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Glycosyl Hydrolases from Extremophiles

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1. INTRODUCTION

A feature of extremophilic organisms is their ability to survive and grow in an environment that can be considered extreme from the anthropocentric point of view. The survival mechanisms of these organisms are partially due to the proper adaptation of the individual components by the organisms. Extremophilic microorganisms are adapted to live at high temperatures (as in volcanic springs), at low temperatures (as in the cold polar seas), at high pressure in the deep sea, at extreme pH values (pH 0–1 or pH 10–11), or at very high salt concentrations. Presently, more than 60 species of hyperthermophilic bacteria and archaea have been isolated and characterized. They consist of anaerobic and aerobic chemolithototrophs and heterotrophs. Various heterotrophs are able to utilize various biopolymers such as starch, hemicellulose, chitin, proteins, and peptides.

Several hyperthermophilic archaea have been isolated with growth temperatures (103–110°C) and have been classified as members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus,* and *Methanopyrus* (Stetter, 1996). Within the bacteria, *Aquifex pyrophilus* and *Thermotoga maritima* exhibit the highest growth temperatures.

Metabolic processes and specific biological functions of these microorgan-

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isms are facilitated by enzymes and proteins that function under extreme conditions. The enzymes that have been recently isolated from these exotic microorganisms show unique features. Generally, the enzymes are: (1) extremely thermostable; and (2) resist chemical denaturants such as detergents, chaotropic agents, organic solvents, and pH extremes (Friedrich and Antranikian 1996; Jørgensen *et al.,* 1997; Leuschner and Antranikian 1995; Rüdiger *et al.,* 1995). Therefore, these enzymes offer an exceptional opportunity to be used as models, where it is possible to study their features in terms of stability, specificity, and enzymatic mechanisms, in order to learn how to design and construct proteins with properties that are of particular interest for industrial applications.

Biotechnological processes at elevated temperatures have many advantages. The increase of temperature has a significant influence on the bioavailability and solubility of organic compounds. The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Consequently, higher reaction rates, due to smaller boundary layers, are expected (Becker *et al.,* 1997; Krahe *et al.,* 1996). Of special interest are reactions involving less soluble hydrophobic substrates such as polyaromatic, aliphatic hydrocarbons, and fats and polymeric compounds such as starch, cellulose, hemicellulose, chitin, and proteins. The bioavailability of hardy biodegradable and insoluble environmental pollutants also can be improved dramatically at elevated temperatures allowing efficient bioremediation. Furthermore, by performing biological processes at temperatures above 60°C, the risk of contamination is reduced and controlled processes under strict conditions can be carried out.

The determination of the mechanism of enzyme adaptation to extreme conditions is strategic, since extremophiles are unique models for investigations on how biomolecules are stabilized and constitute a valuable resource for the exploitation of novel biotechnological processes. The number of genes from thermophiles that were cloned and expressed in mesophiles is sharply increasing (Ciaramella *et al.,* 1995). The proteins produced in mesophilic hosts are able to maintain their thermostability, are correctly folded at low temperature, are not hydrolyzed by host proteases, and can be purified by using thermal denaturation of the mesophilic host proteins. The obtained degree of enzyme purity is generally adequate for most industrial applications.

In this chapter we will briefly discuss the enzymatic action and properties of starch, pullulan, cellulose, xylan, pectin, and chitin hydrolases and focus only on those enzymes that have been isolated and characterized from extreme thermophilic (optimal growth 70–80°C) and hyperthermophilic (optimal growth 85–100°C) archaea and bacteria. We also are going to discuss their biotechnological significance. Some of these aspects already have been presented in recent reviews (Antranikian, 1992; Ladenstein and Antranikian, 1998; Moracci *et al.,* 1998; Müller *et al.,* 1998; Rüdiger *et al.,* 1994; Sunna and Antranikian, 1997).

2. STARCH-DEGRADING ENZYMES FROM EXTREMOPHILIC MICROORGANISMS

Starch is a widespread natural nutrient storage polysaccharide consisting of glucose residues. In plant cells or seeds, starch is usually deposited in the form of large granules in the cytoplasm. Starch occurs in two forms: (1) α-amylose (15–25% of starch), which is a linear polymer of α-1,4-linked glucopyranose residues, and (2) amylopectin (75–85% of starch), which is highly branched containing α-1,6-glycosidic linkages at branching points (Fig. 1). α-Amylose chains are polydisperse and vary in molecular weights from a few to thousands. They are not soluble in water, but form hydrated micelles. In amylopectin the average length of the branches is from 24 to 30 glucose residues, depending on the species, and in solution, amylopectin yields colloidal or micellar forms. The molecular weight of amylopectin may be as high as 100 million.

2.1. Starch-Degrading Enzymes

In order to be able to utilize starch cells must employ a number of enzymes for its degradation and bioconversion to smaller sugars and oligosaccharides, such as glucose and maltose (Antranikian, 1992, Rüdiger *et al.*, 1994). Starch-hydrolyzing enzymes can be distinguished as endo-acting or endo-hydrolases and exo-acting enzymes or exo-hydrolases, as summarized in Fig. 2.

Endo-acting starch-degrading enzyme is α-amylase (E.C.3.2.1.1) or α-1,4 glucan glucanohydrolase, which hydrolyzes α-1,4 glucosidic linkages in the interior of the starch polymer or oligosaccharides in a random manner. The action of this enzyme leads to the formation of linear and branched oligosaccharides and the sugar-reducing groups are liberated in the α-anomeric configuration.

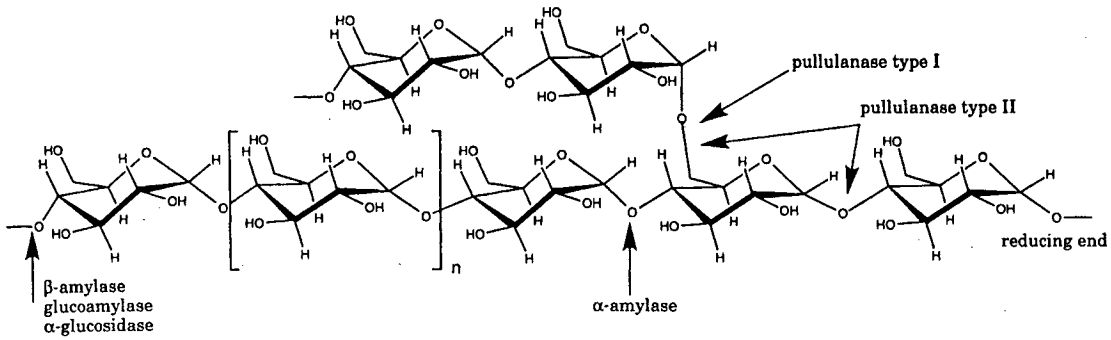

Figure 1. Chemical structure of starch and the enzymes involved in its degradation.

