

FINAL REPORT for the DOE grant (DE-FG09-89ER-14059): Hemicellulases from the ethanogenic thermophile *Thermoanaerobacter ethanolicus* and related anaerobic thermophiles (Sept. 1992-June 1996)*).

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* A No-Cost Extension was granted for the period from August 1996 until August 1997.

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A. STATED GOALS FROM APPLICATION SUBMITTED IN 1991:

The SHORT TERM GOALS of this application were to characterize hemicellulases from anaerobic thermophiles on the biochemical and molecular level to extend the presently limited knowledge of hemicellulases in anaerobic thermophilic bacteria. This objective includes the following TASKS:

1) Traditional purification and biochemical/biophysical characterization of xylanases from the newly isolated, slightly alkalitolerant strain NDF190, and the slightly acid-tolerant strain YS485, both with high xylanolytic activities, and of the 4-O-methyl glucuronidase and arabinosidase from strain NDF190 and the acetyl (xylan) esterase from *T. ethanolicus*. This also includes determining the N-terminal sequences and obtaining gene probes.

2) Elucidation of the regulation of hemicellulolytic enzymes in anaerobic thermophiles.

3) To clone into *E. coli* and identify the multiplicity of the enzymes involved in hemicellulose degradation by *T. ethanolicus* and other suitable organisms.

4) To purify and characterize the recombinant enzymes with the goal of identifying the best enzymes for cloning into the ethanologenic *T. ethanolicus* to obtain an optimized hemicellulose utilization by this bacterium (one of our long term goals).

GENERAL COMMENTS ON MODIFICATIONS MADE IN REGARD TO THE ABOVE STATEMENTS DURING THE GRANT PERIOD:

- 1) Due to the significantly reduced budget awarded (down to 65%), less manpower was available and not all of the above listed tasks could be done even within the extended grant period, i.e., not all of the enzymes were studied, e.g., arabinosidase. Also, the genetically trained postdoctoral candidate could not be hired until 10 months after the grant started, thus delaying the start of the cloning tasks.
- 2) Encouraged by the comments of reviewers, and our preliminary results, we chose one of the thermophilic anaerobes, (*Thermoanaerobacterium* spec. strain JW/SL-YS485) exhibiting high activities of the various enzymes, and started to characterize all its hemicellulolytic enzymes as possibility for later cloning their genes into the weakly hemicellulolytic *Thermoanaerobacter ethanolicus*.
- 3) We started with the development of a genetic system for *T. ethanolicus* (development of an autoplating and regeneration system, shuttle vector and expressing heterologous genes in the thermophile with the goal of manipulating the genes in our thermophilic bacteria). Since no genetic system exists for any anaerobic thermophiles and *T. ethanolicus* was resistant to all antibiotics for which resistance genes were available, we focused on the hemicellulolytic but less ethanologenic *Thermoanaerobacterium*, JW-SL-YS485.
- 4) Furthermore, we sequenced several genes for some of the purified enzymes and also obtained novel genes for hemicellulolytic enzymes not purified.
- 5) The grant was extended for additional 12 months (from September 1995 to August 1996) and a No-Cost Extension was granted until August 1997 in order to publish more of the work. Although several manuscripts were published during this time, there are at least two more which are presently still under preparation.

B. REPORT

TASK 1: PURIFICATION AND CHARACTERIZATION OF HEMICELLULOLYTIC ENZYMES:

1) DESCRIPTION OF NEW MICROORGANISM AND ISOLATED STRAINS.

In connection with this grant and during this grant period a total of eight very different thermophilic anaerobes (4 new genera) have been validly published. This research and the publications were at least in part funded by this DOE. Furthermore, the description of three additional novel bacteria are in preparation (DeBlois, MS. thesis 1996: '*Thermotorquereum fastidiosum*' JW/SD EPP100 gen. nov., sp. nov. and '*C. pseudocellulolyticum*' JW/SD-ND190; Dashti, MS thesis 1997: '*Thermoanaerobacter sulfurignens*', sp. nov. We are still working on the determination of the systematic positions of several of the over 100 xylanolytic isolates obtained (Liu, unpubl.).

Seven of the xylanolytic isolates, all obtained from geothermal heated pools in New Zealand, were characterized. They constitute a new species, *Thermoanaerobacterium aotearoense* sp. nov. (Liu et al., 1996a). This species represents the most acidophilic anaerobic thermophilic (eu)bacterium described, so far. At the temperature optimum for growth of 60 to 62°C, its pH optimum is around pH 5.2. On the other side, looking for bacteria containing small alkaline xylanases, we isolated and described the most alkaline thermophile, *Clostridium paradoxum* (Li et al., 1993) isolated from sewage sludge able to grow optimally with a doubling time of around 16 min at pH^{25°C} 10.3 and 55°C. We further isolated one of the fastest growing bacteria *Thermobrachium celere* gen. nov. sp. nov.; (Engle et al. 1996) with doubling times at around 60°C and pH^{60°C} 8.5 of as short as 10 min..).

2) ENZYMES PURIFIED FROM THE PARENTAL STRAINS

The following enzymes were purified and described from the following anaerobic thermophilic bacteria:

***Thermoanaerobacter ethanolicus* JW 200:**

A bi-functional xylosidase/arabinosidase (Shao and Wiegel, 1992)

***Thermoanaerobacter* (bas. *Clostridium*) *thermohydrosulfuricus* JW 102:**

A bi-functional xylosidase/arabinosidase similar to the one in *T. ethanolicus* JW 200 (in preparation).

'*Thermotorquereum fastidiosum*' gen. nov., sp. nov. ND190:

A mono-functional xylosidase some what similar to one of the two(three) enzyme from *Thermoanaerobacterium* JW/SL-YS485 (in preparation, to be published together with sequence data).

