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Thermozymes and Their Applications

A Review of Recent Literature and Patents

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Abstract

Enzymes from thermophilic microorganisms, thermozymes, have unique characteristics such as temperature, chemical, and pH stability. They can be used in several industrial processes, in which they replace mesophilic enzymes or chemicals. Thermozymes are often used when the enzymatic process is compatible with existing (high-temperature) process conditions. The main advantages of performing processes at higher temperatures are reduced risk of microbial contamination, lower viscosity, improved transfer rates, and improved solubility of substrates. However, cofactors, substrates, or products might be unstable or other side reactions may occur. Recent developments show that thermophiles are a good source of novel catalysts that are of great industrial interest. Thermostable polymer-degrading enzymes such as amylases, pullulanases, xylanases, proteases, and cellulases are expected to play an important role in food, chemical, pharmaceutical, paper, pulp, and waste-treatment industries. Considerable research efforts have been made to better understand the stability of thermozymes. There are no major conformational differences with mesophilic enzymes, and a small number of extra salt bridges, hydrophobic interactions, or hydrogen bounds seem to confer the extra degree of stabilization. Currently, overexpression of thermozymes in standard *Escherichia coli* allows the production of much larger quantities of enzymes, which are easy to purify by heat treatment. With wider availability and lower cost, thermophilic enzymes will see more application in industry.

Index Entries: Thermophilic; thermozyme; enzyme; hyperthermophilic; thermostability.

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Introduction

Since the discovery of thermophiles in the 1970s by Brock *(1)*, the study of thermophilic and hyperthermophilic microorganisms has become a major domain of research. Increasingly attention is being paid to these microorganisms because of their unusual properties. Their enzymes (thermozymes) have unique characteristics such as temperature, chemical, and pH stability. Thermozymes are being used more frequently in several industrial processes.

In this review, we focus on thermozymes and their applications as described in the literature. We discuss the background of their high (thermo)stability and production of thermozymes in mesophiles. Furthermore, we consider the application of thermozymes in industrial processes and their benefits over mesophilic enzymes. We also review examples of current and future processes using various enzymes and summarize recent utilization of thermozymes in the section on patents.

Thermophilic Organisms

Early characterizations of thermophiles were limited to spore-forming aerobes such as *Bacillus stearothermophilus* and anaerobes such as *Clostridium thermosaccharolyticum*. These organisms were not thought to possess inherently stable enzymes and were assumed to have evolved from mesophiles. More recently, microorganisms that grow optimally above 60°C and that do possess thermostable enzymes, such as *Thermus aquaticus* and *Methanobacterium thermoautotrophicum*, a member of the Archaea, have been isolated. Furthermore, hyperthermophiles that grow from 80 to above 100°C have now been isolated, such as *Pyrococcus furiosus*, *Thermotoga neopolitana*, and *Thermotoga maritima*. These types of microbes are thought to be among the first forms of life to have evolved on earth. A phylogenetic tree, showing hyperthermophiles and their place in the three-domain model, is depicted in Fig. 1. With the exception of *Thermotogales* and *Aquifex*, all organisms that grow optimally above 80°C are Archaea *(2)*.

Thermozymes

Enzymes from thermophiles and hyperthermophiles are thermostable and display irreversible protein denaturation only at high temperatures. Thermozymes also feature a high temperature for maximum activity. In this article, we define thermozymes as enzymes that have their maximum activity above 60°C. Hyperthermophilic enzymes have their maximum activity above 80°C.

Thermophilic enzymes can be used in several industrial processes, in which they can replace mesophilic enzymes or chemicals. This opens new possibilities for process optimization. These enzymes, particularly those that are active and stable at temperatures up to 100°C, are used to study protein adaptation to high temperatures and to obtain a better understanding of protein stability in general *(5)*.

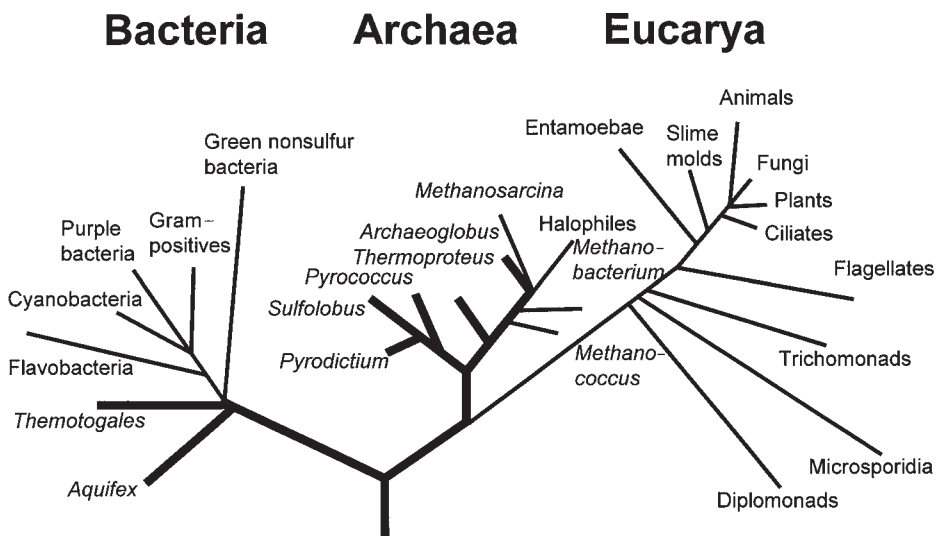

Fig. 1. Phylogenetic tree according to Woese et al. *(3)*. The tree has been rooted by analysis of duplicates in protein sequences *(4)*. Hyperthermophilic genera are depicted as thick lines.

Stability of Thermozymes

The stability of proteins is the result of a delicate balance between large stabilizing and large destabilizing forces *(6–8)*. Consequently, relatively small changes in either the stabilizing or destabilizing forces can result in large changes in stability. A small number of extra salt bridges, hydrophobic interactions, or hydrogen bounds can confer this extra degree of stabilization. There are, therefore, no systematic structural differences between extremely stable and normal proteins *(9)*.

The stability of enzymes is interesting from a fundamental as well as an industrial point of view. Thermozymes can function as examples for improved stability. A better understanding of the stability of thermozymes could reveal ways to stabilize other enzymes. Engineering of mesophilic enzymes might be an option when specific functionality cannot be found among thermozymes.

The first studies on thermostability focused on amino acid sequences *(10)*. More recently, crystal structures of thermozymes have been compared with those of their mesophilic counterparts. This gives extra insight into their mechanism of stability *(11)*.

Conformational stability of proteins is the result of compromise between two opposing factors: flexibility, for the catalytic function of the enzyme, and rigidity, for conformational stability. Thermozymes are significantly more rigid than their mesophilic counterparts at room temperature. Their high rigidity protects them from unfolding and preserves their catalytically active structure. Therefore, they can be optimally active under more denaturing conditions (e.g., higher temperatures).

Enzymes that have been characterized or cloned from (hyper)-thermophilic organisms have been listed by Vieille et al. *(2)*. They included thermostability properties of these enzymes, when available.

Mechanisms of Stability

Basic mechanisms of stability are high packing density, optimum charge patterns or ion pairs, minimization of accessible hydrophobic surface area, helix stabilization, and subunit assembly *(8,11,12)*. Oligomer formation and other environmental factors can further stabilize the enzyme. These mechanisms are discussed next.

Internal Stabilization

Maximum packing efficiency of an enzyme can be achieved by filling cavities in the molecular structure and increasing core hydrophobicity. Higher levels of isoleucine, alanines, and prolines are suggested, which should provide tighter packing in hydrophobic cores and extra stability to loops. Vieille and Zeikus *(12)* listed several thermozymes with an increased hydrophobicity, but only two of them have an increased packing efficiency.

Enhanced helix stabilization can contribute to thermostability in various ways. Most important is the loss of conformational entropy of amino acid residues in helical arrangements. Amino acids that are branched on C^{β} (i.e., valine, isoleucine, and threonine) cause more conformational restraints, and, therefore, they occur less frequently in thermozymes *(13)*. Additionally, specific amino acid compositions at the helical ends can influence stability, but this phenomenon is not unique to thermozymes *(11)*.

Another way to stabilize enzymes is to increase the number of stabilizing interactions in the folded protein. Disulfide bridges can stabilize the enzyme by increasing the conformational rigidity. Hydrogen bonds might also stabilize the enzyme *(14)*. Recently, the increasing numbers of known three-dimensional structures has made it possible to see the importance of ion pairs, organized in large networks. It is believed that these networks at the surface of proteins are a major stabilizing factor for thermostability *(5)*. Arginines frequently occur in ion-pair networks, and, indeed, they occur in higher numbers in enzymes from hyperthermophiles *(15)*.

