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EXTREMELY THERMOPHILIC MICROORGANISMS AND THEIR POLYMER-HYDROLYTIC ENZYMES

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MINI-REVIEW

ABSTRACT

Thermophilic and hyperthermophilic microorganisms are found as normal inhabitants of continental and submarine volcanic areas, geothermally heated sea-sediments and hydrothermal vents and thus are considered extremophiles. Several present or potential applications of extremophilic enzymes are reviewed, especially polymer-hydrolysing enzymes, such as amylolytic and hemicellulolytic enzymes. The purpose of this review is to present the range of morphological and metabolic features among those microorganisms growing from 70°C to 100°C and to indicate potential opportunities for useful applications derived from these features.

Key words: Archaea, extremophiles, amylases, xylanases, pullulanases, thermostability

INTRODUCTION

In recent years it became obvious that extremophilic microorganisms differ from eucaryotic cells because they have adapted to grow under extreme conditions such as high temperature (>100°C), high salinity (saturated NaCl), extremes of pH (<2.0, >10.0), and substrate stress. These kinds of extreme microbial growth conditions are found in exotic environments which were more widespread on primitive Earth. Extreme environments include also high pressure (> 50 MPa) and the presence of organic solvents (e.g. > 1% toluene) or heavy metals.

The evolution and taxonomy of extremophiles, especially the thermophiles, is an area that is receiving increasing attention. In general, moderate thermophiles are primarily bacteria and display optimal growth temperature between 60°C and 80°C. Hyperthermophiles are primarily archaea and growth optimally at 80°C or above, being unable to grow below 60°C (47).

The hyperthermophiles are now well characterised taxonomically at the DNA-DNA hybridisation level, and their evolutionary relatedness has been examined. By using 16S rRNA sequence comparison, an archaeal phylogenetic tree has been proposed (54), with a tripartite division of the living world consisting of the domains Eucarya, Bacteria, and Archaea. In this

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division *Sulfolobales* and *Thermoproteales* form one branch (the Crenarchaeota) and the remaining thermophiles form another branch containing methanogens, and extreme halophiles (the Euryarchaeota). Currently, the only hyperthermophilic organisms within the Bacterial domain are members of the genus *Thermotoga* and *Aquifex* (47). Until now, no hyperthermophilic microorganisms in the domain Eucarya have been reported.

Hyperthermophiles are represented at the deepest and shortest lineages, including both genera of hyperthermophilic bacteria and the genus *Pyrodictium*, *Pyrobaculum*, *Desulfurococcus*, *Sulfolobus*, *Methanopyrus*, *Thermococcus*, *Methanothermus*, *Archaeoglobus* within the Archaea. Recently, genetic elements, e.g. viruses and plasmids (excluding IS elements and transposons) have been described in the kingdom Crenarcheota (*Thermoproteales* and *Sulfolobales*) and in the kingdom Euryarchaeota (*Thermococcales* and *Thermoplasmales*) of the archaeal domain (57). Some similarities between the archaeal virus FH and the bacterial phage P1 strongly indicate that this temperate phage type already existed before the separation of the Archaea from the Bacteria, which was the first documented lineage diversion in cellular evolution (57). Based on these observations, hyperthermophiles may still be rather primitive and the last common ancestor, the progenota, may have been a hyperthermophile (47, 54).

In the last decades thermophilic and hyperthermophilic anaerobes have been isolated from continental and submarine volcanic areas, such as solfatar fields, geothermal power plants, geothermally heated sea sediments and hydrothermal vents (14, 18, 47, 50). Sites from which hyperthermophilic organisms have been isolated comprises solfataric fields; steam-heated soils, mud holes, surface waters; deep hot springs; geothermal power plants as well as submarine hot springs and fumaroles; hot sediments and vents,"black smokers" or "chimneys"; and active sea-mounts.

It is interesting that some organisms have been isolated from areas with temperatures much higher than their maximum growth temperature, e.g., *Hyperthermus butilicus* (56) and *Pyrococcus abyssi* (18), which suggests that in these environments the organisms may not be actively growing. The same could be true for the organisms isolated from temperatures much below their growth temperature optimum, such as *Archaeoglobus profundus* (7).

