THERMOACTIVE ENZYMES IN BIOTECHNOLOGICAL APPLICATIONS

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Summary

Extremophilic microorganisms have developed a variety of molecular strategies that help them to survive in ecological niches such as high temperatures, extremes of pH, high salt concentrations, and high pressure. It has been demonstrated that these microorganisms produce novel organic compounds and stable biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes. Some of the enzymes have already been purified and their genes successfully cloned in the mesophilic hosts. In this chapter, we will focus on enzymes such as amylases, pullulanases, cyclodextrin glycosyltransferases, cellulases, xylanases, chitinases, pectinase proteases, alcohol dehydrogenase, esterases, and DNA-modifying enzymes which are of potential use in various biotechnological processes including food, chemical, and pharmaceutical industries.

1. Introduction

Enzymes (biocatalysts) are present in all biological systems including bacteria, archaea, and yeast, and are needed to control vital cellular processes. The industrial application of biocatalysts began in 1915 with the introduction of the first detergent enzyme by Dr.

Röhm. Since that time, enzymes have found wider application in various industrial processes and production. The most important fields of enzyme application are nutrition, pharmaceuticals, diagnostics, detergents, textiles, and leather industries. There are more than 3 000 enzymes known that catalyze different biochemical reactions among the estimated 7 000 known enzymes. Interestingly, only few hundred enzymes are being used industrially. The world market for industrial enzymes, which includes enzymes for research and diagnostic, is estimated to be around 1 billion US dollars. The products derived from these enzymes are estimated to represent a value of more than US$100 billion. For various industrial applications, there is a great demand for enzymes of high specificity and stability. In many cases, microbial biocatalysts, especially of extremophiles, are superior to the traditional catalysts, because they allow the performance of industrial processes even under harsh conditions under which conventional proteins are completely denatured. By virtue of their positive properties, stability, specificity, selectivity, and efficiency, enzymes already occupy a prominent position in modern biotechnology. For many processes in the chemical and pharmaceutical industries, suitable microbial enzymes can be found that have the potential to optimize or even replace chemical processes. By using robust enzymes in biotechnical processes one is often able to better utilize raw materials, minimize pollutant emissions, and reduce energy consumption while simultaneously improving quality and purity of products. The additional benefits in performing industrial processes at high temperature include reduced risk of contamination, improved transfer rates, lower viscosity, and higher solubility of substrates. The recent exciting results in the field of extremophile research, the high demands of the biotech industries for tailor-made novel biocatalysts and the simultaneous rapid development of new techniques (e.g., genomics, proteomics, DNA-chip technology) will stimulate the development of innovative processes on the basis of biocatalysts from extremophiles. Extremophiles are unique microorganisms that are adapted to survive in ecological niches such as high or low temperatures, extremes of pH, high salt concentrations, and high pressure. Accordingly, biological systems and enzymes can even function at temperatures between –5 and 130 °C, pH 0–12, salt concentrations of 3–35%, and pressures up to 1000 bar. The majority of the organisms that grow in these extreme environments belong to a group with distinct characteristics. Carl Woese named this group archaea, and postulated the archaea as the third domain of life on earth, different from bacteria and eukarya. As of 2002, more than 60 species of hyperthermophilic archaea (growth at 80 to 110 °C) have been isolated and characterized.

Several enzymes from hyperthermophiles were purified, their genes cloned and expressed in mesophilic hosts (e.g., *E. coli* and *B. subtilis*). As a general rule, they show extraordinary heat stability, are resistant to chemical reagents, detergents, urea, and guanidinium hydrochloride. The hyperthermophilic archaeon *Pyrococcus woesei* (growth at 100 °C) harbors an amylase that is active at 130 °C. Even complex enzymes such as DNA-dependent RNA polymerases or glutamate dehydrogenases show a remarkable heat stability. The principles of heat stabilization of thermoactive enzymes are so far not elucidated. The overall amino acid composition is very similar to homologous mesophilic enzymes. However, trends commonly associated with elevated thermostability in proteins include relatively small solvent-exposed surface area, increased packing density that reduces cavities in the hydrophobic core, an increase in core hydrophobicity, decreased length of surface loops, and increased hydrogen bonds between polar residues. The three-dimensional structure of the first archaeal amylase

