CURRENT MICROBIOLOGY Vol. 29 (1994), pp. 343–347

Current Microbiology
An International Journal
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Xylanolytic Activities of *Spirochaeta thermophila*

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Abstract. Spirochetes capable of degrading xylan or cellulose have not been commonly isolated, nor have their polysaccharolytic activities been characterized. *Spirochaeta thermophila* strain RI 19.B1 is xylanolytic and grows well at 65°C with oatspelt (OX), birchwood (BX), corncob (CCX-A) xylans, or glucuronoxylan (MGX) as the energy source. All xylans were extensively degraded and utilized during growth. About 72–82% of the initial hexuronic acids and 57–79% of initial pentoses disappeared during growth. *S. thermophila* possessed xylanase, xylosidase, and arabinofuranosidase enzyme activities. Low levels of these activities were detected with growth on glucose, but high expression of these activities occurred during growth on OX. All three activities were cell-associated and were more stable in cells than cell extracts. Xylan-degrading activities were measured with cells or cell extracts exposed (60 min) to a variety of temperatures (65°–85°C) and pHs (5.0–8.0). More than 50% loss of activities occurred at temperatures above 75°C. Although pH stability was affected by buffer, the optimal range was pH 6.5–7.5. These temperature and pH profiles for xylan-degrading activities coincide with those found for the growth of *S. thermophila*.

Spirochetes are a group of bacteria that commonly possess a helical cell shape and are motile by means of periplasmic flagella. Phylogenetic studies with 16s RNA oligonucleotide cataloging methods showed spirochetes to be a phylogenetically valid group of microorganisms of ancient origin and one of the ten major eubacterial phyla to have evolved [15, 16]. Members of the genus *Treponema* are host-associated obligate anaerobes and numerous nonpathogenic *Treponema* species have been isolated from the rumen and colon of domesticated animals [5, 6, 13, 14, 23]. *Spirochaeta* species are free-living anaerobes or facultative anaerobes isolated from marine or freshwater habitats. Isolation of polysaccharolytic strains of these species has not been common, but a few strains isolated from the rumen, such as *T. saccharophilum* [14], can be amylolytic or pectinolytic, but none have been reported to be cellulolytic or xylanolytic. Likewise, these traits have not been found with *Spirochaeta* species isolated from a variety of aquatic habitats.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Recently, anaerobic *Spirochaeta*-like species were isolated from thermal springs and differed from previously described species by being not only thermophilic, but also cellulolytic and xylanolytic [17]. These isolates, now designated as *Spirochaeta thermophila* [1], are of interest in numerous ways. Given the ancient origin of spirochetes, these organisms may possess some of the earliest evolved enzymes for cellulose and xylan degradation. These enzymes and associated genes in spirochetes could be significantly different from homologous ones found with bacteria of separate phylogenetic lines of evolution or possibly a source for horizontal gene transfer to these bacteria. On a practical basis, *S. thermophila* might serve as a source of thermostable xylanases that have value in wood pulping and other commercial uses. The present study was undertaken to determine what xylan-degrading activities are present in *S. thermophila* and to characterize their basic biochemical properties.

Materials and Methods

Strains and growth conditions. *S. thermophila* strains were kindly provided by Hugh W. Morgan (Thermophile and Microbial Biochemistry and Biotechnology Unit, University of Waikato, New

Table 1. Cell yields of *S. thermophilia* on various growth substrates

Substrate	Cell protein (μg/ml)[a]
Glucose	260
Cellobiose	180
Cellulose	170
Arabinose	170
Xylose	200
OX	210
BX	290
MGX	300
CCX-A	140

[a] Maximal levels after 36–48 h growth, 0.4% initial substrate concentration.

Table 2. Utilization of xylans and xylan components by *S. thermophila*

Xylan	Percent utilization[a]		
	Hexuronic acids	Pentoses	Neutral sugars
OX	71.6	78.7	83.0
BX	81.0	62.8	78.2
MGX	83.8	71.1	84.0
CCX-A	81.8	57.4	79.2

[a] Determined after 48 h growth.