Stabilizing interactions between domains and subunits also contribute significantly to the intrinsic stability of proteins. Enzymes from (hyper)thermophilic organisms are known to exist as higher-order association states compared with their mesophilic analogs, suggesting that the formation of oligomers is one way of increasing thermostability *(8,11,15,16)*.

At temperatures above 100°C, inactivation of enzymes is mainly caused by chemical modification of the protein, rather than by irreversible unfolding. Examples of nonenzymatic irreversible reactions or irreversible covalent modifications are deamidation of asparagine and glutamine and oxidative degradation of cysteine, methionine, and tryptophan *(17,18)*. Enzymes with lower levels of asparagine, glutamine, cysteine, methionine, and tryptophan are less susceptible to degradation by chemical modification.

Environmental Factors

Although most enzymes from (hyper)thermophilic organisms are intrinsically very stable, some intracellular enzymes obtain their thermostability from intracellular environmental factors. The presence of salts; high-protein concentrations; coenzymes; substrates; activators; or general stabilizers such as thermamine, sorbitol, or cyclic polyphosphates can stabilize the enzyme. By manipulating the environmental conditions, one can sometimes achieve higher thermostabilities than by application of genetic engineering *(18)*. Cell-bound thermozymes such as saccharidases and proteases are active at temperatures (far) above the optimal growing temperature of the organism and are, as a rule, highly stable.

Some other environmental factors that can be important are chemical crosslinking, immobilization, and glycosylation. Glycosylation gives various effects, but only in some cases is protein stability improved *(19)*. Glycosylation can also improve the efficiency of tertiary protein assembly. The latter effect involves the stabilization of the nascent rather than the native protein *(8)*.

A final method for enzyme stabilization is the use of ultrahigh pressure (up to 50 MPa), which may lead to more compact structures and thus higher thermostability *(20,21)*.

Protein Engineering

Research into the mechanisms of thermostability provides fundamental knowledge on enzyme structure. It allows us to specifically modify enzymes to improve their stability. Enzymes with specific catalytic functions that cannot be found among thermozymes may be engineered from the appropriate mesophilic enzymes.

Different approaches can be used to stabilize a (mesophilic) enzyme against inactivation by unfolding. Promising strategies are often directed at protein surface loops and turns. Surface residues are typically involved in fewer intermolecular interactions than internal residues and are less likely to create volume interferences.

Stabilizing or creating ion pairs and reduction of conformational strain in helices can be successful. Additional disulfide bridges have been introduced with varying success. They should not be introduced where they put too much strain on the protein structure. Also, cysteine residues are not stable at about or above 100°C, which renders them less suitable for this purpose *(17)*. Various strategies to improve core packing, such as stabilization of α-helices, have shown varying results and the gain in stability is usually small.

To stabilize thermophilic enzymes further, the inactivation mechanism must first be identified. As discussed, inactivation above 100°C is often caused by covalent modification rather than by unfolding, as is the case with mesozymes. This type of inactivation may be prevented by substituting specific surface amino acids such as asparagine, glutamine, cys-

teine, methionine, and tryptophan with residues that are less susceptible to degradation *(12,14)*.

Until now protein engineering of thermozymes has mainly focused on improving and understanding (thermo)stability. The main goal is to improve the activity of (mesophilic) enzymes at higher temperatures and other extreme conditions. Less attention is given to engineering thermozymes for the purpose of changing activity or enlarging selectivity.

Production of Thermozymes in Mesophilic Organisms

In early purification schemes of enzymes from thermophilic bacteria, the growth of large quantities of these thermophiles under unconventional conditions was required *(22)*. Now overexpression of enzymes from thermophiles in standard *Escherichia coli*, or other organisms such as *Saccharomyces cerevisiae (23)*, allows the production of much larger quantities of enzymes, which are easy to purify by heat treatment. Thermal denaturation will affect only the host's mesophilic enzymes, and the precipitated proteins can simply be eliminated by centrifugation, leaving the thermophilic enzyme in solution. Despite being synthesized in a mesophilic host, recombinant thermophilic enzymes usually have kinetic and thermal characteristics identical to those of the native protein *(23)*.

Reasons for Using Thermozymes

The main reason for selecting enzymes from thermophiles is their high stability, which makes them attractive for several industrial processes. It is not only their thermostability but also their greater stability under other extreme conditions such as high pH or low water concentrations that is useful in many applications. Stable enzymes are also more likely to allow the use of organic solvents and detergents and are more resistant to proteolytic attack. For example, proteases are used as an additive in household laundry detergents, where they have to be resistant to denaturation by surfactants and alkaline conditions *(24)*. Sellek and Chaudhuri *(25)* have reviewed thermophiles to be used as biocatalysts in organic solvents.

A further advantage of using higher temperatures is the reduced risk of contamination. Temperatures above 70°C are sufficiently high to kill almost all pathogenic bacteria and greatly reduce the numbers of the bacteria most likely to cause troublesome contamination of food processes.

Another advantage of applying thermostable enzymes for production purposes is lower viscosity of process fluids. At higher temperatures viscosity is usually reduced, which lowers shear and, consequently, the costs of pumping, filtration, and centrifugation, or allows the use of lower water levels during processing. Heat and mass transfer rates are also improved. At higher temperatures diffusion rates will be higher and mass transfer is less limiting *(26)*. In addition, more substrate will dissolve at higher temperatures, which can shift the equilibrium to a higher product yield. This is the case, e.g., in reversed hydrolysis reactions in water *(27)*.

It is often speculated that thermozymes should have higher maximal catalytic rates *(28)* since classic reaction rate theory states that the rate of a chemical reaction increases with temperature. These higher maximal catalytic rates, however, are not found experimentally. Despite their activity at high temperatures, thermophilic enzymes catalyze reactions at these temperatures with K_m and V_{max} values similar to those of their mesophilic counterparts at their respective optimal temperature *(29)*. As expected, thermozyme activity is driven by variation in temperature-dependent substrate kinetic energy alone. If the enzyme structure were to change significantly with different temperatures, one would expect to find nonlinear Arrhenius plots, which is not the case *(30)*. However, thermozymes do have a broader temperature range in which they are active *(26)*.

An important disadvantage of enzymatic processes at higher temperatures is the loss of selectivity and formation of byproducts. Furthermore, possible required cofactors, substrate *(27)*, or products might be unstable, and side reactions, such as Maillard reactions in sugar-enzyme mixtures, may occur.

Some processes will have higher costs owing to additional heating, and other processes are less likely to need cooling *(26)*. It depends on the overall process and its implementation whether operation at higher temperatures is beneficial or not. Often applications with thermozymes are found when the enzymatic process is compatible with existing (high-temperature) process conditions.

Applications

Recent developments clearly show that thermophiles are a good source of novel catalysts that attract great industrial interest. Thermostable polymer-degrading enzymes such as amylases, pullulanases, xylanases, proteases, and cellulases are expected to play an increasingly important role in food, chemical, pharmaceutical, paper, pulp and waste-treatment industries. Table 1 gives a short summary of thermozymes and their (future) applications.

Proteases

Proteases hydrolyze proteins into amino acids and peptides. Thermostable proteases are already used in several industrial processes. In most cases, they are used for their stability at higher pHs or in organic solvents rather than their stability at elevated temperatures. Daniel et al. *(31)* listed several thermostable proteases. The best-characterized thermostable protease is probably thermolysin, produced by *Bacillus thermoproteolyticus*, first described by Endo *(32)*.

