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Thermophilic Enzymes: The Key for Greener Industrial Processes

Matilde F. Viegas[1], Maria J. Ramos[2], Pedro A. Fernandes[3]

[1]LAQV@REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal (matilde.viegas@fc.up.pt) ORCID 0000-0002-4472-9691; [2]LAQV@REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal (mjramos@fc.up.pt) ORCID 0000-0002-7554-8324; [3]LAQV@REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal (pafernan@fc.up.pt) ORCID 0000-0003-2748-4722

Abstract

Substantial improvements in the industrial production of goods led to a widespread feeling of unlimited access to food, commodities, and energy. As greener alternatives for industrial processes are in demand, scientists have turned to enzymes, looking for apt biocatalysts. Focusing on extremophiles, this mini review draws a comparison between thermophiles and their mesophilic counterparts, exploring what features are instrumental to their thermostability. A higher number of ion-pairs, hydrophobicity of buried side chains, compact tertiary structure cores, and a complex network of hydrogen bonds are the four main characteristics responsible for the robustness of thermophilic enzymes.

Author Keywords. Enzymes, Mesophiles, Thermophiles, Extremophiles, Protein Engineering.

Type: Review Article

1. Introduction

The world, as we know it, is tightly bound to the industrial advances of the twentieth century. The First and Second Industrial Revolutions, aligned with the use of fossil fuels in the nineteenth century, have prompted the world to evolve at a violent rate (Wrigley 2013). Substantial improvements in the industrial production of goods led to a widespread feeling of unlimited access to food, commodities, and energy. However, behind these are polluting industrial processes with major drawbacks to the environment, contributing to the rise of greenhouse gas emissions we have been witnessing during the last century (American Chemical Society 2016; United Nations 2008; 2015).

The concerns about the environment (Lewis 2016), and our future, have led scientists and industry sectors to invest both time and funds on research to find greener alternatives to industrial processes. Enzymes, the catalysts of a myriad of chemical reactions essential to life, offer high specificity and reaction rate enhancement, posing themselves as the scaffold for the search of greener solutions (Cipolatti et al. 2019).

Enzymes' best performance is typically achieved under physiological conditions as they are tremendously sensitive to any environmental fluctuations. However, a group of enzymes, called extremozymes, possesses the ability to perform the same biological tasks under severe settings. These enzymes belong to extremophile organisms that live in extreme conditions (high temperature, pressure, salt concentration, pH, among others) (Cavicchioli et al. 2011;

Deppe et al. 2005). Their adaptability makes them indisputably suited to harsh, industrial environments.

2. Enzymes, Nature's Manufacturers

Enzymes are responsible for accelerating (catalyzing) chemical reactions, lowering their activation energy, thus being crucial for life maintenance in living organisms (Berg, Tymoczko, and Stryer 2002). The use of enzymes in human-made products started 5000 years ago, in ancient Greece, when people ingeniously took advantage of rennet, a set of enzymes present in animal stomachs, to turn milk into cheese (Dekker 2019). Soon after, the use of microbial enzymes was extended to brewing and baking. In the past century, science has turned its interest to these macromolecules, intending to maximize its potential, and increasing its usage manifolds (Hao et al. 2011).

An archetype case of the use of enzymes is that of *Saccharomyces cerevisiae*. Despite the existence of thousands of microorganisms catalyzing glucose fermentation, *S. cerevisiae* has been extensively used in fermentation. It has since been studied, and genetic and metabolically engineered, garnering it its title of Baker's yeast (Deák 2003).

