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Thermostable enzymes and their industrial application: a review

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ABSTRACT

Enzymes produced by hyperthermophilic microorganisms are stable at high temperature. Most of the hyperthermophiles are Archaea but there are two groups of bacteria: Thermotogales and Aquificales. Activity of thermostable enzymes can be at temperature close to the organism's growth temperature or at above the organism's growth temperature or can be below the organism's optimum growth temperature. Archaeal transcription systems are more closely related to eukarial than bacterial transcription system. Thus cloning of genes from hyperthermophiles in fast-growing mesophilic eukaryotes (*Saccharomyces cerevisiae*) by recombinant DNA technology can show stability as native enzyme. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes in the food and paper industry, detergents,

drugs, toxic wastes removal and drilling for oil is being studied extensively. In this review, the source of microorganisms and properties of thermostable starch hydrolyzing amylases, xylanases, cellulases, chitinases, pectinases, lipases, DNA polymerases and DNA ligases have been discussed.

Key words: Thermostable, enzyme, application, industry

1. INTRODUCTION

Hyperthermophilic microorganisms are organisms capable of producing thermostable enzymes. Hyperthermophiles have been isolated almost exclusively from both hot natural and industrial environments with temperatures in the range of 80 to1l5^{0}C (Eichler, 2001). Most of the hyperthermophiles are included in the domain Archaebacteria. Thermotogales and Aquificales are the only bacteria. Enzymes from these organisms developed unique structure-function properties of high thermostability and optimal activity at temperatures above 70^{0}C. Some of these enzymes are active at temperatures as high as 110^{0}C and above (Vieille et al., 1996). Hyperthermophilic enzymes can serve as model systems for use by biologists, chemists, and physicists interested in understanding enzyme evolution, molecular mechanisms for protein thermostability, and the upper temperature limit for enzyme function. Intrinsically stable and active at high temperatures, thermophilic and hyperthermophilic enzymes offer major biotechnological advantages over mesophilic enzymes (Bouzas et al., 2006).The molecular determinants of extreme protein thermostability and the thermophilic and hyperthermophilic enzymes with the highest commercial relevance will be focused.

Hyperthermophile diversity

The growing interest is demonstrated by the increasing number of hyperthermophilic species that have been described and by the major central place occupied by hyperthermophiles in worldwide genome sequencing projects. Studies of environmental 16S rRNA sequences analysis suggest that known hyperthermophiles represent only afraction of hyperthermophilic species diversity (Barns et al., 1996). A striking example of this difficulty isthe bacterium *Thermocrinis ruber*. This pink-filament-forming bacterium was described as early as 1967 by Brock, but it took more than 25 years to successfully cultivate this organism (Vielle and Zeikus, 2001).

Hyperthermophiles have been isolated almost exclusively from environments with temperatures in the range of 80 to ll5^{o}C. Hyperthermophiles have also been isolated from hot industrial environments (e.g. the outflow of geothermal power plants and sewage sludge systems) (Satyanarayana et al., 2005). Deep-sea hyperthermophiles thrive in environments with hydrostatic pressures ranging from 200 to 360 atm. The most thermophilic organism known, *P.fumarii*, grows in the temperature range of90 to 113^{0}C (Rothschild and Manicineli, 2001).

Hyperthermophile communities are complex systems of primary producers and decomposers of organic matter. All hyperthermophilic primary producers are chemolithoautotrophs (i.e., sulfur oxidizers, sulfur reducers and methanogens) (Lowe et al., 1993). In relation to the high sulfur content of most hot natural biotopes, most hyperthermophiles are facultative or obligate chemolithotrophs: they either reduce S^0 with H_2 to produce H_2S (the anaerobes) or oxidize S^0 with O_2 to produce sulfuric acid (the aerobes). Extremely acidophilic hyperthermophiles belong to the order *Sulfolobales*. They are all strict aerobes (e.g., *Sulfolobus*) or facultative aerobes (e.g., *Acidianus*) (Fujiwara, 2002).