The use of proteases at higher temperatures gives rise to a specific problem: heat enhances autolysis, i.e., self-digestion of proteases. The resulting conformational unfolding leads to much faster autolysis, because the (partially) unfolded form of the enzyme is a particularly good sub-

Table 1
Summary of Thermozymes, Their Possible Applications, and Main Reason That a Specific Thermophilic Enzyme Is of Interest for an Industrial Process

Enzyme	Applications	Reason for thermozyme use	Reference no./patent no.
Alcohol dehydrogenase	Chiral synthesis	Improved stability	WO 9921971
Amylase	Production of high-glucose syrup	Compatibility with an existing high-temperature process	*(43,52)*, US 5714369
Cellulase	Pulp and paper processing	Compatibility with an existing high-temperature process	WO 9744361, WO 9714804
	Laundry detergents	Stable at high pH	WO 9744361, WO 9743381, WO 9714804
Cyclodextrin glycosyl transferases	Cyclodextrin production	Compatibility with an existing high-temperature process	*(52,60,61)*, JP 10234387
DNA polymerase	DNA amplification, reverse transcription	Reaction at high temperature	*(78)*, US 6054301, WO 0020629, WO 9953074, WO 9845452, WO 9814590, WO 9735988
	DNA labeling	Reaction at high temperature	—
	DNA sequencing, cycle sequencing method	Reaction at high temperature	*(79)*, EP 892058, WO 9814588
Glucoamylase	Starch conversion	Compatibility with an existing high-temperature process	*(52)*
Glycosidase	Hydrolysis of lactose	Less microbial growth at high temperature	*(69)*
	Oligosaccharide synthesis	Better substrate solubility at high temperature	*(27,70,72)*
	Synthesis of alkyl glycoside detergents	Compatible with organic solvents	*(71)*

Laccase	Textile bleaching	—	WO 9725469
	Laundry detergents	Stable at high pH	WO 9743381
Ligase	Ligase chain reaction	Reaction at high temperature	*(81)*, WO 0026381, US 5830711
	Construction of sequencing primers	Reaction at high temperature	*(82)*
Lipase	—	Melting of substrate at high temperature	—
	Detergents	Stable at high pH	—
Protease	Synthesis of aspartame precursor	Compatibility with organic solvents	*(32,36,37)*
	Cleanup of DNA before PCR	Compatibility with an existing high-temperature process	—
	Meat tenderization	High temperature	*(39)*
	Peptide synthesis	Compatibility with organic solvents	*(35)*
	Detergents	Stable at high pH	US 5714373
	Leather soaking	Stable at extreme pH	*(24)*
	Membrane cleaning	High temperature, low viscosity	*(38)*
Pullulanases	High-glucose syrup production	Compatibility with an existing high-temperature process	*(52)*, US 5714369
Xylanase	Bleaching	Compatibility with an existing high-temperature process	*(74)*, US 5922579, EP 828002, WO 9736995, WO 9722691, WO 9714803
Xylose/glucose isomerase	High-fructose syrup production	Equilibrium shifted by high temperature	*(28,63)*

strate for the protease molecules that are still active. The better susceptibility of the substrate to proteolytic attack results in higher specific activities for the proteases from thermophilic origin *(29,33)*. Because most commercial substrates for proteases are mesophilic proteins, proteases have a high industrial potential. A method to prevent autolysis might be immobilization of the enzyme *(34)*.

Thermostable proteases are used in peptide synthesis, mainly because of their compatibility with organic solvents *(35)*. The major currently used thermostable protease is thermolysin, which is used in the synthesis of the dipeptide N-CBZ-L-Asp-L-Phe methyl ester, which is the precursor in the preparation of the sweetener aspartame *(36)*. In the process that employs immobilized thermolysin, the compatibility of the enzyme to organic solvents is crucial to the process rather than its use at high temperature *(37)*.

Another actively marketed thermostable protease is a *Thermus* protease, Pretaq, used to clean up DNA before amplification in the polymerase chain reaction (PCR). The commercial success of this application may be owing to the relatively cost-intensive nature of the application.

Proteases that are resistant to alkalinity and anionic or nonionic surfactants are used as additives in domestic laundry detergents. Improved stability is desirable, but thermostable proteases are not yet being produced in sufficient quantities to be cost competitive. Moreover, current trends in laundering processes toward lower temperatures may present another barrier. The use of thermozymes in laundry detergents for institutional (e.g., hospital) use may well be a future application because in those processes especially high temperatures are required.

A larger application might be the use of thermophilic enzymes in (domestic) dishwashing detergent. In this case, much lower pHs are applied (about 9), combined with temperatures of about 60°C. A thermostable protease would easily be able to withstand this pH and temperatures above 60°C.

Proteases showing high keratinolytic and elastolytic activities are used for soaking in the leather industry *(24)*. Thermophilic proteases could be better applicable at the bating of hides and skins at acid (pH 2.5–5.0) and alkaline (pH 12.0–13.0) conditions.

Coolbear et al. *(38)* tested several proteases for application in the cleaning of ultrafiltration membranes. Thermostable proteases at high temperatures (and low viscosities) may allow these products to be reused, and this may lead to a reduction in chemical use and waste production. Presently, no thermophilic proteases are available for this application.

Other potential applications are found in meat tenderizing, in which the ideal meat-tenderizing enzyme would be active only during preparation, and not during storage *(39)*.

Starch-Converting Enzymes

Starch is a mixture of amylose and amylopectin. Amylose consists solely of α-1,4-linked glucose polymers, whereas amylopectin also has

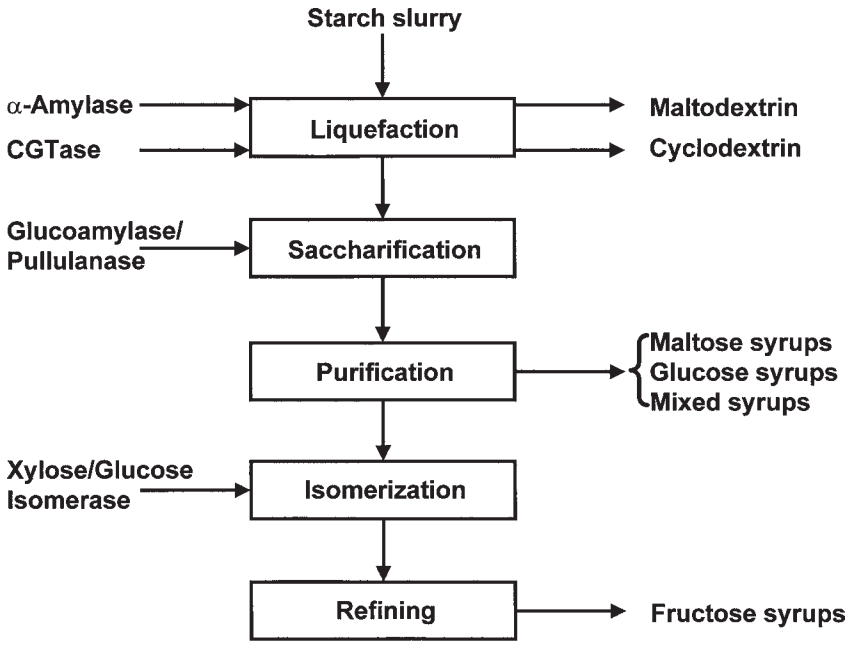

Fig. 2. Enzymatic starch conversion into various sugars. (Adapted from ref. *41*.)

α-1,6 linkages. Several enzymes are involved in the conversion of starch; endo- and exoacting amylases that act primarily on α-1,4-linkages; debranching enzymes, such as pullulanases, that act on α-1,6-linkages; and cyclodextrin glycosyltransferases that degrade starch by catalyzing cyclization and disproportionation reactions. The industrial conversion of starch is a multistage process that involves a range of enzymes in successive steps (*see* Fig. 2). Enzymes from hyperthermophiles that can act at the boiling point of water and preferably at lower pHs are regarded as interesting candidates for starch conversion. Lévêque et al. *(40)* have listed several thermophilic archaeal amylolytic enzymes.

α-Amylases

α-Amylases are endoacting enzymes that cleave α-1-4 bonds. They are used in the first stage of starch conversion, called liquefaction. Raw starch consists of insoluble semicrystalline granules on which most enzymes are poorly active. The first process step consists of heating the granules in water, which causes swelling and gelatinization *(42)*. Then starch is liquefied by, e.g., the thermostable α-amylase from *Bacillus licheniformis* (Thermamyl) or *Bacillus amyloliquefaciens*. Various enzymes can be used, depending on the desired product *(43)*. Several thermostable α-amylases have already been characterized *(44–49)*. The most thermostable α-amylase to date is from *Pyrococcus woesei*. It remained active after autoclaving for 4 h at 120°C *(50)*.

Glucoamylases

Glucoamylases are exoacting enzymes that cleave both α-1-4 and α-1-6 bonds. The branching points, however, are hydrolyzed at a rather slow rate. These enzymes are currently used in the second step of starch conversion in which they are used for the production of high-glucose syrup from starch-originated polysaccharides. For industrial production, the glucoamylase from *Aspergillus niger* is generally used. Because of the lack of commercially available thermostable enzymes, the process conditions after starch liquefaction must be changed. For the production of glucose, the pH and temperature must be lowered. Owing to the variation in pH, large amounts of salts are added, which need to be removed.

