Smriti Shrivastava *Editor*

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To My Family and Research Group

Foreword

Glycoside hydrolases (glycosidases or glycosyl hydrolases) are specific catalysts involved in breaking down glycosidic bonds of complex polysaccharides, such as cellulose, hemicelluloses, and starch. The cellulases, amylases, xylanases, and arabinases are examples of few enzymes of the group, and complete classification data of these enzymes describe their diverse mode of action, making them capable of acting on varied substrate range and thus breaking down the complex molecules to simple components that can be promisingly used for the production of a wide range of value-added products. It is this feature of the enzymes which makes them highly industrially significant. Alongside they also function as antibacterial defense proteins and help in normal cellular function and pathogenesis in the case of viruses. These enzymes have been studied since the last 60–70 years and, though being highly utilized in industries, still find numerous applications and high scope for research. This work has been designed focusing on industrial applications of glycoside hydrolases and covers reports on recent tools and techniques for enhancing enzyme performance.

Since they are numerous in number and found in diverse cells, a comparative analysis of the same will give a clear picture of the utilization of these enzymes in various industrial targets, benefiting the large research group working on glycoside hydrolases. With this concept in cognizance, chapter 1 describes a formatted detail on all glycoside hydrolases with specific characteristics in their three-dimensional structures, thus enabling their classification into 166 families that can again be grouped under 16 superfamilies. After considering the base classification of glycoside hydrolases, an understanding of structure and applications of glycoside hdyrolases is highly essential and has been brought forward as chapter 2, with a

note on recent tools and synergistic effect of enzymes for improving catalytic activity depicted in chapters 3 and 9.

This book features industrial applications of glycoside hydrolases, which have been enumerated well, in paper and pulp industries, bioenergy segment, and food industries, and the use of marine glycoside hydrolases (described in chapters 5–8). Glycoside hdyrolases find major applications in these industrial segments. The last section of the book covers functional and comparative genomic analysis of glycoside hydrolases and gives a description of metagenomics and its application for studying cellulases (Chapter 10).

It is anticipated that this book will be an excellent knowledge source for different sections of the scientific community to understand the functioning of diverse glycoside hydrolases and further exploring the promising applications of these enzymes.

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Preface

Energy and environmental security are two major concerns of the present world. We at our laboratory are working on the production of value-added components through enzymatic hydrolysis of Indian agricultural residues. In the process, I came across a few hydrolytic enzymes (belonging to glycoside hydrolases), responsible for saccharification of polysaccharides (hemicelluloses), and subsequent reading on the subject improved my understanding on the wide utility of glycoside hydrolases and its application in numerous industries, thus initiating work on compiling details of glycoside hydrolases with its major applications.

Glycoside hydrolases are resourceful tools in glycobiology and an important catalyst for industrial and biotechnological processes. They have a high level of structural diversity and due to their exquisite specificity and excellent catalytic efficiency, they find a wide range of distinct applications from biomass degradation to cell surface engineering.

This volume summarizes and updates both the state of knowledge and theories on structure, function, and biotechnological applications of glycoside hydrolases. It will be of great interest to diverse research groups working on glycoside hydrolases, in particular to the group working on industrial applications of these enzymes.

While planning this book, invitations for contributors were extended to subject experts. I would like to express my deep appreciation to each contributor for their patience and attention during the production process. This book contains 10 chapters, grouped under 3 sections that include (A). Introduction, (B). Industrial Applications, and (C). Functional and Comparative Genomic Analysis of Glycoside Hydrolases.

I am extremely delighted to edit this volume, due to the stimulating cooperation of the contributors. I wish to generously thank Dr. Bhavik Shawney and Mr. Lenold Christ Raj, Springer Nature, SPi Global for their munificent assistance in finalizing the volume. My special thanks to family, friends, colleagues, and students and sincere thanks to all contributors.

Noida, India — Smriti Shrivastava

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I would like to express my sincere and heartfelt thanks to my family, for their constant support throughout my career. My special thanks to Prof. Ashok Chauhan (Founder President, Amity University) for giving us an excellent platform to work and thanks to each member of the Amity University Uttar Pradesh, Noida. I would certainly not have been able to compile this book without the wonderful contributions of each author, who has excellently given their inputs. My sincere thanks to all the authors for sharing their knowledge and expertise through this book. My special thanks of gratitude to all my mentors throughout my journey to build me day by day. My earnest thanks to the editorial team, Springer to help in this journey all the way through. And last of all, I would be extremely thankful to all readers of my book and believe that this book will prove to be of high interest and significance to them.

Contents

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Smriti Shrivastava is an Assistant Professor at Amity Institute of Biotechnology, Amity University, Uttar Pradesh, India. She was previously an Assistant Professor at P.D.D.Y. Patil University, Navi Mumbai (2010–2011) and as DST Young Scientist at Amity University, Uttar Pradesh. Her research focuses on enzyme technology and applied microbiology, particularly the targeted production of value-added products. She also serves as a reviewer for a number of applied microbiology and enzyme technology journals. She has edited one book and published 17 research/review articles and 6 book chapters. She has been reviewer to various journals and have presented her work in numerous conferences.

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Abbreviations

AD	Anaerobic digestion
ADH	Alcohol dehydrogenase
AFEX	Ammonia fiber expansion
AGX	Arabinoglucuronoxylans
AI	Arabinose isomerase
AR	Arabinose reductase
AXs	Arabinoxylans
BOD	Biological oxygen demand
CAGR	Compound annual rate of growth
CAZy	Carbohydrate-Active EnZymes
CBD	Cellulose binding domain
CBM/CBD	Carbohydrate binding module/domain
CBP	Consolidated bioprocessing
CDH	Cellobiose dehydrogenase
CE	Carbohydrate esterase
CHS	Chitosan
CMC	Carboxymethyl cellulase
COS	Chitooligosaccharides
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DP	Degree of polymerization
EC	Enzyme Commission
EMP	Embden–Meyerhof–Parnas
Eno	Enolase
EPS	Extracellular polymeric substances
ERA-NET (ERA-MBT)	European Marine Biotechnology
FA	Fructose bisphosphate aldolase
FAD	Flavin adenine dinucleotide
FCR	Feed conversion rates
FE	Feruloyl esterase
FOS	Fructooligosaccharides
GAXs	Glucuronoarabinoxylans
GDH	3-phosphate dehydrogenase
GH	Glycoside hydrolases

GlcNAc	*N*-acetyl glucosaminidases
GOS	Galactooligosaccharides
GPDH	Glucose-6-phosphate dehydrogenase
GT	Glycosyltransferase
GXs	Glucuronoxylans
HEWL	Hen egg-white lysozyme
HK	Hexokinases
HMF	Hydroxymethyl furfural
IUBMB	International Union of Biochemistry and Molecular Biology
KDNase	*Aspergillus fumigatus* Sialidase
KDPG	2-keto-3-deoxy-6-phosphogluconate
KDPGA	2-keto-3-deoxy-6-phosphogluconate aldolase
Lac	Lactonase
LAD	L-arabitol dehydrogenase
LPMO	Lytic polysaccharide mono-oxygenase
L-XuR	L-xylulose reductase
MLF	Malolactic fermentation
MOS	Mannooligosaccharides
NAD	Nicotinamide adenine dinucleotide
NAD(H)	Nicotinamide adenine dinucleotide (reduced form of NAD)
NAG	N-acetyl-D-glucosamine
NAM	N-acetylmuramic acid
NSPs	Non-starch polysaccharides
O-GlcNAcase	(protein)-3-*O*-(*N*-acetyl-D-glucosaminyl)-L-serine/threonine *N*-acetylglucosaminyl hydrolase
PDB	Protein Data Bank
PDC	Pyruvate decarboxylase
PFK	Phosphofructokinase
PGD	6-phosphogluconate dehydratase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PI	Phosphoglucose isomerase
PK	Pyruvate kinase
PKL	Phosphoketolase
PL	Polysaccharide lyase
POS	Pectin oligosaccharides
PPE	Phosphopentose epimerase
PPP	Pentose phosphate pathway
PUGNAc	O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate
R5PE	Ribulose-5-phosphate-4-epimerase
RK	Ribulokinase
SSCF	Simultaneous saccharification and co-fermentation

SSF	Solid state fermentation
TA	Transaldolase
TIM	Triosephosphateisomerase
TK	Transketolase
TPI	Triosephosphate isomerase
VDECH	Endochitinase from *Verticillium dahlia*
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulose kinase
XOS	Xylooligosaccharides
XR	D-xylose reductase

Part I

Introduction

Introduction to Glycoside Hydrolases: Classification, Identification and Occurrence

Smriti Shrivastava

Abstract

Glycoside hydrolases are group of enzymes belonging to class 3 enzymes as per classification by IUBMB. They specifically break down glycosidic bonds of complex polysaccharides and are generally named upon the substrates on which they act (e.g. lactase acting on lactose; chitinase acting on chitin, sucrase acting on sucrose, etc). Present chapter introduces glycoside hydrolases, its identification and occurrence in nature, highlighting the diverse existence of these enzymes. It covers a detailed report on classification and available three-dimensional structures of the enzymes of the group. Glycoside hydrolases have been classified under 166 families which are grouped in 16 different superfamilies and three added clans. The chapter gives a complete documentation of all available data on glycoside hydrolases, which will be beneficial for researchers working in the domain.

Keywords

Glycoside hydrolases · Enzyme classification · Enzyme nomenclature · Glycoside hydrolase superfamilies

Introduction

Glycoside hydrolases (Glycosidase or glycosyl hydrolases) are specific catalyst that breaks down glycosidic bonds of complex polysaccharides, such as cellulose, hemicelluloses, starch etc. These enzymes with their specific functions are called cellulase, amylases, xylanases, arabinases and includes several others. In addition to

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degradation of plant polysaccharides they also function in anti bacterial defense mechanism (lysozymes), in normal cellular functioning (such as biosynthesis of N-linked glycoprotein through mannosidases), pathogenesis through viral neuraminidase (Bourne and Henrisatt 2001; Henrisatt and Davies 1997)

Occurrence and Importance

Present in almost all domains of life, glycoside hydrolases are found both as intracellular and extracellular enzymes in prokaryotes and are majorly involved in hydrolysis of glycoside molecules, nutrient acquisition, regulation of expression of operon, post translational modification, lysosomal storage in higher organisms, biosynthesis and degradation of glycogen. In prokaryotes and lower eukaryotes they are present intracellular as well as secreted as extracellular enzymes. In higher organisms they are found within endoplasmic reticulum and golgi apparatus (processing of N-linked glycoproteins); in lysosome (degradation of carbohydrate structure) in intestinal tract and in saliva as carbohydrate degraders (amylase), in gut as glycosylphosphatidyl anchored enzymes on endothelial cells as enzyme lactase (degradation of milk sugar lactose), enzyme O-GlcNAcase (removal of N-acetylglucosamine groups from cytoplasmic and nuclear located serine and threonine residues)

Mechanisms of Glycoside Hydrolases

Basis their mechanism of action, Glycoside hydrolases are broadly classified as inverting glycoside hydrolases and retaining glycoside hydrolases.

Inverting enzymes utilize two enzymic residues (generally carboxylate), one act as acid and other as base. Figure 1 shows mechanism of action of a β-glucosidase.

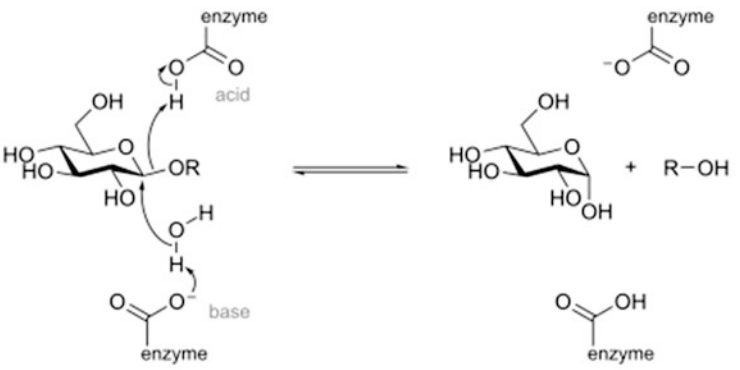


Fig. 1 Mechanism of action of a β-glucosidase

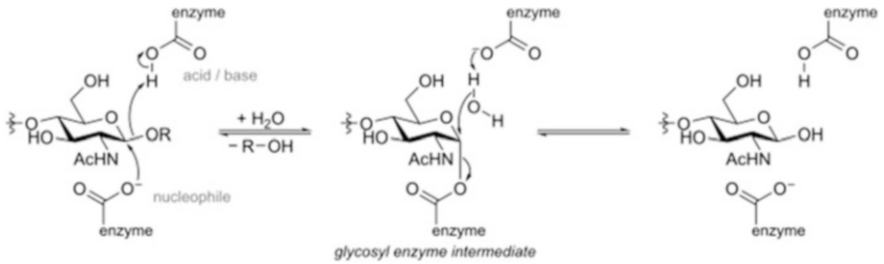


Fig. 2 Mechanism of action for hen egg white lysozyme

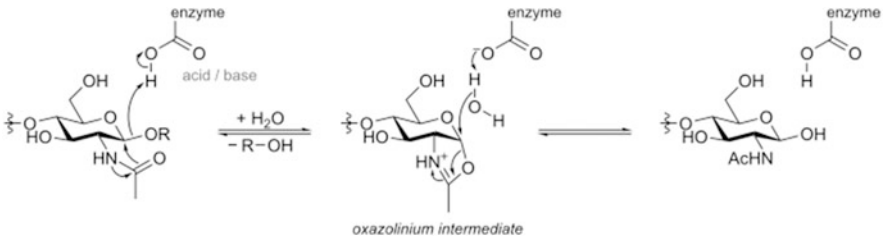


Fig. 3 Mechanism of action of N-acetyle Hexoaminidases

Retaining glycoside hydrolases conduct hydrolysis through two main mechanisms, with each step resulting in inversion. Two residues involved are generally enzyme accepted carboxylates; of which one acts as a nucleophile and the other as acid/base. First step involves attack of nucleophile to the anomeric center forming a glycosyl enzyme intermediate (acidic assistance provided by acid carboxylate), followed by hydrolysis of glycosyl-enzyme transitional state through nucleophilic water (assisted by deprotonated acidic carboxylate, that acts as a base). This process results in net retention of stereochemistry. Mechanism has been illustrated as example for hen egg white lysozyme (Fig. 2) (Vocadlo et al. 2001).

Enzyme mechanisms that carried out hydrolysis with retention of stereochemistry occurs through a substrate bound nucleophilic residue, rather than being directly attached to the enzyme. Such mechanism can be seen for certain N-acetylhexosaminidases (Fig. 3), where an acetamido group present on the enzyme can participate with neighboring group and forms and intermediate oxazoline or oxazolinium ion following two steps mechanism of distinct inversions making net retention of configuration.

Applications of Glycoside Hydrolases

Glycoside hydrolases are one of the major catalysts of ensuing generation owing to its varied applications in biorefining processes. These includes hydrolysis of plant materials (cellulases, xylanases) for production of value added components,

applications in food industries (invertase for production of invert sugars; amylase for production of maltodextrins), usage in paper and pulp industry, in detergent manufacturing (cellualses for washing of cotton fabrics for maintenance of fabric colour by removing microfibre) (Linares-Pastén et al. 2014).

These enzymes are used as synthetic catalysts (performing reverse hydrolysis/transglycosylation); reversing equilibrium and enabling the retaining glycoside hydrolase to catalyze transfer of glycosyl moiety from an activated glycoside to an acceptor alcohol. Glycosynthases (formed from retaining glycoside hydrolases by site directed mutagenesis of enzymic nucleophile to some less nucleophilic group like alanine/glycine) are enzymes catalyzing high yield of glycosides from activated glycosyl donors like gycosyl fluorides. Thioglycoligases also are mutant glycoside hydrolases, formed by site directed mutagenesis of the acid base residue of retaining glycoside hydrolases and catalyze condensation of activated glycosides and thiol comprising acceptors.

Glycoside hydrolases show usefulness in matrix polysaccharide within extracellular polymeric substances (EPS) of microbial biofilm (Fleming and Rumbaugh 2017). Degrading microbial biofilm, increases antibiotic efficacy, potentiating host immune function (Fleming et al. 2017).

Inhibitors if Glycoside Hydrolases

There are various natural and synthetic compounds that have been reported to act as inhibitors of glycoside hydrolases. This includes naturally occurring nitrogen containing sugar shaped heterocycles (deoxynojirimycin, swainsonine, australine, castanospermine) working as natural templates for developing modified inhibitors (e.g. isofagomine, deoxygalactonojirimycin, unsaturated compounds such as PUGNAc). Glycoside hydrolase inhibitors finding clinical usage includes antidiabetic drugs (acarbose and miglitol) and antiviral drugs (oseltamivir and zanamivir). Few proteins have also been identified as Glycoside hydrolase inhibitors.

Classification of Glycoside Hydrolases

According to enzyme nomenclature by International Union of Biochemistry and Molecular Biology (IUBMB), Glycoside hydrolases are classified into EC 3.2.1 as enzymes catalyzing the hydrolysis of O- or S-glycosides (Sinnott 1990). They are also classified based on stereochemical outcome of hydrolysis reaction (retaining or inverting enyzmes); on exo (non-reducing end) and endo (middle of molecule) acting, on sequence and structure based classification. Sequence based classification has suggested more than 150 different families of glycoside hydrolases (Henrissat et al. 1995; Henrissat and Davies 1995; Bairoch 1999), these are available on CAZy Carbohydrate-Active Enzymes web site, supported by CAZypedia [Cazy family Glycoside hydrolase; Cazypedia; Henrissat and Coutinho 1999]. Sequence based

classification significantly helps in prediction of mechanism of action of enzyme, active site residues and possible substrates. Enzymes further classified as clans of related structure based on three dimensional structural similarities obtained from available sequences (Naumoff 2006, 2011).

Glycoside hydrolases (O-Glycosyl hydrolases) EC 3.2.1.X catalyzing hydrolysis of glycosidic bond are classified based on sequence similarity to be most reliable and led to the definition of 128 families and 14 clans (based on folds of proteins) of the same (Henrissat et al. 1995; Henrissat and Davies 1995; Henrissat and Bairoch 1996). This sequence based classification is available on the CAZy (Carbohydrate-Active Enzymes) (Cantarel et al. 2009). Glycoside hydrolases are also classified based on their localization in cell (secreted, monotopic, peripheral, lysosomal, located in eukaryotic plasma, inner membrane of Gram positive bacteria).

Following section in the chapter will deal with the major features and mode of action of all glycoside hydrolases classified. One prominent known structure from each family is depicted in Fig. 4a-r5.

Glycoside Hydrolase Family 1

Enzymes of family 1 follow the IUBMB EC 3.2.1 nomenclature and catylzes hydrolysis of glycosidic bond between two carbohydrate molecules or a carbohydrate and non carbohydrate molecule (Lombard et al. 2014; Cazypedia.org and Cazypedia Consortium). Major enzymes of Glycoside Hydrolase family 1 are; beta-glucosidase (EC 3.2.1.21); beta-galactosidase (EC 3.2.1.23); 6-phospho-beta-galactosidase (EC 3.2.1.85); 6-phospho-beta glucosidase (EC 3.2.1.86); lactase-phlorizin hydrolase (EC 3.2.1.62), lactase (EC 3.2.1.108); beta mannosidase (EC 3.2.1.25); myrosinase (EC 3.2.1.147). Figure depicts general structure of Glycoside hydrolase family 1. According to the PROSITE documentation (https://prosite.expasy.org/cgi-bin/prosite/prosite-search-ac?PDOC00495#description) (Henrissat 1991a, b; Gonzalez-Candelas et al. 1990; El Hassouni et al. 1992) classifies β-glucosidases (EC 3.2.1.21); β-Galactosidases (EC 3.2.1.23); 6-phospho-β-Galactosidases (EC 3.2.1.85); β-Glucosidases (EC 3.2.1.86); plant myroniases (EC 3.2.1.147; e g. synigrinases or thioglucosidases); Mammalian lactase-phlorizin hydrolase (LPH; EC 3.2.1108/EC 3.2.1.62) in family 1 of Glycoside hydrolases. Conserved regions are central glutamic acid residues, acting as nucleophile during glycosidic bond cleavage. This is marked as signature pattern for this group of enzymes with another along with a conserved region containing glutamic acid found at their N-terminal extremity (Withers et al. 1990). This group belongs to the TIM Barrel glycoside hydrolase superfamily contains the range of enzymes belonging to group that posses a TIM barrel fold merging clans GH-A, GH-D, GH_H and GH-K. It contains 57 families and 259,156 domains and was built by A Bateman (Naumov and Karreras 2009). All members of family 1 glycoside hydrolases for which localization is known are typically restricted to plasma membrane and endoplasmic reticulum membrane (Davies and Henrissat 1995; Henrissat et al. 1995) (Fig. 4a).

Glycoside Hydrolase Family 2

Glycoside hydrolases family 2 enzymes comprises enzymes with β-galactosidases (EC 3.2.1.23); β-mannosidases (EC 3.2.1.25), β-glucuronidase (EC 3.2.1.31). Glutamic acid residue is the general acid/base catalyst at active sites of these enzymes (Gebler et al. 1992). The catalytic domain of β-galactosidases contains TIM barrel core surrounded beta domains, with sugar binding domain forming jelly–roll fold, containing immunoglobulin like beta-sandwich domain (Jacobson et al. 1994). General structure of glycoside hydrolase family 2 enzymes is elaborated as its

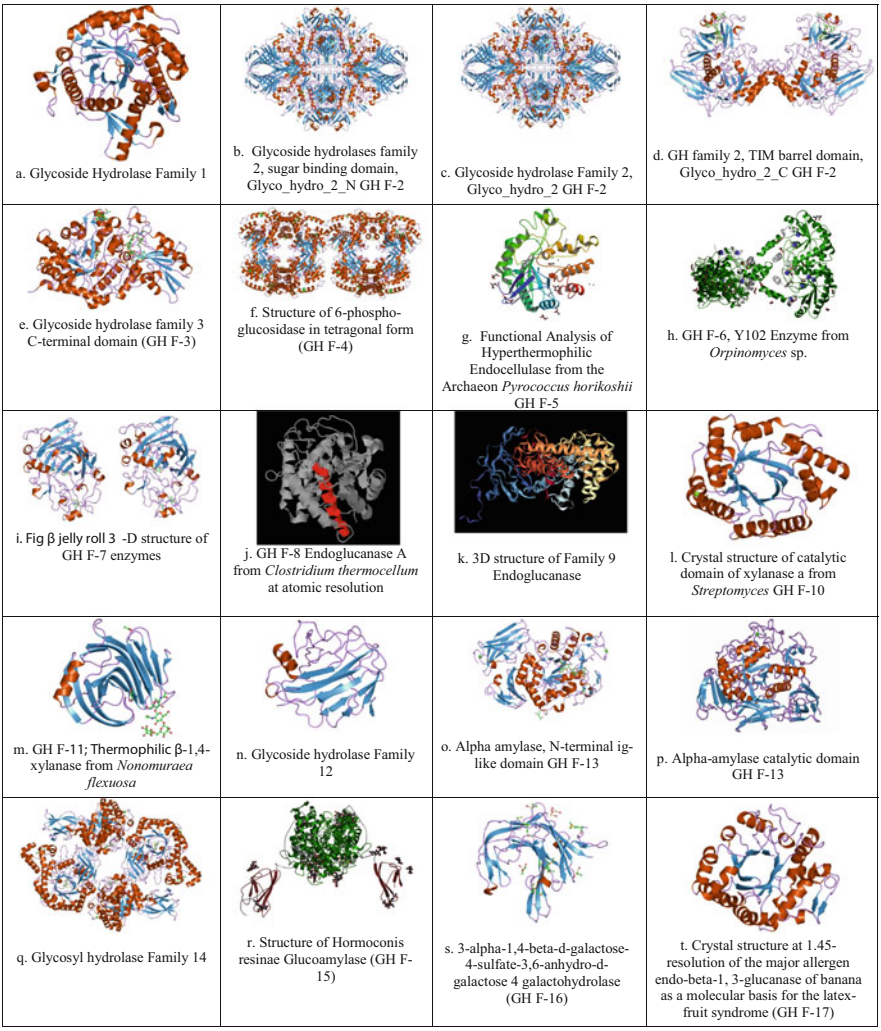


Fig. 4 Glycoside hydrolase family 1-Family 166 (One PDB accession for each family)

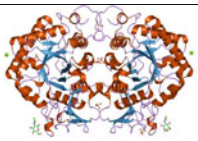
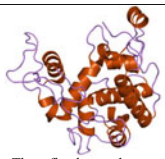
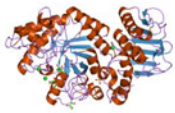
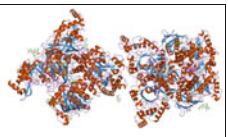
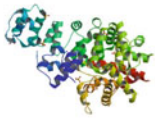
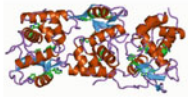
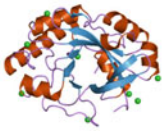
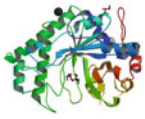
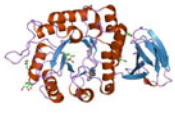
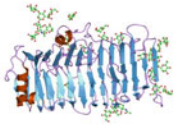
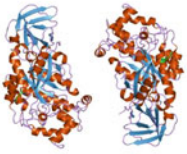
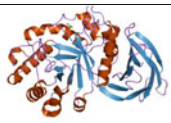
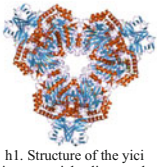
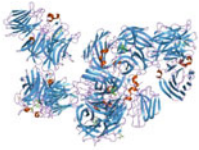
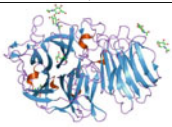
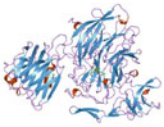
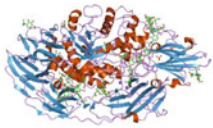
 u. Crystal structure of a native chitinase from aspergillus fumigatus yj-407 (GH F-18)	 v. The refined crystal structure of an endochitinase from hordeum vulgare l. seeds to 1.8 angstroms resolution (GH F-19)	 w. Wildtype streptomyces plicatus beta-hexosaminidase in complex with product (glcnac) [GH F-20; Catalytic domain]	 x. Crystallographic structure of human beta-hexosaminidase a [GH F-20, Domain 2]
GH F-21 (No strcuture available)	 y. C-type lysozyme/alpha-lactalbumin family (Bobwhite quail lysozyme) GH F-22	 z. Crystal structure of Cj0843 lytic transglycosylase of Campylobacter jejuni at 1.87A resolution (GH F-23)	 a1. Lysozyme from bacteriophage lambda (GH F-24)
 b1. Crystal structure of the bacterial lysozyme from *Streptomyces coelicolor* at 1.65 a resolution (GH F-25)	 c1. Structure of *Bacillus subtilis* mannanase man 26 (GH F-26)	 d1. Melibiase (Crystal structure of rice alpha-galactosidase) GH F-27	 e1. Rhamnogalacturonase a from *Aspergillus aculeatus* (GH F-28)
 f1. Crystal structure of thermotoga maritima alpha-fucosidase; GH F-29	 g1. Structure of a xylanase from glycoside hydrolase family 5 (O-glycosyl hydrolase family 30)	 h1. Structure of the yici thiosugar michaelis complex (GH F-31)	 i1. Beta-fructosidase from *Thermotoga maritima* in complex with raffinose (Glycoside hydrolase Family 32, N-Terminal Domain)
 j1. Crystal structure of exo-inulinase from *Aspergillus awamori* in spacegroup p21 (GH F 32, C- Terminal Domain)	 k1. Sialidase, N-terminal domain (GH F-33)	 l1.Crystallographic structure of influenza neuraminidase in complex with the inhibitor 4-acetamido-3-hydroxy-5-nitro-benzoic acid (GH F-34)	 m1. Native structure of beta-galactosidase from penicillium sp. (Glycoside hydrolase family 35)

Fig. 4 (continued)

complete picture, sugar binding domain and TIM Barrel domain as depicted in Fig. 4b–d respectively.

Sugar binding domain belongs to the galactose binding domain like superfamily, the clan that contains 70 families and 124,105 domains. This superfamily has prominent sandwich domains with a jelly roll topology and is mainly involved in

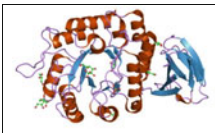
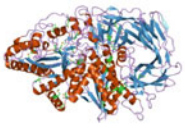
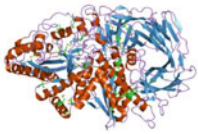
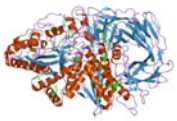
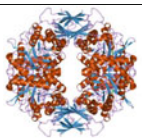
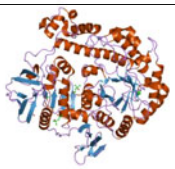
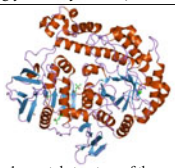
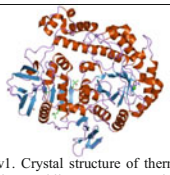
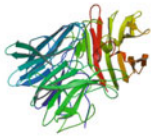
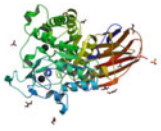
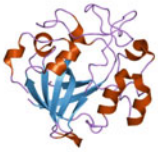
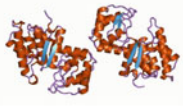
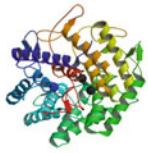
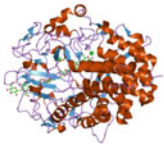
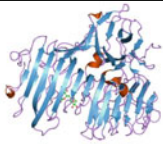
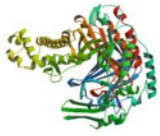
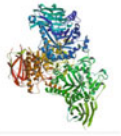
 n1. Crystal structure of rice alpha-galactosidase (Melibiase, GH F-36)	 o1. Structure of periplasmic trehalase from Diamondback moth gut bacteria complexed with validoxylamine (GH F-37)	 p1. Golgi alpha-mannosidase II (GH F-38, N terminal domain)	 q1. Golgi alpha-mannosidase II (Alpha mannosidase middle domain; GH F-38)
 r1. Golgi alpha-mannosidase II (GH F-38, C-terminal domain)	 s1. Crystal structure of beta-d-xylosidase from *Thermoanaerobacterium saccharolyticum*, a family 39 glycoside hydrolase (GH F-39)	GH F-40 (No strcuture available)	GH F-41 (No strcuture available)
 t1. Crystal structure of thermus thermophilus a4 beta-galactosidase (GH F-42)	 u1. crystal structure of thermus thermophilus a4 beta-galactosidase (Trimerization domain; GH F-42)	 v1. Crystal structure of thermus thermophilus a4 beta-galactosidase (C-terminal domain; GH F-42)	 *w1.* Crystal structure of xylan beta-1,4-xylosidase from Bacillus Halodurans C-125 (GH F-43)
 x1. Crystal Structure of a Glycoside Hydrolase Family 44 Endoglucanase produced by *Clostridium acetobutylium* ATCC 824 (GH F-44)	 y1. Endoglucanase from *Humicola insolens* at 1.7a resolution (GH F-45)	 z1. *Streptomyces* n174 chitosanase ph5.5 298k (Glycoside hydrolase family 46)	 a2. Structure of The GH47 processing alpha-1,2-mannosidase from *Caulobacter* strain K31 *(GH F-47)*
 b2. X-tal structure of the mutant e44q of the cellulase cel48f in complex with a thiooligosaccharide (GH F-48)	 c2. dex49a from *Penicillium minioluteum* complex with isomaltose (GH F-49)	 d2. The crystal structure of an agarase, AgWH50C (GH F-50)	 e2. Elucidation of the substrate specificity and protein structure of AbfB, a family 51 alpha-L-arabinofuranosidase from *Bifidobacterium longum* (GH F-51)

Fig. 4 (continued)

carbohydrate recognition. They share very little sequence similarity and weak sequence motif with conserved bulge (possibly helps in bending of beta sheets) in the C-terminal beta sheet, enabling curvature of sheet that forms a sugar binding site (Murzin and Bateman 1998).

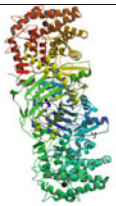
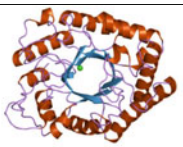
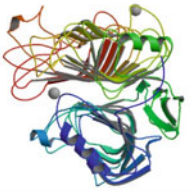
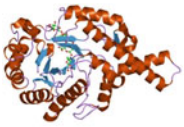
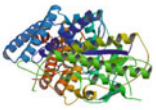
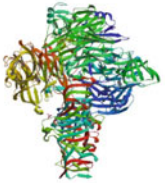
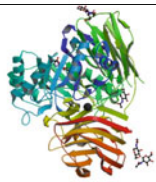
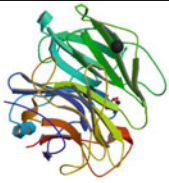
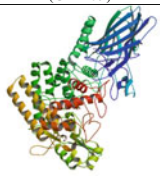
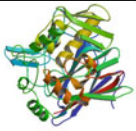
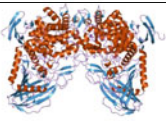
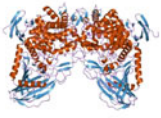
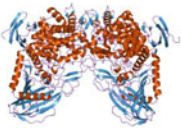
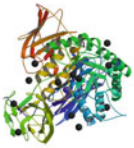
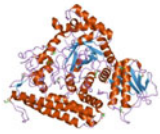
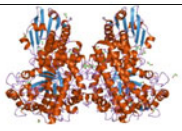
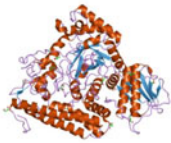
 f2. Crystal structure of the catalytic mutant Xyn52B2-E335G, a GH52 Beta-D-xylosidase from *Geobacillus stearothermophilus* T6 (GH F-52)	 g2. Crystal structure of beta-1,4-galactanase from *Aspergillus aculeatus* at 100k (GH F-53)	 *h2. Crystal structure of arabinofuranosidase (GH F-54)*	 *i2. Structure of sacteLam55A from Streptomyces sp. SirexAA-E* (GH F-55)
 j2. Crystal structure of bee venom hyaluronidase in complex with hyaluronic acid tetramer (Hyaluronidase; GH F-56)	 k2. Crystal structure of GH57-type branching enzyme from hyperthermophilic archaeon Pyrococcus horikoshii (GH F-57)	 l2. Endosialidase of Bacteriophage K1F (GH F-58)	 m2. Structure of *Galactocerebrosidase* from Mouse (GH F-59)
GH F-60 (Structure not available)	GH F-61 (Structure not available)	 n2. Crystal Structure of *Streptomyces coelicolor* alpha-L-arabinofuranosidase (GH F-62)	 *o2. Crystal structure of E. coli YgjK, a member of glycosyl hydrolase family 63*
 p2. Crystal structure of a beta-1,3-glucanase domain (GH64) from Clostridium beijerinckii (GH F-64)	 q2. Maltose phosphorylase from lactobacillus brevis (GH F-65; N terminal domain)	 r2. maltose phosphorylase from lactobacillus brevis (GH F-65, central catalytic domain)	 s2. maltose phosphorylase from lactobacillus brevis (GH F-65; C-terminal domain)
 t2Crystal Structure of *Bacillus circulans* T-3040	 u2. The 1.7 a crystal structure of alpha-d-glucuronidase, a family-67 glycoside hydrolase from	 v2. *Pseudomonas cellulosa* e292a alpha-d-glucuronidase mutant complexed with	 w2. The 1.7 a crystal structure of alpha-d-glucuronidase, a family-67

Fig. 4 (continued)

Glycoside Hydrolase Family 3

Glycoside hydrolase family 3 enzymes are two domain globular proteins N-glycosylated at 3 sites (Varghese et al. 1999). This family comprises of

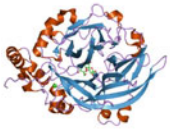
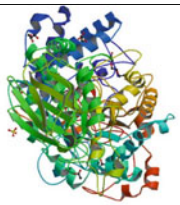
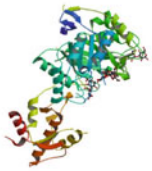
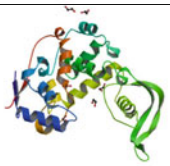
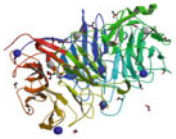
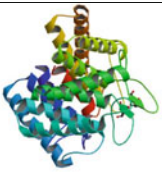
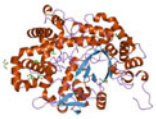
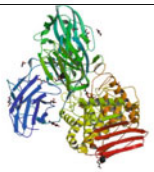
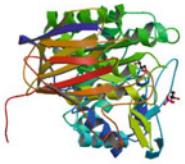
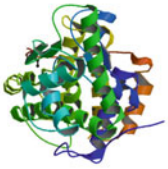
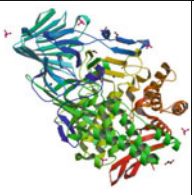
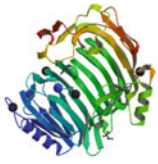
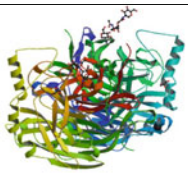
cycloisomaltooligosaccharide glucanotransferase *(GH F 66)*	bacillus stearothermophilus t-1 (GH F-67, N-terminal domain)	aldotriuronic acid (GH F-67 Middle domain)	glycoside hydrolase from bacillus stearothermophilus t-1 (GH F-67; C Terminal domain)
 x2. crystal structure of levansucrase (e342a) complexed with sucrose (GH F-68)	GH F-69 (No strcuture available)	 y2. 4,6-alpha-glucanotransferase GTFB from *Lactobacillus reuteri* 121 (GH F-70)	GH F-71 (No strcuture available)
 Z2. *Saccharomyces cerevisiae* gas2p (e176q mutant) in complex with laminaritetraose and laminaripentaose *(gh f-72)*	 *a3. X-ray structure of catalytic domain of autolysin from Clostridium perfringens* (GH F73)	 b3. The structure of Acidothermus cellulolyticus family 74 glycoside hydrolase (GH F-74)	GH F-75 (No strcuture available)
 c3. Crystal structure of Bacillus circulans TN-31 Aman6 in complex with mannobiose (GH F 76)	 d3. structure determination and refinement at 1.8 a resolution of disproportionating enzyme from potato (GH F-77)	 e3. Crystal structure of GH78 family rhamnosidase of *Bacillus* SP. GL1 AT 1.9 A *(GH F-78)*	 *f3.* Crystal structure of beta-glucuronidase from *Acidobacterium capsulatum* (GH F-79)
 *g3. C*rystal Structure of GH80 chitosanase from *Mitsuaria chitosanitabida* (GH F-80)	 *h3.* Crystal structure of SeMet derivative BhGH81 (GH F-81)	 *i3. 1,3-Alpha-1,4-Beta-D-Galactose-4-Sulfate- 3,6-Anhydro-D-Galactose-2-Sulfate 4 Galactohydrolase (GH F-82)*	 j3. Structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase *(GH F-83)*

Fig. 4 (continued)

β-glucosidases (EC 3.2.1.21); β-xylosidase (EC 3.2.1.37), N-acetyl β-glucoseaminidase (EC 3.2.1.52), Glucan β-1,3-glucosidase (EC 3.2.1.58), cellodextrinase (EC 3.2.1.74); exo 1,3-1,4-glucanase (EC 3.2.1.). Crystal structures for N-terminal and C-terminal domain of family 3 enzymes are available (Fig. 4e). Conserved region of these enzymes have been centered on a conserved aspartic acid residue depicted in β-glucosidase A3 in *Aspergillus wentii* (Bause and Legler 1980).

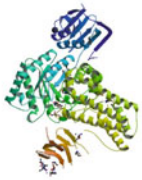
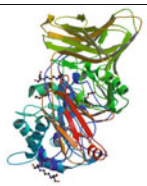
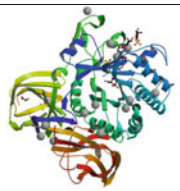
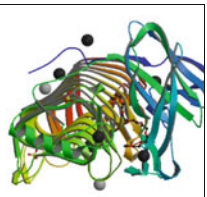
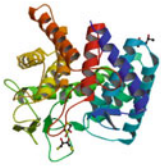
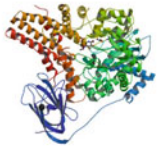
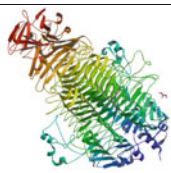
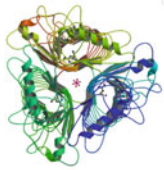
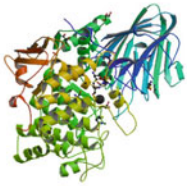
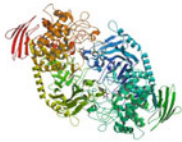
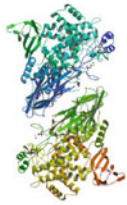
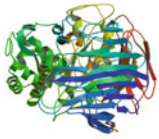
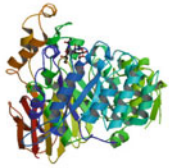
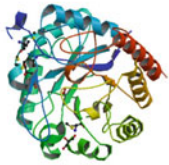
 k3. Bacteroides thetaiotaomicron hexosaminidase with O-GlcNAcase activity- NAG-thiazoline complex *(GH F-84)*	 l3. X-ray crystal structure of the Endo-beta-N-acetylglucosaminidase from Arthrobacter protophormiae E173Q mutant reveals a TIM barrel catalytic domain and two ancillary domains (GH F-85)	 *m3*. BpGH86A: A beta-porphyranase of glycoside hydrolase family 86 from the human gut bacterium Bacteroides plebeius (GH F-86)	 *n3*. Crystal structure of *Agl*-KA catalytic domain (GH F-87)
 *o3*. Crystal Structure of Unsaturated Glucuronyl Hydrolase, Responsible for the Degradation of Glycosaminoglycan, from Bacillus sp. GL1 at 1.8 A Resolution (GH F-88)	 p3. Family 89 Glycoside Hydrolase from *Clostridium perfringens* in complex with 2-acetamido-1,2-dideoxynojirmycin *(GH F-89)*	 *q3*. Siphovirus 9NA tailspike receptor binding domain (GH F-90)	 r3. Crystal structure of insulin fructotransferase in the absence of substrate (GH F-91)
 *s3*. Structure of the Family GH92 Inverting Mannosidase BT3990 from *Bacteroides thetaiotaomicron* VPI-5482 (GH F-92)	 t3. structure of the GH93 alpha-L-arabino furanosidase of *Fusarium graminearum* (GH F-93)	 u3. Crystal Structure of *Cellvibrio gilvus* Cellobiose Phosphorylase Crystallized from Ammonium Sulfate *(GH F-94)*	 *v3. Crystal structure of a putative glycoside hydrolase family protein from Bacillus halodurans* (GH F-95)
GH F-96 (No strcuture available)	 w3. Crystal structure of BT1871 retaining glycosidase (GH F-97)	 x3. Crystal structure of the catalytic module of a family 98 glycoside hydrolase from *Streptococcus pneumoniae* SP3-BS71 in complex with the A-trisaccharide blood group antigen. (GH F-98)	 *y3. Selenomethionine derivative of the GH99 endo-alpha-mannosidase from Bacteroides thetaiotaomicron (GH F-99)*

Fig. 4 (continued)

Glycoside Hydrolase Family 4

Major enzyme activities of this group comprise 6-phospho-β-glucosidase (EC 3.2.1.86); 6-phospho-α-glucosidase (EC 3.2.1.122), α-galactosidase (EC 3.2.1.22); of these 6-phospho-α-glucosidase requires both NAD(H) and divalent

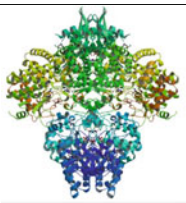
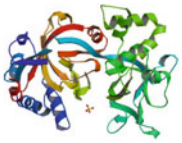
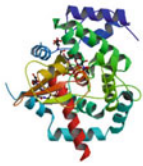
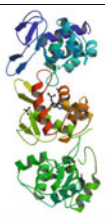
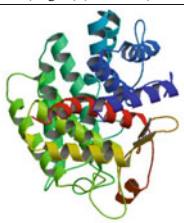
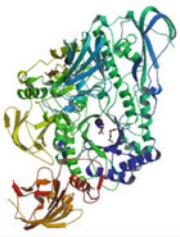
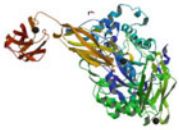
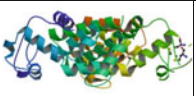
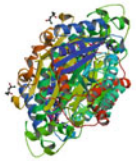
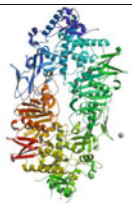
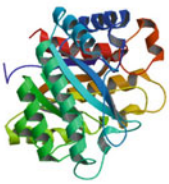
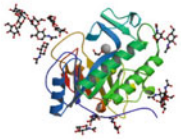
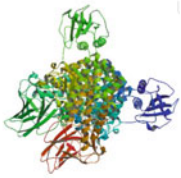
 z3. Crystal structure of alkaline invertase InvA from *Anabaena* sp. PCC 7120 complexed with fructose (GH F-100)	 a4. Crystal structure of endo-alpha-N-acetylgalactosaminidase from *Bifidobacterium longum* (EngBF) (GH F-101)	 b4. Crystal structure of membrane-bound lytic murein transglycosylase from *Agrobacterium tumefaciens* (GH F-102)	 C4. The *Escherichia Coli* Lytic Transglycosylase Slt35 In Complex With Two Murodipeptides (Glcnac-Murnac-L-Ala-D-Glu) (GH F103)
 *d4*. Lysozyme from bacteriophage lambda (GH F-104*)*	 *e4*. Structure of Protein of Unknown Function of YteR from *Bacillus Subtilis* (GH F-105)	 f4. Glycoside hydrolase BT_0986 *(GH F-106)*	 *g4*. Endo-fucoidan hydrolase MfFcnA4 from glycoside hydrolase family 107
 *h4*. Crystal Structure of a Protein of Unknown Function NMB1012 from *Neisseria meningitides (*GH F-108)	 *i4*. A-zyme, N-acetylgalactosaminidase (GH F-109)	GH F-110 (No strcuture available)	GH F-111 (No strcuture available)
 j4. Crystal structure of Galacto-N-biose/Lacto-N-biose I phosphorylase *(GH F-112)*	 k4. Crystal structure of the endo-beta-1,4-mannanase from *Alicyclobacillus acidocaldarius* (GH F-113)	 l4. Crystal Structure of *Aspergillus fumigatus* Ega3 (GH F-114)	 m4. Evidence that GH115 alpha-glucuronidase activity is dependent on conformational flexibility *(GH F-115)*

Fig. 4 (continued)

metal (Mn^{2+}, Fe^{2+}, Co^{2+}, or Ni^{2+}) for activity (Thompson et al. 1998). Figure shows structure of 6-phospho-β-glucosidase from *Thermotoga maritime* in tetragonal form with manganese, NAD+ and glucose-6-phosphate (GH F-4), belonging to FAD/NAD(P)-binding Rossmann fold Superfamily (CL0063) (Fig. 4f).

These are redox enzymes containing a catalytic domain (provides substrate specificty) and Rossmann-fold domain (contains alpha-beta folds with central beta-sheet surrounded by 5 alpha-helices in order 654123; binds to NAD+), where NAD+ reversibly binds to hydride ion (lost/gained during redox process). Inter sheet

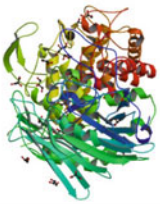
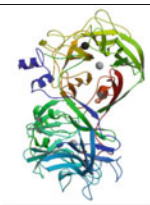
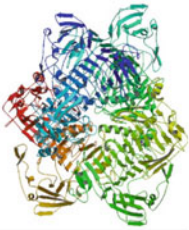
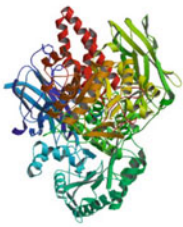
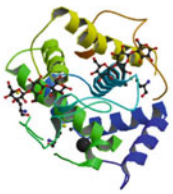
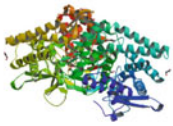
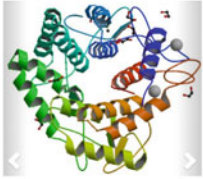
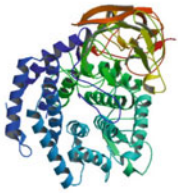
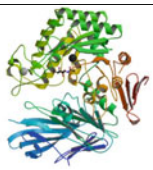
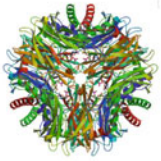
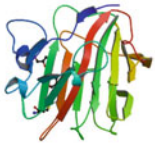
 n4. Crystal structure of *Thermoanaerobacterium xylolyticum* GH116 beta-glucosidase (GH F-116)	 *o4. Native crystal structure of BpGH117 (GH F-117)*	GH F-118 (No strcuture available)	GH F-119 (No strcuture available)
 p4. The complex structure of XylC with Tris (GH F-120)	GH F-121 (No strcuture available)	GH F-122 (No strcuture available)	 q4. Crystal structure of BvGH123 (GH F-123)
 r4. CtCel124: a cellulase from *Clostridium thermocellum* (GH F-124)	 s4. Crystal structure of an exo-alpha-1,6-mannosidase (bacova_03347) from *bacteroides ovatus* at 1.60 a resolution (GH F-125)	 *t4.* CPF_2247, a novel alpha-amylase from *Clostridium perfringens (*GH F-126*)*	 *u4.* Glycoside hydrolase BT_1003 GH F-127
GH F-128 (No strcuture available)	 v4. Alpha-N-acetylgalactosaminidase NagBb from *Bifidobacterium bifidum* - GalNAc complex (GH F-129)	 *w4.* Crystal structure of 4-O-beta-D-mannosyl-D-glucose phosphorylase MGP complexed with Man-Glc+PO4 (GH F-130)	 x4. Crystal structure of the catalytic domain of the glycoside hydrolase family 131 protein from *Coprinopsis cinerea GH F-131*

Fig. 4 (continued)

crossover of the stands in the sheet form the NAD+ binding site (Bashton and Chothia 2002) and in some distantly related Rossmann NAD+ is replaced by FAD. Clan has been buit by RD Finn.

Structure of GH F-4 C terminal domain belongs to LDH C-terminal domain-like superfamily (CL 0341). This superfamily includes the C-terminal domain of lactate/malate dehydrogenase as well as the C-terminal domain of the GH F-4.

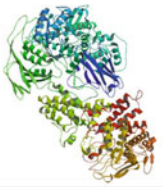
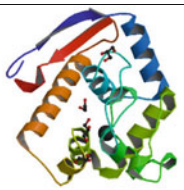
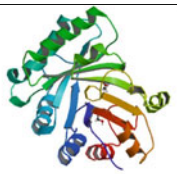
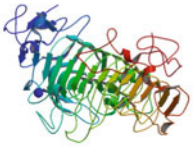
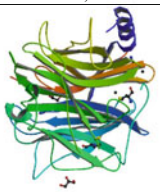
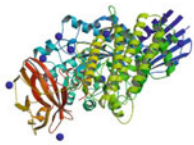
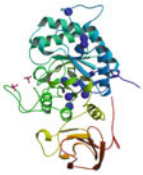
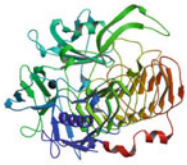
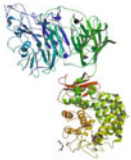
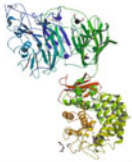
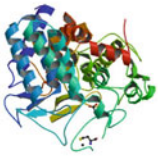
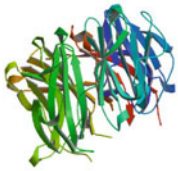
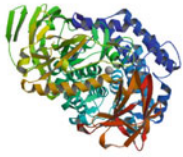
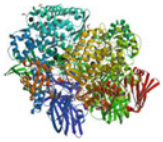
GH F-132 (No strcuture available)	 y4. Crystal Structure of the *Candida Glabrata* Glycogen Debranching Enzyme (GH F-133)	 z4. Structure of a beta-1,4-mannanase, SsGH134 (GH F-134*)*	 *a5.* Crystal Structure of *Aspergillus clavatus Sph3* (GH F-135)
 b5. Crystal structure of lacto-N-biosidase LnbX from *Bifidobacterium longum* subsp. longum, ligand-free form (GH F - 136)	 c5. Glycoside hydrolase BT_0996 (GH F-137)	 d5. BP0997, GH138 enzyme targeting pectin rhamnogalacturonan II (GH F138)	GH F-139 (No strcuture available)
 e5. Glycoside hydrolase BT_1012 (GH F-140)	 f5. Glycoside hydrolase BT_1002 (GH F-141)	 g5. Sialidase BT_1020 (GH F-142)	 h5.Sialidase BT_1020 (GH F-143)
 *i5.* Crystal structure of a putative glucoamylase (BACCAC_03554) from *Bacteroides caccae* ATCC 43185 at 2.05 A resolution (GH F-144)	 j5. Glycoside Hydrolase BACCELL_00856 (GH F-145)	 k5. Beta-L-arabinofuranosidase (GH F-146)	GH F-147 (No strcuture available)
GH F-148 (No strcuture available)	 l5. Bacterial beta-1,3-oligosaccharide phosphorylase from GH F- 149	GH F-150 (No strcuture available)	GH F-151 (No strcuture available)

Fig. 4 (continued)

Glycoside Hydrolase Family 5

They are membrane localized enzymes, basically involved in polysaccharides like cellulose and xylan and have been reported to be produced from fungi as well

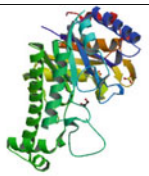
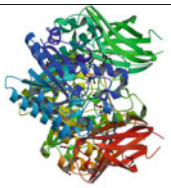
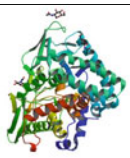
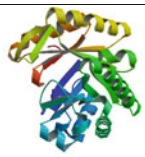
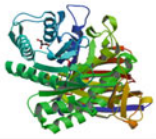
 m5. Recombinant thaumatin I at 0.9 Angstrom (GH F-152)	 n5. Structure of the PgaB (BpsB) glycoside hydrolase domain from *Bordetella bronchiseptica* (GH F-153)	GH F-154 (No strcuture available)	GH F-155 (No strcuture available)
 o5. Crystal structure of an inverting family GH156 exosialidase from uncultured bacterium pG7 (GH F-156)	GH F-157 (No strcuture available)	GH F-158 (No strcuture available)	GH F-159 (No strcuture available)
GH F-160 (No strcuture available)	GH F-161 (No strcuture available)	 p5. The apo-structure of endo-beta-1,2-glucanase from *Talaromyces funiculosus* (GH F-162)	GH F-163 (No strcuture available)
GH F-164 (No strcuture available)	GH F-165 (No strcuture available)	GH F-166 (No strcuture available)	 Fig. 4q5. Structure of the glycoside hydrolase domain of PelA from Pseudomonas aeruginosa (GH F-166)
 r5. Crystal structure of glucosidase from *Croceicoccus marinus* at 1.8 Angstrom resolution (GH F-Non classified)			

Fig. 4 (continued)

bacteria. This family comprises of enzymes namely endoglucanase (EC 3.2.1.4); beta-mannanase (EC 3.2.1.78); exo-1,3-glucanase (EC 3.2.1.58); endo-1,6-glucanase (EC 3.2.1.75); xylanase (EC 3.2.1.8); endoglycoceramidase (EC 3.2.1.123). Active site includes conserved glutamic acid residue (Henrissat

1991a; Py et al. 1991). Relationship between thermal stability and structural rigidity of members of GH F-5 enzymes have been reported after being studied through molecular dynamics (Bedieyan et al. 2012). It belongs to TIM Barrel Glycoside hydrolase Superfamily (CL0058) (Fig. 4g).

Glycoside Hydrolase Family 6

They were formerly known as Cellulase family B including endoglucanses (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91). They are alpha-beta proteins (Fig. 4h) having similar folds as that seen in triose phosphate isomerase. This was studied in 3D structure of cellobiohydrolase II (CBHII) from the fungus *Trichoderma reesei*, having active site located at C-terminal end of a parallel beta barrel present in an enclosed tunnel. Probable catalytic residues are two aspartic acid residues (positioned in center of tunnel) (Rouvinen et al. 1990).

Glycoside Hydrolase Family 7

Known activities of enzymes (formerly known as cellulase family C) classified under this group are endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91, end-acting cellobiohydrolase (EC 3.2.1.176); chitosanase (EC 3.2.1.132); endo-β-1,3-1,4-glucanase (EC 3.2.1.73) from eukaryotes catalyzing glycosidic bond using double-displacement mechanism. This mechanism helps in net retention of the conformation at the anomeric carbon. Endoglucanases cleaves the beta-1,4 linkages of cellulose and CBH cleaves off cellobiose disaccharide units from the reducing end of the chain. Their catalytic region is generally bound to cellulose binding domain (CBD) via a proline and/or hydroxy-amino acids rich linker region. In type I exoglucanases, the CBD domain is found at the C-terminal extremes (this domain forms a hairpin loop structure stabilized by 2 disulphide bridges). Active site contains glutamic acid residue as catalytic nucleophile/base as well as proton donor and catalysis progress through retaining mechanism (Divne et al. 1994; Mackenzie et al. 1998; Ducros et al. 2003; Klarskov et al. 1997; Sulzenbacher et al. 1997; Viladot et al. 1998).

GH F-7 enzymes belong to Concanavalin-like lectin/glucanase superfamily (contains 49 families and 153254 domains) (Bateman..). Conserved protein domain of endoglucanase and cellobiohydrolase are grouped under O-glycosyl hydrolases domain family (cd07999) contains a total of 95 families which includes enzymes of glycoside hydrolase family 7 as well.

Beta-jellyroll folded framework, containing two antiparallel beta-sheet packed face-to-face forming a curved beta-sandwich is the predominant three dimensional feature (Fig. 4i). These sandwich structures are extended along both the ends through several loops, resulting in an elongated assembly of ~50 Å, with a substrate binding structure running perpendicular to the β-strands of the inner, concave β-sheet. Some loops have short alpha-helical segments present at the periphery. Endoglucanases

specifically contains open substrate binding domain, while cellobiohydrolases have additional elongated loops that bend around the active site giving it a closed tunnel form. Some key studies include, boat confirmation required prior to hydrolysis (Sulzenbacher et al. 1997), presence of discrete glycoside binding subsites (Divne et al. 1998); product ejection mechanism during hydrolysis of cellulose also suggesting prior release of cellobiose products (Ubhayasekera et al. 2005); flexibility of sugar binding within tunnel of cellobiohydrolase (Parkkinen et al. 2008). Numerous commercial enzyme of this group is also available produced through Megazymes (Company).

Glycoside Hydrolase Family 8

These enzymes are of prokaryotic/eukaryotic origin belonging to clan GH-M of six hairpin glycosidase superfamily (CL0059). Enzymes classified under this superfamily share common structure composed of six helical hairpins and are chitosanase (EC 3.2.1.132), cellulase (EC 3.2.1.4), licheninase (EC 3.2.1.73), endo-1,4-β-xylanase (EC 3.2.1.8) and reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156). GH F-8 enzymes cleaves β-1,4 linkages in β-1,4 glucans, xylans (or xylooligosaccharides), chitosans, and lichenans (1,3-1,4-β-D-glucan). They are majorly endo acting with few having exo-activity. They were classified by hydrophobic cluster analysis, and was previously known as "Cellulase Family D" (Henrissat et al. 1989; Gilkes et al. 1991). These enzymes have inverting mechanism of action (Fierobe et al. 1993; Petersen et al. 2009). General acid (proton donor to the leaving group; located at N-terminal end of α4 helix), base of GH8a (proton acceptor from the nucleophilic water) and base of GH8b subfamily was first identified in CelA from *C. thermocellum* as Glu95 (Alzari et al. 1996; Adachi et al. 2004), CelA from *C. thermocellum* as Asp278 (Alzari et al. 1996) and chitosanase from *Bacillus* sp. K17 as Glu309 (based on its crystal structure and by making E309Q mutant) (Adachi et al. 2004) respectively. They are divided in three subfamilies based on positions of general base. Subfamily 'a' has Aspartate at the N-terminal end of α8 helix (cellulases, xylanases); 'b' has Asparagine, and the general base is a Glu residue located in a long loop inserted between α7 and α8 helices (chitinosases, lichenisases, cellulases). They have $(\alpha/\alpha)_6$ fold like Glycoside Hydrolase Family 48. Atomic (0.94 Å) resolution structure of CelA in complex with substrate (PDB ID 1kwf) and has been determined (Guérin et al. 2002; Honda and Kitaoka 2006; Béguin et al. 1985) (Fig. 4j).