Thermophiles and hyperthermophiles: physiological and morphological aspects. Most of the anaerobic thermophilic bacteria are chemoorganotrophic in their metabolism. The bacterial thermophilic thermoanaerobes, for example, belong to nearly the same range of nutritional categories as do mesophilic bacteria. The hyperthermophilic bacteria *Thermotoga* are able to ferment various carbohydrates like glucose, starch and xylans, forming acetate, L-lactate, H_2 and CO_2 as end product (47), while the hyperthermophilic *Aquifex* is strictly chemolithoautotrophic, using molecular hydrogen, thiosulfate and elemental sulphur as electron donors and oxygen (at low concentrations) and nitrate as electron acceptors (22).

In general, the physiological processes for adaptation to environmental stress in anaerobic bacteria seem to have involved different factors from those in aerobic bacteria. First, anaerobes are energy limited during the chemoorganotrophic growth because they can not couple dehydrogenation reaction to oxygen reduction and gain a high level of chemical free energy. Second, growth of most chemoorganotrophic anaerobes (except for methanogens) is naturally associated with the generation of toxic end products (e.g., organic acids or alcohol's, HS^-), which requires that anaerobic species develop some sort of dynamic adaptation mechanism or tolerance to their catabolic end products.

The most interesting group of thermophiles is the hyperthermophiles, since the isolation of these organisms has caused a revaluation of the possible habitats for microorganisms and has increased the high-temperature limits at which life is known to exist. The hyperthermophilic anaerobic archaea have almost the same size as one typical procaryotic cell, about 0.5 - 2.0µm, although some of them have unusual morphological features (47). Hyperthermophiles are rather diverse with respect to their metabolism, since they include methanogens, sulphate-reducers, nitrate-reducers and also the aerobic respirers. However the majority of the species know at the present are strictly anaerobic heterotrophic S^0 reducers (24). Among the terrestrial Archaea, three groups can be distinguished. Acidophilic extremethermophiles, which are found exclusively within continental solfataric fields. The organisms are coccoid-shaped, strict and facultative aerobes, and require acidic pH (opt. approx. pH 3.0) to grow. Phylogenetically, they belong to the archaeal

genera *Sulfolobus*, *Metallosphaera*, *Acidianus*, and *Desulfurolobus* (47). On the other hand, the slightly acidophilic and neutrophilic thermophiles are found both in continental solfataric fields and in submarine hydrothermal systems. All of them are strict anaerobes. Solfataric fields contain members of the genera *Thermoproteus*, *Pyrobaculum*, *Thermophilum*, *Desulfurococcus*, and *Methanothermus*. *Pyrobaculum islandicum* is able to grow autotrophically by anaerobic reduction of S^0 with H_2 as electron donor (35), but is also able to grow heterotrophically by sulphur respiration (47). Strains of *Thermophilum* and *Pyrobaculum organotrophum* are obligate heterotrophs. They grow by sulphur respiration using different organic substrates. Interestingly, *Thermophilum pendens* shows an obligate requirement for a lipid fraction of *Thermoproteux tenax* (117).

The variety of hyperthermophilic archaea that are adapted to the marine environment is represented by the crenarchaeal genera *Archaeoglobus*, *Pyrodictium*, *Thermodiscus*, *Staphylothermus*, *Hyperthermus*, *Methanopyrus*, *Pyrococcus*, *Thermococcus*, and some members of *Methanococcus*. From these organisms, Optimum growth temperatures range from 75° to 105°C, and the maximum temperature of growth can be as high as 113°C (*Pyrobolus*) or even up to 110°C (*Pyrodictium occultum).* They are so well adapted to high temperatures that they are unable to grow below 80°C (47).

Like all Archaea, Crenarchaeota are prokaryotic, and are bounded by ether-linked lipid membranes which contain isoprinoid side chains instead of fatty acids. Cells range in size from cocci <1μm in diameter to filaments over 100μm in length. Species display a wide range of cell shapes, including regular cocci clustered in grape-like aggregates (*Staphylothermus*), irregular, lobed cells (*Sulfolobus*), discs (*Thermodiscus*), very thin filaments (<0.5μm diameter; *Thermofilum*), and almost rectangular rods (*Thermoproteus*, *Pyrobaculum*). Most species possess flagella and are motile. A few members of the Crenarchaeota exhibit strange morphologies: *Pyrodictium occultum* and *Pyrodictium brockii* grow as a mold-like layer on sulphur, and have uncommon cells, which are irregularly disc shaped and dish shaped, with granules of sulphur frequently seen sticking to the fibbers, whose production may confer an adaptation advantage to the organism in trapping nutrients. The cells are connected by a network of ultra thin hollow tubules (47). Strains of *Pyrodictium* are usually chemolitoautotrophs gaining energy by reduction of S^0 by H_2. Although growth is stimulated by yeast extract, both species of *Pyrodictium* are strictly dependent upon H_2.