Zealand). Strains RI 19.B1 and Z-1203 were grown at 65°C in CBM4 and CMB15 media, respectively [17]. However, both media were supplemented with 0.2% yeast extract and 0.3% Trypticase, L-cysteine HCl was added in place of the sodium sulfide, and the growth substrate was increased to 0.4%. Growth was routinely monitored by optical density (660 nm), and cell yields were quantitated by protein determinations.

Cell suspensions and extracts. Cells were harvested at late log to early stationary phase (ca. 20–26 h) by centrifugation (12,000 *g*, 15 min, 4°C), and the cell pellet was washed once with 50 mM sodium phosphate–1.0% sodium chloride buffer (pH 7.0). Cells were resuspended in 20 mM potassium phosphate buffer (pH 7.0) and held on ice until needed for enzyme assays or other uses. Cell extracts were prepared from cell suspensions concentrated 10- to 20-fold over culture levels. After addition of dithiothreitol to 1 mM, suspensions were passed through a chilled French pressure cell (16,000 psi), centrifuged (10,000 *g*, 15 min, 4°C), and the resulting supernatant fluid was used as the extract.

Enzyme and chemical assays. Unless indicated otherwise, all enzymes were assayed at 65°C incubation temperature, with incubation times of 10–25 min. Xylosidase, arabinofuranosidase, and other glycosidase activities were determined by the release of nitrophenol from *p*-nitrophenyl glycoside substrates [10]. Xylanase activity was determined by release of pentose sugars from soluble oatspelt xylan as described previously [10]. The soluble oatspelt xylan fraction was obtained by heating a 10% oatspelt xylan suspension in water at 45°C for 60 min followed by centrifugation (22,000 *g*, 30 min, 22°C) to remove the particulate matter. Total carbohydrates and pentoses in samples were measured by the phenol sulfuric acid [2] and orcinol [19] methods with glucose and xylose, respectively, as standards. The dimethylphenol method [20] with glucuronic acid as the standard was used to estimate uronic acid concentrations in various samples. After hydrolysis of xylans with trifluoroacetic acid, neutral sugar compositions were determined by gas liquid chromatography of alditol acetate derivatives [22]. Xylans were obtained from Sigma Chemical Company (St. Louis, Mo) or from Megazyme (Sydney, Australia), except for corncob CCX-A, which was provided by Shelby Freer (NCAUR) and was obtained by alkaline extraction of corncobs. Protein levels were determined by the Bradford dye-binding method [4] with commercial reagents (Bio Rad, Richmond, Calif.) with bovine serum albumin as a standard. Whole cells were digested (70°C, 10 min) in 0.1 M NaOH and diluted in water prior to protein assays. One unit of enzyme activity was defined as release of 1 μmole nitrophenol or sugar/h, and specific activity as units/mg protein.

Results

Growth on sugars and xylans. *S. thermophila* strains RI 19.B1 and Z-1205 grew slowly (ca 3.5 h doubling time) on the basal CBM4 and CBM10 media, with maximal growth between 50–60 h. Addition of yeast extract and Trypticase decreased doubling times to ca. 3.0 h and increased cell yields by two- to threefold. Once cultures entered stationary growth phase, spherical cells formed, and cell lysis became evident. Thus, all experimental work was done with cultures in late logarithmic (ca. 48 h) or earlier growth phase. Since initial experiments indicated strain RI 19.B1 grew faster and with better yields than strain Z-1205 and both strains are highly related [1], all subsequent results are from experiments done with strain RI 19.B1 unless indicated otherwise.

