Hyperthermophilic Enzymes with Industrial Applications

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Abstract — **Hyperthermophilic enzymes are typically thermostable and are optimally active at high temperatures. Hyperthermophilic enzymes are very similar to their mesophilic homologues. No single mechanism that is responsible for the remarkable stability of hyperthermophilic enzymes. Increased thermo stability must be found in a small number of highly specific alterations. In this review are described current uses and potential applications of thermophilic and hyperthermophilic enzymes as catalysts for industrial processes.**

Keywords - enzymes; hyperthermopilic, starch, molecular biology

I. INTRODUCTION

Hyperthermophiles grow optimally at temperatures between 80 and 110 °C. These organisms have been isolated from all types of terrestrial and marine hot environments. Enzymes from these organisms developed unique structure-function properties of high thermostability and optimal activity at temperatures above 70 °C. Some of these enzymes are active at temperatures as 110 °C and above [1, 2]. Thermophilic organisms grow optimally between 50 and 80 °C. These thermophilic enzymes are usually optimally active between 60 and 80 °C. Active at high temperatures, thermophilic and hyperthermophilic enzymes typically do not function well below 40 °C.

Thermophilic and hyperthermophilic enzymes are part of another enzyme category called extremozymes, which evolved in extremophiles. Extremozymes can function at high salt levels, under highly alkaline conditions and under other extreme conditions (pressure, acidity, etc.) [3]. Intrinsically stable and active at high temperatures, thermophilic and hyperthermophilic enzymes offer major biotechnological advantages. We will focus on the latest findings that explain the thermophilic and hyperthermophilic enzymes with the highest commercial relevance. The interest shown by the scientific community in hyperthermophiles has constantly increased over the last 30 years [4-7].

Hyperthermophiles have been isolated almost exclusively from environments with temperatures in the range of 80 to 115 °C. Hyperthermophiles have also been isolated from hot industrial environments. The most thermophilic organism known, *P. fumarii*, grows in the temperature range of 90 to 113 °C. The upper temperature at which life is possible is still unknown, but it is probably not much above 113 °C. Hyperthermophiles are represented in the Crenarchaeota and Euryarchaeota [8]. Hyperthermophile communities are complex systems of primary producers and decomposers of organic matter [9].

Most enzymes characterized from hyperthermophiles are optimally active at temperatures close to the host organism's optimal growth temperature, usually 70 to 125 °C [10]. Two types of protein stability (thermodynamic and long term) are of interest from an applied perspective. Industrialists need active enzymes rather than enzymes that are in a reversibly inactivated state. For other enzymes, for example diagnostics enzymes, it is often long-term stability that needs to be improved [11]. While improving thermodynamic thermal stability can have a beneficial effect on long-term stability. Directed evolution is a powerful engineering method, and it is now often used to design enzymes with increased thermostability [12]. This method has also been used for a variety of other needs, such as developing enzymes active in solvents or thermostable enzymes with high activity at 20 to 37 °C. Enzymes improved by directed evolution have already been commercialized [12, 13].

Only a few of today's industrial and specialty enzymatic processes utilize thermophilic and hyperthermophilic enzymes. The ever-growing number of enzymes characterized from hyperthermophilic organisms and the recent advent of powerful protein engineering tools suggest that thermophilic and hyperthermophilic enzymes will see more and more use in a variety of applications.

II. INDUSTRIAL APPLICATIONS OF HYPERTHERMOPHILIC ENZYMES

A. Applications in Molecular Biology

The cloning and expression of *T. aquaticus Taq* DNA polymerase in *E. coli* was instrumental in the development of the polymerase chain reaction (PCR) technology. Thermophilic DNA polymerases have since been cloned and characterized from a number of thermophiles and hyperthermophiles. Proofreading enzymes are preferred when high fidelity is required. These applications have been extensively reviewed [13].

Thermophilic DNA ligases are commercially available. Optimally active in the range 45 to 80 °C, they represent an excellent addition to PCR technology.