As an exception, *Pyrodictium abyssi* is a heterotroph growing by fermentation of peptides and is unable to grow chemolitotrophically on H_2/CO_2 either in the presence of S^0 or $S_2O_3^{-2}$. Similar to the other members of the genus, the cells of *Pyrodictium abyssi* are highly polymorphous, often disk-shaped, and display ultra flat areas. The cell envelope consists of the cytoplasmic membrane, a periplasmic space, and a surface layer protein. The ultra thin sections also reveal a zigzag structure of the S-layer (40). Usually S-layer proteins are highly stable, maintain the structural integrity of bacterial cells under extreme environmental conditions, and resist dissociation by high temperature, chemical treatment, or mechanical disruption (32). The existence of such a coat suggests an adaptative mechanism to the extreme environment in which these organisms live and could have a barrier function against both external and internal factors, that would affect the stability of the cells.

All of the hyperthermophilic heterotrophs can use complex peptide mixtures, like peptone, tryptone, or yeast extract, as carbon and energy source. Relatively few hyperthermophiles are, however, saccharolytic. Nevertheless, this number is increasing steadily, especially because several species that were originally described as growing solely on peptides, recently were shown to grow also on carbohydrates (24).

Biotechnological features of thermophiles and hyperthermophiles. In addition to the heterotrophic extremophiles, many autotrophic hyperthermophiles are able to grow by fermentation or respiration of organic matter too, and are, therefore opportunistic heterotrophs. They are able to synthesise heat stable molecules, including enzymes. The current biotechnological interest in enzymes from these microorganisms is motivated by their ability to work under conditions that are normally denaturing for mesophilic enzymes. Particular attention has been focused on enzymes from extremely thermophilic archaea (1, 30). A wide range of enzymes from hyperthermophilic archaea, both intracellular and extracellular, has been investigated and data on isolation, purification and

structural/functional characterization have been presented (Tables 1 and 2).

Whereas conventional enzymes are irreversibly inactivated by heat, the enzymes from these extremophiles show not only great thermostability, but also enhanced activity in the presence of common protein denaturants such as detergents, organic solvents and proteolytic enzymes (26, 30). Enzymes from thermophilic and extreme thermophilic microorganisms have received

Table 1. Hydrolases, Polymerases and Isomerases from thermophilic and extremely thermophilic Archaea belonging to the *Sulfolobales*, *Termoproteales*, *Methanopyrales* and *Pyrodictiales* groups, which have been isolated and/or investigated in some detail.

Species	T-opt. [°C]	Habitat	Enzyme	References
Sulfolobales				
Sulfolobus acidocaldarius	75	t	DNA polymerase	(17)
			Proteinase	(31)
			RNA polymerase	(3)
			Topoisomerase I	(20)
			Topoisomerase II	(34)
Sulfolobus solfataricus	80	t	DNA polymerase	(37)
			s-Adenosyl-homocysteine-hydrolase	(38)
Thermoproteales				
Thermoproteus tenax	88	t	RNA polymerase	(3)
Pyrobaculum aerophilum	100	m	Proteinase	(51)
Methanopyrales				
Methanopyrus kandleri	98	m	Topoisomerase type I	(46)
Pyrodictiales				
Pyrodictium occultum	105	m	DNA polymerase	(50)

m, marine; t, terrestrial

Table 2: Hydrolases, Polymerases and Isomerases from thermophilic and extremely thermophilic Archaea belonging to the *Thermococcales*, *Desulfurococcales* and *Thermoplasmales* groups, which have been isolated and/or investigated in some detail.