***Thermoanaerobacterium* JW/SL-YS489:**

Xylose isomerase (Liu et al. , 1996) this enzyme is the xylose isomerase (GI-activity) with the most acidic pH. Thus the enzyme is presently under industrial evaluation.

***Thermoanaerobacterium* JW/SL-YS485:**

- i) a high molecular weight, cell-bound xylanase (Shao et al., 1995) the largest xylanase found so far (see below for sequence analysis and special features).
- ii) two acetyl xylan esterases (Shao and Wiegel, 1995a),
- iii) the α -(O-methyl)glucuronidase (Shao et al., 1995b), the first one from a thermophile
- iv) two xylosidases (mono-functional enzymes) (to be published together with sequence analysis; Lorenz et al., in preparation)

Most of the results have been published (see below list of publications). Thus, only a few points are highlighted or discussed in the following paragraphs. As expected, all the enzymes we have isolated exhibit relatively high thermal stabilities, e.g., the acetyl xylan esterase II has a 1 h-half life time at 100°C.

i) XYLANASES

As stated in the grant application for 1992-1995, the activity of the xylanase from *T. ethanolicus* strain JW200 was too low and unstable to be purified. Thus, we have identified several new anaerobic xylanolytic thermophiles exhibiting xylanase activity up to 9 U/ml culture at various pH ranges. For further studies we chose -- for the alkaline pH range-- the enzymes from '*To fastidiosum*' JW/SD-ND190 and *Clostridium 'pseudocellulolyticum'* JW/SD-EPP100) and --for the acidic pH range-- the enzyme from *Thermoanaerobacterium* JW/SL-YS485.

a) Xylanase from *Thermoanaerobacterium* JW/SL-YS 485 (Liu et al., 1996): We encountered only one enzyme in this organism; it is an unusually large (>100,000 MW) high molecular weight (Family F) and cell-bound enzyme. The determined molecular weights were about 350,000 (pI around 5) using gel filtration and after SDS-treatment using denaturing SDS gel electrophoresis around 180,000 suggesting a dimeric enzyme; the sequence analysis lead to a deferred value of about 150,000. We assume the difference is due to the 6% glucosylation of the native enzyme, which is missing in the recombinant enzyme. The recombinant enzyme stained readily with various protein stains. However, we had to use increase of the amount protein in the gel by more than 10-fold to be able to stain the enzyme from the parental strain. Thus we assume that the staining problem could be due to the glycosylation of the enzyme. The extremely high molecular mass of the xylanase activity from *Thermoanaerobacterium* was surprising and puzzling but is explained by the sequence analysis showing that the enzyme contains three repeated units which are used to anchor the enzyme in the S-layer of the organisms. The catalytic region, which is similar to other xylanases, is connected via a spacer unit (Liu et al. 1996).

b) Xylanases from '*C. pseudocellulolyticum*' JW/SD-EPP100 and *To. fastidiosum*-JW/SD-ND190. The determined sizes (gel filtration and/or gradient PAGE) for the extracellular xylanases from the different organism were: strain JW/SDB-ND190: 97,000 (pI 5.7); 40,000 (pI 8.5) (similar values were obtained using non-denaturing [so that activity still can be measured] SDS gels); strain JW/SDB-EPP100: 125,000 (gel filtration) and 115 (SDS-PAGE). The 115 kD xylanase from strain JW/SD-EPP100 was partially (to about 80% purity) purified by DEAE cellulose, hydroxyapatite, phenyl superose, and gel filtration chromatography (unpubl. results; due to termination of the grant this project could not be finished, the student left without her final degree). The enzyme retains 50% of maximum activity between pH values of 6.5 and 10.2, with up to 80% activity remaining up to pH 9.5. The temperature optimum, based on a 2 min assay, was around 80°C. The regulation of the xylanase from *C. 'pseudocellulolyticum'* JW/SD-EPP100 is typical for a cellulase (DeBlois and Wiegel, 1995; see below the paragraph on REGULATION).

ii) ACETYL(XYLAN)ESTERASES

Acetyl xylan esterase activity for deacetylation of xylan was detected in cultures of *T. ethanolicus* JW200 (very low activities), *T. thermohydrosulfuricus*, *To. fastidiosum*. JW/SDB-ND190, and *Thermoanaerobacterium* JW/SL-YS485. The latter one exhibited the highest acetyl xylan esterase activity, which was determined by measuring liberation of acetate from acetylated larch xylan as well as with p-nitrophenylacetate. During the purification we found that two different enzymes are formed having different molecular weights (110,000 and 170,000) and pI, and substrate specificity. Interestingly, one of the enzymes is glycosylated and the other one is not (see below for sequence

analysis). The enzymes were both mainly extracellular and only to some degree cell-bound, i.e., only slight activity was found inside the cells. This suggests that the acetyl group is removed before the xylose unit is transported into the cell. Again it is assumed that the two enzymes function at different substrate concentrations and that one of them is using preferentially the polymeric substrate, whereas acetylase II reacts more with acetylated oligomers (see also below the paragraph on synergistic interactions).

iv) 4-O METHYL α -GLUCURONIDASE

This is the first time that this enzyme has been purified from a bacterium. The 4-O-methyl α -glucuronidase activity could not be detected in *T. ethanolicus*. The absence of this enzyme was evident from the accumulation of acidic xylooligomers in growing cultures (Wiegel et al. 1985) which also demonstrated the importance of this enzyme for an economical utilization of hemicellulolytic biomass (see also below paragraph on regulation and synergistic interactions). From the biochemical characterization, we could not deduce to which enzyme family this enzyme belongs, thus we have to wait for this until the sequence is known (not done due to termination of grant).