A number of thermophilic and hyperthermophilic proteases are now used in molecular biology and biochemistry procedures. Proteases can be used in DNA and RNA purification procedures. This enzyme can be used as an adjunct to PCR to break down cellular structures prior to PCR. Numerous thermophilic restriction endonucleases are now commercialized. Most of them, isolated from Bacillus and Thermus strains, are optimally active in the range of 50 to 65 °C. Examples of thermophilic and hyperthermophilic enzymes with applications as molecular biology reagents are presented in Table I.

Table I. Thermophilic and hyperthermophilic enzymes with applications in molecular biology

Enzyme	**Origin**	**Applications**	**Properties**
Taq polymerase	*T. aquaticus*	PCR technologies	Optimal activity at 75°C, pH 9.0
C. *therm* DNA polymerase	*Carboxydothermus hydrogenoformans*	Reverse transcription-PCR	Reverse transcriptase activity, 3′→5′ proofreading activity; optimal activity as 60–70°C
Tcs DNA ligase	*Thermus scodoductus*	Ligase chain reaction	Optimal activity at 45°C
Alkaline phosphatase	*T. neapolitana*	Enzyme-labeling applications where high stability is required	Optimal activity at 85°C, pH 9.9; $t_{1/2}$, 4 h (90°C) (+ Co^{2+})
Protease S	*P. furiosus*	Protein fragmentation for sequencing	Optimal activity at 85–95°C, pH 6.0–8.0; 80% active after 3 h (95°C)

B. Applications in Starch Processing

Most industrial starch processes involve starch hydrolysis into glucose, maltose, or oligosaccharide syrups. These syrups are then used as fermentation syrups to produce a variety of chemicals (ethanol, lysine, and citric acid). High-fructose corn syrup is produced by the enzymatic isomerization of high-glucose syrup. Starch bioprocessing usually involves two steps, liquefaction and saccharification, both run at high temperatures. During liquefaction, starch granules are gelatinized in a jet cooker at 105 to 110 °C for 5 min in aqueous solution (pH 5.8 to 6.5) and then partially hydrolyzed at α-1,4 linkages with a thermostable α-amylase at 95 °C for 2 to 3 h. If the gelatinization temperature drops below 105 °C, incomplete starch gelatinization occurs, which causes filtration problems in the downstream process. If the gelatinization temperature increases much above 105 °C, the α-amylases are inactivated. During saccharification, the liquefied starch is converted into low-molecular-weight saccharides and ultimately into glucose or maltose. Glucose syrups are produced using pullulanase and glucoamylase in combination, while maltose syrups are produced using pullulanase and β-amylase [14].

The pullulanase, isoamylase, β-amylase, and glucoamylase used in industrial starch processing originate from mesophilic organisms and are only marginally stable at 60 °C. α-Amylases which do not require added Ca^{2+} and which operate above 100 °C at acid pH values are also targeted for improved processing. Increasing the saccharification process temperature would result in many benefits:

- higher substrate concentrations
- limited risks of bacterial contaminations
- longer catalyst half-life
- increased reaction rates and decrease of operation time

- decreased viscosity and lower pumping costs and
- lower costs of enzyme purification.

Able to grow at temperatures in the range 80 to 110 °C, hyperthermophiles are great potential sources for α-amylases functioning in the same temperature range. Their optimal activities range from 80 to 100 °C at pH 4.0 to 7.5. An optimal catalyst for starch liquefaction should be optimally active at 100 °C and pH 4.0 to 5.0 and should not require added Ca^{2+} for stability. Greater characterization of some of these enzymes is needed to determine if they are stable and retain significant activity at pH 4.0. With the recent development of powerful engineering tools can expect that an α-amylase with these features will soon be available. Some of the α-amylases were initially believed to be independent of calcium. EDTA has no effect on *P. furiosus* α-amylase activity and stability at temperatures below 90 °C. Full activity is restored by adding $CaCl_2$ to the enzyme and heating the enzyme solution for 30 min at 90 °C. At least 80% α-amylase enzymes are calcium dependent. Examples of thermophilic and hyperthermophilic enzymes with potential applications in starch processing are presented in Table II.

