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Review Article

Carbohydrate active enzyme system in rumen fungi: a review

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Abstract: Hydrolysis and dehydration reactions of carbohydrates, which are used as energy raw materials by all living things in nature, are controlled by Carbohydrate Active Enzyme (CAZy) systems. These enzymes are also used in different industrial areas today. There are different types of microorganisms that have the CAZy system and are used in the industrial sector. Apart from current organisms, there are also rumen fungi within the group of candidate microorganisms with the CAZy system. It has been reported that xylanase (EC3.2.1.8 and EC3.2.1.37) enzyme, a member of the glycoside hydrolase enzyme family obtained from *Trichoderma* sp. and used especially in areas such as bread, paper, and feed industry, is more synthesized in rumen fungi such as *Orpinomyces* sp. and *Neocallimastix* sp. Therefore, this study reviews *Neocallimastix*sp., *Orpinomyces* sp., *Caecomyces* sp., *Piromyces* sp., and *Anaeromyces* sp., registered in the CAZy and Mycocosm database for rumen fungi to have both CAZy enzyme activity and to be an alternative microorganism in the industry. Furthermore the CAZy enzyme activities of the strains are investigated. The review shows that*Neocallimax* sp. and *Orpinomyces* sp. areconsidered as candidate microorganisms.

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1. INTRODUCTION

Carbohydrates have different chemical forms in nature such as mono-, di-, poly-, and oligo- (Asp, 1996). Monosaccharides of these chemical forms can be converted into more complex carbohydrates with the help of α and β glycoside bonds of covalent character (Yuan *et al*., 2018). Complex carbohydrates, although they have different tasks in living things, constitute the structure of the cell wall of plantsand are the most abundant in nature as a source of renewable energy (Guo *et al*.,2018; Singh *et al*.,2017). Plants can be called lignocellulosic biomass due to the complex carbohydrates they have (Vu *et al*., 2020; da Costa *et al*., 2019; Tsapekos *et al*., 2018). At the same time, this structure includes carbohydrates such as cellulose, hemicellulose, and pectin (Bhutto *et al*., 2017). The change of this chemical bond found in the structure of complex carbohydrates such as lignocellulose occurs with the help of the Carbohydrate Active Enzyme (CAZyme) family (Bredon *et al*., 2019). Due to the enzyme families contained in the CAZyme system, assimilation, inheritance, and modification

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processes related to the bond in the structure of carbohydrates occur (Lombard, 2010). It is thanks to symbiotic microorganisms living in the digestive tract of the CAZyme family herbivores that provide hydrolysis of plant-based complex carbohydrates (Gruninger *et al*., 2014). The digestive tracts of ruminants and monogastrics contain a complex microbiome consisting of bacteria, archaea, protozoa, viruses, and anaerobic gut fungi. Although anaerobic fungi were reported early, their discovery was delayed because they resembled protozoa. Since its first discovery in 1975, 18 genera have been described. The life cycles of AGFs vary according to the species. The life cycle of AGFs takes approximately 23-32 hours(Lowe *et al*.,1987; Ozkose *et al*., 2001).

The rhizoidal structure of anaerobic fungi is the most important feature that distinguishes them from each other(Orpin, 1975; Ozkose, 2001; Kar *et al.,* 2021). Rumen fungi, which are among these microorganisms and produce digestive enzymes belonging to the CAZyme enzyme family, can deconstruct approximately half of the vegetable substrate and form different products for other microorganisms (Solomon *et al*., 2016; Youssef *et al.,*2013; Ekinci *et al*., 2006). In addition, it has been observed that some enzymes belonging to the CAZyme family of rumen fungi are higher than some commercially used strains such as *Trichoderma* sp. (Solomon *et al*., 2016).

1.1. Rumen Fungi

The existence of rumen fungi was first discovered by Colin Orpin in 1975, when he concluded that a previously identified protozoan flagellate (*Callamastix frontalis*) found in the sheep rumen is the motile stage of an obligate anaerobic rumen fungus(Hess *et al*., 2020; Wood &Wilson, 1995; Trinci,1994).Rumen fungi are found in the digestive tract of ruminant and monogastric herbivores as a habitat. Also, it has important functions, both mechanical and enzymatic. Rumen fungi are classified taxonomically at the genus level according to their morphological characteristics (Orpin, 1977).However, today, morphological features are not sufficient for the classification of rumen fungi. Therefore, molecular approaches targeting at specific phylogenetic marker genes are utilized to facilitate taxonomic classification of the complex life cycles of rumen fungi (Hess *et al*., 2020). Recently, a large number of new, yet uncultured rumen fungus taxa have been identified in culture-independent diversity studies. Many rumen fungi species still wait to be identified in intestinal ecosystems(Hanafy *et al*., 2021; Hess *et al*., 2020; Haitjema *et al*., 2014).

