Developments in industrially important thermostable enzymes: a review

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Abstract

Cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also thermostable. Apart from high temperature they are also known to withstand denaturants of extremely acidic and alkaline conditions. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes in maximising reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal and drilling for oil is being studied extensively. The enzymes can be produced from the thermophiles through either optimised fermentation of the microorganisms or cloning of fast-growing mesophiles by recombinant DNA technology. In this review, the source microorganisms and properties of thermostable starch hydrolysing amylases, xylanases, cellulases, chitinases, proteases, lipases and DNA polymerases are discussed. The industrial needs for such specific thermostable enzyme and improvements required to maximize their application in the future are also suggested.

Keywords: Thermostable enzymes; Thermophilic microorganisms

1. Introduction

The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzymes the number of applications has increased manyfold, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan et al., 2001). Advances in this area have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them (Burrows, 1973; Antranikian et al., 1987; Groboillot, 1994; Bharat and Hoondal, 1998; Bauer et al., 1999; Kohilu et al., 2001).

While the most widely used thermostable enzymes are the amylases in the starch industry (Poonam and Dalel, 1995; Crab and Mitchinson, 1997; Emmanuel et al., 2000; Sarikaya et al., 2000), a number of other applications are in various stages of development. In the food related industry, they have been used in the synthesis of amino acids (Satosi et al., 2001). In the petroleum, chemical and pulp and paper industries, for example, thermostable enzymes have been used for the elimination of sulphur containing pollutants through the biodegradation of compounds like dibenzothiophene (Bahrami et al., 2001), in the production of 1,3-propanediol from glycerol and in replacing polluting chemical reagents causing toxic products (Peter et al., 2001). Currently, a number of publications have extensively discussed developments in this area. Adaptation of extremophiles to hot environments (Danson et al., 1992; Stetter, 1999), production of heat-stable enzymes from thermophiles and hyperthermophiles (Knor, 1987; Jakob, 1989; Huber and Stetter, 1998; Niehaus et al., 1999), structure and function relationships of thermozymes (heat-tolerant enzymes) (Zeikus et al., 1998a,b).
and biotechnological and industrial applications of thermostable enzymes (Franks, 1993; Lasa and Berengué, 1993; Leuschner and Antranikan, 1995; Cowan, 1996; Holst et al., 1997; Hough and Danson, 1999; Eichler, 2001) are among the topics that have been studied.

In the present review, an attempt is made to document the research activities conducted in the area and indicate the source microorganisms of some important thermostable enzymes. The need for thermostable catalysts, the optimum conditions for an efficient catalytic activity of the enzymes and current industrial applications are presented. Besides discussing the reason for the thermostable character of the enzymes, possible improvements and a number of expected developments are also suggested in the article.

2. Geothermal sites as sources of extremophiles

In situ temperatures between 80 and 115 °C were found to be conducive biotopes for a number of hyperthermophiles (Huber and Stetter, 1998). Some examples are mentioned in Table 1. Extremophiles are also adapted to live at low temperatures in the cold polar regions, at high pressure in the deep sea and at a very low and high pH values (pH 0–3 or 10–12), or at a very high (5–30%) salt concentration (Herbert and Sharp, 1992).

In the last decade, a number of hyperthermophilic archaea, the least understood domain of life (Woese et al., 1990; Rotschild and Manicinelli, 2001) have been isolated and are able to grow around the boiling point of water (Niehaus et al., 1999). The organisms with the highest growth temperatures (103–110 °C) are members of the genera *Pyrobaculum*, *Pyrodictium*, *Pyrococcus* and *Methanopyrus*. Within the bacteria, *Thermotoga maritima* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95 °C respectively (Herbert and Sharp, 1992). These properties imply extremely important industrial and biotechnological implications due to the fact that enzymes from such microorganisms can be employed for use in harsh industrial conditions where their specific catalytic activity is retained.

3. Resistance of thermophiles to high temperatures and denaturation

Microorganisms, like all living things, adapt to the condition in which they have to live and survive. Thermophiles are reported to contain proteins which are thermostable and resist denaturation and proteolysis (Kumar and Nussinov, 2001). Specialized proteins known as ‘chaperons’ are produced by these organisms, which help, after their denaturation to refold the proteins to their native form and restore their functions (Everly and Alberto, 2000). The cell membrane of thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures (Herbert and Sharp, 1992). The archaea, which compose most of the hyperthermophiles, have lipids linked with ether on the cell wall. This layer is much more heat resistant than a membrane formed of fatty acids (De Rosa et al., 1994).