Glycoside Hydrolase Family 9

These enzymes were earlier known as cellulase family E. Enzymes include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) (exoglucanases), or xylanases (EC 3.2.1.8) (Beguin, 1990; Gilkes et al. 1991). Enzymes from diverse group of organisms (fungi, bacteria, amaebozoa, invertebrate metazoan, ferns,

mosses, gymnosperms and angiopserms) are reported to belong to this family. They belong to six-hairpin glycosidase super family and major localized to plasma membrane (Fig. 4k).

Glycoside Hydrolase Family 10

Enzymes of this family were formerly known as cellulase family F and comprise of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) (exoglucanases), or xylanases (EC 3.2.1.8) activities. All family 10 xylanases hydrolyze the glycosidic bond in a double-displacement 'retaining' mechanism using two catalytic acidic residues, where one residue acts a nucleophile (base) and the other acts as a general acid/base (Fig. 4l). They undergo retaining type catalytic mechanism, with conserved glutamic acid residues acting as catalytic nucleophile/base and catalytic proton donor. Three dimensional structure has prominent $(\beta/\alpha)_8$ barrels.

Glycoside Hydrolase Family 11

This family was earlier known as cellulase family G (Henrissat and Bairoch 1996) and is monspecific, consisting of xylanases. They belong to Concanavalin-like lectin/glucanase superfamily (CL0004)**.** Major enzymes are xylanases from *Aspergillus awamori, Bacillus circulans, Clostridium stercorarium, Fibrobactor succinogenes* etc. Domains folds into a jelly roll shaped, and anti-parallel β strands bending almost 90 °C to produce a substrate binding grooves are characteristics of this family. Three dimensional structure has two catalytic Glu residues facing each other from opposite sides of the groove and catalysis is carried through double-displacement mechanism, with one Glu residue acting as a general acid/base catalyst and the other as a nucleophile. Two catalytically active conserved regions are centrally located on glutamic acid residues (studied in *Bacillus* pumilis, Lombard et al. 2014) (Fig. 4m).

Glycoside Hydrolase Family12

Family 12 comprises of enzymes Concanavalin-like lectin/glucanase superfamily (CL0004). These include endoglucanase (EC 3.2.1.4), xyloglucan hydrolase (EC 3.2.1.151), β-1,3-1,4-glucanase (EC 3.2.1.73) and xyloglucan endotransglycosylase (EC 3.2.1.207). These enzymes were formerly known as cellulase family H. Three dimensional structures has β-jelly roll and glutamic acid residues acts as catalytic site nucleophile/base and proton donor (Fig. 4n).

Glycoside Hydrolase Family 13

These are maltogenic alpha-amylase, catalyzing hydrolysis of (1-4)-alpha-D-glucosidic linkages in polysaccharides that helps in removal of successive alpha-maltose residues from the non-reducing ends of chains, during conversion of starch to maltose. Other enzymes in this family include neopullulanase, which hydrolyses pullulan to panose, and cyclomaltodextrinase, which hydrolyses cyclodextrins. They belong to TIM Barrel glycosyl hydrolase family (CL0058) (Fig. 4o,p).

Glycoside Hydrolase Family 14

Enzymes of this family have β-amylase (EC 3.2.1.2) activities. They belong to TIM Barrel glycoside hydrolase superfamily (CL0058) containing catalytic glutamic acid residues and have three highly conserved regions (Mikami et al. 1988; Friedberg and Rhodes 1988). First on N-terminal (contains catalytic aspartic acid, Nitta et al. 1989) and second conserved domain is centrally located (catalytic glutamic acid, Totsuka et al. 1994) (Fig. 4q). Various three dimensional structures of enzymes belonging to this family have been studied; like soyabean beta-amylase with an inhibitor, alpha-dextrin etc (Table 1).

Glycoside Hydrolase Family 15

Major enzymes of family 15 are glucoamylase (EC 3.2.1.3; catalyze release of D-glucose from the non-reducing end of starch and other oligo and polysaccharaides); alpha-glucosidase (EC 3.2.1.20) and glucodextranase (EC 3.2.1.70). Glucoamylases have three closely clustered acidic conserved residues at catalytic site (Sierks et al. 1990 and Ohnishi et al. 1992) and 3-D structure have been reported to belong to mainly alpha-class and contains 19 helices and 9 strands (Aleshin et al. 1994). They belong to Six- hairpin glycosidase superfamily (Fig. 4r).

Glycoside Hydrolase Family 16

Enzymes of family 16 belong to Concanavalin-like lectin/glucanase superfamily (CL0004) and are group with functionally heterogenous members, like lichenase (EC 3.2.1.73); xyloglucan xyloglucosyltransferase (EC 2.4.1.207); agarase (EC 3.2.1.81); kappa-carrageenase (EC 3.2.1.83); endo-beta-1,3-glucanase (EC 3.2.1.39); endo-beta-1,3-1,4-glucanase (EC 3.2.1.6); endo-beta-galactosidase (EC 3.2.1.103). All these enzymes have a common ancestor and have diverged significantly in their primary sequences (Fig. 4s).

Catalytic domain have this family has sandwich-like β-jelly roll fold formed by two anti-parallel beta sheet that are closely packed. All enzymes of GH F-16 feature a common catalytic motif E-[ILV]-D-[IVAF]-[VILMF](0,1)-E. The two glutamic

Table 1 Glycoside hydrolase superfamilies

Sl no:	Super family	Clan number	Clans included	Family	Number of families	Number of domains
1	TIM Barrel glycoside hydrolase superfamily	CL0058	GH-A, GH-D, GH-H and GH-K, GH-R	GH F-1, GH F-2 TIM Barrel Domain, GH F-3 N terminal domain; GH F-5; GH F-10; GH F-13; GH F-14; GH F-17; GH F-18, GH F-20; GH F-25; GH F-26; GH F-27; GH F-29; GH F-30; GH F-31; GH F-35; GH F-36; GH F-39; GH F-42; GH F-44; GH F-50; GH F-51; GH F-53; GH F-56; G F-59; GH F-66; GH F-70; GH F-72; GH F-77; GH F-79; GH F-85; GH F-86; GH F-89; GH F-97; GH F-101; GH F-107; GH F-113; GH F-128; GH F-147; GH F-148; GH F-157; GH F-158	57	259156
2	Galactose binding domain like superfamily	CL0202		GH F-2 Sugar Binding Domain	70	124105
3	FAD/NAD(P)-binding Rossmann fold superfamily	CL0063		GH F-4	204	2203085
4	LDH C-terminal domain-like superfamily	CL0341		GH F-4 C Terminal Domain	2	17217
5	–	–		GH F-6	–	1 (PF01341)
6	Concanavalin-like lectin/ glucanase superfamily	CL0004		GH F-7; GH F-11; GH F-12; GH F-16	49	153254

(continued)

Table 1 (continued)

Sl no:	Super family	Clan number	Clans included	Family	Number of families	Number of domains
7	Six-hairpin glycosidase superfamily	CL0059	GH-L, GH-M and GH-G	GH F-8; GH F-9, GH F-15; GH F-37; GH F-47; GH F-48; GH F-63; GH F-65 (central catalytic domain); GH F-76; GH F-78; GH F-88; GH F-100; GH F-125	29	105072
8	Lysozyme-like superfamily	CL0037	GH-I	GH F-19; GH F-22, GH F-24; GH F-46; GH F-73; GH F-108	19	71609
9	Pectate lyase-like beta helix superfamily	CL0268	GH-N; GH-I	GH F-28; GH F-49; GH F-80; GH F-92	29	92337
10	Beta-fructosidase/ Furanosidase superfamily	CL-0143	GH-J, GH-F	GH F-32; GH F-62; GH F-68	9	30592
11	Sialidase superfamily	CL0434	GH-E	GH F-33, GH F-34; GH F-83; GH F-93	16	21224
12	Glycoside hydrolase/ deacetylase superfamily	CL0158		GH F-38 (N-terminal domain); GH F-57	11	48380
13	Galactose mutarotase like superfamily	CL0103		GH F-38 (C-terminal domain); GH F-65 (N-Terminal domain)	31	78752
14	Class-I Glutamine amidotransferase superfamily	CL0014		GH F-42 (Trimerisation domain)	18	138858
15	Glycosyl hydrolase domain superfamily	CL0369		GH F-42 (C-terminal Domain)	39	76165
16	Double Psi beta barrel glucanase superfamily	CL0199		GH F-45	5	16903
17			GH-O	GH F_52; GH F-116		

(continued)

Table 1 (continued)

Sl no:	Super family	Clan number	Clans included	Family	Number of families	Number of domains
18			GH-Q	GH F-94; GH F-149; GH F-161		
19			GH-P	GH F-127; GH F-146		

Clan and Superfamily not known for GH F-40, GH F-41, GH F-54, GH F-55, GH F-58, GH F-60; GH F-61; GH F-64; GH F-67; GH F-69; GH F-71; GH F-74, GH F-75; GH F-81; GH F-82; GH F-84; GH F-87; GH F-90; GH F-91; GH F-95; GH F-96; GH F-98; GH F-99; GH F-102; GH F-103; GH F-104; GH F-105; GH F-106; GH F-109; GH F-110; GH F-111; GH F-112; GH F-114; GH F-115; GH F-117; GH F-118; GH F-119; GH F-120; GH F-121; GH F-122; GH F-123; GH F-124; GH F-126; GH F-129; GH F-130; GH F-131; GH F-132; GH F-133; GH F-134; GH F -135; GH F-136; GH F-137; GH F-138; GH F-139; GH F-140; GH F-141; GH F-142; GH F-143, GH F-144; GH F-145; GH F-150; GH F-151; GH F-152; GH F-153; GH F-154; GH F-155; GH F-156; GH F-159; GH F-160; GH F-162; GH F-163; GH F-164; GH F-165; GH F-166; GH F-Non classified

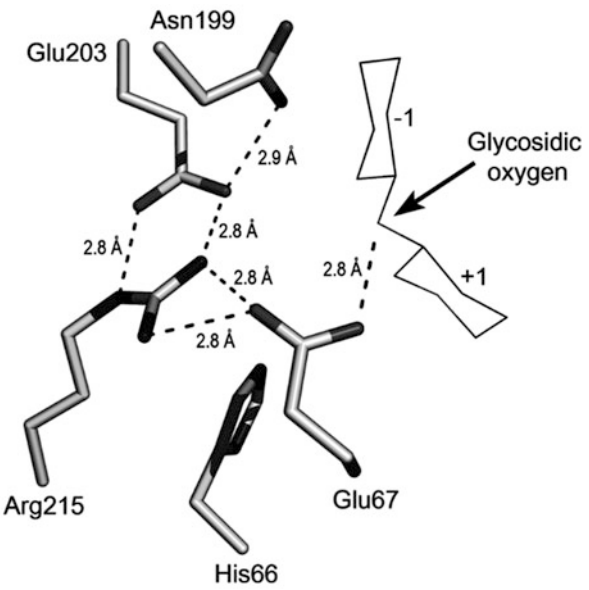


Fig. 5 Active site of glycoside hydrolase family 19 papaya chitinase (PDB: 3cql). Conserved residues are shown surrounding the catalytic acid, Glu67. A GlcNAc2 unit binding in the -1 and +1 subsites is shown in narrow stick representation and an arrow indicates the position of the glycosidic oxygen (Eijsink et al. 2010)

acid residues in the conserved motif act as nucleophile and base in the catalytic reaction and aspartic acid residue maintains relative position of catalytic amino acids (Allouch et al. 2003; Michel et al. 2001) (Fig. 5).

Glycoside Hydrolase Family 17

This family comprise of enzymes endo-1,3-beta-glucosidase (EC 3.2.1.39); lichenase (EC 3.2.1.73); exo-1,3-glucanase (EC 3.2.1.58) and have been reported only in eukaryotes namely plants and fungi. Central section has the conserved region, containing tryptophan residue, which is involved in interaction with the glucan substrate (Ori et al. 1990). It also contains a glutamate residue acting as nucleophile in the catalytic reaction (Varghese et al. 1994) (Fig. 4t).

Glycoside Hydrolase Family 18

Chitinase class II group, including chitinase and chitodextrinase are enzymes belonging to this family, falling under TIM-barrel Glycoside hydrolase superfamily (CL0058) and located on endoplasmic reticulum membrane. They majorly catalyze hydrolysis of chitin oligosaccharides. This family also includes chitinase like protein that binds to chitin but does not hydrolyse it, and also varied glycoproteins (from mammals, cartilage and oviduct specific) (Fig. 4u).

Glycoside Hydrolase Family 19

Like GH F-18, this family also has enzymes only with chitinase activity [EC 3.2.1.14], catalyzing hydrolysis of beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers (Flach et al. 1992). These enzymes also known as class IA/I and IB/II are of plant origin functioning as defense tool against fungal and insect pathogen. Catalytic domain of these enzymes consists of 220-230 residues (Henrissat 1991b) of which class IA/I has an extra N-terminal chitin domain. There are two highly conserved regions on this enzyme, of which one is located N-terminal section (containing cysteines involved in disulphide bond formation). They belong to Lysozyme like superfamily having structurally invariant core consisting of two-helices and a three stranded beta sheet that forms substrate binding and catalytic cleft (Monzingo et al. 1996) (Fig. 4v).

Glycoside Hydrolase Family 20

Beta-hexosaminidase (EC 3.2.1.52); lacto-N-biosidase (EC 3.2.1.140) are major enzymes of this family belonging to TIM-Barrel glycosyl hydrolase superfamily (CL0058). Catalytic nucleophile/base of these enzymes are carbonyl oxygen of the C-2 acetamide group. Beta-hexosaminidase degrade GM2 gangliosides by hydrolysing the terminal non-reducing N-acetyl-D-hexoamine residues. This enzyme is found in three forms; hexosaminidase A is a trimer, with one alpha, one beta-A and one beta-B chain; hexosaminidase B is a tetramer of two beta-A and two beta-B chains; and hexosaminidase S is a homodimer of alpha chains (Fig. 4w,x).

Glycoside Hydrolase Family 21

There is no entry of enzyme been classified under family 21 of glycoside hydrolases as per source of cazypedia.org and a detailed study of enzymes to be classified under this family is required.

Glycoside Hydrolase Family 22

Lysozyme type C (EC 3.2.1.17) lysozyme type I (EC 3.2.1.17) and alpha-lactalbumins are enzymes classified as GH Family 22. Specifically based on activities they belong to Lysozyme like superfamilies and the catalytic nucleophile/base for the same is aspartate and/or carbonyl oxygen of the C-2 acetamido group. Five different classes of lysozymes are known; namely C (chicken) type, G (goose) type, Phage type, fungal and bacterial and all these exhibit minor sequence similarities. Though being functionally divergent, primary sequence and structure of lysozyme type C and alpha-lactalbumin are very similar, reflecting their common origin (Nitta and Sugai 1989) where approximately 40% of residues and 4 disulphide bonds are conserved. All lactalbumins and few lysozymes have the property of binding to calcium while catalysis (Nitta et al. 1987; Stuart et al. 1986) in order to obtain a stable structure. Catalytic site of these enzymes have three aspartic acid residues (Fig. 4y).

Glycoside Hydrolase Family 23

Lysozyme type G (EC 3.2.1.17) and chitinase (EC 3.2.1.14) are activities of enzymes of glycoside hydrolase family 23. This family also include peptidoglycan lyase (EC 4.2.2.n1) also known as peptidoglycan lytic transglycosylase. Glutamic acid is the catalytic proton donor, mechanism and catalytic nucleophile/base is not known. Enhanced and elaborate studies are still under progress (Fig. 4z).

Glycoside Hydrolase Family 24

Lysozymes (EC 3.2.1.17) are the activity reported in this group and enzymes belong to Lysozyme like superfamily. Lambda phage lysozyme and *Escherichia coli* endolysin (Weaver and Matthews 1987) are included in this family. These enzymes have inverting mechanism of action and glutamic acid acts as a catalytic proton donor. Three dimensional structure of the protein has both alpha helices and beta sheets (Fig. 4a1).

Glycoside Hydrolase Family 25

Family 25 also comprises of enzymes with lysozyme activity (EC 3.2.1.17; cell wall lytic enzymes) (Henrissat 1991b; Croux and García 1991), with retaining mode of catalytic mechanism where aspartic acid is catalytic nucleophile or base and glutamic acid is the proton donor. DxE is a conserved active site motif, helping in catalysis by formation of oxazoline intermediate. Aspartate residue initially protonates the leaving facilitating its departure and subsequently acts as a general base to activate the hydrolytic water molecule; and the glutamic residue stabilized or deprotonated the oxazoline N- atom. Aspartic acid at sixth position might be involved in catalysis with the glutamic acid at DxE motif, provided the hydrolysis has taken place via net inversion of anomeric configuration, with the aspartic acid residue acting as general base activating the nucleophilic water molecule and glutamic acid of DxE motif acting as general acid and protonating the departing oxygen atom in a concerted fashion as the bonds cleaves (Rau et al. 2001; Korczynska et al. 2010; Hermoso et al. 2003; Al-Riyami et al. 2016). Three dimensional structure consists of $(\beta/\alpha)_5$ $(\beta)_3$ TIM-like domain and thus they classified under TIM Barrel glycoside hydrolase super family. Majorly studied enzymes include lysozymes (lysine) from *Streptococcus pneumoniae* bacteriophages of the Cp family, lysozyme (endolysin) from Lactococcus delbrueckii phage mv1, autolytic lysozyme from *Clostridium acetobutylicum*, lysozyme M1 from *Streptomyces globisporus* and N,O-diacetylmuramidase (lysozyme ch) from the fungus *Chalaropsis* (Fig. 4b1).

Glycoside Hydrolase Family 26

Enzymes in this group has activities of mannanase (EC 3.2.1.78) and β-1,3-xylanase (EC 3.2.1.32) of which main enzyme is Mannan endo-1,4-beta-mannosidase catalyzing specific hydrolysis of mannan and galactomannan and showing very little hydrolysis towards 1,4-beta-D-linkages in mannans, galacto-mannans, glucomannans and galactoglucomannans (Braithwaite et al. 1995) (Fig. 4c1).

Glycoside Hydrolase Family 27

Alpha-galactosidases (Melibiase) (EC 3.2.1.22) (Dey and Pridham 1972) α-N-acetylgalactosaminidase (EC 3.2.1.49); isomalto-dextranase (EC 3.2.1.94); β-L-arabinopyranosidase (EC 3.2.1.88); galactan: galactan galactosyltransferase (EC 2.4.1.-), are major activities of GH Family 27 enzymes, catalyzing hydrolysis of melibiose to glucose and galatose. Together with members of family 11 and 36, these enzymes form clan GH-D (alpha-galactosidase; alpha-N-acetylgalactosaminidases, and isomaltodextranases); belonging to TIM Barrel glycoside hydrolase superfamily. NAD and magnesium acts as cofactors for few enzymes of prokaryotic origin. Mode of action for these enzymes is retaining type,

where aspartic acid is catalytic nucleophile and base as well as proton donor. These enzymes find mechanistic commonality with family GH36 demonstrated by Comfort et al. 2007 (Fig. 4d1).

Glycoside Hydrolase Family 28

Activities of enzymes belonging to this group includes polygalacturonase (EC 3.2.1.15); α-L-rhamnosidase (EC 3.2.1.40); exo-polygalacturonase (EC 3.2.1.67); exo-polygalacturonosidase (EC 3.2.1.82); rhamnogalacturonase (EC 3.2.1.171); rhamnogalacturonan α-1,2-galacturonohydrolase (EC 3.2.1.173); xylogalacturonan hydrolase (EC 3.2.1.-); all these enzymes have inverting mechanism for catalysis, with aspartic acid residue as catalytic nucleophile/base and catalytic proton donor. Three dimensional structures have prominent beta- helices. These enzymes belong to pectate-lyase like beta-helix Superfamily. Main feature of this superfamily is presence of right handed beta helix similar to first found in pectate lyase (Jenkins et al. 1998). Polygalatorunoase/pectinase catalyzes hydrolysis of 1,4-alpha-D-galactosiduronic linkages in pectate/galacturonans (Ruttkowski et al. 1990). Exo-poly-alpha-D-galacturonosidase (EC 3.2.1.82) (exoPG) hydrolyzes peptic acid from the non-reducing end, releasing digalacturonate (He and Collmer 1990) (Fig. 4e1).

Glycoside Hydrolase Family 29

Alpha-L-fucosidase (EC 3.2.1.51) and α-1,3/1,4-L-fucosidase (EC 3.2.1.111) are major activities of this family, belonging to the TIM barrel glycoside hydrolase superfamily, these emzymes have retaining mechanism of action and has hexamer structure with $(\beta/\alpha)_8$ domain observed in their three dimensional structures. Aspartic acid and glutamic acid acts as catalytic nucleophile base and proton donor, respectively (Fisher and Aronson 1989; Sulzenbacher et al. 2004). Alpha-L-fucosidase catalyze hydrolysis of alpha-1,6-linked fucose joined to the reducing-end N-acetylglucosamine (glycoproteins) (Fig. 4f1).

Glycoside Hydrolase Family 30

Endo-β-1,4-xylanase (EC 3.2.1.8); β-glucosidase (3.2.1.21); β-glucuronidase (EC 3.2.1.31); β-xylosidase (EC 3.2.1.37); β-fucosidase (EC 3.2.1.38); glucosylceramidase (EC 3.2.1.45); β-1,6-glucanase (EC 3.2.1.75); glucuronoarabinoxylan endo-β-1,4-xylanase (EC 3.2.1.136); endo–β1,6-galactanase (EC:3.2.1.164); [reducing end] β-xylosidase (EC 3.2.1.-) are major activities of enzymes belonging to glycoside hydrolase family 30. These include enzymes of mammalian origin (Dinur et al. 1986). They follow retaining type mechanism of

action, with glutamic acid as catalytic nucleophile/base as well as proton donor. Three dimensional structure has $(\beta/\alpha)_8$ domains (Fig. 4g1).

Glycoside Hydrolase Family 31

Alpha-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22); α-mannosidase (EC 3.2.1.24); α-1,3-glucosidase (EC 3.2.1.84); sucrase-isomaltase (EC 3.2.1.48) (EC 3.2.1.10); α-xylosidase (EC 3.2.1.177); α-glucan lyase (EC 4.2.2.13); isomaltosyltransferase (EC 2.4.1.-); oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161); sulfoquinovosidase (EC 3.2.1.-) are activities of enzymes of family 31 of glycoside hydrolases, with retaining mechanism of action and aspartic acid residues as nucleophile/base as well proton donor, these enzymes have been reported to be of archaea, eukaryotes, prokaryotes (bacteria) origin (Kinsella et al. 1991; Naim et al. 1991; Hermans et al. 1991). These enzymes belong to TIM barrel glycoside hydrolase superfamily clan-D (Fig. 4h1).

Glycoside Hydrolase Family 32

Invertase (EC 3.2.1.26); endo-inulinase (EC 3.2.1.7); β-2,6-fructan 6-levanbiohydrolase (EC 3.2.1.64); endo-levanase (EC 3.2.1.65); exo-inulinase (EC 3.2.1.80); fructan β-(2,1)-fructosidase/1-exohydrolase (EC 3.2.1.153); fructan β-(2,6)-fructosidase/6-exohydrolase (EC 3.2.1.154); sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99); fructan:fructan 1-fructosyltransferase (EC 2.4.1.100); sucrose:fructan 6-fructosyltransferase (EC 2.4.1.10); fructan:fructan 6G-fructosyltransferase (EC 2.4.1.243); levan fructosyltransferase (EC 2.4.1.-); [retaining] sucrose:sucrose 6-fructosyltransferase (6-SST) (EC 2.4.1.-); cycloinulo-oligosaccharide fructanotransferase (EC 2.4.1.-) forms major activities of enzymes of glycoside hydrolase family 32, with retaining mechanisms of action and fivefold β-propeller in their 3-D structure (Alberto et al. 2004), has aspartic acid and glutamic acid residue at their active site that serves as nucleophile/base and proton donor respectively. They belong to clan GH-J of beta-fructosidase/furanosidase superfamily (composed of glycoside hydrolase enzymes having five-bladed beta-propeller fold, built by J Mistry) (Hettwer et al. 1998; Nurizzo et al. 2002a) (Fig. 4i1, j1).

Glycoside Hydrolase Family 33

Sialidase or neuraminidase (EC 3.2.1.18); trans-sialidase (EC 2.4.1.-); anhydrosialidase (EC 4.2.2.15); Kdo hydrolase (EC 3.2.1.-); 2-keto-3-deoxynononic acid hydrolase/KDNase (EC 3.2.1.-) are enzyme activities of family 33.ave. They catalyze through retaining mode of activity and have sixfold β-propeller domain as found in 3-D structure. Tyrosine and glutamic acid acts as catalytic nucleophile/base (Rothe et al. 1991; Luo et al. 1998) (Fig. 4k1).

Glycoside Hydrolase Family 34

Sialidase or neuraminidase (EC 3.2.1.18) are activities of enzymes of glycoside hydrolase family 34, with retaining catalytic mechanism, tyrosine and glutamic acid serves as nucelophile/base, these enzymes have sixfold-beta propellers. They belong to Sialidase superfamily (CL0434). Neuraminidases cleave the terminal sialic acid (negatively charged sugars associated with proteins and lipids portion of the lipoproteins) residues from carbohydrate chains in glycoproteins. These enzymes have been extensively studied and have been widely reported to act as endo and exo-enzymes (Kim et al. 2013) (Fig. 4l1).

Glycoside Hydrolase Family 35

Beta-galactosidase (EC 3.2.1.23); exo-β-glucosaminidase (EC 3.2.1.165); exo-β-1,4-galactanase (EC 3.2.1.-); β-1,3-galactosidase (EC 3.2.1.-), are major enzymes of this group, with retaining mode of action they belong to clan GH-A, where glutamic acid residue acts as catalytic nucleophile/base and proton donor and the three dimensional structure of these enzymes have $(\beta/\alpha)_8$ barrels. They are reported to be produced from various sources including mammals, fungi, plants and bacteria (Fig. 4m1).

Glycoside Hydrolase Family 36

Alpha-galactosidase (EC 3.2.1.22); α-N-acetylgalactosaminidase (EC 3.2.1.49); stachyose synthase (EC 2.4.1.67); raffinose synthase (EC 2.4.1.82) are prominent activities of the family with retaining mechanism of catalysis, they belong to GH-D, containing $(\beta/\alpha)_8$ motif. Aspartic acid residues in the conserved domain work as nucleophile/base and proton donor. They show similarity in catalytic mechanism as glycoside hydrolase family 27 (Fig. 4n1).

Hydrolysis of melibiose into galactose and glucose is done by alpha-galactosidase, prokaryotic enzyme requires NAD and magnesium as co-factor. Glycoside hydrolase family 36 have been subdivided into 11 families GH36A to GH36K (Dey and Pridham 1972; Aslanidis et al. 1989; Wang et al. 1990; Peterbauer et al. 2002; Naumoff 2011).

Glycoside Hydrolase Family 37

Alpha,α-trehalase (EC 3.2.1.28) is the activity of the enzymes of this family, with inverting mode of catalytic mechanism and conserved glutamic and aspartic acid acting as nucleophile/base an proton donor they belong clan GH-G, of six-hairpin glycosidase superfamily. The three dimensional structure of same contains $(\alpha/\alpha)_6$

(Kopp et al. 1993). Trehalases catalyses degradation of disaccharide α,α-trehalose yielding two-glucose subunits (Fig. 4o1).

Glycoside Hydrolase Family 38

Alpha-mannosidase (EC 3.2.1.24); mannosyl-oligosaccharide α-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide α-1,3-1,6-mannosidase (EC 3.2.1.114); α-2-O-mannosylglycerate hydrolase (EC 3.2.1.170); mannosyl-oligosaccharide α-1,3-mannosidase (EC 3.2.1.-) are major activities of enzymes of the group with a prominent $(\beta/\alpha)_7$ domain in their three-dimensional structure. Aspartic acid residue at the catalytic site acts as nucelophile/base and these enzymes show retaining mode of catalytic mechanism. They carry catalysis of N-linked carbohydrates released during glycoprotein turnover, catalyzing hydrolysis of terminal, non-reducing alpha-D mannose residues in α-D-mannosides and are localized in plasma membrane and golgi membrane. N-terminal domain of these enzymes belongs to glycoside hydrolase/deacetylase superfamily (CL0158) and C-terminal domain is categorized in Galactose Mutarotase-like superfamily (CL0103). Both the clans were built by A Bateman. Clan CL0158 contains diverse carbohydrate catalysing enzymes including hydrolases and deacetylases (Heikinheimo et al. 2003). Clan CL0103 is group of enzymes composed of beta-sandwich acting on sugar substrate and can be observed in any location (eg., domain 5 of beta-galatosidase, central domain of copper amine oxidase; c-terminal of chondroitinase and hyaluronate lyase, N terminal of maltose phosphorylase and galactose mutarotase) (Thoden and Holden 2002) (Fig. 4p1, q1, r1).

Glycoside Hydrolase Family 39

Alpha-L-iduronidase (EC 3.2.1.76); β-xylosidase (EC 3.2.1.37), are activities of the family. These enzymes belong to TIM-barrel glycoside hydrolase superfamily (CL0058) with prominent $(\beta/\alpha)_8$ motif in its three dimensional structure. They follow retaining type catalytic mechanism with conserved glutamic acid residue working as nucleophile/base and proton donor (Henrissat and Bairoch 1996; Henrissat et al. 1995) (Fig. 4s1).

Glycoside Hydrolase Family 40

This family does not have specified studied enzymes classified under its group. No data available through CAZypedia.

Glycoside Hydrolase Family 41

This family does not have specified studied enzymes classified under its group. No data available through CAZypedia.

Glycoside Hydrolase Family 42

Beta-galactosidase (EC 3.2.1.23); α-L-arabinopyranosidase (EC 3.2.1.-) are activities of enzymes of this family. They undergo retaining type catalytic mechanism with conserved glutamic acid residue acting as nucleophile/base and proton donor. Three dimensional structure has $(\beta/\alpha)_8$ barrels. They share domains with three superfamilies (beta-galactosidase, beta-galactosidase trimerisation domain and beta-galactosidase C-terminal domain belongs to TIM-barrel glycoside hydrolase, Class I glutamine amidotransferase and glycoside hydrolase domain superfamily respectively). These enzymes catalyze hydrolysis of terminal and non-reducing terminal beta-D-galactosidase residues (Shimizu et al. 1995). Class I glutamine amidotransferase superfamily contains glutaminase enzymes and also the members of DJ-1/PfpI family (peptidases, catalytic triad Cys-His-Glu). This clan was built by A Bateman. Class I glutamine amidotransferase superfamily also has Cys-His-Glu triad, but differs from that of peptidases PfpI. Glycoside hydrolase domain superfamily includes the C-terminal domain of sugar-lytic enymes and was also developed by A Bateman (Fig. 4t1, u1, v1).

Glycoside Hydrolase Family 43

This family consists of numerous enzymes namely with activities β-xylosidase (EC 3.2.1.37); α-L-arabinofuranosidase (EC 3.2.1.55); xylanase (EC 3.2.1.8); α-1,2-L-arabinofuranosidase (EC 3.2.1.-); exo-α-1,5-L-arabinofuranosidase (EC 3.2.1.-); [inverting] exo-α-1,5-L-arabinanase (EC 3.2.1.-); β-1,3-xylosidase (EC 3.2.1.-); [inverting] exo-α-1,5-L-arabinanase (EC 3.2.1.-); [inverting] endo-α-1,5-L-arabinanase (EC 3.2.1.99); exo-β-1,3-galactanase (EC 3.2.1.145); β-D-galactofuranosidase (EC 3.2.1.146). They exhibit inverting mode of catalytic mechanism with aspartic acid and glutamic acid as catalytic nucleophile/base and proton donor respectively. Fivefold β-propellers are prominent feature of three-dimensional structure with long V-shaped groove forming a single extended substrate binding surface across the face of propeller. These enzymes belong to beta-fructosidase superfamily and have membrane localization (Nurizzo et al. 2002b) (Fig. 4w1).

Glycoside Hydrolase Family 44

Endoglucanase (EC 3.2.1.4) and xyloglucanase (EC 3.2.1.151) forms major activities of this family with retaining mechanism of catalytic activity and glutamic acid at conserved domain acting as nucleophile/base and proton donor. They belong to TIM barrel glycoside hydrolase superfamily (CL0058) and has $(\beta/\alpha)_8$ domain in its three-dimensional structure (Kitago et al. 2007; Ariza et al. 2011). This family was formerly known as cellulase family J (Fig. 4x1).

Glycoside Hydrolase Family 45

Endoglucanase (EC 3.2.1.4); xyloglucan-specific endo-β-1,4-glucanase/endo-xyloglucanase (EC 3.2.1.151); endo-β-1,4-mannanase (EC 3.2.1.78) are prominent activities of enzymes of this group and were formerly known to classified under cellulase family K. They exhibit inverting mode of catalytic reaction, with conserved aspartic acid residues (conserved N-terminal domain with several cysteines involved in forming disulphide bonds, referred as signature sequence) working as nucelophile/base and proton donor (Davies et al. 1993). Enzymes of this family belong to Double Psi beta barrel glucanase superfamily (CL0199). This clan represents barwin like barrels and was developed by A Bateman (Mizuguchi et al. 1999; Castillo et al. 1999). Examples of enzymes of this group includes endoglucanase 5 from *Humicola insolens*, *Trichoderma reesei*¸ endoglucanase K from *Fusarium oxysporum* etc (Fig. 4y1).

Glycoside Hydrolase Family 46

Chitosanases (EC 3.2.1.132) is activity of enzymes of this group, they belong to lysozyme like superfamilies (CL0037) and have inverting mode of chitinase activity, with aspartic acid and glutamic acid at probable catalytic conserved site acting as nucleophile/base and proton donor, respectively. Chitosanase catalyze the endohydrolysis of beta 1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partly acetylated chitosan (Fig. 4z1).

Glycoside Hydrolase Family 47

Alpha-mannosidase (EC 3.2.1.113) is activity of enzymes found in this family and these protein have $(\alpha/\alpha)_7$ domain in their three dimensional structure with conserved glutamic acid acting as catalytic proton donor. Alpha-mannosidase catalyzes maturation of Asn-linked oligosaccharides (Lal et al. 1994). Hydrolysis of terminal 1,2-linked alpha-D-mannose residues into oligo-mannose oligosaccharide man(9)(glcnac)(2) is calcium dependent process and mannose residues get trimmed to produce man(8)glcnac(2) followed by man(5)(glcnac)(2) structures. They are

prominently located in endoplasmic reticulum membrane, golgi membrane and cell membrane (Fig. 4a2).

Glycoside Hydrolase Family 48

Reducing end-acting cellobiohydrolase (EC 3.2.1.176); endo-β-1,4-glucanase (EC 3.2.1.4); chitinase (EC 3.2.1.14) are major enzymes of the group of the family (formerly known as cellulase family L). Conserved glutamic acid residues act as catalytic proton donor with the enzyme exhibiting inverting catalytic mechanism. Three dimensional structure has $(\alpha/\alpha)_6$ barrels, these enzymes belong to six-hairpin glycosidase superfamily (CL0059) (Te'o et al. 1995) (Fig. 4b2).

Glycoside Hydrolase Family 49

Dextranase (EC 3.2.1.11); isopullulanase (EC 3.2.1.57); dextran 1,6-α-isomaltotriosidase (EC 3.2.1.95); sulfated arabinan endo-1,4-β-L-arabinanase (EC 3.2.1.-) and catalyze hydrolysis of alpha-1,6-glycosidic bonds in dextran polymers, through catalytic mechanism with conserved aspartic acid residues acting as catalytic nucleophile/base and proton donor. They belong to Pectate lyase-like beta helix with prominent beta helix seen in three dimensional structures (Fig. 4c2).

Glycoside Hydrolase Family 50

Beta-agarase (EC 3.2.1.81) is the activity reported for this group. These enzymes are less explored and probably follow retaining mode of activity with glutamic acid residues as nucleophile/base and proton donor. They belong to clan GH-A of Tim barrel glycoside hydrolase superfamily. Few bacterial enzymes of the group are reported (Fig. 4d2).

Glycoside Hydrolase Family 51

Endoglucanase (EC 3.2.1.4); endo-β-1,4-xylanase (EC 3.2.1.8); β-xylosidase (EC 3.2.1.37); α-L-arabinofuranosidase (EC 3.2.1.55); lichenase/endo-β-1,3-1,4-glucanase (EC 3.2.1.73) are major enzymes of the group with retaining mode of action, and glutamic acid residues at catalytic conserved site acting as nucleophile/base and proton donor. These enzymes belong to Clan GH-A of TIM-barrel glycoside hydrolase superfamily and have $(\beta/\alpha)_8$ motif in their three dimensional structure (Fig. 4e2).

Glycoside Hydrolase Family 52

Beta-xylosidase (EC 3.2.1.37) is the activity of enzymes of this group, having retaining mode of catalytic mechanism, with glutamic acid and aspartic acid residues on conserved domains acting as nucelophile/base and proton donor, respectively. They belong to clan GH-O with $(\alpha/\alpha)_6$ barrels in their three dimensional structure. These enzymes are reported to be from *Bacillus stearothermophilus*, functioning in xylan degradation through hydrolysis of 1,4- beta-D-xylans, through successive removal of D-xylose residues from non-reducing termini. Alongside it also carries out hydrolysis of xylobiose (Baba et al. 1994) (Fig. 4f2).

Glycoside Hydrolase Family 53

Endo-β-1,4-galactanase (EC 3.2.1.89) are major activities of family 53 enzymes. They belong to clan GH-A of TIM barrel glycoside hydrolase superfamily with $(\beta/\alpha)_8$ barrels in their three dimensional structure. They undergo retaining type catalytic mechanism with glutamic acid as catalytic nucleophile/base and proton donor (Ryttersgaard et al. 2002) (Fig. 4g2).

Glycoside Hydrolase Family 54

Alpha-L-arabinofuranosidase (EC 3.2.1.55); β-xylosidase (EC 3.2.1.37) are activities of enzymes of the group. They show retaining mode of catalytic mechanism. This group of enzymes is very less explored and needs detailed study (Fig. 4h2).

Glycoside Hydrolase Family 55

Exo-β-1,3-glucanase (EC 3.2.1.58); endo-β-1,3-glucanase (EC 3.2.1.39) are activities of enzymes belonging to this family and have inverting mode of catalytic mechanism. These enzymes have prominent beta-helix in their three dimensional structure and conserved glutamic acid residue is known to be catalytic proton donor. This group of enzyme is less explored and classified is not available (Fig. 4i2).

Glycoside Hydrolase Family 56

Hyaluronidase (EC 3.2.1.35); chondroitin hydrolase (EC 3.2.1.-) are activities of enzymes of this family (eg., venom of *Apis mellifera*; Gmachl and Kreil 1993). They belong to TIM-barrel glycoside hydrolase superfamily with $(\beta/\alpha)_7$ motif in their three dimensional structure and are reported to be located on plasma membrane. They have retaining type of catalytic mechanism, where carbonyl oxygen of C-2

acetamido group of substrate work as nucleophile/base and conserved glutamic acid residue works as proton donor. Amino acid sequences of hyaluronidases have multiple glycosylation sites and numerous cysteines (Lathrop et al. 1990) (Fig. 4j2).

Glycoside Hydrolase Family 57

Alpha-amylase (EC 3.2.1.1); α-galactosidase (EC 3.2.1.22); amylopullulanase (EC 3.2.1.41); cyclomaltodextrinase (EC 3.2.1.54); branching enzyme (EC 2.4.1.18); 4-α-glucanotransferase (EC 2.4.1.25) are activities of enzymes of the family, they carry out retaining type catalytic mechanism and glutamic acid residues at conserved site working as nucleophile/base. Three dimensional structure has $(\beta/\alpha)_7$ motifs. This family includes highly specific enzymes (Laderman et al. 1993) (Fig. 4k2).

Glycoside Hydrolase Family 58

Endo-N-acetylneuraminidase or endo-sialidase (EC 3.2.1.129) are activities of enzymes found in this family having inverting type catalytic mechanism, water activated by carboxylate of substrate working as catalytic nucleophile/base and conserved glutamic acid working as catalytic proton donor. Three dimensional structures have sixfold β-propellers present. This group of enzyme is highly explored and needs extensive study and classification (Fig. 4l2).

Glycoside Hydrolase Family 59

Beta-galactosidase (EC 3.2.1.23) galactocerebrosidase (EC 3.2.1.46) are activities of enzymes of this family, with $(\beta/\alpha)_8$ motif in their three-dimensional structure, they are classified under clan GH-A of TIM barrel glycoside hydrolase superfamily (CL0058) and have conserved glutamic acid at catalytic region that acts as nucleophile/base and proton donor. These enzymes are responsible for lysosomal catabolism of galactolipids (Rafi et al. 1996; Luzi et al. 1995; Fukushima et al. 1998) (Fig. 4m2).

Glycoside Hydrolase Family 60

Precise data of any particular enzyme of this family is not reported.

Glycoside Hydrolase Family 61

Copper-dependent lytic polysaccharide monooxygenases were activities of enzymes belonging to this family, now reclassified in family AA9 (Auxilary activity Family 9). Classification under GH Family 61 was considered incorrect.

Glycoside Hydrolase Family 62

Alpha-L-arabinofuranosidase (EC 3.2.1.55) are activities of enzymes of this group, belonging to Clan GH-F of Beta-fructosidase/Furanosidase Superfamily. They catalyze hydrolysis of aryl alpha L arabinofuranosides by cleaving arabinosyl side chains from arabinoxylan and arabinan, but the detailed catalytic mechanism of the enzymes of this group is not known (Fig. 4n2).

Glycoside Hydrolase Family 63

Processing α-glucosidase (EC 3.2.1.106); α-1,3-glucosidase (EC 3.2.1.84); α-glucosidase (EC 3.2.1.20); mannosylglycerate α-mannosidase/mannosylglycerate hydrolase (EC 3.2.1.170); glucosylglycerate hydrolase (EC 3.2.1.208) are major enzymes of this family belonging to clan GH-G of Six-hairpin glycosidase superfamily with $(\alpha/\alpha)_6$ motif in their three dimensional structure. They show inverting mode of catalytic mechanism with conserved glutamic acid and aspartic acid working as catalytic nucleophile/base and proton donor respectively. These enzymes are majorly located in endoplasmic reticulum membrane and they catalyze cleavage of non-reducing terminal glucose residue from Glc(3)Man(9)GlcNAc(2). Mannosyl oligosaccharide glucosidase EC 3.2.1.106 is the first enzyme in the N-linked oligosaccharide processing pathway (Fig. 4o2).

Glycoside Hydrolase Family 64

Beta-1,3-glucanase (EC 3.2.1.39) are activities of the group, less explored and are known to show inverting mode of catalytic mechanism, with conserved aspartic acid and glutamic acid working as catalytic nucleophile/base and proton donor respectively (Fig. 4p2).

Glycoside Hydrolase Family 65

Alpha,α-trehalase (EC 3.2.1.28); maltose phosphorylase (EC 2.4.1.8); trehalose phosphorylase (EC 2.4.1.64); kojibiose phosphorylase (EC 2.4.1.230); trehalose-6-phosphate phosphorylase (EC 2.4.1.216); nigerose phosphorylase (EC 2.4.1.279); 3-O-α-glucopyranosyl-L-rhamnose phosphorylase (EC 2.4.1.282);

2-O-α-glucopyranosylglycerol: phosphate β-glucosyltransferase (EC 2.4.1.-); α-glucosyl-1,2-β-galactosyl-L-hydroxylysine α-glucosidase (EC 3.2.1.107); are activities of enzymes of this family. They consist of three structural domains; central catalytic domain, N-terminal domain and C-terminal domain. Central catalytic domain of these families belongs to Clan GH-L of Six-hairpin glycosidase superfamily and the N-terminal domain belongs to Galactose mutarotase like superfamily. They catalyze hydrolysis through inverting mode of catalytic mechanism, through phosphate for phosphorylases; water for hydrolases acting as nucleophile/base and conserved glutamic acid residue acting as proton donor. Maltose phosphorylase (MP) (dimeric enzyme) catalyzes the conversion of maltose and inorganic phosphate into beta-D-glucose-1-phosphate and glucose (Van Tilbeurgh et al. 2001) (Fig. 4q2, r2, s2).

Glycoside Hydrolase Family 66

Cycloisomaltooligosaccharide glucanotransferase (EC 2.4.1.248) and dextranase (EC 3.2.1.11) are activities of this family. They show retaining mode of catalytic mechanism with conserved aspartic acid residues acting as catalytic nucleophile/base and proton donor. They belong to TIM barrel glycoside hydrolase superfamily (CL0058) (Igarashi et al. 1995; Funane et al. 2011) (Fig. 4t2).

Glycoside Hydrolase Family 67

Alpha-glucuronidase (EC 3.2.1.139) and xylan α-1,2-glucuronidase (EC 3.2.1.131) are activities of enzymes of glycoside hydrolase family 67, catalyzing through inverting mode of catalytic mechanism and conserved glutamic acid residues as proton donor. They have $(\beta/\alpha)_8$ barrels in their three dimensional structure. Crystal structures of all the three domains of family are known, C-terminal, N-terminal and middle domain where the central domain contains invariant glutamic acid and aspartic residues making them catalytic centre. They catalyze removal of alpha-1,2 linked 4-O-methyl glucuronic acid from xylans. (Shoham et al. 2001 and Nurizzo et al. 2002b) (Fig. 4u2, v2, w2).

Glycoside Hydrolase Family 68

Levansucrase (EC 2.4.1.10) (beta-D-fructofuranosyl transferase); β-fructofuranosidase (EC 3.2.1.26); inulosucrase (EC 2.4.1.9) are major activities of the family, with retaining mode of catalytic mechanism, these enzymes use conserved aspartic acid and glutamic acid as catalytic nucleophile/base and proton donor. They are classified under clan GH-J of Beta-fructosidase/Furanosidase Superfamily. L catalyze the conversion of sucrose and (2,6-beta-D-fructosyl)(N) to glucose and (2,6-beta-D-fructosyl)(N+1), where other sugars can also act as fructosyl

acceptors. Invertase, or extracellular sucrase (EC 3.2.1.26), catalyses the hydrolysis of terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides (Fig. 4x2).

Glycoside Hydrolase Family 69

No enzyme is classified under this family at present. Earlier classified enzymes have been grouped as family PL16.

Glycoside Hydrolase Family 70

Dextransucrase (EC 2.4.1.5); alternansucrase (EC 2.4.1.140); reuteransucrase (EC 2.4.1.-); α-4,6-glucanotransferase (EC 2.4.1.-); α-1,2-branched dextransucrase (EC 2.4.1.-); α-4,3-glucanotransferase (EC 2.4.1.-) are the activities reported to be classified under glycoside hydrolase family 70. They belong to clan GH-H of TIM Barrel glycoside hydrolase superfamily with $(\beta/\alpha)_8$ motif in their three dimensional structure. They follow retaining type catalytic mechanism with aspartic acid and glutamic acid residues working as catalytic nucleophile/base and proton donor. They catalyze transfer of D-glucopyramnosyl units from sucrose onto acceptor molecules (Croux et al. 1996) (Fig. 4y2).

Glycoside Hydrolase Family 71

Alpha-1,3-glucanase (EC 3.2.1.59) is the activity of enzyme belonging to this family and they are known to catalyze activity through inverting mechanism of reaction. This group of enzymes is not extensively studied. No known three dimensional structures is available.

Glycoside Hydrolase Family 72

Beta-1,3-glucanosyltransglycosylase (EC 2.4.1.-) is the activities of enzyme belonging to glycoside hydrolase family 72, clan GH-A of TIM Barrel glycoside hydrolase superfamily. They exhibit retaining type of catalytic mechanism with glutamic acid residues in the conserved domain acting as catalytic nucleophile/base and proton donor. These enzymes are glycolipid proteins anchored to membrane (Fig. 4z2).

Glycoside Hydrolase Family 73

Lysozyme (EC 3.2.1.17); mannosyl-glycoprotein endo-β-N-acetylglucosaminidase (EC 3.2.1.96); peptidoglycan hydrolase with endo-β-N-acetylglucosaminidase

specificity (EC 3.2.1.-) are activities of enzymes of the group and the conserved glutamic acid acts as catalytic proton donor. They also catalyze hydrolysis of peptidoglycan (Nambu et al. 1999) (Fig. 4a3).

Glycoside Hydrolase Family 74

Endoglucanase (EC 3.2.1.4); oligoxyloglucan reducing end-specific cellobiohydrolase (EC 3.2.1.150); xyloglucanase (EC 3.2.1.151) are activities of enzymes of the group. They follow inverting type catalytic mechanism with conserved aspartic acid residue as catalytic nucleophile/base and proton donor. Three dimensional structures have sevenfold β-propeller. This group of enzymes requires detailed studies (Fig. 4b3).

Glycoside Hydrolase Family 75

Chitosanase (EC 3.2.1.132) is reported activity of enzymes of this group, with conserved aspartic acid and glutamic acid residues working as probable catalytic nucleophile/base and proton donor respectively. They catalyze through inverting mode of catalytic mechanism (Cheng et al. 2017). No known three dimensional structures are available.

Glycoside Hydrolase Family 76

Alpha-1,6-mannanase (EC 3.2.1.101) and α-glucosidase (EC 3.2.1.20) are activities of enzymes of this family. They belong to Six-hairpin glycosidase superfamily, and catalyses through retaining mode of action through conserved aspartic acid residues working as catalytic nucleophile/base and proton donor (Fig. 4c3).

Glycoside Hydrolase Family 77

Amylomaltase or 4-α-glucanotransferase (EC 2.4.1.25) are activities of enzymes of this family, they belong to clan GH-H of TIM Barrel glycoside hydrolase superfamily and have $(\beta/\alpha)_8$ motifs in their three dimensional structure. They undergo catalysis through retaining type mechanism with conserved aspartic acid and glutamic residues working as catalytic nucleophile/base and proton donor respectively. They catalyze transfer of a segment of a (1,4)-alpha-D-glucan to a new four-position in an acceptor, which may be glucose or (1,4)-alpha-D-glucan. They belong to the disproportionating family of enzymes (Okada et al. 1993) (Fig. 4d3).

Glycoside Hydrolase Family 78

Alpha-L-rhamnosidase (EC 3.2.1.40); rhamnogalacturonan α-L-rhamnohydrolase (EC 3.2.1.174); L-Rhap-α-1,3-D-Apif -specific α-1,3-L-rhamnosidase (EC 3.2.1.-) are activities of enzymes of this family belonging to Six-hairpin glycosidase superfamily having $(\alpha/\alpha)_6$ motif in their three dimensional structures (Zverlov et al. 2000). They catalyze hydrolysis through inverting mode of catalytic mechanism (Fig. 4e3).

Glycoside Hydrolase Family 79

Beta-glucuronidase (EC 3.2.1.31); hyaluronoglucuronidase (EC 3.2.1.36); heparanase (EC 3.2.1.166); baicalin β-glucuronidase (EC 3.2.1.167); β-4-O-methyl-glucuronidase (EC 3.2.1.-) are activities of enzymes reported to be classified under this family. They belong to clan GH-A of TIM Barrel glycoside hydrolase superfamily with $(\beta/\alpha)_8$ domains in its three dimensional structure. They undergo catalysis through retaining mechanism with conserved glutamic acid residues inferred to be catalytic nucleophile/base and proton donor. These enzymes play vital role in the self- assembly, insolubility and barrier properties of basement membranes and extracellular matrices and are also involved in cell migration associated with inflammation and autoimmunity (Vlodavsky et al. 2001) (Fig. 4f3).

Glycoside Hydrolase Family 80

Chitosanase (EC 3.2.1.132) are activities of enzymes belonging to this family. They belong to clan GH-I of Pectate lyase-like beta helix superfamily and process through inverting mode of catalytic mechanism (Shimono et al. 2002). Detailed catalytic conserved domain and three dimensional structures are yet to be explored (Fig. 4g3).

Glycoside Hydrolase Family 81

Endo-β-1,3-glucanase (EC 3.2.1.39) are the activities of enzyme exhibiting inverting mode of catalytic mechanism. Detailed catalytic mechanism and complete analysis of conserved motifs in the three dimensional structures is yet to be explored (Fig. 4h3).

Glycoside Hydrolase Family 82

I-carrageenase (EC 3.2.1.157) are the activities of enzymes belonging to the family with a prominent (β)-helix in their three dimensional structure and showing inverting

mode of catalytic mechanism through conserved aspartic acid and glutamic acid working as catalytic nucleophile/base and proton donor, respectively (Fig. 4i3).

Glycoside Hydrolase Family 83

Neuraminidases (EC 3.2.1.18) are the enzyme activities reported for this family, belonging to clan GH-E of sialidase superfamily. With sixfold β-propeller motif in their three dimensional structure, these enzymes show retaining mode of catalytic mechanism (conserved tyrosine + glutamic acid working as catalytic nucleophile/base). Neuraminidase domain is the glycoside hydrolase domain of Hemagglutinin-neuraminidase (unit viral protein) containing both hemagglutinin and (endo) neuraminidase (Zaitsev et al. 2004 and Yuan et al. 2005) (Fig. 4j3).

Glycoside Hydrolase Family 84

N-acetyl β-glucosaminidase (EC 3.2.1.52); hyaluronidase (EC 3.2.1.35) and [protein]-3-O-(GlcNAc)-L-Ser/Thr β-N-acetylglucosaminidase (EC 3.2.1.169) are prominent activities of enzymes belonging to this family with $(\beta/\alpha)_8$ in their three dimensional structures. They exhibit retaining mode of catalytic mechanism where carbonyl oxygen of C-2 acetamido group of substrate acts as catalytic nucleophile/base and aspartic acid residues are proton donors (Macauley et al. 2005) (Fig. 4k3).

Glycoside Hydrolase Family 85

Endo-β-N-acetylglucosaminidases (EC 3.2.1.96) are activities of enzymes of GH family 85, belonging to clan, GH-A of TIM barrel glycoside hydrolase superfamily. They exhibit retaining type of catalytic mechanism with carbonyl oxygen of C-2 acetamido group of substrate acting as catalytic nucleophile/base and glutamic acid residues as proton donor. These enzymes are secretary enzymes and work on broad spectrum of substrates (Fig. 4l3).

Glycoside Hydrolase Family 86

Beta-agarase (EC 3.2.1.81); β-porphyranase (EC 3.2.1.178) are major activities of enzymes of this group. They belong to clan GH-A of TIM Barrel glycoside hydrolase superfamily, with $(\beta/\alpha)_8$ barrels in their three dimensional structures. Studies have reported probable retaining mode of catalytic mechanism with conserved glutamic acid residues as catalytic nucleophile/base and proton donor (Fig. 4m3).

Glycoside Hydrolase Family 87

Mycodextranase (EC 3.2.1.61) and α-1,3-glucanase (EC 3.2.1.59) are major enzymes of this family. These enzymes are not well explored need extensive study (Fig. 4n3).

Glycoside Hydrolase Family 88

Major activities of enzymes of this group involves d-4, 5-unsaturated β-glucuronyl hydrolase (EC 3.2.1.-) (Hashimoto et al. 1999). They belong to six-hairpin glycosidase superfamily (CL0059) containing $(\alpha/\alpha)_6$ motif determined in their three dimensional structure. Mechanism of catalysis by the group of enzymes has not been explored and needs detailed study (Fig. 4o3).

Glycoside Hydrolase Family 89

Alpha-N-acetylglucosaminidase (EC 3.2.1.50) are activities of enzyme reported in GH Family 89, with specific $(\beta/\alpha)_8$, they belong to TIM-Barrel glycoside hydrolase superfamily (CL0058). They exhibit retaining mode of catalytic mechanism with conserved glutamic acid residues working as catalytic nucleophile/base and proton donor. Three structural domains of these enzymes are N-terminal domain (alpha-beta fold); central domain (TIM barrel fold) and C-Terminal domain (alpha-helical fold). Improper functioning of these enzymes leads to various disorders (Ficko-Blean et al. 2008; Li et al. 1999; Villani et al. 2002) (Fig. 4p3).

Glycoside Hydrolase Family 90

Endorhamnosidases (EC 3.2.1.-) are activities of enzymes of this group, with prominent (β)-helix in their three-dimensional structure. They carry out catalytic mechanism through inverting mode, where conserved domain with glutamic and aspartic acid acts as nucleophile/base and aspartic acid separately acts as proton donor (Fig. 4q3).

Glycoside Hydrolase Family 91

Inulin lyase [DFA-I-forming] (EC 4.2.2.17); inulin lyase [DFA-III-forming] (EC 4.2.2.18); difructofuranose 1,2′:2,3′ dianhydride hydrolase [DFA-IIIase] (EC 3.2.1.-) are major activities of enzymes of this family having β-helix domain in its three dimensional structure. They follow inverting mode of catalytic mechanism and was temporarily changed into PL19 until it was recently realized that these

"PLs" are analogous to GH23 SLTs/lysozymes (there is at least one hydrolase in the GH91 family) and therefore are better classified in the GH category (Fig. 4r3).

Glycoside Hydrolase Family 92

Mannosyl-oligosaccharide α-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide α-1,3-mannosidase (EC 3.2.1.-); mannosyl-oligosaccharide α-1,6-mannosidase (EC 3.2.1.-); α-mannosidase (EC 3.2.1.24); α-1,2-mannosidase (EC 3.2.1.-); α-1,3-mannosidase (EC 3.2.1.-); α-1,4-mannosidase (EC 3.2.1.-) and mannosyl-1-phosphodiester α-1,P-mannosidase (EC 3.2.1.-) are activities of enzymes belonging to this family and pectate-lyase like beta-helix superfamily (CL0268). The conserved catalytic site consists of aspartic acid and glutamic acid residues working as nucleophile/base and proton donor for carrying out catalysis through inverting catalytic mechanism. These enzymes are reported to be critical for maturation of N- linked oligosaccharides and endoplasmic reticulum associated degradation (Liu et al. 1999) (Fig. 4s3).

Glycoside Hydrolase Family 93

Exo-α-L-1,5-arabinanase (EC 3.2.1.-) are activities classified under this family and with six-bladed β-propeller motif they belong to Clan GH-E of Sialidase superfamily (CL434). They carry out catalysis through retaining mode of action with conserved Glutamic acid residues working as catalytic nucleophile/base and proton donor (Fig. 4t3).

Glycoside Hydrolase Family 94

Cellobiose phosphorylase (EC 2.4.1.20); laminaribiose phosphorylase (EC 2.4.1.31); cellodextrin phosphorylase (EC 2.4.1.49); chitobiose phosphorylase (EC 2.4.1.-); cyclic β-1,2-glucan synthase (EC 2.4.1.-); cellobionic acid phosphorylase (EC 2.4.1.321); β-1,2-oligoglucan phosphorylase (EC 2.4.1.-) are activities of enzymes belonging to family and with $(\alpha/\alpha)_6$ barrels they are classified under clan GH-Q. Catalysis involves inverting mode of mechanism and conserved phosphate and aspartic acid residues work as catalytic nucleophile/base and proton donor. These enzymes were formerly known as glycosyltransferase family GT36; and have been assigned to GH family by Hidaka et al. 2004 that reported evolutionary, structural and mechanistic relationship of these phosphorylases with glycoside hydrolases of clan GH-L (Fig. 4u3).

Glycoside Hydrolase Family 95

α-L-fucosidase (EC 3.2.1.51); α-1,2-L-fucosidase (EC 3.2.1.63); α-L-galactosidase (EC 3.2.1.-) are major enzymes with inverting catalytic mechanism containing conserved $(\alpha/\alpha)_6$ motif in three dimensional stcrutures. Asparagine activated by aspartic acid works as catalytic nucleophile/base and Glutamic acid as proton donor (Katayama et al. 2004) (Fig. 4v3).