The rumen fungi, which taxonomically belong to the *Neocallimastimycota phylum*, physically break down the plant cell wall with the mycelium in their structure, thus increasing the energy source for other microorganisms in the rumen (Yanuartono *et al*., 2019; Hibbet *et al.*, 2007; Heath *et al*., 1983). Rumen fungi are one of the microorganisms that play a vital role in the deterioration of the fibrous structure in the rumen, as they can produce cellulase enzyme and penetrate the feed particles(Agustına *et al*., 2022).In addition, they have great potential in the biofuel production process, as they hydrolyze lignocellulose with this enzyme and can convert this substrate into H_2 and ethanol (Saye, 2021).In other words, due to the fact that rumen fungi are very successful in the hydrolysis ofcarbohydrates with their hydrolysis enzyme, the CAZy system is thought to be present in these microorganisms and may be a candidate microorganism for this system (Kameshwar *et al*., 2019).

1.2. Carbohydrate Active Enzymes

CarbohydrateActive Enzymes provide control of chemical reactions such as hydrolysis, dehydration, and modification (glycoside transferases, glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases) of complex carbohydrates (Lombard *et al*., 2010). The concept of Carbohydrate-Active Enzymes (CAZymes), first used in the late 1990s, are based on structurally similar, related, or functional areas, and since then related studies have been

carried out to provide a database about the enzym system (Lombard *et al*., 2014; Cantarel *et al*., 2009). It is known that CAZymes, which are especially effective on glycosidic bonds, are necessary for significant biotechnological progress in the bioenergy and biobased (such as food, feed, materials, and chemical) industry sectors (Kameshwar *et al*., 2018). Apart from bioenergy and agricultural industries, CAZymes also have a very important place for human health. As a result of metagenomic studies conducted on symbiotics, which are responsible for decomposing various dietary and which host carbohydrates found in the digestive tract of humans, it has been found that these microorganisms encode more than one hundred CAZyme genes in their genomes (Huang *et al*., 2017). It is expected that these metabolic enzymes secreted by both aerobic and anaerobic fungi that perform the hydrolysis of biopolymer compounds such as cellulose, hemicellulose, pectin, and chitin represent a fairly rich and diverse enzyme pool (Lange *et al*., 2019).

The CAZymes database also provides online and up-to-date access to a sequence-based enzyme family classification that demonstrates the specificity and 3D structure of biological catalysts that assemble, alter, and degrade the sequence encoding these enzymes (Lombard *et al.*, 2014; Benson *et al*.,2004). In other words, the CAZy database is uptodate with sequence studies from the National Center for Biotechnology Information (NCBI), including taxonomic, sequence, and reference information, enzymatic family classification, and known functional information. These data allow an enzyme (CAZyme) to be searched for all CAZyme in an organism or a CAZyme protein family. The addition of new family members and the inclusion of biochemical information from the literature are regularly updated after a careful review.

The classification system of the CAZyme family covers all taxonomic groups, providing basic commonality(Davies *et al*.,2005). It has various enzymes involved in obtaining nutrients from substrates, hydrolysis, or dehydration, especially those that play a key role in the degradation of substrates and all known variants in databases and related bioinformatics tools of CAZymes (Davies & Williams, 2016) associated with the hydrolysis of polysaccharides in six main groups classified as Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), Auxiliary Activities (AAs), and Carbohydrate-Binding Modules (CBMs) (Lombard *et al*., 2014; Lombard *et al*., 2013; Levasseur *et al*., 2013).

