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REVIEW

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Glycosyl hydrolases from hyperthermophiles

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Introduction

Extreme thermophilic and hyperthermophilic microorganisms are classified as those which are adapted to grow optimally at temperatures ranging from 70°C to 110°C. A number of such microorganisms have been found to produce thermostable enzymes which are capable of hydrolyzing natural polymers such as starch, cellulose, and xylan (Leuschner and Antranikian 1995; Antranikian et al. 1995).

Glycosyl hydrolases are a group of enzymes which share the ability to hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Recently, glycosyl hydrolases have been classified into more than 45 families according to amino acid sequence similarities (Henrissat 1991; Henrissat and Bairoch 1993). In starch the glucose residues are linked via α-1,4- and α-1,6-glycosidic bonds, whereas in xylan and cellulose the constituent sugars are linked via β-1,4-glycosidic bonds. Microorganisms capable of producing enzymes that hydrolyze these glycosidic linkages play the most important role in the biodegradation of natural polysaccharides.

We briefly review glycosyl hydrolases involved in the hydrolysis of natural polysaccharides. In this review we focus on α- and β-glycosyl hydrolases from *Bacteria* and *Archaea* that grow optimally above 70°C. Glycosyl hydrolases from microorganisms that grow below 70°C have been described elsewhere (Rüdiger et al. 1995b).

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Starch-degrading enzymes

Starch is, after cellulose, one of the most abundant polysaccharides produced by plants and is composed of amylose (15%–25%) and amylopectin (75%–85%). Amylose is a linear molecule consisting of 1,4-linked α-D-glucopyranose residues. Amylopectin is a branched polymer and contains α-1,6 glycosidic linkages in addition to the α-1,4 bonds. The hydrolysis of starch requires the coordinate action of several enzymes, such as α-amylase, β-amylase, glucoamylase, debranching enzymes (pullulanases), and α-glucosidase.

The biodegradation of starch is widespread among *Bacteria*, fungi, and yeast (Antranikian 1992). Several extreme thermophilic and hyperthermophilic *Bacteria* and *Archaea* have been found to grow at high temperatures (>70°C) on starch and produce extremely thermostable starch-degrading enzymes (Leuschner and Antranikian 1995). Table 1 summarizes some properties of selected thermostable starch-hydrolyzing enzymes.

α-Amylase (1,4-α-D-glucan, 4-glucanhydrolase, EC 3.2.1.1)

This endoacting enzyme produces oligosaccharides and glucose as end-products by randomly hydrolyzing the α-1,4-glycosidic linkages of starch.

The most thermoactive α-amylases that have been purified and characterized are those from the archaeon *Pyrococcus woesei* (Koch et al. 1991) and *P. furiosus* (Brown et al. 1990; Koch et al. 1990; Ladermann et al. 1993a,b). The extracellular α-amylase of *P. woesei* is a monomeric protein with a molecular mass of 68 kDa. The enzyme is active between 40°C–130°C, with an optimum at 100°C and pH 5.5. The intracellular enzyme from *P. furiosus* has been characterized as a homodimer with a molecular mass of 129 kDa [66 kDa/subunit (SU)]. The enzyme is active in the range 40°C–120°C with an optimum at 100°C and pH 6.5–7.5; the half-life at 110°C is 4 h. To inactivate the α-amylase of *P. woesei* completely, autoclaving at 120°C for 6 h is necessary. An additional characteristic of

Table 1. Physicochemical properties of selected α-glycosyl hydrolases from extremely thermophilic *Bacteria* and hyperthermophilic *Archaea*

Organism	Growth temperature (°C)	Enzymes	Sample[a]	Optimal temperature (°C)	Optimal pH	M_r[b] (kDa)	Remarks	Reference
Archaea:								
Desulfurococcus mucosus	85	α-Amylase	Crude extract	100	5.5			Canganella et al. 1994
		Pullulanase type II	Crude extract	100	5.0			Canganella et al. 1994
Pyrococcus furiosus	100	α-Amylase	Purified	100	6.5–7.5	66/subunit (129)	Intracellular/ cloned	Ladermann et al. 1993a,b
		α-Amylase	Purified/ clone	100	7.0	68	Extracellular/ cloned	Jorgensen et al. 1996
		Pullulanase type II	Purified	98	5.5	110	Extracellular/ glycoprotein	Brown and Kelly 1993
		α-Glucosidase	Purified	115	5.5	125	Intracellular	Costantino et al. 1990
Pyrococcus woesei	100	α-Amylase	Purified	100	5.5	68	Extracellular	Koch et al. 1991
		Pullulanase type II	Purified/ clone	100	6.0	90	Cell-associated/ cloned	Rüdiger et al. 1995a
		α-Glucosidase	Purified	110	5.0–5.5		Intracellular	Linke et al. 1992
Pyrodictium abysii	98	α-Amylase	Crude extract	90	5.0			Andrade et al. 1996
		Pullulanase type II	Crude extract	100	9.0			Andrade et al. 1996
Staphylothermus marinus	90	α-Amylase	Crude extract	100	5.0			Canganella et al. 1994
Sulfolobus solfataricus MT-4	88	Glucoamylase	Partially purified	70	5.5		Cytoplasmic fraction	Lama et al. 1991
Sulfolobus solfataricus 98/2	80	α-Amylase	Purified			120/subunit (240)	Extracellular	Haseltine et al. 1996
		α-Glucosidase	Purified	105	4.5	80/subunit (400)	Intracellular	Rolfsmeier and Blum 1995
Thermococcus celer	85	α-Amylase	Crude extract	90	5.5			Canganella et al. 1994
		Pullulanase type II	Crude extract	90	5.5			Canganella et al. 1994
Thermococcus litoralis	90	Pullulanase type II	Purified	98	5.5	119	Extracellular/ glycoprotein	Brown and Kelly 1993
Thermococcus profundus DT5432	80	α-Amylase	Purified	80	5.5–6.0	42	Extracellular	Chung et al. 1995
Thermococcus strain AN1	75	α-Glucosidase	Purified	75[c]	7.0	60	Extracellular	Piller et al. 1996
Thermococcus strain TY	85	α-Amylase	Crude extract	100	5.5			Canganella et al. 1994
		Pullulanase type II	Crude extract	100	6.5			Canganella et al. 1994
Thermococcus strain TYS	85	α-Amylase	Crude extract	100	6.5			Canganella et al. 1994
		Pullulanase type II	Crude extract	100	6.5			Canganella et al. 1994
Bacteria:								
Dictyoglomus thermophilum H-6-12	78	α-Amylase	Purified/ clone	90	5.5	75	Cytoplasmic fraction	Fukusumi et al. 1988
Thermotoga maritima MSB8	80	Glucoamylase/ α-amylase	Partially purified	90	6.0	60	Toga-associated	Schumann et al. 1991
Thermus caldophilus GK-24	75	Pullulanase type I	Purified	75	5.5	65	Cell-associated	Kim et al. 1996

[a] Pure enzymes or crude extracts were used for enzyme characterization.
[b] Values in brackets represent the size of the native enzyme.
[c] Assay temperature.

these two enzymes is that, unlike mesophilic α-amylases, they do not require Ca^{2+} for activity or thermostability.

The *P. woesei* α-amylase is capable of hydrolyzing randomly the α-1,4-glycosidic linkages in various substrates such as soluble and native starch, glycogen, amylopectin, and amylose. The results of hydrolysis are various oligosaccharides, but unlike other α-amylases, glucose is not formed as an end-product. Maltoheptaose is the smallest substrate that can be hydrolyzed by this enzyme. The intracellular α-amylase of *P. furiosus* hydrolyzes starch to glucose and maltose in addition to a mixture of oligosaccharides.