Glycoside Hydrolase Family 96

Alpha-agarase (EC 3.2.1.158) is activity classified under this family. No proper literature of three dimensional structure and catalytic mechanism for the same is reported.

Glycoside Hydrolase Family 97

Glucoamylase (EC 3.2.1.3); α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22) are the activities of the family, with $(\beta/\alpha)_8$ motif they are classified under TIM Barrel glycoside hydrolase Superfamily (CL0058). They exhibit both inverting and retaining mode of catalytic mechanism where Glutamic acid and Aspartic acid residues acts as catalytic nucleophile/base for inverting and retaining mechanisms respectively. Catalytic proton donor in both the cases is conserved glutamic acid residues (Hughes et al. 2003). N-terminal and C-terminal of the enzyme has pre-dominant beta-strands and the non catalytic domains are involved in oligomerization and carbohydrate binding (Naumoff 2005) (Fig. 4w3).

Glycoside Hydrolase Family 98

Blood-group endo-β-1,4-galactosidase (EC 3.2.1.102); blood group A- and B-cleaving endo-β-1,4-galactosidase (EC 3.2.1.-); endo-β-1,4-xylanase (EC 3.2.1.8); endo-β-1,4-xylanase (EC 3.2.1.8) are activities of enzymes belonging to this group. Two domains of these enzymes are known, of which putative catalytic domain is believed to be present at N-terminus of the non catalytic region (Anderson et al. 2005). They follow inverting catalytic mechanism with Aspartic acid and Glutamic acid diad as catalytic nucleophile/base and conserved glutamic acid residues as proton donor (Fig. 4x3).

Glycoside Hydrolase Family 99

Glycoprotein endo-α-1,2-mannosidase (EC 3.2.1.130) and mannan endo-1,2-α-mannanase (3.2.1.-) are activities of the group with $(\beta/\alpha)_8$ motifs in their

three dimensional structure. They undergo retaining type catalytic mechanism with unclear, possible neighboring group participation from O_2 (Thompson et al. 2012) working as catalytic nucleophile/base and conserved glutamic acid residues as proton donor (Spiro et al. 1997) (Fig. 4y3).

Glycoside Hydrolase Family 100

Alkaline and neutral invertase (EC 3.2.1.26) are activities of the enzymes classified under this family, with a $(\alpha/\alpha)_6$ motif in their three dimensional structure, they belong to clan GH-G of Six-hairpin glycosidase superfamily. Catalytic mechanism takes place through inverting mode with glutamic acid and aspartic acid acting as nucleophile/base and proton donor respectively (Gallagher and Pollock 1998; Lee and Sturm 1996; Sturm 1999) (Fig. 4z3).

Glycoside Hydrolase Family 101

Endo-α-N-acetylgalactosaminidases (EC 3.2.1.97) are activities reported, and contains $(\beta/\alpha)_8$ motifs, belonging to TIM barrel Glycoside hydrolase Superfamily (CL0058). They carry retaining type of catalytic mechanism with conserved Aspartic acid and glutamic acid residues working as catalytic nucleophile/base and proton donor (Fujita et al. 2005). These enzymes can be split into several subfamilies (Naumoff 2010) (Fig. 4a4).

Glycoside Hydrolase Family 102

Peptidoglycan lytic transglycosylases (EC 3.2.1.-) are activities of enzymes belonging to GH family 102, exhibiting retaining type catalytic mechanism. These enzymes corresponds to family 2 of the peptidoglycan lytic transglycosylases (Blackburn and Clarke 2001) (Fig. 4b4).

Glycoside Hydrolase Family 103

Peptidoglycan lytic transglycosylases (EC 3.2.1.-) are activities of enzymes belonging to GH family 102, exhibiting retaining type catalytic mechanism with carbonyl oxygen of C-2 acetamido group of substrate inferred to be catalytic nucleophile/base and conserved glutamic acid residues working as proton donor. These enzymes corresponds to family 3 of the peptidoglycan lytic transglycosylases (Blackburn and Clarke 2001) (Fig. 4C4).

Glycoside Hydrolase Family 104

Peptidoglycan lytic transglycosylases (EC 3.2.1.-) are activities of enzymes belonging to GH family 102, exhibiting retaining type catalytic mechanism. These enzymes corresponds to family 4 of the peptidoglycan lytic transglycosylases (Blackburn and Clarke 2001) (Fig. 4d4).

Glycoside Hydrolase Family 105

Unsaturated rhamnogalacturonyl hydrolase (EC 3.2.1.172); d-4,5-unsaturated β-glucuronyl hydrolase (EC 3.2.1.-); d-4,5-unsaturated α-galacturonidase (EC 3.2.1.-) are activities reported belonging to this family with predominant $(\alpha/\alpha)_6$ in their three dimensional structures. Catalytic mechanism for this group of enzymes is not known (Itoh et al. 2006) (Fig. 4e4).

Glycoside Hydrolase Family 106

Alpha-L-rhamnosidase (EC 3.2.1.40); rhamnogalacturonan α-L-rhamnohydrolase (EC 3.2.1.174) are activities of this family of enzymes containing $(\beta/\alpha)_8$ barrels in their three-dimensional structure. They exhibit inverting mode of catalytic mechanism (Miyata et al. 2005) (Fig. 4f4).

Glycoside Hydrolase Family 107

Sulfated fucan endo-1,4-fucanase (EC 3.2.1.-) are activities of enzymes belonging to GH Family 107, with $(\beta/\alpha)_8$ motif in their three dimensional structure, they belong to Clan GH-R of TIM Barrel Glycoside Hydrolase Superfamily. They exhibit retaining mode of catalytic mechanism with conserved Aspartic acid residues and Histidine acting as catalytic nucleophile/base and proton donor (Colin et al. 2006 and Vickers et al. 2018) (Fig. 4g4).

Glycoside Hydrolase Family 108

N-acetylmuramidase (EC 3.2.1.17) are the activities of enzymes belonging to this family and belong to Lysozyme like superfamily (CL0037). Conserved glutamic acid acts as catlytic proton donor. One pf their probable activities is activation of secretion of large proteins via the breaking and rearrangement of bacterial peptidoglycan layer during secretion (Stojković and Rothman-Denes 2007; Pei and Grishin 2005; Kondo et al. 1994; Emina and Lucia 2007) (Fig. 4h4).

Glycoside Hydrolase Family 109

Alpha-N-acetylgalactosaminidase (EC 3.2.1.49) are the activities of enzymes related to this family, with their catalytic mechanism unusually involving NAD+, catalyzing through retaining mode (Liu et al. 2007) (Fig. 4i4).

Glycoside Hydrolase Family 110

Alpha-galactosidase (EC 3.2.1.22); α-1,3-galactosidase (EC 3.2.1.-) are activities of enzymes reported for this family and they carry out catalysis through inverting mechanism (Liu et al. 2007). No structure is reported till date for enzymes of this family.

Glycoside Hydrolase Family 111

keratan sulfate hydrolase (endo-β-N-acetylglucosaminidase) (EC 3.2.1.-) are the activities reported with no further details available.

Glycoside Hydrolase Family 112

Lacto-N-biose phosphorylase or galacto-N-biose phosphorylase (EC 2.4.1.211); D-galactosyl-β-1,4-L-rhamnose phosphorylase (EC 2.4.1.247) and galacto-N-biose/lacto-N-biose phosphorylase (EC 2.4.1.-) are activities reported for this family and studies show that they undergo inverting mode of catalytic mechanism with Aspartic acid residues (through site directed mutagenesis) acting as proton donor (Fig. 4j4).

Glycoside Hydrolase Family 113

Beta-mannanase (EC 3.2.1.78) are activities of enzymes belonging to this family and with specific $(\beta/\alpha)_8$ barrels in their three dimensional structure they are classified under clan GH-A of TIM Barrel Glycoside hydrolase Superfamily. They exhibit retaining mode of catalytic mechanism with conserved glutamic acid residues working as catalytic nucleophile/base and proton donor (Zhang et al. 2008) (Fig. 4k4).

Glycoside Hydrolase Family 114

Endo-α-1,4-polygalactosaminidase (EC 3.2.1.109) are activities of enzymes belonging to this group, exhibiting retaining mode of catalytic mechanism (Tamura et al. 1995) (Fig. 4l4).

Glycoside Hydrolase Family 115

Xylan α-1,2-glucuronidase (3.2.1.131) and α-(4-O-methyl)-glucuronidase (3.2.1.-) are activities of enzymes belonging to this family and they catalyze reaction through inverting mechanism (Ryabova et al. 2009) (Fig. 4m4).

Glycoside Hydrolase Family 116

Beta-glucosidase (EC 3.2.1.21); β-xylosidase (EC 3.2.1.37); acid β-glucosidase/β-glucosylceramidase (EC 3.2.1.45) and β-N-acetylglucosaminidase (EC 3.2.1.52) are activities of enzymes classified under this family, with $(\alpha/\alpha)_6$ motif in their three dimensional structure, they belong to clan GH-O of glycoside hydrolases superfamily. They exhibit retaining mode of catalytic mechanism through glutamic acid and aspartic acid residues working as catalytic nucleophile/base and proton donor (Ferrara et al. 2013) (Fig. 4n4).

Glycoside Hydrolase Family 117

Alpha-1,3-L-neoagarooligosaccharide hydrolase (EC 3.2.1.-) and α-1,3-L-neoagarobiase/neoagarobiose hydrolase (EC 3.2.1.-) are major activities of the enzymes classified under this family. These enzymes are distantly related to GH Family 43 (Fig. 4o4).

Glycoside Hydrolase Family 118

This family contains enzymes with activities of β-agarase (EC 3.2.1.81), found to exhibit inverting mode of catalytic mechanism.

Glycoside Hydrolase Family 119

Alpha-amylases (EC 3.2.1.1) are enzymes classified under GH Family 119. They are distantly related to GH family 57 and process through inverting catalytic mechanism (Watanabe et al. 2006)

Glycoside Hydrolase Family 120

Beta-xylosidase (EC 3.2.1.37) are activities of enzymes exhibiting retaining type catalytic mechanism with conserved aspartic acid and glutamic acid residues working as catalytic nucleophile/base and proton donor (Shao et al. 2010) (Fig. 4p4).

Glycoside Hydrolase Family 121

Beta-L-arabinobiosidases (EC 3.2.1.-) are activities belonging to this group and enzymes catalyze through retaining mechanism (Fujita et al. 2011).

Glycoside Hydrolase Family 122

Alpha-glucosidase (EC 3.2.1.20) is activity of enzymes belonging to this family (Comfort et al. 2008).

Glycoside Hydrolase Family 123

β-N-acetylgalactosaminidase (EC 3.2.1.53) and glycosphingolipid β-N-acetylgalactosaminidase (EC 3.2.1.-) are the activities for the family, exhibiting retaining mode of catalytic mechanism through carbonyl oxygen of C-2 acetamido group of substrate acting as catalytic nucleophile/base and conserved glutamic acid residues as proton donor (Sumida et al. 2011) (Fig. 4q4).

Glycoside Hydrolase Family 124

Endoglucanase (EC 3.2.1.4) is activity for the enzymes belonging to this family and they carry catalysis through inverting mechanism (Bras et al. 2011) (Fig. 4r4).

Glycoside Hydrolase Family 125

Exo-α-1,6-mannosidase (EC 3.2.1.-) is the activity reported for this group of enzymes belonging to clan GH-G with specific $(\alpha/\alpha)_6$ of Six-hairpin glycosidase superfamily. They carry out catalysis through inverting mechanism with glutamic acid and aspartic acid residues working as catalytic nucleophile/base and proton donor (Gregg et al. 2011) (Fig. 4s4).

Glycoside Hydrolase Family 126

Alpha-amylase (EC 3.2.1.-), with $(\alpha/\alpha)_6$ are classified under this family (Ficko-blean et al. 2011) (Fig. 4t4).

Glycoside Hydrolase Family 127

Beta-L-arabinofuranosidase (EC 3.2.1.185) and 3-C-carboxy-5-deoxy-L-xylose (aceric acid) hydrolase (EC 3.2.1.-) are activities of the enzymes reported under this family and with specific $(\alpha/\alpha)_6$ motif, they belong to clan GH-P of Glycoside hydrolase superfamilies exhibiting retaining mode of catalytic mechanism (Fujita et al. 2011) (Fig. 4u4).

Glycoside Hydrolase Family 128

Beta-1,3-glucanase (EC 3.2.1.39) are activities of enzymes belonging to GH Family 128, with $(\beta/\alpha)_8$ motif in their three dimensional structures, they are classified under Clan GH-A of TIM barrel glycoside hydrolase superfamily (Sakamoto et al. 2011). They exhibit retaining mode of catalysis with conserved glutamic acid residues working as catalytic nucleophile/base and proton donor.

Glycoside Hydrolase Family 129

Enzymes of this family exhibit α-N-acetylgalactosaminidase (EC 3.2.1.49) with retaining mode of action and Aspartic acid residues as catalytic nucleophile/base (Kiyohara et al. 2012) (Fig. 4v4).

Glycoside Hydrolase Family 130

Activities of enzymes of this family includes β-1,4-mannosylglucose phosphorylase (EC 2.4.1.281); β-1,4-mannooligosaccharide phosphorylase (EC 2.4.1.319); β-1,4-mannosyl-N-acetyl-glucosamine phosphorylase (EC 2.4.1.320); β-1,2-mannobiose phosphorylase (EC 2.4.1.-); β-1,2-oligomannan phosphorylase (EC 2.4.1.-) and β-1,2-mannosidase (EC 3.2.1.-). These enzymes have fivefold β-propeller in their three dimensional structure and exhibit inverting mode of catalytic mechanism (Senoura et al. 2011) (Fig. 4w4).

Glycoside hydrolase Family 131

Enzymes with broad specificity exo-β-1,3/1,6-glucanase with endo-β-1,4-glucanase activity (EC 3.2.1.-) belong to this family (Lafond et al. 2012) (Fig. 4x4).

Glycoside Hydrolase Family 132

Activity on β-1,3-glucan (curdlan) shown for the *Aspergillus fumigatus* Sun4 protein; activity on laminarioligosaccharides shown for *Aspergillus fumigatus* Sun4 protein and *Candida albicans* Sun41 protein; transglycosylation activity are activities of enzymes belonging to this family and they show retaining type of catalytic mechanism (Gastebois et al. 2013).

Glycoside Hydrolase Family 133

Enzymes of this exhibit amylo-α-1,6-glucosidase (EC 3.2.1.33) activity through inverting mechanism with conserved aspartic acid residues working as catalytic nucleophile/base and proton donor (Nakayama et al. 2001) (Fig. 4y4).

Glycoside Hydrolase Family 134

These are enzymes with endo-β-1,4-mannanase (EC 3.2.1.78) with inverting mode of action (a unique feature compared to all other beta-mannanses) (Shimizu et al. 2015; Jin et al. 2016) (Fig. 4z4).

Glycoside Hydrolase Family 135

Alpha-1,4-galactosaminogalactan hydrolase (EC 3.2.1.-) are activities of enzymes of this family, showing retaining type catalytic mechanism (Bamford et al. 2015) (Fig. 4a5).

Glycoside Hydrolase Family 136

Lacto-N-biosidase (EC 3.2.1.140) is the activity reported for the group, with prominent (β)-helix in their 3-D structure. They catalyze through retaining mode of catalytic mechanism with conserved aspartic acid residues as catalytic nucleophile base and proton donor (Yamada et al. (2017) (Fig. 4b5).

Glycoside hydrolase Family 137

Beta-L-arabinofuranosidase (EC 3.2.1.185) are major activities of the enzymes belonging to GH Family 137, having fivefold β-propeller in their three dimensional structure and conserved glutamic acid residues acting as catalytic nucleophile/base and proton donor (Ndeh et al. 2017) (Fig. 4c5).

Glycoside Hydrolase Family 138

This family of glycoside hydrolase have activities of rhamnogalacturonan α-1,2-galacturonohydrolase (EC 3.2.1.173); with enzymes having $(\beta/\alpha)_8$ barrels in their three dimensional structure, exhibiting retaining mode of catalytic mechanism with conserved glutamic acid residues acting as catalytic nucleophile/base and proton donor (Ndeh et al. 2017; Labourel et al. 2019) (Fig. 4d5).

Glycoside Hydrolase Family 139

Alpha-2-O-Me-L-fucosidase (EC 3.2.1.-) is the activity of enzymes reported for this group (Ndeh et al. 2017).

Glycoside Hydrolase Family 140

Beta-1,2-apiosidase (EC 3.2.1.-) are the activities of enzymes reported in this group, having $(\beta/\alpha)_8$ barrels in three dimensional structures and exhibiting retaining mode of catalytic mechanism (Ndeh et al. 2017) (Fig. 4e5).

Glycoside Hydrolase Family 141

Alpha-L-fucosidase (EC 3.2.1.51) and xylanases (EC 3.2.1.8) are activities of enzymes of this group, specifically containing (β)-helix in their three dimensional structures; and conserved aspartic acid acts as catalytic nucleophile/base and proton donor (Ndeh et al. 2017 and Heinz et al.) (Fig. 4f5).

Glycoside Hydrolase Family 142

Beta-L-arabinofuranosidase (EC 3.2.1.185) is the reported activity for the enzymes of this family with prominent $(\alpha/\alpha)_6$ barrel in their three-dimensional structure. Conserved aspartic acid residues acts as catalytic nucleophile/base (Ndeh et al. 2017) (Fig. 4g5).

Glycoside Hydrolase Family 143

Enzymes with activity of 2-keto-3-deoxy-D-lyxo-heptulosaric acid hydrolase (EC 3.2.1.-) belong to this family and have prominent fivefold β-propeller in their three dimensional structures. They catalyze through retaining mode of reaction with conserved tyrosine and glutamic acid residues acting as nucleophile/base and individual glutamic acid acting as catalytic proton donor (Fig. 4h5).

Glycoside Hydrolase Family 144

Endo-β-1,2-glucanase (EC 3.2.1.71) and β-1,2-glucooligosaccharide sophorohydrolase (EC 3.2.1.-) are activities of enzymes belonging to this group with specific $(\alpha/\alpha)_6$ motif in their three dimensional structures catalyzing enzymatic reaction through inverting mode (Abe et al. 2017) (Fig. 4i5).

Glycoside Hydrolase Family 145

L-Rhα-α-1,4-GlcA α-L-rhamnohydrolase (EC 3.2.1.-) are activities reported for this family, having seven-bladed β-propellerin their three dimensional structures. These enzymes are reported to exhibit retaining mode of catalytic mechanism with conserved Histidine residues working as catalytic nucelophile/base. They carry out hydrolysis of alpha-L-rhamnose residues linked to position 4 of a glucuronic acid. One of the sites of these enzymes which is still under study shows string resemblance to family PL25 suggesting polysaccharide lyase activity (Fig. 4j5).

Glycoside Hydrolase Family 146

Beta-L-arabinofuranosidases (EC 3.2.1.185) are activities of the enzymes having $(\alpha/\alpha)_6$ barrels in their three dimensional structure, they are classified under clan GH-P with retaining type of catalytic mechanism (Luis et al. 2018) (Fig. 4k5).

Glycoside Hydrolase Family 147

Beta-galactosidase (EC 3.2.1.23) is activity reported for this group, with specific $(\beta/\alpha)_8$ (inferred) present in their three dimensional structures, they have been classified under Clan GH-A of TIM Barrel glycoside hydrolase superfamily. Catalysis is carried out through retaining mode with conserved glutamic acid residues acting as catalytic nucleophile/base and proton donor (Luis et al. 2018).

Glycoside Hydrolase Family 148

Enzymes of this family are reported to have β-1,3-glucanase (EC 3.2.1.-) activities, and with specific $(\beta/\alpha)_8$ (inferred) present in their three dimensional structures, they have been classified under Clan GH-A of TIM Barrel glycoside hydrolase superfamily. Conserved glutamic acid residues act as catalytcic nucleophile/base and proton donor (Angelov et al. 2017).

Glycoside Hydrolase Family 149

β-1,3-glucan phosphorylases (EC 2.4.1.97) are activities reported for enzymes belonging to family GH F-149, having $(\alpha/\alpha)_6$ barrels in their three dimensional structures, they are classified under clan GH-Q of glycoside hydrolase superfamily sharing this family GH F-94. They carry out inverting mode of catalytic mechanism, with phosphate ion and conserved aspartic acid residues acting as catalytic nucleophile/base and proton donor (Kuhaudomlarp et al. 2018) (Fig. 4l5).

Glycoside Hydrolase Family 150

l-carrageenase (EC 3.2.1.-) are activities of enzymes belonging to this family, and have been reported to carry out catalysis through inverting mechanism (PMID:16926183).

Glycoside Hydrolase Family 151

Alpha-L-fucosidase (EC 3.2.1.51) are activities of enzymes belonging to this family (Sela et al. 2012), and have been reported to carry out catalysis through retaining mechanism (inferred through transglycosylation activity) (Benesová et al. 2013).

Glycoside Hydrolase Family 152

Beta-1,3-glucanase (EC 3.2.1.39) are activities reported of this group of enzymes with only few structural details available for the same (Sakamoto et al. 2006) (Fig. 4m5).

Glycoside Hydrolase Family 153

Poly-β-1,6-D-glucosamine hydrolase (EC 3.2.1.-) are activities of enzyme belong to this family. They have specific $(\beta/\alpha)_8$ barrels in their three dimensional structures (Little et al. 2018) (Fig. 4n5).

Glycoside Hydrolase Family GH F-154

Beta-glucuronidase (3.2.1.31) is the activity of enzymes reported for this group (Cartmell et al. 2018).

Glycoside Hydrolase Family GH F-155

This family was reported by Cartmell et al. (2018) with retaining type catalytic mechanism, but presently this family has been deleted.

Glycoside Hydrolase Family 156

Exo-α-sialidase (EC 3.2.1.18) is the reported activity for the enzymes belonging to this family, catalyzing reaction through inverting mode and have been found through functional metagenomics study of DNA from thermal springs (Chuzel et al. 2018) (Fig. 4o5).

Glycoside Hydrolase Family 157

Endo-β-1,3-glucanase (EC 3.2.1.39) and endo-β-1,3-glucanase/laminarinase (EC 3.2.1.39) are activities reported for this family, they carry out enzymatic reaction through retaining mode and conserved glutamic acid residues in their active site acts as catalytic nucleophile/base and proton donor. They belong to clan GH-A of TIM Barrel glycoside hydrolase superfamily (Helbert et al. 2019).

Glycoside Hydrolase Family 158

Like family 158, endo-β-1,3-glucanase (EC 3.2.1.39) are also activities of enzymes of family 158, belong to clan GH-A of Barrel glycoside hydrolase superfamily, carrying out catalytic mechanism through retaining mode with conserved glutamic acid residues working as catalytic nucleophile/base and proton donor (Helbert et al. 2019).

Glycoside Hydrolae Family 159

β-D-galactofuranosidases (EC 3.2.1.146) are the activities reported for this group of enzymes (Helbert et al. 2019).

Glycoside Hydrolase Family 160

Endo-β-1,4-galactosidase (EC 3.2.1.-) are the activities reported for enzymes belonging to this family and are known to have probable folding similarities with several PL families (Helbert et al. 2019).

Glycoside Hydrolase Family 161

Beta-1,3-glucan phosphorylase (EC 2.4.1.-) are the activities of enzymes reported for this family of enzymes, with $(\alpha/\alpha)_6$ (inferred) motif in their three dimensional structure they are classified under clan GH-Q. They carry out catalysis through inverting mechanism with phosphate ion and conserved aspartic acid acting as catalytic nucleophile/base and proton donor respectively (Kuhaudomlarp et al. 2019).

Glycoside Hydrolase Family 162

Endo-β-1,2-glucanase (EC 3.2.1.71) is the activity of enzyme of this family, having $(\alpha/\alpha)_6$ motif in their three dimensional structure, exhibiting inverting mode of catalytic mechanism, with conserved aspartic acid and glutamic acid residues working as catalytic nucleophile/base and proton donor (Tanaka et al. 2019) (Fig. 4p5).

Glycoside Hydrolase Family 163

Endo-β-N-acetylglucosaminidase cleaving GlcNAc-β-1,2-Man (EC 3.2.1.-) are the activities of enzymes reported to belong to this family (Briliute et al. 2019).

Glycoside Hydrolase Family 164

Beta-mannosidase (EC 3.2.1.25) are activities of enzymes of this family (Helbert et al. 2019) (Note: it appears alpha-mannosidase in reference due to uncorrected typo in Table 2)

Glycoside Hydrolase Family 165

Beta-galactosidase (EC 3.2.1.23) is activity of enzymes determined through metagenomics study (Cheng et al. 2017)

Table 2 Active Site pattern and PDB Data and crystal structure availability of Glycoside hydrolase Family 1-166 and non classified group

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
1	Family 1	[LIVMFSTC]-[LIVFYS]-[LIV]-[LIVMST]-E-N-G-[LIVMFAR]-[CSAGN] E is the active site residue	PS00572	Archaea, eukaryotes, prokaryotes (bacteria)	95	0
2	Family 2	N-x-[LIVMFYWD]-R-[STACN](2)-H-Y-P-x(4)-[LIVMFYWS](2)-x(3)-[DN]-x(2)-G-[LIVMFYW](4)	PS00719	Eukaryotes, prokaryotes (bacteria)	63	2
3	Family 3	[LIVM](2)-[KR]-x-[EQKRD]-x(4)-G-[LIVMFTC]-[LIVT]-[LIVMF]-[ST]-D-x(2)-[SGADNIT]	PS00775	Eukaryotes, prokaryotes (bacteria)	27	4
4	Family 4	[PS]-x-[SAC]-x-[LIVMFY](2)-[QN]-x(2)-N-P-x(4)-[TA]-x(9,11)-[KRD]-x-[LIV]-[GN]-x-C	PS01324	Prokaryotes (bacteria)	4	0
5	Family 5	[LIV]-[LIVMFYWGA](2)-[DNEQG]-[LIVMGST]-{SENR}-N-E-[PV]-[RHDNSTLIVFY]	PS0659	Eukaryotes, prokaryotes (bacteria)	55	0
6	Family 6	–	PS00655, PS00656	Eukaryotes, prokaryotes (bacteria)	76	0
7	Family 7			Eukaryotic	86	0
8	Family 8	A-[ST]-D-[AG]-D-x(2)-[IM]-A-x-[SA]-[LIVM]-[LIVMG]-x-A-x(3)-[FW]	PS00812	Prokaryotes (bacteria)	5	1
9	Family 9	[HLY]-[AILMV]-[FIL]-G-x-[NSTW]-x(2,4)-[SCTV]-[FY]-[LIVMFY]-[SITV]-G-x(1,5)-[GSY]-x(2)-[AFPSTY]-[FLPSV]-x(2)-[AILPQVM]-[HV]-[DHLS]-[KRS]	PS00592	Eukaryotes, prokaryotes (bacteria)	13	0

10	Family 10	–	PS51760 (Domain profile)	Eukaryotes, prokaryotes (bacteria)	82	2
		[GTA]-{QNAG}-{GSV}-[LIVN]-x-[IVMF]-[ST]-E-[LIY]-[DN]-[LIVMF]	PS00591		67	
11	Family 11	–	PS51761 (Domain profile)	Eukaryotes, prokaryotes (bacteria)	82	2
		[PSA]-[LQ]-x-E-[YF]-Y-[LIVM](2)-[DE]-x-[FYWHN]	PS00776 (Active Site Signature I)		85	
		[LIVMF]-x(2)-E-[AG]-[YWG]-[QRFGS]-[SG]-[STAN]-G-x-[SAF]	PS00777 (Active Site Signature II)		64	
12	Family 12			Eukaryotes, prokaryotes (bacteria)	43	1
13	Family 13		α-amylase, N-terminal ig-G like domains		34	2
			α-amylase catalytic domain		419	
14	Family 14		PF01373	Eukaryotes, prokaryotes (bacteria)	43	0
15	Family 15		PF00723	Eukaryotes, prokaryotes (bacteria)	25	1
16	Family 16		PS51762 GH F-16 Domain Profile)	Eukaryotes, prokaryotes (bacteria)	35	1
		E-[LIV]-D-[LIVF]-x(0,1)-E-x(2)-[GQ]-[KRNF]-x-[PSTA]	PS01034 (GH F-16 Active Site)		21	
17	Family 17	[LIVMKS]-x-[LIVMFYWA](3)-[STAG]-E-[STACVI]-G-[WY]-P-[STN]-x-[SAGQ]	PS00587	Eukaryotes	4	1
18	Family 18		PF00704	Eukaryotes, prokaryotes (bacteria)	281	3

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
19	Family 19	C-x(4,5)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA]	PS00773 (Signature 1)	Eukaryotes	11	0
		[LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM]	PS00774 (Signature 2)	Eukaryotes		
20	Family 20		PF02838 (Domain 2)	Eukaryotes, prokaryotes (bacteria)	85	0
			PF00728 (Catalytic domain)		78	
21	Family 21					0
22	Family 22		PS51348 (Domain Profile)	Eukaryotes	530	0
		C-x(3)-C-x(2)-[LMF]-x(3)-[DEN]-[LI]-x(5)-C	PS00128 (Domain Signature)		1091	
23	Family 23			Eukaryotes and prokaryotes	76	0
24	Family 24		PF00959 (pfam domain)	Prokaryotes (phage lysozymes)	666	1
25	Family 25		PS51904 (Glycosyl hydrolase family 25 (GH25) domain profile)	Bacteriophages, eukaryotes, prokaryotes (bacteria)	7	0
		D-[LIVM]-x(3)-[NQ]-[PGE]-x(9,15)-[GR]-x(4)-[LIVMFY](2)-K-x-[ST]-E-[GS]-x(2)-[FYL]-x-[DN]	PS00953 (Glycosyl hydrolases family 25 active site signature)		7	
26	Family 26		PF02156	Eukaryotes, prokaryotes (bacteria)	42	2

27	Family 27	G-[LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-[DF]-x(1,2)-W-x(3,7)-[RV]-[DNSF]	PS00512	Eukaryotes and prokaryotes (bacteria)	40	0
28	Family 28	[GSDENKRH]-x(2)-[VMFC]-x(2)-[GS]-H-G-[LIVMAG]-x(1,2)-[LIVM]-G-S	PS00502 (Polygalacturonase catalytic site)	Eukaryotes, prokaryotes (bacteria)	6	3
29	Family 29	P-x(2)-L-x(3)-K-W-E-x-C	PS00385 (alpha-l-fucosidase putative active site)	Eukaryotes	6	0
30	Family 30		PF02055	Eukaryotes	45	1
31	Family 31	[GFY]-[LIVMF]-W-x-D-M-[NSA]-E	PS00129 (active site)	Archaea, eukaryotes, prokaryotes (bacteria)	31	0
		G-[AVP]-[DT]-[LIVMTAS]-[CG]-G-[FY]-x(3)-[STP]-x(3)-L-[CL]-x-R-W-x(2)-[LVMI]-[GSA]-[SA]-[FY]-x-P-[FY]-x-R-[DNA]	PS00707 (Signature 2)		27	
32	Family 32	H-x(2)-[PV]-x(4)-[LIVMA]-N-D-P-N-[GA]	PS00609 (active site)	Eukaryotes, prokaryotes (bacteria)	13	2
33	Family 33		PF02973	Eukaryotes and prokaryotes	60	0
34	Family 34		PF00064	Viruses	149	0
35	Family 35	G-G-P-[LIVM](2)-x(2)-Q-x-E-N-E-[FY]	PS01182 (putative active site)	Eukaryotes, prokaryotes (bacteria)	17	0
36	Family 36		PF02065 (Melibiase)	Eukaryotes, prokaryotes (bacteria)	48	0
			PF05691 (Raffinose)		0	
37	Family 37	P-G-G-R-F-x-E-x-Y-x-W-D-x-Y	PS00927 (Trehalase signature 1; located centrally)	Eukaryotes, prokaryotes (bacteria)	8	0
		Q-W-D-x-P-x-[GAV]-W-[PAS]-P	PS00928 (Trehalase signature II) (C-terminal region)		8	

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
38	Family 38		PF01074 (N-terminal Domain)	Eukaryotes, prokaryotes (bacteria)	63	0
			PF09261 (alpha-mannosidase middle domain)		63	
			PF01074 (C-terminal Domain)		63	
39	Family 39	W-x-F-E-x-W-N-E-P-[DN]	PS01027 (Active Site)	Eukaryotes, prokaryotes (bacteria)	15	0
40	Family 40	Not defined				0
41	Family 41	Not defined				0
42	Family 42		PF02449 (beta-galactosidase)	Prokaryotes	15	1
			PF08532 (Beta-galactosidase trimerisation domain)		9	
			PF08533 (Beta-galactosidase C terminal domain)		7	
43	Family 43		PF04616	Eukaryotes and prokaryotes (bacteria)	75	2
44	Family 44		PF12891	Prokaryotes	18	0
45	Family 45	[STA]-T-R-Y-[FYW]-D-x(5)-[CA]	PS01140 (Active Site)	Eukaryotes and prokaryotes (bacteria)	3	0
46	Family 46		PF01374	Prokaryotes	7	0

47	Family 47		PF01532	Eukaryotes and prokaryotes (bacteria)	19	0
48	Family 48		PF02011	Prokaryotes	28	0
49	Family 49		PF03718	Eukaryotes and prokaryotes (bacteria)	6	0
50	Family 50			Prokaryotes	6	0
51	Family 51			Eukaryotes and prokaryotes (bacteria)	19	
52	Family 52		PF0312	Prokaryotes	1	0
53	Family 53		PF07745	Eukaryotes and prokaryotes (bacteria)	14	0
54	Family 54			Eukaryotes and prokaryotes (bacteria)	4	0
55	Family 55			Eukaryotes and prokaryotes (bacteria)	13	0
56	Family 56		PF01630	Eukaryotes and prokaryotes	6	0
57	Family 57		PF03065	Eukaryotes and prokaryotes (bacteria)	9	0
58	Family 58			Prokaryotes	7	0
59	Family 59			Eukaryotes, prokaryotes (bacteria)	13	0

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
60	Family 60					0
61	Family 61					0
62	Family 62		PF03664	Eukaryotes, prokaryotes (bacteria)	12	1
63	Family 63		PF03200	Eukaryotes and prokaryotes	1	0
64	Family 64			Eukaryotes and prokaryotes	5	0
65	Family 65		PF03633 (C-terminal domain)	Eukaryotes and prokaryotes	5	1
			PF03632 (Central catalytic domain)			
			PF003636 (N-terminal domain)			
66	Family 66		PF13199	Prokaryotes	11	0
67	Family 67		PF07477 (C _terminus domain)	Eukaryotes and prokaryotes	12	0
			PF03648 (N-terminus domain)			
			PF07488 (middle domain)			
68	Family 68		PF02435 (Levansucrase/ invertase)	Prokaryotes	22	0
69	Family 69					0
70	Family 70		PF02324	Prokaryotes	16	0

71	Family 71		PF03659	Eukaryotes and prokaryotes (bacteria)	0	0
72	Family 72		PF03198	Eukaryotes and prokaryotes (bacteria)	4	0
73	Family 73		PF01832—Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	Prokaryotes	17	0
74	Family 74			Eukaryotes and prokaryotes (bacteria)	18	1
75	Family 75		PF07335	Eukaryotes and prokaryotes (bacteria)	0	0
76	Family 76		PF03663	Eukaryotes and prokaryotes	12	0
77	Family 77		PF02446—4-alpha-glucanotransferase	Eukaryotes and prokaryotes	20	0
78	Family 78		PF05592—Bacterial alpha-L-rhamnosidas	Eukaryotes and prokaryotes	4	0
79	Family 79		PF03662—Glycosyl hydrolase family 79, N-terminal domain	Eukaryotes and prokaryotes	8	0
80	Family 80		PF13647 (Chitosanase A)	Prokaryotes	1	0
81	Family 81		PF03639	Eukaryotes and prokaryotes	3	0
82	Family 82			Prokaryotes	3	0
83	Family 83		PF00423—Haemagglutinin-neuraminidase	Prokaryotes (viruses)	42	0

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
84	Family 84			Eukaryotes and prokaryotes	70	0
85	Family 85		PF03644	Eukaryotes and prokaryotes (bacteria)	6	0
86	Family 86			Prokaryotes	5	0
87	Family 87			Prokaryotes	8	0
88	Family 88		PF07470	Eukaryotes and prokaryotes	21	0
89	Family 89		PF12972—Alpha-N-acetylglucosaminidase (NAGLU) C-terminal domain	Eukaryotes and prokaryotes	7	0
			PF12971—Alpha-N-acetylglucosaminidase (NAGLU) N-terminal domain			
			PF05089—Alpha-N-acetylglucosaminidase (NAGLU) tim-barrel domain			
90	Family 90			Prokaryotes	18	0
91	Family 91			Prokaryotes	9	0
92	Family 92		PF07971	Eukaryotes and prokaryotes (bacteria)	8	0
93	Family 93			Eukaryotes and prokaryotes (bacteria)	7	0

94	Family 94			Eukaryotes and prokaryotes	31	0
95	Family 95			Eukaryotes and prokaryotes	6	0
96	Family 96			Prokaryotes	0	0
97	Family 97		PF10566	Eukaryotes and prokaryotes	8	0
98	Family 98		PF08306	Prokaryotes	5	0
			PF08307—Glycosyl hydrolase family 98 C-terminal domain			
99	Family 99			Eukaryotes and prokaryotes	30	0
100	Family 100		PF12899—Alkaline and neutral invertase	Eukaryotes and prokaryotes (bacteria)	4	0
101	Family 101		PF12905—Endo-alpha-N-acetylgalactosaminidase	Prokaryotes	8	0
			PF11308		0	
102	Family 102			Prokaryotes	10	0
103	Family 103			Eukaryotes and prokaryotes (bacteria)	13	0
104	Family 104			Prokaryotes	3	0
105	Family 105			Eukaryotes and prokaryotes	11	0

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
106	Family 106			Eukaryotes and prokaryotes	4	0
107	Family 107			Prokaryotes	4	0
108	Family 108		PF05838	Prokaryotes	3	0
109	Family 109			Prokaryotes	2	0
110	Family 110			Prokaryotes	0	0
111	Family 111			Prokaryotes	0	0
112	Family 112			Prokaryotes	6	0
113	Family 113			Eukaryotes and prokaryotes	9	0
114	Family 114			Eukaryotes and prokaryotes	2	0
115	Family 115			Eukaryotes and prokaryotes	4	1
116	Family 116			Eukaryotes and prokaryotes	10	0
117	Family 117			Prokaryotes	11	0
118	Family 118			Prokaryotes	0	1

119	Family 119			Prokaryotes	0	0
120	Family 120			Eukaryotes and prokaryotes (bacteria)	3	0
121	Family 121			Prokaryotes	0	0
122	Family 122			Prokaryotes	0	0
123	Family 123			Eukaryotes and prokaryotes (bacteria)	8	0
124	Family 124			Prokaryotes	3	0
125	Family 125			Eukaryotes and prokaryotes	12	0
126	Family 126			Prokaryotes	2	0
127	Family 127			Eukaryotes and prokaryotes	9	0
128	Family 128			Eukaryotes and prokaryotes	0	0
129	Family 129			Prokaryotes	5	0
130	Family 130			Eukaryotes and prokaryotes	22	0
131	Family 131			Eukaryotes	3	0
132	Family 132			Eukaryotes	0	0

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
133	Family 133			Eukaryotes and prokaryotes	2	0
134	Family 134			Eukaryotes and prokaryotes (bacteria)	9	0
135	Family 135			Eukaryotes and prokaryotes	2	0
136	Family 136			Eukaryotes and prokaryotes (bacteria)	3	0
137	Family 137			Prokaryotes	3	0
138	Family 138			Prokaryotes	4	0
139	Family 139			Eukaryotes and prokaryotes (bacteria)	0	0
140	Family 140			Prokaryotes	1	0
141	Family 141			Eukaryotes and prokaryotes	1	0
142	Family 142			Eukaryotes and prokaryotes (bacteria)	2	0
143	Family 143			Prokaryotes	2	0

144	Family 144			Prokaryotes	5	0
145	Family 145			Eukaryotes and prokaryotes	5	0
146	Family 146			Eukaryotes and prokaryotes (bacteria)	1	0
147	Family 147			Prokaryotes	0	0
148	Family 148			Prokaryotes	0	0
149	Family 149			Eukaryotes and prokaryotes (bacteria)	3	0
150	Family 150			Prokaryotes (bacteria)	0	0
151	Family 151			Prokaryotes (bacteria)	0	0
152	Family 152			Eukaryotes and prokaryotes	11	0
153	Family 153			Prokaryotes (bacteria)	7	0
154	Family 154			Eukaryotes and prokaryotes	0	0
155	Family 155	Deleted		–	0	0
156	Family 156			Prokaryotes (bacteria)	5	0
157	Family 157			Prokaryotes	0	0

(continued)

Table 2 (continued)

Sl no	Glycoside hydrolase family	Consensus pattern/active site residue	Prosite number	Taxonomic range	PDB/Pfam domain/ PDB accessions DATA entries	Crystal structure
158	Family 158			Prokaryotes	0	0
159	Family 159			Prokaryotes	0	0
160	Family 160			Prokaryotes	0	0
161	Family 161			Eukaryotes and prokaryotes	0	0
162	Family 162			Eukaryotes and prokaryotes (bacteria)	3	0
163	Family 163			Eukaryotes and prokaryotes (bacteria)	0	0
164	Family 164			Prokaryotes	0	0
165	Family 165			Prokaryotes (bacteria)	0	0
166	Family 166			Prokaryotes (bacteria)	1	0
167	Non classified			Eukaryotes and prokaryotes	55	0

Data as per PROSITE and EMBL-EBI (Pfam Domain)
https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/
https://prosite.expasy.org/

Glycoside Hydrolase Family 166

Alpha-1,4-galactosaminogalactan hydrolase (EC 3.2.1.-) is the activities of enzymes belonging to this family, exhibiting retaining type catalytic mechanism (Le Mauff et al. 2019) (Fig. 4q5).

Glycoside Hydrolase Family Non Classified

Group of glycoside hydrolase enzymes to which family has yet not been designated are reported here (Fig. 4r5).

Conclusions

Glycoside hydrolases, as stated earlier in this chapter, finds varied applications and this feature is much supported by the presence of diverse catalytic sites on these enzymes. They have been classified under 166 families which are grouped in 16 different superfamilies and three added clans (not specified yet under any superfamily). Being broadly studied for its action on numerous substrates, glycoside hydrolases like amylases, cellulases, chitinases, xylanases, glucanases, glucuronidases are few in humungous list finding usage in numerous technologies involved in production of various value added components. Specific region of catalytic domain, mode of action (retaining/inverting) and three dimensional structures for majority are available with added data on crystal structures of few specifically studied (like family 2, 3, 10, 11 etc.) and all figures have been reported in this chapter. In the present book few extensively studied reports on glycoside hydrolases have been brought forward, which broadly includes, structure, mechanism and applications of glycoside hydrolases, followed by a note on directed evolution of glycoside hdyrolases, its applications in degradation of lignocellulosic biomass, bioethanol production, food industries, paper and pulp industries, marine glycoside hydrolases as industrial tool and role of metagenomics in discovering industrially significant cellulases. With this minute initiative we bring to you an insight on industrial applications of glycoside hydrolases, an initial approach.

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Glycosyl Hydrolases: Biochemistry and Applications

Thankamani Marar and Deepa Garg

Abstract

Glycoside hydrolases, cleave the glycosidic linkage of glycosides, forming a sugar hemiacetal or hemiketal and a free aglycon. It also catalyzes the hydrolysis of carbohydrates with O-, N- and S-linkages. Since these glycosidases hydrolyze specific linkages they are used for elucidating the structure and function of polysaccharides.

Glycoside hydrolases can be classified in several ways based on their substrate specificity, their molecular mechanism and amino acid or nucleotide sequence similarity which reflects their evolutionary relationship. Lysozyme, (glycoside hydrolase, muramidase), is a small, monomeric protein and was the first protein which underwent extensive structural studies. Antibacterial property of lysozyme against Gram positive organism is being exploited by different industries like food processing, food preservation and pharmaceutical industry making it an commercially important enzyme.

Keywords

Glycoside hydrolase · Muramidase · Lysozyme · Antibacterial · Retaining hydrolases · Inverting hydrolases

Introduction

Carbohydrates are the most abundant biomolecule on Earth. They play a diverse roles in living organisms from being structural elements (cellulose, chitin) and energy molecules (starch, glycogen) to being involved in cell recognition processes.

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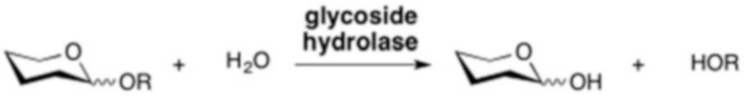

Fig. 1 Reaction catalysed by glycosidases

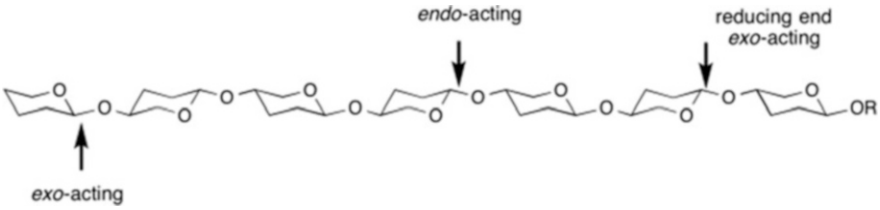

Fig. 2 Position of hydrolysis of endo- and exo-glycosidases

A covalent bond that joins a carbohydrate (sugar) molecule to another group, which may or may not be another carbohydrate, leading to formation of long polymers or polysaccharides is termed a glycosidic bond or glycosidic linkage.

Glycoside hydrolases (EC 3.2.1. . .) are a broad group enzymes that hydrolyze the glycosidic linkage of glycosides, forming a sugar hemiacetal or hemiketal and the free aglycon (Fig. 1). Glycoside hydrolases alternately are also referred to as glycosidases, or glycosyl hydrolases. The glycosidic bond can link two or more sugars, or link a sugar and a non-sugar moiety. Glycoside hydrolases can also hydrolyse linkages of carbohydrates with O-, N- and S-linkages. Since glycosidases hydrolyze definite linkages in polysaccharides they are used for elucidating their structures and functions.

Classification

Glycoside hydrolases are classified in several ways based on their substrate specificity, their molecular mechanism and amino acid or nucleotide sequence similarity which reflects their evolutionary relationship.

One of the easiest ways of classification is as endo- and exo-glycosidases based on the capability of the enzyme to hydrolyse the reducing end of the substrate (mostly, but not always) or in the middle of the chain (Fig. 2). Most cellulases are *endo*-acting, whereas Lac Z β-galactosidase from *E. coli* is *exo*-acting (Davies and Henrissat 1995).

The glycosyl hydrolases can be classified based on sequence or evolutionary similarity. This has divided the glycosyl hydrolases into 128 families and 14 clans. Glycoside hydrolase family 1 (GH1) comprises some common enzymes like beta-glucosidase (EC: 3.2.1.21); beta-galactosidase (EC: 3.2.1.23); 6-phospho-beta-galactosidase (EC: 3.2.1.85); 6-phospho-beta-glucosidase (EC: 3.2.1.86);

lactase-phlorizin hydrolase (EC: 3.2.1.62), (EC: 3.2.1.108); beta-mannosidase (EC: 3.2.1.25); myrosinase (EC: 3.2.1.147) etc.

Nomenclature can also be based on substrate that they act upon. Thus glucosidases act on glucosides and xylanases catalyze the cleavage of the homopolymer of xylose called xylan. Other common examples are lactase, amylase, chitinase, sucrase, maltase, neuraminidase, invertase, hyaluronidase and lysozyme.

Depending on the reaction mechanism, glycoside hydrolases can be classified based on the stereochemistry of the product formed. They can be classified as either *retaining* or *inverting* enzymes. These two most common reaction mechanisms that was first outlined by Koshland. Enzymatic hydrolysis of the glycosidic bond takes place via a general acid base catalysis that requires two vital residues: a proton donor and a nucleophile/base. Some variations of these mechanisms have been found and one catalyzed by an NADH cofactor, has been identified. The reaction takes place by two key mechanisms giving rise to either retention, or an inversion, of the anomeric configuration at the end of the reaction (Gebler et al. 1992).

Retaining Glycoside Hydrolases

Retaining glycosidases catalyze a two-step reaction, each step resulting in inversion, for a net preservation of stereochemistry (Fig. 3). In retaining enzymes, the nucleophilic catalytic base is in close vicinity of the sugar anomeric carbon. This base is more distant in inverting enzymes because a water molecule is accommodated between the base and the sugar. This difference results in an average distance between the two catalytic residues of −5.5 Å in retaining enzymes as against −10 Å in inverting enzymes (Gloster et al. 2008).

Inverting Glycoside Hydrolases

The catalysis of the glycosidic bond by inverting glycoside hydrolases results in a net inversion of the anomeric configuration. This is a single step, single-displacement mechanism involving oxo-carbenium ion-like transition states (Fig. 4). The reaction normally occurs with a general acid–base catalysis with glutamic or aspartic acids usually being two amino acids involved in the catalysis process (McCarter and Withers 1994).

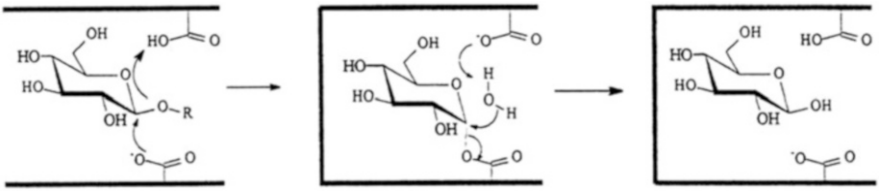

Fig. 3 Mode of action of retaining glycoside hydrolases

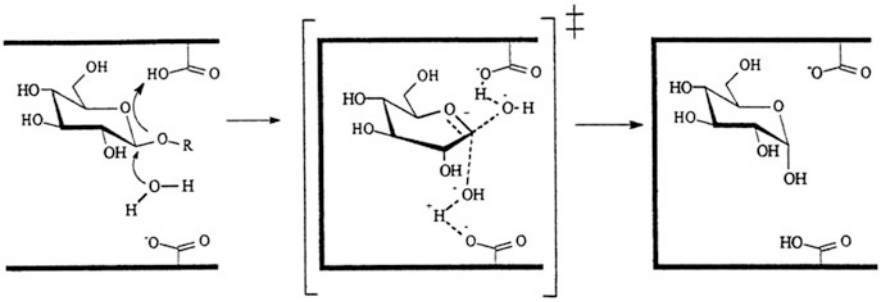

Fig. 4 Mode of action of inverting glycoside hydrolases

Lysozyme: A Representative Glycosyl Hydrolase

Introduction

Lysozyme, also known as muramidase or N-acetylmuramic hydrolase, is a monomeric protein of 129 amino acids, stabilized and cross-linked by four disulfide bridges among the eight cysteine residues of its polypeptide chain. Alexander Fleming accidentally discovered that a drop of his nasal mucus could cause the lysis of bacteria, which enabled him to detect a 'remarkable bacteriolytic element' that he later named lysozyme (Fleming 1922).

Lysozyme, N acetyl-hexosaminidase is ubiquitous in nature. Lysozyme is a naturally occurring enzyme found in bodily secretions such as tears, saliva, serum, and milk. It is also present in cytoplasmic granules of the macrophages and the polymorphonuclear neutrophils (PMNs).

Chicken egg white is the richest source of lysozyme with a concentration ranging between 3400 and 5840 mg/L (Sauter and Montoure 1972; Wilcox and Cole 1957). Similar enzymes are also found in organs and secretions of various vertebrates, invertebrates, bacteria, and even plants (e.g., Papaya latex), thus making lysozymes the most widely distributed substance (Ogawa et al. 1971). Based on different characteristics like structure, catalysis and immunization, lysozymes are classified into three families: chicken-type (c-type), goose-type (g-type) and invertebrate-type (i-type). Several other types of lysozymes, including phagetype, bacterial-type and plant-type lysozyme, have also been documented (Callewaert and Michiels 2010; Cao et al. 2015).

The bacteriolytic properties of hen egg white lysozyme (HEWL) were first described by the Russian scientist Laschtchenko in 1909. It was given the name, lysozyme in 1921 by Sir Alexander Fleming, since it causes bacterial lysis. Lysozyme catalyzes the breakdown of certain carbohydrates found in the cell walls of certain bacteria (e.g., cocci) and functions, in the case of lacrimal fluid, to protect the cornea of the eye from infection. Lysozyme has many firsts to its credit too. Lysozyme being first enzyme having all the 20 usual amino acids to be sequenced

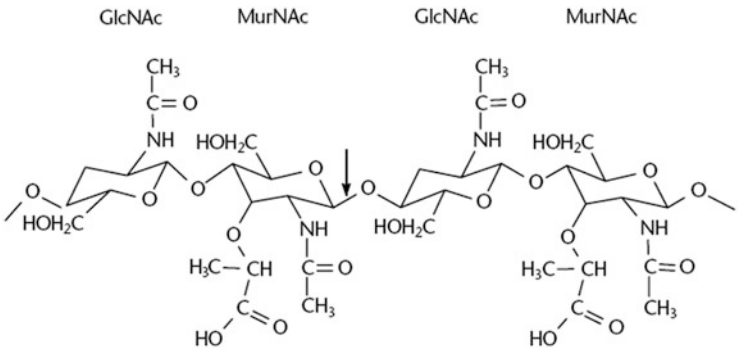

Fig. 5 Substrate for lysozyme is a copolymer of N-acetylmuramic acid (MurNAc, NAM) and N-acetyl glucosamine (GlcNAc, NAG), constituent of bacterial cell walls. The bond hydrolysed by lysozyme is indicated by the arrow (Imoto 2001)

(1963), and the first enzyme whose reaction mechanism and precise X-ray crystallographic study was accomplished (1961–1966). Thus, lysozyme is one of the most thoroughly characterized enzymes and serves as a model enzyme in biochemistry and biology.

Lysozyme being a small, stable enzyme makes an ideal molecule for research into protein structure and function and the recent development of structural biology owes much to it. Brian Matthews performed hundreds of mutations on the bacteriophage lysozyme by replacing one or more amino acids in the protein chain, removed large residues inside the protein, leaving a hole, or crammed a large amino acid inside, where it would not normally fit to study the consequence of such changes in the function of the enzyme. He has also attempted to create new active sites by creating new molecule-shaped pockets. Structures of hundreds of these mutant lysozymes are available at the PDB (Kuroki et al. 1993; Pjura et al. 1990).

Lysozyme catalyzes the breakdown of 1, 4-beta-linkages between C-1 of N-acetylmuramic acid (NAM) and C-4 of N-acetyl-D-glucosamine (NAG) residues in a peptidoglycan, a major component of gram-positive bacterial cell wall. This hydrolysis in turn compromises the integrity of bacterial cell walls causing lysis of the bacteria. It exerts lytic action mainly on Gram-positive bacteria and thus is effective against bacterial and viral infections (Fig. 5).

Hen Egg White Lysozyme (HEWL)

Hen egg white has abundant quantities if lysozyme and hence has been the most researched one. It is a small protein consisting of 129 amino acids, with a molecular weight of 14,307 Da. The 10 carboxyl, 7 amino, 11 guanidyl groups, six tryptophyl residues, and four disulfide bridges in locations between Cys 6-Cys127, Cys30-Cys115, Cys 64-Cys 80 and Cys 76-Cys 94 helps its folding into a compact globular structure and lends stability and unusual compaction. Lysozymes were the first

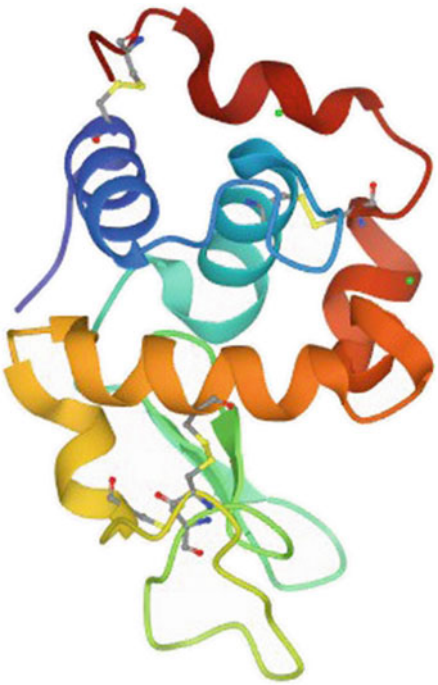

Fig. 6 Ribbon diagram of hen egg white lysozyme

enzyme to have their three-dimensional (3D) structures resolved by X-ray crystallographic method in 1965 by David Philips (Deborah et al. 1984; Fleming 1922). X-ray studies of the lysozyme-inhibitor complex helped identify the structure and location of the active site which is located in a deep cleft of the protein structure. It has both alpha and beta folds, consisting of five to seven alpha helices and a three-stranded antiparallel beta sheet. The enzyme is roughly ellipsoidal in shape, comprising of two domains joined by a long alpha helix forming a cleft. The upper lip of the active site cleft confers potent antimicrobial activity. The N-terminal domain (residues 40–88) has some helices and beta parallel sheets and the second domain (1–39 and 89–129) has mostly alpha helical structure (Fig 6).

Catalytic Mechanism

The two catalytic amino acids residues were identified as aspartate-52 and glutamate-35. At pH 5 (optimum pH for lysozyme), Asp-52 is deprotonated, while Glu-35 is in its undissociated form, the enzymatic reaction is possible due to this successful co-existence. Glu-35 acts as general acid in the reaction donating the proton to oxygen atom of glycosidic bond to accelerate the reaction. Asp-52 stabilizes the oxy-carbonium ion generated at the site of the reaction.

In most of the glycosyl hydrolases studied aspartate and/or glutamate residues have been found to be involved in catalysis.

Glycosyl hydrolases have innumerable industrial and biotechnological applications from biofuel production to drug design. Cellulases are involved in several industries like biofuel, food, feed, beverages, paper, textile, pharmaceutical, agricultural etc. Enzymic hydrolysis of cellulosic biomass results in the generation of sugars that are the starting materials for production of various value added products of commercial interest, such as bioethanol, organic acids, sugars and animal feeds (Kuhad et al. 2011).

Genetically inherited or, in rare cases acquired deficiency or malfunction of glycosyl hydrolases, such as lactase deficiency or break down of cell–cell communication can lead to serious health problems, from allergies and autoimmune diseases to severe recessive lysosomal storage diseases like Tay-Sachs, Hunter, Fabry, Gaucher, and Krabbs diseases, caused by lack of hexosaminidase A, iduronate-2-sulfatase, α-galactosidase A, and glucocerebrosidase, respectively. Glycosyl hydrolases, glycosyl hydrolases inhibitors, and chaperones are used to treat these diseases, but chances of a complete cure are still a long way off.

Applications of Lysozyme

Antibacterial characteristic of lysozyme against Gram-positive organisms is employed by a myriad of industries like food processing, pharmaceutical, and medicine. The food processing industry benefits primarily by its application as a natural preservative. Lysozyme is widely used as a preservative for meat, fish, milk, dairy products, as well as for fruits and vegetables. The pharmaceutical industry utilizes lysozyme for creating adjuvant drugs for antibiotics and analgesics to be used in microbial infections. It is also used in the treatment of leukemia and neoplastic diseases. Furthermore, lysozyme can act as an indicator of the progression of pathological variations in humans and animals.

Lysozyme has the characters of a ferment. There is an increase in activity up to 60 °C, but above 65 °C, it is destroyed more rapidly. Neutral medium is best to act optimally. Lysozyme resists peptic or tryptic digestion thus increasing the superfluity of applications. Lysozyme can be preserved for a long duration, when kept dry. Commercial dried egg albumen is considered as very rich source of lysozyme.

The protein engineering of lysozyme has substantially extended its practical applications. The engineered enzyme, being a dimeric protein exhibits a new specific activity in relation to Gram-negative bacteria and Gram-positive bacteria. In the monomeric form it is more active against Gram-positive bacteria, as reported by Ibrahim et al. (1991, 1996), Lesnierowski et al. (2004) and Kijowski et al. (2006). A drug produced on the basis of lysozyme dimer exhibited immune stimulating and immune corrective activity and was more potent in the treatment of bacterial and viral diseases.