1.2.1. *Glycoside hydrolases*

It forms a family of proteins responsible for the hydrolysis (Park *et al*., 2017) or transglycosylation (Manas *et al*., 2018) of glycosidic bonds. Glycoside hydrolases (EC3.2.1.-), a common group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate compound and make up almost half of the CAZyme family of genes encoding GH. Glycoside hydrolases are among the key enzymes of carbohydrate metabolism found in all three main domains (archaebacteria, bacteria, and eukaryotes). As it is actively used in biotechnological and biomedical applications, it creates the most successful set of biochemically characterized enzymes available in the CAZyme database (Henrissat *et al*., 1991).

1.2.2. *Polysaccharide lyases*

Polysaccharide lyases (PLs) function as a mechanism of hydrolysis of glycosidic bonds or acidic group elimination mechanism in acidic polysaccharides such as polysaccharides containing uronic acid. PLsdisrupt the structure of organic compounds such as glycosaminoglycans and pectin found in some microorganisms (Cantarel *et al*., 2009; Yip *et al*., 2006). Although many PLs are involved in biotechnological and biomedical fields and their total number is low compared to other enzymes belonging to the CAZyme family, there are biochemically characterized examples in the database (Yip *et al*., 2006; Coutinho *et al*., 1999).

1.2.3. *Carbohydrate esterases*

Carbohydrate esterases eliminate ester-based modifications found in mono-, oligo- and polysaccharides, thereby facilitating the action of GHs on complex polysaccharides. Because of the low barrier of specificity between carbohydrate esterases and other esterase activities, sequence-based classification is likely to include some enzymes that can act on non-carbohydrate esters (Coutinho *et al*., 1999).CEs also catalyze the -O or de-N-acylation of esters or amides and other substituted saccharides, where sugars play the role of alcohol and amine (Biely *et al*., 2012).

1.2.4. *Carbohydrate binding modules*

CarbohydrateBinding Modulesaim at a long-term interaction with other enzymes involved in the hydrolysis of some polysaccharides such as cellulose, which generally forms the structure of the water-insoluble plant cell wall.At the same time, CBMs help hydrolysis of these insoluble polysaccharides (Boraston *et al*., 2004). CBMs are known to be most likely associated with other carbohydrate active enzyme catalytic modules within the same polypeptide and can target at different substrate forms due to their different structural properties (Biely *et al*.,2012).

1.2.5. *Glycosyl transferases*

Glycosyltransferase enzymes (EC2.4.x.y) are involved in the biosynthesis of disaccharides, oligosaccharides, and polysaccharides. These enzymes transfer sugar groups from activated giver molecules to specific recipient molecules by forming a glycosidic bond (Campbell *et al*., 1998).

1.2.6. *Auxiliary activities*

Carbohydrateactive enzymes are the first described families of enzymes that break down or form complex carbohydrates, namely glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), Glycosyltransferases (GT), and non-catalytic carbohydrate-binding modules (CBM) added to them.The recent discovery that members of some families in this group are lytic polysaccharide mono-oxygenases (LPMO) has necessitated the reclassification of these families into an appropriate category.Since lignin is always present in the plant cell wall together with polysaccharides and lignin fragments which are likely to act in concert with (LPMO), the families of lignin degradation enzymes were decided to be added to the LPMO familiesand initiate a new CAZy class.For this reason, the so-called "auxiliary activities" group has been established to accommodate several enzyme mechanisms and substrates (Levasseur *et al*., 2013).

1.3. Numbering of the Carbohydrate Active Enzymes System

The enzyme commission number (EC) is also used scientifically to name enzymes and other enzymes that are bound to the CAZyme system. In EC, according to the terminology, the first three digits indicate enzymes that hydrolyze O-glycosyl bonds, while the last digit indicates the substrate, and sometimes reflects the molecular mechanism. This classification provides a unique classification that provides ease of use, especially to avoid ambiguities and to prevent the proliferation of unimportant names (Henrissat *et al*., 1991). Table1 shows the functions and EC numbers of enzymes belonging to the CAZyme system.

Table 1. The enzyme groups that are members of the CAZyme system: the Enzyme Commission Number (EC), the enzyme family to which they are attached (function), and the types of glycoside bonds by which they act (naming) (http://www.cazy.org).

