaving L-arabinose residues attached at 2 and 3 positions of arabinoxylan backbone (**Figure 1.3a**). Depending on its mode of action it is categorized into two distinct types, exo and endo arabinofuranosidases. Exo-type α-L-arabinofuranosidase (EC.3.2.1.55) hydrolyzes *p*-nitrophenyl-α-L-arabinofuranosides

and branched arabinans from the non-reducing end, whereas endo-type α-L-arabinanase (EC.3.2.1.99) primarily act upon linear arabinans (Kaneko et al., 1993; de Vries et al., 2000).

1.4.4 α-Glucuronidase

α-Glucuronidase (EC.3.2.1.139) is another accessory xylanolytic enzyme which play an important role in complete xylan hydrolysis. It is active only in presence of glucuronoxylan substrate since it can only hydrolyze α-1,2 linkages present between glucuronic acid residues and β-D-xylopyranosyl backbone (**Figure 1.3a**). However, substrate specificity varies with the microbial sources and some glucuronidases can cleave intact polymer. The activity of these enzymes can be partially hindered by the presence of the acetyl group in close vicinity of glucuronic residues on the xylan backbone (Harris and Ramalingam, 2010).

1.4.5 Acetyl xylan esterase

Acetyl xylan esterase (EC.3.1.1.72) can hydrolyze only acetylated xylan. In acetylated xylan molecule, it hydrolyses bonds present between *O*-acetyl group attached at positions 2 and/or 3 on xylan backbone (**Figure 1.3a**). This enzyme plays a crucial role in increasing accessibility for main xylanolytic enzymes to hydrolyze acetylated xylan linear backbone. Acetyl residues which create a steric hindrance are eliminated by acetyl xylan esterase.

1.5 Xylanolytic enzymes in paper and pulp industry

The global paper consumption is at a record high level and eventually, it will continue to increase. As per studies, the global average paper consumption is about 55 kilograms per person. Such a high level of paper consumption led the world to a major type of industry which is paper and pulp industry. Pulp manufacturing is essentially a process in which cellulose fibres are extracted from the plant material and residual large quantities of organic waste are discarded (Pearson, 1972). These industries are so massive that these became one of the major environment polluting industries. Conventional pulp and paper industries follow two major chemical processes, the 'Kraft' or alkaline process and the sulphite or acidic process (Pearson, 1972). The effluent released from these industries varies by both, volume and type. Depending upon the type of pulping process being used, these industries generate varieties of pollutants which include highly hazardous chlorinated compounds (United

States Environmental Protection Agency, Office of Water 4303, EPA-821-F-97-011November 1997) and various chloro-lignin derivatives. It also includes problems such as pH difference, increase in suspended solids and dissolved solids, lower oxygen concentration etc. (Giri et al, 2014) of water bodies in which untreated effluent is released. Dioxins and furans (Rathna et al., 2018; Singh and Chandra, 2019) which are highly persistent in the environment have a strong affinity for sediments and are bioaccumulative, were also a concern for communities living downstream from mills. These are some serious issues related to this industry which cause serious public concern.

For lessening this elevating pollution problem xylanolytic enzymes with industrial process benevolent characteristics can be employed for the bio-bleaching of cooked kraft pulp which can reduce the use of otherwise required conventional chlorinated compounds which lead to environmental pollution. Colour imparting lignin can be released by hydrolysis of xylan which ultimately enhances the brightness of pulp with a reduced level of chlorine during the bleaching process.

1.7 Aim, outline and scope of the present research

Enzymes assisted biochemical reactions carried out under optimum conditions generate negligible or no hazardous and toxic by-products, which is a major advantage over chemical technologies contributing to environmental pollution. Enzymes could be used in the agricultural sector for processing and recycling of agro-waste and also as an alternative for several chemical technologies creating a massive amount of pollution. Based on recent developments in enzymatical processes these could be considered as practically feasible technologies of the future.

For industrial-scale production of xylanolytic enzymes bacterial isolates such as *Bacillus* sp. are often used as production organisms. The natural ability to produce enzymes even on abundantly available inexpensive substrates makes them ideal organisms for this task. Due to rich bacterial diversity in dynamic environments such as mangroves the anticipation of finding enzymes of novel characteristics is very high. The individual xylanolytic enzyme production by different microorganisms and their applications in different processes has been previously reported. Another set of reports provides statistics about the use of more than one xylanolytic enzymes from the different origin for application purpose underlining limitations of using a single xylanolytic enzyme.

Moving forward from previous research, current research is focused on the isolation of bacteria producing multiple xylanolytic enzymes or xylanolytic enzyme consortia, their characterization and application in kraft pulp pre-treatment. The characteristics of xylanolytic enzymes were marked with special emphasis on paper and pulp industry. In order to fulfil the above-mentioned aspects of research work following major objectives were set:

- ***Isolation and screening of marine bacteria for xylanolytic enzymes with extremophilic characteristics***

  The rationale of this objective was to obtain potential xylanolytic enzyme producers from the mangroves of Goa. Bacterial isolate exhibiting the natural ability to produce xylanolytic enzymes consortia was desirable. As per the literature survey, there are hardly any studies in which xylanolytic enzymes consortia production from single bacterial isolate has been shown.

- ***Extraction, purification and characterization of xylanolytic enzymes***

  The investigation will give insights about optimum physio-chemical conditions required for highest enzyme titer volume production. Also, the knowledge of xylanolytic enzyme characteristics will enable the user to use it at its maximum potential.

- ***Bio-bleaching of pulp using xylanolytic enzyme***

  A comparative study of the cumulative effect of xylanolytic enzymes consortia and solo xylanase on kraft pulp in terms of reduction in kappa number and increase in brightness will impart the efficacy of both enzyme systems. The overall chemical consumption during chemical bleaching process will mark the finest enzyme system through this assessment.

Chapter 2

Review of Literature

2.1 Introduction

Enzymes are biomolecules, mostly proteins with catalytic domains. These are also known as bio-catalyst due to their active role in lowering the activation energy and increasing rate of reaction of various metabolic as well as biochemical reactions. The potential and application of these bio-molecules are not new to the humankind. These are used almost in all kinds of small-scale household as well as large scale industrial processes. The demand for microbial enzymes in recent decades has been increased from close to a billion USD in 1990 to over 2 billion USD in 2005 (Sharma and Kumar, 2013). As per recent reports published by Research and Markets (https://www.researchandmarkets.com, 2020) at the end of 2019, the global enzyme market estimated value was USD 10.0 billion and it is forecasted to reach USD 14.7 billion by 2025. Rapid growths in industrialization worldwide within a short span of timeline led to haphazard accumulation of pollutants and elevation in pollution. To address these issues modern technologies are required which can either replace completely or partially environmental pollution causing agents. Use of enzymes in various industrial processes to replace or to reduce the use of chemical agents is one of the major accomplishments in green technology. In lieu of several recent developments in the field of green technology, particularly applications of enzyme there are still some lacunae which need to be addressed for the smooth functioning of these technologies.

Inadequate supply versus high demand is one of the leading factors of limited usage of the enzyme at an industrial scale, which leads to fierce competition for maximum production of superior quality enzymes by enzyme manufacturing companies. At a global scale enzyme market, xylanolytic enzymes are one the leading shareholder in this segment. The prime reason backing this is their variety of application in various industries. Based on applications these industries are segmented into food (bakery and fermentation), feed and livestock, additive (fodder), agro waste treatment, and xylitol production (Polizeli et al., 2005), human health (Harris and Ramalingam, 2010) and bleaching of wood (paper and pulp).

By considering the quality and the quantity aspects microbial community which consists of bacteria, fungi, yeast, actinomycetes etc. affiliated to different genera and type (Bajaj and Singh, 2010; Khandeparker and Bhosle, 2006a) with a natural ability to produce xylanolytic enzymes using cheaper sources of the substrate are considered

for xylanolytic enzyme production. The production cost of any microbial origin enzyme is primarily dependent on the type of substrate provided to growing microorganism. For industrial-scale production of xylanolytic enzymes, hemicelluloses could be the elite substrate provided to growing microbial isolates. Hemicellulose is the second inexhaustible sustainable biomass in nature and it predominantly exists in all agro-industrial remnants. The agro-industrial remainder is nothing but the residues generated from the growing and processing of raw agricultural products such as crops, fruits, vegetables, meat, poultry and dairy products (Obi et al. 2016). The disposal of these invoked by-products due to infeasibility to convert them into beneficial products for mankind is becoming a major concern in agricultural countries since it causes environmental pollution (Wang et al. 2016). Utilization of these for production of xylanolytic enzymes could become an add-on application in terms of reduction in environmental pollution along with a reduction in the production cost of xylanolytic enzymes.

2.2 Xylanolytic Enzymes

A xylanolytic enzyme is a syndicate of xylan hydrolyzing enzymes which have congregated attention due to their immense potential in many industries. As described in the previous chapter xylanolytic enzyme consortia include primary xylanolytic enzymes, comprising endo-xylanase and β-xylosidase and accessory xylanolytic enzymes comprising α-L-arabinofuranosidase, α-glucuronidase and acetyl xylan esterase.

Endo-1,4-β xylanases (EC-3.2.1.8) of bacterial origin have been reported in numerous research articles and still search for novel xylanase producers is carried out throughout the world. Endo-xylanases with various characteristics such as molecular weight, temperature and pH tolerance have been reported in the literature. There are even reports of extremophilic xylanases extracted from bacterial isolates. Khandeparker and Bhosle, (2006 a,b) reported production of two different endo-xylanases from 2 different bacterial isolates having a molecular weight of ~43 kDa and ~20 kDa respectively. Both the xylanases were most active at temperature and pH optima of 100 °C and 9.0 respectively. Broad range temperature and pH tolerating endo-xylanase have been described by Kapoor and Kuhad., (2007). The xylanase reported was most active in temperature and pH range of 60-68 °C and 7.0-10.0 respectively. Similar findings were also reported by Battan et al., (2007), in which

xylanase was active at broad temperature and pH range of 55-70 °C and 6.0-10.0 respectively. Xylanase with high-temperature optima of 70 °C and broad pH tolerance of 6.0-8.0 was reported by Yang et al., (2007). Similarly, Han et al., (2009) reported the production of xylanase which was having broad range pH tolerance of 5.0-9.0 and temperature optima of 55 °C. Xylanases described by Anuradha et al., (2007); Phitsuwan et al., (2010) and Shanthi and Roymon, (2018) showed maximum activity at alkaline pH (8.0-9.0) and high temperature (50-60 °C). In another report, Mohana et al., (2008) described the production of xylanase with temperature and pH optima of 50 °C and 8.6 respectively. Xylanases with a molecular weight of 24.6 kDa and 43.1 kDa, with maximum activity at 60 °C and 8.0 was reported by Chaturvedi and Khurana, (2016) and Liew et al., (2019) respectively. Some xylanases which were most active at neutral pH are also been reported. Waeonukul et al., (2009) reported 142.726 kDa xylanase with temperature optima of 60 °C; similarly, 55 °C was the optimum temperature for xylanase described by Boucherba et al., (2017). Production of another neutral pH active xylanases having optimum temperature 60 °C and 40 °C and molecular weight of 78.15 kDa and 48.15 kDa were reported by Sari et al., (2018) and Adiguzel et al., (2019) respectively. Acidic pH tolerating xylanases has got numerous applications and with this regard, several reports have been mentioned in literature. Ninawe et al., (2008) described the production of xylanase having a molecular weight of 20.5 kDa and isoelectric point of 8.5. It exhibited maximum activity at pH 6.0 at an incubation temperature range of 60-65 °C. Bouanane-Darenfed et al., (2011) and Seo et al., (2013) reported xylanases having an optimum pH value of 6.5 and 5.0, whereas temperature optima of 70 and 50°C respectively. Xylanase with 50 and 23.3 kDa molecular weight most active at pH 6.4 and 6.0 and temperature 63 and 60 °C were reported by Hung et al., (2011) and Khandeparker et al., (2017) respectively. Cold active xylanase was also been reported with maximum activity at only 30 °C (Han et al., 2018).

β-xylosidase (EC. 3.2.1.37) is produced by microorganisms along with other xylanolytic enzymes as an accessory enzyme but there are some bacterial cultures which produce β-xylosidase as the main enzyme along with other hydrolytic enzymes. Jordan and Wagschal, (2010) and Gharib et al., (2019) described exclusive production of β-xylosidase by bacterial isolates. Similarly, Shao et al., (2011) described the production of β-xylosidase having a molecular size of 73 kDa and pH and temperature

optima of 6.0 and 65 °C. Production of β-xylosidase with a molecular weight of 58 kDa, exhibiting optimum activity at pH 6.5 and temperature 70 °C was reported by Bhalla et al., (2014).

α-L-Arabinofuranosidases (EC.3.2.1.55)are not very commonly produced, unlike xylanases and β-xylosidases. There are very limited reports available which describe exclusive production of AFases by bacterial isolates. Canakci et al., (2007) described the production of AFase having molecular weight 58 kDa. It exhibited the highest enzyme activity at temperature optima between 75-80 °C and pH 6.0. AFase bearing molecular size of ~79 kDa and temperature and pH optima of 65 °C and 6.0 respectively was reported by Raweesri et al., (2008). Similarly, Fujita et al., (2014) also reported production of AFase from a bacterial isolate.

Studies describing the production of α-Glucuronidase (EC.3.2.1.139) from bacterial isolates are very limited. These enzymes are accessory in nature hence most commonly produced in association with other xylanolytic enzymes. Iihashi et al., (2009) reported the production of α-glucuronidase from bacterial isolate with native protein bearing a molecular mass of 115 kDa. The enzyme activity was highest at 30 °C, at pH range of 6.0-7.0. There are other reports describing the production of α-glucuronidase by Wang et al., (2016); Rhee et al., (2017) and Yan et al., (2017) but in these reports, complete characterization was not carried out.

Acetyl xylan esterase (EC.3.1.1.72) of bacterial origin has been reported by Alalouf et al., (2011) and Till et al., (2013). AXE bearing the molecular weight of 31.75 kDa exhibited maximum activity at pH and temperature 8.0 and 50 °C respectively (Hettiarachchi et al., 2019). Kim et al., (2020) reported production of two AXEs from different bacterial isolates which were active at 50 °C and pH 7.0-8.0.

Apart from individual xylanolytic enzyme production, there are several reports in which production of xylanolytic enzyme consortia by bacterial isolates have been described. The number of enzymes produced by bacterial isolates may vary based on metabolic requirements. Pason et al., (2006) described the production of four different xylanolytic enzymes along with some cellulolytic enzymes from single bacterial isolates. All the enzymes reported were active between pH range of 4.0-9.0, whereas temperature range was 50-60 °C. Production of β-xylosidase and arabinosidase having temperature optima of 45 and 40 °C pH optima of 5.6 and 5.0 respectively was

reported by Zeng et al., (2007). Similarly, Lee and Yoon, (2008) reported the production of xylanase and xylosidase. Set of three xylanolytic enzymes production was also reported by Dodd et al., (2009). Anand et al., (2013) and Mi et al., (2014) reported the production of xylanase and xylosidase enzymes. Wherein former article reported xylanase to be most active at pH 7.5 and temperature 70 °C, β-xylosidase was having pH and temperature optima of 7.0 and 60 °C. The latter article measured pH of 7.0 and 5.0 for xylanase and β-xylosidase respectively at a temperature of 75 °C. Khandeparker and Jalal, (2015) described the production of three different xylanolytic enzymes including xylanase, AFase and AXE from two different bacterial isolates which exhibited temperature and pH optima of 80 °C and 8.0 respectively. A similar set of enzymes from different bacteria were also reported by Wang et al., (2018). Reports suggesting the production of a set of four xylanolytic enzymes from solo bacterial isolate have also been available (Baramee et al., 2015; Teeravivattanakit et al., 2016; Liew et al., 2018). The natural ability to produce multiple xylanolytic enzymes describes the significance and potential of bacterial isolates in xylanolytic enzymes production at commercial scale.

2.3 Xylanolytic enzyme producers

There are a plethora of living organisms in nature which are reported in the literature for their natural ability to produce varieties of biomolecules including enzymes. Based on the geographical location and environmental physio-chemical conditions the spectrum of enzyme production is very diverse. According to literature, microbes, molluscs and protozoans possess natural ability to produce varieties of hydrolytic enzymes including xylanolytic enzymes. But among all living forms, microbial communities are extensively studied for xylanolytic enzyme production. Xylanolytic enzymes are one of those industrially valued biomolecules which have significant applications and tremendous demand in enzyme market.

Microbial communities which have diverse genera and species of bacteria, fungi, yeast and actinomycetes are a major stakeholder in xylanolytic enzyme production. All of these produce xylanolytic enzymes along with other enzymes to follow metabolic pathways essential for their conventional functioning. Bacterial and fungal isolates are the most favoured microorganisms for commercial-scale production of xylanolytic enzymes since they exhibit high titer volume production of xylanolytic enzymes (Motta et al., 2013). Although there are several reports of the exorbitant

level of xylanolytic enzyme production using fungal isolates the major problems associated with these enzymes is their low temperature and pH optima. Since most of the fungal isolates are acidophilic in nature the initial pH of growth and enzyme production media need to be maintained less than 7.0. For industrial-scale production of xylanolytic enzymes, bacterial isolates are the mean competitors for fungal isolates due to their uncomplicated nature. The elementary physio-chemical requirements for xylanolytic enzyme production are add-ons of using bacterial isolate. There are numerous reports of production of xylanolytic enzymes by bacteria of superior characteristics, which can be employed in various industrial processes.

In the past few decades, numerous tactics have been developed to improve the natural ability of xylanolytic enzyme production by bacteria, which includes optimization of physio-chemical conditions required for higher enzyme production and development of gene cloning techniques. Both the strategies seem to be productive in their respective way, wherein gene cloning techniques provide freedom of choosing required characteristics and production level of enzyme; physio-chemical conditions optimization technique is more feasible for production of xylanolytic enzymes at commercial scale.

In literature *Bacillus* sp. is the most prominently studied bacterial genus for the production of xylanases. Kapoor and Kuhad, (2007) reported xylanase from *Bacillus pumilus* strain MK001 with broad temperature and pH optima. Similarly, Battan et al., (2007) and Anuradha et al., (2007) described the production of xylanase from *Bacillus* sp. which also showed broad range temperature and pH tolerance characteristics. Boucherba et al., (2017) described xylanases which were active at neutral pH and moderately high temperatures. Endo-xylanase active at acidic pH produced by *Bacillus licheniformis* was reported by Seo et al., (2013), which was having temperature optima of 50 °C. Other xylanolytic enzymes such as β-xylosidase (Gharib et al., 2019) and acetyl xylan esterase (Kim et al., 2020) have also been reported to be produced by *Bacillus* sp. Khandeparker and Bhosle, (2006a, b) reported production of endo-xylanase from *Enterobatcter* and *Arthrobacter* sp. which had extremophilic characteristics. Multiple xylanolytic enzymes from *Paenibacillus curdlanolyticus* strain B-6 with activity over a wide range of pH and temperature was described by Pason et al., (2006). Highly alkalithermostable AFase and AXE produced by *Arthrobacter* and *Lactobacillus* sp. were reported by Khandeparker and Jalal, 2015.

Likewise, there are several reports of xylanolytic enzyme production by bacterial isolates mentioned in **Table 2.1.** To enhance xylanolytic enzyme characteristics recombinant DNA technology is also employed. Wirajana et al., (2010) used *Saccharomyces cerevisiae* as a host organism for expression of a thermostable α-L-Arabinofuranosidase gene.

Fungal isolates are also reported to produce genre of xylanolytic enzymes. *Aspergillus* sp. has been reported by several researchers for production of xylanase (Botella et al., 2007; Dobrev et al., 2007; Koseki et al., 2007; Chidi et al., 2008; Tamayo et al., 2008; Betini et al., 2009; Miao et al., 2015). Similarly, Chávez et al., (2006); Knob and Carmona, (2008) and Terrasan et al., (2010) reported for xylanase production from *Penicillium* sp. *Trichoderma* sp. (Azin et al., 2007; Goyal et al., 2008; Zhang et al., 2011) along with few other fungal species are also reported for xylanase producing ability. GH51 α-L-arabinofuranosidase from *Talaromyces leycettanus* strain JCM12802 was reported by Tu et al., (2019). Wu et al., (2016) reported rice blast disease-causing fungus *Magnaporthe oryzae* producing arabinofuranosidase B protein, which helps it to invade into the plant cell wall. Iembo et al., (2002) reported the production of β-Xylosidase from a strain of *Aureobasidium* sp. Similarly, *Aspergillus versicolor* was reported by de Vargas et al., (2004) for its ability to produce β-xylosidase. Recently Komiya et al., (2017) and Yang et al., (2017) described about the production of acetyl xylan esterase from *Aspergillus luchuensis* and *Penicillium chrysogenum* respectively. The productivity of fungal xylanolytic enzymes has been improved by using *Pichia* sp. yeast cells (Chung et al., 2002; Zheng et al., 2018).

Yeast isolates are another microbial community which actively produces xylanolytic enzymes. In literature *Cryptococcus* sp. has been intensively studied and reported for the production of xylanase enzyme (Iefuji et al., 1996; Petrescu et al., 2000; Gomes et al., 2000). Yanai and Sato, (2000) described production and characterization of AFase from *Pichia capsulata* X91, whereas *Rhodotorula glutinis* is reported for the production of AFase by Martínez et al., (2006). Extracellular Acetyl xylan esterase production from *Rhodotorula mucilaginosa* is described by Lee et al., (1987). From various species of yeast production of β-xylosidase has also been reported (Manzanares et al., 1999; La Grange et al., 2000, 2001; Heo et al., 2004; López et al.,

2015; Mhetras et al., 2016). For scale-up production of α-glucuronidase use of recombinant *Saccharomyces cerevisiae* described by Anane et al., (2013).

Some of the actinomycetes species are also known for the production of xylanolytic enzymes. *Streptomyces* sp. is most often studied for its ability to produce xylanase. Reports described by Raweesri et al., (2008); Ninawe et al., (2008) and Chungool et al., (2008) prove their ability of xylanase production. This species is also reported for the production of AFase (Kaji and Sato, 1981; Olajide et al., 2020), AXE (Tsujibo et al., 1997) and β-xylosidase (Flores et al., 1997). Another actinomycete *Thermomonospora fusca* is also reported for the production of AFase enzyme (Tuncer, 2000; Tuncer and Ball, 2003).

Apart from microorganisms, there are also few reports which describe the isolation of xylanolytic enzymes genes from the rumen of herbivorous animals and environmental samples, which are cloned in a suitable host for the production of xylanolytic enzymes with novel characteristics (Palaniswamy et al., 2008; Zhou et al., 2012; Gruninger et al., 2014; Matsuzawa et al., 2015).

Recent developments in xylanolytic enzyme production have raised the bar of the search for novel xylanolytic enzyme producers. Along with conventional culturing technique metagenomic approach should also be given equal weight.

2.4 Synergistic action of xylanolytic enzymes

The heterogeneous nature of xylan drives xylanolytic enzyme system function in a more organized, cooperative and synergistic way in order to completely hydrolyze xylan into monomeric sugars, acids and monophenols. Due to inaccessible catalytic sites in highly branched xylan molecule xylanase cannot catalyze linear xylan chain and requires other accessory xylanolytic enzymes for de-branching. For better and efficient functioning of xylanase, other xylanolytic enzymes need to function in a synergistic manner. There are two modes of synergisms between xylan specific CAZymes is reported. The first mode being homeosynergy, in which two linear chains hydrolyzing or two side branches cleaving xylanolytic enzymes execute hydrolysis. In the second mode, which is heterosynergy the main chain hydrolyzing and a side branch cleaving xylanolytic enzyme work in conjunction (Malgas et al., 2019).

It is prominent from the literature that linear xylan chain is hydrolyzed by the catalytic action of endo-xylanase. Thus, the random site of action of xylanase produces diverse products such as fragmented and/or branched xylo-oligomers, xylo-biose, triose and tetraose. Arabinoxylanases are a type of xylanases which are active against arabinose sugar substituted in xylan molecule. Similarly, glucuronoxylanases are the type of xylanases whose activity is dependent on methyl-glucuronic acid substitutions. Reducing end-xylanases (Rexs) are responsible for the hydrolysis of xylan linear chain or xylooligosaccharides (XOS) from reducing end which results in the production of short XOS and xylose.

Hydrolysis of XOS which are resultant products of xylanase and Rexs activities are dealt with by β-xylosidases. Unlike xylanases and Rexs it removes D-xylose residues from the non-reducing terminal. α-Arabinofuranosidases enhances saccharification of arabinoxylan in association with other xylanolytic enzymes by specifically cleaving α-L-arabinofuranoside substituent present in arabinoxylan and arabinosyl substituted XOS. α-Glucuronidases exhibits its activity on α-(1,2)-D-(4-O-methyl)-glucuronosyl side chains and its resultant product is glucuronic acid moieties. Acetyl xylan esterases are the type of Carbohydrate Esterases (CEs) which mainly belong to CE families 1-7 and 16 in CAZy database. These enzymes are primarily active against ester bonds of acetylated polysaccharides (acetylated xylan) which results in the liberation of acetic acid.

2.5 Applications

The applications of xylanolytic enzyme revolve around their ability to hydrolyze xylan from lignocellulosic materials present in bulk in nature. This property is utilized in many industries at a commercial level, although during initial stages its use was limited up to animal feed preparation which slowly expanded to food, textiles and paper industry. As per current statistics xylanases and cellulases along with pectinases, account for a total 20% world enzyme market. Intensive use of xylanolytic enzymes in some of the major industries are briefed below.

2.5.1 Bakery and fruit juice industry

Xylanolytic enzymes are employed in bread making industry in association with other enzymes such as alpha-amylase, glucose oxidase, malting amylase and proteases (Polizeli et al., 2005). During recent decade application of these enzymes in bread

making has been tremendously increased due to their potential effectiveness (Butt et al., 2008). Like other hemicelluloses, these enzymes also break down wheat-flour hemicellulose and facilitate water redistribution which makes the wheat dough softer and easy to knead. Delayed crumb formation, allowing the dough to grow, bread volume increase, greater water absorption and improved resistance to fermentation are some the other salient advantages of using xylanolytic enzymes during bread baking process (Polizeli et al., 2005; Butt et al., 2008; Bajaj and Manhas, 2012).

The important processes in the fruit juice industry are extraction, clearing and stabilization. The main problem faced by any fruit juice industry is turbidity in the juice after the extraction procedure. Turbidity reduces the quality and subsequently the value of extracted fruit juice, to enhance its quality juice filtration technique was adopted which resulted in low yields. Nowadays, xylanases, in conjunction with cellulases, amylases and pectinases, lead to an improved yield of juice through liquefaction of fruit and vegetables; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate (Polizeli et al., 2005, Bajaj and Manhas, 2012). With respect to above characteristics requirements of xylanolytic enzymes Sari et al., (2018) and Adiguzel et al., (2019) described potential application of xylanolytic enzyme produced using bacterial isolate in juice and baking processes.

2.5.2 Animal feed

Xylanolytic enzymes are used in the animal feed industry along with other hemicellulolytic and cellulolytic enzymes. Arabinoxylans of the feed ingredients are broken down by these enzymes which reduce the viscosity of raw material (Twomey et al., 2003). Arabinoxylan has got an anti-nutrient effect in poultry since in its soluble form it increases the viscosity of the ingested feed, which can interfere with mobility and absorption of other components (Polizeli et al., 2005). Young swine and fowl produce smaller quantities of endogenous enzymes as compared to adult which are not sufficient enough for digestion, in those conditions feed, supplemented with exogenous enzymes may increase the digestibility. An add-on advantage of this type of diet is that it reduces the undesired residues passed through excreta (Polizeli et al., 2005).

In an experiment conducted by Café et al., (2002), poultry birds were fed with a nutritionally rich diet with or without the 0.1% Avizyme 1500 (xylanase, protease and amylase). Significant higher body weights, less mortality and a greater amount of energy were observed in a group of birds which were supplemented with Avizyme 1500 as compared to the control group. A report by Dhivahar et al., (2020) suggests the production of a xylanolytic enzyme which has got immense potential in partial digestion of poultry feed.

2.5.3 Biofuel

The biofuel industry is still in its naïve stage due to cost-intensive physical, chemical, biological pretreatment operations and slow enzymatic hydrolysis which makes the overall process of lignocellulosic conversion into biofuels less economical than available fossil fuels. There is constant progress in this industry due to extensive research carried out with these xylanolytic enzymes, along with other hemicellulolytic enzymes. Lignocellulosic material is the potential source for the production of biofuel using xylanolytic enzyme for hydrolysis. The hydrolytic products of lignocellulosic material obtained after xylanolytic treatment are a mixture of xylose monomer and oligomers, these monomeric forms of sugar can be converted to xylitol or ethanol (Sreenath and Jeffries, 2000, Paul et al., 2020). Ethanol produced by this process can be utilized as combustible fuel in combination with available fossil fuel.

2.5.4 Neutraceutical industry and Human health

Pre-biotics are the compounds which facilitate the better growth of human intestinal microbiota. Xylooligosaccharides (XOS) has emerged in recent years as pre-biotics and are of great neutraceutical value. These are supplemented in the human diet with lower digestion rate. Experimental studies on *Bifidobacterium* (intestinal bacteria) proved that XOs has beneficial effects on micro-biota as growth-promoting factors, hence xylanolytic enzymes are used to produce these xylooligosaccharides which has potential in nutraceutical industry (Vásquez et al., 2000; Rycroft et al., 2001; Hsu et al., 2004; Yang et al., 2007; Kapoor and Kuhad, 2007; Khandeparker et al., 2017). Along with XOSs arabinoxylans (AXs) as well as arabinooligosaccharides (AXOs) are also of great neutraceutical value. With the interest in a healthful diet, dietary fibres such as arabinoxylans and arabinooligosaccharides are fetching increasing popularity in the food and feed industry. Arabinoxylans and arabinoxylan oligosaccharides show pre-biotic activity.

They also exhibit immunomodulatory and antitumor activity (Mendis and Simsek, 2014). Han et al., (2018) reported production of a xylanolytic enzyme which is found advantageous in saline food processing industry.

2.5.5 Paper and pulp industry

These are one of the largest industries in the world. Xylanolytic enzymes find its major use in this industry during Kraft process, which is a crucial process in terms of quality of pulp obtained and hence the quality of paper produced. Most of the industries follow chemical oriented bleaching process for removal of a brown colour imparting compound called lignin since it is considered as more efficient. Along with the advantage of getting good quality pulp and recycling of chemicals used from black liquor, the major disadvantage come in the form of organo-chlorine compounds, which include chlorolignins which are very complex which comes out into effluents, thus polluting water bodies and water table in close vicinity of effluent discharge. Along with water and soil pollution, air pollution is another concern since there are massive amount of chemicals used and gases liberated has a very strong smell. Another disadvantage of a chemical process is low yield (40-50%) and high cost of bleaching.

The process of biological delignification of pulp using enzymes was first introduced by Viikari et al. (1994). Xylanolytic enzymes in recent years have been widely studied by considering these aspects of paper and pulp industry. These enzymes are used for pre-bleaching of unbleached pulp, which on later stages bleached with chemical processes. The main idea behind this is to reduce the use of chlorinated chemicals used for the bleaching process. This method can reduce the amount of chlorine chemicals up to 30% and 15 to 20 % reduction in organo-chlorines which are liberated in the effluents (Polizeli et al., 2005). It is not necessary to have xylanolytic enzymes in their purest form for these processes but it has to be thermostable and halotolerant since most of the paper and pulp industries run at higher level temperature and higher values of pH.

The efficiency of bacterial xylanolytic enzymes for enzymatic pre-treatment of kraft pulp has been studied worldwide by many research groups (Walia et al., 2017). As compared to other microbial sources bacterial isolates are the most favoured organisms. Multiple reports on xylanolytic enzyme production from *Bacillus* sp. have been available in the literature (Kulkarni and Rao 1996; Shah et al., 1999; Bim and

Franco 2000; Duarte et al., 2000; Dhillon et al., 2000; Poorna and Prema., 2007; Battan et al., 2007) which has potential application in paper and pulp industry. *Streptomyces* sp. is also reported for its potential to produce a xylanolytic enzyme which can be used for delignification of kraft pulp (Beg et al., 2000; Georis et al., 2000; Kansoh and Nagieb, 2004). Khandeparker and Bhosle, (2007) reported the use of xylanase from *Arthrobacter* sp. in biobleaching of kraft pulp. Along with the kraft process, these enzymes also have got an application in the recycling of used paper, in which deinking of paper is a major problem. As per experiments conducted by Poorna and Prema, (2007), the enzymatic extracts were used in mixed waste paper recycling, which resulted in a considerable improvement of the paper strength with high drainage and easy drying up. The results of enzyme application with recycled paper indicated that the effective use of enzymes in fibre separation could reduce the cost of carton paper production.

Table 2.1. List of bacterial isolates producing xylanolytic enzymes (2006-2020)

Bacterial culture	Xylanolytic enzyme produced	Optimum Temperature (°C)	Optimum pH	Reference
Arthrobacter sp.MTCC 5214	Endo- Xylanase	100	9.0	Khandeparkar and Bhosle, 2006a
Enterobacter sp. MTCC 5112	Endo-xylanase	100	9.0	Khandeparkar and Bhosle, 2006b
Paenibacilluscurdlanolyticus strain B-6	Endo-xylanase β-xylosidase Arabinofuranosidase Acetyl esterase	50 -60	4.0-9.0	Pason et al., 2006
Bacillus pumilus	Endo-xylanase	-	-	Poorna and Prema, 2007
Bifidobacterium adolescentis	β-xylosidase Arabinosidase	45	5.6	Zeng et al., 2007
Bacillus pumilus strain MK001	Endo-xylanase	60-68	7.0-10	Kapoor and Kuhad, 2007
Bacillus pumilus	Endo-xylanase	55-70	6.0-10	Battan et al., 2007
Thermobifida fusca	Endo-xylanase	70	6.0-8.0	Yang et al., 2007
Bacillus subtilis ASH	Endo-xylanase	-	-	Sanghi et al., 2008
*Bacillus*sp. St_A	Endo-xylanase	55	9.0	Anuradha et al., 2007
*Bacillus*sp. St_B		50	9.0	
*Bacillus*sp. St_C		55	8.0	
Geobacillus caldoxylolyticus TK4	Arabinofuranosidase	75-80	6.0	Canakci et al., 2007
Paenibacillus woosongensis sp.	Endo-xylanase β-xylosidase	-	-	Lee and Yoon., 2008
Burkholderia sp.	Endo-xylanase	50	8.6	Mohana et al., 2008

Bacterial culture	Xylanolytic enzyme produced	Optimum Temperature (°C)	Optimum pH	Reference
Streptomyces sp.	Arabinofuranosidase	65	6.0	Raweesri et al, 2008
Streptomyces cyaneus SN32	Endo-xylanase	60-65	6.0	Ninawe et al., 2008
Streptomyces sp. PC22	Acetyl esterase	50	6.5-7.0	Chungool et al., 2008
Cellulosimicrobium sp. strain HY-13	Endo-xylanase	55	5.0-9.0	Han et al., 2009
Paenibacillus curdlanolyticus B-6	Endo-β-1,4-Xylanase	60	7.0	Waeonukul et al., 2009
Prevotella ruminicola 23	β-D-Xylosidase Endo-xylanase Ferulic Acid Esterase	-	-	Dodd et al., 2009
Paenibacillus sp. TH501b	α-glucuronidase	30	6.0-7.0	Iihashi et al., 2009
Tepidimicrobium xylanilyticum BT14	Endo-xylanase	60	9.0	Phitsuwan et al., 2010
Selenomonas ruminantium	β-D-xylosidase	-	-	Jordan and Wagschal., 2010
Caldicoprobacter algeriensis sp.	Endo-xylanase	70	6.5	Bouanane-Darenfed et al., 2011
Thermoanaerobacterium saccharolyticum NTOU1	Endo-xylanase	63	6.4	Hung et al., 2011
Thermoanaerobacterium saccharolyticum JW/SL-YS485	β-D-xylosidase	65	6.0	Shao et al., 2011
Geobacillus stearothermophilus	Acetyl xylan esterase	-	-	Alalouf et al., 2011
Geobacillus thermodenitrificans	Endo-xylanase β-D-xylosidase	70 60	7.5 7.0	Anand et al., 2013
Bacillus licheniformis	Endo-xylanase	50	5.0	Seo et al., 2013
Butyrivibrio proteoclasticus	Acetyl xylan esterase	-	-	Till et al., 2013
Caldicellulosiruptor owensensis	Endo-xylanase	75	7.0	Mi et al., 2014

Bacterial culture	Xylanolytic enzyme produced	Optimum Temperature (°C)	Optimum pH	Reference
	β-D-xylosidase		5.0	
Geobacillus sp.	β-xylosidase	70	6.5	Bhalla et al., 2014
Bifidobacterium longum	Arabinofuranosidase	-	-	Fujita et al., 2014
Cellulosibacter alkalithermophilus	Endo-xylanase β-xylosidase Arabinofuranosidase Acetyl esterase	-	-	Baramee et al., 2015
Arthrobacter sp. MTCC 5214, *Lactobacillus* sp.	Endo-xylanase Arabinofuranosidase Acetyl xylan esterase	80	8.0	Khandeparker and Jalal, 2015
Bacillus licheniformis	Endo-xylanase	60	8.0	Chaturvedi and Khurana, 2016
Paenibacillus curdlanolyticus B-6	Endo-xylanase β-xylosidase ArabinoxylanArabinofuranohydrolase	-	-	Teeravivattanakit et al., 2016
Saccharophagus degradans	α-glucuronidase			Wang et al., 2016
*Bacillus tequilensis*BT21	Endo-xylanase	60	6.0	Khandeparker et al., 2017
*Bacillus oceanisediminis*SJ3	Endo-xylanase	55	7.0	Boucherba et al., 2017
Paenibacillus sp. JDR-2	α-Glucuronidase	-	-	Rhee et al., 2017
Amphibacillus xylanus	α-Glucuronidase	-	-	Yan et al., 2017
Geobacillus galactosidasius BS61	Endo-xylanase	60	7.0	Sari et al., 2018
Bacillus sp. MCC2727 & MCC2728	Endo-xylanase	-	-	Shanthi and Roymon, 2018

Bacterial culture	Xylanolytic enzyme produced	Optimum Temperature (°C)	Optimum pH	Reference
Rhodothermaceae bacterium RA	Endoglucanase Endo-xylanase β-glucosidase β-glucosidase β-xylosidase	-	-	Liew et al., 2018
Caldicoprobacter sp. CL-2	Endo-xylanase	-	-	Widyasti et al., 2018
Ruminiclostridium josui Abf62A-Axe6A	Arabinofuranosidase Endo-xylanase Acetyl xylan esterase	-	-	Wang et al., 2018
Luteimonas sp.	Endo-xylanase	-	-	Han et al., 2018
Ochrovirga pacifica	Acetyl xylan esterase	50	8.0	Hettiarachchi et al., 2019
Pediococcus acidilactici GC25	Endo-xylanase	40	7.0	Adiguzel et al., 2019
Rhodothermaceae bacterium RA	Endo-xylanase	60	8.0	Liew et al., 2019
Bacillus sp.	Endo-xylanase	-	-	Dhivahar et al., 2019
Bacillus sp.	β-xylosidase	-	-	Gharib et al., 2019
Acidobacteria bacterium AB60	Arabinofuranosidase Rhamnosidase	-	-	Rodrigues et al, 2020
Bacillus halodurans	Acetyl xylan esterase	50	8.0	Kim et al., 2020
Lactobacillus antri	Acetyl xylan esterase	50	7.0	
Streptomyces fulvissimus CKS7	Endo-xylanase	-	-	Mihajlovski et al., 2020
Pseudomonas nitroreducens	Endo-xylanase	-	-	Dhivahar et al., 2020
Pseudomonas mohnii	Endo-xylanase	-	-	Paul et al., 2020

Chapter 3

Isolation and screening of marine bacteria for xylanolytic enzyme production with extremophilic characteristics

3.1 Introduction

Mangroves are coastal wetland, salt-tolerant forest ecosystem which covers approximately 60-75% of the tropical and subtropical coastline globally (Thatoi et al., 2013; Sahoo and Dhal, 2009; Liang et al., 2007). Asia continent has the largest amount (42%) of the world's mangroves, of which India itself owns 3.3% (4921 km^2). Goa is the smallest state of India and has got total coastal belt approximately 101km which bestowed with 26 km^2 mangrove regions (Giri et al., 2015, http://fsi.nic.in/isfr2017/isfr-mangrove-cover-2017, India State Forest Report, 2015), which includes two major estuaries and several creeks and backwaters, all of which are densely covered with mangrove vegetation. Mangrove forest ecosystems are mainly restricted to intertidal zones of estuaries, sheltered shores, backwaters, deltas, tidal creeks, lagoons, mudflats and marshes of above-mentioned regions (Saddhe et al., 2016; Liang et al., 2007).

These are among the most productive and biologically diverse ecosystems in nature and to support this biodiversity availability of nutrients at the beginning of the food web is necessary. Microbial communities play a crucial role in maintaining productivity and nutritional supply in these ecosystems (Gomes et al., 2011; Soares Junior et al., 2013). Microbes which are found in these regions are adapted towards various physiological conditions such as, periodical change in temperature, water level, salinity, anoxic condition (Saddhe et al., 2016). Adaptation towards these conditions strives them to follow unique pathways which ultimately results in nutrient cycling and mineral transformation by the degradation of organic matter. As per a report by Thatoi et al., (2013) this role is majorly carried out by bacteria, which has many folds higher population than fungi followed by algae and protozoa.

Mangrove regions are full of nutrient-rich organic matter. The major carbon sources such as, celluloses and hemicelluloses are utilized by bacteria for their growth which simultaneously leads to the production of many commercially valuable bio-molecules including xylanolytic enzymes. The unique environmental conditions present in mangroves selectively promote xylanolytic bacteria which can degrade xylan, which is major hemicellulose, by producing consortia of xylanolytic enzymes and also tolerate these harsh conditions.

A Xylanolytic enzyme is a set of enzymes which cleaves complex xylan molecule into smaller oligosaccharides and monomers. Member enzymes of xylanolytic enzyme consortia play a crucial role in the complete hydrolysis of heterogenous xylan molecule by cleaving side chains, followed by a linear chain and fragmented oligosaccharides. Xylanolytic enzymes are classified based on several physicochemical and functional characteristics. One of the classifications is based on amino acid sequence similarity which categorizes them into different glycoside hydrolase (GH) families. Xylanases are classified into GH families 5, 7, 8, 10, 11 and 43 based on the sequence similarities of the catalytic domain (Collins et al., 2005). Among these, GH10 and GH11 xylanases are the most abundant families found in nature, they have unique three-dimensional structures and mechanisms of action (Biely et al., 1997; Jeffries, 1996), and they also differ in substrate specificity to xylan. Members of GH11 are considered as true xylanases due to their stringent substrate specificity on xylans. While in the case of GH10 xylanases, reports available in the literature indicate that these can hydrolyze other polysaccharides along with xylan (Hu et al., 2013; Hu and Saddler, 2018). Similarly, α-arabinofuranosidases are classified into GH families 3, 43, 51, 54 and 62. The acetyl xylan esterases are broadly classified as Carbohydrate-Active enzymes (CAZymes) which catalyze the hydrolysis of acetylated hemicellulose. As per CAZy database AXE belong to 8 families out of all carbohydrate esterases (CE) families (Alalouf et al., 2011). These enzymes are of immense potential in several industries worldwide, such as paper and pulp industry, cattle feed and fodder industry for agro-waste treatment, food industry, bioethanol production, breweries. Since all these industries work under different physiochemical conditions it is important to provide enzymes as per industrial process requirement. There are high possibilities of finding xylanolytic enzymes with unique properties from isolates obtained from mangrove regions.

3.2 Materials and Methods

3.2.1 Collection of samples

Mangrove ecosystem sites from different parts of Goa (India) were selected for sediment and water sample collection, which included 7 sites from North Goa and 6 sites from South Goa (**Fig. 3.1, Table 3.1**). From each site, 2 sediment and 2 water samples were collected. The superficial layer of sediment (0–5 cm) was collected in an aseptic zip lock bag. Surface water samples from different mangrove regions were

collected in sterile plastic tubes. In all sampling locations, the temperature was around 28 °C (±2 °C), whereas surface water temperature was found to be 25 °C (±2 °C). The samples were immediately transported to the laboratory under cold conditions (4 °C) for further processing.

3.2.2 Isolation and purification

Serial dilutions of sediment (10^0-10^{-5}) and water (10^0-10^{-3}) samples were made using sterile 0.8% NaCl solution, and 200 µL volume was inoculated on sterile medium. Zobell marine agar (ZMA) (HiMedia, India), MacConkey agar (HiMedia, India), thiosulfate citrate bile salts sucrose (TCBS) agar (HiMedia, India), Eosin methylene blue (EMB) agar (HiMedia, India), cetrimide agar (HiMedia, India), and xylose lysine deoxycholate (XLD) agar (HiMedia, India) were used as cultivation media. The media plates with bacterial inoculum were incubated at room temperature (RT) (28 ± 2 °C) for 24-48h. Based on morphological differences such as colony colour, shape, size, and appearance (glossy or matt), the individual colony was isolated on a fresh sterile ZMA plate. The obtained isolates were stored at 4 °C for further processing.

3.2.3 Screening for a xylanolytic enzyme-producing bacteria

Screening for potential xylanase producer was carried out since xylanase is contemplated to be a primary xylanolytic enzyme. Isolated colonies were screened for xylan degrading ability by inoculating them on sterile xylan-agar plates (**Table 3.2**). The composition of the basal salt solution (BSS) medium (w/v) is mentioned in **Table 3.3**. The inoculated plates were incubated at RT for 24-48h. After the incubation period, the plates were flooded with 1% Congo Red. Excess stain was discarded, and the plates were washed with 1.5 M NaCl to observe the zone of clearance (Khandeparker et al., 2017).

3.2.4 Estimation of xylanase activity

Isolates showing zone of clearance on xylan containing plates as mentioned in the above paragraph were further grown at RT in 5mL of the same medium for 24-48h under shaking conditions. Cultures grown in broth were centrifuged (10625×g, 4 °C for 10 min), and the supernatant was used for determining xylanase activity. Xylanase assay was performed in triplicate for each bacterial supernatant, and the activity was

determined by the 3,5-di-nitrosalicylic acid (DNS) method described by Khandeparker and Bhosle, (2006a).

3.2.5 DNS method for estimation of xylanase activity

In brief, crude xylanase along with 1% beechwood xylan solution as substrate, was mixed in a test tube and incubated at RT for 20 min, control tube was maintained without adding substrate solution. At the end of the incubation period, DNSA solution was added to the reaction tube to terminate the enzyme-substrate reaction. Substrate solution (1% beechwood xylan) added to control tubes post addition of DNSA solution to avert reducing sugars formation as a result of the enzyme-substrate reaction. All the tubes were kept in boiling water bath for 5 min until tube content changed its colour from orange to wine red. The reaction mixture was cooled and absorbance was measured using a spectrophotometer at 510nm. One micromole of reducing sugar (xylose) produced under standard assay conditions in 1 min is considered as 1 international xylanase unit (IU).

3.2.6 Genomic DNA extraction

All the bacterial isolates were inoculated in 5 mL ZMB separately and incubated for 24h at RT under shaking conditions. Cell pellet of grown culture was collected by centrifugation at 10625×g. Genomic DNA extraction was carried out using GenElute bacterial genomic DNA kit (Sigma-Aldrich, USA) as per the manufacturer's instructions and stored at -20 °C until further use.

3.2.7 Gene amplification and sequencing

16S rRNA gene of all bacterial isolates was amplified using genomic DNA as a template and set of gene-specific universal primers (**Table 3.4**) (Wang et al., 2012). PCR 96-well thermal cycler (Veriti 9902, Applied Biosystems, USA) was used to amplify the gene. The PCR reaction mixture of 50 µL consist 27F, 3 µL; 1492R, 3 µL; genomic DNA template, 6 µL, Readymix (Sigma-Aldrich, USA), 25 µL and nuclease-free water, 13 µL. PCR reaction program was set, which comprised 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1min (Khandeparker et al., 2014). The amplified PCR products were electrophoresed on 1% agarose gel to confirm gene amplification and cleaned up to remove unused dNTPs and primer-dimer using GenElute PCR clean-up kit (Sigma-Aldrich) as per manufacturers' instructions. Gene sequencing was carried out using the Taq Dye Deoxy Terminator

cycle sequencing kit (PerkinElmer, USA) and analyzed in 373A automated DNA sequencer (3130x/Genetic analyzer, Thermo Fisher Scientific, USA) (Khandeparker et al., 2017).

Since xylanase is a primary xylanolytic enzyme glycoside hydrolase (GH) family-based identification of xylanase was carried out. This experiment was performed to check the type of xylanase (GH family) produced by bacterial isolates from mangrove regions. Genes responsible for the production of GH10 and GH11 xylanases were amplified using genomic DNA as a template and set of gene-specific primers (**Table 3.4**). PCR amplified *xylanase* gene products were processed and nucleotide sequence determined as mentioned in the above paragraph.

