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Bioinformatic Analysis of Glycoside Hydrolases in the Proteomes of Mesophilic and Thermophilic *Actinobacteria*

Abstract

Petroleum reserves are rapidly depleting and alternative renewable sources of energy need to be developed to meet the energy demands of the planet. Lignocellulose has been recognized as a highly promising and renewable resource for the development of clean energy. Thermophilic microbes and thermostable enzymes are being sought for biological conversion of lignocellulose into biofuels. The phylum *Actinobacteria* includes several efficient cellulose-degrading microorganisms. Genomes of several *Actinobacteria* have been completely sequenced and deposited in public databases, which are a great resource for uncovering new enzymes and targets for biotechnology. We searched the predicted proteomes of 69 *Actinobacteria* for the homologs of 20 glycoside hydrolase families relevant to lignocellulose degradation and identified 589 glycoside hydrolase homologs. We analyzed (1) the distribution of the glycoside hydrolase homologs across mesophilic and thermophilic *Actinobacteria* (2), the domain architecture of cellulases (from GH5 and GH6 families) and xylanases (from GH10 and GH11 families) from mesophilic and thermophilic *Actinobacteria* , and (3) asymmetric amino acid substitutions between mesophilic and thermophilic glycoside hydrolases. Overall, our data provide new insights into the distribution of different glycoside hydrolases in *Actinobacteria* as well as into the thermostability features of cellulases and xylanases from *Actinobacteria*. Our findings provide a basis for genetic engineering of glycoside hydrolases as well as new targets for biotechnology.

Keywords: Thermophiles; Enzymes; Cellulases; Xylanases; Biofuel; Lignocellulose; Genome; Proteome

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Abbreviations: GH: Glycoside Hydrolases; CBM: Carbohydrate Binding Module; OGT: Optimal Growth Temperature

Introduction

Petroleum fuels are finite and non-renewable and they pose a significant concern for global climate, sustainability, and international security [1]. Alternative renewable sources of energy are urgently needed to meet the current global challenges. Plants are the most abundant source of renewable carbon on Earth. Plant cell wall (lignocellulose) can be used for the production of renewable, sustainable, and environmentally -clean biofuels [2]. Lignocellulose is mainly composed of polymers of sugars (cellulose and hemicellulose) and phenolic units (lignin). While complex lignocellulose can be converted into liquid fuels thermo-chemically, biological transformation of lignocellulosic polysaccharides using microorganisms and microbial enzymes is an economical and environmentally benign process for sustainable production of biofuels [3,4]. Several microorganisms produce glycoside hydrolase enzymes such as cellulases and xylanases that break down cellulose and xylan (hemicellulose), respectively [5]. Efficient lignocellulose-degrading microorganisms and catalytically- superior cellulases and xylanases are of very high value in the bioconversion of lignocellulose into biofuels [6,7].

Actinobacteria are a phylum of Gram-positive bacteria that are found abundantly in soil [8]. They include some of the most prolific lignocellulose-degrading bacteria [9]. *Actinobacteria* include both mesophilic and thermophilic members. Many new *Actinobacteria* continue to be isolated and sequenced in bioprospecting studies aimed at identifying new biotechnological targets [10]. Growing number of completely sequenced genomes are being steadily deposited in public databases, which provide an expanding resource for discovering novel targets for biotechnology. Systematic bioinformatic mining of the genomes and predicted proteomes of sequenced *Actinobacteria* has the potential to reveal novel insights into lignocellulose-degrading enzymes for bioenergy applications [11].

Thermophilic microbes and thermostable enzymes are most useful for the development of cost- effective, industrial scale technologies [12]. Thermostability of enzymes increases their shelf life, reduces reaction times, improves industrial productivity, and lowers manufacturing costs [12]. Thus, enzyme thermostability is a highly desirable property for industrial enzymatic deconstruction of lignocellulose. Valuable insights can be gleaned about factors that contribute to thermostability by performing comparative analysis of amino acid sequences of proteins from mesophilic and thermophilic organisms [13]. Such

insights can be exploited for designing and genetically engineering enhanced enzymes for industrial applications.