Antimicrobial Activity

Lysozyme being a muramidase, hydrolyses the β-l, 4-glycosidic linkage of peptidoglycans, and leads to breakdown of the murein layer which decreases the mechanical strength of the bacterial cell wall. Hence, nucleic acids do not bind to the cell wall resulting in the disintegration of bacterial genetic material, finally leading to the death of the bacteria (Wang et al. 2005).

Non-lytic activity of lysozyme was recognized by the fact that partially or completely denatured lysozymes deficient in enzymatic activity could still work against both classes of bacteria. This property has been attributed to the cationic and hydrophobic properties associated with the conformational changes (Ibrahim et al. 2002; Masschalck et al. 2000).

Lysozyme modified with polysaccharides by the Maillard reaction, exhibits many enhanced characters such as improved solubility, stability, emulsifying characteristics and antimicrobial activity. These improved characteristics make lysozyme an ideal molecule in food matrices, especially in the fish, meat, dairy, fruit, vegetable and wine processing industries.

Lysozyme from egg white is widely used in food industries. It is effective mainly against Gram-positive bacteria, but through denaturation, chemical modifications, or by combining it with other preservatives its spectrum can be broadened for Gram-negative bacteria also. The stability and safety of lysozyme makes it an ideal preservative for other food applications such as food packaging too (Min and Krochta 2005). It is also used as a preservative for food like sausages, broiler, raw marine products, as well as an antibutyric acid blowing agent in semi-hard cheese production. It increases shelf life of nominally processed and non-sterile foods like meats.

It can be added to infant formula milk and significance of lysozyme is in the improvement of digestibility of the milk by the normalization of the intestinal flora, and also it contributes in the improvement of natural immunity through the augmentation of properdin, r-globulin and agglutinins.

Saliva shows strong antimicrobial effect on the oral pathogens due to the imminent presence of antibodies as well as proteins. One such protein is lysozyme which is one of the most powerful natural antibacterial and antiviral activity compound. According to erstwhile studies, it has been reported that lysozyme may bind and aggregate Gram-positive bacteria and Gram-negative periodontopathic bacteria such as *Capnocytophaga gingivalis* (Jesse Joel et al. 2016). Lysozyme plays a vital role in improvement of digestive function in ruminants (Dobson et al. 1984), leaf-eating monkeys, and birds (Kornegay et al. 1994). In many invertebrates, bacteria represent a substantial part of their diet. Worms and flies often feed on decomposing organic matter including the large biomass of microorganisms, digestion of which is aided by midgut lysozyme (Lemos and Terra 1991).

Lysozyme, the enzyme produced by polymorphonuclear and mononuclear inflammatory cells, is a strong antibacterial molecule that inhibits the growth of pathogens. Lysozyme and β defensin-2 can act synergistically against some microbial strains which is in consistent with the concept that secreted antimicrobial

peptides and other components of innate immunity constitute the first line of defense consequently protecting the host mucosal surfaces (Lee et al. 2004). It is used prophylactically for dental caries, lozenges for sore throat, in contact lens decontamination solutions, and eye drops, topical creams for dystrophic and inflammatory conditions of skin and tissues. It is also used as a prognostic marker for male prostate cancer.

Industrial Applications

Lysozyme, from egg white is extensively used in soluble form to control lactic acid bacteria in different foods. It can be used to control malolactic fermentation (MLF) during winemaking. MLF is only considered necessary in red and in some white wine but requires to be controlled in all other types of wine (Liburdi et al. 2014).

Lysozyme can effectively control the growth of beer spoilage bacteria, chiefly lactic acid bacteria (Commission Regulation 2012). Lysozyme added to beer exerts a strong inhibitory action on any lactic acid bacteria that may be present and is very stable throughout the shelf life without any unfavorable impact on beer savor or foam stability (Silvetti et al. 2010). It is a good alternative to sulphites and prevents malo-lactic fermentation. But it has no effect against other spoilage agents like yeasts.

Lysozyme (as the hydrochloride) is used in cheese production to prevent "late blowing" (cheese spoilage). This phenomenon is caused by the growth of *C. tyrobutyricum* which carries out butyric acid fermentation. Lysozyme exhibits high specificity by preventing microbial contamination without inhibiting starter and secondary cultures required for the ripening process (Mine 2008). *C. tyrobutyricum* metabolize lactate and produce carbon dioxide, hydrogen, butyric acid and acetic acid. In curing process the accumulation of these gases builds up pressure and causes splits or cracks in the cheese. Lysozyme influences the acceleration of cheese ripening by the lysis of starter bacteria leading to the release of cytoplasmic proteolytic enzymes, which plays a key role in the cheese ripening process (Abdou et al. 2013).

Thus lysozyme obtained from comestible sources that is commonly used as food, can be considered acceptable for use in food processing when used in accordance with good production practice.

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Lignocellulolytic and Chitinolytic Glycoside Hydrolases: Structure, Catalytic Mechanism, Directed Evolution and Industrial Implementation

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Abstract

Lignocellulolytic and chitinolytic glycoside hydrolases are commonly used enzymes in commercial processes and thus are well documented for their vital role in agriculture, textile, paper, food, biofuel and healthcare sectors. Ongoing research is targeted to further enhance the applicability of these vital enzymes in the above mentioned sectors. In this regard, an up-to-date knowledge of enzymatic structure, catalytic mechanism and several modern approaches like recombinant technology, chemical and enzymatic pre-treatment, enzyme immobilization and directed evolution for the improvement of enzymes production, activity and stability is highly required. This review provides an up-to-date information and discussion on the various lignocellulolytic and chitinolytic glycoside hydrolases regarding the vital aspects of the enzymes *viz*. catalytic mechanism, structural features, directed evolution approaches, current applications and under research potential applications.

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Introduction

Lignocellulose and chitin biomass are the top two naturally most abundant polymeric biomass on earth (Bar-On et al. 2018). Due to the renewable nature, both of them are highly explored for the generation of wide range of value-added products and bioenergy generation. The conversion of these lignocellulosic and chitin containing biomass has been carried out by various technologies involving chemical and enzymatic means (Ngernyuang et al. 2018; Yadav 2017). However, enzymatic conversion is always preferred due to environmental safety and highly specific product development. In this regard, lignocellulolytic and chitinolytic glycoside hydrolases (GH) has been of prime interest for the scientific and industrial community due to their specific natural affinity towards lignocellulose and chitin, respectively. The lignocellulolytic GH are well documented for their application in, food, beverages, animal feed, laundry, textile, paper and pulp as well as in agriculture and biofuel generation (Isikgor and Becer 2015). Whereas, chitinolytic GH has shown their potential applicability in healthcare, agriculture, and food industries (Patil et al. 2000). These vast range of applications has resulted in enormous demand of lignocellulolytic and chitinolytic GH production with improved characteristics. In order to achieve cost-effective and industrial-scale production of these enzymes a lot of techniques have been developed. These include exploration of novel sources for enzyme production, utilization of chemical and molecular techniques to boost the production and properties as well as genetic manipulation in order to fulfil the requirement of industries i.e. high activity and productivity over a wide range of pH and temperature along with eco and cost-effective production (Liu and Kokare 2017). In the present study, detailed discussion is included about various lignocellulolytic and chitinolytic GH in term of their structure, occurrence, catalytic mechanism, directed evolution strategies and various developed as well as under developing industrial applications.

Glycoside Hydrolases

GH are the group of widely spread enzymes existing in all living organisms with an exception of some Achaeans and unicellular parasitic eukaryotes (Naumoff 2011). GH catalyzes the hydrolysis of glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. In order to enhance the ease of study and understanding, the GH were classified on various basis like substrate specificity, mode of action and amino acid sequence similarities (Henrissat 1991). GH classification on the basis of substrate specificity is simplest one and is the basis of recommendation of the International Union of Biochemistry and

Molecular Biology (IUBMB). The IUBMB classification of GH is expressed as EC 3.2.1.x, where x signifies the substrate specificity as well as molecular mechanism or type of linkages in some cases. However, the intrinsic problem associated with substrate specificity based classification is in accommodating the enzymes that can act upon several substrates. Additionally, the classification is also unable to reflect about the 3D structural features of these enzymes. Another extensively used GH classification is on the basis of mode of action i.e. *exo* (attacks specifically at one of the termini of polymer chain) or '*endo*' (attacks randomly within the polymer chain) (Davies and Henrissat 1995). Although the dissimilarity assessment on the basis of mode of action is a powerful mean of classification but the distinction measurement of many enzymes with processive or multiple-attack nature is extremely challenging. The drawbacks of the conventional classification system leads to the development of amino acid sequence similarities based classification. The amino acid sequence similarities based classification is based on the fact that there is direct relationship between sequence and folding similarities and hence, it provides structural, evolutionary and mechanistic information of enzymes (Henrissat 1991). According to the Carbohydrate-Active Enzyme database (CAZy), GH are divided in 156 family with 5,99,654 modules or in 18 clans on the basis of conserved protein folds (Henrissat and Bairoch 1996) (www.cazy.org). The clans reflects about a group of families with significant similarity in their tertiary structure, catalytic residues and mechanisms.

Catalytic mechanism: GH catalyzes the hydrolysis of glycosidic bond by the involvement of two amino acid residues of the enzymes, among which one be the proton donor commonly glutamate or aspartate while the other be a nucleophile or general base residue like glutamic or aspartic acid (Davies and Henrissat 1995). These catalytic residues spatial position are accountable for the hydrolysis via overall retention or overall inversion of the anomeric configuration. In case of some enzymes the catalytic nucleophile are also replaced by the acetamido group at C-2 of the substrate (Terwisscha van Scheltinga et al. 1995). In GH, the inversion of anomeric configuration takes place through single-nucleophilic substitution whereas, the retention occurs due to double-displacement mechanism involving covalent glycosyl-enzyme intermediate (Ardèvol and Rovira 2015). During the retention, first glycosylation takes place in which one residue act as nucleophile that attacks on the anomeric centre to displace the aglycon resulting to the formation of glycosyl enzyme intermediate (Koshland 1953). Simultaneously, the other residue acts as an acid catalyst and protonates the glycosidic oxygen upon bond disruption. Following the second step of deglycosylation, the GH is hydrolysed by water and the other residue serves as base catalyst and deprotonate the water molecules (Koshland 1953). GH families 18, 20, 25, 56, 84 and 85 contains enzymes with no catalytic nucleophile and hence the intramolecular nucleophile is provided through the neighbouring group participation mechanism by 2-acetamide groups that results in the formation of an oxazolinium ion intermediate (Terwisscha van Scheltinga et al. 1995; Vocadlo and Withers 2005). However, the GH of families 33, 34 and 143 uses a tryosine as a catalytic nucleophile which has to be activated by an adjacent base residue (Ndeh et al. 2017; van Aalten et al. 2001).

Directed Evolution of Enzymes

Enzymes based biocatalysis has emerged as one of the most promising and widely accepted means over the chemical processes for product generation (Turner 2009). Nowadays, enzymes are used in detergent, textile, pulp and paper, food and cosmetic industries (Cherry and Fidantsef 2003). These rapid increasing industrial applicability of enzymes are possible due to the day to day biotechnological advancements made in the field of recombinant DNA technology, fermentation techniques and directed evolution. Among these the directed evolution has promptly emerged as a prevailing approach for refining the characteristics of enzymes in a targeted manner. It can be explained as an artificial approach to mimic and speed up the natural evolutionary process in laboratory with the help of molecular biology techniques (Turner 2009). Directed evolution can alter enzymes to act in new reaction environments, optimize their catalytic activity towards substrates and enable them to catalyze new chemical reactions. The basic concept of directed evolution has shown in Fig. 1. The major steps in the directed evolution of enzymes, includes identification of preliminary state protein, modification of its gene, an expression and

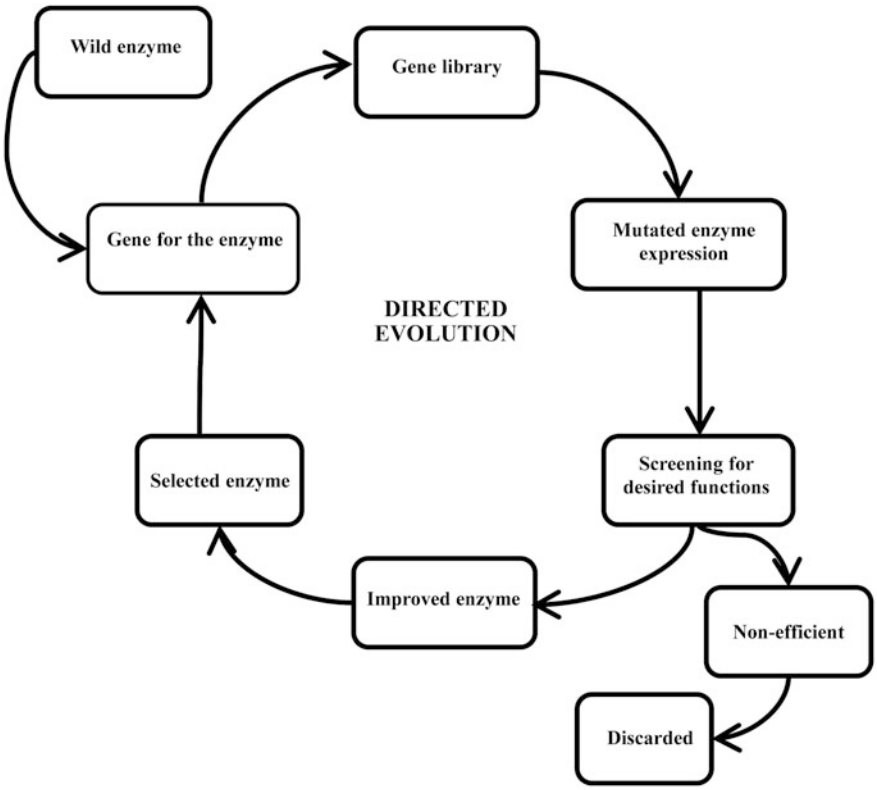

Fig. 1 Steps involved in the directed evolution of enzymes

screening strategy, re-modification, re-screening, and so on till an acceptable performance level in term of enzymatic activity, binding affinity or specificity is achieved (Turner 2003).

Lignocellulolytic Glycoside Hydrolases

Lignocelluloses, the major structural components of all plants is the most abundant organic compound present on the earth. About 10 to 50 $\times$ 10^9 tons of lignocelluloses are produced annually that is approximately 50% of the world's total biomass (Kuhad and Singh 1993). Lignocelluloses consist mainly cellulose (35–50%), hemicellulose (20–35%) and lignin (10–25%) which in different species, tissues and according to the maturity of plant cell wall (Isikgor and Becer 2015). Apart from them lignocelluloses also contain proteins, oils, and ash in less amount. Cellulose is a semi-crystalline, linear syndiotactic polymer of D-glucopyranose subunits linked together through intra- and intermolecular β-(1 → 4)–glycosidic bonds and provides strength to the material (Menon and Rao 2012). While, hemicellulose comprises of shorter, branched polymer chains of various C_6 and C_5 sugars that function as glue around and between the cellulose bundles. The C_6 sugar mainly contains glucose together with mannose and galactose while, C_5 sugars includes xylose and arabinose (Lange 2007). The next major contributor of lignocellulosic biomass is lignin which is a complex amorphous tri-dimensional polymer of propyl-phenol that is embedded in and bound to the hemicellulose and provides rigidity to the structure (Andlar et al. 2018). The detail structure of lignocellulose and their enzymatic degradation can be easily understand by Fig. 2. The enormous amount of lignocelluloses upon degradation has shown a wide range of applicability in various industries like biofuels, paper and pulp, agriculture, food etc. (Malherbe and Cloete 2002). The conversion of biomass into value-added products has become so effective and popular due to the practice of different lignocellulolytic enzymes i.e. pectinases, cellulases, hemicellulases and ligninases (Arevalo-Gallegos et al. 2017). The tremendous research success in the field of lignocellulolytic GH has resulted into several patents, some of recent patents publications are summarised in Table 2. As the present study is centered on GH, thus, the authors will focus only on cellulases and hemicellulases which are the members of GH.

Cellulases

Cellulases belongs to family 5, 6, 7, 9, 45 and 48 of GH and it catalyzes the endohydrolysis of (1 → 4)-β-D-glucosidic linkages (www.cazy.org). Cellulases mainly consist of three group of enzymes i.e. endo-(1,4)-β-D-glucanase (EC 3.2.1.4) which randomly attacks on the internal O-glycosidic bonds leading to the generation of glucan chains with various lengths, second is exo-(1,4)-β-D-glucanase (EC 3.2.1.91) that acts on the ends of cellulose chain releasing β-cellobiose and the third one is β-glucosidases (EC 3.2.1.21) which shows specific

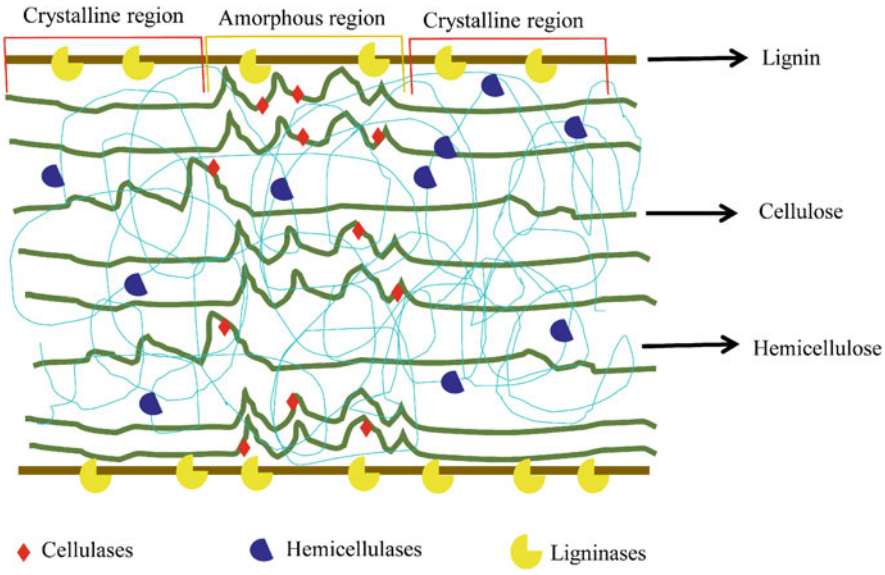

Fig. 2 Lignocellulose structure and enzymatic degradation

affinity towards the β-cellobiose disaccharides and generates glucose (Kuhad et al. 2011). The catalysis of GH family 5 and 7 cellulases follows retaining mechanism while GH family 6, 9, 45 and 48 shows inverting mechanism. Commercial production of cellulases has been in practice since last three decades. Several fungal genera like *Aspergillus* (Devi and Kumar 2017), *Fusarium* (Ramanathan et al. 2010), *Penicillium* (Jung et al. 2015), *Trichoderma* (Ellilä et al. 2017) has been reported to be high cellulolytic abilities. In case of bacteria *Bacillus* (Shajahan et al. 2017), *Pseudomonas* (Gautam et al. 2010), *Clostridium* (Xiong et al. 2018) has been reported to be produced cellulases on higher amount.

Hemicellulases

Hemicellulases are a diverse group of enzymes that hydrolyze hemicelluloses and are grouped in family 1, 2, 3, 4, 5, 10, 11, 26, 27, 39, 43, 53, 62, 67 and 116 of GH on the basis of amino acid sequence similarities (www.cazy.org). The GH families 43 and 67 of hemicellulases shows inverting mechanism for catalysis while, rest all the families with hemicelluloses follow retaining mechanism for catalysis. However, hemicellulases of GH family 62 catalysis mechanism is still not clear. These GH families mainly contains hemicelluloses *viz.* endo-β-1,4-xylanase, exo-β-1,4-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, endo-β-1,4-mannanase, exo-β-1,4-mannosidase and β-glucosidase (Shallom and Shoham 2003). Hemicellulases are mainly reported to be produced from *Aspergillus* (Tallapragada and Venkatesh 2017), *Trichoderma* (Inoue et al. 2016), *Thermomyces*

(Gramany et al. 2016), *Streptomyces* (Phuengmaung et al. 2018), *Bacillus* (Tariq et al. 2018) etc.

Advancement in the Directed Evolution of Cellulases and Hemicellulases

Cellulases and hemicellulases have well established significant commercial applications in textile industries *viz*. as biopolishing agents, in the pilling of fabrics, in the reduction of fuzz, and substituting in stonewash (Mojsov 2011; Schülein 2000). But, from the last three decades the research is concentrated on the degradation of lignocellulosic biomass for development of value-added products or the generation of biofuels (Mansour et al. 2016). However, these highly anticipated applications of converted lignocellulosic biomass has not yet been economically feasible. The major interruption for the development of an economical process is the cost and less efficiency of lignocellulose-degrading enzymes. In this regard, various bioprocess development strategies like exploration of higher lignocellulolytic enzyme producers, optimization of production media and conditions, biological or chemical pretreatment of the biomass and molecular means for high enzymes expression along with computational approach have been developed and checked for its efficiency to convert into industrial scale (Koutinas et al. 2012). However, all traditional approaches have not resulted into much success. So, the current scientific community is focusing on the employment of directed evolution along with other traditional developed techniques in order to enhance the enzyme production level as well as to improve its catalytic activity by altering their natural molecular and physical behaviors (Table 1) (Guo et al. 2018).

Diogo et al. (2015), developed a chimeric hemicellulase and reported three fold enhancement in the xylose production and improved thermotolerance as shift of optimum temperature was from 35 to 50 °C. The developed chimeric enzyme was the molecular fusion of two enzymes i.e. GH11 endo-1,4-β-xylanase and GH43 β-xylosidase from *Bacillus subtilis* in order to alter the substrate cleavage rate. Similarly, GH family 26 β-mannanase from *Bacillus sp*. MK-2 was altered through random mutagenesis in *B. subtilis* WB800 and the three positive mutants namely K291E, Q112R and L211I were selected on the basis of improved specific activities (Zhang et al. 2019). The single acid substitution in K291E was resulted into the 3.5 fold increment in catalytic efficiency while, the mutants Q112R (200%) and L211I (80%) showed increase in catalytic efficiency towards konjac glucomannan. In a study, Goedegebuur et al. (2017) reported improved thermal stability of cellobiohydrolase of GH family 7 from *Hypocrea jecorina* through the employment of directed evolution. By the directed evolution a variant FCA398 was developed and it was exhibiting 10.4 °C enhancement in T_m with a 44-fold increased half-life compared to the wild-type enzyme. Similarly, thermal stability was enhanced by improving the activity α-L-arabinofuranosidase of *Geobacillus vulcani* GS90 through directed evolution carried by one round error-prone PCR (Sürmeli et al. 2018). In the study, the selected enzyme variants GvAbf L307S and GvAbf Q90H/

Table 1 Recent directed evolved lignocellulolytic and chitinolytic glycoside hydrolases with improved properties and potential applications

Enzyme	Enzyme source	Technique used	Improved properties	Proposed application	References
Chitinase	*Bacillus subtilis* WB600	Site-directed mutagenesis	Expression level and specific activity	COS production	Pan et al. (2019)
β-Mannanase	*Bacillus sp.* MK-2	Random mutagenesis	Catalytic efficiency and thermal stability	Industry competent β-mannanase	Zhang et al. (2019)
Endo-β-1,4-glucanase	*Streptomyces sp.* G12	Random mutagenesis	Hydrolytic activity	Bioconversion of lignocellulosic biomass	Cecchini et al. (2018)
Chitinase	Serratia marcescens B4A	Site-directed mutagenesis	Kinetic and thermal stability	Suitable for industrial and biotechnological use	Emruzi et al. (2018)
Xylanase	*Aspergillus niger* ATCC1015	Site-directed mutagenesis	Catalytic activity and thermostability	Potentiality for industrial application	Wu et al. (2018)
Xylanase	*Bacillus circulans*	Random mutagenesis	Enzyme activity and thermal stability towards alkaline pH	For pulping and bleaching processes	Shah et al. (2018)
Chitosanase	*Bacillus sp.* MN	Rolling-circle PCR	Substrate specificity	Partially acetylated CHS COS production	Regel et al. (2018)
Cellobiohydrolase	*Hypocrea jecorina*	Site-directed mutagenesis	Thermal stability	Conversion of cellulosic biomass to fermentable sugars	Goedegebuur et al. (2017)
Chitosanase	*Renibacterium sp.* QD1	Site-directed mutagenesis	Thermostability	Hydrolysis of CHS to produce COS	Han et al. (2017)
Xylanase β-xylosidase	*T. reesei* ATCC66589	Disparity mutagenesis	Enzyme activity	Disparity mutagenesis for improving enzyme activity	Watanabe et al. (2019)

L307S showed 2.5-fold increase in the specific activities compared to the α-L-arabinofuranosidase of *G. vulcani* GS90. Irfan et al. (2018), developed six mutants (R81P, H82E, W185P, D186E, W185P/D186E and H82E/W185P/D186E) through site-directed mutagenesis by interchanging the xylanase producing residue of *G. thermodenitrificans* C5 with proline and glutamic acid. Both the mutant and wild type enzymes were expressed in *E. coli* BL21 and upon comparing to wild type (control) the mutant, H82E/W185P/D186E showed increment in half-life for thermal inactivation i.e. 13 times at 60 °C, 15 times at 65 °C, 9 times at 70 °C and 5 times at 75 °C. Due to the incredible growing exploration in the field of directed evolution of lignocellulolytic GH, the economical utilization of lignocellulosic biomass can be attained in the near future and hence there is also need of alike attention towards other naturally abundant biomass such as chitin containing waste.

Chitinolytic Glycoside Hydrolases

Chitin is the second most ubiquitous natural structural polymer after cellulose which is mainly present in the exoskeletons of arthropods, crustaceans, mollusks as well as in the cell wall of fungi and algae (Kumar et al. 2018d). Chitin is mainly produced from the seafood processing industries and is posing a serious environmental concern to the coastal areas (Yadav et al. 2019a). Chitin is an ordered crystalline microfibrils made up of β (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucose units and is found in three forms i.e. α, β and γ (Dutta et al. 2004). Among these α-chitin is the predominant and is formed by the antiparallel organization of microfibrils. However, sheets are present in antiparallel organization in β-chitin. In γ-chitin sheets are in both parallel as well as antiparallel fashion (Rinaudo 2006). Alike lignocellulosic biomass, the chitin has enormous applicability upon its transformation into derivatives with novel and improved properties i.e. chitosan (CHS), chitooligosaccharides (COS) and *N*-acetylglucosamine (GlcNAc) (Kumar et al. 2018a). CHS is the deacetylated form of chitin and is composed of α (1 → 4)-linked 2-amino-2-deoxy-β-D-glucopyranose. The vital properties of chitin and its derivatives that boosts its necessity of exploitation for human wellbeing *viz*. mucoadhesive, hemostatic, antimicrobial, antioxidant, antitumor, biodegradable and biocompatible (Younes and Rinaudo 2015). The chitin containing biomass can be converted into the valuable products through the chitinolytic enzymes *viz*. chitinases, chitosanases, chitin deacetylases and *N*-acetyl glucosaminidases (NAG). The cleavage action pattern of different chitinolytic enzymes has been diagrammatically represented through Fig. 3. The majority of chitinolytic enzymes are placed in GH class of CAZy database except chitin deacetylase (EC 3.5.1.41) which is grouped in family 4 of carbohydrate esterase class of CAZy database. There are a lot of research work has done related to the chitinolytic GH and many of them has patented. Some of the related patent publications with claimed application are discussed in Table 2.

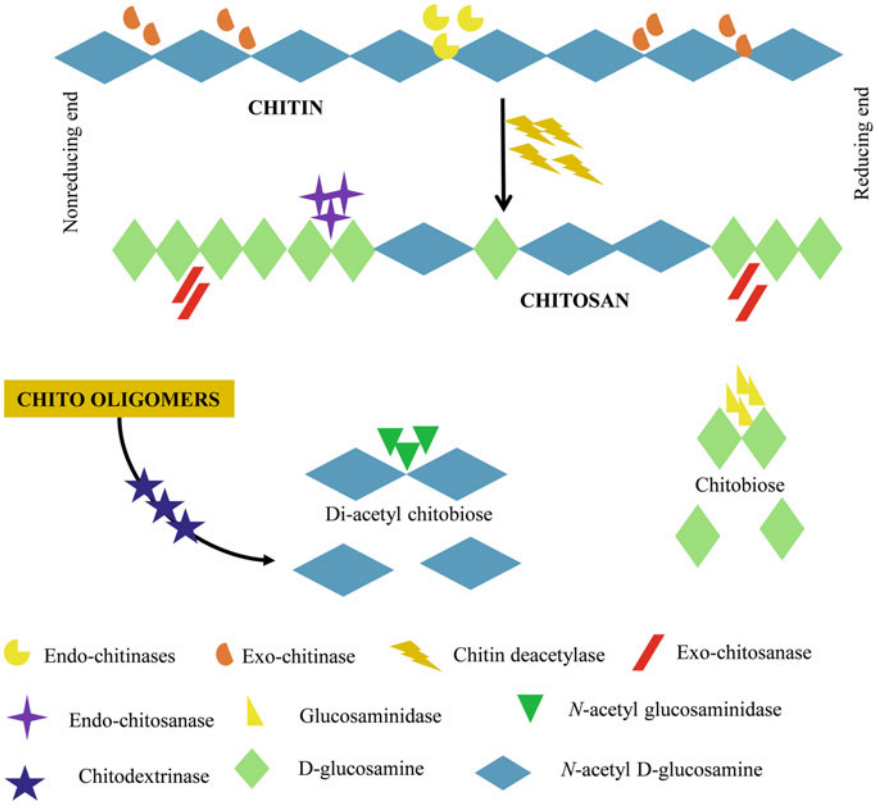

Fig. 3 Action pattern of chitinolytic enzymes

Chitinases

Chitinases (EC 3.2.1.14) are GH with specific affinity towards chitin and it randomly hydrolyse glycosidic linkages in the chitin and chitodextrins in a non-progressive manner to generate low molecular weight chitooligomers and free ends on which exochitinases and exochitodextrinases can act (www.cazy.org). On the basis of mode of action chitinases can be either endochitinases or exochitinases (Kumar et al. 2018b). The endochitinases randomly cleave the chitin chain at internal sites, generating soluble low molecular mass chitooligomers *viz.* chitobiose, chitotriose and chitotetraose (Hamid et al. 2013). However exochitinases contains two major enzymes groups: chitobiosidases (EC 3.2.1.29), involved in the catalysis of progressive release of di-acetylchitobiose starting at the non-reducing end of the chitin chain, and 1–4-β-glucosaminidases (EC 3.2.1.30), splitting the oligomeric products of endochitinases and chitobiosidases with the formation of GlcNAc monomers (Hamid et al. 2013). Chitinases has been mainly placed into family 18 and 19 of GH in the CAZy classification (Henrissat and Bairoch 1996). Family 18 chitinases

Table 2 Recent patent publication related to lignocellulolytic and chitinolytic glycoside hydrolases

Title	Patent/publication no.	Lignocellulolytic/ chitinolytic GH involved	Application claimed	Reference
Method of producing nanofibrillar cellulose with high absorptivity to fat and cholate	US9149064B2	Xylanase Cellulase	Enhanced fat and cholate absorbing capacity	Zhao et al. (2015)
Chitinase-3-like protein 1 as a biomarker of recovery from kidney injury	US2014/0200184A1	Chitinase-3-like 1/Brp-39/YKL-40	Biomarker for kidney injury and its reparative response	Elias et al. (2014)
Liquid detergent composition comprising cellulosic polymers and cellulase	US2018/006621A1	Cellulase	Formulation of liquid detergent for laundry	Pickering et al. (2018)
Insecticidal chitinase protein its encoding nucleotide and application thereof	US10006014B2	Chitinase	In insect control	Singh et al. (2018)
Preparation of a baked products comprising fibers treated by a cellulase	US20190029272A1	Cellulase Xylanase Cellobiohydrolase	Bakery products with improved anti-staling properties	Niemann (2019)
Method and system for preparing pulp for paper with grass straws as raw materials	US20180105851A1	Hemicellulases	Pulp preparation from grass straws	Sun and Zhongyu (2018)
Process for treating wastewater	US201900399932A1	Xylanase Cellulase Hemicellulase	Sludge treatment	Flannery (2019)
Cosmetic use of chitinase-type proteins	US9926587B2	Chitinase-type protein (YKL-40)	Stimulate terminal epithelial differentiation	Bernard and Donovan (2018)

follows the retention mechanism for catalysis while the family 19 chitinases shows inverting mechanism for catalysis. Family 18 chitinases utilizes *N*-acetamido carbonyl oxygen (i.e. through the neighboring group participation) as nucleophile in the double-displacement reaction instead of the common enzyme derived nucleophile. Family 19 chitinases contains chitinases of class I, II, and IV whereas, the family 18 contains chitinases of class III and V (Patil et al. 2000). Chitinases are found in a varied organisms *viz*. bacteria, fungi, yeasts, plants, actinomycetes, arthropods, and humans. The chitinases from microbial sources are of prime concern due to greater possibilities of converting them into commercial-scale production. In this regard, some chitinases producing fungi like *Thermomyces lanuginose* (Zhang et al. 2015b), *Aspergillus niveus* (Alves et al. 2018), *Humicola grisea* (Kumar et al. 2017), *T. viride* (Omumasaba et al. 2001) and bacteria such as *Paenibacillus barengoltzii* (Yang et al. 2016), *Serratia marcescens* (Horn et al. 2006), *B. pumilus* (Rishad et al. 2016) can be consider for industrial-scale production.

Chitosanases

Chitosanases (EC 3.2.1.132) are the GH that catalyzes the endohydrolysis of β-(1 → 4) linkages between D-glucosamine (GlcN) residues of partly acetylated CHS from its reducing end. However, there are also exo-β-D-glucosaminidase (EC 3.2.1.165) that attack CHS from its non-reducing end. Chitosanases are placed into families 5, 8, 46, 75 and 80 of GH on the basis of amino acid sequence (Lombard et al. 2014). However, on the basis of specificity of cleavage positions for the partly acetylated CHS, chitosanases are grouped into three subclasses i.e. subclass I (act on both GlcN-GlcN and GlcNAc-GlcN linkages), subclass II (split only GlcN-GlcN linkages) and subclass III (cleave both GlcN-GlcN as well as GlcN-GlcNAc linkages) (Thadathil and Velappan 2014). Families 8, 46, 75, and 80 chitosanases followed inverting mechanism for catalysis while, only family 5 chitosanases has been reported to use retaining mechanism for catalysis (www.cazy.org). Generally the molecular mass of chitosanases are present in the range of 20–75 kDa. However, the molecular mass of chitosanases from *A. fumigatus* KH-94 has been reported to be 108 kDa (Kim et al. 1998). Mostly, chitosanases are produced from bacteria, fungi, cyanobacteria and plants. Recently, chitosanases are reported to be produced from bacteria like *B. mojavensis* (Liaqat et al. 2018), *P. macerans* (Doan et al. 2018), *Pseudoalteromonas* sp. (Zhou et al. 2019) and from fungi like *Penicillium* sp. (Aktuganov et al. 2019), *Gongronella butkeri* (Seki et al. 2018), *Aspergillus* sp. (Zhang et al. 2015a).

N-Acetyl Glucosaminidases

NAG (EC 3.2.1.96) are the GH that catalyzes the endohydrolysis of *N,N″*-diacetylchitobiosyl units. In this reaction one GlcNAc residue remains attached to the protein and the rest of oligosaccharides are released in an intact manner.

However, β-*N*-acetylhexosaminidase (EC 3.2.1.52) hydrolyzes the terminal non-reducing *N*-acetyl hexosamine residues. On the basis of amino acid sequence similarities NAG are placed into families 3, 20, 73, 84 and 85 of GH in CAZy database (Lombard et al. 2014). The GH families 3, 20, 84 and 85 follows retaining mechanism for catalysis whereas, the family 73 catalytic mechanism is not clear. NAG is present in various tissues in human body and helps in breaking chemical bonds of glycosides and amino sugars that forms the structural components of several tissues and NAG also serve vital role in the degradation and disposal of many parts of cell (Wen and Kellum 2012). NAG has been detected in a range of bacteria, fungi, insects, plants and animals. Recently, NAG has been reported from bacteria like *Streptomyces alfalfa* (Lv et al. 2019), *B. subtilis* (Nayyab et al. 2017), *Corynebacterium glutamicum* (Matano et al. 2016) and from fungi like *A. versicolor* (Bojarová et al. 2019), *T. reesei* (Chen et al. 2015).

Update on the Directed Evolution of Chitinolytic Glycoside Hydrolases

Chitinolytic GH has been well explored for their applications in the conversion of chitin and CHS into their oligomers and monomers that has a huge potential applicability a wide range of sectors *viz*. medicine, agriculture, wastewater treatment, food and cosmetics. Apart from the polymer degradation ability chitinolytic GH also has a lot of application in agriculture in fighting against phytopathogens and various insects (Kumar et al. 2018c, d). In order to strive the demand there is much more want of developing processes having enhanced level of chitinolytic enzyme production with better efficiency in terms of catalytic power, temperature stability and wide range of pH stability. In this regard, a lot of notable work has been done and still ongoing with addition like finding of novel strains, improving fermentation conditions, effective pretreatment strategies and recombinant DNA technology, but still the desired level of output has not achieved (Devi and Kumar 2017; Gramany et al. 2016; Inoue et al. 2016). Recently, the combination of these conventional techniques along with the directed evolution approaches are promising a lot for the commercial scale production of chitinolytic GH and their valuable bioactive oligomers and monomers (Table 1) (Abdul Manas et al. 2018). The secretion efficiency and thermal stability of chitosanaseA from *Mitsuaria chitosanitabida* 3001 was significantly enhanced through the directed evolution and reported 1.5 fold increment in secretion efficiency and 17% (at 50 °C) enhancement in thermal stability as compared to the wild –type chitosanaseA (Yun et al. 2006). The study used inactive chitosanaseA mutant gene (G151D) in order to perform mutation through an error-prone PCR technique and the gene that restored chitosanase activity were selected. Fan et al. (2007), reported an enhancement in the catalytic ability of chitinase produced from *Beauveria* bassiana through error prone PCR and DNA shuffling. The amino acid alterations were performed outside the two putative substrate binding sites and the catalytic region namely SXGG and DXXD XDXE in the Bbchit1 gene. DNA shuffling technique was also used to bring

betterment in pH performance and activity with two chitosanase gene from *B. cereus* KNUC51 and *B. cereus* KNUC55 (Park and Ghim 2009). The DNA shuffled products i.e. YM18 and YM20 were reported to enhance chitosanase specific activity as compared to the native up to 250% and 350%, respectively. The study also reported that the shuffle product MY20 was exhibiting a shift in the optimal pH level from 5.5 to 6.5. Similarly, the properties of chitinase from *B. licheniformis* were improved through the directed evolution carried out through error prone PCR and DNS shuffling (Songsiriritthigul et al. 2009). The study reported 2.7 and 2.3 fold increment in the average catalytic efficiency at pH 3.0 and 6.0, respectively. Yu and Xu (2012), reported 1.8 fold increased endochitinase activity towards 2-nitrophenyl-*N*-acetyl-β-D-glucosaminide and 3.5 fold increment in endochitinase activity towards colloidal chitin through an error prone PCR directed evolution. The thermostability of chitinase produced from *S. marcescens* B4A was improved by the site-directed mutagenesis of G191V (Emruzi et al. 2018). The study suggested an increase in thermostability of 5 and 15 fold at 50 and 60 °C respectively with a decrease in the K_m and V_{max} of about 1.3 and three fold, respectively. Recently, a study suggested that the N-terminal sequence is essential for the optimum temperature, pH stability, thermostability and catalytic efficiency of chitosanase CsnA from *Renibacterium* sp. QD1 (Han et al. 2018). The used extra 7-residue *N*-terminal sequence was not from the regular secondary structure in chitosanase. The above-mentioned studies suggests the possibilities directed evolution for the improvement of chitinolytic GH in term of their catalytic efficiency, thermostability and pH stability. But, these works are in their initial stage in concern of commercial level output so, there is a lot of improvement and studies required.

Application in Industries

The lignocellulolytic and chitinolytic GH have shown immense applicability in a wide range of sectors (Sarrouh et al. 2012). Lignocellulolytic GH are utilized commonly in paper and pulp, textile and laundry, beverages and biofuel industries while the chitinolytic GH has more explored for medicine, agriculture and food industries (Fig. 4). At present development of techniques for the generation of biofuel from the naturally available biomass is one of the prime concern of the scientific community due to the rapid utilization of the conventional fossils fuels. The lignocellulolytic and chitinolytic GH possess specific affinity towards the most abundant biomasses of the earth and hence are widely applied in the value-added product generation. There are some success achieved in the form of commercial product development which are presented through Table 3 with their application. In the forthcoming section of the study, authors are going to discuss about the various utilization of the lignocellulolytic as well as the chitinolytic GH in different industries.

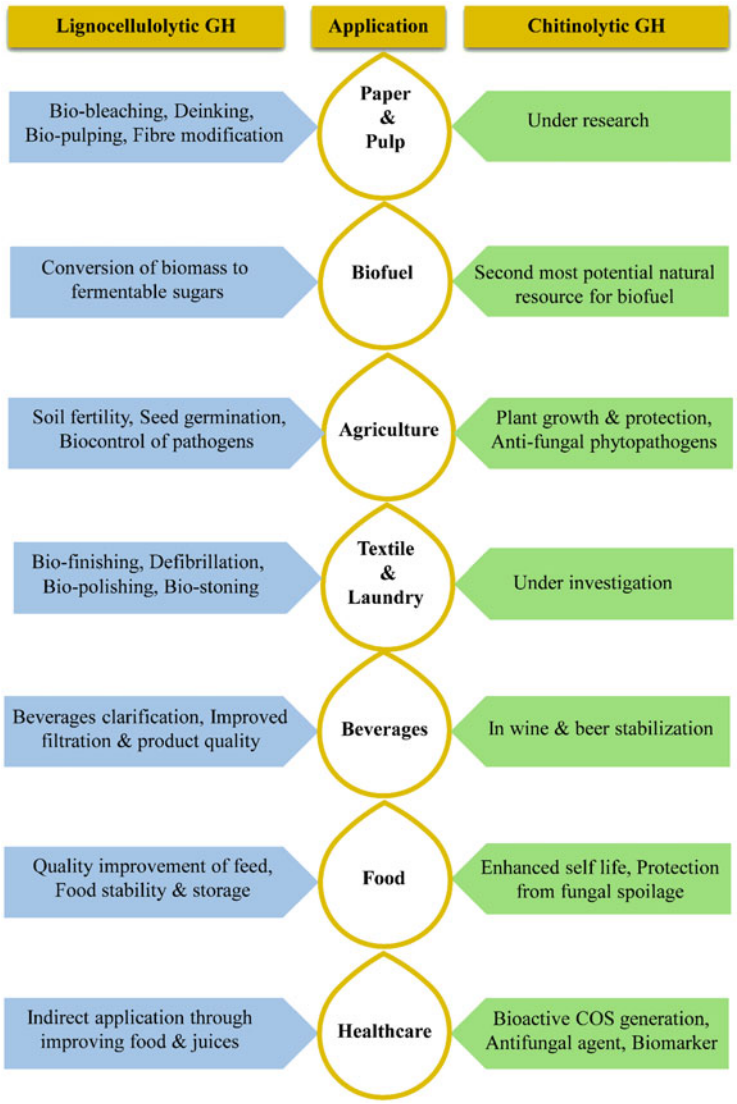

Fig. 4 Application of lignocellulolytic and chitinolytic glycoside hydrolases

Paper and Pulp

Lignocellulolytic GH have been tremendously used in the pulp and paper industries due to their vast range of applicability *viz*. de-inking, pulping, bleaching, fibers modification, debarking, pulp fiber characterization, and drainage improvement (Imran et al. 2019; Singh et al. 2016). These enzymes has imparted crucial role in lowering the overall production cost of paper with improved qualities. The

Table 3 Commercially available lignocellulolytic and chitinolytic glycoside hydrolases and their application

Product name	Enzymes	Source	Application
Celluclast®	Cellulase	*Trichoderma ressei*	Enhanced conversion of cellulosic materials into fermentable sugars
Cellic®CTec2	Cellulase β-glucosidases Hemicellulase	Not reported	Degrade cellulose to fermentable sugar Cellulosic ethanol production
Accellerase®TRIO™	Exoglucanase Endoglucanase Hemicellulase β-glucosidases	*T. ressei*	Accelerates biomass conversion processes
Cellic®HTec2	Endoxylanase Cellulase	Not reported	Boost conversion of hemicellulose to fermentable sugar Cellulosic ethanol generation
Fibrezyme®G4	Cellulase	*Myceliophthora thermophila*	Reduces energy requirement and increase production rate for pulp and paper industries
AlternaFuel®CMAX™	Cellulase β-glucosidase Hemicellulase Arabinase	*M. thermophila*	Degradation of lignocellulosic biomass Effective in both acidic and neutral processes
Chitinase (by Sigma-Aldrich, USA and Megazyme, Ireland)	Chitinase	*Streptomyces griseus* *Clostridium thermocellum* *T. viride* *S. marcescens*	For research only
Chitosanase (by Sigma-Aldrich, USA)	Chitosanase	*S. griseus*	For research only

utilization of lignocellulolytic GH in paper and pulp industries have attained their commercial position around two decades ago due to the remarkable research in this field. Thus there we are describing only about the latest improvements made in the paper and pulp industries related to lignocellulolytic GH. Buzała et al. (2016), investigated the effect of xylanases on kraft pulp and reported saving of refining energy without reducing the paper characteristics. The study used enzyme preparation made up of xylanase from *T. lanuginosus*, cellulase from *Aspergillus* sp., and a multienzyme preparation NS-22086 which contained the both enzymes and reported shorter time for freeness (30°SR) with greater water retention value. The modern industrial enzymatic process are also targeting on the recycling of enzyme as some amount of active enzyme are left after the process can be used in order to reduce the production cost. Wang et al. (2016), demonstrated the use of fresh cellulase addition in recycling cellulase for the industrial level enzymatic treatment of the kraft-based dissolving pulp. The study also reported recovery of about 48.8–35.1% of cellulase

activity from the filtered liquor in five recycle rounds and the recovered cellulase were can be reused in the treatment of pulp. Similarly, some combined process like involvement of pulp fractionation and cellulase treatment of each fraction has also investigated (Duan et al. 2017). The combined approach at a given viscosity resulted into lower polydispersity index (3.71 vs 4.98) with a greater fock reactivity (85.6 vs 76.3%) compared to the cellulase treatment alone. Recently, scientific community is also paying its key attention on the paper waste management and their reusability techniques development. Jain et al. (2017), showed the utilization of thermostable cellulase from *Thermoascus aurantiacus* RCKK for the hydrolysis of office waste paper, algal pulp (*Gracillaria verulosa*) and biologically treated wheat straw at 60° with release of significant amount of substrate i.e. 830 mg/ml, 285 mg/g and 260 mg/g, respectively. The formulation of cellulase and xylanase from *Escherichia coli* SD5 was found to be promoting deinking efficiency and paper pulp modification with reduction of kappa number and hexenuronic acid (Kumar et al. 2018e). Kaschuk and Frollini (2018), investigated the impact of different cellulosic material properties like average molecular mass, crystallinity index, and hemicelluloses content on the efficiency of the enzyme derived conversion of cellulose to glucose. The study suggested highest yield for the conversion of 88% in case of sisal pulp, followed by 64% for microcrystalline cellulose and 52% conversion for filter paper. Recently, significant reduction in the refining energy with improved fiber freeness was achieved at lab as well as plant scale (Tripathi et al. 2019). The study reported reduction in refining energy of enzymatic treated pulp by 29.3% in lab scale as well as 20% in plant scale trials. The remarkable research progress in the field of lignocellulosic GH has resulted in the low cost and better quality production in pulp and paper industries. However, there is still need of attention on the utilization of other natural polymer like chitin and their degrading enzymes in these industries.

Textile and Laundry

Lignocellulolytic GH has gained attention in textile and laundry from last two decades due to their ability of modifying cellulosic fibers in desired manner as well as capacity of enhancing fabric quality. Nowadays, these enzymes are best-known for their bio-stoning and bio-polishing applications. They applicability has widely accepted in washing powders because of their capability of enhancing detergent performance and in removal of small, fuzzy fibrils from fabric surfaces in order to improve the appearance and color brightness (Bhat 2000). Battan et al. (2012), evaluated the impact of thermostable xylanase from *B. pumilus* ASH in the processing of textiles and found 0.9% higher whiteness values of micro poly fabrics after bioscouring with the xylanase as compared to the chemical scoured fabrics. Similarly, the bioscouring of flax fibers through the cellulase-free xylano-pectinolytic enzymes was carried out and resulted in the 1.84% sugar release, 4% weight loss, 19.46% increase in brightness along with 8.2% whiteness enhancement (Kaur et al. 2016). The study also suggested that the enzymatic pretreatment of flax fibers reduced the scouring chemical consumption up to 70% with 30% reduction in

the bleaching chemicals as compared to the control flax fiber. Gumel et al. (2018), extracted cellulase from *Aspergillus niger* by using pineapple peel as the substrate and the produced cellulase was applied to cotton fabrics at different concentration at pH 5.5 and temperature 55 °C. The study reported that the cellulase was able to remove staple fibers from the fabrics leading to glabrous appearance and soft touch as well as the better dye uptake. Xylanases from *T. longibrachiatum* KT693225 has been reported to be highly effective in wet processing stages in textile industries like desizing, bioscouring and biofinishing (El Aty et al. 2018). Although, the chitinolytic GH has not reported to have direct utilization in textile and laundry but, the deacetylated form of chitin (i.e. CHS) has gained tremendous applicability in textile industry due to its antimicrobial activity.

Biofuel

Lignocellulose being the most abundant renewable organic material is considered as the most promising alternate of the conventional petroleum fuel. A lot of attempts has been carried out in the conversion of the enormous biomass into biofuel (Himmel et al. 2007; Yadav et al. 2019b). In this regard, lignocellulolytic GH are proposing to play major role due to their specific affinity towards the lignocellulosic biomass. The enzyme derived energy production always has an upper hand on the fossils fuels in term of environmental management. The utilization of lignocellulolytic GH obtained from fungi are highly explored for biofuel production (Srivastava et al. 2018). For the enhanced biofuel production strategies are developed in order to reduce enzyme dose. Liu et al. (2016), engineered a cellulose adherent *Saccharomyces cerevisiae* and it was capable of directly producing ethanol from rice straw with 40% less enzyme dose. The developed strain showed clear cell-to-cellulose adhesion with a tearing pattern of cellulose degradation resulting into the enhanced hydrolysis efficiency. Another study demonstrated the improved efficiency of bioethanol production from *Eichhornia crassipes* by the mean of statistical optimization (Das et al. 2016). The study reported two fold increase in ethanol production compared to the unoptimized condition. Yarbrough et al. (2017), compared the capability for coproduction of nanocellulose and fermentable sugars by the utilization of two different enzyme system. The first cellulase enzyme system was the "free enzyme" system of *Trichoderma reesei* and second one was a complex multifunctional enzymes produced from *Caldicellulosiruptor bescii*. The study also suggested the complex enzyme system better than the free enzyme system in term of total cellulose conversion, sugar production, and nanocellulose generation (Yarbrough et al. 2017). Gil and Maupoey (2018), developed in integrated design for the simultaneous saccharification and fermentable of pineapple waste for the enhanced ethanol generation as well as extraction of bromelain. The study reported increase in ethanol production ($4.7 \pm 0.3\%$ v/v) by direct fermentation, ($5.4 \pm 0.1\%$ v/v) through simultaneous saccharification and fermentation and ($4.9 \pm 0.4\%$ v/v) from saccharification and fermentation of the solid waste. β-xylosidase from *Aspergillus niger* has also been reported to have potential of bioethanol generation from

lignocellulose as the hydrolysis of pretreated straw at 70 °C by the lignocellulosic enzyme cocktail resulted in 19-fold increment in xylose level after 6 h (Boyce and Walsh 2018). Enzyme immobilization on various matrix has also been explored to enhance the enzyme efficiency for biofuel production. Cellulase from *Bacillus subtilis* UniMAPKB01 was immobilized on a multi-walled carbon nanotubes and the immobilized enzyme was capable of producing 0.129 mg/0.5 ml of glucose which serves as the precursor for bioethanol production (Naresh et al. 2018). The chemical pretreatment of grasses like vetiver grass and switchgrass has shown enormous potentiality of biofuel production. Subsamran et al. (2019), reported 90 FPU/ml of fermentable sugar from enzymatic hydrolysis with yield of 21.10 and 5.85 g/l of bioethanol production from the 1% (w/v) NaOH and 0.5% (v/v) H2SO4 pretreatment of vetiver grass, respectively at 121 °C for 60 min. Recently, switchgrass was pretreated with (3 g/l) acetic acid and the simultaneous saccharification and fermentation approach was employed by the help of *Clostridium saccharoperbutylacetonicum* N1–4 to produced 8.6 g/l butanol. There are some success in the conversion of biofuel production from lignocellulosic biomass through lignocellulolytic GH but still it is lacking behind in term of cost efficiency and produced biofuel application related engineering. Nowadays, researchers are also paying attention the chitinous biomass for energy generation but research in this field in its primitive due to the vast applicability of chitin oligomers and chitin derived products in the field of medicine and agriculture.

Agriculture

The lignocellulolytic GH has been extensively exploited for their use in agriculture and reported to have application in enhancing soil fertility, promoting cell growth, improvement of seed germination and protection, biocontrol of pathogens and diseases, extraction of bioactive compounds, production of protoplast (Phitsuwan et al. 2013). The utilization of plant cell wall disrupting lignocellulolytic GH for the extraction of novel natural bioactive compounds from plants with plenty of beneficial properties has been extensively reviewed (Puri et al. 2012). Chamani et al. (2012), evaluated different cellulase and pectinase enzymes treatments on the production of protoplast and its viability in *Lilium ledebeourii* and reported the significant effect of cellulase treatment on the protoplast production. The cellulase at 4% level was the most effective treatment with 3.71×10^5 protoplast/g FW. Lignocellulolytic GH producing microorganisms has been well documented for their application in the biological control of phytopathogenic fungi. Cellulase from *T. harzianum* Th22 was reported to stimulate 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxzzin-3(4H)-one (DIMBOA) as well as the defense-related gene expression in maize root against *Fusarium graminearum* (Saravanakumar et al. 2018). The study suggested that the cellulase gene *Thph1* and *Thph2* were accountable for the biocontrol of *F. graminearum* and its associated plant diseases by activating jasmonate acid, ethylene, systemic acquired resistance, DIMBOA and plant innate immunity-related gene expression in the maize root. Recently, cellulase has also

been reported to increase the endophytism of *Metarhizium brunneum* CB15 in potato plants (Krell et al. 2018). The beads treated with cellulase and inactivated baker's yeast was reported to upsurge mycelial progress by 13.6% with a shift from mycelial growth to spore formation i.e. maximum numbers of $2.5 \times 10^8 \pm 6.1 \times 10^7$ per bead (Krell et al. 2018).

Similarly, chitinolytic GH has been also well investigated in agriculture sector for their widespread employment as an agent of plant growth and protector. The chitinolytic GH have natural affinity towards the hydrolysis of chitin, which is the major component of cell wall of fungi. The intrinsic property of chitinolytic GH made it the most demanding agent to fight against the phytopathogens and helps in improving the crop cultivation and productivity (Kumar et al. 2018c). Chitinases has reported to be effective in controlling crop diseases like rot, blight, rust, spot and wilt diseases of various crop plants as well in the development of resistance against plant diseases (Sharma et al. 2011). Awad et al. (2017), investigated partial pure fungal exochitinase on mortality of *Galleria mellonella*, *Spadoptera littoralis* and *Agrotis ipsilon* and it was reported to be 92, 86.67 and 65.67%, respectively. A transgenic tomato was developed *via in vitro* and *in planta* transformation technique with enhanced salt and drought tolerance through the expression of osmotin-like protein and *Chi11* genes (Kumar et al. 2016). Insect chitinase gene, *CsCht10* was reported to control *Chilo suppressalis* Walker which is one of the most destructive pest for rice crop (Zhao et al. 2018). Cheng et al. (2017), cloned an endochitinases, VDECH from *Verticillium dahlia* strain Vd080 that showed high thermostability. The study also suggested that VDECH was able to triggered plant defense responses with hypersensitive response, oxidative burst, and elicited increased expression of defense-related genes in *Arabidopsis* as well as in cotton. Chitinase I gene from barley was expressed in *E. coli* Rosetta strain under the control of T7 promoter in pET 30a vector and the expressed chitinase was more effective as compared to control against phytopathogenic fungi like *Alternaria solani*, *Fusarium spp*., *Rhizoctonia solani* and *V. dahlia* (Toufiq et al. 2018). Recently, chitinase and chitinolytic GH derived CHS and COS based products has been developed in the form of biopesticides and biofertilizers by various companies and their industrial-scale production and field trials are in progress that reflects the possibilities of presence of such products in market in near future.

Beverages

Lignocellulolytic GH are extensively used in the preparation of beverages products like wine, beer, and fruits as well as vegetables juices (Bhat 2000). The GH application improves the primary fermentation and extraction processes along with enhancing the quality of the products. Moreover, they are also known for parting significant role in the advancement of clarification and aroma of beverages (Kaur and Gill 2019). Recently, a lot of successful attempts has been made for the utilization of GH enzymes in winemaking from grapes (Gao et al. 2019). Several lignocellulolytic GH enzymes are also utilized in the processing of fruit and vegetables and in the

improving the quality of the processed juices (Kumar 2015; Toushik et al. 2017). Zhao et al. (2013), reported the production of thermophilic xylanase from *Achatomium* sp. Xz-8 with catalytic efficiency of 3710 ml/s/mg. The produced xylanase showed improved filtration of 20.24% and 38.50% when combined with the commercial β-glucanase under the stimulated mashing conditions which was comparable to Ultraflo, Novozymes. Recently, a magnetic biocatalyst of pectinase and cellulase were prepared and compared to the glutaraldehyde-activated magnetite in term of magnetic properties, immobilization parameters, stability and grape juice clarification (Dal Magro et al. 2018). The study suggested about the increased possibility of recovery of biocatalysts (i.e. cellulase, 33.4%) applicable in juice industries when the magnetic technology was integrated with enzyme technology. However, the chitinolytic GH has been less studied in the field of beverages industries but, the chitinolytic GH derived products of chitin has been reported to be applicability in providing stability and production to the beverages (Rocha et al. 2017; Yang et al. 2017).

Food

The lignocellulolytic and chitinolytic GH has a wide range of application in food industries *viz*. quality improvement, extraction, clarification, stability and protection. Lignocellulolytic GH has been extensively explored for their function utilization in fruits and vegetables processing industries (Toushik et al. 2017). Cellulase and pectinase treated *Ecklonia cava* extract showed anti-obesity effects in C57BL/6 N mice with high-fat diet induced obesity (Kim et al. 2018). The anti-obesity effects were monitored through evaluating change in body weight, fat, serum lipid levels and lipogenic enzymes levels and the results of the study suggested that the *Eckloni cava* supplementation reduces high-fat diet induced obesity and following metabolic disorders (Kim et al. 2018). Cellulase and hemicellulase has also been applied for the enhanced utilization of the leftover processed by-products i.e. okara from the soymilk and tofu production (Vong et al. 2017). The total nutritional value of okara was achieved through the sequential saccharification by Celluclast® (cellulase) and Viscozyme® L (cellulase and hemicellulase), followed by *Yarrowia lipolytica* derived fermentation. The resulted okara was reported to have higher antioxidant activity with enhanced volume of total amino acids and ferulic acid. Lignocellulolytic GH has also been well documented for their valuable role in improving animal feed. The impact of xylanases on the nutrient digestibility and gut microbiota of growing pigs were investigated in a study conducted by Zhang et al. (2018). The study found that xylanase C from *Bacillus subtilis* more effective when applied to wheat-based diets whereas, xylanase A from *Fusarium verticilliodes* was showing better result with corn-based diets. Similarly, xylanases were reported to improve digestibility of dietary fibers in the stomach and hindgut along with the enhancement in the energy status of pigs fed based on wheat diets (Abelilla and Stein 2018). Xylanases and other lignocellulolytic GH has been investigated in the quality and nutritive value enhancement of wheat and other

grains used for poultry industries. Nourmohammadi et al. (2018), reported the better result of wheat-based feed diet from xylanase supplementation on male broilers in the terms of growth performance, energetic efficiencies, nitrogen balance and energy partitioning. The chitinolytic GH are more linked with the preservation of food mainly against post-harvest spoilage causing fungal pathogens like *Monilima spp*., *Botrytis cinerea*, *Penicillium expansum* (da Silva 2019). Chitinases with vicilins and lectins from legume flours were reported to have antifungal activity which resulted into the enhanced shelf-life of wheat bread (Rizzello et al. 2017). Moreover, the degraded enzymatic products of chitin i.e. CHS and COS are widely applied in the food packing industries due to their bioactive properties and improving physical qualities of the packing materials (Dutta et al. 2009).