The DNA of thermophiles contains a reverse DNA gyrase which produces positive super coils in the DNA (Lopez, 1999). This raises the melting point of the DNA (the temperature at which the strands of the double helix separate) to at least as high as the organ-

<table>
<thead>
<tr>
<th>Source</th>
<th>Microorganism</th>
<th>Enzyme</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot spring</td>
<td><em>Thermus</em> sp.</td>
<td>α-Amylase</td>
<td>Shaw et al. (1995)</td>
</tr>
<tr>
<td>Hot spring</td>
<td><em>Bacillus</em> sp. WN.11</td>
<td>α-Amylase</td>
<td>Mamo and Gessese (1999)</td>
</tr>
<tr>
<td>Deep sea hydrothermal vent</td>
<td><em>Staphylothermus marinus</em></td>
<td>α-Amylase</td>
<td>Canganella et al. (1994)</td>
</tr>
<tr>
<td>Decomposed plant samples from a lake</td>
<td><em>Clostridium absonum</em> CFR-702</td>
<td>Cellulase free xylanase</td>
<td>Swaroopa and Krishna (2000)</td>
</tr>
<tr>
<td>Hot spring</td>
<td><em>Bacillus thermoleovorans</em> ID-1</td>
<td>Lipase</td>
<td>Dong-Woo et al. (1999)</td>
</tr>
<tr>
<td>Compost of fermenting citrus peels, coffee and tea extract residues</td>
<td><em>Bacillus</em> strain MH-1</td>
<td>Endochitinase</td>
<td>Kenji et al. (1998)</td>
</tr>
<tr>
<td>Korean salt fermented anchovy</td>
<td><em>Bacillus</em> steatorrhophilus CH-4</td>
<td>β-N-acetylmuramidase</td>
<td>Kenji et al. (1994)</td>
</tr>
<tr>
<td>Deep sea hydrothermal vent</td>
<td><em>Bacillus</em> sp. KY963</td>
<td>β-Amylase</td>
<td>Young et al. (2001)</td>
</tr>
<tr>
<td>Sediments of hot springs</td>
<td><em>Pyrococcus abyssi</em></td>
<td>Alkaline phosphatase</td>
<td>Sebastian et al. (2001)</td>
</tr>
<tr>
<td>Garbage dump</td>
<td><em>Bacillus</em> sp. 3183</td>
<td>β-Amylase-like pullulanase</td>
<td>Badal et al. (1989)</td>
</tr>
<tr>
<td>Compost treated with artichoke juice</td>
<td><em>Bacillus</em> circulans</td>
<td>Xylanase</td>
<td>Ashita et al. (2000)</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus</em> sp.</td>
<td>Inulinase</td>
<td>Jean-Jacques et al. (1987)</td>
</tr>
</tbody>
</table>
isms' maximum temperature for growth. Thermophiles also tolerate high temperature by using increased interactions that non-thermotolerant organisms use, namely, electrostatic, disulphide bridge and hydrophobic interactions (Kumar and Nussinov, 2001).

Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Saboto et al., 1999). These authors indicated that relatively few studies have been carried out in this regard and the only available information is that thermophilic enzymes are more rigid proteins than their mesophilic counterparts. A clearer understanding of this capacity should be possible with new methodologies that clearly indicate changes in protein structure.

The effect of structural fluctuations of α-amylases of mesophilic and thermophilic sources were studied and reported by Fitter et al. (2001). While determining the conformational entropy of both enzymes, the folded state showed a higher structural flexibility for the thermophilic protein than the mesophilic homologue. It has, thus, been assumed that a mechanism characterized by entropic stabilization could be responsible for the higher thermostability of the thermophilic enzyme.

4. General advantages of enzymes from thermophiles

Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes (Leuschner and Antranikan, 1995; Fredrich and Antrakian, 1996; Diane et al., 1997; Zeikus et al., 1998a,b). Some biocatalytic conversions and industrial applications of thermostable enzymes are presented in Table 2.

One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient bioremediation (Becker, 1997). Other values of elevated process temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions (Mozhaev, 1993; Krahe et al., 1996; Kumar and Swati, 2001). Such enzymes can also be used as models for the understanding of thermostability and thermo-activity, which is useful for protein engineering. The following sections describe specific thermostable enzymes, their sources, characteristics and application requirements of high temperature resistance.

Table 2
Bioconversion reactions and applications of thermostable enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature range (°C)</th>
<th>Bioconversions</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylase (bacterial)</td>
<td>90–100</td>
<td>Starch → dextrose syrups</td>
<td>Starch hydrolysis, brewing, baking, detergents</td>
</tr>
<tr>
<td>α-Amylase (fungal)</td>
<td>50–60</td>
<td>Starch → dextrose syrups</td>
<td>Production of maltose</td>
</tr>
<tr>
<td>Pullulanase</td>
<td>50–60</td>
<td>Starch → dextrose syrups</td>
<td>Production of glucose syrups</td>
</tr>
<tr>
<td>Xylanase</td>
<td>45–65, 105a</td>
<td>Craft pulp → xylan + lignin</td>
<td>Pulp and paper industry</td>
</tr>
<tr>
<td>Chitinase</td>
<td>65–75b</td>
<td>Chitin → chitobiase</td>
<td>Food, cosmetics, pharmaceuticals, agrochemicals</td>
</tr>
<tr>
<td>Chitinase</td>
<td></td>
<td>Chitin → N-acetyl glucosamine (chitobiase)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-acetyl glucosamine → glucosamine (deacylation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellulose → glucose</td>
<td>Cellulose hydrolysis, polymer degradation in detergents</td>
</tr>
<tr>
<td>Cellulase</td>
<td>45–55, 95c</td>
<td>Protein → amino acids and peptides</td>
<td>Baking, brewing, detergents, leather industry</td>
</tr>
<tr>
<td>Protease</td>
<td>65–85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>30–70</td>
<td>Fat removal, hydrolysis, interesterification, alcholysis, aminolysis</td>
<td>Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>90–95</td>
<td>DNA amplification</td>
<td>Genetic engineering/PCR</td>
</tr>
</tbody>
</table>

*a Xylanase from Thermotoga sp.
*b Within this range enzyme activity was high.
*c Cellulases from Thermotoga sp.

