The Isolation and Characterization of Novel Pectinolytic Organisms

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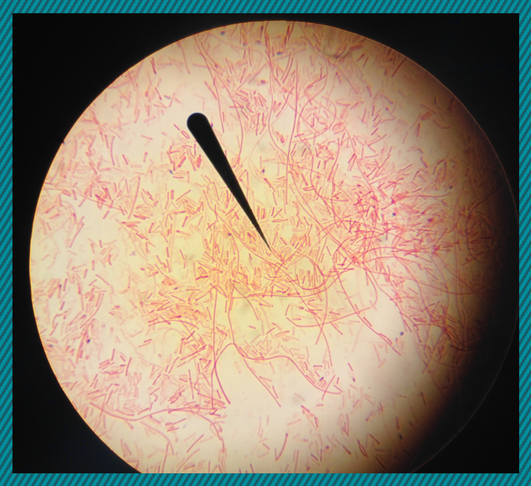

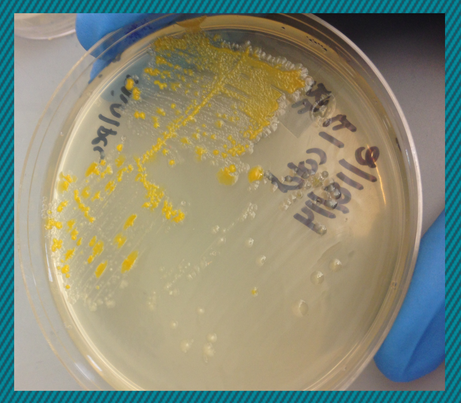

Abstract:

Pectinolytic enzymes can be used for the conversion of agricultural and other wastes containing pectin to produce simpler sugars, which can then be fermented for the production of biofuels. Bacterial strains with highly active pectinolytic enzymes that function at high temperatures can be engineered to reduce pectin more effectively. Bacterial organisms were isolated from aquatic and terrestrial environments within the Frederick area. Environmental samples were grown at 55°C in minimal pectin broth and then plated on minimal pectin plates and 12-15 distinct strains of bacteria have been isolated. The isolated bacteria were identified through PCR of the 16s rDNA sequence and several strains of *Bacillus licheniformis* and Bacillus subtilis as well as others have been discovered. The growth patterns and other physical characteristics (colony morphology etc.) of the isolated organisms have been investigated and have produced interesting results. A particular strain that has yet to be identified, which grows in small, red colonies, has been noted to grow faster and more prolifically in pectin-based mediums as opposed to Luria Broth, a very unique and valuable trait that will be looked into with greater detail.

Introduction:

Biomass covers about 10% of the world's primary energy demand (Antoni et al. 2007). With rising crude oil prices, depletion of natural resources, environmental instability and pollution challenges, only biofuels have the potential to supply the most realistic, sustainable transportation energy. Bioethanol production in the United States has risen from 4.0 million cubic meters annually in 1996 to 18 million cubic meters in 2006 (Antoni et al. 2007) Biomass in the form of agricultural and plant waste (such as sugar beet pulp) can be turned into fuel by way of certain microbes that can break down the polysaccharides found in the cell walls of plants into simple sugars. These can be fermented to alcohol or other molecules and then be refined and turned into transportation fuel.

The ultimate goal in the production of biofuels is to produce fuels to replace petroleum-derived fuels in commercial quantities. Most biofuel production is done by saccharification at mesophilic temperatures (25-37°C), but thermophilic processes (45-55°C) seem to be more productive, and are increasingly researched and developed. To allow for thermophilic saccharification, biologists have been engineering microbes to treat biomass at optimal conditions, by working with enzymes that can remain functional at high temperatures and pressure. It is possible to create 'designer proteins' from these microorganisms to aid in the degradation of pectin or other complex sugars in plant materials.

To be able to engineer these temperature-stable, pectinolytic enzymes, bacteria that are tolerant to high temperatures, and can degrade pectin readily, must be discovered and characterized. The purpose of this research is to collect environmental samples where pectinolytic bacteria are hypothesized to be found, and characterize their phenotypic qualities and growth patterns at high temperatures in environments where pectin is the only energy source, to then be used to create biofuels more efficiently.

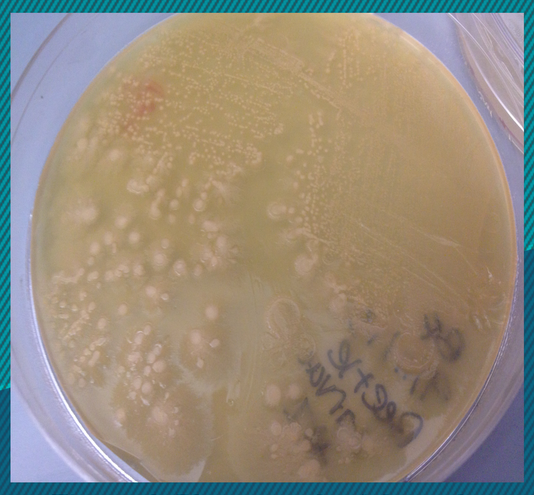

Figure 1. Photograph of the unkown terrestrial microbe isolated from Beetle Larvae feces.