3.2.8 Nucleotide data analysis, identification and accession number

Obtained nucleotide sequence data were further processed and used for alignment with corresponding *16S rRNA* gene as well as GH10 and GH11 xylanase genes using BLAST from the NCBI database. This was carried out to obtain the closest bacterial sequence match present within the respective database. Short sequences (<500bp), sequences with low similarity (<90%), and chimeric sequences were removed. All the nucleotide sequences (*16S rRNA* gene and *xylanase* gene) obtained in this study were submitted to the NCBI BankIt database to obtain accession numbers.

3.2.9 Phylogenetic analysis

16S rRNA gene sequences were analyzed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A single multifasta file was created containing a single representative sequence of each genus along with its reference sequence. All the sequences were aligned, and the phylogenetic tree was constructed using MEGA version 6, with a bootstrap value of 1000. Nucleotide sequences of GH10 and GH11 xylanase gene fragments were translated into amino acids. ExPASy translation tool (https://web.expasy.org/translate/) was used for translation. Similarities in protein sequences were assessed using the BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned with known sequences from the GenBank database at the protein level using ClustalW. Phylogenetic trees were constructed with MEGA using the neighbor-joining method. Confidence for tree topologies was estimated by bootstrap values based on 1,000 replicates. A total of 10 representative sequences were selected and used as references

for the GH10 gene, and one GH11 sequence was used for phylogenetic tree construction.

3.2.10 Characterization of GH10 xylanase

The effect of pH and temperature on GH10 xylanase activity was studied using extracellular xylanase from representative *Bacillus* isolates. 50 mM (pH 5.0 - citrate, pH 6.0-8.0 – Na-phosphate and pH 9.0-10.0 – glycine-NaOH) buffer solutions were used to make crude xylanase aliquots in 1:1 ratio. These aliquots were used for xylanase assay. Similarly, the effect of temperature on GH10 xylanase activity was also studied by varying incubation temperatures of the enzyme-substrate reaction mixture from 30 to 80 °C. GH10 xylanase activity was measured as per the xylanase assay protocol mentioned above.

3.3 Results and Discussion

Mangroves are complex, unique, and extremely productive ecosystems found in tropical and subtropical intertidal regions of the world. Microbial populations in mangrove sediments have a significant effect on biogeochemical cycles of the coastal ecosystem (Thorsten et al., 2001). Although the mangrove ecosystem is rich in microbial diversity, from total species present less than 5% only have been described. In many cases, neither their ecological role nor their technological potential is known (Thatoi et al., 2013).

Lignocellulosic materials are difficult to degrade due to their compact structural features, (Kumar et al., 2008) and microorganisms that specialize in lignocellulose degradation are expected to find in specific environments, such as the mangrove ecosystem, which is rich in such biomass.

In an attempt to explore lignocellulose-degrading bacteria which produce xylanases, 303 bacteria were isolated from mangrove areas along the west coast of India, Goa. Accession numbers for all *16S rRNA* gene were obtained from GenBank with serial number MH767087-MH767389. GH10 gene nucleotide sequences were also submitted to GenBank, and accession numbers assigned were MK911042-MK911051.

Sequence data analysis showed a total of 22 cultivable bacterial genera, of which the top 15 genera are represented in **Figure 3.2**. The evolutionary history of culturable bacterial isolates is represented in the form of a phylogenetic tree (**Figure 3.3a, b**).

Mangrove habitat showed the dominance of *Proteobacteria* phylum (57%) comprising of genus *Vibrio* (36%) and *Photobacterium* (9%). Oliveira et al., (2014) studied the metagenomic bacterial community of mangrove sediment of Goa and reported that the *Proteobacteria* is dominant in the sediment samples comprising 43-46% of the total population. There are reports showing 88% of bacterial communities belong to *Proteobacteria* in mangrove sediment of southeastern Brazil (Mendes and Tsai, 2014). Also**,** a previous study conducted on mangrove sediments of Sanya, Hainan Island, China reported predominant bacterial phylotypes to cluster within *Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria* and *Firmicutes* where proteobacteria dominated the community (Zhang et al., 2009). *Proteobacteria* have been suggested to have an important role in the nitrogen, phosphorous and sulphur cycles in mangrove sediments, such as nitrogen fixation, phosphate solubility and sulfate reduction (Holguin and Bashan, 1996). *Firmicutes* was the second dominant phylum reported in my study with *Bacillus* (32%) genera being the most abundant. Similar observations were also reported from estuarine and mangrove environments (Holguin and Bashan, 1996). Phylum *Actinobacteria* (1%) and *Fusobacteria* (2%) were also reported in the current study.

Mangrove sediments studied here showed a high abundance of *Vibrio,* and *Bacillus,* while *Vibrio* dominated. High numbers of pathogenic bacteria such as *Escherichia coli*, *Listeria, Salmonella,* and *Vibrio* sp. are previously reported from Goan mangroves by Poharkar et al., (2016). Family *Vibrionaceae* is one of the most fundamental 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. The genus *Bacillus* contains phylogenetically and phenotypically diverse species, which are ubiquitous in all the habitats, including terrestrial, freshwater also widely distributed in seawater.

Preliminary screening of xylan-degrading bacteria based on Congo red assay revealed 94 isolates (31%) representing 6 genera (*Bacillus, Vibrio, Fusobacterium, Catenococcus*, *Staphylococcus,* and *Klebsiella)* with xylan-degrading capacity (**Figure 3.4**). A total of 17 representative isolates from 6 genera were further studied for the quantitative estimation of xylanase activity (**Table 3.5**). It was seen that *Bacillus* strains produced a high amount of xylanase compared to *Vibrio* and other

genera (**Figure 3.5**). *Bacillus* alone represented 86% of xylan degraders in the study area. *Bacillus* genus has been reported as a potential source of xylanases production, and a large number of bacilli, such as *B. circulans, B. stearothermophilus, B. amyloliquefaciens, B. subtilis, B. pumilus, B. halodurans* and *B. tequilensis* (Khandeparker et al., 2017; Gupta et al., 2015) have been reported to have significant xylanase activity. In this study, 10 strains of *Bacillus* were found to be involved in hemicellulose degradation. *B. megaterium, B. cereus, B. subtilis, B. proteolyticus, B. tropicus, B. tenquilitis, B. amyloliquefaciens, B. safensis, B. vietnamensis and B. plakortidis* are among them. Some of the Bacillus species reported in this study are already reported for hemicellulose degradation (Khandeparker et al., 2017).

Most of the previous investigations were focused on microbial ecology-based taxonomic studies, which help in understanding microbial populations in a particular environmental niche (Haegeman et al., 2013), although few efforts have been made to study the microbial diversity by exploring the functional potential of microbes (Engel et al., 2012). Our understanding of the functional diversity of microbial xylanases from different mangrove regions can possibly help to elucidate the mechanisms of the transformation of organic substances in marine ecosystems and to fulfil the requirements of modern biotechnology. To study the functional diversity of *Bacillus* screened in the study area, xylan-degrading *Bacillus*, comprising 10 representative strains, were further screened for the presence of GH10 and GH11 xylanase gene fragments. DNA of each bacterial sample was PCR amplified using GH10 and GH11 gene primers (**Table 3.4**). Amplification with GH10 primers indicated the presence of the GH10 xylanase gene in all ten representative *Bacillus* strains isolated from mangrove sediments of Goa. The PCR product was sequenced. BLASTx analysis inferred all sequences showing high similarities to GH10 xylanase gene fragments. Partial sequences of GH10 xylanases obtained in this study and GH11 xylanase sequence from *Bacillus* sp. BT21 reported earlier (Khandeparker et al., 2017) were used to construct a phylogenetic tree (**Figure 3.6**). All the sequences reported in this study shared high identity with GH10 xylanases from *Bacillus* sp. whereas GH11 xylanase was seen as out-group (**Figure3.7**). According to the Pfam database (http://pfam.sanger.ac.uk/), GH11 xylanase is mainly distributed in fungi, while GH10 sequences are mainly from bacteria. This suggests that bacteria and fungi are the main producers of GH10 and GH11 xylanases, respectively (Wang et al., 2012). Although

GH11 xylanases are most frequently chosen for the industrial processes, GH10 enzymes display certain enzymological characteristics that can make them better candidates for enzymatic degradation of lignocelluloses biomass (Beaugrand et al., 2004). According to Hu and Saddler, (2018), GH10 xylanases have higher accessibility towards the xylan backbone within pre-treated biomass, thus, enhancing biomass degradation. This characteristic makes GH10 xylanases a potential enzyme for biomass utilization.

As per the earlier report, bacterial isolates from mangrove regions have the potential to produce a broad spectrum of xylanases. According to Guo et al., (2013), xylanases from marine bacterium *Glaciecola mesophila* are stable at a lower temperature (25 °C). On the contrary, Khandeparker and Bhosle, (2006a, b) reported xylanases from *Enterobacter* sp. and *Arthrobacter* sp. isolated from an estuarine environment had temperature optima of 100 °C. Alkaline pH tolerance of xylanases of bacterial isolates from mangrove reported by Khandeparker and Bhosle, (2006a, b) and Annamalai et al., (2009) was found to be 9.0, whereas, Khandeparker et al., (2017) revealed *Bacillus* sp. xylanase having pH optimum of 6.0 which was also isolated from bacteria of mangrove sediment. Palavesam and Somanath, (2017) reported xylanase, which had organic solvent tolerance ability up to 25%. Xylanases with organic solvent tolerance can be of great potential in industries involved in the saccharification of sugars to produce bioethanol. Salt tolerance of xylanase obtained from bacterial isolates from mangroves have also been widely studied and reported.

Extracellular GH10 xylanases from marine *Bacillus* sp. reported in the current study showed optima pH from 5.0 to 9.0 and temperature optima in the range of 50 to 60 °C (**Table 3.5, Figure 3.8**). *Bacillus* sp. possessing accession number MH767108 and MH767158 produced xylanase with optimum pH of 9.0 and 8.0, respectively, and temperature optimum at 60 °C. Xylanases active at high temperatures and alkaline pH are applicated in biobleaching of pulp (Polizeli et al., 2005). The effectiveness of bacterial xylanase in pulp bleaching has been studied previously for *Bacillus* sp. (Kulkarni and Rao, 1996; Shah et al., 1999), where *Bacillus* sp. NCIM 59 xylanases had a temperature and pH optima of 60 °C and 6.0, respectively, while Bacillus sp. Sam-3 xylanase showed optimum pH 8.0 and temperature optimum of 60 °C. *Bacillus circulans* is reported for its xylanase application in biobleaching of

eucalyptus pulp (Dhillon et al., 2000) the optimum pH for the enzyme was 6.0–7.0, while temperature optimum was 80 °C.

In this study, acidic xylanase isolated from *Bacillus sp.* (MH767110) and having optima at pH 5.0 and 50 °C can also be a potential candidate for the food industry as the main desirable properties for xylanases for this use are high stability and optimum activity at acidic pH. As per my knowledge, there are fewer reports on bacterial xylanases in this area; mostly fungal xylanases are reported in the food industry (Camacho and Aguilar, 2003). Singh and Singh, (2018) have reported *B. subtilis* for improving the nutritional value of poultry feed with an optimum pH of 4.0 and temperature optimum of 35 °C. Looking at the properties of the *Bacillus* sp. isolated from mangroves, the potential application of these bacterial cultures in various industries which require acidic/alkaline conditions and elevated temperatures during processing can be targeted which includes fruit juice, bakery, seafood processing industries, and paper and pulp industry (Teo et al., 2019).

Table 3.1 Name and location details of sampling site (Parab et al., 2020)

North-Goa, India			***South-Goa, India***		
Sr. No.	**Name of the sampling site**	**Location on map**	**Sr. No.**	**Name of the sampling site**	**Location on map**
1.	Vagalim	15°38'06.1"N 73°46'13.0"E	1.	Cortalim	15°24'17.7"N 73°53'49.9"E
2.	Shiollim	15°36'55.1"N 73°45'16.6"E	2.	Chandor	15°19'08.7"N 74°00'23.5"E
3.	Sarmanas (Piligao)	15°32'39.8"N 73°57'37.7"E	3.	Navelim	15°14'31.8"N 73°58'29.1"E
4.	Marcel	15°32'42.0"N 73°56'39.4"E	4.	Assolna	15°10'00.7"N 73°57'06.7"E
5.	Diwar	15°30'18.6"N 73°53'39.8"E	5.	Talpona	14°59'06.9"N 74°02'35.7"E
6.	Chorao	15°30'42.4"N 73°51'29.6"E	6.	Galgibag	14°57'38.1"N 74°03'03.7"E
7.	4-Pillar (Panjim)	15°29'13.1"N 73°50'02.9"E			

Table 3.2 Composition of xylan-agar media plates.

Xylan	0.5 g
Yeast extract	0.2 g
Peptone	0.03 g
Bacteriological agar	1.5 g
BSS	100 ml
pH*	7.00

*The pH of the xylan-agar media was adjusted using 1N NaOH.

Table 3.3 Chemical composition of Basal salt solution (BSS) and trace metal solution (TMS)

Chemical composition	**Per liter**	**Chemical composition**	**Per liter**
Basal salt solution		****Trace Metal Solution (TMS)***	
NaCl	30.00 g	H_3BO_3	2.85 g
KCl	0.75 g	$MnCl_2.7H_2O$	1.80 g
$MgSO_4$	7.00 g	$FeSO_4.7H_2O$	2.49 g
NH_4Cl	1.00 g	Na-Tartarate	1.77 g
K_2HPO_4 (10%)	7.00 mL	$CuCl_2$	0.03 g
KH_2PO_4 (10%)	3.00 mL	$ZnCl_2$	0.02 g
Trace metal solution*	1.00 mL	$CoCl_2$	0.04 g
Distilled water	1000 mL	$Na_2MoO_4.2H_2O$	0.02 g
pH**	7.00		

**The pH of the BSS solution was adjusted using 1 N NaOH.

Table 3.4 List of primers used for gene amplification (Parab et al., 2020)

Sr. No.	Gene	Primer	Nucleotide sequence of primer
1.	16S rRNA gene	27F	5’-AGAGTTTGATCCTGGCTCAG-3’
		1492R	5’-GGTTACCTTGTTACGACTT-3’
2.	Glycosyl Hydrolase 10 gene (GH10)	X10-F	5’-CTACGACTGGGAYGTNIBSAAYGA-3’
		X10-R	5’-GTGACTCTGGAWRCCIABNCCRT-3’
3.	Glycosyl Hydrolase 11 gene (GH11)	X11-F	5’-CCGCACGGACCAGTAYTGNKIRAANGT-3’
		X11-R	5-’AACTGCTACCTGKCNITNTAYGGNTTGG-3’

Table 3.5 Xylanase activity and pH and temperature optima of representative bacterial isolates (Parab et al., 2020)

Accession number	Nearest neighbor in NCBI database	Similarity (%)	Xylanase activity (IU/mL)	Standard error	Optimum pH	Optimum temperature, (°C)
MH767089	*Bacillus altitudinis* 41KF2b	99.69	0.722	0.015	6.0	50
MH767093	*Fusobacterium varium*	81.78	0.046	0.017	6.0	60
MH767098	*Bacillus subtilis* strain NBRC 101239	97.40	0.473	0.020	7.0	50
MH767099	*Vibrio alginolyticus* strain NBRC 15630	99.21	0.239	0.013	-	-
MH767100	*Bacillus proteolyticus* strain MCCC 1A00365	99.86	0.732	0.016	6.0	55-60
MH767101	*Fusobacterium necrogenes*	83.19	0.047	0.022	6.0-7.0	40
MH767105	*Staphylococcus edaphicus* strain CCM 8730	99.89	0.214	0.110	-	-
MH767158	*Bacillus safensis* strain FO-36b	99.48	0.841	0.041	8.0	60
MH767108	*Bacillus aerius* strain 24K	99.62	0.946	0.010	9.0	50
MH767109	*Bacillus subtilis* strain JCM 1465	95.80	0.353	0.009	6.0	60
MH767110	*Bacillus cereus* strain IAM 12605	99.85	0.134	0.031	5.0-6.0	50
MH767164	*Bacillus amyloliquefaciens* strain MPA 1034	99.04	0.617	0.023	6.0	55-60
MH767266	*Vibrio areninigrae* strain J74	96.54	0.306	0.012	-	-
MH767274	*Catenococcusthiocycli* strain TG 5-3	97.67	0.072	0.030	7.0	60
MH767341	*Klebsiella oxytoca* strain ATCC 13182	97.65	0.281	0.017	-	-
MH767342	*Bacillus plakortidis* strain P203	96.29	0.513	0.012	7.0-8.0	55-60
MH767343	*Bacillus vietnamensis* strain NBRC 101237	97.82	0.189	0.010	7.0	50

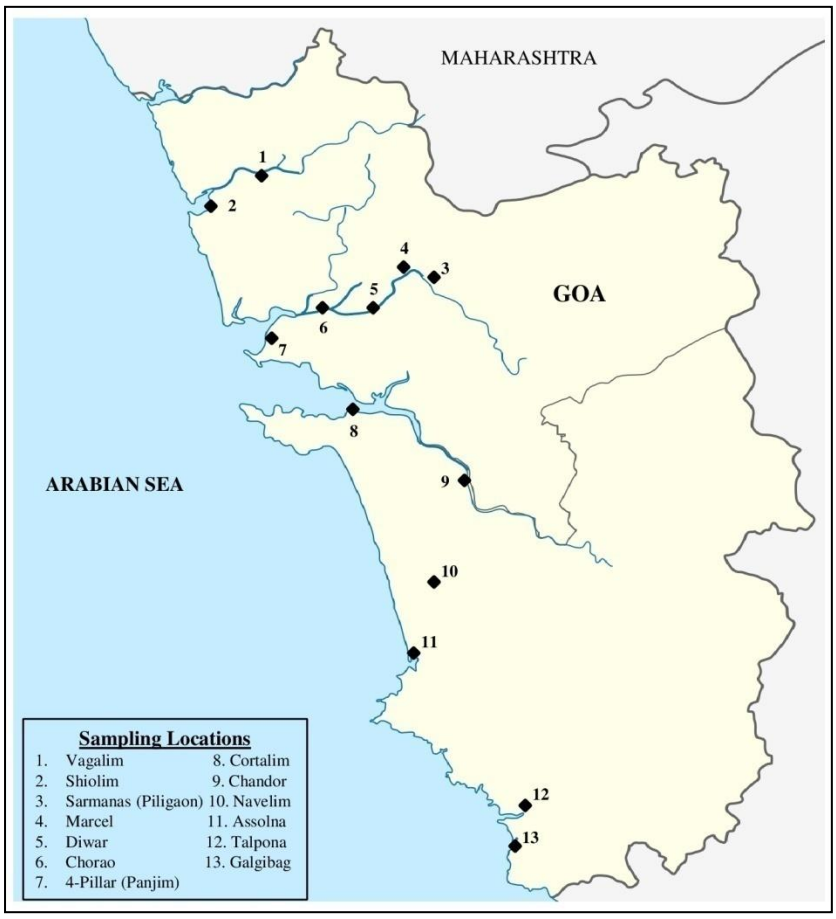

Fig. 3.1 Sediment and water sampling sites (edited from https://en.wikipedia.org/wiki/Ponda,Goa)

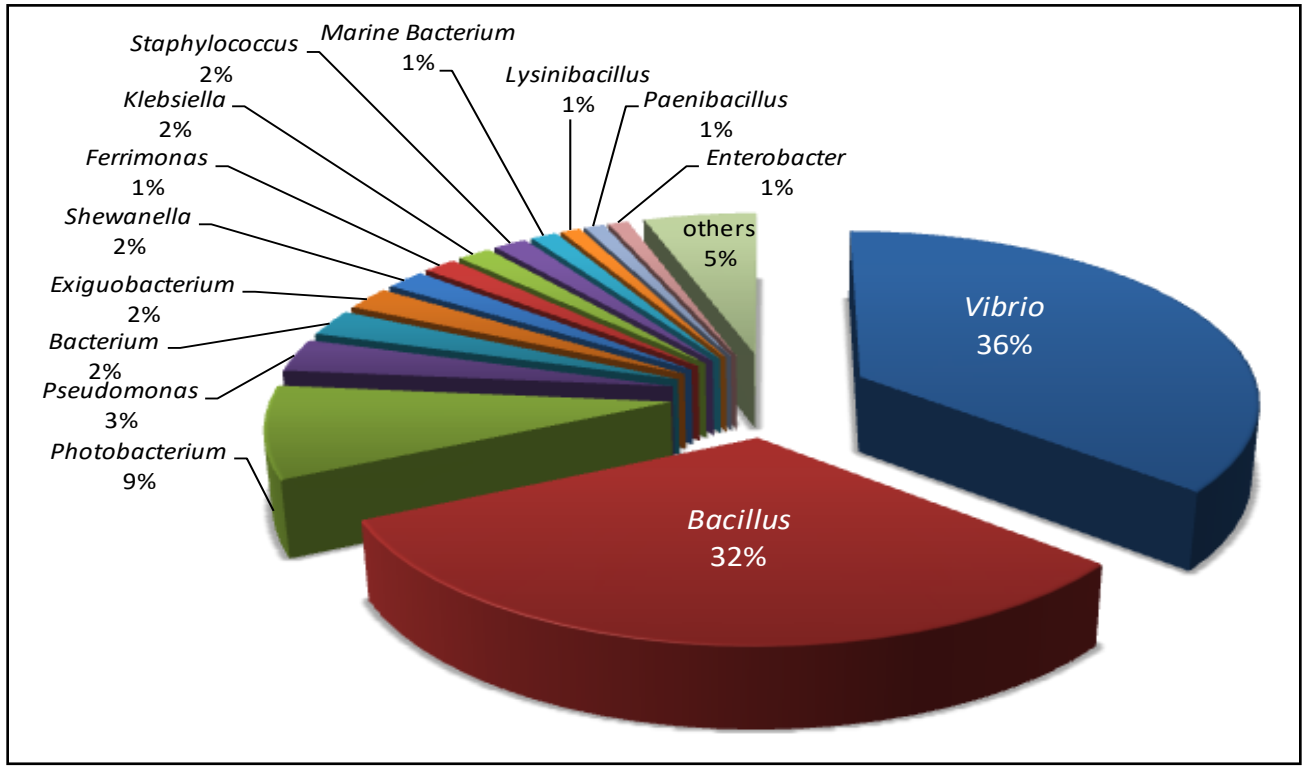

Fig. 3.2 Bacterial diversity in mangrove regions of Goa (Parab et al., 2020).

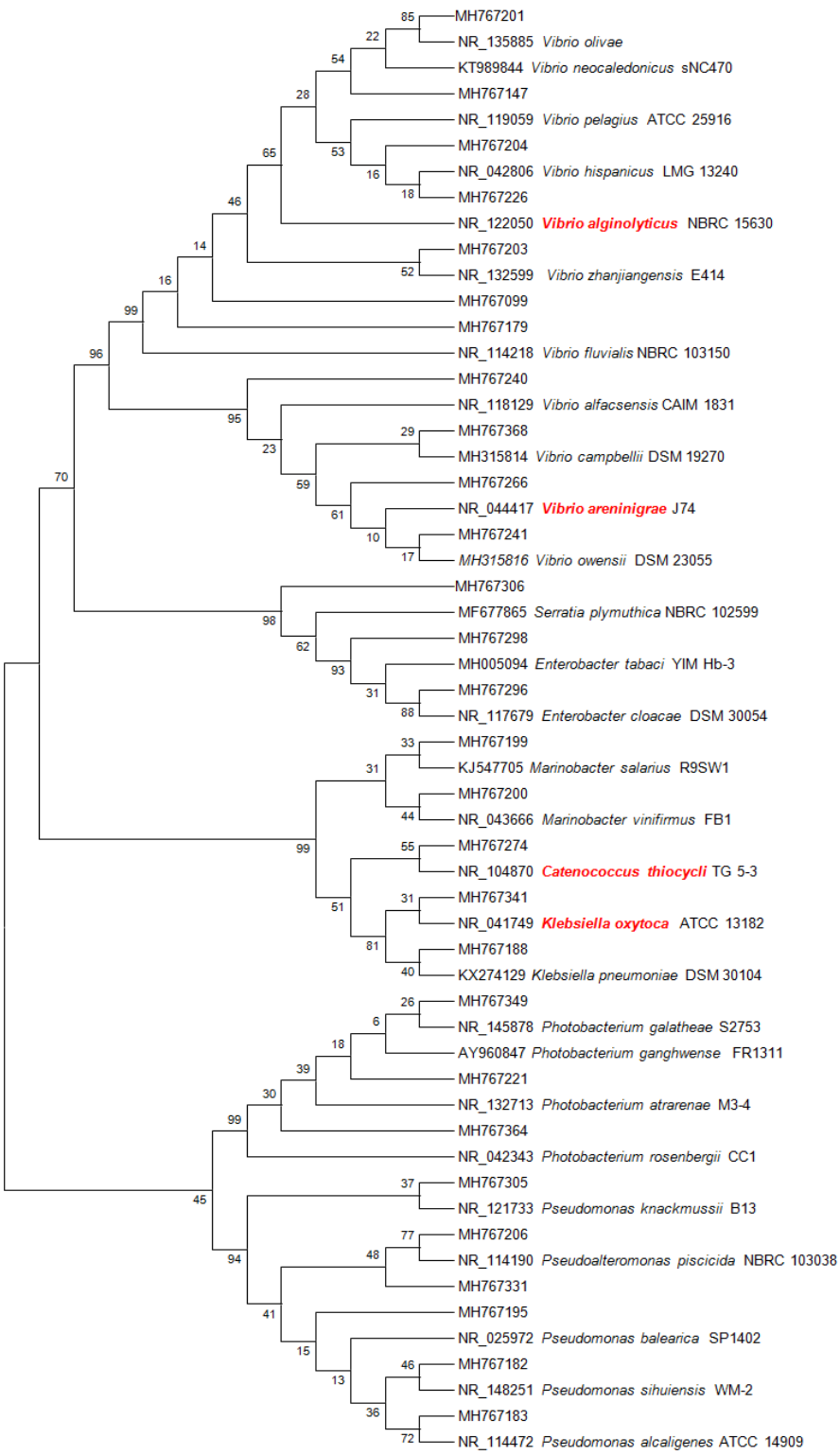

Fig. 3.3a Evolutionary history of culturable bacterial isolates from Mangroves of Goa (Proteobacteria) (Parab et al., 2020).

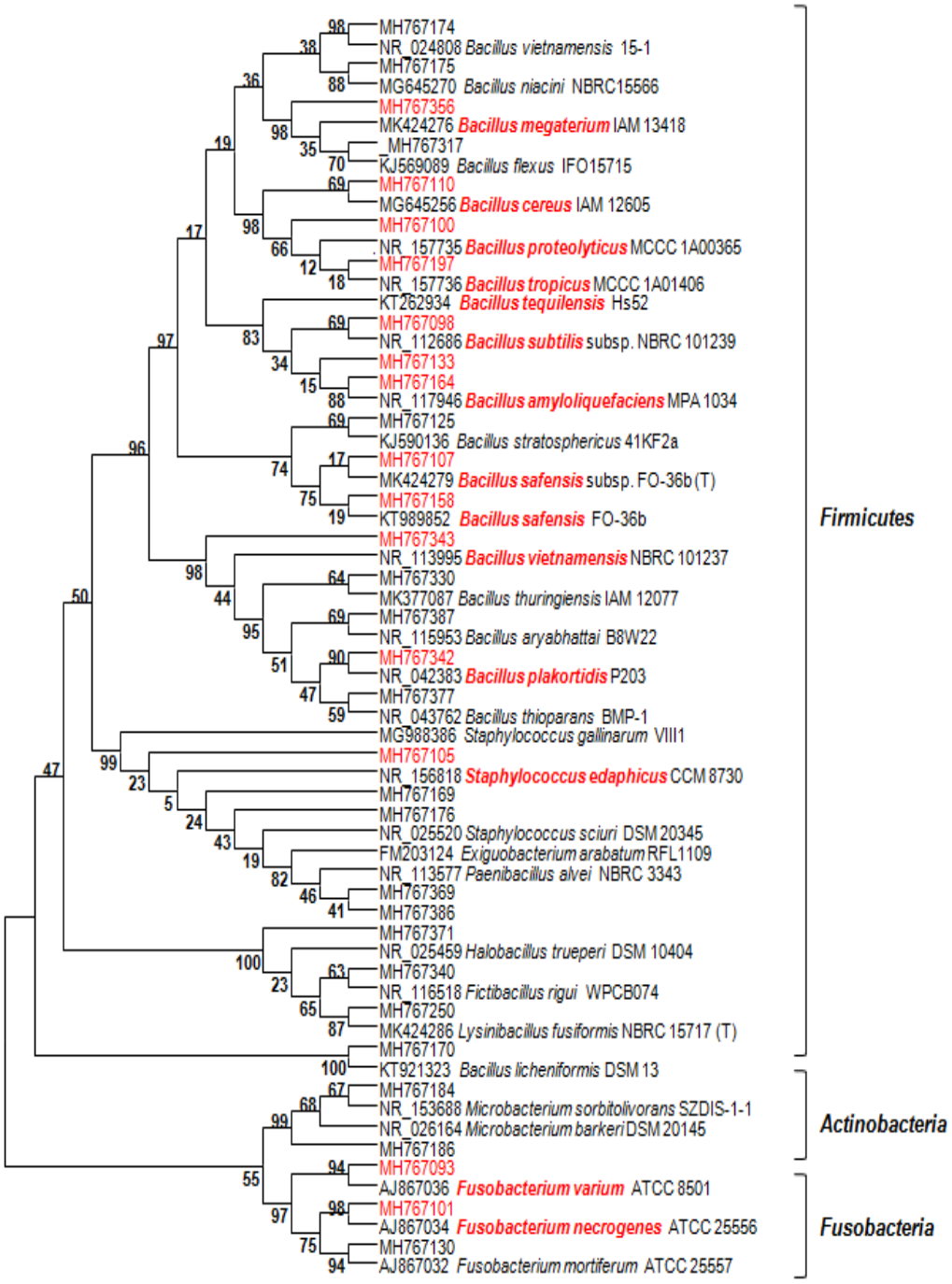

Fig. 3.3b Evolutionary history of culturable bacterial isolates from Mangroves of Goa (Fermicutes and others); *Highlighted strains possess xylan degrading capacity (Parab et al., 2020).

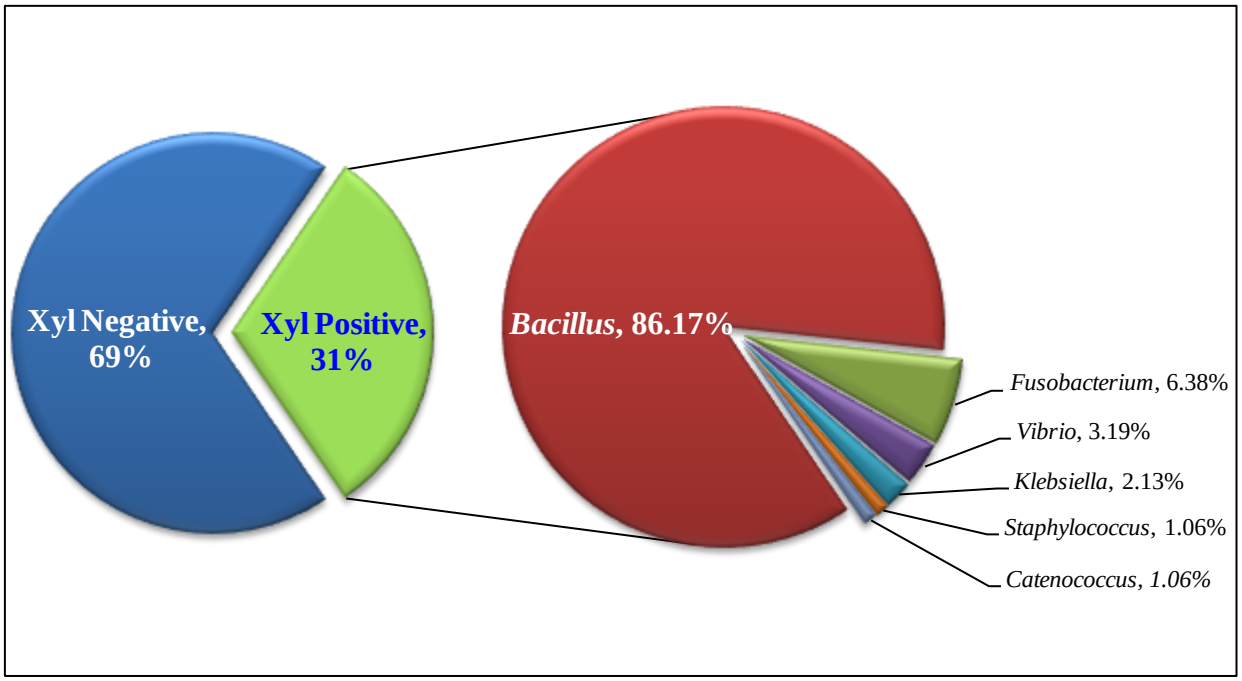

Fig. 3.4 Xylanase producing bacterial communities in mangroves of Goa region (Parab et al., 2020).

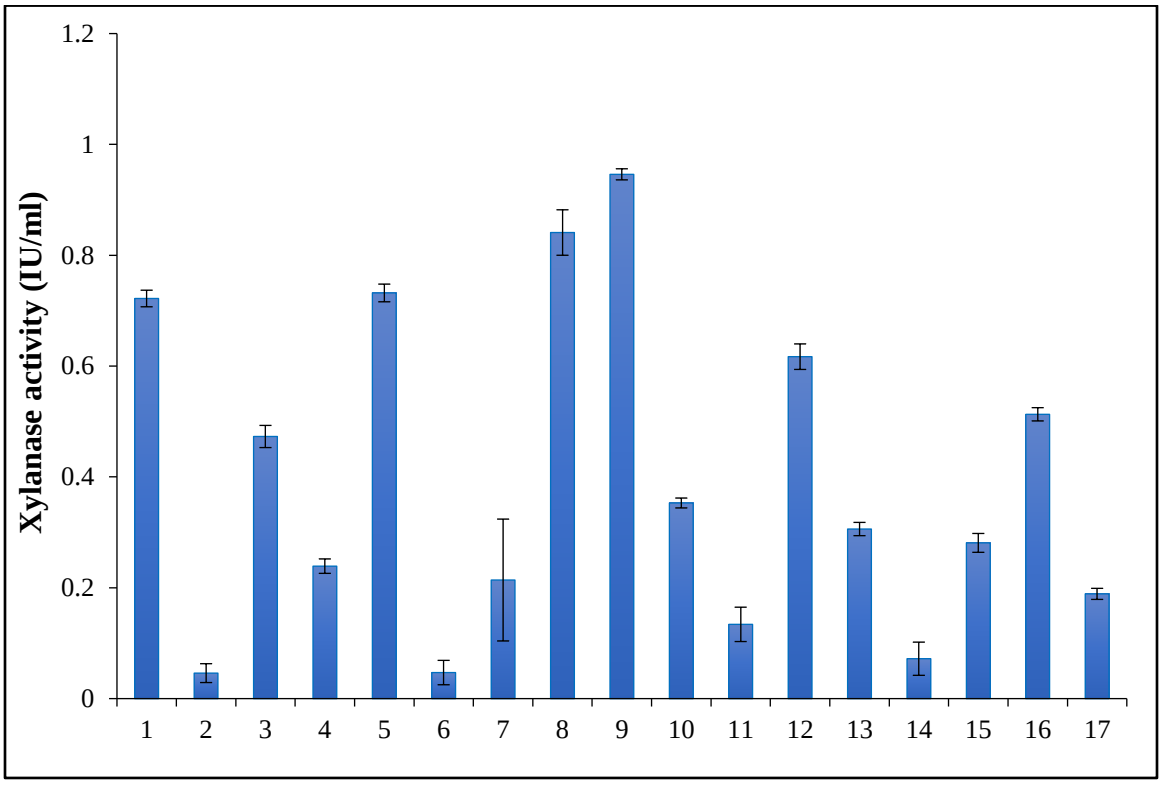

Fig. 3.5 Xylanase production by xylanase-producing bacterial representatives. 1 - *Bacillus* MH767089; 2 - *Fusobacterium* MH767093; 3 - *Bacillus* MH767098; 4 - *Vibrio* MH767099; 5 - *Bacillus* MH767100; 6 - *Fusobacterium* MH767101; 7 - *Staphylococcus* MH767105; 8 - *Bacillus* MH767158; 9 - *Bacillus* MH767108; 10 - *Bacillus* MH767109; 11 - *Bacillus* MH767110; 12 - *Bacillus* MH767164; 13 - *Vibrio* MH767266; 14 – *Catenococcus thiocycli* MH767274; 15 - *Klebsiella* MH767341; 16 - *Bacillus* MH767342; 17 - *Bacillus* MH767343.

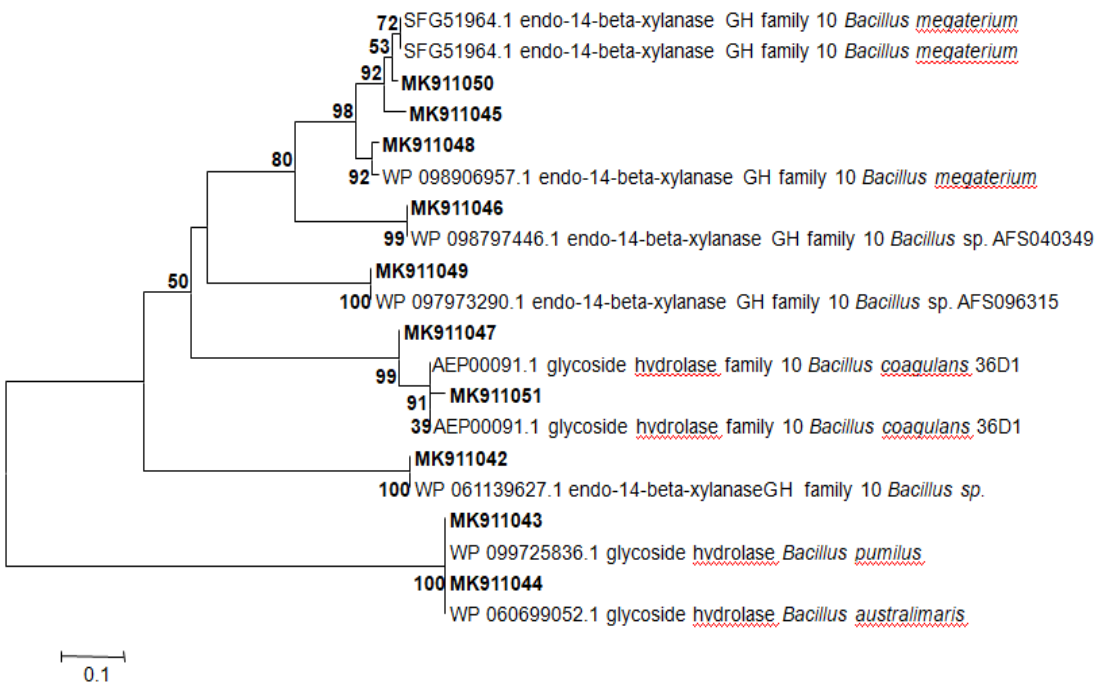

Fig. 3.6 Phylogenetic tree constructed by MEGA using the neighbor-joining method. The lengths of the branches indicate the relative divergence among the amino acid sequences (Parab et al., 2020).

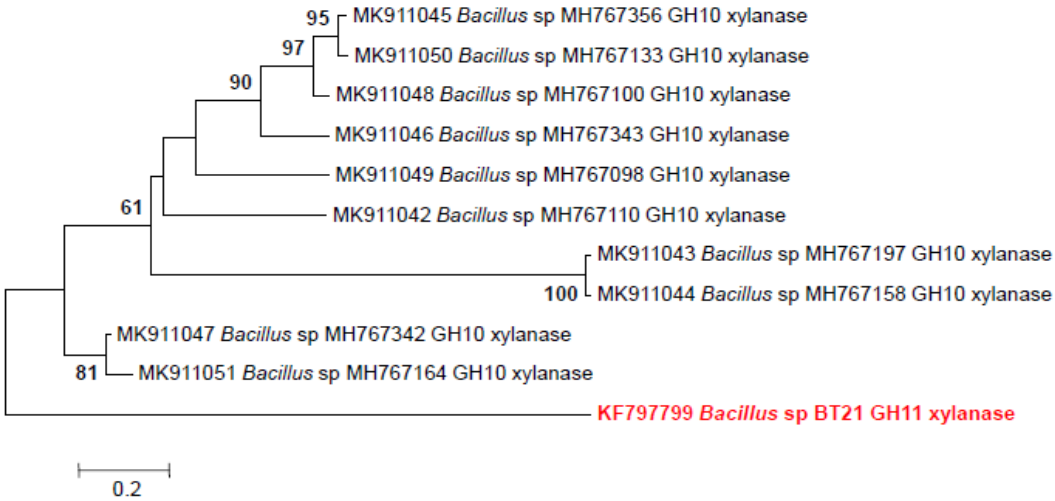

Fig. 3.7 Phylogenetic relationships of a GH10 xylanase of *Bacillus* species from mangroves with GH11 xylanase of previously reported *Bacillus* species constructed using NJ-tree method with bootstrap values (1000 replicates) as a percentage at the nodes(Parab et al., 2020).

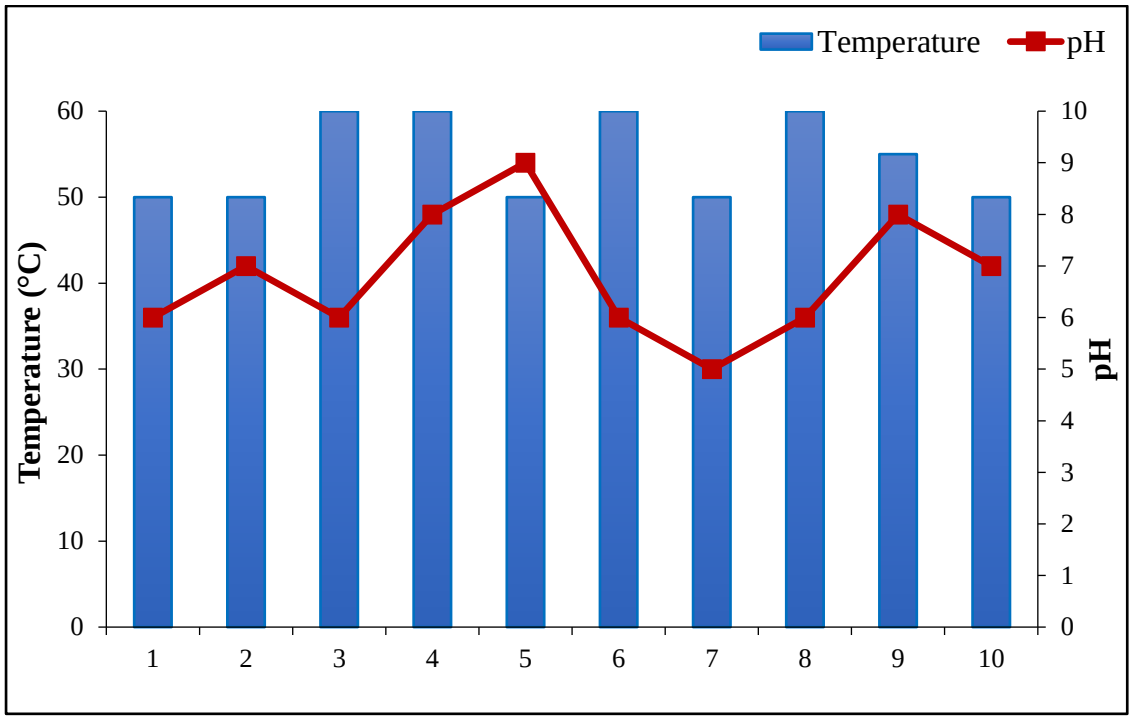

Fig. 3.8 Temperature and pH optima of a GH10 xylanase from *Bacillus* species, 1 – *Bacillus* MH767089; 2 -*Bacillus* MH767098; 3 - *Bacillus* MH767100; 4 - *Bacillus* MH765158; 5 - *Bacillus* MH767108; 6 - *Bacillus* MH767109; 7 - *Bacillus* MH767110; 8 - *Bacillus* MH767164; 9 - *Bacillus* MH767342; 10 - *Bacillus* MH767343 (Parab et al., 2020).

Chapter - 4

Extraction, Purification and

Characterization of Xylanolytic Enzyme

4.1 Introduction

Xylan together with cellulose (1,4-β-glucan) and lignin (a complex polyphenolic compound) make up the major polymeric constituents of the plant cell wall (Kulkarni et al., 1999). Xylans are straight homopolymers that contain D-xylose monomers connected through β-1, 4–glycosyl bonds (Kulkarini et al., 1999; Beg et al., 2001). Because of its heterogeneity and intricacy, the total hydrolysis of xylan requires a huge assortment of agreeably acting catalysts (Puls et al., 1987; Biely, 1985; Subramaniyan and Prema, 2002). Xylanolytic enzymes are a consortium of xylan hydrolyzing enzymes which mainly includes endo-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55) and acetyl xylan esterase (EC 3.1.1.72). These are the bio-catalysts which can break down complex xylan molecule into its oligomers and further monomers. Endo-1,4-β-D-xylanases (EC 3.2.1.8) arbitrarily separate the xylan spine and β-D-xylosidases (EC 3.2.1.37) sever xylose monomers from the non-reducing end of xylooligosaccharides and xylobiose. Arabinofuranosidases (AFase) and acetyl xylan esterases (AXE) are accessory xylanolytic enzymes which increases the accessibility of linear xylan backbone for hydrolysis by xylanase enzymes by trimming side branches of arabinose and acetylated residues (if present). There is a vast number of applications and subsequent advantages of these xylanolytic enzymes in various industries such as paper and pulp industry, animal fodder, food and beverage, fruit juice, vegetable processing, textile, detergent, leather, bioethanol production, bioconversion of lignocellulosic matter etc. (Khandeparker and Bhosle, 2006b; Helianti et al., 2016). The major limitation of industrial usage of the xylanolytic enzyme is its production cost. The most prominent reason for the high cost of the xylanolytic enzyme is the high purity xylan used as a substrate during the production of these enzymes. If the inexpensive alternate source of xylan is used for the production of xylanolytic enzymes, the total enzyme cost can be reduced significantly (Knob et al., 2014).

An agro-industrial waste is nothing but the residues generated from the growing and processing of raw agricultural products such as crops, fruits, vegetables, meat, poultry and dairy products (Obi et al., 2016). The disposal of these invoked by-products is becoming a major concern in agricultural countries since it causes environmental pollution (Wang et al., 2016). Agro-industrial residues majorly comprised of lignocellulosic biomass containing hemicelluloses and treated as waste

in many agricultural countries due to infeasibility to convert them into beneficial products for mankind (Obi et al., 2016). Developments of new and potential bio-catalytical processes which can utilize these agro-industrial residues and convert it into valuable products, simultaneously competing with economically well-established chemical processes are required (Knob et al., 2014). An agro-industrial residue mainly consists of lignocellulosic biomass such as wheat bran, rice bran, sugarcane bagasse, corn cob, straw, stem, deciduous wood etc. Among all, wheat bran is readily available cheap lignocellulose biomass which is a rich source of xylan but utilized least possibly and often considered to be waste. Biotechnological utilization of wheat bran for production of xylanolytic enzymes under solid state-fermentation (SSF) not only serves as an economical substrate but it will also seize environmental advantages. SSF has got several unambiguous advantages over submerged fermentation such as economy of space, media simplicity, comprehensible machinery, equipment and control systems, lower water volume due to compact fermentation vessels, greater product yields, reduced energy demand, lower capital and recurring expenditures in industry, easy scale-up of the fermentation process, simple product recovery with fewer solvents, superior yields, absence of foam build-up and easy control over contamination in the system due to low moisture level (Khandeparker and Bhosle, 2006a; Kamble and Jadhav, 2012).

By considering all aspects, the prime objective of my current study was to isolate, partially purify and characterize xylanolytic enzyme produced by *Bacillus* sp. NIORKP76 strain when grown on SSF.

4.2 Materials and methods

4.2.1 Microbial strain and growth conditions

The bacterial strain used in this study was obtained from sediment and water samples collected from mangrove regions of Goa, India and it was identified as *Bacillus* sp. NIORKP76 strain with GenBank accession number MH767158. It was isolated based on morphological characteristics of the colony and screened qualitatively for xylan hydrolyzing ability. Various biochemical tests were performed for selected isolate by using HiBacillus identification kit (HiMedia, India). By understanding its potential as xylanolytic enzymes producer it was further stored at in house culture repository facility. For its revival, the culture was inoculated in sterile

Zobelle Marine broth (ZMB) and incubated at room temperature (RT, 28 ± 2 °C) for 24 hours (h) under shaking conditions (150 rpm).

4.2.2 Media and growth conditions optimization

Bacillus sp. NIORKP76 strain inoculum was prepared in Erlenmeyer flasks (250 mL) containing 100 mL sterile Basal salt solution (BSS) with 0.5% xylan as sole carbon source, 0.2% yeast extract and 0.03% peptone. At regular time intervals, bacterial growth in inoculated media was measured spectrophotometrically (600 nm) as well as by counting colony-forming units (cfu).

Wheat bran, an inexpensive agro-industrial residue was collected from a local vendor to use as growth media for xylanolytic enzyme production.