5. Amylolytic enzymes

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such
polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, gluco-amylases or β-amylases and isoamylases or pullulanases (Poonam and Dalel, 1995). The enzymes are classified into endo-acting and exo-acting enzymes. α-amylase is an endo-acting enzyme and hydrolyses linkages in a random fashion and leads to the formation of linear and branched oligosaccharides, while the rest are exo-acting enzymes and attack the substrate from the non-reducing end, producing oligo and/or monosaccharides. The starch hydrolytic enzymes comprise 30% of the world’s enzyme consumption (Van der Maarel et al., 2002).

The enzymatic conversion of all starch includes gelatinization, which involves the dissolution of starch granules thereby forming a viscous suspension, liquefaction, which involves partial hydrolysis and loss in viscosity, and saccharification, involving the production of glucose and maltose via further hydrolysis. Gelatinisation is achieved by heating starch with water, and starch is water-soluble only at high temperatures which are dependent on the source (Rakshit, 1998). For hydrolysis of the starch to proceed immediately after gelatinization, hence, among other things avoiding a lot of cooling time, the enzyme has to be thermostable.

A number of attempts were made to isolate and characterize amylolytic enzymes from diversified sources (Daniel, 1979; Norman, 1979; Fogarty and Kelly, 1995; Medda and Chandra, 1980; Morgan and Priest, 1981; Fogarty, 1983; Krishnan and Chandra, 1983; Rolfsmeir and Blum, 1995; Piller et al., 1996; Crab and Mitchinson, 1997; Rama et al., 1998; Jin et al., 2001; Min Ha et al., 2001; Stanford et al., 2001; Wojciechowski et al., 2001; Andersson et al., 2002; Carlsen et al., 2002; Dijkhuizen et al., 2002; Miura et al., 2002) to meet the requirements of the starch industry. Table 3 shows thermostable microbial starch hydrolyzing enzymes and their properties. Thermostable α-amylases were isolated a long time ago from Bacillus subtilis, Bacillus amylo-liquefaciens and Bacillus licheniformis (Underkofler, 1976). The commercially available β-amylases with a catalytic activity up to 60°C were isolated from various Bacillus species (Brown, 1987). They can be used in conjunction with debranching enzymes to produce pure maltose syrups.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Organism</th>
<th>Enzyme properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylase</td>
<td>Bacillus amylo-liquefaciens</td>
<td>70</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Bacillus licheniformis</td>
<td>100</td>
<td>6.0–6.5</td>
</tr>
<tr>
<td></td>
<td>Bacillus steatorrhophilus</td>
<td>70–80</td>
<td>5.0–6.0</td>
</tr>
<tr>
<td></td>
<td>Bacillus steatorrhophilus</td>
<td>70</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bacillus subtilis</td>
<td>70</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus manihotivorans</td>
<td>55</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Myceliophthora thermophila</td>
<td>100</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Pyrococcus furiosus</td>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Pyrococcus woesei</td>
<td>100</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td></td>
<td>Staphylothermus marinus</td>
<td>65</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Sulfolobus solfataricus</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Thermococcus aggregatans</td>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermococcus celer</td>
<td>90</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermococcus fumicola</td>
<td>95</td>
<td>4.0–6.3</td>
</tr>
<tr>
<td></td>
<td>Thermococcus hydrothermalis</td>
<td>85</td>
<td>4.8–7.8</td>
</tr>
<tr>
<td></td>
<td>Thermomyces lanuginosus</td>
<td>60</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Thermomycopsis profundus</td>
<td>80</td>
<td>4.0–5.0</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>Bacillus circulans</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bacillus cereus var. mycoides</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>50</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Clostridium thermosulphurogenes</td>
<td>75</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Clostridium thermosulfurogenes</td>
<td>60</td>
<td>5.8–6.0</td>
</tr>
<tr>
<td>Pullulanase</td>
<td>Bacillus sp.</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Pyrococcus furiosus</td>
<td>98</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Pyrococcus woesei</td>
<td>100</td>
<td>5.5–6.0</td>
</tr>
<tr>
<td></td>
<td>Thermococcus aggregans</td>
<td>100</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Thermus caldophilus GK24</td>
<td>75</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermococcus celer</td>
<td>90</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermococcus hydrothermalis</td>
<td>95</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermococcus litoralis</td>
<td>98</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Thermotoga maritima MSB8</td>
<td>90</td>
<td>6.0</td>
</tr>
</tbody>
</table>
The pullulanases that were produced in earlier work on different microorganisms were not very suitable for operation under the conditions prevailing in the industry (Nakamura et al., 1975; Konishi et al., 1979; De Mot et al., 1984a,b; Takizawa and Murooka, 1985). However, many thermophilic microorganisms have since been found to produce pullulanases (Suzuki and Chishoro, 1983; Jensen and Norman, 1984; Plant et al., 1986; Antranikian et al., 1987; Koch et al., 1987; Saha et al., 1988; Canganella et al., 1994; Swamy and Seenayya, 1996) and β-amylases (Shen et al., 1988; Nipkow et al., 1989; Swampus et al., 1994; Rina and Geeta, 1996; Rama et al., 1999; Erra-Pujada et al., 2001).

