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THE HEMICELLULASES FROM THE ETHANOLOGENIC THERMOPHILE,
THERMOANAEROBACTER ETHANOLICUS AND
SIMILAR ANAEROBIC THERMOPHILES

Annual Technical Progress Report

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**Report after 6 months in the 1st year after a 3-year
renewal of the DOE - grant (DE-FGo9-89ER-14059)
Hemicellulases from the ethanologenic thermophile
T.ethanolicus and similar thermophiles**

1. Goals and General Comments

A: The SHORT TERM GOAL for the first year of the renewal grant is to further purify and characterize the xylanases and other hydrolytic enzymes required for an efficient hemicellulose degradation and start with the cloning of the enzymes into an appropriate host for sequencing purposes.

The ULTIMATE GOAL of this grant is to clone suitable genes into *T. ethanolicus* to supplement the activities which it lacks for an efficient degradation of xylan.

B: Due to the reduced budget and the prior commitment to graduate students on this project, I have not hired the desired postdoctoral candidate trained in molecular biology. The postdoctoral candidate is expected to begin in May or June. With respect to the genetic work we only have obtained further N-terminal sequences to make probes for identifying the clones.

Though cloning of the target genes has not begun, the studies on xylanase regulation are pertinent to the goal of expressing xylanolytic enzymes in *T. ethanolicus* because the stoichiometry of conversion of monomeric sugars to ethanol by *T. ethanolicus* is not stable with increasing sugar concentrations, so the rate of depolymerization of xylan will be an important factor in regulating the proportion of xylan which is converted to ethanol rather than to acetate and/or lactate, which are the predominant products when the sugar concentration is high.

Xylanases were sought from newly isolated thermophilic bacteria whose growth conditions ranged from the acidic to alkaline pH scale, in accordance with the broad pH range for growth of *T. ethanolicus* (see previous report). The new isolates were characterized for xylanase production, with respect to temperature, H^+ , Na^+ , and K^+ concentrations, and inducer/repressor substances. So far we have continued with the purification and characterization of the previously identified xylanases, xylosidases, arabinosidases, O-methyl glucuronisidase and acetyl (xylan) esterase (in short: acetylase) from organisms other than *T. ethanolicus* since the activities were either too low or unstable in this ethanologen. The systematic position of these organisms are still under investigation and proceeds slowly since it is a side project of this grant application. However, all new strains are only minor ethanol forming strains and belong probably to the newly defined genera *Thermoanaerobacterium* and *Thermoanaerobacter*. All three strains NDF190, Y485 and EPPH100 probably present new species.

2. Regulatory Properties of Xylanases:

INDUCTION OF XYLANASE ACTIVITY BY SUBSTRATES

Strain EPP100: Xylanase is inducible by xylan (presumably by xylooligomers), xylobiose, lactose, and cellobiose. As has been reported for *Clostridium thermolacticum*, xylanase also appears upon the depletion of glucose, xylose, or arabinose in the medium, and a low level of induction accompanies growth on salicin. The levels of xylanase production are modified by culture temperature and pH, and the pattern of induction/repression are influenced by Na^+/K^+ concentrations. Of 13 compounds tested, cellobiose > lactose > oat spelt xylan > salicin > (galactose) > xylobiose, in order of decreasing strength, induced xylanase (induction doesn't occur with galactose alone, but it can stimulate induction). The presence of certain sugars reduced the level of induction (Table I).

Table I. Induction of EPP xylanase in the presence of other sugars, reported as a percentage of the unit activity/unit growth by the inducer alone.

sugar added	% induction						
	cellobiose		lactose		salicin		oat spell xylan
	15h	24h	15h	24h	15h	24	
inducer	100	100	100	100	100	100	100
xylose	29	27	4	2	24	10	1194
glucose	87	91	0	0	48	58	416
maltose	64	72	9	18	-	-	395
galactose	76	85	59	190	39	57	-

Though the trend is consistent, the magnitude of repression is variable, depending on the physiological state of the cell, and the carbon source of the inoculum. For cells with inducer alone, half maximal induction of xylanase occurred at between 0.01-0.05% (ca. 0.02%) of cellobiose or lactose. While the velocity curves for induction by xylan and xylobiose were biphasic, half maximal induction occurred in the same range.

Fig. 1. Concentrations of from 0.01 to 0.5% inducer were added to cell suspensions inoculated with washed log phase xylose-grown cells into fresh medium. Xylanase activity was assayed following incubation for 6h at 60 C, and activity was corrected for growth.