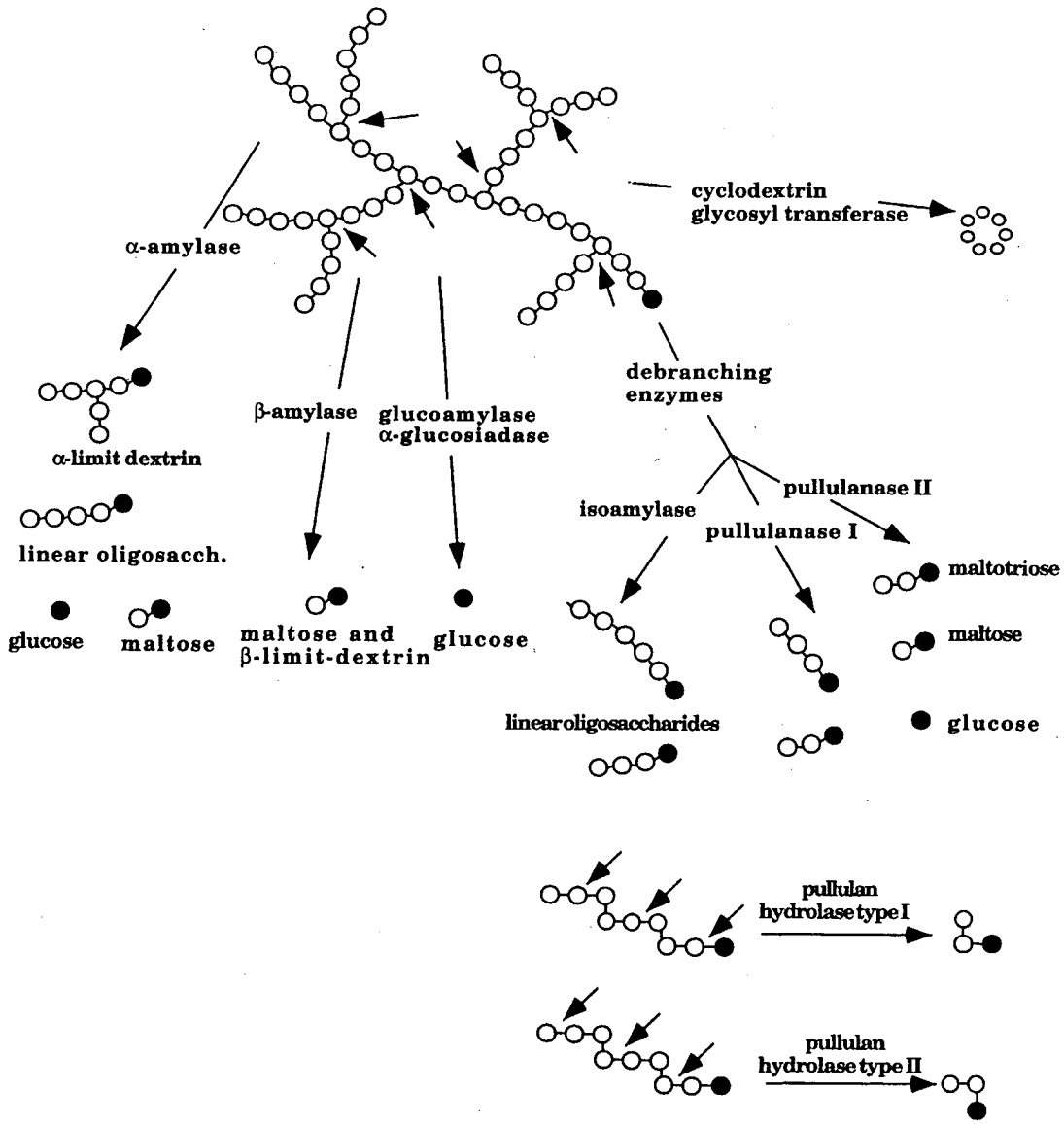

Figure 2. Schematic presentation of the action of starch hydrolyzing enzymes and their degradation products.

α-Amylases belong to the families, numbers 13 and 57 of the glycosyl hydrolases superfamily (Henrissat, 1991). Family 13 has around 150 members from eukaryotae and prokaryotae, unlike family 57, which has only three members from prokaryotae and archaea. In the structural level there are several X-ray structures determined and the mode of the enzymatic action is well studied.

Exo-acting starch hydrolases are β-amylases, glucoamylases, α-glucosidases, and isoamylase. These enzymes attack the substrate from the nonreducing end, producing small and well-defined oligosaccharides.

β-Amylase (E.C.3.2.1.2) or α-1,4-D-glucan maltohydrolase or saccharogen amylase hydrolyzes α-1,4 glucosidic linkages to remove successive maltose units from the nonreducing ends of the starch chains producing β-maltose by an inver-

sion of the anomeric configuration of the maltose (Fig.1). β-Amylase belongs to family 14 of the glycosyl hydrolases, having 11 members from eukaryotae and prokaryotae.

Glucoamylases (E.C.3.2.1.3) hydrolyze terminal α-1,4-linked-D-glucose residues successively from nonreducing ends of the chains with release of β-D-glucose (Fig. 2). The enzyme has several names: α-1,4-D-glucan glucohydrolase, amyloglucosidase, and γ-amylase and it is a typical fungal enzyme. Most forms of the enzyme can hydrolyze α-1,6-D-glucosidic bonds when the next bond in sequence is α-1,4. However, some preparations of this enzyme hydrolyze α-1,6- and α-1,3-D-glucosidic bonds in other polysaccharides. In contrast to α-glycosidase, glucoamylases preferentially degrade polysaccharides with high molecular weights.

α-Glucosidases (E.C.3.2.1.20) or α-D-glucoside glucohydrolase attacks the α-1,4 or α-1,6 linkages from the nonreducing end in short saccharides that are produced by the action of other amylolytic enzymes (Fig. 2). Unlike glucoamylase, α-glucosidase liberates glucose with an α-anomeric configuration. α-Glucosidases are members of the family 15 and the very diverse family 31 of the glycosyl hydrolases (Henrissat, 1991). Isoamylase (E.C.3.2.1.68), or glycogen-6-glucanohydrolase, is a debranching enzyme specific for α-1,6 linkages in polysaccharides such as amylopectin, glycogen, and β-limit dextrins, but it is unable to hydrolyze the α-1,6 linkages in pullulan; therefore, it has limited action on α-limit dextrins.

2.2. Pullulan-Degrading Enzymes

Pullulan is a linear α-glucan consisting of maltotriose units joined by α-1,6 glycosidic linkages and it is produced by *Aureobasidium pullulan* with a chain length of 480 maltotriose units (Fig. 3). Enzymes capable of hydrolyzing α-1,6 glycosidic bonds in pullulan and amylopectin are defined as debranching enzymes or pullulanases. On the basis of substrate specificity, pullulanases have been classified into pullulanases types I and type II. Pullulanase type I (E.C.3.2.1.41) specifically hydrolyzes the α-1,6-linkages in pullulan and in the branched oligosaccharides, and its degradation product is α-limit dextrin (Fig. 2). These type I enzymes are unable to attack α-1,4-linkages in α-glucans and belong to family 13 of the glycosyl hydrolases. Pullulanase type II or amylopullulanases attack α-1,6-glycosidic linkages in pullulan and α-1,4-linkages in other oligosaccharides (Fig. 2). This enzyme has a relatively multiple specificity and is able to fully convert polysaccharides to small sugars in the absence of other enzymes such as α-amylases or β-amylases.

