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Review Article

Glycoside hydrolases from (hyper)thermophilic archaea: structure, function, and applications

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(Hyper)thermophilic archaeal glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds to break down complex sugars and polysaccharides at high temperatures. These enzymes have an unique structure that allows them to remain stable and functional in extreme environments such as hot springs and hydrothermal vents. This review provides an overview of the current knowledge and milestones on the structures and functions of (hyper)thermophilic archaeal glycosidases and their potential applications in various fields. In particular, this review focuses on the structural characteristics of these enzymes and how these features relate to their catalytic activity by discussing different types of (hyper)thermophilic archaeal glycosidases, including β-glucosidases, chitinase, cellulases and α-amylases, describing their molecular structures, active sites, and mechanisms of action, including their role in the hydrolysis of carbohydrates. By providing a comprehensive overview of (hyper)thermophilic archaeal glycosidases, this review aims to stimulate further research into these fascinating enzymes.

Introduction

Glycosidases (GHs) (EC 3.2.1.-) are enzymes, present in all living organisms and some types of viruses, able to break the glycosidic bonds, the most stable covalent bond in nature (half-life of 4.7×10^9 years) [1], accelerating its hydrolysis up to 17 orders of magnitude if compared to the spontaneous reaction. This peculiarity makes the GHs reaction one of the most efficient catalytic mechanisms known [2].

Traditionally, GHs are classified according to their substrate specificity, by the IUBMB Enzyme nomenclature, and by their reaction mechanism. A further classification, based on the anomeric specificity of the hydrolysed glycosidic bond, allows classifying the GHs as 'α-D-/β-L-glycosidases' and 'β-D-/α-L-glycosidases'. This macro-distinction is deepened by further structural characteristics of the substrate, such as the shape of the hemiacetal/ hemiketal ring (pyranosides or furanosides) and by the position of the cleavage site, within the substrate molecule, allowing the classification into exo- and endo-glycosidases. Exo-glycosidases release monosaccharides from the terminals of the substrates, while the endo-glycosidases act within the chains of glycan and glycoconjugates [3]. In addition to the classification based on the anomeric bond, Koshland's studies allowed to classify GHs, depending on their catalytic mechanism, as *retaining* and *inverting* [4]. Briefly, *retaining* GHs act through a two-step mechanism (SN2) with the formation of a covalent intermediate, leading to preserve the configuration of the anomeric carbon from the substrate to the product. Conversely, *inverting* GHs act according to a single-step acid/base catalysis (SN1) with the formation of a product having an inverted configuration compared to the original substrate. For further details and insights on the molecular mechanisms of glycosidases please refer to Chapter 8 of the volume 'Comprehensive Natural Products II' [5]. Retaining glycosidases also arouse considerable interest in the chemo-enzymatic synthesis of glycoconjugates, both

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through transglycosylation [6] and protein engineering, which has allowed the development of glycosynthases. Indeed, glycosynthases are engineered GHs able of synthesizing glycans in quantitative yields, overcoming the intrinsic limitations of glycosidases during transglycosylation, and which have proven to be convenient alternatives to the more expensive glycosyltransferase. For an in-depth reading of the topic, see the review *'Glycosynthases in Biocatalysis'* [7].

A world-known classification of GHs is found in Carbohydrate Active enZymes database (CAZy, www.cazy.org) which classifies GHs into families, based on their primary structure, and into clans based on their tertiary structure. The CAZy database is constantly updated and currently (April 2023) presents 180 GHs families (from GH1 to GH180) and 18 clans (from GH-A to GH-R), representing the main resource for the study and characterization of these biocatalysts, and for other CAZymes, namely glycosyltransferases, polysaccharide lyases, carbohydrate esterases, as well as carbohydrate-binding modules and auxiliary activities cover redox enzymes that act in conjunction with CAZymes [8].

Among the GHs, those identified in (hyper)thermophilic archaea are of great applicative interest. The use of (hyper)thermophilic GHs allows working in conditions in which mesophilic enzymes would undergo rapid denaturation at high temperatures (above 50°C), extreme of pHs and high concentrations of detergents [9]. In particular, the possibility of operating at high temperatures, without incurring denaturation, provides several advantages such as a reduced possibility of contamination, a decrease in viscosity, and an increase in the diffusion coefficient, necessary to increase the solubility and availability of organic substrates during the reaction, allowing more efficient biocatalysis [10]. To date, in the CAZy database, the 120 archaeal (hyper)thermophilic GHs are distributed in 21 families (Table 1) including enzymes active on both anomeric configuration bonds. These enzymes are grouped into different CAZy families (Figures 1 and 2) and taxonomically assigned to a total of 17 genera, with the most present in the *Saccharolobus*, *Pyrococcus* and *Thermococcus* genera (Table 2). In the last decade, the impact of the 'omics' approaches on enzyme discovery, applied to geothermal environments characterized by extreme temperatures, has recently allowed the identification of new GHs in a completely culture-independent way, demonstrating that the limits of discoveries in this area are very far from being reached [11,12]. For further details regarding the possible "omics" strategies of enzyme discovery from (hyper)thermophilic microorganisms, possible advantages and limitations, we refer the reader to De Lise et al. 2023 and Strazzulli et al. 2017 [13–15].

Exo-glycosidases

Exo-glycosidases active on α-D-glycosides

α-D-glucosidases and glucan 1,4-α-D-glucosidases

The α-glucosidases (EC 3.2.1.20) are a large group of enzymes that catalyze the hydrolysis of the α-glycosidic bond from the non-reducing end of oligosaccharide chains such as starch and glycogen, as well as from free disaccharides such as maltose, and are of particular biotechnological interest, together with glucoamylase and α-amylases, for the saccharification and liquefaction of starch [16]. At the level of (hyper)thermophilic archaea, these activities are mainly reported in the GH31 and GH122 families. While the first family (clan GH-D, $(\alpha/\beta)_8$ barrel structure) groups different (hyper)thermophilic exo-glycosidase activities such as α-mannosidase and α-xylosidase (see below), and numerous other activities widely distributed in the three domains of life [17]; the GH122 family currently includes 79 sequences, all of (hyper)thermophilic archaeal origin and of which only one has been characterized, the α-glucosidase PF0132 from *Pyrococcus furiosus*. PF0132, purified and characterized in native form, is a homodimer of about 125 kDa with an optimal pH of 5.5 and an optimal temperature between 105 and 115°C. Although never expressed and purified in recombinant form, the native enzyme showed remarkable thermostability with a half-life of 48 h at 98°C and with a residual activity of 80% after 30 min at 98°C in the presence of 1.0 M urea or 100 mM DTT [18]. PF0132 showed activity on pNP-α-Glc as well as on natural disaccharides of glucose such as bready, isomaltose and maltose on which it has the best specificity constant (225 s^{-1} $mM^{-1)}$. On the contrary, to date, there are six α-glucosidases of the GH31 family characterized from (hyper)thermophilic archaea [19–23]. MalA α-glucosidase from *Saccharolobus solfataricus* (strains 98/2 and P2, respectively), formerly *Sulfolobus solfataricus* [24], is a 480 kDa homohexamer composed of a monomer structured in four main structural domains (N, A, C, and D) whose catalytic domain (A) is positioned in the center, and two subdomains B and B' (PDB ID: 2G3M) [17]. MalA preferentially hydrolyses maltose and small maltooligosaccharides and is also moderately active on isomaltose. This enzyme, however, was particularly active both on pNP-α-Glc and on pNP-α-Man showing comparable K_M values (1.74 vs 3.82 mM, respectively) but a specificity toward the α -glucosidic substrate 1000-fold higher than the α-mannosidic one [19,20]. It is important to underline that MalA from *S. solfataricus* represents, to date, the only member of the GH31 family to have been engineered in glycosynthase (Asp320Gly mutant) and capable of synthesizing mannoinositols starting from β-D-Man-F

Table 1 Distribution of characterized archaeal (hyper)thermophilic GHs[1] in CAZy Database families (www.cazy.org)