SUBSTRATE	ECNUMBER	FUNCTION	NAMING
CELLULOSE	3.2.1.91	endo-β-1,4-glucanase / cellulase	GH5, GH6, GH7, GH8, GH45, GH9, GH10, GH12, GH44, GH48, GH51, GH74, GH124
	3.2.1.4	cellulose 1,4-β-cellobiosidase	GH5, GH6, GH9, GH51
	3.2.1.176	cellulose 1,4-β-cellobiosidase	GH7, GH48
HEMICELULOSE	3.2.1.37	xylan 1,4-β-xylosidase	GH1, GH2, GH3, GH30, GH39, GH43, GH51, GH52, GH54, GH116, GH120
	3.2.1.55	α-L-arabinofuranosidase	GH2, GH3, GH5, GH39, GH43, GH51, GH54, GH62
	3.1.1.72	acetylxylan esterase	CE1, CE2, CE3, CE4, CE5, CE6, CE7, CE12
PECTIN	3.1.1.11	Pectinesterase	CE8
	3.2.1.23	β-galactosidase	GH1, GH2, GH35, GH39, GH42, GH59, GH147, GH165
	4.2.2.2	pectate lyase	PL1, PL2, PL3, PL9, PL10
STARCH	3.2.1.1	α-amylase	GH13, GH57, GH119
	3.2.1.20	α-glucosidase	GH4, GH13, GH31, GH63, GH76, GH97, GH122
	3.2.1.28	α,α-trehalase	GH15, GH37, GH65
MANNAN	3.2.1.24	α-mannosidaz	GH38, GH31, GH92
	3.2.1.78	mannan endo-1,4-β mannosidaz	GH5, GH26, GH45, GH113, GH134
	3.2.1.113	mannosyl-oligosaccharide 1,2-α-mannosidase	GH38, GH47, GH92

1.4. Rumen Fungi and Carbohydrate Active Enzyme Activity

Herbal substrates, which are the main nutrition source of herbivores, have an average of 65% carbohydrates in their structure, and these organic compounds are mainly cellulose, hemicellulose, and pectin(Pettersen *et al*., 1984).A large number of species and genus levels continue to be added to the *Neocallimastimycota* phylum, which includes rumen fungi discovered by Orpin (1975) in the middle of the 20th century (Hanafy *et al*., 2020). The fact that rumen fungi have a morphologically filamentous structure positively affects the surface of attachment to the plant material contained in the habitat of these microorganisms. It is also known that these microorganisms have a high degree of enzyme activity, such as lignocellulosic (Meng *et al*., 2021; Liang *et al*., 2020). Complex carbohydrates which rumen fungi use both as a habitat and as a substrate increase the activity of carbohydrate-active enzymes (CAZy) of these microorganisms (Solden *et al*., 2018; Haitijema *et al*., 2017; Cantarel *et al*., 2009). Due to this property of rumen fungi, it has a symbiotic positive effect on the rumen ecosystem by