from *Pyrococcus woesei* has been resolved. Due to the high biodiversity of extremophilic archaea, and their existence in various biotopes a variety of other biocatalysts with different physicochemical properties were discovered. The thermoacidophilic archaeon *Picrophilus oshimae* produces a glucoamylase, which is even active at pH 0 and 90 °C. A thermostable endoglucanase, which is able to hydrolyze cellulose, has been found in *Pyrococcus furiosus* (maximal enzyme activity at 105 °C). A xylanase with a maximal activity at 110 °C has been purified from the hyperthermophilic marine archaeon *Pyrodictium abysii*. Xylanase treatment of wood at elevated temperatures opens up the cell wall structure thereby facilitating lignin removal. Chitin with an annual worldwide formation rate of 100 billion tons is also a substrate for extremophilic archaea such as *Thermococcus chitinophagus* and *Pyrococcus kodakaraensis* (growth 85 °C). In addition, a number of heat-stable proteases have been identified in hyperthermophilic archaea such as *Pyrobaculum* sp. and *Staphylothermus* sp., and thermoacidophilic archaea (growth pH 3 and 75 °C) such as *Sulfolobus* sp. and extreme thermophilic bacteria belonging to the order of Thermotogales. Some of these serine proteases have a residual activity even at 135 °C after 10 min of incubation. In order to be able to find application for various extremozymes it is essential, however, to ensure overexpression of these proteins in mesophilic hosts such as the gram-positive bacteria *Bacillus* sp. and *Staphylococcus*, or yeast (*Pichia* sp.) followed by the optimization of the cultivation process.

Intracellular enzymes from thermophiles (e.g., thermostable DNA polymerases) play a key role in a variety of molecular biological applications (e.g., polymerase chain reaction (PCR)). *Taq* polymerase, the first thermostable DNA polymerase characterized and applied in PCR, has a 5´-3´-exonuclease activity, but no detectable 3´-5´-exonuclease activity. This enzyme, which is derived from the thermophilic bacterium *Thermus aquaticus*, is unable to excise mismatches and as a result, the base insertion fidelity is low. Archaeal proofreading polymerases such as *Pwo* pol from *Pyrococcus woesei*, *Pfu* pol from *Pyrococcus furiosus*, Deep Vent™ pol from *Pyrococcus* strain GB-D or Vent™ pol from *Thermococcus litoralis* have an error rate that is up to 10-fold lower than that of *Taq* polymerase.

In addition to the mentioned extremozymes, thermophilic archaea and bacteria are a resource for unique chemical compounds such as ether-lipids, and compatible solutes. Very little, however, is known on the ability of extremophiles to produce bioactive compounds that are of value for the pharmaceutical and food industries. There is a need to develop fast and intelligent screening techniques for the identification of new products from extremophiles. New far-reaching ideas and problem-solving potential are expected to emerge in future from the sectors of genome sequence analysis, functional genomics, proteomics, and directed evolution. These techniques are expected to give decisive impulses to extremophilic biotechnology in the near future.

2. Extreme Environments as a Source of Novel Thermoactive Enzymes

2.1. Biology at the Boiling Point of Water

Microorganisms that are adapted to grow optimally at high temperatures (60–108 °C) have been isolated from high-temperature terrestrial and marine habitats. The most common biotopes are volcanically and geothermal heated hydrothermal vent systems