EC number[2]	Activity	GH0[3]	GH1	GH116	GH12	GH122	GH13	GH15	GH16	GH18	GH2	GH29	GH3	GH31	GH35	GH36	GH38	GH5	GH53	GH57	GH176	GH179
3.2.1.-	**Glycosidase**	1																				
3.2.1.1	**α-amylase**						5													7		
3.2.1.100	**mannan 1,4-mannobiosidase**																	1				
3.2.1.108	**lactase**		1																			
3.2.1.133	**glucan 1,4-α-maltohydrolase**																			3		
3.2.1.14	**chitinase**									2												
3.2.1.141	**4-α-D-{(1→4)-α-D-glucano} trehalose trehalohydrolase**						3															
3.2.1.165	**exo-1,4-β-D-glucosaminidase**														2							
3.2.1.177	**α-xylosidase**													1								
3.2.1.20	**α-glucosidase**					1								6								
3.2.1.200	**exo-chitinase (non-reducing end)**									1												
3.2.1.21	**β-glucosidase**		17	2									1									
3.2.1.22	**α-galactosidase**															2				1		
3.2.1.23	**β-galactosidase**		8																			
3.2.1.24	**α-mannosidase**													2			2					
3.2.1.25	**β-mannosidase**		3																			
3.2.1.28	**trehalase**							5														
3.2.1.3	**glucan 1,4-α-glucosidase**							5														
3.2.1.31	**β-glucuronidase**										1											
3.2.1.37	**xylan 1,4-β-xylosidase**			1									1									
3.2.1.38	**β-fucosidase**		1																			
3.2.1.39	**glucan endo-1,3-β-D-glucosidase**								1													
3.2.1.4	**cellulase**				5													4				
3.2.1.41	**pullulanase**						1													10		
3.2.1.51	**α-fucosidase**											1										
3.2.1.52	**β-N-acetylhexosaminidase**			1																		1
3.2.1.54	**cyclomaltodextrinase**						3													2		
3.2.1.55	**α-L-arabinofuranoside**												1									
3.2.1.68	**isoamylase**						2														1	
3.2.1.89	**endo-1,4-β-galactanase**																		1			
	Total eznymes for GH family	1	30	4	5	1	14	10	1	3	1	1	3	9	2	2	2	5	1	23	1	1

[1]GHs from hyperthermophilic archaea
[2]Data taken from in CAZy Database on April 2023.
[3]Non-classified sequences

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Table 2 Microbial distribution of achaeal (hyper)thermophilic GHs[1] at genera level

EC number[2]	Activity	Acidilobus	Caldivirga	Desulfurococcaceae	Desulfurococcus	Ignisphaera	Metallosphaera	Methanocaldococcus	Picrophilus	Pyrococcus	Saccharolobus	Staphylothermus	Sulfolobus	Sulfurisphaera	Thermococcus	Thermofilum	Thermoplasma	Thermosphaera	uncultured
3.2.1.-	**Glycosidase**														1				
3.2.1.1	**α-amylase**							1		4	1		2		4				
3.2.1.100	**mannan 1,4-mannobiosidase**																		1
3.2.1.108	**lactase**																		1
3.2.1.133	**glucan 1,4-α-maltohydrolase**									2					1				
3.2.1.14	**chitinase**									1					1				
3.2.1.141	**4-α-D-{(1→4)-α-D-glucano} trehalose trehalohydrolase**						1				1		1						
3.2.1.165	**exo-1,4-β-D-glucosaminidase**									1					1				
3.2.1.177	**α-xylosidase**										1								
3.2.1.20	**α-glucosidase**									1	2		1	1			1		1
3.2.1.200	**exo-chitinase (non-reducing end)**														1				
3.2.1.21	**β-glucosidase**	1	2							3	6		1		3	1	1	1	1
3.2.1.22	**α-galactosidase**									1	1			1					
3.2.1.23	**β-galactosidase**	1								3	1				2		1		
3.2.1.24	**α-mannosidase**								2		2								
3.2.1.25	**β-mannosidase**									3									
3.2.1.28	**trehalase**												3				2		
3.2.1.3	**glucan 1,4-α-glucosidase**							1	1		1			1			1		
3.2.1.31	**β-glucuronidase**										1								
3.2.1.37	**xylan 1,4-β-xylosidase**										2								
3.2.1.38	**β-fucosidase**																		1
3.2.1.39	**glucan endo-1,3-β-D-glucosidase**									1									
3.2.1.4	**cellulase**		1	1						3	3								1
3.2.1.41	**pullulanase**		1		2					1		1	1		4	1			
3.2.1.51	**α-fucosidase**										1								
3.2.1.52	**β-N-acetylhexosaminidase**										1								1
3.2.1.54	**cyclomaltodextrinase**		1							1		1			2				
3.2.1.55	**α-L-arabinofuranoside**										1								
3.2.1.68	**isoamylase**										1		1		1				
3.2.1.89	**endo-1,4-β-galactanase**					1													
	Total enzymes	**2**	**5**	**1**	**2**	**1**	**1**	**1**	**3**	**25**	**26**	**2**	**10**	**3**	**21**	**2**	**6**	**1**	**7**

[1]GHs from hyperthermophilic archaea.
[2]Data taken from in CAZy Database on April 2023.

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(*donor)* and myo-inositol (*acceptor)* with a final yield of 41% [19]. A member of GH31 active on maltooligosaccharides has also been identified in the thermophilic archaea *Thermoplasma acidophilum* (AglA). AglA was able to perform hetero-condensation by transglycosylation by using maltose as *donor* and arbutin as *acceptor*, in contrast with the α-galactosidase of *Sulfurisphaera tokodaii* of which, to date, only homo-condensation on maltose is reported [22]. Particularly interesting was the inhibitory effect of aesculin on AglA, which showed a non-competitive mixed inhibition mechanism by both [EI] and [ESI] complex formation, while maintaining a classic competitive profile in the presence of acarbose as well as other mesophilic GH31 enzymes [25]. More recently, a novel α-glucosidase (Aglu1) from the GH31 family has been identified by a metagenomic approach from laboratory enrichment on lignocellulosic biomass. Aglu1, taxonomically associated with an uncultured archaeon belonging to the Sulfolobaceae family, has shown to date to be the archaeal α-glucosidase member with the highest specificity towards kojibiose (352.49 s^{-1} mM^{-1}) [23].

Another class of enzymes involved in the hydrolysis of α-glucosidic bonds is that of glucan 1,4-α -D-glucosidases also known as glucoamylases (EC 3.2.1.3). These enzymes are capable of hydrolysing a glucoside bound in α-(1,4) at the non-reducing end of starch, amylopectin, amylose, glycogen and maltooligosaccharides, producing a β-D-glucose unit through an *inverting* mechanism. In the CAZy database, these activities are present in the GH15 and GH97 families. While the GH97 family mainly groups sequences of bacterial origin and from halophilic archaea, the GH15 family (GH-D clan, structure $(\alpha/\alpha)_6$ barrel), to date contains several sequences of (hyper)thermophilic archaeal origin, of which only five characterized and belonging to *Methanocaldococcus jannaschii* (MGA), *Picrophilus torridus* (PTO1492), *S. solfataricus* (SSG), *S. tokodaii* (STGA), and *T. acidophilum* (TaGA). Unlike the extracellular fungal glucoamylases predominantly active on polysaccharides containing α-(1,4) bonds, the (hyper)thermophilic archaeal glucoamylases of the GH15 family are intracellular and show high specificity towards short-chain matooligosaccharides such as Glc3 (SSG, PTO1492, and STGA), Glc4 (MGA) and Glc5 (TaGA) [26–30]. It is important to underline that, despite its intracellular nature, STGA is able to convert starch into glucose at 90°C in 4 h of reaction with a final yield of 95%, resulting faster than the fungal counterpart which requires 24–60 h to reach the same yields [28].

α-D-mannosidases

The α-D-mannosidases (EC 3.2.1.24) are exo-glycosidases capable of hydrolysing a mannose unit from the non-reducing end of α-D-mannosidic substrates. They are of particular interest in the pharmaceutical and diagnostic fields as α-D-mannosidases are involved in the glycosylation and maturation of glycoproteins [31]. According to the CAZy classification, these enzymes are grouped into the GH31, GH38, and GH92 families, of which only the first two families (*retaining*) include archaeal (hyper)thermophilic activities.