□ cellobiose, + lactose, ▲ xylobiose,
◆ oat spelt xylan

The variability in repressor behavior may reflect the physiological state of the cells. Internal pH has been implicated in the regulation of sugar transport in *Clostridium thermocellum*. The growth of strain EPP on cellobiose or xylose differs dramatically in regimes where the Na^+/K^+ ratio is low. Growth with xylose is little affected when the $[\text{Na}^+]$ is low, whereas a lag of 6-7 days exists when $[\text{Na}^+]$ is low and cellobiose is the carbon source, suggesting that the internal physiological state may be important for inducer transport.

Strain NDF190: Xylanase is inducible only by growth on xylan. Xylose and xylobiose neither repress nor induce xylanase activity. Temperature affects the rate of xylanase production.

Strain YS485: Numerous exceptions to the rule that extracellular enzymes are induced by their products have been found among fungi whose xylanases are induced by xylose. YS485 also produces lignocellulolytic enzymes, including xylanase in response to xylose. In no case has a transglycosidase activity been demonstrated, but the addition of xylobiose to cultures did not induce xylanase in YS485.

EFFECT OF GROWTH TEMPERATURE ON XYLANASE FORMATION

The level of xylanase expression varies according to growth temperature in all 3 strains investigated, with the xylanase of NDF190 being expressed at the highest temperature, and over the narrowest range, and EPP100, and YS485 xylanase production covering the lower temperatures, down to ca. 40 C. Fig. 2.

T induction of xylanase activity

Fig. 2. Regulation of xylanase induction by temperature. % max. activity
-□- NDF190; -+- EPP100; -◆- YS485

pH-profile for enzyme activities
pH optimum of xylanase activity

Fig. 3. pH_{opt} of xylanase activity
% max. activity
-◆- NDF190; -□- EPP100; -+- YS485

3. Enzyme purification:

pH-Optima: Xylanases from the 3 isolates are active over the ranges indicated in Fig. 3. The initial report of a pH_{opt} of 10-11 for EPP100 xylanase activity was due to incomplete purification of the newly isolated strain. Separation of the 2 strains resulted in 2 xylanase activities, one

alkalizable, but of low activity, and a major activity which had more activity at pH 9 where only 50% of its optimal activity remained than the alkalizable enzyme had at its optimum. We have pursued work on the strain which produced high activity.

The xylanases from NDF190, EPP100, and YS485 have been at least partially purified and characterized.

NDF190 xylanases focus to a highly active acidic protein on IEF gels, (s.a. not available because protein cannot be determined colorimetrically, by UV absorption, or by double staining with Coomassie and Silver on a gel, though activity is readily detected by reducing sugar assay or using an overlay gel) with a pI of ca. 5.5 - 6, and a MW of ca. 40k. After DEAE, CM Sepharose, and MonoQ column chromatography, this protein appears to be pure because of the disappearance of all contaminating bands on double stained gels, while a single activity band was detected in an overlay gel. On IEF gels, other bands of activity migrated to positions equivalent to pI's of 9, 8.6, 8, 7.7. Whether these bands represent the dissociation of an aggregate of basic proteins, or are separate xylanases has not been distinguished. The basic and acidic xylanases were separated from in the first step of purification, and because the xylanase associated with the basic proteins was of low activity, compared to the acidic protein, it was not further purified.

The xylanases induced in strain **EPP100** by different sugars all migrate to the same position in 4% polyacrylamide gels, suggesting that the same proteins are induced. Activity focusses to 2 bands of pI 4.5, and 6.6 on IEF gels.

Strain YS485 produced an acidic xylanase with a pI between 4.5-5.5 which was purified 150 times to a spec. activity of 882 U/mg by DEAE, CM, and MonoQ ion exchange chromatography. A basic xylanase of pI 8.4 has not been further characterized.

N-terminal sequencing has been attempted for the acidic NDF190 xylanase, and will be re-tried following an amino acid analysis of the activity to determine protein concentration.

During the next 6 month we will especially focus on the final purification and characterization of the xylanases.

Xylosidases:

***T. ethanolicus*:** We are presently repeating the determinations of $K_{0.5}$ values for the true substrates xylooligomers (X_2 - X_6). We could not find any significant difference in the affinity for the different oligomers. This could be due to the longer assay times required for the HPLC-type analysis. We are also repeating the enzyme assay for the arabinoxylan. The defined oligomers are not available and the Arabinoxylopentamer which was purchased was obviously not the designated substance.