Species	T-opt. [°C]	Habitat	Enzyme	References
Thermococcales				
Pyrococcus furiosus	100	m	Proteinase(14)	
			DNA-polymerase	(50)
Pyrococcus woesei	100	m	Proteinase	(25)
Thermococcus stetteri	75	m	Proteinase	(26)
Thermococcus AN1	75-80	t	Proteinase	(25)
Thermococcus celer	87	m	Proteinase	(25)
Thermococcus litoralis	88	m	Proteinase	(25)
Desulfurococcales				
Desulfurococcus amylolyticus	90	t	Topoisomerase type I	(46)
			Proteinase	(13)
Desulfurococcus mucosus	88	t	RNA polymerase	(3)
Staphylothermus marinus	92	m	Proteinase	(25)
Thermoplasmales				
Thermoplasma acidophilum	55-60	T	DNA polymerase	(3)
			RNA polymerase	(3)
			Topoisomerase	(20)

m, marine; t, terrestrial

considerable attention from industry, because of their special characteristics such as high stability to changes in pH. Reasons for targeting these enzymes include their suitability as models for investigating protein thermostability and their potential as biocatalysts in modern biotechnology. Thus, these molecules have considerable industrial potentialities, giving better yields under extreme operational conditions.

For instance, the proteolytic archaea *Thermococcus litoralis* and *Thermococcus celer* showed good growth on starch. Also species belonging to the *Desulfurococcales* (*D. mucosus* and *D. mobilis*) which were thought to use only peptides, were found to grow on starch (8). Moreover, some species (*Thermophilum pendens*) were found to produce amylase or glucosidase, due their potential for growth on carbohydrates (4).

Hyperthermophiles, that are saccharolytic, either perform a complex oxidation to CO_2, and energy is gained from aerobic respiration or anaerobic S^0-respiration (*Sulfolobales*, *Archaeoglobales*, *Thermoproteales*), or they exhibit a fermentative metabolism, leading to acetate, alanine or lactate as predominant products, in addition to H_2 and CO_2 (members of the *Thermococcales*, the *Pyrodictiales*, the *Desulfurococcales* and the eubacterial *Thermotogales*). The latter incomplete oxidisers are mostly facultatively S^0-dependent, S^0 being used as a sink for reductant (24). However, the exact type of metabolism is often difficult to judge because of the limited information that is available on the end products formed. Beside *Pyrococcus furiosus* (88), *Sulfolobus* species (24), *Thermoproteus tenax* (91) and *Thermotoga maritima* (24), few organisms have been investigated in more detail. Therefore, little is know on the metabolism of a number of carbohydrate utilising hyperthermophiles.

Biopolymer degradation at high temperatures. Polysaccharides must be initially hydrolysed prior to transport into the periplasmic space, because of the size of substrate which can be transported into the cell is severely restricted; The size limit in most cases is a molecular weight (MW) of ~600 kDa (112). Extracellular enzymes are hence necessary for the degradation of macromolecules like cellulose, hemicellulose (xylan), pectin, pullulan and starch. In addition, polysaccharides have tertiary structures (ribbons, loops, coils), which may aid or impede enzymatic access to hydrolytic sites.

Enzymes from thermophiles and extremethermophiles can replace their mesophilic counterparts in different industrial processes and thereby reduce the need for cooling. For instance, a variety of industries employ microbial amylolytic enzymes in the enzymic conversion of starch into different sugar solutions, representing an important growth area of industrial enzyme usage. The bioprocessing of starch into malto-oligosaccharides is gaining importance because of their potential uses in food, pharmaceutical and fine chemical industry (50). A high value is placed on thermostable and thermoactive amylases in these processes, since the bioprocessing of starch at elevated temperature improves the solubility of starch, decreases its viscosity, limits microbial contamination, and reduces reaction times. Another hydrolytic enzyme, pullulanase, is used in combination with saccharifying amylases for the improved production of various sugar syrups (15). In addition, pullulanase has gained significant attention as a tool for structural studies of carbohydrates.