As mentioned above, concerning the GH31 family, MalA of *S. solfataricus* represents the only archaeal glycosidase of the family possessing also an α-mannosidase activity, exploited in particular for the chemoenzymatic synthesis of mannoinositols [19]. On the contrary, in the GH38 family, which includes several enzymes active on α-mannosidic substrates, such as mannosyl-oligosaccharide α-1,2-mannosidase (EC 3.2.1.113), mannosyl-oligosaccharide α-1,3-1,6-mannosidase (EC 3.2.1.114), mannosyl-oligosaccharide α-1,3-mannosidase (EC 3.2.1.207), and exo-α-1,6-mannosidase (EC 3.2.1.163), to date, the only characterized archaeal α-mannosidases are ManA from *P. torridus* (ManA) and SsαMan from *S. solfataricus*. ManA is a homodimeric α-mannosidase of approximately 220 kDa, active on different types of mannobioses, and, in particular with a k_{cat} /K_M of 210 s^{-1} mM^{-1}, on pNP-α-D-Man [32]. SsαMan is an α-mannosidase of class II (a class of mannosidases active on α-1,3, α-1,6, and α-1,2 linkages [33]) that has been purified and characterized in both native and recombinant form. SsαMan showed a k_{cat} /K_M on pNP-α-D-Man of 351 and 54 s^{-1} mM^{-1} for the native and recombinant form, respectively. In addition, SsαMan recognizes as substrate also the *N*-glycosylation typical oligosaccharides, such as $Man_3GlcNAc_2$ and $Man_7GlcNAc_2$ and is also directly active on glycoproteins, removing sequentially mannose units from the glycosidic antenna of RNAseB, allowing to hypothesize for this enzyme a role in the modification of glycoproteins *in vivo* [34].

α-D-xylosidases

The α-D-xylosidases (EC 3.2.1.177), which catalyze the removal of a xylose unit from an α-xylosidic substrate, within the CAZy database, are exclusively classified in the GH31 family and are of particular interest for biotechnological applications related to the degradation of xyloglucan as in second generation biorefineries [35].

Of the 13 α-xylosidases currently characterized (http://www.cazy.org/GH31_characterized.html), XylS *from S. solfataricus* is the only archaeal α-xylosidase, while the remaining belong to bacteria (7) and eukarya (5). XylS, unlike the other GH31 from the same microorganism (the α-glucosidase MalA), has a native monomeric structure of ∼72 kDa and is active on isoprimeverose (xylopyranosyl-α(1,6)-glucopyranose) and pNP-β-isoprimeveroside showing a specificity 4- and 33-fold higher than that on pNP-α-Xyl, respectively, suggesting that XylS could be involved in the

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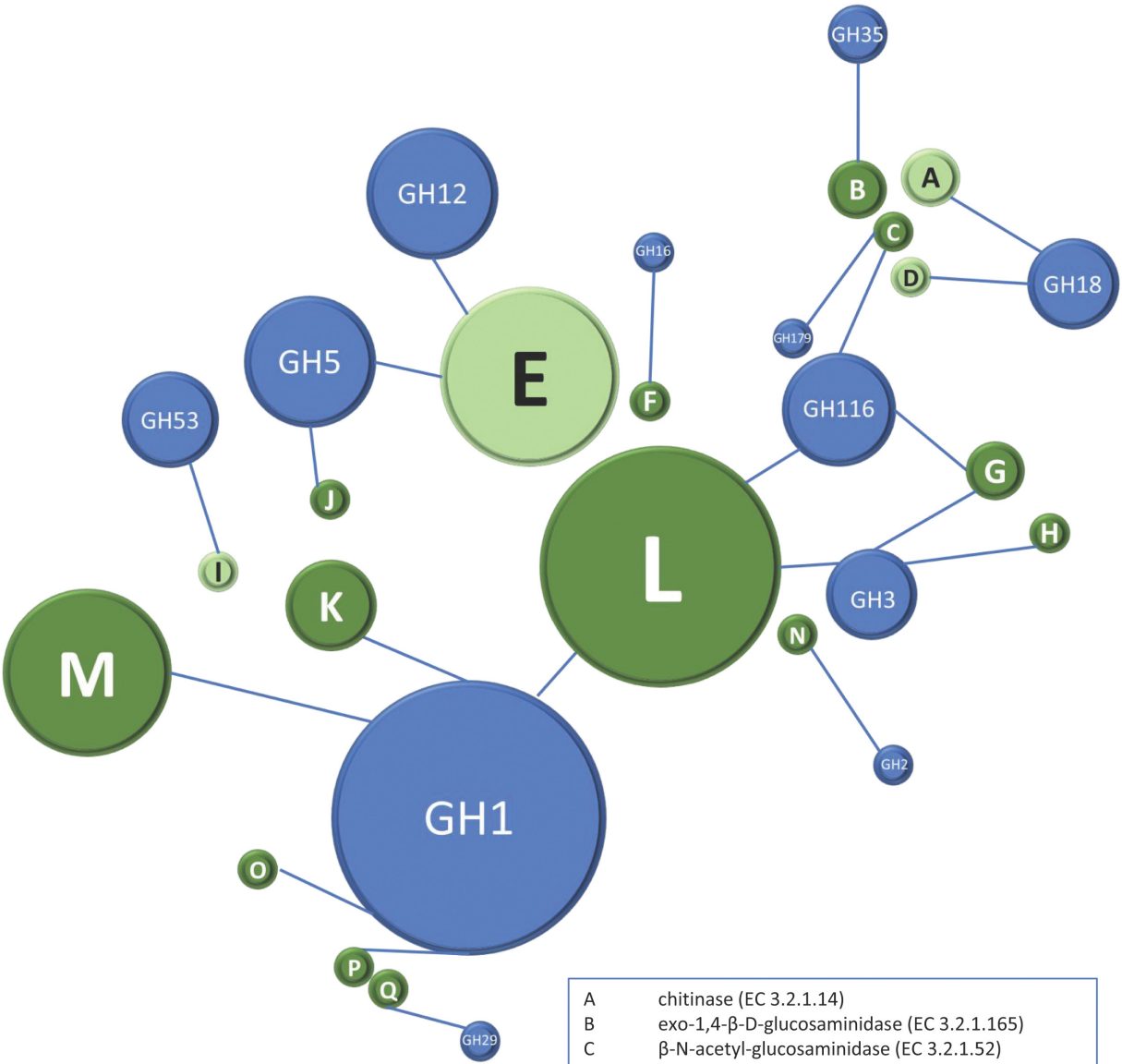
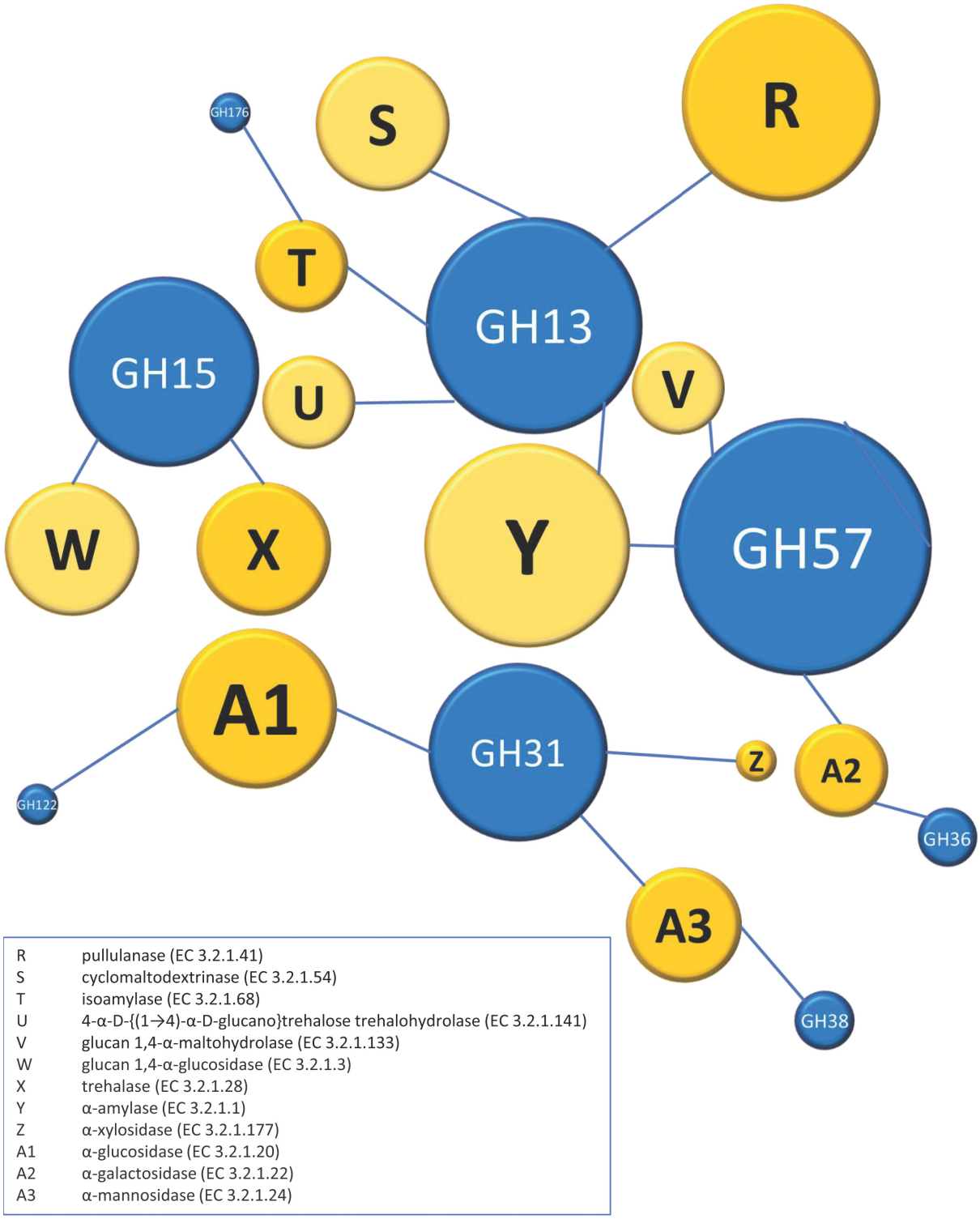