TASK 2: Regulatory Properties

C. pseudocellulolyticum JW/SD-EPP100 is an interesting organism. From its 16S rDNA sequence analysis, it belongs to the group of cellulolytic organisms around *C. thermocellum* and *C. stercorarium*, but is the only organism in this group which lacks cellulase activity. When we analyzed regulation of the hemicellulolytic enzymes, we found that the regulation pattern for the xylanase was a pattern otherwise only described for cellulase complexes, i.e., activity is induced when grown in the presence of cellobiose and lactose beside xylan (DeBlois and Wiegel, 1995).

Several of the purified enzymes exhibited synergistic actions when mixed with each other. An example is the combination of the purified 4-O-methyl- α -glucuronidase and the β -1,4-xylosidase. The purified xylosidase alone exhibits some activity with the glucuronyl xylotetraose but has no activity with the glucuronyl-substituted xylobiose, whereas the glucuronidase alone has a lower activity with the on the glucuronyl-substituted tetraose but a higher activity with the glucuronyl xylobiose and -triose formed by the xylosidase. Thus a fast hydrolysis to the unsubstituted xylose was observed when both enzymes were incubated together with the substituted glucuronyl-tetraose (Shao et al., 1995).

Also the acetyl (xylan) esterases reacted synergistically with the xylanase plus the xylosidase /arabinosidase from *T. ethanolicus* and *Thermoanaerobacterium* on acetylated xylan. This was also evident during the purification of the esterases since the relatively high activity of the acetyl xylan esterase towards the acetylated polymer decreased by a factor of more than 2.

TASK 3: Cloning into *E. coli* and sequence analysis

We have obtained genomic libraries in the vector λ ZAP Express (Stratagene) for two organisms:

***T. ethanolicus* JW200 and for *Thermoanaerobacterium spec.* JW/SL-YS485.**

Two different methods were required for isolating high molecular weight DNA from these two organisms. The best method for *Thermoanaerobacter* was a guanidineisothiocyanate method similar to the one used for the isolation of mRNA from eukaryotic sources (GITC-method from Judy D.Wall, Biochemistry Univ. Missouri-Columbia, pers. comm.) whereas for *Thermoanaerobacterium* the presence of elevated salt and hexadecyltrimethyl ammonium bromide concentration and the inclusion of

ultracentrifugation in CsCl gradients was required. The libraries were constructed using either *Sau3A*-digested DNA which had been partially digested and size selected. The libraries are judged to be viable based on total plaque forming units (10⁻⁷ pfu/ml) and on relative insert sizes.

Since we are interested in several of the *Thermoanaerobacterium* JW/SL-YS485 enzymes involved in hemicellulose degradation, we constructed plasmid libraries using randomly sheared and restriction digested DNA ligated into *SmaI* site of pUC18 allowing us to screen for clones which expressed active hemicellulolytic enzymes using indicator plate.

We have sequenced the genes for the following enzymes:

1) The xylose isomerase from *Thermoanaerobacterium* JW/SL-YS-489 (Liu et al. 1996):

We hypothesize that we have elucidated the critical amino acids to render the pH-optimum of the enzyme to the desired acid pH. Further experimental documentation. e.g., site directed mutagenesis, was in progress but is on hold due to lack of funds.

2) Two xylosidases and some ORFs from *Thermoanaerobacterium* JW/SL-YS485 (1 sequence submitted to the genebank):

2a) The **xylosidase 1** gene codes for the xylosidase I which we had isolated and characterized previously (Lorenz and Wiegel, 1997; Shao and Wiegel, unpubl. results). The deduced amino acid sequence for one of the genes shows greater than 90% identity to the enzyme from *T. saccharolyticum* (Lee et al., 1993 J. Gen. Microbiol. 139:1235-1243).

2b) However, the second sequence does not represent the second enzyme (Xylosidase II) we have described from this organism, but represents a third and novel xylosidase (**putative xylosidase III**), which has no homology to genes from other xylosidases in the genebank (Lorenz and Wiegel, in preparation). Xylosidase III gene (*xylC*) was isolated on a 3.5-kb *HindIII* fragment by expression cloning on 4-methylumbelliferyl xyloside plates. The *xylC* gene encodes a polypeptide containing 638 residues with a MW = 72.7 kDa. Removal of 5'-noncoding sequence (*PstI* / *HindIII* subclone) leads to very high levels of xylosidase expression in *E. coli* under the control of an endogenous promoter. Thermostable activity has been demonstrated with p-nitrophenyl xyloside. Further work is needed to elucidate the total number of genes for xylosidases and enzymes exhibiting this activity as an unspecific reaction.

3) Various ORFs with putative functions: **i)** An ORF designated *xylR* is located immediately 5-prime to *xylC* and is transcribed divergently. *xylR* has significant similarity to bacterial regulatory proteins of the *araC/xylS* family signature, most of which are positive transcriptional regulators. These proteins all bind DNA by a helix-turn-helix motif located in the C-terminal region. Effector molecules are presumed to interact with N-terminal and central regions of these proteins. Most of these regulatory genes are located next to, and transcribed divergently from, the genes they regulate. *xylR* likely is involved in the regulation of *xylC* transcription. These appear to be important and interesting enzymes for the hemicellulolytic apparatus in this thermophilic anaerobe and should be further investigated.

ii) A partial ORF is located immediately 3' to the *xylC* gene. The deduced sequence has no similarity to any published protein sequence. We speculate that this gene encodes a third xylosidase gene. So far, we were not able to obtain the gene for the second purified xylosidase from this organism.