Improvement of the process by the introduction of new and more efficient (e.g., thermostable) enzymes would significantly lower the costs. To date, only few thermostable glucoamylases have been found. Recently, a thermostable glucoamylase was purified from *Thermoanaerobacterium thermosaccharolyticum* DSM 571 *(51)*.

Pullulanases

Type I pullulanase cleaves α-1-6 bonds in starch and is an example of a debranching enzyme. It is used in the production of high-glucose syrup from polysaccharides. For industrial purposes, the pullulanase from *Bacillus acidopullulyticus* is often used, in combination with the glucoamylase from *A. niger (52)*. Thermostable type I pullulanase from *Thermus caldophilus* and *Fervidobacterium pennavorans (53)* has been characterized, and the latter has recently been cloned into *E. coli* by Bertoldo et al. *(54)*.

In addition to α-1-6 bonds, Type II pullulanase cleaves α-1-4 bonds and is therefore able to saccharify starch directly into maltotetraose, maltotriose, and maltobiose. Type II pullulanase is usually referred to as amylopullulanase *(28)*.

Several thermostable type II pullulanases have already been described *(47,55)* and purified *(56,57)*. Two hyperthermophilic type II pullulanases from *P. woesei (58)* and *Pyrococcus furiosus (59)* have been expressed in *E. coli*.

Cyclodextrin Glycosyl Transferases

Cyclodextrin glycosyl transferases (CGTases) attack α-1-4 linkages in polysaccharides and convert starch by transglycosylation to α-, β-, and γ-cyclodextrins. Cyclodextrins can form inclusion complexes with small hydrophobic molecules, and this ability has provided a number of applications in the food, cosmetic, pharmaceutical, and agrochemical industries as well as in analytical chemistry *(60)*.

Normally, cyclodextrin production is a two-stage process. The first step consists of starch liquefaction by a heat-stable α-amylase followed by the cyclization reaction by a CGTase from a *Bacillus* sp. Because of the low stability of this enzyme, the second process step is carried out at a lower temperature than the previous liquefaction step *(24)*.

New thermostable CGTases have been found that are able to solubilize starch without the need for α-amylase pretreatment *(61)*. In addition, thermostable CGTases have been found in *Thermoanaerobacter* sp. *(61)* and *Thermoanaerobacterium thermosulfurogenes (62)*. By using thermostable CGTases, the overall time and cost of cyclodextrin production can be reduced significantly. This is necessary in order to open up the application of cyclodextrins on a commercially attractive scale *(52)*.

Other Important Sugar-Converting Enzymes

Xylose Isomerase and Glucose Isomerase

Thermophilic xylose isomerases have been characterized from a *Thermoanaerobactererium* strain *(63)* and from *Thermus flavus (64)*.

Xylose isomerase is used for the isomerization of glucose into fructose. This process transfers high-glucose corn syrup into high-fructose corn syrup, which is used as a sweetener. The current process is operated at 58°C with mesophilic xylose isomerases and yields syrup with 40–42% fructose. This necessitates an additional chromatographic step to obtain the required 55% syrup concentration.

Because the conversion is endothermal, the equilibrium for the isomerization reaction is shifted to fructose at higher temperatures. The same concentration of 55% fructose syrup can be obtained by performing the reaction at 95°C with a thermophilic xylose isomerase without the chromatographic concentration step *(28,65)*.

Isomerization of glucose into fructose can also be done by a glucose isomerase. The most acid-stable and thermostable glucose isomerase available is the glucose isomerase of *Bacillus coagulans (52)*.

β-Glycosidases

Glycosidases hydrolyze sugar bonds. Bauer et al. *(66)* listed several glycosidases from hyperthermophiles. The best-known β-glycosidases are from *P. furiosus (67)* and *Sulfolobus solfataricus (68)*.

Most industrial applications of β-glycosidases have employed thermostable glycosidases for hydrolytic purposes. For example, β-glycosidase is used in the hydrolysis of lactose into glucose and galactose. The monosaccharides that are formed from this reaction have a higher solubility and sweetening effect than lactose and are more easily digested by humans. The main reasons for using thermozymes in this process are increased substrate and product solubility and reduced risk of microbial growth *(69)*.

Glycosidases are also used in the reverse reaction of oligosaccharide synthesis. The thermostable *P. furiosus* β-glycosidase produces tri- and tetrasaccharides with lactose as a substrate. These oligosaccharides may be used as prebiotics in functional foods. The increase in the substrate solubility at higher temperatures and the subsequent lower water activity improves the oligosaccharide yield. The occurrence of Maillard reactions between the sugar and the enzyme hinders this reaction at higher temperature *(70)*.

The low acceptor specificity of glycosidases can be further exploited for the synthesis of alkyl glycoside detergents and other novel products. The β-glycosidase of *P. furiosus* can accept primary and secondary alcohols, diols and cyclic diols, and sometimes tertiary alcohols *(71)*.

Lowering the water activity for specific synthesis may also be achieved by using solvents, since thermozymes are often compatible with organic solvents. They further permit higher concentrations of hydrophobic donor substrates *(27,72)*.

Xylanases

Xylanases catalyze the hydrolysis of xylan. Kulkarni et al. *(73)* listed some xylanases from thermophilic bacteria. Xylan is the main polymeric compound of hemicellulose. Xylanases can be used in the biobleaching of wood or bagasse pulp. This is of great interest because the use of chlorine chemicals can be reduced, thereby minimizing environmental concerns. Minimal attack on the cellulose fiber is a prerequisite, and for this reason, the xylanases should be cellulase free. During the enzymatic bleaching process, the polysaccharide chains attached to the lignin hydrolyze and the pulp structure opens up. In this way, the consumption of bleaching chemicals can be reduced while a higher final optical brightness is obtained.

Commercial enzymes such as Pulpzyma and Cartazyme are not thermostable. In the treatment of pulp, several stages before the chlorine bleaching step are performed at 70°C and higher. Consequently, pulp must be cooled before the enzymatic treatment and reheated for subsequent processing steps *(74)*. In addition to obtaining improved thermostability, it is also desirable to have enzymes that are active at the alkaline pH of the pulp *(75–77)*.

DNA-Processing Enzymes

DNA Polymerases

DNA polymerases are the key enzymes in the replication of DNA. Currently, thermostable polymerases play a major role in several molecular biologic applications such as DNA amplification, sequencing, and labeling. A major advance in molecular biology is the development of the PCR. In the first PCR reaction, a heat-labile *E. coli* DNA polymerase was used, which had to be added during each cycle following the denaturation and primer hybridization steps. The availability of thermostable DNA polymerases enormously facilitated the automation of the thermal cycling. The polymerase from *T. aquaticus*, *Taq* polymerase, is the first characterized thermophilic polymerase *(22)* and is still widely applied in PCR.

Thermostable DNA polymerases can also be used for reverse transcription, in which RNA is used as a starting template by first converting RNA to cDNA. This can then be used to generate cDNA libraries. With the original mesophilic reverse transcriptase that was used at low temperatures, there was the problem of formation of stable secondary RNA structures *(78)*. However, many thermostable polymerases can also use RNA as

a template. The DNA polymerase from *Thermus thermophilus* HB8 is commonly used for this reaction.

The introduction of thermostable DNA polymerases in sequencing was a major step forward leading to the cycle sequencing method. This is a PCR-like amplification of the sequencing products. The advantages are that less template DNA is required, no separate primer annealing step is needed, and dissolution of unwanted secondary structures within the template can be achieved by high-temperature elongation *(79)*.

Ligases

DNA ligase catalyzes the linking of polynucleotides. The first thermostable ligase was discovered in *T. thermophilus* HB8 in 1984 *(80)*. Several thermostable DNA ligases have been discovered since then *(60)*. An important application for thermophilic ligases is the ligase chain reaction. In this reaction, DNA ligase amplifies DNA, but a single-base substitution prevents ligation and is thus distinguished. Therefore, it can be used in DNA diagnostics for the detection of single-base mismatches. The specificity of this reaction is highest at the melting point of the primers *(81)*, which is typically 50–65°C. Another application lies in the construction of sequencing primers by high-temperature ligation of hexameric primers *(82)*.

Lipases

Lipases hydrolyze tri-, di-, and monoglycerides into fatty acids and glycerol. Several (hyper)thermophilic lipases have been isolated and characterized. Biologic treatment of fats at high temperatures is advantageous, since fats above their melting point, in the liquid state, become more accessible to the enzyme. However, not many specific applications for thermophilic lipases are discussed in the literature. Although oil modification is often performed under virtually anhydrous conditions, and at temperatures of up to 70°C, most processes still use mesophilic enzymes *(83)*.