The gene encoding for a distinct thermostable extracellular α-amylase from *P. furiosus* has been cloned, sequenced, and expressed in *E. coli* and *B. subtilis* (Jorgensen et al. 1996 unpublished work). This recombinant α-amylase shows sequence homology to the α-amylases belonging to family 13 of glycosyl hydrolases. This enzyme, however, is not homologous to the intracellular α-amylase of the same archaeon (Ladermann et al. 1993a,b). The gene encoding for an intracellular α-amylase from *P. furiosus* has also been cloned and expressed in *E. coli* (Ladermann et al. 1993a). The recombinant α-amylase has no leader sequence and its amino acid sequence is homologous to that of the α-amylase from *Dictyoglomus thermophilum* (Fukusumi et al. 1988).

The hyperthermophilic archaeon, *Thermococcus profundus* DT5432, has been reported to produce extracellular thermostable amylases. One of these enzymes, namely amylase S, has been purified and characterized (Chung et al. 1995). This enzyme consists of a single subunit with a molecular mass of 42 kDa. The enzyme exhibits catalytic activity between 40°C and 100°C with an optimum at 80°C and pH 5.5–6.0. The half-life of the enzyme at 90°C is 15 min. At the same temperature in the presence of Ca^{2+}, however, the half-life is increased to 4 h.

The amylase S of *T. profundus* DT 5432 hydrolyzes soluble starch, glycogen, amylose, and amylopectin producing mainly maltose and maltotriose as major end-products. Maltose is not hydrolyzed and, like the α-amylases from *P. furiosus*, amylase S possesses transferase activity.

Although only few archaeal amylases have been purified and characterized, the production of amylases seems to be widely distributed among *Archaea*. Canganella et al. (1994) and Andrade et al. (1996) reported on the presence of thermostable amylases in the crude extracts of two archaea belonging to the order Thermoproteales (*Desulfurococcus mucosus*, *Staphylothermus marinus*), four archaea belonging to the order Thermococcales (*Thermococcus celer*, *T. litoralis*, *Thermococcus* strain TYS, and *Thermococcus* strain TY), and one archaeon belonging to the order Pyrodictiales (*Pyrodictium abyssi*). The enzymes are optimally active between 90°C and 100°C and at pH values of 5.0–6.0. All the enzymes exhibit remarkable thermostability in the absence of substrate and calcium ions.

The extreme thermoacidophilic archaeon *Sulfolobus solfataricus* is able to grow on several sugars, including starch and maltose, as carbon and energy sources (*Grogan* 1989). A thermostable amylolytic enzyme from the cytoplasmic fraction of *S. solfataricus* strain MT-4 was partially purified and characterized (Lama et al. 1991). The enzyme is optimally active at 70°C and pH 5.5 and shows no calcium requirement for thermostability. The main products resulting from the hydrolysis of soluble starch, amylose, and amylopectin were glucose and trehalose, indicating that the amylolytic activity is of the glucoamylase type. Recently, an α-amylase from the culture supernatant of *Sulfolobus solfataricus* 98/2 grown on starch was purified (Haseltine et al. 1996). The enzyme is a homodimer with a subunit size of 120 kDa. The enzyme is an endoacting amylase capable of hydrolyzing starch, dextrin, and α-cyclodextrin with similar efficiencies. The α-amylase is produced constitutively at low levels and the addition of starch induces the synthesis of higher levels of enzyme. Glucose, on the other hand, represses the synthesis of α-amylase by catabolite repression.

The anaerobic thermophilic bacterium *Dictyoglomus thermophilum* produces a heat-stable amylase complex when grown on starch (Kobayashi et al. 1988). The preliminary characterization of the crude extract revealed an optimal temperature and pH for activity at 90°C and 5.0, respectively. In the absence of substrate the amylolytic activity was stabilized in the presence of calcium. Chromatographic fractionation of the crude extract showed the presence of at least four fractions with different amylolytic activities. One amylase gene (*amyA*) from *Dictyoglomus thermophilum* has been cloned and expressed in *E. coli* (Fukusumi et al. 1988). The recombinant enzyme did not possess an NH_2-secretion signal peptide and was found to be localized in the cytoplasmic fraction of *E. coli*. The purified recombinant amylase shows a molecular mass of 75 kDa and is optimally active at 90°C and pH 5.5. The amylase hydrolyzes starch to produce mainly maltotriose, maltose, and glucose.

The extreme thermophilic anaerobic bacterium *Thermotoga maritima* is able to grow on starch as a carbon source without releasing amylase into the culture medium (Schumann et al. 1991; Jaenicke 1993). Two distinct amylolytic fractions were found to be associated with the "toga" (the outer sheath covering the cells). Fraction I seems to possess β-amylase that is optimally active at 95°C and pH 5.0. Fraction II, on the other hand, seems to contain α-amylase and glucoamylase activities with an optimum at 90°C and pH 6.0.

Pullulanase type I (α-dextrin 6-glucanohydrolase, EC 3.2.1.41)

Pullulanase type I hydrolyzes exclusively the α-1,6-glycosidic linkages in branched oligosaccharides, polysaccharides, and pullulan to form linear oligosaccharides as end-products. Most of the thermoactive pullulanases studied so far belong to the type II pullulanases, and attack both α-1,4- and α-1,6-glycosidic linkages in branched substrates.

The aerobic thermophilic bacterium *Thermus caldophilus* GK-24 grows optimally at 75°C and produces thermostable pullulanase of type I when grown on starch (Kim et al. 1996). The enzyme has a molecular mass of 65 kDa and is optimally active at 75°C and pH 5.5. The

pullulanase is thermostable up to 90°C and Ca^{2+} ions do not have any effect on the enzyme activity. The pullulanase attacks the α-1,6-linkages of pullulan and produces maltotriose. The α-1,6-linkages of α,β-limit dextrins, amylopectin, and glycogen are hydrolyzed less efficiently. A debranching enzyme with similar properties was purified from a newly isolated anaerobic bacterium which was identified as *Fervidobacterium pennavorans* strain Ven 5. The enzyme, with a molecular mass of 240 kDa (77 kDa/SU), is optimally active at 85°C and pH 5.0–7.0 (Leuschner and Antranikian 1995).

Pullulanase type II (amylopullulanase)

This class of enzyme is capable of randomly hydrolyzing α-1,4-linkages in addition to the branching points (α-1,6-linkages) in polysaccharides and limit dextrins. The action of pullulanase type II on poly- and oligosaccharides results in the formation of glucose, maltose, and maltotriose as major products (Antranikian 1992). Unlike pullulanase type I, pullulanase type II is widely distributed among extreme thermophilic *Bacteria* and hyperthermophilic *Archaea*.

The most thermostable and thermoactive pullulanase type II reported to date are derived from the hyperthermophilic archaeon *Pyrococcus woesei* (Linke et al. 1992; Rüdiger et al. 1995a) and *P. furiosus* (Brown et al. 1990; Brown and Kelly 1993). Unlike the pyrococcal α-amylase, the pullulanase was found to be cell-associated. The gene encoding the pullulanase of *P. woesei* was cloned and expressed in *E. coli*. This is the first archaeal pullulanase that has been cloned and expressed in a mesophilic host (Linke et al. 1992; Rüdiger et al. 1995a). The purification of the recombinant pullulanase was achieved by thermal denaturation of the host protein and recovery of the thermostable pullulanase in the supernatant. The enzyme possesses a signal sequence and is composed of a single polypeptide chain with a molecular mass of 90 kDa. The pullulanase is optimally active at 100°C and pH 6.0. The half-life of the enzyme at 110°C in the presence of maltodextrin (0.25%) or Ca^{2+} (1 mM) is raised from 7 to 20 min. Enzyme activation (up to 370%) is achieved in the presence of Ca^{2+} (0.3 mM) and reducing agents such as β-mercaptoethanol (10 mM) and dithiothreitol (10 mM).