In contrast to the previously described pullulanases, pullulan hydrolases type I and type II are unable to hydrolyze α-1,6-glycosidic linkages in branched substrates or in pullulan. Because they can hydrolyze the α-1,4-linkages in pullulan,

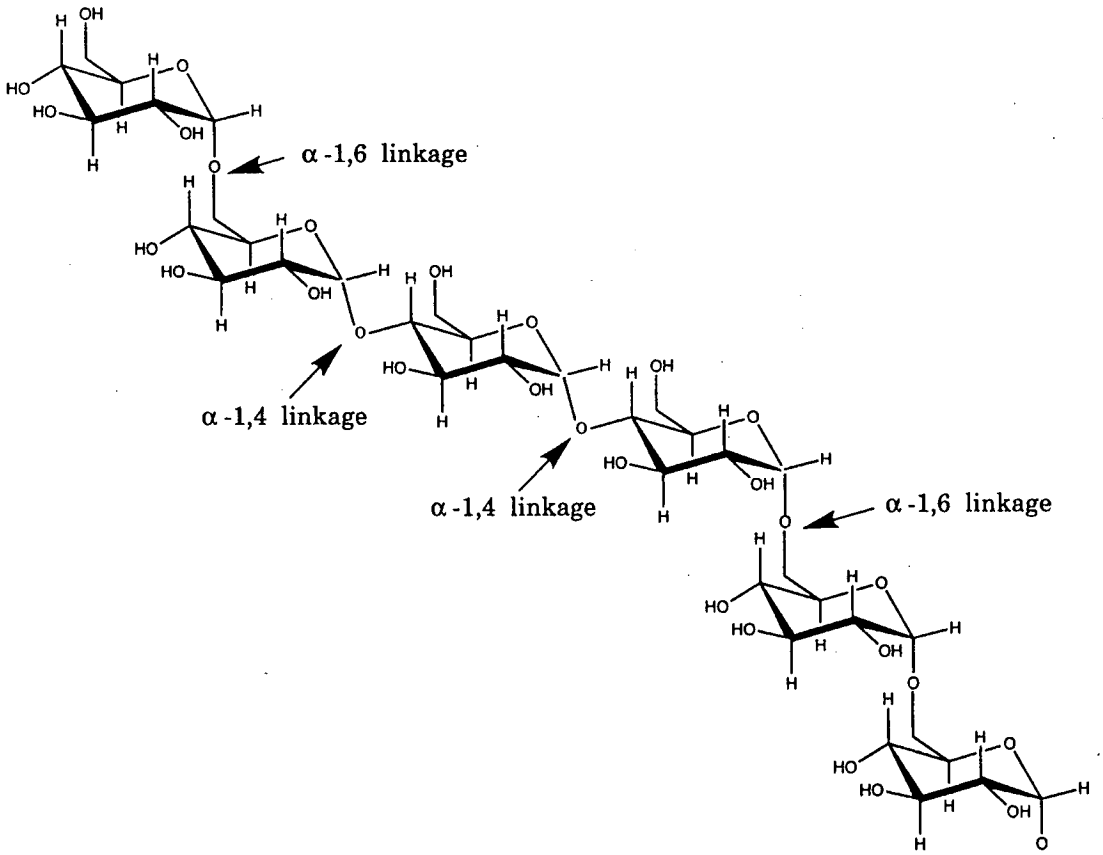

Figure 3. Chemical structure of pullulan.

they were incorrectly named pullulanases. They can attack α-1,4-glycosidic linkages but not α-1,6-linkages in pullulan. Pullan hydrolase type I or neopullulanase (E.C.3.2.1.135) hydrolyzes pullulan to panose (α-6-D-glucosylmaltose). Pullulan hydrolase type II or isopullulanase (E.C.3.2.1.57) hydrolyzes pullulan to isopanose (α-6-maltosylglucose) (Fig. 2).

Finally, cyclodextrin glycotransferase (E.C.2.4.1.19) or α-1,4-D-glucan α-4-D-(α-1,4-D-glucano)-transferase is an enzyme that has been found only in bacteria. This enzyme produces a series of nonreducing cyclic dextrins from starch, amylose, and other polysaccharides (Fig. 2). α-, β-, and γ-Cyclodextrins are rings formed by 6, 7, and 8 glucose units that are linked by α-1,4-bonds, respectively.

2.3. Ability of Hyperthermophilic Microorganisms to Produce Starch-Degrading Enzymes

Many hyperthermophilic organisms are known to utilize natural carbohydrates polymers for nutritional purposes. Therefore, it is apparent that these

organisms can facilitate the enzymatic degradation of carbohydrates, producing enzymes that are capable of hydrolyzing them very effectively at elevated temperatures.

Hyperhermophilic bacteria and archaea able to grow on starch at temperatures over 70°C have been identified and the corresponding starch-degrading enzymes have been isolated and characterized. In several cases genes encoding these enymes have been isolated, cloned, and overexpressed in heterologous hosts.

2.3.1. α-AMYLASES

Extremely thermostable α-amylases have been characterized from *Pyrococcus woesei, Pyrococcus furiosus* (Koch *et al.,* 1991), and *Thermococcus profundus* (Chung *et al.,* 1995; Lee *et al.,* 1996), with optimum activities at temperatures of 100°C, 100°C, and 80°C, respectively (Table I). In the hyperthermophilic archaea of the genera *Sulfolobus, Thermophilum, Desulfurococcus, Thermococcus,* and *Staphylothermus* (Bragger *et al.,* 1989; Canganella *et al.,* 1994) amylolytic activities have been detected. Due to the high activity of these hydrolases, the cells do not produce high amounts of protein, and therefore it is essential to clone the corresponding genes and express them in heterologous hosts.

The gene encoding an extracellular α-amylase from *P. furiousus* recently has been cloned and the recombinant enzyme expressed in *Escherichia coli* and *Bacillus subtilis* (Jørgensen *et al.,* 1997). This enzyme is an interesting one be-

Table I
α-Amylases Produced by Extremely Thermophilic and Hyperthermophilic Archaea and Bacteria

Organism	Growth Temp. (°C)	Enzyme Optimal Temp. (°C)	Enzyme Optimal pH	Enzyme MW (kDa)
Desulfurococcus mucosus	85	100	5.5	
Pyrococcus furiosus	100	100	6.5–7.5	129
		100	6.5–7.5	129
Pyrococcus sp. KOD1		90	6.5	68
Pyrococcus woesei	100	100	5.5	—
Pyrodictium abyssi	98	100	5.0	—
Staphylothermus marinus	90	100	5.0	240
Sulfolobus solfatatrcus	88			
Thermococcus celef	85	90	5.5	
Thermococcus profundus DT5432	80	80	5.5	42
Thermococcus profundus	80	80	4.0–5.0	42
Thermococcus aggregans	85	100	5.5	
Dictyoglomus thermophilum Rt46B.1		90	5.5	75
Thermotoga maritima MSB8	90	85–90	7.0	61

cause besides its high thermostability (thermal activity even at 130°C) it does not require metal ions either for stability or for optimal activity. Pyrococcal α-amylase also has a unique product pattern and substrate specificity that makes it a unique enzyme and an interesting candidate for industrial applications. The intracellular α-amylase gene from *P. furiousus* also has been cloned and sequenced (Laderman *et al.,* 1993).

α-Amylases with lower thermostability and thermoactivity have been isolated and characterized from the archeon *Pyrococcus* sp. KOD1, and the bacterium *Thermotoga maritima.* The genes encoding these enzymes have been well-expressed in *E. coli* (Liebl *et al.,* 1997; Tachibana *et al.,* 1996). *T. maritima* amylase requires the presence of Ca^{2+} for its enzymatic activity (Liebl *et al.,* 1997), similar to amylase from *Bacillus licheniformis.*

Recent investigations have shown that the hyperthermophilic archaeon *Pyrodictium abyssi* can grow anaerobically on various polymeric substrates and it secretes a heat-stable amylase that is active even above 100°C and in a wide pH range (Table 1).

2.4. Pullulan-Degrading Enzymes from Hyperthermophilic Organisms

Thermostable and thermoactive pullulanases from extremophilic microorganisms have been detected in *Thermococcus celer, Desulfurococcus mucosus, Staphylothermus marinus,* and in the novel archaeal strain *Thermococcus aggregans* (Table II). These pullulanases show temperature optima between 90°C and 105°C and high thermostability in the absence of substrate and calcium ions (Canganella *et al.,* 1994). Most of the thermophilic pullulanases studied to date belong to type II and have been purified from *P. woesei, P. furiosus,* and *Thermococcus litoralis* (Brown and Kelly, 1993) and ES4 (Schuliger *et al.,* 1993). The extreme thermostability of these enzymes, coupled with their ability to attack both α-1,6 and α-1,4 glycosidic linkages, may improve the industrial starch hydrolysis process.

