

Journal Pre-proof

A review of the principles and biotechnological applications of glycoside hydrolases from extreme environments

Ellie Ashcroft, Jose Munoz-Munoz



PII: S0141-8130(24)00030-8

DOI: <https://doi.org/10.1016/j.ijbiomac.2024.129227>

Reference: BIOMAC 129227

To appear in: *International Journal of Biological Macromolecules*

Received date: 28 July 2023

Revised date: 27 December 2023

Accepted date: 2 January 2024

Please cite this article as: E. Ashcroft and J. Munoz-Munoz, A review of the principles and biotechnological applications of glycoside hydrolases from extreme environments, *International Journal of Biological Macromolecules* (2023), <https://doi.org/10.1016/j.ijbiomac.2024.129227>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2024 Published by Elsevier B.V.

A review of the principles and biotechnological applications of glycoside hydrolases from extreme environments.

Authors

Ellie Ashcroft^a, Jose Munoz-Munoz^{a,b}

Affiliations:

^aMicrobial Enzymology Lab, Department of Applied Sciences, Ellison Building A, Northumbria University, Newcastle Upon Tyne, NE1 8ST, United Kingdom.

ellie.ashcroft@northumbria.ac.uk ^bjose.munoz@northumbria.ac.uk

Abstract

It is apparent that Biocatalysts are shaping the future by providing a more sustainable approach to established chemical processes. Industrial processes rely heavily on the use of toxic compounds and high energy or pH reactions, factors that both contribute to the worsening climate crisis. Enzymes found in bacterial systems and other microorganisms, from the glaciers of the Arctic to the sandy deserts of Abu Dhabi, provide key tools and understanding as to how we can progress in the biotechnology sector. These extremophilic bacteria harness the adaptive enzymes capable of withstanding harsh reaction conditions in terms of stability and reactivity. Carbohydrate-active enzymes, including glycoside hydrolases or carbohydrate esterases, are extremely beneficial for the presence and future of biocatalysis. Their involvement in the industry spans from laundry detergents to paper and pulp treatment by degrading oligo/polysaccharides into their monomeric products in almost all detrimental environments. This includes exceedingly high temperatures, pHs or even in the absence of water. In this review, we discuss the structure and function of different glycoside hydrolases from extremophiles, and how they can be applied to industrial-scale reactions to replace the use of harsh chemicals, reduce waste, or decrease energy consumption.

Keywords: Glycoside Hydrolases; Extremozymes; Biotechnological Applications; Biocatalysis, AlphaFold[®], Pymol[®]

Introduction

The desire to take advantage of enzymatic activity for biotechnological applications has increased exponentially in recent years. Enzymes provide a sustainable substitute for industrial catalysts such as metals, polymers, and single-atom catalysts. These inorganic catalysts have many limitations such as low activity and stability without additional support or their dependency on high-temperature treatment [1, 2]. Biocatalysts, on the other hand, provide an alternative method of synthesis without requiring high-energy reactions but still increasing activity rate and maintaining stability. Through directed evolution, optimised biocatalysts such as nitrilases and ketoreductases have expanded the biological library of intermediates, which can be used in metabolic pathways and pharmaceutical syntheses [3]. Furthermore, the financial burden of single-use, high-waste chemicals contribute to continuous economic pressure on the scientific industry. The alternative use of enzymes can not only increase activity but once immobilized they are both reusable and more thermo-dynamically stable [4]. As there is a great fascination with using biocatalysts in an abundance of reaction conditions, investigating catalytic proteins from extremophilic microorganisms (microorganisms from environments with extreme conditions, Figure 1) is the focus of many researchers today [5-7]. Extremophilic enzymes or 'extremozymes' provide a plethora of protein structures with the ability to work in harsh conditions such as high temperatures or low pH environments denoting their potential as successful biocatalysts.

As mentioned above, the extreme environments refer to locations around the world where there are harsh climates and almost inhospitable settings. Microorganisms, and their catalytic proteins, harbour unique chemical properties and advanced adaptations to survive by both thermodynamic and kinetic means. The survivability of the proteins differs depending on their habitual origin, but it has been widely described that the internal bonding and amino acid residues of all

extremozymes play a crucial role in their ability to remain folded and active because of the increase in their stability [8, 9].

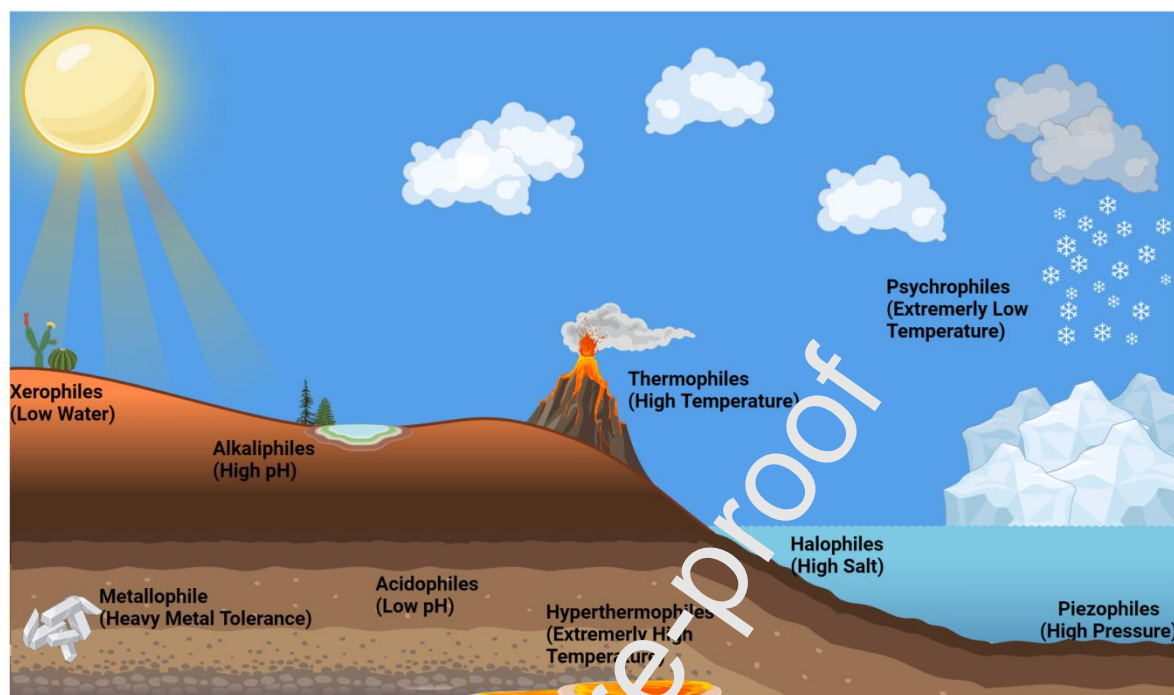


Figure 1. Habitats of distinct extremophiles, i.e., hot springs salt lakes (Image created with Biorender®).

The CAZy database (<http://www.cazy.org/Home.html>) documents the many carbohydrate-active enzymes (CAZymes) currently discovered to date [10]. This database is highly populated and well-described. According to the catalytic activity, CAZymes are classified into five categories: glycosyl transferases, when they synthesize oligo/polysaccharides, and glycoside hydrolases, polysaccharide lyases, carbohydrate esterases and auxiliary activities. For the purpose of clarity, this review will focus on the most described CAZymes to date, glycoside hydrolases [11]. As these proteins are known to have high catalytic abilities and substrate specificities, their biocatalytic potential has been proven successful by their use in many industrial processes today. They have been identified in over 26,000 bacterial genomes, which include many different extremophilic bacteria such as 163 strains of *Bacillus subtilis*, classified in the CAZy database (<http://www.cazy.org/b.html>). The Bacillus genus categorises many bacterial species which are commonly found in extremophilic environments [12-14].

It is noteworthy to mention that some extremophiles have more than one adaptation, indicated by the term polyextremophiles and their respective polyextremozymes. For example, using psychrophilic β -galactosidases from *Halorubrum lacusprofundi* (adapted to be active at temperatures lower than 15°C) which are also halophilic, enables them to be used in organic solvent reactions but at low temperatures which would prevent the threat of contamination [15]. These glycoside hydrolases or glycosidases will therefore have multiple evolutions to their structure and function, which are all essential for their stability in biocatalytic industries.

Although there are numerous different extremozymes, there is a clear focus on research academically and industrially on thermophiles, halophiles and alkaliphiles. With an additional mention of xerophiles, which although the understanding of these extremozymes is limited they could still be employed in the industry [16] once evidence of scale-up to at least 150g/L is achieved. In this review, we will discuss the different glycoside hydrolases from several extremophilic bacteria and how extensive categorization of these proteins has influenced the biotechnology sector. For this purpose, we have searched for the words “extremophiles and industrial applications” into PubMed obtaining 262 results. In addition, this result was reduced to only 20 results when we incorporated “glycoside hydrolase” into the above search. Finally, this explore was repeated using the same words in Web of Science and we obtained 109 and only 1 citation. We have revised all the citations obtained from PubMed and Web of Science and we have incorporated the most cited applications with these enzymes from extreme environments.

Biotechnological applications of thermophilic Glycoside Hydrolases

Thermophiles are bacteria with the capability to survive at temperatures around 40°C to 55°C. Hyperthermophiles such as the anaerobic bacteria *Thermotoga* spp. are adapted to even higher temperature environments from 70°C to 100°C [17]. It has been proven that the enzymes, specifically glycoside hydrolases in this case, found in these thermophiles tend to also have thermostable properties [18-20]. Thermophilic enzymes derived from these bacteria have subjective characteristics, often including a tightly packed hydrophobic core [21]. This core, not

necessarily the active site, reduces the exposure of hydrophobic regions to solvents. One prediction was associated with fluctuations in the protein water interface, an interaction which aided stability in higher temperatures [22]. In the interest of CAZymes, these temperature-resistant functions enable the degradation of sugars for the generation of biofuels, production of biochemicals or even use in the food industry with the added benefit of high temperatures which can prevent any risks of contamination [23].

Bioethanol Production

The degradation of lignocellulose biomass to produce ethanol as a biofuel is a core focus of biotechnological research, especially in our energy crisis era. This cellulosic biomass is the most abundant and sustainable material for biofuel production worldwide [24]. Cellulose, hemicellulose and lignin can be valorised in lignocellulosic biorefineries to produce useful biofuel and industrial bioproducts [25]. Although, there is still the requirement to use high-temperature reactions whether that be chemically or biochemically.