In this study, we systematically analyzed the predicted proteomes of 69 *Actinobacteria* for homologs of glycoside hydrolase enzymes that are relevant to lignocellulose degradation. We analyzed the distribution of the homologs across the phylum. We identified homologs from mesophilic and thermophilic *Actinobacteria* and analyzed their domain architecture to decipher thermophilic patterns. Finally, we analyzed the amino acid sequences of cellulases and xylanases from mesophilic and thermophilic *Actinobacteria* and identified asymmetric amino acid substitution patterns in the thermophilic enzymes.

Methodology

Predicted proteomes of known lignocellulose-degrading *Actinobacteria* were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/). Optimal growth temperature (OGT) information was obtained through literature. Organisms were classified as mesophilic (OGT < 40 °C) or thermophilic (OGT > 40°C). Glycoside hydrolase families that contain lignocellulose degradation enzymes were identified from the CAZy database [14]. Representative Actinobacterial sequences from the CAZy families were used to identify homologs in the proteomes of the *Actinobacteria* using BLAST [15]. Domains in the glycoside hydrolase proteins were identified using the NCBI's CDD-search tool [16]. Amino acid substitutions between homologs of mesophilic and thermophilic *Actinobacteria* were identified using multiple alignments as described previously [17]. Briefly, for each GH family, orthologs from mesophilic and thermophilic *Actinobacteria* were aligned using CLUSTAL [18,19]. Each substitution was counted only once per position in the alignment. For each amino acid substitution pair (e.g., A_MB_T and A_TB_M where A and B represent amino acids and the subscripts M and T represent mesophilic and thermophilic organisms, respectively), the total number of substitutions over the entire alignment was summed and the percentage of each substitution within the pair was calculated. Statistical significance (*p*-value) of asymmetric amino acid substitutions between the two groups of organisms was calculated using a binomial function. The asymmetry (i.e., bias) in A_MB_T and A_TB_M substitutions was considered significant if their p-value was below the threshold.

Results and Discussion

Distribution of glycoside hydrolases in *Actinobacteria*

We identified a total of 1133 *Actinobacteria* in the NCBI database. Of these, genomes of only 236 (21%) *Actinobacteria* have been completed sequenced. Within the 236 sequenced *Actinobacteria* , we identified 69 (29%) organisms that have been described in literature to have cellulolytic activity. We analyzed the predicted proteomes of the 69 *Actinobacteria* for the presence of glycoside hydrolases relevant to lignocellulose degradation. In addition, we collected information on their optimal growth temperature for each organism from literature. Using the CAZy database, we identified 20 glycoside hydrolase families that contain enzymes known to hydrolyze various plant cell wall polysaccharides [14]. A total of 589 glycoside hydrolase homologs were identified in the proteomes of the 69 *Actinobacteria* (Table 1). Of the 69 *Actinobacteria*, 61 organisms are mesophilic and only 8 are thermophilic. This highlights the need to sequence more thermophilic *Actinobacteria*.

We analyzed the relationship between optimal growth temperature and glycoside hydrolases encoded in the proteomes of the *Actinobacteria* (Figure 1). In general, there was very poor correlation ($R^2 < 0.1$) between optimal growth temperature and glycoside hydrolase content of the proteomes. However, this may be partly due to the overrepresentation of mesophilic *Actinobacteria* in the dataset. The 61 mesophilic *Actinobacteria* encoded between 0 and 13 glycoside hydrolase families with an average of 4.0 ±3.9, while they encoded between 0 and 47 homologs of glycoside hydrolases with an average of 8.4±10.9. The 8 thermophilic