Termamyl and Fungamyl are two well-known amylolytic enzymes which are now available commercially. These enzymes are used world-wide for the production of glucose syrups and syrups with different level of dextrose equivalent. Hetero-oligosaccharides have been synthesized by Termamyl obtained from B. licheniformis (Chitradon et al., 2000). In the presence of soluble starch and added non-starch sugars, oligosaccharides were produced by the reversed catalytic reaction.

One of the concerns of the starch industry is the calcium requirement of industrially important starch degrading enzyme. Table 4 illustrates some important thermostable α-amylases, aspergillus oryzae, which was produced via site-directed mutagenesis, has shown high calcium independence (Hashida and Bisgaard-Frantzen, 2000).

Although, raw starch dominates in nature there are few enzymes that can catalyze its hydrolysis efficiently (Hamilton et al., 1999). Saccharification of liquefied starch is carried out at low pH values. However, currently used thermostable α-amylases are not stable at such low pH (Crab and Mitchinson, 1997). An economical process could be attained through the use of amylases stable at the saccharification stage. In addition to this, a one-step process of starch hydrolysis using amylolytic enzymes would decrease the costs of glucose production. The anticipated diversity of species of thermophiles within high temperature environments (Huber and Stetter, 1998) and the requirements of the new and improved technological operations for enzymes with novel and fitting characteristics (Emmanuel et al., 2000) have placed a challenge both for the scientific and business community.

### Table 4
Calcium requirement of industrially important starch degrading enzyme

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Application temperature range (°C)</th>
<th>Application pH range</th>
<th>Minimum Ca²⁺ dosage (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial mesophilic α-amylase</td>
<td>Bacillus subtilis</td>
<td>80–85</td>
<td>6.0–7.0</td>
<td>150</td>
</tr>
<tr>
<td>Bacterial thermophilic α-amylase</td>
<td>Bacillus licheniformis</td>
<td>95–105</td>
<td>6.0–7.0</td>
<td>20</td>
</tr>
<tr>
<td>Fungal α-amylase</td>
<td>Aspergillus oryzae</td>
<td>55–70</td>
<td>4.0–5.0</td>
<td>50</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>Aspergillus niger</td>
<td>55–65</td>
<td>3.5–5.0</td>
<td>0</td>
</tr>
<tr>
<td>Pullulanase</td>
<td>Bacillus acidopoluliticus</td>
<td>55–65</td>
<td>3.5–5.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Source: Hans (1995).*

6. Thermostable xylanases

Xylan, which is the dominating component of hemicelluloses, is one of the most abundant organic substances on earth. It has a great application in the pulp and paper industry (Dekker and Linder, 1979; Chen et al., 1997; Lee et al., 1998). The wood used for the production of the pulp is treated at high temperature and basic pH, which implies that the enzymatic procedures require proteins exhibiting a high thermostability and activity in a broad pH range (Jacques et al., 2000). Treatment with xylanase at elevated temperatures disrupts the cell wall structure. This, as a result, facilitates lignin removal in the various stages of bleaching. Xylanases for such a purpose (1) must lack cellulytic activity to avoid hydrolysis of the cellulose fibers, (2) need to be of low molecular mass to facilitate their diffusion in the pulp fibers, and (3) most importantly, high yields of enzyme must be obtained at a very low cost (Niehaus et al., 1999). According to these authors all commercially available xylanases can only partially fulfill these requirements, and the optimum temperature for the activity of most xylanases is reported to be 50–60 °C with a half-life of about 1 h at 55 °C (Jacques et al., 2000). However, some xylanases have been reported to exhibit higher thermal stability and optimal activity ranging from 80 to 100 °C (Saul et al., 1995; Zverlov et al., 1996; Morris et al., 1998).
These enzymes have offered a major step in the reduction of chlorine consumption in the bleaching process of kraft pulp, thus, lowering environmental pollution by organic halogens (Vikari et al., 1994; Eriksen and Heitmann, 1998). The enzymes are generally classified into two families. Family 10 groups (EXs 10) having high molecular weights (>30 Kda) and Family 11 (EXs 11) which are low molecular weight (<30 Kda). Xylanases (Henrissat and Bairoch, 1993; Dominguez et al., 1995). Among the two families, the EX 10-fold is found to be more thermostable (Fontes et al., 1995). Due to their capacity to enhance the bleaching of kraft paper, however, EXs 11 have remained more attractive (Clarke et al., 1997; Morris et al., 1998; Georis et al., 2000).