The enzyme from *P. woesei* has been overexpressed in *E. coli.* The recombinant purified enzyme has a temperature optimum at 100°C and it is extremely thermostable, with a half life of 7 min at 110°C (Rüdiger *et al.,* 1995). The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 produces a thermostable pullulanase of type I when grown on starch. This pullulanase is optimally active at 75°C and pH 5.5, and is thermostable up to 90°C and does not require Ca^{2+} ions either for activity or stability.

The first starch-debranching enzyme from an anaerobe was identified in the thermophilic bacterium *Fervidobacterium pennavorans* Ven5 (Koch *et al.,* 1997), and the corresponding gene was cloned and expressed in *E. coli* (unpublished results). In contrast to the pullulanase from *P. woesei,* the enzyme from *F. penavo-*

Table II

Pullulanases (Type I and II) from Extremely Thermophilic and Hyperthermophilic Archaea and Bacteria

Enzymes	Organism	Growth Temp. (°C)	Enzyme Optimal Temp. (°C)	Enzyme Optimal pH	Enzyme MW (kDa)
Pullulanase type I	*Fervidobacterium pennavorans Ven5*	75	80	6.0	190 (93 sub)
	Thermotoga maritima MSB8	90	90	6.0	93
	Thermus caldophilus GK24	75	75	5.5	65
Pullulanase type II	*Desulfurococcus*	—	100	5.0	—
	Pyrococcus woesei	100	100	6.0	90
	Pyrodictium abyssi	98	100	9.0	—
	Thermococcus celer	85	90	5.5	—
	Thermococcus litoralis	90	98	5.5	119
	Thermococcus aggregans	85	100	6.5	—

rans Ven5 attacks exclusively the α-1,6-glycosidic linkages in polysaccharides. This is the only thermostable debranching enzyme known to date that hydrolyzes amylopectin leading to the formation of long linear chain polysaccharides, which are the ideal substrates for the enzymatic action of glucoamylase.

2.5. Cyclodextrin Glycosyl Transferases

Cyclodextrin glycosyl transferase (CGTase) attacks α-1,4-linkages in polysaccharides in a random fashion and acts on starch by an intramolecular transglycosylation reaction. The nonreducing cyclization products of this reaction are α-, β-, or γ-cyclodextrin consisting of 6, 7, or 8 glucose molecules, respectively. Thermostable CGTases already have been found in *Thermoanaerobacter* and *Thermoanaerobacterium thermosulfurogenes* (Petersen *et al.*, 1995; Wind *et al.*, 1995). Recently a heat- and alkali-stable CGTase (65°C, pH 4–10) was purified from a newly identified strain that was isolated from Lake Bogoria, Kenya (Prowe *et al.*, 1996).

2.6. Biotechnological Relevance

Industrial production of fructose from starch, consists of three steps: liquefaction, saccharification, and isomerization. This multistage process (step 1: pH 6.5, 98°C; step 2: pH 4.5, 60°C: step 3; pH 8.0, 65°C) leads to the conversion of starch to fructose with concurrent formation of high concentrations of salts that have to be removed by ion exchangers. The application of thermostable enzymes such as amylases, glucoamylases, pullulanases, and glucose isomerases that are active and stable above 100°C and at acidic pH values can simplify this complicated process. Therefore, strong efforts have been invested in the isolation of thermostable and thermoactive amylolytic enzymes from hyperthermophiles, since they could improve the starch conversion process and lower the cost of sugar syrup production.

The predominant biotechnological application of CGTase occurs in the industrial production of cyclodextrins. Due to the ability of cyclodextrins to form inclusion complexes with a variety of organic molecules, they improve the solubility of hydrophobic compounds in aqueous solutions. Cyclodextrin production occurs in a multistage process in which in the first step starch is liquefied by a heat-stable amylase and in the second step the cyclization reaction with the CGTase from *Bacillus* sp. takes places. Due to the low stability of this enzyme, the process must run at lower temperatures. The finding of heat-stable and more specific

CGTases from extremophiles will solve this problem. The application of heat-stable CGTase in jet cooking, where temperatures up to 105°C are used, will allow the liquefaction and cyclization to take place in one step.

3. CELLULOSE-DEGRADING ENZYMES FROM EXTREMOPHILIC MICROORGANISMS

Cellulose is the most abundant organic biopolymer in nature since it is the structural polysaccharide of the cell wall in the plant kingdom. It consists of glucose units linked by β-1,4-glycosidic bonds in a polymerization grade up to 15,000 glucose units in an absolutely linear mode. The minimal molecular weight of cellulose from different sources has been estimated to vary from about 50,000 to 2,500,000 in different species, which is equivalent to 300 to 15,000 glucose residues (Fig. 4). Although cellulose has a high affinity for water, it is completely insoluble in it.

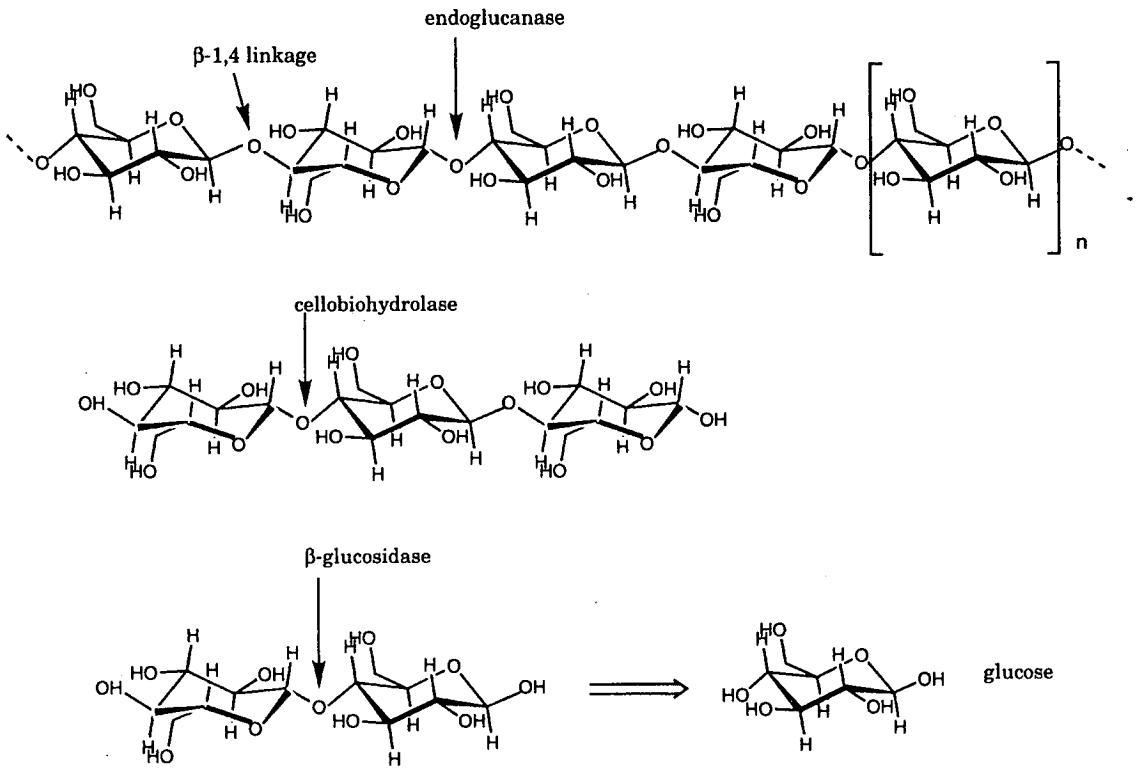

Figure 4. Chemical structure of cellulose and the action of the enzymes involved in its degradation.