Other thermophiles: Due to the interesting property of being a bifunctional enzyme (the two activities have two different pH and T-optima) we were interested whether this enzyme is also

Other thermophiles: Due to the interesting property of being a bifunctional enzyme (the two activities have two different pH and T-optima) we were interested whether this enzyme is also present in the other organisms, and if so how much do they differ. In the long run, we will be interested whether the bifunctional and monofunctional xylosidases and arabinosidases are from the same family and are highly similar, or whether this are totally different enzymes.

Beside the xylosidase/arabinosidase enzyme from *T. ethanolicus* we have isolated and partly characterized the xylosidase from:

i) *C. thermohydrosulfuricum* JW 102. This is also a combined xylosidase/arabinosidase enzyme, Using p-nitrophenylxyloside and p-nitroarabinoside as substrate the pH_{opt} are 5,0 and 6,0, and the $T_{\text{opt; 2min assay}}$ 85 and 75°C, respectively. The molecular weight is around 175 kD. We have obtained 10 aminoacid residues from the N-terminal which are similar to the sequence of *T. ethanolicus*. Thus, the properties are relatively close to the enzyme from *T. ethanolicus*.

ii) strain NDF190. This enzyme has only xylosidase activity and the organism contains a separate arabinosidase which has not yet been purified. This enzyme whose pH_{opt} of 5.5 and $T_{\text{opt; 2min assay}}$ of 65°C are similar to the monofunctional xylosidase of YS485, is distinguished by its large size of greater than 400kD. has a molecular weight of above 400kD. The N-terminal residues are Met-Lys-?-Tyr-Tyr-Val-?-?-Asn and thus totally different from the above enzymes.

iii) strain YS485. The organism contains 2 different xylosidases, however, so far, we have only isolated the major enzyme, a monofunctional xylosidase with a MW around 160kD, pH_{opt} 5.5, $T_{\text{opt; 2min}}$ 65°C and an isoelectric point of 4.6 (which is similar to the bifunctional enzyme). The N-terminal residues determined are Met-Ile-Ser-Lys-Ser/Asp-Phe/Ile-?-Ala- and thus different from the above mono and bifunctional enzymes.

Future tasks:

With respect to the xylosidases we will work towards purifying the second xylosidase from YS485 (so we get as many hemicellulolytic enzymes as possible from this organism; this is also suggested by several reviewers of the grant). We will prepare from all enzymes DNA probes so we can later clone and sequence the enzymes. The enzyme from T. ethanolicus will be the first to be cloned and sequenced to find whether two separate binding sites can be identified (A more detailed structure/function analysis among the various enzymes should be done later as a separate project). Since we have at least three different "types" of xylosidases identified, it will be interesting to see the relationship on the sequence level to each other and to mesophilic bacterial and fungal enzymes.

Glucuronidase

The glucuronidase from strain YS485 (the only one so far further investigated) was partially purified. The molecular weight, as determined by native gradient gel, is 160kD and similar to the acetylases in strain YS485, is intracellular. This fact is somewhat puzzling to us. The $T_{\text{opt; 10 or 40 min assay}}$ is about 70°C and the pH_{opt} is around 6.3. We are still in the process of purifying this enzyme.

Acetyl(xylan) esterase (acetylase):

We have continued to investigate this enzyme in strain YS485 and have isolated two different acetylerases, acetylase I and acetylase II and purified to gel-electrophoretic homogeneity. The enzymes were separated on DEAE-sepharose chromatography. To our knowledge these are the first acetyl(xylan) esterases isolated from thermophilic anaerobes.

Table: Comparison between Acetylase I and Acetylase II *

	Acetyl(xylan) esterase I	Acetyl(xylan) esterase II
Isoelectric point	pH 4.3	4.3
Apparent mol. weight gel-filtration, native gradient gel)	194 kD	106 kD
SDS-PAGE	32 kD	25 kD
specific activity with 4-methylumbelliferyl acetate	75 μ mol/min and mg protein	80 μ mol/min and mg protein
Activity with acetylated xylan	+	+++
pH _{opt}	7.0	7.5
T _{opt,2min assay}	80°C	84°C
T-stability	half life: at 70°C 18 min	stable at 84 for >2 h
Arrhenius energy	29 kJ/mol	27 kJ/mol

* Graphs attached.

