Abstract

ENRICHMENT FROM VARIOUS HABITATS OF BACTERIA CAPABLE OF DEGRADING XYLAN AT HIGH TEMPERATURE AND HIGH pH

Lee Hughes
MBL Microbial Diversity, 1993

The ability to enzymatically remove xylan from wood pulp has important industrial implications since current chemical methods of removal create toxic by-products, i.e. dioxins. Treatment conditions require an enzyme capable of xylan-degradation at pH 9.0 and 70 degrees Celsius.

In an attempt to isolate organisms able to degrade xylan under these conditions, samples were chosen from several freshwater and marine habitats in the Woods Hole, MA area. Freshwater samples were: 1) household compost; and 2) Garden Pond sediment. Marine samples from the Great Sippewisset Salt Marsh were: 3) decaying algal matter; 4) soil from the fringe of the salt marsh tidal zone; and 5) mud flat sediment rich in organic material. Enrichments were carried out in a basal salts medium at pH 9.0 with xylan as the sole carbon source under both aerobic and anaerobic conditions. Incubation temperatures were 50, 60 and 70 degrees Celsius.

Growth was found in four of the samples under aerobic conditions at 50 degrees, the only exception being Sample 3. No growth was found in the aerobic samples at higher temperatures or in any of the anaerobic samples. The enriched organisms were primarily a number of Gram-positive rods, many of which were observed to form spores. One filamentous organism, presumably an actinomycete, was also found.

Although the organisms were not isolated and characterized, this study shows that a variety of habitats, including those not obviously meeting the conditions sought, can harbor organisms with diverse growth tolerances. Careful selection of inocula with this in mind can expand the range of potential sample sites from which enrichments can be drawn.
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The ability to enzymatically remove xylan from wood pulp has important industrial implications since current chemical methods of removal create toxic by-products, i.e. dioxins. Treatment conditions require an enzyme capable of xylan-degradation at pH 9.0 and 70 degrees Celsius.

This report will describe an experiment aimed at isolating such organisms from the available inocula in the Woods Hole, MA area. It was presumed that these organisms should be found in sampled areas that do not necessarily appear to exhibit either or both of the conditions (pH 9.0 or 70 degrees Celsius) sought. It is likely that microenvironments exist in many areas that at times might meet these conditions. Therefore, organisms able to survive and grow at high pH and at high temperature should be isolatable from such inocula.

Materials and Methods

Inocula:

The main criteria used in selecting inocula for this study were the presence of organic material likely to contain xylan (namely any plant debris) and the possibility of localized heating (i.e. active composting, solar irradiation). The pH was not considered.

Five samples were collected (two freshwater and three marine):

#1 - Compost collected from the interior base of a compost pile located at the home of Dr. Ed Leadbetter. No active heating was noted.

#2 - Anoxic sediment from Garden Pond (freshwater).

(Marine samples all obtained from the Great Sippewisset Salt Marsh)

#3 - Residue from the interior of a decaying pile of algae. The outside of the pile was in dried by direct sunlight.

#4 - Fringe soil taken at the edge of the salt marsh/non-marsh upper tidal boundary.

#5 - Sediment from an exposed mud flat which was high in organic material.
Media:

The same base media was used for all liquid and solid media. Slight modifications (listed below) were made depending on whether the media needed to be anaerobic, marine, or at a different pH:

Modified Methanogen/Acetogen Freshwater Medium for Xylan Degraders (per 1 liter)

First prepare salts solution (in final volume of 897 ml - aerobic
895 ml - anaerobic)

KH$_2$PO$_4$ 0.2 g
NH$_4$Cl 0.25 g
KCl 0.5 g
CaCl$_2$-2H$_2$O 0.15 g
NaCl 1.0 g
MgCl$_2$-6H$_2$O 0.62 g

Autoclaved above [For solid media: adjusted pH and added buffer before autoclaving. Agar added to 1.5%] and then cooled (cool under N$_2$ gas for anaerobic). Add remaining constituents in order.