3.1. Cellulose-Degrading Enzymes

Cellulose can be hydrolyzed to its monomeric glucose units by the synergistic action of at least three different enzymes: endoglucanases, exoglucanase (cellobiohydrolase), and β-glucosidase (Fig. 4). Cellulose hydrolyzing enzymes are widespread in fungi and bacteria, but until now have not been found in hyperthermophilic archaea.

3.1.1. CELLULASES

Cellulase (EC 3.2.1.4) or β-1,4-D-glucan glucanohydrolase or endo-β-1,4-glucanase or carboxymethyl cellulase, is an endoglucanase that hydrolyzes cellulose in a random manner, producing oligosaccharides, cellobiose, and glucose. The enzyme catalyzes the endohydrolysis of β-1,4-D-glucosidic linkages in cellulose, but it also will hydrolyze 1,4-linkages in β-D-glucans containing some 1,3-linkages. Cellulases belong to the family 5 of the glycosyl hydrolases (Henrissat, 1991).

3.1.2. CELLOBIOHYDROLASE

Exoglucanase (or β-1,4-cellobiosidase) or exocellobiohydrolase (or β-1,4-cellobiohydrolase) (EC 3.2.1.91) hydrolyses β-1,4 D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the nonreducing end of the chain; they belong to family 6 of the glycosyl hydrolases.

3.1.3. β-GLUCOSIDASE

β-Glucosidase (EC 3.2.1.21) or gentobiase, cellobiase, or amygdalase catalyze the hydrolysis of terminal, nonreducing β-D-glucose residues with release of β-D-glucose. The enzymes belong to family 3 of the glycosyl hydrolases and have a broad specificity for β-D-glucosides. They are able to hydrolyze β-D-galactosides, α-L-arabinosides, β-D-xylosides, and β-D-fucosides.

3.2. Distribution of Cellulose-Degrading Enzymes in Hyperthermophilic Microorganisms

Several cellulose-degrading enzymes from various thermophilic organisms have been detected, purified, characterized, and cloned. Table III summarizes the properties of these enzymes. A thermostable cellulase from *Thermotoga maritima*

Table III
Cellulose-Hydrolyzing Activities from Various Thermophic and Thermoacidophilic Microorganisms

Organism	Growth Temp. (°C)	Enzyme Optimal Temp. (°C)	Enzyme Optimal pH	Enzyme MW (kDa)
Thermotoga maritima MSB8	80	95	6.0–7.0	29
Thermotoga neapolitana	80	110	—	
Thermotoga sp FjSS3-B1	80	115	6.8–7.8	36
Pyrococcus furiosus	100		6.8–7.8	224(56 sub)
Sulfolobus sulfataricus MT4	88		6.8–7.8	224(56 sub)
Sulfolobus shibatae	95		6.8–7.8	224(56 sub)
Sulfolobus acidocaldaricus	88		6.8–7.8	224(56 sub)
Caldocellum saccharolyticum	—		6.8–7.8	200 (1751 aa)
Anaerocellum thermophilum	85	85–95	5.0–6.0	230

MSB8 has been characterized (Bronnenmeier *et al.,* 1995). The enzyme is rather small, with a molecular weight of 27 kDa, and it is optimally active at 95°C and pH between 6.0 and 7.0. Another marine eubacterium, *Thermotoga neapolitana,* on cultivation in the presence of cellobiose produces two endoglucanases, the endoglucanases A and B (Bok *et al.,* 1994). Purified endoglucanase B shows a remarkable thermostability between 100 and 106°C, and both enzymes show high specificity for carboxy-methyl (CM)-cellulose (CMC).

Cellulase and hemicellulase genes have been found clustered together on the genome of the extremely thermophilic anaerobic bacterium *Caldocellum saccharolyticum,* which is capable of growing on cellulose and hemicellulose as sole carbon sources (Teo *et al.* , 1995). The gene for one of the cellulases (*Cel*A) was isolated and consists of 1751 amino acids. This is the largest known cellulase gene (Teo *et al.,* 1995).

A large cellulolytic enzyme (*Cel* A) with the ability to hydrolyze microcrystalline cellulose was isolated from the extremely thermophilic, cellulolytic bacterium *Anaerocellum thermophilum* (Zverlov *et al.,* 1998). The enzyme has an apparent molecular mass of 230 kDa. It exhibits significant activity toward Avicel and is most active toward soluble substrates such as CMC and β-glucan. Maximal activity was observed between pH values of 5 and 6 and temperatures of 95°C (CMC) and 85°C (Avicelase).

A thermostable cellobiohydrolase was also reported from *Thermotoga maritima* MSB8 (Bronnenmeier *et al.,* 1995). The enzyme has a molecular weight of 29 kDa, an optimal activity at 95°C at pH 6.0–7.5, and the half-live of 2 hr at 95°C in the absence of substrate. Cellobiohydrolase hydrolyzes Avicel with main product cellobiose and cellotriose, as well as CMC and β-glucan.

Thermotoga sp. FjSS3-B1 (Ruttersmith and Daniel, 1991) produces cellobiase. The enzyme is highly thermostable and shows maximal activity at 115°C, pH:

6.8–7.8. The thermostability of this enzyme is salt dependent; at 0.5 M NaCl the life time is almost doubled, from 70 min to 130 min at 108°C and from 7 min to 15 min at 113°C. This cellobiase is active against amorphous cellulose and CMC.

β-Glucosidases have been detected in *Sulfolobus sulfataricus* MT4, *Sulfolobus acidocaltaricus,* and *Sulfolobus shibatae* (Grogan, 1991), as well as in *Pyrococcus furiosus* (Kengen *et al.,* 1993). Among these enzymes the β-glucosidase from *Sulfolobus sulfataricus* MT4 has been purified and characterized (Pisani *et al.,* 1990; Nucci *et al.,* 1993). This β-glucosidase is a homotetramer with 56 kDa for each subunit; it very resistant to various denaturants with activity up to 85°C (Pisani *et al.,* 1990). The gene for this β-glucosidase has been cloned and overexpressed in *E. coli* (Cubellis *et al.,* 1990; Moracci *et al.* 1992; Prisco *et al.,* 1994).

Pyrococcus furiosus β-glucosidase is expressed in high levels in cells grown on cellobiose (Kengen *et al.,* 1993). β-Glucosidase is a homotetramer with molecular weight of 58 kDa for each subunit. It is very stable and shows optimal activity at 102°C to 105°C, while the half life is 3.5 days at 100°C and 13 hr at 110°C. Previously, the gene of *P. furiosus* β-glucosidase had been cloned and expressed in *E. coli* (Voorhorst *et al.,* 1995)

3.3. Biotechnological Relevance

Cellulases have found various applications in several biotechnological applications. The most effective commercial cellulase is the one produced by *Trichoderma* sp. Other cellulases of commercial interest are obtained from strains of *Aspergillus, Penicillium,* and *Basidiomycetes.* Fungal cellulases are used in alcohol production. Cellulolytic enzymes also can be used to improve juice yields and effective color extractions of juices. The presence of cellulases in detergents cause color brightening and softening and improve particulate soil removal. A novel application of cellulases in textile industry is the use of Denimax (Novo Nordisk) for the "biostoning" of jeans instead of the use of abrasive stones in stonewashed jeans. Also, other significant applications of cellulases include the pretreatment of cellulosic biomass and forage crops to improve nutritional quality and digestibility, enzymatic saccharification of agricultural and industrial wastes, and production of fine chemicals.