Thermostable xylanases were isolated from a number of bacterial and fungal sources (Table 5). Members of the Bacillus sp., Streptomyces sp., Thermoascus aurantiacus and Fusarium proliferatum have been reported to produce xylanases which are active at temperatures between 50 and 80 °C. While the Dictyoglomus sp. were described to produce xylanases operating at an optimum temperature of 90 °C, a number of Thermotogaes sp. were reported to secrete thermostable xylanases which can function at higher temperatures. Alkaliphilic and cellulase-free xylanases with an optimum temperature of 65 °C from Thermoactinomyces thalophilus subgroup C (Kohilu et al., 2001) and cellulase-free xylanases from Clostridium absonum CFR-702 (Swaroopa and Krishna, 2000) were also reported recently. From one Thermotoga sp. an enzyme active at 115 °C was produced and characterized (Simpson et al., 1991). It was also possible to produce xylanases in solid-state fermentation, where a decreased cultivation time and an increased concentration of basal medium improved its production. This is a promising aspect from an economic point of view (Park et al., 2002).

The pulp and paper technology is one of the fastest growing industries and the use of thermostable xylanases seems attractive since they provide global environmental benefits. However, scaling up of the enzyme production from the respective microorganisms to the level required by the industry remains to be seen. It is also worth mentioning that, extreme thermophiles that are able to secrete xylanase are few. The search for a thermophile with high yield of enzyme and the desired characteristics is still being pursued.

7. Thermostable cellulases

Cellulose, the most abundant organic source of feed, fuel and chemicals (Spano et al., 1975) consists of glucose units linked by β-1,4-glycosidic bonds in a linear
mode. The difference in the type of bond and the highly ordered crystalline form of the compound between starch and cellulose make cellulose more resistant to digest and hydrolyze. The enzymes required for the hydrolysis of cellulose include endoglucanases, exoglucanases and β-glucosidases (Matsui et al., 2000). While cellulase is an endoglucanase that hydrolyzes cellulose randomly, producing oligosaccharides, cellobiose and glucose, exoglucanases hydrolyze β-1,4-D-glucosidic linkages in cellulose releasing cellobiose from the non-reducing end. On the other hand, β-glycosidases of thermophilic origin, which have received renewed attention in the pharmaceutical industry hydrolyze cellobiose to glucose.

In the current industrial processes, cellulolytic enzymes are employed in the color extractions of juices, in detergents causing color brightening and softening, in the biostoning of jeans, in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage and in the pretreatment of industrial wastes (Buchert et al., 1997; Niehaus et al., 1999; Bhat, 2000; Nakamura et al., 2001; Van wyk et al., 2001; Zhou et al., 2001). In order to attack the native crystalline cellulose, which is water insoluble and occurs as fibers of densely packed structures, however, thermostable cellulases active at high temperature and high pH are required.

Thermostable cellulases of archaean origin include those isolated from Pyrococcus furiosus (Kengen et al., 1993) and Pyrococcus horikoshi (Ando et al., 2002). While the latter has an optimum temperature of 97 °C, the enzyme from Pyrococcus furiosus has shown optimal activity at 102–105 °C (Table 6). Sulfolobus solfataricus MT4, Sulfolobus acidocaldarius and Sulfolobus shibatae were also described as producers of β-glucosidases (Grogan, 1991). From Thermotoga maritima MSB8 optimally active cellulase acting at 95 °C and between pH 6.0 and 7.0 was reported (Bronnenmeier et al., 1995). Other endocellulases (CelA and CelB) from Thermotoga neapolitana, were purified and characterized (Bok et al., 1998). Optimal pH for CelA is 6.0 at 95 °C, and the pH range of CelB is broad (6.0–6.6) at 106 °C. CelA, with the ability to hydrolyze microcrystalline cellulose, was isolated from the extremely thermophilic bacterium Anaerocellum thermophilum (Zverlov et al., 1998) and maximal activity of this enzyme was observed at pH 5.0–6.0 and 85–95 °C. A highly thermostable cellobiose (115 °C at pH 6.8–7.8) was also produced from Thermotoga sp. FjSS3-B1 (Ruthersmith and Daniel, 1991, 1992).

It is obvious that, the successful utilization of cellulose is dependent on the development of economically feasible technologies for the production of cellulase. The biopolishing process of cotton in the textile industry, for example, requires cellulase stable at high temperature close to 100 °C (Ando et al., 2002). Presently used enzymes for this purpose, however, are active only at 50–55 °C. Cellulase production is also found to be the most expensive step during ethanol production from cellulose biomass, and accounted for approximately 40% of the total cost (Spano et al., 1975). In the food industry, degradation of cellulose by acids is still unsatisfactory and results in the decomposition of the sugars. Finally, even though many cellulolytic enzymes of thermophilic origin are known their function under physiological condition remains unclear.