such as solfataric fields, neutral hot springs, and submarine hot vents. Submarine hydrothermal systems are situated in shallow and abyssal depth. They consist of hot fumaroles, springs, sediments, and deep-sea vents with temperatures up to 400 °C ("black smokers"). Shallow marine hydrothermal systems are located at the beaches of Vulcano, Naples, and Ischia (Italy), Sao Miguel (Azores), and Djibouti (Africa). Examples of deep-sea hydrothermal systems are the Guaymas Basin (depth 1500 m), the East Pacific Rise (depth 2500 m), both off the coast of Mexico, the Mid-Atlantic Ridge (depth 3700 m) and the Okinawa Trough (depth 1400 m). Because of their ability to convert volcanic gases and sulfur compounds at high temperatures, hyperthermophilic communities living in such hydrothermal vents are expected to play an important role in marine ecological, geochemical, and volcanic processes. Shallow as well as deep-sea hydrothermal systems harbor members of various genera including *Pyrococcus*, *Pyrodictium*, *Igneococcus*, *Thermococcus*, *Methanococcus*, *Archaeoglobus*, and *Thermotoga*. So far, members of the genus *Methanopyrus* have been found only at greater depths, whereas *Aquifex* was isolated exclusively from shallow hydrothermal vents. Recently, interesting biotopes of extreme and hyperthermophiles were discovered in deep, geothermally heated oil reservoirs around 3500 m below the bed of the North Sea and the permafrost soil of North Alaska.

Most of the hyperthermophiles grow optimally between 80 and 108 °C. Extreme thermophiles, which grow optimally between 60 and 80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Thermotoga*, and *Aquifex*. Microorganisms capable of growing optimally at temperatures between 50 and 60 °C are designated as moderate thermophiles. Most of these microorganisms belong to many different taxonomic groups of eu- and prokaryotic microorganisms such as protozoa, fungi, algae, streptomycetes, and cyanobacteria, which comprise mainly mesophilic species. The relative abundance of archaea and bacteria in high-temperature environments was, until recently, mainly studied by cultivation-based techniques. Because of the frequent isolation of archaea from these habitats, it was assumed that archaea dominate the high-temperature biotopes. Recently, the application of molecular-biological methods revealed that also bacterial communities are abundant in these environments. These results suggest that archaea may generally be of lower abundance in hot environments than could be assumed from cultivation-based experiments. However, the factors that allow bacteria to dominate in high temperature habitats, that were once believed to be the realm of archaea, remain unknown.

2.2. Microbial Life at High Temperatures and at Extremes of pH

Solfataric fields are the most important biotopes of microorganisms that prefer to live under both thermophilic and acidic conditions. Solfataric soils consist of two different layers which can be easily distinguished by their characteristic colors: the upper, aerobic layer has an ochre color due to the presence of ferric iron. The layer below, which is anaerobic, appears rather blackish-blue owing to the presence of ferrous iron. According to the chemical parameters of the two layers, different kinds of microorganisms can be isolated from these habitats. Thermophilic acidophiles, belonging to the genera *Sulfolobus*, *Acidianu*s, *Thermoplasma*, and *Picrophilus*, with growth optima between 60 and 90 °C and pH 0.7–5.0 are commonly found in the aerobic upper layer, whereas slightly acidophilic or neutrophilc anaerobes such as

Thermoproteus tenax or *Methanothermus fervidus* can be isolated from the lower layer. Species of *Thermoplasma* (growth optima: pH 2.0 and 60°C) have been found in hot springs, solfataras, and coal refuse piles. Their closest known phylogenetic relatives, also found in solfataras, are species of the genus *Picrophilus*, which are so far the most extreme acidophiles with growth close to pH 4.0. *Picrophilus oshimae* and *P. torridus* are both aerobic, heterotrophic archaea that grow optimally at 60 °C and pH 0.7 and utilize various polymers such as starch and proteins as carbon source.

Members of the genus *Sulfolobus* are strict aerobes growing either autotrophically, heterotrophically or facultatively heterotrophically. During autotrophic growth, S^0, S^{2-}, and H_2 are oxidized to sulfuric acid or water as end products. *Sulfolobus metallicus* and *S. brierley* are able to grow by oxidation of sulfidic ores. A dense biofilm of these microorganisms is responsible for the microbial ore leaching process, in which heavy metal ions such as Fe^{2+}, Zn^{2+}, and Cu^{2+} are solubilized. Other thermoacidophiles have been affiliated to the genera *Metallosphaera* (growth range: 50–80 °C, pH 1–4.5), *Acidianus* (growth range: 60–95 °C, pH 1.5–5), and *Stygioglobus* (growth range: 57–90 °C, pH 1–5.5).