During the hydrolysis phase of bioethanol production, multiple glycoside hydrolases from thermophiles are used in synergy to break down the cellulose polysaccharide into its monomeric β -D-glucose units. This synergy is between a large volume of different enzymes, including cellobiohydrolase, endoglucanase, β -glucosidase, endo- β -mannanase and β -mannosidase [26], as shown in Table 1. The advantages of the use of these enzymes are that there is low energy consumption and reduced levels of waste providing a much more sustainable approach than that of acid hydrolysis or other (chemo)physical approaches [27]. Although this cocktail of enzymes is also commercially available from companies such as Novozymes[®], Dupont[®] and Abengoa[®] [28], there are some drawbacks to this process. While these hydrolases are from thermophiles, they must not only remain stable at exceedingly high temperatures, but they also must retain viable activity for polysaccharide degradation. Understanding glycoside hydrolase kinetics and structure is only the first step in industrial development because the demand for scale-up requires a focus on the optimisation of enzyme stability and volume. One example of a thermostable enzyme is the α -amylase shown in Figure 2, from the thermophile *Anoxybacillus sp. GXS-BL*. α -amylase fold into a $(\beta/\alpha)_8$ -TIM barrel and target the α -

1-4-linkages of starch and smaller maltooligosaccharides usually at 37°C. This α -amylase has properties which reflect closely to its homologues in terms of optimum thermostability at 60°C. However, with calcium ions as a co-factor, this enzymatic activity was retained between 45°C and 70°C [29]. The addition of calcium ions prevented the denaturation of the protein which is usually caused by an increase in helical content and the formation of pleated sheets [29]. Although the use of cofactors is a common method of protein folding optimisation, the benefits it possesses can always be improved for the enzymes shown in Figure 2. Scale-up requires promising activity and accessibility to large volumes of protein for such large-scale methods of biorefinery.

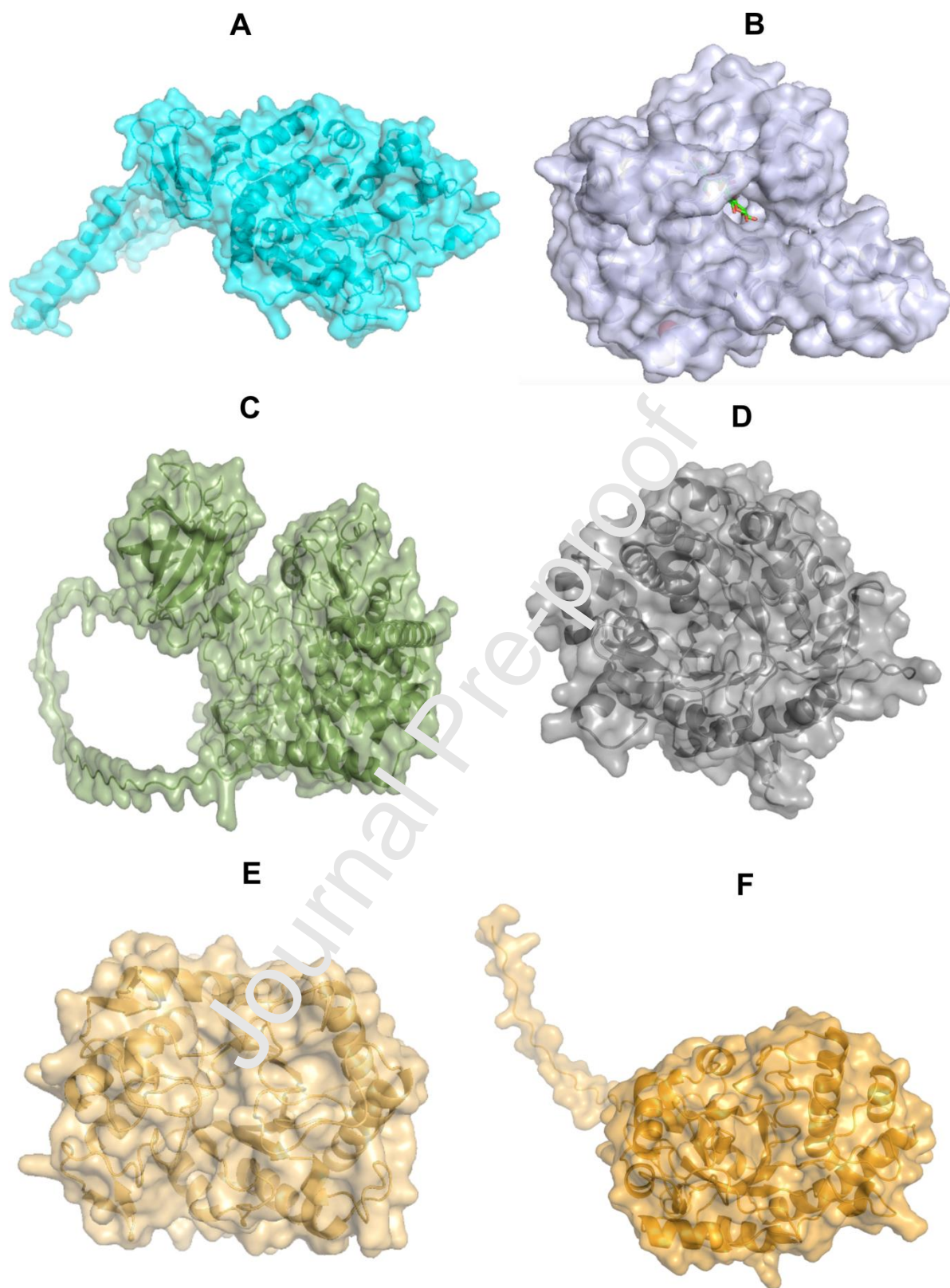


Figure 2. Structures of thermostable glycoside hydrolases for the degradation of starch, cellulose and hemicellulose. (a) *Anoxybacillus sp. GXS-BL*. α -amylase. (b) *Thermobifida fusca* Cellobiohydrolase Cel6B (c) *Spirochaeta thermophila*

Endoglucanase (d) *Halothermothrix orenii* β -glucosidase (e) *Dictyoglomus turgidum* Endo-mannanase (f) *Bacillus licheniformis* Endo-1,4- β -mannosidase. Structural prediction was made using AlphaFold[®] and visualized using Pymol[®].

Enzyme	Function	Origin	Optimum Temp	Reference
Cellobiohydrolase	Cleavage of cellobiose through inverting mechanism on one end of the cellulosic polymer.	<i>T. fusca</i> Glycoside Hydrolase Family 6	50°C	[30, 31]
Endoglucanase	Cleave β -glycosidic bonds in a cellulosic polymer.	<i>S. thermophila</i> Glycoside Hydrolase Family 9	45°C	[32]
β-glucosidase	Conversion of lactose to glucose and galactose	<i>H. orenii</i> Glycoside Hydrolase Family 2	65- 75°C	[33]
Endo-mannanase	Hemicellulose hydrolysis by cleavage of β -1,4 D-manno-pyranosyl bonds.	<i>D. turgidum</i> Glycoside Hydrolase Family 5	70°C	[34]
β-mannosidase	Hydrolysis of β -D-mannose residues at the non-reducing end	<i>B. licheniformis</i> Glycoside Hydrolase Family 9	50°C	[35]

Table 1. Thermophilic enzyme candidates, with their origin and function, for the potential use in the industrial degradation of lignocellulose.

Bleaching

Paper and pulp bleaching requires strenuous use of chlorine gas which is highly bio-accumulative and toxic [36]. The demand to overcome this use of chlorine for the bleaching process is difficult in terms of the high reaction temperature required, between 60 °C-80 °C [37]. Replacing this pre-bleaching step with enzymes requires proteins with the stability to maintain their activity under these conditions-high pH and temperatures. The pulping process results in xylan depositing on fibres causing a barrier between the bleaching chemicals and the fibre surface [38] which increases consumption of chlorine dioxide. In this instance, bacterial thermophilic xylanases, which also function under alkaline conditions, can be used to hydrolyse precipitated xylans to allow the bleaching agents to access the fibres. These glycoside hydrolases also reduce the cost of bleaching, improve the pulp properties such as brightness and reduces Adsorbable organic halides (AOX) formation, a common environmental pollutant produced in this industry [39]. By reducing the bleaching agents and improving the rate of delignification, the AOX formation is reduced up to 40% [40, 41].

For example, a thermophilic xylanase from *Bacillus* species was isolated from Sapan Sungai Aro Hot Spring, South Solok [42]. Xylanase enzymes from thermophilic *Bacillus* sp. can be classified by their molecular mass and isoelectric point [43] under different GH families such as GH10 or GH11 [44, 45]. Xylanases are endo-acting proteins which target the 1,4- β -D-xylosidic linkages in xylan through a retaining mechanism [46]. These GH's derived from thermophilic bacteria found in the hot springs have the potential to increase pulp brightness by removing the xylan layer from fibres at a temperature of 60 °C [47]. Examples of other thermophilic bacteria have been well studied specifically to understand their catalytic potential for paper and pulp bleaching. Extracellular endo-1,4- β -xylanases isolated from *Thermotoga* sp. strain FjSS3-B.1, another strain from hot springs in Savu-Savu Beach, Fiji, showed optimal thermostability at 105 °C which increased to 110 °C once immobilised. The immobilisation showed an increase in protein rigidity and resistance to thermal inactivation [48] making this hyper-thermophilic protein another potential candidate for scaled pre-bleaching processes.

Beverages

Glycoside hydrolase families 5 (GH5) and 26 (GH26) classify a particular type of CAZyme activity called β -mannanase. GH5 includes many enzymatic activities described in the family including endo- β -mannanases and β -mannosidase which use a classical Koshland double-displacement mechanism as discussed by Withers and Williams [49]. This retaining mechanism relies on two amino acid residues as the acid/base and nucleophile, both found at the C-terminal of β -strands 5 and 7 [50]. GH26, on the other hand, includes endo- β -1,4-mannanases which degrade mannose-derived polysaccharides into simple sugars through a glutamic acid residue acting as an acid/base with a retaining mechanism [46]. However, exo-acting mannanases have also been reported in this GH family [51]. Mannanases can be utilised in the fruit juice industry to reduce the high viscosity levels produced in fruit juice developments. The reaction presented in Figure 3 shows the β -mannanase active site's role in the breakdown of the mannan fraction of hemicellulose found in fruit juices [52]. Two glutamate residues are used as an acid/base and nucleophile in this reaction. The method would be used in an attempt to reduce the costings of viscosity reduction conventionally used for fruit juice clarification [53].