4.2.2a Effect of salts

Effect of varying concentration of nutrients such as phosphate ions (PO_4^{3-}), sodium chloride (NaCl) and ammonium chloride (NH_4Cl) in growth media on xylanase production and bacterial growth was monitored. The optimum concentration of nutrients required for maximum xylanase production was determined. Phosphate ion concentrations were varied from 0 mM-128 mM, whereas NaCl and NH_4Cl concentrations were varied from 1 to 4% and 0 to 0.11% respectively. Pre-grown bacterial inoculum was added to sterile fermentation media with appropriate nutrient concentration and incubated at room temperature for varying time intervals (0-120h). Blank was maintained for each fermentation tube without inoculating bacterial cells.

4.2.2b Effect of moisture content

A substrate to moisture content (w/v) for maximum xylanase production was optimized using the modified basal salt solution (MBSS). An equal amount of wheat bran was weighed and sterilized in glass tubes. Sterile MBSS with optimum nutrient concentration for maximum xylanase production was added aseptically with a varying substrate to moisture ratio of 1:1.5 to 1:4 (w/v). Pre-grown *Bacillus* sp. NIORKP76 strain was inoculated in all fermentation tubes and incubated at RT for varying time intervals (0-120h).

4.2.3 Xylanase production and bacterial growth determination

After each time interval, phosphate buffer (pH 7.0) was added to the fermentation tube and vortex to homogenize entire content. The homogenized mixture was centrifuged at 12,000 rpm for 5 min to obtain clear supernatant (SN) and pellet. SN

was used as crude enzyme mixture to determine extracellular xylanase activity. The collected pellet was used for extraction of total protein (biomass determination) as per the method described by Khandeparker et al., (2008). In brief, the pellet was treated with 0.1N NaOH and kept in boiling water bath for 30 min, this was done to extract total proteins, further sample was cooled and neutralized by adding an equal volume of 0.1N HCl. The sample was vortex and supernatant was collected which was used for protein estimation. Protein estimation was carried out using protein assay dye reagent concentrate (BioRad, India) as per the manufacturer's instructions.

4.2.4 Growth and xylanolytic enzymes production under optimized conditions

Growth and xylanolytic enzyme production by *Bacillus* sp. NIORKP76 strain was monitored on wheat bran using MBSS. Erlenmeyer flasks (25 mL) containing 1g sterile wheat bran each, were moistened with sterile 3 mL MBSS. All the flasks were inoculated with 1% pre-grown bacterial culture and incubated at RT under the static condition for varying time intervals. At each time interval flasks were selected in duplicate to extract crude enzyme and total protein as per protocol mentioned above. Production of xylanolytic enzymes such as xylanase (xyl), arabinofuranosidase (AFase) and acetyl xylan esterase (AXE) was measured by carrying out respective enzyme assays.

4.2.4a Xylanase assay

The activity of xylanase was measured as per the method described by Khandeparker and Bhosle, (2006a) with minor modifications. 1% beechwood xylan (SRL chemicals, India) solution was used as a substrate for xylanase. The reaction mixture containing the crude enzyme and substrate was incubated at RT for 20 min. The reducing sugars liberated were measured by 3, 5-dinitrosalicylic (DNS) method (Miller, 1959).

4.2.4b AFase assay

Arabinofuranosidase (AFase) activity was measured as per the method described by Khandeparker and Jalal, (2015). The reaction mixture containing crude enzyme and 2.5 mM *p*-nitrophenyl α-L arabinofuranoside (Sigma-Aldrich, USA) solution as substrate was incubated at RT for 15 min. The *p*-nitrophenol released as a result of enzyme catalysis was measured using a spectrophotometer at 420 nm.

4.2.4c AXE assay

Acetyl xylan esterase activity was measured as per the method described by Mai-Gisondi and Master, (2017) with minor modifications as per requirement. The reaction mixture containing crude enzyme and 500 mM *p*-nitrophenyl acetate (Sigma-Aldrich, Switzerland) was incubated at RT for 20 min. *p*-nitrophenol liberated during enzymatic hydrolysis of substrate was measured at a wavelength of 405 nm using UV-Vis spectrophotometer (Shimadzu, UV-1800, Japan).

4.2.5 Scale-up production of the xylanolytic enzyme under SSF

1% overnight grown bacterial inoculum was inoculated in 500mL Erlenmeyer flasks containing 5g sterilized fermentation media moistened with sterile 15mL MBSS. Fermentation flasks were incubated at RT for 72h under static conditions. After incubation period 100 mL sodium phosphate buffer (pH 7.0) was added to each flask containing fermented product. Fermentation medium completely homogenized using vortex and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant containing extracellular crude enzymes was collected and refrigerated until further use.

4.2.6 Partial purification of xylanolytic enzymes

The total protein content from the supernatant was precipitated by adding ammonium sulphate up to saturation point (80%). The precipitate was collected after overnight refrigeration at 4 °C. The collected precipitate was re-dissolved in a sodium-phosphate buffer (50 mM, pH 7.0) and dialyzed using cellulose membrane centrifugal devices (Merck-Millipore, Amicon ultra centrifugal filters) with molecular weight cutoff 13 kDa. The dialyzed fractions were concentrated using a rotary evaporator (Roteva, Equitron Medica Pvt. Ltd, India) and stored at 4 °C. The partially purified, concentrated protein fraction was subjected to ion-exchange chromatography for further purification.

The anion exchanger resin (DEAE-Sepharose FF, Sigma-Aldrich) was packed in column (10 mm diameter and height 150 mm) and the flow rate was adjusted to 0.5 mL/min. The packed column was equilibrated with 50 mM Na-phosphate buffer to remove unbound impurities. 1 mL of concentrated partially purified enzyme sample was loaded onto the column and linear gradient of NaCl from 0 to 3 M applied. Protein concentration in collected fractions was measured spectrophotometrically at

280 nm. Xylanolytic enzyme activity in collected fractions was also determined using standard enzyme assays. All active fractions were pooled together, concentrated and stored at 4 °C for further analysis.

4.2.7 Molecular weight and zymogram analysis

Crude and partially purified xylanase sample was electrophoresed on SDS-PAGE (stacking gel-6%, resolving gel-12%) along with low range protein marker (BioRad, 14.4 kDa to 97.4 kDa) to determine the molecular weight of xylanolytic enzymes. The electrophoresed gel was stained with silver stain technique to visualize protein bands. Zymogram analysis of crude and partially purified xylanase was carried out on SDS-PAGE (stacking gel, 6%; resolving gel, 12%) as per the method described by Khandeparker et al., (2017). In brief, 0.1% xylan was added to resolving gel (12%) as a substrate for xylanase. The electrophoresed gel with separated protein bands was washed thoroughly (4 times) in sodium phosphate buffer (50 mM, pH 8.0), with initial two washes contained 25% isopropanol to remove SDS from the gel and re-nature protein followed by two washes with sodium phosphate buffer (50 mM, pH 8.0). The washed gel was incubated at an optimum temperature of xylanase for 60 min and soaked in 0.1% Congo red stain solution for 2 min. Excess stain was washed with 1% NaCl solution until active xylanase band were visible. The visibility of active xylanase band was improved by adding 0.5% acetic acid solution which changed the gel background colour to dark blue and clear colourless active xylanase band were visible. AFase and AXE zymograms were obtained as per the method described by Khandeparker and Jalal, (2015). The electrophoresed gel after washing process as described above containing re-natured protein was overlaid on a fresh gel containing substrate. The *pNP*-arabinofuranoside and *pNP*-acetate were used as substrates for AFase and AXE respectively. The gels were incubated at optimum temperature until active bands were visible.

4.2.8 Effect of pH and temperature on xylanolytic enzymes

4.2.8a Effect of pH on xylanolytic enzyme activity and stability

The effect of pH on xylanolytic enzymes was measured by determining the enzyme activity of crude enzyme aliquots made using 50 mM buffer solutions of pH range 5.0 to 10.0. For acetyl xylan esterase, pH range of 4.0 to 7.0 was selected since at alkaline pH *p*-nitrophenyl acetate was found to be unstable and displayed decomposition.

Evaluation of xylanolytic enzyme stability at different pH was carried out by preparing the mixture of a crude enzyme in a respective buffer (4.0-5.0, Citrate buffer; 6.0-8.0, sodium phosphate buffer; 9.0-10.0, Glycine-NaOH buffer). The prepared crude enzyme-buffer mixtures were incubated at RT for 24h. At varying time intervals aliquots from mixture were drawn out to determine residual enzyme activity (%). All the enzyme assays were carried out as per protocols mentioned above.

4.2.8b Effect of Temperature on xylanolytic activity and stability

The influence of temperature on xylanolytic enzymes was determined by incubating enzyme-substrate reaction mixture at different temperatures (30 - 80 °C). The stability of xylanolytic enzymes at different temperatures was determined by incubating enzyme solution at a range of 30 – 80 °C for 24h. The xylanolytic enzyme solution prepared using one part of the crude enzyme and equal volume of respective buffer, in which enzyme showed maximum stability. At different time intervals, aliquots from mixture were taken to determine residual enzyme activity. All the enzyme assays were carried out as per protocols mentioned above. Under optimum assay conditions, the activity of all xylanolytic enzymes was considered to be 100%.

4.3 Results and Discussion

Bacillus sp. NIORKP76 strain (GenBank accession number: MH767158) used in this study displayed morphological colony characteristics such as circular in shape with entire margin, raised elevation, moist consistency and white colour upon growing on sterile ZMA plates. It showed cell properties such as Gram-positive, rod-shaped, motile and facultatively anaerobic. It exhibited the ability to produce β-galactosidase and catalase. It was able to utilize sugars such as sucrose, mannitol, glucose, xylose, arabinose and trehalose but showed negative results for utilization of malonate and citrate. Acetoin production and nitrate reduction were not observed with this bacterial strain (**Table 4.1**). Based on the morphological, biochemical and molecular analysis (*16S rRNA* gene sequencing) it was identified and confirmed to be *Bacillus* sp. and designated as *Bacillus* sp. NIORKP76 strain. *Bacillus* sp. has been previously reported for the production of the xylanolytic enzyme, where most of the work is focused on the production of the key hemicellulolytic enzyme that is xylanases (Helianti et al., 2016; Kamble and Jadhav, 2012; Irfan et al., 2016; Gowdhaman et al., 2014; Kumar et al., 2017; Ho et al., 2014; Boucherba et al., 2017; Virupakshi et al., 2004). There are limited reports where other xylanolytic enzymes

such as arabinofuranosidase, acetyl xylan esterase, β-xylosidase etc. are also studied along with xylanases (Khandeparker and Jalal, 2015; Lindner et al., 1994; Seo et al., 2013; Lee et al., 2006; Chaturvedi et al., 2015). Xylan molecule is complex due to its heterogeneity and for the total hydrolysis of xylan, knowledge of other xylanolytic enzymes, their mode of working synergistically is crucial as it will direct our understanding to regulatory machinery in that particular organism.

Growth curve of *Bacillus* sp. NIORKP76 was studied with respect to absorbance at 600 nm as well as colony-forming unit (cfu) count and it clearly displayed three different phases of bacterial growth (Lag phase: 0-3h, exponential phase: 3-15h, stationary phase: 15-24h) (**Figure 4.1**). At the late lag phase, the average cfu count was found to be $1.85*10^6$ mL^{-1} with corresponding average absorbance of 0.097, which increased by 98% at end of exponential phase, making cfu count $94.7*10^6$ mL^{-1} and absorbance of 1.853. Bacterial biomass as well as xylanase production was significantly influenced by varying salt concentrations in growth medium (BSS). The substrate to moisture ratio also played a very crucial role in enzyme production. It was observed that low moisture containing growth media was more favourable for bacterial growth as well as xylanase production as compared to a high content of free moisture. The culture showed maximum xylanase production in growth media supplemented with modified BSS containing 64 mM phosphate ion, 1.5% NaCl, 0.03% NH_4Cl and with the substrate to moisture ratio of 1:3 (w/v) (**Figure 4.2**).

Bacillus sp. NIORKP76 isolate used in this study exhibited an ability to produce three extracellular xylanolytic enzymes which are xylanase, AFase and AXE. Lee et al., (2006) described xylanase from *Bacillus* sp. K-1 which produced four xylanolytic enzymes, among which xylanase, β-xylosidase and AXE were extracellular in nature, whereas AFase was reported as intracellular. Apart from *Bacillus* sp. there are other bacterial strains such as *Arthrobacter* sp., *Lactobacillus* sp., *Caldicellulosiruptor* sp. and *Paenibacillus* sp. which are also reported for the production of xylanolytic enzymes (Khandeparker and Jalal, 2015; Mi et al., 2014; Teeravivattanakit et al., 2016). Bifunctional and trifunctional property of xylanolytic enzymes was also proposed in Khandeparker and Jalal, (2015) and Teeravivattanakit et al., (2016) respectively.

Xylanolytic enzyme production was reported under submerged fermentation (SmF) as well as solid-state fermentation (SSF). Although SSF gives superior yields

of xylanolytic enzyme production (Khandeparker and Bhosle, 2006b; Kamble and Jadhav, 2012) there are still reports found which describes the use of SmF for xylanolytic enzyme production (Kalim and Ali, 2016; Nawawi et al., 2017). There are various substrates used for the production of xylanolytic enzymes which include wheat bran, tea dust, sawdust, paper waste, cassava bagasse, rice straw, rice husk, palm kernel cake, barley husk, corn cob, sugar cane bagasse, oat bran, oat spelt, beet pulp and pineapple peel, among which wheat bran reported to be the best carbon source for xylanolytic enzyme production (Khandeparker et al., 2008; Gowdhaman et al., 2014; Ho et al., 2014). Xylanase being primary xylanolytic enzyme, optimized physio-chemical parameters were used for scaling up xylanolytic enzyme production by SSF. While growing in fermentation media using inexpensive agro-industrial wastes that is wheat bran as carbon source *Bacillus* sp. NIORKP76 strain showed maximum growth at 84h. The observed growth was of diauxic (Schmiedel and Hillen, 1996) pattern. Such a pattern observed when bacterial isolate undergoes stress conditions in growing media. Utilization of simpler sugars leads to first log phase which is followed by lag phase due to exhaustion of simple sugars and switching over of cellular mechanism to utilize more complex nutrients available in fermentation media. First log phase was observed for 12h (24h to 36h) followed by lag phase of 12h (36h to 48h) and further second log phase of 36h (48h to 84h). Maximum xylanase production was observed at 72h, whereas, maximum AFase and AXE production was seen at 48h (**Figure 4.3**).

As per the report of Raimbault, (1998) the free moisture content in SSF is mainly influenced by the type of substrate used and its water-binding ability, which subsequently has a pronounced effect on growth kinetics. In our current study optimum substrate to moisture ratio was found to be 1:3 (w/v) which was similar to the reports described by Kumar et al., (2017), Khandeparker et al., (2008), Khandeparker and Bhosle, (2006b). In other reports such as Sindhu et al., (2006), Kamble and Jadhav, (2012) reported 1:1.5 and 1:1.8 (w/v) ratio for production of xylanase under SSF (Wheat bran) respectively, whereas Sanghi et al., (2008) (Wheat bran) and Virupakshi et al., (2004) (Rice bran) used 1:2 (w/v) ratio for the same. This clearly indicates that substrate to moisture ratio may vary with microbial strain used in the study.

Bacillus sp. NIORKP76 isolate produced highest xylanase titer at the end of 72h incubation period using wheat bran as a substrate which was also reported by Kumar et al., (2017), Kamble and Jadhav, (2012), Khandeparker and Bhosle, (2006b) and Virupakshi et al., (2004) in their studies. While all other reported enzyme production in 72 hours the major advantage of *Bacillus* sp. NIORKP76 isolate is its ability to produce optimum AFase and AXE in 48h of incubation period that is before xylanase reaches its optimum concentration.

Xylanolytic enzymes reported by Khandepaerker and Jalal, (2015) showed a similar trend for *Lactobacillus* sp. while in *Arthrobacter* sp. AFase and AXE reached its maximum concentration after xylanase. The major difference between the previously reported xylanolytic enzymes by Khandeparker and Jalal, (2015) and the present study is that *Bacillus* sp. NIORKP76 showed high production of AFase during the bacterial growth which is almost 10 to 100 times higher than the reported one. For an industrial application which includes utilization of agricultural residues, bacterial isolate which produces a high amount of all xylanolytic enzymes in given set of conditions will be preferred rather than the isolate which produces a low amount of enzymes under same conditions.

The crude xylanolytic enzyme consortium produced by *Bacillus* sp. NIORKP76 strain under SSF was precipitated, dialyzed and partially purified by ion-exchange chromatography.

Dialyzed protein sample loaded on to anion exchanger resin column (DEAE-Sepharose) delivered two major protein peaks. First protein peak showed maximum xylanase activity along with AXE activity, whereas AFase activity was measured in the beginning fractions of second major peak (**Figure 4.4**). The xylanolytic enzymes containing fractions were pooled together concentrated and further used for molecular weight determination and zymogram analysis.

As purification of xylanolytic enzymes preceded the decrease in the number of protein bands observed thus confirming partial purification of xylanolytic enzymes. Crude xylanolytic enzyme consortia showed multiple protein bands as compared to very few protein bands after partial purification by anion exchanger column. Zymogram analysis revealed active xylanase band corresponding to a molecular weight of ~31 kDa, whereas active AFase and AXE bands were observed with molecular weight ~66 kDa and ~45 kDa respectively (**Figure 4.5**).

The optimum pH of the crude xylanase produced by *Bacillus* sp. NIORKP76 strain was 8.0 (sodium phosphate buffer), while AFase and AXE activity was highest at pH 6.0 (sodium phosphate buffer) (**Figure 4.6**). Xylanase retained 92% of its activity at pH 8.0, whereas AFase and AXE showed remarkable stability over a wide range of pH (5.0 to 8.0) and retained 100% of their activity at all pH values for 24h. Interestingly, AFase and AXE showed an increase in activity after 2h and 6h of incubation period respectively which further subsided and lasted up to 100% over the period of 24h (**Figure 4.7**).

The optimum temperature for all xylanolytic enzymes was found to be 60 °C at their respective optimum pH value (**Figure 4.8**). Standard assay of crude enzyme mixture diluted in suitable buffer incubated at various temperatures unveiled the influence of temperature on xylanolytic enzymes. Over the incubation period, xylanase from crude enzyme aliquot incubated at 40 °C displayed an increase in activity and retained its 100% activity for 24h. AFase and AXE also exhibited superior properties in terms of thermal stability at a wide range of temperature (30°C and 40 °C). While AFase retained 100% of its activity for 24h AXE managed to retain 80% activity at all incubation temperatures (**Figure 4.9**).

The xylanase enzymes produced by *Bacillus* sp. NIORKP76 isolate exhibited optimum pH and temperature of 8.0 and 60 °C respectively. Xylanases reported by Khusro et al., (2016) showed optimum pH 7.0 and temperature optima of 35 °C. Chaturvedi et al., (2015) reported xylanase having pH and temperature optima of 6.5 and 45 °C respectively. Teeravivattanakit et al., (2016) also reported xylanase with pH and temperature optima of 7.0 and 50°C respectively. Similarly, Boucherba et al., (2017) reported xylanase with pH and temperature optima of 7.0 and 55 °C. Adigüzel and Tunçer, (2016) reported xylanase having temperature optima of 60 °C which was similar to our reported xylanase but pH optima was only 6.0.

The xylanase enzyme was found to be very stable, retaining 92% of residual activity when incubated in buffer pH 8.0 and 137% residual activity was recorded when incubated at 40 °C for 24h, unlike xylanase reported by Adigüzel and Tunçer, (2016) which was having a half-life of 10h when incubated at pH 7.0 and temperature 40 °C. Xylanase reported by Khusro et al., (2016) was able to retain approximately 80% activity, when incubated for 4h in pH 8.0 and at 40 °C.

The AFase and AXE isolated in this study were having pH and temperature optima of 6.0 and 60 °C respectively. Also, AFase and AXE retained more than 100% residual activity over a wide range of pH (5.0 to 8.0) for 24h incubation period. Although Khandeparker et al., (2008) described AFase retaining 100% activity at pH 9.0 for 24h, there was a major loss in residual activity observed when lower pH values were considered, making it alkalophilic in nature but not accustomed to wide pH range unlike AFase isolated in our study. In the case of thermal stability, AFase retained 100% residual activity for 24h at 50 °C which is similar to AFase property reported by Khandeparker et al., (2008). AXE retained 80% of residual activity at 50 °C for 24h which was superior to AXE reported by Hettiarachchi et al., (2019) which retained 100% activity at 45 °C only for 2h.

In most of the previous studies conducted so far individual xylanolytic enzyme production and their optimization was targeted (Virupakshi et al., 2004; Chaturvedi et al., 2015; Nawawi et al., 2017). To the best of our knowledge, this study is one among the few reports where the characterization and optimization of the xylanolytic enzyme are studied simultaneously.

Table 4.1 Morphological, physiological and biochemical characteristics of the bacterial isolate NIORKP76.

Tests	**Results**	**Tests**	**Results**
Colony Morphology		***Carbohydrate Utilization***	
Shape	Circular	Xylose	+
Margin	Entire	Glucose	+
Elevation	Raised	Sucrose	+
Consistency	Moist	Mannitol	+
Color	White	Arabinose	+
Opacity	Opaque	Trehalose	+
Gram Nature	Gram Positive	Lactose(ONPG)	+
Shape of the Cell	Rods	Malonate	-
Growth Conditions		Arginine	-
Temperature	30 °C-40 °C	VP	-
pH	7.0-8.0	Citrate	-
Aerobe/Anaerobe	Facultative anaerobe	Nitrate Reduction	-
Motility	Motile	Catalase	+

+ : Positive, **- :**Negative

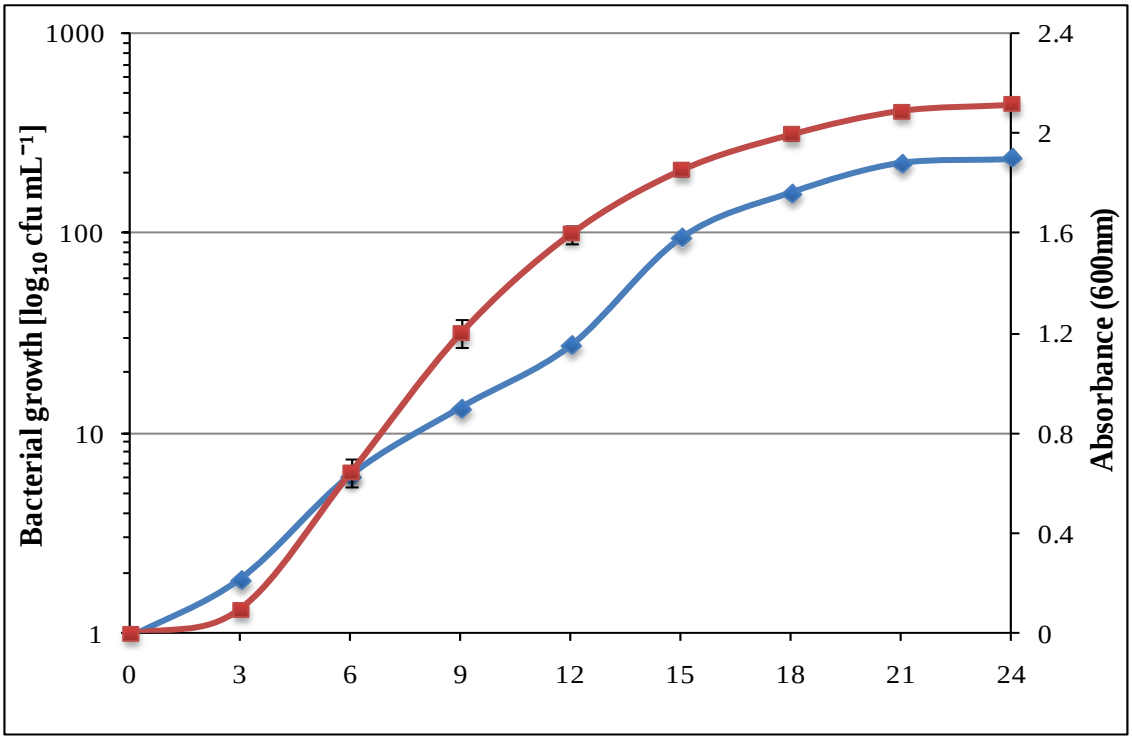

Figure 4.1 Growth measurement of *Bacillus* sp. NIORKP76,

-■- Average absorbance at 600 nm, -♦- Log_{10} of average cfu mL^{-1}

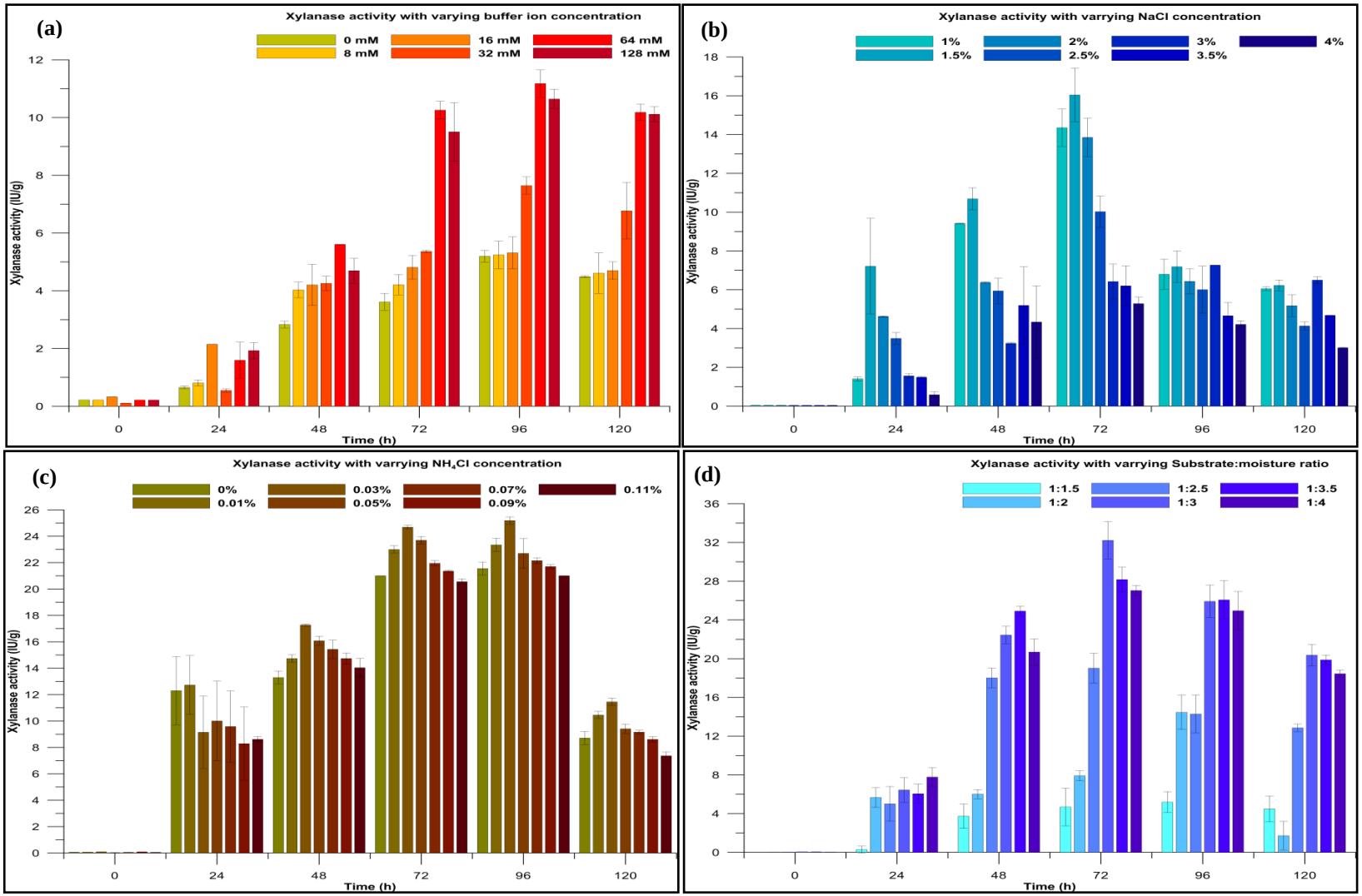

Figure 4.2 Media optimization for xylanase production**;** (a) buffer ion, (b) NaCl, (c) NH_4Cl and (d) substrate to moisture ratio (w/v).

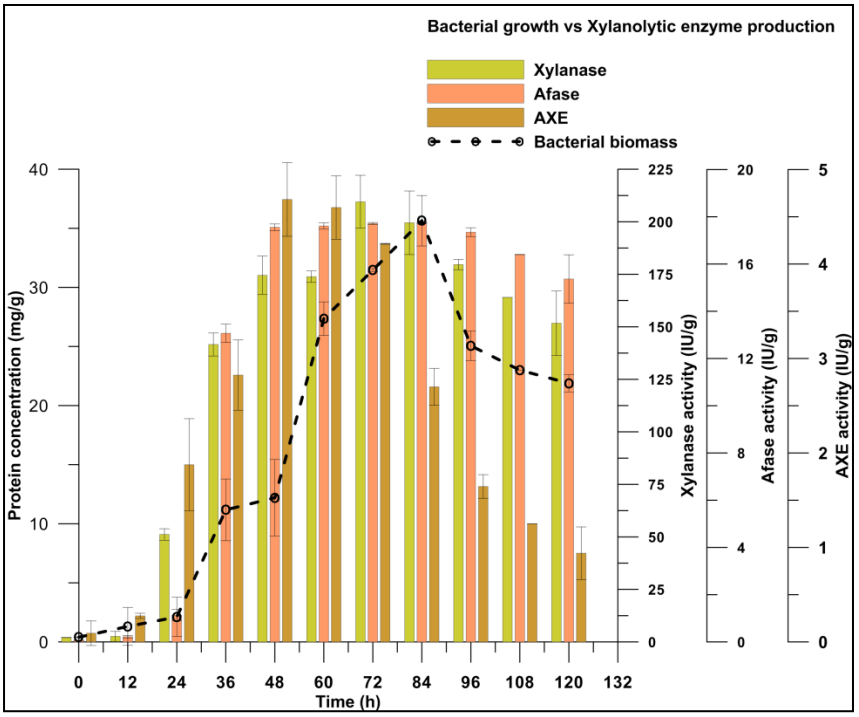

Figure 4.3 Bacterial growth and xylanolytic enzyme production.

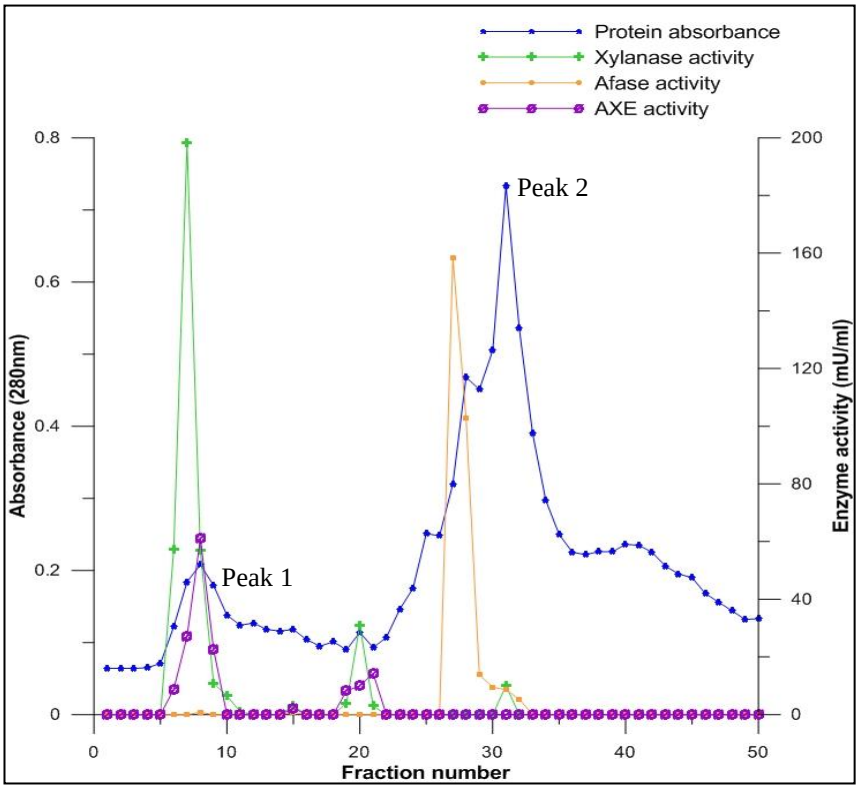

Figure 4.4 Xylanolytic enzyme elution profile on DEAE-Sepharose column.

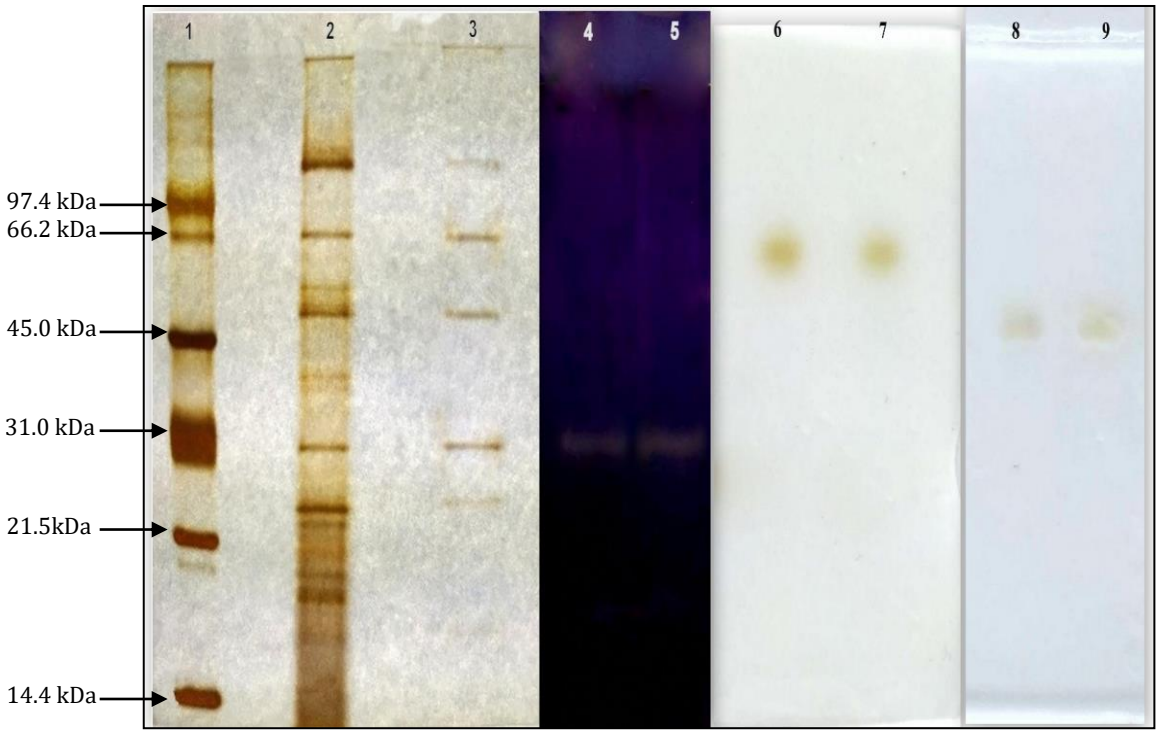

Figure 4.5 Molecular weight and zymogram analysis; Lane 1: Low range protein marker (BioRad) Lane 2: Crude protein mixture; Lane 3: Partially purified proteins; Lane 4 and 5: Xylanase zymogram; Lane 6 and 7: AFase zymogram; Lane 8 and 9: AXE zymogram.

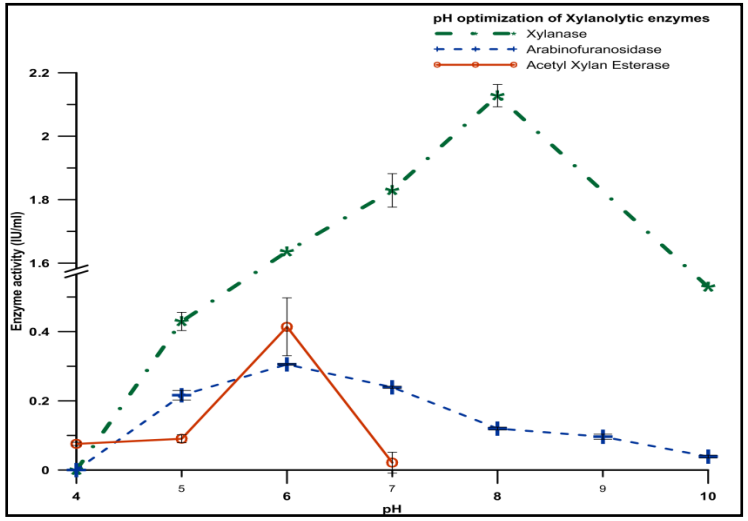

Figure 4.6 Effect of pH on Xylanolytic enzyme activity

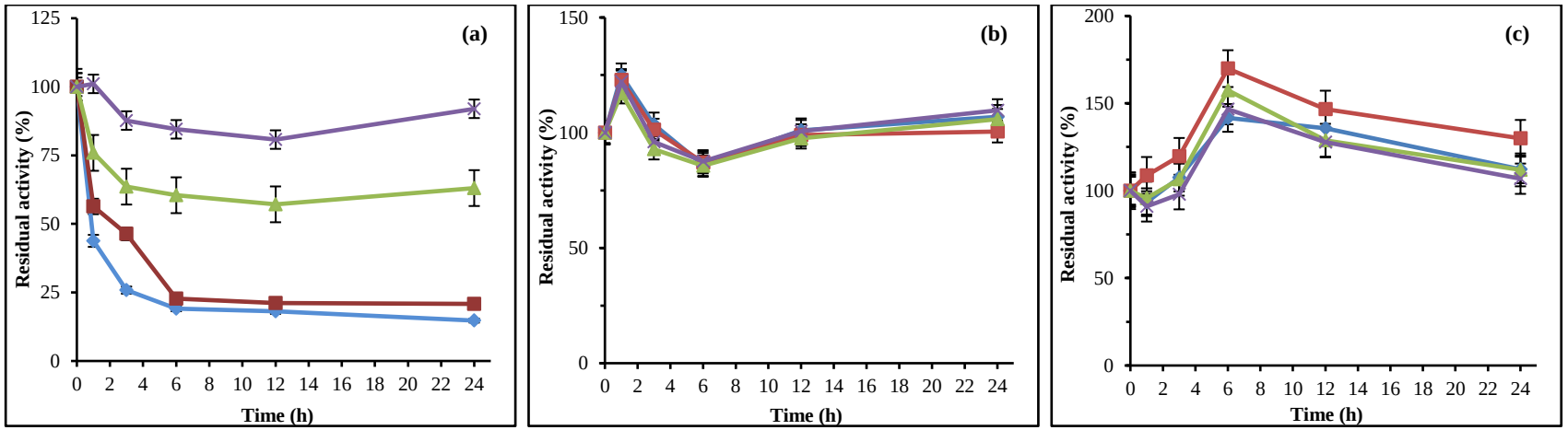

Figure 4.7 Effect of pH on stability of xylanolytic enzymes; (a) Xylanase, (b) AFase, (c) AXE

(-♦- -pH 5.0, -■- -pH 6.0, -▲- -pH 7.0, -×- -pH 8.0)

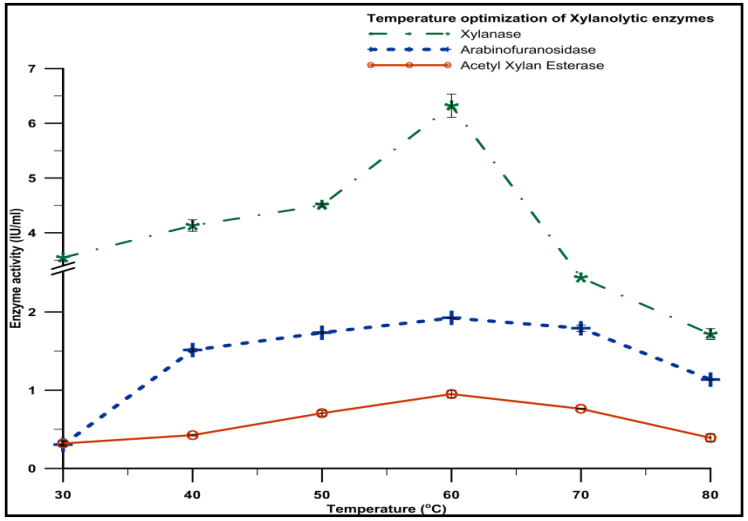

Figure 4.8 Effect of temperature on Xylanolytic enzyme activity

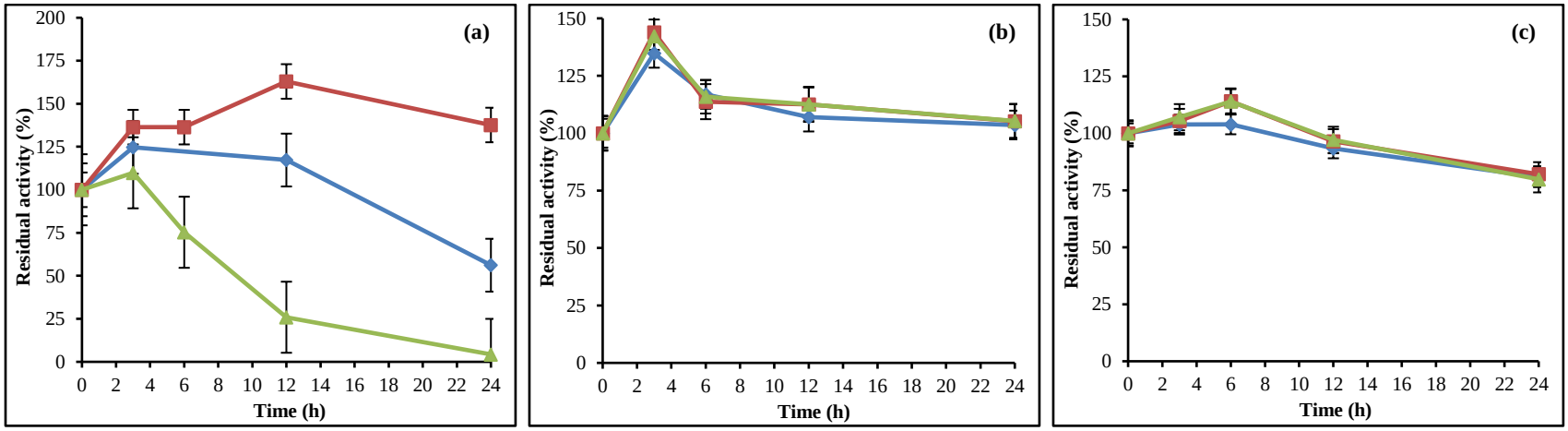

Figure 4.9 Effect of temperature on stability of xylanolytic enzyme; (a) Xylanase, (b) AFase, (c) AXE

(-♦- -30 °C, -■- - 40 °C, - ▲ - -50 °C)

Chapter - 5

Bio-bleaching of Pulp using Xylanolytic Enzymes

5.1 Introduction

The pulp and paper industry which is considered to be one of India's rapid emerging business sector has shown remarkable development in the last few years. Government policies are building enormous pressure on paper industries so as to maintain a pollution-free and clean environment (Singh et al., 2019). Pulp bleaching irrespective of their origin uses large amounts of chlorinated compounds. The byproducts formed while chemical processing are mutagenic, toxic and persistent thus are the reason for numerous problems in the biological systems. In view of this pressure, the pulp and paper mills/industries are trying to change chlorine-based chemicals and move towards environment-friendly molecules (Beg et al., 2000). Environment-friendly bleaching enzymes mainly xylanases and laccases have great potential for bio-bleaching of agro-based pulps at an industrial scale, due to this, the interest in xylan degrading enzymes have developed extensively over the past few years.

Viikari et al., (1994) first introduced the process where biological delignification of pulp was attempted using enzymes. Xylanase, which is a hydrolytic enzyme, is mainly used to breakdown the lignin–carbohydrate complex, which acts as a physical barrier during chemical bleaching. Pre-bleaching of kraft pulp using xylanase was reported to minimize the amount of chlorine required for bleaching and thus dipping chloro-organic discharges (Koponen, 1991; Viikari et al., 1994). There are several articles on the application of xylanases for pre-treatment of unbleached pulp, which proved to be efficient to reduce the use of chlorinated compounds in the subsequent bleaching process (Nagar and Gupta, 2020; Dutta et al., 2020; Khandeparker and Bhosle, 2007; Nair et al., 2010; Chawannapak et al., 2012; Sridevi et al., 2016).

Due to the heterogeneity of hemicellulose, its hydrolysis requires the action of a complex enzyme system. This usually composed of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterases. All these enzymes act cooperatively to convert xylan to its constituent sugar. The presence of such multifunctional xylanolytic enzyme systems is quite widespread among bacteria and fungi (Woodward, 1984; Wong et al., 1988; Coughlan and Hazlewood, 1993; Clarke et al., 2000; Bajpai et al., 2006; Mi et al., 2014; Chaturvedi et al., 2015; Liao et al., 2015; Teeravivattanakit et al., 2016; Nawawi et al., 2017). The combined effect of hemicellulases on different pulp woods are published (Elegir et al., 1995). It was noticed that synergism does exist between

the two types of endoxylanases reported from *Streptomyces* sp., which resulted in the enhancement of brightness in softwood (Elegir et al., 1995). However, the bioleaching process needs further development; different xylanolytic enzymes and their synergistic effect need to be studied; this was also suggested by Dhiman et al., (2009). According to the author, the combined use of xylanase with other xylanolytic enzymes can be a better option for kraft pulp treatment.

Given this, the present chapter is mainly focused on the application of cellulase-free xylanolytic enzyme consortia (xylanase, arabinofuranosidase and acetyl xylan esterase) isolated from *Bacillus* sp. NIORKP76 strain on pre-treatment of unbleached pulp samples (Hardwood pulp and Chemical bagasse pulp).

5.1 Materials and methods

5.1.1 Microorganism

As described in chapter 3 bacterial isolate identified as *Bacillus* sp. NIORKP76 strain (GenBank accession number: MH767158) (Parab et al., 2020) was isolated from the mangroves of Goa and used in this study for xylanolytic enzymes production under SSF technique (Chapter 4). The application of cellulase free crude xylanolytic enzyme consortia in kraft pulp pre-treatment process is presented in this chapter.

5.1.2 Characteristics of xylanolytic enzymes

The xylanolytic enzyme consortium (*B*-XEC) production was carried out under solid-state fermentation (SSF) using a modified basal salt solution (MBSS) and wheat bran as substrate. Optimum pH of xylanase, arabinofuranosidase (AFase), and acetyl xylan esterase (AXE) was found to be 8.0, 6.0, and 6.0, respectively (**Figure 4.6**), whereas optimum temperature for all xylanolytic enzymes studied was 60 °C (**Figure 4.8**). All xylanolytic enzymes were stable for 24h at pH 8.0 and 40 °C (**Figure 4.7 and 4.9**).

Xylanase enzyme (*B*-X) (free from other xylanolytic enzymes) was produced under submerged fermentation (SmF) using MBSS containing 0.5% xylan as sole carbon source along with 0.2% yeast extract and 0.03% peptone. BSS medium composed of: NaCl, 15.00g; $MgSO_4$, 7.00g; NH_4Cl, 0.3g; KCL, 0.75g; KH_2PO_4 (10 mM), 6.00 mL; K_2HPO_4 (10 mM), 14.00 mL; trace metal solution, 1 mL; distilled water, 1 liter. The composition of trace metal solution is as follows: H_3BO_3, 2.85g; $FeSO_4.7H_2O$, 2.49g; $MnCl_2.7H_2O$, 1.80g; $CuCl_2$, 0.03g; Na-tartrate, 1.77g; $ZnCl_2$,

0.02g; $Na_2MoO_4.2H_2O$, 0.02g; $CoCl_2$, 0.04g; distilled water, 1 liter. Commercially available xylanase (CM-X) was purchased from Sigma-Aldrich, USA (*Trichoderma longibrachiatum*, X2629).

5.1.3 Pulp samples

Two different types of unbleached kraft pulp samples, hardwood pulp (HW pulp) and chemical bagasse pulp (CB pulp) were used in the current study. The kraft pulp samples were kindly spared by Tamilnadu Newsprint and Papers Limited, Tamilnadu, India. Prior to any experimental usage, both the kraft pulp samples were thoroughly washed with tap water (until neutral pH), oven-dried and stored for further use.

The initial kappa number was found to be 21.51 and 14.13, whereas brightness was 41.3 and 53.1 (% ISO) of hardwood and chemical bagasse pulp respectively.

5.2.4 Optimization of xylanolytic enzyme pre-treatment conditions on kraft pulp

The enzymatic pre-treatment studies were carried out on kraft pulp using a crude xylanolytic enzyme from *Bacillus* sp. NIORKP76 strain and commercial xylanase at Biological Oceanography Division of National Institute of Oceanography, Goa, India. The enzyme activity of xylanase, AFase and AXE in one millilitre of a crude xylanolytic enzyme consortium was measured to be 2.5U, 0.67U, and 0.45U respectively. The enzyme activities were determined before carrying out experimental work.