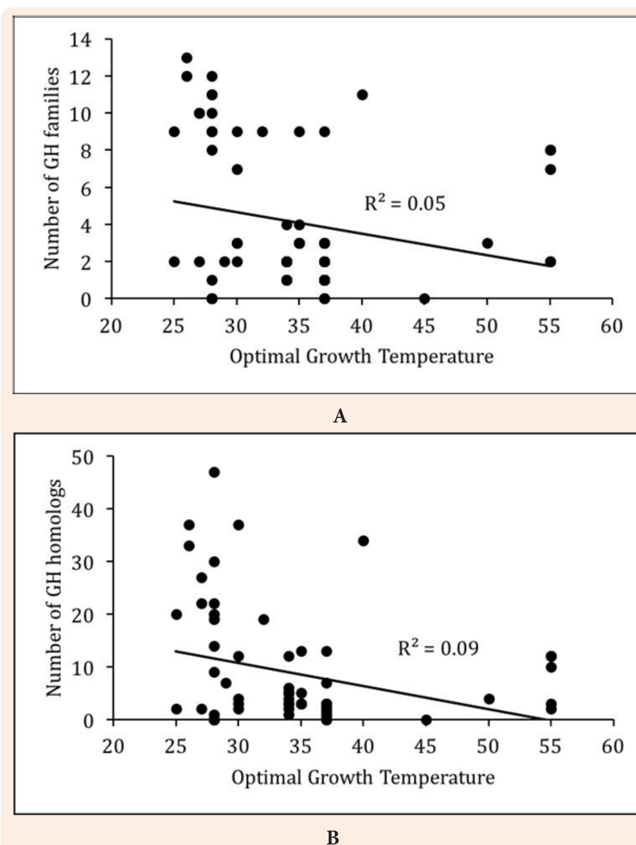

Figure 1: Relationship between optimal growth temperature and glycoside hydrolases in *Actinobacteria*.
(A) Scatter plot of number of glycoside hydrolases (GH) families versus optimal growth temperature.
(B) Scatter plot of number GH homologs versus optimal growth temperature. Best-fit line with R-squared value is shown.

Actinobacteria encoded between 0 and 11 glycoside hydrolase families with an average of 5.1±3.9, while they encoded between 0 and 34 homologs of glycoside hydrolases with an average of 9.6±10.9. There were no statistically significant differences in the distribution of glycoside hydrolases between mesophilic

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and thermophilic *Actinobacteria*. However, substantially greater numbers of thermophilic Actinobacteria need to be sequenced before deciphering any underlying biases between the two groups of *Actinobacteria*.

Table 1: Summary of the analysis of *Actinobacteria* used in this study.

Organism Name	OGT (°C)	# of GH Families	Total GH Homologs
Acidothermus cellulolyticus 11B (ATCC 43068)	55	7	12
Actinosynnema mirum DSM 43827	28	9	30
Amycolatopsis mediterranei S699	26	13	37
Amycolatopsis mediterranei U32	26	12	33
Bifidobacterium adolescentis 15703	50	3	4
Bifidobacterium animalis AD011	37	1	1
Bifidobacterium animalis ATCC 25527	37	1	1
Bifidobacterium animalis B420	37	1	1
Bifidobacterium animalis Bb12	37	2	2
Bifidobacterium animalis Bi-04	37	1	1
Bifidobacterium animalis Bi-07 37 1	37	1	2
Bifidobacterium animalis BLC1	37	1	1
Bifidobacterium animalis CNCM I-2494	37	2	3
Bifidobacterium animalis DSM 10140	37	1	1
Bifidobacterium animalis V9	37	1	1
Bifidobacterium bifidum PRL2010	37	1	2
Bifidobacterium bifidum S17	37	1	2
Bifidobacterium breve ACS-071-V-Sch8b	37	2	2
Bifidobacterium breve UCC2003	37	0	0
Bifidobacterium dentium Bd1	29	2	7
Bifidobacterium longum 157F	34	2	6
Bifidobacterium longum BBMN68	34	1	3
Bifidobacterium longum DJO10A	34	1	2
Bifidobacterium longum F8	34	1	1
Bifidobacterium longum JCM 1217	34	2	5
Bifidobacterium longum JCM 1222 (ATCC 15697)	34	2	3
Bifidobacterium Longum JDM301	34	2	5
Bifidobacterium longum KACC 91563	34	2	4
Bifidobacterium longum NCC2705	34	2	5
Cellulomonas fimi ATCC 484	40	11	34
Cellulomonas flavigena DSM 20109	30	9	37
Cellvibrio gilvus ATCC 13127	25	9	20
Clavibacter michiganensis NCPPB 382	37	3	7
Clavibacter michiganensis sepedonicus	37	1	2
Jonesia denitrificans DSM 20603	37	9	13