### Table 6
Source microorganisms and properties of thermostable cellulases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimal temperature (°C)</td>
<td>Optimal pH</td>
</tr>
<tr>
<td>Anaerocellum thermophilum</td>
<td>85–90</td>
<td>5.0–6.6</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>65–70</td>
<td>5.0–6.5</td>
</tr>
<tr>
<td>Pyrococcus horikoshi</td>
<td>97</td>
<td>–</td>
</tr>
<tr>
<td>Rhodothermus marinus</td>
<td>95</td>
<td>6.5–8.0</td>
</tr>
<tr>
<td>Thermotoga maritima MSB8</td>
<td>95</td>
<td>6.0–7.0</td>
</tr>
<tr>
<td>Thermotoga neapolitana (EndocellulaseA)</td>
<td>95</td>
<td>6.0</td>
</tr>
<tr>
<td>Thermotoga neapolitana (EndocellulaseB)</td>
<td>106</td>
<td>6.0–6.6</td>
</tr>
</tbody>
</table>

8. Thermoactive chitinases

Chitin, the second most abundant natural biopolymer after cellulose, consists of a linear β-1,4-homopolymer of N-acetylgalactosamine residues. In nature, it is usually found attached to other polysaccharides and proteins. The covering layer of insects, cell walls of various fungi and crab and shrimp wastes are the main sources of chitin (Majeti and Kumar, 2000; Nwe and Stevens, 2002; Suntornsuk et al., 2002). The major applications of chitosan include wastewater clearing, preparation of cosmetics, paper production, textile finishes, photographic products, cements, heavy metal chelating agents.
and for medical and veterinary purposes (Spindler et al., 1990; Georgopapadakou and Tkacz, 1995; Hirano, 1996; Cohen and Chet, 1998; Majeti and Kumar, 2000).

The production of chitosan from chitin involves a deacetylation reaction that is done using 40% sodium hydroxide solution. This is a highly corrosive stream which is difficult to control. Attempts are thus being made to do this conversion using deacetylase enzyme. Perhaps a thermostable deacetylase enzyme will help in increasing yields and conversion rates and allow reuse of the enzyme.

Endo-acting chitin hydrolase chitinase-A, the exo-acting hydrolase chitinase-B and N-acetyl-D-glucosaminidase are responsible for chitin degradation (Kenji et al., 1994). However, chitin is not easily accessible to chitinases and chitin deacetylases. Evidences from X-ray diffraction studies have shown that chitin is a highly ordered crystalline structure and does not dissolve in water (Roberts, 1992). This property of chitin has shown the need for thermostable enzymes.

The thermophilic organisms Bacillus licheniformis X-7u (Takayanagi et al., 1991), Bacillus sp. BG-11 (Bharat and Hoondal, 1998) and Streptomyces thermoviolaceous OPC-520 were reported to be the major sources of chitinases (Tsujibo et al., 1995). Chitinase and N-acetyl-glucosaminidase were produced from the extreme thermophilic anaerobic arachaeon Thermococcus chininophagus (Huber et al., 1995). A thermophilic bacterium strain producing three different endochitinases in its culture fluid was isolated from chitin-containing compost (Kenji et al., 1994). The strains belonged to the genus Bacillus and three isoforms of endochitinases (L, M and S) were purified and characterized. They had different molecular masses (71, 62 and 53 Kda) and showed temperature optima of 75, 65 and 75 °C at a pH of 6.5, 5.5 and 5.5 respectively. The iso-electric points were 5.3, 4.8, and 4.7. Thermostable exochitinases were also isolated from Bacillus steaethermophilus CH-4, isolated from a compost of organic solid wastes (Kenji et al., 1998).

The basic parts of shrimp and prawns used for consumption are the muscle tissues. The resulting waste of the products, thus, is vast (Healey et al., 1994). In some developing countries it was reported that more than 2.5 million tones of shrimp and prawns are produced per year (Anon., 1996). Therefore, there is a high need to develop methods of producing value added products from this waste. As the major way to exploit the potential from this waste is the production of chitin and its derivative chitosan, improved and economically feasible chemical, enzymatic and novel biotechnological conversion bioprocesses are required. Chitin together with its derivatives can also be used as agrochemicals (Kenji et al., 1998). However, there is only little effort to clarify its utilization in the food industry.

9. Thermostable proteases

Proteases, which are generally classified into two categories (exopeptidases, that cleave off amino acids from the ends of the protein chain and endopeptidases, which cleave peptide bonds within the protein) are becoming major industrial enzymes, and constitute more than 65% of the world market (Rao et al., 1998). These enzymes are extensively used in the food, pharmaceutical, leather and textile industries (Cowan, 1996; Fan et al., 2001; Mozersky et al., 2002). The applications will keep increasing in the future as will the need for stable biocatalysts capable of withstanding harsh conditions of operation.