5.2.4a Influence of xylanolytic enzyme dose

The effect of varying xylanolytic enzyme dosages was studied by treating oven-dried pulp samples (HW and CB) with different doses of xylanolytic enzymes. The xylanolytic enzyme dosages were varied by changing the volume of crude *B*-XEC. The enzyme doses ranging between 2.5 to 25 Ug^{-1} pulp of xylanase, 0.67 to 6.7 Ug^{-1} pulp of AFase, and 0.45 to 4.5 Ug^{-1} pulp of AXE were used. The pulp samples were mixed with suitably diluted *B*-XEC and sodium phosphate buffer (pH 8.0) with 5% consistency (Clarke et al., 2000) in zip lock polyethylene bags and incubated in a water bath maintained at a constant temperature of 40 °C for 2h. Pulp sample was prepared and treated using the same physiochemical parameters in the absence of any xylanolytic enzyme used as control. At the end of the incubation time, the pulp samples were washed with distilled water and air-dried.

5.2.4b Influence of incubation period

To study the influence of the incubation period on bio-bleaching, the reaction mixture containing an optimized dose of *B*-XEC and pulp sample prepared in sodium phosphate buffer (pH 8.0) with 5% consistency was incubated in a water bath maintained at a constant incubation temperature of 40 °C for varying time intervals (0-6h). At the end of a given time interval, pulp samples were washed with an equal volume of distilled water and air-dried.

5.2.4 c Influence of pulp consistency

The effect of pulp consistency on xylanolytic enzyme bleaching was studied by varying pulp concentration. Pulp samples with 2.5% to 10% consistency were prepared in sodium phosphate buffer (pH 8.0). Optimized *B*-XEC dose and the optimized incubation period were used to treat the enzymatic treatment of unbleached pulp samples under constant incubation temperature of 40 °C. Following the incubation period, pulp samples were washed with an equal volume of distilled water and air-dried.

5.2.5 Efficacy of xylanolytic enzyme consortium

The efficiency of enzymatic pre-treatment for bio-bleaching of unbleached pulp samples (HW and CB) was examined by treating pulp samples with crude xylanolytic enzyme consortium, crude xylanase (free from other xylanolytic enzymes) isolated from *Bacillus* sp. NIORKP76 and commercially available xylanase (Sigma-Aldrich, USA). The optimized *B*-XEC dose containing 5 Ug^{-1} pulp of xylanase, 1.34 Ug^{-1} pulp of AFase, and 0.90 Ug^{-1} pulp of AXE was used. Equivalent dose (5 Ug^{-1} pulp) of commercial xylanase (CM-X) and *Bacillus* sp. NIORKP76 xylanase (*B*-X) was used for the treatment of pulp samples. The pulp samples were mixed thoroughly with optimized enzyme dose and sodium phosphate buffer (pH 8.0) at a 5% consistency in a polyethylene zip lock bag and plunged in a water bath, maintaining a constant temperature of 40 °C for an incubation period of 2h. After enzyme pre-treatment, pulp samples were washed thoroughly with distilled water, and the filtrate was collected to carry out analysis as per the protocol mentioned above. Controls were prepared similarly, devoid of the enzyme. The pulp obtained was air-dried and stored for further analysis.

5.2.6 Spectrophotometric analysis of the filtrate

The absorbance of the filtrates collected after enzymatic pre-treatment was measured spectrophotometrically at a wavelength ranging from 200 nm to 465 nm. The release of phenolic and hydrophobic compounds was estimated by measuring the absorption at wavelength 237 nm and 465 nm respectively (Khandeparker and Bhosle, 2007). The reducing sugar released in the filtrate was measured using dinitrosalicylic (DNS) acid method (Miller, 1959).

The maximum decrease in κ number and an increase in the release of the above-said products were considered to be the optimum conditions required for enzymatic pre-treatment.

5.2.7 Chemical bleaching

An air-dried kraft pulp samples pre-treated with xylanolytic enzymes were used for hypochlorite and followed by hydrogen peroxide treatment. For hypochlorite treatment, the pulp samples were made to 5% consistency and divided into three equal parts. Hypochlorite solution of 4%, and 2% concentration was prepared, and each portion of the pulp sample was treated with the respective solution and incubated at 40 °C for 1h (Khandeparker and Bhosle, 2007). At the end of the first chemical treatment step, pulp samples were washed and air-dried. All pulp samples obtained after hypochlorite treatment were treated with 1% hydrogen peroxide in a consecutive step. The pulp consistency, incubation period, and temperature were the same as used for the previous step. The pulp samples were washed with distilled water and air-dried. Kappa number, brightness, and viscosity of bleached pulp samples obtained at the end of both chemical treatment stages were determined.

5.2.8 Analysis of pulp properties

Kraft pulp samples (HW and CB) obtained during optimization of pre-bleaching conditions, after pre-bleaching by various sources of xylanolytic enzyme and followed by chemical bleaching were used for determination of κ number as per Technical Association of Pulp and Paper Industry (TAPPI) Test Methods (T-236 m-60). The effect of initial xylanolytic enzyme pre-treatment and final chemical treatment on brightness and viscosity of pulp was also determined.

5.2.6a Kappa number of pulps

The amount of lignin content present in pulp is called kappa number (κ number). The relative degree of delignification of enzyme pre-treated pulp samples and control pulp sample was determined using standard TAPPI protocols (Khandeparker and Bhosle, 2007). Pulp samples obtained after each experiment were converted to handmade pulp sheets (~5-10g) and oven-dried. Three grams of the test specimen (moisture-free pulp) was accurately weighed, transferred to a 1 L beaker and disintegrated completely in 250 mL distilled water till fibres get separated. Rinse the apparatus with 130 mL distilled water. A solution comprising 50 mL 0.1 N $KMnO_4$ and 50mL 4.0 N H_2SO_4 was prepared separately and added to the above solution. The solution containing apparatus was rinsed with 10 mL distilled water and added to the above beaker. Precisely after 10min of continuous stirring 10 mL 1.0 N KI solution was added to reaction mixture mixed and immediately titrated against 0.2 N $Na_2S_2O_3$ solution filled in burette using 1% starch as an indicator.

Kappa number was calculated using the following formula:

$$\text{Kappa Number} = \frac{P \times f}{W} [1 \times 0.013 (25 - t)]$$

$$\text{And,} \quad P = \frac{(b - a) \times N}{0.1}$$

Where,

f = Factor for correction to a 50% $KMnO_4$ consumption

W = Weight of moisture-free pulp (in gram)

P = Volume of 0.1 N $KMnO_4$ consumed by specimen

b = Volume of $Na_2S_2O_3$ consumed in the blank determination

a = Volume of $Na_2S_2O_3$ consumed in the test sample

N = Normality of $Na_2S_2O_3$

t = Actual reaction temperature

5.2.8b Brightness and Viscosity of pulp

The analysis of brightness and viscosity was carried out at Paper and Pulp Research Institute (PAPRI), Rayagada, Odisha-India. The brightness of pulp samples was determined as per the ISO 2470 method using L & W Elrepho spectrophotometer (model-SE070, Kista-Sweden). The whiteness, yellowness, fluorescence, colour,

opacity and tint of pulp samples were measured using above said instrument to ensure ISO optical standards for paper production. Viscosity was measured by standard TAPPI test method T230.

5.3 Result and Discussion

Optimization of bio-bleaching conditions is an essential step to maximize lignin removal and enhance pulp characteristics. The xylanolytic enzyme consortia extracted from *Bacillus* sp. NIORKP76 was optimized for pre-bleaching parameters such as enzyme dose, incubation period, and pulp consistency. The different parameters were optimized separately to improve the efficacy of enzyme treatment. Enzymatic biobleaching reported by Nair et al., (2010), Khandeparker et al., (2007) and Sridevi et al., (2016, 2017) also provided the importance of pertaining to use optimization parameters prior to enzymatic pre-treatment of pulp.

The xylanolytic enzymes consortium comprising 5 Ug-1 of xylanase (1.34 Ug-1 and 0.9 Ug-1 of AFase and AXE, respectively) was most efficient for bio-bleaching pulp sample (HW and CB) when incubated for 2h at 40 °C with 5% pulp consistency. It was seen that the kappa number (κ) was reduced to 54.34%, and a 4-5-fold increase in the release of reducing sugar was observed for the HW pulp sample. Similarly, a 26.89% reduction in κ number and increase in the release of reducing sugar by 7-8-fold was measured for CB pulp sample at 5U of xylanase dose. The release of phenolic (237 nm) and hydrophobic (465 nm) compounds was also found to be elevated in both types of pulp samples with the reaction conditions above-mentioned (**Figure 5.1a, b**). The decrease in κ number and the simultaneous increase in the release of reducing sugar, phenolic, and hydrophobic compounds reveal the delignification process by *B*-XEC. The effect of xylanolytic enzyme dose on pulp bio-bleaching was highly significant, which is evident from a one-way analysis of variance (ANOVA) ($p < 0.05$).

The effect of time concerning enzyme treatment on pulp was investigated using an optimized enzyme dose. After 1h of the incubation period, HW and CB pulp samples showed a 30.8% and 3.9% reduction in κ number, respectively. A significant decrease in κ number was seen after 2h of the incubation period. A steep reduction in κ number of 54.43% and 27.10% in HW and CB pulp, respectively, was observed. Similarly, the maximum release of reducing sugar, phenolic, and hydrophobic

compounds was observed at a 2h incubation period for both types of pulp samples (**Figure 5.2a, b**). It was also observed that the incubation time did not show a further reduction in κ number with further increase in time. The effect of incubation time on bio-bleaching of both types of pulp was significant and evident from one way ANOVA ($p < 0.05$).

Pulp consistency optimization is a must to attain a proper and effective spread of the enzyme to improve the enzyme treatment efficiency. During the enzymatic pre-bleaching experiment, pulp consistency was varied from 2.5 to 10%. Pulp consistency of 2.5% with 5U enzyme dose and at 2h incubation time could deliver up to 33.5% and 8.28% reduction in κ number for HW and CB, respectively. A maximum reduction in κ number was observed when the pulp consistency of 5% was subjected to the pre bleaching process by keeping treatment conditions at an optimized level. The reduction in κ number was 54.67% for hardwood pulp, whereas it was 27.24% for chemical bagasse pulp. Pulp consistency higher than 5% did not contribute to much reduction in κ number (**Figure 5.3a, b**). The $p < 0.05$ from one-way ANOVA reveals the significant effect of pulp consistency on pulp properties such as κ number, the release of phenolic, hydrophobic compounds, and release of reducing sugars.

The release of colour from the pulp treated with xylanase (*B*-X and CM-X) as well as xylanolytic enzyme consortia (*B*-XEC) was studied using UV spectrum analysis. Enzymatically pre-treated pulp filtrate displayed an increase in absorbance and peak at 280 nm, which confirms the release of lignin from the pulp (Nissan et al., 1992). The correlation between the nature of chromophore released and the enzyme is described in the literature (Kulkarni and Rao, 1996; Patel et al., 1993; Khandeparker and Bhosle, 2007). Xylanolytic consortia from *Bacillus* sp. NIORKP76 showed the absorption of ≅ 0.5 when treated with 5 Ug^{-1} of oven-dried hardwood and softwood pulp (**Figure 5.4a, b**). While commercial xylanase as well as single xylanase from *Bacillus* sp. NIORKP76 showed absorption of ≅ 0.3. High absorbance at 280 nm when XEC was used compared to XE gives a clear indication of the effectiveness of XEC during pre-treatment. The xylanase from *Arthrobacter* sp. showed absorption of 1.5 when treated with 20 Ug^{-1} of oven-dried pulp (Khandeparker and Bhosle, 2007), the absorption of 0.8 was recorded by Kulkarni and Rao, (1996) using 20 Ug^{-1} of

oven-dried pulp, while the absorption of 0.4-0.5 was noted when Dwivedi et al., (2010) used 16 Ug^{-1} of oven-dried pulp.

The present work demonstrated that an improved delignification could be achieved by using xylanolytic enzyme consortia when compared to xylanase alone. In enzymatic pre-bleaching of hardwood and chemical bagasse pulp, *B*-XEC delivered excellent results in terms of *κ* number reduction compared to *B*-X, which was partially purified xylanase from *Bacillus* sp. NIORKP76 and CM-X, which is commercial xylanase. In the case of HW pulp samples, *B*-XEC treatment could reduce *κ* number by 55%**,** while 44.6% and 45.8% reduction in *κ* number were seen by *B*-X and CM-X treatment, respectively (**Figure 5.5a**). In the case of CB pulp, enzyme pre-treatment showed a reduction in *κ* number as 27.53%, 20.6%, and 19.0% by *B*-XEC, *B*-X, and CM-X, respectively (**Figure 5.5b**) (**Table 5.1**). Along with a reduction in *κ* number, the release of phenolic compounds, hydrophobic compounds and reducing sugars were highest in *B*-XEC treatment (**Figure 5.5a, b**). The enzymatic biobleaching showed the highest reduction in *κ* number with an increase in brightness index, and without much alteration in viscosity by using xylanolytic consortia compared to xylanase alone, observed differences were highly significant as evident from one-way ANOVA analysis ($p<0.05$).

Biobleaching of eucalyptus pulp has been reported to reduce the kappa number by 3.0 and 3.3 units by using xylanase doses of 2 and 4 Ug^{-1} dried pulp after 1h treatment respectively, thus suggesting that *P. janczewskii* xylanases can be a promising candidate for biobleaching of kraft pulp (Terrasan et al., 2013) which is around 20 % reduction in kappa number. Enzymatic pre-bleaching of kraft pulp reported by Khandeparker and Bhosle, (2007) also showed a 20% decrease in kappa number of the kraft pulp without compromising with viscosity. As per their claim, a 29% reduction in chlorine requirement can be reduced by enzymatic treatment without any decrease in brightness. The biobleaching efficiency of xylanase obtained from *Streptomyces* sp. QG-11-3 was maximum after 2h of incubation using a xylanase dose of 3.5 Ug^{-1}, at 50 °C and at 6% pulp consistency. The reduction in kappa number was found to be 23% (Beg et al., 2000). Sridevi et al., (2017) reported a reduction in kappa number by 4.2 units, and increase in brightness by 4 units as compared to untreated pulp samples when xylanase produced by *Trichoderma asperellum* with enzyme dose of 100 Ug^{-1} and 1h incubation period was used for pre-

treatment. Xylanase from *Aspergillus niger* with an optimum dose of 60 Ug^{-1}, 1h incubation period and pulp consistency 3% enabled a reduction in kappa number by 3.5 units and increase in brightness by 3.1 units as compared to untreated pulp sample (Sridevi et al., 2016).

There are reports in the literature which describes about use of enzymes other than xylanolytic enzymes for biobleaching purpose. Bhoria et al., 2012 reported production and application of mannanase for enzymatic pre-treatment of wheat straw-rich soda pulp samples. In this report with optimum enzyme dose of 5 Ug^{-1} and time interval of 4h showed an increase in brightness of 8.7% ISO and reduction in kappa number by 16% with 6% overall reduction in chlorine consumption.

Report by Gupta et al., (2015) describes the use of enzyme consortia (xylanase and laccase) obtained from two different bacterial species grown with co-culturing technique. Using eucalyptus kraft pulp mixture of enzyme pre-treatment raised brightness and reduced kappa number by 5% and 9.5% respectively which after the final chemical bleaching stage improved to 13% and 15%.

Recently, Angural et al., (2020) used a mixture of xylanase, laccase, and mannanase enzymes for pulp biobleaching on mixed wood pulp and got a 46.32/40.25% reduction in kappa number while 13.21/10.01% improvement in brightness. Xylanolytic consortia reported in the present study could reduce the kappa number of hardwood pulp by 55%, making it a suitable candidate for its application in pulp and paper mill.

Apart from microbial origin, there are reports in which metagenomic approach for novel xylanase is used. Chawannapak et al., 2012, reported metagenomic endoxylanase, with optimum enzyme dose of 50 Ug^{-1} used for bio bleaching which was able to fetch 4.5-5.1% ISO increase in brightness.

To evaluate the potential of *B*-XEC, *B*-X, and CM-X in reducing chlorine consumption, enzyme pre-treated pulp was treated with sodium hypochlorite followed by H_2O_2. Results showed *B*-XEC significantly reduced hypochlorite consumption without compromising the brightness and viscosity compared to control after processing the pulp sample.

Pulp samples (HW and CB) pre-treated with *B*-XEC resulted in a 50% reduction in hypochlorite consumption, by keeping the brightness level same as that of control

(**Figure 5.6a, b** and **5.7a, b**). Pulp samples pre-treated with xylanolytic enzyme consortia utilized 2% hypochlorite and proffered brightness and reduction in kappa number results superior to that of control pulp sample bleached with 4% hypochlorite solution (**Table 5.2**). The xylanolytic enzyme pre-treatment was more prominently effective against HW pulp as compared to CB pulp samples. When compared with kappa number of untreated kraft pulp, solo xylanase (*B*-X and CM-X) pre-treatment followed by 2% hypochlorite treatment led to 84-85.5% reduction in kappa number. The reduction in kappa number was ~87% when the same procedure was followed using *B*-XEC. The control pulp sample treated with 4% hypochlorite displayed only 81.77% reduction in kappa number. In case of CB pulp sample highest reduction in kappa number was recorded for pulp samples pre-treated using *B*-XEC followed bleaching using 2% hypochlorite which was ~89%, whereas, control pulp sample bleached using 4% hypochlorite harnessed only 86%. Further, hypochlorite treated pulp was subjected to H_2O_2, which increased the brightness index with a mild reduction in κ number of pulp samples without affecting viscosity values (**Figure 5.8a, b** and **5.9a, b**).

Beg et al., (2000) reported pulp treated with xylanase when exposed to 4.5% chlorine; it reduced kappa number by 25% and also the brightness was enhanced (% ISO) by 20%. Ninawe and Kuhad, (2006) reported xylanase isolated from *Streptomyces cyaneus* SN32, which was used for biobleaching of pulp. The resulted bleached pulp on treating with a 10% less hypochlorite (5.4%) the brightness was similar to fully bleached pulp (6% hypochlorite). Xylanase enzyme from *Arthrobacter* sp. MTCC 5214 could reduce the chlorine use by 29% without much change in the brightness of kraft pulp (Khandeparker and Bhosle, 2007). Xylanase and laccases mediator system reported by Kapoor et al., (2007) facilitated a 15% reduction in hypochlorite consumption as compared to controls. With the use of optimum conditions such as xylanase dose of 25 Ug^{-1}, 5h incubation period, and 10% pulp consistency, Nair et al., (2010), reported elemental chlorine reduction of 14.3%. Chawannapak et al., (2012) reported a 20% decrease in hypochlorite consumption by using 50 Ug^{-1} of endoxylanase for biobleaching of pulp samples. 6% overall reduction in chlorine consumption was reported by Bhoria et al., (2012) by using 50 Ug^{-1} of endoxylanase for biobleaching of pulp samples. The demand of chlorine-based chemicals was reported to be reduced up to 15% with the use of xylanase for pre-

treatment of kraft pulp (Thakur et al., 2012). Nagar et al., (2013) applied xylanase for pretreatment of pulp which achieved 29.16% reduction in chlorine consumption while maintaining brightness equivalent to control.

As per our understanding, this is one among the few reports where the effect of sole xylanase and consortia of xylanolytic enzymes on bio-bleaching of pulp is shown. This study is unique because the entire xylanolytic enzyme consortia is isolated from a single bacterial strain and used for bio-bleaching of different unbleached pulp samples.

Table 5.1 Effect of xylanolytic enzyme pre-treatment on kappa number, brightness and viscosity of different kraft pulp. Control: Treatment only with buffer; CM-X: Treatment with commercial xylanase; *B*-X: Treatment with *Bacillus* sp. NIORKP76 xylanase; *B*-XEC: Treatment with *Bacillus* sp. NIORKP76 xylanolytic enzyme consortia. (Parab and Khandeparker, 2021)

Bleaching state		Hardwood pulp			Chemical bagasse		
		Kappa number	**Brightness (%)**	**Viscosity (cP)**	**Kappa number**	**Brightness (%)**	**Viscosity (cP)**
Untreated		21.51±0.749	41.30±0.94	6.30±0.021	14.13±0.645	53.10±0.82	7.70±0.041
Pre-treated	Control	20.37±0.652	45.70±0.75	6.20±0.024	13.36±0.583	56.30±0.65	7.50±0.021
	CM-X	11.64±0.559	61.00±0.45	6.00±0.031	11.22±0.562	64.70±0.52	7.30±0.0.23
	B-X	11.92±0.575	60.80±0.63	6.00±0.012	11.44±0.550	63.40±0.63	7.30±0.021
	B-XEC	9.70±0.485	71.00±0.23	5.80±0.026	10.24±0.515	71.70±0.24	6.90±0.035
2% NaOCl	Control	7.56±0.175	70.00±0.23	3.90±0.045	2.70±0.090	64.70±0.35	5.80±0.036
	CM-X	3.11±0.160	77.65±0.15	3.70±0.031	2.16±0.060	75.20±0.12	5.40±0.031
	B-X	3.38±0.170	76.95±0.76	3.70±0.033	2.43±0.036	74.90±0.19	5.50±0.029
	B-XEC	2.84±0.150	82.15±0.36	3.50±0.028	1.49±0.070	76.00±0.24	4.70±0.041
1% H_2O_2	Control	4.05±0.125	72.45±0.51	3.70±0.027	2.53±0.080	67.20±0.58	4.60±0.048
	CM-X	2.23±0.168	78.40±0.62	3.60±0.035	1.95±0.060	76.80±0.51	4.80±0.026
	B-X	2.73±0.132	78.15±0.31	3.60±0.043	2.34±0.070	76.20±0.37	4.20±0.036
	B-XEC	1.75±0.149	83.70±0.85	3.40±0.021	1.36±0.050	78.40±0.54	4.40±0.019

4% NaOCl	Control	3.92±0.149	73.50±0.62	3.70±0.035	1.98±0.075	75.50±0.32	5.30±0.021
	CM-X	2.84±0.140	79.30±0.90	3.50±0.044	1.49±0.045	76.70±0.31	4.80±0.025
	B-X	2.90±0.180	78.95±0.30	3.65±0.019	1.62±0.029	75.90±0.61	5.10±0.028
	B-XEC	2.30±0.170	84.40±0.21	3.45±0.022	1.22±0.035	77.70±0.37	4.30±0.027
1% H_2O_2	Control	3.04±0.142	77.65±0.25	3.60±0.021	1.75±0.050	76.00±0.24	4.10±0.036
	CM-X	1.82±0.154	80.25±0.23	3.45±0.031	1.36±0.040	77.70±0.31	3.90±0.016
	B-X	1.95±0.186	79.15±0.26	3.50±0.048	1.56±0.080	77.50±0.19	3.95±0.031
	B-XEC	1.56±0.157	85.60±0.34	3.35±0.046	1.17±0.070	79.20±0.53	3.90±0.034

Table 5.2 Effect of xylanolytic enzyme pre-treatment on kappa number, brightness and viscosity of different kraft pulp after chlorine reduction. Control: Treatment only with buffer; CM-X: Treatment with commercial xylanase; *B*-X: Treatment with *Bacillus* sp. NIORKP76 xylanase; *B*-XEC: Treatment with *Bacillus* sp. NIORKP76 xylanolytic enzyme consortia; 4C: 4% hypochlorite treatment on pre-treated pulp; 2C: 2% hypochlorite treatment on pre-treated pulp; H: 1% hydrogen peroxide treatment on pre-bleached pulp. (Parab and Khandeparker, 2021)

Bleaching state		Hardwood pulp			Chemical bagasse		
		Kappa number	Brightness (%)	Viscosity (cP)	Kappa number	Brightness (%)	Viscosity (cP)
Untreated		21.51±0.749	41.30±0.94	6.30±0.021	14.13±0.645	53.10±0.82	7.70±0.041
Pre-treated	Control	20.37±0.652	45.70±0.75	6.20±0.024	13.36±0.583	56.30±0.65	7.50±0.021
	CM-X	11.64±0.559	61.00±0.45	6.00±0.031	11.22±0.562	64.70±0.52	7.30±0.0.23
	B-X	11.92±0.575	60.80±0.63	6.00±0.012	11.44±0.550	63.40±0.63	7.30±0.021
	B-XEC	9.70±0.485	71.00±0.23	5.80±0.026	10.24±0.515	71.70±0.24	6.90±0.035
NaOCl	Control-4C	3.92±0.149	73.50±0.62	3.70±0.035	1.98±0.075	75.50±0.32	5.30±0.021
	CM-X-2C	3.11±0.160	77.65±0.15	3.70±0.031	2.16±0.060	75.20±0.12	5.40±0.031
	B-X-2C	3.38±0.170	76.95±0.76	3.70±0.033	2.43±0.036	74.90±0.19	5.50±0.029
	B-XEC-2C	2.84±0.150	82.15±0.36	3.50±0.028	1.49±0.070	76.00±0.24	4.70±0.041
1% H_2O_2	Control-4CH	3.04±0.142	77.65±0.25	3.60±0.021	1.75±0.050	76.00±0.24	4.10±0.036
	CM-X-2CH	2.23±0.168	78.40±0.62	3.60±0.035	1.95±0.060	76.80±0.51	4.80±0.026
	B-X-2CH	2.73±0.132	78.15±0.31	3.60±0.043	2.34±0.070	76.20±0.37	4.20±0.036
	B-XEC-2CH	1.75±0.149	83.70±0.85	3.40±0.021	1.36±0.050	78.40±0.54	4.40±0.019

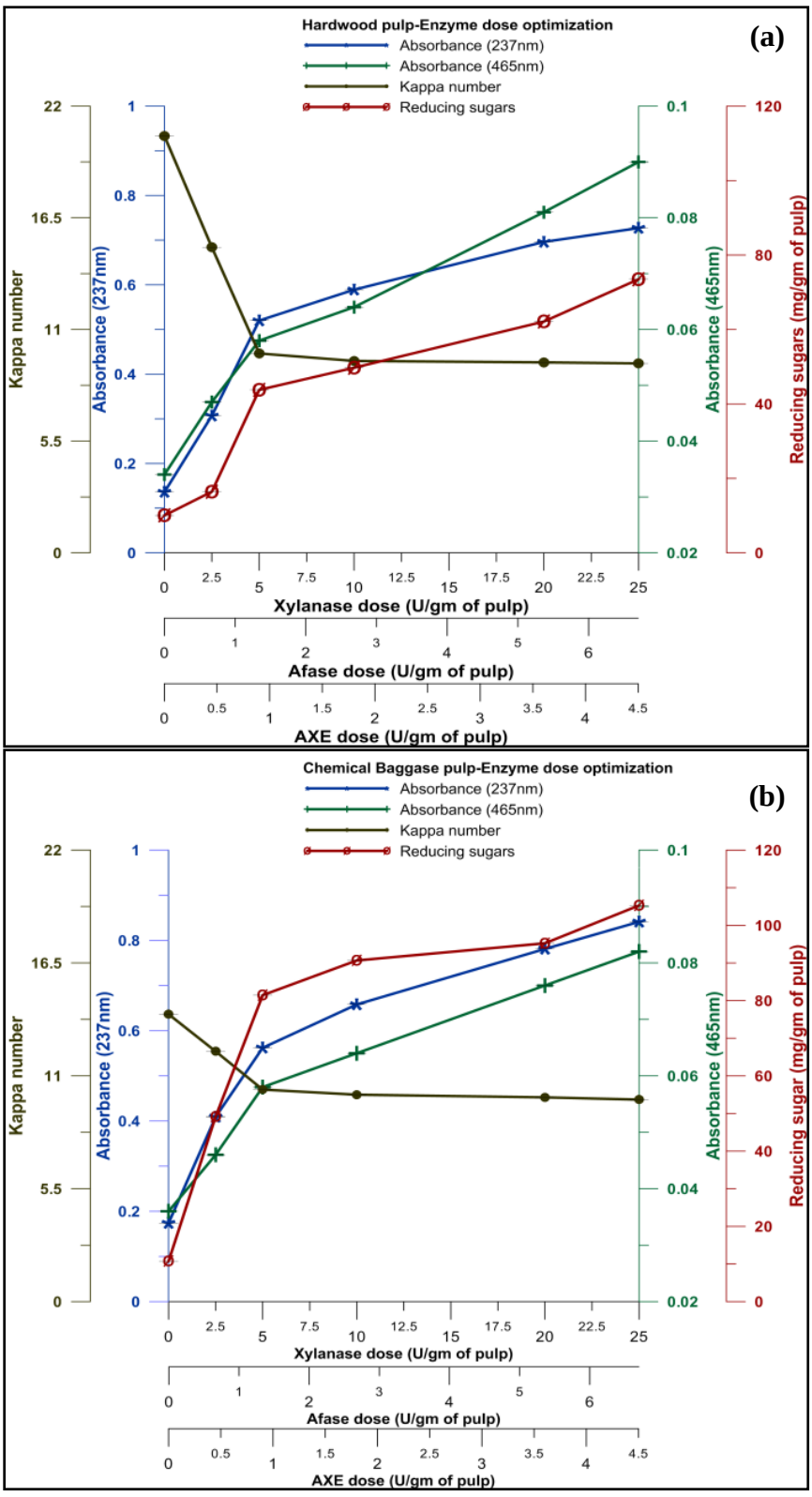

Figure 5.1 Xylanolytic enzyme dose optimization (Ug^{-1} pulp) for pre-treatment of pulp.

a) Hardwood pulp, b) Chemical bagasse pulp

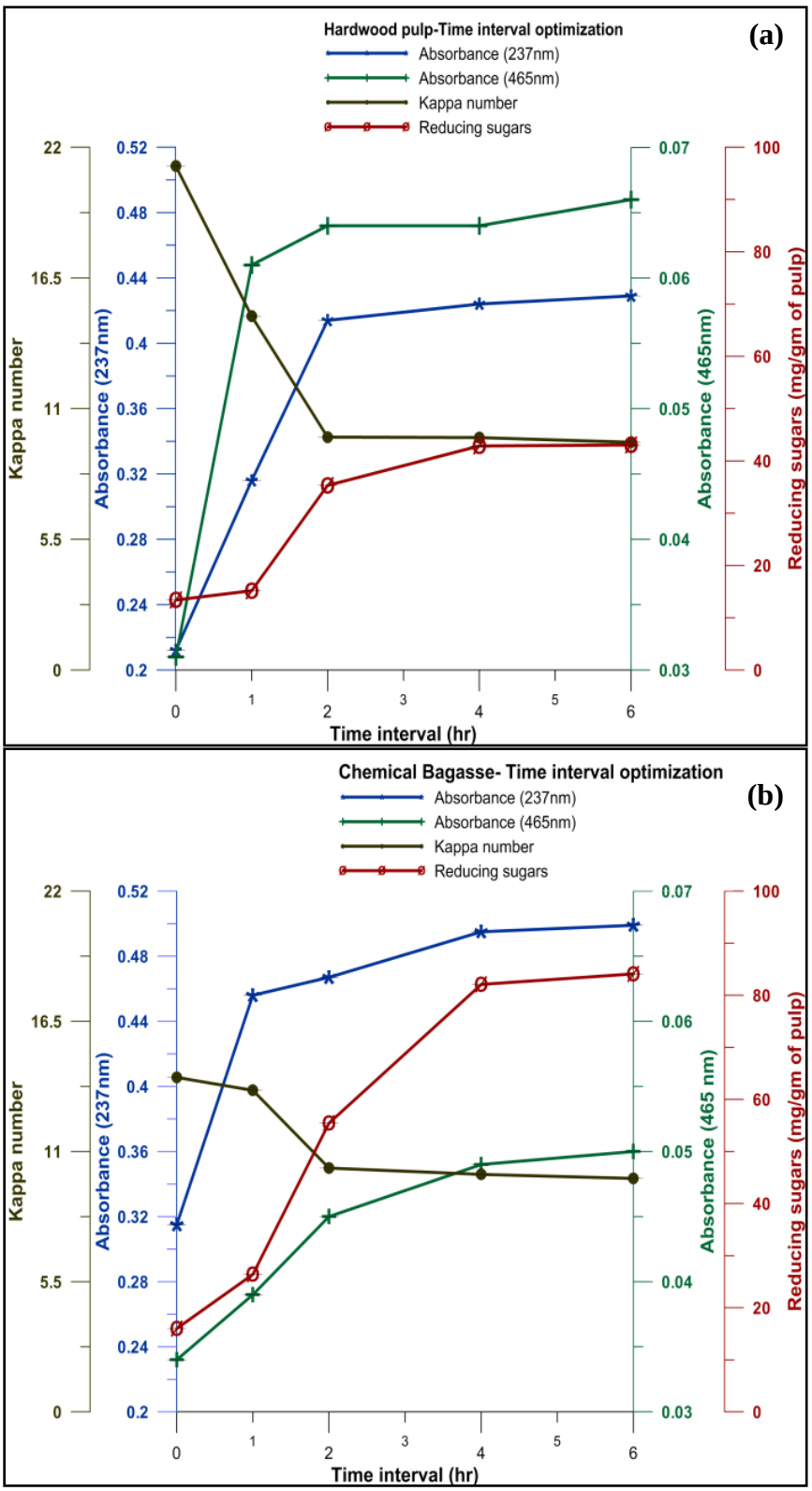

Figure 5.2 Time interval optimization (h) for pre-treatment of pulp.

a) Hardwood pulp,b)Chemical bagasse pulp

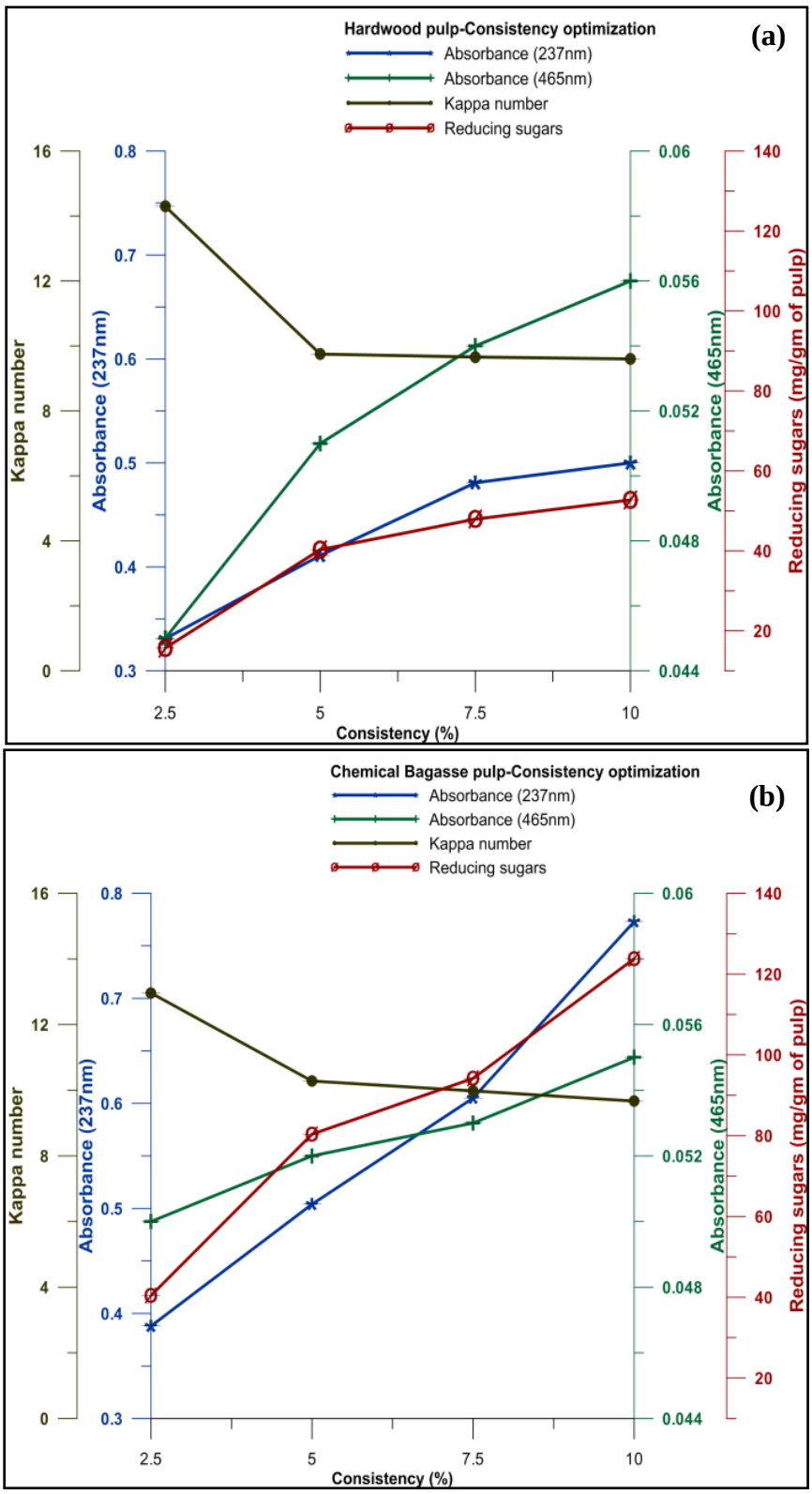

Figure 5.3 Pulp consistency optimization (%) for pre-treatment of pulp.

a) Hardwood pulp, b) Chemical bagasse pulp

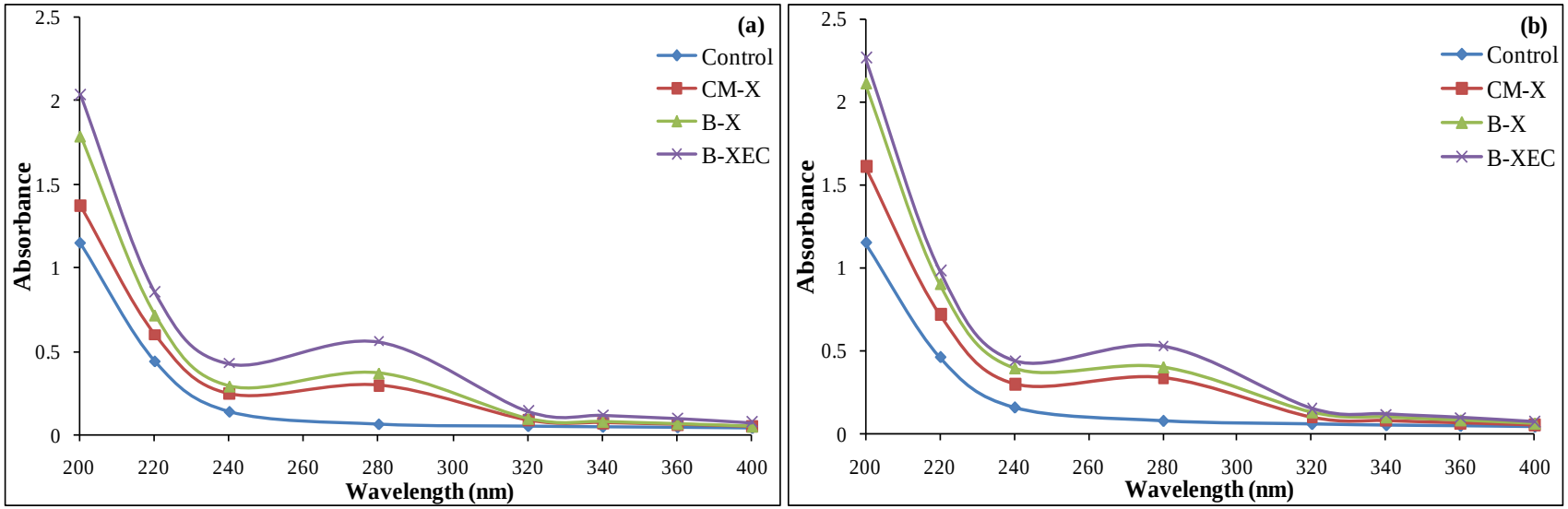

Figure 5.4 UV spectrum of colored compounds released during xylanolytic enzyme treatment.

a) Hardwood pulp, b) Chemical bagasse pulp

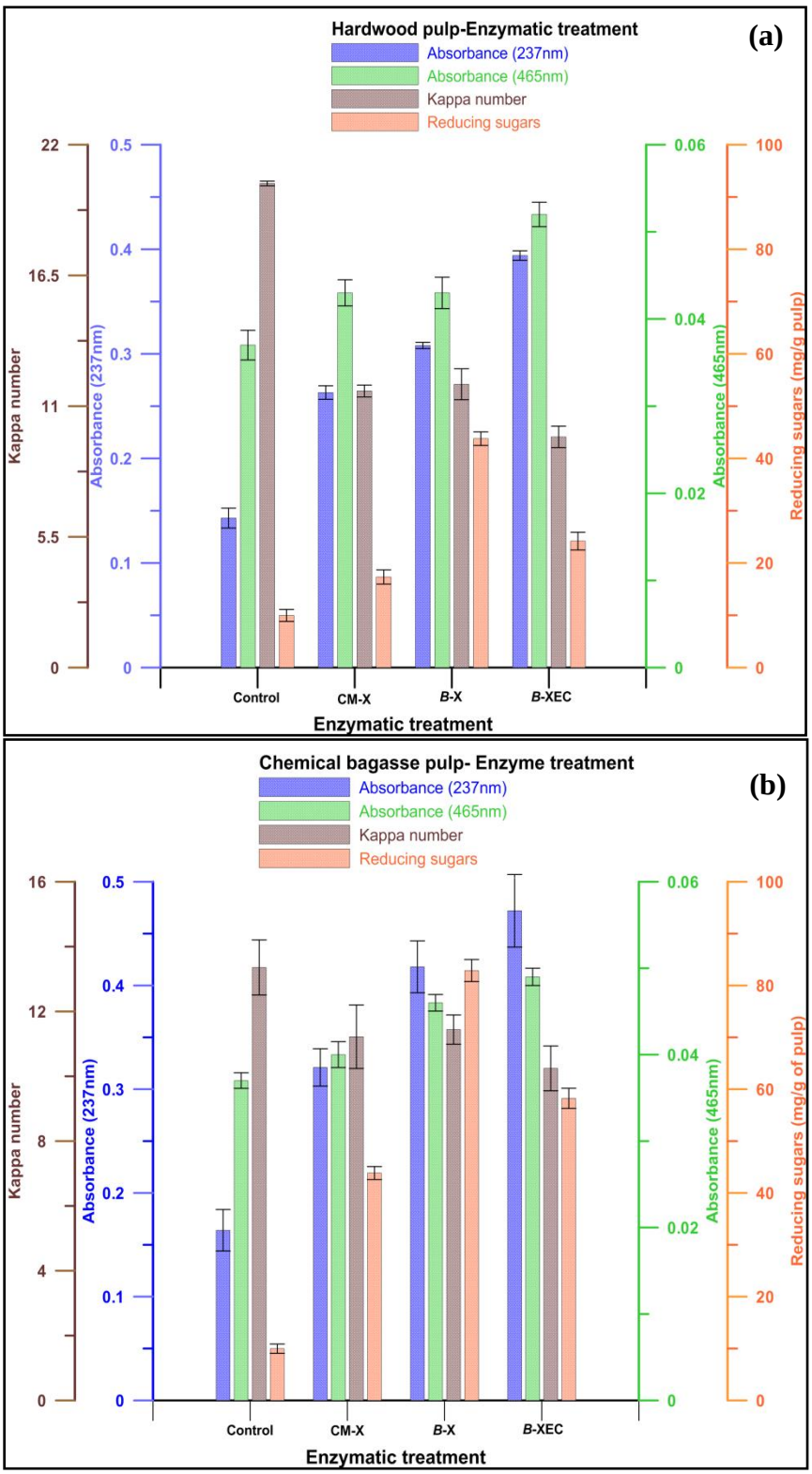

Figure 5.5 Enzymatic pre-treatment of pulp.

a) Hardwood pulp, b) Chemical bagasse pulp

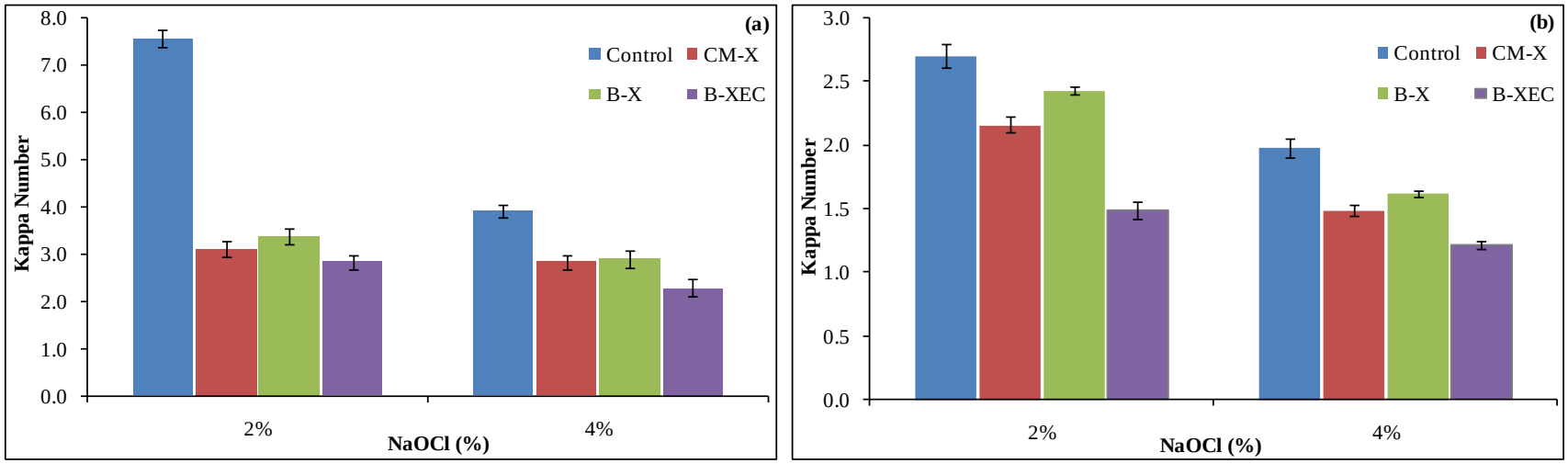

Figure 5.6 Effect of hypochlorite treatment on kappa number of enzymatically pre-bleached pulp: (a) Hardwood, (b) Chemical bagasse

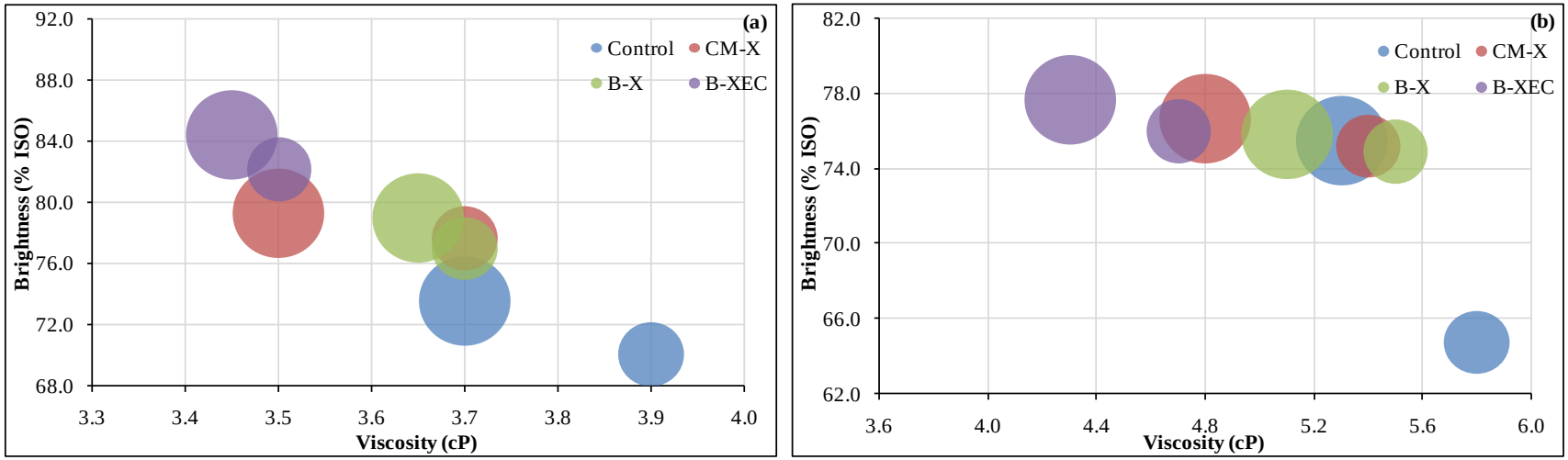

Figure 5.7 Effect of hypochlorite treatment on brightness and viscosity of enzymatically pre-bleached pulp: (a) Hardwood, (b) Chemical bagasse

(Note: Size of the bubble represents the concentration of hypochlorite used in the bleaching of enzymatically pre-treated pulp samples)

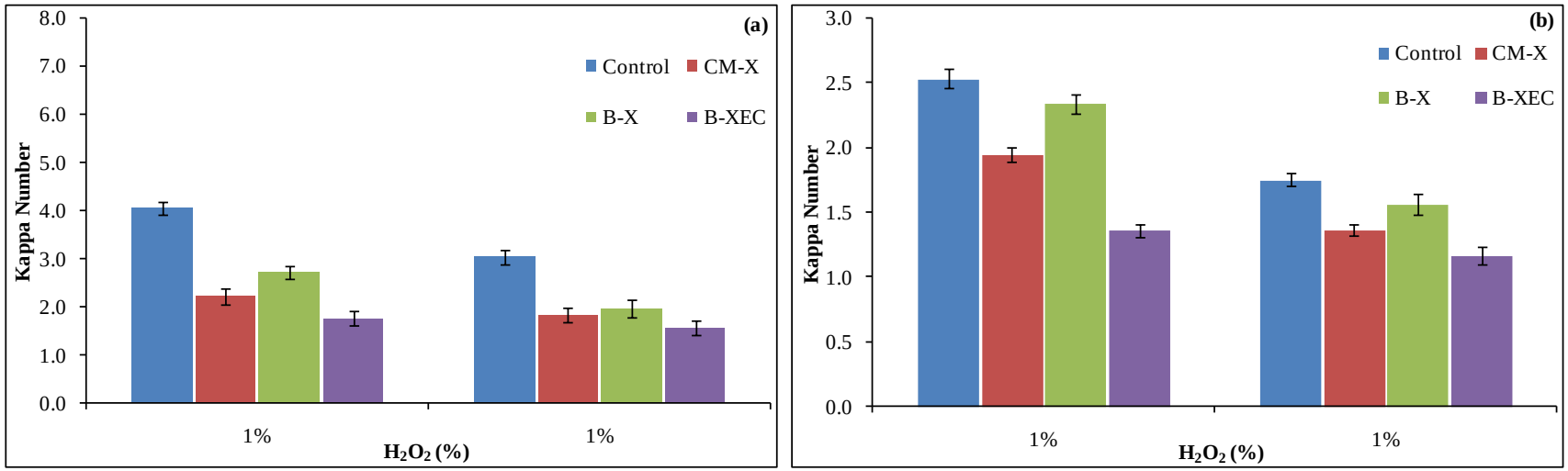

Figure 5.8 Effect of hydrogen peroxide treatment on kappa number of enzymatically pre-bleached pulp: (a) Hardwood, (b) Chemical bagasse

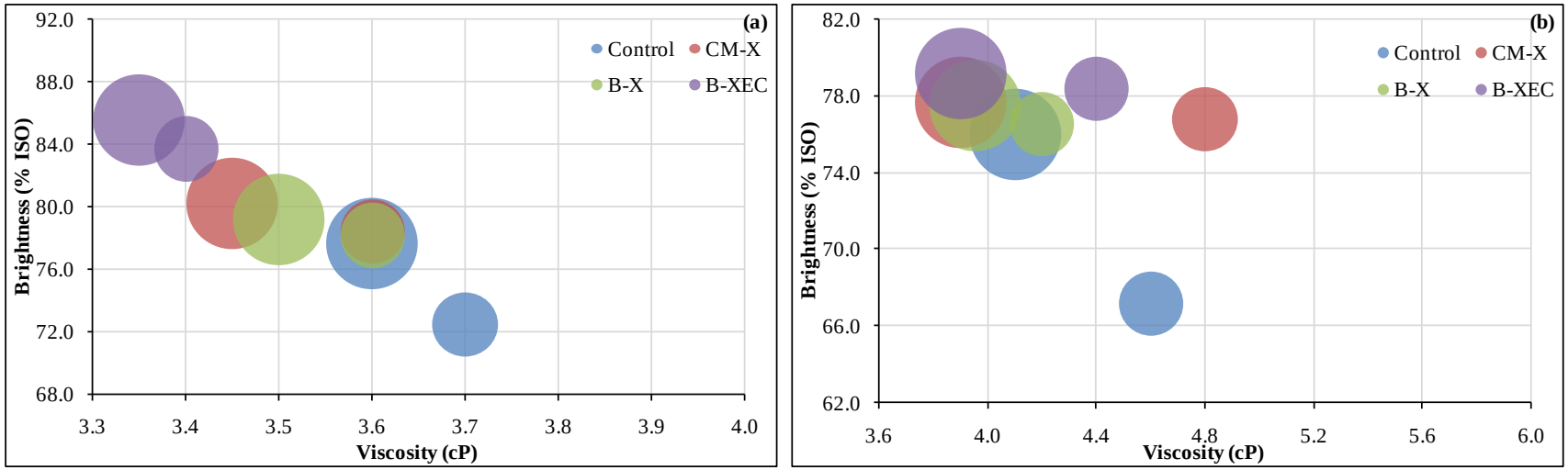

Figure 5.9 Effect of hydrogen peroxide treatment on brightness and viscosity of enzymatically pre-bleached pulp: (a) Hardwood, (b) Chemical bagasse

(Note: Size of the bubble represents the concentration of hypochlorite used in the bleaching of enzymatically pre-treated pulp samples)

Chapter 6

Summary

Currently, the entire world seems to be going through a battle against scorching issues such as global warming, rising sea level, irregular climate pattern, novel pathogen pandemics, freshwater crises etc. The prevalent reason for these problems is environmental pollution. In order to control environmental pollution, numerous amendments and treaties are made worldwide among nations with common interests. Development of green technologies is the chief agenda of most of the countries globally and India is among one of those.