Figure 1. Distribution of glycosidases active on β-D- and α-L-glycosides (as EC numbers) in GHs families

In dark green are exo-activities. In light green are endo-activities. The size of the circles is proportional to number of enzymes present inside. In the box, the legend of the letter code.



R	pullulanase (EC 3.2.1.41)
S	cyclomaltodextrinase (EC 3.2.1.54)
T	isoamylase (EC 3.2.1.68)
U	4-α-D-{(1→4)-α-D-glucano}trehalose trehalohydrolase (EC 3.2.1.141)
V	glucan 1,4-α-maltohydrolase (EC 3.2.1.133)
W	glucan 1,4-α-glucosidase (EC 3.2.1.3)
X	trehalase (EC 3.2.1.28)
Y	α-amylase (EC 3.2.1.1)
Z	α-xylosidase (EC 3.2.1.177)
A1	α-glucosidase (EC 3.2.1.20)
A2	α-galactosidase (EC 3.2.1.22)
A3	α-mannosidase (EC 3.2.1.24)

Figure 2. Distribution of glycosidases active on α-D-glycosides (as EC numbers) in GHs families

In dark yellow are exo-activities. In light yellow are endo-activities. The size of the circles is proportional to number of enzymes present inside. In the box, the legend of the letter code.

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degradation of xyloglucan in archaea [36].More recently it has been shown that XylS, together with two other enzymes of *S. solfataricus*, the GH1 β -glucosidase/β-galactosidase SsβGly and the GH29 α-L-fucosidase SsαFuc (see below), allows the synergistic degradation of xyloglucan oligosaccharides from tamarind and apple pomace [37]. It is worth noting that, besides the activity on (hemi)cellulosic substrates, XylS is also active on α-dystroglycan together with β-glucuronidase (Bgus) from *Thermotoga maritima* [38].

α-D-galactosidases

In the CAZy database, α-D-galactosidases (EC 3.2.1.22) are distributed in seven different families, namely, GH4, GH27, GH31, GH36, GH57, GH97, and GH110. The α-galactosidases can find use in various biotechnological applications such as food processing, pulp and paper industry, for the synthesis of oligosaccharides by chemo-enzymatic approaches, and in medical applications [7,39].

To date, the α-galactosidases from (hyper)thermophilic archaea characterized belong exclusively to the GH36 and GH57 families, both presenting a *retaining* catalytic mechanism.

The GH36 family, which belongs to the GH-D clan, includes not only the α-galactosidases but also other activities such as α-*N*-acetylgalactosaminidase (EC 3.2.1.49), stachyose synthase (EC 2.4.1.67), and raffinose synthase (EC 2.4.1.82), none of them archaeal. The two α-galactosidases characterized to date are GalS from *S. solfataricus* and GalSt from *S. tokodaii*, which share 64% identity. GalS and GalSt represent two of the most thermoactive α-galactosidases characterized to date and, although they are significantly different at the level of amino acid sequence, their catalytic properties, such as the pH_{opt} 5.0 and the T_{opt} 90°C, are comparable [40]. However, about the GH36 family, it is interesting to note that most of the enzymes belonging to this family show a pH_{opt} neutral or slightly alkaline. This peculiarity has been related to the presence, in these enzymes, of an Asp residue opposed to the acid/base catalyst and which could increase the p*K*a by requiring a higher pH for the catalysis to take place. Conversely, since GalS and GalSt contain an Ile residue at the corresponding location, their optimal activity could therefore be at a slightly acidic pH [40].

The GH57 family currently includes, in addition to α-galactosidases, also other members in particular associated with the hydrolysis of polysaccharides and oligosaccharides containing α-(1,4)-and α-(1,6)-glycosidic linkages and characterized by a $(\beta/\alpha)_7$ barrels structure (see below).

To date, the only archaeal (hyper)thermophilic α-galactosidase characterized and belonging to this family is Gal57 from *P. furiosus*. This enzyme shows the highest homology with other members of the GH57 family in the N-terminal part of the protein, which is considered the characterizing domain of the family itself (usually also associated with one or more additional domains). This conserved domain was also shared with class I α-mannosidases of family 38 [41].

α,α-Trehalases

The α,α-trehalases (EC 3.2.1.28) hydrolyse trehalose, a disaccharide consisting of two glucose units joined by an α,α-(1,1) bond, to produce two glucose molecules and have potential applications in several fields, including food additives, development of insecticides and transgenic plants [42]. In the CAZy database, these are grouped into three families: GH15, GH37, and GH65. To date, the only archaeal (hyper)thermophilic trehalases belong to the GH15 family and are respectively from *Thermoplasma volcanium*, *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*. In *S. acidocalarius*, in particular, two trehalases have been identified, namely SaTreH1 and SaTreH2 which share 44% identity. Although these two enzymes belong to the same microorganism and the same GH family, they present peculiar features in terms of T_{opt} and thermostability. In detail, SaTreH1 has its maximum activity at 60°C while SaTreH2 has an optimum of 70°C. With regards to thermostability, SaTreH1 is stable for 60 min at temperatures between 30 and 70°C, although it exhibits a loss of activity after 60–80°C. Conversely, SaTreH2 showed greater stability than SaTreH1 maintaining more than 80% residual activity after 60 min at 80°C. Although SaTreH2 shows a 15-fold greater affinity for trehalose than SaTreH1, the two specificity constants on this substrate are comparable (1.0 vs 1.8 s^{-1} mM^{-1}, respectively). Moreover, SaTreH2 is the only of the two to show a substrate inhibition effect as already observed for fungal glucoamylases of the same family GH15 [43]. Similar kinetic constants were also measured for the GH15 trehalases identified in species belonging to the genus Thermoplasma TVN1315 (*T. volcanium)* and Ta0286 (*T. acidophilum)*, which showed trehalose specificity values of 1.29 and 1.66 s^{-1} mM^{-1}, respectively [43]. These values are considerably lower if compared with the trehalases belonging to the GH37 and GH65 families. Indeed, it has been suggested that this effect, mainly due to the ∼10-fold higher K_M values for GH15, could be structurally related to a portion of the substrate binding site in GH15 that differs substantially from that of the other two families [43].

Exo-glycosidases active on β-D- and α-L-glycosides

β-Glucosidases and β-N-acetyl-glucosaminidases

The β-glucosidases (EC 3.2.1.21) catalyze the hydrolysis of β-glycosidic bonds present in either disaccharides, oligosaccharides, or so-called conjugated glucoside resulting in release of β-D-glucose [44,45]. These enzymes are extensively studied for their applications in the food, feed, textile, and paper industries [46]. Moreover, β-glucosidases have been used for ethanol production in the process of saccharification of cellulose [47].