Healthcare

Lignocellulolytic GH and chitinolytic GH has shown immense potential in the healthcare of human beings. They are reported to have a wide range of biomedical applications. Although the lignocellulolytic GH are not directly applicable for maintaining human healthcare. However, they are indirectly involved in promoting human health through their valuable application in juice, alcohol, tobacco, bakery, poultry, piggery and fishery (Kunamneni 2016). In contrast, the chitinolytic GH has huge role in human welfare directly as well as indirect manner. Indirectly, chitinolytic GH helps in the generation of bioactive CHS and COS, which are well known for their well-established applications in tissue engineering, drug delivery, wound healing, and as nutraceuticals (Kumar et al. 2019). However, chitinolytic GH has also direct involvement in human healthcare *viz*. as antifungal agent and as biomarker for cancer and lung diseases (Nagpure et al. 2014). In this regard, the role of human chitinases and chitinase-like proteins to serve as indicators for inflammation and cancer has been well studied (Kzhyshkowska et al. 2007). Chitinase present in human beings are considered as to play crucial role in the establishment of type 2 innate immunity. Vannella et al. (2016), showed the role of acidic mammalian chitinase in initiating protective type 2 responses to gastrointestinal nematodes *Nippostrongylus brasilliensis* and *Heligmosomoides polygyrus* through using acidic mammalian chitinase deficient mice. Similarly, Cohen et al. (2017) reported that cancer-associated fibroblasts derived chitinase 3-like 1 facilitated tumor progression growth and imitated type-2 immunity. The study, also reported signaling axis between fibroblasts, cancer cells and immune cells in breast tumors. Chitinase 3 like 1 was also explored for its pathological role in promoting mimicry formation leading to tumor cell-mediated vascularization and it was found that the chitinase 3-like 1 expression was correlated with the formation of tumor cell-associated vascular channels in the absence of endothelial cells (Ngernyuang et al. 2018). Xing et al. (2017), showed that chitinase 3-like 1 secreted by peritumoral macrophages could serve as a suitable biomarker for the forecast of esophageal squamous cell carcinoma. Seibold et al. (2008), reported that the chitinase activity in the lung was due to the activity of chitotriosidase with the presence of acidic

mammalian chitinase in inactive form. The study also suggested that the chitinase activity trends to reduce in subjects with asthma however, there was high level of chitinase activity detected in the habitual smokers that resulted in the upregulation of chitotriosidase gene expression in macrophages. Moreover, chitinolytic GH obtained from microbial sources also has been explored for their potential application in controlling human pathogenic bacteria and fungi but the research is still in its primitive stage (Allonsius et al. 2019). Despite of a lot of research work done, still there is need of more study in order to understand the role and applicability of chitinases and other chitinolytic GH present in human beings and also to measure the impact of chitinolytic GH enzymes when applied from external sources on the complex human system.

Conclusions and Future Prospects

Although lignocellulolytic and chitinolytic GH has been well explored for their potential applications but still their industrial level utilization has been achieved in limited sectors. Lignocellulolytic GH has achieved their competent utilization in the industries like paper and pulp, textiles, detergents, and beverages. On the other hand chitinolytic GH has proved their applicability in food, agriculture and healthcare. However, still there is a lot of work is required in the utilization of these GH for the cost-effective generation of bioenergy from the two most naturally abundant renewable biomass of the earth in the form of lignocellulose and chitin. There is also a lot of work and studies required for the application of lignocellulosic and chitinolytic GH for the biomedical industries. At present researchers are paying a lot of attention on these two most demanding area so, we can hope economical and highly effective solution in the near future.

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Part II

Industrial Applications

Role of Glycosyl Hydrolases in Breakdown of Lignocellulosic Waste and Its Industrial Applications

Pamela Jha, Moksha Pathan, Akshara Iyer, Renitta Jobby, and Neetin Desai

Abstract

Due to depletion of non-renewable resources at an alarming rate, the need for utilization of renewable resources to make valuable chemicals is of profound importance. Though lignocellulose is most widely available renewable resource on earth, yet it is utilized much below its potential. The conversion of lignocellulose, especially from agricultural wastes and forest residues to vast array of fuels and polymeric precursors is of crucial relevance in the sustainability and development of energy and chemical industries. But, there are technical and economical hindrances to the development of a commercial processes utilizing lignocellulose. There are few promising technologies developed and many are being developed which will allow the conversion of lignocellulose commercially viable. The conversion of lignocellulose to simple sugars requires daunting biological processes which includes delignification and depolymerization. In the second step, the liberation of free sugar can be done by enzymes which can efficiently carry out the process. Hence, understanding the mechanisms, limitations and improvement of these key enzymes, especially the glycosyl hydrolases dictate the efficiency and viability of the entire conversion.

Keywords

Glycosyl Hydrolases · Lignocellulose · Cellulase · Mechanism · Applications

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Introduction

The accumulation of large quantities of lignocellulosic wastes over the years, cause big environmental concerns. However, due to their uniques chemical composition based on sugars and other compounds of commercial interest, they could be utilized for the production of a number of value added products, such as ethanol, food additives, organic acids, enzymes etc. The reuse of carbon obtained from breakdown of lignocellulose can be one of the approach towards decreasing the carbon footprint (Isikgor and Becer 2015). Therefore, along with the associated environmental hazards caused by their accumulation, the failure of the use of these materials encompasses a huge loss of potentially useful sources.

Lignocellulose Composition

Chemical Composition

The Lignocellulosic biomass is an organic material representing the most abundant source of biomass on earth (Lin and Tanaka 2006). The cells of woody and non-woody plant are majorly composed of lignocellulosic material, which includes cellulose, hemicellulose and lignin (Yang et al. 2001). The major component of lignocellulose biomass is cellulose, which is the potential substrate as it is renewable, biocompatible and biodegradable (Ahn et al. 2012). These major polymer constituents are closely associated with each other, constituting the cellular complex of the plant biomass (Fig. 1). Hemicellulose wraps around the cellulose backbone. This core is guarded by the lignin molecule forming the lignocellulosic structure and it act like cellular glue (Isikgor and Becer 2015).

Cellulose, hemicellulose and lignin consists 30–60%, 20–40% and 15–25% of the lignocellulosic material, respectively (Hamelinck et al. 2005). The properties of each component of lignocellulose is mentioned in Table 1.

The lignocellulosic biomass is a major component of disposed wastes obtained from industries such as agriculture, forestry and municipal waste treatment (Perez et al. 2002). These wastes are plentiful, renewable and of meagre expense as energy sources, and has been mostly under utilized. The percentage of each component in lignocellulose varies, depending on the source of biomass. The wastes include sponge gourd fibers, bagasse, banana waste, wheat straw, grasses, newspaper, nut shells, rice straw, corn stover, corn cobs, softwood, hardwood, sweet sorghum, sugarcane bagasse (Fig. 2).

Tertiary Architecture

The management of the tertiary architecture of lignocellulose structures is done by a diverse covalent and non-covalent linkages between its major components. As cellulose is enmeshed with hemicellulose, lignin, and other components, hence

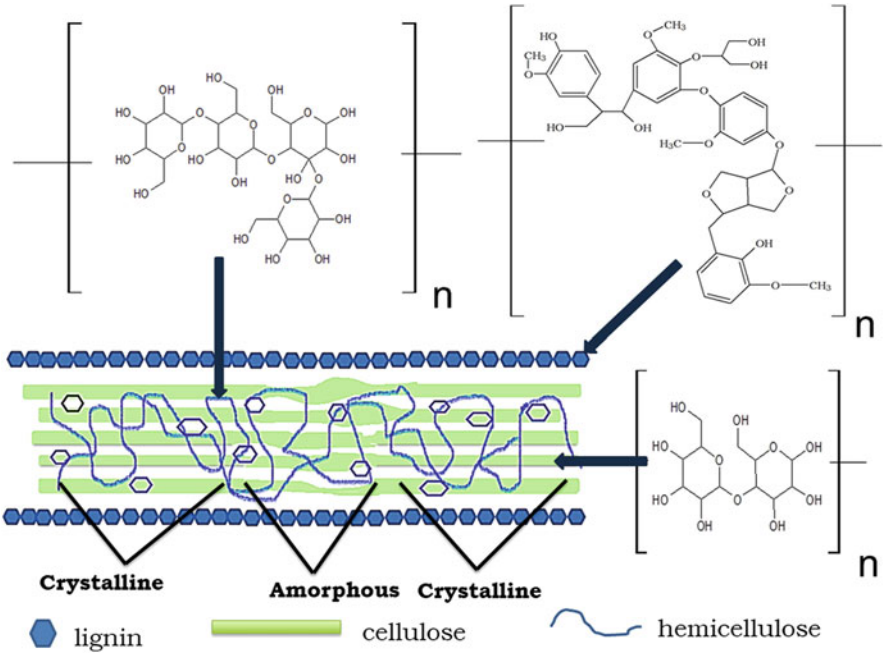

Fig. 1 Representation of lignocellulose structure showing cellulose, hemicellulose and lignin fractions

their hydrolysis is a daunting task (Leonowicz et al. 1999; Tomme et al. 1995). The microfibrils of cellulose are stabilized by intra- and intermolecular H-bonds and encircled by hemicellulose (mannans and xylans) which linked to cellulose by covalent and H-bonds (Heredia et al. 1995). The complicated H-bonding network found within and between glycan units merged with high degree of polymerization containing thousands of glucose monomers promote additional limitation of the linkages for the.

hydrolysis. These covalent bonds are intensely resistant to chemical and biological hydrolysis (Ezeilo et al. 2017). On contrary, amorphous regions within the cellulose crystalline structure have a heterogeneous composition characterized by a various types of bonds. Finally, this asymmetrical arrangement, which characterizes amorphous regions, is critical to the biodegradation of cellulose. The accessibility of enzymes towards cell wall polysaccharides is directed by the extent to which they are interconnected with phenolic polymers (Chesson 1981; Kuhad et al. 1997).

Table 1 Various components of the lignocellulosic biomass

Components	Composition (%)	Monomeric units	Type of polymer	Nature	Degree of polymerisation	References
Cellulose	30–60	d-glucopyranose	Homopolymer	Mostly crystalline	500–25,000	Himmel et al. (1994)
Hemicellulose	20–40	Xylans, mannans glucans and acid sugars	Heteropolymer	Amorphous	100–200	Saha (2000)
Lignin	15–25	Phenylpropanoid precursors	Heteropolymer	Amorphous	6–41	Demirbas (2005), Balat (2011), Molina-Sabio and Rodríguez-Reinoso, (2004), Yang et al. (2009)

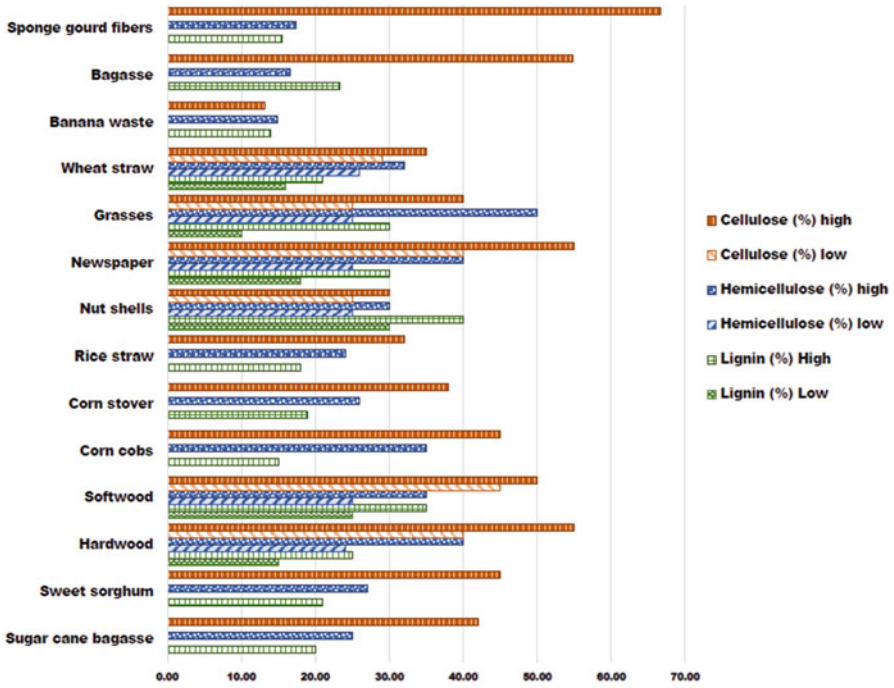

Fig. 2 Percentage of each component variation in lignocellulose from different sources (Reproduced from Iqbal et al. 2013)

Lignocellulose Breakdown

The conversion of lignocellulose to simple sugars requires daunting biological processes i.e.:

1. Delignification—release of free cellulose and hemicellulose from the lignocellulosic biomass;
2. Depolymerization—conversion of cellulose and hemicellulose to generate free sugars.

In the second step, the liberation of free sugar can be done by enzymes which can efficiently carry out the process. But, a complicated and detailed sequence arrangement between polysaccharides (hemicellulose and cellulose) and lignin makes the lignocellulosic material a challenge for carbohydrase and ligninase enzyme systems (de Siqueira et al. 2010).

Delignification

There are many conventional pretreatment techniques reported for the delignification of lignocelluloses which includes physical (grinding and milling), chemical (acid and alkali treatment), physicochemical (use of steam, hot air or ammonia fibres) and biological (fungi) treatments (Sun and Cheng 2002). But conventional methods have several disadvantages: they are time consuming, elaborate and cause damages to the environment by generating secondary pollutants. Of late, lignin modifying enzymes have been used such as laccases, peroxidases and enzymes hydrolyzing lignin-carbohydrate complexes.

Depolymerization

The hydrolysis of lignocellulose is a major obstruction in the technology of utilization of lignocellulose to valuable products. Presently, there are two major processes known for hydrolysis of lignocellulose into free sugars, includes

- catalytic hydrolysis and
- enzymatic hydrolysis.

Catalytic

The catalytic hydrolysis is usually carried out with homogeneous acid or solid acid. The catalysts used are relatively inexpensive. These catalysts are mineral acids such as acetic acid, oxalic acid etc. and organic acids such as H_2SO_4, HCl for hydrolysis of cellulose (Amiri and Kamiri 2013). However, there are multiple disadvantages of their use such as energy-inefficiency, requires separation, recycling, and treatment of the waste acids. Of late, recyclable solid acid catalysts are being used to replace mineral acids for cellulose hydrolysis (Zhou et al. 2013).

Enzymatic

The enzymatic hydrolysis is a promising method to obtain fermentable sugars from lignocellulosic biomass, which can proceed under mild conditions with high selectivity. But, the high cost and low efficiency of recycling enzymes are a problem for the application of enzymatic hydrolysis (Mascal and Nikitin 2008).

Main Enzymes in Cellulose and Hemicellulose Hydrolysis

The enzymes employed in depolymerization of cellulose and hemicellulose are as follows:

Cellulases

- Endo-β-1,4-glucanases, cellobiohydrolases, β-glucosidases
- Fungal cellulases e.g. *Trichoderma, Humicola, Acremonium*
- Bacterial cellulases e.g. *Clostridium thermocellum*

Hemicellulases

- Backbone degrading enzymes
- Enzymes removing the side groups
- β-xylosidases

Other helper enzymes/proteins

- Swollenin

Inhibitors of Enzymatic Degradation

The inhibitors of enzymatic degradation are as follows:

- Phenolics

Aromatic compounds show varied effects depending on the functional groups attached and it is related to their interference with the cell membrane composition and function (Larsson et al. 2000).

- Aliphatic acids

Acids diffuse through the cell membranes and dissociate in the neutral environment of the cytoplasm which causes the pH of the cell to go down and ultimately lead to cell death. It may also compromise on the formation of biomass by producing ethanol (to reverse the lowered pH) (Verduyn et al. 1992).

Glycosyl Hydrolases (GH)

The enzymes are usually classified on the type of the bond that they break. The class of enzymes which hydrolytically break the bonds are grouped under class 3 enzymes and are called hydrolases (Fig. 3) (IUBMB 1992). In this class, the enzymes that hydrolyze glycosidic bonds between two or more sugars and non-sugars within carbohydrate and oligonucleotides are called GH. There are 115 families of these enzymes known, on the basis of mode of action and amino acid sequence (Sathya and Khan 2014). These enzymes hydrolytically breaks the bonds such as ester, glycosidic, ether, and peptide bonds, and also transfers the specific group to water, which acts as an acceptor (IUBMB 1992). GHs are key enzymes involved in the depolymerization of cellulose, which is the most crucial step in formation of fermentable sugars for production of fuels and chemicals. GH catalysed reactions are accelerated by 17 folds compared to spontaneous hydrolysis, which makes them lucrative biocatalysts (Wolfenden et al. 1998).

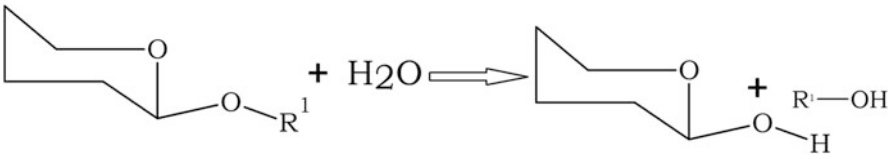

Fig. 3 Hydrolysis reaction of glycosidic bond

Classification of GH

GH also known as glycosidases or carbohydrases (EC 3.2) comprises of a wide spectrum group of enzymes that has the catalytic properties to cleave N-, S- and O-glycosidic bonds. They either cleave two sugar moieties or a sugar and a non-sugar region of carbohydrate. There are 115 families (based on the amino acid sequence and action) of GH, which have been identified (Lehninger 2004). There are few glycosidic bond, which are most stable covalent bonds found between monomers in biopolymers, especially between two glucose residues in some polysaccharides (Wolfenden et al. 1998). The domain, E.C., CATH, CAZY FAM, and CAZY clan cells, show different entries could be assigned to the given classes (Table 2).

Structure of GH

The 3D structure of Lysozyme was the first to be resolved and it was found that aspartate and glutamate are the major amino acids involved in catalysis. Further studies proved that most glycosyl hydrolases has conserved regions of aspartate or glutamate that were involved in catalysis. Recent examples of neuraminidase show that tyrosine is used in stabilising the intermediates of the reaction (Burmeister et al. 1993; Crennel et al. 1993).

GH catalyse all the hydrolytic reactions of glycosidic bonds and are studied thoroughly. They cleave the C–O exo-cyclic acetal bond.

Study of the spontaneity of the reaction reveal their extremely slow action with the half life a hydrolytic cleavage estimated to be 4.7 million years. Thus GH act as an efficient and powerful catalyst speeding up the reaction by more than 1000× (Stick and Williams 2009).

The most important structural aspect of an enzyme is its active site conformation and GH is found to have three distinct conformation in its active site (Davies and Henrissat 1995).

Active Site Topology

22 families of the protein have been studied and it has been found that the active sites can be classified into three classes:

Table 2 Summary of the structurally homologous classes of GH and their sources

Domains	Class	Domains	EC	CATH	Cazy FAM	CAZY CLAN	Representative enzymes	Sources
N-glycosyl catalytic (A)	1	25	1	1	–	–	Shiga Toxin A subunit	*Shigella dysenteriae*
	2	30	1	4	–	–	Ricin A chain	Castor beans
	3	14	1	1	–	–	ADP-ribosyl cyclase	Sea urchins
	4	10	2	1	–	–	Pyrimidine nucleoside hydrolase	*E. coli*
	5	5	2	2	–	–	3-Methyladenine DNA glycosylase	Recombinant human
	6	2	1	1	–	–	Uracil-DNA glycosylase	Recombinant human
	7	2	1	1	–	–	MTA/SAH nucleosidase	*Escherichia coli*
N-glycosyl binding (B)	8	5	2	2	1	–	Endo-1,4-beta-xylanase A	*Trichoderma longibrachiatum*
O-(S)-glycosyl catalytic (C)	1	171	2	2	3	–	Lysozyme	Albumin
	2	12	2	4	2	1	Chitosanase	*Penicillium chrysogenum, E. coli*
	3	13	1	1	1	1	Polygalactouronidase	Agro wastes (pectin)
	4	303	33	35	28	4	Beta-galactosidase	*Aspergillus niger*
	5	48	2	2	3	2	Endo-1,4-beta-Xylanase	*Trichoderma longibrachiatum*
	6	54	5	7	6	2	Cellobiohydrolase	*Saccharomyces cerevisiae*
	7	34	2	6	4	2	Neuraminidase	*Influenza virus*
	8	2	2	–	1	–	6-Phospho-beta-glucosidase	*Escherichia coli*
	9	3	1	1	1	–	Endoglucanase	*Aspergillus terreus*
	10	17	4	2	2	1	Beta-Agarase B	*Alteromonas sp. C-1*

(continued)

Table 2 (continued)

Domains	Class	Domains	EC	CATH	Cazy FAM	CAZY CLAN	Representative enzymes	Sources
O-(S)-glycosyl binding (D)	11	3	1	1	1	–	Endo-1,4-beta-Xylanase Y	*Clostridium thermocellum*
	12	2	2	1	1	–	Glucoamylase	*Aspergillus niger*
	13	2	1	–	1	–	Cellobiohydrolase	*Trichoderma reesei*
	14	8	3	2	1	–	Endo-1,4-beta-Xylanase D	*Talaromyces funiculosus* (*Penicillium funiculosum*) (*Bacteroides ruminicola*)
	15	2	1	1	1	–	Endo-1,4-beta-Xylanase A	*Trichoderma longibrachiatum*
	16	10	2	3	4	–	Endoglucanase C	*Cellulomonas fimi*

Reproduced from Fülöp and Ponyi (2015)

- Pocket or crater
  This is mainly when non-reducing saccharides are substrates (example: monosaccharidases like neuraminidase and galactosidase and exopolysaccharidases like amylase). These enzymes act on substrates which have exposed non-reducing ends. They have limited activity on fibrous substrates with no free ends (Davies and Henrissat 1995).
- Cleft or groove
  These are the binding points for endo-acting polymeric substrates like lysozymes and chitinase (Davies and Henrissat 1995).
- Tunnel
  These are modified clefts with the protein loops covering the clefts to form a tunnel shaped structure. Cellobiohydrolases are the only class of enzyme with this structure and they pass through the site for the reaction to occur (Rouvinen et al. 1990). Products are released while the polysaccharide chain is still bound to the enzyme enabling better processivity. Although the exact mechanism of action is unknown, it is observed that the direction of enzyme motion changes during the reaction based on the enzyme class (Divne et al. 1994).

Mechanism of Glycosyl Breakdown

During enzyme hydrolysis of the glycosidic bond, two essential components required are proton donor and a nucleophile (Fig. 4).

GH in Lignocellulose Breakdown

The breakdown of lignocellulosic biomass for the production of valuable products can add to the economics as well as alleviate the detrimental effect on environment caused due to their disposal into soil or landfills (Taherzadeh and Karimi 2008). The cellulose-degrading systems are pertinent for industrial cellulose breakdown as well as to sustain the carbon cycle. The class of enzymes which are crucial in liberation of free sugars after delignification of lignocellulosic biomass is cellulase. Cellulases are the class of enzymes that are associated with the breaking down of cellulose and other related polysaccharides. Although produced by various organisms like bacteria, fungi and yeast, termites and their intestinal symbionts form the major source of cellulase (Watanabe and Tokuda 2001).

Classes of GH in Cellulosic Breakdown

They exist as complexes of various enzymes from the class exocellulase, endocellulase, cellobiolases, cellulose phosphorylases and oxidative cellulases (Tsai et al. 2012). The families of GH with different types of cellulases is mentioned in Table 3.

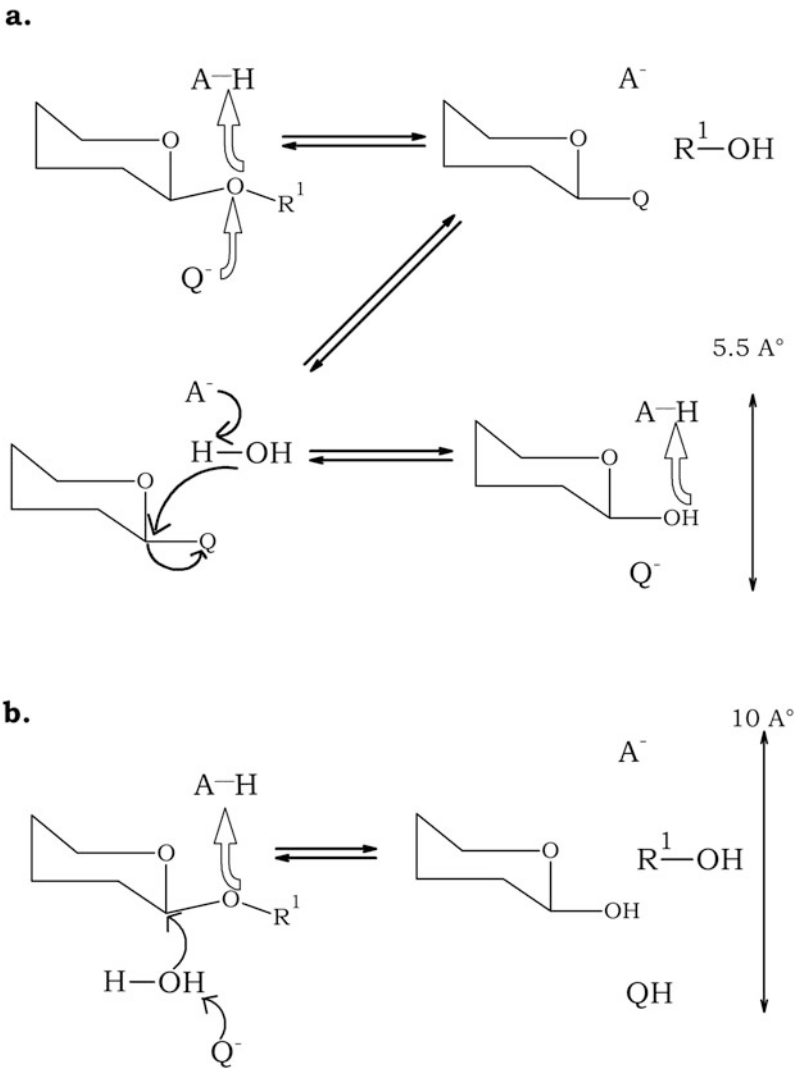

Fig. 4 Mechanism of breakdown of lignocellulose. (**a**) The retaining method, the acid catalyst AH protonate the oxygen in the glucose and base Q- avails the nucleophilic function to knockout the aglycon group. The glycosyl enzyme is then undergoes hydrolysis. (**b**) The inverting method, the oxygen of the glucose is protonated and aglycon group knockout occurs simultaneously by the attack of the water molecule which is activated by the base Q- (Reproduced from Davies G. and Henrissat B. 1995)

The major families of Cellulases belong to GH class of 5,9,17 and 48 (Ezeilo et al. 2017). These cellulase of particular class, have the following properties:

- Enzymes of GH 5—These are characterized by the presence of conserved glutamic acid residues which are the major catalytic groups. They exhibit the retaining mechanism

Table 3 Cellulases, functions and their corresponding GH families

Enzyme	EC number	Functions	GH family
1. Name: β-1,4-glucanase Systematic name: 4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase	EC 3.2.1.4	Endohydrolysis of (1 → 4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. Randomly hydrolyses b-(1,4)-glycosidic bonds exposing reducing and non-reducing ends of the linear polymer of glucose units	GH5, GH9, GH12, GH44, GH45, GH48, GH51, GH74, GH124
2a. Name: glucan 1,4-β-glucosidase Systematic name: 4-β-D-glucan glucohydrolase	EC 3.2.1.74	Hydrolysis of (1 → 4)-linkages in (1 → 4)-β-D-glucans, to remove successive glucose units Cleaves glucose successively from the non-reducing ends of the glucan to liberate glucose units, with preference for substrates of longer chain length and giving rise to inverted products	GH3, GH5, GH6, GH7, GH9, GH48
2b. Name: β-1,4-glucan cellobiohydrolase Systematic name: 4-β-D-glucan cellobiohydrolase (non-reducing end)	EC 3.2.1.91	Hydrolysis of (1 → 4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains Acts at the reducing ends of -b-(1,4)-glucans, produced by endoglucanases, cleaving cellobiose and cellooligosaccharides	GH5, GH6, GH7, GH9, GH48
3. Name: β-1,6-glucosidase Systematic name: β-D-glucoside glucohydrolase	EC 3.2.1.21	Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose Hydrolyses cellobiose and other very short chain b-D-oligosaccharides up to cellohexaose to form glucose; unlike exoglucosidases the rate of hydrolysis decreases markedly as the degree of polymerization of the substrate increases	GH1, GH3, GH5, GH9, GH30, GH116

Reproduced from Saini et al. (2015), Gilbert and Hazlewood (1993), and Xiros et al. (2016)

- Enzymes of GH 9—These are subdivided as E1 (source: aerobic and anaerobic bacteria) and E11 (Source: mainly non-bacterial). They exhibit the inverting mechanism.
- Enzymes of GH 17—These exhibits retaining mechanism. It includes 2 major enzyme families: 1,3-β-D-glucan endohydrolases and 1,3;1,4-β-D-glucan endohydrolases
- Enzymes of GH 48—These have high efficiency for amorphous or crystalline cellulose than carboxymethylcellulose or cellobiose as the latter inhibits its activity. They too exhibit inverting mechanism.

The mechanisms stated above for each class were obtained from CAZYPedia.

Model of Cellulose Degradation

The model known for enzymatic degradation of cellulose is depicted (Fig. 5).

The diffusion of enzymes into the substrate has been simulated and the biological conditions understood. The model is based on (Luterbacher et al. 2013a, b):

1. The dependency of the hydrolysis on the particle size (it can be modified by a suitable pretreatment)
2. Sudden decrease in the reaction rate after the initial rush of the enzyme into the substrate.

Cellulosome Complex

Cellulosomes have the capability to breakdown cellulose. The understanding of structure, mechanism and functions of this multi-enzyme complex began from *Clostridium thermocellum* (Lamed et al. 1983). The key features of cellulosome are stability and flexibility, composed of many subunits, its organization promotes synergistic action, and contains catalytic and non catalytic units. The components of cellulosome are the scaffoldin subunit (1800 amino acids), cohesin modules,

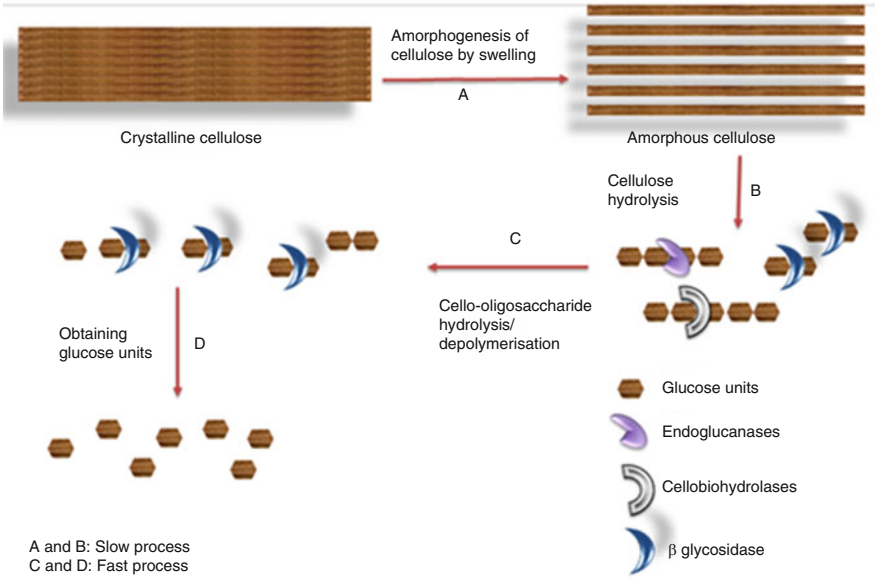

Fig. 5 Enzymatic cellulose degradation model (Reproduced from Lynd et al. 2002)

dockerin modules, and catalytic subunits (Bayer et al. 2004). The catalytic subunits of cellulosomes consist of enzymes such as endoglucanases, xylanases and cellobiohydrolases. These enzymes portray array of substrate specificities and catalytic mechanisms Apart from the catalytic module, the dockerin module modulates enzyme interaction alongside the scaffoldin protein, responsible for assembly of the cellulosome complex (Bayer et al. 2004). The synergism of different subunits ensures a well-harmonized enzymatic coordinations, thus making cellulosomes the most efficient system for the breakdown of cellulose (Xiros et al. 2016). Recently, artificial cellulosomes are being built for the dedicated aim of enhancing the catalytic efficiency for the depolymerization of cellulose (Gefen et al. 2012).

Factors Influencing the Enzymatic Hydrolysis of Lignocellulose

Complexity of the Substrate

Cellulose is a complex and heterogeneous molecule which makes its breakdown difficult to achieve. Thus achieving a fool-proof model for its breakdown is an issue. Cellulose structure has both intra and inter-chain bondings forming a tightly packed crystalline structure interspaced with some amorphous regions each of which are at different complexity and availability (Yang et al. 2011; Bubner et al. 2013).

The enzymatic breakdown of the biomass is affected by various factors like (Fox et al. 2013; Luterbacher et al. 2013a, b):

- Biomass content
- Available surface area
- Bond accessibility
- Pretreatment used (minimal inhibitors produced)
- Degree of polymerization
- Organization of cellulose

Heterogeneity of the Enzymes

Microbial cellulases have been in use since the 1990s. The demand for the enzymes has only increased with time making it imperative to find other sources for the bulk production of the enzymes. The search led to a finding that both bacteria and fungi are efficient cellulase producers with organisms like *Clostridium, Bacillus, Erwinia* and *Streptomyces* being some of them (Yang et al. 2011; Bubner et al. 2013).

Based on the enzyme source they can be generally classified as (Coughlan and Ljungdahl 1988; Sun and Cheng 2002; Elkins et al. 2010; Bayer et al. 1998):

1. Non-complexed system: Produced by fungi and aerobic bacteria.
2. Complexed system: Produced by anaerobic organisms.

Recently, the study on cellulases produced by *Trichoderma reesei* has gained momentum due to the large amount of enzyme released which is beneficial economically. It is the only non-complexed system studied as the degradation of the biomass

by the organism occurs simultaneously (Coughlan and Ljungdahl 1988); Sun and Cheng 2002; Elkins et al. 2010; Bayer et al. 1998). On the other hand complexed system has various advantages like:

- A coordinated process
- Only specific interaction

Applications of Cellulase

The addition of novel cellulases to the existing list of GH in lignocellulosic breakdown is increasing the applications of these enzymes in various industries (Fig. 6).

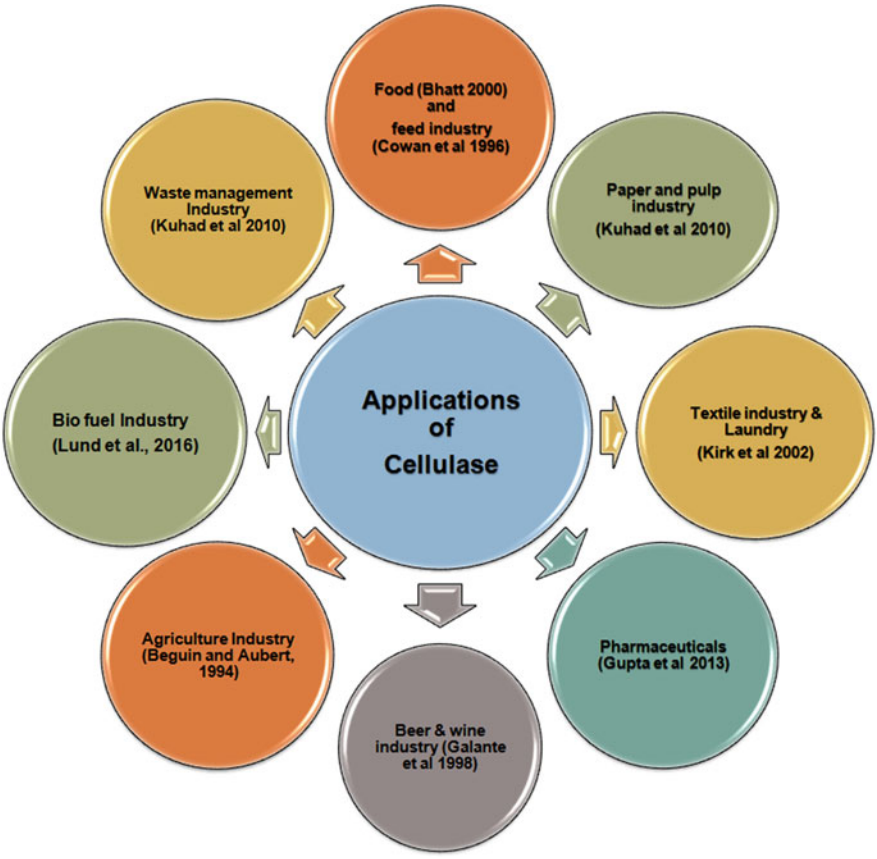

Fig. 6 Diverse applications of cellulases

Conclusion

The requirement of huge energy and chemicals demand is compelling the human race to find alternative to renewable clean technologies. In this context, one of the most lucrative substrate which promises to partially fulfil our demand is lignocellulosic biomass. The usage of lignocellulose at industrial scale is limited due to the huge expenses incurred in enzymes used for their hydrolysis. Thus, in present scenario the focus is being targeted to improve enzymes with respect to increased stability, wider substrate affinity, and magnus increase in catalytic activity at wide range of pH and temperature.

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Marine Glycosyl Hydrolases as Tool for Industrial Application

I. D. Antonio Trincone

Abstract

Usually biocatalysts for industrial applications are required to possess optimum properties to adapt to the harsh conditions utilized. Discovering better enzymes in nature (bioprospecting) is one of the approach to overcome this problem. The biodiversity value of different natural environments has always been important and marine environment seems to be the most promising for the properties of marine biocatalysts linked to the habitat where they thrive. Salt tolerance, hyperthermostability, barophilicity and cold adaptivity are indeed properties of great interest for industry. Industrial applications of marine glycosyl hydrolases cover a broad range of individual sectors from biorefinery to food industry. Importance is also recognized to the biobased extraction or modification of structurally complex marine molecules, etc. Patents are a valuable source of technical scientific knowledge showing the current or past state of technology. Their analysis is capable of fully presenting the whole potential of a scientific topic with ready cases for industrial applications. A recent quick analysis of patents (2016–2017) in this field, as sorted from the Espacenet patent database, helped to point out that the literature in the scientific journals is at the moment, showing only a partial result of different applicative projects. In this chapter, an analysis of patents using a search engine of larger coverage such as Google patents (120 million patent publications from 100+ patent offices around the world) is reported as it has been adapted to a wider period from 2011 to 2019 and the results deeply analysed in detail. Different aspects are presented in tabulated forms and conclusions drawn for geographical origin of patenting activity and for scientific issues such as novelty of biocatalysts discovered, correlated in a field/polymer network map presented.

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Keywords

Marine enzymes · Biocatalysts · Bioprocesses · Glycosyl hydrolases · Carbohydrate hydrolyzing enzymes · Marine polysaccharides

Introduction

Enzymes acting on carbohydrate-based materials are important tools from an industrial point of view. Although the term "acting on carbohydrates" is rather vague, the enzymes most studied and applied in industry are glycosyl hydrolases, naturally devoted to the hydrolysis of glycosidic bonds that is industrially required mainly to change/adjust chemical characteristics of natural polysaccharides. Natural enzymes devoted to the synthesis of glycosidic bonds are glycosyl transferases that can be classified according to the nature of their mechanisms; Leloir type, acting by sugar nucleotides, non-Leloir type A acting by sugar phosphates and transglycosidase acting via non-activated sugars such as sucrose, lactose or starch. Even glycosyl hydrolases in appropriate conditions could act as synthesizing enzymes and this is an aspect of potential interest for industry being these biocatalysts easily available, robust and easy to be used. A complete survey of industrial applied enzymes has been compiled (Polaina and McCabe 2007); in it an important percentage of the thirty-four chapters is dedicated to glycosyl hydrolases. From the analysis of industrial sectors potentially interested one can learn that classical current major large-scale applications of glycosyl hydrolases pertain to detergent industries (ca. 25% of total sales), starch and fruit juices production (15%), animal feed, textiles and pulp and paper (ca 10–15% each) and others.

In general, natural enzymes lack those optimum properties required in the industrial process, as they evolved in relatively mild conditions *in vivo* compared to harsh industrial conditions. Therefore, bioprospecting better enzymes suitable for industrial applications is one of the approach to overcome this problem and the biodiversity value of different natural environments is important. In this context from the analysis of review articles, carbohydrate-hydrolyzing enzymes of marine origin appear today as an important aspect (Giordano et al. 2006; Parte et al. 2017). Knowledge of marine biocatalysts and analysis of their habitat-related properties (salt tolerance, hyperthermostability, barophilicity and cold adaptivity) of interest for industry, is necessary for exploitation of their bioprocesses (Trincone 2010, 2011). Marine enzymes cover a broad range of individual application sectors such as the food industry to deal with the enzymatic procedures adopted in food manipulation or in the biorefinery value-chain, where supplying of biomass is one of the most important aspects. The selective and easy extraction and or modification of structurally complex marine molecules, where enzymatic treatments enable technology to improve efficiency and selectivity and the topic of marine biomarkers are also important (Trincone 2017).

From the analysis of the references during the preparation of a recent review (Trincone 2018) it was evident that only a half of the published reports resulted from

scientific literature search are original research articles, being the remaining ones represented by books or reference works. One conceivable reason of this high amount of reference material was seen in temporal terms i.e. in the pipeline of applicative research, from idea to product. In agreement to two leading experts in the patent field (Gordon and Cookfair 2000), scholars following patent literature are often able to find information for basing or expanding their research. However, patents and similar literature are usually neglected in a normal reference material and not analysed in detail. They are instead a valuable font of technical knowledge showing the current or past state of technology, fully presenting the potential of a scientific topic in its entirety, with ready cases for industrial application. A quick analysis of patents related to 2016–2017 in the field of marine carbohydrate hydrolysing enzymes was indeed reported (Trincone 2018) and only few patents sorted revealing that the literature in the scientific journals is showing only a partial result of different funded research. Additional time is necessary to appreciate in full the complete output of these research pipelines in worldwide governmental boosts in marine biotechnology, from basic research sector, proof of concept up to products. It is hopeful that early grand results are at the moment pre-commercialization reports, patents etc. with primary applicative aim.

The present chapter shows a more in-depth analysis of patents about marine glycoside hydrolases with respect to the previous report (Trincone 2018). Google patents covering over 120 million patent publications from 100+ patent offices around the world (UpCounsel 2019) was used as search engine and products from 2011 to 2019 analyzed in details.

Modern Focuses on Applications of Glycosyl Hydrolases

A search in Science Direct for review articles published only in 2019 using keywords depicting the general concept "industrial application of glycosyl hydrolases" furnished a dozen of hits that seems to be important to analyse in this introductory part to learn about a flavour of the modern focuses on applications of glycosyl hydrolases from different origins. It is noticeable that five of these review articles have the word thermophilic in the title (Bala and Singh 2019; Varshney et al. 2019; Patel et al. 2019; Chadha et al. 2019; Kumar et al. 2019).

However, within this highly selected pool of articles one is specifically dedicated to green technologies for recovery of compounds of interest from seafood by-products and discards. Fermentative and enzymatic technologies are listed among the techniques used as the main biotechnological approach. Enzymes from different sources (animal, plant, microbial) are described and indeed those originating from marine organisms play a critical role (Bruno et al. 2019).

In marine biotechnology oriented studies, as revealed from results of European projects in the field, the analysis related to enzymatic processes of industrial interest (Trincone 2017, 2018) revealed a a network correlations based on polymers and research topics expressed as keywords, as shown by the matrix resulted in Table 1.

Table 1 A network correlations based on polymers (columns) and current research topics/fields expressed as keywords (lines)

	LAM	ULV	MAN	PEC	CAR	CEL	STA	CHI	XYL	AGA	FUC	ALG
Food and pharma	X	X									X	X
Fine chemistry		X										
Profiling presence											X	
Decoration catalysts											X	
Chemical procedures											X	X
Homogeneous fractions											X	
Recombinant biocatalysts												X
Bioprospecting new enzymes		X	X	X			X	X	X	X	X	
Extremophilic biocatalysts					X	X	X					
Biofuel						X		X	X	X		
Immobilized biocatalysts					X							

Research on marine polysaccharides and marine carbohydrate-hydrolyzing enzymes is clearly of interdisciplinary nature. Successes in the biochemical understanding of the machinery for bond cleavage guided the bioprospecting of marine agarases, marine chitinases and xylanases and it embraces many other polymers (Karthik et al. 2017). Marine enzymes processing alginates is another big area where research seems to be very active during the last years. Degradation and general metabolism of these polymers have been studied in details; recombinant alginate lyases are ready for applications. Generally speaking seems that the biocatalysts acting on various marine only-glucose polymers are of great importance. Both small oligosaccharides of pharmaceutical interest and biofuel domain represent interesting fields. At first amylases of extremophilic nature are interesting as model biocatalysts to assess the molecular basis of their biochemical resistance. Production of laminarin-type oligosaccharides is catching attention for their applications in food. Marine fungi and actinobacteria originating cellulases and other glucanases are of great interest in biofuel applications. Profiling presence, structural details and natural functions of fucoidans are aspects intensely studied for these sulfated glycans. Not only enzymes acting on fucosidic linkages are important but also others acting on decorations (deacetylation, desulfation, etc.) of these polysaccharides deserve attention. There is a need for preparation and structural elucidation of homogeneous fractions by new enzymes. Standardization of extraction and purification methods are also topics present (Trincone 2018).

In continuing this bibliographic research effort, further refining of the scientific literature could be of interest. Exploration of the fields individuated above in Table 1 should be continued in depth, in specialized journals, in a manner allowing revealation of sub-fields and more details pointing to a single process, with room to discuss a single enzymatic activity, but this is out of the scope of the present chapter.

At the end of one of the recent transnational calls of ERA-NET (ERA-MBT), an action funded under the EU FP7 program focused on biorefinery, a key area where development is still needed. The very conclusion was the final consideration drawn about the importance of an interdisciplinary network in setting up successful research projects, thus enabling the identification of an arsenal of enzymes and pathways greatly in demand for biotechnological applications.

Patents retrieved here are those from 2011 to 2019 containing in the text the words “marine glycosyl hydrolases.” After a first screening of suitable hits based on the titles, their abstracts were analysed for selection. The following two paragraphs below related to analysis of enzymatic activities, biodiversity in bioselection of the marine environment and bioprocesses acted by marine enzymatic activities.

Analysis

In the general mass of patents found for this search two interesting ones were present both regarding the detection of enzymatic activity. The first one concerning modern fluorogenic substrates, with their synthesis and methods of use for the detection of a

glycosidase enzyme in vivo, in a human or animal (Hasserodt 2014). In particular, the compounds reported in the invention have selected glycosyl groups acting as a molecular probe capable of identifying the presence of specific glycosidase activities. Known fluorescent probes are soluble in the cytoplasm, this causes a fail to provide information about biocatalytic activity in situ. Diffusing away from the site of their generation, products of enzyme conversion produce poor imaging resolution and low contrast. Compounds studied here have chemical structures securing that they are water soluble thus can easily reach the sites, cells and tissues of interest for the presence of the enzymes to be detected. However the aglycon part of these structures is devised in such a manner that after hydrolysis, cyclization of the free aglycone occurs giving rise to both intense fluorescence signal, obtained from the solid precipitated form of aglycon, and securing water-insolubility limiting diffusive dilution from reaction sites (Liu et al. 2017).

The second patent is about a methodology for a high throughput screening of glycoside hydrolases. Among claims authors declared that the invention may be used to identify glycosidases for use in the production of second generation biofuels (Willats and Melgosa 2013). The method is based on naturally specific proteins that bind to polysaccharides so called carbohydrate binding module (CBM) which are typically highly specific for epitopes composed by 3–8 sugar residues. The enzymatic activity, if present, acting on that sugar assembly will prevent or reduce the binding of the CBM. Quantifying the CBM-binding before and after the enzyme action it is possible to assess the enzyme activity in the sample.

Biodiversity

Patents describing new enzymes or new organisms with interesting activities from marine environment are relatively more abundant. They are listed in a concise tabulated manner (Table 2) specifying enzyme type along with some explanatory notes. Many types of marine glycosyl hydrolases acting on a portfolio of various polysaccharides of both marine and terrestrial origin are reported; many biocatalysts possess enzymatic characteristic interesting for harsh industrial applications.

It is remarkable to mention the patent related to a method to increase the expression of a specific, thermostable, thermotolerant and pressure stable enzyme originating from Thermotogales (Tan et al. 2013) with cellulolytic activity in an industrial field very far from what considered life science discipline, such as oil and gas exploration, in particular for the recycling of the flow-back liquids from drilling operations. Gel based fracturing fluids utilize guar, guar derivatives, cellulose, or synthetically derived polymers to increase the apparent viscosity of the fracturing fluid and allows for increased fracture propagation. These gels must be degraded to remove any blockage to the flow of oil or gas being extracted. Industry uses viscosity breakers (such as oxidizers, acids, or enzymes) to degrade the fracturing fluid and to remove any solid gel residue from the fractures. The disclosed cellulase can be used as a high temperature viscosity breaker to enhance oil and gas operations.

Table 2 New enzymes or new organisms with interesting activities from marine environment

Patent	Enzyme/organism	Notes
CN-103131685-A	β-Galactosidase	β-galactosidase in decomposing milk lactose at low temperature
CN-102220302-A	β-Glucosidase mutant	Marine uncultured microbial origin
CN-108220189-A	Xylanase	Marine bacterium for producing xylanase
CN-102586150-A	Alginate lyase	Bacterial strain classified as *Tamlana* sp. capable of generating alginate lyase
CN-103540579-A	β-Agarase and application	Can degrade agarose to produce pure neoagarobiose
WO-2013024021-A1	Cellulase activity	From *Terebella lapidaria*, a polychaete living in marine environments
CN-102971426-A	Alginate lyase	Bacterial enzyme from a metagenome library
CN-103194420-A	β-Agarase	Recombinant expression strain producing β-agarase
CN-106811451-A	Chitosanase	Cryogenic enzyme
WO-2013148163-A1	Cellulase	Increased expression of a specific, thermostable, thermotolerant, pressure stable enzyme originating from Thermotogales
KR-101483182-B1	endo β-1,3-Glucanase	–
WO-2015077744-A1	Xylanase and application	Genetically modified bacterial strains
US-2016030528-A1	Muramidase	Antimicrobial activity
CN-104245929-A	α-Glucuronidase	Marine genus Bacillus (Oceanobacillus) originating enzyme
CN-104388411-A	Agarase	Provide a marine microbial origin agarase gene
KR-101780234-B1	Chitinase	Antifungal and biodegradation properties
JP-5605331-B2	β-Glucosidase activity	Originated from *Thermotoga maritima*
KR-101618765-B1	β-Agarase	From marine *Pseudoalteromonas*
KR-101653340-B1	β-Glucosidase	Thermostable
CN-103384678-B, CN-103562384-A, CN-103958672-A, CN-103649308-A, CN-108467877-A, CN-104334572-A, CN-105452271-A,	Cellulolytic, endoglucanase and other hydrolases acting on glycosyl compounds	Marine genus Bacillus (Oceanobacillus) originating enzyme

(continued)

Table 2 (continued)

Patent	Enzyme/organism	Notes
CN-104968781-A, CN-104736698-A, CN-107532154-A, CN-103930438-A, CN-107750275-A, CN-104640874-A, CN-105283546-A, CN-105838698-A		
CN-105950640-A	k-Carrageenase	Marine *Cellulophaga lytica* DSM 7489
CN-102311944-B	Mannase	Low temperature optimal activity
CN-108350443-A	Xanthan degrading activity	–
CN-105567606-A	Hyaluronidase	Marine hyaluronidase produced, isolated and purified from marine *Arthrobacter globiformis* Al52
US-2018044654-A1	Agarase	–
CN-103525790-B	Mannanase	High-temperature resistant mannanase
CN-106467901-A	Agarase, amylase and xylanase	
CN-103343099-A	Glucosidase	Low-temperature acting glucosidase
CN-103194435-B	β-agarase	Thermoactive agarose from *Catenivulum agarivorans*
CN-103997902-A	Xylanase	Hyperthermophilic and hyperthermostable xylanase in an animal feed composition
CN-105586288-A	Efficient degradation of agar	*Marinobacter adhaerens* G4 producing β-agarase
CN-107922933-A	Pullulanase	Mutants expressed in host cells such as marine *Bacillus*
CN-103958674-A	Xylanase	Expressed in host cell such as marine *Bacillus*
CN-103517986-B	Cellobiohydrolase	Expressed in host cell such as marine *Oceanobacillus*
CN-103710325-B	Amylase	Amylase of marine bacterial origin
CN-103957929-A	Lysozyme	Expressed in host cell such as marine *Bacillus*
CN-103789241-A	Iota-carrageenan degrading activity	From marine marine *Cytophaga* strain
CN-105518142-A	Endoglucanase	Expressed in host cell such as marine *Bacillus*
CN-103194414-B	Dextran enzyme	From *Catenovulum* sp.

(continued)

Table 2 (continued)

Patent	Enzyme/organism	Notes
CN-102174439-B	β-Galactosidase	Cold active enzyme from marine *Halomonas* strain
CN-105164254-A	Cellobiohydrolase	Expressed in host cell such as marine *Bacillus*
WO-2018091836-A1	α-1,3-(3,6-anhydro)-D-galactosidase	From *Zobellia galactanivorans* DsiJ
CN-105683370-A, CN-104145016-A	α-Xylosidase, β-Xylosidase	Expressed in host cell such as marine *Oceanobacillus*
CN-108350044-A	Cellobiohydrolase	Expressed in host cell such as marine *Bacillus*
CN-104245926-A	Cellulases	Expressed in host cell such as marine *Oceanobacillus*
CN-102732462-A	Chitosan digestive enzyme	High-yield production of enzyme as extracellular protein by marine bacteria *Renibacterium* sp. QD1
CN-104962503-B	Amylase	Cold active
CN-108018246-A	Chitosanase	From marine *Bacillus safensis* LZ303
CN-108138153-A	Arabinofuranosidase	Expressed in host cell such as marine *Bacillus*
CN-105296448-A	Agarase	from the marine environment producing strains
CN-103060289-B	Xylanase	Salt tolerant activity from *Bacillus cellulosilyticus*
KR-20180047493-A	β-Agarase	novel β-agarase AgaJ9 derived from *Gayadomonas joobiniege*
CN-106957832-A	β-Galactosidase	marine bacterium *Paenicibacillus barengoltzii*
CN-105316301-A	Xylanase	Thermostable enzyme
CN-102154239-B	β-Galactosidase	Galactosyl transfer activity
CN-106906161-A	Glycosaminoglycan lyase	Firstly obtained from the genome of *Microbacterium* WS15
CN-102586296-B	β-Glucosidase	Derived from the marine bacterium *Aeromonas* (*Marinomonas* MWYLI)

Many other patents are present from new organisms from marine environments originating from genus Bacillus. Microorganisms of the genus Paenibacillus are widely distributed in the environment including marine sediment samples (Lee et al. 2013) thus it is important to notice about a patent related to agarase enzyme (Lin et al. 2016) from *Paenibacillus agarexedens* and a method to provide a novel enzyme for digesting agarose.

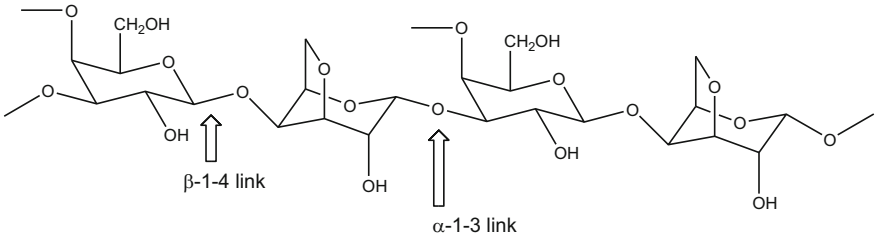

Fig. 1 Glycosidic linkages in carrageenans subjected to enzymatic hydrolysis

Another interesting patent to be mentioned is related to an α-galactosidase capable of acting on α-1,3 bond in carrageenans (Ficko-Blean et al. 2018). Carrageenans polymers are polysaccharides of high molecular weight made up of repeating galactose and 3,6-anhydrogalactose. Units are linked by α-1,3 and β-1,4 alternating glycosidic linkages (Fig. 1) and can be sulfated and nonsulfated. The enzyme is originating from *Zobellia galactanivorans* DsiJ, a marine microorganism that has been isolated from the red alga *Delesseria sanguinea*. The products of hydrolysis of the β-1-4 in the κ- and ι-carrageenans by κ- and ι-carrageenases are oligosaccharides of the neo-carrabiose series characterized by the a residue 3,6-anhydro-D-galactose at non-reducing end and D-galactose residue at the reducing end. The need solved by this patent is for enzymes attacking the α-1-3 linkage that can be useful for (1) a complete sustainable hydrolysis of carrageenan producing up to the monomer 3,6-anhydro-D-galactose that can be utilized as building block in chemical synthesis, and (2) production of new oligosaccharides. Chemical hydrolysis using strong acid can degrade 3,6-anhydro-D-galactose generating toxic by-products. In view of known biological effects of oligosaccharides from carrageenans (Bhattacharyya et al. 2010) the importance of enzymes acting on α-1,3 galactosidic bonds is in the context of reducing inflammatory response. Moreover authors of the patent claimed application also in saccharification and possible production of bioethanol from carrageenophytes red algae.

The backbone of xyloglucan is composed by a cellulose chain of (1-4)-linked β-D-glucose units, most of these units carry an α-D-xylose residue on position 6, forming the isoprimeverose disaccharide moiety. Enzymes that can modify xyloglucan include glycanase, and several glycosidases (α-xylosidase and β-glucosidase) catalysing exo-hydrolysis, and others. The importance of including α-xylosidases in cellulolytic cocktails resides in the possibility of a complete hydrolysis of lignocellulosic material. The interest for marine originating α-xylosidases is based upon possible presence of suitable features that they may possess in terms of resistance and action in presence of salts. A patent is listed in Table 1 regarding these enzymes (Berte et al. 2016), with some other having no worldwide application documents. In general there is presence of patents about other hemicellulosic components such as xylan. In particular the patent (Preston 2013) describes genetically modified marine microorganisms that produces secreted endo-xylanases for production of xylooligosaccharides with or without arabinofuranosyl substitutions

and acidic substituents. These compounds are in fact of interest for applications as prebiotics, anti-inflammatory agents and other interesting biomedical activities (Chen et al. 2012).

Another interesting patent is the one describing the muramidase activity from the extremophilic archaea *Aciduliprofundum boonei* isolated from acidic hydrothermal vent environments and the antibacterial activity of this enzyme (Metcalf and Bordenstein 2014) that is explicated by the hydrolysis of 1-4-β-glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in peptidoglycans. These inventors correlated the presence of such enzyme with selective evolutionary action over more vulnerable relatives. Indeed, members of the genus Aciduliprofundum are widespread thermoacidophiles in deep-sea hydrothermal vent chimney biofilms. They concluded that a systematic surveys of antibacterial peptides from archaea could uncover a broad range of antibacterial activities and may eventually offer novel therapeutics. In fact, the patent is centered on a pharmaceutical composition comprising an *Aciduliprofundum boonei* GH25 muramidase domain disposed in a pharmaceutically acceptable diluent, carrier or excipient.