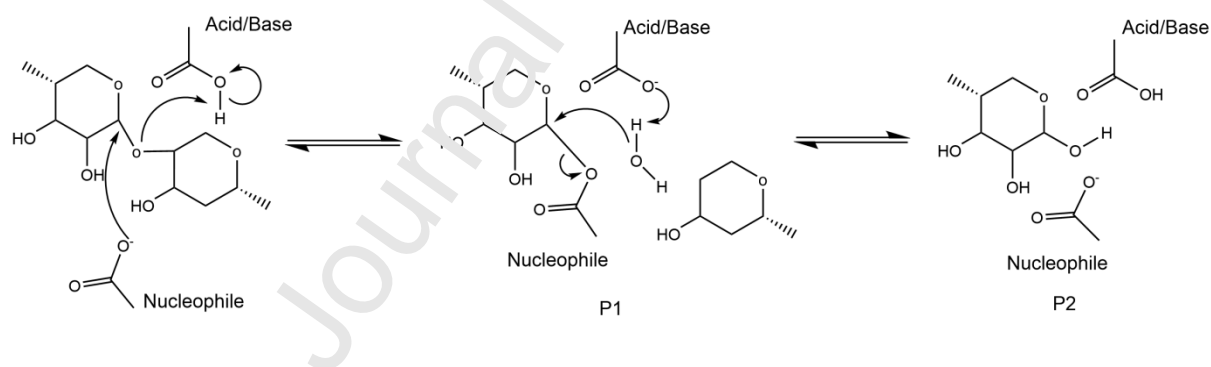


Figure 3. The Koshland double-displacement mechanism of β -mannanases as discussed by Sharma, Dhillon and Goyal [54]. P1 and P2 identify the reaction products.

Bacillus pumilus is a prevalent aerobic bacteria that can grow in temperatures of up to 55 °C [55]. Unlike other thermophiles, this bacterial species is not only found in high-temperature environments. Due to its wide range of growth temperature, it can be found in soil, water, fermented food and the gastrointestinal tract [56]. As the optimum temperature for the high-mannan hydrolysis activity in the fruit juices was

found to be 60 °C [57], properties of β -mannanases from this *B. pumilus* mirror the high growth temperature of their host, making them an ideal candidate for viscosity optimisation in the fruit juice industry.

Other thermostable endo- β -1,4-mannanases have proved to reduce viscosity in grape juice clarification increasing 12.6% in volume when compared with the control [53].

Extreme pH-resistant glycoside hydrolases

pH-resistant bacteria are called acidophiles, which can be found in low pH environments, and alkaliphiles found in high pH environments, which are known to grow best at pH higher than 9 [58]. Their ability and consequently their enzyme's ability to handle solvents and detergents allows them to be utilised in almost all areas of biotechnology from pharmaceuticals to alkali-treated wood pulp. Alkaliphiles uptake protons to maintain cytoplasmic pH homeostasis exploiting a combination of symporters and antiporters [59]. They are used in conjunction with proton and sodium ion transfer to maintain a low pH outside of the cell to aid the generation of ATP. The antiporters are essential for cell survival in low hydrogen conditions whereby the Na^+ ions are extruded from the cell generating energy for the H^+ to cross the cytoplasmic membrane [60]. Although there are many cellular adaptations, the enzymes, especially the ones secreted into the environment, specifically have advanced structures to retain their stability.

Although the first studies of alkaliphilic enzymes were over 50 years ago [61], understanding of how their structural adaptations benefited protein stability in alkaline conditions was still not well studied until the crystal structure of phosphoserine amino transferase from alkaliphile *Bacillus alcalophilus* was determined [62]. Solving the 3D-structure by crystallisation promoted others to determine alkaliphilic protein structures to understand better their industrial uses.

As we discussed before, GH5 possesses many subfamilies from GH5_1 to GH5_57. β -mannanases, as mentioned previously, fall in the GH5_8 subfamily until today. Like the other extremozymes discussed in this review, glycoside hydrolases, such as mannanases, have properties that mirror their alkaliphilic bacterial hosts. As

shown in Figure 4B, β -mannanases from *Bacillus* sp. N16-5, for example, can maintain their stability in high pH conditions due to less polar residues and an increase in hydrophobic and arginine residues [63]. Adaptations like this make alkaliphilic β -mannanases and other glycoside hydrolases suitable for use in wastewater treatment, degumming and most commonly in detergents- a process we will discuss in more detail in the next section.

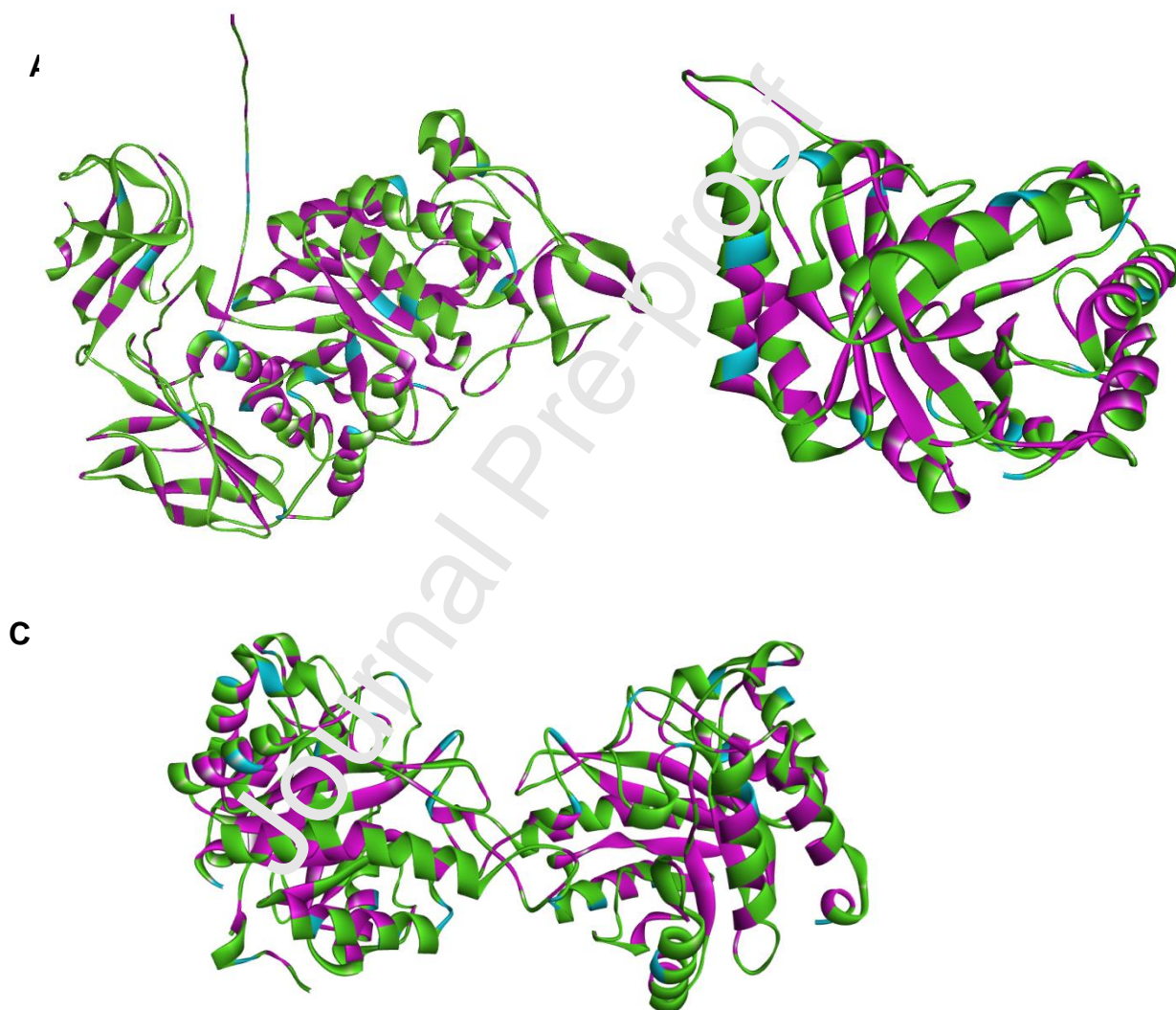


Figure 4. Protein structure predictions of (A) amylase from *Alkalimonas amylolytica* N10 [64] (B) β -mannanase 3JUG from *Bacillus* sp. N16-5 [65] (C) Endo- β -1,4-mannanase 7DVZ from *Bacillus* sp. N16-5 [66]. Residues distinguished by colours,

Pink = Hydrophobic Non-Polar Residues. Blue= Arginine Residues, created using Discovery Studio®.

Alkaline α -amylases in detergents

It is well known that alkaline enzymes are the most common bio-degraders used in detergents today [67]. Specifically, glycoside hydrolases, such as cellulases and α -amylases, but also other enzyme families such as proteases. There is an abundance of literature showing alkaline enzymes are perfect for laundry processing as detergents often have a pH of 8.0 to 11.0 [68, 69]. An example candidate for an alkaliphile in the laundry would be α -amylase from *Streptomyces gulbargensis* [70]. Products formed from this protein are glucose, maltose and malto-oligosaccharides indicating endo-acting activity of this amylase specifically. This glycoside hydrolase would traditionally be classified into the GH family 13 however, the full characterisation is not yet available and therefore is absent in the CAZy database. Nevertheless, it is known that this particular protein has optimum activity at pH 10.0 to 11.0 [70] and could show enhanced activity similar to *Bacillus* species adding the cofactor Ca^{2+} by 48% [71]. These characteristics are not only useful for stability but it would be fair to suggest the the Ca^{2+} which is found in household water at ranges of 1-150 mg/L [72], could help increase the activity of the enzyme through the washing cycle.

Soil and lake environments are often alkaline in nature due to sulphate reduction and the presence of sodium carbonate. Many *Bacillus* sp. isolated from soil and lakes provide some promising amylases for use in laundry detergent. *Bacillus* sp. SP-CH7 isolated from Chilika lake (India) contains alkaline amylase with both activity and stability optimum between pH 7.0 and 13.0 [73]. This amylase's ability to remove stains was tested according to the stain removal protocol used to assay cold-adapted lipase from psychrophiles [74]. The results showed that the amylase removed 96.1% of the stain, higher than other detergents lacking this enzyme [73].

There are many other examples of amylases from bacteria found in the soil, such as the shown in Figure 4A, from *Alkalimonas amylolytica* N10, which can withstand pHs around 9.5 but with the added benefit of thermostability at 50°C [64], optimized by the authors through site-directed mutagenesis. Although soil

environment is shown to contain many extremophilic and polyextremophilic microbes with their encoded proteins, recent discoveries have shown soil contaminated with household waste might have even more suitable contenders for laundry products. Some of these amylases are active at pH from 7.0 to 9.0. Amylases from one bacterial isolate, *Bacillus* sp. C37PLCA showed good compatibility with detergents due to an optimum activity level at alkaline pH and in the presence of detergents such as Ariel (Procter and Gamble®) and OMO Matik (Unilever®) [75].