β-Glucosidases have been found in 10 GHs families [8], but only characterized members of GH1, GH3, and GH116 families came from (hyper)thermophilic archaea [48–55].

The most known enzymatic activities from (hyper)thermophilic archaeal GH1 are β-glucosidases (EC 3.2.1.21) and β-galactosidases (EC 3.2.1.23). However, other commonly found activities are β-mannosidase (EC 3.2.1.25), β-D-fucosidase (EC 3.2.1.38) and lactase (EC 3.2.1.108). The β-glycosidase (Ssβgly) isolated from *S. solfataricus*, represents an interesting example of a well-characterized GH1. This enzyme has broad substrate specificity for β-D-glycosides, such as galacto-, gluco-, and fucosides, under a wide range of temperature and pH conditions [49]. Also, Ssβgly showed high efficiency on glucose oligomers in the following order cellotetraose > cellotriose > cellobiose. This enzyme, as well as CelB from *P. furiosus* [56] and Asβ-Gly from *Acidilobus saccharovorans* [57], crystallizes as an asymmetric homotetramer, in which a dimer is present in the asymmetric unit [58]. The monomer shows a typical structure $(\beta/\alpha)_8$ barrel of GH-A clan. The active site is located at the center of the tetramer at the top face of the barrel, connected to the surface by a radial channel, probably acting as the binding site for extended oligosaccharide substrates [58]. The length of this channel is consistent with the observation of efficient exo-glycosidase activity against substrates as long as five sugar units [58,59]. The structure of SsβGly has been used as model to understand the molecular bases of the protein thermostability [58,60]. Indeed, some features of Ssβgly significantly differ from the mesophilic members of GH1: (i) a large proportion of surface involved in ion-pairs networks, (ii) many solvent molecules buried in the hydrophilic cavities of protein core, and (iii) a non-conserved region of 28 amino acid residues (Pro91– Asp119) with unordered structure, which buried two α-helices of the barrel. These features suggest a model for thermostability via resilience rather than rigidity [58]. SsβGly was the first thermophilic glycosidase engineered into a glycosynthase, by removing the carboxylic nucleophile group in the enzyme [61]. Ssβgly mutant Glu387Gly, based on different external nucleophiles used in the reaction, can act as both *retaining* and *inverting* glycosynthase [62]. The same approach has been extended to two other β-glycosynthases, from the (hyper)thermophilic archaea *Thermosphaera aggregans* and *P. furiosus* (Taβ-gly and CelB, respectively) [63].

In family GH3 only the β-glucosidase Tpbgl from the archaeon *Thermophilus pendens* has been characterized [53]. Similarly to GH1, Tpbgl is active on β-glucosides and β-mannosides and, as member of GH3, it is classified into GH Clan-A. Due to its high stability in the presence of DMSO, Tpbgl is a promising candidate for the production of the phytoestrogen genistein by enzymatic conversion of the soybeans [53].

As reported above, β-glucosidases are also included in the GH116 family [8]. GH116 contains mammalian non-lysosomal bile acid β-glucosidase GBA2 with glucosylceramidase activity (EC 3.2.1.45), but also β-xylosidase (EC 3.2.1.37) and β-N-acetylglucosaminidase (EC 3.2.1.52) activities [8]. This family has been defined based on the characterization of the bifunctional β-glucosidase/β-xylosidase SSO1353 from *S. solfataricus* [55]. Although the common characteristic of family GH116 enzymes is the specificity for β-glucosides, SSO3039 from *S. solfataricus* had higher specific activity on β-N-acetyl-glucosaminide and, to the best of our knowledge, this enzyme is the first β-*N*-acetylglucosaminidase (EC 3.2.1.52) characterized from (hyper)thermophilic archaea so far [54], and it is putatively involved in the turnover of *N*,*N*-diacetylchitobiose in *N*-glycans [64]. GH116 family is composed of three subfamilies with distinct substrate specificities and inhibitor sensitivities [54]: subfamily 1 contains TxGH116 and the human non-lysosomal glucosylceramidase (GBA2) only sensitive to *N*-butyldeoxynojirimycin (NB-DNJ) [55,65]; subfamily 2 includes SSO3039, sensitive to both NB-DNJ and conduritol β-epoxide (CBE), but with different IC_{50} (μM vs mM, respectively) [54]; subfamily 3 contains SSO1353, which has mM sensitivity to both inhibitors [54,55]. In family GH116, only crystallographic structure of TxGH116 from bacterium *Thermoanaerobacterium xylanolyticum* has been solved. This family belong to Clan GH-O and shows no structural similarity to other retaining β-glucosidases from clan GH-A families, including GH1. Indeed, in GH116 the active site is found in $(\alpha/\alpha)_6$ solenoid domain at C-terminus. Moreover, the general acid/base residue in TxGH116 is in a completely different position in the active site compared to the general acid/base in GH1. While in GH116 this residue is found above carbons 3 and 4 (C3-C4) of the pyranose ring, in clan GH-A enzymes it is located lateral to the ring on the C1-C2 bond side [66].

Recently a novel β-*N*-acetylglucosaminidase (GH109_Pool2) from an uncultured archaeon was identified by sequence-based metagenomic approach from a solfataric mud pool at 92°C and pH 1.5 [11]. This enzyme, initially

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assigned to the GH109 family, has recently been reannotated in the CAZy database as belonging to the newly introduced GH179 family and of which it represents (April 2023) the only characterized entry (http://www.cazy.org/GH179_characterized.html). GH109_Pool2, expressed in recombinant form with the NusA fusion tag was found to be active on 4NP-β-D-GlcNAc and 4NP-β-D-Glc with a k_{cat}/K_M of 50.91 and 39.58 mM^{-1} S^{-1}, respectively, with a T_{opt} of 85°C and pH_{opt} 8.0. The enzyme, whose structure has not yet been determined to date, also exhibits a hydrolytic activity strictly dependent on NAD^+ with a Kd of 32 μM suggesting a clear analogy, both regarding the catalytic mechanism and the three-dimensional structure, with members of the GH4 family and GH109 [11].

β-Galactosidases and β-glucuronidases

Most of characterized (hyper)thermophilic β-glucosidases showed also β-galactosidase activity, such as Asβ-Gly from *A. saccharovorans* [67]. CelB from *P. furiosus* [56], Tkβgly from *Thermococcus kodakarensis* [48], Ssβgly from *S. solfataricus* [59], TVG0691226 from *T. vulcanium* [68] and Bgl1 from a hydrothermal spring metagenome collected in Azores (Portugal) [69]. β-Galactosidases (EC 3.2.1.23) catalyze the hydrolysis of β-1,4-D-galactosidic linkages resulting in release of β-D-galactose. Asβ-Gly, CelB and Ssβgly showed the highest specific activity (U/mg) on β-galactosides but a lower specificity constant (k_{cat}/K_M) compared with β-glucosides [56,59,67]. Instead, Bgl1 had a similar specific activity on both substrates [69]. Nowadays, only β-galactosidases from family GH1 have been characterized from (hyper)thermophilic archaea. As reported above, they have a well-conserved structure defined by a $(\beta/\alpha)_8$-barrel fold.

The most common natural substrate for β-galactosidases is lactose, the main sugar of milk and dairy products. A large number of people suffer from intestinal dysfunctions after the consumption of milk. Then, β-galactosidases are employed to produce lactose-free milk products [70]. Li and collaborators used β-glucosidases from *P. furiosus* to hydrolase lactose in milk under pasteurization conditions. The authors were able to obtain the 90% of lactose hydrolysis in 30 min at 65°C by using 498 U/ml of the recombinant β-glucosidase [71]. β-Galactosidases can be also used to produce high-sweetness syrup, an additive in ice creams and desserts [72]. In addition, these enzymes can be employed to produce bioactive constituents. In fact, Noh and co-workers reported the efficient production of the ginsenoside compound K, a metabolite with anti-tumor, anti-inflammatory, anti-allergic, and hepatoprotective effects, by using β-glycosidase from *S. solfataricus* on Ginseng root extract [73]. In molecular biology, the gene of Ssβgly is used as a reporter at high temperature by using the well-known substrate X-Gal that is converted into a blue-colored product and can be detected readily on Gelrite plates [74]. Similarly, the β-glucuronidase (EC 3.2.1.31) GusB from *S. solfataricus*, belonging to family GH2 and catalyzing the hydrolysis of β-D-glucuronic acid residues, has been used as reporter for thermophilic microorganism [75].