On the other hand, the alkaliphiles that grow at high pH values are widely distributed throughout the world. They were found in carbonate-rich springs and alkaline soils, where the pH can be around 10.0 or even higher, although the internal pH is maintained around 8.0. In such places, several species of cyanobacteria and *Bacillus* are normally abundant and provide organic matter for diverse groups of heterotrophs. Alkaliphiles require alkaline environments and sodium ions not only for growth but also for sporulation and germination. Sodium ion-dependent uptakes of nutrients have been reported in alkaliphiles. Many alkaliphiles require various nutrients for growth; few alkaliphilic *Bacillus* strains can grow in simple minimal media containing glycerol, glutamic acid, and citric acid. In general, cultivation temperature is in the range of 20 to 55 °C. Furthermore, many haloalkaliphiles isolated from alkaline hypersaline lakes can grow in alkaline media containing 20% NaCl. The soda lakes in the Rift Valley of Kenya and similar lakes found in a few other places on earth are highly alkaline with pH values between 11.0 or 12.0 and represent a typical habitat where alkaliphilic microorganisms can be isolated. Thermophilic anaerobic spore-forming alkaliphiles, thermoalkaliphilic *Clostridia*, were isolated from sewage plants. Very recently, two thermoalkaliphilic bacteria, *Anaerobranca gottschalkii* and *Anaerobranca horikoshii* have been isolated from Lake Bogoria in Kenya and from Yellowstone National Park, respectively. The new isolates represent a new line within the *Clostridium/Bacillus* subphylum. The two archaeal thermoalkaliphiles identified to date are *Thermococcus alcaliphilus* and *Thermococcus acidoaminivorans*, both growing at 85 °C and pH 9.0. The main industrial application of alkaliphilic enzymes is in the detergent industry, where they account for ~30% of the total worldwide enzyme production. Alkaline enzymes have been also used in the hide-dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0.

3. Cultivation of Extremophilic Microorganisms

In order to obtain higher levels of enzymes and other cell components of extremophiles, high cell-density cultivation is necessary. However, until recently only low cell yields could be obtained making application studies very difficult. The main reason for this

has to be ascribed to the difficulties related to produce and purify large quantities of biocatalysts and cell components. Moreover, extremophilic microorganisms require special equipment to reach and maintain the optimal cultivation temperatures and extreme pH. There are two different approaches to overcome this problem: recombinant DNA technique for increasing enzyme production in mesophilic hosts and innovative bioreactor design to improve biomass yield. Because the accumulation of toxic compounds is thought to be responsible for low biomass yields, dialysis fermentation with a number of extremophiles has been performed for effective removal of low-molecular-mass components from fermentation broth. This lead to a dramatic increase in cell yields. The cultivation of the hyperthermophilic Archaeon *Pyrococcus furiosus* (growth at 90 °C) and the thermoacidophile *Sulfolobus shibatae* (growth at 75 °C, pH 3.5) resulted in cell yields of 2.6 g L^{-1} and 114 g L^{-1} (cell dry weight), respectively. In the case of *S. shibatae*, which grows at low pH values, the choice of an appropriate membrane was crucial. Cuprophan membranes, which consist of regenerated cellulose and polyamide membrane, were damaged after 2 days of operation, probably due to enzyme action. A porous, nontransparent polyethersulphonic membrane was found to be stable. The fermentation processes can be scaled up from 3 L over 30 L and up to 300 L. The pilot plant scale offers the possibility of transferring the fermentation performance into industrial standards. It was also shown that even the results of the 1-L dialysis reactor could be reproduced in the 30-L reactor using external dialysis modules.