Under mammoth size green technology flagship, a modest yet noteworthy attempt is made in current research work to address environmental pollution problems associated with paper and pulp industry and how it can be reduced. For this, an approach was made towards retrieving bacterial isolates from their natural habitats with a natural ability to produce xylanolytic enzymes. Obtained enzymes were further characterized and utilized for improving the already established protocols of enzymatic pre-treatment of kraft pulp in order to reduce chemical consumption during bleaching process which ultimately lessens the release of hazardous chemical compounds from effluents into environment.

Major observations from current research work are as follows:

- Mangroves which are possible hotspots for xylanolytic enzyme producers were selected for water and sediment collection. A total of 303 bacterial isolates from all 13-mangrove sediment and water samples were isolated, screened and identified. The accession numbers for all bacterial *16S rRNA* gene nucleotide sequences were obtained from the NCBI database.

- A tally of 22 cultivable bacterial genera was found. There was a clear dominance of phylum *Proteobacteria* (57%) comprising of genus *Vibrio* (36%) and *Photobacterium* (9%) observed in mangrove regions followed by phylum *Firmicutes* (32%). Phylum *Actinobacteria* (1%) and *Fusobacteria* (2%) were also reported in the current study. Mangrove sediments studied here showed a high abundance of *Vibrio* and *Bacillus,* while *Vibrio* dominated the study area. Preliminary screening for xylan-degrading potential of bacterial isolates based on Congo red assay revealed 94 isolates (31%) representing 6 genera (*Bacillus, Vibrio*, *Fusobacterium*, *Catenococcus*, *Staphylococcus*, and

Klebsiella) to be exhibiting the same. *Bacillus* genus alone represented 86% of xylan degraders in the study area.

- A quantitative estimation of xylanase activity of 17 representatives affiliated to 6 genera revealed *Bacillus* sp. to be superior in terms of xylanase production as compared to *Vibrio* and other genera. To study the functional diversity of *Bacillus* screened in the study area, xylan-degrading *Bacillus*, comprising 10 representative strains, were further screened for the presence of GH10 and GH11 xylanase gene fragments. All the sequences reported in this study shared high identity with GH10 xylanases from *Bacillus* sp. where GH11 xylanase was seen as out-group.

- Based on preliminary results *Bacillus* sp. NIORKP76 isolate was selected for further studies. The concentration of various nutrients such as phosphate, NaCl and NH_4Cl in growth media and growth conditions like substrate to moisture ratio was optimized for maximum xylanase production by *Bacillus* sp. NIORKP76 isolate under solid-state fermentation (SSF). The optimum concentrations of salts required for maximum xylanase production were 1.2%, 1.5% and 0.03% of phosphate ions, NaCl and NH_4Cl respectively, whereas 1:3 (w/v) substrate to moisture ratio was optimum for maximum xylanase production. Using optimized growth media and growth conditions, growth curve as well as xylanolytic enzyme production was monitored. The selected isolate showed diauxic growth pattern with maximum cellular biomass production at 84h time interval while maximum xylanase production was observed at 72h, whereas maximum AFase and AXE production was seen at 48h. Mass production of xylanolytic enzymes was achieved under SSF.

- Under preliminary characterization, the effect of pH and temperature on activity and stability of xylanase, AFase and AXE was observed. The pH optimum for xylanase was found to be 8.0 and AFase and AXE were most active at pH6.0. Xylanase retained 92% of its activity at pH8.0, whereas AFase and AXE showed remarkable stability over a wide range of pH (5.0 to 8.0) and retained 100% of their activity at all pH values for 24h at room temperature (28 ± 2°C). The optimum temperature for all xylanolytic enzymes was found to be 60 °C. Over temperature range of 30-40 °C, while xylanase

and AFase retained 100% of their activities for 24h, AXE managed to retain 80% at the same temperature range for 24h.

- Partial purification of xylanolytic enzymes was achieved by performing protein precipitation (using a saturated solution of ammonium sulphate) followed by dialysis and ion-exchange chromatography. SDS-PAGE and zymogram analysis revealed active xylanase, AFase and AXE bands corresponding to a molecular weight of ~31kDa, ~66kDa and ~45kDa respectively.

- Optimization of the biobleaching conditions is an essential step to maximize lignin removal and enhance pulp characteristics. The xylanolytic enzymes consortia dose comprising 5 Ug^{-1} of xylanase (1.34 Ug^{-1} and 0.9 Ug^{-1} of AFase and AXE, respectively) was most efficient for bio-bleaching pulp sample (HW and CB) when incubated for 2h at 40°C with 5% pulp consistency. Similarly, the maximum release of reducing sugar, phenolic, and hydrophobic compounds was observed at a 2h incubation period for both types of pulp samples. A maximum reduction in κ number was observed when the pulp consistency of 5% was subjected to the pre bleaching process by keeping treatment conditions at an optimized level. Biobleaching of pulp samples using xylanolytic enzyme isolated from *Bacillus* sp. NIORKP76 under optimized conditions was successfully achieved, which displayed remarkable results as compared to single xylanase enzyme. Pulp samples (HW and CB) pre-treated with *Bacillus* sp. NIORKP76 xylanolytic enzyme consortium (*B*-XEC) resulted in 50% reduction in hypochlorite consumption (2% hypochlorite), by procuring brightness level and reduction in kappa number superior to that of control (4% hypochlorite). When compared with kappa number of untreated kraft pulp, solo xylanase (*B*-X and CM-X) pre-treatment followed by 2% hypochlorite treatment led to 84-85.5% reduction in kappa number for HW and 82-84% for CB pulp. The reduction in kappa number was ~87% and ~89% for HW and CB pulp respectively when the same procedure was followed using *B*-XEC. The control pulp sample treated with 4% hypochlorite displayed only 81.77% reduction in kappa number for HW and 86% for CB pulp thus proving the xylanolytic enzyme pre-treatment was more prominently effective against HW pulp as compared to CB pulp samples.

Further, sodium hypochlorite treated pulp was subjected to H_2O_2, which increased the brightness index with a mild reduction in κ number of pulp samples without affecting viscosity values.

- All the above results indicate that the consortia of a xylanolytic enzyme is extra suitable for pre-treatment of pulp samples, which significantly reduced consumption of NaOCl in subsequent chemical bleaching steps without negotiating the brightness and viscosity results.

Future prospects

Until now various studies have been carried out on extraction of the xylanolytic enzyme from microbial sources and its application in pre-treatment of kraft pulp. From the development carried out in current research work, it is evident that this is one of the few reports in which consortium of xylanolytic enzymes was extracted from single bacterial isolate and used for pre-treatment of kraft pulp. It can definitely become a head turner and help other researchers to develop a keen interest in this subject area.

The data generated in the current study can be a roadmap for future investigation to target various other microbial communities from mangrove regions producing multiple xylanolytic enzymes or consortium with novel characteristics. Due to the dynamic nature of mangrove regions the probability of finding microbes with novel biomolecules arises. Furthermore, exploration of these regions is required not merely in terms of microbial diversity but also with respect to functional diversity.

Among microbial communities found in nature, bacterial isolates can be effortlessly grown on artificial medium with minimal nutrient requirements. Despite this majority of the fraction is still unexplored because of their stringent physio-chemical requirements, due to which biomolecules of novel characteristics remain unearthed. Rather than following only conventional approach, specific gene amplification directly from environmental samples should be encouraged. The metagenomic approach which is merely a three-decade-old technique had already garnered popularity among the researcher's community. The xylanolytic enzymes obtained by using the above approaches may not be always industrial process friendly. The natural characteristics can be enhanced by various molecular biology

techniques. Protein engineering can improve the properties of xylanolytic enzymes and make them suitable for various biotechnological processes. Recombinant DNA technology is another way of escalating the innate characteristics of xylanolytic enzymes. High fold production of the xylanolytic enzyme using their genes can be achieved by extraction and artificial insertion of enzyme-specific genes into hosts cells.

Efficient utilization of renewable biomass for scale-up production of these xylanolytic enzymes needs to be addressed. The production cost xylanolytic enzyme is primarily influenced by substrate fed as growth media to bacterial isolate. Although different agro-residues are already proved to be very efficient in xylanolytic enzyme production, optimization of additional nutrients requirements and physiological conditions can improve xylanolytic enzyme production many folds higher than native lignocellulosic biomass. Few nutrients concentration and substrate to moisture ratio is already optimized in the current study which can be taken further by optimizing a greater number of parameters.

In the current study experiment on application of xylanolytic enzymes in pre-treatment of kraft pulp is conducted at pilot scale. The reciprocation and feasibility of the same should be checked at a higher scale so as to know whether it can be functional at an industrial level.

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- Parab, P., Khandeparker, R., Xylanolytic enzymes from marine bacteria and their application in paper and pulp industry. Poster presentation. "7th Indian Chitin and Chitosan Society Meeting, Pune", CSIR-National Chemical Laboratory, Pune, India, October 11th to 13th, 2018.

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RESEARCH PAPER

Xylanolytic enzyme consortia from *Bacillus* sp. NIORKP76 for improved biobleaching of kraft pulp

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Abstract

A cellulase-free xylanolytic enzyme consortia consisting of a xylanase, arabinofuranosidase, and acetyl xylan esterase produced by *Bacillus* sp. NIORKP76 isolate under solid-state fermentation was assessed for its bio-bleaching ability on kraft pulp. In the biobleaching analysis, the xylanase dose of 5 Ug^{-1} dry pulp denoted the optimum bleaching of pulp at 40 °C and pH 8.0 after 2 h of treatment. The reduction in kappa number of pre-treated hardwood pulp using xylanolytic enzyme consortium (XEC) was found to be ~ 55%, while solo xylanase could reduce the kappa number to 44–46%. In the case of chemical bagasse pulp, a reduction of ~ 27.5% and 19–20% was seen in kappa number using XEC and solo xylanase, respectively. Enzyme-treated pulp (HW and CB) showed a 50% reduction in hypochlorite consumption during the chlorine treatment. The current study results reveal the significant potential of xylanolytic enzyme consortium from *Bacillus* sp. NIORKP76 on the environmentally friendly bio-bleaching process.

Keywords *Bacillus* sp. · Bio-bleaching · Xylanolytic enzymes · Kappa number · Brightness · Viscosity

Introduction

The pulp and paper industry which is considered to be one of India's rapid emerging business sector, has shown remarkable development in the last few years. Government policies are building enormous pressure on paper industries so as to maintain a pollution-free and clean environment [1]. Pulp bleaching irrespective of their origin use large amounts of chlorinated compounds. The byproducts formed while chemical processing are mutagenic, toxic, and persistent and thus are the reason for numerous problems in the biological systems. In view of this pressure the pulp and paper mills/industries are trying to change chlorine-based chemicals and move towards environment friendly molecules [2]. Environment friendly bleaching enzymes mainly xylanases and laccases have great potential for bio-bleaching of agro-based pulps at an industrial scale, due to this, the interest in xylan degrading enzymes have developed extensively over the past few years.

Viikari et al. [3] first introduced the process where biological delignification of pulp was attempted using enzymes. Xylanase, which is a hydrolytic enzyme, is mainly used to breakdown the lignin–carbohydrate complex, which acts as a physical barrier during chemical bleaching. Pre-bleaching of kraft pulp using xylanase was reported to minimize the amount of chlorine required for bleaching and thus dipping chloro-organic discharges [3, 4]. There are several reports on the application of xylanases for pre-treatment of unbleached pulp, which proved to be efficient to reduce the use of chlorinated compounds in the subsequent bleaching process [5–10].

Due to the heterogeneity of hemicellulose, its hydrolysis requires the action of a complex enzyme system. This usually composed of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterases. All these enzymes act cooperatively to convert xylan to its constituent sugar. The presence of such multifunctional xylanolytic enzyme systems is quite widespread among bacteria and fungi [11–20]. The combined effect of hemicellulases on different pulp woods are published [21]. It was noticed that synergism does exist between the two

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types of endoxylanases reported from *Streptomyces* sp., which resulted in the enhancement of brightness in softwood [21].

However, the bioleaching process needs further development; different xylanolytic enzymes and their synergistic effect need to be studied. With this objective, the present study is mainly focused on the application of cellulase-free xylanolytic enzyme consortia (xylanase, arabinofuranosidase, and acetyl xylan esterase) isolated from *Bacillus* sp. NIORKP76 strain on pre-treatment of unbleached pulp samples (Hardwood pulp and Chemical bagasse pulp). As per our understanding, this is one among the few reports where the effect of sole xylanase and consortia of xylanolytic enzymes on bio-bleaching of pulp is shown. This report is unique because the entire xylanolytic enzyme consortia is isolated from a single bacterial strain and used for bio-bleaching of different unbleached pulp samples.

Materials and methods

Microorganism, growth conditions and enzymatic assays

Xylanolytic enzymes used for this study were isolated from *Bacillus* sp. NIORKP76 strain (GenBank accession number: MH767158) [22]. The bacterial isolated was obtained from mangrove sediments of Goa. The xylanolytic enzyme consortium (*B*-XEC) production was carried out under solid-state fermentation (SSF) using modified basal salt solution (MBSS) [7] supplemented with 0.2% yeast extract and 0.03% peptone and wheat bran (substrate to moisture ratio 1:3) as a substrate. Xylanase enzyme (*B*-X) was produced under submerged fermentation (SmF) by replacing carbon source with 0.5% xylan.

The detailed composition of the MBSS medium (w/v) was as follows: NaCl, 30.00 g; $MgSO_4$, 7.00 g; NH_4Cl, 0.5 g; KCl, 0.75 g; KH_2PO_4 (10%), 7.00 mL, K_2HPO_4 (10%), 3.00 mL; trace metal solution, 1 mL; distilled water, 1 L. The composition of trace metal solution is as follows: H_3BO_3, 2.85 g; $FeSO_4.7H_2O$, 2.49 g; $MnCl_2.7H_2O$, 1.80 g; $CuCl_2$, 0.03 g; Na-tartrate, 1.77 g; $ZnCl_2$, 0.02 g; $Na_2MoO_4.2H_2O$, 0.02 g; $CoCl_2$, 0.03 g; distilled water, 1 L. Commercially available xylanase (CM-X) was purchased from Sigma-Aldrich, USA (*Trichoderma longibrachiatum*, X2629).

Xylanase, arabinofuranosidase (AFase), and acetyl xylan esterase (AXE) assays were performed using 1% xylan, 2.5 mM *p*-nitrophenyl-α-l-arabinofuranoside [7], and 50 mM *p*-nitrophenyl acetate [23], respectively.

Optimization of xylanolytic enzyme pre-treatment conditions on kraft pulp

The enzymatic treatment studies were carried out on kraft pulp using a crude xylanolytic enzyme from *Bacillus* sp. NIORKP76 strain and commercial xylanase at Biological Oceanography Division of National Institute of Oceanography, Goa, India. Xylanase activity 2.5U, AFase activity 0.67U, and AXE activity 0.45U were measured in one milliliter of a crude xylanolytic enzyme mixture. The enzyme activities were determined before carrying out experimental work. Two different pulp samples, hardwood pulp (HW pulp) and chemical bagasse pulp (CB pulp) provided by Tamilnadu Newsprint and Papers Limited (TNPL), Tamilnadu, India, was used in this study. Unbleached pulp samples were washed thoroughly with tap water, oven-dried, and used for further studies.

Influence of xylanolytic enzyme dose

The effect of varying xylanolytic enzyme dosages was studied by treating oven-dried pulp samples (HW and CB) with different doses of xylanolytic enzymes. The xylanolytic enzyme dosages were varied by changing the volume of crude *B*-XEC. The enzyme doses ranging between 2.5 U to 25 Ug^{-1} pulp of xylanase, 0.67 U to 6.7 Ug^{-1} pulp of AFase, and 0.45 U to 4.5 Ug^{-1} pulp of AXE were used. The pulp samples were mixed with suitably diluted *B*-XEC and sodium phosphate buffer (pH 8.0) with 5% consistency [14] in zip lock polyethylene bags and incubated in a water bath maintained at a constant temperature of 40 °C for 2 h. Pulp sample was prepared and treated using the same physio-chemical parameters in the absence of any xylanolytic enzyme used as control. At the end of the incubation time, the pulp samples were washed with distilled water, filtered through a mesh sieve no. 200 [24] and filtrate was collected.

Influence of incubation period

To study the influence of the incubation period on bio-bleaching, the reaction mixture containing an optimized dose of *B*-XEC and pulp sample prepared in sodium phosphate buffer (pH 8.0) with 5% consistency was incubated in a water bath maintained at a constant incubation temperature of 40 °C for varying time intervals (0–6 h). At the end of a given time interval, pulp samples were washed with an equal volume of distilled water, filtered through a mesh sieve no. 200 [24] and filtrate was collected.

Influence of pulp consistency

The effect of pulp consistency on xylanolytic enzyme bleaching was studied by varying pulp concentration. Pulp samples with 2.5–10% consistency were prepared in sodium phosphate buffer (pH 8.0). Optimized *B*-XEC dose and the optimized incubation period were used to treat the enzymatic treatment of unbleached pulp samples under constant incubation temperature of 40 °C. Following the incubation

period, pulp samples were washed with an equal volume of distilled water, filtered through a mesh sieve no. 200 [24] and filtrate was collected.

Pulp properties

Kappa number is used to determine the lignin content in pulp. Kappa number of pulp was measured as per Technical Association of Pulp and Paper Industry (TAPPI) Test Methods T236 cm-85. Kappa number is defined as the volume of (mL) 0.1 N $KMnO_4$ solution consumed by 1 g moisture-free pulp under the conditions specified. The analysis of brightness and viscosity was carried out at Paper and Pulp Research Institute (PAPRI), Rayagada, Odisha-India. The brightness of pulp samples was determined as per the ISO 2470 method (L & W Elrepho, model-SE070, Kista-Sweden). Viscosity was measured by standard TAPPI test method T230. The relative degree of delignification of enzyme pre-treated pulp samples and control pulp sample was determined using standard TAPPI protocols [7].

Analysis of pulp filtrates

The absorbance of the filtrates collected in the above experiments was measured spectrophotometrically at a wavelength ranging from 200 to 465 nm to determine the release of phenolic and hydrophobic compounds [7]. The reducing sugar released in the filtrate was measured using dinitrosalicylic acid method [25].

Efficacy of xylanolytic enzyme consortium

The efficiency of enzymatic pre-treatment for bio-bleaching of unbleached pulp samples (HW and CB) was examined by treating pulp samples with crude xylanolytic enzyme consortium, crude xylanase (free from other xylanolytic enzymes) isolated from *Bacillus* sp. NIORKP76 and commercially available xylanase (Sigma-Aldrich, USA). The optimized *B*-XEC dose containing 5 Ug^{-1} pulp of xylanase, 1.34 Ug^{-1} pulp of AFase, and 0.90 Ug^{-1} pulp of AXE was used. Equivalent dose (5 Ug^{-1} pulp) of commercial xylanase (CM-X) and *Bacillus* sp. NIORKP76 xylanase (*B*-X) was used for the treatment of pulp samples. The pulp samples were mixed thoroughly with optimized enzyme dose and sodium phosphate buffer (pH 8.0) at a 5% consistency in a polyethylene zip lock bag and plunged in a water bath, maintaining a constant temperature of 40 °C for an incubation period of 2 h. After enzyme pre-treatment, pulp samples were washed thoroughly with distilled water, and the filtrate was collected to carry out analysis as per the protocol mentioned above. Controls were prepared similarly, devoid of the enzyme. The pulp obtained was air-dried and stored for further analysis.

Chemical bleaching

An air-dried kraft pulp samples pre-treated with xylanolytic enzymes were used for hypochlorite and followed by hydrogen peroxide treatment. For hypochlorite treatment, the pulp samples were made to 5% consistency and divided into three equal parts. Hypochlorite solution of 8%, 4%, and 2% concentration was prepared, and each portion of the pulp sample was treated with the respective solution and incubated at 40 °C for 1 h [7]. At the end of the first chemical treatment step, pulp samples were washed and air-dried. All pulp samples obtained after hypochlorite treatment were treated with 1% hydrogen peroxide in a consecutive step. The pulp consistency, incubation period, and temperature were the same as used for the previous step. The pulp samples were washed with distilled water and air-dried. Kappa number, brightness, and viscosity of bleached pulp samples obtained at the end of both chemical treatment stages were determined.

Result and discussion

Optimization of bio-bleaching parameters

Optimization of bio-bleaching conditions is an essential step to maximize lignin removal and enhances pulp characteristics. The target properties were the kappa number, brightness and viscosity of the pulp.

The xylanolytic enzyme consortia extracted from *Bacillus* sp. NIORKP76 was optimized for pre-bleaching parameters such as enzyme dose, incubation period, and pulp consistency. The different parameters were optimized separately to improve the efficacy of enzyme treatment. Enzymatic biobleaching reported by Nair et al. [8], Khandeparker et al. [7] and Sridevi et al. [10] also provided importance of pertaining to use optimization parameters prior to enzymatic pre-treatment of pulp. The original kappa number of hardwood and chemical bagasse pulp was 21.51 and 14.13 and brightness was 41.3 and 53.1 (% ISO), respectively. As the kappa number is an estimation of the lignin content in pulp, it is evident that hardwood pulp has more lignin compared to chemical bagasse. The increase in delignification usually results in increased brightness as the brightness is directly linked to the residual lignin in the pulp [26]. The pulp brightness has been reported to have a linear relationship with the kappa number [27, 28]. Brightness of paper have a strong effect on the print quality, more the brightness better is the

quality thus kappa number and brightness play important role in pulp properties [29].

Optimization enzyme dose

The xylanolytic enzymes consortia comprising 5 Ug^{-1} of xylanase (1.34 Ug^{-1} and 0.9 Ug^{-1} of AFase and AXE, respectively) was most efficient for bio-bleaching pulp sample (HW and CB) when incubated for 2 h at 40 °C with 5% pulp consistency.

It was seen that the kappa number (κ) was reduced to 54.34%, and a 4–5-fold increase in the release of reducing sugar was observed for the HW pulp sample. Similarly, a 26.89% reduction in κ number and increase in the release of reducing sugar by 7–8-fold was measured for CB pulp sample at 5U of xylanase dose. The release of phenolic (237 nm) and hydrophobic (465 nm) compounds was also found to be elevated in both types of pulp samples with the reaction conditions above-mentioned (Fig. 1a, b). The decrease in κ number and the simultaneous increase in the release of reducing sugar, phenolic, and hydrophobic compounds reveals the delignification process by *B*-XEC. It was seen that *B*-XEC could delignify HW pulp better then CB pulp. We know that the original kappa number of HW pulp was high, treatment with *B*-XEC brought the HW pulp at equivalent level to CB pulp in term of lignin content. The effect of xylanolytic enzyme dose on pulp bio-bleaching was highly significant, which is evident from a one-way analysis of variance (ANOVA) ($p < 0.05$).

Optimization of the incubation period

The effect of time concerning enzyme treatment on pulp was investigated using an optimized enzyme dose. After 1 h of the incubation period, HW and CB pulp samples showed 30.8% and 3.9% reduction in κ number, respectively. A significant decrease in κ number was seen after 2 h of the incubation period. A steep reduction in κ number of 54.43% and 27.10% in HW and CB pulp, respectively, was observed. Similarly, the maximum release of reducing sugar, phenolic, and hydrophobic compounds were observed at a 2 h incubation period for both types of pulp samples (Fig. 2a, b). It was also observed that the incubation time did not show further reduction in κ number with further increase in time. The effect of incubation time on bio-bleaching of both types of pulp was significant and evident from one-way ANOVA ($p < 0.05$).

Optimization of pulp consistency

Pulp consistency optimization is a must to attain proper and effective spread of the enzyme so as to improve the enzyme treatment efficiency. During enzymatic prebleaching experiment, pulp consistency was varied from 2.5 to 10%. Pulp consistency of 2.5% with 5U enzyme dose and at 2 h incubation time could deliver up to 33.5% and 8.28% reduction in κ number for HW and CB, respectively. A maximum reduction in κ number was observed when the pulp consistency of 5% was subjected to the pre-bleaching process by keeping treatment conditions at an optimized level. The reduction in

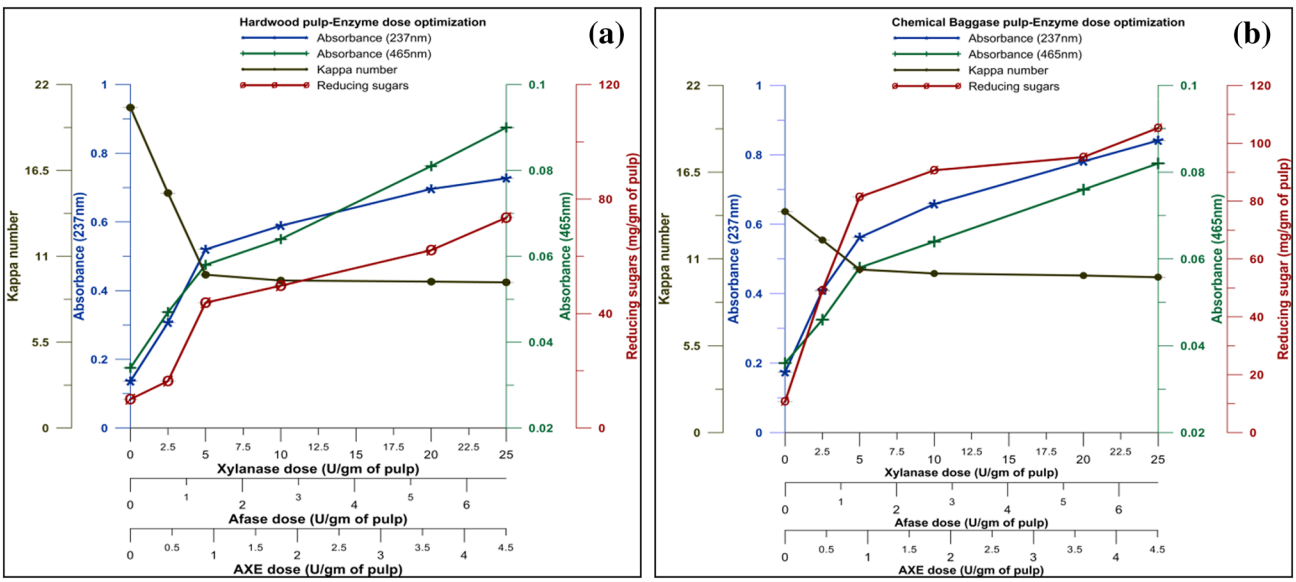

Fig. 1 Xylanolytic enzyme dose optimization (Ug^{-1} pulp) for pre-treatment of pulp. **a** Hardwood pulp, **b** Chemical bagasse pulp

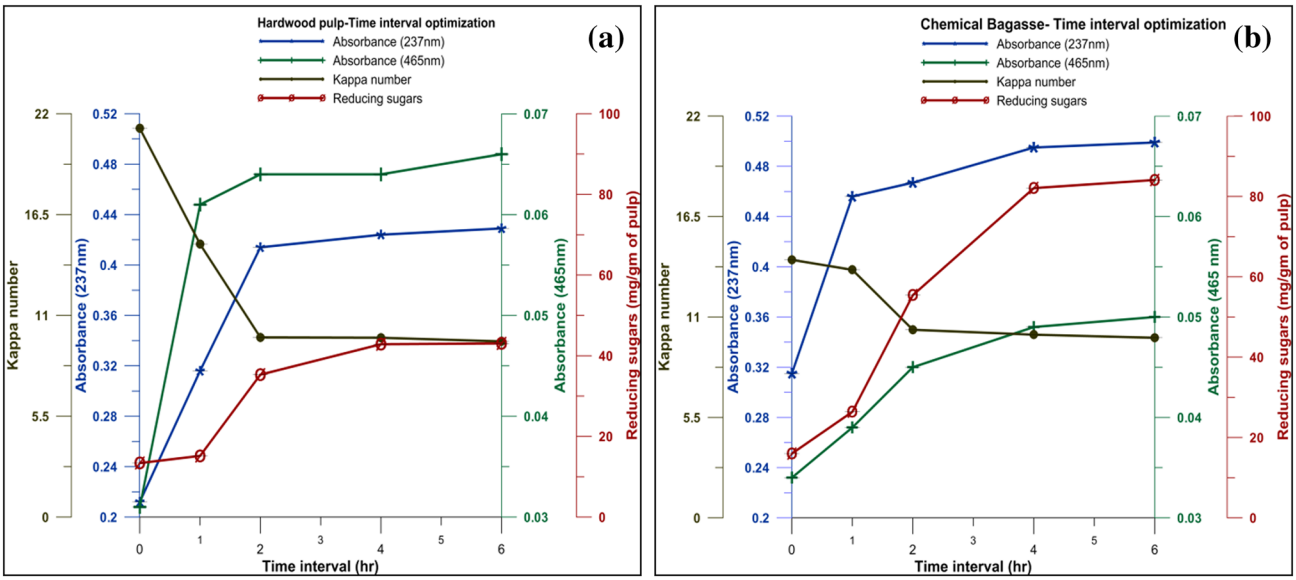

Fig. 2 Time interval optimization (h) for pre-treatment of pulp. **a** Hardwood pulp, **b** Chemical bagasse pulp

κ number was 54.67% for hardwood pulp, whereas it was 27.24% for chemical bagasse pulp. Pulp consistency higher than 5% did not contribute to much reduction in κ number (Fig. 3a, b). The $p < 0.05$ from one-way ANOVA reveals the significant effect of pulp consistency on pulp properties such as κ number, the release of phenolic, hydrophobic compounds, and release of reducing sugars.

Color removal from the kraft pulp

The release of color from the pulp treated with xylanase (*B*-X and CM-X) as well as xylanolytic enzyme consortia (*B*-XEC) was studied using UV spectrum analysis. Enzymatically pre-treated pulp filtrate displayed an increase in absorbance and peak at 280 nm, which confirms the release of lignin from the pulp [30]. The correlation between the

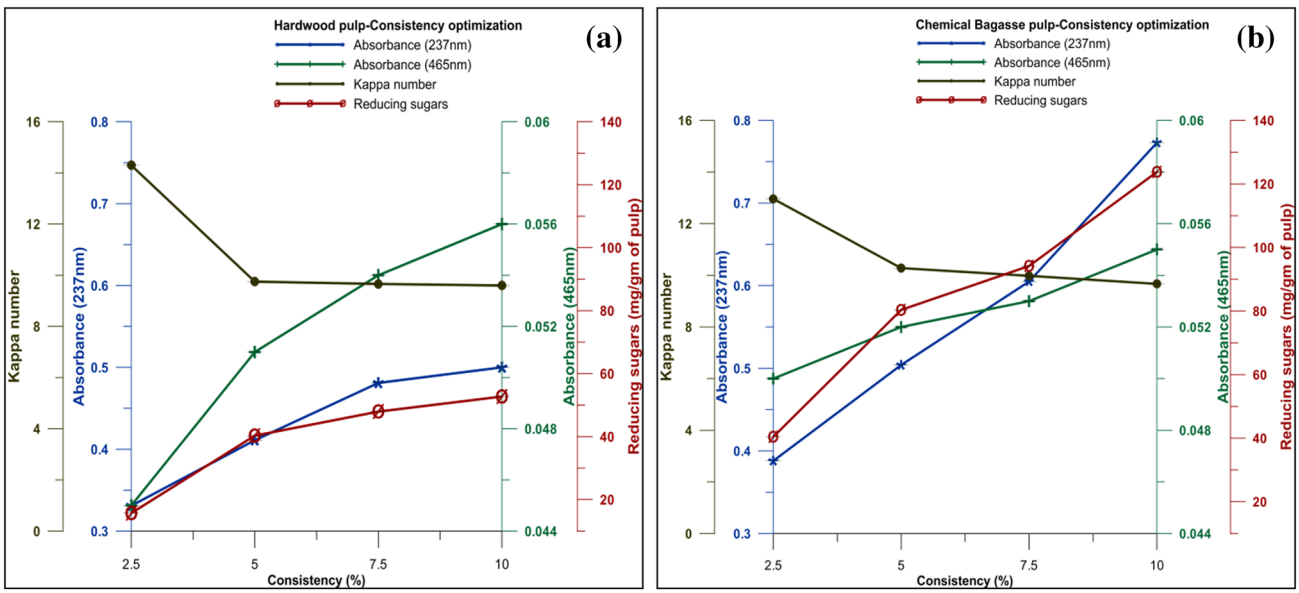

Fig. 3 Pulp consistency optimization (%) for pre-treatment of pulp. **a** Hardwood pulp, **b** Chemical bagasse pulp

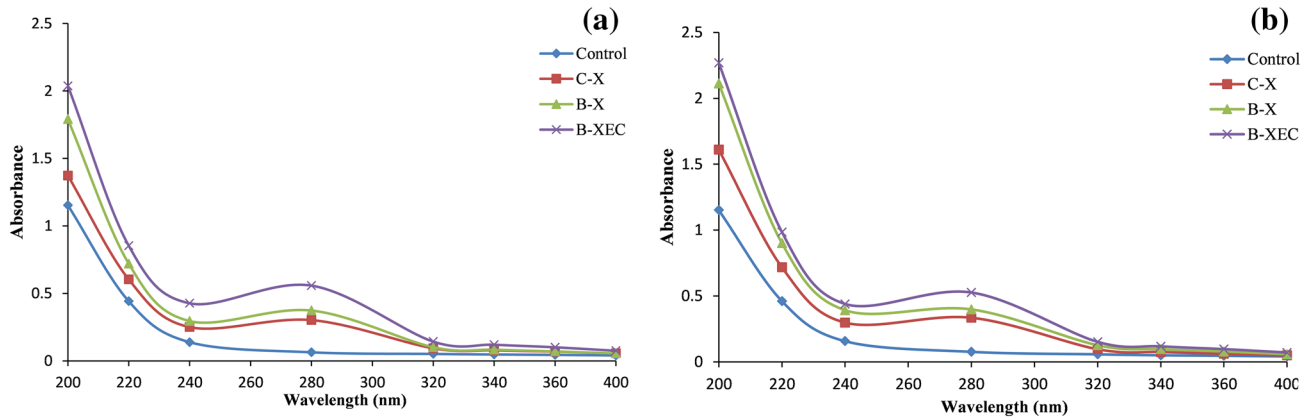

Fig. 4 UV spectrum of colored compounds released during xylanolytic enzyme treatment, **a** Hardwood pulp, **b** Chemical bagasse pulp

nature of chromophore released and enzyme is described in the literature [7, 31, 32]. Xylanolytic consortia from *Bacillus* sp. NIORKP76 showed the absorption of ≅0.5 when treated with 5 Ug^{-1} of oven-dried hardwood and chemical bagasse pulp (Fig. 4a, b). While commercial xylanase as well as single xylanase *Bacillus* sp. NIORKP76 from showed absorption of ≅0.3. High absorbance at 280 when XEC was used compared to XE gives a clear indication of the effectiveness of XEC during pre-treatment. The xylanase enzyme from *Arthrobacter* sp. showed absorption of 1.5 when treated with 20 Ug^{-1} of oven-dried pulp [7], the absorption of 0.8 was recorded by Kulkarni and Rao, [31] using 20 Ug^{-1} of oven-dried pulp, while the absorption of 0.4–0.5 was noted when Dwivedi et al. [33] used 16 Ug^{-1} of oven-dried pulp.

Bio-bleaching of kraft pulp

The present work demonstrated that an improved delignification could be achieved by using xylanolytic enzyme consortia when compared to xylanase alone. In enzymatic prebleaching of hardwood and chemical bagasse pulp, *B*-XEC delivered excellent results in terms of κ number reduction compared to *B*-X, which was partially purified xylanase from *Bacillus* sp. NIORKP76 and CM-X, which is commercial xylanase. In the case of HW pulp samples, *B*-XEC treatment could reduce κ number by 55%, while 44.6% and 45.8% reduction in κ number were seen by *B*-X and CM-X treatment, respectively (Fig. 5a). In the case of CB pulp, enzyme pre-treatment showed a reduction in κ number as 27.53%, 20.6%, and 19.0% by *B*-XEC, *B*-X,

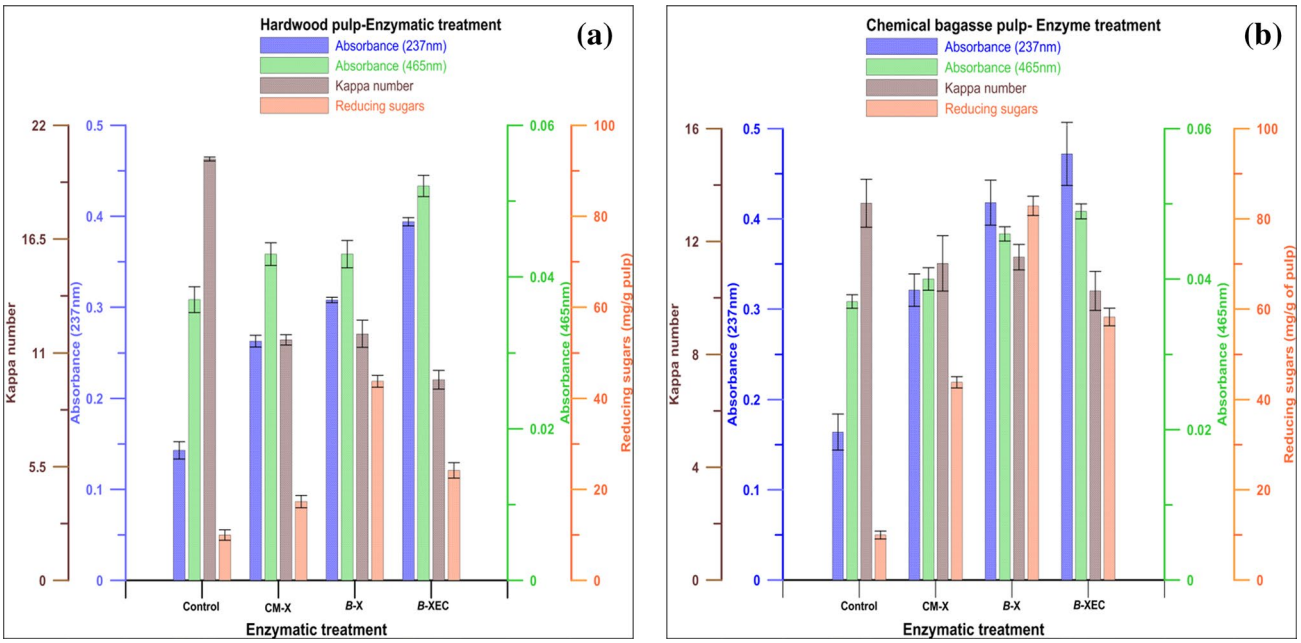

Fig. 5 Enzymatic pre-treatment of pulp, **a** Hardwood pulp, **b** Chemical bagasse pulp

Table 1 Effect of xylanolytic enzyme pre-treatment on kappa number, brightness and viscosity of different kraft pulp

Bleaching state	Hardwood pulp			Chemical bagasse		
	Kappa number	Brightness (%)	Viscosity (cP)	Kappa number	Brightness (%)	Viscosity (cP)
Untreated	21.51 ± 0.749	41.30 ± 0.94	6.30 ± 0.021	14.13 ± 0.645	53.10 ± 0.82	7.70 ± 0.041
Pre-treated						
Control	20.37 ± 0.652	45.70 ± 0.75	6.20 ± 0.024	13.36 ± 0.583	56.30 ± 0.65	7.50 ± 0.021
CM-X	11.64 ± 0.559	61.00 ± 0.45	6.00 ± 0.031	11.22 ± 0.562	64.70 ± 0.52	7.30 ± 0.0.23
B-X	11.92 ± 0.575	60.80 ± 0.63	6.00 ± 0.012	11.44 ± 0.550	63.40 ± 0.63	7.30 ± 0.021
B-XEC	9.70 ± 0.485	71.00 ± 0.23	5.80 ± 0.026	10.24 ± 0.515	71.70 ± 0.24	6.90 ± 0.035
2% NaOCl						
Control	7.56 ± 0.175	70.00 ± 0.23	3.90 ± 0.045	2.70 ± 0.090	64.70 ± 0.35	5.80 ± 0.036
CM-X	3.11 ± 0.160	77.65 ± 0.15	3.70 ± 0.031	2.16 ± 0.060	75.20 ± 0.12	5.40 ± 0.031
B-X	3.38 ± 0.170	76.95 ± 0.76	3.70 ± 0.033	2.43 ± 0.036	74.90 ± 0.19	5.50 ± 0.029
B-XEC	2.84 ± 0.150	82.15 ± 0.36	3.50 ± 0.028	1.49 ± 0.070	76.00 ± 0.24	4.70 ± 0.041
1% H_2O_2						
Control	4.05 ± 0.125	72.45 ± 0.51	3.70 ± 0.027	2.53 ± 0.080	67.20 ± 0.58	4.60 ± 0.048
CM-X	2.23 ± 0.168	78.40 ± 0.62	3.60 ± 0.035	1.95 ± 0.060	76.80 ± 0.51	4.80 ± 0.026
B-X	2.73 ± 0.132	78.15 ± 0.31	3.60 ± 0.043	2.34 ± 0.070	76.20 ± 0.37	4.20 ± 0.036
B-XEC	1.75 ± 0.149	83.70 ± 0.85	3.40 ± 0.021	1.36 ± 0.050	78.40 ± 0.54	4.40 ± 0.019
4% NaOCl						
Control	3.92 ± 0.149	73.50 ± 0.62	3.70 ± 0.035	1.98 ± 0.075	75.50 ± 0.32	5.30 ± 0.021
CM-X	2.84 ± 0.140	79.30 ± 0.90	3.50 ± 0.044	1.49 ± 0.045	76.70 ± 0.31	4.80 ± 0.025
B-X	2.90 ± 0.180	78.95 ± 0.30	3.65 ± 0.019	1.62 ± 0.029	75.90 ± 0.61	5.10 ± 0.028
B-XEC	2.30 ± 0.170	84.40 ± 0.21	3.45 ± 0.022	1.22 ± 0.035	77.70 ± 0.37	4.30 ± 0.027
1% H_2O_2						
Control	3.04 ± 0.142	77.65 ± 0.25	3.60 ± 0.021	1.75 ± 0.050	76.00 ± 0.24	4.10 ± 0.036
CM-X	1.82 ± 0.154	80.25 ± 0.23	3.45 ± 0.031	1.36 ± 0.040	77.70 ± 0.31	3.90 ± 0.016
B-X	1.95 ± 0.186	79.15 ± 0.26	3.50 ± 0.048	1.56 ± 0.080	77.50 ± 0.19	3.95 ± 0.031
B-XEC	1.56 ± 0.157	85.60 ± 0.34	3.35 ± 0.046	1.17 ± 0.070	79.20 ± 0.53	3.90 ± 0.034
8% NaOCl						
Control	3.11 ± 0.190	78.20 ± 0.41	3.70 ± 0.032	1.62 ± 0.050	76.90 ± 0.41	4.60 ± 0.038
CM-X	2.57 ± 0.180	80.15 ± 0.38	3.50 ± 0.014	1.22 ± 0.080	78.40 ± 0.32	3.80 ± 0.051
B-X	2.63 ± 0.150	79.65 ± 0.54	3.50 ± 0.019	1.22 ± 0.055	78.60 ± 0.16	4.20 ± 0.057
B-XEC	1.76 ± 0.140	85.35 ± 0.36	3.45 ± 0.024	1.08 ± 0.035	79.10 ± 0.72	3.70 ± 0.036
1% H_2O_2						
Control	1.82 ± 0.156	79.80 ± 0.21	3.45 ± 0.023	1.56 ± 0.090	77.00 ± 0.63	3.90 ± 0.022
CM-X	1.42 ± 0.135	81.20 ± 0.32	3.40 ± 0.035	1.17 ± 0.080	78.65 ± 0.34	3.55 ± 0.015
B-X	1.56 ± 0.175	81.15 ± 0.38	3.50 ± 0.016	1.17 ± 0.050	79.30 ± 0.26	3.75 ± 0.011
B-XEC	1.36 ± 0.137	86.60 ± 0.60	3.25 ± 0.011	1.07 ± 0.040	80.80 ± 0.12	3.45 ± 0.019

Control: treatment only with buffer; CM-X: treatment with commercial xylanase; *B*-X: treatment with *Bacillus* sp. NIORKP76 xylanase; *B*-XEC: treatment with *Bacillus* sp. NIORKP76 xylanolytic enzyme consortia

and CM-X, respectively (Fig. 5b) (Table 1). Along with a reduction in κ number, the release of phenolic compounds, hydrophobic compounds and reducing sugars were highest in *B*-XEC treatment (Fig. 5a, b). The enzymatic biobleaching showed the highest reduction in κ number with an increase in brightness index, and without much alteration in viscosity using xylanolytic consortia compared to xylanase alone, observed differences were highly significant as evident from one-way ANOVA analysis ($p < 0.05$). Kappa number was negatively correlated to brightness for HW as well as CB pulp $r = -0.9803$; $p < 0.01$, and $r = -0.9870$; $p < 0.01$), respectively (Supplementary Fig. 1).