The pullulanases of type II which are produced by *Thermococcus litoralis* and *P. furiosus* are glycoproteins with a molecular mass of 119 and 110 kDa, respectively (Brown and Kelly 1993). Both enzymes are optimally active at around 100°C and pH 5.5. The presence of Ca^{2+} (5 mM) had a positive effect on the activity of both enzymes at high temperatures. At 120°C in the presence of substrate (maltoheptaose) and Ca^{2+}, the pullulanases of *T. litoralis* and *P. furiosus* have half-lives of 5 and 12 min, respectively. In addition to thermal stability, calcium stimulates the pullulan-hydrolyzing activity of these enzymes while inhibiting the starch-hydrolyzing activity. At high temperatures both enzymes show higher specificity towards pullulan than towards starch or glycogen.

Thermoactive pullulanases of type II also have been reported from *Thermococcus celer*, *Thermococcus* strain TYS, *Thermococcus* strain TY, *Desulfurococcus mucosus*, and *Staphylothermus marinus* (Canganella et al. 1994). Only recently, the pullulanase from the hyperthermophile *Pyrodictium abyssi* was characterized (Andrade et al. 1996). The enzyme is optimally active at 100°C and at alkaline pH (pH 9.0).

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20)

α-Glucosidases hydrolyze terminal, nonreducing 1,4- and, to a lesser extent, 1,6-linked α-glucose residues of disaccharides, oligosaccharides, and aryl-glucosides. They are involved in the last step of the degradation of starch, forming glucose as an end-product. Organisms growing on starch produce intracellular α-glucosidases together with extracellular amylolytic enzymes; these activities constitute the starch-degrading system. Among *Archaea*, α-glucosidases have been characterized from two species of the genus *Pyrococcus*: *P. furiosus* and *P. woesei* (Costantino et al. 1990; Leuschner and Antranikian 1995).

The α-glucosidase activity in *P. furiosus* is mostly intracellular, and its expression, as for other starch-degrading enzymes from this source, is induced by carbohydrates containing α-1,4- linkages (Costantino et al. 1990). However, the mechanism of induction remains unknown. This α-glucosidase has highest activity toward maltose and isomaltose, but significant activity against aryl-glucosides is also displayed. The enzyme is a monomer with a molecular mass of 125 kDa, it has optimal activity over a broad temperature range, about 105°C–115°C, and it exhibits remarkable thermal stability (2 days at 98°C). The observed inactivation in the presence of EDTA suggests that metal ions may be important for activity and/or stability.

Recently, a study by subtractive hybridization of the regulation of the genes for maltose fermentation in *P. furiosus* was reported (Robinson et al. 1994). Although some of the genes isolated with this technique are required for maltose utilization (i.e., pyruvate dikinase), being typical of the pyroglycolysis pathway, no α-glycohydrolase-encoding genes were isolated.

An intracellular α-glucosidase activity has been purified to homogeneity in *S. solfataricus* strain 98/2 (Rolfsmeier and Blum 1995). The enzyme is made of four to five identical subunits of 80 kDa; it displays maximal activity on maltose, and is active to a lesser extent on aryl-glucosides, but not on starch. Maltosaccharides of up to seven glucose units are substrates of the enzyme that is also moderately active on α-1,6 disaccharides. Interestingly, the enzyme is optimally active at 105°C, more than 20°C above the optimal growth temperature of this *S. solfataricus* strain, and has an half-life of 3 h at 95°C. These data suggest that the enzyme is structurally different from *P. furiosus* enzymes although they show similar specificities. The identification of maltose as preferred substrate would suggest

that the *S. solfataricus* α-glucosidase is a proper maltase that allows the use of this sugar as a carbon source in vivo.

The purification and characterization of an extracellular α-glucosidase from the archaeon *Thermococcus* strain AN1 has been recently reported (Piller et al. 1996). The enzyme is a monomer with a molecular mass of 60 kDa and a half-life of 35 min at 98°C; the substrate preference is aryl-α-D-glucoside > nigerose > panose > palatinose > isomaltose > maltose > turanose. These authors reported that the enzyme reduces its thermostability on purification; however, its half-life is increased in the presence of several additives such as sorbitol, dithiothreitol, or bovine serum albumin (BSA) and by co-cross-linking with BSA. The biochemical data reported show a certain variety among the α-glucosidases from hyperthermophilic *Archaea*: α-glucosidases from *P. furiosus*, *P. woesei*, and *S. solfataricus* are intracellular and oligomeric, whereas the enzyme from *Thermococcus* is smaller, monomeric, and mainly extracellular. However, in the absence of the genes coding for these enzymes, it is not possible to analyse in detail the structural similarities among archaeal α-glucosidases.

Xylan-degrading enzymes

Xylan is the major component of plant hemicelluloses. Plant xylans are heterogeneous polysaccharides consisting of a main chain of β-1,4-linked D-xylopyranosyl residues. The backbone structure contains, depending on the origin, acetyl, arabinosyl, and glucuronosyl substituents. The depolymerization action of the endoxylanase results in the conversion of the polymeric substrate into xylooligosaccharides, which can be further hydrolyzed to xylose by the action of the β-xylosidase. The side-chain substituents are removed by a set of enzymes which include α-arabinofuranosidase, acetylxylan esterases, and α-glucuronidase. In Table 2 some properties of thermostable xylan-hydrolyzing enzymes are presented.

Endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8)

β-1,4-Endoxylanases catalyze the hydrolysis of the internal glycosidic linkages of a heteroxylan backbone resulting in a decreased degree of polymerization of the substrate. The most thermostable endoxylanases that have been described so far are those purified from the anaerobic thermophilic species of the genus *Thermotoga*, namely *Thermotoga* sp. strain FjSS3-B.1 (Simpson et al. 1991), *T. maritima* (Winterhalter and Liebl 1995), *T. neapolitana* (Bok et al. 1994), and *T. thermarum* (Sunna et al. 1996). Like the α-amylase produced by *T. maritima*, all the thermostable xylanases produced by these microorganisms are mainly cell-associated and most probably localized within the toga (Schumann et al. 1991; Ruttersmith et al. 1992; Winterhalter and Liebl 1995; Sunna and Antranikian 1996).

Thermotoga sp. strain FjSS3-B.1 produces a thermostable endoxylanase with a molecular mass of 31 kDa (Simpson et al. 1991). The endoxylanase is optimally active at 105°C and pH 5.4. The enzyme is thermostable at 95°C and exhibits a half-life of 8 min at 100°C. At 105°C the half-life of the enzyme is less than 2 min; however, in the presence of 0.25% xylan alone or 0.25% xylan plus 0.2% BSA, the half-life of the enzyme increases to 9 and 11 min, respectively. The endoxylanase hydrolyzes oat spelts xylan to mainly xylobiose and xylotriose.

Three xylanase genes (*xynA*, *xynB*, and *xynC*) have been found in *Thermotoga* sp. strain FjSS3-B.1 (Saul et al. 1995). One gene, namely *xynA*, has been isolated and overexpressed in *E. coli*. The *xynA* gene encodes a thermostable xylanase with a molecular mass of 40.5 kDa. This enzyme belongs to the glycosyl hydrolase family 10 and is closely related to XynZ from *Clostridium thermocellum* (Grépinet et al. 1988) and XynA from the thermophilic anaerobe *Caldicellulosiruptor* Rt8B.4 (Dwivedi et al. 1996). However, the *xynA* gene from *Thermotoga* sp. strain FjSS3-B.1 shows only low homology to the *T. maritima xynA* gene reported by Winterhalter et al. (1995). The pH optimum and thermostability of the recombinant XynA differ from those reported previously for the purified endoxylanase from *Thermotoga* sp. strain FjSS3-B.1. Thus, the recombinant

Table 2. Physicochemical properties of selected β-glycosyl hydrolases from extremely thermophilic *Bacteria* and hyperthermophilic *Archaea*