10X NaHCO$_3$-Na$_2$CO$_3$ Buffer, pH 9.0 100 ml
[Note: For plates, substituted either Tris or AMPSO, final concentration 20 mM, for pH 9.0. Used MOPS for pH 7.0 media and Tris for pH 8.0]
0.5 M Na$_2$S Solution (under 100% N$_2$) 2 ml [for anaerobic only]
SL-10 Trace Element Solution 1 ml
Six Vitamin Solution 1 ml
Vitamin B Solution 1 ml

Anaerobic media pH was adjusted to 9.0 using sterile NaOH (1N). Dispensed as follows to bottles containing washed xylan (final concentration 0.2%) [for plates, 10X xylan slurry autoclaved separately and then added to media before pouring]:

Anaerobic - 50 ml per bottle (1/2 full). Gassed headspace with N$_2$ and stoppered.

Aerobic - 25 ml per bottle (1/4 full). Stoppered (leaving normal atmosphere, but minimizing CO$_2$ exchange which will lower pH of media).

For Marine samples: Added appropriate volume of 10X Marine Salts Solution. Added Na$_2$SO$_4$ to a final concentration of 28 mM.

Washed Xylan Preparation: 5 g of Oat Spelt Xylan was washed in 50 ml of 70% ethanol. After mixing well, the particles were allowed to settle and the supernatant drained off. This process was repeated. A final wash of 95% Ethanol was done, and then the xylan was air dried in a 37 degree incubator with frequent mixing to promote even drying.
10X NaHCO3-Na2CO3 Buffer, pH 9.0: Made stock solutions of 250 mM NaHCO3 and Na2CO3. While checking the pH, added Na2CO3 into the NaHCO3 solution. Approximately 15 ml were required to bring a 250 ml volume to pH 9.0. 50 ml aliquots in bottles were gassed with N2, stoppered, crimped, and autoclaved.

10X Marine Salts: 19% NaCl and 2.4% MgCl2 in a final volume of 100 ml dH2O. 50 ml aliquots were bottled, gassed with N2, stoppered, crimped, and autoclaved.

Media Code Key for designating enrichments: X= aerobic, freshwater, xylan
MX= aerobic, marine, xylan
AX= anaerobic, freshwater, xylan
MAX= anaerobic, marine, xylan

Procedure:

Initial Enrichment

Inoculated 7 bottles per sample. One anaerobic and one aerobic per sample at 50 °C, 60 °C, and 70 °C (stationary water baths), plus another aerobic bottle each in a 70 °C shaker. Designated by Sample # - Media - Temperature

Inocula were prepared by adding 5 ml of sterile media (no xylan, used aerobic media) to a small amount (~1 g) of sample in a sterile 16X150 mm tube. Stoppered and vortexed each sample twice for 1 min. (total 2 min.). Allowed sediment to settle. Pour supernatant into a surface-sterile beaker (95% ethanol, flamed). Drew sample into a 5 ml syringe (~3.5 ml/sample). Aseptically dispensed 0.4-0.5 ml in each bottle per sample. Incubated samples at appropriate temperatures.

Isolation Attempts

Streaked to plates after removing a drop from the growing enrichments and placing on the plate. The initial streaks were all done to a freshwater xylan plate regardless of sample origin. This set of plates also lacked the trace element and vitamin solutions (unintentional). Another set of plates were made subsequently and contained all the appropriate elements, as well as marine salts for the marine samples. In addition, identical plates were made which contained xylose as carbon source.

Growth rate at different pH and temperature

Balch tubes containing 10 ml of media (three different pH media, 7.0, 8.0, and 9.0) were prepared and inoculated with a loop full of the chosen isolate, 5-MX-50(2). One tube at each pH was incubated at the following temperatures: 30, 37, 50 and 60 °C. Growth was measured by absorbance at 600 nm.
Xylan degradation - growth for analysis by HPLC

10 ml of the appropriate aerobic media at pH 9.0 was dispensed into sterile 50 ml Erlenmeyer flasks containing xylan (5mM). Flasks were inoculated from the following enrichments/isolates:

1-X-50 - 0.1 ml from primary enrichment  
2-X-50 - loop from initial plate, streak area (not isolated)  
4-MX-50 - loop from fine colony growth.  
5-MX-50(1) - isolated colony picked with loop from isolate #1  
5-MX-50(2) - isolated colony picked with loop from isolate #2

Stoppered flasks were incubated on shaker at 50 °C.