Biobleaching of eucalyptus pulp have been reported to reduce the kappa number by 3.0 and 3.3 units by using xylanase doses of 2 and 4 Ug^{-1} dried pulp after 1 h treatment, respectively, thus suggesting that *P. janczewskii* xylanases can be a promising candidate for biobleaching of kraft pulp [34] which is around 20% reduction in kappa

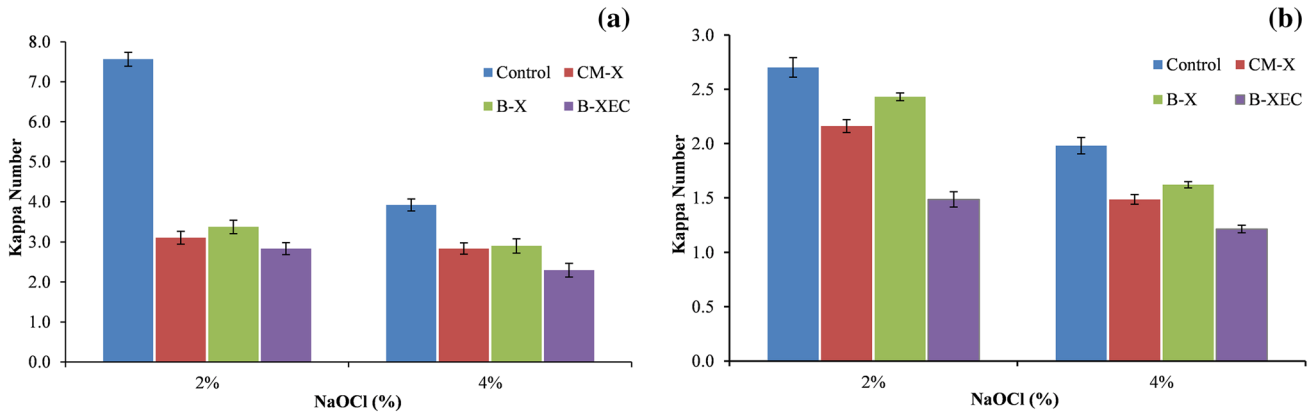

Fig. 6 Effect of hypochlorite treatment on kappa number of enzymatically pre-bleached pulp: **a** Hardwood, **b** chemical bagasse

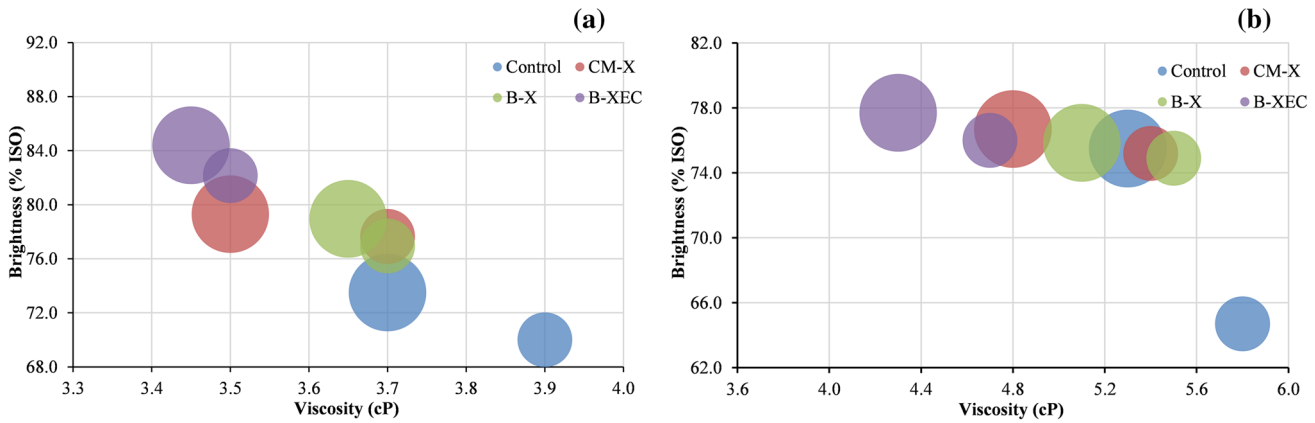

Fig. 7 Effect of hypochlorite treatment on brightness and viscosity of enzymatically pre-bleached pulp: **a** Hardwood, **b** Chemical bagasse. (Note: size of the bubble represents the concentration of hypochlorite used in the bleaching of enzymatically pre-treated pulp samples)

number. Enzymatic prebleaching of kraft pulp reported by Khandeparker and Bhosle, [7] also showed a 20% decrease in kappa number of the kraft pulp without compromising with viscosity. As per their claim, 29% reduction in chlorine requirement can be reduced by enzymatic treatment without any decrease in brightness. The biobleaching efficiency of xylanase obtained from *Streptomyces* sp. QG-11-3 was maximum after 2 h of incubation using a xylanase dose of 3.5 Ug^{-1}, at 50 °C and at 6% pulp consistency. The reduction in kappa number was found to be 23% [2]. Sridevi et al. [35] reported reduction in kappa number by 4.2 units, and increase in brightness by 4 units as compared to untreated pulp samples when xylanase produced by *Trichoderma asperellum* with enzyme dose of 100 Ug^{-1} and 1 h incubation period was used for pre-treatment. Xylanase from *Aspergillus niger* with optimum dose of 60 Ug^{-1}, 1 h incubation period and pulp consistency 3% enabled reduction in kappa number by 3.5 units and increase in brightness by 3.1 units as compared to untreated pulp sample [10].

There are reports in literature which describes about use of enzymes other than xylanolytic enzymes for biobleaching purpose. Bhoria et al. [36] reported production and application of mannanase for enzymatic pre-treatment of wheat straw-rich soda pulp samples. In this report with optimum enzyme dose of 5 Ug^{-1} and time interval of 4 h showed increase in brightness of 8.7% ISO, and reduction in kappa number by 16% with 6% overall reduction in chlorine consumption.

Report by Gupta et al. [37] describes use of enzyme consortia (xylanase and laccase) obtained from two different bacterial species grown with co-culturing technique. Using eucalyptus kraft pulp mixture of enzyme pre-treatment raised brightness and reduced kappa number by 5% and 9.5%, respectively, which after final chemical bleaching stage improved to 13% and 15%. Several research articles.

Recently, Angural et al. [38] used a mixture of xylanase, laccase, and mannanase enzymes for pulp biobleaching on mixed wood pulp and got a 46.32/40.25% reduction in kappa

Table 2 Effect of xylanolytic enzyme pre-treatment on kappa number, brightness and viscosity of different kraft pulp after chlorine reduction

Bleaching state	Hardwood pulp			Chemical bagasse		
	Kappa number	Brightness (%)	Viscosity (cP)	Kappa number	Brightness (%)	Viscosity (cP)
Untreated	21.51 ± 0.749	41.30 ± 0.94	6.30 ± 0.021	14.13 ± 0.645	53.10 ± 0.82	7.70 ± 0.041
Pre-treated						
Control	20.37 ± 0.652	45.70 ± 0.75	6.20 ± 0.024	13.36 ± 0.583	56.30 ± 0.65	7.50 ± 0.021
CM-X	11.64 ± 0.559	61.00 ± 0.45	6.00 ± 0.031	11.22 ± 0.562	64.70 ± 0.52	7.30 ± 0.0.23
B-X	11.92 ± 0.575	60.80 ± 0.63	6.00 ± 0.012	11.44 ± 0.550	63.40 ± 0.63	7.30 ± 0.021
B-XEC	9.70 ± 0.485	71.00 ± 0.23	5.80 ± 0.026	10.24 ± 0.515	71.70 ± 0.24	6.90 ± 0.035
NaOCl						
Control-4C	3.92 ± 0.149	73.50 ± 0.62	3.70 ± 0.035	1.98 ± 0.075	75.50 ± 0.32	5.30 ± 0.021
CM-X-2C	3.11 ± 0.160	77.65 ± 0.15	3.70 ± 0.031	2.16 ± 0.060	75.20 ± 0.12	5.40 ± 0.031
B-X-2C	3.38 ± 0.170	76.95 ± 0.76	3.70 ± 0.033	2.43 ± 0.036	74.90 ± 0.19	5.50 ± 0.029
B-XEC-2C	2.84 ± 0.150	82.15 ± 0.36	3.50 ± 0.028	1.49 ± 0.070	76.00 ± 0.24	4.70 ± 0.041
1% H_2O_2						
Control-4CH	3.04 ± 0.142	77.65 ± 0.25	3.60 ± 0.021	1.75 ± 0.050	76.00 ± 0.24	4.10 ± 0.036
CM-X-2CH	2.23 ± 0.168	78.40 ± 0.62	3.60 ± 0.035	1.95 ± 0.060	76.80 ± 0.51	4.80 ± 0.026
B-X-2CH	2.73 ± 0.132	78.15 ± 0.31	3.60 ± 0.043	2.34 ± 0.070	76.20 ± 0.37	4.20 ± 0.036
B-XEC-2CH	1.75 ± 0.149	83.70 ± 0.85	3.40 ± 0.021	1.36 ± 0.050	78.40 ± 0.54	4.40 ± 0.019

Control: treatment only with buffer; CM-X: treatment with commercial xylanase; *B*-X: treatment with *Bacillus* sp. NIORKP76 xylanase; *B*-XEC: treatment with *Bacillus* sp. NIORKP76 xylanolytic enzyme consortia; 4C: 4% hypochlorite treatment on pre-treated pulp; 2C: 2% hypochlorite treatment on pre-treated pulp; H: 1% hydrogen peroxide treatment on pre-bleached pulp

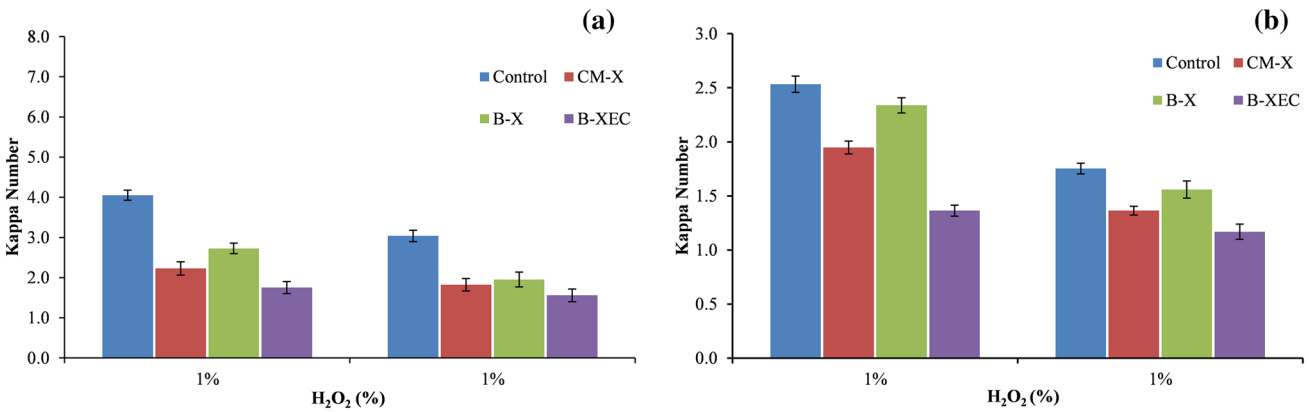

Fig. 8 Effect of hydrogen peroxide treatment on kappa number of enzymatically pre-bleached pulp: **a** Hardwood, **b** Chemical bagasse

number while 13.21/10.01% improvement in brightness. Xylanolytic consortia reported in the present study could reduce the kappa number of hardwood pulp by 55%, making it a suitable candidate for its application in pulp and paper mill.

Apart from microbial origin there are reports in which metagenomic approach for novel xylanase is used. Chawannapak et al. [9], reported metagenomic endoxylanase, with optimum enzyme dose of 50 Ug^{-1} used for bio-bleaching which was able to fetch 4.5–5.1% ISO increase in brightness.

Chemical bleaching of pre-treated pulp

To evaluate the potential of *B*-XEC, *B*-X, and CM-X in reducing chlorine consumption, enzyme pre-treated pulp was treated with sodium hypochlorite followed by H_2O_2. Results showed *B*-XEC significantly reduced hypochlorite consumption without compromising the brightness and viscosity compared to control after processing the pulp sample.

Pulp samples (HW and CB) pre-treated with *B*-XEC resulted in a 50% reduction in hypochlorite consumption, by

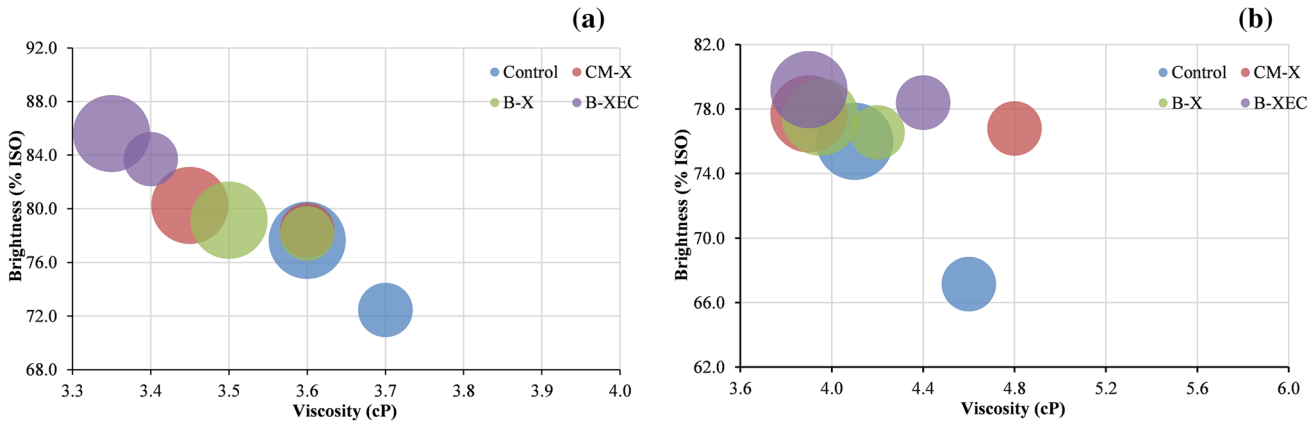

Fig. 9 Effect of hydrogen peroxide treatment on brightness and viscosity of enzymatically pre-bleached pulp: **a** Hardwood, **b** Chemical bagasse. (Note: size of the bubble represents the concentration of hypochlorite used in the bleaching of enzymatically pre-treated pulp samples)

keeping the brightness level same as that of control (Figs. 6a, b, 7a, b). Pulp samples pre-treated with xylanolytic enzyme consortia utilized 2% hypochlorite and proffered brightness and reduction in kappa number results superior to that of control pulp sample bleached with 4% hypochlorite solution (Table 2). The xylanolytic enzyme pre-treatment was more prominently effective against HW pulp as compared to CB pulp samples. When compared with kappa number of untreated kraft pulp, solo xylanase (*B*-X and CM-X) pre-treatment followed by 2% hypochlorite treatment lead to 84–85.5% reduction in kappa number. The reduction in kappa number was ~ 87% when same procedure was followed using *B*-XEC. The control pulp sample treated with 4% hypochlorite displayed only 81.77% reduction in kappa number. In case of CB pulp sample highest reduction in kappa number was recorded for pulp samples pre-treated using *B*-XEC followed bleaching using 2% hypochlorite which was ~ 89%, whereas, control pulp sample bleached using 4% hypochlorite harnessed only 86%. Further, hypochlorite treated pulp was subjected to H_2O_2, which increased the brightness index with a mild reduction in κ number of pulp samples without affecting viscosity values (Figs. 8a, b, 9a, b).

Beg et al. [2] reported pulp treated with xylanase when exposed to 4.5% chlorine; it reduced kappa number by 25% and also the brightness was enhanced (% ISO) by 20%. Ninawe and Kuhad, [39] reported xylanase isolated from *Streptomyces cyaneus* SN32, which was used for biobleaching of pulp. The resulted bleached pulp on treating with a 10% less hypochlorite (5.4%) the brightness was similar to fully bleached pulp (6% hypochlorite). Xylanase enzyme from *Arthrobacter* sp. MTCC 521 could reduce the chlorine use by 29% without much change in the brightness of kraft pulp [7]. Xylanase and laccases mediator system reported by Kapoor et al., [40] facilitated 15% reduction in hypochlorite consumption as compared to controls. With the use of optimum conditions such as xylanase dose of 25 Ug^{-1}, 5 h incubation period, and 10% pulp consistency, Nair et al. [8], reported elemental chlorine reduction of 14.3%. Chawannapak et al. [9] reported 20% decrease in hypochlorite consumption by using 50 Ug^{-1} of endoxylanase for biobleaching of pulp samples. 6% overall reduction in chlorine consumption was reported by Bhoria et al. [36] by using 50 Ug^{-1} of endoxylanase for biobleaching of pulp samples. Demand of chlorine-based chemicals was reported to be reduced up to 15% with use of xylanase for pre-treatment of kraft pulp [41]. Nagar et al. [5] applied xylanase for pretreatment of pulp which achieved 29.16% reduction in chlorine consumption while maintaining brightness equivalent to control.

Conclusion

The current study gave us the clear view, that if the pulp is treated with xylanolytic enzyme consortia, the amount of chlorine required during bleaching of pulp can be reduced to a great extent and also it gives better results when compared to single xylanase. The reduction in chlorine requirement due to prebleaching of pulp with xylanase enzyme and xylanolytic enzyme consortia has made the bio-bleaching process not only economical but also eco-friendly.

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Declarations

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

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Phylogenetic Diversity of Culturable Marine Bacteria from Mangrove Sediments of Goa, India: a Potential Source of Xylanases Belonging to Glycosyl Hydrolase Family 10

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Abstract—Xylan is one of the most abundant polysaccharides present in softwoods and hardwoods. Microbial xylanases mainly mediate its degradation. Xylanase has biotechnological potential with enormous demand in industries. The present study was initiated to investigate the diversity of culturable bacteria in mangrove sediments of Goa (India) and study the xylanase-coding gene in the isolated bacteria. Phylogenetic diversity was determined, in which proteobacteria was the dominant phylum (57%) with the abundance of *Vibrio* (36%) and *Photobacterium* (9%), while the second most abundant phylum *Firmicutes* (40%) was dominated by *Bacillus* (32%). Among all the culturable isolates screened for xylan degradation, the *Bacillus* genus was dominant (86%) xylan degrader. *Bacillus* isolates were further screened for the xylanase gene. The xylanase gene fragments of representative *Bacillus* were sequenced. All sequences matched to glycoside hydrolase (GH) family 10, thus, showing the dominance of GH10 xylanases in the mangroves regions of Goa (India). GH10 xylanases isolated from *Bacillus* sp. showed a broad pH optima ranged from pH 5.0 to 9.0 and temperature optima ranged from 50 to 60°C. These properties can be of potential interest for a variety of industrial processes where degradation of lignocelluloses is a crucial process.

Keywords: isolation, screening, 16S rRNA gene, dominance, phylum

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Mangroves are coastal wetland, salt-tolerant forest ecosystems that cover approximately 60–75% of the tropical and subtropical coastline globally [1]. Mangroves are known to be biologically diverse and productive ecosystems. Microbes found in mangroves are adapted to various physiological conditions such as periodical changes in temperature, water level, salinity and anoxic conditions [2]. Adaptation to the conditions strives them to follow unique metabolic pathways, which ultimately results in nutrient cycling and mineral transformation by the degradation of organic matter. Microorganisms present in such a dynamic environment can be a potential source of biotechnological resources to be exploited [3].

Xylan is a linear polymer of xylose molecules linked through β-1,4-glycosyl bonds. Xylanase breaks down xylan to give xylose and xylo-oligomers. Xylanase has enormous demand in paper and pulp industry, cattle feed and fodder industry for agro-waste treatment, food industry, bioethanol production, breweries, etc. [4]. Xylanases classified into GH families 5, 7, 8, 10, 11 and 43 based on the sequence similarities of the catalytic domain [5]. Among these, GH10 and GH11 xylanases are the most abundant families found in nature, they have unique three-dimensional structures and mechanisms of action [6], and they also differ in substrate specificity to xylan. Members of GH11 considered as true xylanases due to their stringent substrate specificity on xylans. In the case of GH10 xylanases, previous reports indicate that GH10 xylanases can hydrolyze other polysaccharides along with xylan [7].

The nutritive enrichment of the mangrove swamp soils due to rich leaf litter and high organic materials increases the possibility harvesting bacterial community with GH10 and GH11 xylanases. Also, owing to the adaptation to a harsh environment such as high

Table 1. Name and location details of bacterial sampling sites

North-Goa, India		South-Goa, India	
name of the sampling site	location on map	name of the sampling site	location on map
Vagalim	15°38′06.1″ N 73°46′13.0″ E	Cortalim	15°24′17.7″ N 73°53′49.9″ E
Shiollim	15°36′55.1″ N 73°45′16.6″ E	Chandor	15°19′08.7″ N 74°00′23.5″ E
Sarmanas(Piligao)	15°32′39.8″ N 73°57′37.7″ E	Navelim	15°14′31.8″ N 73°58′29.1″ E
Marcel	15°32′42.0″ N 73°56′39.4″ E	Assolna	15°10′00.7″ N 73°57′06.7″ E
Diwar	15°30′18.6″ N 73°53′39.8″ E	Talpona	14°59′06.9″ N 74°02′35.7″ E
Chorao	15°30′42.4″ N 73°51′29.6″ E	Galgibag	14°57′38.1″ N 74°03′03.7″ E
4-Pillar (Panjim)	15°29′13.1″ N 73°50′02.9″ E		

salinity, mangrove bacteria produce compounds that are different from those produced by their terrestrial counterparts [8]. Thus, this study aimed to reveal the distribution of xylanase-producing bacteria in the mangroves regions of Goa, to investigate their phylogenetic diversity and properties and to look at marine bacteria in decayed mangrove leaves as sources of new and interesting xylanases that could be used in the processing of seafood and saline food.

MATERIALS AND METHODS

Collection of samples. Mangrove ecosystem sites from different parts of Goa (India) were selected for sediment and water sample collection, which included 7 sites from North Goa and 6 sites from South Goa (Table 1). From each site, 2 sediment and 2 water samples were collected. The superficial layer of sediment (0–5 cm) was collected in an aseptic zip lock bag. Surface water samples from different mangrove regions were collected in sterile plastic tubes. In all sampling locations, the temperature was around 28–30°C, whereas surface water temperature was found to be 25–27°C. The samples were immediately transported to the laboratory under cold conditions (4°C) for further processing.

Isolation and screening of xylanase-producing bacteria. Serial dilutions of sediment (10^0–10^{-5}) and water (10^0–10^{-3}) samples were made using sterile 0.8% NaCl solution, and 200 µL volume was inoculated on sterile medium. Zobell marine agar (ZMA) (HiMedia, India), MacConkey agar (HiMedia, India), thiosulfate citrate bile salts sucrose (TCBS) agar (HiMedia, India), Eosin methylene blue (EMB) agar (HiMedia, India), cetrimide agar (HiMedia, India), and xylose lysine deoxycholate (XLD) agar (HiMedia, India) were used as cultivation media. The media plates with bacterial inoculum were incubated at room temperature (RT) (28 ± 2°C) for 24–48 h. Based on morphological differences such as colony color, shape, size, and appearance (glossy or matt), the individual colony was isolated on a fresh sterile ZMA plate. The obtained isolates were stored at 4°C for further processing.

Isolated colonies were screened for xylan-degrading ability by inoculating them on sterile 1.5% agar plates containing basal salt solution (BSS) supplemented with 0.5% beechwood xylan (Sigma-Aldrich, USA) as sole carbon source, along with 0.2% yeast extract (HiMedia, India) and 0.03% peptone (HiMedia, India). The composition of the BSS medium (wt/vol) was as follows (g/L): NaCl—30.0, KCL—0.75, $MgSO_4$—7.0, NH_4Cl—1.0, K_2HPO_4—0.7, KH_2PO_4—0.3; trace metal solution, 1.00 mL. Trace metal solution contained (g/L): H_3B0_3—2.85, $MnCl_2 \cdot 7H_2O$—1.8, $FeS0_4 \cdot 7H_2O$—2.49, Na-tartrate—1.77, $CuCl_2$—0.03, $ZnCl_2$—0.02, $CoCl_2$—0.04, $Na_2MoO_4 \cdot 2H_2O$—0.02 [9]. The inoculated plates were incubated at RT for 24–48 h. After the incubation period, the plates were flooded with 1% Congo Red. Excess stain was discarded, and plates were washed with 1.5 M NaCl to observe the zone of clearance [10].

Xylanase assay. Isolates showing zone of clearance on xylan containing plates as mentioned in the above paragraph were further grown at RT in 5 mL of the same medium for 24–48 h under shaking conditions. Cultures grown in broth were centrifuged at 11000×*g*, 4°C for 10 min, and supernatant was used for determining xylanase activity. Xylanase assay was per-

Table 2. List of primers used for gene amplification

Gene fragment to be amplified	Set of primer used	Nucleotide sequence of primer
16S rRNA gene	27F	5'-AGAGTTTGATCCTGGCTCAG-3'
	1492R	5'-GGTTACCTTGTTACGACTT-3'
Glycosyl hydrolase 10 gene (GH10)	X10-F	5'-CTACGACTGGGAYGTNIBSAAYGA-3'
	X10-R	5'-GTGACTCTGGAWRCCIABNCCRT-3'
Glycosyl hydrolase 11 gene (GH11)	X11-F	5'-CCGCACGGACCAGTAYTGNKIRAANGT-3'
	X11-R	5'-AACTGCTACCTGKCNITNTAYGGNTTGG-3'

formed in triplicate for each bacterial supernatant, and activity was determined by the 3,5-di-nitrosalicylic method described by Khandeparker and Bhosle [9].

Genomic DNA extraction. All the bacterial isolates were inoculated in 5 mL ZMB separately and incubated for 24 h at RT. Cell pellet of grown culture was collected by centrifugation at 11000×*g*. Genomic DNA extraction was carried out using GenElute bacterial genomic DNA kit (Sigma-Aldrich, USA) as per the manufacturer's instructions and stored at −20°C until further use.

Gene amplification and sequencing. 16S rRNA gene and genes responsible for the production of GH10 and GH11 xylanases were amplified using genomic DNA as a template and set of gene-specific primers (Table 2) [11]. PCR 96-well thermal cycler (Veriti 9902, Applied Biosystems, USA) was used to amplify genes. PCR reaction program was set, which comprised 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min [12]. Gene sequencing was carried out by the Taq Dye Deoxy Terminator cycle sequencing kit (PerkinElmer, USA) and analyzed using 373A automated DNA sequencer (3130x/Genetic analyzer, Thermo Fisher Scientific, USA) [10].

Nucleotide data analysis, identification and accession number. Obtained nucleotide sequence data were further processed and used for alignment with corresponding 16S rRNA gene as well as GH10 and GH11 xylanase genes using BLAST from the NCBI database. This was carried out to obtain the closest bacterial sequence match present within the respective database. Short sequences (<500 bp), sequences with low similarity (<90%), and chimeric sequences were removed. The nucleotide sequences obtained in this study were submitted to the NCBI BankIt database under accession numbers MH767087-MH767389. GH10 gene nucleotide sequences were also submitted to GenBank, and accession numbers assigned were MK911042- MK911051.

Phylogenetic analysis. 16S rRNA gene sequences were analyzed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A single multifasta file was created containing a single representative sequence of each genus along with its reference sequence. All the sequences were aligned, and the phylogenetic tree was constructed using MEGA version 6, with a bootstrap value of 1000. Nucleotide sequences of GH10 and GH11 xylanase gene fragments were translated into amino acids. ExPASy translation tool (https://web.expasy.org/translate/) was used for translation. Similarities in protein sequences were assessed using the BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned with known sequences from the GenBank database at the protein level using ClustalW. Phylogenetic trees were constructed with MEGA using the neighbor-joining method. Confidence for tree topologies was estimated by bootstrap values based on 1000 replicates. A total of 10 representative sequences were selected and used as references for the GH10 gene, and one GH11 sequence was used for phylogenetic tree construction.

Characterization of GH10 xylanase. The effect of pH and temperature on GH10 xylanase activity was studied using extracellular xylanase from representative *Bacillus* isolates. Fifty mM (pH 5.0—citrate, pH 6.0–8.0—Na-phosphate and pH 9.0–10.0—glycine- NaOH) buffer solutions were used to make crude xylanase aliquots in 1 : 1 ratio. These aliquots were used for xylanase assay. Similarly, the effect of temperature on GH10 xylanase activity was also studied by varying incubation temperatures of the enzyme-substrate reaction mixture from 30 to 80°C. GH10 xylanase activity was measured as per the xylanase assay protocol mentioned above.

RESULTS AND DISCUSSION

Mangroves are complex, unique, and extremely productive ecosystems found in tropical and subtropical intertidal regions of the world. Microbial populations in mangrove sediments have a significant effect on biogeochemical cycles of the coastal ecosystem [13]. Although the mangrove ecosystem is rich in microbial diversity, less than 5% of the species present have been described; in many cases, neither their ecological role nor their technological potential is known [14].

Lignocellulosic materials are difficult to degrade due to their compact structural features, [15] and microorganisms that specialize in lignocellulose degradation are expected to find in specific environments, such as the mangrove ecosystem, which is rich in such biomass.

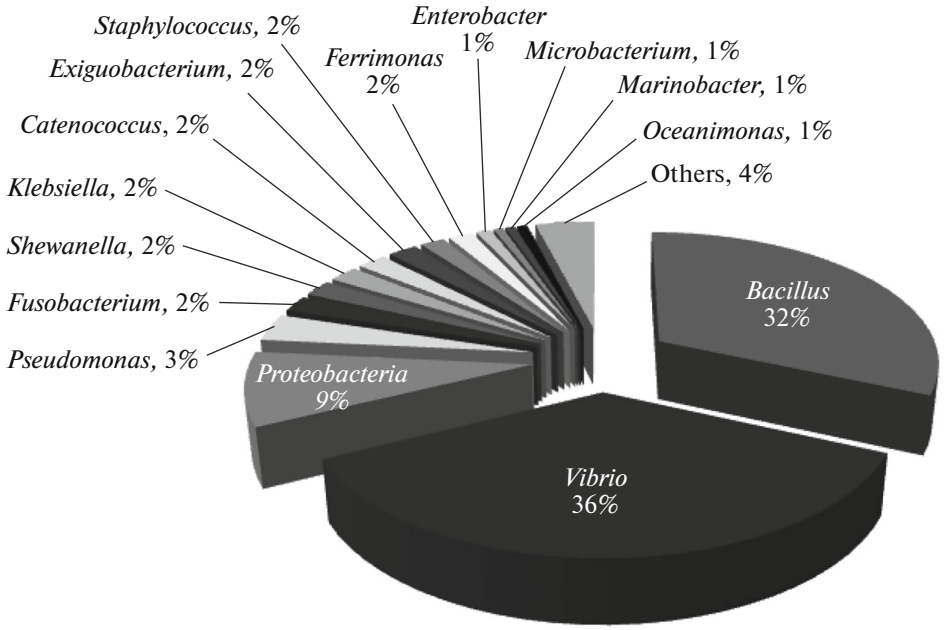

Fig. 1. Total bacterial diversity in mangroves of Goa region.

In an attempt to explore lignocellulose-degrading bacteria which produce xylanases, 303 bacteria were isolated from mangrove areas along the west coast of India, Goa. Sequence data analysis showed a total of 22 cultivable bacterial genera, of which the top 15 genera are represented in Fig. 1. The evolutionary history of culturable bacterial isolates is represented in the form of a phylogenetic tree (Fig. 2). Mangrove habitat showed the dominance of *Proteobacteria* phylum (57%) comprising of genus *Vibrio* (36%) and *Photobacterium* (9%). Oliveira et al. [16] studied the metagenomic bacterial community of mangrove sediment of Goa and reported that the *Proteobacteria* is dominant in the sediment samples comprising 43–46% of the total population. There are reports showing 88% of bacterial communities belong to *Proteobacteria* in mangrove sediment of southeastern Brazil [17]. Also, a previous study conducted on mangrove sediments of Sanya, Hainan Island, China reported predominant bacterial phylotypes to cluster within *Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria* and *Firmicutes* where proteobacteria dominated the community [18]. Proteobacteria have been suggested to have an important role in the nitrogen, phosphorous and sulphur cycles in mangrove sediments, such as nitrogen fixation, phosphate solubility and sulfate reduction [19]. *Firmicutes* was the second dominant phylum reported in our study with *Bacillus* (32%) genera being the most abundant. Similar observations were also reported from estuarine and mangrove environments [19]. Phylum *Actinobacteria* (1%) and *Fusobacteria* (2%) were also reported in the current study.

Mangrove sediments studied here showed a high abundance of *Vibrio*, and *Bacillus*, while *Vibrio* dominated. High numbers of pathogenic bacteria such as *Escherichia coli*, *Listeria*, *Salmonella*, and *Vibrio* sp. are previously reported from Goan mangroves by Poharkar et al. [20]. Family *Vibrionaceae* is one of the most fundamental 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. The genus *Bacillus* contains phylogenetically and phenotypically diverse species, which are ubiquitous in all the habitats, including terrestrial, freshwater also widely distributed in seawater.

Preliminary screening of xylan-degrading bacteria based on Congo Red assay revealed 94 isolates (32%) representing 6 genera (*Bacillus*, *Vibrio*, *Fusobacterium*, *Catenococcus*, *Staphylococcus*, and *Klebsiella*) with xylan-degrading capacity (Fig. 3). A total of 17 representative isolates from 6 genera were further studied for the quantitative estimation of xylanase activity (Table 3). It was seen that *Bacillus* strains produced a high amount of xylanase compared to *Vibrio* and other genera (Fig. 4). *Bacillus* alone represented 86% of xylan degraders in the study area. *Bacillus* genus has been reported as a potential source of xylanases production, and a large number of bacilli, such as *B. circulans*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. halodurans* and *B. tequilensis*; [10, 21] have been reported to have significant xylanase activity. In this study, 10 strains of *Bacillus* were found to be involved in hemicellulose degradation. *B. megaterium*, *B. cereus*, *B. subtilis*, *B. proteolyticus*, *B. tropicus*, *B. tenquilitis*, *B. amyloliquefaciens*, *B. safensis*, *B. vietnamensis* and *B. plakortidis* are

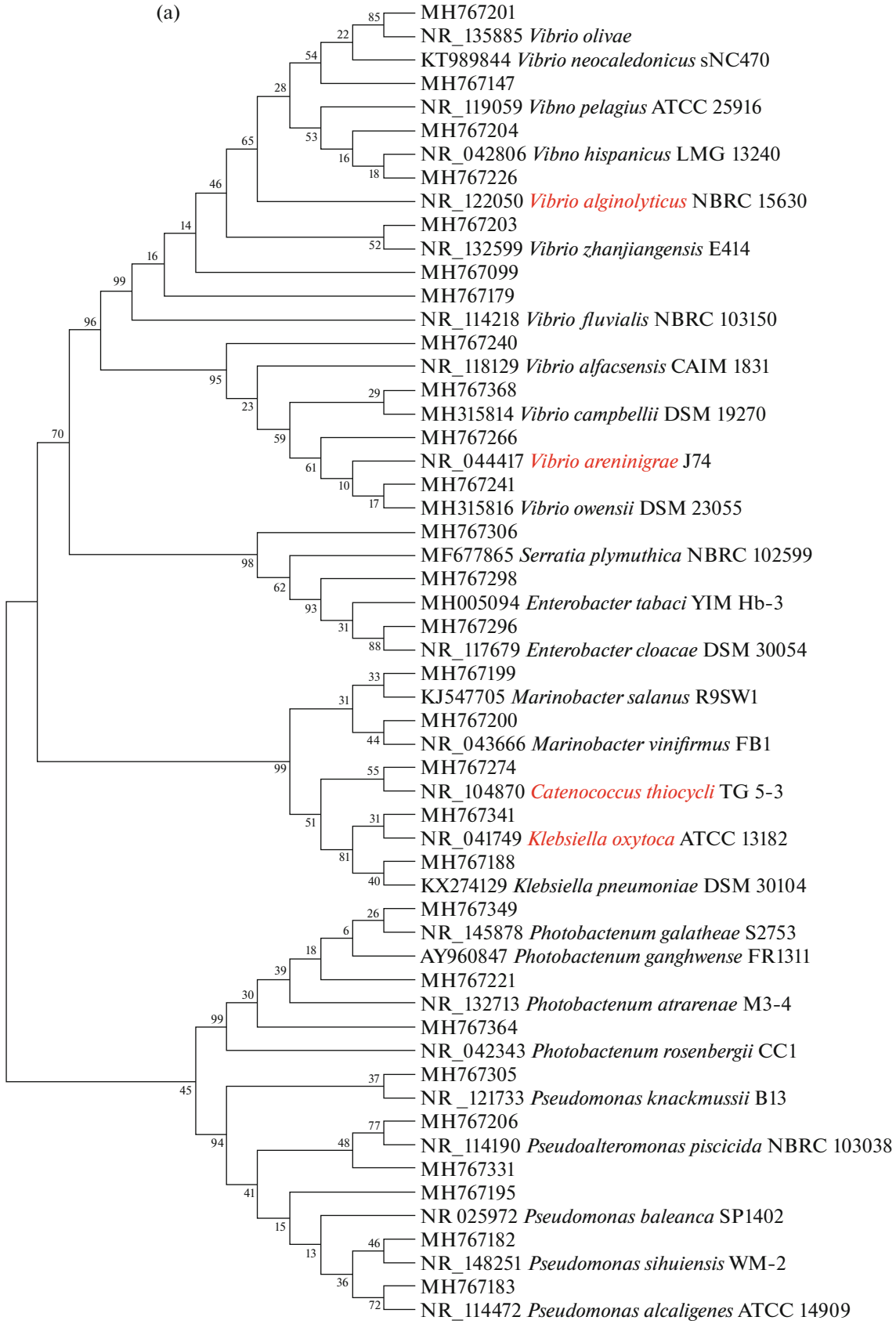

Fig. 2. The evolutionary history of culturable *Proteobacteria* (a) and *Firmicutes* and other bacterial (b) isolates from mangroves of Goa. *Highlighted strains possess xylan-degrading capacity.

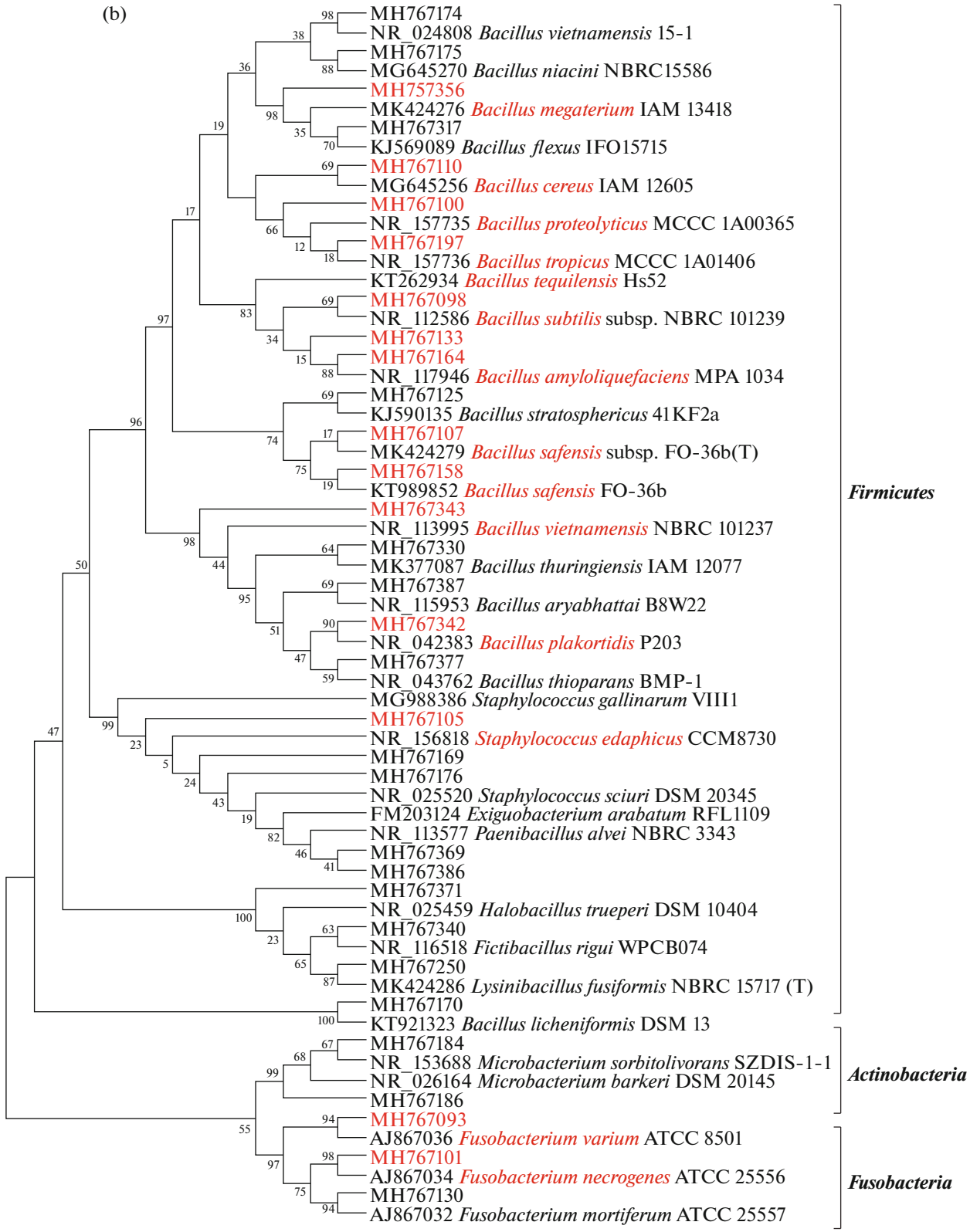
(b)
MH767174
NR_024808 Bacillus vietnamensis 15-1
MH767175
MG645270 Bacillus niacini NBRC15586
MH757356
MK424276 Bacillus megaterium IAM 13418
MH767317
KJ569089 Bacillus flexus IFO15715
MH767110
MG645256 Bacillus cereus IAM 12605
MH767100
NR_157735 Bacillus proteolyticus MCCC 1A00365
MH767197
NR_157736 Bacillus tropicus MCCC 1A01406
KT262934 Bacillus tequilensis Hs52
MH767098
NR_112586 Bacillus subtilis subsp. NBRC 101239
MH767133
MH767164
NR_117946 Bacillus amyloliquefaciens MPA 1034
MH767125
KJ590135 Bacillus stratosphericus 41KF2a
MH767107
MK424279 Bacillus safensis subsp. FO-36b(T)
MH767158
KT989852 Bacillus safensis FO-36b
MH767343
NR_113995 Bacillus vietnamensis NBRC 101237
MH767330
MK377087 Bacillus thuringiensis IAM 12077
MH767387
NR_115953 Bacillus aryabhattai B8W22
MH767342
NR_042383 Bacillus plakortidis P203
MH767377
NR_043762 Bacillus thioparans BMP-1
MG988386 Staphylococcus gallinarum VIII1
MH767105
NR_156818 Staphylococcus edaphicus CCM8730
MH767169
MH767176
NR_025520 Staphylococcus sciuri DSM 20345
FM203124 Exiguobacterium arabatum RFL1109
NR_113577 Paenibacillus alvei NBRC 3343
MH767369
MH767386
MH767371
NR_025459 Halobacillus trueperi DSM 10404
MH767340
NR_116518 Fictibacillus rigui WPCB074
MH767250
MK424286 Lysinibacillus fusiformis NBRC 15717 (T)
MH767170
KT921323 Bacillus licheniformis DSM 13
MH767184
NR_153688 Microbacterium sorbitolivorans SZDIS-1-1
NR_026164 Microbacterium barkeri DSM 20145
MH767186
MH767093
AJ867036 Fusobacterium varium ATCC 8501
MH767101
AJ867034 Fusobacterium necrogenes ATCC 25556
MH767130
AJ867032 Fusobacterium mortiferum ATCC 25557
Firmicutes
Actinobacteria
Fusobacteria

Fig. 2. (Contd.)

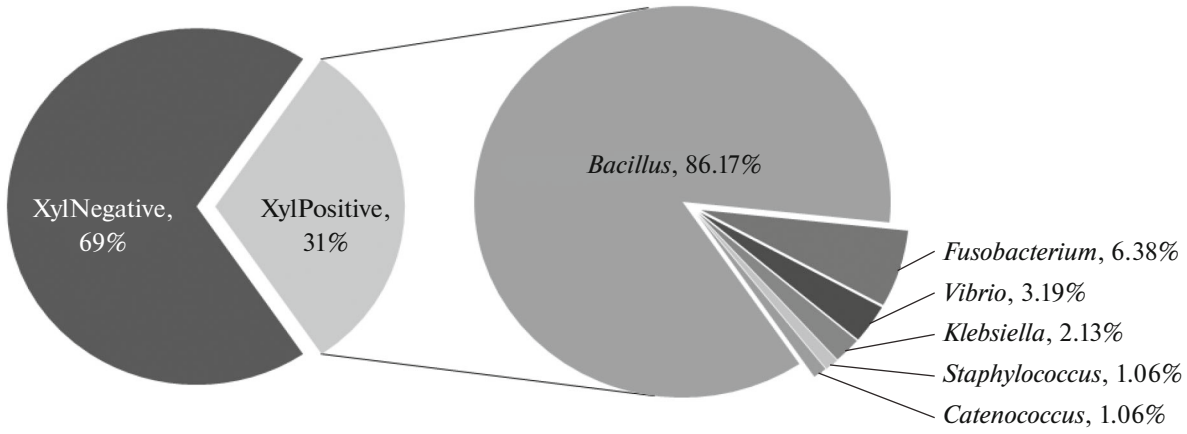

Fig. 3. Diversity of xylanase-producing bacteria in mangroves of Goa region.

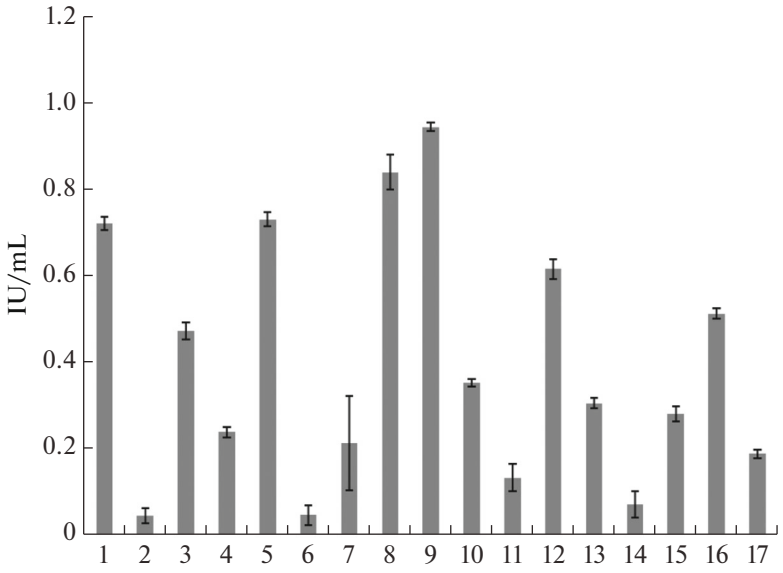

Fig. 4. Xylanase production by xylanase-producing bacterial representatives. 1—*Bacillus* MH767089; 2—*Fusobacterium* MH767093; 3—*Bacillus* MH767098; 4—*Vibrio* MH767099; 5—*Bacillus* MH767100; 6—*Fusobacterium* MH767101; 7—*Staphylococcus* MH767105; 8—*Bacillus* MH767158; 9—*Bacillus* MH767108; 10—*Bacillus* MH767109; 11—*Bacillus* MH767110; 12—*Bacillus* MH767164; 13—*Vibrio* MH767266; 14—*Catenococcusthiocycli* MH767274; 15—*Klebsiella* MH767341; 16—*Bacillus* MH767342; 17—*Bacillus* MH767343.

among them. Some of the *Bacillus* species reported in this study are already reported for hemicellulose degradation [10].