4. XYLAN-DEGRADING ENZYMES FROM EXTREMOPHILES

Xylan is a heterogeneous molecule that comprises the major polymeric compound of hemicellulose. Hemicellulose is a fraction of plant cell walls; is associated with cellulose, lignin, and other polysaccharides; and functions as the major

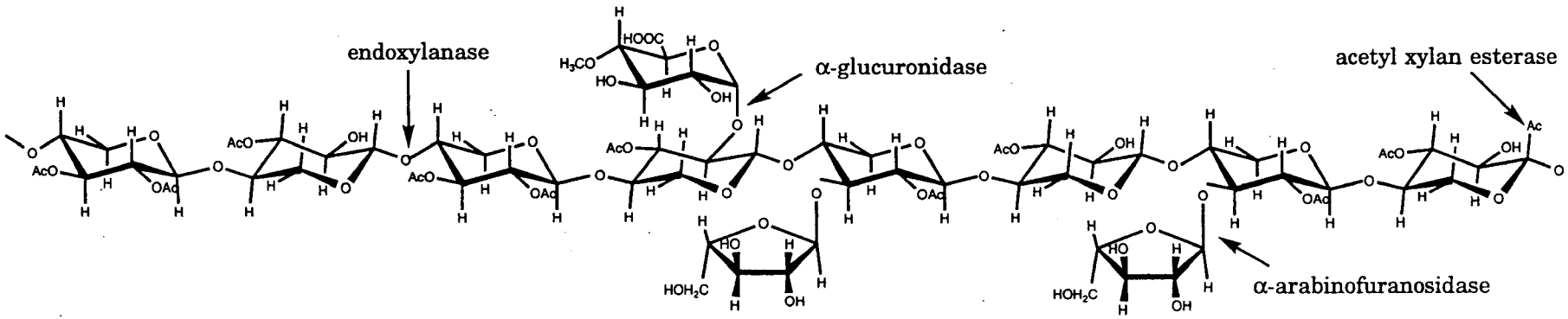

Figure 5. Chemical structure of substituted xylan and the enzymes involved in its degradation.

reservoir of fixed carbon in nature. The main chain of the xylan heteropolymer is composed of xylose residues linked by β-1,4-glycosidic bonds. Approximately half the xylose residues have substitutions at O-2 or O-3 positions of acetyl, arabinosyl and glucuronosyl groups, as shown in Fig. 5. Due to the heterogeneity of xylan, its degradation requires the synergistic action of a xylanolytic enzyme system, which consists of five distinct activities with endoxylanase as the major enzyme. Xylanolytic enzymes are widespread in the terrestrial as well as the marine environment. The concerted action of these enzymes degrades xylan to its constituent sugars; for a detailed description, see the reviews of Sunna *et al.* (1996b) and Sunna and Antranikian (1997).

4.1. Xylan-Degrading Enzymes

The endo-β-1,4-xylanase (E.C.3.2.1.8) or β-1,4-xylan xylanohydrolase hydrolyzes β-1,4-xylosidic linkages in xylans, whereas β-1,4-xylosidase, β-xylosidase, β-1,4-xylan xylohydrolase, xylobiase, or exo-β-1,4-xylosidase (E.C.3.2.1.37) hydrolyzes β-1,4-xylans and xylobiose by removing the successive xylose residues from the nonreducing termini. α-Arabinofuranosidase or arabinosidase (E.C.3.2.1.55) hydrolyzes the terminal nonreducing α-L-arabinofuranoside residues in α-L-arabinosides. The enzyme also acts on α-L-arabinofuranosides, α-L-arabinans containing either (1,3) or (1,5)-linkages, arabinoxylans, and arabinogalactans. Glucuronoarabinoxylan endo-β-1,4-xylanase, feraxan endoxylanase, glucuronoarabinoxylan β-1,4-xylanohydrolase (E.C.3.2.1.136) attacks β-1,4-xylosyl links in some glucuronoarabinoxylans. This enzyme also shows high activity toward feruloylated arabinoxylans from cereal plant cell walls. Acetyl xylan esterase (E.C.3.1.1.6) removes acetyl groups from xylan. Xylanases from prokaryotae and eukaryotae comprise families 10 and 11 of the glycosyl hydrolases.

4.2. Formation of Xylan-Degrading Enzymes by Hyperthermophilic Microorganisms

So far, only a few extreme thermophilic microorganisms are able to grow on xylan and secrete thermoactive xylanolytic enzymes (Table IV). The members of the order of *Thermotogales* and the species *Dictyoglomus thermophilum* have been described to produce xylanases that are active and stable at high temperature (Sunna *et al.,* 1996a; Sunna and Antranikian, 1996; Gibbs *et al.,* 1995).

The most thermostable endoxylanases described so far are those derived from *Thermotoga* sp. strain FjSS3-B.1 (Simpson *et al.,* 1991), *Thermotoga maritima* (Winterhalter and Liebl, 1995), *Thermotoga neapolitana* (Bok *et al.,* 1994), and

Table IV
Production of Heat-Stable Xylanases by Extreme Thermophilic and Hyperthermophilic Archaea and Bacteria

Organism	Growth Temp. (°C)	Enzyme Optimal Temp. (°C)	Enzyme Optimal pH	Enzyme MW (kDa)
Pyrodictium abyssi	98	110	5.5	
Dictyoglomus thermophilum Rt46B.1	73	85	6.5	31 (recombinant)
Thermotoga maritima MSB8	80	92	6.2	120 Xyn A
		105	5.4	40 XynB
Thermotoga sp strain FjSS3-B.1	80	105	5.3	31 (wild type)
		85	6.3	40 (recombinant)
Thermotoga neapolitana	80	85	5.5	37 (wild type)
		102	5.5–6.0	119 (recombinant)
Thermotoga thermarum	77	80	6.0	105/150 Endoxyl 1
		90–100	7.0	35 Endoxyl 2
Caldocellum saccharolyticum	—	70–75	5.5–6.0	40 XynA

Thermotoga thermarum (Sunna *et al.,* 1996b). These enzymes, active between 80 and 105°C, are mainly cell associated and most probably localized within the toga (Ruttersmith *et al.,* 1992; Schumann *et al.,* 1991; Sunna *et al.,* 1996a; Winterhalter and Liebl, 1995).

Several genes encoding xylanases have been cloned and sequenced. The gene from *T. maritima,* encoding a thermostable xylanase, has been cloned and expressed in *E. coli.* Comparison between the *T. maritima* recombinant xylanase and the commerially available xylanase, Pulpenzyme TM (Yang and Eriksson, 1992), indicates that the thermostable enzyme has properties that make it an attractive candidate for application in pulp and paper industry (Chen *et al.,* 1997) .

Archaea growing at temperatures above 90°C seem to be unable to utilize xylan as a carbon source. Recently, however, it was shown that the hyperthermophilic archaeon *Pyrodictium abyssi* is able to produce a unique thermostable endoxylanase upon growth in the presence of xylan, xylose, or arabinose (Andrade *et al.,* 1996). This extracellular enzyme, which is inducible, displays optimal activity at 110°C and pH 5.5. Several years ago, Luthi *et al.* (1990) reported the isolation of a clone from the extremely thermophilic anaerobe *Caldocellum saccharolyticum.* Five open reading frames were found in this clone that appear to code for a xylanase (*Xyn* A; 40.4 kDa) and β-xylosidase (*Xyn* B; 56.3 kDa). The *xyn*A gene product shows significant homology to the xylanases from the alkalophilic *Bacillus* sp. strain C125 and *Clostridium thermocellum.* The enzymes of the *Caldocellum saccharolyticum,* however, have not yet been biochemically characterized.

4.3. Biotechnological Relevance

Xylanases have a wide range of potential biotechnological applications. They already are produced on industrial scale and are used as food additives in poultry, for increasing feed efficiency diets (Annison, 1992; Classen, 1996), and in wheat flour for improving dough handling and the quality of baked products (Maat *et al.*, 1992).