Relative ease of isolation of Bacilli from diverse sources has made these organisms the focus of attention in biotechnology (Johnevelsy and Naik, 2001). So far, however, few thermophilic Bacillus sp. that produce proteases have been isolated, the earliest isolate being Bacillus steaethermophilus (Sulleh et al., 1977) which is stable at 60 °C. Another Bacillus sp. has produced a thermostable protease that has an optimum activity at 60 °C (Razak et al., 1993), while a different Bacillus steaethermophilus sp. produced an alkaline and thermostable protease which is optimally active at 85 °C (Razak et al., 1995, 1997; Rahman et al., 1994). A species of Bacillus steaethermophilus TP26 that has been isolated produces an extra cellular protease having an optimum temperature of 75 °C (Gey and Unger, 1995). Enhancement of protease activity excreted from Bacillus steaethermophilus had also been possible using economical chemical additives in the proteolysis reactions involved in waste activated sludge (Kim et al., 2002a,b). In a chemically defined medium, thermophilic and alkaliphilic Bacillus sp. JB-99 was also reported to produce thermostable alkaline proteases. (Johnevelsy and Naik, 2001). Dominant producers of proteases in fact, are the microorganisms of the genera Pyrococcus, Thermococcus and Staphylothermus (Table 7). Extremely thermostable serine proteases are produced by the hyperthermophilic archaean Desulfurococcus strain (Hanazawa et al., 1996), and thermostable metalloproteases are reported from a gram-negative thermophilic bacterium (Pawinee and Wipapat, 1996).

Major area of focus in the future concerning the production of proteases is the optimization of media (Johnevelsy and Naik, 2001). Synthetic media have a great advantage over complex media in that consistency of processes and production is enhanced through avoiding the variability of complex substrates. Thus, synthetic media provides better control and monitoring, improved product recovery and quality, and simplified purification systems (Zhang and Greasham, 1999). It is for this reason that reports on the production of protease enzymes using synthetic media are available recently (Mao et al., 1992; Ferrero et al., 1996).
There is considerable current interest on the exploration of proteases that can catalyze reactions in cold water (Demirjian et al., 2001). This will allow their use in detergents which can be used in normal tap water without the requirement for increasing the temperature of the water. The search for such enzymes is very much a challenge at this time.

10. Heat stable lipases

Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions (Vulfson, 1994), which includes hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Jaeger et al., 1994; Pandey et al., 1999; Nagao et al., 2001; Kim et al., 2002a,b). Their unique characteristics include substrate specificity, stereospecificity, regioselectivity and ability to catalyze a heterogeneous reaction at the interface of water soluble and water insoluble systems (Brockman and Borgstorm, 1984; Jaeger and Reetz, 1998).

The esters produced play a relevant role in the food industry as flavor and aroma constituents (Gandhi et al., 1995; Pandey et al., 1999). Whereas long chain methyl and ethyl esters of carboxylic acid moieties provide valuable oleo chemical species that may function as fuel for diesel engines, esters of long chain carboxylic acid and alcohol moieties (waxes) have applications as lubricants and additives in cosmetic formulations (Linko et al., 1994). Other applications include the removal of the pitch from pulp produced in the paper industry, for the hydrolysis of milk fat in the dairy industry, removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, drug formulations in the pharmaceutical industry and in the removal of subcutaneous fat in the leather industry (Pandey et al., 1999; Traore and Buschle-Diller, 2000). A biodiesel was derived from vegetable oils using immobilized *Candida antarctica* lipase (Shimada et al., 1999).

Most of the industrial processes in which lipases are employed function at temperatures exceeding 45°C. The enzymes, thus, are required to exhibit an optimum temperature of around 50°C (Sharma et al., 2002). According to some reports there are fats exhibiting higher melting points and which are able to inhibit enzymatic reactions at a low temperature (Dong-Woo et al., 1999). Some enzymatic processes for the physical refining of seed oils have four distinct requirements. These include pH of 5.0 and optimal temperature of around 65°C, adding an enzyme solution and enzyme reaction followed by the separation of the lysophosphatide from the oil at about 75°C (Klaus, 1998). These reactions, therefore, are enhanced through the utilization of thermo-tolerant lipases.

Lipases are of widespread occurrence throughout the earth’s flora and fauna. More abundantly, however, they are found in bacteria, fungi and yeasts (Wu et al., 1996). Several *Bacillus* sp. were reported to be the main source of lypolytic enzymes (Kim et al., 1994; Schmidt et al., 1994; Luisa et al., 1997). While most of these enzymes are active at a temperature of 60°C and pH of 7.0, lipases from *Bacillus thermoleovorans* and a thermo-tolerant *Rhizopus oryzae* strain can moderately function at extreme pH and temperature values (Dong-Woo et al., 1999; Abel et al., 2000). The pH and temperature optima for the catalytic activity of some thermostable lipases are given in Table 8.

Reports of thermostable lipases from archaeal origin, however, are very few. Phospholipase A$_2$ which was secreted from the archaea *Pyrococcus horikoshii*
11. Thermostable DNA polymerases

The polymerase chain reaction (PCR) process has led to a huge advance in genetic engineering due to its capacity to amplify DNA. The three successive steps in this process include denaturation or melting of the DNA strand (separation) obtained at a temperature of 90–95 °C, renaturation or primer annealing at 55 °C followed by synthesis or primer extension at around 75 °C (Mullis et al., 1986; Erlich et al., 1988; Saiki et al., 1988). Development in this process has been to a large extent facilitated by the availability of thermostable DNA polymerases, which catalyse the elongation of the primer DNA strand (Mullis and Falouona, 1987).