Is There a Future for Thermozymes?

Thermozymes are already found in several existing processes and have the potential for much wider application. They can replace enzymes from mesophiles in processes that benefit from a higher operating temperature. At higher temperatures, synthesis processes can be operated at other, beneficial conditions, such as higher substrate concentrations. In addition, thermozymes play a central role in newly developed processes, such as PCR, that could not efficiently be performed before the discovery of thermozymes.

Thermozymes can be applied as biocatalysts in existing industrial processes to replace presently used, often polluting, chemical reagents. This is especially true for the pulp and paper industry, which has problems with aggressive waste from the bleaching process.

Daniel *(18)* states that the theoretical upper limit of enzyme thermal stability might be well above 130°C. If these enzymes cannot be found in

nature, then they might be developed by engineering. This, and the enhanced stability under other extreme conditions, will further broaden the field in which thermozymes can be used.

The production of thermozymes in mesophilic organisms makes large-scale production and easy purification of these enzymes possible, and it is therefore expected that with wider availability and lower costs, thermophilic enzymes will be increasingly applied in industry.

Recent Patents on Utilization of Thermozymes

In this section, we have summarized the latest developments achieved by both academic and industrial research groups in the area of thermozymes. Patents reported cover the time period from January 1997 to May 2000.

HIGH-FIDELITY THERMOSTABLE LIGASE AND THEIR USES
Inventors: Barany, F., Cao, W., and Tong, J.
Assignee: Cornell Research Foundation (United States)
Issued: May 11, 2000
Patent no.: WO 0026381

The present invention is directed to a thermostable ligase having substantially higher fidelity than either T4 ligase or *T. thermophilus* ligase. The DNA molecule encoding this enzyme as well as expression systems and host cells containing it are also disclosed. The thermostable ligase of the present invention is useful in carrying out a ligase detection reaction process as well as a ligase chain reaction process.

METHODS OF AMPLIFICATION
USING A THERMOSTABLE DNA POLYMERASE
FROM THE HYPERTHERMOPHILIC ARCHAEON STRAIN KOD1
AND REAGENT KIT FOR THIS PURPOSE
Inventors: Kitabayashi, M., Arakawa, T., Inoue, H., Kawakami, B., Kawamura, Y., Imanaka, T., Takagi, M., and Morikawa, M.
Assignee: Toyo Boseki Kabushiki Kaisha (Japan)
Issued: April 25, 2000
Patent no.: US 6054301

A nucleic acid amplifying enzyme having a short reaction time and high fidelity is provided. The enzyme of this invention is a thermostable DNA polymerase having a nucleic acid extension rate of at least 30 bases/s and a 3'-5' exonuclease activity. Also provided are a method and kit for amplifying nucleic acid.

QUANTITATION OF RNA BY REVERSE TRANSCRIPTASE PCR
Inventors: Parrington, M., Caterini, J. E., and Michel, H.
Assignee: Connaught (Canada)
Issued: April 13, 2000
Patent no.: WO 0020629

An accurate method of determining the quantity of specific RNA in a tissue sample permits analysis of rare transcripts, such as cytokines, and is based on a modified RNA isolation procedure, reverse transcriptase-PCR in a single enzyme reaction, detection, and quantification, preferably employing an RNA standard.

RECOMBINANT THERMOSTABLE ENZYME
THAT RELEASES TREHALOSE
FROM NONREDUCING SACCHARIDE
Inventors: Mitsuzumi, H., Kubota, M., and Sugimoto, T.
Assignee: Kabushiki Kaisha Hayashibara Seibutsu Kagaku Kenkyujo (Japan)
Issued: February 22, 2000
Patent no.: US 6027918

Disclosed is a recombinant thermostable enzyme that has a mol wt of about 54–64 kDa and a p*I* of about 5.6–6.6 and releases trehalose from nonreducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily high thermostability; that is, it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85°C for 60 min, and this facilitates the production of trehalose on an industrial scale and in a satisfactorily high yield.

EXPRESSION OF ENZYMES INVOLVED
IN CELLULOSE MODIFICATION
Inventors: Himmel M. E., Schaaf, D. J., Stalker, D. M., and Thomas, S. R.
Assignee: Calgene (United States)
Issued: January 11, 2000
Patent no.: US 6013860

Novel compositions and methods useful for genetic engineering of plant cells to provide expression in the plastids of a plant or plant cell of cellulose-degrading enzymes is provided.

CARBOXYMETHYL CELLULASE FROM *T. Maritima*
Inventors: Mathur, E. J. and Lam D. E.
Assignee: Diversa (United States)
Issued: December 28, 1999
Patent no.: US 6008032

A purified thermostable enzyme is derived from the eubacterium *T. maritima*. The enzyme has a mol wt as determined by gel electrophoresis of about 35 kDa and has cellulase activity. The enzyme can be produced from native or recombinant host cells and can be used to aid in the digestion of cellulose, when desired.

THERMOPHILIC PHOSPHOLIPASES
AND METHOD FOR THEIR PRODUCTION
Inventors: Kosugi, Y., Higuchi, K., Ishikawa, K., Matsui, I., and Yong-Goe, J.

Assignee: Agency of Industrial Science and Technology (Japan)
Issued: December 14, 1999
Patent no.: US 6001626

Disclosed are a thermophilic phospholipase having an optimum temperature range of 95–105°C that is useful in high-temperature degumming processes in the oil-refining process and high-temperature processing of phospholipids, and a method for producing a thermophilic phospholipase comprising the culturing of a microorganism capable of producing the phospholipase in a culture medium and collecting the phospholipase from the resultant culture.

ENZYME DERIVED FROM THERMOPHILIC ORGANISMS THAT FUNCTIONS AS A CHROMOSOMAL REPLICASE, AND ITS PREPARATION AND USES
Inventors: Yurieva, O., Kuriyan, J., O'Donnell, M. E., and Jeruzalmi, D.
Assignee: Rockfeller University (United States)
Issued: October 21, 1999
Patent no.: WO 9953074

A DNA polymerase has been identified in a thermophile that functions as a chromosomal replicase. The specific enzyme is a holoenzyme III that has been identified in *T. thermophilus* and corresponds to polymerase III in *E. coli*. The genes and the polypeptides corresponding to *T.th.* γ-, τ-, ε-, α-, and β-subunits that they encode are disclosed, as are probes, vectors, methods of preparation, and the methods of use. The enzymes of the present invention and their components are particularly well suited for use in procedures for the preparation of DNA, such as PCR, because of the speed and accuracy that they are able to achieve.

CLONING OF GENE FOR HIGHLY THERMOSTABLE AMINOTRANSFERASE FROM A HYPERTHERMOPHILIC ARCHAEBACTERIUM, *Pyrococcus horikoshii*
Inventors: Matsui, I., Ishikawa, K., Ishida, H., Kosugi, Y., and Matsui, E.
Assignees: Agency of Industrial Sciences and Technology (Japan)
Issued: September 7, 1999
Patent no.: JP 11239485

The gene encoding a novel highly thermostable aminotransferase is isolated from a hyperthermophilic archaebacterium, *P. horikoshii* strain JCM9974. The deduced protein sequence comprises 389 amino acids. The enzyme exhibits a pH optimum of 6.0, temperature optimum of 90°C, p*I* of 5.2, and mol wt of 44 kDa (homodimer). The half-life of the enzyme at pH 6.5 and 110°C is 30 min. Expression of the gene in *E. coli* is also shown.

MISMATCH CLEAVAGE ENZYMES FROM EXTREME THERMOPHILES AND THEIR USES IN MOLECULAR BIOLOGY TECHNIQUES
Inventors: Chirikjian, J. G., Bazar, L. S., and George, A. L.

Assignees: Trevigen (United States)
Issued: August 26, 1999
Patent no.: WO 9942595

The present invention is directed to extreme thermophilic mismatch cleavage enzymes and their uses. The gene sequence encoding *T. maritima* endonuclease V (TM-EndoV) is provided. TM-EndoV may be used for cleavage of mismatches in detecting mutations by probe hybridization, detecting a sequence in a target polynucleotide, and cleaving mismatches created during PCR.

THERMOSTABLE Mut PROTEIN
Inventors: Mikawa, T., Kato, R., and Kuramitsu, S.
Assignee: Toyobo (Japan)
Issued: August 3, 1999
Patent no.: JP 11206375

The gene *MutM* encoding a novel 266 amino acid thermostable repair enzyme for oxidative DNA is isolated from extremely thermophilic *T. thermophilus* strain HB8. The protein exhibits a mol wt of 25 kDa (gel filtration) and is able to remove 8-oxoguanine from a single-stranded DNA or a double-stranded DNA (paired with cytosine). The enzyme remains >80% active after incubating at 75°C, pH 7.5 for 5 min. The *MutM* protein may also be prepared from other extremely thermophilic bacteria such as *Thermococcus* and *Pyrococcus*.