Acidophiles

Acidophiles can oftentimes be overshadowed by the popularity of alkaliphiles in literature and applied industry. Acidophilic bacterial cellulases appear to be the most used glycoside hydrolases from this extremophilic group for industrial applications [76, 77], such as the degradation of lignocellulose as previously mentioned. These enzymes work best for this application at high temperatures but also at low pHs, around pH 5.0 to 6.0 [2]. Cellulolytic and hemicellulolytic enzymes found in *Alicyclobacillus cellulosilyticus* sp. nov. included β -glucosidase, α -galactosidase, α -glucosidase, α -mannosidase or α -fucosidase [78], which are all categorised as glycoside hydrolase families 1, 4, 31 and 95, respectively (<http://www.cazy.org/Home.html>). These enzymes were the first reported proteins to have the potential to function at pH as low as 3.0 with only a slight decrease in hemicellulose conversion efficiency [2]. Acid cellulases require high thermostability for the process of lignocellulose degradation to ensure complete saccharification and ethanol production concurrently [79]. This would make some of them potentially ideal for use in the biofuel industry, through further research and optimisation.

High saline environments and the development of halophilic glycosidase biocatalysts

Halophilic bacteria and other microorganisms can survive at high salt concentrations. These concentrations can be variable based on the bacteria's salt tolerance as seen in Edbeib's review [80] based on the earlier studies of halophiles [81]. Concentrations of salt can range from slightly halophilic (0.2 to 0.5M), moderate (0.5 to 2.5M), severe (2.4 to 4.0M) or extremely halophilic (4.0 to 5.9M) [80]. Bacterial isolates from salterns across Spain included two moderate halophiles,

Salibacillus salexigens ATCC 700290 *Halomonas meridiana* DSM 5425, both of which grew optimally with a total salt concentration of 5 to 15% (w/v), and showed amylase hydrolytic activity [82]. Contrarily, amylase identified in a *Bacillus* sp. strain H7 isolated from marine water in Harihareshwar beach (India) had activity at a maximum of 6M NaCl denoting the glycoside and its host as extremely halophilic [83].

It is due to this high salt environment that these halophiles are adapted to survive high osmotic stress, freeze-thaw, desiccation and rehydration cycles and finally low water activity conditions [84]. Halophilic enzymes (halozymes) can handle the highly saline environments by creating osmotic equilibrium in the aqueous surroundings creating a high intracellular ionic concentration using K^+ or Na^+ ions [85]. The focus on research of halophilic enzymes has developed studies that have unveiled the key adaptation of these enzymes to maintain stability and osmoregulation with a limiting amount of water and highly saline conditions. This adaptation has highlighted the abundance of polar residues within the protein's amino acid fingerprint including asparagine, glutamine, serine, and threonine [86]. Examples of structures for halophilic cellulase and α -amylase can be seen in Figure 5A and Figure 5B, respectively.

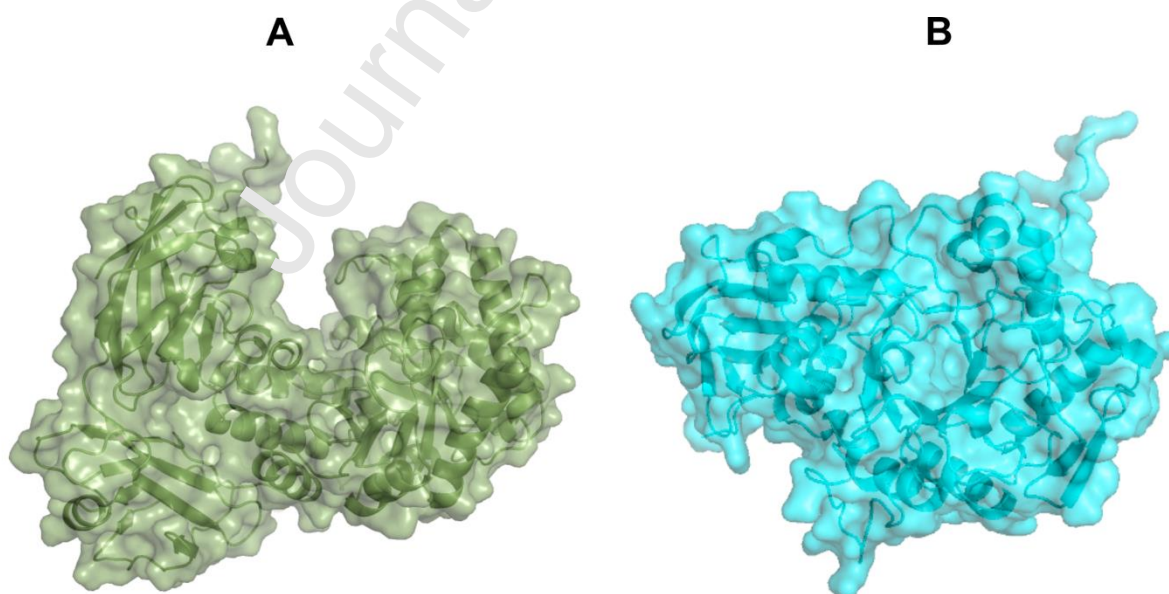


Figure 5. (A) *Bacillus* sp. BG-CS10 cellulase [87] (B) *Halomonas meridiana* α -amylase [88]. Protein structures were predicted by AlphaFold[®] and visualised with Pymol[®].

The addition of salt has proven to increase the activity and thermostability of some glycoside hydrolases. The shift in protein structure in the presence of NaCl can be hard to detect as shown by Zhang et al [87] even when the activity increased ten-fold. These structural shifts have been well-known since the early 2000's [88], but further study is needed to decipher specific natural structural changes to aid the current focus on protein engineering. Beneficial modifications indicate a potential for these halozymes to be used in biotechnology.

Halophilic α -amylase in Detergents

There is significant interest in halozyme's ability to handle high-salt concentrations in detergents. As mentioned previously, the common starch degrading α -amylase is often categorized into glycoside hydrolase family 13, although, it also can be found in the CAZy database under glycoside hydrolase families 57 and 126. High salt concentrations come with the lack of water availability and yet the enzymes, in particular α -amylase, still maintain stability and conformation. This can be very useful in laundry to minimise the use of water required for every wash cycle and add extra value such as the production of bioactive metabolites [89]. For example, α -amylase from *Bacillus* sp. TSCVKK, isolated from soil in Chennai [90], shows promising characteristics for its survivability and maintenance of activity through the wash. This is due to the optimal NaCl concentration at 10% (w/v) for the enzymatic activity. This α -amylase showed little to no loss of activity in the presence of detergents and surfactants such as 0.1% of Triton X-100, Tween 20, Tween 40, and Tween 80 [90]. However, many other bacterial strains from hypersaline environments harness α -amylases which have other extreme catalytic properties such as resistance to temperatures and organic solvents, making them one of the more popular commercially available enzymes [91]. In addition, α -amylase from marine *Streptomyces* sp. retained almost 80% of its activity in the presence of 12% (w/v) of NaCl with activity remaining around 50% at temperatures near 85 °C [92]. These properties, as well as its ability to maintain stability in the presence of metal ions, corroborate that α -amylases can be polyextremophilic enzymes and ideal candidates for laundry detergents [93, 94].

Carbohydrate-active enzymes in the absence of water

Xerophiles are microorganisms found in dry environments and their glycoside hydrolases, like the other extremophilic enzymes, are useful for multiple biotechnological applications. However, there is a lack of literature available compared to other extremophiles due to the water-dependant nature of proteins, and enzymes in particular, as hydration is the driving force of protein folding and stability conservation [95]. Glycoside hydrolases like other enzymes are able to function in the absence of water by ensuring that hydration is maintained in the active site region and enzyme surface [96]. As discussed by Karan et al [16], hydration is maintained based on an excess of acidic residues, in particular, glutamate residues on the protein surface which are known to have efficient water binding capability. The adaptations of halophilic enzymes sometimes are related to xerophiles. These adaptations are reflected by changing some hydrogen bonding in the active site or changing the water dynamics caused by salt ions [97].

Although insight into enzyme function, particularly lipases, in low water was heavily studied towards the end of the 20th century [98-100], more recent studies into other extremozymes have dominated the biotechnological sector. In this section we will discuss the advantages of using xerophilic glycoside hydrolases and how they would be of benefit to scaled industrial processes following further study and optimisation.

Glycosidases with organic solvents

Glycosidases require a nucleophile in their catalytic mechanism usually placed by water. These mechanisms could be inverting or retaining, relating to the inversion or retention of the anomeric carbon in the product. Retaining and inverting glycosidase mechanisms opt for different approaches and transition states, as shown in Figure 6A and 6B, respectively.

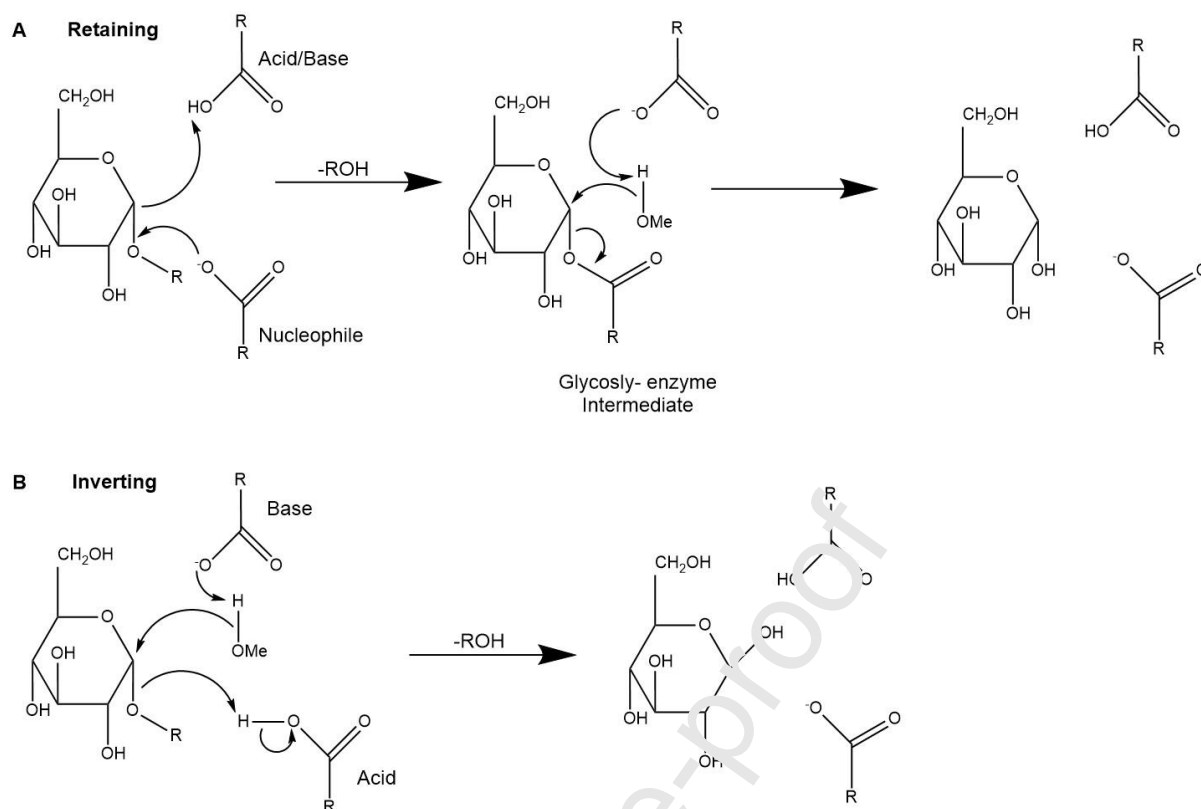


Figure 6. Mechanistic prediction of both retaining (A) and inverting (B) mechanism of α -amylase with glutamate and aspartate residues as the acid/base and nucleophile respectively.