In earlier PCR procedures, DNA polymerases which were isolated from Escheria coli were utilized (Mullis et al., 1986; Erlich et al., 1988). These enzymes, however, lost their enzymatic activities at elevated temperatures and, thus, adding a new polymerase enzyme after each cycle following the denaturation and primer hybridization steps was necessary. This process made the thermal cycling a time-consuming and costly procedure.

Taq polymerase from the bacterium Thermus aquaticus was the first thermostable DNA polymerase characterized (Chien et al., 1976; Kaledin et al., 1980). Repeated exposure to 98 °C in a reaction buffer had little effect on the enzyme activity and significant activity remained after exposure to 99 °C. Although, Taq polymerase exhibits a 5'-3' exonuclease activity, a 3'-5' exonuclease activity was not detected. Thus, the base insertion fidelity is low as the enzyme is unable to correct misincorporated nucleotides (Longley et al., 1990; Ling et al., 1991). It had also been possible to determine the error rates of some polymerases in terms of base pairs (Alkami Biosystems, 1999).

Table 9 shows a variety of thermostable polymerases with 3'-5'-exonuclease-dependent proof reading activity required from a high fidelity polymerase. Optimization of the PCR procedure can be done by mixing a standard polymerase, such as Taq polymerase with high fidelity polymerases (Pfu, Vent and Deep Vent) (Ling et al., 1991).

While the PCR process is developing rapidly through the invention of better instruments (Mariella, 2001), lack of fidelity has remained to be a serious challenge. The five distinct activities in which errors can occur are the rate of phosphodiester bond formation, the binding of dNTP by the polymerase, the rate of pyrophosphate release, contamination after misincorporation and the
3′-5′-exonuclease proof reading capacity of the enzyme (Alkami Biosystems, 1999). Some issues that nowadays have stimulated scientists in the area thus, are improvements required in the fidelity of the PCR, development of strategies for more economical use of the enzymes and ensuring rapid multiplication from fewer strands. A thermostable DNA polymerase having these characteristics will indeed improve the results obtained by PCR machine.

12. Molecular cloning of thermophilic genes into mesophilic hosts

Genetic and protein engineering are the modern techniques for the commercial production of enzymes of improved stability to high temperatures, extremes of pH, oxidizing agents and organic solvents. Cloning and expression of genomic information available in a thermophile in a suitable and faster growing mesophilic host has also provided possibilities of producing the specific thermostable enzyme required for a particular biotransformation process (Blackebrough and Birch, 1981; William and Dennis, 1988; James, 1995; Ikeda and Clark, 1998; Hough and Danson, 1999).

Genes encoding α-amylase (amyA), pullulanase (pulA), maltodextrin phosphorylase (aspA) and α-glucosidase (aglA) were identified in the gene library of Thermotoga maritima (Bibel et al., 1998). The enzymes produced after expression in Escheria coli are reported to demonstrate extreme thermostability. Pyrococcus furiosus α-amylase gene, which was cloned by activity screening in Escheria coli was reported to be optimally active at 100 °C, pH 5.5–6.0 and not to require Ca^{2+} for its activity (Dong et al., 1997a). Genes encoding pullulanases from Pyrococcus furiosus (Dong et al., 1997b) and Ferribacterium pennivorans (Bertoldo et al., 1999), Thermotoga neapolitana genes encoding for xylanases (Velikodvorskaya et al., 1997), Protease genes from Pyrobaculum aerophilum and Bacillus stearothermophilus (Volkl et al., 1995; Kubo and Imanaka, 1988), lipases from Bacillus thermocatenulatus (Schmidt et al., 1994), esterases from Bacillus licheniformis (Alvarez-macarie et al., 1999) and cellulase genes from the archaon Pyrococcus furiosus (Bauer et al., 1999) were expressed from an Escheria coli recombinant strain. The production of cellulase enzyme was further enhanced through DNA recombinant technology by encoding the genes for cellulase in various species of Clostridium (Beguin, 1992). In addition to this, the nucleotide sequence and the cloning of CelA and CelB genes from C. thermocellum were studied and reported (Bergquist et al., 1992). Cloning and expression of chitinase genes was also successfully done (Duffner et al., 1996; Chernin et al., 1997). In cases where a relevant gene of a thermostable enzyme has been identified and once the gene has been transferred into a mesophilic host the resultant enzyme production can be facilitated through appropriate incubation processes.

13. Conclusion

Recent investigations have demonstrated that extremophilic archaea, bacteria and fungi have colonized environments that were believed to be inhospitable for survival. Their true diversity in fact, is not yet been fully explored. The thermostable enzymes isolated from these organisms have just started providing conversions under conditions that are appropriate for industrial applications. The conditions required by these thermostable enzymes which bring about specific reactions not possible by chemical catalysts are still mild and environmentally benign, as compared to the temperatures and pressures required for chemical conversions. Thus, with the availability of thermostable enzymes a number of new applications in the future are likely. Although, believed to provide tremendous economical benefits, production of the enzymes to the level required by the industries has remained a challenge.
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