In recent years the major interest in thermostable xylanases is in enzyme-aided bleaching of paper (Viikari *et al.*, 1994). More than 2 million tons of chlorine and chlorine derivatives are used annually in the United States for pulp bleaching. The chlorinated lignin derivatives generated by this process constitute a major enviromental problem caused by the pulp and paper industry (McDonough, 1992). Recent investigations have demonstrated the feasibility of enzymatic treatments as alternatives to chlorine bleaching for removal of residual lignin from pulp (Viikari *et al.*, 1994). Treatment of kraft pulp with xylanase leads to a release of xylan and residual lignin without undue loss of other pulp components. Xylanase treatment opens up the cell wall structure, thereby facilitating lignin removal in subsequent bleaching stages. In addition, fragmentation of the xylan polymer allows free diffusion of those portions of the residual lignin that are covalently attached to xylan.

Candidate xylanases for this important, potential market would have to satisfy several criteria: (1) they must lack cellulolytic activity, to avoid hydrolysis of the cellulose fibers; (2) their molecular weight should be low enough to facilitate their diffusion in the pulp fibers; (3) they must be stable and active at high temperature and at alkaline pH; and (4) one must be able to obtain high yields of enzyme at very low cost. All the xylanases currently available from commercial suppliers can only partially fulfill the criteria. Xylanases from moderate thermophilic microorganisms are rapidly denaturated at temperatures above 70°C. Several nonchlorine bleaching stages used in commercial operations are performed well above this temperature; consequently, pulp must be cooled before treatment with the available enzymes and reheated for subsequent processing steps. This adds substantially to the cost of bleaching processes. Thus, xylanases active and stable at temperatures of 90°C or more have enormous potential for use in the pulp and paper industry (Chen *et al.*, 1997).

5. PECTIN-DEGRADING ENZYMES FROM EXTREMOPHILIC ORGANISMS

Pectin is a branched heteropolysaccharide consisting of a main chain of α-1,4-D-polygalacturonate, which is partially methyl esterified (Fig. 6). Along the

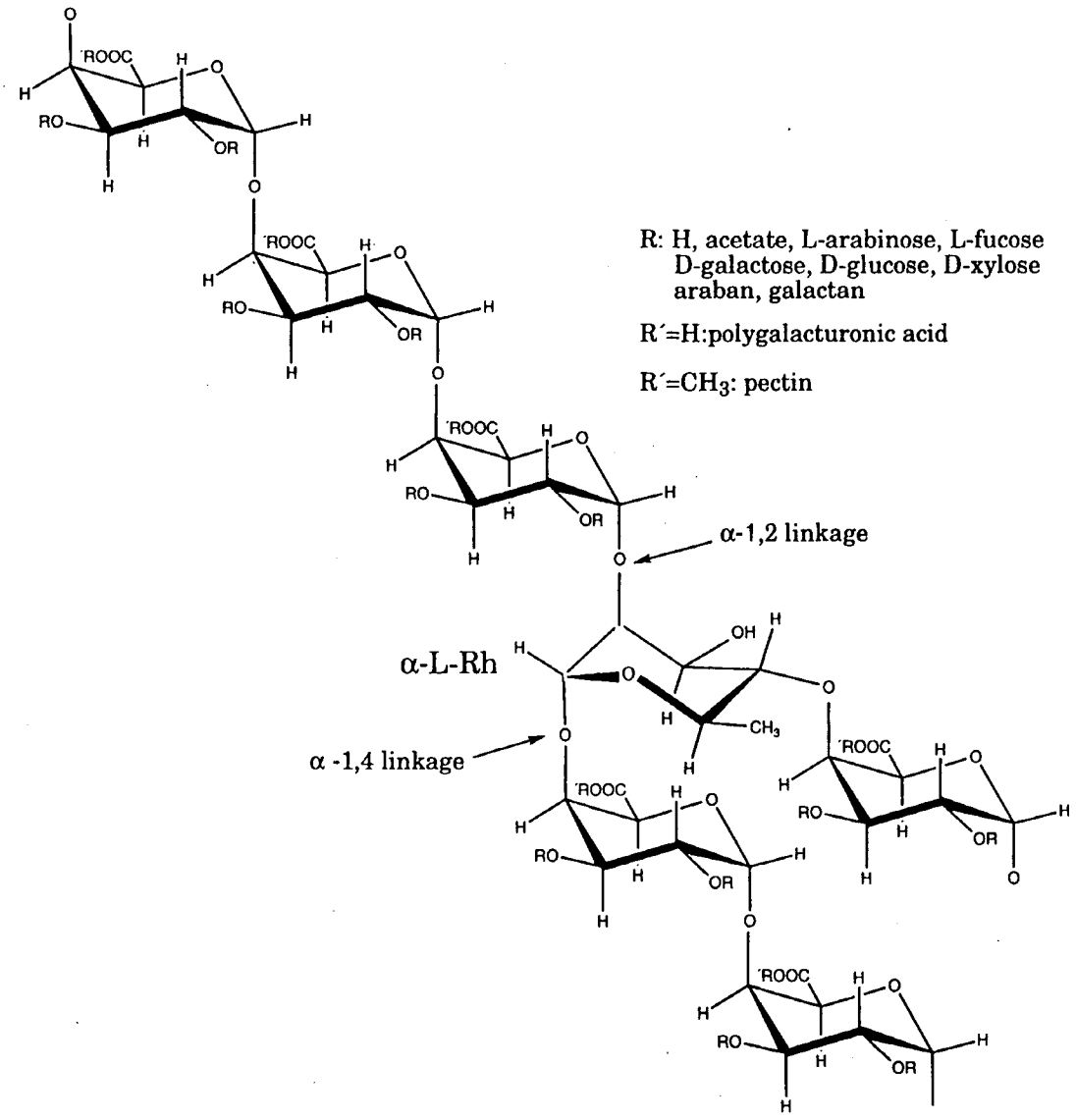

Figure 6. Chemical structure of pectin.

chain, L-rhamnopyranose residues are present that are the binding sites for side chains composed of neutral sugars. Pectin is an important plant material that is present in the middle lamellae as well as in the primary cell walls.

5.1. Pectin-Degrading Enzymes

Pectin is degraded by pectinolytic enzymes that can be classified into two major groups. The first group comprises methylesterases, whose function is to remove the methoxy groups from pectin. The second group comprises the depolymerases

(hydrolases and lyases), which attack both pectin and pectate (polygalacturonic acid).

Pectinase, pectin depolymerase, or polygalacturonase (E.C.3.2.1.15) randomly hydrolyzes α-1,4-galactosiduronic linkages in pectate and other galacturonans. Exopolygalacturonase, Galacturan α-1,4-galacturonidase or poly-galacturonate hydrolase (E.C.3.2.1.67) removes one molecule galacturonate from polygalacturonate. Exo-poly-α-galacturonosidase (E.C.3.2.1.82) hydrolyzes pectic acid from the nonreducing end of pectin, releasing digalacturonate. Pectinesterase, pectin methylesterase, pectin demethoxylase, or pectin methoxylase (E.C.3.1.1.11) removes methyl groups from pectin, producing methanol and pectate. Oligogalacturonide lyase (E.C.4.2.2.6) catalyzes the eliminative removal of unsaturated terminal residues from oligosaccharides of D-galacturonate. Pectin lyase (E.C.4.2.2.10) catalyzes the eliminative cleavage of pectin to give oligosaccharides with terminal 4-deoxy-6-methyl-α-D-galact-4-enuronosyl groups. This enzyme does not act on deesterified pectin.