Enzymatic reactions in alkyl glycoside hydrolases with water produce low yield with regards to industrial processes, making scale-up gruelling [101]. However, if these proteins can adopt a nucleophile from micro-aqueous media, their potential use in industry would be massively enhanced. However, the use of organic solvents can cause issues with reactivity in terms of denaturation and decreased rates of catalysis, therefore method and reaction optimization are required if we would like to use them in different harsh processes as happens in industry. Bansal et al [102] suggested these changes could be due to a change in the polarity of the active site by the stripping of water and the addition of solvent affecting the structural folding, particularly after long solvent exposure. In this sense, many researchers have attempted to overcome this issue over previous years. For example, the use of lipid coating on glycosidases like β -mannanases and β -glucosidases were assessed in transglycosylation assays with dry isopropanol ether [103] to combat this polarity shift.

More recently, it has been shown that the type of organic solvent used can either increase or decrease the activity of glycoside hydrolases, such as the amylopullulanases from *Thermoanaerobacter brockii subsp. brockii*. These glycoside hydrolases are endo-acting multi-domain glycoside hydrolases from families 13 and 57 with the same starch substrate as α -amylases [104]. Amylopullulanase showed an enhanced activity with 50% hexane or acetone but a decreased activity with dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and butanol at 20% to 50% [105]. In addition to this, although amylases from *Brachybacterium* sp. showed a slight decrease in activity in the presence of ethanol, maltotetraose oligosaccharide was formed, which was absent in the reactions without the organic solvent [106]. This introduces an additional benefit to using organic solvents instead of water to produce a wider range of new oligosaccharides. If this approach continues to be developed in the future, these enzymes will be used more frequently in industry with the adaptation to aid the solubility of substrates and reduce levels of contamination. This discovery has made a large impact in the enzymology field as organic solvent methods are favoured to emulate low water assays and even yield additional products.

Other Low water Enzyme Catalytic Applications

Glycoside hydrolases are known to target many food stains, hence their previous mention for their use in detergents. Therefore, they have the potential to be used for stain removal under 'dry' conditions, which could be used to reduce dependency on high water volume washing.

Enzymes, such as α -amylases, generally play a key role in breast milk to compensate for the low pancreatic function of newborns [107]. For parents depending on baby milk formula, these enzymes could play an important role because, although the formula is not considered dry when used, it is made from powder and therefore, the enzymes inside must be able to maintain their folding to work successfully in the formula.

As we stated before, xerophilic glycoside hydrolase studies are very limited, but this does not disregard them as potential industrial biocatalysts and could still be the focus of extremophilic CAZyme research in years to come.

Advantages of extremozymes in industrial applications

As we stated before, enzymes encoded in extremophiles are called “extremozymes”. These extremozymes have multiple advantages to be applied in industrial applications as they can survive in extremely harsh conditions, such as high or low temperatures (some of them even at 100 °C or 4 °C, respectively), high or low pH (even at pH 4 or 13), etc. Industrial applications, such as the detergent industry where the pH of these products can be as high as pH 13 due to the presence of carbonates and other additives, can benefit from the use of these natural alkaline-adapted enzymes. There are multiple examples in the literature, including cellulases, α -amylases, pullulanase or xylanase [108]. In addition, these extremozymes have been used in the biofuel industry for sugar hydrolysis and lignocellulosic biomass to convert it into bioethanol or in the paper industry to hydrolyse starch coatings to make the paper smoother for writing [109]. Most of these industrial applications require a high temperature and high pressure, which make these extremozymes very important biocatalysts.

The molecular adaptations described for these enzymes have been recently reviewed [108]. The authors described the adaptations according to the extreme environment that they are adapted to. Enzymes adapted to high temperature (thermophilic) have been described to have a higher number of salt bridges, electrostatic interactions, strong hydrophobic protein core and a high number of proline residues, compared with the mesophilic counterparts [108]. These adaptations contrast with the ones described in the opposite environment (low temperature or psychrophilic environments) where the proteins have high hydrophilicity in the protein core, a low number of proline and a high number of glycine residues.

Psychrophilic α -amylase from the bacterium *Alteromonas haloplanctics* has a low content in proline in loops and turns [110]. This adaptation increases the flexibility of secondary structures facilitating the activity of the protein at low temperatures. In addition, it has a low number of arginines in the core, which produces weaker electrostatic interactions and thus a reduced number of interdomain interactions decreasing the rigidity of the protein core. Finally, this α -amylase has fewer surface charged residues thus having a smaller number of

hydrogen bonds and therefore increasing the flexibility of the protein at its outer surface. In a similar manner to this α -amylase, cellulases can also be adapted to low temperature activities [111]. In this study, the authors established that the cellulase isolated from a camel rumen metagenome exhibited lower activation energy (E_a), Gibbs free energy of activation (ΔG), and enthalpy of activation (ΔH), as compared to mesophilic and thermophilic enzymes. A lower ΔG may be explained as a lower energy barrier that has to be overcome by the ground-state enzyme–substrate complex (ES) to reach the activated state (ES^*) [111].

In contrast to psychrophilic α -amylase, thermophilic enzymes have internal domain regions and interdomain salt bridges that allow the stabilization of the protein at temperatures of 100 °C compared with the mesophilic enzyme [112, 113]. In addition, the thermophilic α -amylase has a high number of histidine residues, decreasing the entropy of the unfolded state and thus stabilizing the native conformation [112-114].

Finally, less is known about protein adaptations described for alkaliphilic enzymes, such as cellulases or α -amylase [115]. Only a few common features have been described for these extremozymes. Alkaliphilic α -amylases show loss of Lys-Asp pairs but gain in Arg-Asp/Glu pairs as an adaptative characteristic [115].

Limitations and Future Perspectives

Recent emerging technologies are enabling the discovery of novel glycoside hydrolases from extreme environments. In the past, researchers faced the challenges of the isolation of interesting bacteria or archaea because of the absence of alternative microbiological methods. Because of this lack, the discovery and characterization of interesting biocatalysts from extreme environments, such as volcanoes or deep-sea hydrothermal sites, didn't take off in the application of these enzymes in industrial applications. Enzymes have been identified directly from the microorganisms themselves or by screening for enzyme-encoding genes. However, in the last few years, an innovative approach has been developed where the researchers are screening with a sequence-based approach for these biotechnological enzymes [116]. In this approach, the amino acid sequence for the enzyme of interest is used as a reference to look at metagenomes collected in

several environmental samples with the extreme conditions required for the industrial application. This approach gives multiple targeting sequences in those (meta)genomes and the authors only need to obtain the synthetic gene, which price has been massively reduced with the novel sequencing technologies [116]. This approach gives the researchers the ability to obtain a large panel of enzymes from these environments. After this panel discovery, the development of high-throughput methods for activity screening, such as microfluidics, has enabled the expansion of structural/activity studies for important CAZymes [117]. With this novel approach, in 2019, Strazzulli et al applied the metagenomics method to mine for novel glycoside hydrolases from volcanic sites [118]. In this study, they discovered two novel enzymes, a β -mannanase/ β -1,3-glucanase (GH5_19) and a novel NAD⁺-dependent GH109 with a previously unreported β -N-acetylglucosaminidase/ β -glucoside specificity from unknown archaeon [118]. We envisage that this new metagenomics approach will uncover the potential of extreme sites where the condition for bacterial isolation is not yet developed. In another recent study, Amin et al described the use of thermophilic archaeon for the discovery of glycoside hydrolases and transferases with potential applications in industry [119]. They pointed out the paucity in the discovery and application of glycoside transferases from extreme environments for the production of interesting industrial oligo- and polysaccharides that require high temperatures [119].

Finally, huge efforts have been recently made in the mining and engineering of pullulanases, which have given many thermostable biocatalysts for efficient starch processing. Wang et al in 2019 [120] reviewed all the structures solved for pullulanases and identified the different domains that have been engineered to enhance the stability of the protein, such as different critical loops near the active site. They postulated that introducing this loop into other mesophilic enzymes will increase the stability to high temperatures [120]. In addition, many authors have introduced different point mutations in the active site of these pullulanases to increase their efficiency and stability. Jian-Xiu et al (2018) used a new bioinformatic method called Simulated Protein Thermal Detection for the detection of mutations, which use molecular simulations to identify the critical mutations to increase the thermostability of the pullulanase from *Bacillus deramificans* [121]. They showed that the triple mutant D332H/D398Y/V390N was more thermostable than other mutants

and the wild type, reserving more than 60% of the activity at 60 °C for 4 h. Wang et al. (2018) also engineered a pullulanase from *Bacillus naganoensis* via tailoring of the active sites lining the catalytic pocket and the mutant D787C was obtained with improved activity and stability [122].

Conclusions

CAZymes, specifically glycoside hydrolases, have proven their use in industry to save on chemical waste and unsustainable energy. More focus and insight into their optimisation are interesting next steps in the field. These glycoside hydrolases can be optimised to degrade sugars at higher rates under harsh conditions or produce higher quantities or additional types of products using mutagenesis, co-factors, immobilization or other protein engineering approaches.

Polyextremophiles are renowned for their multifunctional use in biocatalytic industries indicating they will continue to be prosperous in this field. Further enzyme discovery in several novel extreme environments worldwide, some of them never explored by human beings, such as the deep sea where piezophiles and psychrophiles are dominants (Figure 1) and will unlock the potential of all their CAZymes to screen candidates for large-scale reactions, taking advantage of the natural evolution and adaptation of those biocatalysts to perform in the natural harsh conditions. We anticipate that this approach will gain more interest in the future as these enzymatic candidates would not need further protein engineering or reaction optimizations of because the physiological adaptation they have been exposed. To gain more understanding of their catalytic mechanism and folding abilities in these challenging conditions, we need to develop novel bioinformatic tools to explore those metagenomes trying to deeply uncover those natural resources as an alternative search for novel biocatalysts, specifically glycoside hydrolases. In addition, we need to progress with the understanding of the structure/activity studies of these novel enzymes to map the specific aminoacidic residues that allow them to perform better under those extreme environments, particularly with xerophilic glycoside hydrolases.