Quantitative measurements of growth (Table 1) showed the highest cell yield on sugars with glucose. Both growth rates and yields were lower (25%) with cellobiose or cellulose as the growth substrate. Regardless of their physical and chemical properties, all xylans supported growth. Cell yields greater than those with glucose were observed with the BX and MGX glucuronoxylans. Poor growth occurred with the insoluble corncob CCX-A xylan, and growth with the oatspelt OX xylan was slightly better than that with xylose. The results of other experiments indicated wheat or rye arabinoxylans also supported growth rates and yields that approximated those found with glucose.

Analysis of the xylans remaining after growth indicated xylan components were utilized to varying extents (Table 2). Utilization of pentoses reflected total carbohydrate utilization, as expected since pentoses are the major component (80–90%) of xylans. Neutral sugar analyses indicated about 88–100% of the non-pentose sugars (2–7% xylan weight; mainly glucose and galactose) were utilized during growth,

Table 3. Xylanolytic activities of *S. thermophila* growth on various substrates[a]

Substrate	Enzyme activity[b]		
	Xylanase	Xylosidase	Arabinofuranosidase
Glucose	6.4	7.6	6.1
Xylose	38.5	11.9	4.6
Arabinose	46.1	10.1	6.7
OX	84.9	19.0	14.4

[a] Determined with log phase cultures (20–48 h).
[b] Units/mg protein.

whereas about 54% and 70% of the initial arabinose and xylose, respectively, of OX and BX were used during growth (data not shown). For all xylans, another component that disappeared extensively during growth was the hexuronic acid (Table 2), which is usually in the form of 4-O-methylglucuronic acid in these xylans. However, other experiments showed neither galacturonic or glucuronic acid alone would support growth of *S. thermophila* (data not shown).

Changes in xylanolytic activities with growth substrate. Xylanase, xylosidase, and arabinofuranosidase were detected in cultures of *S. thermophila* grown on any one of a variety of substrates (Table 3). For xylanase, there were 12-fold or greater increases in level and specific activity with growth on OX versus glucose, but only about two- to threefold increases in these values for xylosidase and arabinofuranosidase. The specific activities and levels of these two enzymes usually increased with growth on xylose or arabinose compared with growth on glucose, but were less than those found with OX cultures.

Distribution and stability of xylanolytic activities. Almost all of the xylanase, xylosidase, and arabinofuranosidase activities were found to be cell-associated with growth of strain RI 19B.1 or Z-1205 on OX (Table 4). In many instances, the activities with the cells obtained after centrifugation of the cultures were greater than those measured with whole cultures. However, in stationary phase these activities, particularly xylanase, became increasingly fluid-associated, probably owing to spherical body formation and cell lysis. When buffered cell suspensions were treated with cetyltrimethylammonium bromide (50–100 μg/ml) or Triton X-100 (0.05–0.2%) at 4°C, 22°C, or 65°C, no release of enzymes from cells occurred. There were substantial losses of xylanase and xylosidase activities along with cell lysis at the higher concentrations of the test compound. In other experiments, enzyme activities of cell suspensions stored for 1–5 days on ice were found to be stable, but 50–75% losses were observed with frozen cells thawed in buffer.

Table 4. Distribution of xylanolytic activities in cultures of *S. thermophila*[a]

Strain	Fraction	Enzyme activity[b]		
		Xylanase	Xylosidase	Arabinofuranosidase
RI-19-B-1	Total culture	11.4	3.3	2.0
	Cells	14.6	2.6	1.9
	Fluid	3.1	0.4	0.3
Z1203	Total culture	19.3	5.6	3.6
	Cells	22.7	5.4	3.4
	Fluid	4.2	0.7	0.4

[a] Determined after 20 h growth on OX.
[b] Units/ml culture volume.