Organism	Growth temperature (°C)	Enzyme	Sample[a]	Optimal temperature (°C)	Optimal pH	M_r[b] (kDa)	Remarks	Reference
Archaea:								
Pyrococcus furiosus	100	β-Glucosidase	Purified	102–105	5.0	58/subunit (230)	Intracellular/ cloned	Kengen et al. 1993
Pyrodictium abysii	98	Endoxylanase	Crude extract	110	5.5			Andrade et al. 1996
Sulfolobus solfataricus MT-4	88	β-Glycosidase	Purified	>90	6.5	56/subunit (240)	Intracellular/ cloned/broad specificity	Nucci et al. 1993
Sulfolobus solfataricus P2	80	β-Glycosidase	Purified	77[c]	5.0	60–65/ subunit (>200)	Intracellular/ broad specificity	Grogan 1991

Table 2. *Continued*

Organism	Growth temperature (°C)	Enzyme	Sample[a]	Optimal temperature (°C)	Optimal pH	M_r[b] (kDa)	Remarks	Reference
Bacteria:								
Dictyoglomus thermophilum Rt46B.1		Endoxylanase (XynA)	Purified/ clone	85	6.5	33	Cloned	Gibbs et al. 1995
Thermotoga sp. strain FjSS3-B.1	80	Endoxylanase	Purified	105	5.3	31	Toga-associated	Simpson et al. 1991
		Endoxylanase (XynA)	Purified/ clone	85[c]	6.3	40	Cloned	Saul et al. 1995
		β-Xylosidase	Purified	80[c]	7.0	92/subunit (174)	Toga-associated	Ruttersmith and Daniel 1993
		β-Glycosidase	Purified	80[c]	7.0	75/subunit (100)	Toga-associated	Ruttersmith and Daniel 1993
		α-Arabino-furanosidase	Purified	>80	7.0	92	Toga-associated	Ruttersmith et al. 1992
		Exoglucanase	Purified	105	6.8–7.8	36	Cell-associated	Ruttersmith and Daniel 1991
Thermotoga maritima MSB8	80	Endoxylanase (XynA)	Purified	92	6.2	120	Toga-associated/ cloned	Winterhalter and Liebl 1995, Winterhalter et al. 1995
		Endoxylanase (XynB)	Purified	105	5.4	40		Winterhalter and Liebl 1995
		β-Xylosidase	Crude extract	90	5.0			Sunna et al. 1996 (in press)
		α-Arabino-furanosidase	Crude extract	80	5.0			Sunna et al. 1996 (in press)
		Endoglucanase (cellulase I)	Purified	95	6.0–7.5	27	Cell-associated	Bronnenmeier el al. 1995
		Exoglucanase (cellulase II)	Purified	95	6.0–7.5	29	Cell-associated	Bronnenmeier et al. 1995
		β-Glucosidase (BglA)	Purified/ clone	75[c]	6.2	47/subunit (95)	Cloned	Gabelsberger et al. 1993
		β-Galactosidase (BgaA)	Purified/ clone	80	5.3	120/subunit (240)	Cloned	Gabelsberger et al. 1993
Thermotoga neapolitana	80	Endoxylanase	Purified	85	5.5	37		Bok et al. 1994
		Endoxylanase (XynA)	Purified/ clone	102	5.5–6.0	119		Zverlov et al. 1996
		β-Xylosidase	Crude extract	90	6.0			Sunna et al. 1996 (in press)
		α-Arabino-furanosidase	Crude extract	80	6.0			Sunna et al. 1996 (in press)
		Endoglucanase (Endo B)	Purified	110				Bok et al. 1994
Thermotoga thermarum	77	Endoxylanase (endo 1)	Purified	80	6.0	105/150 (266)	Toga-associated	Sunna et al. 1996
		Endoxylanase (endo 2)	Purified	90–100	7.0	35	Toga-associated	Sunna et al. 1996
		β-Xylosidase	Crude extract	90	6		Toga-associated	Sunna et al. 1994, 1996 (in press)
		α-Arabino-furanosidase	Crude extract	70	6.0		Toga-associated	Sunna et al. 1994, 1996 (in press)

[a] Pure enzymes or crude extracts were used for enzyme characterization.
[b] Values in brackets represent the size of the native enzyme.
[c] Assay temperature.

XynA seem to be similar but not identical to the enzyme originally purified by Simpson et al. (1991).

During growth with xylan or xylose as the sole carbon source, *Thermotoga maritima* MSB8 produces two distinct thermostable endoxylanases referred to as XynA and XynB (Winterhalter and Liebl 1995). XynA is a membrane-bound, multidomain enzyme with a molecular mass of 120 kDa and optimal activity at 92°C and pH 6.2. XynB on the other hand, has a molecular mass of 40 kDa and is optimally active at 105°C and pH 5.4. Both enzymes produce mainly xylotriose, xylobiose, and low amounts of xylose upon hydrolysis of oat spelts xylan. Unlike XynA, XynB activity is stimulated twofold by the addition of 500 mM NaCl. XynA is able to adsorb to microcrystalline cellulose, indicating the presence of a cellulose-binding domain. Both enzymes show high substrate specificity towards different xylans and none of the enzymes was able to hydrolyze cellulose, mannan, arabinogalactan, pullulan, amylose, or starch. However, in contrast to XynA, XynB was able to cleave aryl β-D-xylosides.

XynA and XynB are encoded by two different genes. The *xynA* gene encoding for the thermostable endoxylanase XynA has been cloned and expressed in *E. coli* (Winterhalter et al. 1995). XynA is a modular enzyme composed of five domains. The XynA of *T. maritima* MSB8 is most closely related to the xylanase of *Thermoanaerobacterium saccharolyticum* B6A-RI (Lee et al. 1993). The catalytic domain of XynA shares amino acid sequence similarity with xylanases of the glycosyl hydrolase family 10. Cellulose-binding studies indicate that the C-terminal domains C1 and C2 promote cellulose-binding. These two domains represent cellulose-binding domains of a novel type that do not share amino acid sequence similarity to any other known cellulose-binding domain.

Thermotoga neapolitana is capable of growth on xylan at up to 90°C and produces several thermostable xylanases (Sunna et al. 1992). One endoxylanase from *T. neapolitana* has been purified and characterized (Bok et al. 1994). The enzyme is a monomeric protein with a molecular mass of 37 kDa. The temperature and pH optima are 85°C and 5.5, respectively. The xylanase is thermostable and exhibits at 82°C a half-life of 130 h. The main products resulting from the hydrolysis of xylan are xylooligosaccharides ranging from xylobiose to xyloheptaose.

The genes encoding for several carbohydrate-degrading enzymes of *T. neapolitana* have been cloned and expressed in *E. coli* (Dakhova et al. 1993). Recently, the nucleotide sequence of the *xynA* gene, encoding for the thermostable extracellular xylanase (XynA) of *T. neapolitana*, has been determined and the recombinant enzyme has been characterized (Zverlov et al. 1996). The molecular mass of the enzyme is 119 kDa and the optimal temperature and pH for activity are 102°C and 5.5–6.0, respectively. The enzyme exhibits remarkable thermal stability at 100°C with a half-life of 120 min. The endoxylanase displays low β-1,3- and β-1,4-glucanase activity as well as cellobiohydrolase and xylosidase activities. The XynA from *T. neapolitana* is composed of three different domains, namely A, B, and C, with domain C being responsible for cellulose binding. The enzyme displays almost 89% identity to the multidomain XynA of *T. maritima* (Winterhalter et al. 1995). Furthermore, high similarity was found between the XynA from *T. neapolitana* and the N-terminal catalytic domain of CelB of *Caldocellum saccharolyticum* (Saul et al. 1990; renamed to *Caldicellulosiruptor saccharolyticus*).