As is the case of *S. cerevisiae*, we can find a widespread use of enzymes in various industries. – food (Fernandes 2010), textile (Selvarajan, Veena, and Manoj Kumar 2018), animal feed (Walsh, Power, and Headon 1993; Cowieson, Hruby, and Pierson 2006; Cowieson, Masey-O´Neill, and Bedford 2013), paper (Singh et al. 2016; Srinivasan and Rele 1999; Bajpai 1999), biofuel production (d'Errico et al. 2016; Poppe et al. 2015; Alvira et al. 2010; Abin-Fuentes et al. 2013), pharmaceutical (Shrivastava, Shrivastava, and Singh 2018), and other markets – whose prime-matters are biomolecules that can be degraded, modified, or produced by enzymes. Enzymatic catalysis brings many advantages to the table, allowing for reduced energy input, lower costs, less polluting processes, and efficient product recovery at the end. Despite this evidence, only in the last two decades there has been a drastic increase in the use of biocatalysts (Choi, Han, and Kim 2015; Schmid et al. 2001). Currently, only about 20 enzymes are produced on an industrial scale, and 75% of these enzymes used in industrial settings are hydrolytic, degrading its substrate (Li et al. 2012). As technological advances allowed for its production and purification in industrial quantities, the global enzyme market size is proposed to reach USD 6.30 billion by 2022 (Selvarajan, Veena, and Manoj Kumar 2018). It is expectable that this industry will increase enormously in the fore coming decades as the scientific accomplishments start to raise interest from investors and industry.

In this review, we highlight and discuss structural characteristics of extremozymes, in particular, thermophilic enzymes, that allow for the maintenance of their function and structure in high or low temperatures.

3. Mesophilic vs. Extremophilic Enzymes

One may question why the sudden interest in enzymes when industrial processes have already been optimized using inorganic catalysts. Enzymes are a valuable and even preferable alternative: they are fast, specific, efficient, cost-effective, biodegradable, and do not produce as many by-products, resulting in a low environmental impact (Johnson 2013). However, the most commonly used enzymes come from fungi or mesophiles (organisms that grow best in temperatures between 20 °C and about 40°, typically 37ºC) (Rao, Boorgula, and Leitão 2011). Mesophilic enzymes exhibit their best performance under physiological conditions, still, in severe conditions such as the ones often encountered in industrial settings (extreme temperatures, pH and/or ionic strength) they are rendered useless. To overcome this, scientists have turned to extremophiles: organisms from the *Archea* and *Bacteria*, that thrive

in stark conditions such as the ones found in hot vents, deep sea, volcanoes, among others. The enzymes produced by these organisms, extremozymes, are heat- or cold-stable, and solvent-tolerant biocatalysts (Adrio and Demain 2014). Extremozymes possess useful native metabolic functions with potential for application in industrial and biotechnological processes. These include: cellulases (Ueda et al. 2010; Li et al. 2018), and other carbohydrate-degrading enzymes such as esterases (Charavgi et al. 2013), amylases (Agrawal, Dwevedi, and Kayastha 2019; Wu et al. 2018), xylanases (Basit et al. 2018; Sunna and Bergquist 2003), proteases (Jayakumar et al. 2012; Barzkar et al. 2018), pectinases (Kaur and Satyanarayana 2004), keratinases (Wu et al. 2017; Kornitłowicz-Kowalska and Bohacz 2011), lipases (Schreck and Grunden 2014), peroxidases (Merlino et al. 2010) and phytases (Jatuwong et al. 2020; Berka et al. 1998).

In the past decades, the progress made in molecular genetics has allowed for extremozymes to be produced in recombinant mesophilic hosts, making it feasible to engineer and produce such peculiar enzymes for industrial and biotechnological use.

3.1. Extremozymes

Extremozymes are enzymes found in organisms deemed as extraordinary, given the conditions in which they can proliferate. When temperatures rise above 62ºC, mesophilic organisms perish, and only thermophilic prokaryotic organisms can survive. Above 80ºC, hyperthermophilic *Archea* organisms become dominating (Stetter 2011).