Most of the earlier investigations are focused on microbial ecology-based taxonomic studies, which help in understanding microbial populations in a particular environmental niche [22], although few efforts have been made to study the microbial diversity by exploring the functional potential of microbes [23]. Our understanding of the functional diversity of xylanases of microorganisms from different mangrove regions can possibly help to elucidate the mechanisms of the transformation of organic substances in marine ecosystems and to fulfil the requirements of modern biotechnology. To study the functional diversity of *Bacillus* screened in study area, xylan-degrading *Bacillus*, comprising 10 representative strains, were further screened for the presence of GH10 and GH11 xylanase gene fragments. DNA of each bacterial sample was PCR amplified using GH10 and GH11 gene primers (Table 2). Amplification with GH10 primers indicated the presence of the GH10 xylanase gene in all ten representative *Bacillus* strains isolated from mangrove sediments of Goa. The PCR product was sequenced. BLASTx analysis inferred all sequences showing high similarities to GH10 xylanase gene fragments. Partial sequences of GH10 xylanases obtained in this study and GH11 xylanase sequence from *Bacil-*

Table 3. Xylanase activity and pH and temperature optima of representative bacterial isolates

Accession number obtained	Nearest neighbor in NCBI database	Similarity, %	Xylanase activity, IU/mL	Standard error	Optimum, pH	Optimum temperature, °C
MH767089	*Bacillus altitudinis* 41KF2b	99.69	0.722	0.015	6.0	50
MH767093	*Fusobacterium varium*	81.78	0.046	0.017	6.0	60
MH767098	*Bacillus subtilis* subsp. spizizenii strain NBRC 101239	97.40	0.473	0.020	7.0	50
MH767099	*Vibrio alginolyticus* strain NBRC 15630	99.21	0.239	0.013		
MH767100	*Bacillus proteolyticus* strain MCCC 1A00365	99.86	0.732	0.016	6.0	55–60
MH767101	*Fusobacterium necrogenes*	83.19	0.047	0.022	6.0–7.0	40
MH767105	*Staphylococcus edaphicus* strain CCM 8730	99.89	0.214	0.110		
MH767158	*Bacillus safensis* strain FO-36b	99.48	0.841	0.041	8.0	60
MH767108	*Bacillus aerius* strain 24K	99.62	0.946	0.010	9.0	50
MH767109	*Bacillus subtilis* strain JCM 1465	95.80	0.353	0.009	6.0	60
MH767110	*Bacillus cereus* strain IAM 12605	99.85	0.134	0.031	5.0–6.0	50
MH767164	*Bacillus amyloliquefaciens* strain MPA 1034	99.04	0.617	0.023	6.0	55–60
MH767266	*Vibrio areninigrae* strain J74	96.54	0.306	0.012		
MH767274	*Catenococcusthiocycli* strain TG 5-3	97.67	0.072	0.030	7.0	60
MH767341	*Klebsiella oxytoca* strain ATCC 13182	97.65	0.281	0.017		
MH767342	*Bacillus plakortidis* strain P203	96.29	0.513	0.012	7.0–8.0	55–60
MH767343	*Bacillus vietnamensis* strain NBRC 101237	97.82	0.189	0.010	7.0	50

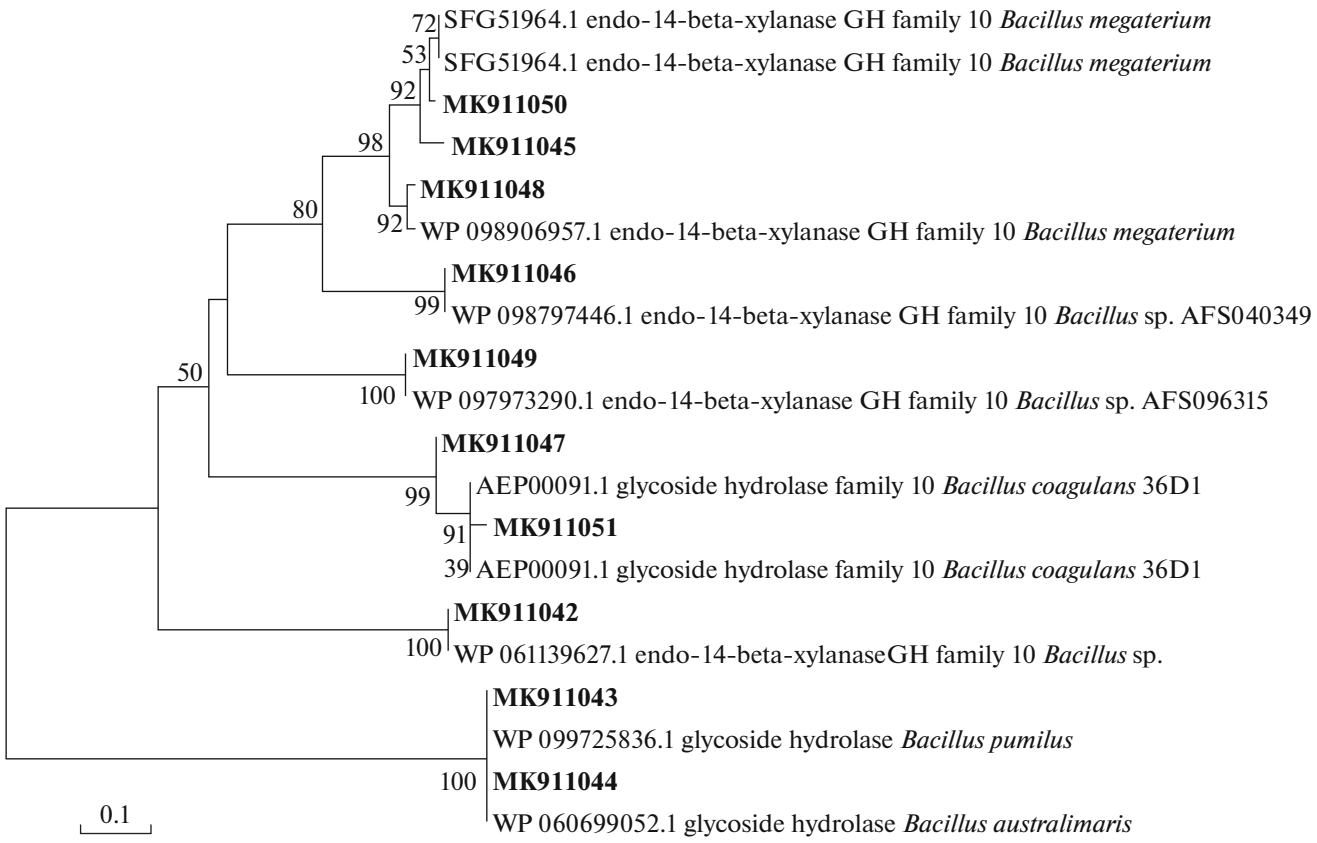

Fig. 5. Phylogenetic tree constructed by MEGA using the neighbor-joining method. The lengths of the branches indicate the relative divergence among the amino acid sequences.

lus sp. BT21 reported earlier [10] were used to construct a phylogenetic tree (Fig. 5). All the sequences reported in this study shared high identity with GH10 xylanases from *Bacillus* sp. where GH11 xylanase was seen as out-group (Fig. 7). According to the Pfam database (http://pfam.sanger.ac.uk/), GH11 xylanase is mainly distributed in fungi, while GH10 sequences are mainly from bacteria. This suggests that bacteria and fungi are the main producers of GH10 and GH11 xylanases, respectively [11]. Although GH11 xylanases are most frequently chosen for the industrial processes, GH10 enzymes display certain enzymological characteristics that can make them better candidates for enzymatic degradation of lignocelluloses biomass [24]. According to Hu and Saddler [7], GH10 xylanases have higher accessibility towards the xylan backbone within pre-treated biomass, thus, enhancing biomass degradation. This characteristic makes GH10 xylanases a potential enzyme for biomass utilization.

As per the earlier report, bacterial isolates from mangrove regions have potential to produce a broad spectrum of xylanases. According to Guo et al. [25], xylanases from marine bacterium *Glaciecola mesophila* are stable at a lower temperature (25°C). On the contrary, Khandeparker and Bhosle [9, 26] reported xylanases from *Enterobacter* sp. and *Arthrobacter* sp. isolated from an estuarine environment had temperature optima of 100°C. Alkaline pH tolerance of xylanases of bacterial isolates from mangrove reported by Khandeparker and Bhosle [9, 26] and Annamalai et al. [27] was found to be 9.0, whereas, Khandeparker et al. [10] revealed *Bacillus* sp. xylanase having pH optimum of 6.0 which was also isolated from bacteria of mangrove sediment. Palavesam and Somanath [28] reported xylanase, which had organic solvent tolerance ability up to 25%. Xylanases with organic solvent tolerance can be of great potential in industries involved in the saccharification of sugars to produce bioethanol. Salt tolerance of xylanase obtained from bacterial isolates from mangroves have also been widely studied and reported.

Extracellular GH10 xylanases from marine *Bacillus* sp. reported in the current study showed optima pH from 5.0 to 9.0 and temperature optima in the range of 50 to 60°C (Table 3, Fig. 7). *Bacillus* sp. possessing accession number MH767108 and MH767158 produced xylanase with optimum pH of 9.0 and 8.0, respectively, and temperature optimum at 60°C. Xylanases active at high temperatures and alkaline pH are applicated in biobleaching of pulp [29]. The effectiveness of bacterial xylanase in pulp bleaching has been studied previously for *Bacillus* sp. [30, 31], where *Bacillus* sp. NCIM 59 xylanase had a temperature and

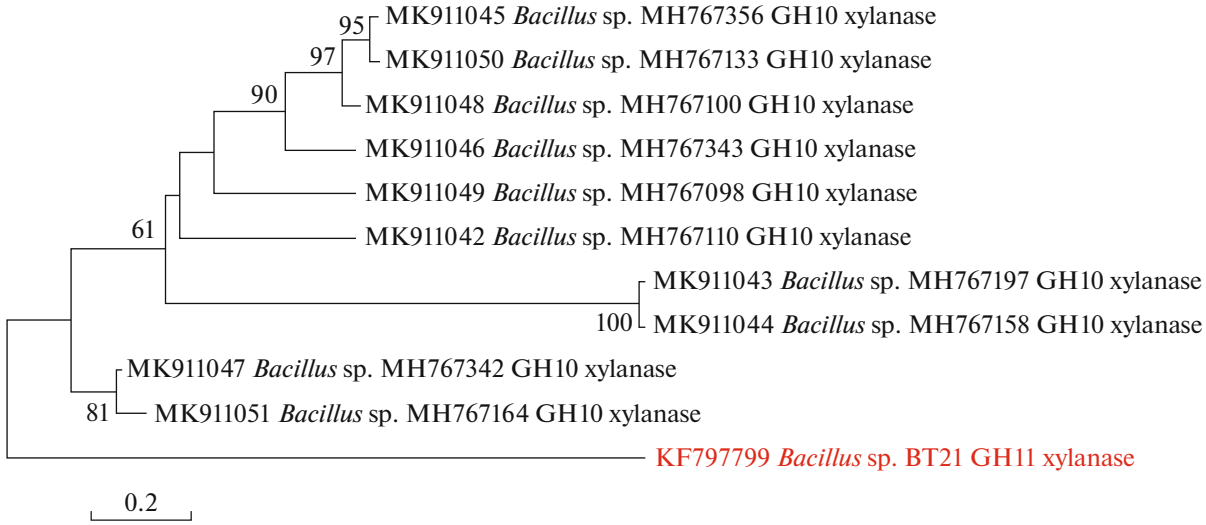

Fig. 6. Phylogenetic relationships of a GH10 xylanase of *Bacillus* species from mangroves with GH11 xylanase of previously reported *Bacillus* species constructed using NJ-tree method with bootstrap values (1000 replicates) as a percentage at the nodes.

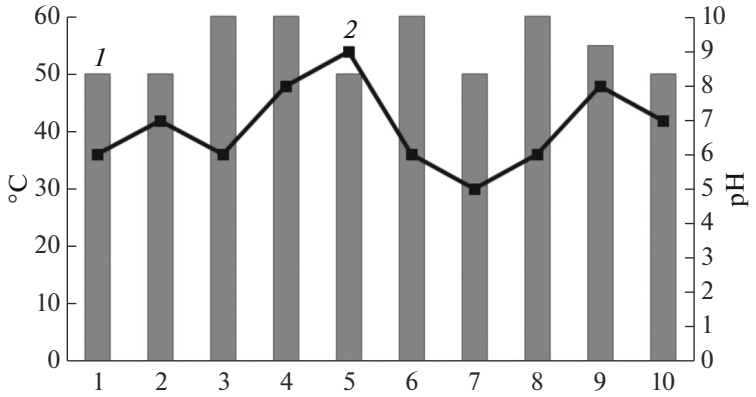

Fig. 7. Temperature (*1*) and pH optima (*2*) of a GH10 xylanase from *Bacillus* species. 1—*Bacillus* MH767089; 2—*Bacillus* MH767098; 3—*Bacillus* MH767100; 4—*Bacillus* MH765158; 5—*Bacillus* MH767108; 6—*Bacillus* MH767109; 7—*Bacillus* MH767110; 8—*Bacillus* MH767164; 9—*Bacillus* MH767342; 10—*Bacillus* MH767343.

pH optima of 60°C and 6.0, respectively, while *Bacillus* sp. Sam-3 xylanase showed optimum pH 8.0 and temperature optimum of 60°C. *Bacillus circulans* is reported for its xylanase application in biobleaching of eucalyptus pulp [32], the optimum pH for the enzyme was 6.0–7.0, while temperature optimum was 80°C.

In this study, acidic xylanase isolated from *Bacillus* sp. MH767110 and having optima at pH 5.0 and 50°C can also be a potential candidate for the food industry as the main desirable properties for xylanases for this use are high stability and optimum activity at acid pH. As per our knowledge, there are fewer reports on bacterial xylanases in this area; mostly fungal xylanases are reported in the food industry [33]. Singh and Singh [34] have reported *B. subtilis* for improving the nutritional value of poultry feed with optimum pH of 4.0 and temperature optimum of 35°C. Looking at the properties of the *Bacillus* sp. isolated from mangroves, the potential application of these bacterial cultures in various industries which require acidic/alkaline conditions and elevated temperatures during processing can be targeted which includes fruit juice, bakery, seafood processing industries, and paper and pulp industry [35].

Mangroves are natural terrains which contain diverse microbial communities with immense potential. Mangrove bacteria play a very important role in these dynamic conditions as they are adapted to extreme conditions, like the higher salinity and the low availability of oxygen, which make them a rich source for novel metabolite and enzyme discovery. The current study was carried out to investigate the bacterial community in mangrove sediments of Goa (India) and to explore the potential GH10 xylanase

producers from this community. Results obtained clearly indicate that mangrove regions could be potential hotspots for xylan-hydrolyzing bacteria, mainly *Bacillus* strains producing GH10 xylanase. Halophilic, alkalophilic, thermophilic and acidophilic xylanases reported from mangroves areas can be further studied for their novel applications.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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ORIGINAL ARTICLE

Enzymatic saccharification of seaweeds into fermentable sugars by xylanase from marine *Bacillus* sp. strain BT21

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Abstract Enzymatic hydrolysis of seaweed biomass was studied using xylanase produced from marine bacteria *Bacillus* sp. strain BT21 through solid-state fermentation of wheat bran. Three types of seaweeds, *Ahnfeltia plicata*, *Padina tetrastromatica* and *Ulva lactuca*, were selected as representatives of red, brown, and green seaweeds, respectively. Seaweed biomass was pretreated with hot water. The efficiency of pretreated biomass to release reducing sugar by the action of xylanase as well as the type of monosaccharide released during enzyme saccharification of seaweed biomass was studied. It was seen that pretreated biomass of seaweed *A. plicata, U. lactuca*, and *P. tetrastroma*, at 121 °C for 45 min, followed by incubation with 50 IU xylanase released reducing sugars of 233 ± 5.3, 100 ± 6.1 and 73.3 ± 4.1 µg/mg of seaweed biomass, respectively. Gas chromatography analysis illustrated the release of xylose, glucose, and mannose during the treatment process. Hot water pre-treatment process enhanced enzymatic conversion of biomass into sugars. This study revealed the important role of xylanase in saccharification of seaweed, a promising feedstock for third-generation bioethanol production.

Keywords Enzyme · Xylanase · Pre-treatment · Hydrolysis · Seaweed · *Bacillus* sp.

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Introduction

Xylan, a major component of hemicelluloses, is a heterogeneous molecule having a linear backbone consisting of β-(1,4)-linked D-xylosyl residues and several side branches of different groups attached to the main chain. Due to heterogeneous nature of xylan, its hydrolysis requires complex enzyme system in which group of enzymes work together synergistically, which mainly includes the main-chain enzyme and side-chain enzyme. In general, depolymerisation of xylan is accomplished by the action of *endo*-xylanases and β-xylosidases. *Endo*-1,4-β-xylanases (EC 3.2.1.8) hydrolyze β-1,4-glycosidic linkages of the xylan backbone to produce short-chain xylooligosaccharides of various lengths. Hence, *endo*-xylanases are the crucial enzyme components of the microbial xylanolytic systems (Frederick et al. 1981; Beg et al. 2001).

From the biotechnological point of view, xylanases have applications in animal feed, aroma, fruit juices, baking, textile, paper industries, ethanol (Polizeli et al. 2005; Khandeparkar and Bhosle 2006b; Khandeparker et al. 2017), and human health (Harris and Ramalingam 2010). Production of fuel ethanol from renewable lignocellulosic materials has been extensively studied in the last decades (Eriksson et al. 2002; Sun and Cheng 2002). Currently, bioethanol is mainly derived from sucrose and starch crops (e.g., sugarcane and corn) as well as lignocellulosic materials (e.g., rice straw and switchgrass). Major drawback faced here is limited cultivable lands, as well as the high costs involved in converting lignocellulosic materials into ethanol due to the presence of lignin. In view of these problems, algae have recently been considered as a third-generation feedstock for biofuel production (Nigam and Singh 2010). Algal feedstocks have several advantages over other types of feedstock. These include high area

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productivity, no competition with conventional agriculture for land, utilization of different water sources (e.g., seawater, brackish water, saline water, and wastewater), recycling of carbon dioxide, and compatibility with integrated production of fuels and co-products within bio refineries. Hence, algal feedstocks are considered one of the most promising non-food feedstocks for biofuels (Wijffels and Barbosa 2010; Wang et al. 2011; Borines et al. 2011; Wei et al. 2013). However, seaweed polysaccharides are structurally complex and diverse in chemical composition and differ from land plants with respect to the abundance of matrix and skeletal components. Thus, an efficient hydrolysis for sustainable production of biofuels from different macroalgal feedstocks is required (Trivedi et al. 2013). The objective of this study is to study the efficiency of hot water pre-treatment process to enhance enzymatic conversion of seaweed biomass using xylanase.

Materials and methods

Microorganism, culture condition, and chemicals

Bacterial culture was isolated from Chorao island of Mandovi estuary, Goa, India. The culture was grown at room temperature in basal salt solution (BSS) supplemented with xylan (0.5%) as sole carbon source. The composition of the BSS medium (w/v) was as follows: NaCl, 30.00 g; KCL, 0.75 g; $MgSO_4$, 7.00 g; NH_4Cl, 1.00 g; K_2HPO_4 (10%), 7.00 mL; KH_2PO_4 (10%), 3.00 mL; trace metal solution, 1.00 mL; distilled water, 1000 mL; the pH of the medium was adjusted using 1 N NaOH. Trace metal solution has the following composition: H_3B0_3, 2.85 g; $MnCl_2{\cdot}7H_2O$, 1.80 g; $FeS0_4{\cdot}7H_20$, 2.49 g; Na-tartrate, 1.77 g; $CuCl_2$, 0.03 g; $ZnCl_2$, 0.02 g; $CoCl_2$, 0.04 g; $Na_2MoO_4{\cdot}2H_2O$, 0.02 g; distilled water, 1000 mL (Khandeparkar and Bhosle 2006a). Xylan (beech wood) and 3,5-dinitrosalicylic acid, and gas chromatography (GC) standards were purchased from Sigma-Aldrich Co., St Louis, MO, USA.

Production and preparation of enzyme

The culture was grown for 48 h in the BSS as above; this was used to inoculate 500-mL flasks each containing 30 mL of BSS medium and 10 g (substrate to moisture ratio 1:3) of wheat bran. The flasks were incubated at room temperature. The culture was harvested in the stationary growth phase, i.e., after 4 days. The content of the flask was suspended in 100 mL of 50 mM glycine–NaOH buffer (pH 9) vortexed thoroughly and centrifuged (10,000 rpm for 10 min, 4 °C). The enzyme was precipitated from the culture supernatant by adding ammonium sulfate to 80% saturation. This was left overnight and the precipitate was collected by centrifugation at 10,000 rpm for 10 min. The precipitate obtained was dissolved in phosphate buffer (50 mM, pH 8.0) and dialyzed against the same buffer for 24 h. Dialysis was carried out using cellulose tubing (molecular weight cut-off 13,000 Da). The enzyme was partially purified using ion-exchange chromatography (Khandeparkar and Bhosle 2006b).

Enzyme assay

Xylanase assay was carried out using 3,5-dinitrosalicylic acid method (DNS method) (Miller 1959). 1% solution of xylan was used as substrate for xylanase. The reducing sugars released due to enzymatic hydrolysis were measured at 510 nm using spectrophotometer.

Estimation of carbohydrate

10 mg of dried seaweed powder was taken and boiled for 2 h in 1 mL of 2.5 N HCl in water bath. The mixture was then cooled and centrifuged. The supernatant was used for carbohydrate estimation using 5% phenol solution and concentrated sulphuric acid (Dubois et al. 1956). The color intensity was measured at 490 nm. Sugar content was calculated by referring to a standard D-glucose and the results have been expressed as μg/mg sugar.

Collection of seaweed sample

Seaweeds such as *A. plicata, P. tetrastromatica,* and *U. lactuca* were collected from the coast of Goa (15.5809°N, 73.7448°E), India. The seaweed samples were washed thoroughly with fresh water to remove salts and debris, and were dried at 50 °C temperature. After drying, the seaweed samples were powdered using a grinder.

Pre-treatment of seaweed

Seaweed pre-treatment was carried out with 10% macroalgal biomass at 121 °C for 45 min. Pre-treatment was performed as per the reports of Yazdani et al. (2015). After the pre-treatment, solids were separated from the solutions by vacuum filtration and washed several times with distilled water.

Optimization of hydrolysis condition for effective saccharification of seaweed

Xylanase doses and incubation period were optimized for enzymatic hydrolysis of seaweed. Dried seaweed biomass (1%) was hydrolyzed with different concentrations of xylanase from 10 to 50 U/mg dry wt. Seaweed biomass was

incubated for different time intervals from 0 to 8 h at 30 °C on an orbital shaker with a speed of 140 rpm. Samples were taken out periodically after an interval of 2 h each and centrifuged. The reducing sugar was measured spectrophotometrically using 3,5-dinitrosalicylic acid (DNS) method (Miller 1959).

Enzymatic hydrolysis of seaweed

Pretreated and untreated macroalgal biomass was added to a phosphate buffer solution (0.05 M), pH 7, and the enzymatic hydrolysis was initiated by adding xylanase (50 IU/mg) to seaweed biomass. The hydrolysis was performed at 30 °C for 6 h. The product was centrifuged and RS (reducing sugar) from hydrolysate was analyzed by DNS method which was then further processed for GC analysis.

GC analysis of hydrolytic product

The monosaccharide composition of seaweed hydrolysis product after hot water treatment and after enzyme treatment was studied using gas chromatography (GC) method described in Khodse et al. (2008). Briefly, the sample was treated with 12 M H_2SO_4 at room temperature for 2 h. It was diluted with 1.2 M H_2SO_4 using cold distilled water, flushed with N_2, sealed and hydrolyzed for 3 h at 100 °C. After cooling, an internal standard (inositol) was added. The sample was neutralized, treated with $NaBH_4$, acetylated and analyzed using a Shimadzu GC Model-GC-2010 equipped with a flame ionization detector (FID), a programmable on-column injector and a fused silica column coated with CPSil-88 (25 m, i.d. 0.32 mm). The response factors were calculated using standard sugar alditol acetates and myoinositol as an internal standard and were used for the quantification of the results.

Result

Identification of the bacterial isolate

Morphological and biochemical analysis showed that isolate BT21 was similar to members of the genus *Bacillus*. 16S rRNA analysis showed that isolate BT21 was equidistantly related to *Bacillus tequilensis* strain IARI-BHI-20 and members of the representatives of the *Bacillus subtilis* cluster (similarity value of 99%). Our results are very similar to that of Gatson et al. (2006) who had also reported that *B. tequilensis* is closely related to *B. subtilis*, but could be differentiated on the basis of DNA homology. Given this, we are unable to report on its precise taxonomic position and will refer to it as *Bacillus* sp. strain BT21. The 16S rRNA gene sequence has been submitted to GenBank under accession number KF797798.

Carbohydrate content of seaweed

The carbohydrate content of seaweeds is listed in Table 1. *A. plicata* red seaweed had a total carbohydrate content of 43.05 ± 0.7%. *P. tetrastromatica* brown seaweed showed 14.2 ± 0.8%, and *U. lactuca* green seaweed had carbohydrate content of 39.2 ± 0.8% on dry weight basis.

Optimization of enzymatic treatment on seaweed biomass

Dry biomass of *P. tetrastromatica, A. plicata*, and *U. lactuca* was saccharified by incubating it with different doses of enzyme (0–100 U). Treatment resulted in attaining maximum reducing sugar yield of 39.5 ± 2.4, 9.16 ± 1.9, and 25.36 ± 1.3 µg/mg, respectively, at an enzyme dose of 50 U for 2 h of incubation (Fig. 1). The enzyme dosage of 50 U/mg was employed for the hydrolysis of biomass in subsequent optimization of incubation period. All three seaweeds showed increase in reducing sugar yield with increase in incubation period from 2 to 6 h, while it remained steady for the next 2 h. Reducing sugar release from *P. tetrastromatica* increased from 39.5 ± 2.4 to 59.56 ± 2.9 µg/mg when incubated for 6 h, while *A. plicata* showed release of reducing sugars from 9.16 ± 1.9 to 12.16 ± 2.4 µg/mg, and reducing sugar yield from *U. lactuca* indicated to increase from 25.36 ± 1.3 to 45.84 ± 3.4 µg/mg after 6 h of incubation (Fig. 2).

Saccharification by optimizing pre-treatment conditions on seaweed biomass

In this study, macroalgal biomass was pretreated with hot water and the effect of pre-treatment on reducing sugar yield was studied. It was seen that the amount of reducing sugar release by all three seaweeds was maximum when the seaweed was pretreated with hot water for 45 min and with further increase in pre-treatment time, sugar yield remained almost constant (Fig. 3).

It was seen that hot water-pretreated dry biomass of *A. plicata* showed drastic increase in reducing sugar (RS) yields of 233 ± 5.3 µg/mg (Fig. 3) when treated with 50 IU of xylanase, while untreated dry biomass of *A. plicata* showed 12.17 ± 0.3 µg/mg of sugar release after enzyme treatment. Carbohydrate content of pretreated *A. plicata* biomass was 406 ± 3.92 µg/mg on dry weight basis (Table 1) and it was seen that the enzymatic hydrolysis released around 57% of RS from this macroalgae. Dry biomass of *U. lactuca* showed reducing sugar (RS) yields of 100 ± 6.1 µg/mg (Fig. 3), while untreated

Table 1 Carbohydrate composition of seaweeds during biomass processing (mean ± SD)

Seaweeds	Total carbohydrate (μg/mg)			
	Untreated biomass	30-min pre-treatment	45-min pre-treatment	60-min pre-treatment
P. tetrastromatica	142.1 ± 8.32	139.07 ± 4.56	136.36 ± 5.12	129.12 ± 5.78
A. plicata	430.5 ± 7.56	419 ± 6.33	406.00 ± 3.92	390 ± 4.68
Ulva lactuca	393.4 ± 12.31	357 ± 8.02	343.00 ± 6.88	306 ± 5.11

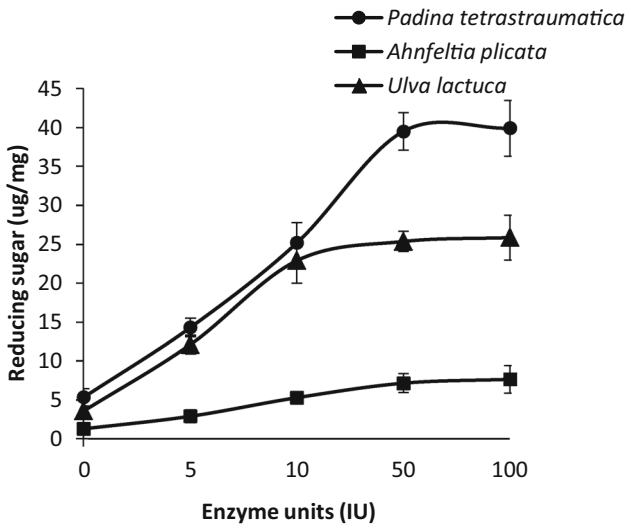

Fig. 1 Saccharification of untreated solid waste (1%) of *A. plicata* (*filled square*), *P. tetrastromatica* (*filled circle*), and *U. lactuca* (*filled triangle*) using different concentrations (10–50 U/g biomass) of *Bacillus* sp. strain BT21 xylanase at 30 °C. *Error bars* indicate the standard deviation of three replicates

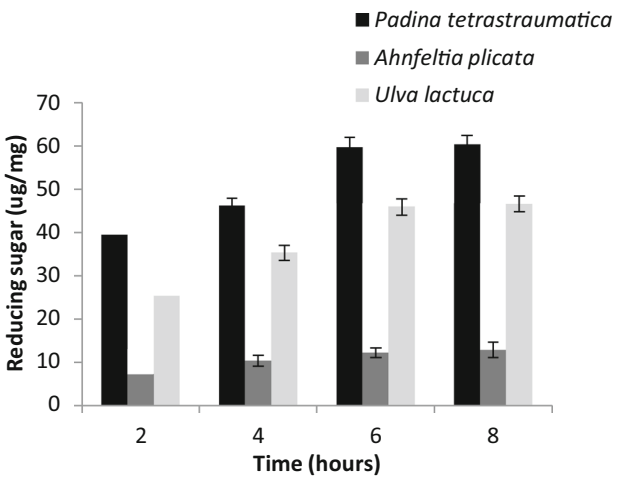

Fig. 2 Optimization of enzymatic hydrolysis of *A. plicata*, *P. tetrastromatica*, and *U. lactuca* with respect to different incubation periods (2–8 h) at 30 °C, pH 7, using 50 U of *Bacillus* sp. strain BT21 xylanase. *Error bars* indicate the standard deviation of three replicates

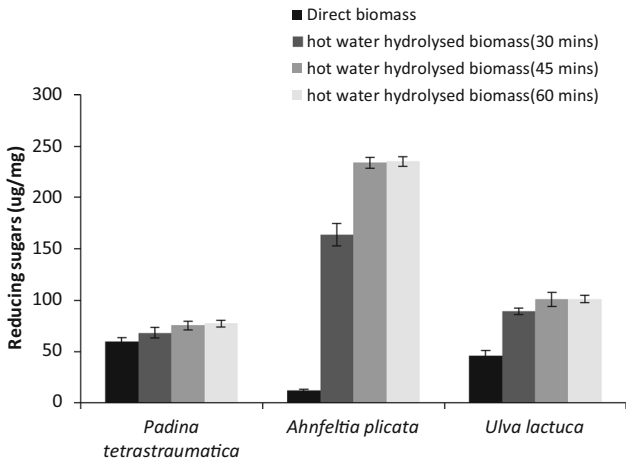

Fig. 3 The effect of hot water pre-treatment for different time intervals (30, 45, and 60 min) on the yield of reducing sugars from biomass *of A. plicata, P. tetrastromatica*, and *U. lactuca.* Pre-treatment was followed by enzymatic hydrolysis for 6 h and using 50 U of *Bacillus* sp. strain BT21 xylanase per gram of biomass at 30 °C, pH 7. *Error bars* indicate the standard deviation of three replicates

dry biomass of *U. lactuca* showed 45.8 ± 2.4 μg/mg of sugar release with enzyme treatment. Carbohydrate content of pretreated *U. lactuca* biomass was 343 ± 6.88 μg/mg on dry weight basis (Table 1), here the release of 29% of RS was observed. RS yield from dry biomass of *P. tetrastromatica* was seen to be 73.3 ± 4.1 μg/mg (Fig. 3), while untreated dry biomass of *P. tetrastromatica* showed 59.5 ± 2.3 μg/mg of sugar release. Carbohydrate content of pretreated *P. tetrastromatica* biomass was 136.36 ± 5.12 μg/mg on dry weight basis (Table 1), thus 53% of RS was released due to enzyme treatment.

Monosaccharide composition of sugars released during hydrolysis

The monomeric sugar yield in the hydrolyzate derived from hot water-pretreated biomass was analyzed by gas chromatography, results are shown in Fig. 4. It was observed that xylanase released xylose, glucose, and mannose from the hot water-pretreated biomass. It was noticed that the amount of xylose monomers released

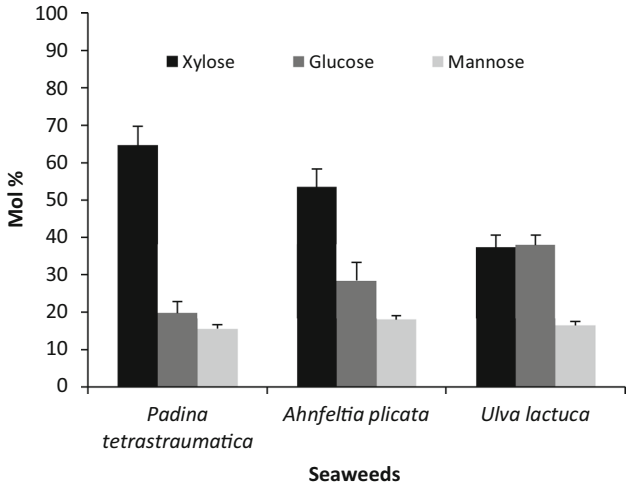

Fig. 4 Variation in the average monosaccharide composition (as Mol%) released due to enzymatic hydrolysis of *Bacillus* sp. strain BT21 xylanase on hot water-pretreated biomass (pretreated for 45 min) of *A. plicata, P. tetrastromatica*, and *U. lactuca* at 30 °C, pH 7, for 6 h. *Error bars* indicate the standard deviation of three replicates

during hydrolysis was more in *Padina* sp. (64%) and *Ahnfeltia* sp. (53%), while *Ulva* sp. released 37% of xylose and glucose in hydrolysate along with small amount of mannose. It is observed that *P. pavonia* contain fucose (35%) and xylose (8%) as major monosaccharides in cell wall followed by glucose (5%), mannose (2%), and galactose (1%) (Fig. 4).

Discussion

In our study, red seaweed had higher carbohydrate content compared to green seaweed, while brown seaweed had least carbohydrates. Carbohydrate content of seaweeds ranging from 10.63 to 28.58% is reported by Parthiban et al. (2013), authors reported maximum carbohydrate content in the green seaweed *Enteromorpha intestinalis* and the brown seaweed *Dictyota dichotoma* was recorded with minimum value. Dhargalkar et al. (1980) from Maharashtra coast and Sobha et al. (2001) from Kovalam coast noted maximum value of carbohydrate content in Rhodophycean members than in Phaeophycean and Chlorophycean members. Kumar et al. (2011) reported slightly higher yields of carbohydrates ranging from 46 to 57% on dry weight basis in different taxa of *Ulva* from tropical seaweeds. Carbohydrate content of Hawaiian seaweeds reported by McDermid and Stuercke (2003) showed 20% carbohydrate content in *Ulva* sp., while other green seaweeds ranged from 4.5 to 39.9% of carbohydrate. They also reported *Ahnfeltiopsis* sp. to have carbohydrate content around 30–35%, while in other red seaweeds carbohydrate content ranged from 10 to 35%. Brown seaweeds reported in this study had least carbohydrates ranging from 7 to 12%. The variations in the carbohydrate contents in seaweeds may be attributed to species difference and to the differences in their habitat and metabolic preferences (Pádua et al. 2004).

The saccharification of seaweed is an essential unit operation for ethanol fermentation and has been widely studied in recent years. Various physical, chemical, and biological pre-treatment have been shown to increase saccharification efficiency (Lu et al. 2010). One of the primary steps to increase the hydrolysis rate of the macroalgal biomass is to apply a pre-treatment to enhance the biodigestibility of the seaweed, thus increasing accessibility of biomass to hydrolytic enzymes. Okuda et al. (2008) showed that hydrothermal pre-treatment can improve the rate of enzymatic hydrolysis of glucan in red and green macroalgae.

Enzymatic pre-treatment of seaweed is mainly influenced by the biochemical composition, physiological structure, life-cycle period, and type of seaweed. It was noticed that the amount of xylose monomers released during hydrolysis was more in *Ahnfeltia* sp. and *Padina* sp, while *Ulva* sp. released similar amount of xylose and glucose in hydrolysate along with small amount of mannose. *Ahnfeltia* sp. and *Padina* sp. also released substantial amount of glucose and mannose. A high percentage of xylose release is quite natural as xylanase was used for hydrolysis which cleaves xylose from xylan backbone. We have not found any traces of fucose in enzymatic hydrolysate of *Padina* sp., may be xylanase was not effective in releasing fucose from cell wall polysaccharides. We have reported *U. lactuca,* green algae, releases 37% of glucose and xylose during enzymatic action. *U. lactuca* has been reported to contain 44% of glucose and 31% of xylose monomer (Jiao et al. 2012). In this study, action of xylanase releasing xylose as well as substantial amount of glucose during enzyme hydrolysis indicates the possibility of xyloglucan-like structure in seaweeds, mainly in *U. lactuca* which shows 37% of glucose release. Xyloglucan has a backbone of β1 → 4-linked glucose residues, most of which are substituted with 1–6-linked xylose side chains. Roelofsen et al. (1953) proposed the idea of xyloglucan as a polysaccharide in cell wall of algae while working on *Halicystis osterhouti* as they found both xylose and glucose in alkaline extract of these algae. Glucose and xylose yields of 93.2 and 79.5% at 15 FPU/g cellulose, respectively, are also reported when corn stover was pretreated with lime and saccharified with cellulase (Kim and Holtzapple 2005). They termed the polysaccharides in corn stover as holocellulose (cellulose and hemicellulose). Xylan is a main constituent of seaweed. Algal biomass has been reported to have high hemicelluloses (16–20%) content compared to

cellulose (7–9%) (Ververis et al. 2007; Yaich et al. 2011), but there are no reports on enzyme hydrolysis of seaweeds by hemicellulases, thus it becomes mandatory to focus on the role of xylanase in the conversion of biomass to sugars and further to bioethanol.

Conclusion

Algae are emerging as one of the most promising long-term, sustainable sources of biomass for fuel, food, feed, and other co-products. Improved saccharification of seaweed will help in producing high concentrations of ethanol. Algal biomass has high hemicellulose content compared to cellulose. This research and previous study suggest that enzymatic hydrolysis followed by hot water pre-treatment of seaweed biomass could be effectively employed for higher yield of reducing sugar if we use cocktail of cellulase and xylanase. Further focus on using enzyme cocktail for potential utilization of seaweed biomass as feedstock for sustainable energy is required.

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Compliance with ethical standards

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

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Recombinant Xylanase from *Bacillus tequilensis* BT21: Biochemical Characterisation and Its Application in the Production of Xylobiose from Agricultural Residues

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Summary

Bacterial strain *Bacillus tequilensis* BT21 isolated from marine sediments was found to produce extracellular xylanase. The *xynBT21* gene encoding xylanase enzyme was cloned and expressed in *Escherichia coli*. The gene encoded a protein consisting of 213 amino acid residues with calculated molecular mass of 23.3 kDa. Purified recombinant xylanase had optimum activity at 60 °C and pH=6. The enzyme was highly stable in alkaline pH, at pH=7 it remained 100 % active for 24 h, while its activity increased at pH=8 and 9 during incubation. *B. tequilensis* BT21 xylanase had alkaline pI of 9.4 and belongs to glycosyl hydrolase family 11. The mode of action of XynBT21 on beechwood xylan and xylooligosaccharides was studied. It hydrolysed xylooligosaccharides and beechwood xylan yielding mainly xylobiose (X_2) with a small amount of xylose (X_1), indicating that XynBT21 was probably an endo-acting xylanase. Enzymatic hydrolysis using wheat bran as a substrate revealed that xylanase reported here has the potential to produce xylobiose from wheat bran. Xylooligosaccharides, especially xylobiose, have strong bifidogenic properties and are increasingly used as a prebiotic. This is the first report that describes this novel xylanase enzyme from marine *B. tequilensis* BT21 used for the release of xylobiose from wheat bran.

Key words: enzyme, xylanase, alkaline pI, characterisation, *Bacillus tequilensis*, xylobiose

Introduction

Xylan, the most abundant hemicellulose, consists of β-1,4-linked xylose residues in the backbone to which O--acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues are attached. Endoxylanases degrade β-1,4--xylan randomly, yielding a chain of linear and branched oligosaccharide fragments. Various microorganisms are known to produce endoxylanases (*1,2*). Regarding the amino acid sequence similarities, xylanases are mostly classified into families 10 and 11 of the glycoside hydrolases. Family GH10 xylanases have high molecular mass (≥30 kDa) and low pI, while GH11 xylanases are normally smaller (≤20 kDa) and have a high pI (*3*).

Xylanases have a range of applications in textile, paper and pulp industries as well as in clarification of fruit juices, aroma production, animal feed, baking industry and production of ethanol. Industrial process conditions are harsh due to extremes of pH, temperature, inhibitors, *etc*. Sufficiently strong enzymes able to withstand such conditions are recommended for these processes. Most of the reported xylanases do not meet such criteria, therefore, enzymes that satisfy these requirements need to be found (*4,5*). The marine environment is highly complex and dynamic with high salinity, high pressure, low or high temperature and unique light conditions, which may explain the significant variations in the enzymes pro-

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duced by marine and terrestrial microorganism. Xylanases from the marine sources can have some remarkable qualities with respect to stability at high temperature and pH, which needs attention. There are a few studies reported on marine xylanases (*6–13*) and it is important to look into more potential xylanases from the marine sources. Recently, the interest in application of endoxylanases in the production of xylooligosaccharides from xylan sources is growing rapidly. Xylobiose stimulates the growth of human intestinal bifidobacteria, which are essential part of sound intestinal microflora. In this study, we report the characterisation of xylanase from a marine bacterium *Bacillus tequilensis* BT21 and propose its efficacy in the nutraceutical industry.

Materials and Methods

Chemicals

Xylooligosaccharides were purchased from Megazyme (Bray, Ireland). Xylan (birchwood and beechwood), 3,5--dinitrosalicylic acid, buffers (citrate, phosphate and glycine, Tris-HCl), solvents (acetonitrile, ethyl acetate, 2-propanol), protein molecular marker, acrylamide, agarose, sucrose, orcinol, serum albumin, bicinchoninic acid and Coomassie Brilliant Blue were purchased from Sigma--Aldrich, St. Louis, MO, USA. Ethanol and thin layer chromatography plates were purchased from Merck, Darmstadt, Germany, while sulphuric and acetic acids, and sodium chloride from SD Fine-Chem Ltd., Mumbai, India.

Bacterial strains, culture conditions and vectors

Bacillus tequilensis was isolated from sediment samples from Chorao island located in Mandovi estuary, Goa, India. The culture was grown in basal salt solution (BSS) medium at room temperature along with 0.5 % of xylan (*13*). *Escherichia coli* strain JM109 was used as the host for DNA manipulation. The plasmid pCR® 2.1-TOPO® TA cloning vector (Invitrogen, Carlsbad, CA, USA) was employed for cloning and DNA sequencing.

PCR amplification of the 16S rRNA for strain identification

DNA was isolated from cells according to the method described by Khandeparker *et al*. (*13*). The 16S rRNA gene fragment was amplified by a PCR 96 well thermal cycler (Veriti 9902; Applied Biosystems, Foster City, CA, USA) with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Gene sequence was determined using a Taq Dye Deoxy terminator cycle sequencing kit (PerkinElmer, Foster City, CA, USA) and were analysed with 373A automated DNA sequencer (model 3130xl; Applied Biosystems) (*13*). The obtained sequence was aligned with corresponding sequences of 16S rRNA from the database using BLAST (*14*).

Molecular cloning, expression and sequencing of xylanase gene

Gene cloning was carried out using standard cloning method (*15*). The primers xynF and xynR were obtained from a previously reported study (*13*). Xylanase gene was amplified using PCR and the amplified gene fragment was then ligated into TOPO TA cloning vector (Thermo Fisher Scientific, San Jose, CA, USA) and transformed into *E. coli* cells. Plasmid was extracted and the xylanase gene was sequenced.

Determination of amino acid composition of xylanase gene

Amino acid sequence of *B. tequilensis* BT21 xylanase gene was determined by translating the gene sequence into the protein sequence using ExPASy server (*16*). Theoretical molecular mass, isoelectric point (pI) and the total number of positive and negative residues were calculated using the ExPASy ProtParam server (*16*).

Enzyme production and fractionation

Recombinant enzyme production was enhanced by incorporating 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the growth medium of transformant cells and incubating at 140 rpm and 37 °C for 24 h. Cells were collected by centrifugation (4000×*g* for 10 min, 4 °C) using centrifuge 5810R (Eppendorf, Hamburg, Germany) and the pellet was rinsed twice in Tris-HCl (10 mM, pH=8). The pellet obtained after centrifugation was resuspended in 25 % sucrose solution. The suspension was shaken with 0.5 M EDTA for 10 min at room temperature. This was again centrifuged and the cell pellet was collected. The supernatant obtained in the above three steps was extracellular enzyme fraction (F1). Ice-cold water was added to the pellet and shaken vigorously for 10 min. After centrifugation at 5976×*g* for 10 mins, the supernatant was collected, forming periplasmic enzyme fraction (F2). The remaining cell pellet was suspended in 10 mM Tris-HCl buffer (pH=7) and lysed by sonication (sonicator model GT-1730QTS; GT Sonic, Guangdong, PR China). Intracellular enzyme fraction (F3) was collected as supernatant by removing the cell pellet by centrifugation (*17*). For native enzyme cell suspension of *B. tequilensis* BT21 grown on xylan, the medium was centrifuged and the supernatant containing extracellular xylanase was collected.

Native and recombinant xylanase purification

Native and recombinant xylanases were precipitated using ammonium sulphate (80 %). The precipitated enzyme was dissolved in phosphate buffer and dialysed for 24 h (50 mM, pH=6). The crude enzyme was further purified using anion exchange resins followed by cation exchange resins DEAE and CM Sepharose fast flow (Sigma--Aldrich) columns respectively (*11*). The native enzyme was used only to study the kinetic properties, otherwise recombinant enzyme was used.

Enzyme assay and protein estimation

The activity of xylanase enzyme was determined by using 3,5-dinitrosalicylic acid (DNS method) (*18*). One unit of xylanase activity was calculated as μmol of xylose released per min. Proteins were analysed by the method of bicinchoninic acid with bovine serum albumin as a standard (*19*).

Effect of temperature and pH on xylanase activity

Optimal temperature for xylanase activity was studied by analysing it at different temperatures ranging from 15–75 °C at pH=6, while the optimal pH was analysed by measuring its activity at 60 °C using different pH buffers (0.05 M): citrate buffer for pH=4–6, phosphate buffer for pH=6–8, and glycine-NaOH buffer for pH=8–11. Thermal stability of the enzyme was verified by incubating the enzyme at 40, 50 and 60 °C for 6 h (pH=6) in an incubator (model LSI-125R; Labtop Instrument, Thane, Maharashtra, India). The pH stability was studied using 100-fold diluted xylanase enzyme and respective buffers (pH=7–9) and incubating it for 24 h at 60 °C. Residual activity was analysed at regular intervals during incubation.

Isoelectric focusing

Isoelectric focusing (IEF) was performed using Ready-Prep® 2-D starter kit (Bio-Rad, Hercules, CA, USA). IPG strips (pH range 3–10, 11 cm; Electrophoresis GmbH SERVA, Heidelberg, Germany,) were rehydrated for 12–16 h in the enzyme sample (10 μg of protein) and mixed with rehydration buffer provided with the starter kit. PROTEAN® IEF chamber (Bio-Rad) was programmed at a constant temperature of 10 °C and 50 Vh/strip for 20 min at 250 V, with 2-hour linear increase to 4000 V and finally obtaining the value of 10 000 Vh. Strips were equilibrated on the rocker in equilibration buffers I and II (provided with the kit) for 10 min each, and then sealed on the top of 12 % SDS-PAGE gels (separating gel) using 0.5 % agarose. SDS-PAGE was run on 12 % acrylamide gel at 120 V and 4 °C for 110 min. Protein molecular mass standard (medium range) was used for electrophoresis in the 2nd dimension.

Molecular mass estimation and zymogram analysis

The xylanase molecular mass was assessed by SDS-PAGE (12 %) electrophoresis (*20*) using medium range molecular mass markers (14.3–97.4 kDa). Protein bands were stained with Coomassie Brilliant Blue stain. Zymogram was obtained using SDS–PAGE (12 %) electrophoresis as described by Nakamura *et al.* (*21*). The gel with separated protein bands was thoroughly washed (four times) using 50 mM phosphate buffer (pH=7.0). Initial two washes contained 25 % isopropanol (to get rid of the SDS and renature the protein). The gel was further incubated at an optimum temperature of the enzyme for 60 min. The gel was stained with 0.1 % (by mass per volume) Congo Red dye for 30 min and then washed with 1.0 % (by mass per volume) NaCl until the remaining dye was washed off from the active band. Finally, the gel was flooded in 0.5 % acetic acid, thus changing the background of the gel to dark blue, and the activity bands were observed as clear colourless areas.

Kinetic determinations

Purified native and recombinant xylanases were incubated with birchwood and beechwood as substrates in 50 mM phosphate buffer at pH=7. The rate of xylan hydrolysis was determined at pH=9 and 60 °C. Various masses of xylan ranging from 0.5 to 8 mg were used in this study. The kinetic constants K_m and v_{max} were determined using the Lineweaver and Burk method (*22*).