Hyperthermophilic Enzymes with Commercial Applications

The characterization of *T. aquaticus* Taq DNA polymerase followed by the quick popularization of PCR-related technologies was instrumental in the ever-growing interest of the scientific and industrial communities in thermophilic and hyperthermophilic enzymes. The ever-growing number of enzymes characterized from hyperthermophilic organisms and the recent advent of powerful protein engineering tools suggest thatthermophilic and hyperthermophilic enzymes will see more and more use in a variety of applications.

Applications in Molecular Biology

DNA polymerases. The cloning and expression of *T. aquaticus* Taq DNApolymerase in *E. coli* was instrumental in the development of the PCR technology. Thermophilic DNA polymerases have since been cloned and characterized from a number of thermophiles and hyperthermophiles. The multiple applications of the PCR technology make use of two major properties of these DNA polymerases: processivity and fidelity (Pandey et al., 2016). Taq DNA polymerase's high processivity, make it the enzyme of choice for sequencing or detection procedures. Proofreading enzymes (such as Vent and Deep Ventpolymerases (Table 1) are preferred

when high fidelity is required. While thermophilic DNA polymerases have partially replaced mesophilic enzymes in a few applications, most applications were developed after the advent of PCR (e.g., PCR *in situ* hybridization and reverse transcription-PCR) (Niehaus et al., 1999).

DNA ligases. Thermophilic DNA ligases are commercially available (Table1). Optimally active in the range 45 to 80^{0}C, they represent an excellent addition to PCR technology (Mesbah and Sarmiento, 2016). Ligating property of these enzymes can be used for ligase chain reaction(a DNA amplification method), for mutational analysis (by oligonucleotideligation assay), or for gene synthesis (from overlapping oligonucleotides) (Pandey et al., 2016).

Other enzymes. A number of thermophilic and hyperthermophilicproteases are now used in molecular biology and biochemistry procedures. Some proteins, in particular thermophilic proteins, resist proteolytic digestion at moderate temperatures (20 to 60^{0}C). They only start to unfold and to become sensitive to proteolytic attack above 70^{0}C (Bruins et al., 2001). Proteases like the *Thermus* Rt41A serine protease PRETAQ (Table 1), which is rapidly inactivated by EGTA, can be used in DNA and RNA purification procedures. Once inactivated, PRETAQ will not interfere with other enzymes during further treatment of the DNA or RNA (Covan et al., 1985; Gomes and Steiner, 2004). The *P. furiosus* protease S has a broad specificity, so it is used to fragment proteins before peptide sequencing. 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^{0}C (Huber and Stetter, 1998; Haki and Rakshit, 2003).

Table 1 Examples of thermophilic and hyperthermophilic enzymes with applications as molecular biology reagents

Enzyme	Source	Applications	Properties
Taq polymerase	*Thermus aquaticus*	PCR technologies	Optimal activity at 75^{0}C, pH 9.0
DNA polymerase	*Thermus thermophilus*	Roche molecular biochemical	Reverse transcriptase activity
Pfu DNA ligase	*Pyrococcus furiosus*	Ligase chain reaction and DNA ligations	Active at 45-80^{0}C; $t_{1/2}$- 60 min (95^{0}C)
Serine protease (PRETAQ)	*Thermus strain Rt41A*	DNA and RNA purifications; cellular structure degradation prior to PCR	Optimal activity at 90^{0}C, pH 8.0
Protease S	*Pyrococcus furiosus*	Protein fragmentation for sequencing	Optimal activity at 85-95^{0}C
Carboxypeptidase	*Sulfolobus solfataricus*	C-terminal sequencing	Broad specificity (can release basic, acidic, and aromatic residues); stable in solvents at 40^{0}C
Alkaline phosphatase	*Thermococcus neapolitana*	Enzyme-labeling applications where high stability is required	Optimal activity at 85^{0}C, pH 9.9; $t_{1/2}$- 4 h (90^{0}C)