3) The large cell-bound xylanase from *Thermoanaerobacterium* JW/SL-YS485 (Liu et al., 1996). The most interesting feature of this largest --so far described-- xylanase is the spacer region followed by three different motifs from S-layer proteins at the C-terminal. As mentioned above, we proposed the repeats are responsible for the attachment of the enzyme to the outside of the cell by intercalating the C-terminal of the enzyme into the S-layer matrix. Thus the extracellular enzymes of this bacterium

can attack the insoluble xylan but the soluble hydrolysis products remain in proximity to the cells. Whether there are special direct channeling processes between the cells and the enzyme to move the xylose and xylose oligomers into the cell is not known and needs to be further investigated. This study will show the extent to which one could use these sequences to modify genes so that the extracellular enzymes become cell associated (DeBlois et al. unpubl. results). Using a probe for S-layer proteins (obtained from H. Bahl, Göttingen) made according to the sequence region from the surface protein gene these repeats were also observed in other hydrolytic, extracellular but partly cell-bound enzymes (pullulanases) from *Thermoanaerobacterium sulfurigenes* EM1. Using the S-layer probe, 5 bands were identified in partially hydrolyzed DNA from strain JW/SL-YS485 and 2 in *T. ethanolicus* JW 200. Thus, we speculate that the S-layer proteins have very similar binding sites so that the enzymes from *Thermoanaerobacterium* JW/SL-YS485 can also bind to the S-layer from *T. ethanolicus* JW200 and thus keeps the enzyme closer to the ethanologenic organism and facilitates a more efficient xylan utilization. Our present hypothesis is that the enzyme is so large due to the need to separate the catalytic domain from the S-layer interacting (anchoring) C-terminal domain by the "spacer" sequence. In the pullulanase gene the sequence shows a high similarity to genes coding for fibrin.

4) One of the purified acetyl(xylan)esterases from *Thermoanaerobacterium* JW/SL-YS 485 (Lorenz and Wiegel, 1997). This enzyme shows an interesting cephalosporin-C deacetylase activity. It represents a novel acetyl(xylan) esterase due to its sequence which does not match the gene sequences of other acetyl(xylan)esterase activity. Unfortunately, we have not yet cloned the second purified enzyme.

5) The novel bi-functional xylosidase/arabinosidase from *T. ethanolicus* (Lorenz and Wiegel in preparation): The *xylA* gene was reconstructed from two clones isolated from a lac ZAP library by oligonucleotide screening. The reconstructed clone, pPXA-1, is 4.5 kb. The *xylA* gene encodes a polypeptide containing 784 residues having a MW = 87.8 kDa. A PCR-generated clone containing only the xylosidase/arabinosidase (XA) coding region and a ribosome binding site has been used for expression in *E. coli*. Both thermostable xylosidase and arabinosidase activities have been demonstrated with p-nitrophenyl derivatives. The clone pXA23/19 contains 1.4 kbp of 5' noncoding and 2 kbp of coding sequence encoding 668 amino acid residues. *E. coli* cells transformed with this plasmid express arabinosidase activity, but not xylosidase activity. There is significant amino acid sequence similarity with some of the β -glucosidases, but none with any published xylosidase or arabinosidase sequences. A putative active site has been identified based on these analyses. Thus this sequence identifies this as another novel xylosidase which is different from the one previously identified by Ut et al., 1991 (Appl. Environ. Microbiol. 57:1227-1234) from *Butyrivibrio fibrisolvens*.

9) Summary of a Partial Heme Biosynthetic Operon Isolated from *Thermoanaerobacter ethanolicus*. (unpubl. results). During expression screening of a pUC18 library on 4-MU xyloside plates, a single colony exhibited brilliant orange fluorescence rather than the blue fluorescence seen with xylosidase expressing colonies. The clone, named pOR-1 has an insert of 3.8 kb but has been only partially characterized. Sequence analysis of the ends of this clone and the ends of an internal *EcoRI* subclone, pOR-2, has shown that the insert contains at least two complete genes: **glutamyl tRNA reductase, *hemA***, and **porphobilinogen deaminase *hemC***, and part of a third gene, *hemD*, which encodes uroporphyrinogen III. Together these probably represent a partial operon. The enzymes catalyze the early steps in the 5-carbon tetrapyrrole (heme) biosynthetic pathway. The result of the overexpression of *hemA* and *hemC* is buildup of protoheme, likely uroporphyrinogen I, which

E. coli cannot utilize. As result of this buildup, the cells are deeply pigmented and brilliantly fluorescent when exposed to a handheld UV light (350nm).

This fortuitous isolation may turn out to be quite fruitful for two reasons. One is that to date, no isolation of this operon has been reported from an anaerobic thermophile and uroporphyrinogen III synthase from other sources is susceptible to heat denaturation. Thus we have made this clone available to interested researchers in the field of heme biosynthesis (Prof. Samuel Beale, Brown University). The more important reason is that the glutamyl tRNA reductase is critical to heme biosynthesis with deletions being lethal in unless the media is supplemented with δ -aminolevulinic acid. This gene may be an ideal candidate for developing a nutritional mutant in *T. ethanolicus* which would further our attempts to develop a reliable transformation system in this organism.

5) "Putative permease" from *Thermoanaerobacter ethanolicus* JW200: An open reading frame, complete with a putative ribosome binding domain and initiating methionine, has been found in the 5' region of pbKXA-23. The translated sequence has a high degree of similarity with the multiple sugar transport system permease from *Streptococcus mutans*. The close proximity of the putative permease gene to the xylosidase/arabinosidase gene (33 nucleotides 5' to the xylosidase/arabinosidase initiating ATG) indicates that these two genes may be transcribed coordinately and could be part of an operon.