β-Mannosidases and exo-β-1,4-mannobiohydrolases

β-Mannosidases (EC 3.2.1.25) and exo-β-1,4-mannobiohydrolases (EC 3.2.1.100) are exo-acting enzymes, which act on the non-reducing ends of mannooligosaccharides or mannobiose to hydrolyse the terminal β-D-mannopyranosyl residues. In family GH1, β-glucosidase also displayed β-mannosidase activity, although with lower specificity constant compared with β-glucosides and β-galactosides [56,59,67]. However, GH1s from Pyrococcus species, BmnA from *P. furiosus* [76] and BglB from *P. horikoshii* [77], showed the highest activity on β-mannosides and, then, they have been classified as β-mannosidases (EC 3.2.1.25). Although this activity (EC 3.2.1.25) has been found in families GH1, GH2, GH5, GH113, and GH164, β-mannosidases from (hyper)thermophilic archaea have been characterized only in family GH1. In *P. furiosus*, both GH1s CelB and BmnA act as β-glucosidase and β-mannosidase with different specificities. Based on both amino acid sequence (46.5% of sequence identity), and substrate specificity, the β-glucosidase CelB and β-mannosidase BmnA are closely related [56]. However, it is not clear whether the two enzymes in *P. furiosus* represent a case of gene duplication, in which an original gene constitutes a template for constructing an enzyme with activity directed to a new stereochemically similar substrate [78], or if they share a common predecessor [76]. Instead, GH1 β-mannosidase BglB from *P. horikoshii* shares 56% and 37% of sequence identity with BmnA and CelB, respectively [77]. Moreover, this enzyme displayed very low hydrolytic activity if compared with BmnA and CelB [77]. Remarkably, in the sequence of BglB, the residues Gly77 and Asp206 have been found, differently from other GH1 members in which these residues are a highly conserved as arginine and asparagine, respectively. Kaper and co-workers published the characterization of mutants in which these unique residues of BglB were introduced in CelB and vice versa. CelB R77Q/N206D showed an increased efficiency for the hydrolysis of mannosides, but β-glucosidase activity was still the most dominant activity. Surprisingly, in BglB D206N mutant, the hydrolytic activity on all substrates increased about 10-fold and the affinity constant for the hydrolysis of β-glucosides was reduced 45-fold, making BglB D206N, effectively a β-glucosidase [77].

Recently, an exo-β-mannanase (EC 3.2.1.100) from a hydrothermal pool metagenome collected in Solfatara Pisciarelli (Pozzuoli, Italy) has been characterized [11]. This enzyme, named GH5_Pool2, belongs to family GH5 subfamily 19. GH5 is one of the largest CAZy families, including a plethora of different activities, acting as *retaining* enzymes. Despite that, GH5_Pool2 represents the first thermophilic member from this subfamily, possibly belonging to the archaea domain, showing an exo-β-mannanase activity [11]. As member of the GH5 family, this enzyme belongs to Clan-A having a classical $(\beta/\alpha)_8$ TIM barrel fold, as well as GH1 members [8].

In the last decade, promising industrial applications requiring β-mannanosidases have been suggested like enzymatic treatment of coffee beans, biobleaching of pulp, detergent additive, synthesis of maltooligosaccharides and production of fermentable sugars for the bioethanol industry [79].

Exo-β-D-glucosaminidase

Exo-β-D-glucosaminidase or GlcNase (EC 3.2.1.165) catalyzes the hydrolysis of the β(1– 4) linkage of chitosan oligosaccharides to remove a GlcN residue from the non-reducing termini, and they are classified into families GH2 and GH35 [8]. No archaeal GlcNases from family GH2 have been characterized. Instead, in family GH35, only members from (hyper)thermophilic archaea showed GlcNase activity while the characterized enzymes from bacteria and eukaryota have been classified as β-galactosidase. To the best of our knowledge, only GlcNases $GlmA_{Tk}$ from *T. kodakarensis* [80] and $GlmA_{Ph}$ from *P. horikoshii* [81] have been characterized. Both enzymes are involved in the last steps of the chitinolytic pathway, in which the disaccharide GlcNAc2 is hydrolysed into GlcN by a concerted action of the GlcNase and GlcNAc deacetylase [82]. The sequence identity between $GlmA_{Tk}$ and $GlmA_{Ph}$ is 63%, and both enzymes show the same substrate specificities and exist as dimers in solution, suggesting that their tertiary structures and catalytic mechanisms are probably identical. In 2017, Mine and collaborators published the crystal structures of both enzymes [83]. The monomer contains three distinct domains: the N-terminal domain composed by a $(\beta/\alpha)_8$-barrel, typical of GHs clan-A, in which active site is found; the second domain is an α/β fold domain, while the C-terminal domain contains antiparallel β-sheets. Interestingly, in the surface area between the two monomers, 29 hydrogen bonds and 16 salt bridges are created upon dimer formation, which intimately associated both monomers and might contribute to thermostability [83]. Remarkably, $GlmA_{Tk}$ shares structural and mechanistic features with β-galactosidases from both families GH35 and GH42. In particular, $GlmA_{Tk}$ has a cleft-type active site in their monomeric forms similar to GH42 β-galactosidases. In addition, a high number of conserved active site residues are shared between GH35 β-galactosidase and $GlmA_{Tk}$, allowing to discriminate glucosamine from galactose based on a little difference in the structure. Indeed, Asp178 of $GlmA_{Tk}$ plays an essential role in the discrimination of GlcN from galactose, whereas the equivalent in GH35 β -galactosidase is an Asn residue. These features suggested that GH35 and GH42 β -galactosidases have evolved by taking advantage of the structural features of archaeal β-D-glucosaminidase [83].

From a biotechnological point of view, chitinolytic enzymes are gaining great interest in the production of chitooligosaccharides (COS). COS have important biological effects, including antimicrobial, antiviral, antitumor, and antioxidant activities. Indeed, the global market for Chitin and Chitosan Derivatives estimated at US$7.9 Billion in the year 2022, and it is projected to reach a size of US$24.9 Billion by 2030 according to the 2023 report 'Chitin and Chitosan Derivatives: Global Strategic Business Report' (https://www.researchandmarkets.com/reports/338576).

β-Xylosidases and α-L-arabinofuranosidase

According to the CAZy database, β-xylosidases (EC 3.2.1.37) are currently grouped into 11 GHs families [8], but β-xylosidases from (hyper)thermophilic archaea have been characterized only in GH3 and GH116 families [55,84].Although the bifunctional aryl β-glucosidase/β-xylosidase SSO1353 in GH116 is defined as β-xylosidase based on its activity on aryl β-xylosides, the enzyme likely does not hydrolyse xylooligosaccharides [55]. Instead, GH3 XarS from *S. solfataricus* is involved in xylan degradation, as demonstrated by the combined action of a xylanase and XarS [84]. To the best of our knowledge, this enzyme is the only thermophilic archaeal bifunctional β-xylosidase/α-L-arabinofuranosidase (EC 3.2.1.55). XarS, together with the endo-xylanase SSO1354, makes *S. solfataricus* able to degrade xylan [84].

β-Xylosidases could be employed in many biotechnological processes: in enzymatic cocktails for the saccharification process in bioethanol production [85], for deinking recycled paper [86], processing wood pulp [87], improving bread dough baking and nutritional quality [88], hydrolysis of bitter xylosylated compounds from grape juice during extraction and liberation of aroma derived from xylosylated compounds of grapes during wine making [89] and hydrolysis of xylan to D-xylose residues for subsequent reduction to xylitol [90].