An additional application for themophilic enzymes is the development of new processes to reduce the release of environmentally harmful chemicals by replacement of existing chemical reactions with enzymatic reactions. A good example can be found in the paper-pulping industry. Kraft pulping, a process widely used in paper manufacture, removes about 95% of the lignin by alkaline sulphate cooking. The remaining lignin gives the pulp a brown colour which is removed in a multistage bleaching process with a variety of agents (35). Currently, there is concern about the environmental impact of some of the compounds used in the process, particularly chlorine and chlorine dioxide. The traditional chemical bleaching of paper pulp can be reduced, however, by introducing a biobleaching step using thermostable xylan-degrading enzymes from thermophilic organisms (35). By adding thermostable xylanases to the unbleached pulp it is possible to remove parts of the lignin by hydro lysing the bonds that link the lignin, via xylan, to the cellulose fibbers. The use of hemicellulases in bleaching is considered as one of the most important, new large scale industrial applications of enzymes (35). Indeed the mesophilic enzymes currently in use have limitations because of the high temperatures used in bleaching.The current prices of the enzymatic treatment, therefore, are expected to decrease as more efficient production strains and technologies are adopted.

Xylanases can also be used in clarification of

juices, preparation of dextran for use as food thickeners, production of fluids and juices from plant materials, in processes for the manufacture of liquid coffee, adjustment of wine characteristics and enhancement of astaxanthin extraction (19).

Xylanolytic enzymes from hyperthermophiles. A very large number of reports on the production, properties and applications of xylanases has been published in the last 25 years. The characteristics of these enzymes from bacterial and fungal sources have been dealt with detail in several review (19, 49). However the knowledge about the hemicellulases from extreme thermophilic bacteria (*Aquifex* sp. and *Thermotoga* sp.) are still limited and little is know about this enzyme in archaea (Crenarcheota and Euryarchaeota).

The first description about the occurrence of xylanases in extreme thermophilic bacteria was made by Bragger *et al.* (4). Screening was performed on solid media including 0.1% of polymer. All *Thermotoga* strains were able to degrade xylan forming clear zones on the plates against a red background, after staining with aqueous congo red and destaining with NaCl. The endoxylanase of *Thermotoga* sp. strain FjSS3B.1 exhibited maximum activity at 105°C and the main hydrolysis products of oatspelt xylan by the enzyme were xylobiose, xylotriose and medium-sized oligomers (45). The strain produces also a heat stable ß-D-xylosidase, which was largely cell-associated, probably associated with the "toga" structures of the organism (42). This might indicate that the substrate is hydrolysed at the toga prior to uptake of the carbohydrates into the cells. Furthermore, the gene expressing xylanase activity was isolated from a genomic library of *Thermotoga* sp. strain FjSS3-B1 (43). The sequence of the gene shows that it encodes a single domain, and belongs to family 10 of xylanases. The plasmid expression vector pJLA602 was used for overexpression of xyn A in *E. coli.* The temperature optimum of the recombinant enzyme of 85°C is the highest value reported for a recombinant xylanase to date.

Recently, two extremely thermostable endoxylanases designated Xyn A and Xyn B, were purified from another member of *Thermotogales*, *Thermotoga maritima* (52). The primary structure of Xyn A from *T. maritima* indicated that this enzyme is also a member of family 10 of glycosyl hydrolases, which corresponds to ß-glycanase family F (53). It is interesting to note that most of the highly thermostable xylanases investigated so far belong to this enzyme family. The gene that encodes the thermostable xylanase was cloned in *E. coli* by screening and expression library of *T. maritima* DNA (10). The enzyme was active at 100°C for several hours and efficient in releasing lignin from the kraft pulp, releasing reducing sugars and aromatic materials from the pulp suspensions over a pH range from 3.5-10.

Almost at the same time, two endoxylanases were purified and characterised from the enzyme complex of *Thermotoga thermarum* (100). While the crude xylanase from *T. thermarum* showed a half-life of 40 min at 90°C, the purified endoxylanase 1 showed a half-life of 16 min at 70°C and 80°C; the more thermostable endoxylanase 2 had a half-life of 18 min at 90°C.

Interestingly, in the last decade, xylanolytic enzymes in archaea (Table 3) have been reported only in two *Thermophilum* strains isolated in New Zealand, which grow at 88°C and pH 6.0 (4). Indeed no characterization or detailed studies were made on xylanases from these archaea. This may be partially due to the difficulties involved in growing thermophilic archaea, especially *Thermophilum* strains (55).

Recently, an archaeal xylanase has been detected in extracts of the hyperthermophilic archaeon *Pyrodictium abyssi* (2). The enzyme displays optimal activity at 110°C and pH 6.0, and is very thermostable, showing activity even after 100 min of incubation at 105°C. The analysis of hydrolysis products performed by HPLC showed as main product xylotriose and xylotetraose, indicating the presence of an endoxylanase.