TASK: DEVELOPMENT OF A GENETIC SYSTEM FOR

***T. ethanolicus* and *Thermoanaerobacterium* JW/SL-YS 485:**

Autoplast Formation and Regeneration: For *Thermoanaerobacter* (syn. *Clostridium* syn.) *thermohydrosulfuricus* JW102 we developed and optimized a method to obtain autoplasts and successfully applied this method to *T. ethanolicus* (Peteranderl et al., 1993). We now have obtained even higher rates of autoplast formation and more than 20% regeneration to walled cells within 48 h. The shortening of the regeneration was important since the antibiotics which can be used are not particular stable under the regeneration conditions and *T. ethanolicus* frequently becomes resistant after two to three days (e.g., Kanamycin). This method was developed because an efficient host-vector system for our organism does not exist and will be an essential tool for later experiments.

Development of a shuttle vector between *E. coli* and *Thermoanaerobacterium* JW/SL YS485 and subsequent transformation of the thermophile (Mai et al., 1997).

Because to the above mentioned and also other problems, *T. ethanolicus* was not suitable to develop the first genetic system for an anaerobic thermophilic bacterium. Thus, we changed from the ethanologenic but weakly hemicellulolytic bacterium to a strong (see above) hemicellulolytic but weak ethanol producer. We have been successful and developed --although it is just the beginning-- a useful genetic system for *Thermoanaerobacterium* JW/SL-YS 485 and thus, exceeded by far the goal of the present grant application.

We spent quite some time on isolating one of the plasmids mentioned in the literature, however, we were not able to isolate and characterize them. All those we analyzed appeared to be chromosomal fragments or artifacts. Thus we evaluated various existing plasmids which have been used as shuttle vectors and modified them accordingly for our purposes. Our presently functioning genetic system in these thermophiles is based on the developed shuttle vector PIKM1 which is based on the pIMP1 vector from *Clostridium acetobutylicum* which in turn is based on the staphylococcal plasmid pUB10 as described by Mai et al, (1997). The vector contains the kanamycin resistance cassette from *Streptococcus faecalis* and the genes for ampicillin resistance and a Gram-type negative and a Gram-type positive origin of replication.

Transformation is obtained by electroporating the plasmid DNA into the above described autoplasts which after the electroporation are allowed to regenerate into walled cells. Kanamycin resistance was transformed successfully into *Thermoanaerobacterium* cells for verification. The plasmid was re-isolated from the thermophile and used for transforming *E. coli*.

Subsequently we have successfully expressed an active mannanase from *Cellulosiruptor* in our thermophile. Other enzymes will follow. However, the "ethanologenic genes" i.e. the genes for the pyruvate decarboxylase and ethanol dehydrogenase from the Gram-type negative mesophilic *Zymomonas* (gift from L.O. Ingram) could be transferred into the thermophile but no functional protein was expressed at all. We hope to obtain in the near future some funds to continue this work and being able to modify the organisms accordingly for the industrial ethanol production or for hemicellulose breakdown in the production of other feed stock chemicals.

C. SUMMARY:

- * During the grant period we have successfully purified to gel electrophoretic homogeneity and characterized six "hemicellulolytic" enzymes from the xylanolytic, non-cellulolytic thermophile *Thermoanaerobacterium* JW/SL-YS485 and three additional xylosidases from the ethanologenic *Thermoanaerobacter ethanolicus*, *T. thermohydrosulfuricus* JW102 and *Clostridium* spec. JW/SD190. The characterization includes the determination of NH₂-terminal sequences, and for some enzymes, internal peptides sequences.
- * We have investigated in part the regulatory properties of the enzymes and shown that several are acting synergistically, i.e., combined actions are required for efficient hydrolysis of the substituted xylooligomers and xylan as they are present in hemicellulolytic biomass. We have analyzed in more detail the regulation of xylanase activities in *Clostridium pseudocellulolyticum* JW/SD-EPP100 (DeBlois and Wiegel, 1995). We are still missing some of the purified enzymes (arabinosidase, mannosidase, mannanase) for a complete study of the interactions and dependencies of the hemicellulolytic enzymes in a mix and match experiment.
- * We have obtained genomic gene libraries (in vector λ ZAP Express (Stratagene) from the *Thermoanaerobacter ethanolicus* JW200 and *Thermoanaerobacterium* spec JW/SL-YS485. We also have obtained plasmid libraries for analysis of expression.
- * We have sequenced the gene for the bi-functional xylosidase/arabinosidase from *T. ethanolicus* and obtained clones which only exhibit the arabinosidase activity but not the xylosidase activity. We are presently screening for the opposite clone. Interestingly, the overall comparison of sequences in the gene bank reveals glucosidases as the most related enzymes (Lorenz and Wiegel, unpubl. results), and a detailed comparison of sequence segments is under way.
- * We have sequenced the genes for the xylosidase/arabinosidase from *Thermoanaerobacter ethanolicus* JW200, two xylosidases, acetyl(xylan)esterase, and the cell-bound xylanase from *Thermoanaerobacterium* JW/SL-YS485 as well as some putative genes of interest, and the glucose isomerase from *Thermoanaerobacterium* JW/SL-YS 489. The recombinant proteins were expressed and characterized and compared to the originally purified (parental) enzymes.
- *) We have developed a reliable method to generate and regenerate at high frequencies autoplasts from *Thermoanaerobacter* (Peteranderl et al. 1993) and subsequently improved the process to obtain regeneration within 48 h. We employed this method to develop successfully a gene transfer system for the thermophile *Thermoanaerobacterium* JW/SL-YS485. We developed a shuttle vector containing the kanamycin resistant gene cassette thermostable to above 60°C and which is stable in the thermophiles for many generation even without antibiotic selection. Subsequently we have expressed a heterologous enzyme activity (mannanase) in this thermophile and thus demonstrated that the present system can function to modify the thermophile.

