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Very stable enzymes from extremely thermophilic archaebacteria and eubacteria

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Summary. Thirty-six thermophilic archaebacteria and nine extremely thermophilic eubacteria have been screened on solid media for extracellular amylase, protease, hemicellulase (xylanase), cellulase, pectinase and lipase activities. Extracellular enzymes were detected in 14 archaebacteria belonging to three different orders. Twelve of these were able to degrade starch and casein and the two *Thermofilum* strains were able to degrade starch, xylan and carboxymethylcellulose. Three of the eubacteria could degrade only starch. The other six (including four *Thermotoga* strains) all had activity against starch, xylan and carboxymethylcellulose, and one also had activity against casein. Some of the amylolytic archaebacteria released α-glucosidase, β-glucosidase, amylase and transglucosylase activities into liquid media containing starch or maltose. *Thermotoga* strain FjSS3B.1 released amylase, xylanase, cellulase and β-glucosidase activities into the medium when grown in the presence of substrates. When the partially purified enzymes from *Thermotoga* and some of the archaebacteria were compared with known thermostable enzymes the majority were found to be the most thermostable of their type. The β-glucosidase, xylanase and cellulase from *Thermotoga* and two α-glucosidases, a β-glucosidase, an amylase and a pullulanase from archaebacteria all have half-lives of at least 15 min at 105° C.

Introduction

The rapid growth of the enzyme industry reflects the advantages of using enzymes as industrial catalysts, and the advantages (real and potential) of using enzymes from thermophiles have been well documented (Zeikus 1979; Sonnleitner and Fiechter 1983; Brock 1985; Daniel et al. 1986). The properties which allow thermostable enzymes to withstand high temperatures also confer resistance to denaturing agents (Fujita et al. 1976; Cowan and Daniel 1982), solvents (Zaks and Klibanov 1984) and proteolytic enzymes (Daniel et al. 1982). In general the higher the growth temperature of the organism the more stable are its enzymes (Singleton and Amelunxen 1973; Langworthy et al. 1979), and extracellular enzymes are more stable than intracellular enzymes. The most stable enzymes are therefore likely to be extracellular enzymes from the most extreme thermophiles.

To date, the highest growth temperature for an organism is 110° C for the archaebacterium *Pyrodictium brockii* (Stetter et al. 1983). Until recent reports of the eubacterium *Thermotoga maritima* growing optimally at 80°-85° C (Huber et al. 1986; Huser et al. 1986) all organisms known to grow optimally above 75° C were archaebacteria. The extremely thermophilic archaebacteria are therefore likely sources of the most stable enzymes. Little is known of their extracellular enzymes, although Cowan et al. (1987) have reported an extremely thermostable extracellular protease from an extremely thermophilic archaebacterium.

In this paper we report the screening for extracellular enzymes of a number of strains of extremely thermophilic archaebacteria isolated from thermal areas in and around New Zealand. Data is presented on the relative thermal stabilities of the enzymes.

Materials and methods

Organisms, media and screening. The organisms used in this survey, their sources and the growth conditions are listed in

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Table 1. Organisms, their sources and the growth conditions used in this study

Organisms		Source	Growth conditions		
			pH	Temperature	Medium
Archaebacteria					
Sulfolobus acidocaldarius		DSM639	2.5	70° C	DSM88
S. acidocaldarius (type 7)		Japan	3.0	75° C	DSM88
Strain WhIlS.1		NZ	3.0	75° C	DSM88
S. solfataricus	T	DSM1616	4.0	80° C	DSM182
Acidianus brierleyi		DSM1651	2.0	70° C	DSM150
Pyrodictium brockii	T	DSM2708	5.5	95° C	DSM283
Thermoplasma acidophilum	T	DSM1728	1.8	60° C	DSM158
Thermoproteus tenax Kra 1	T	DSM2078	5.8	88° C	DSM184[a]
T. tenax H_3		W. Zillig	5.8	88° C	DSM184[a]
Local isolates (15 strains)		NZ and Fiji	6.8	88° C	DSM184[a]
Thermophilum (2 strains)		NZ	6.0	88° C	DSM184[a]
Desulfurococcus mobilis	PT	DSM2161	5.8	88° C	DSM184[a]
D. mucosus	PT	DSM2162	5.8	88° C	DSM184[a]
Local isolates (6 strains)		NZ and Fiji	6.0	88° C	DSM184[a]
Thermococcus celer	T	DSM2476	5.8	80° C	DSM266
Strain AN1 (NZ isolate)		DSM2770	7.3	75° C	AN1 Medium
Thermophilic eubacteria					
Thermotoga (4 strains)		NZ and Fiji	7.0	80° C	YEmm
Strain TP8T6.3.3.1		NZ	7.2	70° C	DSM184[a]
Glycolytic anaerobes (4 strains)		NZ	7.2	70° C	DSM144[b]