Cultivation of the nonmarine bacterium *T. thermarum* on xylan or xylose is accompanied by the induction of high-molecular-mass (HMM) and low-molecular-mass (LMM) thermostable endoxylanases. In the absence of these substrates, however, only the LMM xylanase was present in low amounts (Sunna and Antranikian 1996). From both fractions, two thermostable enzymes from *T. thermarum* were purified and identified as endoxylanase 1 and endoxylanase 2 (Sunna et al. 1996). Endoxylanase 1 is a dimeric enzyme with a native molecular mass of 266 kDa (105 and 150 kDa/SU). The enzyme displays maximal activity at 80°C and pH 6.0. Endoxylanase 2, on the other hand, is composed of a single subunit with a molecular mass of 35 kDa. Endoxylanase 2 is optimally active between 90°C and 100°C and at pH 7.0. Both enzymes do not require metal ions for activity and were not active against carboxymethyl (CM)-cellulose. Unlike endoxylanase 1, endoxylanase 2 was active towards galactomannan indicating the presence of a mannanase activity. Both enzymes hydrolyze partially soluble and insoluble xylans to produce mainly xylobiose as end-product.

The xylanases from the anaerobic thermophilic bacterium *Dictyoglomus thermophilum* and those from several *Dictyoglomus* strains have been characterized by Mathrani and Ahring (1992). All these enzymes have an optimum temperature for activity at around 80°C and are active between pH 5.5 and 9.0. Recently, a xylanase gene (*xynA*) from *D. thermophilum* Rt46B.1 was cloned and expressed in *E. coli* (Gibbs et al. 1995). The recombinant enzyme is a single-domain xylanase which belongs to family 10 of glycosyl hydrolases and shows the highest homology with other thermophilic xylanases of the same family. The thermostable xylanase, Xyn A, is a single protein with a molecular mass of 33 kDa. The enzyme is optimally active at 85°C with an optimum pH at 6.5. The XynA was thermostable in the absence of substrate at 85°C. At this temperature and at pH 6.8 and 9.3 the enzyme displayed half-lives of 24 and 6.5 h, respectively. Substrate hydrolysis experiments with xylan indicate that XynA is an endoxylanase producing xylobiose and xylotriose as main products.

Archaea growing at high temperatures seem to be unable to utilize xylan as a carbon source or to produce thermostable xylanases (Sunna and Antranikian [in press]). Recently, however, it was shown that the hyperthermophilic archaeon *Pyrodictium abysii* is capable of producing a unique thermostable endoxylanase when grown in the presence of xylan, xylose, or arabinose (Andrade et al. 1996). The enzyme, which is inducible, displays optimal activity at 110°C and pH 5.5. The main products resulting from the hydrolysis of xylan are xylose, xylobiose, and xylotriose.

β-Xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37)

β-Xylosidases can be classified according to their relative affinities towards xylobiose and larger oligosaccharides; they generate D-xylose from short oligosaccharides and xylobiose in a concerted action with xylan-degrading enzymes. In this respect, they play an important role in degrading xylobiose, an inhibitor of endoxylanases.

β-Xylosidase activities from hyperthermophilic *Archaea* have been found so far to be one of the enzymatic activities of β-glycosidases with broad specificity (see later). By contrast, true β-xylosidases have been purified and characterized from the thermophilic bacterium *Thermotoga* sp. strain FjSS3-B.1 (Ruttersmith and Daniel 1993). The β-xylosidase from this microorganism is associated with the toga, and it is a homodimer (92 kDa/SU), showing remarkable stability to heat (half-life 2 hours at 80°C) and an optimum of activity at pH 7.0. This enzyme shows high activity with xylobiose, aryl-α-L-arabinofuranoside and aryl-α-L-arabinopyranoside, but it is inhibited by aryl-β-D-xylopyranoside. Interestingly, the β-glucosidase from *Thermotoga* sp. strain FjSS3-B.1 is active on β-D-xylopyranoside, but not on xylobiose, and it differs from the β-xylosidase in its structural properties (Ruttersmith and Daniel 1993).

Other β-xylosidases that are active between 50°C and 100°C and at pH values from 4.0 to 9.0 have been detected in the crude extract of *T. maritima, T. neapolitana*, and *T. thermarum* (Sunna et al. [in press]).

α-L-Arabinofuranosidase (EC 3.2.1.55)

α-Arabinofuranosidases are active against branched arabinoxylans, arabinose-substituted xylooligosaccharides, and *p*-nitrophenyl-α-L-arabinofuranoside. The hydrolysis of arabinoxylans with α-arabinofuranosidase results in the release of arabinose residues. An α-arabinofuranosidase from *Thermotoga* sp. strain FjSS3-B.1 has been purified to homogeneity and characterized (Ruttersmith et al. 1992). The enzyme has a molecular mass of 92 kDa and is thermostable at 98°C. The enzyme displayed high activity towards xylobiose and is also classified as a β-xylosidase.

Also, thermostable α-arabinofuranosidase activities have been detected in the crude extracts of *T. maritima*, *T. neapolitana,* and *T. thermarum* when grown on xylan as the carbon source (Sunna et al. 1994; Sunna et al. [in press]). The enzymes from *T. maritima* and *T. neapolitana* are optimally active at temperatures between 80°C and 90°C and pH values between 5.0 and 6.0. On the other hand, the α-arabinofuranosidase from *T. thermarum* is less thermoactive, showing optimal activity at 70°C and pH 6.0.

Cellulose-degrading enzymes

Cellulose is the most abundant organic biopolymer in nature. Cellulose consists of up to 14000 glucose units, linked together by β-1,4-glycosidic bonds. The enzymatic hydrolysis of cellulose to its monomer glucose requires the synergistic action of at least three different enzymes, namely endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase. Cellulases are widespread in fungi and *Bacteria* but interestingly, until now, they have not been found in hyperthermophilic *Archaea*. Table 2 summarizes some properties of selected thermostable cellulose-hydrolyzing enzymes.

Endoglucanase (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4)

This enzyme hydrolyzes cellulose randomly, producing oligosaccharides, cellobiose, and glucose as end-products. Recently, a thermostable cellulase (cellulase I) from *T. maritima* MSB8 has been characterized (Bronnenmeier et al. 1995). The enzyme was purified to homogeneity and has a molecular mass of 27 kDa. The enzyme is optimally active at 95°C and at pH values between 6.0 and 7.5. When incubated without substrate at 95°C, cellulase I exhibits a half-life of about 120 min. The purified cellulase I is active against CM-cellulose and is accordingly classified as an endoglucanase.

Thermotoga neapolitana produces two endoglucanases, Endo A and Endo B, when grown in a medium containing cellobiose as the carbon source (Bok et al. 1994). The purified Endo B exhibits a temperature optimum for activity at 110°C. The enzyme has a remarkable thermostability, with half-lives at 100°C and 106°C of 8 and 2.2 h, respectively. Both enzymes show high specificity towards CM-cellulose.

Cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91)

Cellobiohydrolases are exoglucanases that attack the nonreducing end of cellulose to produce cellobiose. The most thermostable cellobiohydrolase that has been reported so far is the one purified from the thermophilic bacterium *Thermotoga* sp. strain FjSS3-B.1 (Ruttersmith and Daniel 1991). The enzyme has a molecular mass of 36 kDa and shows maximal activity at 105°C and pH values between 6.8 and 7.8. At 108°C and 113°C the cellobiohydrolase exhibits half-lives of 70 and 7 min, respectively. Thermostability is not enhanced by the addition of glycerol, sorbitol, CM-cellulose, or $CaCl_2$. However, in the presence of NaCl (0.8 M) at temperatures of 108°C and 113°C the half-lives of the enzyme increased almost twofold. The cellobiohydrolase from *Thermotoga* sp. strain FjSS3-B.1 is active against amorphous cellulose and CM-cellulose, with the main hydrolysis products being cellobiose.