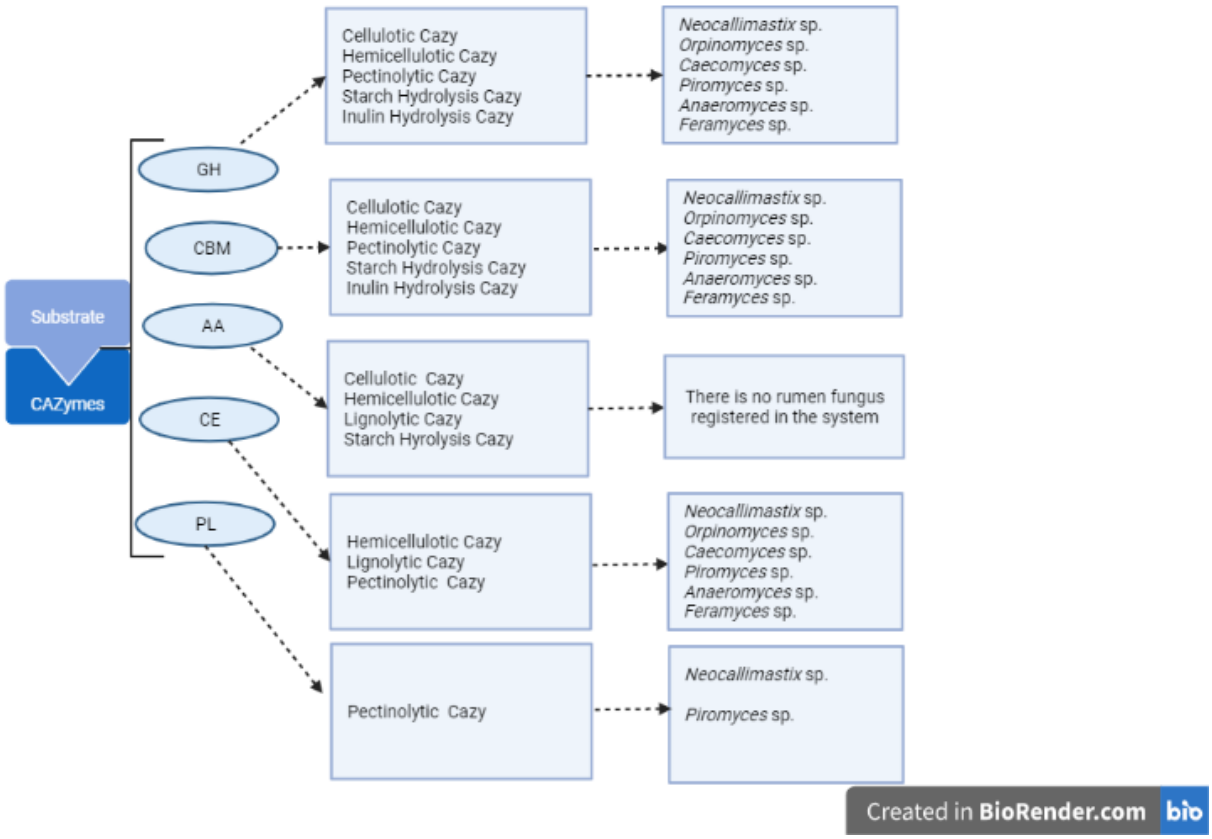
completely hydrolyzing the plant material in the rumen (Terry *et al*., 2019). The complete genome sequencing of some of the microorganisms belonging to the *Neocallimastimycota* phylum has been performed and is given in Table 2 (Wilken *et al*., 2021; Haitijema *et al*., 2017). As a result of the sequencing process, a JGI based database (fungal genomic database) is used in relation to the CAZy enzyme system belonging to these microorganisms (Barrett & Lange, 2019).

Table 2. The identified rumen fungi, registered in the MycoCosm database:microorganism name; summation of nucleotide length; number of genes; and authors who published them

Microorganism Name	Nucleotide Lenght	Number of Genes	Published by Author
Anaeromyces robustus	71.685.009	12.832	Haitijema *et al*.,2017
Caecomyces churravis	165.495.782	15.009	Brown *et al*.,2021
Neocallimasix california	193.032.485	20.219	Haitijema *et al*.,2017
Neocallimastix lanati	200.974.851	27.677	Wilken *et al*.,2021
Orpinomyces sp.	100.954.185	18.936	Yussef *et al*.,2013
Piromyces finnis	56.455.805	10.992	Haitijema *et al*.,2017
Piromyces sp.	71.019.055	14.648	Haitijema *et al*.,2017

(https://mycocosm.jgi.doe.gov/neocallimastigomycetes/neocallimastigomycetes.info.html)

Figure 1. Enzyme group and corresponding substrates of rumen fungi according to Cazyme system (http://cazy.org) (http://mycocosm.jgi.doe.gov) (https://biorender.com/)



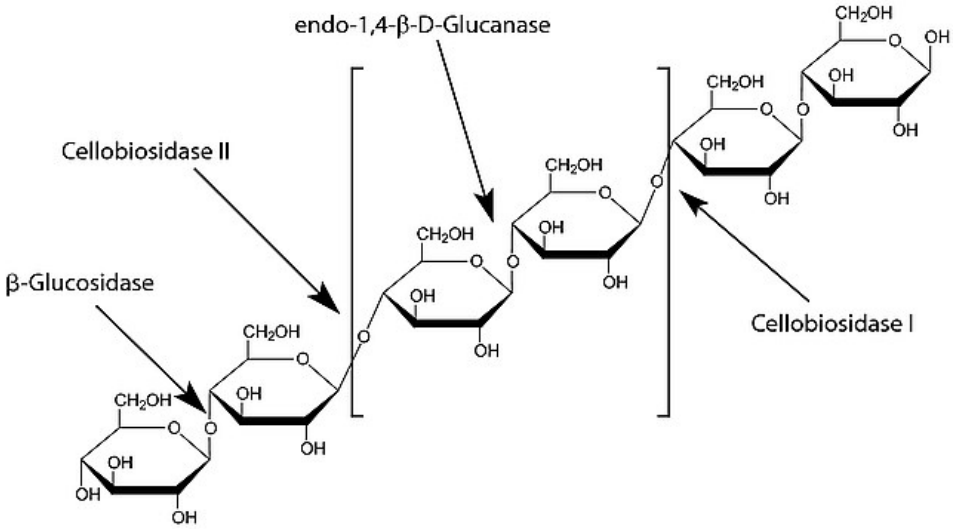
The CAZy system is classified into glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), auxiliary activity enzymes (AA), and carbohydrate-binding modules (CBM) (Lombard, 2013). Although the CAZy system is studied under different groups, they are generally named according to the substrate they act on. The