PROCESS FOR TEXTILE WARP SIZING
USING ENZYMATICALLY MODIFIED STARCHES
Inventors: Hendriksen, H. V., Pedersen, S., and Bisgard-Frantzen, H.
Assignee: Novo-Nordisk A/S (Denmark)
Issued: July 15, 1999
Patent no.: WO 9935325

A new method for sizing textile fibers that involves treating a suspension of gelatinized starch with a CGTase, glycosyltransferase, or branching enzyme, in order to reduce its viscosity, and applying the suspension obtained to the fiber is claimed. This new process may be useful for increasing the strength of fibers and decreasing the risk of damage resulting from knitting or weaving. In an example, a glucanotransferase modified starch suspension was produced via the incubation of a 30% DS potato (*Solanum tuberosum*) starch suspension at 90°C and pH 6.0 with 50 µg of an enzyme from *Thermococcus litoralis* for 24 h.

XYLANASES AND THEIR USE
Inventors: Fagerstrom, R. B., Paloheimo, M., Lantto, R., Lahtinen, T., and Suominen, P.
Assignee: Rohm Enzyme Finland OY (Finland)
Issued: July 13, 1999
Patent no.: US 5922579

Enzyme compositions containing thermostable xylanases of *Chaetomium thermophilum*, purified enzyme preparations of such xylanases, and the use of such compositions and preparations in the bleaching of plant pulp and in feed and baking applications are described.

RECOMBINANT PRODUCTION
OF THERMOSTABLE ALCOHOL DEHYDROGENASES
FOR USE IN CHIRAL SYNTHESES
Inventors: Allen, L., Brikun, I., Alkens, J. H., Demirjian, D. C., Matur, R., Nikolsky, Y., and Rozzell, J. D.
Assignees: Thermogen (United States)
Issued: May 6, 1999
Patent no.: WO 9921971

The present invention provides proteins having alcohol dehydrogenase activity. New enzymes from thermophilic organisms that are suitable for use as stable off-the-shelf reagents for selectively and mildly installing chiral centers from corresponding carbonyl groups are provided. These enzymes are characterized and show a variety of substrate specificities and enantioselectivities that indicate they are useful biocatalysts that can be used economically to prepare fine chemicals and intermediates. Phagemid lpMYF is a high-capacity phagemid vector lpSL5 derivative used for cloning and expression of a number of alcohol dehydrogenases from several thermostable microorganisms.

MUTANT CHIMERIC *Thermus Tma* DNA POLYMERASES
WITH IMPROVED PROPERTIES
FOR NUCLEIC ACID SEQUENCING
Inventors: Gelfand, D. H. and Reichert, F. L.
Assignees: F. Hoffmann-La Roche Ag (Switzerland)
Issued: January, 20 1999
Patent no.: EP 892058

This invention provides mutant, chimeric thermostable DNA polymerase enzymes consisting of an N-terminal region derived from the 5'-nuclease domain of a *Thermus* species DNA polymerase and a C-terminal region derived from the 3' to 5' exonuclease and polymerase domains of *Tma* DNA polymerase. These mutant chimeric thermostable DNA polymerase enzymes have improved properties in nucleic acid–sequencing reactions.

METHODS OF PREPARATION
OF NUCLEIC ACID–FREE THERMOSTABLE ENZYMES
SUCH AS DNA POLYMERASES
AND RESTRICTION ENDONUCLEASES
Inventors: Goldstein, A. S. and Hughes, A. J. Jr.
Assignees: Life Technologies (United States)
Issued: January 19, 1999
Patent no.: US 5861295

This invention provides thermostable enzymes, such as DNA polymerases and restriction endonucleases, that are substantially free from contamination with nucleic acids. The method of purification of the thermostable enzymes comprises permeabilizing a thermophilic bacterial cell with an aqueous solution containing a chaotropic agent and nonionic surfactant to form a spheroplast, and isolating the thermostable enzyme preparation under conditions favoring the partitioning of nucleic acid from the thermostable enzyme preparation. Purification and characterization of DNA-free *Taq* DNA polymerase from *T. aquaticus* is described as an example.

DIRECTED EVOLUTION OF THERMOPHILIC ENZYMES
Inventors: Short, J. M.
Assignee: Diversa (United States)
Issued: November 3, 1998
Patent no.: US 5830696

Thermostable enzymes are subjected to mutagenesis to produce a thermophilic enzyme that is stable at thermophilic temperature and that has increased activities at least twofold higher than the activity of the wild-type thermostable enzyme at lower temperatures, i.e., 50°C or lower.

THERMOSTABLE LIGASE-MEDIATED
DNA AMPLIFICATION SYSTEM
FOR DETECTION OF GENETIC DISEASES
Inventors: Barany, F., Zebala, J., Nickerson, D., Kaiser, R. J. Jr., and Hood, L.
Assignees: Cornell Research Foundation; California Institute of Technology (United States)
Issued: November 3, 1998
Patent no.: US 5830711

This invention relates to the cloning of the gene of a thermophilic DNA ligase, from *T. aquaticus* strain HB8, and the use of this ligase for the detection of specific sequences of nucleotides in a variety of nucleic acid samples, and, more particularly, in those samples containing a DNA sequence characterized by a difference in the nucleic acid sequence from a standard sequence including single nucleic acid base pair changes, deletions, insertions, or translocations.

A DNA POLYMERASE III HOMOLOG
OF THE THERMOPHILIC BACTERIUM *T. thermophilus*
INVOLVED IN CHROMOSOMAL REPLICATION
Inventors: Yurieva, O., Kuriyan, J., O'Donnell, M. E., and Jeruzalmi, D.
Assignees: Rockfeller University (United States)
Issued: October 15, 1998
Patent no.: WO 9845452

A DNA polymerase holoenzyme III that is identified in *T. thermophilus* and corresponds to DNA polymerase III of *E. coli* is described for use in

primer-mediated amplification of DNA. In particular, the clamp structure of DNA polymerase III can be used to extend a primer over a long stretch of single-stranded DNA.

ULTRATHERMOSTABLE CYCLODEXTRIN SYNTHETASE OF ULTRATHERMOPHILIC BACTERIA *Thermococcus*
Inventors: Kuramura, A., Utsura, K., Moriwaki, M., Shirasaka, N., Kojima, I., Suzuki, Y., and Tachibana, Y.
Assignees: Nagase and Co.; Nagase Biochemical Industry (Japan)
Issued: September 8, 1998
Patent no.: JP 10234387

A novel ultrathermostable cyclodextrin synthetase is prepared from the culture of ultrathermophilic bacteria *Thermococcus* and characterized. The enzyme prepared from *Thermococcus* strain B-100 exhibits a pH optimum of 5.0–5.5 (at 110°C), temperature optimum of 110°C (at pH 5.0), p*I* of <3.5, and mol wt of 72 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It remains 90% active after heating in the absence of starch and calcium ions at pH 5.0 and 100°C for 40 min. The enzyme is useful for the production of α-cyclodextrin from starch.

CLONING AND EXPRESSION OF GENE FOR THERMOSTABLE ENZYME EXHIBITING AMINOACYLASE AND CARBOXYPEPTIDASE ACTIVITIES FROM *P. horikoshii*
Inventors: Ishikawa, K., Matsui, I., Ishida, H., Kosugi, Y., and Higuchi, K.
Assignees: Agency of Industrial Sciences and Technology (Japan)
Issued: August 11, 1998
Patent no.: JP 10210976

The gene encoding a novel thermostable enzyme exhibiting aminoacylase and carboxypeptidase activities is isolated from *P. horikoshii* strain JCM 9974, a hyperthermophilic archaebacterium. The enzyme comprises 388 amino acids and exhibits a pH optimum of 6.5–8.0, temperature optimum of 90–95°C, and mol wt of 40 kDa by SDS-PAGE. It remains active after incubating at 95°C and pH 7.5 for 3 h.