Regarding pH conditions, acidophiles grow optimally at pH values of 3.0 or below (Johnson and Schippers 2017). They are used in bioleaching, a process employing microorganisms such as bacteria or archaea for the extraction of valuable metals from low-grade ore, has long depended on mesophilic acidophiles such as *Acidithiobacillus, Ferroplasma, Leptospirillum* (Bosecker 1987; Jafari et al. 2019). Similarly, thermoacidophiles (growing at pH values around zero, and temperatures up to 65ºC) and thermoalkaliphiles (pH 6.0 - 10.0) exhibit clear applicability for industrial purposes, particularly hydrolases for the degradation of biomass for biofuel production (Sharma, Kawarabayasi, and Satyanarayana 2012; Zhao et al. 2018; Maruthamuthu and Van Elsas 2017; Zaparty and Siebers 2011). The group of extremozymes extends beyond pH and temperature adaptations; organisms thriving in high salinity environments are called halophiles (DasSarma and DasSarma 2015), while barophiles proliferate in extreme pressure environments (Horikoshi 1998).

Given the diverse conditions organisms live on planet Earth, investigating their adaptive mechanisms and structural features shine a light on how scientists can improve the enzyme's functionality and resilience for biotechnological applications.

3.2. Thermostability

The amino acid sequence of a protein dictates not only its function but also its three-dimensional structure. With this premise, genetically engineering a protein to improve its reaction rate, or alter its catalysis altogether, seems to be a straightforward process. To do so, scientists can use two distinct approaches, either rational design using site-directed mutagenesis based on prior knowledge or directed evolution, which simulates evolution (Balabanova et al. 2015; Ribeiro et al. 2019). However, when genetically tuning a protein, engineers will most often destabilize it, therefore facing the challenge of maintaining protein stability at the cost of desired functionality (Rubingh 1997).

Thermophiles carry with themselves envy-worth characteristics for biotechnological applications, such as temperature stability, solvent-tolerance, high affinity to the substrate. Instead of genetically altering a given mesophilic enzyme, tuning it for a specific industrial

application, using thermophiles as biocatalysts is an attractive and time-saving alternative (Elleuche et al. 2014).

From a general perspective, there are many different interactions and structural features contributing to thermal stability and denaturation resistance - a greater number of ionic interactions, a higher number of hydrophobic residues at the core of the protein, as well as a higher number of disulfide bridges (Yu and Huang 2014) and proline residues (Takano et al. 2009), resulting in greater packing, shorter surface loops, to name a few. Much like baking itself, it appears that thermal stability results from a careful balance of different ingredients, and not from a single one. Comparative studies have shown four significant determinants of structural stability weighing in the thermostability and resilience of thermophilic enzymes.

3.2.1. Ion pairs

Ion pairs are like- and oppositely charged groups, at a ≤4 Å distance, involved in the folding and stabilization of the tertiary structure of the protein and, consequently, playing an essential role in the protein's function. According to a study by Barlow and Thornton (1983), results show that from a selection of 38 globular proteins, one-third of the charged residues found in a protein are involved in ion-pairs, and 76% are responsible for stabilizing the tertiary structure of the protein.

Szilágyi and Závodszky (2000) established a close correlation between ion-pairs and thermophilic enzymes - extremely thermophilic proteins are characterized by stronger ion pairs than their mesophilic counterparts. These ion pairs were separated by less than 4 Å.

In 2013, another study focused on the ribosomal protein L7Ae from the hyperthermophilic *Aeropyrum pernix,* with a melting temperature of 110ºC, has detailed an extensive ion-pair network in the hyperthermophilic protein when compared to the human homolog (19 out of 24 ion pairs are involved in network *vs.* no ion-pairs in the human protein) (Bhuiya et al. 2013).

3.2.2. Hydrophobicity of buried side chains

The second most striking difference between thermophilic and mesophilic enzymes is the hydrophobic environment at their core. Gromiha et al. (2013) have shown that "80% of the thermophilic proteins examined were characterized by higher hydrophobicity than their mesophilic counterparts". Surprisingly, this was observed only at the core of the proteins, while the average hydrophobicity of the exterior residues was comparable between the two types of enzymes.