So far we have only obtained the N-terminal sequence (20 residues) from Acetylase I: Gly-Leu-Phe-?-Met-Met-?-Trp-Leu-Gln-Las-Leu-Arg-Glu-Tyr-Thr-Gly-Thr-?-Pro. We are in the process of obtaining the sequence from acetylase II.

Future work: We still have to continue for the next 5 month with the enzyme purification and characterization, but assume that the acetylases and glucuronidase will be purified and/or characterized fully within the next 6 months. When in May or June the new post doctoral candidate will start to work, we will begin with obtaining gene libraries (at least for *T. ethanolicus* and YS485) and do the cloning and sequencing of the xylosidases. Otherwise we will proceed as outlined in the grant application except that the genetic work is delayed due to less available manpower caused by the reduced budget.

Fig. 4: Effect of Temperature on Acetylase I and II from strain YS485

a) Temperature curve and b) Arrhenius graph: -●- acetylase I; -○- acetylase II;
Assay conditions: 25 mM phosphate buffer pH 7.0, 2min assay; assay was started by adding
acetylase I (3.5 μg/ml) and acetylase II (2.1 μg/ml).

Figure 5: Effect of pH on activity and stability of acetylase I and II from strain YS485

a) Enzyme activity, -●- acetylase I; -○- acetylase II; and b) stability at various pH and at 70°C - □- acetylase I and -♦- acetylase II remaining after 1hr of incubation. Assay conditions: 25mM phthalate buffer, pH 4.9-6.3 or 25mM phosphate buffer, pH 6.0-8.0. assay temperature 70°C, 2 min; assay was started with adding acetylase I (2.6μg/ml) and acetylase II (1.6 μg/ml)

5. Publications and Presentations since updated report (January 1992)

PRESENTATIONS WITH ABSTRACTS

- F. Canganella, M. Zirletta and J. Wiegel. Induction and Regeneration of autoplasts from *Thermoanaerobacter ethanolicus*. Ann. Meet. Italian Soc. Microbiol. and Appl. Biotechnol. October 1991. Viterbo (Italy).
- F. Canganella and J. Wiegel. Cultivation of *C. thermobutyricum* in a rotary fermentor system. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. p. 119.
- S. DeBlois and J. Wiegel. Comparison of thermostable xylanases having optimal activities at acidic, neutral and alkaline pH values. Ann. Meet. Am. Soc. Microbiol. New Orleans, May 1992.
- S.-Y. Liu, S. DeBlois and J. Wiegel. Isolation and characterization of xylose utilizing anaerobic thermophiles. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. IV-P-06 (p. 123).
- W. Shao, S. DeBlois and J. Wiegel. Hemicellulases from thermophilic anaerobic thermophiles. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. IV-P-09 (p. 125).
- W. Shao, S. DeBlois and J. Wiegel. Acetyl xylan esterases from an anaerobic thermophile. Ann. Meet. Am. Soc. Microbiol., Atlanta, May 1993.

BOOKCHAPTERS:

- F. Canganella and J. Wiegel. 1992. The potential of thermophilic clostridia in biotechnology. IN: The Clostridia and Biotechnology (D. R. Woods, Ed.) Butterworths Publ. Stoneham, MA (in press).
- DeBlois, S. and J. Wiegel. 1992. Effect of temperature and pH on xylanase production in three newly isolated strains of highly xylanolytic anaerobic thermophiles. In: Progress in Biotechnology 7 Proceedings 1st Intern. Symp. 1991 Wageningen, The Netherlands (Visser et al., eds.), pp. 487-490. Elsevier Science Publishers, Amsterdam.
- J. Wiegel. 1992. The anaerobic thermophilic bacteria. Chapter 6 In: Thermophilic Bacteria. J. K. Kristjansson (ed.), CRC-Press, Boca Raton, FL, pp. 105-184. *front matter only submitted (Removed)*

JOURNAL PUBLICATIONS

- F. Bryant, J. Wiegel and L. G. Ljungdahl. 1992. Comparison of alcohol dehydrogenases from wild type and mutant strain, JW200 Fe4, of *Thermoanaerobacter ethanolicus*. Appl. Microbiol. Biotech. 37:490-495.
- W. Shao and J. Wiegel. 1992. Purification and characterization of a thermostable β -xylosidase from *Thermoanaerobacter ethanolicus*. J. Bacteriol. 174:5848-5853.

In preparation:

- W. Shao and J. Wiegel. Comparison of xylosidases from thermophilic anaerobes
- W. Shao and J. Wiegel. Purification and characterisation of two acetyl (xylan) esterases from a thermophilic anaerobe.