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Micrococcus luteus	37	0	0
Micromonospora aurantiaca ATCC 27029	27	10	22
Modestobacter marinus BC501	28	0	0
Mycobacterium abscessus	30	2	2
Mycobacterium avium 104	37	3	3
Mycobacterium avium K-10	37	3	3
Mycobacterium bovis AF2122/97	35	4	5
Mycobacterium bovis BCG str. Mexico	35	3	3
Mycobacterium bovis Pasteur 1173P2	35	3	3
Mycobacterium bovis Tokyo 172	35	3	3
Mycobacterium gilvum PYR-GCK	30	3	3
Mycobacterium marinum	37	2	2
Mycobacterium smegmatis MC^2 155	30	3	4
Rhodococcus erythropolis PR4 (NBRC 100887)	25	2	2
Rhodococcus opacus B4	27	2	2
Saccharomonospora glauca	45	0	0
Saccharomonospora viridis DSM 43017	55	2	2
Streptomyces avermitilis MA-4680	32	9	19
Streptomyces bingchenggensis BCW-1	28	12	47
Streptomyces cattleya DSM 46488	34	4	12
Streptomyces clavuligerus	28	0	0
Streptomyces coelicolor A3(2)	28	11	20
Streptomyces flavogriseus ATCC 33331	28	11	19
Streptomyces hygroscopicus jinggangensis 5008	35	9	13
Streptomyces pristinaespiralis	28	0	0
Streptomyces scabiei 87.22	27	10	27
Streptomyces sirex AA3	28	8	9
Streptomyces sviceus	28	1	1
Streptomyces violaceusniger Tu 4113	28	10	22
Streptosporangium roseum DSM 43021	28	9	14
Thermobifida fusca YX	55	8	12
Thermobispora bispora 43833	55	8	10
Thermomonospora curvata 43183	55	2	3
Xylanimonas cellulosilytica DSM 15894	30	7	12

OGT (°C): optimal growth temperature (degrees Celsius); GH: glycoside hydrolase.

We analyzed relative abundances of the 20 glycoside hydrolase families across *Actinobacteria* (Figure 2). The data show that GH5 was the most highly represented family in the *Actinobacteria*. It was the only family that was found in majority (70%) of the organisms analyzed. The GH6 and GH43 families were the next most represented families and were found in 48% of the *Actinobacteria*. The GH5 family is known to contains cellulose- and hemicellulose-degrading enzymes, while the GH6 family contains cellulases and the GH43 family contains hemicellulases [14]. The GH45, GH51, and GH128 families were not represented in any of the *Actinobacteria* in our dataset. Other GH families showed intermediate representation.