Utilization of various carbon sources

Tested the ability of isolates to grow on other polysaccharides. Used Starch Agar (Nutrient agar + 1% soluble potato starch) and Freshwater agar media (pH 9.0) with either 0.2% cellulose or 0.1% laminarin. The same inocula was used as in the immediately previous description. Starch hydrolysis was visualized by flooding the plate with Gram’s Iodine and looking for zones of clearing. Similarly, Congo Red (1% aqueous solution) was used to stain original xylan plates to look for clear zones indicative of xylan utilization.

Observations

Growth was found only in the aerobic bottles at 50 °C for samples 1, 2, 4 and 5. Some growth was seen in bottle 2-X-60, but pH was at 8.0. When adjusted back to 9.0, no further growth was apparent. Nothing grew on a xylan streak plate from this sample incubated at 60 °C. Isolations were attempted from all those bottles which contained growth. Results of these isolation plates and other tests are summarized in the attached chart. Note that the HPLC analysis of supernatants was performed only for sample 2-X-50 because it was the only one with moderate growth in the shaken incubation. No sugars (xylose or arabinose) were seen in the supernatant. The growth curve experiment at different pH and temperatures achieved no results. Most likely, based on other results, isolate 5-MX-50(2) does not grow well on xylose.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Organism Morphologies</th>
<th>Feat/ed for</th>
<th>Gram Stain</th>
<th>Catalase</th>
<th>Growth on: Xylan</th>
<th>Xylose</th>
<th>Cellulose</th>
<th>Laminarin</th>
<th>Starch Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compost</td>
<td>Short, thin rods; endospores</td>
<td>-</td>
<td>+</td>
<td>(mixed)</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Medium rod</td>
<td>-</td>
<td>+</td>
<td></td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Very long rod</td>
<td>-</td>
<td>+</td>
<td></td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Filamentous, branched with terminal swellings</td>
<td>-</td>
<td>ND</td>
<td></td>
<td>All Growth in Medium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Garden Pond</td>
<td>Small rod, endospores</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>t+ Cleaning</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salt Marsh Fringe Soil</td>
<td>Thin rods, - more than one type</td>
<td>?</td>
<td>+</td>
<td>(F)</td>
<td>- (M)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Salt Marsh Mud Flat</td>
<td>(1) Short rod, oval endospores</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Mixed</td>
</tr>
<tr>
<td></td>
<td>(2) Rod, no visible spores</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Conclusions and Recommendations

Although the original goal of isolating a xylan-degrading organism which grows at pH 9.0 and 70 °C was not realized, the success in enriching aerobic organisms at pH 9.0 and 50 °C holds promise that inocula of the type used can supply organisms outside the expected range of tolerances. With this in mind, studies of this type to find a xylan-degrader who can fulfill the original requirements should be continued.

Based on the difficulties encountered in this study, the following recommendations are made to anyone attempting to continue this work. First would be a careful consideration of the buffering system used for the aerobic media. The carbonate buffering system was difficult to maintain at pH 9.0 due to exchange of CO₂ from the atmosphere. The bottles were sealed, but air had to be exchanged periodically to maintain oxygen availability. The fluctuations in pH appeared to allow for some growth that would otherwise have been unable otherwise (note the results for bottle 2-X-60). Another observation worth noting due to the sealed bottles is that microaerophilic conditions may have been produced in some of the bottles. Since some of the isolation plates grew poorly (for example 4-MX-50), it would be worth investigating for the possibility that a microaerophile had been enriched. It should also be noted that, based on information on alkalophilic bacilli in Bergey's Manual which was read after this study was completed, that Na⁺ is required for their growth. The growth media used here should be scrutinized to determine if it satisfies that requirement.