genome information belonging to other microorganisms, especially rumen fungi, is provided by Interpro (Blum *et al*., 2021; Richardson *et al*., 2019) and is also registered in different databases such as dbCAN (Ausland *et al*.,2021;Huang *et al*.,2018). In this study, research was carried out on the CAZy system of rumen fungi registered in current databases. The preferred web databases for analysis are cazy.org (http://cazy.org) and mycocosm (http://mycocosm.jgi.doe.gov).Data on different CAZy enzymes belonging to rumen fungi are shown in Figure 1. Accordingly, it is observed that *Neocallimastix* sp. and *Piromyces* sp. have activity in all groups belonging to the CAZy enzyme system (AA: Except Auxiliary Activity) in six different rumen fungi registered in the cazy database with mycocosm. It was determined that no rumen fungi were registered for the enzyme activity known as auxiliary activity (AA).

1.4.1. *Carbohydrate active enzymes system in Neocallimastix* sp.

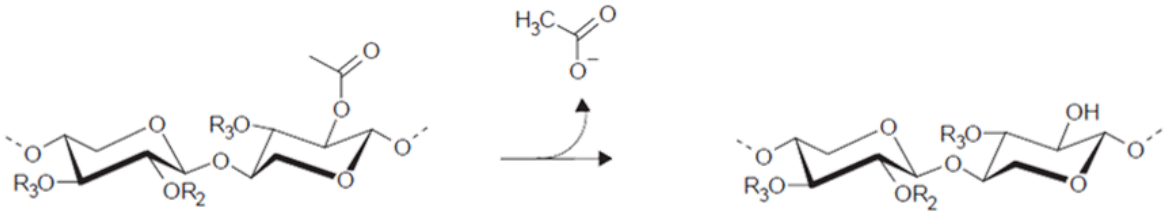
Endo-1,4-β-D-glucanase (EC3.2.1.4) registered to the glycoside hydrolase (GH) enzyme family of Neocallimastix sp. or CAZy enzyme activity related to the cellulase family has a higher rate than that of microorganisms with high activity such as aerobic Trichoderma reesei. known to have a secretoma (Wood *et al*., 1986). Currently, glucanase enzymes from *Trichoderma* sp. are used commercially and the mechanism of action of this enzyme is shown in Figure 2.

Figure 2. Mechanism of action (glucosidase) of *Trichoderma* sp. enzymes registered in Sigmaaldrich database (https://www.sigmaaldrich.com/TR/en/product/sigma/g4423)



Chen *et al*. (2012) determined that the optimum pH range of β-glucosidase, which is defined with the number EC3.2.1.21 related to the CAZy enzyme family in the literature, is in the range of 5-6 and its molecular weight is 85.1 kDa. Mountfort *et al*. (1989) reported that the optimum pH for its activity is 5 and the optimum temperature is 55ºC for the xylanase enzyme (EC 3.2.1.8), a member of the GH family.After the commercial use of the xylanase enzyme (Hemi-Cellulase) became widespread, Huang *et al*. (2021) conducted immobilization studies on the gene region related to the xylanase enzyme belonging to *Neocallimastix* sp. Zhang *et al*. (2019) reported the optimum in vitro working conditions of acetyl xylan esterase (CE - EC3.1.1.72) enzyme, a member of the CAZy enzyme family (Figure 3), which hydrolyzes the ester bonds of acetyl groups in the xylose parts of naturally acidified xylan substrates. Kwon *et al*. (2016) also reported that this enzyme has a molecular weight of 36.5kDa.