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Figure 2. The 16s rDNA sequence of the unknown microbe pictured in Figure 1.

Methods:

Terrestrial samples were collected in late August/early September from the Baker Park area of Frederick, Maryland. Wooly caterpillars, beetle larvae, and grasshoppers, were caught and identified. The insects were placed in captivity and fecal samples were collected over the following days. Aquatic samples were collected from Baker Park during early February, from different microenvironments throughout Carroll Creek, a tributary of the Monocacy River. Samples from the water column, and sediment were collected from: an area of riffle, a pooled area (including leaf piles), a runoff lake, and a flooded nearby grassy field.

The aquatic and terrestrial samples were inoculated in 125 mL flasks containing a minimal pectin broth (!% citrus pectin as sole carbon source) and incubated at 55°C for 2-3 days. Alternatively, all water samples were incubated at 4°C in 125mL flasks containing the same minimal pectin broth. After samples were allowed to incubate, they were streaked onto minimal pectin plates and allowed to incubate at 55°C for 2-3 days. The plates were then examined, and physical characteristics, such as colony morphology, were noted and recorded. Once examined, individual colonies of varying bacteria were re-streaked onto fresh plates for incubation of homogeneous colonies. The resulting strains of unknown organisms were then Gram stained, and the bacterial cell morphology was determined.

To identify the unknown pectinolytic bacterial strains, polymerase chain reaction (PCR) was used to amplify the 16s rDNA sequence. The PCR conditions were as follows: 4 minutes at 96°C; 35 cycles of 45 seconds at 94°C, 30 seconds at 55°C and 60 seconds at 72°C; 10 minutes at 72°C. After completion of the PCR, a 1.5% Agarose gel was performed to assure that the PCR was successful. To perform the agarose gel analysis, a 100 base pair marker from TaKara was used and the gel was allowed to run for an hour and stained with ethidium bromide for 10 minutes. The gel was analyzed using a G:Box with UV light. The PCR products were then sent out for sequencing.

After all analysis was complete, the samples were preserved. To preserve the unknown bacteria, they were grown in a 15mL tube in Luria Broth overnight at 55°C. The samples were then spun, and the supernatant was poured off and the cells were rinsed with a salt solution. After rinsing, the samples were put into labeled, small glass sample vials and stored in a freezer at -80°C.

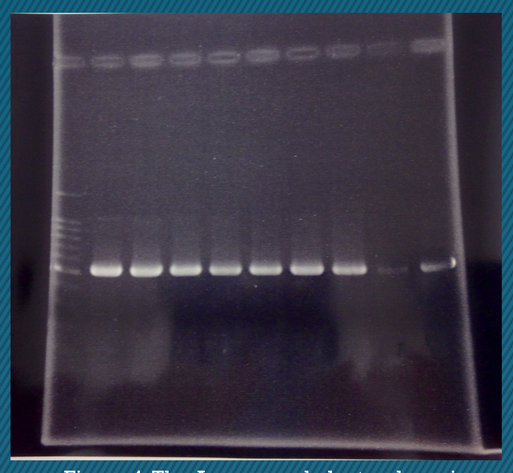

Figure 4. The Agarose gel electrophoresis performed on the unknown bacterial strains. The 100bp ladder is shown in the far left oflane of the gel. The negative control is showin in lane 9 and the positive control in lane 10.

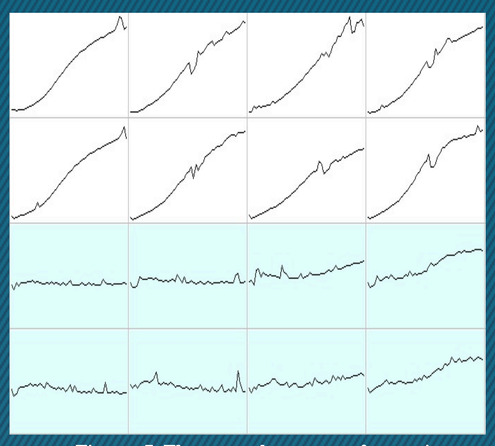

Figure 5. The growth curves of aquatic samples C2-1, C2-2, C2-3, and F1-1. Blue shaded curves are from growth in pectin minimal media, white are from glucose. Each color in each column contains two replicates