Starch-hydrolysing enzymes from thermophiles and hyperthermophiles. Numerous microorganisms, including bacteria, fungi and yeasts are able to degrade starch and related polysaccharides by the action of enzymes that split α -1,4- or α -1,4- and/or α -1,6-linkages of α -glucan. Thermophilic and hyperthermophilic microorganisms have been found to grow on starch indicating that they posses starch-degrading enzymes (Tab. 3).

Amylolytic activity was detected in two *Sulfolobales* (*S. acidocaldarius* and *S. solfataricus*), and in strains of *Thermophilum*, *Desulfurococcus*, *Thermococcu*s and in the thermophilic bacteria *Thermotoga* (4). After growth on starch, the thermophilic bacteria *Thermotoga maritima* produced amylolytic enzymes, which contained three different specificities, β-amylase, α-amylase and glucoamylase (44). The amylases from *T. maritima*

Table 3: Enzymes from thermophilic and extremely thermophilic Archaea involved in carbohydrate hydrolysis.

Species	T-opt. [°C]	Habitat	Enzyme	References
Sulfolobales				
Sulfolobus solfataricus	80	t	α-Amylase	(21), (24)
			β-Glycosidase	(33)
Thermoproteales				
Thermoproteus tenax	88	t	α-Amylase	(4), (24)
			Cellulase	(4), (24)
			Xylanase	(4), (24)
Pyrodictiales				
Pyrodictium abyssi	97	m	α-Amilase	(2)
			Pullulanase	(2)
			Xylanase	(2)
Desulfurococcales				
Desulfurococcus mobilis	88	t	α-Amylase	(8)
Desulfurococcus mucosus	88	t	α-Amylase	(8)
			Pullulanase	(8)
			Transglucosylase	(3)
Staphylothermus marinus	92	m	α-Amylase	(8)
Thermococcales				
Pyrococcus furiosus	100	m	α-Amylase	(5)
			α-Glucosidase	(12)
			β-Glucosidase	(28)
			β-Mannosidase	(24)
			Pullulanase type II	(6)
Pyrococcus abyssi	96	m	β-Glycosidase	(24)
Pyrococcus woesei	100	m	α-Amylase	(27)
			α-Glycosidase	(30)
			Pullulanase type II	(41)
Thermococcus celer	87	m	α-Amylase	(8)
			Pullulanase	(4)
			α-Glucosidase	(24)
			β-Glucosidase	(29)
Thermococcus hydrothermalis	80	t	α-Amylase	(29)
			Pullulanase	(29)
			α-Glucosidase	(29)
Thermococcus litoralis	88	m	α-Amylase	(6)
			Pullulanase type II	(6)
			α-Glucosidase	(24)
Thermococcus profundus	80	m	α-Amylase	(11)

m, marine; t, terrestrial

showed high thermal stability with an upper temperature limit at 95°C.

Extremely thermostable amylolytic enzymes were reported to be produced by the hyperthermophile *Pyrococcus woesei* and *P. furiosus* (11, 27). The amylolytic enzymes are produced by *P. furiosus* in response to the presence of complex carbohydrates in the growth medium (5). The very stable α-glucosidase from *P. furiosus* exhibited remarkable thermostability in the presence of various denaturing agents, like 100 mM dithiothreitol and 1.0 M urea (12). The α -amylase from *P. furiosus* was described as a homodimer with a subunit molecular mass of 66 kDa. The enzyme displayed optimal activity, with substantial thermal stability at 100°C. The gene encoding this highly thermostable

amylase was cloned and expressed in *E. coli* (28). The amylase expressed in *E.coli* exhibited the temperature-dependent activation characteristic of the original enzyme from *P. furiosus*, but a higher apparent molecular weight which was attributed to the improper formation of the native quaternary structure. It was not possible, however, to determine whether this improper assembly was due to translation at lower temperature or to unidentified aspects of production in *E. coli.* On the other hand, the α -amylase from *P. woesei* has showed catalytic activities at a temperature range between 40°C and 130°C. The purified enzyme consisted of a single subunit with a molecular mass of 70 kDa (27).