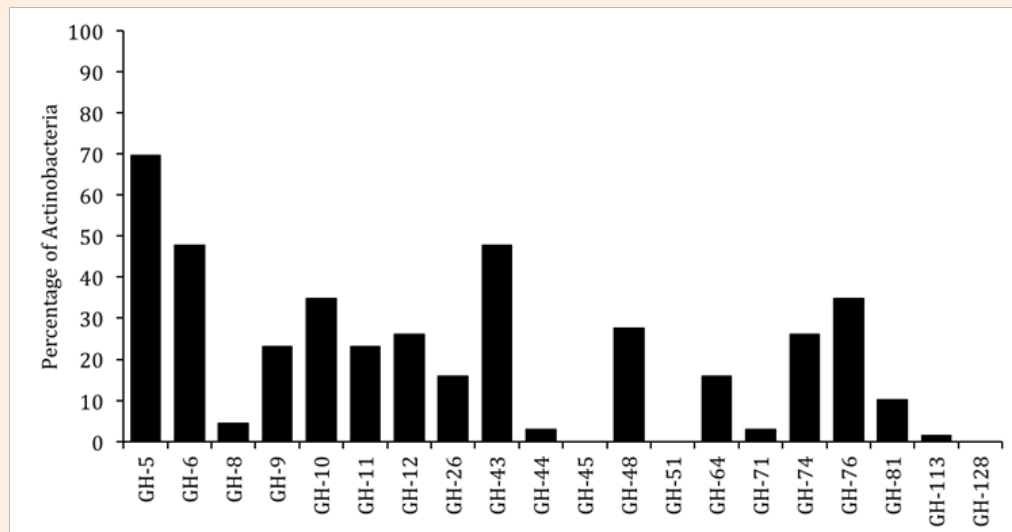

Figure 2: Relative abundance of glycoside hydrolase (GH) families in *Actinobacteria*. Percentage of *Actinobacteria* containing homologs of the different GH families are plotted.

Domain architecture of glycoside hydrolases in *Actinobacteria*

To minimize over-representation of mesophilic *Actinobacteria* in the dataset, we selected one representative species per genus and also retained saprophytic free-living bacteria while removing animal and human pathogens. This yielded a more balanced set of *Actinobacteria* (6 thermophiles and 8 mesophiles). We focused our analysis on four GH families - cellulases from GH5 and GH6 families and xylanases from GH10 and GH11 families. There were 113 glycoside hydrolases from the four families across the 14 *Actinobacteria* (Table 2). There were 77 homologs in the 8 mesophilic bacteria, and 36 homologs in the 6 thermophilic bacteria. Six organisms contained representatives from all four families, while five organisms contained representatives from only three families and two organisms contained homologs from just one family. We analyzed the domain architecture of the 113 glycoside hydrolases using the NCBI's CDD-search tool [16]. At least five different types of carbohydrate binding modules (CBMs - CBM-2, CBM -3, CBM-X2, CBM -9, and CBM- 4 -9) were found fused to the catalytic domains of glycoside hydrolases (Table 3). Further analysis revealed a bias in the presence and location of certain CBMs. For example, CBM-2 was found fused on the C -terminal side of the catalytic domain in all four glycoside hydrolase families, while it was found on the N -terminal side of the catalytic domain in GH5 and GH6 cellulases. CMB -3 was only found in homologs from thermophilic *Actinobacteria*, and it always occurred C-terminal to the catalytic hydrolase domain. CBM- 9 and CMB-4-9 were found attached to only GH10 xylanases. CBM-9 occurred C- terminal to the catalytic domain, while CBM-4-9 was found on the N-terminal side of the hydrolase domain. CBM-X2 was found only in GH5 hydrolases from mesophilic *Actinobacteria* and was found C-terminal to the hydrolase domain. These data suggest that there are positional constraints for CBM domains in glycoside hydrolases. Certain domains may be required for the functioning and stability of the enzymes, while others may be specific to the substrates hydrolyzed by the associated catalytic domains.

Table 2: Distribution of glycoside hydrolases in mesophilic and thermophilic Actinobacteria.

Organism Name	OGT	GH5	GH6	GH10	GH11
(A) Mesophilic *Actinobacteria*					
Actinosynnema mirum DSM 43827	28	5	3	4	1
Amycolatopsis mediterranei S699	26	4	2	7	1
Cellvibrio gilvus ATCC 13127	25	2	4	6	0
Jonesia denitrificans DSM 20603	37	0	2	4	1
Micromonospora aurantiaca ATCC 27029	27	4	2	4	1
Streptomyces coelicolor A3(2)	28	1	3	2	2
Streptosporangium roseum DSM 43021	28	1	3	1	0
Xylanimonas cellulosilytica DSM 15894	30	0	2	4	1

(B) Thermophilic *Actinobacteria*					
Acidothermus cellulolyticus 11B	55	2	2	2	0
Cellulomonas fimi ATCC 484	40	0	6	8	1
Saccharomonospora viridis DSM 43017	55	0	0	1	0
Thermobifida fusca YX	55	2	2	2	1
Thermobispora bispora DSM 43833	55	1	2	2	1
Thermomonospora curvata DSM 43183	55	0	1	0	0

Table 3: Domain architecture of glycoside hydrolases in *Actinobacteria*.