[a] For solid media the sulphur was replaced by cystine·HCl 0.4 g/l
[b] The tryptone and glucose was omitted and 0.2% w/v starch added

Abbreviations used: T, type strain; PT, proposed type strain; NZ, New Zealand; DSM, Deutsche Sammlung von Mikroorganismen, YEmm and AN1, see Materials and methods

Table 1. Each organism was reconstituted from freeze-dried cells using the medium and temperature recommended by the Deutsche Sammlung von Mikroorganismen (DSM) or the researcher who made the isolation.

YEmm medium contained (per litre): $(NH_4)_2SO_4$, 0.65 g; $CaCl_2 \cdot 2H_2O$, 0.074 g; $MgSO_4 \cdot 7H_2O$, 0.28 g; KH_2PO_4, 0.28 g; NaCl, 7.0 g; resazurin, 0.001 g; $Na_2S \cdot 9H_2O$, 0.24 g; yeast extract (Merck, USA), 5.0 g; 3-(N-morpholino) propane sulphonic acid, 4.0 g; trace elements (Brock et al. 1972) 1 ml; vitamin solution (Wolin et al. 1963) 1 ml. The pH was adjusted to 7.0 and the vessels filled in an anaerobic chamber, then autoclaved.

AN1 medium contained (per litre): trypticase peptone, 8 g; NaCl, 2.5 g; K_2HPO_4, 1.5 g; sodium thioglycollate, 1.0 g; sulphur (cystine·HCl for solid medium), 0.6 g. Sulphur was added after autoclaving. Other ingredients were made up to 1 l and adjusted to pH 7.3, autoclaved, the sulphur added, and vessels filled anaerobically.

For screening, the organisms were streaked onto solid media incorporating the substrate at 0.1% w/v (0.5% w/v for butter). Gelrite (1.1% w/v, Kelco, Merck and Co, USA) plus $MgSO_4 \cdot 7H_2O$ (0.1% w/v) was used for solid media (Chi and Casida 1984). When preparing the high ionic strength solid media for *Thermococcus* and the acidic media for *Sulfolobus* and *Thermoplasma* it was necessary to autoclave double strength Gelrite and double strength media separately, mixing immediately before pouring and using small volumes which could be handled quickly. After incubation for sufficient time to produce moderate to heavy growth the plates were examined for zones of degradation of substrate, using appropriate staining if necessary to reveal the zones. Negative controls of uninoculated medium and medium without the substrate added, and positive controls of known positive organisms or purified enzymes were included.

Staining. For media containing casein and butter no staining was required as clear zones were easily observed in the opaque medium. For media containing starch the plates were flooded with aqueous I_2/KI solution (I_2, 1g; KI, 2 g/300 ml) for 15 min. Colourless zones in a deep blue background indicated starch degradation (Hopkins and Bird 1954). Plates of media containing carboxymethylcellulose and xylan were flooded with aqueous Congo Red (0.1% w/v, BDH, UK) for 30 min, then destained for 15 min with 1 *M* NaCl (Teather and Wood 1982). Clear zones indicating degradation appeared against a red (or purplish) background. The pectin- and pectate-containing media were flooded with aqueous Ruthenium Red (0.2% w/v, BDH) for 60 min, then destained for 15 min with distilled water. Pectin methyl esterase activity produced darker red staining against a pink background on the pectin substrate, and polygalacturonase activity produced clear zones against a red background on the pectate substrate (Hagerman et al. 1985).

Enzyme assays. All assays were carried out at 75° C in 0.1 M 2(N-morpholino)ethane sulphonic acid (MES) buffer adjusted to pH 6.2 at 75° C and containing 2 m*M* $CaCl_2$. Carboxymethylcellulase and xylanase activities were determined by measuring the production of reducing sugar using *p*-hydroxyben-

zoic acid hydrazide (Lever 1973). The α- and β-glucosidase activities were determined by following the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-β-D-glucopyranoside respectively, as in Patchett et al. (1987).

Amylase and transglucosylase activities were determined by the dextrinogenic assay of Pfueller and Elliot (1969). The substrate was 0.02% Lintners starch, with the addition of 0.25% D-glucose for the transglucosylase assay. In the presence of amylase the transglucosylase activity is taken as the difference in the reduction in blue colour of the starch-iodine complex in the presence and absence of glucose.