Table 2 Examples of thermophilic and hyperthermophilic enzymes with potential applications in starch processing

Enzyme	Origin	Properties
α-Amylase	*Desulfurococcusmucosus*	Optimal activity at 100^{0}C, pH 5.5
	Pyrococcus furiosus	Optimal activity at 100^{0}C, pH 5.5-6.0
	Pyrococcus woesei	Optimal activity at 100^{0}C, pH 5.5
	Pyrodictiumabyssi	Optimal activity at 100^{0}C, pH 5.0

	Staphylothermusmarinus	Optimal activity at 100^{0}C, pH 5.0
	Thermococcus profundus	Optimal activity at 80^{0}C, pH 4.0-5.0
	Dictyoglomusthermophilum	Optimal activity at 90^{0}C, pH 5.5
Pullulanase	*Bacillus flavocaldarius*	Optimal activity at 75-85^{0}C, pH 6.3
	Fervidobacteriumpennavorans	Optimal activity at 80-85^{0}C, pH 6.0
Amylopullulanase	*Desulfurococcusmucosus*	Optimal activity at 100^{0}C, pH 5.0
	Pyrococcus furiosus	Optimal activity at 105^{0}C, pH 6.0
	Thermococcus celer	Optimal activity at 90^{0}C, pH 5.5
	Thermococcus litoralis	Optimal activity at 117^{0}C, pH 5.3
	Thermoanaerobacterethanolicus	Optimal activity at 90^{0}C, pH 5.5
Glucoamylase	*Clostridium thermosaccharolyticum*	Optimal activity at 70^{0}C, pH 5.0
α-Glucosidase	*Thermoanaerobacterethanolicus*	Optimal activity at 75^{0}C, pH 5.0-5.5
β-Amylase	*Thermotoga maritima*	Optimal activity at 95^{0}C, pH 4.3-5.5
	Thermococcus sp.	Optimal activity at 95^{0}C, pH 5.5-6.0

Applications in Starch Processing

Most industrial starch processes involve starch hydrolysis into glucose, maltose, or oligosaccharide by the enzymatic isomerization of high-glucose syrup. Temperature and pH controls are critical at gelatinization stage (Ding, 2006). If the gelatinization temperature drops below105^{o}C, incomplete starch gelatinization occurs, this causes filtration problems in the downstream process. If the gelatinization temperature increases much above l05^{o}C, the α-amylases typically used (from *Bacillus licheniformis* and *B. stearothermophilus*) are inactivated (Kristjansson, 1989; Littlechild, 2015a). The pullulanase, isoamylase, b-amylase, and glucoamylase used in industrial starch processing originate from mesophilic organisms and are only marginally stable at60^{0}C. There is a need today for thermostable pullulanases, b-amylases, and glucoamylases (Table 2) (Lasa and Berenguer, 1993; Pantazaki et al., 2002). α-Amylases which operate above 100^{0}C at acid pH values are also targeted for improved processing. Increasing the saccharification process temperature would result in many benefits: (i) higher substrate concentrations, (ii) decreased viscosity and lower pumping costs, (iii) limited risks of bacterial contaminations, (iv) increased reaction rates and decrease of operation time,(v) lower costs of enzyme purification. And (vi) longer catalyst half-life, due to increased enzyme thermostability (Zamost et al., 1991; Littlechild, 2015b).

Applications in Cellulose degradation and ethanol production

Cellulose requires an alkaline pretreatment to become accessible to enzyme action. An enzymatic saccharification step makes cellulose and its degradation products suitable for ethanologenic yeast or bacterial fermentations. Since cellulose's alkaline pretreatment is performed at high temperatures, hyperthermophilic cellulases should be the best candidate, catalysts for cellulosedegradation (Zeikus et al., 1998). The production of cellulases by hyperthermophiles is rare, however. Only recently have endoglucanases and cellobiohydrolases been characterized in the *Thermotogales* (Table 2). Endoglucanase and cellobiohydrolase are optimally active either at 95 or at 105^{0}C, represent interesting enzyme combinations to be tested in cellulose processing. Industrial ethanol production is currently based on corn starch that is first liquefied and saccharified. The oligosaccharide syrup is then used as feed stock for ethanologenic yeast fermentation (Synowiecki, 2010).