In addition to dialysis fermentation technique, a novel microfiltration (MF) bioreactor, based on a microfiltration hollow-fiber module located inside the traditional fermentation vessel, has been designed for improving both biomass yield and enzyme productivity. Cultivating of the thermoacidophilic Archaeon *Sulfolobus solfataricus* as a model, a biomass of 35 g L^{-1} dry weight was obtained which was almost 20-fold higher than results obtained in conventional batch fermentors.

4. Screening Strategies for Thermoactive Enzymes

Biotechnologically relevant enzymes can be detected in wild-type or recombinant host strains by classical or molecular biological methods. Hydrolytic enzymes are mostly screened by phenotypic detection of the corresponding activity using dyed substrates such as red-amylopectin, red-pullulan, or azo-casein. Positive strains—either wild-type or recombinant—can easily be distinguished from inactive ones by the formation of clearing zones around the colonies. In contrast to enzyme assays using crude extracts, this technique allows for the screening of a high number of strains in reasonable time. However, the described method can only be successfully applied when the enzyme activity is fully expressed in the strain of interest. Novel screening techniques at the DNA level have been shown to overcome this obstacle and supplement the classical screening methods. For example, genes encoding a desired enzyme can be detected and isolated by specific (non-) radioactive probes. Screening of gene libraries is another promising strategy for the detection of biotechnologically relevant genes and their corresponding enzymes. A genomic library consists of a pool of recombinant microorganisms (bacteria or yeast) or bacteriophages derived from total genomic DNA. The clones should carry as inserts large number of (possibly overlapping) fragments covering the entire genome several times over. A cDNA cloning experiment is appropriate if it is known that the organism or cell type in question is transcribing the relevant gene at reasonably high levels. If it is known that a particular gene is well

expressed, a cDNA cloning experiment is more likely to lead to a successful outcome than genomic DNA cloning, at least as far as the cloning of mammalian genes is concerned. However, a cDNA clone will never give details about important signal structures such as promoters, enhancers and transcription factor binding sites, which are present upstream of a coding region, because these elements are never transcribed and are therefore not part of the mRNA. Nowadays, kits for the construction of gene libraries and even certain types of ready-to-go libraries are commercially available but in any case, the screening still has to be carried out by the individual researcher. Another approach is to collect environmental samples containing heterogeneous populations of uncultured microbes from diverse ecosystems. The genetic material is extracted from these organisms, eliminating the need to grow and maintain the organisms in cultures in the laboratory. Since even small samples yield sufficient DNA, the impact on sensitive environments is minimized. Gene expression libraries created from microbial DNA are subsequently used for the production of biomolecules.

5. Starch-Processing Enzymes

5.1. Heat-Stable α-Amylases, Glucoamylases, and α-Glucosidases

Extremely thermostable α-amylases have been characterized from a number of hyperthermophilic archaea such as *Pyrococcus woesei* and *Thermococcus profundus*. α-Amylase (α-1,4-glucan-4-glucanohydrolase), hydrolyzes linkages in the interior of the starch polymer in a random fashion which leads to the formation of linear and branched oligosaccharides. The sugar-reducing groups are liberated in the α-anomeric configuration. Most of starch-hydrolyzing enzymes belong to the α-amylase family which contains a characteristic catalytic (β/α)8-barrel domain. Throughout the α-amylase family, only eight amino acid residues are invariant, seven at the active site and a glycine in a short turn. The optimal temperatures for the activity of these enzymes range between 80 °C and 100 °C. Thermoactive amylolytic enzymes have been also detected in hyperthermophilic archaea of the genera *Sulfolobus, Thermophilum, Desulfurococcus, and Staphylothermus*. Molecular cloning of the corresponding genes and their expression in heterologous hosts circumvent the problem of insufficient expression in the natural host. The gene encoding an extracellular α-amylase from *Pyrococcus furiosus* has been recently cloned and the recombinant enzyme has been expressed in *Bacillus subtilis* and *E. coli*. This is the first report on the expression of an archaeal gene derived from an extremophile in a *Bacillus* strain. The high thermostability of the pyrococcal extracellular α-amylase (thermal activity even at 130 °C) makes this enzyme an interesting candidate for industrial application. α-Amylases with lower thermostability have been isolated from the archaea *Thermococcus profundus, Pyrococcus kodakaraensis*, and the bacterium *Thermotoga maritima* and *Dictioglomus thermophilum*. The genes encoding these enzymes were successfully expressed in *E. coli*. Similar to the amylase from *Bacillus licheniformis*, which is commonly used in liquefaction of starch in the industry the enzyme from *T. maritima* requires Ca^{2+} for activity. Further investigations have shown that the extreme marine hyperthermophilic archaeon *Pyrodictium abyssi* can grow on various polysaccharides and also secretes a heat stable α-amylase.