CLONING AND EXPRESSION OF GENE FOR THERMOSTABLE ACYLPEPTIDE HYDROLASE FROM *P. horikoshii*
Inventors: Ishikawa, K., Matsui, I., Ishida, H., Kosugi, Y., and Higuchi, K.
Assignees: Agency of Industrial Sciences and Technology (Japan)
Issued: August 11, 1998
Patent no.: JP 10210977

The gene encoding a novel thermostable acylpeptide hydrolase is isolated from *P. horikoshii* strain JCM 9974, a hyperthermophilic archaebacterium. The enzyme comprises 632 amino acids and exhibits a pH optimum of 5.0–6.0, temperature optimum of 90–95°C, and mol wt of 60 kDa by SDS-PAGE. It remains active after incubating at 95°C and pH 7.5 for 3 h.

THERMOPHILE GENE TRANSFER
USING A CHROMOSOMAL GENE
AND A THERMOSTABLE DERIVATIVE
OF THE KANAMYCIN-RESISTANCE GENE
Inventors: Weber, J. M., Demirjian, D. C., Casadaban, M. J., Pagratis, N. C., and Vonstein, V.
Assignees: Thermogen (United States)
Issued: July 28, 1998
Patent no.: US 5786174

A new gene transfer system is provided for extreme thermophiles of the genus *Thermus*, including *T. flavus*, using a chromosomal gene and a thermostable derivative of the kanamycin-resistance gene. A plasmid-mediated gene-replacement process is used to insert it into the chromosome, resulting in the production of Leu-Kmr transformants. This system not only allows stable, single-copy gene insertion into the chromosome of an extreme thermophile, but also can be used in the thermogenetic process described to generate thermostabilized enzymes and proteins for industrial processes. This host-vector environment makes it possible to generate further thermostabilizing mutations in the *kan* gene beyond those levels previously reported.

FEN-1 ENDONUCLEASES, MIXTURES,
AND CLEAVAGE METHODS
Inventors: Kaiser, M. W., Lyamichev, V. I., and Lyamicheva, N.
Assignees: Third Wave Technologies (United States)
Issued: June 4, 1998
Patent no.: WO 9823774

This invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. It also relates to improved cleavage means for the detection and characterization of nucleic acid sequences. Structure-specific nucleases derived from a variety of thermostable organisms are provided. These structure-specific nucleases are used to cleave target-dependent cleavage structures, thereby indicating the presence of specific nucleic acid sequences or specific variations thereof. The cleavage method was used for the identification of hepatitis C virus and human *ras* gene.

THERMOSTABLE DNA POLYMERASE
FROM *Anaerocellum thermophilum*
Inventors: Ankenbauer, W., Schmitz-Agheguian, G., Bonch-Osmolovskaya, E., Svetlichny, V., Markau, U., Angerer, B., and Reiser, A.
Assignees: Boehringer Mannheim G.m.b.H. (Germany)
Issued: April 9, 1998
Patent no.: WO 9814588

A thermostable DNA polymerase that is derived from the microorganism *A. thermophilum* is provided. The enzyme has a mol wt of 96–

100 kDa and shows DNA polymerase activity and reverse transcriptase activity in the presence of Mg^{2+}. The enzyme may be native or recombinant and may be used with selected primers and nucleoside triphosphates in a temperature-cycling PCR on DNA or RNA as template with or without additional DNA polymerases as an enzyme mixture.

THERMOSTABLE NUCLEIC ACID POLYMERASE
FROM *Thermococcus gorgonarius*
Inventors: Ankenbauer, W., Svetlichny, V., Bonch-Osmolovskaya, E., Ebenbichler, C., Angerer, B., Schmitz-Agheguian, G., and Laue, F.
Assignees: Boehringer Mannheim G.m.b.H. (Germany)
Issued: April 9, 1998
Patent no.: WO 9814590

A purified thermostable DNA polymerase enzyme is derived from the thermophilic archaebacterium *T. gorgonarius*. The enzyme can be native or recombinant, retains ~90% of its activity after incubation for 2 h at 95°C in the presence of stabilizing agents, and possesses 3'-5' proofreading exonuclease activity. It has at least a twofold greater replication fidelity than DNA polymerase obtainable from *P. furiosus*. Thermostable DNA polymerases are useful in many recombinant DNA techniques, especially nucleic acid amplification by PCR.

MODIFICATION OF XYLANASES
TO IMPROVE THERMOPHILICITY, ALKALOPHILICITY,
AND THERMOSTABILITY FOR PULP BLEACHING
Inventors: Sung, W. L., Yaguchi, M., and Ishikawa, K.
Assignees: National Research Council of Canada (Canada)
Issued: March 11, 1998
Patent no.: EP 828002

Amino acid–substituted analogs of family II xylanases with superior performance in the bleaching of pulp are described. More specifically, these xylanases show improved thermophilicity, alkalophilicity, and thermostability compared with the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *T. reesei* xylanase II or the corresponding amino acids of another family II xylanase to histidine, methionine, and leucine, respectively; (2) substituting amino acids in the N-terminal region with amino acids from another xylanase enzyme; and (3) extending the upstream region of the N-terminus up to 10 amino acids.

Fervidobacterium AMYLASE AND PULLULANASE
Inventors: Sjøholm, C. and Antranikian, G.
Assignee: Novo Nordisk A/S (Denmark)
Issued: February 3, 1998
Patent no.: US 5714369

This invention relates to *Fervidobacterium* amylase and pullulanase preparations and their use in producing sweeteners and ethanol from starch. In particular, the enzymes are derived from *F. pennavorans*.

Thermococcus AV4 AND ENZYMES PRODUCED BY IT
Inventors: Stetter, K. O.
Assignees: Recombinant BioCatalysis (United States)
Issued: February 3, 1998
Patent no.: US 5714373

An isolated preparation of a new species of thermophilic bacteria, *Thermococcus* AV4, is disclosed. Also described is a substantially pure preparation of a protease and a substantially pure preparation of a lipase produced by *Thermococcus* AV4. The thermostable enzymes are useful for industrial applications such as for the manufacture of detergents.

ENDOGLUCANASE GENE SEQUENCES
FROM THERMOPHILIC ARCHAEL BACTERIA
Inventors: Lam, D. E. and Mathur, E. J.
Assignees: Recombinant Biocatalysis (United States)
Issued: November 27, 1997
Patent no.: WO 9744361

This invention provides a purified thermostable enzyme derived from the archael bacterium AEPII1a. The enzyme has a mol wt of ~60.9 kDa and has cellulase activity. The enzyme can be produced from native or recombinant host cells and can be used to aid in the digestion of cellulose, when desired. Also included are an additional 23 genes and their encoded endoglucanases having homology to the AEPII1a enzyme. The cellulase enzymes may be used for degradation of cellulose for the conversion of plant biomass into fuels and chemicals, as well as in detergents, in the textile industry, in animal feed, in waste treatment, and in the fruit juice/brewing industry for the clarification and extraction of juices.

DETERGENT COMPOSITION COMPRISING
A CELLULASE ENZYME AND A LACCASE ENZYME
Inventors: Herbots, I. M. A. J., Barnabas, M. V., Busch, A., and Denoodt, K.
Assignees: Procter & Gamble (United States)
Issued: November 20, 1997
Patent no.: WO 9743381

This invention relates to detergent compositions comprising a laccase enzyme and a cellulase enzyme that provide effective and efficient cleaning of colored stains and soils and the sanitization of the treated surface. In a preferred embodiment, it relates to a laundry detergent composition comprising a laccase and a cellulase for excellent fabric care and cleaning properties.

BIOLOGICALLY ACTIVE FRAGMENT
OF *B. stearothermophilus* DNA POLYMERASE
Inventors: Swaminathan, N. and Wilkosz, R. K.
Assignees: Molecular Biology Resources (United States)
Issued: October 23, 1997
Patent no.: WO 9739113

This invention is directed to an isolated and purified DNA encoding a biologically active fragment of a thermostable, full-length DNA polymerase I enzyme of *B. stearothermophilus*. More particularly, the invention is directed to a DNA encoding an approx 66 kDa DNA polymerase that lacks 273 amino acids from the N-terminus of the approx 96 kDa *B. stearothermophilus* DNA polymerase I, and to the protein encoded thereby, which has been designated the *B. stearothermophilus* DNA polymerase I exo-fragment. The enzyme fragments are useful in DNA sequencing, cDNA preparations, thermophilic strand displacement amplification, and other molecular biology applications.