Another approach to consider is approaching the original enrichment from one of the two aspects, either temperature or alkalinity, and then performing a secondary enrichment at gradations of the other factor to find the upper tolerances of the organisms. This approach might prove more effective in finding organisms closer fitting to the original goal of pH 9.0 and 70 °C. Overall, due to the success of the primary enrichments in 4 out of 5 samples, it appears that many environments are capable of containing organisms which may not fit the expectations one might have based on the macro features of the environment. One difficulty with this study, especially in the limited time available, was the broad-based attempt to characterize all the organisms which were enriched. A more successful approach, in terms of characterizing specific organisms, would be to concentrate on isolation and characterization of a single, promising organism from the primary enrichment.
Relevant References


Abstract

EXAMINATION OF XYLAN-DEGRADERS IN A HIGHER TERMITE HINDGUT

Lee Hughes
MBL Microbial Diversity, 1993

The goal of this project was to determine the role of xylan-degrading bacteria in the hindgut of a higher termite, *Nasutitermes nigriceps*. Anaerobic and aerobic serial dilutions were prepared in media containing 0.5% Yeast Extract, 0.5% Casamino Acids, and washed xylan. Dilutions were carried out to $10^{-6}$ hindgut equivalents.

After 4 days incubation, growth was observed in all samples. They were incubated an additional 2 days to watch for increases in turbidity above basal growth and for degradation of xylan. Aerobic growth did not appear to increase, but several of the anaerobic dilutions looked promising. A shake series was performed on those samples, utilizing xylose in place of xylan. Isolated colonies were obtained for the $10^{-4}$, $10^{-5}$, and $10^{-6}$ samples. An additional isolate was obtained from a Pasteurized series of the $10^{-5}$ dilution.

Microscopic observation revealed that 3 types of organisms had been isolated (the $10^{-4}$ sample and the $10^{-5}$ un-Pasteurized isolate appeared to be the same). HPLC analysis of the growth media for samples grown in xylan did not show any release of xylose or buildup of fermentation products. On xylose, neither the $10^{-6}$ or $10^{-5}$ Pasteurized isolates appeared to utilize xylose. The $10^{-5}$ un-Pasteurized isolate proved to be a heterofermentative lactic acid bacterium, presumably *Lactobacillus*.

The results of this experiment were inconclusive as to the role of xylan-degraders in the higher termite hindgut. Further study is necessary to determine if these isolates do indeed degrade xylan or, if not, which organisms in the primary enrichments were responsible for utilization of xylan.
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The goal of this project was to determine the role of xylan-degrading bacteria in the hindgut of a higher termite, *Nasutitermes nigriceps*. Since termites feed on wood, of which xylan is a major constituent, bacteria which can degrade this polysaccharide could play a major role in the termite hindgut. Serial dilutions were done to determine the relative number of xylan-degraders present in the hindgut. Both anaerobic and aerobic conditions were used.

Materials and Methods

Inoculum

In the anaerobic glovebox, a homogenate of hindguts from *Nasutitermes nigriceps* was prepared to a concentration of 10 hindguts/ml. This inoculum was used for the anaerobic samples. Another homogenate was prepared at the lab bench to a concentration of 2 hindguts/ml. This inoculum was used for the aerobic samples.

Media

Anaerobic: Used Freshwater Anaerobic Media (from Methanogen/Acetogen enrichments) with the following changes:

- omit Na2S
- add Na2SO4 to 1 mM final concentration

Prior to dispensing, added 0.1 ml of 100X PdCl2 and 0.1 ml of 100X YE/CAA stock (see aerobic media) to a sterile bottle containing 0.009g washed xylan (0.1% final concentration) [Note: Xylose media for isolation was prepared by replacing the xylan with 0.1 ml of 1M Xylose]. Added 9 ml of media to the bottle while gassing with H2/CO2. Crimped and sealed.

Aerobic: 0.1% CYE (per liter)

100X Yeast Extract (5%)/Casamino Acids (5%) 2 ml
1M Tris pH 7.5 (to final concentration 20 mM) 20 ml
d H2O 978 ml

Dispensed 4.5 ml into a sterile Erlenmeyer flask containing 0.009g washed xylan (0.2% final concentration).
Shake Series Media: Same anaerobic base media, substituting 0.1 ml of sterile 1M Xylose for xylan. Each agar tube contained 3 ml of 3% washed agar to which 5 ml of media was added. All additives (PdClz, YE/CAA, Xylose) were added after base media mixed with liquefied agar and placed at 40 °C.