Competing Interests Statement

All authors declare that they don't have any competing interest to declare.

Author contributions

E.A. reviewed and wrote the article. J.M-M obtained the funding, designed and directed the review focus and co-wrote the article with input from E.A.

Acknowledgements

E.A. is supported via a BBSRC case PhD studentship (number BB/X511298/1). In addition, JM-M received financial support from an internal grant from Northumbria University. We would like to thank Michael Gollan from Northumbria University for his help using Discovery Studio[®] to make Figure 4.

References

- [1] H. Jeong, S. Shin, H. Lee, Heterogeneous Atomic Catalysts Overcoming the Limitations of Single-Atom Catalysts, *ACS Nano* 14(11) (2020) 14355-14374.
- [2] Q.-Q. Shi, J. Sun, H. Yu, C. Li, J. Bao, J.-H. Xu, Catalytic Performance of Corn Stover Hydrolysis by a New Isolate *Penicillium* sp. ECU0913 Producing both Cellulase and Xylanase, *Applied biochemistry and biotechnology* 164 (2011) 819-30.
- [3] B. Hauer, Embracing Nature's Catalysts: A Viewpoint on the Future of Biocatalysis, *ACS Catalysis* 10(15) (2020) 8418-8427.
- [4] E.A. Karam, W.A. Abdel Wahab, S.A.A. Saleh, M.E. Hassan, A.L. Kansoh, M.A. Esawy, Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase, *Int J Biol Macromol* 102 (2017) 694-703.
- [5] A. Bankar, S. Patil, M. Shinde, S. Shinde, B. Kowligi, Chapter 7 - Potential of microbial extremophiles for biotechnological applications: An overview, in: M. Kuddus (Ed.), *Microbial Extremozymes*, Academic Press 2022, pp. 89-109.
- [6] M. Alam, B.K. Tiwary, *Extremophiles: Diversity, Adaptation and Applications*, Bentham Science Publishers 2023.
- [7] K. Vivek, G.S. Santhosh, S. Subramaniyan, Extremophilic lipases for industrial applications: A general review, *Biotechnology Advances* 60 (2022) 108002.
- [8] F. Sarmiento, R. Perata, J.M. Blamey, Cold and Hot Extremozymes: Industrial Relevance and Current Trends, *Front Bioeng Biotechnol* 3 (2015) 148.
- [9] S. Elleuche, C. Schröder, K. Sahm, G. Antranikian, Extremozymes—biocatalysts with unique properties from extremophilic microorganisms, *Current Opinion in Biotechnology* 29 (2014) 116-123.
- [10] E. Drula, M.-L. Garron, S. Dogan, V. Lombard, B. Henrissat, N. Terrapon, The carbohydrate-active enzyme database: functions and literature, *Nucleic Acids Research* 50(D1) (2021) D571-D577.
- [11] B. Henrissat, A classification of glycosyl hydrolases based on amino acid sequence similarities, *Biochem J* 280 (Pt 2)(Pt 2) (1991) 309-16.
- [12] H. Wu, Q. Gu, Y. Xie, Z. Lou, P. Xue, L. Fang, C. Yu, D. Jia, G. Huang, B. Zhu, A. Schneider, J. Blom, P. Lasch, R. Borriss, X. Gao, Cold-adapted *Bacilli* isolated from the Qinghai-Tibetan Plateau are able to promote plant growth in extreme environments, *Environmental Microbiology* 21(9) (2019) 3505-3526.
- [13] X. Wu, H. Wu, R. Wang, Z. Wang, Y. Zhang, Q. Gu, A. Farzand, X. Yang, M. Semenov, R. Borriss, Y. Xie, X. Gao, Genomic Features and Molecular Function of a Novel Stress-

- Tolerant *Bacillus halotolerans* Strain Isolated from an Extreme Environment, *Biology* 10(10) (2021) 1030.
- [14] R. Malhotra, S.M. Noorwez, T. Satyanarayana, Production and partial characterization of thermostable and calcium-independent α -amylase of an extreme thermophile *Bacillus thermooleovorans* NP54, *Letters in Applied Microbiology* 31(5) (2000) 378-384.
- [15] R. Karan, S. Mathew, R. Muhammad, D.B. Bautista, M. Vogler, J. Eppinger, R. Oliva, L. Cavallo, S.T. Arold, M. Rueping, Understanding High-Salt and Cold Adaptation of a Polyextremophilic Enzyme, *Microorganisms* 8(10) (2020) 1594.
- [16] R. Karan, M.D. Capes, S. DasSarma, Function and biotechnology of extremophilic enzymes in low water activity, *Aquatic Biosystems* 8(1) (2012) 4.
- [17] B.M. Zeldes, M.W. Keller, A.J. Loder, C.T. Straub, M.W. Adams, R.M. Kelly, Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals, *Front Microbiol* 6 (2015) 1209.
- [18] I. Gomes, J. Gomes, W. Steiner, Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization, *Bioresour Technol* 90(2) (2003) 207-14.
- [19] E. Legin, C. Ladrat, A. Godfroy, G. Barbier, F. Duchiron, The most stable amylolytic enzymes of thermophilic microorganisms from deep-sea hydrothermal vents, *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie* 320(11) (1997) 893-898.
- [20] T. Pozzo, J.L. Pasten, E.N. Karlsson, D.T. Logan, Structural and Functional Analyses of β -Glucosidase 3B from *Thermotoga neapolitana*: A Thermostable Three-Domain Representative of Glycoside Hydrolase 3, *Journal of Molecular Biology* 397(3) (2010) 724-739.
- [21] C. Brininger, S. Spradlin, L. Cobani, C. Etila, The more adaptive to change, the more likely you are to survive: Protein adaptation in extremophiles, *Seminars in Cell & Developmental Biology* 84 (2018) 158-169.
- [22] S. Melchionna, R. Sinibaldi, G. Briganti, Explanation of the stability of thermophilic proteins based on unique micromorphology, *Biophys J* 90(11) (2006) 4204-12.
- [23] R. Mehta, P. Singhal, H. Singh, D. Damle, A.K. Sharma, Insight into thermophiles and their wide-spectrum applications, *3 Biotech* 6(1) (2016) 81.
- [24] S. Nanda, J.A. Kozinski, A. Danti, Biomass—an overview on classification, composition and characterization, *Biomass Processing, Conversion and Biorefinery* (2013) 1-35.
- [25] Z. Usmani, M. Sharma, Y. Karpichev, A. Pandey, R. Chander Kuhad, R. Bhat, R. Punia, M. Aghbashlo, M. Tabatabaei, V.K. Gupta, Advancement in valorization technologies to improve utilization of bio-based waste in bioeconomy context, *Renewable and Sustainable Energy Reviews* 131 (2020) 109965.
- [26] J.S. Van Dyk, B.I. Fleischke, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy, *Biotechnology Advances* 30(6) (2012) 1458-1480.
- [27] E.C. Bensah, M. Mensah, Chemical Pretreatment Methods for the Production of Cellulosic Ethanol: Technologies and Innovations, *International Journal of Chemical Engineering* 2013 (2013) 719607.
- [28] M. Valdivia, J.L. Galan, J. Laffarga, J.L. Ramos, Biofuels 2020: Biorefineries based on lignocellulosic materials, *Microb Biotechnol* 9(5) (2016) 585-94.
- [29] S.M. Liao, G. Liang, J. Zhu, B. Lu, L.X. Peng, Q.Y. Wang, Y.T. Wei, G.P. Zhou, R.B. Huang, Influence of Calcium Ions on the Thermal Characteristics of α -amylase from Thermophilic *Anoxybacillus* sp. GXS-BL, *Protein Pept Lett* 26(2) (2019) 148-157.
- [30] J. Klinger, R. Fischer, U. Commandeur, Comparison of *Thermobifida fusca* Cellulases Expressed in *Escherichia coli* and *Nicotiana tabacum* Indicates Advantages of the Plant System for the Expression of Bacterial Cellulases, *Front Plant Sci* 6 (2015) 1047.
- [31] M. Sandgren, M. Wu, S. Karkehabadi, C. Mitchinson, B.R. Kelemen, E.A. Larenas, J. Ståhlberg, H. Hansson, The structure of a bacterial cellobiohydrolase: the catalytic core of the *Thermobifida fusca* family GH6 cellobiohydrolase Cel6B, *J Mol Biol* 425(3) (2013) 622-35.