XYLANASE FROM *Dictyoglomus thermophilum*
AND ITS USE IN BLEACHING OF CELLULOSE PRODUCTS
Inventors: Bergquist, P. L., Gibbs, M. D., and Morris, D.
Assignees: Pacific Enzymes (New Zealand)
Issued: October 9, 1997
Patent no.: WO 9736995

An enzyme preparation for application in the bleaching of cellulose products is provided comprising an enzyme-derived *D. thermophilum*, the enzyme further being contained within the family of enzymes known as G-xylanases, and having β-1,4-xylanase activity at elevated temperatures. The enzyme may be isolated from *D. thermophilum* or, alternatively, produced from a recombinant vector contained within a host microorganism (such as *E. coli* strain JM101). Thus, the G-xylanase-encoding gene *xynB* was isolated from *D. thermophilum* strain Rt46B.1 by standard PCR amplification using amplification primers. Cloning of *xynB* into the controlled expression vector pJLA602 and expression in *E. coli* yields an enzyme with a pH optimum of about 6.5 and a temperature optimum of ~85°C. The exceptional temperature stability of Rt46B.1 xylanase enhances its use in the bleaching of eucalyptus kraft.

SPECIFIC AMPLIFICATION
OF LONG NUCLEIC ACIDS USING PCR
Inventors: Frey, B. and Kuebler, H.
Assignees: Boehringer Mannheim G.m.b.H. (Germany)
Issued: October 2, 1997
Patent no.: DE 19612779

This invention discloses an enzyme mixture comprising two thermostable DNA polymerases with and without proofreading activity plus a thermostable pyrophosphatase with other substances for PCR. This mixture is useful for amplification of long single- and double-stranded nucleic acid fragments. Preferred embodiments of the invention use a tricine-NH_3 buffer and an elongation temperature of 68°C.

THERMOSTABLE DNA POLYMERASE
FROM *Thermococcus* spec. TY
Inventors: Niehaus, F., Antranikian, G., and Frey, B.

Assignees: Boehringer Mannheim G.m.b.H. (Germany)
Issued: October 2, 1997
Patent no.: WO 9735988

This invention concerns a thermostable enzyme with DNA polymerase and 3'-5'-exonuclease activity obtainable from *Thermococcus* spec. TY (DSM 10597). The gene sequence and protein sequence are reported. In addition, this invention involves a process for producing the recombinant thermophilic enzyme and can be used for specific amplification of nucleic acid sequences.

ENZYME WITH GALACTANASE ACTIVITY
Inventors: Kofod, L. V., Kauppinen, M. S., Andersen, L. N., Clausen, I. G., and Mullertz, A.
Assignees: Novo Nordisk A/S (Denmark)
Issued: September 4, 1997
Patent no.: WO 9732014

Complementary DNAs encoding galactanases with optimal pH above 5.9 are cloned from *Myceliophthora thermophilum* CBS 117.65 and *Humicola insolens* DSM 1800 and expressed in *A. niger*, *A. oryzae*, and *S. cerevisiae*. A method of purification of the recombinant *M. thermophilum* and *H. insolens* galactanases is described. The enzyme can be used in compositions for digestion of plant cell wall–derived material in a number of industrial applications, such as in detergents, in food or feed additives, and in the production of wine or juices. Thus, inclusion of galactanase in a rat diet improved the apparent protein and dry matter digestibility.

ENZYME WITH XYLANASE ACTIVITY
Inventors: Sandal, T., Kofod, L. V., Kauppinen, M. S., Andersen, L. N., and Dybdal, L.
Assignees: Novo Nordisk A/S (Denmark)
Issued: July 31, 1997
Patent no.: WO 9727292

This invention relates to an *M. thermophila* xylanase, cDNA encoding the xylanase, a method of producing the enzyme, an enzyme preparation comprising the xylanase, and the use of the enzyme and enzyme preparation for food and feed preparation and pulp and paper manufacture. The cDNA for *Myceliophtora thermophila* xylanase was cloned, sequenced, and expressed in *Aspergillus oryzae*. The recombinant enzyme had a mol wt of 43 kDa, p*I* of 4.5 and 5.5, pH optimum of 4.0–6.0, temperature optimum of 65–75°C, and specific activity of 425–575 mmol/(min · mg) of enzyme. The enzyme was used in the preparation of dough and bread baking.

BLEACHING AND BRIGHTENING OF TEXTILES
Inventors: Vollmond, T.
Assignees: Novo Nordisk A/S (Denmark)
Issued: July 17, 1997
Patent no.: WO 9725469

This invention deals with a process for providing a bleached and brightened look in the color density of the surface of dyed fabric. The process consists of (1) contacting, in an aqueous medium, a dyed fabric with a phenol-oxidizing enzyme system and enhancing agent; and (2) simultaneously or subsequently treating the fabric with a brightening agent.

NOVEL XYLANASES AND THEIR USES
Inventors: Fagerstrom, R., Paloheimo, M., Lantto, R., Lahtinen, T., and Suominen, P.
Assignees: Primalco (Finland)
Issued: June 26, 1997
Patent no.: WO 9722691

Enzyme components containing thermostable xylanases of *Chaetomium thermophilum*, purified enzyme preparations of such xylanases, and the use of such components and preparations in the bleaching of plant pulp and in feed and baking applications are described. Bleaching experiments were done to determine the usefulness of culture filtrates containing *C. thermophilum* xylanase activity in both elementary chlorine-free and totally chlorine-free bleaching of pulp. Enzyme pretreatments enhanced lignin removal. Also, brightness values of the final pulps were higher compared with the reference although chlorine dioxide consumption had not increased.

CELLULASES, THE GENES ENCODING THEM,
AND THEIR USES
Inventors: Miettinen-Oinonen, A., Londesborough, J., Vehmaanperae, J., Haakana, H., Maentylae, A., Lantto, R., Elovainio, M., Joutsjoki, V., Paloheimo, M., and Suominen, P.
Assignees: Primalco (Finland)
Issued: April 24, 1997
Patent no.: WO 9714804

New cellulases and a protein with a carbohydrate-binding domain that is synergistic for cellulases are described and purified from a number of species of fungi, and the genes encoding them are cloned and characterized. Two forms of the enzymes with molecular weights of approx 20 and 50 kDa have mildly acid pH optima and temperature optima of 60°C. The enzyme may be manufactured for use in textile processing, laundry detergents, and pulp and paper processing by cultivation of the microorganism or by expression of the cloned gene in a suitable host. Uses of the enzymes in biostoning and biofinishing are demonstrated. It was found that backstaining during biostoning was a function of the enzyme rather than of the pH. Purification of the enzymes from *Melanocarpus albomyces* and cloning of the genes are described.

XYLANASE, OLIGONUCLEOTIDIC SEQUENCE ENCODING IT,
AND ITS USES
Inventors: Perez, S., Breton, C., and Debeire, P.
Assignees: Institut National de La Recherche Agronomique (France)

Issued: April 24, 1997
Patent no.: WO 9714803

A xylanase that is stable at 60°C is obtained from a thermophilic *Bacillus* and the gene encoding it is cloned and characterized. The enzyme is suitable for use in pulp processing. Cloning of the gene by PCR using primers derived from conserved peptides of microbial xylanases is described.

ALKALIPHILIC AND THERMOPHILIC MICROORGANISMS AND ENZYMES OBTAINED FROM THEM
Inventors: Jones, B. E., Herweijer, M. A., Danson, M. J., Hough, D. W., and Thompson, C. R.
Assignees: Genencor International (United States)
Issued: March 20, 1997
Patent no.: WO 9710342

A novel species of thermophilic alkalophilic bacteria, *Thermopallium natronophilum*, is identified, and thermostable enzymes with possible commercial use are characterized. The bacterium was characterized from two independent isolates by standard biochemical markers and assigned its taxonomic position on the basis of 16S rRNA sequences. Genes encoding some of these enzymes are cloned for manufacturing of the enzyme in convenient expression hosts. These enzymes may be useful in the detergent, paper and pulp, and textile industries. Two amylases identified with temperature optima of 95 and 80°C and pH optima of 10.2 and 9.6 are characterized. One is further characterized as a pullulanase.

DNA ENCODING THERMOSTABLE PYROPHOSPHATASE
Inventors: Gelfand, D. H. and Wang, A. M.
Assignees: F. Hoffmann-La Roche Ag (Switzerland)
Issued: March 19, 1997
Patent no.: EP 763599

The thermostable pyrophosphatase of *T. thermophilus* is manufactured by expression of the cloned gene in *E. coli*. The enzyme is useful in pyrophosphate-generating reactions such as PCR because the hydrolysis of pyrophosphate drives the reaction in the direction of polymerization. The construction of expression vectors for the gene is described. The cloned gene can be used as a probe to identify pyrophosphatase genes from other species of *Thermus*. The thermostable pyrophosphatase prepared in accordance with this invention is preferably packed in a kit and can be used in a nucleic acid amplification method.

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