Partial purification of enzymes. Cells were removed from well-grown cultures (4 l) using hollow fibre filtration (Amicon, USA). The resulting cell-free broth was concentrated 100-fold and dialysed using an ultrafiltration apparatus (YM10 membrane, Amicon). The concentrate was applied to a column of DEAE-Sepharose (25 ml) equilibrated in 20 m*M* MES, pH 6.2. Under these conditions the enzyme activities of interest remained bound to the column and were subsequently eluted using a gradient of 0–1.0 *M* NaCl (150 ml). Fractions (2 ml) were collected and those containing enzyme activity were pooled appropriately and used for subsequent assays and thermal inactivation studies.

Thermal inactivation. Aliquots of enzyme (100 μl) were incubated in an oil bath for set intervals using polypropylene micro-tubes with tight-fitting caps. After heating, the tubes were stored in ice for up to 20 min so that all samples could be assayed for remaining enzyme activity at the same time.

Sonication. The cells were collected from 1 ml culture by centrifugation, washed, and resuspended in assay buffer. The resuspended cells were kept on ice and disrupted using the needle probe on the Dynatech sonic dismembranator Model 300 (Artek Systems Corp, USA) at full power for 2 min.

Results and discussion

We have screened thirty-six thermophilic archaebacteria and nine extremely thermophilic eubacteria (all anaerobes) for the secretion of extracellular protease, amylase, cellulase, hemicellulase (xylanase), pectinase and lipase. The archaebacteria comprised seven type (or proposed type) strains, three other overseas isolates and twenty-six isolates from thermal areas in New Zealand and Fiji. The eubacteria comprised one isolate from Fiji and eight isolates from New Zealand. No evidence was found for the production of pectinase or lipase by any of the organisms tested. Extracellular enzymes were produced by 14 of the archaebacteria and all nine of the eubacteria tested (Table 2).

Extracellular enzymes were found in three different orders of archaebacteria, the *Sulfolobales, Thermoproteales* and *Thermococcales. Sulfolobus solfataricus* and *S. acidocaldarius* (DSM639) were both proteolytic and amylolytic. However, *S. acidocaldarius* (type 7), the local isolate WhI1S.1,

Table 2. Production of extracellular enzymes

Organisms	Extracellular enzymes			
	Protease	Amylase	Cellulase	Xylanase
Archaebacteria				
S. acidocaldarius (DSM639)	†	†	–	–
S. acidocaldarius (type 7)	–	–	–	–
WhI1S.1	–	–	–	–
S. solfataricus	†	†	–	–
A. brierleyi	–	–	–	–
P. brockii	–	–	–	–
Thermoplasma acidophilum	–	–	–	–
Thermoproteus (17 strains)	–	–	–	–
Thermofilum (2 strains)	–	†	†	†
Desulfurococcus (8 strains)	†	††	–	–
Thermococcus (2 strains)	†	††	–	–
Thermophilic eubacteria				
Thermotoga (4 strains)	–	††	†	†
Glycolytic anaerobes (3 strains)	–	††	–	–
Strain TP8T6.3.3.1	–	††	††	††
Strain Rt381B.B.1	††	†	†	†

After incubation of organisms on solid media to produce moderate to heavy growth, plates were examined for zones of degradation of the substrate, using appropriate staining if necessary (see Materials and methods): –, no zones of clearing around colonies; †, small zones of clearing around colonies; ††, large zones of clearing around colonies

and *Acidianus brierleyi* (formerly grouped with *Sulfolobus*) were all unable to degrade any of the substrates. Neither *Thermoplasma acidophilium* nor *Pyrodictium brockii* showed any activity against the substrates.

The order *Thermoproteales* is widely represented in thermal areas in New Zealand and all isolates have growth optima between 85° C and 90° C (Jasperse-Herst 1984; Patel et al. 1986). No extracellular enzymes were detected from any of the 17 *Thermoproteus* strains tested. Cellulolytic, xylanolytic and amylolytic activites were observed for the two *Thermofilum* isolates. The local isolate, strain AN1, identified only as a sulphur-dependent archaebacterium has been shown to belong with *Thermococcus celer* in the newly described order Thermococcales (Zillig et al. 1987). *Thermococcus celer,* AN1 and all the *Desulfurococcus* strains tested produced strong amylolytic and some proteolytic activity. All these strains required protein or peptides for growth.

Table 2 shows no consistent pattern for the occurrence of extracellular enzymes among archaebacteria. The family *Thermoprotaceae* is the only one where a significant number of isolates have been tested, but since 15 of the 17 strains have been isolated by us from a limited range of habitats in New Zealand, they may show less variation than the family as a whole. The same is true for six of the *Desulfurococcus* isolates tested.