Bronnenmeier et al. (1995) reported the purification of a thermostable cellobiohydrolase from *T. maritima* MSB8. The enzyme has a molecular mass of 29 kDa and an optimal activity at 95°C and pH 6.0–7.5. In the absence of substrate, at 95°C, cellobiohydrolase retains 50% of its initial activity after 120 min. The enzyme is able to hydrolyze Avicel, CM-cellulose, β-glucan, and *p*-nitrophenyl-β-D-cellobioside. The

main products resulting from the hydrolysis of Avicel are cellobiose and cellotriose.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)

β-Glycosidases catalyse the hydrolysis of *O*-glycosidic bonds with β-anomeric configuration in disaccharides or between a saccharide and an aglycon group. Different classes of enzymes are able to cleave β-glycosidic bonds: (1) phospho-β-glucosidases and -galactosidases, specific for phosphorylated substrates; (2) typical β-galactosidases, relatively specific for β-galactosides; and (3) β-glucosidases. The latter group contains β-glycosidases, mostly microbial, with rather broad specificity toward β-glucosides, galactosides, fucosides, and xylosides; and cellobiases, the enzymes hydrolyzing cellobiose to glucose in the last step of cellulose degradation. Cellobiose-degrading enzymes are often described as the least-stable member of the cellulolytic enzyme system and this is one of the reasons for the noticeably widespread expectation that these enzymes from hyperthermophiles will find important applications.

High levels of β-glucosidase activity (5% of total cell protein) were found in *P. furiosus* cells grown on cellobiose (Kengen et al. 1993). Interestingly, two enzymes contribute, to different extents, to the total activity found in the cell extract. The major β-glucosidase activity isolated in *P. furiosus* (Pfuβ-gly) is optimally active at 102°C–105°C and is very thermostable, with half-lives of 3.5 days and 13 hours at 100°C and 110°C, respectively. Pfuβ-gly is a tetramer of 230 kDa (58 kDa/SU); it shows broad substrate specificity, hydrolyzing aryl-galactoside, -xyloside, and -mannoside substrates; and it does not require metal ions for activity. The enzyme is unable to produce glucose from cellulose or CM-cellulose, but remarkable activity is found on disaccharides such as cellobiose and salicin. The enzyme is intracellular and, since the organism is unable to grow on glucose, cellobiose is expected to be transported into the cell. The gene coding for Pfuβ-gly has recently been isolated and expressed in *E. coli* (Voorhorst et al. 1995); its transcription and translation initiation sites have been determined. The organization of the gene indicates that it is transcribed in a single unit and no genes coding for hypothetical sugar transporters could be found.

β-Glucosidase has been detected in different Sulfolobales species such as *S. acidocaldarius* DSM 639; *S. solfataricus* MT4 and P2, and *S. shibatae* B12 (Grogan 1991) and a gene for a putative β-galactosidase from *S. solfataricus* DSM 1616 has been cloned by directed expression in *E. coli* (Little et al. 1989). Interestingly, further studies indicated that the true source of this gene was a *S. acidocaldarius* strain of unknown origin (Grogan 1991). Among these enzymes, the only ones that were purified to homogeneity were those from *S. solfataricus* strains. The β-glycosidase from *S. solfataricus* MT4 strain (Ssβ-gly) was originally classified as a β-galactosidase (Pisani et al. 1990), but further biochemical characterization demonstrated a broad substrate specificity (Nucci et al. 1993). This is in agreement with the results previously found for the strain P2 (Grogan 1991): the β-galactosidase and the β-glucosidase activities reside on the same enzyme. Hence, MT4 and P2 strains are closely related, and the corresponding enzymes show similar characteristics.

The native molecular mass of Ssβ-gly is 240 kDa, being a tetramer of 56 kDa identical subunits, and it does not need any cofactors for activity. Aryl-glycosides are the preferred substrates: the enzyme hydrolyzes aryl-glucosides, -fucosides, -galactosides, and -xylosides. Noteworthy, its catalytic efficiency is β-1,3 > β-1,4 > β-1,6, and cellotetraose > cellotriose > cellobiose, for glucose dimers and oligomers, respectively. Cellopentaose, the longest oligosaccharide tested, is hydrolyzed less efficiently. Ssβ-gly, like other enzymes from hyperthermophiles, shows noticeable resistance to heat and other common protein denaturants (alcohols, detergents, etc.), and enhanced activity at temperatures up to 85°C (Pisani et al. 1990). The gene coding for Ssβ-gly (*lacS*) has been cloned and overexpressed in *Saccharomyces cerevisiae* and *E. coli*, and its organization in the *S. solfataricus* genome was determined (Cubellis et al. 1990; Moracci et al. 1992, 1995; Prisco et al. 1994). A gene encoding a putative membrane protein homologous to the major facilitator superfamily was found with the same orientation upstream from *lacS* (Prisco et al. 1994). Each gene has its own archaeal consensus promoter, but the terminator sequences between them are absent. Northern blot analysis of RNA prepared from *S. solfataricus* reveals transcripts of the dimension expected for the two genes; however, they show different relative abundances, and no larger transcripts are evident. Hence, the putative permease-encoding gene is transcribed in vivo, but it is still not clear if these two genes are cotranscribed and/or regulated in a proper operon.

The physiological role of these β-glycohydrolases in *Archaea* is still obscure: growth on cellulose has never been reported. Furthermore, typical cellulolytic enzymes, such as endoglucanases and cellobiohydrolases, are unknown in *Archaea*; presumably, other cellulolytic organisms should provide cellobiose in vivo. *Sulfolobus solfataricus* P2 and MT4 strains can grow on cellobiose as a source of carbon and energy, but this does not significantly induce the enzyme (Grogan 1991). In *P. furiosus*, the mechanism used by cells growing on cellobiose to regulate the expression of the enzyme is still unknown. In future, the availability of well-established molecular genetic techniques in hyperthermophilic *Archaea* will certainly improve these studies in vivo.

Among the enzymatic activities typical of the cellulolytic system that have been reported from Thermotogales, there are several β-glucosidases. In *T. maritima* strain MSB8, one β-galactosidase and two β-glucosidases were found (Gabelsberger et al. 1993). Unlike the specific β-glucosidase (BglB), the β-glucosidase (BglA) shows a broad substrate specificity. β-Galactosidase (BgaA) and β glucosidase (BglA) were studied by direct expression in *E. coli*, and biochemically characterized. BglA and BgaA are both dimers of 95 and 120 kDa, respectively; the BglA enzyme hydrolyzes aryl-glucoside, -galactoside, and -fucoside substrates and several disaccharides such as cellobiose, lactose,

arbutin, and salicin, while BgaA is active on aryl-galactosides and lactose substrates. These enzymes show similar optimal temperatures for activity, but different optimal pHs: 6.2 and 5.3 for BglA and BgaA, respectively. Recently, the coding sequence of BglA was reported (Liebl et al. 1994; Moore et al. 1994).

The β-glucosidase from *Thermotoga* strain FjSS3-B.1 (Ruttersmith and Daniel 1993) shows a broad specificity by hydrolysing aryl-glucosides, -fucosides, and -xylosides, and β-linkages in the order β-1,2 > β-1,6 > β-1,4 in disaccharides. This enzyme resembles BglA from strain MSB8, but the molecular cloning of the gene would allow a better structural comparison of these enzymes. Interestingly, in FjSS3-B.1 strain, a second β-glucosidase activity was detected.

Several enzymatic activities involved in carbohydrate degradation have been identified in *T. neapolitana*, strain Z2706-MC24, by directly cloning and expressing their genes in *E. coli* (Dakhova et al. 1993). Along with amylolytic, endo- and exo-glucanase, laminarase, and xylanase activities, two classes of clones expressing β-glucosidase activities were isolated: one more active on cellobiose, and one on aryl-glucosides. However, no further biochemical information is available on these enzymes.