Hydrolysis studies

The partially purified periplasmic xylanase fraction was dissolved in phosphate buffer (pH=7) and used for hydrolysis study. Substrates and the enzyme were incubated at 60 °C. Samples were collected at intervals of 0, 1, 3, 6, 12 h. Xylooligosaccharides were also incubated with the enzyme and the samples were collected at different time intervals ranging from 1 to 3 h. Enzyme-substrate reaction was terminated by putting the reaction mixture into the boiling water for 5 min (*23*). The unused polysaccharide was precipitated using isopropanol and centrifuged. The supernatant was collected. Hydrolysis products were identified by thin layer chromatography (TLC) with the mixture of acetonitrile/ethyl acetate/2-propanol/water as a solvent system, and then spotted by spraying the TLC plates with orcinol spray, a mixture of ethanol, sulphuric acid and orcinol, and heating them for 5 min at 150 °C. Similarly, hydrolytic products obtained by enzymatic saccharification of wheat bran were analysed by TLC. Here xylanase enzyme was incubated with wheat bran (1 %) used as substrate at 60 °C. Samples were taken in triplicates in 24-hour intervals.

Results

Identification of B. tequilensis BT21 by 16S rRNA sequence analysis

Biochemical analysis of *B. tequilensis* BT21 isolate revealed that it belonged to the Gram-positive group, its cells are short motile rods, oxidase positive and catalase positive. As described in Bergey's manual of systematic bacteriology (*24*), these characteristics show close similarity to *Bacillus* sp. The amplified 16S rRNA sequence matched to the 16S rDNA sequence in the GenBank (*25*), and the results showed 99 % identity with *Bacillus tequilensis* (GenBank accession no. KF054870). Thus, the strain BT21 identified as a strain of *B. tequilensis* (GenBank accession no. KF797798).

Nucleotide sequence analysis of the xylanase gene

The PCR product of total chromosomal DNA acquired from *B. tequilensis* strain BT21, using primers xynF and xynR, was ligated with TOPO® TA cloning vector (Invitrogen). The recombinant plasmid DNA was introduced into *Escherichia coli* JM109. A complete nucleotide sequence was acquired (GenBank accession no. KF797799), with a 639-bp open reading frame encoding a protein of 23 324.6 Da having 213 amino acid residues with pI=9.44. The gene sequence is 99 % similar to that of xylanase gene sequence of *B. subtilis* R5 (GenBank accession no. AB 457186.1). The catalytic domain of *xynBT21* (from *B. tequilensis*) showed good homology with xylanase that is classified into family 11 glycosyl hydrolases according to hydrophobic cluster analysis (*26*).

Amino acid composition of recombinant xylanase

Recombinant xylanase from *B. tequilensis* showed a relatively high percentage of glycine (12.2 %), threonine (12.2 %) followed by serine (10.8 %) and asparagine (8.9 %), while there were no traces of cystine and pyrrolysine (Table 1). Highly conserved Glu104 and Glu196, which are crucial for the catalytic activity of family 11 glycosyl

Table 1. Amino acid composition of xylanase from *Bacillus tequilensis* BT21

Amino acid	*w*/%
Alanine	6.6
Arginine	3.3
Asparagine	8.9
Aspartic acid	3.3
Cystine	0.0
Glutamine	2.3
Glutamic acid	0.9
Glycine	12.2
Histidine	0.9
Isolucine	3.3
Leusine	4.2
Lysine	3.8
Methionine	1.9
Phenylalanine	3.3
Proline	2.8
Serine	10.2
Threonine	12.2
Trptophan	5.2
Tryosine	7.0
Valine	7.0
Pyrrolysine	0.0

Total number of negatively charged residues (aspargine+glutamic acid) is 9
Total number of positively charged residues (arginine+lysine) is 16

hydrolases, were found in two conserved regions (Fig. 1). The *B. tequilensis* BT21 xylanase contained double the number of acidic amino acids compared to basic amino acid, also the total number of negatively charged residues in the enzyme was lower (asparagine+glutamic acid=9), than of positively charged residues (arginine+lysine= 16). The enzyme had 41 % polar amino acids and 59 % nonpolar (hydrophilic) amino acids.

Characterisation of xylanase from B. tequilensis BT21

Xylanase had optimal pH=6.0 and optimal temperature of 60 °C (Fig. 2). At pH=6 and 7, enzyme retained 100 % of activity when incubated for 24 h, while at pH=8 and 9 it showed an increase in the activity during incubation (Fig. 3). At 40 °C, the enzyme activity increased with the incubation time, while at 50 °C it remained stable for almost 1 h. At 60 °C, the enzyme showed 100 % activity for up to 20 min and remained active for 2 h retaining 75 % activity (Fig. 4).

Kinetic parameters

The K_m and v_{max} values of native and recombinant xylanase were obtained from Lineweaver-Burke plot. The K_m of native and recombinant xylanases on birchwood xylan was 11.1 and 3.3 g/L, while v_{max} was 2222 and 3125 μmol/(mg·min) respectively. The K_m of native and recombinant xylanases on beechwood xylan was 16.6 and 5.0 g/L while v_{max} was 2500 and 4347 μmol/(mg·min) respectively.

Molecular mass estimation, zymogram analysis and isoelectric focusing

Molecular mass of recombinant xylanase enzyme deduced from amino acid sequence was 23 324.6 Da, which is in full agreement with the results of the zymographic analysis (*7*). The molecular mass of recombinant xylanase obtained by zymogram analysis is approx. 23 kDa (Fig. 5). Isoelectric focusing of the enzyme run under denaturing and non-denaturing conditions gave a band at pI value above 9. SDS-PAGE zymograms showed a single clearance zone coinciding with a molecular mass of 22 to 23 kDa (Fig. 5).

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B. tequilensis BT21      MLKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN   60
B. subtilis 168          MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
B. subtilis R5           MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
B. circulans             MFKFKKNFLVGLSAALMRIILFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN

Query 61                 TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG   120
B. subtilis 168          TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
B. subtilis R5           TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
B. circulans             TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWPRSPLIEYYVVDSWGTYRPTG
                                                          ♦ Glu 104
Query 121                TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA  180
B. subtilis 168          TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA
B. subtilis R5           TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA
B. circulans             TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNA

Query 181                WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW  213
B. subtilis 168          WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
B. subtilis R5           WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
B. circulans             WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
                                                  Glu 197
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Fig. 1. Alignment of the amino acid sequence of xylanase gene from *Bacillus tequilensis* BT21 (AHN14743.1) with xylanases from other *Bacillus* sp. strains: *B. subtilis* 168 (AOA11206.1), *B. subtilis* R5 (AB457186.1) and *B. circulans* (AAM08360.1). The Glu residues corresponding to our xylanase Glu-104 and Glu-196, essential to the catalytic activity, are marked with diamond and triangle, respectively

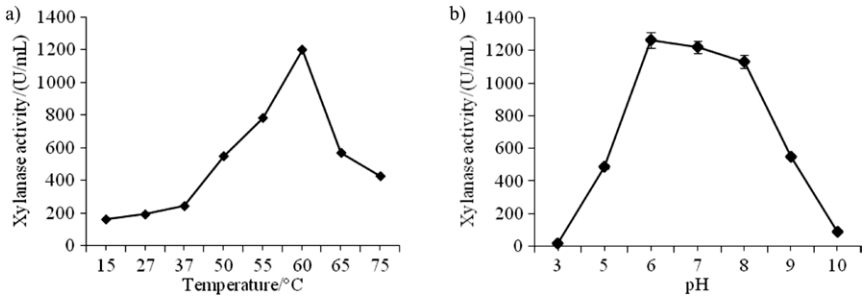

Fig. 2. The effect of: a) temperature (at pH=6), and b) pH (at 60 °C) on the activity of xylanase from *Bacillus tequilensis* BT21

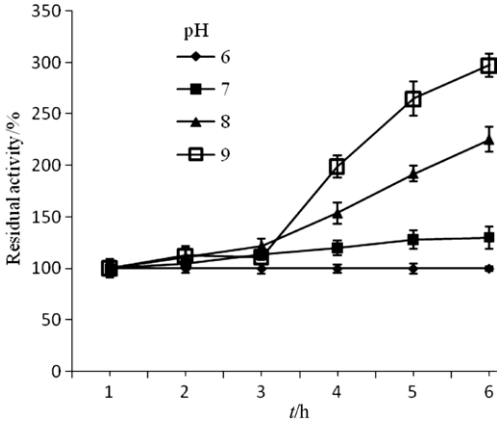

Fig. 3. Effect of pH on xylanase stability. The enzyme was diluted with phosphate buffers of various pH values and incubated at room temperature for *t*=24 h. Residual activity was essayed at pH=6 and 60 °C

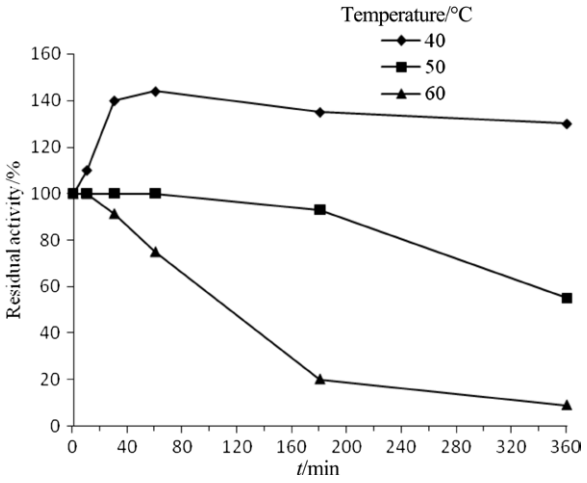

Fig. 4. Effect of temperature on xylanase stability. The pure enzyme was incubated in phosphate buffer (pH=6) at 40, 50 and 60 °C for different intervals and residual activity was determined

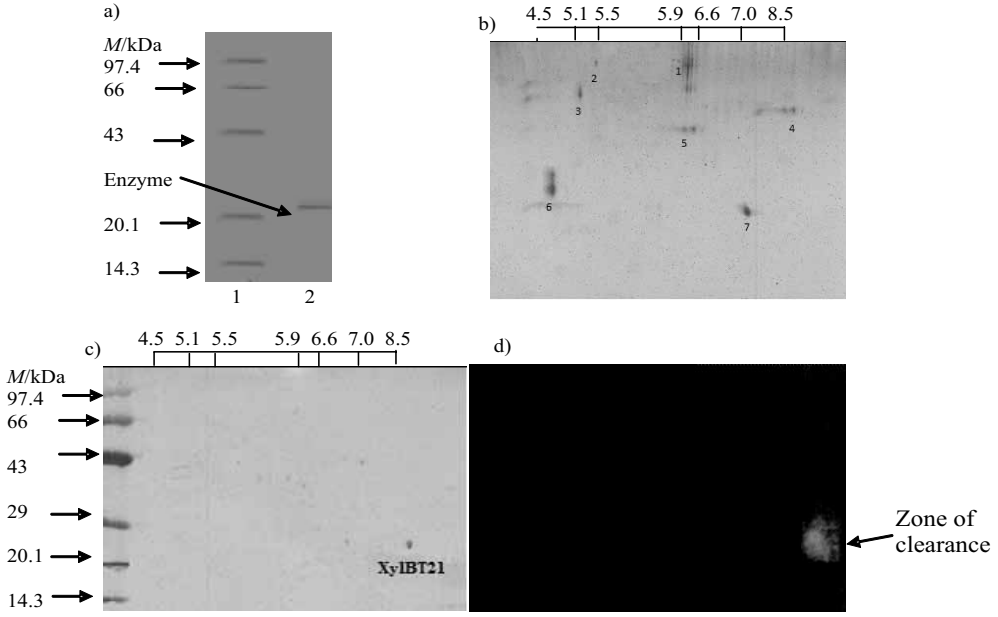

Fig. 5. SDS-PAGE analysis of recombinant xylanases XynBT21: a) lane 1: molecular markers in kDa, lane 2: pure xylanase enzyme. Isoelectric focusing of: b) pI standards, c) enzyme sample, and d) zymogram analysis of recombinant xylanases from *Bacillus tequilensis* BT12 grown on 1 % birchwood xylan and separated by two-dimensional gel electrophoresis. Proteins (10 μg) were first separated in 6 % native PAGE, the lane was stripped and then run in 8 % SDS-PAGE. Xylanase activity was examined by Congo Red. The position of XynBT21 in native PAGE is indicated

Enzymatic hydrolysis of xylan, xylooligosaccharides and wheat bran

The mechanism by which recombinant xylanase acts on xylan was followed by allowing it to react with beechwood xylan and different xylooligosaccharides. Beechwood hydrolysis released the xylooligosaccharides of higher molecular mass during initial stages (t_1=30 min, t_2=1 h). At the end of 3 h (t_3), 6 h (t_4) and finally at 12 h (t_5), xylooligosaccharides were completely broken to xylobiose and xylose (Fig. 6).

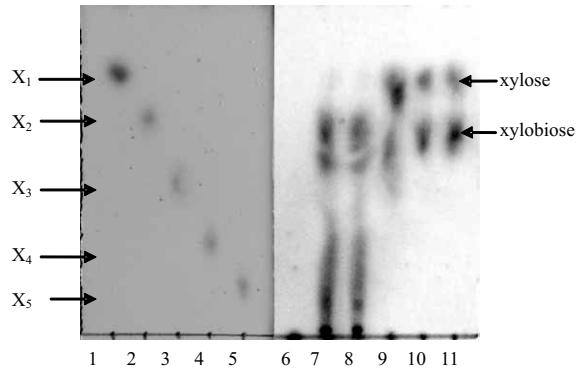

Fig. 6. Time course of the hydrolysis of beechwood xylan by the recombinant xylanase. Beechwood xylan was incubated at 60 °C with 20 U/mL of XynBT21 and analysed by thin-layer chromatography. Lanes 1 to 5 contain standards: xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), respectively. Lanes 6 to 11: samples were taken at time 0, 0.5, 1, 1, 3, 12 h. The reaction was carried out at 60 °C

To understand the hydrolysis pattern more clearly, xylooligosaccharides (X_2–X_5) were treated with XynBT21 at time interims (t_1=1 h, t_2=3 h). Observations after 1 h of incubation revealed that there was no breakdown of xylobiose (X_2), while xylotriose (X_3) was partially degraded to xylose (X_1) and xylobiose (X_2), xylotetraose (X_4) released xylobiose (X_2), while xylopentaose (X_5) was partially hydrolysed to give xylose (X_1), xylobiose (X_2) and xylotriose (X_3). After 3 h of incubation, xylotriose, xylotetraose and xylopentaose produced xylose (X_1) and xylobiose (X_2), while xylobiose was not cleaved at all (Fig. 7).

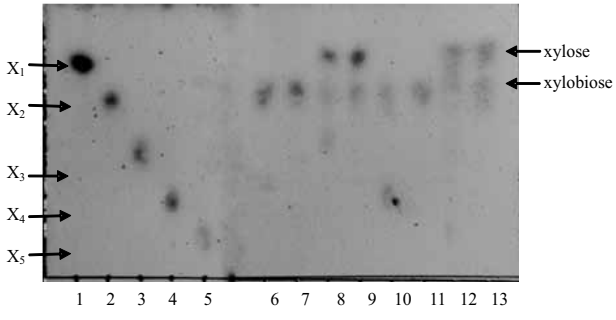

Fig. 7. Time course of xylooligosaccharide hydrolysis by the recombinant xylanase. Xylooligosaccharides were incubated at 60 °C with 20 U/mL of XynBT21 at different time intervals (t_1=1 h, t_2=3 h) and analysed by thin-layer chromatography. Lanes 1 to 5 contain standards: xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentaose (X_5), respectively. Hydrolysis of xylobiose at t_1 (lane 6) and t_2 (lane 7), xylotriose at t_1 (lane 8) and t_2 (lane 9), xylotetrose at t_1 (lane 10) and t_2 (lane 11), xylopentose at t_1 (lane 12) and t_2 (lane 13) was determined

Hydrolysis products of wheat bran were also analysed by TLC. By the end of 24 h, wheat bran released xylobiose and xylose when treated with partially purified native xylanase enzyme (Fig. 8).

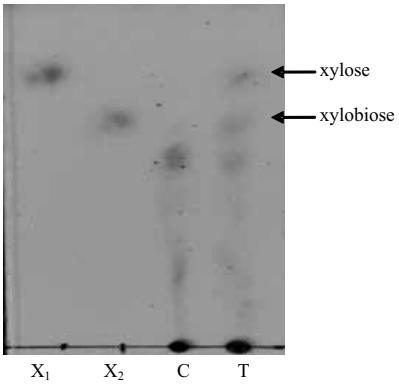

Fig. 8. Time course of wheat bran hydrolysis by the recombinant xylanase. Wheat bran was incubated at 60 °C with 20 U/mL of XynBT21 for 24 h and analysed by thin-layer chromatography. Xylose (X_1) and xylobiose (X_2) are standards, while C is control (sample without enzyme fraction) and T is the test sample with active enzyme

Discussion

A potential xylanolytic *B. tequilensis* strain BT21 isolated from mangrove area is reported. Genus *Bacillus* is known to produce industrially valuable xylanases, a number of which are reported (*13,16,19,27–29*). The catalytic domain of *xynBT21* (from *B. tequilensis*) showed high homology with xylanases belonging to glycosyl hydrolase family 11 according to the hydrophobic cluster analysis (*27*). There is a widespread occurrence of xylanase family 11 in *Bacillus* sp. (*13,27–29*). The amino acid sequence of the *B. tequilensis* BT21 xylanase was compared with other reported xylanases in the NCBI database with the help of BLAST search program (*14*). It showed 99 % similarity with xylanase from *Bacillus subtilis* R5 (AB457-186.1), which belongs to GH family 11, but it was 98 % similar to *Bacillus subtilis* 168 (AOA11206.1). It also had high amino acid sequence identity (98 %) with xylanase from *Bacillus circulans* (X07723.1). Sequence comparison of *B. tequilensis* with *B. subtilis* showed that only one amino acid L (lysine) at position 2 replaced F (phenylalanine), while when compared with amino acid sequence of *B. circulans*, L, S (serine), S and T (threonine) replaced F, R (arginine), I (isoleucine) and S, respectively (Fig. 2). With the difference in one single amino acid in xylanase gene of the *B. tequilensis* strain BT21 and *Bacillus subtilis*, enzymes showed diverse properties. Single amino acid mutations influence the structure of the protein complex because of the changes in binding affinity of the amino acid. More than half of monogenic diseases are caused by single mutations, in which amino acid substitution causes changes in protein stability (*30*). The optimum temperature for *B. tequilensis* BT21 xylanase activity was 60 °C while for *B. subtilis* R5 xylanase it was 40 °C. Although that of *B. subti-*

lis 168 xylanase was 65 °C, the stability of XynBT21 was better than of *B. subtilis* 168 xylanase. *B. tequilensis* BT21 xylanase had pH optimum of 6.0 and temperature optimum of 60 °C. Xylanase from *Bacillus subtilis* cho40 reported previously by Khandeparker *et al*. (*13*) also had the same pH and temperature optima, but *B. subtilis* cho40 xylanase showed drastic loss of activity after it reached optimum pH of 6.0, while *B. tequilensis* BT21 xylanase remained active over a range of pH (from pH=6 to 8) and its activity increased with the incubation time. *Arthrobacter* sp. reported by Khandeparker and Bhosle (*12*) showed similar properties when studying the pH effect on xylanase enzyme. The residual activity of xylanase from *Bacillus pumilus* SV-85S also increased after 1 h of incubation at alkaline pH (*31*). Bai *et al.* (*32*) reported that high molecular mass xylanase (42.5 kDa) from *Alicyclobacillu*s sp. retained 80 % of enzyme activity after incubation at pH=2.6 to 12.0 for 1 h at 37 °C. Xylanase isolated from *Streptomyces actuosus* was reported to retain 80 % of its activity in the pH range of 5–8 when incubated for 30 min (*33*). The protein stability is controlled by the pH mainly by changing the net charge of the protein. Denaturation of many proteins takes place at extreme pH due to destabilising repulsive interactions that are present between similar charges in the native protein. The behaviour of a given protein at low or high pH depends mainly on stabilising and destabilising forces, which are sensitive to the environment (*34*). If we compare the pH stability of earlier reported xylanases, pH stability of *B. tequilensis* BT21 is exceptionally superior.

Kinetic studies performed on *B. tequilensis* BT21 revealed that K_m of the recombinant enzyme was much lower than of the native enzyme, which clearly shows that the recombinant enzyme had better affinity than the native enzyme. K_m values of recombinant xylanase enzyme reported in *B. tequilensis* BT21 were 3.3 and 5.0 g/L on birchwood xylan and beechwood xylan, respectively, which indicates that birchwood xylan was more efficiently utilised than beechwood xylan. K_m of the recombinant xylanase II by *Trichoderma reesei* was 13.8 g/L using birchwood xylan as the substrate, while *Bacillus alcalophilus* xylanase enzyme showed K_m of 4.9 and 4.5 g/L using beechwood xylan and birchwood xylan, respectively.

IEF analysis and zymograms showed a sole and prominent activity band in the alkaline pH range indicating that BT21 xylanase had a pI of 9.0 or higher with a molecular mass of around 23 kDa. *Bacillus* sp. (NCL 87-6-10) produces two xylanases, A, with the molecular mass of 44 kDa and pI=5.3, and C, with 25 kDa and pI=8.9 (*35*). Similarly, there are reports that *B. circulans* and *B. polymyxa* produce high- and low-molecular mass xylanases with acidic and basic pI respectively (*20*,*35*). Proteins with acidic isoelectric points degraded faster than those with neutral or basic isoelectric points (*36*), which might be because of chemical properties of the acidic or basic polypeptides. As a result, xylanase from *B. tequilensis* BT21, with basic pI and high pH stability, has many advantages, although there is an exception to this behaviour as when an acidic protein with acidic pI degrades extremely slowly (*37*).

Xylanase from *B. tequilensis* BT21 predominantly releases xylobiose from the xylan backbone suggesting the endo-acting nature of XynBT21. In the first stage, the mixture of oligomers was also detected, which might be because of random hydrolysis of xylan. Hydrolysis products similar to XynBT21 have been reported such as XynB from *Thermotoga maritima*, XynC from *Clostridium stercorarium*, and an endoxylanase from *Bacillus* sp. (*38–40*). Endoxylanase from *Streptomyces* sp. S27 has also been reported to release xylobiose (>75 %) as a hydrolysis product of xylan by XynBS27 (*41*), while xylanase from *Streptomyces thermocyaneoviolaceus* is reported to release diverse xylooligosaccharides (X_1 to X_5) from birchwood xylan (*42*).

XynBT21 with an ability to hydrolyse polymeric substrates such as beechwood xylan into dimer and monomer may find helpful application in saccharifying xylan-rich materials. A lignocellulosic material wheat bran, which is a by-product of conventional wheat milling, is available in large quantities (*43*) and a good source of raw material for xylooligosaccharide production. Enzymatic hydrolysis by xylanase from *B. tequilensis* BT21 on wheat bran released xylobiose and xylose. Koga *et al.* (*44*) reported uses of xylobiose in cosmetics, drugs or quasi-drugs, hair-care products and detergents. There are reports of xylobiose used as prebiotics (*45*). Xylooligosaccharides obtained on wheat bran improve blood lipid metabolism and antioxidant status in rats that feed on the high-fat diet, suggesting that wheat bran xylooligosaccharides might be useful in protecting humans against high-fat diet-induced oxidative stress (*46*). Accordingly, streamlining the conditions such as enzyme dose and pre-treatment strategies, large-scale production of xylobiose from wheat bran can be achieved with *B. tequilensis* BT21 xylanase.

Conclusion

The recombinant xylanase from *Bacillus tequilensis* BT21 is a low-molecular-mass enzyme with alkaline pI, that belongs to the group of glycanase family 11. XynBT21 has excellent pH stability and also the ability to produce xylobiose from agricultural residues. With the growing interest in the application of xylobiose in nutraceutical industries, we look forward to using this novel enzyme for industrial purposes.

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Nitrate Reductase Gene Expression in *Idiomarina* Strain cos21 Obtained from Oxygen Minimum Zone of Arabian Sea

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Abstract
This study analyses the induction and repression of nitrate reduction activity in a batch culture of *Idiomarina* strain cos21. On a change from aerobic to anaerobic respiration, the culture entered a stationary phase. The onset of this phase showed 3.75 fold increase in mRNA levels for the nitrate reductase enzyme. mRNA accumulated very rapidly during a short period, after which its overall concentration declined to reach a lower value. The level of nitrite reductase protein reached a maximum value at 36 h of growth when the oxygen concentration dropped below 10 µM. The data set provided here confer new insights into the understanding of the physiological response of *Idiomarina* strain cos21 to change in oxygen concentration allowing the bacterium to survive and adapt to a new environment by dissimilatory reduction of nitrate to nitrite, which serves to provide energy as the bacteria adapt to anaerobiosis. Main strategy used here is to induce, measure, and track the expression of microbial genes, while they grow in culture conditions to better mimic interaction in a natural environment. This study will help us with a better understanding of the nitrate reduction process in the oxygen minimum zone.

Introduction

Arabian Sea covers only ~2% of the global oceanic area but accounts for ca. 20% of oceanic denitrification and, thus, a very important factor playing major role in the marine nitrogen budget [9]. It is observed that water column denitrifying zones are major sources to the atmospheric nitrous oxide, if denitrification is partial and nitrous oxide is not converted to N_2. Nitrous oxide is a strong greenhouse gas which is also involved in ozone layer depletion [12, 24, 46].

Denitrification in the ocean is not entirely understood, but it is reported that O_2 levels less than or equal to 20 µM is the maximal O_2 level which prompts utilization of an alternative electron acceptor [23, 44]. With low oxygen concentrations, nitrate and nitrite serve as the key hydrogen acceptors which produce molecular nitrogen [8, 14, 48]. During suboxic conditions presence of nitrite maxima signifies that denitrification is the principle respiratory pathway occurring in that area, which utilizes fixed nitrogen and reduces it to free nitrogen gas [14, 31].

Nitrate reduction is catalyzed by nitrate reductase (NR). NR has complex network governing regulation and expression [20]. In order to study the network of denitrification, it is essential to study this process by combining physiological, molecular genetics, and biochemical tools [1]. Some microorganisms have the ability to use both oxygen and nitrogenous oxides, such as *P. denitrificans*, which can survive in both aerobic or anaerobic conditions. Signals and mechanisms controlling the switches between aerobic and anaerobic conditions are not well known, although some regulatory genes have been found to be involved in this process and are characterized [15, 35, 40, 45].

In this study, we illustrate the use of gene probes for the nitrate reductase of strain cos21 to characterize the dynamics of nitrate reduction in a continuous culture depicting the low oxygen environment in the oxygen minimum zone, to understand the nitrate reduction process in this region.

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Materials and Methods

Bacterial Strain and Culture Conditions

Idiomarina strain cos21 was isolated from water samples collected from the oxygen minimum zone (OMZ) of Arabian Sea during an interdisciplinary cruise of FORV Sindhu Sankalp (SSK046). Samples were collected along a time series transect off Arabian Sea (20°59.87′N; 67°59.92′E) at 500 m depth using rosette niskin sampler. Isolation of bacteria from water sample was done immediately after onboard sampling by plating on Zobell Marine Agar (ZMA) plate. Strain cos21 was grown at room temperature at neutral pH for 48 h in nitrate broth containing peptone, 5.0 g; meat extract, 3.0 g; potassium nitrate, 1.0 g; sodium chloride, 30.0 g, and distilled water, 1000 mL.

Total DNA Extraction, PCR Amplification of 16S rRNA, and Phylogenetic Tree Construction

Bacterial cells were grown overnight in Zobell marine broth and DNA extraction was carried out using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Extraction of DNA was confirmed by gel electrophoresis. The 16S rRNA gene fragments were amplified by PCR using the universal 16S primers [47]. PCR and sequencing details were followed as [21]. The 16S rDNA sequence of *Idiomarina* strain cos21 was aligned along with the sequences of type strains of closest taxa from GenBank database using clustal W [43]. Phylogenetic tree was constructed by using the neighbor-joining algorithm using software, MEGA6 [42]. To confirm the reliability of the phylogenetic tree, bootstrap tests (1000 replicates) were done.

Cloning and Sequencing of Denitrification Gene

The gene responsible for nitrate reduction process (*narG*) was also amplified by PCR using gene specific primers (Table 1). Real-time PCR primers (Table 1) were used for the amplification and sequencing of the *narG* gene [10]. Amplified *narG* gene was purified using the PCR purification kit (QIAGEN) and cloned using the TOPO TA cloning kit (Invitrogen). Luria–Bertani agar plates containing 50 µg/mL of ampicillin, 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 mg/mL of 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (XGal) were used to select transformants. Nucleotide sequences of clones were determined using a Taq Dye Deoxy terminator cycle sequence kit (Perkin–Elmer). Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems). Analysis of the *narG* gene sequence was done through Databases (GenBank).

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from bacterial cells using Pure Link RNA Mini kit (Ambion by Life technologies). Cells were grown for 48 h and the pellet was collected by centrifugation. Cells were suspended in lysis buffer containing 1% of 2-mercaptoethanol, followed by homogenization. The clear supernatant was obtained by centrifuging at 12,000×*g* for 5 min. The homogenate was mixed with 70% ethanol to form a clear visible precipitate and then it was loaded onto the binding columns provided in RNA mini kit. RNA was eluted by the addition of RNase-free water and stored at − 20 °C. Extracted RNA was then quantified by using NanoDrop. RNA was converted to cDNA by using ReadyScript cDNA Synthesis Mix (Sigma-Aldrich).

Table 1 Primer and probe sets used for PCR

Target gene	Amplicon size (bp)	Primer or probe		PCR cycle annealing temp (°C)	References
		Name	Sequence (5′→3′)		
narG	650	narG1F	TAYGTSGGGCAGGARAAACTG	63	[28]
		narG1R	CGTAGAAGAAGCTGGTGCTGT		
nap	414	nap A V67F	TAY TTY YTN HSN AAR ATH ATG TAY GG	55	[39]
		nap A V67R	DAT NGG RTG CAT YTC NGC CAT RTT		
For Q-(RT)-PCR					
narG	174	Nar G-1F	TCGCCSATYCCGGCSATGTC	60	[10]
		Nar G-1R	GAGTTGTACCAGTCRGCSGAYTCG		
GAPDH	63	GAPDH_ 1F	CGACAGTCAGCCGCATCTT	60	[17]
		GAPDH_1R	CCCCATGGTGTCTGAGCG		

Culture Conditions for Gene Expression Studies

Strain cos21 was grown in tightly screw capped 100 mL glass bottles (30 numbers) containing nitrate broth (NB). The temperature was maintained at 30 °C ± 2 °C. The culture shifted from aerobic to anaerobic growth conditions during its growth due to the uptake of oxygen by bacteria which were not replenished due to the tight closure of experimental glass bottles. Each time three bottles (triplicates) were collected in the interval of 12 h to obtain sample during each growth phase. The culture was sampled for analysis of optical density, dissolved oxygen, nitrate, nitrite, nitrate reductase enzyme, and mRNAs. Gene Expression of the nitrate reductase gene was studied by RT-PCR at each growth stage.

Analytical Determinations and Enzyme Assays

Dissolved oxygen from the screw capped glass bottles was measured using a galvanic dissolved oxygen probe (PCD650, Oakton, USA). Cell growth was measured by taking the absorbance of the cultures at 600 nm. The culture was centrifuged. Centrifugation was done at 5000 rpm for 10 min. Supernatant and pellet were separated. Nitrite and nitrate in the supernatant were determined by the method described in [41]. The pellet was further homogenized with phosphate buffer (50 Mm, pH 7) and then centrifuged at 0 °C at 2000 rpm for 15 min. The supernatant was used for the nitrate reductase activity assay. The supernatant was incubated with ß-NADH for 2 min at 30 °C and the reaction was stopped by adding sulphanilamide and NED reagent (*N*-(1-naphthyl) ethylenediamine dihydrochloride). Activity was recorded spectrophotometrically at 540 nm [33]. Nitrate reduction rate was calculated using following equation [modified from 25].

$$\text{Nitrate reduction rate} = A - B/V \times T$$

A is the initial concentration of nitrate in NB, B is the final concentration of nitrate in NB, V is the volume of NB, T is the hours of incubation.

The data reported are representative of three independent experiments, with standard deviations < 10%.

Real-Time PCR Quantification of Nitrate Reductase Gene Expression

Gene expression analysis for nitrate reductase transcript was carried out using Real Time PCR (qPCR). Primers used in the qPCR study are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was an internal control during all the qPCR run. There was barely any dissimilarity in the mean CT values of GAPDH gene expression during different growth stages of bacteria from 0 h (CT = 28.3) to 72 h (CT = 28.2). All the PCRs had an efficiency between 95 and 100%. The amplifications were carried out in a 96 well plate in a total volume of 20 µL using 1 µL of cDNA, 200 nM of each gene-specific primer pair with 5X qARTA. Cycling parameters were 95 °C for 15 min and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. $^{\Delta\Delta}$CT method was used to determine the relative fold of induction [27].

Quantification of *narG* mRNA by Using an External *narG* Standard Curve in One-Step RT-PCR

Serial dilutions from 1000 to 0.1 ng of total RNA was prepared using RNasefree H_2O. The amplification was carried by using gene-specific primer pair with 5X qARTA Green qPCR Mix (QARTA Bio) in an ABI 7500 Real-Time PCR system (as described above). Standard curves were plotted using the threshold cycle for each standard. By using the slope of the standard curve, the efficiency (E) of amplification was estimated. Second derivative maximum method was used to quantity *narG* mRNA in different stages of growth by using external *narG* standard curve [32]. RT PCR products were checked for the specificity by melting-curve analyses.

Results

Identification of *Idiomarina* Strain cos21 by 16S rRNA Sequence

Bacterial strain cos21 was isolated from water samples collected from oxygen minimum zone (OMZ) of Arabian Sea. The isolate is gram-negative, straight rod, motile, non-spore forming, positive for oxidase, catalase, DNase, gelatinase, and nitrate reductase activities, while negative for amylase activity. Growth is seen in sea salt concentrations of 1–20% (w/v). These characteristics of the culture are similar to those reported for *Idiomarina seosinensis* [7]. The amplified 16S rRNA gene sequence was compared to the 16S rRNA sequence in gene bank and the results indicated an identity of 99% with *Idiomarina* sp. (GenBank Accession No. KC762311.1) and 99% similar to *I. seosinensis* (Go0027002) (GenBank Accession No. AY635468) [7]. Therefore, the strain cos21 was considered as one strain of *Idiomarina* sp. (GenBank Accession No. KP663369). The phylogenetic tree created on the 16S rRNA gene of strain cos21 (Fig. 1) shows the location of strain cos21 within the genus *Idiomarina*.

Amplification and Sequencing of *narG* Gene from *Idiomarina* strain cos21

Amplification product of *narG* gene was obtained by PCR using total chromosomal DNA as a PCR template, and primers listed in Table 1. While *Nap* gene could not amplify.

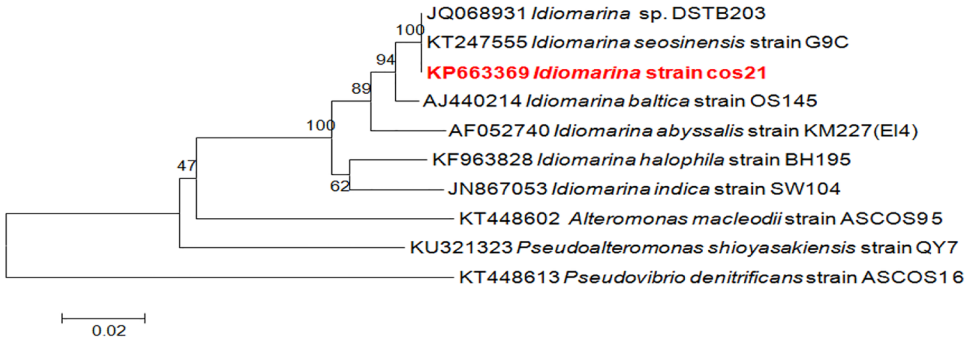

Fig. 1 Phylogenetic tree of *Idiomarina* strain *cos21* based on 16S rRNA gene sequence analysis, constructed through neighbor-joining method with bootstrap values as percentage at the nodes

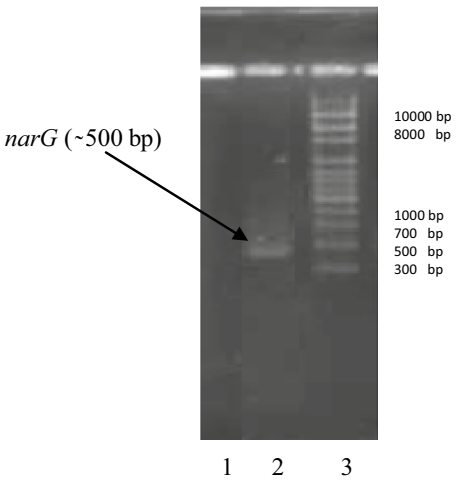

Fig. 2 Agarose gel electrophoresis of PCR product by amplification of genes in *Idiomarina* strain *cos21* isolated from OMZ region using gene specific primers. Lane 1: *nap* (no amplification), lane 2: *narG*, and lane 3: molecular markers (Genaxy, India)

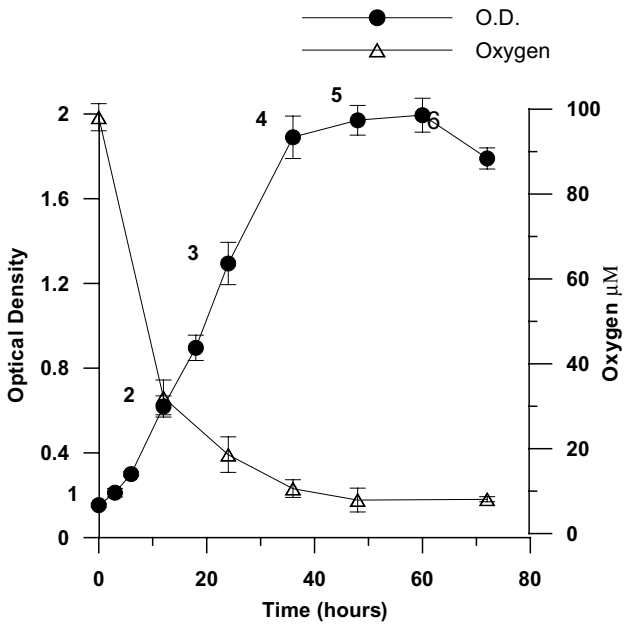

Fig. 3 The growth of *Idiomarina* strain cos21 (KP663369). Cells were grown in a synthetic minimal medium and samples for total RNA preparation were taken at indicated points (1, lag phase; 2, logarithmic growth phase; 3, negative acceleration phase; 4, early stationary phase; 5, late stationary phase; 6, death phase)

The PCR product was ~500 bp (Fig. 2). *narG* gene was separately amplified using RT-PCR primers (Table 1). TOPO Vector (invitrogen) was used for ligating PCR product and the recombinant plasmids were transformed into *E. coli* JM109. The partial nucleotide sequence of the insert in TOPO was determined. The sequence obtained with amplicon size of 174 bp, when compared to the protein sequences in gene bank. The results indicated that the short sequence of *narG* gene from strain cos21 (KU921436) is 98% identical to the nitrate reductase alpha subunit of *Hahella ganghwensis* (GenBank Accession No WP_020407638.1).

Response of *Idiomarina* Strain cos21 to a Change from Aerobic to Suboxic Growth Conditions

Strain cos21 was cultivated in a nitrate-rich medium with peptone as a carbon source. Within a few hours, the cells increased gradually over a cultivation period. Growth was measured by checking culture density. Growth reached a maximum by 36 h and remained steady for the next few hours and declined after 48 h (Fig. 3). Samples for nutrient analysis, enzyme activity, and total RNA isolation were taken during the different phases of growth (lag, logarithmic, Negative acceleration phase, early stationary phase, late stationary phase and death phase). The production of nitrate reduction metabolites was seen along the growth curve. Nitrate concentration decreased with an increase in cell growth while nitrite concentration increased with the time. Nitrate was almost completely consumed by 36 h. After the exhaustion of nitrate, the culture switched to the stationary phase (Fig. 4). Stationary phase was also followed by low oxygen concentration that is around 10 µM, which is considered to be suboxic (1–20 µM) condition (Fig. 3). Maximum nitrate reduction rate was reported during early stationary phase of strain cos21 which was 3.5 µM NO_3^- h^{-1} (Fig. 4).

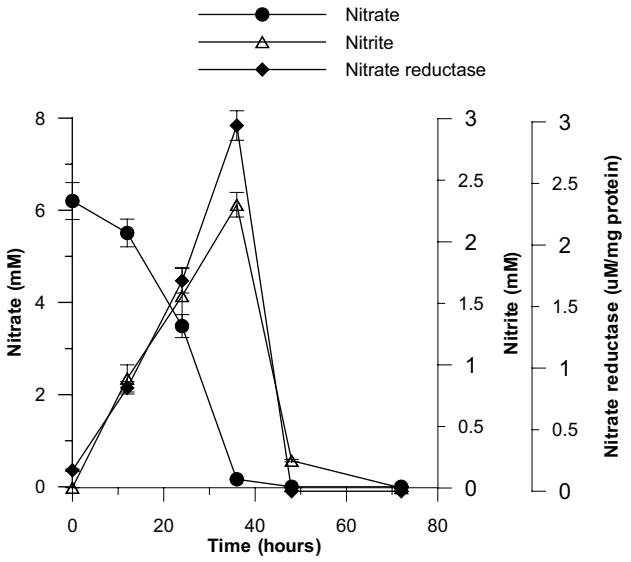

Fig. 4 Utilization of nitrates during growth of *Idiomarina* strain cos21

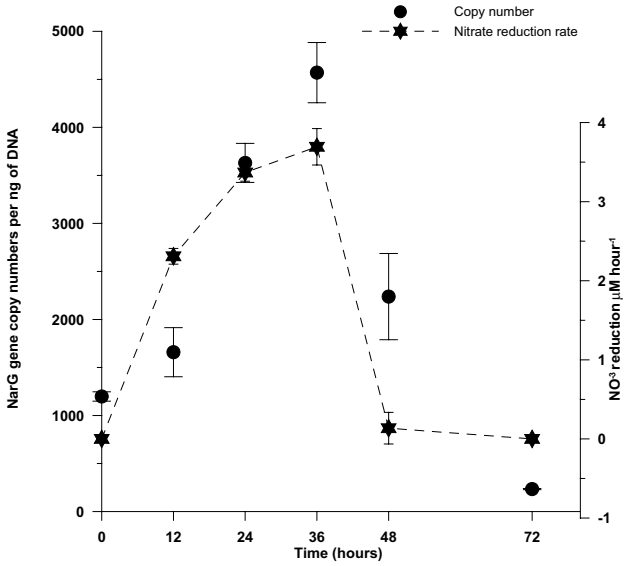

Fig. 5 Variation in gene abundance (copy number per ng of DNA) of nitrate reduction genes of *Idiomarina* strain cos21 during the change from aerobic to anaerobic growth. Standard errors are indicated ($n=3$). Gene copy numbers were calculated from the standard curve for *narG* showing, r2 0.995, *y*-intercept, 26.83, *E* (amplification efficiency) 108.2%

Quantification of the *narG* Gene Copy and Its Expression in *Idiomarina* Strain cos21

The *narG* transcripts were quantified by using the standard curve method. For *narG* mRNA was isolated from lag growth phase, a value of approximately 1.1×10^3 molecules was calculated; for mRNA isolated from the logarithmically growing cells, 1.6×10^3 molecules while mRNA isolated from negative acceleration phase showed 3.6×10^3 molecules. A value of 4.5×10^3 and 2.2×10^3 molecules was calculated for mRNA isolated from early stationary phase and stationary phase, respectively. While death phase was reported with 2.3×10^2 molecules (Fig. 5).

The expression of the genes for the nitrate reduction process was examined by checking the levels of nitrate specific mRNAs in the cell culture as well as nitrate reductase enzyme activity in the culture medium. The gene expression levels obtained by real-time quantitative RT-PCR analysis were normalized to that of the *GAPDH* gene since its expression was found to be invariant under different oxygen concentration changes. Quantitative values were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method recommended by Applied Biosystems. The relative expression of the *narG* gene was determined three times in each of the five experimental RNA samples and is expressed as the fold difference in the quantity of cDNA molecules present at different growth stages. *narG* gene expression gradually increased over time until it upregulated by 3.75 folds at the early stationary phase of growth of strain cos21 after which *narG* gene expression with late stationary and death phase decreased drastically (Fig. 6). This time course study of gene expression shows that a *narG* gene was differentially expressed during the different growth stage of strain cos21, and also at different oxygen concentrations.

Discussion

The high abundance of *Idiomarina* sp. was observed in a metagenomic fraction of the surface layers during spring intermonsoon in the OMZ region of the central Arabian Sea

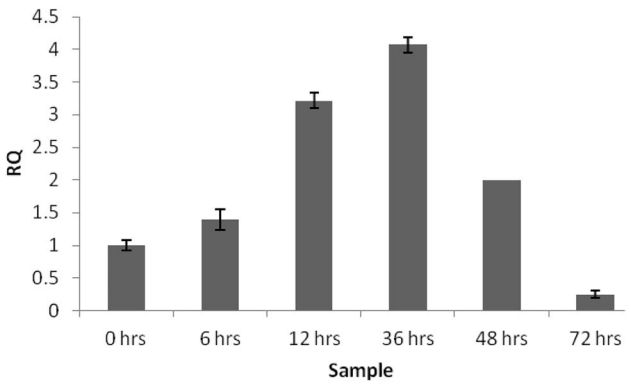

Fig. 6 Gene expression plot of analyzed transcripts. Relative expression of the *narG* gene of *Idiomarina* strain *cos21*. Expressions normalized with respect to the internal control gene *GAPDH*. The error bars in the histogram represent the maximum and minimum fold changes of relative expression

[18]. *Idiomarina* sp. seems to be one of the potent nitrate reducing bacterial genera in OMZ region of Arabian Sea.

The switch from aerobic to suboxic growth conditions seems to necessitate the onset of nitrate respiration in strain cos21. To study the physiological role of dissimilatory nitrate reductase and the involvement of membrane-bound and/or periplasmic nitrate reductase in this process, we analyzed nitrate reductase gene expression over time, at six different stages of bacteria growth.

Nitrate reduction process in continuously growing culture was studied by analysing the products such as the concentration of nitrite, synthesis of the nitrate reductase enzyme, and expression of mRNA for nitrate reduction in growing media. It was observed that bacteria showed highest nitrate reductase enzyme activity during early stationary phase accompanying low oxygen concentration; also the nitrate reduction rate of strain cos21 was maximum when the oxygen concentration dropped down below 10 µM, drop in oxygen levels seems to trigger bacteria to use alternate electron acceptors like nitrate and hence enhancing nitrate reduction process. It has been well documented that dissimilative nitrate reductase is membrane bound proteins inhibited by oxygen and synthesized under anaerobic conditions [4]. Another class of respiratory enzyme which is periplasmic in nature have been reported to take part in the dissimilatory nitrate reduction. This periplasmic nitrate reductase (nap) is constitutively expressed in both oxic and anoxic conditions. The role of Nap is not fully understood, yet it might be significant during the transition of aerobic to anaerobic growth [4]. PCR analysis of strain cos21 did not show amplification of nap gene ruling out the possibility of nap gene playing any role during nitrate reduction in this strain. Presence of two to three nitrate reductases are reported in bacteria, even assimilatory and dissimilatory nitrate reductases are found in same bacteria with independent function and gene regulation namely *Klebsiella pneumoniae* [6, 26], *Pseudomonas aeruginosa* [16, 19, 37] and *AlcaIigenes eutrophus* [38]. While others have two dissimilative nitrate reductases like *Rhodobacter capsulatus* [29, 30], *R. sphaeroides, R. denitrificans* [5, 34], *Paracoccus denitrificans* [11, 36], *Thiosphaera pantotropha* [2], *A. eutrophus* [38], and *Escherichia coli* [3, 16].

Idiomarina strain cos21 was seen reducing nitrate while growing in suboxic condition as the conversion of nitrate to nitrite increased as the oxygen concentration dropped. These are important aspects for the organisms growing in rapidly changing environmental conditions from aerobic conditions to anoxia. Inhibition of nitrate reductase activity due to oxygen (aerobic) in actively growing cultures of *M. tuberculosis* while the approximately fourfold increase in nitrite production when incubated anaerobically [13, 22] has been previously reported.

Bacteria undertake a range of strategies to monitor and coordinate the respiratory shift form oxic to anoxic environments. The process is based on the complex regulatory network, which controls the expression of target genes. Thus in order to understand this process, it's important to understand signalling and the response of the cells to these signal in different bacterial groups which will be useful to comprehend how bacteria integrate different signals and respond to these signals and coordinate to change in environment.

Studying denitrification process using mRNA gene expression is not been broadly looked into. The change in denitrification gene mRNA levels can directly reflect the dynamics of changing environmental conditions including OMZ region. It would be appealing to extend this approach for *nirS* and *nosZ* genes involved in the denitrification pathway to understand the complete denitrification process which has recently been depicted as an important process for the removal of fixed nitrogen in the OMZ's of the Arabian Sea.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest with regard to this manuscript.

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