- [32] V. Hämäläinen, J.D.D. Barajas-López, Y. Berlina, R. Álvarez-Rafael, K. Birikh, New thermostable endoglucanase from *Spirochaeta thermophila* and its mutants with altered substrate preferences, *Applied Microbiology and Biotechnology* 105(3) (2021) 1133-1145.
- [33] N. Hassan, T.H. Nguyen, M. Intanon, L.D. Kori, B.K. Patel, D. Haltrich, C. Divne, T.C. Tan, Biochemical and structural characterization of a thermostable β -glucosidase from *Halothermothrix orenii* for galacto-oligosaccharide synthesis, *Appl Microbiol Biotechnol* 99(4) (2015) 1731-44.
- [34] F.A. Fusco, R. Ronca, G. Fiorentino, E. Pedone, P. Contursi, S. Bartolucci, D. Limauro, Biochemical characterization of a thermostable endomannanase/endoglucanase from *Dictyoglomus turgidum*, *Extremophiles* 22(1) (2018) 131-140.
- [35] C. Songsiriritthigul, B. Buranabanyat, D. Haltrich, M. Yamabhai, Efficient recombinant expression and secretion of a thermostable GH26 mannan endo-1,4-beta-mannosidase from *Bacillus licheniformis* in *Escherichia coli*, *Microb Cell Fact* 9 (2010) 20.
- [36] K.R. Solomon, Chlorine in the Bleaching of Pulp and Paper, *Pure and Applied Chemistry* 68(9) (1996) 1721-1730.
- [37] P. Bajpai, Chapter 19 - Pulp Bleaching, in: P. Bajpai (Ed.), *Biermann's Handbook of Pulp and Paper* (Third Edition), Elsevier 2018, pp. 465-491.
- [38] A. Kantelinen, B. Hortling, J. Sundquist, M. Linko, L. Viikari, Proposed Mechanism of the Enzymatic Bleaching of Kraft Pulp with Xylanases, 47(4) (1993) 318-324.
- [39] X. Lin, Z. Wu, C. Zhang, S. Liu, S. Nie, Enzymatic pulping of lignocellulosic biomass, *Industrial Crops and Products* 120 (2018) 16-24.
- [40] S. Nie, S. Yao, S. Wang, C. Qin, Absorbable Organic Halide (AOX) Reduction in Elemental Chlorine-Free (ECF) Bleaching of Bagasse Pulp from the Addition of Sodium Sulphide, *Bioresources* 11 (2016) 713-723.
- [41] D. Senior, J. Hamilton, Xylanase treatment for the bleaching of softwood kraft pulps: the effect of chlorine dioxide substitution, *TAPPI Journal* 76(8) (1993) 200-206.
- [42] I. Irdawati, S. Syamsuardi, A. Agustien, Y. Rilda, Screening of Thermophilic Bacteria Produce Xylanase from Sapan Sungai Aro Hot Spring South Solok, *IOP Conference Series: Materials Science and Engineering* 335 (2018) 012021.
- [43] K.K. Wong, L.U. Tan, J.N. Saddler, Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications, *Microbiol Rev* 52(3) (1988) 305-17.
- [44] S. Withers, Glycoside Hydrolase Family 11, 2015. <http://www.cazypedia.org/>. (Accessed 11 May 2023 2023).
- [45] S. Withers, Glycoside Hydrolase Family 10 2015. <http://www.cazypedia.org/>. (Accessed 11 May 2023 2023).
- [46] H. Gilbert, Glycoside Hydrolase Family 26 2021. <https://www.cazypedia.org/>. (Accessed 11 May 2023 2023).
- [47] Irdawati, N.D. Putri, Syamsuardi, A. Agustien, Y. Rilda, Potential of Xylanase Thermophilic Bacteria in the Pulp Biobleaching Process, *Atlantis Press*, 2020, pp. 23-27.
- [48] H.D. Simpson, U.R. Haufler, R.M. Daniel, An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*, *Biochem J* 277 (Pt 2)(Pt 2) (1991) 413-7.
- [49] S.W.a.S. Williams, Glycoside Hydrolases 2021. <https://www.cazypedia.org/>. (Accessed 09 May 2023 2023).
- [50] B. Henrissat, I. Callebaut, S. Fabrega, P. Lehn, J.P. Mornon, G. Davies, Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases, *Proc Natl Acad Sci U S A* 92(15) (1995) 7090-4.
- [51] A. Cartmell, E. Topakas, V.M.A. Ducros, M.D.L. Suits, G.J. Davies, H.J. Gilbert, The *Cellvibrio japonicus* Mannanase CjMan26C Displays a Unique exo-Mode of Action That Is Conferred by Subtle Changes to the Distal Region of the Active Site*, *Journal of Biological Chemistry* 283(49) (2008) 34403-34413.
- [52] A. Dawood, K. Ma, Applications of Microbial β -Mannanases, *Front Bioeng Biotechnol* 8 (2020) 598630.
- [53] G. Adiguzel, Z. Sonmez, A. Adiguzel, H. Nadaroglu, Purification and characterization of a thermostable endo-beta-1,4 mannanase from *Weissella viridescens* LB37 and its

- application in fruit juice clarification, *European Food Research and Technology* 242(5) (2016) 769-776.
- [54] K. Sharma, A. Dhillon, A. Goyal, Insights into Structure and Reaction Mechanism of β -Mannanases, *Current protein & peptide science* 17 (2016).
- [55] D. Lindsay, R. Collin, R. van Hekezen, Microorganisms in Milk Powders, in: P.L.H. McSweeney, J.P. McNamara (Eds.), *Encyclopedia of Dairy Sciences* (Third Edition), Academic Press, Oxford, 2022, pp. 329-337.
- [56] J.L. Reyes-Cortes, A. Azaola-Espinosa, L. Lozano-Aguirre, E. Ponce-Alquicira, Physiological and Genomic Analysis of *Bacillus pumilus* UAMX Isolated from the Gastrointestinal Tract of Overweight Individuals, *Microorganisms* 9(5) (2021).
- [57] A. Adiguzel, H. Nadaroglu, G. Adiguzel, Purification and characterization of β -mannanase from *Bacillus pumilus* (M27) and its applications in some fruit juices, *Journal of Food Science and Technology* 52(8) (2015) 5292-5298.
- [58] K. Horikoshi, Alkaliphiles: some applications of their products for biotechnology, *Microbiol Mol Biol Rev* 63(4) (1999) 735-50, table of contents.
- [59] R. Salwan, V. Sharma, Chapter 2 - Physiology of extremophiles, in: R. Salwan, V. Sharma (Eds.), *Physiological and Biotechnological Aspects of Extremophiles*, Academic Press 2020, pp. 13-22.
- [60] K. Dhakar, A. Pandey, Wide pH range tolerance in extremophiles: towards understanding an important phenomenon for future biotechnology, *Applied Microbiology and Biotechnology* 100(6) (2016) 2499-2510.
- [61] K. Horikoshi, Production of alkaline enzymes by alkaliphilic microorganisms 11. Alkaline amylase produced by *Bacillus*, No A-40-2. *Agric. Biol. Chem.*, 35, 1783–1791, 1971.
- [62] A.P. Dubnovitsky, E.G. Kapetaniou, A.C. Papageorgiou, Enzyme adaptation to alkaline pH: atomic resolution (1.08 Å) structure of phosphoserine aminotransferase from *Bacillus alcalophilus*, *Protein Sci* 14(1) (2005) 97-110.
- [63] Y. Zhao, Y. Zhang, Y. Cao, J. Qi, L. Mao, Y. Xue, F. Gao, H. Peng, X. Wang, G.F. Gao, Y. Ma, Structural analysis of alkaline β -mannanase from alkaliphilic *Bacillus* sp. N16-5: implications for adaptation to alkaline conditions, *PLoS One* 6(1) (2011) e14608.
- [64] Z. Deng, H. Yang, J. Li, H.-d. Shi, C. Du, L. Liu, J. Chen, Structure-based engineering of alkaline α -amylase from alkaliphilic *Alkalimonas amylolytica* for improved thermostability, *Applied Microbiology and Biotechnology* 98(9) (2014) 3997-4007.
- [65] Y. Ma, Y. Xue, Y. Dou, Z. Xu, W. Tao, P. Zhou, Characterization and gene cloning of a novel β -mannanase from alkaliphilic *Bacillus* sp. N16-5, *Extremophiles* 8(6) (2004) 447-454.
- [66] W. Liu, C. Ma, W. Liu, Y. Zheng, C.C. Chen, A. Liang, X. Luo, Z. Li, W. Ma, Y. Song, R.T. Guo, T. Zhang, Functional and structural investigation of a novel β -mannanase BaMan113A from *Bacillus* sp. N16-5, *Int J Biol Macromol* 182 (2021) 899-909.
- [67] S. Ito, *Alkaline Enzymes in Current Detergency*, 2011, pp. 229-251.
- [68] S. Ito, S. Shikata, K. Ozaki, S. Kawai, K. Okamoto, S. Inoue, A. Takei, Y.-i. Ohta, T. Satoh, Alkaline Cellulase for Laundry Detergents: Production by *Bacillus* sp. KSM-635 and Enzymatic Properties, *Agricultural and Biological Chemistry* 53(5) (1989) 1275-1281.
- [69] H.S. Olsen, P. Falholt, The role of enzymes in modern detergency, *Journal of Surfactants and Detergents* 1(4) (1998) 555-567.
- [70] D.G. Syed, D. Agasar, A. Pandey, Production and partial purification of α -amylase from a novel isolate *Streptomyces gulbargensis*, *Journal of Industrial Microbiology and Biotechnology* 36(2) (2009) 189-194.
- [71] A. Burhan, U. Nisa, C. Gökhan, C. Ömer, A. Ashabil, G. Osman, Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6, *Process Biochemistry* 38(10) (2003) 1397-1403.
- [72] Y. Yang, H. Kim, A. Starikovskiy, Y. Cho, A. Fridman, Mechanism of Calcium Ion Precipitation from Hard Water Using Pulsed Spark Discharges, *Plasma Chemistry and Plasma Processing* 31 (2011) 51-66.
- [73] S. Priyadarshini, P. Ray, Exploration of detergent-stable alkaline α -amylase AA7 from *Bacillus* sp strain SP-CH7 isolated from Chilika Lake, *International Journal of Biological Macromolecules* 140 (2019) 825-832.

- [74] A. Maharana, P. Ray, A novel cold-active lipase from psychrotolerant *Pseudomonas* sp. AKM-L5 showed organic solvent resistant and suitable for detergent formulation, *Journal of Molecular Catalysis B: Enzymatic* 120 (2015) 173-178.
- [75] K. Inan Bektas, A. Nalcaoğlu, E. Ceylan, D.N. Colak, P. Caglar, S. Agirman, N.S. Sivri, S. Gunes, A. Kaya, S. Canakci, A.O. Belduz, Isolation and characterization of detergent-compatible amylase-, protease-, lipase-, and cellulase-producing bacteria, *Brazilian Journal of Microbiology* (2023).
- [76] R.C. Kuhad, R. Gupta, A. Singh, Microbial cellulases and their industrial applications, *Enzyme research 2011* (2011).
- [77] N.A. Ibrahim, K. El-Badry, B.M. Eid, T.M. Hassan, A new approach for biofinishing of cellulose-containing fabrics using acid cellulases, *Carbohydrate Polymers* 83(1) (2011) 116-121.
- [78] M. Kusube, A. Sugihara, Y. Moriwaki, T. Ueoka, Y. Shimane, H. Minegishi, *Alicyclobacillus cellulosilyticus* sp. nov., a thermophilic, cellulolytic bacterium isolated from steamed Japanese cedar chips from a lumbermill, *Int J Syst Evol Microbiol* 64(Pt 7) (2014) 2257-2263.
- [79] B. Kumar, N. Bhardwaj, A. Alam, K. Agrawal, H. Prasad, P. Verma, Production, purification and characterization of an acid/alkali and thermotolerant cellulase from *Schizophyllum commune* NAIMCC-F-03379 and its application in hydrolysis of lignocellulosic wastes, *AMB Express* 8(1) (2018) 173.
- [80] M.F. Edbeib, R.A. Wahab, F. Huyop, Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments, *World J Microbiol Biotechnol* 32(8) (2016) 135.
- [81] Kushner, Kamekura, Physiology of halophilic bacteria, *Halophilic Bacteria* 1 (1988) 87-103.
- [82] C. Sánchez-Porro, S. Martín, E. Mellado, A. Ventosa, Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes, *Journal of Applied Microbiology* 94(2) (2003) 295-300.
- [83] J.N. Bandal, V.A. Tile, R.Z. Sayyed, H.P. Jadhav, N.I.W. Azelee, S. Danish, R. Datta, Statistical Based Bioprocess Design for Improved Production of Amylase from Halophilic *Bacillus* sp. H7 Isolated from Marine Water, *Molecules* 26(10) (2021).
- [84] T.P. Thompson, J. Megaw, S.A. Kelly, J. Hopps, B.F. Gilmore, Chapter One - Microbial communities of halite deposits and other hypersaline environments, in: G.M. Gadd, S. Sariaslani (Eds.), *Advances in Applied Microbiology*, Academic Press 2022, pp. 1-32.
- [85] J.K. Lanyi, Salt-dependent properties of proteins from extremely halophilic bacteria, *Bacteriol Rev* 38(3) (1974) 272-300.
- [86] S. Fukuchi, K. Yoshimura, M. Wakayama, M. Moriguchi, K. Nishikawa, Unique Amino Acid Composition of Proteins in Halophilic Bacteria, *Journal of Molecular Biology* 327(2) (2003) 347-357.
- [87] G. Zhang, S. Li, Y. Xue, L. Mao, Y. Ma, Effects of salts on activity of halophilic cellulase with glucomannanase activity isolated from alkaliphilic and halophilic *Bacillus* sp. BG-CS10, *Extremophiles* 16(1) (2012) 35-43.
- [88] M.A. Coronado, C. Vargas, E. Mellado, G. Tegos, C. Drainas, J.N.J. Nieto, A. Ventosa, The alpha-amylase gene amyH of the moderate halophile *Halomonas meridiana*: cloning and molecular characterization, *Microbiology (Reading)* 146 (Pt 4) (2000) 861-868.
- [89] S. DasSarma, P. DasSarma, Halophiles and their enzymes: negativity put to good use, *Curr Opin Microbiol* 25 (2015) 120-6.
- [90] K. Kanthi Kiran, T.S. Chandra, Production of surfactant and detergent-stable, halophilic, and alkali-tolerant alpha-amylase by a moderately halophilic *Bacillus* sp. Strain TSCVKK, *Applied Microbiology and Biotechnology* 77(5) (2008) 1023-1031.
- [91] S. Kumar, J. Grewal, A. Sadaf, R. Hemamalini, S. Khare, Halophiles as a source of polyextremophilic α -amylase for industrial applications, *AIMS Microbiology* 2 (2016) 1-26.
- [92] S. Chakraborty, A. Khopade, C. Kokare, K. Mahadik, B. Chopade, Isolation and characterization of novel α -amylase from marine *Streptomyces* sp. D1, *Journal of Molecular Catalysis B: Enzymatic* 58(1) (2009) 17-23.