D. PUBLICATIONS ON BY ANAEROBIC THERMOPHILES UTILIZING XYLOSE AND XYLAN published during the present grant period (1992 -1997) and supported by the grant:

INVITED SEMINAR AND PRESENTATIONS (1992-1997)

69. Xylose utilizer and xylose-isomerase. Novo Industries, Copenhagen, KD, August 1992.
70. Thermophilic (eu) bacterial anaerobes. Intern. Conference on Thermophiles: Science and Technology, (invited plenum talk), Reykjavik, Iceland, August, 1992.
71. Conversion of added acetate to butyrate by *C. thermobutyricum*. International Workshop Clostridium II, Blacksburg, VA, August 1992.
76. Thermophilic Clostridia. Symposium talk at Annual Meeting American Society for Microbiology, Atlanta, May 1993.
77. Anaerobic alkali-thermophiles, a novel group of organisms. University of Bonn., June 1993.
78. Xylose isomerase from thermophilic anaerobes. Techn. Univ. Hamburg (Germany) Novo-Meeting, June, 1993.
79. Anaerobic thermophiles growing at pH values below 5.0 and xylose isomerase. Technical University Hamburg, June 1993.
81. Extremophiles from not so extreme environments (in German) 3. Göttinger Mikrobiologen Forum. University of Göttingen, Germany October 1993 (in honor of Prof. H.G. Schlegel; invited plenum talk).
83. Alkalithermophiles, a novel group of extremophiles. University of Bergen, Norway October 1993 (invited seminar).
84. Alkalithermophiles. Deutsche Sammlung for Microorganisms (DSM) Braunschweig (Germany), October 1993 (invited seminar).
85. Novel alkali-thermophilic anaerobes-potential industrial organisms. Genencor, San Francisco, CA, May 1994 (invited seminar).
86. Alkalithermophiles, a novel group of thermophilic anaerobic (eu)bacteria. (Soc. Industrial Microbiol. Ann. Meet. Boston, August 1994 (invited seminar).
87. Alkalithermophiles, a novel group of thermophilic anaerobes. Div. of Biol. Sciences. Cornell University. September 1994 (inv. seminar)
89. The novel group of extremely fast growing alkalithermophiles. Seminar at the Von Humboldt Univ. Berlin , Germany. Juli 1995
90. Diversity and Distribution of Thermoanaerobacterium species (Inv. Lecture at 1st Yellowstone Nat.Park Symposium on Biodiversity, Ecology, and Evolution of Thermophiles in Yellowstone National Park: Overview and Issues. Old Faithful, Aug. 1995
92. Novel thermophilic anaerobes. JAMSTEC, Tokyo (Japan) (invited seminar); July 1996
93. Alkalithermophiles, a novel interesting group of fast growing bacteria (inv. seminar) Tokyo Univ. of Pharmacy and Life Science, Japan; July 1996
96. Horizontal gene transfer. Intern. Workshop "Thermophiles Key to Molecular Evolution and Origin of Life. Athens, GA. (Plenum presentation)
97. Isolation of some novel anaerobic bacteria. Thermophiles '96, Intern. Conference Athens, GA. (Plenum presentation)
98. Thermophilic alkaliphiles and novel thermophilic anaerobes. University of Naples. June 1997
99. Novel thermophilic anaerobes for industrial applications. University of Tuscia, June 1997

POSTERS AND TALKS PRESENTED AT MEETINGS (with printed Abstracts)

73. 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.
80. F. Canganella and J. Wiegel. Cultivation of *C. thermobutyricum* in a rotary fermenter system. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. p. 119.
81. Y. Li and J. Wiegel. Isolation and characterization of thermophilic alkalitolerant and alkaliphilic anaerobes. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. 11-P-10 (p. 66).
82. 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).
83. 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).
86. W. Shao, S. DeBlois and J. Wiegel. Acetyl xylan esterases from an anaerobic thermophile. Ann. Meet. Am. Soc. Microbiol., Atlanta, May 1993.
87. M. Engle, Y. Li and J. Wiegel. Isolation and characterization of various alkalitolerant and alkaliphilic anaerobic thermophiles from various habitats. Ann. Meet. Soc. for Industrial Microbiology and Canadian Soc. of Microbiologists. Toronto, August 1993 P-186.
88. M. Engle, Y. Li and J. Wiegel. A novel group of anaerobic (eu)bacteria: alkalitolerant and alkaliphilic thermophiles isolated from various mesobiotic and thermobiotic environments. Thermophiles '93. Intern. Meeting in Hamilton, NZ, December 1993 (invited plenum talk; Ext. Abstr. A10).
89. *Clostridium thermoalkalophilum*, a novel anaerobic alkaliphilic thermophilic isolated from mesobiotic sewage sludge. Thermophiles '93. Intern. Meeting in Hamilton, NZ, December 1993. Ext. Abstr. P43.
92. M. Engle, S. DeBlois, V. Mai, J. Brill, F. Rainey, and J. Wiegel. Anaerobic alkalithermophiles, a novel group of fast growing bacteria. IUMS 7th Intern. Congress of Bacteriology and Applied Microbiology Division, Prague July 1994. Abstr. BC-4-228.
94. J. Wiegel, Y. Li, and M. Engle, Novel anaerobic thermophiles growing at alkaline pH. Ann. SIM. Boston. Abstr. S 107.
96. V. Svetlitsnyi and J. Wiegel. Isolation and characterization of an obligately anaerobic lipolytic thermophilic alkalitolerant bacterium. Ann. Mee. Am. Soc. Microbiol. Washington DC, May 1995. Abstr. II
98. J. Brill, P. Youngman, and J. Wiegel. Detection of sporulation-specific genes in thermophilic, nonsporeforming isolates. Ann. Mee. Am. Soc. Microbiol. Washington DC, May 1995. Abstr. R32
99. S.-Y. Liu, F.C. Gherardini, M. Matuschek, H. Bahl, and J. Wiegel. Cloning, sequencing, and expression of an unusually large xylanase gene from *Thermoanaerobacter* sp. strain JW/SL-YS485. Ann. Mee. Am. Soc. Microbiol. Washington DC, May 1995
102. V. Mai, W.W. Lorenz and J. Wiegel. Development of a genetic system for the gram-type positive thermophilic anaerobe *Thermoanaerobacterium* strain YS485. Ann. Mee. Am. Soc. Microbiol. New Orleans, May 1996, O-75
103. A. Slobodkin and J. Wiegel. Fe(III) reduction with oxidation of H₂ by enrichment cultures of thermophilic microorganisms (Thermophiles '96, Intern. Conference Athens, GA. Sept. 96. (Abstr. GA. P1, A.17)