Figure 3. The mechanism of action of the enzyme acetyl xylan esterase (Krastanova *et al*., 2005).



1.4.2. *Carbohydrate active enzymes system* in *Orpinomyces* sp.

The activity of endo-1,4-β-D-glucanase has a very important place in terms of microorganisms. It is known that the activity of this enzyme in the species Trichoderma reesei is significantly higher and endo-1,4-β-D-glucanase is used in the textile industry. Because of this, it has been observed that the effectiveness of this enzyme of Orpinomyces sp. is also significantly important in studies conducted for living beings alternative to this microorganism (Jin & Xia, 2011). In studies on the characterization of Orpinomyces sp, its properties such as acetyl xylan esterase enzyme, optimum pH, temperature and molecular weight were determined (Blumm *et al*., 1999; Razeq *et al*., 2011).The enzyme xylanase is actively used in the production of biofuels and different industrial fields, and *Orpinomyces* sp. studies on the production of thermo-stable form have been reported (Passarinho *et al*.,2019; Ventorim *et al*., 2018).

1.4.3. *Carbohydrate active enzymes system in Caecomyces* sp.

Breton *et al*. (1995) reported that *Caecomyces* sp. has the enzyme activity of β-glucosidase (EC 3.2.1.21), while β-galactosidase (EC 3.2.1.23 related to the GH family) has no activity.Brown *et al*. (2021) preferred *Caecomyces* sp. in co-culture studies with methanogenic microorganisms because CAZy enzyme activity and the effect of carbohydrate-binding module (CBM) are significantly increased.

1.4.4. *Carbohydrate active enzymes system in Piromyces* sp.

Ali *et al*. (1995) reported that the molecular weights of xylanase, endoglucanase, and aviselase enzymes, which are members of the cellulase and hemicellulase enzyme family, are in the range of 50kDA to 190kDa.Thanks to its CAZy activities,*Piromyces* sp. can be used among the microorganisms used in silage production (Wang *et al*., 2019).Characterization and immobilization studies of the enzyme β-glucosidase, which is involved in the hydrolysis of cellulose, a renewable polysaccharide, have been reported (Chu *et al*.,2011; Tseng *et al*.,2015).

1.4.5. *Carbohydrate active enzymes system in Anaeromyces* sp.

The presence of enzymes such as endoglucanase, xylanase, and β-glucosidase, which are members of the *Anaeromyces* sp. enzyme family, and whose patterns range from 26 kDa to 130 kDa, has been reported (Wen *et al*.,2021; Novotná *et al*., 2010).Qi *et al*. (2011) reported that cloning and purification of the enzyme was achieved as a result of isolation and characterization of the enzyme ferulic acid esterase (EC 3.1.1.73), which belongs to the carbohydrate esterase (CE) enzyme group, from *Anaeromyces* sp.

2. CONCLUSION

In the CAZy family of enzymes, there are such groups of enzymes as Cellulase, Hemi-Cellulase, Pectin (galactosidase, pectinesterase, etc.), and Chitin (Chitinase) (Lange *et al*.,2019).Most of these enzymes are involved in the hydrolysis of substrates found in the plant cell wall (Dally *et al*.,2017).The rumen fungi living in the digestive tract of herbivores are a group with an important role in the hydrolysis of the plant cell wall and have a high ligninolytic enzyme activity (Kar *et al*., 2021; Henske *et al*., 2018; Zahang *et al*., 2016). In previous studies,

it was reported that microorganisms with CAZy enzyme activity can hydrolyze plant biomass at a high rate (Dally *et al*., 2017; Min *et al*., 2017).

Research studies show that rumen fungi have a high degree of lignocellulolytic enzyme activity, these enzymes are present in studies such as isolation or cloning of rumen fungi, and there are also numerous enzyme groups in the cazy and mycocosm databases that belong to the CAZy enzyme family.

Futhermore, based on our review of the related research, the rumen fungi can be reported to have CAZy activity, Neocallimaxsp can be used for the xylanase enzyme, which is industrially important, and Orpinomyces sp. can be considered as candidate microorganisms.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Halit Yucel: investigation, visualization, and writing**. Kubra Ekinci:** investigation, literature compilation, and writing**.**

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