Unlike α-amylase, the production of glucoamylase seems to be very rare in extremely thermophilic and hyperthermophilic bacteria and archaea. Glucoamylases hydrolyze terminal α-1,4-linked-D-glucose residues successively from nonreducing ends of the chains, releasing β-D-glucose. Among the thermophilic anaerobic bacteria, glucoamylases have been purified and characterized from *Clostridium thermohydrosulfuricum* 39, *Clostridium thermosaccharolyticum*, and *Thermoanaerobacterium thermosaccharolyticum* DSM 571. Seour and Antranikian (personal communication) have shown that the thermoacidophilic archaea *Thermoplasma acidophilum*, *Picrophilus torridus*, and *Picrophilus oshimae* produce heat and acid stable glucoamylases. The purified archaeal glucoamylases are optimally active at pH 2 and 90 °C. Catalytic activity is still detectable at pH 0.5 and 100 °C.

α-Glucosidases attack the α-1,4 linkages of oligosaccharides that are produced by the action of other amylolytic enzymes. Unlike glucoamylase, α-glucosidase prefers smaller oligosaccharides (e.g., maltose, maltotriose) and liberates glucose with an α-anomeric configuration. α-Glucosidases are present in thermophilic archaea and bacteria. An intracellular α-glucosidase has been purified from *P. furiosus*. The enzyme exhibits optimal activity at pH 5.0 to 6.0 over a temperature range of 105 to 115 °C; the half life at 98 °C is 48 h.

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Biographical Sketches

Costanzo Bertoldo has a degree in Biology Summa cum Laude from the University of Naples (1988); his thesis was on the presence of antibiotic substances in Bryophytae. After postgraduate studies at the Botanical Institute at the University of Naples, he became a Fellow at the Institute of Proteins and Enzymology, Arco Felice, Italy and then at the Department of Biochemistry of Macromolecules, Faculty of Medicine, II university of Naples, where his PhD (1996) was on ”5´Methylthioadenosine phosphorylase: a model enzyme for the study of molecular basis of thermophilicity and thermostability of proteins.” He currently holds a postdoctoral position in Biotechnology at the Technical University Hamburg-Harburg: his research has included studies on: antibiotic substances from vegetable microorganism; purification and characterization of enzymes from thermophilic a microorganisms; the effect of microwave radiation on the stability of enzymes; cloning and expression of the thermoactive proteases, and starch hydrolyzing enzymes.

Garabed Antranikian graduated from the American University of Beirut in 1974 with a BSc in Biology and in 1976 with a MSc in Biology. He received his PhD in Microbiology in 1980 at Gerog-August, University of Göttingen, Institute of Microbiology, under the supervision of Prof. G. Gottschalk. He stayed at the University in Göttingen as a postdoctoral fellow until 1989. In 1990, he became Professor of Microbiology at the Technical University Hamburg-Harburg and, subsequently, leader of the Technical Microbiology team there. He was coordinator of the 39-partner European Network Project ”Biotechnology of Extremophiles” from 1993 to 1996, and then coordinator of the 58-partner European Network Project ”Extremophiles as Cell Factories” from 1997 to 1999. Since April 2000, he has been coordinating the 30 partner Network Project ”Biocatalysis,” supported by the German Federal Environmental Foundation. He is currently managing editor of the journal ”Extremophiles” and the Director of Innovation Center Biocatalysis.