Bioprocesses

In Table 3 are listed those patents classified under this section dedicated to bioprocesses. All material is listed with a note in the table quickly describing the bioprocess, while just most important ones are discussed here as follows.

The preparation of one or more human milk oligosaccharides is described in a patent (Gyula et al. 2011). These compounds are based on five monosaccharides glucose, galactose, N-acetylglucosamine, fucose and N-acetylneuraminic acid. A hundred or more different human milk oligosaccharides have been identified so far; their composition is related to the expression of certain glycosyltransferases and is linked to blood group characteristics. With lactose as base disaccharidic acceptor (in vitro or in vivo), fucosyltransferases can be used for the production of different regioisomers of fucosyllactose, or more complex fucosylated core structures. Sialylated structures can be produced by sialyltransferases and trans-sialidases. It is current opinion of experts that the state of the art for an industrial production of human milk oligosaccharides will be based on whole cell biotransformation that is the most promising choice to provide human milk oligosaccharides as food additive (Sprenger et al. 2017). Human milk oligosaccharides are recognized of great importance in the maturation of the immune system and as immunomodulators and this patent describes the synthesis in a cost efficient manner and possibly on an industrial scale using trans-fucosidase and other enzymes of at least one organism of marine origin (*Thermotoga*). In particular the α-L-fucosidase from *Thermotoga maritima* (Tmαfuc) was converted into variants by directed evolution, with increase of the yield of the enzymatic reaction from 7 to more than 60%. Many other patents focused on the preparation of these oligosaccharides from the same inventors were retrieved.

Table 3 Patents on bioprocesses using marine glycosyl hydrolases

Patent	Bioprocess
WO-2012156898-A1, WO-2012156897-A1, US-10005807-B2, WO-2013190531-A1, WO-2013190530-A1, WO-2013190529-A1	Preparation of one or more human or bovine milk oligosaccharides
CN-104768391-A, CN-103797126-A, CN-103608461-A, CN-103459605-B, CN-104685052-A, CN-105492601-A, CN-103930555-A	Enzymatic treatment of cellulosic material to increase digestibility using Bacillus marinus or other marine derived enzymes
KR-101787331-B1	A process for agar decomposition by marine thermotolerant agarase (*Saccharophagus degradans*)
CN-104862294-B	Marine bacterium *Catenovulum agarivorans*
CN-102618451-B	Synthesis of alkyl glycosides using enzymes from *Arthrobacter* sp
CN-105249434-A	Preparation of alginate oligosaccharides by enzymatic hydrolysis using abalone enzymes
CA-2920768-A1	Chitinolytic enzymes against parasitic infestation in fish
CN-102178938-A	Production of a suppository used against infections incorporating a marine lysozyme preparation
KR-20140139118-A	Marine cellulase from *Thermotoga maritima* for hydrolysis of β-1,4 linkages within guar network
CN-106987574-A	Preparation of oligosaccharides from K-carrageenan using *Cellulophaga lytica* strain N5-2
CN-102653747-A	A process of fermentative production of a cold adapted β-galactosidase
CN-103834593-A	Application of important glycosyl hydrolases from marine strain of Paracoccus
CN-108165541-A	Application of a β-galactosidase from marine bacteria for agar degradation
WO-2018127486-A1	Enzymatic process for dehusking of pulses by pretreatment with GH11 or GH8 xylanase or a GH5 endo glucanase or a polygalacturonase
EP-2907504-A1	Hyaluronidases (including marine originated) in pharmaceutic preparation
WO-2012161250-A1	Halomonas derived α-glucosidase application for production of gingerol glucosides
CN-103805541-A	Alginate lyase from *Photobacterium phosphoreum*
CN-108431220-A	Composition for dish washing containing β-glucanase of various origin including marine Oceanobacillus
CN-103014041-A	Production of recombinant chitinase originating from *Tilapia nilotica*

Another patent from *Thermotoga maritima* (Tan et al. 2012) is related to a marine cellulase for hydrolysis of β-1,4 linkages within guar network useful in fracturing liquid as above discussed.

A process for agar decomposition by marine thermotolerant agarase is reported (Kim et al. 2016). *Saccharophagus degradans* is a known microorganism able to grow using many of the complex polysaccharides found in the marine environment (Hutcheson et al. 2011). Many genes and enzymes of the *S. degradans* are likely to function in the system for the utilization of agar, alginate, α- and β-glucans, chitin, mannans, pectins, and xylans. The agarase gene of this patent is expressed in *E.coli*.

An important bioprocess of practical value is the dehusking of pulses (lentils or many others) aided by enzymatic pretreatment using three types of glycosyl hydrolases (Tripathy 2017) originated at least by one psychrophilic marine microorganism included in the long list of suitable endoglucanases reported in the patent from planta, archaea, bacterial or fungal origin.

Gingerols are pungent phenolic substances contained in ginger, *Zingiber officinale*, known as anticancer, anti-inflammation, and anti-oxidative (Wang et al. 2014) agents. An enzyme was found (Ojima et al. 2012) in a marine microorganism belonging to the genus *Halomonas* that has great capability to transfer glucose from maltose to glycerol and ethanol; an interesting yield of ca. 60% is reached with gingerol. Glucosylation of gingerol is claimed to increase water solubility of the aglycon molecule from 0 to 43.8 mM (for monoglucoside) an interesting feature for biomedical applications. A Japanese patent describes this α-glucosidase activity for the production of glycosylated derivatives of this aglycon. Of similar nature there is a chinese patent dealing with an enzymatic activity from a marine microorganism (*Arthrobacter*) for the synthesis of alkyl glycosides. Interesting yields around 50% are registered.

Chitinolytic enzymes are used in different bioprocesses. They can be used against infestations in fish, offering a non-toxic, biological tool for controlling sea lice infestation of salmon. The sea lice are capable of infesting a fish attaching to the skin, scales, mucus and blood and parasitically feeding off. This is particularly important in commercial efficacy of fish farming. Marine chitinolytic enzymes of interest are found in fish and production of a recombinant chitinase originating from *Tilapia nilotica* is reported (Hoell et al. 2015).

Conclusion

In a previous report (Trincone 2018) only few patents related to 2016–2017 in the field of marine carbohydrate hydrolysing enzymes were sorted from the Espacenet patent database, leading to the conclusion that the literature in the scientific journals is showing only a partial result of different applicative projects. The analysis of patents reported here using a search engine of larger coverage such as Google patents has been adapted to a wider period from 2011 to 2019 and results deeply analysed in detail. Although this search sorted initially ca. 450 patents, a more precise refining into abstracts and overall texts enabled to delete a consistent amount of them not

related to marine origin of the biocatalysts. Tabulated results are presented with notes for new biocatalysts (Table 2) and bioprocesses (Table 3) envisaged in patents, only important hits are discussed and fully reported in references; all others are easily traceable by patent numbers.

One of the aspects of Table 2 is the preponderance of Asian countries in developing such patents. This is probably based on ethnic and historical interest for marine polysaccharides in these countries, with habit since long time for funding numerous research projects with respect to western countries where general dedicated interest is more recent. From the analysis of both Tables 2 and 3, a first conclusion on research devoted more on new biocatalysts than on the next envisaged bioprocess appears justified. This is the first output about the importance of marine enzymes in biotechnological applications with their features inherited from the environment. This basic scientific interest for them has historical origin since the 1960s. Preponderance of new biocatalysts not yet used in bioprocesses was also anticipated from the analysis of the network correlations between field/polymer reported in Table 1, where bioprospecting new enzymes covers the most of marine saccharidic polymers. Preponderance of Asian countries is evident also in Table 3 with bioprocesses developed on classical topic/enzyme and very few new applications were found. In addition, in this case what can be concluded in temporal terms for Far East originating patents, is the continuing applications of known enzymes or mutants for improvement of already established bioprocesses. In particular, processes for cellulose, agar, alginate and chitin are present.

Registered as a new entry a bioprocess for a well-known application of glycosyl hydrolases is the synthetic aspect using capability of these enzymes to transfer carbohydrate moieties, coupled to their resistance and specificity and ease to develop mutants for increasing reaction yields. These are the case of alkyl glycosides and gingerol glucosides enzymatic syntheses.

At the end of 80s general biocatalysis was already present as tool of chemical industry to achieve sustainability, it was at the core of definition of white biotechnology driven by the demand of sustainable processes as alternative to petroleum-based chemical processes. With bioprospecting from terrestrial and or microbial sources during all these years, a convergence with engineering biotechnology enabled penetration in many sectors. Challenges posed by new environments such as the marine or extremophilic loci have been more demanding for basic research studies with respect to classical biomass sources. Interdisciplinary needs must be organized thus protracting pipelines to study valid substitutes before applications can found room for patenting activity on bioprocesses.

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Glycosyl Hydrolases and Biofuel

Meenal Rastogi and Smriti Shrivastava

Abstract

Paucity of non-renewable energy sources has created a vital requirement for renewable and sustainable biofuels from lignocellulosic biomass. Exploitation of lignocelluloses for the production of second generation fuels such as bioethanol, biodiesel and biogas etc forms an attractive energy alternative since they are naturally abundant and easily accessible throughout the year. Efficient pretreatment methodologies such as alkali, acid treatment, enzymatic hydrolysis and steam explosion are useful for enhancing the digestibility of major lignocellulosic components, cellulose and hemicellulose, followed by the fermentation of obtained sugars exists as a prerequisite for effective transformation of lignocelluloses to diverse value added products. Present chapter compiles different approaches made by eminent scientists and researchers for competent use of celluloses and hemicelluloses for enhancing bioethanol production and also describes recent innovative techniques exploited for the same. The chapter gives an overview of simple sugars utilization by bacteria and fungi and also highlights the influence of consolidated bioprocess systems on ethanol production from various agro-industrial wastes.

Keywords

Bioethanol · Lignocelluloses · Glycoside hydrolases · Xylanases · Cellulases

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Introduction

Glycoside hydrolases (GHs) are catalysts responsible for hydrolysis of the glycosidic bonds in glycoconjugates, oligo- and polysaccharides, thereby forming a reducing sugar and the corresponding free aglycon (Zhang et al. 2010, Van Niël et al. 2017). Sometimes they are also referred to as glycosyl hydrolases and can catalyze the hydrolysis of O-, N- and S-linked glycosides. There are two types of glycoside hydrolases: exo- and endo-, which refers to their ability to cleave a substrate at the end or within the middle of a chain, respectively.

Glycosidases are ubiquitously found in nature. About 1% of the genome of any organism encodes for GHs where they are involved in processes that are essential for life including cell wall metabolism, signaling, biosynthesis of glycans, defence in plants as well as mobilization of storage reserves (Roth 2002; Ardèvol and Rovira 2015). Varying kinds of GHs are involved in different applications such as cellulase, hemicellulase, and amylase are involved in the degradation of biomass, lysozyme in anti-bacterial defense strategies and mannosidases in normal cellular function.

Biofuels eliminate the concerns arising from continuous usage of fossil fuels. Since they are produced through biological processes they offer an ecological and economical alternative for improving energy security. For more than a decade, primary biofuels such as fuel-wood have been used for cooking, heating and generation of electricity. Conversely, biomass can be processed into secondary biofuels like bioethanol and biodiesel to be used in vehicles and industries (Dragone et al. 2010). Biofuels (solid, liquid or gas) can be produced from a variety of biomass including aquatic, agricultural and forestry wastes (Rastogi and Shrivastava 2017). Biofuels can be characterized into first, second, third and fourth generation subject to their source and production technique. First generation biofuels are quite easy to obtain since they are directly processed from food crops such as vegetable oil, starch, sugar and also animal fats. However, utilization of food crops as feed for energy production not only competes with food consumption but also deteriorates the quality of agricultural areas. Inedible crops including lignocellulosic biomass and bio-wastes can be processed into fuels which constitute the second generation biofuels. Algal feedstocks and photobiological solar fuels (presently on paper) are categorized into third and fourth generation biofuels, respectively (Rastogi and Shrivastava 2018).

The abundant supply and low cost of plant biomass favors its usage as energy crops for biofuel production making it one of the highlighted areas for research and industry currently. Mostly, the biotransformation emphasizes on establishing a sugar platform of simple sugar moieties which can be subsequently converted into fuels (such as ethanol, hydrocarbons and butanol) through biological or chemical processes (Cherubini 2010, Singh et al. 2015). Glycoside hydrolases or glycosidases are biocatalysts that play a significant role in industrial and biotechnological processes via hydrolytic degradation of carbohydrates. These versatile enzymes have been applied for a wide variety of processes from biomass degradation to cell surface engineering due to their high specificity and remarkable catalytic efficiency. Moreover, glycosidases aid in the synthesis of glycan through transglycosylation wherein

Fig. 1 Hydrolysis reaction by GHs (cazypedia.org)

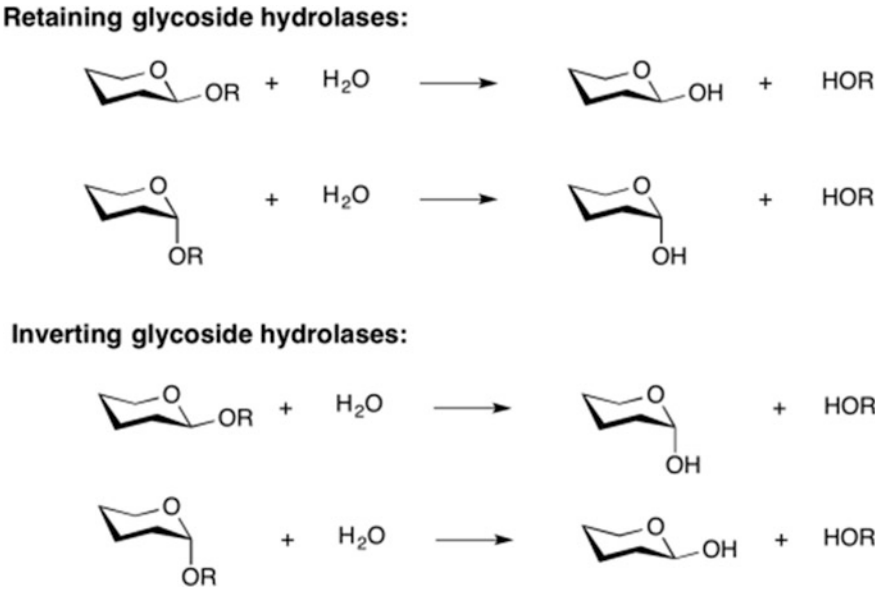

Fig. 2 Reaction mechanism of glycosyl hydrolases (cazypedia.org)

a sugar moiety is transferred from one glycoside to another, rather than to water as in the case of hydrolysis (Wang and Huang 2009) (Fig. 1).

Industrially Important Glycosyl Hydrolases

Glycosyl hydrolases hydrolyze the glycoside bonds by the universal acid catalysis method that entails two main residues: a proton donor and a nucleophile/base as initially suggested by Koshland (1953). The hydrolysis reaction occurs via two different mechanisms resulting in either retaining (via a double-displacement) or inverting of the anomeric configuration of the substrate as shown in Fig. 2 (Van Niël et al. 2017). Hydrolysis of a glycosidic bond via GHs is always stereospecific (Naumoff 2011).

Glycosyl hydrolases have found numerous applications in industrial as well as biotechnological areas. Some applications of these enzymes, ranging from biofuel production to drug designing, have been discussed in brief in Table 1.

Cellulases which are predominantly produced by bacterial, fungal and protozoal organisms are crucial for the hydrolysis of cellulose polymers into monomers of

Table 1 Various applications of commonly used glycosyl hydrolases

Enzyme	GH family	Industry	Applications	References
Cellulase	GH-5, -9, -17, -48	• Biofuel • Detergent • Textile • Food processing • Wine and Brewery • Paper and pulp	• For degradation of cellulose to glucose, to be used for bio-ethanol production • In detergents for washing of cotton fabrics for maintenance of colours • For bio-stoning of denim jeans and bio-polishing of cotton clothes • For maceration and clarification of fruits and vegetable juices, nectars, oils and purees; Olive paste malaxation and olive oil extraction • To improve skin maceration, must clarification, color extraction, filtration, and wine quality and stability • For pulping and deinking of waste papers	Sajith et al. (2016), Kuhad et al. (2011), Sathya and Khan (2014)
Invertase	GH-32, -100	• Food and beverage • Drug and pharmaceutical	• For manufacture of invert sugar; production of non-crystallizable sugar syrup from sucrose for candies and fondants • In the manufacture of artificial honey and plasticizing agents used in cosmetics	Samarth et al. (2013), Willem et al. (2009)
Amylase	GH-13, -14, -15, -31, -57	• Starch • Detergent • Biofuel • Food • Textile • Paper	• For starch hydrolysis in the starch liquefaction process • For laundry and dishwashing to degrade the residues of starchy foods • For bioconversion of starch into ethanol via liquefaction and saccharification	De Souza and De Oliveira Magalhães (2010), Sathya and Khan (2014)

(continued)

Table 1 (continued)

Enzyme	GH family	Industry	Applications	References
			• For production of maltodextrins; in baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups; to improve viscosity of dough for better volume and texture of the product • As a desizing agent to strengthen and prevent breaking of the warp thread during the weaving process • For modification of starch of coated paper	
Xylanase	GH-5, -7, -8, -9, -10, -11, -12,-16, -26, -30, -43, -44, -51, -62	• Biofuel • Paper and pulp • Animal feed • Baking • Fruit juice and Brewery • Detergent • Others	• To convert hemicellulose to simple sugars for bioethanol production • To remove hemicelluloses from paper pulp • To stimulate animal growth rates by improving digestibility and quality of animal litter • To increase the elasticity of the gluten network • To increase yield in the maceration process, reduces viscosity of fruit juice, extraction of more fermentable sugar from barley • In detergents to remove stains • To produce xylo-oligosaccharides to be used in pharmaceuticals, food and feed formulations, agricultural applications	Walia et al. (2017), Goswami Girish and Seema (2015), Garg (2016)

(continued)

Table 1 (continued)

Enzyme	GH family	Industry	Applications	References
Mannanase	GH-5, -26	• Paper • Detergent • Food • Pharmaceutical • Others	• For biobleaching of pulp and paper • For removal of stains of mannan containing gums, ice-creams, hair gels, sauces, shampoos, and tooth-pastes • In coffee processing to reduce viscosity; to produce prebiotic manno-oligosaccharides, maceration of fruits and vegetables • To provide fast dissolving and structure forming properties to the tablets • Gas and oil exploitation, production of animal feed, bioethanol production, textile printing	Chauhan (2012)
Pectinase	GH-28	• Food • Wine • Bioenergy • Textile • Animal feed • Paper and pulp	• To reduce viscosity, increase the yield and juice clarification by liquefaction of pulps; in maceration of vegetables; extraction of vegetable oils in an aqueous process; tea and coffee processing; • To maximise juice extraction, facilitate filtration and strengthen the flavour and colour • To hydrolyze pectin present in agro-wastes into simple sugars to be converted into bioethanol • In bio-scouring, to remove impurities and prevent fiber damage • To decrease feed viscosity for better absorption of nutrients	Garg et al. (2016)

(continued)

Table 1 (continued)

Enzyme	GH family	Industry	Applications	References
			by ruminants and thus reduce amount of faeces • In bio-bleaching of kraft pulp to reduce cationic demand in the filtrate thereby reducing yellowness of paper	
Pullulanase	GH-13	Starch Processing	To hydrolyse the α-1,6 glucosidic linkages in starch, amylopectin and pullulan during starch saccharification; production of high-maltose/fructose corn syrup and cyclodextrins; in dishwashing and laundary detergents	Hii et al. (2012)
Chitinase	GH-18, -19, -20	• Waste management • Medical	• To convert chitinous waste of marine organisms into simple components and reduce water pollution • As an antifungal agent in combination with antifungal drugs to treat fungal infections	Rathore and Gupta (2015)
Inulinase	GH-32, -91	• Food • Others	• Production of high fructose syrup and fructo-oligosaccharides • Assist citric acid, lactic acid, ethanol and butanediol production	Singh et al. (2017)
Agarase	GH-16, -50, -86, -96	• Biotechnological • Others	• To recover DNA bands from the agarose gel; to produce oligosaccharides having antioxidant properties; preparation of seaweed protoplasts • Used as low-calorie additives to improve qualities of food; in cosmetics and medical fields	Fu and Kim (2010), Sathya and Khan (2014)

fermentable sugars (glucose) to produce biofuels. They break the β-1,4-d-glucan bonds present in the cellulose and liberate glucose, cellobiose and cello-oligosaccharides. This enzymatic complex includes endo-glucanases (EG; EC 3.2.1.4), exoglucanases (cellobiohydrolases, CBH; EC 3.2.1.91) and β-glucosidases (BGL; EC 3.2.1.21) (Rawat et al. 2014; Srivastava et al. 2017). These enzymes are required to work synergistically for efficient conversion of celluloses to sugar monomers for effective biofuel production. The enzyme hydrolysis commences with the release of nicks in the cellulosic structure by the endoglucanases thereby revealing the reducing and non-reducing ends, in order for the cellobiohydrolases to liberate cellobioses and cello-oligosaccharides by acting upon both the ends. Subsequently, β-glucosidases breakdown the cellobioses into glucosemonomers during the hydrolysis reaction (Bhat and Bhat 1997; Srivastava et al. 2014, 2017).

Owing to the structural heterogeneity of hemicellulosic constituent of plant biomass, complete degradation requires an array of hemicellulolytic enzymes. These enzymes include endo-1,4-β D-xylanase (E.C.3.2.1.8) which randomly cleave the xylan backbone to produce xylooligomers; xylan-1,4-β-xylosidase (E.C.3.2.1.37) cleaves these xylooligomers into xylose monomers; α-l-arabinofuranosidase (E.C. 3.2.1.55) remove the side groups from the main chain; acetylxylan esterases (E.C. 3.1.1.72) and α-D glucuronidases (E.C. 3.2.1.139) act synergistically to remove phenolic and acetyl side branches from the complex polymer (Ahmed et al. 2009; Uma Shankar et al. 2016). Xylanases are ubiquitous in both eukaryotes and prokaryotes as they have been accounted from bacterial (terrestrial, marine or rumen), fungal, protozoans and algal sources in addition to snails, insects and crustaceans (Walia et al. 2017).

Lignocelluloses comprise nearly 5% mannan apart from softwoods or coniferous sources that usually contain more mannan (10%) than xylan. Mannans are polysaccharides that consist of β-1,4-linked backbone of mannose as the major constituent unit. Mannans are non-starch carbohydrate reserves and are one of the constituents of hemicellulose. There are two major mannan-degrading enzymes: β-mannanase (1,4-β-D-mannan mannohydrolase, EC 3.2.1.78), an endo-acting enzyme catalyzing the random cleaving of β-1,4-linked internal linkages of the mannan, galactomannans and glucomannans via double displacement mechanism and an exo-acting β-mannosidase enzyme (1,4-β-D-mannopyranoside hydrolase, EC 3.2.1.25) which works on the non-reducing ends of the chain to release β-1,4-linked mannosides (Moreira and Filho 2008). The two enzymes work in collaboration with each other on mannans and its oligosaccharides to form mannose, which is utilized as a sugar substrate by some microbes for subsequent fermentation (Ishii et al. 2016). In addition to the key enzymes (cellulases and xylanases), β-mannanases are essential for the efficient bioconversion of lignocellulose biomass to fermentable sugars (Yamabhai et al. 2016).

Pectinases remain as one of the significant enzymes in the current biotechnological perspective due to their wide-ranging application. Based upon the mode of action, they are broadly categorized into three types: pectin esterase, hydrolases and lyases. Pectin esterases are responsible for the de-esterification of methoxyl

moieties present in pectin to form pectic acid. The polygalacturonases and polymethylgalacturonases hydrolases cleave the α-1,4-glycoside bonds in pectic acid and pectin, respectively, while lyases (polygalacturonate lyase and polymethylgalacturonate lyase) disintegrate the α-1,4-glycosidic linkages in pectic acid and pectin, respectively by trans-elimination reaction forming unsaturated galacturonates and methyl galacturonates, respectively (Garg et al. 2016; Ismail et al. 2016). Pectins, being the main components of the middle lamella in the cell wall, not only hamper second generation bioethanol production via inhibition of release of sugars but also obstruct the dilapidation of cells from tissues in first generation bioethanol process (Latarullo et al. 2016).

Biodiesel is manufactured via transesterification of vegetable oils. The presence of precipitates in biodiesel is of utmost importance as it declines the quality. These precipitates have been accounted to contain steryl glucosides (SGs) which give a hazy appearance to biodiesel while forming white sediments during storage. Thus, SGs need to be selectively removed to circumvent the blockage of filters as well as engine failures, hence producing biodiesel of superior quality to be accepted by the consumers. At present, distillation is the solitary means for complete removal of SGs from biodiesel that happens to be an expensive process requiring a lot of energy. This compromises with the cost efficiency and net energy gain of biodiesel production. In such scenario, glycoside hydrolases provide an alternative for cost-effective industrial methods to eradicate these compounds. Steryl-beta-glucosidase (EC 3.2.1.104) enzymes have been proposed to catalyze the hydrolysis of SGs while forming glucose and a sterol. This enzyme belongs to the family of O- and S-glycosyl hydrolases. The sterols thus generated completely solubilize in biodiesel, whereas the glucose is subsequently eliminated during the water-washing steps after transesterification (Peiru et al. 2015).

Agricultural Residues/Wastes/Industrial Effluents (Sources of Carbon)

A variety of wastes including agricultural residues, municipal solid waste and industrial effluents can be utilized for efficient production of biofuels. Lignocellulosic biomass can either be derivatives of agricultural practices or related industries. Lignocelluloses entail agro-wastes such as sugarcane bagasse, corn cobs, wheat and rice straw, cotton stalks, jute sticks, rice husks, coconut shells; forest wastes like wood chips, bark and sawdust; and organic fractions of sewage treated sludge and municipal solid wastes (MSW). Cellulose forms a major fraction of lignocellulosic feedstocks with 40–60% of the total dry weight followed by hemicellulose and lignin constituting about 20–40% and 10–25%, respectively (Kang et al. 2014). These are present in the cell walls of practically all plant materials. Cellulose is a homopolysaccharide of glucose residues attached to each other by β-1,4-glycosidic linkages. The linear cellulose chains are packed into crystalline microfibril bundles by means of hydrogen bonds (internal and external). Hemicelluloses are considered to be heterogeneous as these complex polysaccharides constitute different hexose

Table 2 Composition of various lignocellulosic biomass (Rastogi and Shrivastava 2017)

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Sugarcane bagasse	32–48	19–24	23–32	1.5–5
Corn stalk	39–47	26–31	3–5	12–16
Rice husk	31.3	24.3	14.3	23.5
Rice straw	28–36	23–28	12–14	14–20
Wheat straw	33–38	26–32	17–19	6–8
Groundnut shell	35.7	18.7	30.2	5.9
Coconut shell	29.7	NA	44.0	0.5
Corn stover	38–40	28	7–21	3.6–7.0
Cotton waste	80–95	5–20	–	–
Softwoods	45–50	25–35	25–35	NA
Hardwoods	40–55	24–40	18–25	NA
Newspaper	40–55	25–40	18–30	8.8–1.8
Algae (green)	20–40	20–50	NA	NA

NA Not available. Composition is represented in percent weight on dry weight of the samples

and pentose sugars including glucose, galactose, mannose, xylose, arabinose and glucuronic acid. Hemicelluloses differ in plants depending upon their species and type of tissues. Some of the side residues in hemicellulosic structures have been found to be acetylated or methylated. Hemicelluloses are covalently linked to lignin, which comprises of three aromatic monomers- guaiacyl (G-lignin), syringyl (S-lignin), and *p*-hydroxyphenyl (H-lignin). The enzyme accessibility to cellulose is blocked due to the orientation of the cellulose microfibrils between the lignin and hemicellulose matrix which is a major problem regarding biofuel production (Xiao et al. 2016). Plant species differ in lignocellulosic composition (Table 2) with the abundance (on average) of major macromolecules in the following order: glucan > lignin > xylan > mannan > arabinan > galactan (Ishii et al. 2016).

Industries such as paper, pulp, food processing, dairy, sugar, poultry, tanneries, distilleries and biodiesel generate large volumes of inadequately treated solid and liquid wastes that pose a serious threat to ecosystems causing environmental distress. Industrial effluents rich in organic matter having high BOD/COD levels form potential fermentable substrates for bio-ethanol, methane, bio-hydrogen and surfactants production (Diwan et al. 2018). In food industry, waste generation occurs at every stage of food supply chain from production, transportation and storage to processing, packing, distribution and consumption. Fruit and vegetable wastes contain cellulose, hemicellulose and pectin in high quantities and can be used either directly or after pretreatment for production of bioethanol, biodiesel and biogas (Eleren et al. 2018). Industrial effluents act as sustainable feedstock for algal growth which accumulates nutrients and metals and degrading toxic compounds present in the effluents. Algal ponds containing wastewater has been used for production of biodiesel which also aid in sequestering the carbon dioxide emitted by the industries utilizing it for their photosynthesis. Table 3 describes various industrial effluents and its applications.

Table 3 Industrial effluents and its applications

Industries	Wastes generated	Applications	References
Sugar mills	Bagasse	Production of bio-ethanol and bio-methane, heat and electricity in sugar mills, cattle feed and paper making	Bhatnagar (2016)
	Press mud	Fertilizer and wax production; cement and paint manufacturing, foaming agent, animal feed	
	Molasses	Ethanol production	
	Fermentative yeast biomass	Biogas production and digestate	
	Bagasse fly ash	As an adsorbent for the removal of different metal ions, dyes from effluents, phenol and its derivatives, pesticide removal from wastewater	
Slaughter houses	Organs, tissues, hides, blood, animal excreta, carcass	Biogas production; fat for making grease, animal feed, candles, soap and biodiesel; tallow as a lubricant in steel rolling industry	Franke-Whittle and Insam (2013)
Paper mills	Pulp	Biogas production and digestate	Chakraborty (2019)
	Paper shavings	Heat and power	
	Wood wastes and paper boards	Heat and power	
Dairy plants	Whey and milk cream	Biogas production and digestate	Chakraborty (2019)
Sago factories	Starch materials and peels	Biogas production and digestate	
Tanneries	Hides and skins	Biogas production and digestate	
Animal husbandries	Animal excreta and body fluids	Biogas production and digestate	
Fruits and vegetable processing units	Pulp wastes	Biogas production and digestate	

Processing of These Wastes

Biofuels obtained as metabolites of microbial processes (as in the case of bioethanol production) primarily target cellulose component of biomass. A major challenge is to accomplish high titers of fermentable sugars from lignocelluloses that usually requires the aid of different microbial GHs, which catalyze the saccharification of varying polysaccharides present in the biomass. The microbial glycoside hydrolases disintegrate the complex carbohydrate compounds into mono or oligosaccharides, allowing their uptake and subsequent metabolism by a suitable microorganism for their conversion into desirable biofuels. Due to the structural, physicochemical and

compositional complexity, cellulose is resistant to enzymatic degradation. Moreover, many microorganisms are deficient of a competent enzyme machinery essential for the effective degradation of lignocellulosic biomass. Thus, lignocellulosic biomass requires a pretreatment step for their efficient conversion to biofuels, thereby reducing the crystallinity of the cellulose, removing the lignin and hemicellulosic components and improving the permeability of the biomass. The enzyme accessibility to cellulose is further enhanced which leads to improved production of fermentable sugars from cellulose (Mamo et al. 2013). This has led to the development of numerous pre-treatment methods to breakdown the intertwined interaction between the lignocellulosic constituents (cellulose, hemicellulose and lignin), and have been summarized in Table 4.

Bioethanol Production (Native/Biotechnological or Cloning)

The biochemical conversion of lignocelluloses to bioethanol comprises of four major processes: physicochemical pretreatment, enzymatic hydrolysis of the complex sugar polymers, ethanol production via sugar fermentation and distillation (Sánchez and Cardona 2008). This conversion route can be accomplished by utilizing highly proficient microbial strains (native as well as recombinant) that are able to produce both glycosyl hydrolases and ethanol. The celluloses and hemicelluloses in the biomass are acted upon by varying cellulases and hemicellulases for their respective conversion into glucose and xylose. Upon saccharification, the hexose and pentose monomeric sugars so formed can be sequentially fermented to ethanol by diverse microorganisms. Microorganisms like *C. thermocellum* and *T. reesei* have been extensively interrogated for their natural ability to produce ethanol. On the other hand, prospective microorganisms such as *F. oxysporum, C. thermocellum*, *K. oxytoca* and *T. mathranii* that are naturally cellulolytic have been genetically altered to be ethanologenic and potential fermenting microbes such as *P. stipitis*, *S. cerevisiae*, *H. polymorpha*, *E. coli*, *K. marxianus* and *Z. mobilis* have been transformed to be cellulolytic (Jouzani and Taherzadeh 2015).

Different strategies integrating the hydrolysis and fermentation processes have been proposed with the aim of escalating the efficacy of bioethanol production. The process of separate hydrolysis and fermentation (SHF) allows the hydrolysis and fermentation processes to operate distinctly. The pretreated biomass is degraded first into sugar monomers that are subsequently converted to ethanol via fermentation. Although, both enzyme hydrolysis and fermentation operate at their respective optimum conditions during the process, yet accumulation of sugars inhibits the enzymatic activity ultimately affecting the ethanol yield (Jambo et al. 2016). Simultaneous saccharification and fermentation (SSF) permits the saccharification and fermentation to occur simultaneously in a single reactor, that is, as soon as the sugars are released from the biomass, they are quickly converted into ethanol. The major constraint in this process is to optimize the process parameters suitable for both the microorganisms as well as enzymes since they are operating at the same time (Vohra et al. 2014). Simultaneous Saccharification and Co-Fermentation (SSCF) is focused

Table 4 Comparative analysis of various pre-treatment techniques (Rastogi and Shrivastava 2017)

Pretreatment	Mode of action	Advantages	Disadvantages
Mechanical	Milling, grinding, shredding or chipping reduces particle size	• Increase in specific surface area and digestibility of biomass • Reduced crystallinity and degree of polymerization of cellulose	• High energy consumption renders this method economically inefficient
Extrusion/ pyrolysis	Treatment at high temperature (>300 °C) following by mixing and shearing causes defibrillation, fibrillation and shortening of the fiber		Parameters in bioreactor need to be highly efficient
Liquid hot water	Liquid hot water (160–240 °C) under high pressure (>5 MPa) for time ranging from few minutes up to an hour removes hemicellulose from lignocellulosic biomass making the cellulose more accessible	• Better pH control minimizes non-specific degradation of polysaccharides • High pentose recovery and lower formation of inhibitors • No chemicals and corrosion resistant materials are required	• High energy requirement and water demand • Not feasible for commercial scale
Steam explosion (Autohydrolysis)	Exposure of chopped biomass to hot steam (160–260 °C) under high pressure for specific period of time followed by sudden release in pressure causes autohydrolysis of acetyl groups of hemicellulose. Individual fibers are separated disrupting the cell wall structure	• Improved enzymatic hydrolysis • Lower environmental impact • Less hazardous chemicals required • High sugar yield • Feasible for industries	• Less effective for softwoods • Formation of inhibitory products (eg furfural and HMF[a]) • Partial degradation of hemicelluloses and lignin • Additional equipment requirement for acid addition • High cost
Ammonia fiber expansion (AFEX)	Treatment with liquid ammonia at moderate temperature (60–100 °C) for 30–60 min at high pressure (250–300 psi) followed by sudden pressure release causes	• Increases the surface area accessible for enzymes and thus enhanced digestibility • Less inhibitory or	• Not very effective for the biomass with high lignin content • High cost of large amount of ammonia • Hemicelluloses are not significantly

(continued)

Table 4 (continued)

Pretreatment	Mode of action	Advantages	Disadvantages
	disruption of biomass fibers and partial decrystallization of cellulose	toxic compound formation	reduced affecting the sugar yield
Acid	Dilute rather than concentrated acid is used either at high temperature (e.g., 180 °C) for short period of time or lower temperature (e.g., 120 °C) for longer retention time (30–90 min) to solubilize hemicelluloses and lignin	• Enhanced hydrolysis of hemicelluloses and amount of amorphous cellulose • High sugar yield	• High energy input so cost is high • Acids are corrosive so the process requires specific reaction vessels • Formation of inhibitory compounds (furfural, 5-HMF[a], phenolic acids and aldehydes)
Alkali	Treatment with alkali such as sodium, potassium, calcium and ammonium hydroxides disrupts the ester and glycosidic side chains causing alteration in lignin structure, cellulose swelling and its partial decrystallization and partial solubilization of hemicelluloses	• Efficient removal of all lignin • Increased accessibility of hemicelluloses-degrading enzyme • Decrease in the degree of polymerization and crystallinity of cellulose	• Downstream processing costs are high • Not efficient for industrial scale
Organosolv	Organic or aqueous organic solvent mixtures (such as ethanol, ethylene glycol, acetone, methanol, etc.) with inorganic acid catalysts are used to extract lignin	• Improvement in enzymatic digestibility of lignin and hemicelluloses	• Cost of solvent and the catalysts are high • Risk of fires and explosions as organic solvents are inflammable
Ozonolysis	Ozone treatment degrades lignin by attacking aromatic rings structure, hardly affecting cellulose and hemicelluloses	• No toxic residues produced • Reaction is carried out at room temperature and pressure	• Highly expensive due to requirement of large amount of ozone
Biological	Microorganisms specifically white rot fungi such as *P. chrysosporium,C. lacerata, C. stercolerus,* etcproduce lignin peroxidases and	• Low capital cost • No chemicals required • Low energy requirement • Mild environmental	• Slow rate of hydrolysis so inefficient for industrial purposes • Operational costs increase in large scale operation

(continued)

Table 4 (continued)

Pretreatment	Mode of action	Advantages	Disadvantages
	manganese-dependent peroxidases and laccase that causes lignin degradation	conditions required • Improved productivity • Control of pH during sugar utilization	• More microbes need to be identified and isolated to delignify the plant material quickly and efficiently

[a]*HMF* hydroxymethylfurfural

on the microbial assimilation of the entire sugars that are released from the pretreatment in addition to the hydrolytic processes of biomass. For instance, a mixture of yeast cultures can assimilate both type of sugars but a higher rate of hexose conversion to ethanol will be seen as hexose utilizing microbes grow faster than pentose-utilizing microbes (Koppram et al. 2013).

Consolidated bioprocessing (CBP), on the other hand, integrates all the reactions essential for the conversion of lignocellulosic biomass into ethanol. This approach emphasizes on the usage of a single microbe which can carry out all the processes of enzyme production and hydrolysis as well as fermentation in a single step (Vohra et al. 2014). Naturally occurring cellulase-producing microbial strains can be improved for their biofuel yield or cellulolytic microorganisms can be altered to be ethanologenic while ethanologenic strains can be engineered to be cellulolytic to generate CBP organisms. Different bacterial species such as *Clostridium thermocellum* (Maki et al. 2013; Kumagai et al. 2014; Tian et al. 2016), *Clostridium phytofermentans* (Jin et al. 2011), *Clostridium cellulolyticum* (Li et al. 2012), *Thermoanaerobacterium saccharolyticum* (Shaw et al. 2012), *Caldicellulosiruptor bescii* (Chung et al. 2015), *Escherichia coli* (Shin et al. 2014; Luo et al. 2014), *Zymomonas mobilis* (Wu et al. 2014); fungi such as *Fusarium oxysporum* (Ali et al. 2013), *Trichoderma reesei* (Huang et al. 2014), *Paecilomyces variotii* (Zerva et al. 2014), *Aspergillus oryzae* (Hossain 2013); and yeasts including *Kluyveromyces marxianus* (Hu et al. 2012; Chang et al. 2013), *Clavispora* (Liu et al. 2012), *Pichia stipitis* (Watanabe et al. 2011; Puseenam et al. 2015) and *Saccharomyces cerevisiae* (Yamada et al. 2011, Sakamoto et al. 2012, Fan et al. 2016) have been extensively studied, modified by various strategies such as adaptive evolution, directed mutagenesis and engineered genetically and metabolically to enhance ethanol yield and tolerance for their widespread use in bioethanol production (Table 5).

Discussion

Varied families of glycoside hydrolases are responsible for conversion of lignocellulosic polysaccharide chains into oligomeric and monomeric sugars that can be processed into several value added products, specifically biofuels. Different cellulases, xylanases and mannanases act synergistically on complex sugars present

Table 5 Genetically modified microbes for consolidated bioprocess system (Rastogi and Shrivastava 2017)

Substrate	Microorganism	Description	Ethanol concentration/ yield
Inulin Jerusalem artichoke tuber powder	*S. cerevisiae*	Co-expression of exo-inulinase (InuMK1) and endo-inulinase (InuB) genes from *A. niger* and *K. marxianus* respectively, with repression of proteinase gene PEP4 and switch between haploid and diploid strains	2.44 g/l/h 3.13 g/l/h
20 g/l galactose + 10 g/l CMC 20 g/l galactose + PASC (10 g/l)	*S. cerevisiae*	Cell-displayed minicellulosome consisting of endo- and exo-glucanase with intracellular cellodextrin utilization pathway mimicking the one in *C. thermocellum*	62.61 mg/g cell/h 56.37 mg/g cell/h
60 g/l cellulose (Avicel PH105)	*C. thermocellum* strain AG553	Mutation: adaptive evolution strategy. Apparent changes in Clo1313_1831-2, AdhE and GapDH genes in adapted strain (LL1210)	22.4 ± 1.4 g/l (75% of the theoretical maximum)
Natural sorghum Triticale	*S. cerevisiae* strains M2n and MEL2	Integration of codon optimized variants of *T. lanuginosus*glucoamylase (TLG1) and *S. fibuligera*α-amylase (SFA1) genes	62% and 73% (theoretical maximum)
2% (w/v) Avicel 2% (w/v) Switchgrass	*C. bescii*	Expression of bi-functional acetaldehyde/alcohol dehydrogenase (AdhE) from *T. pseudethanolicus* 39E in the *C. bescii* strain lacking lactate dehydrogenase gene	2.3 mM 1.6 mM
Xylan and β-glucan	*S. stipitis* strain BCC15191	Co-expression of endoxylanase and endoglucanase from *Aspergillus niger* and *Aspergillus aculeatus* respectively	2.7 g/l
50 g/l Glucose 50 g/l Sugarcane bagasse	*T. reesei*CICC 40360	Genome shuffling and mutagenesis to improve production of ethanol under aerobic condition and increased ethanol tolerance (4% v/v)	9.7 g/l 3.1 g/l

(continued)

Table 5 (continued)

Substrate	Microorganism	Description	Ethanol concentration/ yield
20 g/L CMC	*S. cerevisiae*	Co-expression of endoglucanase (eg3) and β-glucosidase (bgl1) were obtained from *Trichoderma viride*	4.63 g/l
Wheat straw	*F. oxysporum*	Post-translational gene silencing of the sugar transporter (Hxt) in the fungus	33.8% (theoretical maximum)
10 g/l crystalline cellulose	*C. cellulolyticum*	Inactivation of L-lactate dehydrogenase (*ldh*) and L-malate dehydrogenase (*mdh*) genes	2.7 g/l

in biomass leading to the formation of monosaccharides such as glucose, xylose and mannose, respectively. Other accessory enzymes such as glucuronidases and arabinofuranosidases cleave branching components from the backbone chains forming glucuronic acid and arabinose, respectively as end products (Walker et al. 2017). Hexose sugars (such as glucose, mannose and galactose) and pentose sugars (such as xylose and arabinose) are typically assimilated in all microorganisms through specific metabolic processes, Embden-Meyerhof-Parnas (EMP) and Pentose Phosphate pathway (PPP), respectively. A detailed description and interaction of the pathways has been described in Fig. 3. Xylulose kinase converts D-xylose into D-xylulose-5-phosphate via an intermediary reaction, which is directed to the native pentose phosphate pathway. Xylose conversion to xylulose, an intermediary product, occurs directly in bacteria whereas fungi and yeasts employ xylose reductase and xylitol dehydrogenase through a two-step oxidation-reduction pathway (Koppram et al. 2013; Poszytek et al. 2016). The synergistic action of phosphopentose epimerase, transketolase and transaldolase further converts D-xylose-5-phosphate through non-oxidative rearrangement into fructose-6-phoshate and glyceraldehyde-3-phosphate, the latter being metabolized into pyruvate by EMP.

In most fungi, L-arabinose is metabolized into D-xylulose-5-phosphate through a series of oxidation-reduction reactions involving reductases and dehydrogenases while in bacteria, arabinose assimilation occurs via the formation of ribulose by arabinose isomerase. A series of reactions involving ribulokinase and ribulose-5-phosphate-4-epimerase further convert ribulose into D-xylulose-5-phosphate which enters the PPP. Pyruvate, the end product of EMP has several fates subject to the environmental circumstances befitting the microorganism. In anaerobic environments, pyruvate is converted to acetaldehyde and carbon dioxide via a decarboxylation reaction by pyruvate decarboxylase. The acetaldehyde is subsequently reduced to form ethanol by alcohol dehydrogenase (De Souza et al. 2013). In

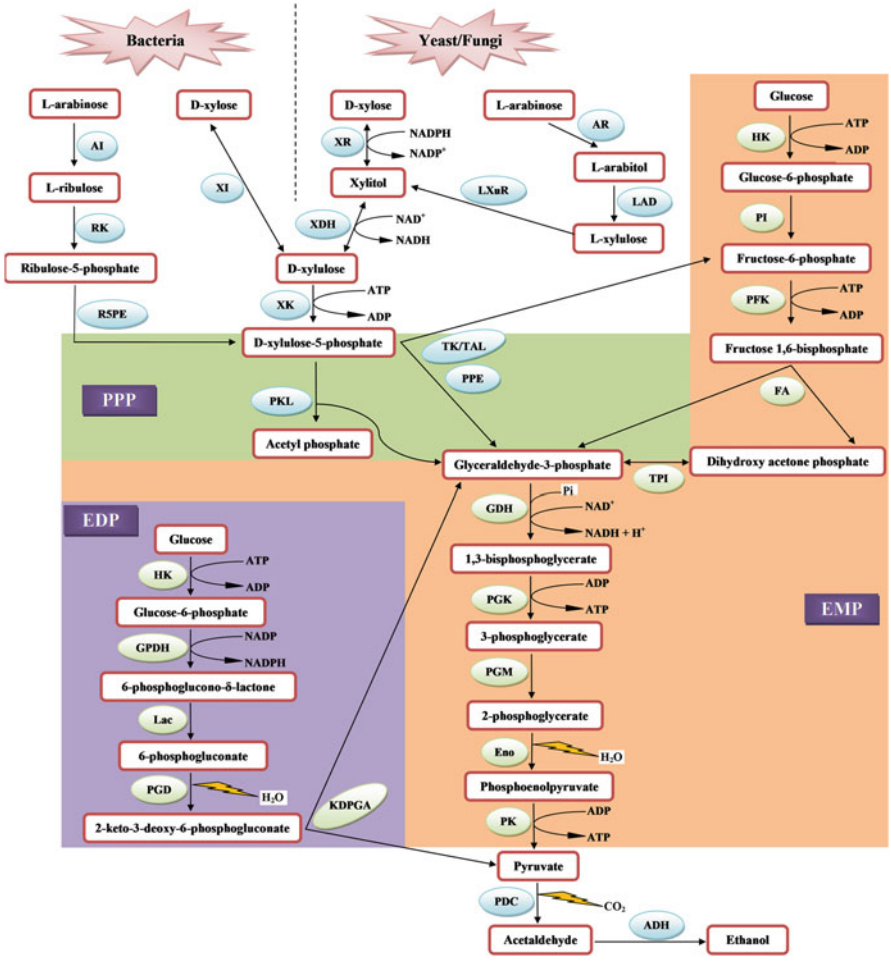

Fig. 3 Hexoses (glucose) and pentoses (xylose and arabinose) assimilation via different pathways in microorganisms. *AI* arabinose isomerase, *RK* ribulokinase, *R5PE* ribulose-5-phosphate-4-epimerase, *XI* xylose isomerase, *XK* xylulose kinase, *XR* D-xylose reductase, *XDH* xylitol dehydrogenase, *AR* arabinose reductase, *LAD* L-arabitol dehydrogenase, *L-XuR* L-xylulose reductase, *PKL* phosphoketolase, *HK* hexokinases, *PI* phosphoglucose isomerase, *PFK* Phosphofructokinase, *FA* fructose bisphosphate aldolase, *TPI* triosephosphate isomerase, *PPE* phosphopentose epimerase, *TA* transaldolase, *TK* transketolase, *GDH* 3-phosphate dehydrogenase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *Eno* enolase, *PK* pyruvate kinase, *GPDH* glucose-6-phosphate dehydrogenase, *Lac* lactonase, *PGD* 6-phosphogluconate dehydratase, *KDPGA* 2-keto-3-deoxy-6-phosphogluconate aldolase, *PDC* pyruvate decarboxylase, *ADH* alcohol dehydrogenase

E. coli instead of decarboxylase, pyruvate formate lyase catalyzes the conversion of pyruvate to ethanol (Maki et al. 2013). In some bacteria such as *Pseudomonas*, *Rhizobium*, *Agrobacterium* and *Zymomonas mobilis* glycolysis is substituted by the Entner-Doudoroff pathway (EDP). Glucose-6-phosphate is formed via same

reactions through the PPP. The serial conversion of glucose-6-phosphate to 2-keto-3-deoxy-6-phosphogluconate (KDPG), a key intermediate, is assisted by glucose-6-phosphate dehydrogenase, lactonase and 6-phosphogluconate dehydratase enzymes. The formation of pyruvate and glyceraldehyde 3-phosphate occurs via the cleavage of KDPG by the action of 2-keto-3-deoxy-6-phosphogluconate aldolase. Glyceraldehyde 3-phosphate is further converted to pyruvate similarly as in the glycolytic pathway followed by its subsequent conversion to ethanol.

One of the most valuable renewable energy source is biogas. Biogas is a mixture of different gases, naturally produced via decomposition of organic matter in the absence of oxygen. This multilateral biofuel can be utilized for the production of heat and power and also as a gaseous fuel in automobiles. Apart from reducing the greenhouse gases emissions, the digestate of anaerobic digestion (AD) technology acts as a high-value fertilizer for crop cultivation thereby replacing the mineral fertilizers (Achinas et al. 2017). The microbial decomposition of organic substances to biogas comprises of four stages: (1) hydrolysis of complex organic polymers to simple soluble compounds, (2) fermentation of hydrolytic products into intermediates such as alcohols and fatty acids (acidogenesis); (3) anaerobic oxidation of these intermediary compounds to produce acetate, hydrogen and carbon dioxide gases (acetogenesis) and lastly (4) methane production by methanogenic Archaea (methanogenesis) (Sun et al. 2013). Lignocellulosic biomass is popularly utilized for biogas production since they do not compete with food but the recalcitrance of plant material results in its slow or incomplete digestion. Lignocelluloses are degraded into sugars which are subsequently utilized by microbial consortium to produce biogas. The complexity of lignocellulosic biomass acts as an obstacle for enzyme accessibility and thus the hydrolysis process is a rate-limiting step. Therefore, the efficacy of anaerobic digestion, and thus yield of biogas is not always adequate. In such scenario, using glycosyl hydrolases either in pure form or as enzyme complexes or utilizing microorganisms producing such enzymes in situ seems to be the potent solution.

Mixed cultures of cellulolytic and non-cellulolytic bacteria have been used. Many species of fungi, especially white rot fungi (e.g., *Phanerochaete chrysosporium, Coriolus versicolor*, *Fusarium* sp., *Cyathus stercoreus*, *Pleurotus ostreatus* and *Ceriporiopsis subvermispora*) have been used in consortia for pretreatment of lignocelluloses. These organisms secrete unique ligninolytic enzymes useful for boosting the enzymatic disintegration of lignocellulosic biomass (Pinto et al. 2012). They require strict anaerobic conditions which is often provided by using pre-reduced media (Poszytek et al. 2016; Prasertsan et al. 2017). Nonetheless, many factors such as type of substrate, environmental conditions (such as pH, temperature), pre-treatment time, restrict the efficiency of enzymatic pretreatment. Also, usage of free enzymes is less effective as compared to the propagation of microbial consortia, which can stably and constantly produce glycosyl hydrolases for the degradation of lignocelluloses (Parawira 2011).

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Role of Glycoside Hydrolases in Pulp and Paper Industries

Maansi Vermani, Vandana Chauhan, Sunishtha S. Yadav, Trina Roy, and Shivani Singh

Abstract

As paper has become an important part of global consumption, there is a necessity to find a balance between production and resources, when an increase in paper production has been observed within past decades. Diverse technologies are being used for paper production one of them being use of enzyme of microbial origin for better bio-pulping and bio-bleaching results along with easier fibre grafting and de-inking. Though there are many applications of such enzymes in this industry, they are still at early development stage. The most essential feature of using enzymes is that it helps in eco-friendly bio-bleaching of pulps. Xylanase, cellulose free xylanase and occasionally use of laccase are the enzymes being used as a better alternative for reduction in the amount of environmental-polluting chlorine. Endo-β-xylanases, belonging to xylanases, is the key enzyme required for the enzymatic bleaching. The main purpose to adopt such techniques is to reduce the requirement of chlorine containing chemicals for bleaching. Enzymes further increases the pulp brightness making it easier for the industry to go chlorine free, along with improving fibre quality, producing better quality fibre. We will now discuss about the pulp industry and how xylanase enzyme helps in improving the productivity and revolution of the industry in detail in this chapter.

Keywords

Paper · Pulp · Xylanases · Cellulases

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Introduction

An important part of global economy is the paper industry but a right balance needs to be maintained between the production (cost and materials used) and profit especially when there has been an over-all increase in paper consumption. Presently a key research topic is to improve paper productivity by finding an alternative method of using the raw material thus, reducing the amount of pollutants released during the manufacturing process. The primary source of paper manufacturing is the plant biomass majorly formed of different assemblies of polysaccharides, structural proteins and phenylpropanoids that majorly contributes in forming the plant biomass (Carpita and Gibeaut 1993; Popper and Fry 2008; Roppolo et al. 2011). During the last 2–3 decades, many advancements have been witnessed in the manufacturing process of paper. Some advancements for improving the efficiency of the manufacturing process include discovery of enzymatic de-inking, fibre engineering, use of "xylanase" in bleaching etc. as it is considered to play important role in limiting the resources required for the production of paper at the same cost. This will aid in reducing the impact of these industries on nature thus, help in its preservation the basis of achieving such a goal depends on understanding the biosynthetic pathways involved in production of various wood components and improving the quality of fibre using conventional breeding methods or using leading-edge biotechnology–based techniques.

Enzyme–based methods have been used as a process aide for fibre modification in the paper industry. A study on patents displayed a high number of documents have been and are being published correlated to this technology. The enzymes like xylanase, cellulase–free xylanase, cellulase etc. show a huge potential of being used in the pulp and paper processes to boost the process of bleaching by fibre engineering. Improving the thermostability of these enzymes along with increasing their alkalinity strength forms the current trends of study and research. Microbial cellulase, contributes to 10% of global enzyme demand and its usage can give rise to newer opportunities in making paper industries grow commercially.

However certain criteria need to be followed before using any enzyme–based method in the manufacturing process. The criteria that must be followed includes:

- Fibre quality and processing of fibre should either be improved or be maintained
- Using enzyme should result in economic benefits
- The enzyme preparation used should be available at reasonable price and in a large quantity for use by industries
- The enzyme should not be responsible for any unlikely changes in the process it is used in

Fibre properties can be improved or newer properties can be created by fibre surface target modification using enzyme treatment or by combined enzymatic and chemical treatment. This can be used not only to improve paper manufacturing but also to improve other non-paper manufacturing processes (Fig. 1).

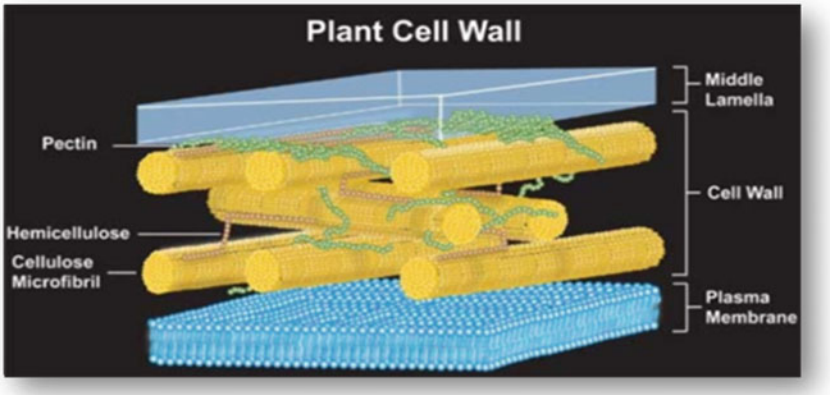

Fig. 1 The structure and composition of plant cell wall (https://images.app.goo.gl/qrhMXZKWcNVxZhp89). Dburkhammer cell project. Cell wall, protects the plant cell, the outermost layer [photograph], Retrieved from: https://dprojectmiller.weebly.com/cell-wall.html

Table 1 The composition of plant primary and secondary cell wall

Cell wall components	Primary cell wall	Secondary cell wall
Cellulose	20–30%	20–50%
Hemicellulose	30–70%	35–50%
Pectin	5–35%	Absent
Lignin	Absent	20%
Others	5–6%	3–4%

Plant Cell Wall

The primary wall of plant cells, play an essential role in its support and other physiological processes, is rich in polysaccharides, as seen in Table 1 (Carpita and Gibeaut 1993; Taiz and Zeiger 2002). The cell wall that develops during the cell differentiation and elongation stages is known as the "primary cell wall" whose composition shows great variation depending on the cell and tissue type and developmental stage the cell is in (Pauly and Keegstra 2008; Höfte and Voxeur 2017).

A cell wall for extra support develops towards the inner surface of the primary wall, displacing it as can be seen in Fig. 2. It is known as the "secondary cell wall" and its constituents are similar in both hardwood and softwood trees. The secondary wall differs from the primary wall in both composition and concentration of components as can be seen in Table 1 (O'Neill and York 2003; Vogel 2008; Chundawat et al. 2011; Meents et al. 2018). The composition and the proportion of components of secondary cell wall vary among different species.

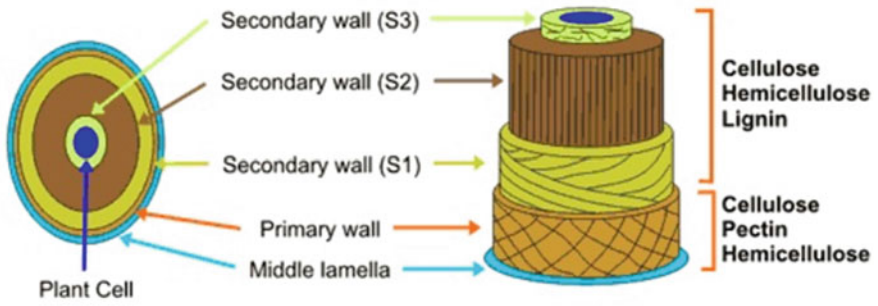

Fig. 2 The difference in composition of plant primary and secondary cell wall (https://www.google.co.in/url?sa=i&rct=j&q=&esrc=s&source=images&cd=&ved=2ahUKEwiasK6jjfjkAhWNb30KHXByA-IQjRx6BAgBEAQ&url=http%3A%2F%2Fwww.ifasonline.com%2FNEET-AIIMS-COACHING%2FBiology%2FNeet-Biology-topic-15.jsp&psig=AOvVaw1PPksFuB5XxC2fWFKHJKgY&ust=1569917497299480).Ifas biology. Cell boundaries, cell wall [photograph], retrieved from: http://www.ifasonline.com/NEET-AIIMS-COACHING/Biology/Neet-Biology-topic-15.jsp

The Components of Cell Wall

Cellulose

It is considered as the most abundant biological polymer with cellobiose as its central repeating unit as can be seen in Fig. 3a. D-glucose monomers connected by β-1,4 glycoside bonds forms the building blocks of cellulose. The component is synthesized by a multi-enzyme complex known as the "cellulose synthase" that can add upto 2000–25000 glucose monomers to form the chains of cellulose (Turner and Kumar 2018). 30–36 linear chains of cellulose polymer connected to each other parallelly by hydrogen bonds forms the cellulose microfibril that provides additional mechanical strength and protects cellulose against enzymatic degradation (Carpita and Mccann 2000).