Table II. Thermophilic and hyperthermophilic enzymes with applications in starch processing

Enzyme	**Origin**	**Properties**
α-Amylase	*Thermococcus profundus*	Optimal activity at 80°C, pH 4.0–5.0
	Pyrococcus woesei	Optimal activity at 100°C, pH 5.5
	Thermococcus profundus	Optimal activity at 80°C, pH 4.0–5.0
Glucoamylase	*Thermoanaerobacterium thermosaccharolyticum*	Optimal activity at 50–60°C, pH 4.0–5.5
β-Amylase	*Thermotoga maritima*	Optimal activity at 95°C, pH 4.3–5.5
	Thermoanaerobacterium thermosulfurigenes	Optimal activity at 75°C, pH 5.5–6.0
Pullulanase	*Thermotoga maritima*	Optimal activity at 90°C, pH 6.0
Amylopullulanase	*Thermococcus celer*	Optimal activity at 90°C, pH 5.5
Xylose isomerase	*Thermus aquaticus*	Optimal activity at 85°C, pH 7.0
	Thermotoga maritima	Optimal activity at 100–110°C
	Thermotoga neapolitana	Optimal activity at 97°C, pH 7.1

Thermophilic β-amylases will improve starch saccharification only if they are active at acidic pH and only if they can reduce the saccharification time. If intermediate temperatures (70 to 80 °C) are required to limit the browning side reactions. Production of maltose syrups using β-amylases would still require a compatible debranching enzyme.

With hyperthermophiles as α-amylase or amylopullulanase typically hydrolyze starch. Oligosaccharides are then degraded intracellularly by an α-glucosidase. Like β-amylases, glucoamylases are rare in thermophiles and hyperthermophiles. Glucoamylases have been purified from only a few anaerobes. The *T. thermosaccharolyticum* glucoamylase represents an alternative catalyst for the development of a starch saccharification process at 70 to 75 °C.

Pullulanases are used as debranching enzymes in starch saccharification. Until recently pullulanases were known only in mesophilic organisms and in thermophilic aerobic bacteria. The *Thermotoga maritima* enzyme is the only one characterized in a hyperthermophile. Pullulanases are all optimally active at acidic pH.

Amylopullulanases show dual specificity for starch α-1,4- and α-1,6-glucosidic linkages [15]. And they cannot be used as debranching enzymes in maltose and glucose syrup productions. However, they can be used as alternative enzymes to replace α-amylases during starch liquefaction for producing fermentation syrups.

Xylose isomerases catalyze the equilibrium isomerization of glucose into fructose. Xylose isomerases represent the first industrial use of immobilized enzymes [16]. The isomerization process is typically run at 58 to 60 °C for 1 to 4 h, and the converted syrup reaches 42 % fructose. Producers are interested in using a more stable enzyme, at a lower pH and temperatures close to those of today's processes.

Highly thermophilic and thermostable xylose isomerases have been characterized from *Thermus thermophilus, Thermus aquaticus, Thermotoga maritima, and Thermotoga neapolitana.* In addition their high catalytic efficiency at 90 °C, the *T. maritima* and *T. neapolitana* xylose isomerase are active only at neutral pH.

C. Other Industrial Applications

Cellulose is the most abundant and renewable no fossil carbon source on Earth [17]. Cellulose requires an alkaline pretreatment to become accessible to enzyme action. An enzymatic saccharification step makes cellulose suitable for yeast or bacterial fermentations. One of the main limitations to this process is the low activity and high cost of the cellulases used. Since alkaline pretreatment is performed at high temperatures, hyperthermophilic cellulases can be the best catalysts for cellulose degradation. The production of cellulases by hyperthermophiles is rare and only the enzymes of endoglucanase and cellobiohydrolase, at 95 to 105 °C, represent interesting enzyme combinations in cellulose processing. Examples of thermophilic and hyperthermophilic enzymes with potential industrial applications are presented in Table III.