Table 5. Temperature stability of xylanolytic activities in cells or extracts of *S. thermophila*[a]

Temperature (°C)	Enzyme activity[b]				
	Xylanase		Xylosidase		Arabinofuranosidase
	Cells	Extract	Cells	Extract	Extract
Control	110.8	40.2	14.7	2.6	3.1
60	103.2	39.4	13.7	2.1	3.1
65	92.4	29.4	13.4	1.0	2.6
70	57.3	23.4	15.3	0.5	2.5
75	56.5	20.2	11.1	0.2	1.4
80	27.2	9.7	3.7	0.1	0.9
85	15.2	7.7	1.2	0.1	0.8

[a] Cells or extract incubated at appropriate temperature for 60 min in potassium–PO_4 buffer (20 mM, pH 7.0) prior to assay.
[b] Units/mg protein.
[c] Cells or extract held on ice and then assayed immediately.

Stability of enzyme activities towards temperature and pH. Incubation of cell suspensions at 65°C for 60 min prior to assay resulted in 10–20% losses in xylanase or xylosidase activity compared with cells held on ice. Incubation at higher temperatures, particularly above 75°C, enhanced loss of activity (Table 5). Measurements made with crude extracts indicated much lower specific activities of these enzymes compared with whole cells and showed greater activity losses, but yielded similar profiles of activity losses with temperature. The arabinofuranosidase activity of crude extracts also declined significantly, particularly at 75°C or higher.

Xylanase stability was significantly affected by both pH and the type of buffer. With cell suspensions, optimal stability and minimal activity losses (less than

Table 6. Effect of pH and buffer on xylanolytic activities in cells or extracts of *S. thermophila*[a]

		Enzyme activity[b]				
		Xylanase		Xylosidase		Arabino-furanosidase
Buffer	pH	Cells	Extract	Cells	Extract	Extract
Control[c]	7.0	111.3	40.1	13.7	2.3	3.2
Citrate-PO_4	5.5	52.0	5.1	8.4	1.8	2.7
Citrate-PO_4	6.0	81.2	6.4	8.2	1.3	2.1
Sodium-PO_4	6.0	78.5	3.1	8.0	0.8	2.7
Sodium-PO_4	6.5	94.5	2.2	8.3	1.2	2.6
Sodium-PO_4	7.0	97.5	5.5	8.8	0.8	2.3
Sodium-PO_4	7.5	51.6	21.3	18.8	0.7	2.4
Tris	7.5	89.3	24.9	10.3	1.2	2.7
Tris	8.0	60.3	3.2	6.6	1.0	2.6

[a] Cells or extract diluted four-to fivefold in appropriate buffer (10 mM) and pH, then incubated for 60 min at 65°C, followed by assays under standard conditions with potassium-PO_4 buffer (50 mM, pH 7.0).
[b] Units/mg protein.
[c] Cells or extract held on ice and then assayed immediately at 65°C.

20%) were around pH 6.5–7.0 with sodium phosphate buffer and about pH 7.5 with Tris buffer (Table 6), whereas other conditions led to about 50% losses in activity. Xylanase activity in crude extracts was quite unstable, with 90% activity losses occurring with most pH and buffer conditions used. However, about 50% of the activity did remain after 60 min at pH 7.5 in Tris or sodium phosphate buffers. Compared with xylanase, xylosidase activity of cells was slightly more stable, and 60% or more of this activity was present in cells held in buffers ranging from pH 5.0 to 7.5 (Table 6). Both xylosidase and arabinofuranoside activities in crude extracts were at relatively low levels. Xylosidase in extracts was most stable at pH 5.0–5.5 in citrate phosphate buffer, but only about 60% of initial activity remained after 60 min with these conditions. Regardless of pH or buffer, about 30–35% of the arabinofuranosidase activity in extracts was lost after 60 min.