A comparative study on adenylate kinases (ADK) from the genus *Methanococcus* has shown that, despite the 68% amino acid sequence identity between thermophilic and mesophilic counterparts of methanococcal ADKs, their melting points were separated by 34ºC. This difference is attributable, in part, to a small number of non-conserved residues with hydrophobic side chains found in the core area, in close proximity with each other (Haney, Stees, and Konisky 1999). Recent studies, using different techniques (Vijayabaskar and Vishveshwara 2010; de Oliveira et al. 2018), support the same hypothesis.

This relevance of a highly hydrophobic core can be explained by the compaction level at the core of the protein. As the hydrophobic residues tightly adhere to one another, acting as an internal support structure counteracting destabilizing influences from the external environment, they provide a higher degree of thermal integrity.

3.2.3. Hydrogen bonds

Studies have shown that the presence and distribution of hydrogen bonds in thermophilic enzymes are distinctive when compared to mesophilic enzymes, as bonds established between exposed and buried residues tighten the protein into itself.

The thermos-stable firefly Luciferase has been widely used as a marker in bioimaging applications (Mezzanotte et al. 2017). In a study aiming to improve its stability by rational design, an amino acid substitution led to a disruption of hydrogen bonds in the mutant. This break of hydrogen bonds caused an increase in the protein's flexibility, therefore destabilizing it (Yu et al. 2015).

Bezsudnova et al. (2015) explored the influence of charged residues on the intramolecular hydrogen-bonding network of a polyextremophilic short-chain alcohol dehydrogenase. This protein is capable of enduring not only high temperature, but also high salinity, and the presence of organic solvents and denaturants. Their study showed that structural analysis showed an increase in the number of Charged-Neutral hydrogen bonds at the surface of the protein, compared to its thermophilic and mesophilic homologs. This indicates that an effective hydrogen bond crosslinking is instrumental in preventing instability when facing denaturation conditions.

3.2.4. Compact tertiary structure cores

Thermophilic proteins present a higher quantity of small amino acids rather than bulky ones (Tompa, Gromiha, and Saraboji 2016). Proline residues, characterized by its fixed dihedral angle, are widely distributed in transmembrane helices of integral membrane proteins (Brandl and Deber 1986; Sansom 1992). They possess a pyrrolidine ring that creates steric constraints in Pro-adjacent regions. Therefore, their role is to provide rigidity, leading to a decrease in the flexibility of the aminoacid chain (Li et al. 1996). Additionally, a higher number of disulfide bonds is found distributed across thermophilic proteins, which contribute to a tighter packing and higher resistance to unfolding (Ladenstein and Ren 2008; Jorda and Yeates 2011).

In a wide study performed with 400 (200 thermophilic and 200 mesophilic homologues) proteins, results demonstrate that factors contributing to core compactness are found across a large number of datasets of thermophilic enzymes. These include higher hydrophobicity, smaller volume nonpolar residues (Ala, Gly and Val), and smaller distribution of bulky polar residues (Panja, Bandopadhyay, and Maiti 2015).

4. Conclusions

Environmental concerns have brought awareness to the importance of optimization of industrial processes in order to reduce pollutants. For the past two decades, there has been an increase in research dedicated to biocatalysis: enzymes' intrinsic stereoselectivity, regioselectivity, and chemoselectivity is appealing for biotechnological applications and are, undeniably, greener solutions to chemical catalysts. Mesophilic enzymes present clear limits on the conditions they can operate on, however, extremophiles possess the characteristics to fit industrial catalysis. With technological advances in protein engineering, it is now possible to alter the enzyme's functionalities. However, these alterations require a fine-tuning of several factors. Extremophiles, with their increased stability and robustness, granter a more appropriate scaffold for protein engineering than mesophilic proteins. As demonstrated in the literature, extremophiles, and in particular thermophilic enzymes, can improve with, and support, the mutations intended to enhance an existing enzyme. In the future, as research progresses, a thorough understanding of the structural properties will enable the

development of equally efficient biocatalysts to replace polluting, costly chemical reactants in industrial processes.

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