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THE HEMICELLULASES FROM THE ETHANOLOGENIC THERMOPHILE: THEMOANAEROBACTER ETHANOLICUS

Progress Report

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REPORT (for the first 19 months)

(Preamble: In contrast to assumptions of some reviewers, we did not have unreported (preliminary) results on this project; thus the experimental work started after the grant was awarded.)

1. Objective: As stated in the grant application,we only had obtained extremely low xylanase activities in cultures of *T. ethanolicus* strain JW200 grown on xylans, despite demonstrated xylan hydrolysis and growth on xylans (Wiegel et al., 1985). Although we spent considerable time and effort on it, we were not able 1) to increase the activity in culture supernatants or cells to higher levels by modifying the growth conditions, (e.g., varying the type and concentrations of xylans used, pH of medium, and growth temperature), 2) to concentrate this low activity in culture supernatants by various methods including ultrafiltration using various type of membrane filters and ultrafiltration fibers, freeze drying, precipitations (ammonium sulfate, alcohols, acids), adsorption on chromatography materials, and electrolytic procedures.

Therefore, we decided **to isolate new strains** of thermophilic anaerobes with a higher xylanase activity as future source for cloning xylanases into *T. ethanolicus*. Among over 50 isolates we obtained several strains exhibiting the highest xylanase activities (5.8 U/ml culture) reported, so far, for anaerobic bacteria (Table 1). We chose two types of organism for further investigation: Organisms able to grow at slightly alkaline (pH 8.5) and organisms to grow at pH 4.5 (with a pH_{opt} below 6.0) to cover the broad pH range for growth of *T. ethanolicus*. The strains produced thermostable xylanases with pH optima between 4.5 and 6.3 (Table 1). The xylanases with a pH_{opt} at 6.3 exhibited still 55% of its maximal activity at pH 8.0. None of the organisms could be immediately classified into existing species, thus a taxonomic characterization is in progress. Although, we now have organisms, exhibiting more than 100-fold higher xylanase activities than *T. ethanolicus* JW200, the enzyme activities were not recoverable at all or at very low yields after precipitations, gel filtrations or adsorption to various chromatography materials including cellulose-based and poly acrylamide-based materials.

However, recently we were able to concentrate and partially purify a **xylanase** from strain N.D. (obtained from compost of urban waste, pH_{opt} of xylanase activity 6 - 6.5) using a preparative, matrix-free ROTOFOR isoelectric focusing cell from Bio-Rad. Presently we are trying to use further purification steps to obtain gel electrophoretic pure enzymes. In SDS-PAGE we have detected 5 activity bands. During the preparative isoelectric focusing, we detected four fractions with activity around pH 4.4, 5.8, 7.1, and 8.8.

In contrast to the xylanase, we have purified and partially characterized a **xylosidase** from *T. ethanolicus* JW200 to homogeneity in SDS and native gradient PAGE and on isoelectric focusing gels (see attached draft for a manuscript). The enzyme consists of two subunits with a molecular weight of 85 kD, is active with p- and o-nitrophenyl-ß-D-xylopyranoside (pNPX) and with the p-nitrophenyl-

α-L-arabinopyranoside and the p-nitrophenyl-α-L-arabinofuranoside. However, we still regard the enzyme as a xylosidase with arabinosidase activity, although the V_{max} with the arabinosides is about 50-times higher than for the xylosides, since the affinity for the xyloside is about 50 and 100-fold higher than for the two arabinosides.

We also tested the **arabinosidase (pNPAP) activity** in *C. thermohydrosulfuricum* and in strain N.D. and found 0.9 and 4.3 nmol/min and ml. This is much higher than the value obtained for *T. ethanolicus* JW200 (0.5 nmol/min and ml). The enzyme in *C. thermohydrosulfuricum* is, as expected, cell associated and not extracellular, and using analytical isoelectric focusing we could detect only one activity band with the arylxyloside in extracts of *T. ethanolicus* JW200, .