104. A. Slobodkin and J. Wiegel. "Thermoanaerobacter ferrireducens, gen. Nov., sp. Nov., and Clostridium glycerini, sp. Nov. New anaerobic glycerol utilizing thermophiles. Thermophiles '96, Intern. Conference Athens, GA. Sept. 96. (Abstr. GA. P1-A.11)
105. W. Lorenz and J. Wiegel. Isolation and expression of a unique xylosidase gene from Thermoanaerobacter sp. JW/SL YS485 and possible regulatory consequences of an adjacent open reading frame encoding a putative transcriptional activator. Thermophiles '96, Intern. Conference Athens, GA. Sept. 96. (Abstr. P.1.D.31.
106. J. Wiegel. Isolation of some novel anaerobic bacteria. Thermophiles '96, Intern. Conference Athens, GA. Sept. 96. (Abstr. S.A.3) (Plenum presentation)
107. J.A. Brill and J. Wiegel. Sporulation genes in non sporulating bacteria and sequence of a small acid soluble protein (SASP). A sporulation protein in the non spore former Thermoanaerobacter ethanolicus. Thermophiles '96, Intern. Conference Athens, GA. Sept. 96. (Abstr. S.B.7)
108. J. Wiegel. Horizontal gene transfer. Intern. Workshop "Thermophiles Key to Molecular Evolution and Origin of Life. Athens, GA. Sept. 96. Abstr.W24) (Plenum presentation).
110. V. Mai and J. Wiegel. Transfer and expression of heterologous genes in Thermoanaerobacterium spec. Strain JW/SL-YS485. Ann. Mee. Am. Soc. Microbiol. Miami, May 1997.

REVIEWS AND BOOK CHAPTERS (*The bolded ones are primary publications dealing directly with the research stated in the goals of the grant whereas the other citations deal with work which was funded in part by the DOE grant but are more indirectly related to the goals of the grant.*)

28. J. Wiegel. 1992. The obligately anaerobic thermophilic bacteria. Chapter 6 In: Thermophilic Bacteria. J. K. Kristjansson (ed.), CRC-Press, Boca Raton, FL, pp. 105-184.
30. F. Canganella and J. Wiegel. 1993. The potential of thermophilic clostridia in biotechnology. IN: The Clostridia and Biotechnology (D. R. Woods, Ed.) Chapter 16, Butterworths Publ. Stoneham, MA pp.391-429.
31. 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.
35. J. Wiegel. 1994. Potentials of acetogenic bacteria for industrial processes. Chapter 18 IN: Acetogenesis (Drake, ed). Chapman and Hall, N.Y. pp. 484-504.
37. J. Wiegel. 1998. Lateral gene exchange, an evolutionary mechanism for extending the upper or lower temperature limits for growth of an microorganism? A hypothesis. IN: Thermophiles: Key to Molecular Evolution and Origin of Life. (J. Wiegel and M. Adams (Eds) Chapter 11. Taylor & Francis, London (in press).

Research Publications in Peer-reviewed Journals (*Selection out of a total of 39 manuscripts published during the grant period 1992-1997*)

45. 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.
47. W. Shao and J. Wiegel. 1992. Purification and characterization of a thermostable β -xylosidase from *Thermoanaerobacter ethanolicus*. *J. Bacteriol.* 174:5848-5853.
50. R. Peterandel, F. Canganella, A. Holzenburg, and J. Wiegel. 1993. Induction and regeneration of autoplasts from *Clostridium thermohydrosulfuricum* JW102 and *Thermoanaerobacter ethanolicus* JW200. *Appl. Environ. Microbiol.* 59:3498-3501.
51. Y. Li, L. Mandelco and J. Wiegel. 1993. Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum*, sp. nov. *Int. J. Syst. Bacteriol.* 43:450-460.
52. Y. Li, M. Engle, L. Mandelco and J. Wiegel. 1994. *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. *Int. J. Syst. Bacteriol.* 44:111-118.
57. W. Shao, S. DeBlois and J. Wiegel. 1995. A high-molecular weight, cell-associated xylanase isolated from exponentially growing *Thermoanaerobacterium* sp. strain JW/SL-YS485. *Appl. Environ. Microbiol.* 61:937-940
58. W. Shao and J. Wiegel. 1995. Purification and Characterization of two thermostable acetyl xylan esterases from *Thermoanaerobacterium* sp. Strain JW/SL-YS485. *Appl. Environ. Microbiol.* 61:729-733.
59. W. Shao, S. K. C. Obi, J. Puls, and J. Wiegel. 1995. Purification and Characterization of the α -glucuronidase from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an important enzyme for the utilization of substituted xylan. *Appl. Environ. Microbiol.* 61:1077-1081
60. S. DeBlois and J. Wiegel. 1995. Cellulolytic vestiges of the xylanase activity in a new strictly xylanolytic thermophile *Clostridium* sp. *Biotechnol. Lett.* 17:89-94
62. M. Engle, Y. Li, C. Woese, and J. Wiegel. 1995. Isolation and characterization of a novel alkalitolerant thermophile, *Anaerobranca horikoshii* gen. nov. sp. nov. *Int. J. Syst. Bacteriol.* 45:454-461
63. S.-Y. Liu, F.A. Rainey, H.W. Morgan, F. Mayer, and J. Wiegel. 1996. *Thermoanaerobacterium aotearoense*, sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand and emendation of the genus *Thermoanaerobacterium*. *Int. J. Syst. Bacteriol.* 46: 388-396
64. Y.-S. Liu, F.C. Gherardini, M. Matuschek, H. Bahl, and J. Wiegel. 1996. Cloning, sequencing, and expression of the gene encoding a large S-layer-associated endoxylanase from *Thermoanaerobacterium* sp. strain JW/SL-YS 485 in *Escherichia coli*. *J. Bacteriol.* 178:1539-1547
65. F.A. Rainey and J. Wiegel. 1996. 16S rDNA sequence analysis confirms the close relationship between the genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter* and reveals a lack of phylogenetic coherence among the species of the genus *Xanthobacter*. *Int. J. Syst. Bacteriol.* 46: 907-610
66. M. Engle, Y. Li, F. Rainey, S. DeBlois, V. Mai, A. Reichert, F. Mayer, P. Messmer, and J. Wiegel. 1996. *Thermobrachium celere*, gen. nov., sp. nov., a fast growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. *Int. J. Syst. Bacteriol.* 46:1025-1033
68. S.-Y. Liu J. Wiegel, F.C. and Gherardini. 1996. Purification and cloning of a thermostable xylose (glucose)isomerase with an acidic pH optimum form *Thermoanaerobacterium* sp. Strain JW/SL-YS489 *J. Bacteriol.* 178:5938-5945.