5.2. Production of Pectin-Degrading Enzymes by Hyperthermophiles

A great variety of pectinolytic bacteria have been isolated from various habitats such as trees, lakes, soil, tumen, mullet gut, and the human intestinal track. Pectin hydrolases are predominantly synthesized by fungi (Albersheim *et al.,* 1960; Itoh *et al.,* 1982; Kamamiya *et al.,* 1974; Schlemmer *et al.,* 1987; Sone *et al.,* 1988), whereas pectate lyases are mostly produced by bacteria and usually act at alkaline pH and are Ca^{2+} dependent (Whitaker, 1991). Pectin degradation from bacteria has been reported for *Thermoanaerobacter thermohydrosulfuricus* (Wiegel *et al.,* 1979), *Thermoanaerobacter thermosulfurigenes* (Schink and Zeikus, 1983), *Clostridium thermocellum* (Spinnler *et al.,* 1986), *Desulfurococcus amylolyticus* (Bonch-Osmolovsskaya *et al.,* 1988), *Clostridium thermosaccharolyticum* (van Rijssel and Hansen, 1989), and *Bacillus stearothermophilus* (Karbassi and Vaughn, 1980).

Although many microorganisms have been screened for pectinolytic activity, little attention has be given to pectinolytic enzymes from thermophilic and hyperthermophilic bacteria (Kozianowski *et al.,* 1997). Previously a novel anaerobic strain from a thermal spa in Italy was isolated that produces two thermoactive lyases that have a very high affinity for polygalacturonate. This is a spore-forming anaerobic microorganism able to grow on citrus pectin and pectate optimally at 70°C, which has been identified as *Thermoanaerobacter italicus.* After growth on citrus pectin, two pectate lyases (α and β) were induced, purified, and biochemically characterized. Pectate lyase α is a single polypeptide of 135 kDa, whereas pectate lyase β is a heterodimer with 93 and 158 kDa molecular weight for the two subunits. Both enzymes display similar catalytic properties and can function at

temperatures up to 80°C. An increase in the enzymatic activity of both pectate lyases was observed upon addition of Ca^{2+} at 1 mM concentration.

Another anaerobic, extremely thermophilic, non–spore-forming bacterium was isolated from a sediment sample taken from Owens Lake, California, and designated strain OLT. It grows between 50 and 80°C, with a temperature optimum at 75°C and at pH range 5.5 to 9.0, with a pH optimum at about 7.5. The isolate utilized pectin, sucrose, xylose, fructose, ribose, xylan, starch, and cellulose. It has been proposed that OLT be designated *Caldicellulosiruptor owensensis* sp. nov., based on 16S rDNA sequence analysis.

Unfortunately, pectin-hydolyzing enzymes from archaea have not yet been identified and characterized (Huang *et al.,* 1998). However, due to the lack of biochemical data on various pectin hydrolases, it is impossible to make proper comparisons from various organisms.

5.3. Biotechnological Relevance

Enzymatic pectin degradation is widely applied in food technology processes, as in fruit juice extraction, in order to increase the juice yield, reduce its viscosity, improve color extraction from the skins, as well as to macerate fruit and vegetable tissues.

6. CHITIN-DEGRADING ENZYMES FROM EXTREMOPHILIC ORGANISMS

Chitin is a linear β-1,4 homopolymer of *N*-acetyl-glucosamine residues and it is the second-most abundant natural biopolymer after cellulose on earth (Fig. 7). Chitin is produced in enormous amounts, particularly in the marine environment (Gooday 1990, 1994), and its turnover is due to the action of chitinolytic enzymes. Chitin is the major structural component of most fungi and invertebrates (Gooday, 1990, 1994), whereas chitin serves as a nutrient for soil or marine bacteria.

6.1. Chitin-Degrading Enzymes

Chitin degradation is known to proceed with the endo-acting chitin hydrolase chitinase A (E.C.3.2.1.14) and the chitin oligomer exo-acting hydrolases chitinase B and *N*-acetyl-D-glucosaminidase (trivial name: chitobiase) (E.C.3.2.1.52). The chemical structure and the site of action of chitinolytic enzymes is shown in Fig. 7.

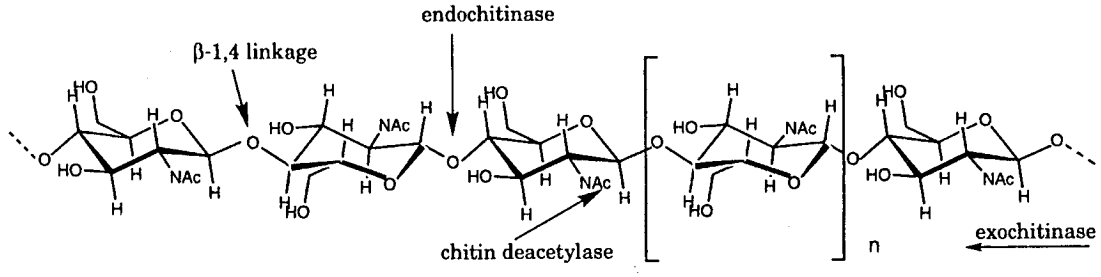

Figure 7. Chemical structure of chitin and the action of the enzymes involved in its degradation.

Endo- and exochitinases comprise three glycosyl hydrolase families: families 18, 19, and 20. Family 18 consists of chitinases, endo-β-*N*-acetyl-D-glucosaminidases (EC 3.2.1.96) and di-*N*-acetylchitobiases from eukaryotae, prokaryotae, and viridae. The *N*-acetyl-D-glucosamine oligomeric product retains its C1 anomeric configuration. Family 19 contains only chitinases from eukaryotae and prokaryotae, and in contrast to the family 18, the product has inverted anomeric configuration. Finally, family 20 contains β-hexosaminidases and chitobiases. Chitobiases degrade only small *N*-acetyl-D-glucosamine oligomers (up to pentamers) and the released *N*-acetyl-D-glucosamine monomers retain their C1 anomeric configuration. The X-ray structure of these enzymes previously has been determined and the enzymatic mechanism of hydrolyis of chitin by chitinase A and chitobiase from *Serratia marcescens* has been resolved at the atomic level (Perrakis *et al.*, 1994; Tews *et al.*, 1996).

6.2. Chitin-Degrading Enzymes from Hyperthermophilic Organisms

Although a large number of chitin-hydrolyzing enzymes have been isolated and their corresponding genes have been cloned and characterized, only a few chitin-hydrolyzing enzymes that are thermostable are known. These enzymes have been isolated from the thermophilic bacterium *Bacillus licheniformis* X-7u (Takayanagi *et al.*, 1991), *Bacillus sp.* BG-11 (Bharat and Hoondal, 1998), and *Streptomyces thermoviolaceus* OPC-520 (Tsujibo *et al.*, 1995)

The extremophilic anaerobic archeon *Thermococcus chitonophagus* has been reported to hydrolyze chitin (Huber *et al.*, 1995). This is the first extremophilic archeon that produces chitinase(s) and *N*-acetylglucosaminidase(s) in order to degrade chitin for nutritional purposes. *Thermococcus chitonophagus* can grow up to 93°C under nitrogen and the chitinolytic enzyme system is cell associated and inducible by chitin. The chitin-degrading enzymes have been identified and their biochemical characterization as well as their molecular cloning is underway (C. Vorgias, unpublished results).

6.4. Biotechnological Relevance

Although chitin and its partially deacetylated derivative chitosan are not well established as products with a particular biotechnological interest, there are a number of scientific works reporting that the natural polymer chitin exhibits interesting properties that make it a valuable raw material for several applications (Cohen-Kupiec and Chet, 1998; Kramer and Muthukrishnan, 1997; Spindler *et al.*, 1990; Muzzarelli, 1997; Shigemasa and Minami, 1996; Georgopapadakou and Tkacz, 1995; Benhamou, 1995; Chandy and Sharma, 1990; Kas, 1997).

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