Most of the archaeal amylases from *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus* as well as in the two novel archaeal isolates from deep-sea hydrothermal vents (TY and TYS strains) displayed optimal activity at 100°C with the exception of *Thermococcus celer*, with an optimum at 90°C (8). One extracellular thermostable amylase from *Thermococcus profundus* exhibited maximal activity at pH 5.5 and was stable in the range of pH 5.9 to 9.8 (11). Recently, it has been described the production of α-glucosidase and α-amylase by *Thermococcus hydrothermalis* after growth on maltose or starch (29). α-Glucosidase seems to be the dominant amylolytic activity in the enzymatic extract and was capable of hydrolysing the α(1-4)linkages of oligosaccharides and maltose.

Another extracellular amylase has been isolated from culture supernatants of *Sulfolobus solfataricus* during growth on starch (21). The secreted protein has an apparent mass of 240 kDa, consisting of two identical subunits. Its levels in crude culture supernatants varied greatly in response to the carbon source used for growth of the organism.

Pullulanases from thermophilic and hyperthermophilic archaea. Since the discovery of *Klebsiella pneumoniae* pullulanase, a number of microbial pullulanases have been purified and characterised from thermophilic bacteria and archaea by many investigators (11, 12, 41, 85). However most enzymes from thermophilic bacteria belong to type II pullulanase. Among the several amylolytic enzymes produced by the hyperthermophilic archaeon *Pyrococcus furiosus*, pullulanase was characterised by temperature optimum of at least 100°C and a high degree of thermostability (5). The pullulanase from *P. furiosus* was purified and reported to be a glycoprotein with an optimum of activity at 100°C (6).

An extracellular amylopullulanase from *Thermococcus litoralis* (6) was optimally active at 110°C, but the presence of Ca^{+2} extended the range at which the activity could be measured (up to 130°C-140°C) Thermoactive pullulanases have been characterised in *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus*, and in the two novel archaeal strains (TYS and TY). The enzymes showed temperature optima between 90°C and 105°C and exhibit remarkable thermostability, even in the absence of substrate and calcium ions (8). An extracellular pullulanase has been found also in the culture medium after fermentation of starch by *Thermococcus hydrothermalis* (29), with an extracellular production represented almost 80% of the total pullulanase production.

The pullulanase from *Pyrococcus woesei* has been purified and the gene has been cloned and expressed in *E.coli* (41). The native and cloned enzymes are identical in their physiochemical properties, being optimally active at 100°C and pH 6.0. The high rigidity of the heat stable enzyme was demonstrated by fluorescence spectroscopy in the presence of denaturing agents (41).

Unlike all thermoactive pullulanase know so far, the pullulanase from *Pyrodictium abyssi* showed highest activity at alkaline pH, at pH 9.0, and very high optimal temperature (100°C). Preliminary results also indicate that *P. abyssi* forms a real debranching enzyme, i.e., pullulanase type I, which is very rare among bacteria and archaea (2).

Thermophily and thermostability. A consistent characteristic of all enzymes from hyperthermophilic microorganisms is their high level of thermostability. The positive correlation between the thermophily of the source organism and thermostability of both intra and extracellular proteins has been demonstrated frequently (16, 40, 51).

Different protein engineering studies, where diverse point mutations can enhance (or reduce) protein thermostability have shown, that there are consequences, both structural and functional, in artificially enhancing protein thermostability. One increase of the thermal stability of a protein may result in reduced conformational flexibility and depending on locality and extent of these changes, this may result in significant (and sometimes detrimental) consequences with respect to the biological function. There is some evidence from various sources including

proteolysis studies, ^{1}H-^{2}H exchange studies, and X-ray diffraction that at room temperature a thermophilic protein will be less flexible than its mesophilic equivalent. At their respective growth temperatures, similar proteins from both mesophilic and thermophilic sources will posses similar levels of molecular flexibility, a consequence that molecular flexibility is critical for function (3, 14).

Moreover, it is not clear what are the upper limits for the thermal stability of proteins. Studies with one protease from *Pyrobaculum aerophilum*, which exhibits strong proteolytic activities with a temperature range of 80°C-130°C, allowed identification of sites potentially contributing to the thermostability of the protein (51). Aspartic acids were found at the N-terminus of several surface helices, possibly increasing stability by interacting with the helix dipole. Several of the substitutions in regions expected to form surface loops were adjacent to each other in the tertiary structure model. A marked increase in glutamic acid residues in the hyperthermostable citrate synthase from *Pyrococcus furiosus* with respect to its mesophilic counterpart, may be related to the high concentration of compatible solutes present within the cell. The percent of aromatic amino acids is also one of the highest in the citrate synthases, which may lead to enhanced stabilising aromatic packing interactions (66).