Applications in Paper pulp bleaching

In the paper production process, pulping is the step during which wood fibers are broken apart and most of the lignin is removed. Pulping often corresponds to a chemical hot-alkali treatment of the wood fibers. The remaining lignin is removed by a multistep bleaching process. Performed with chlorine and/or chlorine dioxide at high temperatures, pulp bleaching generates high volumes of polluting wastes (Podar and Reysenbach, 2006). The amount of chemical used-and, therefore, the resulting pollution-can be reduced if the paper pulp is pretreated with hemicellulases. Since pulping and bleaching are both performed at high temperatures, the paper industry needs thermophilic hemicellulases, preferably those active above pH 6.5 or pH 7.0 (). Hyperthermophilic hemicellulases have only been characterized in the *Thermotogales*. These enzymes are active at pHs around 7.0. It is interesting that the *Bacillus* 3D endoxylanase is at least 100 times more active than the *Thermotoga thermarum* (Schiraldi and Giuliano, 2002).

Applications in Chemical synthesis

Production of the dipeptide aspartame (L-aspartyl-L-phenylalaninemethyl ester) by using thermolysin is the only chemical synthesis process that uses a thermophilic enzyme on an industrial scale (Vielle, 2001). Other thermophilic and hyperthermophilic enzymes have been suggested as potential catalysts for a variety of synthetic processes. A number of thermophilic enzymes (including hydantoinase, cytochrome P450, secondary alcohol dehydrogenase, and various glycosyl hydrolases) show region selective and/or stereo selective reaction mechanisms that are highly desirable for synthetic chemistry (Pandey et al., 2016).

Other applications

The use of enzymes (including horseradish peroxidase, alkaline phosphatase, and glucosephosphate dehydrogenase) in immunoassays in the pharmaceutical and food industries is constantly increasing. Highly stable enzymes are desirable for these diagnostic applications only if they are active at moderate temperatures (Vielle and Zeikus, 2001).The thermostable alkaline phosphatase recently characterized from *Thermotoganeapolitana* is highly active at high temperatures but shows almost no activity at room temperature. This enzyme could become valuable if its activity at room temperature is engineered to levels comparable to currently available mesophilic alkaline phosphatases and if its stability can be retained (Verma and kanwar, 2012).

Pectin is a branched heteropolysaccharide abundant in plant tissues. Its main chain is a partially methyl-esterified (1. 4)-a- D-polygalacturonate chain. There are two types of pectinolytic enzymes: methylesterases and depolymerases (hydrolases and lyases). These enzymes 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 (Antranikian et al., 2005).

Chitin (a linear β-1, 4 homopolymer of N-acetylglucosamine) is also an abundant carbohydrate in the biosphere. Chitinases could be used for the utilization of chitin as a renewable resource and for the production of oligosaccharides as biologically active substances (Gupta et al., 2004). Some chitooligosaccharides can be used in phagocyte activation or as growth inhibitors of certain tumors. A few thermophilic chitinases have been characterized. Their potential for an economically competitive chitin degradation process remains to be tested (Pandey, 2016).

Animal feedstock production processes include heat treatments that inactivate potential viral and microbial contaminants. Using thermophilic enzymes (i.e., arabinofuranosidase and phytase) in feedstock production would enhance digestibility and nutrition of the feed while allowing the combination of heat treatment and feed transformation in a single step (Vielle and Zeikus, 2001; Pandey 2016).

2. CONCLUSION

Use of hyperthermophilic enzymes as molecular templates to design highly stable enzymes that have high activity at low temperatures could greatly enhance the range of applications for hyperthermophilic enzymes in areas including medicine, food, and research reagents.

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