Discussion

Spirochaeta thermophila is able to grow on a number of carbohydrates including cellulose and xylans plus hexoses, pentoses, and disaccharides derived from these polysaccharides [17]. Glucose is metabolized through an Emden–Meyerhof–Parnas pathway [11] and, presumably, arabinose or xylose through standard pentose pathways. A variety of xylans support growth of *S. thermophila* (Table 1), resulting in extensive xylan degradation and utilization of the sugar components (Table 2). The organism possesses the major xylan-degrading enzyme activities, namely xylanase, xylosidase, and arabinosidase (Table 3). The large disappearance of uronic acids during growth, particularly with the BX and MGX glucuronoxylans, suggests *S. thermophila* probably has an α-glucuronidase activity to cleave off the 4-O-methylglucuronic acid residues from the xylose backbone. Although not examined, *S. thermophila* might also have other enzymes involved with microbial xylan degradation such as esterases for removal of ester-linked acetic, ferulic, or coumaric acid residues [3, 8, 12].

Expression of xylanolytic activities, especially xylanase, in microorganisms varies greatly, being constitutively expressed in some species such as *Butyrivibrio fibrisolvens* or totally induced by growth on xylans such as with *Cellulomonas* and other species [24, 25]. Xylanolytic activities were highly expressed with *S. thermophila* growth on xylan, and significant xylanase activity was present in cultures grown on xylose or arabinose (Table 3). Only low levels of these enzymes were present with growth on glucose. The results of other experiments showed *S. thermophila* possessed a cell-bound β-glucosidase activity that followed the same pattern of expression, with high levels with growth on cellulose or OX (data not shown). Low-level constitutive expression of hydrolytic enzymes with growth on sugars is common in microorganisms capable of degrading diverse polysaccharides found in plant cell walls [9, 10, 24]. This type of regulation may be an ecological advantage to the microorganism by providing a constant low-level supply of energy substrates during changes in the types of nutrient sources in the environment. These substrates also include oligosaccharides, which may be needed for high-level induction of hydrolytic enzymes [24].

Although *Bacteroides* species [18] and *Prevotella* (*Bacteroides*) *ruminicola* [25] have cell-associated xylanases, most xylanolytic microorganisms produce xylanases as extracellular enzymes. Xylanolytic activities of *S. thermophila* are predominantly cell associated (Table 4) and could not be released from cells by washing cells or treating them with mild detergents. The helical shape and rotational/translational motility of spirochetes allows them to readily penetrate particulate matter. Thus, being in close proximity to xylan-containing fibers, the production of an extracellular xylanase would not seem advantageous to *S. thermophila*. In addition, spirochetes have a high cell surface to volume ratio that favors efficient transport, and with *S. thermophila* the loss of xylooligosaccharides to the surrounding environment could be mini-

mal. In contrast, the thermophile *Thermoanaerobacterium saccharolyticum* strain B6A is a weakly motile, thin rod, and most of its xylanase is extracellular [5].

Studies on the effects of temperature and pH on stability of xylanolytic activities with cells or extracts of *S. thermophila* indicate optima of about 60°–70°C and pH of 6.5–7.5 (Tables 5 and 6). Considerable activity losses occurred at temperatures above 75°C, which is only 10 degrees above the optimal growth temperature for *S. thermophila* [17]. This was somewhat unexpected, as often enzymes from thermophiles are stable at temperatures that are much higher than their growth temperature. For example, the xylosidase of *Thermoanaerobacter ethanolicus* retains 75% of its activity after 3 h at 82°C, which is 28°C higher than its growth temperature [21]. The pH range for stability of the *S. thermophila* enzyme activities is narrow, but also reflects the pH range (6.25–7.15) for growth of this bacterium.

The results of the present study show that *S. thermophila* has a multiplicity of enzymes for xylan degradation and is capable of growing on different types of xylans. Other polysaccharides such as cellulose, starch, or glycogen support growth of this bacterium. Thus, it would seem that *S. thermophila* might be able to attack and degrade intact plant cell walls. Further work is necessary to examine this aspect and to characterize the other hydrolytic enzyme activities of *S. thermophila*.

ACKNOWLEDGMENTS

The author gratefully acknowledges the technical assistance of Patricia J. O'Bryan. The helpful discussions of M.A. Cotta and T.R. Whitehead are appreciated.

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