Hemicellulose

Hemicellulose provides additional support to the cell wall by preventing sliding–over of cellulose chains and is considered to be the next most abundant plant carbohydrate after cellulose. It is microfibrils to prevent the collapse of cell wall due to cellulose chains sliding over each other. Individual hemicellulose chains (100–200 residue) remains cross-linked to cellulose, in a matrix composed of pectin (primary wall) or lignin (secondary wall) (Abramson et al. 2010; Van Der Weijde et al. 2017). Several hemicellulose variants are present depending on whether it is composed of D–xylose (pentose) or D–mannose or D–glucose (hexose) that are

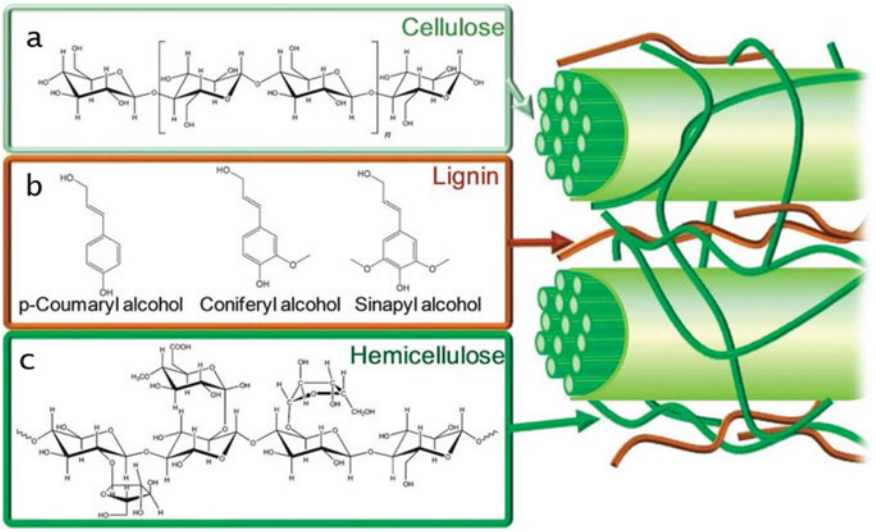

Fig. 3 The molecular structure of different components of the plant cell wall (https://images.app.goo.gl/BitmTaC2yTJD4YCc9). Alonso et al. 2012, Structure of lignocellulosic biomass with cellulose, hemicellulose, and lignin represented, retrieved from: https://www.researchgate.net/publication/321075806_A_study_of_the_uncertainty_associated_with_tar_measurement_and_an_investigation_of_tar_evolution_and_composition_during_the_air-blown_fluidised_bed_gasification_of_torrefied_and_non-torrefied_grassy_b

linked to each other by β-1,4 glycoside bonds as can be seen in Fig. 3b (Meents et al. 2018).

Hemicellulose that is abundant in non-commelinid monocots or eudicots is the 'xyloglucan', characterised by α-(1,6)-bonded xylose monomers branched off from β-1,4-D-glucose backbone that may further be linked to galactose, fucose, arabinose or other sugars (Pauly and Keegstra 2016).

Xylan, primary hemicellulose in Poaceae, is characterised by different branching of glucuronic acid pattern as seen in Fig. 4a and arabinose from the linear β-1,4-D-xylose chain. Four fundamental groups of xylans can be considered (Biswal et al. 2018):

- *Arabinoxylans*, having just side chains of single terminal units of α-L-arabinofuranosyl substituents. In the specific instance of oats, arabinoxylans vary in the level of arabinosyl substitution, with either 2-O-and 3-O-mono-substituted or twofold (2-O-, 3-O-) substituted xylosyl residues
- *Glucuronoxylans*, majorly linear polymers of xylose residues bonded by (1→4) glycoside, has several units substituted at C-2 or C-3 or both by a glucuronate residue, often methylated at C-4
- *Glucuronoarabinoxylan*, formed of linear xylose polymer has α-D-glucuronic (often methylated) or α-L-arabinose substitutions on xylose residues

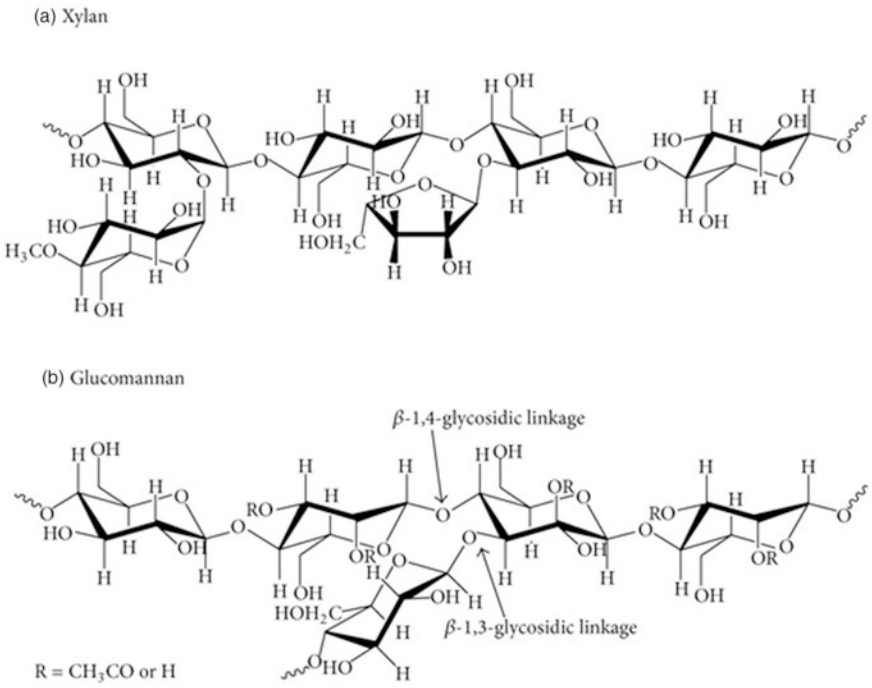

Fig. 4 The molecular structure of the two major classes of Hemicellulose (https://images.app.goo.gl/pmVHSACMfQdNfr2H6). Lee HV, Hamid SB, Zain SK (2014), conversion of Lignocellulosic biomass to nanocellulose: structure and chemical process, retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/25247208

- *Galacto-glucurono-arabinoxylans*, is the hemicellulose of perennial plants, characterised by closeness of the terminal β-D-galacto-pyranosyl residues on the branched chains of xylans

Mixed–linkage glucans or β-glucans are also found in abundance in the cell wall of grasses. They are unbranched glucose polymer characterized by alternating short sequences of β-1,4-glucan with a single residue of β-1,3-glucans (Turner and Kumar 2018; Oliveira et al. 2016).

The third most essential hemicellulose is found in charophytes and are known as mannans and glucomannans. As can be seen in Fig. 4b, glucomannan formed of non-repeating units of mannose and glucose has a linkage of β-1,4-glycoside between them (like in galacto–glucomannans) (Srivastava and Kapoor 2017).

Lignin

Lignin, the most abundant non-polysaccharide in plants, is present in the secondary wall of specialised tissues (vessels, cortex, etc.) of plants and is responsible for about 15–40% plant dry weight. It forms the plant matrix that interact with both cellulosic microfibrils and hemicellulose. It is a phenol derivative as can be seen in Fig. 3c which protects plants against physiological stress, pathogens or other abiotic stress and provides rigidity to plants (Nguyen et al. 2016; Santos and Ferrarese 2008).

Pectin

Pectin, present in the primary wall's middle lamella, is a structural polysaccharide having branched chains formed of neutral sugars (like galactose, arabinose and rhamnose) and acidic sugars (like glucuronic and galacturonic acid). 'Homogalacturonan', the linear, most abundant pectin as represented in Fig. 5 is formed of α-1,4 glycoside linked galacturonic acid monomers. Other pectin types can involve xylogalacturonan, rhamnogalacturonan I and II etc. (Biswal et al. 2018; Mohnen 2008)

Pectin confers plant rigidity and defence against pathogens. It also helps in cell adhesion and maintaining the water content in the plant cell. Pectin has a huge potential to be used in different industries like in paper industry, drug industry, in cosmetics etc. Pectin percentage of about 30% is found in dicots and non-commelinid monocots that is observed to be far greater than its percentage in Poaceae and commelinid monocots (2–10% pectin) (Mohnen 2008; Voragen et al. 2009).

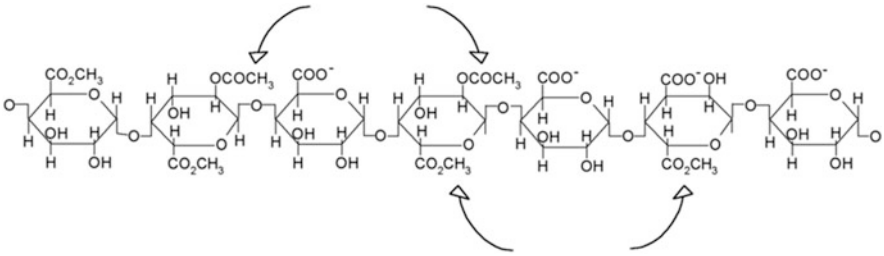

Fig. 5 The molecular structure of pectin (https://images.app.goo.gl/ZRGffnLnceK9EUov7). Mario Aguedo (2011), Pectic oligosaccharides: production and possible applications, retrieved from: https://www.researchgate.net/figure/Structure-primaire-dun-homogalacturonane-Primary-structure-of-a-homogalacturonan_fig1_50235213

The Glycoside Enzymes

CAZymes (Carbohydrate–active enzymes) have the ability to modify complex polysaccharides and are organized into the families of GHs (glycoside hydrolase), GT (glycosyl transferase), CE (carbohydrate esterase), PL (polysaccharide lyase) and auxiliary activities [including oxidative enzymes CDH (cellobiose dehydrogenase) and LPMO (lytic polysaccharide mono-oxygenase) (Lombard et al. 2014; Bourne and Henrissat 2001). CAZymes are examples of modular proteins, formed of different domains like the 'catalytic domain' (for enzyme activity) and a 'carbohydrate–binding domain (CBM)'. The CBM is the site of association of the insoluble substrate to the enzyme and is found in the enzymes that can degrade xylans, mannans, glucomannans, galactans, pectins, and arabinans (Hashimoto 2006; Boraston et al. 2004; Terrapon et al. 2017).

Ligninases

Ligninases are enzymes that can hydrolyse lignin into simpler compounds for easier microbial assimilation. Ligninase is majorly classified into:

(a) Laccases–they are Cu-containing glycoproteins, formed of a single, double or a quadruple polymer, that are involved in lignin degradation by oxidizing phenolic compounds. This results in the yield of compounds with lower molecular weight like quinine and other ions and radicles like phenoxyl radicals (Biswal et al. 2018). Some microbes that are known to produce this enzyme includes *Aspergillus nidulans, Phellinus ribis, Phanerochaete chrysosporium* etc. (Mohnen 2008)
(b) Peroxidases–Lignin Peroxidase, an iron-containing protein, is an oxidoreductase that uses hydrogen peroxide to depolymerise lignin. They are observed to show a high potential of oxidation–reduction at an optimum pH of around 3 (Mohnen 2008), but generally shows a low substrate specificity
(c) Manganese peroxidases, is another type of Peroxidase, that are observed to use Mn^{2+} for oxidation of phenolics to yield phenoxyl radicals (Nguyen et al. 2016)

Cellulase

It is an important enzyme for cellulose degradation, used in different industries, that can hydrolyse the β-1,4-glycosidic bonds between glucose monomers. It has a huge potential to be used in food sectors, paper industries, in cosmetics etc. (Sette et al. 2008). It is characterized by three essential synergistic enzymatic activities of exoglucanase and cellobiohydrolase (removes cellobiose from non-reducing and reducing end respectively from the crystalline portion), endoglucanase (cleaves glycosidic bond from the non-crystalline portion) and β-glucosidase (Segato et al. 2017; Segato et al. 2014).

Cellobiohydrolase can remove cellobiose from cellulose's non-reducing end. It can hydrolyse cellodextrins (intermediate cellulose degradation product), but not the soluble derivatives of cellulose (CMC and hydroxyethyl cellulose) (Segato et al. 2014).

Endoglucanase are known to degrade both carboxymethyl cellulose (CMC) and cellodextrins to form cellobiose (simplest unit of cellulose) and glucose. (Begum et al. 2009).

β-glucosidase cleaves cellobiose thus removing glucose from the non-reducing end of cellulose (end with free -OH group at C-4) and can also hydrolyse the aryl and alkyl β-glucosides.

Fungal Cellulases

Fungi, among the microbes, are the principal cellulose decomposers, responsible for about 80% of cellulose degradation particularly in forest ecosystem. The fungal families that can actively degrade cellulose are found belonging to phylum Ascomycota, Deuteromycota, Basidiomycota and some Chytridiomycota occurring in the animal rumen. Some of the Competent cellulolytic fungal species are *Penicillium, Aspergillus, Trichoderma, Alternaria, Fusarium, Myrothecium* and so on (Sajith et al. 2016). Aerobic fungal cellulases unlike anaerobic fungal cellulase and other microbial cellulase that are membrane–bound, are extracellular, secreted in large quantities and adaptive in nature for which it is economical and are usually preferred by different industries (Mathew et al. 2008).

Aspergillus, Penicillium, Trichoderma and *Sclerotium* are the species that are commercially exploited for cellulase production (Milala et al. 2005). Fungal cellulase like other cellulases, has a 'catalytic domain' (for enzyme activity) and a CBM connected by a poly–linker that is rich in serine and threonine (Payne et al. 2015). The CBD is rich in polar and aromatic amino acid and thus aids in substrate immobilization during catalysis while the catalytic domain houses the active site for substrate hydrolysis (Hildén and Johansson 2004). In the last 2–3 decades, an aerobic fungi *Trichoderma reesei* is extensively studied for its capability to hydrolyse native cellulose due to the presence of genes encoding for cellulase genes encoding exoglucanase, 8 coding endoglucanases and 7 coding glucosidases (Singhania et al. 2006; Sukumaran et al. 2005).

Nowadays, thermophilic fungi are significantly studied because of the tendency of cellulose fibres to bulge out at high temperatures, making it accessible to the hydrolytic enzymes (Li et al. 2011; Murray et al. 2004). *Talaromyces emersonii* is one such thermophilic fungi from which cellulase active at 70 °C can be isolated (Murray et al. 2004). *Chaetomium thermophilum,* two strains of *Penicillium, Thermoascus aurantiacus* and *Sporotrichum thermophile* are among the thermophilic fungal species that have the potential to be used for the commercial production of thermo-stable cellulase enzyme (Picart et al. 2007).

Use in Paper and Pulp Industry

Cellulases are used in paper industries for pulping and de-inking of waste papers. Nowadays, bio-mechanical method using enzymes are being widely used to acquire

the fibre suspensions from wood (i.e. the pulp). Cellulase can improve the physical properties (like inter-fibre bonding and mechanical strength) of the final product thus, increasing the energy efficiency of the process (Chen et al. 2012). It further makes the process environment-friendly by limiting the usage of harmful chemicals. For instance, bleached Eucalyptus globules kraft pulp can be effectively refined by cellulase as it enhances the pulp drain-ability by about 80% without changing the energy consumption (Gil et al. 2009). It can further be used in fibre modification of Kraft (sulphate pulp) to improve the properties of the final sheets (Cui et al. 2015).

Cellulase can be used during deinking of waste papers as it can hydrolyse cellulose thus releasing the ink attached to the surface of cellulose. This results in peeling of individual fibre or bundles (Lee et al. 2011). Waste papers often act as an important raw material for the paper industries, as recycling them reduces amount of solid-waste produced and also helps in forest conservation by lowering deforestation for wood fibres (Singh et al. 2012). Enzymatic deinking using a commercial mixture of cellulase and hemicellulase is used for improving the over-all brightness and quality of recycled paper (Ibarra et al. 2012).

Cellulases can dissolve clogged fibre residues thus improving drainage of paper mills (Kuhad et al. 2011). Furthermore, commercial cellulase preparation have been used in the manufacture of easily biodegradable cardboards, tissue and sanitary papers (Hsu and Lakhani 2002).

Hemicellulose

It is another group of carbohydrate esterases represented by xylanases, β-xylosidases, β-mannanases and arabino-furanosidases. Xylan, constituting about 33% of the total global polysaccharide and 70% of all hemicelluloses known, is hydrolysed by xylanases and β-xylosidases to xylose. Other hemicellulases like mannanases and arabinases can be used for further degradation process depending on the chemical composition of hemicelluloses (Dashtban et al. 2009).

Xylanase

They are glycoside hydrolase (GH) that can break the β-1,4 glycoside bonds of xylosides forming sugar hemiacetal and non-sugar aglycone. Xylanases can be classified depending on its molecular weight, chemical and physical properties, isoelectric point (pI) and structure (Collins et al. 2005). Xylanase are predominantly found in both prokaryotes and eukaryotes. They have been derived from different marine and terrestrial organisms like microbes, protozoa, algae, snails, shellfish, insects and seeds of terrestrial plants and so on (Walia et al. 2013).

Endo-1-4-β-xylanases

The bonds cleaved by endo-xylanase depends on chain length, the degree of branching etc of Xylan (Li et al. 2000) resulting in release of different products (like xylose, xylobiose, xylotriose and arabinose). This forms the basis of classifying different endo-xylanase. Xylanases can both liberate arabinose or not liberate

arabinose, but several organisms tend to produce both of them for maximum hydrolysis efficiency (Wu et al. 2006; Okazaki et al. 2005).

The endo-xylanases are generally observed to show maximum activity when temperature is between 40–80 °C and pH 4.0–6.5. Individually fungi and bacteria can exhibit a fine diversity of endo-xylanases. Three or more enzyme activities were separated from a single culture as was proposed by (Polizeli et al. 2005). It was noted that endo-xylanases fall into two main classes (Corral and Villasenor-Ortega 2006).

- with molecular weight <30 kDa; they are basic in nature
- with molecular weight values >30 kDa; they are acidic in nature

β-Xylosidases
β-Xylosidases are classified based on their affinities to degrade the end products formed when xylan undergoes through successive hydrolysis by other xylanases. Successive hydrolysis often results in accumulation of β-D-xylo-pyranosyl that are further broken down using β-xylosidases to prevent these short oligomers from inhibiting endo-xylanases (Andrade et al. 2004). They are produced by both bacteria and fungi and can cleave artificial substrates (p-nitrophenyl, o-nitrophenyl-β-D-xylo-pyranoside etc.) (Polizeli et al. 2005; Corral and Villasenor-Ortega 2006).

β-Xylosidases can show peak activity when temperature varies from 40 to 80 °C, making them highly thermo-stable depending on their source organism. One such enzyme that can retain 100% activity when stored at 60 °C for 4 h was isolated from *Aspergillus phoenicis*, (Rizzatti et al. 2001).

α-Arabino-Furanosidases
It hydrolyses L-arabinose, without degrading the xylan backbone, from C- 2/C-3 of β-D-xylo-pyranosyl. They have two distinct mode of action (De Vries et al. 2000).

- They can degrade p-nitrophenyl-α-L-arabino-furanosides and branched arabinans (exo-α-L-arabino-furanosidase)
- They can only hydrolyse linear arabinans (endo-1, 5-α-L-arabinase)

Acetyl-Xylan Esterase
It acts on acetylated xylans and removes the O-acetyl group that are present at carbon position 2 or 3 of xylose residues. Removal of acetyl side-groups is necessary as it sterically hinders the enzymatic degradation of the backbone (Corral and Villasenor-Ortega 2006).

α–Glucuronidases
They recognize the α-1, 2 bonds of glucuronoxylan present between glucuronic acid and β-D-xylo-pyranosyl residues. Some glucuronidases can even hydrolyse intact polymer. It has been observed that α-glucuronidase activity can partially be hindered

by the presence of acetyl groups near the glucuronosyl substituents. (Tenkanen and Siika-aho 2000).

Extremophilic Xylanases

Xylanase generally has high activity at mesophilic conditions of 40–60 °C temperature and neutral or slightly acidic pH in case of bacterial xylanase and fungal xylanase respectively (Walia et al. 2014). Extremophilic xylanase are a new group of xylanases that are under study as they are active under extreme conditions of pH 2–11 and temperature from 5 to 105 °C. Some thermophilic microbes like *Thermotoga* sp., *Bacillus stearothermophilus, Rhodothermus marinus*, *Thermoascus aurantiacus* was found to synthesize xylanase that can show stable enxymatic activity at temperature as high as 105 °C (Song et al. 2013). One such xylanase showing better activity at 30% concentration of NaCl and temperature of 55–65 °C was isolated from an extreme halophilic archaeon, *Halorhabdus utahensis* (Wainø and Ingvorsen 2003).

Bacterial Xylanases

Xylanases are observed to be produced by both free–living bacteria as well as endosymbionts like those present in ruminants, insects etc. Bacterial xylanases are mainly isolated from the genera Bacillus, Microbacterium, Staphylococcus, Cellulomonas, Micrococcus, Arthrobacter, and Rhodothermus (Chapla et al. 2012). Some of the bacterial xylanases that are well studied includes:

- Xylanase isolated from *Bacillus subtilis* (CXJZ) is reported to show the highest activity of 36633 IU/mg when the substrate used for its culture is Xylan derived from oat spelt or birchwood. This enzyme was found to show optimum activity at 60 °C temperature and a pH of 5.8 i.e. slightly acidic conditions. When purification of enzyme was initially carried out using ultracentrifugation followed by gel-filtration chromatography, it was found that cellulase-free Xylanase (pI of 9.63) could be isolated that was stable even at a temperature of 70 °C. This bacterial strain was found to be abundant in "ramie stems" that are submerged partially in river (Guo et al. 2012).
- Xylanase that was isolated from a different Bacillus genus—*Bacillus halodurans* (TSEV1), an extremophile, using Xylan derived from oat spelt, birchwood or beechwood as the substrate for its culture was a thermo-stable endo-xylanase which was cellulase-free. It is usually observed to degrade Xylan derived from soft wood. This enzyme was observed to have maximum activity at 80 °C temperature and a pH between 8.0–12 (optima at 9.0). Endo-xylanase isolated from this strain was better in activity and stability as compared to other Bacillus halodurans strains. Enzyme purification of 13.5-fold was reported when anion-exchange was used followed by gel-filtration chromatography along with a yield of 30%. Because of its alkaline nature, it can be used in paper industries for de-inking and bio-bleaching (Kumar and Satyanarayana 2012; Kumar and Satyanarayana 2013).

- Xylanase gene from a different *Bacillus halodurans* strain (C-125), was expressed for its thermo-stability along with its cellulase-free nature in *Pichia pastoris*. This enzyme was observed to have maximum activity at 70 °C temperature and a pH of 9.0. Because of its alkaline nature along with its thermo-stability, it can be used in paper industries for bio-bleaching. It was reported that using this xylanase for the pre-treatment of pulp derived from wheat straw, improved the brightness of the paper along with improving paper strength and quality (Lin et al. 2013).
- An endo-xylanase is synthesized by *Bacillus brevis* (ATCC 8246), when wheat straw is used as the substrate for its culture. It is usually observed to degrade xylan derived from soft wood. This enzyme was observed to have maximum activity at 45–95 °C temperature (optimum at 55 °C) and at a pH of 7.0. Enzyme purification of 2.4-fold was reported when precipitation using ammonium sulphate and anion-exchange was used followed by gel-filtration chromatography. Because of its thermo-stability, it can be used in bio-bleaching in paper industries using kraft pulp (Bai et al. 2014).
- Xylanase isolated from *Paenibacillus macerans* (IIPSP3A) is reported to show an activity of 4170 U/mg approx. when the substrate used for its culture is Xylan derived from beechwood. This enzyme was found to show optimum activity at 50 °C temperature and a pH of 4.5 i.e. acidic conditions, but can also show stable activity for a few hours when the temperature is 90 °C and pH is 9.5. This stability at high temperature and alkaline nature allows it to be used in paper industries. The combination of techniques used for enzyme purification included cation-exchange that was immediately followed by gel-filtration chromatography. This bacterial strain was found to be abundant in the gut of termites (Dheeran et al. 2012).
- *Paenibacillus barcinonensis* (BP-23), produces a Xylanase named as (Xyn11E), that in presence of the lipoprotein, synthesized by the same organism, named as——LppX, shows high activity when *E. coli* (DH5α) is used as the vector for expression. This enzyme was observed to have maximum activity at 50 °C temperature and at a pH of 6.5. Enzyme purification was carried out by metal–affinity chromatography using Histidine–trap columns. It was reported that using this xylanase for the pre-treatment of pulp derived from flax, Eucalyptus and sisal, improved the paper along with quality as it led to formation of pulps free from chlorine and having a high content of cellulose (Valenzuela et al. 2014).
- Xylanase gene isolated from *Thermotoga petrophila* (RKU-1), an extremophile, was expressed in the vector *E. coli* (BL21) to produce a thermo-stable endo-xylanase. The oil reservoirs primarily found in Japan, was observed to be the source of this bacteria. This enzyme was observed to have maximum activity at 95 °C temperature and a pH of 6.0. The combination of techniques used for enzyme purification included thermal treatment that was immediately followed by ion-exchange chromatography. It, due to its stability at higher temperature can be used in paper industries (Ul Haq et al. 2012).
- Soil rich in decomposed products of rice straw was found to be rich in *Streptomyces rochei and S. chromofuscus* both organisms being able to synthesize

Table 2 The commercially available xylanases

S. no	Product	Company	Source	Application
1	"Bleachzyme F"	Biocon India, Bangalore	Not known	Bleaching of pulp
2	"Pulpzyme HA, HB, HC"	Novozymes, Denmark	*Bacillus* sp.	Cellulose and paper industry
3	Luminase	Verenium	Bacteria from thermal spring	Bleaching of pulp
4	Xylanase (bacterial)	Biovet JSC, Bulgaria	*Bacillus subtilis*	Bakery, pulp and paper industries, feed additive

xylanase when the substrate used for its culture is cotton stalk or Papyrus pulp. Enzyme activity was found to be greater in *S. chromofuscus* as compared to *S. rochei*. When *S. chromofuscus* was used for treatment of pulp formed of cotton stalk, it was observed that it can decrease lignin percentage from 24.5% to 11.2% and increase brightness percentage from 9% to 17%, when the pulp was treated using EDTA. These results were superior to the ones received when *S. rochei* was used instead (Nagieb et al. 2014).

- *Streptomyces thermocyaneoviolaceus* (M049), was reported to produce a xylanase that was able to retain 65% of its activity at 70 °C. StxII (S. thermoviolaceus) and XlnB (S. lividans)—was used as probes to bring about amplification of the xylanase–producing gene. This amplified gene was named as "xynB" as its N-terminal sequence shows high similarity to the probes used and was replicated in *E. coli* [BLR(DE3)] for the expression of protein that is much more thermally stable and easier for purification. This enzyme was observed to have maximum activity at 60 °C temperature and a pH of 5.0. A combination of techniques like precipitation using ammonium sulphate which was then followed by ion-exchange along with gel–filtration was used for purification of xylanase. Due to it showing 90% of enzyme activity even at high temperature, it has a potential of being used in different industries including paper industries (Shin et al. 2009).

Some of the commercially available xylanases that are present in market are given in Table 2

Fungal Xylanases

- *Aspergillus usamii* (E001), produces a xylanase—AuXyn11A, which by itself does not show thermal stability but when its N-terminal domain is replaced by that of a "thermo-stable family 11" Xylanase—Syxyn11, that was found to share codon bias with *Pichia pastoris* (GS115). The amplified AuXyn11A along with the synthesized Syxyn11 was expressed in the vector E. coli (JM109). It was reported that about three replaced amino acids of the N-terminal, conferred the thermo-stability of this engineered xylanase. This enzyme was observed to have maximum activity at 75 °C temperature when enzyme purification was carried out

using a combination of different techniques like precipitation using ammonium sulphate immediately followed by gel and ultra-filtration (Zhang et al. 2014).

- *Aspergillus niger*, having a putative gene of Xylanase—xyl10, was replicated in *Pichia pastoris* to optimize the codons before cloning it in a vector for optimization, post which, it was inserted in *Pichia pastoris* (X33), under the promoter–glyceraldehyde-3-phosphate dehydrogenase (GAP), for its expression. This enzyme was observed to have maximum activity of 32000 U/mg at 60 °C temperature and a pH of 5.0 when enzyme purification was carried out using the technique of size–exclusion chromatography. Due to it showing 90% of enzyme activity even at high temperature, it has a potential of being used in different industries including paper industries (Zheng et al. 2013).
- *Aspergillus terricola* xylanase and that of *A. ochraceus*, are observed to be cellulase-free when Xylan derived from oat spelt, wheat bran or birchwood is used as a substrate, wheat bran being the most favourable in comparison. Highest enzyme activity of 50% was reported when the optimum conditions of 60 °C temperature and 6.5 pH was maintained for *A. terricola*, while 65 °C temperature and pH of 5.0 was maintained for *A. ochraceus*. The xylanases, being stable at an alkaline pH shows potential of being used in cellulose pulp bio-bleaching. Delignification of 36.4% and 14.3% in case of *A. ochraceus* and *A. terricola* respectively was observed with an over-all increase in the brightness by 2.4–3.4% (Michelin et al. 2010).
- *Schizophyllum,* found in abundance in rotten wood, was observed to produce an endo-xylanase. Gene was replicated in *Pichia pastoris* (*GS115*) for its expression when it was marked using a Histidine tag at the C-terminal. This enzyme was observed to have maximum activity at 50 °C temperature and a pH of 5.0 when enzyme purification was carried out using the technique of Ni-NTA–affinity chromatography. Due to its stability at high temperature and ability to use cations (potassium, lithium, cobalt, sodium etc.) to enhance its activity, it has the potential of being used in paper industries for degradation of lignocellulose (Song et al. 2013).
- Xylanases was isolated from fungi *Trichoderma harzianum* (mesophilic) and *Chaetomium thermophilum* (thermophilic), when the substrate used was birchwood or oat spelt for their culture. It was observed that enzyme shows high activity when the temperature is 60 °C and pH is 5.0–6.0 when its source was *Trichoderma harzianum* while enzyme isolated from *Chaetomium thermophilum* shows maximum activity at 70 °C at the same pH. They have the potential of being used in bio-bleaching in paper industry as they can reduce the amount of toxic chemicals like chlorine oxides, that are formed as wastes, thus, helping in reducing impact on environment (Ahmed et al. 2012).
- *Talaromyces versatilis*, was found to produce a varied range of cellulases and xylanases (like xynB, xynC). Two novel genes from its genome identified as xynE and xynF have been cloned for its expression in the yeast *Pichia pastoris* to compare their properties with those of previously reported xynC and xynB, previously reported. All four of the recombinant enzymes showed high expression of xylanase that has maximum activity when the pH ranges between 3.5–4.5

and temperature was maintained between 50–60 °C., with XynF showing highest thermal-stability (Lafond et al. 2014).

- *Pichia pinus* (a newly discovered yeast strain), was observed to produce a thermostable xylanase that is cellulase free. This strain of yeast, isolated from decayed wood as its major source, shows best xylanase production when oat spelt or bagasse is used as a substrate. It was observed that enzyme shows highest activity at 55 °C temperature and 4.5 pH even though low concentration of β-xylosidase as well as α-L-arabino-furanoside was reported in the crude preparation. It has the potential of being used for bio-bleaching and improving paper quality in paper industries. It was reported to be able to reduce the requirement of using harmful chemicals in paper manufacturing thus, reducing impact on environment (Ravindra et al. 2007).

Difference Between Bacterial and Fungal Xylanase

The xylanases produced by bacteria are considered to be more effective in industrial processes as compared to xylanases of fungi. Table 3 mentions few of the key points of comparison between the xylanases produced by both bacteria and fungi (Mathur et al. 2015; Subramanian and Prema 2002; Dashtban et al. 2009).

Endo-xylanases of both bacteria and fungi are observed to have their pI (isoelectric point) within the range of 4.0 and 10.3 with majority of them bring glycosylated (Polizeli et al. 2005).

Cellulase-Free Xylanases

Cellulase-free xylanases play a major role in pulp bio-bleaching as a substitute for noxious chlorinated compounds, they caused by releasing organic halogens in atmosphere which is hazards for environmental and cause developing many health issues.

Xylanases, that are cellulase-free, are more favourable agents because of their lesser use of chlorine in bio-bleaching for paper pulps production. Though, there are still need for taking precautions to prevent the unwanted hydrolysis of cellulose on a large scale. It is important to do selective hydrolysis of xylan specifically re-precipitated xylan, without affecting the viscosity of pulp facilitates by exclusion of lignin through using mild oxidative agents (Valls et al. 2010).

The substrate used for production of xylanases plays a very important role in selecting the fermentation process, like purified xylans that are excellent inducers of

Table 3 Comparison between bacterial and fungal xylanases

Characters	Bacterial xylanase	Fungal xylanase
Family of glycoside hydrolase	GH10	GH11
Secondary structure	Beta sheets	Alpha helices
Proton donor	Glutamic acid	Glutamic acid
Cellulase content	Often cellulase-free	High
Time for purification	Less	More
pH required	Neutral or alkaline	Acidic

xylanase production because of their low molecular weight, resulting in higher yield of the enzyme. However, use of purified substrates is not always economically–feasible in processes occurring at large-scale hence, different substitutes must be considered (Singh et al. 2013). Some alternative sources like corn cobs, straw, barley husk and wheat bran, when used as substrates are observed to have resulted in a significant increase in yield. Use of agro-chemical residues that are present in abundance results in lessening the overall cost of bio-bleaching and manufacturing of paper thus making paper industries eco-friendly.

It is rare to find a fungus producing xylanases having low cellulase activity. *Aspergillus fumigatus* (AR1), is one such alkali-tolerant fungus, that is capable of producing xylanases showing no growth in presence of carboxymethyl cellulose (CMC) as a substrate. The enzyme is observed to have a high index of stability but shows highest activity at pH ranging between 6.0–6.5 and at 60 °C temperature (Anthonya et al. 2003).

Use in Paper and Pulp Industry

Bio-pulping

It includes treating the wood chips with combinations of fungi found in decaying wood or other lignocellulosic components before it is sent to conventional pulping for lignin removal. The wood is chipped, screened and debarked and briefly streamed to reduce the amount of natural microorganism in them, after which, they are cooled in order to inoculate with the fungal strains selected for bio-pulping. Pre-treatment of inoculated chips (forming a heap and ventilating with humidified air for 1 month) and loss of yield are the major challenges faced in the paper industries. Dissemination of NaOH from wood can be improved using xylanase pre-treatment thus enhancing conventional process of pulping (Khonzue et al. 2011).

This process is mechanically plausible and financially savvy as it reduces electrical energy utilization and increases the manufactory throughput for mechanical pulping thus improving the quality of paper and minimizes harm caused to the environment (Khonzue et al. 2011). The utilization of bio-pulping as a pre-treatment for the Kraft procedure is as yet an open research issue. This technique can be used for different substrates, for example, non-woody plants like straw, and corn stalks likewise need a lot of consideration (Woldesenbet et al. 2012).

Bio-bleaching

The main concern in using the conventional process of pulping is cellulose degradation as it modifies the cellulosic fibre thus, affecting the paper quality (Shatalov and Pereira 2008). The system of pulp dying by chemical treatment was given in Fig. 6. It clarifies that the utilization of chemical can't completely expel the lignin from the fibre (A, B, C) a few pieces of lignin get re-precipitated on to the fibre surfaces (D). Thus, the resultant mash gets a trademark dark coloured shading because of the nearness of lingering lignin and its derivatives.

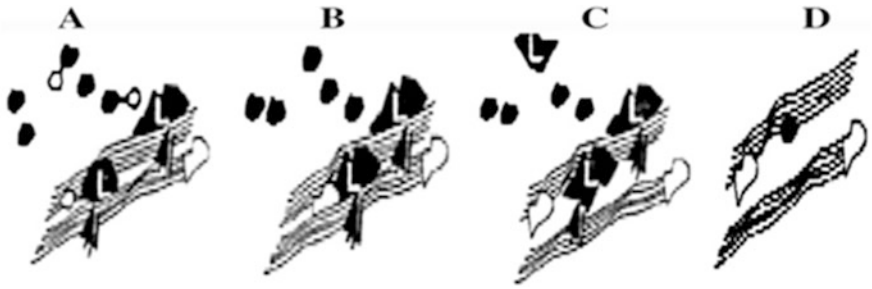

Fig. 6 Diagrammatic representation of chemical treatment on paper pulp (L lignin). Abhishek Walia, Shiwani Guleria, Preeti Mehta, Anjali Chauhan, Jyoti Parkash (2017), microbial xylanases and their industrial application in pulp and paper biobleaching: a review, retrieved from: https://link.springer.com/article/10.1007/s13205-016-0584-6

Enzymatic treatment of pulp using xylanase is 2–3 times more helpful than other glycoside hydrolases as it lowers the costs and improves fibre characteristics (Shatalov and Pereira 2008). To acquire brilliant white pulp necessary for manufacturing high quality paper, it is important to bleach out lignin and its degradation products, resins and metal ions (Azeri et al. 2010). The xylanase pre-treatment before conventional bleaching helps in breaking the linkage hemicellulose forms with residual lignin to enhance the pulp accessibility to bleaching, thus improving lignin removal (Azeri et al. 2010; Walia et al. 2015).

For bio-bleaching process of pulp in paper industries bacterial xylanases from different organism like *Staphylococcus* sp. (SG-13), B. pumilus, B. coagulans, B. subtilis (ASH) and so on have been used (Gupta et al. 2000; Kaur et al. 2011; Choudhury et al. 2006; Sanghi et al. 2009).

- Gupta et al. was able to isolate a *Staphylococcus sp.* (SG-13) xylanase that was alkali-stable and showed high activity when the temperature was maintained at 50 °C and pH at 9.5–10.0. Xylanase pre-treatment was able to enhance bleaching of kraft pulps that resulted in 8% reduction in use of hypochlorite and 30% reduction in kappa particles along with enhancing the brightness and paper quality (Gupta et al. 2000).
- Kaur et al. reported that a decline of 8.5% in kappa particle and 25% reduction in chloride usage without any decline in the brightness was observed when Kraft pulp was pre-bleached using a xylano-pectinolytic enzyme isolated from *Bacillus pumilus.* The action of the enzyme also resulted in an enhancement of properties of pulp like burst factor (by 9%), CED viscosity (by 11.8%), tear factor (by 4.6%) etc (Kaur et al. 2011).
- Torres et al. (2000) was able to isolate a xylanase from *Bacillus sp.* (BP-23) that was able to enhance bleaching of Kraft pulp, made up of eucalyptus, resulting in 30% reduction in use of chlorine and 16% decline in kappa particles along with increasing the brightness by 1% without requirement of any pre-treatment.

- Dhiman et al. carried out an analytical study on the bio-bleaching potential of xylanase used independently or in combination with pectinase in the presence of similar temperature (70 °C) and pH (9.5) conditions. They observed that bio-bleaching using the combination treatment resulted in redeeming of chlorine dioxide, about 35.71% more efficiently, along with improvement of other properties of pulp like strength (by 25.70%), burst factor (by 20.00%), tear factor (by 6.61%) and so on (Dhiman et al. 2009).
- *Arthrobacter* sp. produces a heat and alkali stable, xylanase that is free of cellulase activity Bleaching, using xylanase, of kraft pulp results in 29% reduction in chloride usage and 20% decline in kappa particle, without having any decline in the brightness (Khandeparkar and Bhosle 2007).
- *B. megaterium* produces a heat-stable xylanase, as reported by Sindhu et al., that showed high activity when the conditions maintained was 50 °C temperature and a pH of 8.0. Bleaching of Kraft pulp was enhanced by using Xylanase resulting in 31% reduction in chloride usage, 8.12% increase in the brightness and 13.67% decline in kappa particle (Sindhu et al. 2006).
- Beg et al. was able to isolate a *Streptomyces* sp. (QG-11-3) cellulase-free xylanase that showed high activity when the condition maintained was 50 °C temperature and pH at 8.5. Bleaching, using xylanase, of kraft pulp made from eucalyptus results in 4.5% reduction chloride usage, 20% increase in the brightness, 25% decline in kappa particles and improving the over-all paper quality (Beg et al. 2000).

De-inking of Waste Papers

De-inking is one of the more essential steps carried out during recycling waste-paper which is characterised by dislodging ink from the waste-paper. Traditionally, de-inking of paper required use of large amount of chlorine or its derivatives like NaOH, H_2O_2, hypochlorite, sodium silicate and other agents that has given rise to hazardous effluents creating several problem in its disposal (Maity et al. 2012). Use of enzymes like xylanase and laccase, in recent times, have provided us with an alternative method of removing ink from the effluents that have been generated by the processes carried out in the paper industries (Dhiman et al. 2014).

Gupta et al., attempted to use a combination laccase and xylanase, produced during the co-culturing of *B. halodurans* (FNP135) and *Bacillus sp*., to remove ink from old newsprints. This approach significantly increased the paper brightness (by 11.8%) and whiteness (by 39%) besides enhancing other recycled paper properties like burst factor (by 2.77%), tear factor (by 2.4%) etc. Synergistic action of the combination of laccase and xylanase improves the recycled paper properties unlike the process of chemical deinking that negatively influences its freeness and strength (Gupta et al. 2015).

Kumar and Satyanarayana carried out a similar experimentation using xylanase or commercially available cellulase and xylanase combinations, isolated from Bacillus halodurans (TSEV1). They found out that the combination was efficiently able to de-ink when the conditions of 9.0 pH and 70 °C temperature was maintained (Kumar and Satyanarayana 2014).

Future-Scope

A noteworthy rise in usage of the industrial enzymes like cellulase has been observed in these recent years. The main holdup for the commercialization of cellulase is lacuna in the economically feasible process and to improve the functioning/catalysis of cellulase in tune with the demand. Utilizing lignocellulosic waste has shown a great alternative to be consider for the future. With the increase demand there are still room for exploration of sustainable strategies for substrates, fermentation and microorganisms to get higher quality product with economic feasibility.

The paper industry is constantly growing resulting in the increase in use of different environmentally–harmful chemicals which are not economical feasible. In this chapter we have talked about different microbial enzyme and process that are involve in making paper, it also highlights the hazardous chemical compound that has been used in the industry. Cellulase-free xylanases in bio-bleaching have shown effective results in reducing the consumption of chlorine compounds. Due the high quality of paper with lesser use of deinking chemical, cellulases, xylanases and amylases are now highly in demand. By using rot fungi in bio-pulping and enzymatic refining in cellulases and xylanases, can also help in reducing the mechanical energy consumption. Therefore, applications of microbial technology have been proved to be a cost effective and eco-friendly alternative for chemical intensive steps in the pulp and paper industry

Bacterial xylanases, when paralleled against fungal xylanases, was found to be more efficient due to its heat and alkaline stability and cellulase-free nature. But to produce a xylanase that is stable in a wider temperature and alkaline range along with having low activity of cellulase and higher degradative potential towards xylans, selectively hydrolysing only arabinoxylan is very taxing and is a tedious job. Producing super-xylanases that have all the essential functions is not always possible leading to production of enzymes with very few essential properties that makes industrial processing more expensive. Strain improvement using random UV mutation or through mutations using chemical mutagens has been used for the longest time for improving xylanase by stimulating essential and inhibiting undesirable characters.

Recently, engineering the microbial strains, producing xylanase for industrial usage, was made possible due to the presence of genome sequencing data and information on metabolic pathways. The techniques like molecular dynamics simulation, in-silico mutagenesis etc. can be of great importance in designing of super-xylanases with numerous catalytic activities, required for degradation of biomass.

However, basic research on isolating novel microbial strains having potentially high yield of enzyme is the primary challenge. Using genomic information for selecting the ecological–niche of the target organism is necessary to know about whether genes for xylanase production is present or not. Thus, using different techniques for developing super-xylanase for industrial perspective has become an essential area of research in recent times.

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Application of Glycosyl Hydrolases in Food Industry

Azza Silotry Naik and Roji Waghmare

Abstract

Enzymes that hydrolyse glycosidic bonds between sugars or sugar-nonsugar groups are known as glycosyl hydrolases or glycosidases. This group of enzymes has many members but the most commonly used ones in the food industry are amylases, cellulases, xylanases, β-galactosidases, and pectinases. Bacterial amylases have been used for saccharification and are often engaged in bakery and confectionary based applications. Fungal cellulases have mainly been employed for vegetable and fruit juice processing apart from their crucial role in wine and beer industries. Fungal xylanases on the other hand mainly degrade hemicellulose based agro-waste and are useful in pre-digestion of animal feed. Fungal and yeast β-galactosidases are used to process dairy products into low lactose products that benefit lactose intolerant population. The bacterial β-galactosidases on the other hand are used to formulate prebiotic dairy products. Finally, insect derived pectinases are being use for juice processing and clarification apart from the conventional microbial derived enzymes. The temperature and pH optima of this class of enzymes are variable and depending on the food processing application, the appropriate enzyme is chosen. Thus glycosyl hydrolases are an important class of commercially relevant enzymes that form focus of multiple research endeavours.

Keywords

Glycosyl hydrolases · Amylases · Cellulases · Xylanases · β-galactosidases · Pectinases

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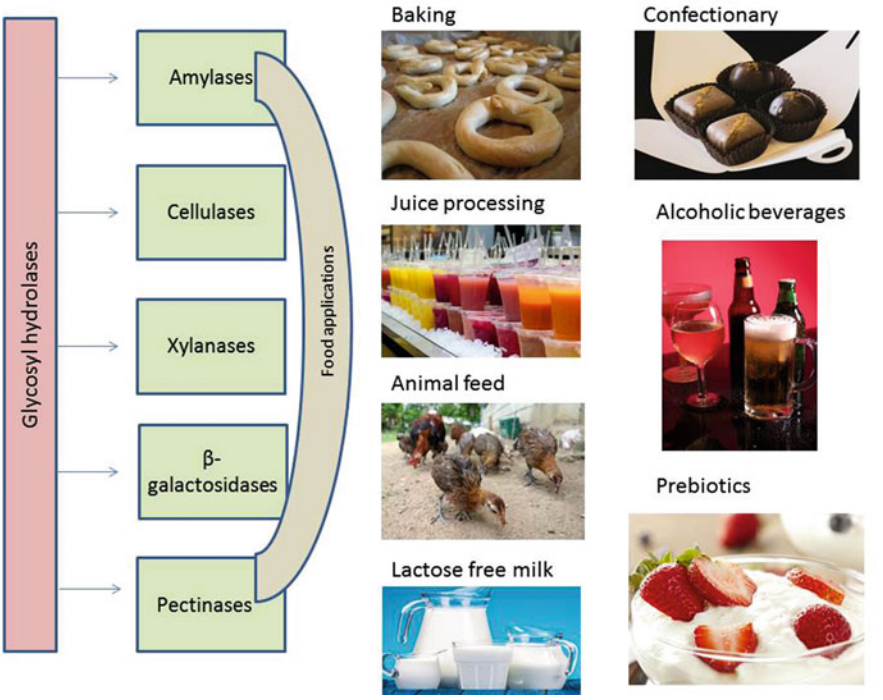

Fig. 1 Graphical abstract representing application of glycosyl hydrolases in food industry

Enzymes as biocatalysts have been used for decades in the food industry. The classification of enzymes has undergone several revisions, and type of substrate, source, and mechanism of action and amino acid sequence has been used to categorise this complex group of molecules (Martínez Cuesta et al. 2015). Enzymes known to hydrolyse glycosidic bonds between sugars or sugar-nonsugar groups are characterised as GHs or glycosidases (Sathya and Khan 2014). Enzymes belonging to this group are found in prokaryotic, eukaryotic, and archaea domains (Cantarel et al. 2009; Henrissat 1991; Henrissat and Bairoch 1993), and are present as 115 distinct families as listed under CAZy classification. This approach of classification is based on the mode of action of the enzyme and amino acid sequence. Glycosyl hydrolases have been used for various food based applications such as food processing, extraction of bioactives, preservation, and food analysis and food product development. Though there are multiple families and several different members belonging to glycosyl hydrolases, the focus of this chapter will be on commonly used enzymes such as amylases, cellulases, xylanases, β-galactosidases, and pectinases. This chapter will review the source, biochemistry, optimum conditions and application of the selected enzymes (Fig. 1).

Amylases

α-Amylases has huge importance in current industries such as food, fermentation, textile, paper, pharmaceutical industries. Presently, in food industry amylases (-α-amylases, β-amylases and glucoamylases) is one of the most importantenzyme groups which have wide applications. This enzyme is extensively dispersed in all living organisms. Amylases degraded starch present in food and efficiently hydrolyze α-1,4 glycosidic bonds of polysaccharides producing short-chain dextrins. Among this α-amylases, most the enzymes present are metalloenzymes which needs calcium ions for their activity, stability and integrity (Sindhu et al. 2017).

Amylases has widely used and has many important applications in food industry. This enzyme is used in the preparation of bakery products, fruit juices, starch syrup, brewing etc. Amylases extensively employed in bakery industry for products such as cake, bread, cookies. Malt and fungal alpha-amylases enzymes play an important role in bread making. These alpha-amylases acts on the starch present in wheat flour and degrade that starch into small dextrins. This causes yeast to work repeatedly during dough fermentation, proofing and the early stage of baking which enhances the bread volume and crumb texture. These enzymes are also responsible for the anti-staling effect in bread baking. It gives an attractive baked flavour, retains the softness and enhances the shelf life of the bakery products (Lundkvist and Olsen 2007). Presently, in bakery industry a thermostablemaltogenic amylase of *Bacillus stearothermophilus* is commercially employed which has another application in clarification of beer or fruit juices. It acts as a pre-treatment and enhances the digestibility of fiber (van der Maarel et al. 2002).

The most extensive use of α-Amylases is in starch industry, in which this emzyme hydrolyse starch and produces glucose and fructose syrups in liquefaction process. The enzymatic conversion has three important steps which includes gelatinization, liquefaction and saccharification. This processing requires more thermostable enzymes. During gelatinization viscous solution is formed by dissolution of starch granules. In liquefaction process, partial hydrolysis occur which reduces the viscosity. In further step of saccharification glucose and maltose are produced (van der Maarel et al. 2002). In another application of amylases on starch, α-amylases convert starch into fermentable sugars and further conversion is carried out by *Saccharomyces cerevisiae* which results into ethanol. α-amylases are also responsible to enhance the yield and for the clarification of fruit juices in the presence of cellulases and pectinases (Kumar 2015).

The thickness of the chocolate syrup can be prevented by treating cocoa slurries with amylases. In this treatment chocolate starch is dextrinized which avoids thick formation of syrup. The high cocoa content cocoa flavoured syrup with improved stability and flow properties at room temperature are produced with the help of amylolytic enzyme and Dutch process cocoa. The syrup is prepared by alternate addition of cocoa and sweetener with water to obtain around 58–65% total solid content. Further, amylolytic enzyme is added and heated to 175–185 °F for 10 to 15 min and finally cooling (El-Aassar et al. 1992).

Lactase (β-Galactosidase)

β-galactosidase enzyme is a part of the family of hydrolases. The two important classes of β-galactosidase are commonly used for commercial applications such as cold-active and thermostable β-galactosidase. This enzyme can be produced from various biological systems ranging from plants, animals to micro-organisms. However, the formation of β-galactosidase from microorganisms sources for example bacteria, fungi and yeast produces greater yield with less cost of production. The final application of the enzyme β-galactosidase decides the source to utilize. Hydrolysis of lactose is an essential treatment which is catalyzes by β-galactosidase enzyme. For the hydrolysis of lactose in milk of whey, the β-galactosidase enzyme produced from yeast source at pH from 6.5 to 7.0 is commonly employed. Whereas, fungal sources are commonly used to produce β-galactosidase enzyme at pH 3.0–5.0 for the hydrolysis of acidic whey. Hence, the final preference of β-galactosidase depends on the application of this enzyme in industry (Harju 1991).

For industrial applications in dairy products, usually β-galactosidase produced using microorganisms with GRAS status are used. Decrease in lactose content in milk and milk-based products products can be performed by various ways. Among these treatments, use of β-galactosidase is widely used for pre-hydrolyzation of dairy products to obtain nutritional rich products. This enzyme is commercially employed from fungal or yeast sources in dairy industry to lower down the lactose level for the lactose intolerant consumers. This enzyme is also available in tablet form and it is swallowing instantly before the milk and milk-based products are consumed. The Aspergillus strain is employed to produce β-galactosidase enzyme, which stays functional even in acidic conditions of human stomach (McNeil-PPC 2003).

The hydrolysis of lactose with β-galactosidase enhances the creaminess of ice cream and reduces the required quantity of sweeteners as it also improves the sweetness of the products. β-galactosidase has another application in the processing of whey. Whey is byproduct produced during the cheese manufacturing and its major constituents are lactose, proteins and minerals. The dumping of whey into environment causes severe environmental concern as lactose present in whey leads to high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Rosenberg 2006). It is treated as a byproduct and disposed into water streams. β-galactosidase provides solution for this situation as it hydrolysis the lactose present in whey. Two important products can be produced from whey such as ethanol and β-galactosidase (Kokkiligadda et al. 2016).

The demand in the production of lactose-free products is increasing with this the demand for β-galactosidases are also growing. β-galactosidases shows several health benefits on human health. One of the important advantage is the formation of galactooligosaccharides which is commonly employed as prebiotic food ingredient which has potential to modify the intestinal microflora by promoting healthy bacteria such as *Bifidobacterium* sp. and *Lacto-bacillus* sp. Galactooligosaccharides are formed together with the hydrolysis of lactose due to transgalactosylation activity of β galactosidase. The percentage of production of oligosaccharides can vary from 1 to 45% of total saccharides present and depends on the source of enzyme. In early

years of 1950s the potential of β galactosidases to develop a series of oligosaccharides containing galactose was discovered. However, in the later studies optimization of conditions required for the manufacturing of oligosaccharides were performed. In recent years, more studies have been focused on the health benefits of oligosaccharides on human (Rabiu et al. 2001).

Xylanases

The much needed property for xylanases to be useful in the food industry is high stability and optimum activity closer to an acidic pH. With advances in the field of molecular biology, other techno-functional properties of xylanases are being studied (Harris and Ramalingam 2010).

Xylanases are commonly used in bread making industry in combination with other enzymes. Many endo-1, 4-xylanases derived from bacterial and fungal sources have been used in baking applications. Enzymatic hydrolysis of non-starch polysaccharides improves rheological traits of dough, bread specific volume, and crumb firmness, resulting in a better end product (Goswani and Rawat 2015).

The hemicellulose component of wheat is water-insoluble and leads to technical issues. Xylanase during baking helps to convert water-insoluble hemicellulose into a soluble form, which aids to enhance the volume and creates fine uniform crumb. Through the bread baking process, it delays crumb formation and allows the dough to grow. With the incorporation of xylanases, there has been an increase in bread volumes, higher absorption of water and enhanced resistance to fermentation (Harris and Ramalingam 2010). Also leads to an increase in arabino-xylo oligosaccharide in bread, which is proven to be beneficial to health. Xylanases and enzymes that hydrolyze cell wall are used to develop dough handling properties, enhance bread quality, extend shelf life by reducing staling and they are seen to be particularly effective in straight dough process (Sharma and Sharma 2017).

Addition of xylanase from *Aspergillus foetidus* led to a significant difference in water absorption of the tested samples as seen from a study. Xylanase also makes the dough less sticky such that it does not stick to any equipment parts, enabling mechanized processing (Harris and Ramalingam 2010).

Xylanases increase the elasticity of the gluten network and are thus used as additives in the baking industry. As mentioned earlier, increased elasticity improves stability and handling of the dough (Goswani and Rawat 2015). In biscuit-making, xylanase ensures the cream crackers are light and have improved texture, palatability and uniformity of the wafers (Sharma and Sharma 2017).

Xylanase along with cellulase and pectinase are used for clarifying must and juices and also for liquefying fruits and vegetables (Goswani and Rawat 2015). In combination with other enzymes, xylanases lead to higher juice recovery from the fibrous fruit and increased extraction of aromas, essential oils, vitamins, mineral salts, pigments, etc. (Raveendran et al. 2018).

Xylanase is also used in coffee bean processing (Mandal 2015). Recently, recombinant strain of yeast was constructed with the gene for xylanase from

Aspergillus nidulans, xlnA, leading to a wine with superior aroma bouquet than found traditionally. During beer manufacturing process, the cell wall of barley is hydrolyzed which releases long chains of arabinoxylans that increase the beer viscosity and affects the appearance. Xylanases are often used to hydrolyze arabinoxylans to lower oligosaccharides lowering beer viscosity and improving the appearance of the final product (Harris and Ramalingam 2010).

Xylanases are also used for non-food applications such as the retting process which is the disintegration of the outer bark of plants like flax, jute etc. before the fibers are processed into any fabric (Mandal 2015). Xylanases along with pectinolytic enzymes are used for the degumming of fibers such as flax, hamp, jute and ramie. The combination of these enzymes is known to be used in the preliminary process of wood debarking. Though pectinases are believed to play a key role in elimination of binding materials from plant tissues, xylanases may also assist in this process (Goswani and Rawat 2015). The processed wood finds application in several industries including food packaging.

A U.S. patent for a novel method of xylanase production was granted in 1979 and the produced xylanase along with other enzymes was incorporated as a dairy cattle feed additive (Garg and Kumar 2010). Adding xylanase to animal feed results in higher animal growth rates and improves digestibility and quality of animal litter (Goswani and Rawat 2015). Another application of xylanase is for chicken feeds containing wheat, rye and other grains which are not completely digested due to the presence of hard seed coat. These grains become too viscous in the intestine of chickens and hence escape complete digestion. Xylanase partially digests the grains and improves digestibility of the feed. It also converts hemicelluloses to small sugars such that essential nutrients formerly locked in the cell walls are released. The chicken gets adequate energy from less feed and leads to enhanced feed conversion efficiency and body weight. Fungal xylanase derived from *Aspergillus niger* helps maintain growth, nutrient digestibility, and non-starch polysaccharide breakdown in broilers (Mandal 2015). The addition of xylanase to animal feed results in partial xylan hydrolysis or pre-digestion of the feed and thus improves cellulose accessibility to ruminal digestion and subsequent nutritional value of the feed (Mandal 2015).

Hemicellulose (xylan) containing agro waste can be enzymatically digested using xylanase to convert xylan into xylose through hydrolysis. Development of an optimized scaled up enzymatic hydrolysis process offers new possibilities for treatment of hemi cellulosic wastes contributing to thrust research area (Goswani and Rawat 2015).

Cellulases

Cellulases have several important applications in food industry. Cellulases derived from two important sources such as fungi (Aspergillus and Trichoderma) and bacteria (Bacillus and Paenibacillus) has widely used in the manufacturing of food products (Kuhad et al. 2011; Raveendran et al. 2018).

The manufacturing of fruit and vegetable juices involves steps like extraction, clarification and stabilization. To improve the efficiency of extraction methods, clarification and stabilization, cellulases enzymes are used in association with other macerating enzymes. This also leads to increase in the performance of the process and improves the yield. Hence in this way by using cellulases and macerating enzymes yield can be increases by avoiding additional capital investment (Kuhad et al. 2011). This type of extraction methods are preferred over conventional treatments as it provides higher yields, less heat damage and less processing time. The potential application of cellulases has also been explored for the extraction of phenolic compounds and flavonoids from grape pomace and flowers/seeds respectively (Raveendran et al. 2018).

Tropical fruits like mango, peach, papaya, plum are widely used to prepare fruit beverages. Similarly like fruit and vegetable juices, cellulases has also involved in the processing of fruit nectars and purees. As the fruits are highly perishable, the preparation and preservation of these products is of extraordinary commercial significance to draw the attention of consumers. The macerating enzyme enhances the cloud stability, texture and promotes easy concentration of the nectars and purees. These enzymes are effective in enhancing the aroma and taste of citrus fruit by reducing the extreme their bitterness (Raveendran et al. 2018).