Molecular cloning of the genes encoding hyperthermophilic archaeal and bacterial β-glucosidases allowed their classification into glycosyl hydrolase families based on amino acid sequence similarities. Bacterial β-glucosidase (*T. maritima* MSB8) and archaeal β-glucosidase (*P. furiosus*), β-glycosidase (*S. solfataricus* MT4), and β-galactosidase (*S. solfataricus* DSM 1616, putative *S. acidocaldarius*) were placed in family 1 along with bacterial β-glycosidases, 6-phospho-β-glucosidases and -galactosidases, cyanogenic β-glycosidases, plant myrosinases, and mammalian gut lactases. Archaeal β-glycosidases show the highest similarity scores (approximately 80%), and much lower scores with enzymes from other domains (Moracci et al. 1994; Voorhorst et al. 1995). Interestingly, lower similarity is found also between archaeal enzymes and *T. maritima* BglA, suggesting that the adaptation to high temperatures followed different strategies in different domains. Sequence motifs surrounding the active site found in these enzymes are common to glycosyl hydrolase families 2, 5, 10, and 17 (Jenkins et al. 1995). Furthermore, by hydrophobic cluster analysis, an even wider structural conservation among glycosyl hydrolases was observed (Henrissat et al. 1995). Family 1 enzymes catalyse the hydrolysis of substrates through a covalent intermediate and without the help of cofactors, whereas family 2 enzymes require Mg^{2+} ions for full activity: this confirms the biochemical data collected so far. Recently, the three-dimensional structures of both family 1 and family 2 mesophilic enzymes have became available (Jacobson et al. 1994; Barret et al. 1995; Wiesmann et al. 1995), and *S. solfataricus* MT4 β-glycosidase has been crystallised (Pearl et al. 1993). When the structures of hyperthermophilic glycosyl hydrolases become available, their detailed inspection and comparison with those of mesophilic homologous enzymes would certainly throw some light on the origins of thermal stability and activity.

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References

Andrade CM, Morana A, de Rosa M, Antranikian G (1996) Production and characterization of amylolytic and xylanolytic enzymes from the hyperthermophilic archaeon *Pyrodictium abyssi*. First International Congress on Extremophiles, Estoril, Portugal, 2–6 June 1996, Book of Abstracts: 98

Antranikian G (1992) Microbial degradation of starch. In: Winkelmann G (ed) Microbial degradation of natural products. VCH, Weinheim, pp 27–56

Antranikian G, Rüdiger A, Canganella F, Klingeberg M, Sunna A (1995) Biodegradation of polymers at temperatures up to 130°C. J M S – Pure Appl Chem A32:661–669

Barret T, Suresh CG, Tolley SP, Dodson EJ, Hughes MA (1995) The crystal structure of a cyanogenic β-glucosidase from white clover, a family 1 glycosyl hydrolase. Structure 3:951–960

Bok JD, Goers SK, Eveleigh DE (1994) Cellulase and xylanase systems of *Thermotoga neapolitana*. ACS Symp Ser 566:54–65

Bronnenmeier K, Kern A, Liebl W, Staudenbauer WL (1995) Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. Appl Environ Microbiol 61:1399–1407

Brown SH, Kelly RM (1993) Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. Appl Environ Microbiol 59:2614–2621

Brown SH, Costantino HR, Kelly RM (1990) Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 56:1985–1991

Canganella F, Andrade CM, Antranikian G (1994) Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new *Fervidobacterium* species. Appl Microbiol Biotechnol 42:239–245

Chung YC, Kobayashi T, Kanai H, Akiba T, Kudo T (1995) Purification and properties of an extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Appl Environ Microbiol 61:1502–1506

Costantino HR, Brown SH, Kelly RM (1990) Purification and characterization of an α-glucosidase from hyperthermophilic Archaebacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105°C to 115°C. J Bacteriol 172:3654–3660

Cubellis MV, Rozzo C, Montecucchi P, Rossi M (1990) Isolation and sequencing of a new β-galactosidase-encoding archaebacterial gene. Gene 94:89–94

Dakhova ON, Kurepina NE, Zverlov VV, Svetlichnyi VA, Velikodvorskaya GA (1993) Cloning and expression in *Escherichia coli* of *Thermotoga neapolitana* genes coding for enzymes of carbohydrate substrate degradation. Biochem Biophys Res Commun 194:1359–1364

Dwivedi PP, Gibbs MD, Saul DJ, Bergquist PL (1996) Cloning, sequencing and overexpression in *Escherichia coli* of a xylanase gene, *xynA*, from the themophilic bacterium *Caldicellulosiruptor* Rt8B.4. Appl Microbiol Biotechnol 45:86–93

Fukusumi S, Kamizono A, Horinouchi S, Beppu T (1988) Cloning and nucleotide sequence of a heat-stable amylase gene from an anaerobic thermophile, *Dictyoglomus thermophilum*. Eur J Biochem 174:15–21

Gabelsberger J, Liebl W, Schleifer KH (1993) Cloning and characterization of β-galactoside and β-glucoside hydrolysing enzymes of *Thermotoga maritima*. FEMS Microbiol Lett 109:131–138

Gibbs MD, Reeves RA, Bergquist PL (1995) Cloning, sequencing, and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1 and activity of the enzyme on fiber-bound substrate. Appl Environ Microbiol 61:4403–4408

Grépinet O, Chebrou MC, Béguin P (1988) Nucleotide sequence and deletion analysis of the xylanase gene (*xynZ*) of *Clostridium thermocellum*. J Bacteriol 170:4582–4588

Grogan DW (1989) Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. J Bacteriol 171:6710–6719

Grogan DW (1991) Evidence that β-galactosidase of *Sulfolobus solfataricus* is only one of several activities of a thermostable β-D-glycosidase. Appl Environ Microbiol 57:1644–1649

Haseltine C, Rolfsmeier M, Blum P (1996) The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J Bacteriol 178:945–950

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarity. Biochem J 280:309–316

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc Natl Acad Sci USA 92:7090–7094

Jacobson RH, Zhang X-J, DuBose RF, Matthews BW (1994) Three-dimensional structure of β-galactosidase from *E. coli*. Nature 369:761–766

Jaenicke R (1993) Structure–function relationship of hyperthermophilic enzymes. ACS Symp Ser 516:53–67

Jenkins J, Lo Leggio L, Harris G, Pickersgill R (1995) β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold β/α architecture and with two conserved glutamates near the carboxy-terminal ends of β-strands four and seven. FEBS Lett 362:281–285

Kengen SWM, Luesink EJ, Stams AJM, Zehnder AJB (1993) Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Eur J Biochem 213:305–312

Kim CH, Nashiru O, Ko JH (1996) Purification and biochemical characterization of pullulanase type I from *Thermus caldophilus* GK-24. FEMS Microbiol Lett 138:147–152

Kobayashi Y, Motoike M, Fukusumi S, Ohshima T, Saiki T, Beppu T (1988) Heat-stable amylase complex produced by a strictly anaerobic and extremely thermophilic bacterium, *Dictyoglomus thermophilum*. Agric Biol Chem 52:615–616

Koch R, Zablowski P, Sprinat A, Antranikian G (1990) Extremely thermostable amylolytic enzyme from the archaebacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 71:21–26

Koch R, Spreinat A, Lemke K, Antranikian G (1991) Purification and properties of a hyperthermoactive α-amylase from the archaebacterium *Pyrococcus woesei*. Arch Microbiol 155:572–578

Ladermann KA, Asada K, Uemori T, Mukai H, Taguchi Y, Kato I, Anfinsen CB (1993a) α-Amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 268:24402–24407

Ladermann KA, Davis BR, Krutzsch HC, Lewis MS, Griko YV, Privalov PL, Anfinsen CB (1993b) The purification and characterization of an extremely thermostable α-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 268:24394–24401

Lama L, Nicolaus B, Trincone A, Morzillo P, Calandrelli V, Gambacorta A (1991) Thermostable amylolytic activity from *Sulfolobus solfataricus*. Biotech Forum Europe 8:201–203

Lee YE, Lowe SE, Henrissat B, Zeikus JG (1993) Characterization of the active site and thermostability regions of endoxylanase from *Thermoanaerobacterium saccharolyticum* B6A-RI. J Bacteriol 175:5890–5898