- [93] P. Kakkar, N. Wadhwa, Extremozymes used in textile industry, *The Journal of The Textile Institute* 113 (2021) 1-9.
- [94] I. Ali, A. Akbar, M. Anwar, S. Prasongsuk, P. Lotrakul, H. Punnapayak, Purification and Characterization of a Polyextremophilic α -Amylase from an Obligate Halophilic *Aspergillus penicillioides* Isolate and Its Potential for Souse with Detergents, *Biomed Res Int* 2015 (2015) 245649.
- [95] W. Kauzmann, Some Factors in the Interpretation of Protein Denaturation 11 The preparation of this article has been assisted by a grant from the National Science Foundation, in: C.B. Anfinsen, M.L. Anson, K. Bailey, J.T. Edsall (Eds.), *Advances in Protein Chemistry*, Academic Press 1959, pp. 1-63.
- [96] M.N. Gupta, I. Roy, Enzymes in organic media. Forms, functions and applications, *Eur J Biochem* 271(13) (2004) 2575-83.
- [97] H.J. Bakker, Ion-ing out the details, *Nature Chemistry* 1(1) (2009) 24-25.
- [98] Y.L. Khmel'nitsky, A.V. Levashov, N.L. Klyachko, K. Martinek, Engineering biocatalytic systems in organic media with low water content, *Enzyme and Microbial Technology* 10(12) (1988) 710-724.
- [99] R. Drapron, Enzyme Activity as a Function of Water Activity, in: D. Simatos, J.L. Multon (Eds.), *Properties of Water in Foods: in Relation to Quality and Stability*, Springer Netherlands, Dordrecht, 1985, pp. 171-190.
- [100] M.T. de Gómez-Puyou, A. Gómez-Puyou, Enzymes in Low Water Systems, *Critical Reviews in Biochemistry and Molecular Biology* 33(1) (1993) 53-89.
- [101] P. Lundemo, E.N. Karlsson, P. Adlercreutz, Preparation of two glycoside hydrolases for use in micro-aqueous media, *Journal of Molecular Catalysis B: Enzymatic* 108 (2014) 1-6.
- [102] V. Bansal, Y. Delgado, E. Fasoli, A. Ferrer, K. Griebenow, F. Secundo, G.L. Barletta, Effect of prolonged exposure to organic solvents on the active site environment of subtilisin Carlsberg, *J Mol Catal B Enzym* 64(1-2) (2010) 38-44.
- [103] T. Mori, Y. Okahata, A variety of lipid-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, *Tetrahedron Letters* 38(11) (1997) 1971-1974.
- [104] M. Nisha, T. Satyanarayana, Recombinant bacterial amylopullulanases: developments and perspectives, *Bioengineered* 4(3) (2013) 388-400.
- [105] H. Mumcu, A. Kayrav, N.D. Isleyen, N.G. Karaguler, Cloning and characterization of thermostable amylopullulanase TbbApu and its C-terminal truncated variants with enhanced activity in organic solvents, *Enzyme and Microbial Technology* 164 (2023) 110176.
- [106] N. Doukyu, W. Yamagishi, H. Kuwahara, H. Ogino, N. Furuki, Purification and characterization of a maltotriose-forming amylase that improves product selectivity in water-miscible organic solvents, from dimethylsulfoxide-tolerant *Brachy bacterium* sp. strain LB25, *Extremophiles* 11(6) (2007) 781-788.
- [107] M. Hamosh, T.R. Henderson, L.A. Ellis, J.-I. Mao, P. Hamosh, Digestive Enzymes in Human Milk: Stability at Suboptimal Storage Temperatures, *Journal of Pediatric Gastroenterology and Nutrition* 24(1) (1997) 38-43.
- [108] A. Ahmad, Rahamtullah, R. Mishra, Structural and functional adaptation in extremophilic microbial α -amylases, *Biophysical Reviews* 14(2) (2022) 499-515.
- [109] N. Gupta, E. Beliya, J.S. Paul, S. Tiwari, S. Kunjam, S.K. Jadhav, Molecular strategies to enhance stability and catalysis of extremophile-derived α -amylase using computational biology, *Extremophiles* 25(3) (2021) 221-233.
- [110] N. Aghajari, G. Feller, C. Gerday, R. Haser, Structures of the psychrophilic *Alteromonas haloplanctis*- α -amylase give insights into cold adaptation at a molecular level, *Structure* 6(12) (1998) 1503-1516.
- [111] K. Khalili Ghadikolaei, J. Gharechahi, K. Haghbeen, K. Akbari Noghahi, G. Hosseini Salekdeh, H. Shahbani Zahiri, A cold-adapted endoglucanase from camel rumen with high catalytic activity at moderate and low temperatures: an anomaly of truly cold-adapted evolution in a mesophilic environment, *Extremophiles* 22(2) (2018) 315-326.

- [112] N. Declerck, M. Machius, G. Wiegand, R. Huber, C. Gaillardin, Probing structural determinants specifying high thermostability in *Bacillus licheniformis* α -amylase11 Edited by A. R. Fersht, *Journal of Molecular Biology* 301(4) (2000) 1041-1057.
- [113] J. Alikhajeh, K. Khajeh, B. Ranjbar, H. Naderi-Manesh, Y.H. Lin, E. Liu, H.H. Guan, Y.C. Hsieh, P. Chuankhayan, Y.C. Huang, J. Jeyaraman, M.Y. Liu, C.J. Chen, Structure of *Bacillus amyloliquefaciens* alpha-amylase at high resolution: implications for thermal stability, *Acta Crystallogr Sect F Struct Biol Cryst Commun* 66(Pt 2) (2010) 121-9.
- [114] K. Hiteshi, R. Gupta, Thermal adaptation of α -amylases: a review, *Extremophiles* 18(6) (2014) 937-944.
- [115] T. Shirai, K. Igarashi, T. Ozawa, H. Hagihara, T. Kobayashi, K. Ozaki, S. Ito, Ancestral sequence evolutionary trace and crystal structure analyses of alkaline alpha-amylase from *Bacillus* sp. KSM-1378 to clarify the alkaline adaptation process of proteins, *Proteins* 66(3) (2007) 600-10.
- [116] C. Burkhardt, L. Baruth, M.-H. Neele, B. Klippel, A. Margaryan, A. Paloyan, H.H. Panosyan, G. Antranikian, Mining thermophiles for biotechnologically relevant enzymes: evaluating the potential of European and Caucasian hot springs, *Extremophiles* 28(1) (2023) 5.
- [117] S. Ladeveze, P.J. Zurek, T.S. Kaminski, S. Emond, F. Hollfelder, Versatile Product Detection via Coupled Assays for Ultrahigh-Throughput Screening of Carbohydrate-Active Enzymes in Microfluidic Droplets, *ACS Catalysis* 13(15) (2023) 10232-10243.
- [118] A. Strazzulli, B. Cobucci-Ponzano, R. Iacono, P. Ciglio, L. Maurelli, N. Curci, C. Schiano-di-Cola, A. Santangelo, P. Contursi, V. Lombardi, B. Henrissat, F.M. Lauro, C. Fontes, M. Moracci, Discovery of hyperstable carbohydrate-active enzymes through metagenomics of extreme environments, *Febs j* 277(6) (2020) 1116-1137.
- [119] K. Amin, S. Tranchimand, T. Benvegnu, Z. Abdel-Razzak, H. Chamieh, Glycoside Hydrolases and Glycosyltransferases from Hyperthermophilic Archaea: Insights on Their Characteristics and Applications in Biotechnology, *Biomolecules* 11(11) (2021) 1557.
- [120] X. Wang, Y. Nie, Y. Xu, Industrially produced pullulanases with thermostability: Discovery, engineering, and heterologous expression, *Bioresource Technology* 278 (2019) 360-371.
- [121] J.-X. Li, S.-Q. Wang, Q.-S. Du, H. Wei, X.-M. Li, J.-Z. Meng, Q.-Y. Wang, N.-Z. Xie, R.-B. Huang, K.-C. Chou, Simulated Protein Thermal Detection (SPTD) for Enzyme Thermostability Study and an Application Example for Pullulanase from *Bacillus deramificans*, *Current Pharmaceutical Design* 24(34) (2018) 4023-4033.
- [122] X. Wang, Y. Nie, Y. Xu, Improvement of the Activity and Stability of Starch-Debranching Pullulanase from *Bacillus naganoensis* via Tailoring of the Active Sites Lining the Catalytic Pocket, *Journal of Agricultural and Food Chemistry* 66(50) (2018) 13236-13242.