Procedure

Initial Enrichment

Anaerobic: Added 1 ml of inoculum (10 hindgut equivalents) to the first bottle of the series with a syringe. Serial dilutions were made by mixing and then transferring 1 ml to each successive bottle until a dilution of 10^-6 hindgut equivalents was reached. Bottles were incubated at room temperature in a drawer (Note: gas in head space was never exchanged for N2/CO2).

Aerobic: Added 0.5 ml of inoculum (1 hindgut equivalent) to the first flask of the series. Serial dilutions were made by mixing and then transferring 0.5 ml to each successive bottle until a dilution of 10^-6 hindgut equivalents was reached. Flasks were incubated at 30 °C on a shaker.

Shake Series

Prepared media as above. Inoculated first tube of the series with 0.1 ml of inoculum from primary anaerobic dilutions (10^-4, 10^-5 {two tubes}, and 10^-6) after 6 days incubation. One tube of the 10^-5 dilution was Pasteurized for 20 min. at 70 °C prior to continuing the shake series. After inoculation, a dilution series was prepared by carefully transferring a small drop (~.1 ml) to the next tube in the series. After careful mixing by inversion, the process was repeated until a total of 5 tubes per series were inoculated. Immediately gassed with H2/CO2 and cooled on a slant. Tubes were incubated in the dark at room temperature (head space replaced with N2/CO2 after overnight incubation).

Isolation

Picked isolated colonies from slant series after 2 days incubation with a drawn out, sterile Pasteur pipette (cotton-plugged). Resuspended in 1 ml of FW medium in a sterile Eppendorf tube. Transferred half of the suspension into a bottle of anaerobic media with xylan as carbon source (media prepared as in original enrichment) and the other half to a bottle containing xylose as carbon source. Incubated at room temperature in a desk drawer.

HPLC Analysis

Used a Differential Refractometer to observe volatile fatty acids and sugars in the supernatant of isolated cultures. Prepared samples by aseptically removing
1 ml with a syringe and placing in an Eppendorf tube which was spun for 2 min. to remove cells and other particles. 200 ul of the supernatant was transferred to a clean tube to which was added 50 ul of 1% H₃PO₄. After mixing, the samples were observed on 15 min. runs of the HPLC and peaks compared to known standards.

Observations

Growth was found in all the enrichments down to the 10⁻⁶ dilutions after 4 days incubation. Incubation was continued to 2 additional days to look for growth above the basal level. None of the aerobic enrichments appeared to grow (examined microscopically - almost all cells seen initially had sporulated by second observation or disappeared). Several of the anaerobic enrichments looked promising so were continued for isolation in a shake series. These were the 10⁻⁴, 10⁻⁵, and 10⁻⁶ anaerobic dilution bottles (see methods). Three types of organisms were isolated from these shake tubes, one from the 10⁻⁶ dilution (designated strain NNX-I) and two from the 10⁻⁵ dilution (one survived Pasteurization, designated NNX-2, the other, NNX-3, did not. The second appeared to be identical to isolates found from the 10⁻⁴ dilution series). Observations of these isolates are summarized in the attached chart.

Conclusions

As seen from the results of the HPLC analysis, it does not appear that any of the isolates is degrading xylan. Isolate NNX-3 fermented xylose to produce acetate, lactate, and ethanol, and is most likely a lactic acid bacterium, presumably Lactobacillus. Further study is necessary to determine if the other isolates do indeed degrade xylan or, if not, to determine which organisms in the original enrichments were responsible for degradation of the xylan. The use of xylose in the shake series may have selected for faster growing xylose-utilizers (although NNX-1 and NNX-2 do not appear to use xylose). A longer incubation period may be necessary to successfully isolate the xylan-degrading organisms.
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Morphologies (Primary Enrichment)</th>
<th>Gram-Stain</th>
<th>Isolated</th>
<th>Survival Pasteurization</th>
<th>Growth on:</th>
<th>HPLC Products:</th>
<th>Products:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
<td>Long, thin rod. Motile, sporeform. Coccobacillus</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>Acetate (trace) Acetate (trace)</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>Large rod, sometimes in chains. Sporeform. Veils in liquid culture.</td>
<td>+</td>
<td>+</td>
<td>ND.</td>
<td>+++</td>
<td>++</td>
<td>Acetate (trace) Acetate (trace)</td>
</tr>
</tbody>
</table>