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α-L-fucosidases

α-L-fucosidases (EC 3.2.1.51) are exo-glycosidases capable of cleaving α-linked L-fucose residues from glycoconjugates. These enzymes are found in families GH29, GH95, GH141 and GH151 [8], but only in GH29 (hyper)thermophilic α-L-fucosidase from *S. solfataricus* (Ssα-fuc) has been identified and characterized [91]. This enzyme is encoded by two ORFs and the full-length form is expressed *in vivo* by a programmed -1 frameshifting, therefore, α-L-fucosidase from *S. solfataricus* represents a unique example of this peculiar mechanisms of gene expression in archaea [92]. To produce the recombinant protein Ssα-Fuc, a single base has been inserted by site-directed mutagenesis in the region of overlap between the two ORFs, restoring a single reading frame. The study of Ssα-fuc allowed, therefore, to demonstrate that the GH29 enzymes follow a *retaining* reaction mechanism and to identify the catalytic nucleophile and acid/base residues [91,93]. In 2009, Cobucci-Ponzano and collaborators converted Ssα-fuc in α-fucosynthase by the mutation of the catalytic nucleophile; the mutant has been chemically rescued by using sodium azide as external nucleophile [94]. In 2021, Curci and co-workers employed Ssα-fuc as part of the enzymatic cocktail for the hydrolysis of xyloglucan oligosaccharides (XGO) from apple pomace; this enzyme alone was able to release 96% of all α1,2-linked fucose available in XGO in 4 h, indicating that SsαFuc recognized complex fucosylated oligosaccharides [37].

Endo-glycosidases

Endo-glycosidases active on β-D-glycosides

Chitinase and exo-chitinase

Chitinases (E.C. 3.2.1.14), classified in GH18, GH19, GH23, and GH48 [8] are enzymes that hydrolyse the β-1,4 linkage between *N*-acetyl-D-glucosamine units in chitin. The characterized members from (hyper)thermophiles could be found only in family GH18, in which also exo-chitinases (E.C. 3.2.1.200) have been included [8]. Exo-chitinase (E.C. 3.2.1.200) cleaves the chitin chain exclusively from the non-reducing end, to form diacetyl-chitobiose (GlcNAc2) [95].

Chitin is the second-most prevalent polysaccharide in biomasses and its derivatives resulted to be relevant as food additives and medicines. Chitinases, in general, bind to chitin and randomly cleave glycosidic linkages in chitin and chitodextrins generating chitooligosaccharides and free ends on which exo-chitinases and exo-chitodextrinases can act. The enzymatic activity is greatly stimulated in the presence of lytic chitin monooxygenase (EC 1.14.99.53), which attacks the crystalline structure of chitin and makes the polymer more accessible to the chitinase. Instead, chitinase from *P. furiosus* PF-Chia, obtained in recombinant form by the frameshifted PF1234 gene, possesses a unique ability to hydrolyse both colloidal and crystalline chitin [96].

The first chitinase from (hyper)thermophilic archaea has been identified in *T. kodakaraensis* (TK-ChiA) [97]. The enzyme showed an interesting multidomain structure containing dual catalytic domains and triple chitin-binding domains. The biochemical characterization demonstrated that the N-terminal and C-terminal catalytic domains functioned as exo- and endo-chitinases, respectively. It is worth noting that the two domains exhibited a synergistic effect in chitin hydrolysis, where the endo-chitinase domain randomly cleaves chitin-producing ends accessible to the exo-chitinase domain [95].

In *Thermococcus chitonophagus*, a (hyper)thermophilic archaeon able to use chitin as the sole carbon and energy source [98], a structurally different chitinase (EC 3.2.1.-), Tc-ChiD, was discovered [99].The protein has two chitin-binding domains, a signal peptide for secretion, and a catalytic domain that differs from other known chitinases. Hence, Tc-ChiD stands for a novel family of chitinases (belonging so far to GH0). When Tc-ChiD recognizes the reducing end of chitin chains, it releases either GlcNAc2 or GlcNAc3 [99]. As a thermostable chitinase that recognizes reducing end of chitin, Tc-ChiD may be useful in a variety of enzyme-based technologies to degrade and utilize chitin.

Cellulases and glucan endo-1,3-β-D-glucosidases

Cellulases (E.C. 3.2.1.4) catalyze the hydrolysis of β-D-(1,4)-glucosidic linkages in cellulose, lichenin, and cereal β-D-glucans. This activity is found in 10 different families [8], but characterized members from (hyper)thermophilic archaea are reported only in GH5 and GH12. The first (hyper)thermostable GH5 cellulase was identified in *P. horikoshii*, and it is reported to hydrolyse celluloses, including Avicel and carboxymethyl cellulose, as well as β-glucose oligomers [100]. One of the most thermoactive GH5 endoglucanase described to date is Vul_Cel5A, identified in the metagenome derived from an anaerobic enrichment culture, grown on cellulose at 90°C. The enzyme showed the highest activity at 115°C and a half-life time of 46 min at 100°C, making this enzyme a promising candidate for high-temperature biotechnological processes, like second-generation biorefineries [101]. Another extremely stable GH5 cellulase was identified from a microbial consortium of three (hyper)thermophilic archaea collected in

Nevada and enriched on crystalline cellulose. This enzyme, named EBI-244, showed a peculiar multidomain structure: indeed, most cellulases have a modular design including a carbohydrate-binding module (CBM) or they form an enzyme complex known as the cellulosome but, typically, multidomain cellulases have not been found in (hyper)thermophilic archaea [102]. The only crystal structure of archaeal GH5 is that of EglB from *P. horikoshii*, with typical GH5 Clan GH-A $(\beta/\alpha)_8$ fold and an atypical substrate-binding pattern with additional subsites -4 and -3 compared to other GH5 members [103].

Moving to cellulases belonging to GH12, only 5 members have been characterized. Three have been identified in *Saccharolobus* species [104–106], one in *Caldivirga maquilingensis* [107] and one in *P. furiosius* [108]. The crystal structure of EglA from *P. furiosius* revealed that the enzyme has a β-jelly-roll fold, typical of GH12 Clan GH-C [109]. EglA is incapable of degrading the β-1,3 bonds but it works in a concerted-synergistic fashion with LamA to efficiently hydrolyse mixed-linkage β-glucans [108]. LamA from *P. furiosius* is the only characterized laminarinase (Glucan endo-1,3-β-D-glucosidases, E.C. 3.2.1.39) [52] from (hyper)thermophilic archaea and it belongs to family GH16 [8]. Crystallographic studies on LamA revealed that this enzyme consists of a classical sandwich-like β-jelly-roll motif (Clan-B) formed by the face-to-face packing of two anti-parallel sheets containing seven and eight strands. LamA contains two additional secondary structural elements, which may contribute to its higher stability compared with the mesophilic counterpart. The presence of an additional β-strand at the N-terminus, and the presence of the six-residue kink may contribute to thermostability [110].

Glucanases can be employed in a plethora of industrial applications; indeed, the global cellulase market is estimated at US$ 1621 Million according to 2022 report 'Cellulase (CAS 9012-54-8) Market Research Report' (https://dataintelo.com/report/cellulase-cas-9012-54-8-market/). The description of all industrial processes is beyond the scope of this review. So, we refer to recent reviews focused on the biotechnological aspect of endo-glucanases [101,111,112].

Arabinogalactan endo-β-1,4-galactanases

Endo-β-1,4-galactanases (E.C 3.2.1.89) are enzymes which cleaves β-D-(1,4)-galactosidic linkages in type I arabinogalactans and are classified into GH53. These enzymes are of particular interest as they can be used, in association with other enzymes, to produce prebiotic oligosaccharides, functional foods, additives for animal feed and for biomass degradation in biorefinery for biofuel production [113]. The only (hyper)thermophilic archaeal enzyme is IaGal from *Ignisphaera aggregans*. The enzyme showed a T_{opt} of 95°C and a temperature of melting of 100°C, and its activity on lupin galactan produces mainly galactopentaose, but galactotetraose and galactotriose are also present. The 3D structure analysis revealed the usual $(\beta/\alpha)_8$-barrel structure of Clan GH-A enzymes but an intermediate substrate binding site length compared to previously characterized galactanases [114].