The studies conducted in the 1980's, proved that single enzyme is not sufficient for the effective maceration and extraction of oil from olives. Majorly three types of enzymes such as pectinases, cellulases and hemicellulases showed potential effectiveness for the extraction of oil from olives. The combination of two enzymes viz. cellulases and hemicellulases (from Trichoderma) achieve improved performance for efficient olive oil extraction than the enzymes from single micro-organism (Raveendran et al. 2018). In commercial application Olivex was the first enzyme combination used to enhance the extraction of olive oil (Kuhad et al. 2011). Maximum yield is obtained with completely ripened fruit when processed at higher ambient temperature. However, this also leads to high acidity, rancidity and poor aroma of the extracted oil. Therefore, improved methods for the extraction high quality olive oil are needed. However, the use of macerating enzymes enhances the antioxidant levels in extra virgin olive oil and decreases the induction of rancidity (Bhat 2000).

Cellulases have one more interesting application in the field of food coloring agents. Cellulases enzymes are used in the extraction of carotenoid from orange peel, sweet potato and carrot (Sukumaran et al. 2005). This enzyme breaks down the cell wall and discharges the carotenoids in the chloroplasts and in cell fluids. Carotenoid stays in their natural state still bound with proteins which prevents oxidation of the pigments and hence stabilizes color. Solvent extraction is used to break the pigments from bound structure with protein and promotes oxidation (Kuhad et al. 2011).

Cellulase has potential applications in the field of beer and wine production. Glucanases are used to enhance the malting of barley in beer manufacturing. Whereas, for efficient maceration and color extraction, exogenous hemicellulases and glucanases are widely used in wine production (Sukumaran et al. 2005). During 1980s, Trichoderma β-glucanase has been commonly applied for the preparation of

wine from grape which was infected with *Botrytis cinerea*. This *Botrytis cinerea* degrade grapes and causing several difficulties by forming high molecular mass while wine filtration. Studies conducted on Endoglucanase II and exoglucanase II from *Trichoderma* sp. proved that it significantly decreases wort viscosity by reducing degree of polymerization. The enhanced aroma of wine is produced by β-glucosidases through alteration of glycosylated precursors (Raveendran et al. 2018).

Cellulases show significant improvements in silage production for cattle feeding. This shows improvements in feed digestibility of grasses which constituents more amounts of digestible nutrients and energy values (Kuhad et al. 2011). The combination of enzymes Hemicellulase and cellulases enhances the nutritive quality of forages. With improvements in feed digestibility, cellulases also shows improvements in animal performance (Sukumaran et al. 2005).

Pectinases

Pectinases are the heterogeneous group of enzymes which break down pectic polymer into parts by different reactions such as hydrolysis, trans-elimination and de-esterification. This pectinases enzyme is a mixture of distinct enzymes including polygalcturonase, pectinesterase and pectin lyase based on their mode of action (Rebello et al. 2017). Pectinases are widely used to decrease the viscosity, to improve the yield and clarification of juice. The growth of Pectinases is increasing exponentially with an annual growth rate of 2.86% and estimated to reach 35.5 million $ by 2021 (Global Pectinase Market Research Report 2017). Pectinases enzyme was firstly commercialized in 1930 for the clarification of apple juice (Garg et al. 2016).

This enzyme has been extensively employed for the clarification of various fruit juices such as apple juice (Joshi et al. 2011); orange juice (Maran et al. 2013); Lemon juice (Maktouf et al. 2014); Mango juice (Kumar et al. 2012); pineapple juice clarification (Patidar et al. 2016); Blueberry juice (Sandri et al. 2013). Pectinase rupture the glycosidic bonds present between the galacturonic acid monomers which reduces the water holding capacity of pectin and subsequently produces higher yield of juice. It is also found that the pectinases helps to extract phenolics from the fruit skin. A study on dragon fruit proves that pectinases enzyme treated dragon fruit beverage yields 15% higher phenolic content than the untreated beverage (Aliaa et al. 2010). Pectinases has been commonly applied in various other food industries. In wine industry, pectinases are used at different stages during the processing especially during the crushing of fruits. Pectinases improves the yield, recues processing time, and enhances the color of the wine (Bigelis 1993). This enzyme is also used to develop essential flavours in different food products. It has application in coffee and instant tea preparation to decrease viscosity and mucilage (Verma et al. 2018). It improves foam formation in instant tea powder by killing tea pectins (Willson and Clifford 1992) (Table 1).

Table 1 Varied glycosyl hydrolases and their applications

Enzyme	Source	Optimum pH and temperature	Food application	Reference
Amylases	Bacterial: *Bacillus amyloliquefaciencs*	pH 7 and 33 °C	Saccharification for HFCS	van der Maarel et al. (2002)
	Bacterial: *Bacillus stearothermophilus*	pH 5–5.8 and 60 °C	Bakery: Convert starch present in dough into smaller fermentable sugars	van der Maarel et al. (2002)
	Bacterial: *Bacillus lentus*	pH 6.1 and 70 °C	Frozen chocolate flavored confections	El-Aassar et al. (1992)
Cellulases	Fungal: *Aspergillus niger*	pH 6–7 and30 °C	Production of fruit and vegetable juices, fruit nectars and purees	Bhat (2000)
	Fungal *Trichoderma harzianum*	pH 6.5 and 45 °C	Beer and wine production	Prabhu et al. (2017)
	Fungal: *Trichoderma* spp.	pH 6.5 and 45 °C	Improvement in animal feed digestibility	Sukumaran et al. (2005)
Xylanases	Fungal: *Aspergillus foetidus*	pH 4–6 and 50 °C	Improvement in baking qualities	Harris and Ramalingam (2010)
	Fungal: *Aspergillus niger*	pH 5.5 and 45 °C	Feed additive, improving digestibility	Mandal (2015)
	Fungal *Thermomyces lanuginosus*	pH 4–6 and 50–60 °C	Degumming of bastfibers such as flax, jute and ramie	Goswani and Rawat (2015)

(continued)

Table 1 (continued)

Enzyme	Source	Optimum pH and temperature	Food application	Reference
β-galactosidases	Fungal: *Aspergillus oryzae*	pH 5 and 50–55 °C	Decrease in lactose content	McNeil-PPC (2003)
	Bacterial: *Lactobacillus thermophilus*	pH 6 and 55 °C	Prebiotic food ingredients	Rabiu et al. (2001)
	Yeasts: *Kluyveromyces lactis*	pH 6.5–7 and 30–35 °C	Lactose hydrolysis	Harju (1991)
Pectinases	*Sphenophorus levis*	pH 4–5, 50 °C for pectin methylesterase and pH 6–8, 40 °C for an endo-polygalacturonase	Fruit juices	Habrylo et al. (2018)

Summary

Enzymes provide value to the raw material and enhance the quality of the final product. In recent years, researchers have focused on the enzymes that showcase higher activity, enhanced stability over wide range of temperature and pH and exhibit longer half-lives. Research endeavours have also been made on cost effective production, recycling through immobilization and reduction in the related waste generated. From the above literature it is clear that glycosyl hydrolases have various applications in food such as bakery, fruit beverages, wine, and confectionary. This book chapter discusses the various types of glycosyl hydrolases enzymes and their applications in food industry.

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The Effects of Xylanase Synergistic Interactions During Lignocellulose Degradation and Their Significance for Industry

Samkelo Malgas, Mpho S. Mafa, and Brett I. Pletschke

Abstract

Xylan, the most abundant hemicellulose and major component in lignocellulosic biomass requires depolymerisation in order for it to be used as a precursor for the production of value added products (VAPs). As global interest in using lignocellulosic feedstocks for VAPs production increases, an accompanying knowledge on how to efficiently depolymerise these feedstocks is required. Thermochemical depolymerisation of xylan in lignocellulose is non-specific and environmentally unfriendly, and as a result, enzymatic means of xylan depolymerisation is favoured. Due to the complex nature of xylans, a consortium of xylanolytic enzymes is required for the efficient degradation of xylans. Interesting applications of xylanolytic enzymes include uses in plant-based industries such as use as feed additives, in waste treatment, biofuel production, bread making, probiotics and fruit juice production, and pulp and paper bleaching. Although many xylanolytic enzymes exhibit certain desirable characteristics for industry, no individual enzyme is capable of meeting the needs of all xylanolytic enzyme requiring industries. In addition, as industry requires cheaper enzyme processing costs, efficient catalysis by xylanolytic enzymes is required. This review examines recent advances in the use of xylanolytic enzymes and their synergistic associations with each other and with other classes of enzymes which can improve their industrial applications.

Keywords

Degradation · Glycoside hydrolases · Hemicellulose · Synergy · Xylanases

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Introduction

Hemicelluloses are the second most abundant polysaccharide in nature, after cellulose, and represent about 20–35% of the dry weight of the lignocellulosic biomass (Saha 2003). Generally, hemicelluloses are named according to the major monosaccharide residues found in their structural backbones and most of these are linked together by β-1,4-glycosidic bonds (Saha 2003; Van Zyl et al. 2010). The classes of hetero-polysaccharides that hemicellulose represents or denotes to includes plant cell wall polysaccharides such as xylans, mannans, β-glucans and xyloglucans (Peng and She 2014).

The distribution of hemicellulose in plants varies greatly—hardwoods and grasses contain xylans as the major hemicellulosic component, whereas softwoods and some specialized structures, such as plant seeds and fruits, are comprised of mannans as a more prominent type of hemicellulose (Moreira and Filho 2008; Saha 2003). Among hemicelluloses, xylan is the major carbohydrate, and is composed of a backbone of β-1,4-D-xylose residues substituted with acetyl, α-L-arabinofuranosyl, α-galactosyl, α-glucuronyl, and 4-O-methylglucuronyl groups.

Xylan-containing lignocellulosic biomass is a renewable and carbon-neutral energy source that has considerable potential as a feedstock for the large scale production of liquid fuels, prebiotics, artificial sweeteners and other value added products (VAPs), with the production of most of these VAPs requiring the xylan portion of lignocellulose to be hydrolysed into its monomers, xylose, or at least into xylo-oligosaccharides (XOS) in some instances (Charoensiddhi et al. 2017; Saha 2003; York and O'Neill 2008). Currently, thermochemical pre-treatment of the xylan-containing lignocellulosic biomass is the predominantly used method for hydrolysing xylan. During this procedure, the lignocellulosic biomass is generally treated with a diluted acid such as 2% (w/v) sulfuric acid by steam-heating to 120 °C or higher for 2 h (Alvira et al. 2010). The solid to liquid ratio in this process is typically around 1:7 to 1:15, respectively (Wang et al. 2017). Technical drawbacks in xylan hydrolysis via thermochemical means include: energy consumption (accounts for 40% of total energy consumption), corrosion of equipment, production of waste water containing acids and salts which accounts for 30% of the total water consumption and can cause heavy environment pollution and production of undesirable by-products resulting from dehydration of xylose and other monosaccharides, which increase the difficulty of subsequent purification of target products (Alvira et al. 2010; Hendriks and Zeeman 2009; Wang et al. 2017).

Recent studies have demonstrated synergistic interactions between various xylanolytic enzymes and other enzyme classes and, as a result, improve xylan and xylan-containing biomass industrial utilization (Malgas et al. 2017a). Here, we summarize recent studies that report on advances in industrial utilization of xylanolytic enzymes for value added products derived from lignocellulosic biomass.

Xylans

Xylans are classified into three subfamilies: glucuronoxylan, arabinoxylan and arabinoglucuronoxylan or glucuronoarabinoxylan. Structural properties and occurrence of these various xylan subfamilies are briefly explained in this review (Fig. 1).

Arabinoxylans

Arabinoxylans (AXs) consist of a main chain of 1,4-linked β-D-xylopyranosyl residues to which mono- or di-α-L-arabinofuranosyl substituents are attached through O-2 and/or O-3 positions of xylose (Revanappa et al. 2015; Schendel et al. 2015). The arabinofuranosyl residues in AXs can be esterified with hydroxycinnamic acid derivatives, such as ferulic and *p*-coumaric acid (Lagaert et al. 2014). AX solubility and viscosity can vary depending on their molecular weight, arabinose: xylose ratio and degree of cross-linking with other polymers in the plant cell wall (Schendel et al. 2015). Arabinoxylans are the main non-starch hemicelluloses found in the cell walls of endosperms of Gramineae such as wheat,

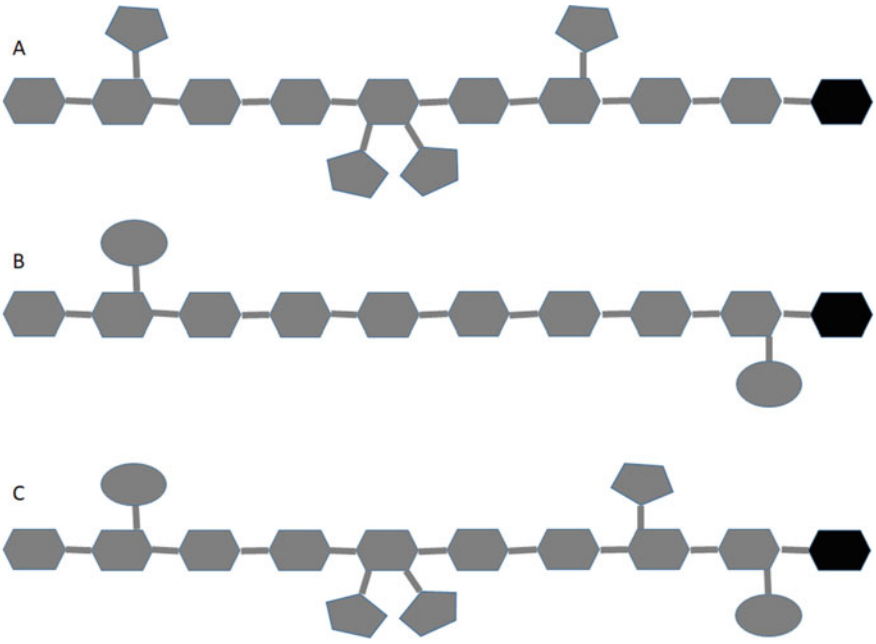

Fig. 1 General structure of hetero-xylans. (**a**) A typical arabinoxylan structure, (**b**) a typical glucuronoxylan structure, and (**c**) a typical arabinoglucuronoxylan or glucuronoarabinoxylan structure. *Grey hexagons* represent xylose while *black hexagons* represent reducing end xylose, *pentagons* represent arabinose, *circles* represent (methyl)-glucuronic acid residues and *lines* represent glycosidic bonds between sugar moieties

bamboo, rice, sorghum, sugarcane and ryegrass (Peng and She 2014; Revanappa et al. 2015).

Glucuronoxylans

Glucuronoxylans (GXs) consist of a main chain of 1,4-linked β-D-xylopyranosyl residues to which α-1,2-D-linked glucupyranosyluronic acid residues are attached to O-2 of each tenth xylose residue (Togashi et al. 2009). Some hardwood species may have some or all of their glucuronic acid substituents methylated at O-4 position; however, in *Eucalyptus globulus* wood, about one third of the 4-O-methyl-α-D-glucuronopyranosyl residues are substituted at O-2 by D-galactopyranosyl and D-glucopyranosyl units (Pinto et al. 2005). Therefore, in hardwood species, generally, the majority of hemicelluloses are O-acetyl-4-O-methylglucurono-β-D-xylans, their content varying between 15 and 30% (wood weight basis) (Pinto et al. 2005). The degree of polymerization of these hemicelluloses in wood is approximately 150–200 (Singh et al. 2003).

Arabinoglucuronoxylan and Glucuronoarabinoxylan

According to Gatenholm and Tenkanen (2003), and Huisman and co-workers (Huisman et al. 2000), arabinoglucuronoxylans (AGX) and glucuronoarabinoxylans (GAXs) consist of a β-D-(1,4)- linked xylopyranoside backbone and can be substituted with α-L-arabinofuranose on C2 and/or C3, α-D-glucopyranosyluronic acid, or its 4-O-methyl derivative on C2, acetyl on C2 or C3 of some xylose residues. These are considered the most complex types of xylans. AGXs are the dominant hemicelluloses in cereals and grasses, and are also present in coniferous species (Ebringerová 2006; Gatenholm and Tenkanen 2003).

Xylan Degradation

Complete degradation of xylans requires the synergistic action of a consortium of glycoside hydrolase (GH) enzymes, including β-xylanase, β-xylosidase, α-glucuronidase and α-arabinofuranosidase, and accessory enzymes, such as acetyl xylan esterase, ferulic acid esterase and *p*-coumaric acid esterase (Fig. 2). Here we give a brief summary of the properties of each xylanolytic glycoside hydrolase enzyme required during xylan degradation—in-depth information on these enzymes has been reviewed elsewhere (Collins et al. 2005; Biely et al. 2016; Juturu and Wu 2012).

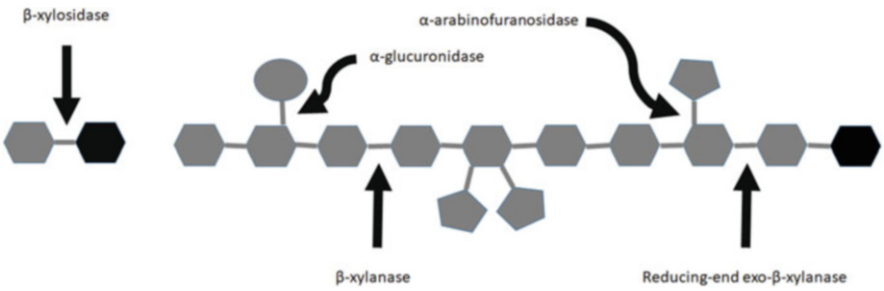

Fig. 2 An illustration of the enzyme sites of the enzymes required to completely degrade hetero-xylans. *Grey hexagons* represent xylose while *black hexagons* represent reducing end xylose, *pentagons* represent arabinose, *circles* represent (methyl)-glucuronic acid residues, *lines* represent glycosidic bonds between sugar moieties and *arrows* represent the enzyme required for cleaving a glycosidic bond between specific sugar moieties

Xylanases

Endo-β-1,4-xylanases (also called 1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) are enzymes which randomly cleave the β-(1,4)-linkages between two D-xylopyranosyl residues in xylan backbones (Mendis and Simsek 2015). Xylanases have been classified into glycoside hydrolase families GH5, 8, 10, 11, 30, 43, 62 and 98 in the CAZy database, with GH10 and 11 being the best characterized families. GH10 fungal and bacterial xylanases often contain a catalytic domain linked to one or more non-catalytic modules (such as CBM22). CBM22 possesses the ability to bind four to five xylosyl residues in insoluble xylans via hydrophobic stacking interactions (Motta et al. 2013; Simpson et al. 2003). According to Paës et al., CBMs probably help the xylanase to indirectly localize the xylan substrate, since it is in close association with cellulose in lignocellulosic biomass (Paës et al. 2012). GH11 xylanases can only hydrolyse xylosidic bonds where the two corresponding xylose moieties in subsites (−1) and (+1) are not branched, while GH10 xylanases attack the glycosidic linkage next to a single or double substituted xylose toward the non-reducing end and require two unsubstituted xylose residues between branched residues (Mendis and Simsek 2015; Paës et al. 2012).

Arabinofuranosidases

Arabinofuranosidases (also called Abfs, EC 3.2.1.55) are enzymes involved in the hydrolysis of terminal non-reducing α-L-arabinofuranoside (L-Araf) residues in α-L-arabinans containing α-(1,2)- or α-(1,3)- and/or α-(1,5)-linkages, arabinoxylans, arabinoxylo-oligosaccharides and arabinogalactans (Lagaert et al. 2014). Abfs belong to the glycoside families GH2, 3, 10, 43, 51, 54 and 62 in the CAZy database. All Abf families perform glycosidic bond hydrolysis with retention of the anomeric configuration, except for GH62 which still has an unknown mechanism (www.cazy.

org). Abfs that release L-Araf units from mono-substituted main-chain D-xylopyranosyl (D-Xylp) motifs are termed AXH-m, while those that release single L-Araf residues from double-substituted main-chain D-Xylp motifs are termed AXH-d. Only GH43 and 51 Abfs have been demonstrated to readily de-branch L-arabinose groups on both mono- and di-substituted D-xylopyranosides (Lagaert et al. 2014).

Glucuronosidases and Glucuronidases

Xylan α-1,2-glucuronosidases and α-glucuronidases (EC 3.2.1.131 and EC 3.2.1.139, respectively) are enzymes that hydrolyse α-(1,2)-D-(4-O-methyl)-glucuronosyl links in the main chains of glucuronoxylans and glucuronoarabinoxylans. According to Nurizzo et al. (2002), some GH67 glucuronidases prefer short glucuronic acid substituted xylo-oligomers and are generally intracellular or membrane associated, while others have specificity for polymeric glucuronic acid-containing polymeric xylans, these being consequently extracellularly produced enzymes. The reaction mechanism of GH67 glucuronidases has been elucidated as net inversion (Shallom and Shoham 2003). Generally, GH115 glucuronidases are more active on polymeric glucuronic acid-containing polymeric xylans (McKee et al. 2016; Tenkanen and Siika-Aho 2000) compared to GH67 glucuronidases (Golan et al. 2004; Nagy et al. 2002).

Xylosidases

According to the CAZy database, xylosidases are presently found in glycoside hydrolase families GH3, 30, 39, 43, 52, 54, 116 and 120. Some GH39 xylosidases exist as tetramers in solution and have also been reported to exhibit glycosylation activity on xylo-oligomers (Lagaert et al. 2014). Xylosidases catalyse the hydrolysis of xylobiose and also attack the non-reducing ends of short xylo-oligosaccharides, but do not hydrolyse xylan, which is a polysaccharide (Huy et al. 2015; Knob et al. 2010; Yan et al. 2008).

Exo-xylanases

Exo-xylanases (also called oligosaccharide reducing-end xylanase (Rex), EC. 3.2.1.156) show a different mode of action when compared to the β-xylanases and β-xylosidases, and are strictly found in GH8 according to the CAZy database. Exo-xylanases hydrolyse the xylan back-bone from the reducing ends producing xylo-oligomers. Interestingly, these enzymes can also produce xylose from short xylo-oligomers with a degree of polymerization of 2 or 3 (Juturu and Wu 2012; Malgas and Pletschke 2019; Lagaert et al. 2014). Rex's are said to recognize the xylose unit of the reducing end in a very strict manner, even discriminating the

β-anomeric hydroxyl configuration from the α-anomer or 1-deoxyxylose (Fushinobu et al. 2005).

Since numerous comprehensive reviews on the characteristics of xylanolytic enzymes have been published (Juturu and Wu 2013; Lagaert et al. 2014; Paës et al. 2012), this review will focus on the role of xylanolytic enzymes and their synergism with other enzymes in industrial applications.

Synergistic Actions of Xylanolytic Enzymes on Xylans

As previously mentioned, an array of xylan specific hemicellulolytic enzymes synergistically acts on xylans, leading to their complete hydrolysis into monomeric sugars. According to Moreira and Filho (2008), two types of synergies have been identified between mannanolytic enzymes, namely homeosynergy and heterosynergy. Based on the complexity of xylan, which includes the presence of substituents on its backbone, we propose that xylanolytic enzymes can also exhibit these two types of synergies during its hydrolysis. Homeosynergy can be defined as the cooperation between two main-chain-cleaving enzymes (e.g. β-xylanase and β-xylosidase) or two side-chain-cleaving enzymes (e.g. α-arabinofuranosidase and α-glucuronidase), while heterosynergy can be defined as the synergistic association between a side-chain-cleaving and a main-chain-cleaving enzyme (e.g. β-xylanase and α-arabinofuranosidase).

Sources of Xylanolytic Enzymes

Xylanolytic enzymes are produced by a diverse variety of microorganisms including archaea, bacteria, fungi, yeast etc. Different niches such as insect gut, manure, waste water, hot environmental samples and chicken cecum have been reported as sources which harbour xylanolytic enzyme producing micro-organisms (Chadha et al. 2019). Some bacterial and fungal strains which produce aggressive xylanolytic enzymes include *Aspergillus* sp., *Bacillus* sp., *Thermoascus auranticus*, *Talaromyces emersonii*, *Thermomyces lanuginosus*, *Melanocarpus albomyces*, *Sporotrichum thermophile*, *Geobacillus stearothermophilus*, *Caldoceum saccharolyticum*, *Clostridium* sp., *Trichoderma* sp. and *Thermomonospora* sp. (Chadha et al. 2019; Collins et al. 2005). Table 1 lists bacterial and fungal sources of xylanolytic machinery and the characterised xylanolytic enzyme gene products the micro-organisms produce as reported in the CAZy database (http://www.cazy.org). In addition to the production of a variety of hydrolytic enzymes, many microorganisms can produce multiple isozymes of the same enzyme. A classic example of this is *Aspergillus niger* which is reported to encode a multiple of up to fifteen extracellular xylanases (Malgas et al. 2017b).

Table 1 A list of selected xylanolytic enzyme producing organisms with their characterized xylanolytic enzymes belonging to GH families 2,3, 4, 5, 8, 10, 11, 30, 39, 43, 51, 52, 54, 62, 67, 98, 115, 116 and 120 (www.cazy.org)

Organism	Enzyme(s)
Bacterial sources	
Bacillus sp.	XlnA, Rex, XylY, XynC, Xyn5B, Xyn30A, XynB, Arf43A, AbfA
Bacteriodes sp.	BCellWH2, Rex8A, Xyn5A, Xyl3A, Xyn30A, Xyl43A, Xyn10B/Ara43A, AsdII, Agu67A, Agu115A
Caldicellulosiruptor sp.	Xyn10B, XynA, XynF, XynD, Agu67A
Cellulomonas fimi	XynC, Xyl3A, AbfCelf
Celvibrio japonicas	Xyn10A, Xyn10B, Xyn10C, Xyn10D, Xyl43A, Abf43, Abf51, Abf62A, GlcA67A
Clostridium sp.	EngB, EngD, ArfA
Geobaccillus sp.	XynA2, XT6, XynB3, SXA, XynB, Abf1, Abf2, XylA, XynB2, AguA
Thermotoga maritima	Agu4A, Agu4B, Xyl10A, Xyl3, AraA
Fungal sources	
Aspergillus sp.	XynA, Xyn10C, XlnD, XynB, XynC, Abf51, Abf54, Axh62A, AguA
Chrysosporium lucknowense	Xyn11A, AXH-d, Xyl7, Xyl8, Abf2, Abf3, Agu1
Humicola insolens Y1	XynA, XynB, XynC, Xyl43A, Xyl43B
Neocallimastix patriciarum	XynA, XynB
Talaromyces sp.	Bxy3A, XylC, Abf3, Abf1, Abf2
Thermomyces lanuginosus	XynA, Xyl43
Trichoderma sp.	Xyn1, XynIII, Xyl3A, Abf1, Abf2

Xylanase Market

It is reported that the global market for industrial enzymes was around $6.3 billion in 2017 and will continue to grow at around 6.8% compound annual rate of growth (CAGR) up to 2024, with xylanases expected to be worth $35 million in the same year (http://www.alliedmarketresearch.com). According to Collins and co-workers, presently the technical industries, dominated by the detergent, starch, textile and fuel alcohol industries, account for the majority of the total enzymes market, with the feed and food enzymes together totalling only about 35% (Collins et al. 2005). The biofuels segment is expected to be the fastest-growing application area for industrial enzymes during the forecast period, with a CAGR of about 7.3% through to 2024. The United States Patent and Trademark Office lists 3975 patents to date with reference to xylanases (http://www.uspto.gov/).

The major global manufacturers and suppliers of industrial enzymes include: AB Enzymes, Adisseo, ABF Group, BioZyme, Danisco, Dyadic, Genencor, DSM, Elanco, Iogen, Roche, Alltech, Basf, Takabio and Novozymes. The top three

companies holding the biggest enzyme market share (approximately 75%) are Novozymes, AB Enzymes and Genencor at 47, 21 and 20%, respectively (Li et al. 2012). These companies are focusing on improving their production capacities and upgrading their solutions to differentiate their services from their competitors.

Industrial Applications of Xylanases

Xylanases and other xylanolytic enzymes are reported to be useful in numerous industrial applications beyond their use as feed additives, waste treatment, biofuel production, bread making, probiotics and fruit juice production, and pulp and paper bleaching. Table 2 lists possible industrial applications of xylanolytic enzymes and the specific roles they play in those certain processes.

Application of Xylanases in Prebiotics Production

According to Yasmin and co-workers, prebiotics are simply defined as non-digestible food components that selectively stimulate the growth or activity of specific indigenous bacteria (prebiotics) in the digestive tract in a manner claimed to be beneficial for the host (Yasmin et al. 2015). Well known probiotics include

Table 2 Potential applications for xylanolytic enzymes as described by Collins et al. (2005), Kumar et al. (2018) and Lei et al. (2016)

Market	Industry	Function
Feed	Animal feeds	Digestion of NSPs leading to lowered digesta viscosity and release of nutrients resulting in improved feed conversion rates
Food	Artificial sweeteners production	Production of xylose from xylan degradation for xylitol production
	Baking	Reduction of the water absorption of whole wheat flour and increased loaf volume and crumb softness by hydrolysing xylan
	Brewing	Extraction of more fermentable sugars from barley for making beer
	Juice and vegetable processing	Increased juice yield from improved maceration process. Reduced viscosity and higher filterability and clarity of juice
	Prebiotics	Production of nutraceutical prebiotic XOS from xylan degradation
Technical	Biofuels	Bioconversion of lignocellulosic biomass into fermentable sugars for bioethanol production
	Paper and pulp	Increased pulp brightness via improved delignification (bleaching)
	Textiles	Bio-polishing of fabric, cotton softening, denim finishing, de-sizing, etc

bacteria such as lactobacilli and Bifidobacterium and non-pathogenic yeast, *Saccharomyces boulardii* (Yasmin et al. 2015; Vandenplas 2016). Prebiotics are also said to decrease the toxin-producing bacteria like *Streptococcus pneumonia*, proteolytic *Clostridia* and *Escherichia coli* (Charoensiddhi et al. 2017). Prebiotics are primarily carbohydrates (oligosaccharides and polysaccharides) in nature. Oligosaccharides included in this category are fructooligosaccharides (FOS), xylooligosaccharides (XOS), galactooligosaccharides (GOS), mannooligosaccharides (MOS) and pectin oligosaccharides (POS) as well as some sugar alcohols (Hutkins et al. 2016; Moreno et al. 2017).

It is reported that prebiotic oligosaccharides can be found naturally in foods or, alternatively, they can be produced by enzymatic or chemical synthesis from disaccharides or other substrates, as well as by hydrolysis of polysaccharides (Moreno et al. 2017). Hydrolysis of polysaccharides is normally the most reliable choice for oligosaccharide production on a large scale, due to its reproducibility and high yield. Lyases such as pectate lyases and glycoside hydrolases (GHs) including cellulases, xylanases, mannanases and amylases are the enzymes required to degrade lignocellulose derived polysaccharides into oligosaccharides which can subsequently be used as prebiotics (Juturu and Wu 2013; Pedrolli et al. 2009). In this section, we shall examine the synergistic use of xylanolytic enzymes for the production of prebiotic XOS.

During prebiotic XOS production, it is noteworthy to mention that homeosynergistic interactions between backbone cleaving enzymes such as xylanases may be required. However, enzymes with xylosidase activity are not desirable as XOS with a degree of polymerisation (DP) of 2 and higher are reported to elicit a prebiotic effect, therefore xylose production is undesirable (Samanta et al. 2015). Synergism between GH10 and 11 xylanases during the degradation of sugarcane bagasse xylan has been reported previously by Gonçalves and co-workers in a protein mass ratio of 50:50%; however, the synergistic effect was enzyme source specific (Goncalves et al. 2015). Malgas and co-workers also recently demonstrated that XT6 and Xyn2A in a mass ratio of 25:75% could synergise and efficiently depolymerize beechwood glucuronoxylan and wheat-flour arabinoxylan into XOS compared to when the enzymes were used alone (Malgas and Pletschke 2019). On the contrary, another study reported that the simultaneous use of XYL10 and XYL11 xylanases from *Thermobacillus xylanilyticus* does not result in a synergistic action on insoluble wheat bran arabinoxylan degradation (Beaugrand et al. 2004).

Malgas and co-workers have postulated that reasons for the differences with respect to both the enzyme ratios and synergism trends (synergy vs anti-synergy/competition) reported in literature could be due to both enzyme and substrate specific properties (Malgas and Pletschke 2019). They further postulated that differences such as the enzyme source and presence or absence of CBMs in enzymes, and solubility or insolubility and/or degree of substitution of substrates have been reported to have an effect on the trend of synergy observed (Malgas and Pletschke 2019; Van Dyk and Pletschke 2012). In summary, it appears as if the synergistic application of xylanases from different GH families holds promise for

improving the production of high yields of XOS from xylan substrates to be used as prebiotics.

Application of Xylanases in Xylose and Xylitol Production

Xylitol, a five carbon sugar alcohol used as an artificial sweetener, can be produced from the second most abundant polysaccharide, xylan, which upon hydrolysis produces xylose (Rao et al. 2016). Xylitol is widely used as a sugar substitute in the food industry because of its properties, such as similarity in sweetness to sucrose, no requirement for insulin, inhibition of dental caries and low calories (Kamat et al. 2013; Li et al. 2015). Xylitol can also serve as a valuable synthetic building block for various chemical compounds such as xylaric acid, succinic acid and glycols (Guo et al. 2013). In this section we describe the role of xylanolytic enzymes in producing xylose from the xylan fraction of lignocellulosic biomass for use as a precursor for xylitol production (Li et al. 2013).

A recent study showed that a sugar-rich effluent from pre-hydrolysis of eucalyptus wood (pre-hydrolysate) composed of XOS is an abundant by-product of paper and pulp industries which could be completely hydrolysed to xylose with concentrations reaching 50.41 g/L after 24 h using a xylanolytic cocktail composed of xylanase (1333.33 U/g), β-xylosidase (60 U/g) and acetyl-xylan-esterase (27 U/g) (Wang et al. 2017). Another study demonstrated that *Aspergillus terreus* derived xylanolytic enzymes, xylanase (722 U/g) and β-xylosidase (196 U/g), could be used to hydrolyse corncob hemicellulose for xylitol synthesis with sugars of 18.03 g/L xylose and 4.87 g/L glucose being obtained after 8 h of hydrolysis (Li et al. 2013). An integrated xylitol production pathway, directly using xylan as the substrate, was constructed in *Candida tropicalis* BIT-Xol-1 which could efficiently convert xylose into xylitol using a xylanase gene (atn) and a xylosidase gene (atl) were cloned from *Aspergillus terreus* which were constructed onto the episomal plasmid pAUR123 of *Candida tropicalis* (Guo et al. 2013).

These studies all showed that a combination of xylanolytic enzymes was more efficient at xylose production from xylan (for use as a precursor for xylitol production) compared to when only xylanases were used for xylose production.

Application of Xylanases in Biofuels

Bioethanol is a renewable liquid fuel that can be produced from lignocellulosic biomass via a combination of physical or chemical pre-treatment, and enzymatic hydrolysis to produce fermentable sugars (e.g. glucose and xylose) from the biomass and their fermentation by microorganisms. The major components of lignocellulose are carbohydrates such as cellulose and hemicellulose (approximately 75% dry weight), and lignin. For maximum utilization of lignocellulosic biomass, efficient hydrolysis of hemicellulose is required. This is mainly based on the following two considerations: (1) the utilization of hemicellulose can increase the theoretical

ethanol yield and improve the economics of lignocellulose conversion and (2) degradation of hemicellulose will further facilitate the access of cellulases to cellulose, leading to more efficient utilization of cellulose, since cellulose is embedded in a matrix of hemicellulose (Van Dyk and Pletschke 2012).

Traditional glucose fermenting organisms such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* cannot ferment other sugars such as xylose to bioethanol efficiently. As a result, research has focused on exploring other yeasts such as *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehate* which have the capability to ferment xylose to ethanol. However, commercial exploitation of these yeasts for bioethanol production from xylose is still not viable due to their low ethanol tolerance, slow rates of fermentation, difficulty in controlling the rate of oxygen supply at the optimal level, in addition to sensitivity to inhibitors generated during pretreatment and hydrolysis of lignocellulosic substrates. Due to this, other efforts have focused on converting xylose to xylulose using the enzyme xylose isomerase and this (xylulose) can be fermented to ethanol by traditional yeasts.

Cellulase to endo-xylanase synergism is so well studied, to such an extent that it is known that glycoside hydrolase (GH) family 10 xylanases show better synergistic cooperation with canonical hydrolytic cellulases than GH11 xylanases on various pre-treated lignocellulosic substrates such as sugarcane bagasse (Hu and Saddler 2018) and wheat straw (Zhang et al. 2011).

From these numerous studies, it appears that an intimate network between cellulose and hemicellulose exists, and as xylanolytic enzymes synergistically interacted with cellulases during the degradation of lignocellulosic biomass both glucose and xylose yields were improved. In this regard, a previous review of ours showed that as the xylan content increases for a particular lignocellulosic substrate, the presence of a xylanase becomes necessary in the enzyme cocktail and the degree of synergy between cellulase and xylanase also increases with respect to both glucose and xylose release (Malgas et al. 2017a, b). Finally, it is clear that the addition of xylanolytic enzymes in cellulolytic cocktails leads to improvement in the specific cellulase performance or activity and results in the reduction of grams of enzyme required for achieving equivalent hydrolysis by the xylanase free cocktail (Malgas et al. 2017a; Pletschke et al. 2016).

Application of Xylanases in Animal Feeds

Xylanases have been documented for their use as feed additives for monogastric animals (particularly poultry and pigs) for more than 20 years (Paloheimo et al. 2012). Monogastric animals are classified as non-ruminant animals which includes pigs, poultry such as turkeys, ducks and chickens (both broilers and layers); fish and crustaceans (e.g. shrimp and prawns). Xylanases have also been reported to be effective feed additives for ruminant animals such as sheep, goats and cattle (e.g. beef cattle and dairy cows). In this review, we will focus on reported advances in the application of xylanases as well as their synergistic associations with non GH enzymes as feed additives for both poultry and pigs.

Cereal grains are the important and major component of animal feeds. In cereal grains, starch is the major nutritional component, however, cereal grains also contain non-starch polysaccharides (NSPs) which cannot be digested by the digestive system of many monogastric animals such as poultry and pigs (Fischer and Petterson 2014; Paloheimo et al. 2012). NSPs in cereal-based feeds include polysaccharides such as xylan (particularly AX), mannan, beta-glucans and cellulose. Grains such as wheat, triticale and maize contain AX as a major anti-nutritional factor which makes up 50% (w/w-dry matter) of the NSPs, whereas for barley, sorghum, rye and rice the AX makes up approximately 25–45% of the NSPs. In grains, AX exists in two forms, water soluble and water insoluble fractions. Insoluble-AX is known for encapsulating nutrients such as starch and protein. The soluble AX, on the other hand, has been shown to lead to an increase in the viscosity of digesta. This encapsulation and high viscosity digesta leads to valuable nutrients bypassing the digestive process in the gut of monogastric animals. Therefore, xylanases are used to improve the feed digestibility of livestock and in cutting expenses with profit gain. Discussed below are recent studies detailing the use of xylanolytic enzymes—either alone or in concert with other classes of enzymes as additives in animal feeds.

The use of xylanases and xylan-debranching enzymes has been reported to solubilize insoluble-AX, and as a result, expose the nutrients which are encapsulated, and also fragments the soluble AX, leading to lowered digesta viscosity (Fischer and Petterson 2014; Lei et al. 2016). Latorre et al. demonstrated that wheat, barley, rye or oat diet increased proliferation of the *Clostridium perfringens* in the guts of the broilers (Latorre et al. 2015). The inclusion of *Bacillus subtilis* (AM1002) and *Bacillus amyloliquefaciens* (AM0938 and JD17), which produced the xylanase in these diets, reduced the viscosity of the feed in the intestinal tract of poultry and significantly reduced *C. perfringens* proliferation. *Bacillus* sp. were incorporated into feed diet through a direct fed microbial method.

In addition, AX is reported to cause gut health problems for broilers, which are fed a wheat-based diet (Fischer and Petterson 2014). AX generates viscous chyme in the guts of broilers, which lead to a proliferation of pathogenic bacteria, intestinal inflammation, impaired intestinal barrier function, and severe intestine lesions (Fischer and Petterson 2014). Lei et al. (2016) demonstrated that xylanase and xylan debranching enzymes Abf and feruloyl esterase (FE) produce XOS which were important for the gut health of the broilers. On the other hand, a GH11 endo-xylanase from *Thermomyces lanuginosus* demonstrated high activity on wheat, rye and barley AX (Ravn et al. 2016). The main products of the xylanase hydrolysate were short oligosaccharides with a degree of polymerization below 100. Microscopy studies revealed that the xylanase led to a complete removal of AX from the cell walls of the grains. These results suggest that this enzyme have potential use in a wide range of monogastric animal feeds.

Nguyen and co-workers used a commercial enzyme cocktail produced by DSM Nutritional Products, Ltd., which consisted of xylanase and proteases, and demonstrated that the addition of these enzymes in corn and soybean diets improved the body weight gain of broilers (Nguyen et al. 2017). The growth performance and body weight gain were attributed to the feed conversion rates and nutrient absorption

by broilers. The xylanase in the cocktail degraded the soluble and insoluble AX, which both reduced the viscosity of the feed and improved feed conversion rates (FCR). A commercial endo-xylanase from *Neocallimastix patriciarum* supplied by Asiapac (Dongguan, China) was added to the wheat diet of the broiler chicken (Lei et al. 2016). The finding showed that the broilers' average daily growth (ADG) increased from day 1 up to 31 days in the study. However, Lei et al. argued that the ADG was even better when xylanase was used in concert with an arabinofuranosidase produced by *Bacillus pumilus* and a feruloyl esterase (EC 3.1.1.73) produced by *Clostridium thermocellum* purchased from Megazyme (Dublin, Ireland). The benefits of this enzyme synergism included (1) significantly decreased digesta viscosity, (2) higher FCR of broilers from day 1 to 36, and (3) reduced intestinal lesions induced by an exclusive wheat-based diet (particularly reduction of duodenum, jejunum, and ileum lesion) (Lei et al. 2016).

From these studies, it is clear that the use of xylanases as feed additives exhibited greater benefit to livestock when the xylanases were used in concert with other xylanolytic enzymes.

Conclusions and Future Perspectives

The diverse industrial uses of xylanolytic enzymes has led to the conduct of several studies to design synergistic tailored xylanolytic enzyme cocktails to improve the hydrolytic potential of enzymes, which, in turn, would lead to the reduction of enzyme loads during industrial applications and, as a result, lower enzyme costs. The two main types of xylanolytic enzyme synergism discussed here were: (1) between xylanolytic enzymes during xylan degradation, and (2) between xylanolytic enzymes and other classes of enzymes during the bioconversion of xylan and other polymers associated with it in lignocellulosic biomass. In both cases, an improvement in the bioconversion of lignocellulosic biomass into VAPs (or precursors thereof) was most critical consideration.

The use of microbial xylanolytic enzymes for biomass hydrolysis is still cost-intensive compared to the use of acids. As a result, more effort is required in producing genetically modified xylanolytic enzymes with improved properties for their large scale production and applications in industry. Biely and co-workers also alluded to another challenge for protein engineers, which is to construct chimeric proteins that would be composed of several catalytic domains which act in synergy, for example xylanases and arabinofuranosidases or xylanases and acetyl-xylan esterases (Biely et al. 2016). They further suggested an alternative approach, which would consist of a combination of catalytic domain(s) with various carbohydrate binding module(s). Empirical elucidation of more synergistic interactions between various GH family xylanolytic enzymes and with other enzyme classes still remains an area of exploration, as enzymes with a similar classification may show a relaxed substrate specificity due to differences in their structure, including active sites, the presence of multifunctional domains and varied carbohydrate-binding domains. Embarking on these future prospects in the study of xylanolytic

enzymes will, without a doubt, further extend the current repertoire, understanding and applications of these enzymes in industry.

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Part III

Functional and Comparative Genomic Analysis of Glycoside Hydrolases

Role of Metagenomics in Discovery of Industrially Important Cellulase

Vijay Kumar and Priyanka Jha

Abstract

Lignocellulose is considered as one of the most copious biopolymer accessible on this planet. Lignocellulosic hydrolysis which yields sugar and phenolics is a must for fermentation processes and pilot scale production of value added products. Cellulases are the class of enzymes which are mainly produced by fungi and bacteria and help in cellulose hydrolysis by acting on the β-1,4 linkages of cellulosic chains. The microbial cellulases have been found to be used in several industries such as biofuel, food, brewing, textile and laundry. Recently, functional metagenomics have been found to be an important strategy for the discovery of cellulose genes. However, the efficiency of such techniques for enzyme discovery from environmental metagenomes is not sufficient to meet the increasing industrial demands. Scientific and industrial advancements, role of metagenomics and future scenario related to the application of several cellulase pertaining to different industries will be discussed in this chapter.

Keywords

Cellulose · Endoglucanase · Exoglucanase · Metagenomics · Microbial enzyme

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Introduction

The genomic examination of a microbial population is called metagenomics which was termed by Handelsman et al. (1998) with an idea of collective analysis of similar but not identical genomes. The thought of exploring and investigating the ecological microbiome has unlocked new prospects with respect to utilization of uncultivated microbial populations. Metagenomics is a comprehensive approach which has provided a path breaking process for recovery of unculturable microbes. One of the most thriving fields in biotechnology is the application of microorganisms for the production of value added products such as antibiotics, enzymes etc. Also, there is a huge requirement for large scale production of enzymes in industries for commercial purpose.

A wider range of biochemical properties and higher growth rate attributed to microorganisms, allow them to become potential candidates to be employed as industrial enzymes (Adrio and Demain 2014). One of the most important commercial applications of metagenomics includes innovations related to antibiotics, bioremediation, and detection of biocatalysts (Gilbert and Dupont 2011). Majority of the industrial catalysts are produced in European Union (60%), whereas the rest of the enzymes production is attributed by USA and oriental nations. However, south east Asian countries such as India, China, Korea are emerging as enzyme production hubs with efficient research and development sector in global catalyst market (World Enzymes 2011).

Cellulase Classification

Cellulose has compact polymeric arrangement which remain enclosed in a highly complex matrix of hemicellulose and pectin (Demain et al. 2005). Majority of enzymes have been observed to take part in cellulosic degradation through microbial genome sequencing. Microbial enzymes are found in huge variety possessing diversified properties, yields, structure and functions, thus differing catalytic properties. Diversified forms of enzyme may be produced as a result of horizontal gene transfer from microbes residing in similar conditions and ecological niche (Jorgensen 2007).

Cellulose degrading enzymes widely known as cellulases are capable of hydrolyzing the cellulosic biomass by attacking the β-1, 4 glycosidic bonds of the polymers (Juturu and Wu 2014). The degradation of cellulosic biomass requires three distinct factors related to extracellular cellulase enzyme system (Acharya and Chaudhury 2012). The components in the enzyme system include β-1, 4 glucosidase, Exo-1, 4-β-glucanase and Endo-1, 4-β-glucanase. The endo-1, 4-β-glucanase also known as carboxymethyl cellulase catalyzes the breakdown of cellulose chain polymers into shorter ones, whereas exo-1, 4-β-glucanase widely known as cellobiohydrolase acts on the non-reducing end of the polymeric chain and β-1, 4 glucosidase disrupts the glucosidic bond of cellodextrins and cellobiose to release free glucose molecules. Endoglucanase attacks at the random sites of the cellulose

polymeric chain, hence producing varying lengths of oligosaccharides (Sharada et al. 2014). Exoglucanase however, attacks on mostly reducing ends of cellulose polymeric chain producing glucose and cellobiose as key products.

Results reported by Schallmey and coworkers suggest identification of polyhydroxyalkanoate synthase encoding genes (Schallmey et al. 2011). The sequence analysis of the cloned samples revealed homology to the sequences (≈61–67%), however significant difference in functions from the PHA (*pha*C and *pha*A) encoding genes. Similar study by Cheema et al. (2012) revealed nine novel PHA synthase genes in a fosmid metagenomic library from oil contaminated soil.

One of the fosmid clones from the metagenomic library showed 76% sequence similarity with *Alcaligenes* sp. synthase. Enzymes like cellulases, xylanases, proteases, amylases and many more have been unclocked from genetically untapped resources via metagenomics.

The present review highlights the latest development in the field of metagenomics related to industrial biotechnology with respect to cellulase.

Role of Metagenomics in Harnessing Cellulases

Cellulose is considered to be one of the most obtained biopolymer on earth. The breakdown of cellulose is catalyzed by cellulose enzyme, hence finding its utilization in industries related to detergent, paper recycling, and juice extraction. Cellulase stands as one of the largest enzymes w.r.t economic turnover. Based on the oxygen availability to the microorganisms, cellulase enzyme can be classified into complexed and non-complexed forms (Lynd et al. 2002). The complexed forms of these enzymes mainly consist of cellulosome which is mostly found in anaerobic cellulose degraders. The cellulosomes are firmly attached to the cell wall and its flexible nature allows it to bind cellulose (Schwarz 2001). However, in non-complexed cellulase system, the enzyme subunits are flexible and can be collected from the supernatant of the aerobic microbial culture (Rapp and Beerman 1991). Presently, non-complexed cellulase enzyme systems are widely utilized for most of the industrial applications (Acharya and Chaudhary 2012). One of the most important industrial producers of cellulase is *Hypocrea jecorina* which hydrolyzes plant biomass to sugars (Kubicek et al. 2009). Such microbes can thrive the unfavorable conditions and produce stable enzyme which potentially help in catalyzing bioconversion reactions (Knapp 1985). Reports from Kanafusa-Shinkai et al. (2013) suggest that the cellulase enzyme system of *Caldicelluloseruptor bescil* are 2× active than *Hypocrea jecorina*.

The technique metagenomics has been employed to discover new cellulose from different environments such as soil samples from cold zones, compost and rumen by preparing metagenomic library clones (Yeh et al. 2013; Lee et al. 2006; Gong et al. 2013; Table 2). These enzymes have achieved special mention in industrial sector as they have potential capacity to convert biomass to renewable energy. Approximately 27.755 potential genes having significant match to catalytic domain were obtained by Hess et al. (2011). The study suggested presence of cellulolytic genes in large

numbers which can be utilized to prepare genome drafts of uncultivated microorganism involved in biomass hydrolysis. In a study by Alvarez et al. (2013), identified and characterized novel cellulase (CelE1), which showed maximum catalytic properties at pH 7.0 and 50 °C and significant activity in alkaline conditions. The ruminal metagenomic library revealed 61 clones expressing cellulase activities were isolated (Duan et al. 2009). The amino acid sequences matched via SMART bioinformatics tool revealed 14 cellulase genes with signal peptide and glycosyl hydrolase family 5 catalytic domain. However, for genes DM1–1 and M8–2 signal peptides were not available (Duan et al. 2009).

In another study by Voget et al. (2006), the biochemical characterization of cellulase (Cel5 A, endoglucanase) from soil sample. The study reported presence of novel cellulase Cel5A extracted from soil samples through biochemcial identification. The obtained sequence of 363 amino acid had functional similarity of 77% with cellulase activity of *Cellvibrio mixtus*. The Cel5A (1092 bp ORF) when cloned in E. coli and the related protein (42.1 kDa) was purified employing chromatography which was found to be active against cellulose contents with −1,4 linkages. The enzyme showed optimum activity at pH 6.5 in an activity range of pH 5.5–9.0. The unusual properties of the enzyme cellulase allow it to be potential candidate in industrial sector for bioprocess and its added values. In a recent study by Wong et al. (2017), metagenomic analysis of gut microbiome of *Castor canadensis* and *Alces americanus* revealed presence of cellulose degrading microbes having phylogenetic origins derived from Firmicutes, Bcateroidetes, and Proteobacteria. Sequences belonging to class Clostridia and Bacteroidia contributed the highest hits (23–52%) for the carbohydrate active enzymes across both the metagenomes.

Study by Ransom-Jones et al. (2017) investigated the lignocellulose-degrading microbial diversity from landfill site. Metagenomic analysis suggested the dominance of Firmicutes, Bacteroidetes, Fibrobacteres and Spirochaetes. Functional analysis revealed presence of ≈3385–4223 CAZymes for Firmicutes and Bacteroidetes respectively. Six distinct CAZyme families were obtained via functional annotation for Spirochaetes. Recent analytical investigation made by Wilhelm et al. (2019) reported the microbial community in forest soil through quantitative stable isotope probing and metagenomic genome assembling analysis across North America. The active cellulolytic populations observed were Deltaproteobacteria, Gammaproteobacteria, Planctomyces, and Ascomycota. Cellulose degrading microbes observed in the functional annotation were *Caulobacter*, *Janthinobacter*, and *Salinibacterium*.

Study by Kanokratana et al. (2015) revealed various types of glycosyl hydrolase from sugarcane bagasse collection site through fosmid metagnomic library preparation. Bioinformatic analysis of the xylan positive fosmids suggested presence of endo- $\beta-1,4$-xylanase of GH11 family. Additionally, two genes (cel9 and xyn11) were observed to be expressed in *E. coli*. The enzymes expressed were found to be active at thermophilic temperatures (75–80 °C) and acidic pH.

Reports from recent study by Wang et al. (2016) suggested involvement of Actinobacteria in lignocellulosic decomposition from compost. Metagenomic analysis reported dominance of Actinobacteria, however, presence of Proteobacteria,

Table 1 Recent metagenomic reports related to novel cellulase function

S. no.	Study type	Findings	References
1	Metagenomics	High forage microbial group showed improved and higher cellulase activity than that of high concentrate group. High forage group included the presence of microbial family Succinivibrionaceae	Wang et al. (2018)
2	Metagenomics	Metagenomic analysis revealed difference in microbial population in conventional and reduced tillage treated soil samples. Predominance of Proteobacteria and Actinobacteria were observed for cellulasic activities	De Vries et al. (2015)
3	Metagenomics and metasecretomics	Microbial consortia obtained from wheat straw, switchgrass, and corn stover suggested predominance of glycosyl-hydrolases for CAZyme functional analysis	Jiménez et al. (2016).
4	Synthetic metagenomics	Characterization of ligno-cellulosic degrading enzymes were accomplished utilizing porcupine microbiome. Sequences with similarity to β-glucosidase, β-xylosidase, endo-1,4-β-xylanase were found	Thornbury et al. (2019)
5	Metagenomics	Most active protein CelA10 was observed to be active at ionic liquid concentration of 30%	Pottkämper et al. (2009)

Bacteroidetes, Firmicutes were also observed. Actinomycetes were observed to have high CAZyme gene disribution (46.1%) which retained enzymes like β-glucosidase, cellobiohydrolase, and ligninase genes (Table 1).

Application of Microbial Cellulases

Cellulosic breakdown occurs in both oxic and anoxic conditions. Various anaerobic microbes as cellulosic degraders have been reported in several studies (Freier et al. 1988; Hamilton-Brehm et al. 2010; Kato et al. 2004; Table 2). The non-complexed cellulose enzyme system subunits are reported from aerobic microbial degraders such as, fungi and bacteria with special mention to fungal cellulases (Resch et al. 2013; Table 2).

Agriculture

Several fungal genus such as Trichoderma, Penicillium, Chaetomium have been observed to act significant in agriculture by increasing crop production, fecilitating plant growth and allowing enhanced seed germination (Phitsuwan et al. 2012). Additionally, it has been reported that few fungal enzymes are potential candidate for attacking the pathogenic strains. Enzymes such as B-1, 3-D-glucanase and N-acetyl glucosaminidase have been reported to degrade the spore germination of

Table 2 List of aerobic and anaerobic microorganisms related to lignocellulosic biodegradation

S. no.	Microorganism specification	Substrate used	References
1	*Fibrobacter* spp., Clostridium cluster III and Clostridium cluster IV	Municipal waste	McDonald et al. (2012)
2	Clostridium straminisolvens CSK1	Compost	Kato et al. (2005)
3	*Clostridium clariflavum*	Biocompost	Sizova et al. (2011)
4	*Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*	Straw	Zhang et al. (2017)
5	Mucilaginibacter L294, Pedobacter O48 and Luteibacter L214	Soil	López-Mondéjar et al. (2016)
6	*Clostridium stercorarium*	Plant cell wall	Zverlov and Schwarz (2008)

B. cinerea (Howell 2003). Several microorganisms die due to scanty nutrient available to them due to the presence of Trichoderma sp. (Waghunge et al. 2016). Hence, nutrient competition to *Trichoderma* sp. is the most common phenomenon responsible for pathogen death. This fungal candidate also helps to promote induced resistance in host plant body by initiating certain chemical production. They promote plant development by initiating an endophytic mode. Fungal cellulases also help in restoring soil quality by degrading the lignocellulosic biomass inside the soil.

Food Processing

Cellulase has managed a strong position in food and feed industry. It is an integral component of maceration unit utilized for extraction and processing of juice and pulp for juice and puree productions (Rai et al. 2007). Cellulase helps in preventing pigment oxidation by generating stable protein bound pigments (Table 3). Report from Kuhad et al. (2011) suggest that cellulase with some more enzymes has been utilized to enhance the taste of citrus fruits.

Study by Çinar (2005) indicated the combination of cellulase with other cell wall degrading enzymes such as pectinase and hemicellulase are employed to improve nutritional level of forages. Another study by Kung et al. (1997) suggested an improved and enhanced digestion of animal fodder and feed in combination with cellulase.

Brewery Industry

Preparation and production of ethanol is enhanced with the help of utilization of cellulase to hydrolyze polymeric substances to simple sugars. Beer quality is dependent on the enzyme activity during malting and subsequent fermentation phases. Seed reserve hydrolysis by B-glucanase and other enzymes (A and B amylase and

Table 3 Cellulase application in industrial sector

Industry	Application	Reference
Detergent	Modify cellulosic microfibrils and help in enhancing the color brightness and remove dirt	Karmakar and Ray (2011)
Waste management	Utilization of cellulosic waste from forests and farming fields	Milala et al. (2005)
Paper and pulp	Combination of endoglucanase I and II alongwith hemicellulase provides improves beatability of the paper pulp	Dienes et al. (2004)
Animal feed	Cellulases with fodder supplement improve the pork meat quality and weight gain in chicken and piglets	Singh et al. (2007)
Others	Improvement in olive oil production, carotenoid synthesis	De Faveri et al. (2008), Çinar (2005)

carboxypeptidase) influence better seed germination which in turn influences malting (Bhat 2000). Though in practice, brewers employ low quality substrate which has poor activity of endo-β-glucanase due to climatic variations. This allows formation of non starch polysaccharide (up to 10% β-glucan), hence gel like formation during brewing process resulting in low level wort filtration and poor alcohol yield.

Textile Industry

Enzyme cellulase has been widely accepted in textile industry for its capability to enhance softness and fabric quality. It has been been employed for biopolishing of cotton fabrics and biostoning of denims to provide the new generation stonewash appearances. The enzyme employed, hydrolyzes the fibre lumps from the fabric surface and removes the dye attached to such protrusions, hence imparting the faded look and development of color gradient to the denim (Arja 2007). This process is called biostoning. Cellulase is widely known for its use in wet processing in textile industry to enhance the appearance of cotton clothings. Cellulase has also been utilized in preparation of detergents to improve the softness, and color appearnaces of fabrics.

Conclusion

Metagenomics imparts a scope to explore the undiscovered microbial biodiversity from a microbiome and utilize the untapped potential of the microbes to generate value added products and processes. Recently, the industrial sector has also gained economically by major innovations such as unlocking of novel enzyme functions made via microbial metagenomics. Recent reports analyzing the sequences via cloning of metagenomic inserts has resulted in isolation and identification of untapped microbial communities for value added products such as significant

fermentation processes and enzyme production. In spite of novel investigations and reports from several studies, a lot remains to be understood about the cellulase enzyme alongwith the microbial mechanisms involved. Metagenomic studies have till date helped in identification of novel cellulolytic biocatalysts amongst other enzyme varieties. However, a vast reserve of enzymes is yet to be characterized. Furthermore, approaches like metatranscriptomic and stable isotope probing should be employed for innovations of enzyme systems. Significant bioprocesses should be developed so that the cellulosic wastes can be efficiently treated and used as cost-effective carbon source.

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