Table III. Thermophilic and hyperthermophilic enzymes with industrial applications

Enzyme	**Origin**	**Potential application**	**Properties**
Endo-1,4-β-glucanase	*T. neapolitana*	Cellulose degradation	Optimal activity at 95 °C, pH 6.0
Cellobiohydrolase	*T. maritima*	Cellulose degradation	Optimal activity at 95 °C, pH 6.0–7.5
Endoxylanase	*Thermoanaerobacterium saccharolyticum*	Paper pulp bleaching	Optimal activity at 70 °C, pH 5.5–6.0
Esterase	*P. furiosus*	Transesterification and ester synthesis	Optimal activity at 100 °C, pH 7.6
Pectin methylesterase	*T. thermosulfurigenes*	Fruit juice clarification; Wine making	Optimal activity at 70 °C, pH 6.5
Polygalacturonate hydrolase	*T. thermosulfurigenes*	Fruit juice clarification; Wine making	Optimal activity at 75 °C, pH 5.5
α-Galactosidase	*T. maritima*	Oil and gas industry; Sugar beet processing; Oligosaccharide synthesis	Optimal activity at 90 °C, pH 5.0–5.5

Industrial ethanol production is currently based on corn starch that is first liquefied and saccharified. The oligosaccharide syrup is then used for yeast fermentation. The use of thermophilic endoglucanases during starch liquefaction and saccharification is performed at high temperatures [18].

Pulping is the step in the paper production process, during which wood fibers are broken and most of the lignin is removed. The remaining lignin is removed by a bleaching process. Performed with chlorine at high temperatures, pulp bleaching generates high volumes of polluting wastes [19]. The amount of chemical used the resulting pollution that can be reduced if the paper pulp is pretreated with hemicellulases. The paper industry needs thermophilic hemicellulases, since pulping and bleaching are both performed at high temperatures, and pH 6.5 - 7.0 [20]. These enzymes are active at pH around 7.0.

Production of the dipeptide aspartame by using thermolysin is the process that uses a thermophilic enzyme on an industrial scale [21]. Thermophilic and hyperthermophilic enzymes have been used as catalysts for synthetic processes.

The use of enzymes in immunoassays in the pharmaceutical and food industries is constantly increasing. Highly stable enzymes are uses for these applications only if they are active in moderate temperatures.

Pectin is a branched heteropolysaccharide abundant in plant tissues. There are two types of pectinolytic enzymes: methylesterases and depolymerases, which are widely used in the food industry. In fruit juice extraction and wine making, pectinolytic enzymes increase juice yield, reduce viscosity, and improve color extraction from fruit skin. A few thermophilic pectinolytic enzymes isolated from thermophilic anaerobes show catalytic and stability properties compatible with industrial needs.

Animal feedstock production processes include heat treatments that inactivate potential viral and microbial contaminants. Using thermophilic enzymes in feedstock production would enhance digestibility and nutrition of the feed.

III. CONCLUSION

The discovery that molecular biology and biochemical studies as protein purification and characterization are facilitated by the cloning and expressing of genes from hyperthermophiles in mesophilic hosts. Hyperthermophilic enzymes have become model to study enzyme stability and activity mechanisms, protein structure and biocatalysis under extreme conditions.

For developing new biotechnological applications there are the great diversity of hyperthermophiles. The use by the hyperthermophilic enzymes whose substrates are stable at very high temperatures come from the discovery of new, natural hyperthermophilic enzymes that are active above 125 °C. The future of this field is fascinating and boundless.

The stability and activity of thermophilic enzymes can be controlled by separate molecular determinants. Such an achievement could greatly enhance the range of applications for hyperthermophilic enzymes in areas including medicine, food, and research reagents.

The study of protein flexibility and thermostability allow average enzyme structure, tools such as molecular dynamics, hydrogen exchange and NMR.

Many algorithms used in computational methods are created using parameters calculated from known protein structures. Despite the many advances in computer algorithms, protein structure prediction remains among the most challenging tasks in computer modeling.

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