Endo-glycosidases active on α-D-glycosides

α-Amylases, pullulanases and cyclomaltodextrinases

α-Amylase (EC 3.2.1.1) enzymes, classified in GH13, GH57 and GH119, act on starch, glycogen and associated poly- and oligosaccharides by hydrolysing α-D-(1,4)-glucosidic linkages in polysaccharides containing three or more α-(1,4)-linked D-glucose units. Amylolytic enzymes play a significant role in a variety of industrial processes, such as those used in the production of foods, textiles, and detergents [115]. Among starch-hydrolysing enzymes, α-amylases, pullulanases, and cyclodextrinases are currently well known, and have been found in several (hyper)thermophilic microorganisms, even though they live in environments where starch is rare. Thermostable α-amylases have been characterized in several archaea, but all belong to GH13 or GH57, while no archaeal representatives are reported in GH119 to date. One of the most deeply characterized α-amylases from family GH13 is PFTA from *P. furiosus* [116–118]. It possesses characteristics of both α-amylase and cyclodextrin-hydrolysing enzyme (EC 3.2.1.54, see below), being able to hydrolyse maltooligosaccharides and starch to produce, mainly, maltotriose and maltotetraose, but it could also attack and degrade pullulan and β-cyclodextrin to produce panose and maltoheptaose (Glc7), respectively [116]. PFTA is one of the most thermostable α-amylases (13 h at 98°C) thanks to the presence of five cysteines. Indeed, Savchenko and co-workers demonstrated that Cys165 is a key residue for enzyme thermostability, since the mutant Cys165Ser was dramatically destabilized [117]. In addition, the aminoacidic sequence of GH13 ApkA from *T. kodakarensis* [119] doesn't contain Cys165, resulting less stable than PFTA [117]. Thanks to its excellent thermostability, α-amylase from *P. furiosus* is currently included in the commercial preparation Termamyl/Liquozyme from Novozymes [9]. While the α-amylase from Thermococcus *sp* is used in the products Fuelzyme® (Verenium Corporation, San Diego, CA, U.S.A.), and Spezyme® (DuPont-Genencor Science, Wilmington, DE, U.S.A.) designed to function at high temperatures (>110°C) during ethanol production [120,121]. The crystallographic structure of PFTA revealed that this enzyme is a monomer with additional N-terminal extension (N′-domain). Its unique ability to act as both α-amylase and cyclomaltodextrinase is probably due to the presence of this N′-domain at the active site,

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which forms an appropriate cavity pocket that holds cyclomaltodextrin substrates between the catalytic domain and the N′-domain [122]. This domain, similar to CBM48, has been identified for the first time in cyclomaltodextrinase (EC 3.2.1.54) SMMA from archaeon *Staphylothermus marinus* [123], and it is common to cyclodextrins-hydrolysing enzymes from (hyper)thermophilic archaea, such as *Staphylothermus hellenicus* [124], *Thermococcus barophilus* [125], *T. kodakarensis* [126], *Thermococcus onnurineus* [127], *T. pendens* [128], and *T. volcanium* GSS1 [129].

Moving on α-amylases belonging to GH57, many enzymes are characterized from (hyper)thermophilic archaea, such as MJA 1 from *Methanococcus jannaschii* [130], *PhGBE from P. horikoshii* [131], SSO1172 from *S. solfataricus* [132], Apu from *Thermococcus litoralis* [133], SMApu from *S. marinus* [134], and PFAPU from *P. furiosus* [135]. By comparing *P. furiosus* α-amylases PFTA (GH13) and PFAPU (GH57), both are extracellular enzymes but, while PFTA is not dependent on Ca^{2+}, PFAPU requires Ca^{2+} for its thermal stability and activity. In addition, only PFAPU is also active on pullulan [135].

α-Amylases (EC 3.2.1.1) are among the most popular enzymes used in industry, together to cellulases. Indeed, α-amylases market reached the value of US$ 1,722.3 million by 2022 according to 2023 report 'Alpha Amylase Market' (https://www.persistencemarketresearch.com/market-research/alpha-amylase-market.asp). This class of enzymes is used in several industrial processes and describing all of them is beyond the scope of this review. Therefore, we refer to reviews focused on this topic [136–138].

Pullulanases (EC 3.2.1.41) are involved in the hydrolysis of α-D-(1,6)-glucosidic linkages in pullulan. Most of them belong to family GH57, while only two pullulanases from the (hyper)thermophilic anaerobic archaea *Desulfurococcus mucosus* [139] and *T. aggregans* [140] belong to family GH13. GH13 pullulanases are able to hydrolyse pullulan and cyclodextrins while most GH57 pullulanases are inhibited by cyclodextrins, except SMApu from *Staphylothermus marinus* [134]. Indeed, SMApu catalyzes the ring-opening reaction by cleaving only one α-1,4-glycosidic linkage of cyclodextrin to produce corresponding single maltooligosaccharide, with the same action pattern of TfMA from *T. pendens* [141] and PFTA from *P. furiosus* [142] of the family GH13. Surprisingly, SMApu mode of action differs to SMMA. Indeed, SMMA catalyzes a ring-opening of the cyclodextrins by cleaving several α-1,4-glycosidic linkages to produce various maltooligosaccharides [143].

Finally, cyclomaltodextrinases (EC 3.2.1.54), classified in GH13 and GH57, de-cyclize cyclodextrins, cyclic maltooligosaccharides composed by at least five α-D-glucopyranoside residues connected by α-1,4glycosidic linkages. The most important representative of this class of enzyme is the cyclodextrinase from *T. kodakarensis* KOD1 CDase-Tk, belonging to GH13, and optimally active at pH 7.5 and 65°C [144] Linear maltodextrins prepared by enzymatic hydrolysis, find widely uses in numerous foods thanks to their typical characteristics, like moderate sweet, rapidly absorption and hygroscopicity [145].

Glucan 1,4-α-maltohydrolase—trehalohydrolase—isoamylase

Enzymes from families GH13 and GH57 also showed activity as glucan 1,4-α-maltohydrolases (EC 3.2.1.133) such as GH13 SMMA and GH57 TCMA from *Thermococcus cleftensis* [146].

In addition, GH13 family includes 4-α-D-{(1,4)-α-D-glucano}trehalose trehalohydrolase (3.2.1.141), that hydrolyses α-D-(1,4)-glucosidic linkage in 4-α-D-[(1,4)-α-D-glucanosyl]n trehalose to yield trehalose and α-D-(1,4)-glucan. Trehalohydrolases characteristics have been already described above; from a biotechnological point of view, the (hyper)thermophilic enzymes able to hydrolyse and produce this disaccharide are very interesting, as their use can improve the amount of glucose obtained from starch hydrolysates and used for the preparation of novel glucose-trehalose syrups starting with low molecular weight trehalosyldextrins [147]. The main thermophilic representatives of this class of enzymes are trehalosyl dextrin-forming enzyme (SsTDFE) and trehalose-forming enzyme (SsTFE), which where purified and characterized from the (hyper)thermophilic archaeon *S. shibatae* and resulted to be quite similar to those isolated in *S. acidocaldarius* and *S. solfataricus* [147,148].

In addition, a maltooligosyltrehalose trehalohydrolase (TreZ) from *S. acidocaldarius* ATCC33909 was identified and resulted to be implicated in the biosynthesis of trehalose, in which also a glycogen debranching enzyme (TreX), and a maltooligosyltrehalose synthase (TreY) are involved [149].

Family GH13 also includes isoamylases (EC 3.2.1.68) activities, that catalyze the hydrolysis of α-D-(1,6)-glucosidic branch linkages in glycogen, amylopectin and their β-limit dextrins. These enzymes, which can hydrolyse both the inner and outer branching points of amylopectin, are frequently employed in conjunction with glucoamylase, α-amylase, and β-amylase to produce amylose, maltose, and glucose from starch [150]. Among the thermophilic representatives, there is the isoamylase TreX from *S. solfataricus;* this enzyme has been shown to have a great potential to be used in industrial applications, being able to degrade the branching points of starch at a high temperature [150].

Summary

- In this review, we have summarized the state-of-the-art on the function, structure, and application of GHs from (hyper)thermophilic archaea.
- Enzymatic investigations of (hyper)thermophilic GHs advanced our knowledge on the strategies used by microbes to adapt to their extreme environments and their applicative potential in industrial processes.
- Glycoside Hydrolase discovery and biochemical and structural characterizations remain the essential step for studying novel enzymes and for validating the new biocatalysts for biotechnology applications.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

β-D-Man-F, β-D-mannosyl-fluoride; GH, glycosidase; Glc3, maltotriose; Glc4, maltotetraose; Glc5, maltopentaose; Glc7, maltoheptaose; GlcNAc2, Chitobiose; GlcNAc3, Chitotriose; pH_{opt}, Optimal pH; pNP-α-Glc, para-nitrophenyl-α-glucopyranoside; pNP-α-Man, para-nitrophenyl-α-mannopyranoside; pNP-α-Xyl, para-nitrophenyl-α-xyloside; T_{opt}, Optimal temperature; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; XGO, xyloglucan oligosaccharides.

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