With regard to the eubacteria, four *Thermotoga* strains, isolated from intertidal hot springs by Huser et al. (1986), were all able to degrade starch, xylan and carboxymethylcellulose. Three glycolytic anaerobes which produced large zones of amylolytic activity have been included in a previous study of starch degradation by thermophilic anaerobic bacteria (Plant et al. 1987). Strain Rt 381B.B.1, which is similar to *Fervidobacterium nodosum* described by Patel et al. (1985), showed good proteolytic activity as well as amylolytic, cellulolytic and xylanolytic activities. Strain TP8T6.3.3.1, known from previous studies to be a good cellulase producer (Reynolds et al. 1986), produced strong amylolytic activity plus cellulolytic and xylanolytic activity.

Three archaebacteria, the *Desulfurococcus* strain Tok12S.1 and the two *Thermococci* AN1 and *T. celer,* and one eubacterium, *Thermotoga* strain FjSS3B.1, were chosen for further study on the basis of their high amylolytic activities and their high growth temperatures. When Tok12S.1, AN1 and *T. celer* were grown in liquid media supplemented with either maltose or starch (both at 0.1%, w/v), amylolytic activity was detected in cell-free supernatants after centrifugation of the cultures. The supernatants also contained α-glucosidase, β-glucosidase and transglucosylase activities, but these enzymes were also detected in the cell pellets after sonication. Given this observation and the fact that the α- and β-glucosidase and transglucosylase activites appeared in the cell-free supernatants late in the growth period, it is probable that these are intracellular enzymes released into the medium after cell lysis. Protease production by these isolates was dependent upon the presence of casein (0.1%, w/v) in the medium.

The *Thermotoga* strain FjSS3B.1 produced low levels of extracellular amylolytic, cellulolytic and xylanolytic activities when grown in liquid basal medium. The amounts of these enzymes found in cell-free culture supernatants were, however, greatly increased when the isolate was grown in medium supplemented with starch, xylan and carboxymethylcellulose (all at 0.1%, w/v).

The thermostabilities of some of these enzymes, partially purified using DEAE-Sepharose, are presented in Table 3. The reported thermostabilities of enzymes from other thermophilic organisms are recorded for comparison. The method we have used for assessing thermostability is convenient, but allows for thermostability to be overestimated if renaturation occurs between the heat treatment and the assay. We have not found this to occur in our previous work on thermophiles and we do not believe it has occurred in this work because those enzymes assayed for significant periods at higher temperatures showed faster reaction rates.

It is clear that *Thermotoga* species and the extremely thermophilic archaebacteria are a potentially rich source of a variety of very stable enzymes. However, because of the difficulties involved in growing archaebacteria (particularly the anaerobes) in large quantities or in high yields, no enzymes from these organisms have been prepared in commercial quantities. The fundamental differences between archaebacteria and the well-studied eubacteria may impose serious difficulties in genetic manipulations to improve yields and, to date, no extremely thermophilic archaebacterial cloned genes have been expressed as active enzymes by a host organism. In contrast, *T. maritima* seems likely to be a more commercially viable source of enzymes of comparable thermostability since it grows more rapidly and with greater cell yields than the thermophilic archaebacteria investigated here.

Table 3. Thermal stability (half-lives) of some archaebacterial and eubacterial enzymes at various temperatures

Enzymes	Half-lives (min)			
	80° C	90° C	95° C	105° C
Archaebacteria				
AN1 (DSM 2770)				
α-Glucosidase			>60[a]	>20[a]
β-Glucosidase			10–15	
Pullulanase			>60[a]	15–20
Transglucosylase			10–20	
Tok12S.1 (NZ isolate)				
Protease (Cowan et al. 1987)			70–90	8–9
Amylase				15–25
Transglucosylase				4–9
Thermococcus celer (DSM 2476)				
Protease			35–45	
α-Glucosidase				>20[a]
β-Glucosidase				20–30
S. solfataricus (DSM 1616)				
Protease			35–40	
Amylase			>60[a]	
Thermophilic eubacteria				
Thermotoga strain FjSS3B.1				
Cellulase				15
β-Glucosidase				15–20
Xylanase				>20
Amylase			20–30	
Strain TP8T6.3.3.1				
Cellulase (Schofield et al. 1988)	50			
β-Glucosidase (Schofield et al. 1988)	20			
β-Xylosidase (Schofield et al. 1988)	12			

[a] More than 80% of the initial activity remained after this time

The half-life is the heating time after which 50% of the original activity remained when the sample was cooled, then assayed in a standard assay

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