		C-Terminal Domain									
(A) Mesophiles		**GH5**	**GH6**	**GH10**	**GH11**	**CBM_2**	**CBM_3**	**CBM_X2**	**CBM_9**	**CBM_4_9**	**No CBM**
N-terminal domain	**GH5**					8		2			2
	GH6					8					6
	GH10					16			2		7
	GH11					4					1
	CBM_2	7	8					2			
	CBM_3										
	CBM_X2										
	CBM_9										
	CBM_4_9			4		1			2		
		C-Terminal Domain									
(B) Thermophiles		**GH5**	**GH6**	**GH10**	**GH11**	**CBM_2**	**CBM_3**	**CBM_X2**	**CBM_9**	**CBM_4_9**	**No CBM**
N-terminal domain	**GH5**					3	2				
	GH6					5	1				3
	GH10					8	1		2		3
	GH11					3					
	CBM_2	1	1								
	CBM_3										
	CBM_X2										
	CBM_9										
	CBM_4_9			2					2		

Asymmetric amino acid substitutions in glycoside hydrolases

We wanted to understand amino acid biases between orthologs from thermophilic and mesophilic *Actinobacteria*. This would help identify amino acid substitutions that may contribute to thermostability of glycoside hydrolases. For each glycoside hydrolase family, we aligned only the hydrolase domains of orthologs from mesophilic and thermophilic organisms identified earlier (Table 2). We calculated the frequencies of all amino acid substitutions between mesophilic and thermophilic homologs at every position and identified the statistically significant asymmetric amino acid substitutions (Table 4). The data revealed 41 pairs of amino acid substitutions that are asymmetric between the homologs from thermophilic and mesophilic *Actinobacteria*. Certain amino acid preferences in the thermophiles were specific to the glycoside hydrolase family, while other amino acid preferences were independent of the glycoside hydrolase family. For example, thermophilic enzymes from GH6, GH10, and GH11 families showed preferences for alanine over glycine. Similarly, thermophilic proteins showed preference for aspartate over thermolabile serine and threonine residues. There was also a biased preference for isoleucine over valine in thermostable homologs. Overall, the data provide several new targets for genetically engineering higher thermostability in glycoside hydrolases [20].

Table 4. Asymmetric amino acid substitution patterns in glycoside hydrolases.

		Amino Acid in the Thermophilic Homologs														
		A	D	E	F	H	I	K	L	N	P	Q	S	T	V	Y
Amino acid in the mesophilic homologs	A		C74	C62	A100	B100				C77	B100	C61, B68				B100
	D	B92								B93						
	E		A62													
	F															C65
	G	B88, C67, D86,	C100					B91								
	K			A86												A100
	L						A62									
	N	B100	D91			D92							D84	D100	C100	
	R			C67				B90				C59				
	S	D86	B77, C67					C100				C64		D85		
	T		A80, C75				B100, C78				B100				B88	D100
	V	B80					A60, C56		D77							
	Y	A100														

Standard single letter amino acid code is use to represent amino acids. Data are represented with a letter followed by a number, where A represents GH5, B represents GH6, C represents GH10, D represents GH11, and numbers represent the percentage of occurrence of the particular substitution. Only statistically significant ($p < 0.1$) asymmetric substitutions are shown.

Conclusion

We analyzed the predicted proteomes of 69 sequenced *Actinobacteria* and identified homologs of 20 glycoside hydrolase families associated with plant cell wall degradation. Some glycoside hydrolase families were well represented across the phylum, while a few families were not represented in any of the *Actinobacteria* we analyzed. The glycoside hydrolases appear to have a constrained domain architecture that likely determines their stability, functioning, and interaction with substrates. Certain carbohydrate binding modules found fused to the glycoside hydrolases were only associated with thermophilic *Actinobacteria*. Finally, glycoside hydrolases from thermophilic *Actinobacteria* showed preferences for certain amino acid substitutions over their mesophilic counterparts. Overall, our data provide new insights into glycoside hydrolases in *Actinobacteria* and provide a basis for genetically enhancing the stability of glycoside hydrolases towards industrial applications.

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