Leuschner C, Antranikian G (1995) Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J Microbiol Biotechnol 11:95–114

Liebl W, Gabelsberger J, Schleifer KH (1994) Comparative amino acid sequence analysis of *Thermotoga maritima* β-glucosidase (BglA) deduced from the nucleotide sequence of the gene indicates distant relationship between β-glucosidases of the BGA family and other families of β-1,4-glycosyl hydrolases. Mol Gen Genet 242:111–115

Linke B, Rüdiger A, Wittenberg G, Jorgensen PL, Antranikian G (1992) Production of heat-stable pullulanase and α-glucosidase from the extreme thermophilic archaeon *Pyrococcus woesei*. DECHEMA Biotech Conf 5:161–163

Little S, Cartwright P, Campbell C, Prenneta A, McChesney J, Mountain A, Robinson M (1989) Nucleotide sequence of a thermostable β-galactosidase from *Sulfolobus solfataricus*. Nucleic Acids Res 17:7980

Mathrani IM, Ahring BK (1992) Thermophilic and alkalophilic xylanases from several *Dictyoglomus* isolates. Appl Microbiol Biotechnol 38:23–27

Moore JB, Markiewicz P, Miller JH (1994) Identification and sequencing of the *Thermotoga maritima lacZ* gene, part of a divergently transcribed operon. Gene 147:101–106

Moracci M, La Volpe A, Pulitzer JF, Rossi M, Ciaramella M (1992) Expression of the thermostable β-galactosidase gene from the archaebacterium *Sulfolobus solfataricus* in *Saccharomyces cerevisiae* and characterization of a new inducible promoter for heterologous expression. J Bacteriol 174:873–882

Moracci M, Ciaramella M, Nucci R, Pearl LH, Sanderson I, Trincone A, Rossi M (1994) Thermostable β-glycosidases from *Sulfolobus solfataricus*. Biocatalysis 11:89–103

Moracci M, Nucci R, Febbraio F, Vaccaro C, Vespa N, La Cara F, Rossi M (1995) Expression and extensive characterisation of a β-glycosidase from the extreme thermoacidophilic archaeon *Sulfolobus solfataricus* in *Escherichia coli*: authenticity of the recombinant enzyme. Enzyme Microb Technol 17:992–997

Nucci R, Moracci M, Vaccaro C, Vespa N, Rossi M (1993) Exoglucosidase activity and substrate specificity of the β-glycosidase isolated from the extreme thermophile *Sulfolobus solfataricus*. Biotechnol Appl Biochem 17:239–250

Pearl LH, Hemmings AM, Nucci R, Rossi M (1993) Crystallisation and preliminary X-ray analysis of the β-galactosidase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. J Mol Biol 229:558–560

Piller K, Daniel RM, Petach HH (1996) Properties and stabilization of an extracellular α-glucosidase from the extremely thermophilic archaebacteria *Thermococcus* strain AN1: enzyme activity at 130°C. Biochim Biophys Acta 1292:197–205

Pisani FM, Rella R, Raia CA, Rozzo C, Nucci R, Gambacorta A, De Rosa M, Rossi M (1990) Thermostable β-galactosidase from the archaebacterium *Sulfolobus solfataricus* – purification and properties. Eur J Biochem 187:321–328

Prisco A, Moracci M, Rossi M, Ciaramella M (1994) A gene encoding a putative membrane protein homologous to the major facilitator superfamily of transporters maps upstream of the β-glycosidase gene in the archaeon *Sulfolobus solfataricus*. J Bacteriol 177:1614–1619

Robinson KA, Robb FT, Schreiner HJ (1994) Isolation of maltose-regulated genes from the hyperthermophilic archaeum, *Pyrococcus furiosus*, by subtractive hybridization. Gene 148:137–141

Rolfsmeier M, Blum P (1995) Purification and characterization of a maltase from the extremely thermophilic crenarchaeote *Sulfolobus solfataricus*. J Bacteriol 177:482–485

Rüdiger A, Jorgensen PL, Antranikian G (1995a) Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl Environ Microbiol 61:567–575

Rüdiger A, Sunna A, Antranikian G (1995b) Polysaccharides-degrading enzymes. In: Drauz H, Waldmann H (eds) Enzyme catalysis in organic synthesis. VCH, Weinheim, pp 316–339

Ruttersmith LD, Daniel RM (1991) Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1. Biochem J 277:887–890

Ruttersmith LD, Daniel RM (1993) Thermostable β-glucosidase and β-xylosidase from *Thermotoga* sp. strain FjSS3-B.1. Biochim Biophys Acta 1156:167–172

Ruttersmith LD, Daniel RM, Simpson HD (1992) Cellulolytic and hemicellulolytic enzymes functional above 100°C. Ann NY Acad Sci 672:137–141

Saul DJ, Williams LC, Grayling RA, Chamley LW, Love DR, Bergquist PL (1990) *celB*, a gene coding for a bifunctional cellulase from the extreme thermophile "*Caldocellum saccharolyticum*." Appl Environ Microbiol 56:3117–3124

Saul DJ, Williams LC, Reeves RA, Gibbs MD, Bergquist PL (1995) Sequence and expression of a xylanase gene from the hyperthermophile *Thermotoga* sp. strain FjSS3–B.1 and characterization of the recombinant enzyme and its activity on kraft pulp. Appl Environ Microbiol 61:4110–4113

Schumann J, Wrba A, Jaenicke R, Stetter KO (1991) Topographical and enzymatic characterization of amylase from the extremely thermophilic eubacterium *Thermotoga maritima*. FEBS Lett 282:122–126

Simpson HD, Haufler UR, Daniel RM (1991) An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*. Biochem J 277:413–417

Sunna A, Antranikian G (1996) Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. Appl Microbiol Biotechnol 45:671–676

Sunna A, Antranikian G (in press) Xylanolytic enzymes from fungi and bacteria. CRC Crit Rev Biotechnol

Sunna A, Stoffregen T, Antranikian G (1992) Xylan-degrading enzymes from thermophilic bacteria. DECHEMA Biotech Conf 5:97–99

Sunna A, Puls J, Antranikian G (1994) Characterization of the xylanolytic enzyme system of the thermophilic eubacterium *Thermotoga thermarum*. In: Alberghino L, Frontali L, Sensi P (eds) Proceedings of the 6th European Congress on Biotechnology. Elsevier, Amsterdam, pp 267–270

Sunna A, Puls J, Antranikian G (1996) Purification and characterization of two thermostable endo-1,4-β-D-xylanases from *Thermotoga thermarum*. Biotechnol Appl Biochem 24:177–185

Sunna A, Puls J, Antranikian G (in press) Characterization of the xylanolytic enzyme system of the extreme thermophilic anaerobic bacteria *Thermotoga maritima, T. neapolitana*, and *T. thermarum*. Comp Biochem Physiol

Voorhorst WGB, Eggen RIL, Luesink EJ, de Vos WM (1995) Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*. J Bacteriol 177:7105–7111

Wiesmann C, Beste G, Hengstenberg W, Schulz GE (1995) The three- dimensional structure of 6-phospho-β-galactosidase from *Lactococcus lactis*. Structure 3:961–968

Winterhalter C, Liebl W (1995) Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl Environ Microbiol 61:1810–1815

Winterhalter C, Heinrich P, Candussio A, Wich G, Liebl W (1995) Identification of a novel cellulose-binding domain within the multidomain 120-kDa xylanase XynA of the hyperthermophilic bacterium *Thermotoga maritima*. Mol Microbiol 15:431–444

Zverlov V, Piotukh K, Dakhova O, Velikodvorskaya G, Borris R (1996) The multidomain xylanase A of the hyperthermophilic bacterium *Thermotoga neapolitana* is extremely thermoresistant. Appl Microbiol Biotechnol 45:245–247