Acetyl esterase for deacetylation of xylans was detected in cultures of *T. ethanolicus* JW200, *C. thermohydrosulfuricum* and in strain N.D. The acetylesterase activity (liberation of acetate from acetylated larch xylan, as well as p-nitrophenylacetate activity) was found to be mainly extracellular and partly cell-bound, very little activity was found inside the cells. This suggest that the acetyl group is removed before the xylose unit is transported into the cell.

Nearly no activity was found, when the cells were grown on xylose instead of beechwood xylan. Highest activity of the acetylesterase was found during mid-logarithmic growth phase. The enzyme was about 4 and 15 times higher at pH 6.5 than at pH 6.0 and 5.5, respectively.
The observed acetyl esterase activities are summarized in table 1.

Table 1: Acetylesterase activity in some thermophilic anaerobes

	Acetylesterase (nmole acetate formed/min and ml)		
Organism	*C. thermohydro-sulfuricum*	*T. ethanolicus*	Strain N.D.
Culture (permeabilized)	2.4	5.0	4.1
Supernatant	1.8	3.6	3.0
Washed cells	0.8	1.5	0.9

Furthermore we started with the analysis the **O-methylglucuronidase** for removal of the inhibiting O-methylglucuronic acid group. Except for demonstrating the presence of enzyme activity in strain N.D. and *C. thermohydrosulfuricum* strain JW102 (very low), we have not obtained further results.

Table 2: some properties of newly isolated thermophilic anaerobes with high xylanase activity

	Xylanase from strain					
	YS 501	YS 485	YS 494/495	YS 460	YS 477	N.D.
pH_{opt} of activity in the culture supernant	n.d.	4.5	4.5 - 5	5.0	6.5	6 -6.3
T-stability for 90 min	n.d.	n.d.	n.d.	n.d.	n.d.	90% at 65°C 80% at 75°C 2% at 85°C
max. Activity (U/ml)	1.03	4.6	2.7	1.0	0.82	5.8
	Properties of the organisms					
pH-range	4.2 - 6.6	4.0 - 6.9	4.5 - 6.6		4.4 - 6.6	6.8 - 8.9
pH_{opt}		5.7 - 6.1	5.4 - 5.7		6.0 - 6.25	7.2 - 7.5
T-range	n.d.	n.d.	n.d.		n.d.	64-66°C
T_{opt}	n.d.	n.d.	n.d.		n.d.	50-69°C
cellobiose sucrose mannose	+	+	+		+	+
mannitol	+	-	-		+	+
galactose	+	+	+		+	-
gluconate	+	-	-		+	-
ribose	+	+	-		-	+
raffinose	+	-	-		+	-
arabinose succinate malate glucuronate	-	-	-		-	-
Spores	+	+	+	+	+	+

1) Organism were isolated at 60°C, but do not grow at 80°C

List of publications (supported under this grant during the reporting period):

Peteranderl, R., E.B. Shotts, and J. Wiegel. 1990. Stability of antibiotics at growth conditions of thermophilic anaerobes. Appl. Environ. Microbiol. 56:1981-1983. (acknowledged erroneously to DE-FG 09-86 ER13614 instead of to this grant)

Deblois, S., and J. Wiegel, 1990. Hemicellulases in Lignocellulose Degradation. In Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants (D.E. Akin et al. eds.) Elsevier, New York 1990, pp. 265-287.

Thesis:

R.Peteranderl. Development of a cell fusion system between mesophilic and thermophilic Clostridia. Univ. of Georgia. August, 1990. (1 publication in prep.)

Abstracts:

Deblois, S., and J. Wiegel. 1991. Thermophilic anaerobes with high xylanolytic activity. Ann. Meet. Am. Soc. for Microbiol. Dallas, Abstr. O-46.

Shao,W., and J. Wiegel. 1991. ß-D-Xylosidase/α-L-arabinosidase from *T. ethanolicus* JW200. Ann. Meet. Am. Soc. for Microbiol. Dallas, Abstr. K-61

In preparation (see included manuscript):

Shao, W. and J. Wiegel. Isolation and characterisation of a highly thermostable xylosidase with strong arabinosidase activity from the ethanologenic anaerobic thermophile *Thermoanaerobacter ethanolicus*.

Peteranderl,R. and J. Wiegel. Formation and regeneration of autoplasts from *C. thermohydrosulfuricum*.

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