The presence of mannosylglycerate, a compatible solute, in two unrelated thermophilic bacteria lead to the speculation that the accumulation of this compound could also be related to the thermophily of organisms. Indeed, until the physiological adaptation to temperature stress has been examined, no final conclusions can be drawn from the relationship between mannosylglycerate and thermophily (36).

Ion pairing also plays a role in protein stabilization (9). This was also confirmed by the determination of the structure of glutamate dehydrogenase (GDH) from *Pyrococcus furiosus*, which was compered with GDH from mesophilic origin. This comparison has revealed that the hyperthermophilic enzyme contains a striking series of networks of ion-pairs which are formed by regions of the protein which contain a high density of charged residues. The ion-pair networks are clustered at both inter domain and inter subunit interfaces. They may well represent a major stabilising feature associated with the adaptation of enzymes to extreme temperatures (39).

Therefore, a study of enzymes from extremely thermophilic archaea, may reveal the existence of enzymes with still greater thermostability. This suggests that enzyme stability does not need to confine the existence of life to 110°C or below. It has also implications for enzyme applications in the industry at high temperatures. At the present the industrial applications of thermostable enzymes are still limited to a few areas. Although the genetic engineering allows the design of "tailor-made" enzymes by altering their amino acid composition, the construction of thermostable enzymes are still highly empirical. This is because little is known concerning the molecular basis of protein thermostability. Enzymes of extremophiles are, however, good starting points for engineering "tailor-made" enzymes. Furthermore, enzymes from thermophiles and especially hyperthermophiles present physiological features and potential technological properties, which must be understood, before an industrial process can be designed or compared with those currently in use.

CONCLUSIONS

There are many existing applications in which more thermally stable versions of enzymes now used will be advantageous. This is especially true in the hydrolysis of corn starch to produce high fructose corn syrup. Amylolytic enzymes are now used at temperatures exceeding 100°C in some cases to hydrolyse liquified starch to oligosaccharides and eventually to glucose. Glucose is then partially isomerized to fructose using immobilized xylose (glucose) isomerase. Many of these same enzyme activities are available in extreme thermophiles. Given the preference of many of these organisms for saccharides, it should be possible to isolate a range of saccharidases for evaluation in starch processing.

There are other hydrolysis reactions that can be catalysed by high temperature enzymes. Cellulose and hemicellulose hydrolysis is important in the processing of renewable resources. Activities to these substrates have been detected among the thermophiles and extreme thermophiles. The isolation of new thermophilic strains on cellulosic substrates is at present an area of great interest.

On the other side, in food processing enzyme use has been limited because of the need to mantain asseptic conditions. However, if enzymes with

sufficient thermostability were available, applications involving modifying the fiber content of foods, perhaps during the baking process, could be considered. The treatment of complex wastes from food processing, such as lactose-laden streams, may also be facilited by decreasing the viscosity and increasing the solubility of lactose at high temperature.

To take advantage of the biotechnological potential of microorganisms growing at extremely high temperatures, there is still a great deal to be learned about their metabolic and genetic characteristics. An interaction between scientists and engineers will be require to assure that fundamental insights are used effectively for technology development.

RESUMO

Microrganismos extremotermofílicos e suas enzimas despolimerizantes

Microrganismos termofílicos e hipertermofílicos são encontrados como habitantes normais de áreas vulcânicas continentais e submarinas, sedimentos marinhos com aquecimento geotermal e ventos hidrotermais; portanto, são considerados extremofílicos. Diversas aplicações presentes ou potenciais de enzimas de extremofílicos são revisadas, especialmente enzimas que hidrolizam polímeros, tais como amilases e hemicelulases. São também apresentadas as variações das características morfológicas e metabólicas entre estes organismos que crescem entre 70ºC e 100°C e indicadas as oportunidades potenciais de aplicações derivadas destas características.

Palavras-chave: Archaea, extremofílicos, amilases, xilanases, termoestabilidade.

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