69. V. Svetlitsnyi, F. Rainey, and J. Wiegel. 1996. *Thermosyntropha lipolytica* gen. nov., sp. nov., a lipolytic, anaerobic, organoheterotrophic, alkalitolerant thermophile utilizing short- and long chain fatty acids in syntrophic coculture with an alkaliphilic and thermophilic bacterium. *Int. J. Syst. Bacteriol.* 46:1131-1137.
70. G. M. Cook, J. B. Russel, A. Reichert, and J. Wiegel. 1996. The intracellular pH of *Clostridium paradoxum*, an anaerobic alkaliphilic, and thermophilic bacterium. *Appl. Environ. Microbiol.* 62:4576-4579
72. V. Mai, W.W. Lorenz, and J. Wiegel. 1997. Transformation of *Thermoanaerobacterium* spec. Strain JW/SL-YS 485 with plasmid pIKM1 conferring kanamycin resistance. *FEMS-Letters* 148:163-167
73. A. Slobodkin and J. Wiegel. 1997 Fe(III) as an electron acceptor for H₂ oxidation in thermophilic anaerobic enrichment cultures from geothermal areas. *Extremophiles* 2:106-109
74. A. Slobodkin, A.-L. Reysenbach, N. Struty, M. Dreier and J. Wiegel (1997) *Thermoterrabacterium ferrireducens*, gen. nov., sp. nov., a thermophilic anaerobic dissimilatory Fe(III)-reducing bacterium from a continental hot spring. *Int. J. Syst. Bacteriol.* 47:541-547
75. A. Slobodkin, A.-L. Reysenbach, and J. Wiegel. 1997. Isolation and Characterization of the homoacetogenic thermophile *Moorella glycerini* sp. nov. *Int. J. Syst. Bacteriol.* 47:969-974
76. J. Brill and J. Wiegel. 1997. Differentiation between spore forming and asporogenous bacteria by a PCR and Southern hybridization based method. *J. Microbiol Methods.* (in press) ***
77. W. Lorenz and J. Wiegel. 1997. Isolation, analysis, and expression of two genes from *Thermoanaerobacterium* sp. strain JW/SL-YS485: a novel acetyl xylan esterase with cephalosporin-C deacetylase activity and a β -xylosidase. *J. Bacteriol.* 179:5436-5441

In addition the following thesis and Dissertations were written from students supported by the DOE grant:

Master thesis:

- 1) Youhong LI, 1993. Isolation and characterization of anaerobic thermophiles growing at alkaline pH.
- 2) Marcella M. ENGLE, 1995. Isolation and characterization of *Anaerobranca horikoshii*, gen. nov., spec. nov., and other similar isolates
- 3) Suzanne DE BLOIS, 1996. Systematics of *Thermotorguereum fastidiosum* and *Clostridium pseudocellulolyticum*. Two new taxa of xylanolytic, thermophilic and anaerobic bacteria.
- 4) Julia BRILL, 1997 The presence of sporulation genes in non-sporulating *Clostridium/Bacillus* and related species.
- 5) Mona G. DASHTI, 1997 Characterization of the anaerobic thermophile *Thermoanaerobacter sulfurigignens* sp. nov.

Ph.D. dissertations:

- 1) Weilan SHAO, (1993). Isolation and characterization of hemicellulolytic enzymes from anaerobic thermophiles.
- 2) Shu-Ying LIU, (1995). Isolation and characterization of *Thermoanaerobacterium aotearoense* sp. nov. and the xylose isomerase and xylanase from a novel *Thermoanaerobacterium* sp.

Due to the unexpected loss of the funds and the leave of the postdoctoral candidate two of the ms are still in preparation: (Lorenz et al. Cloning and sequence analysis of the two-functional arabinosidase/xylosidase from T. ethanolicus; Lorenz et al. Sequence of a novel xylosidase from Thermoanaerobacterium JW/SL-YS 485 and its characterization and comparison of the xylosidases from Thermoanaerobacter thermohydrosulfuricus)