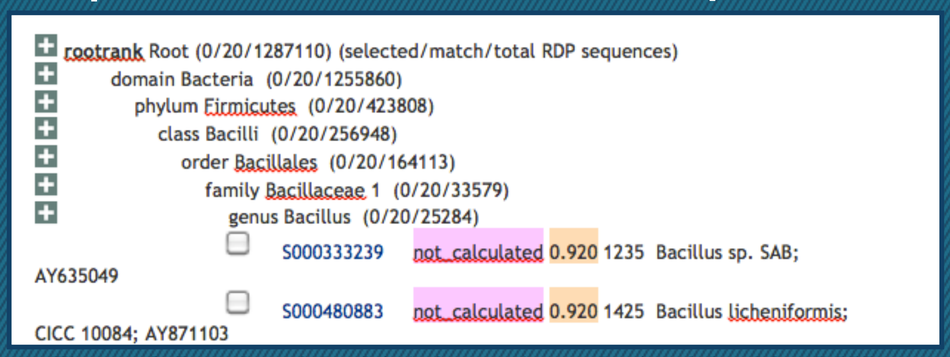

Figure 3. Results of the bioinformatic analysis of the 16s rDNA sequence given in Figure 2. It was discovered that the unknown microbe pictured in Figure 1 was *Bacillus licheniformis*.

References:

Antoni D, Zverlov V, Schwarx W (2007) Biofuels from microbes. Applied Microbiology and Biotechnology 77: 23-25

Ghim C, Kim, T, Mitchell R, Lee S (2010) Synthetic Biology for Biofuels: Building Designer Microbes from Scratch. Biotechnology and Bioprocess Engineering 15: 11-21

Sirotek K, Marounek M, Rada V, Benda V (2001) Isolation and Characterization of Rabbit Caecal Pectinolytic Bacteria. Folia Microbiologica 46(I): 79-82

Tamburnini E, Gordillo A, Perito L, Mastromei G (2003) Characterization of bacterial pectinolyic strains involved in water retting process. Environmental Microbiology 5(9): 730-736

Results:

The terrestrial bacterial strains isolated from insect feces were the first to be characterized. About five strains were isolated that were readily grown in 55°C after two days were noticed and investigated. Three of the strains showed a "fried-egg" shaped colony morphology that was cream colored and flat. It was noted that these three strains grew faster on the pectin minimal plates at 55°C than the other two strains that were isolated (Figure 1). One of the strains of this type seemed to show a pink-colored colony. The other two unknown organisms seemed filamentous, perhaps rhizoid, and were tan/beige colored. The Gram stains of the unknown organisms showed gram positive rods, some in short chains and others singular.

The PCR products were sequenced and matched to known organisms through a "BLAST" search on an online database. An example sequence is shown in Figure 2. It was found that the three faster growing strains were closest matched to *Bacillus licheniformis.* The other two strains that showed fibrous colonies were found to be fungi, and therefore were not of interest to us.

Characterization of colony morphology produced about 6 novel strains. The notable strains were discovered from the aquatic leaf piles, flooded fields, and the water column, and sandy sediment of Carroll Creek (Table 1).

Strain ID #	Colony Morphology
C2-1	Small, red and circular
C2-2	Cream colored, round and fibrous
C2-3	Yellow, round, complete and raised
F1-1	Translucent and splotchy
C4-1	Orange, round w/halo, raised
C4-2	Egg-shaped, cream colored, raised

Table 1. The colony morphology of the aquatic species of unknown bacteria grown at 55°C

The genus and species of the aquatic strains grown at 55°C are given in Table 2.

Strain ID #	16s rRNA Match	Match Score
C2-1	*Geobacillus sp.*	0.921
C2-2	*Bacillus sp.*	0.59
C2-3	*Geobacillus*	0.907
F1-1	*Geobacillus thermoglucosidasius*	0.898
C4-1	*Geobacillus thermoleovorans*	0.915
C4-2	*Bacillus caldotenax*	0.588

Table 2. The 16s rRNA match concluded through BLAST search and Match Score (a score of 1.0 confers a perfect match)

The growth curves of all of the aquatic strains were then analyzed and the data is presented in Table 3.

Strain ID#	Glucose Media (Avg. Slope over 16 hrs)	Pectin Media (Avg. Slope over 16 hrs.)	Pec/Gluc Ratio
K1 (4°C)	0.03	0.001	0.03
C4-1 (4°C)	0.03	0.002	0.07
C1 (4°C)	0.02	0.006	0.30
C4-2 (4°C)	0.03	0.01	0.33
C2-1	0.08	0.00765	0.10
C2-2	0.06	0.007	0.12
C2-3	0.067	0.0028	0.04
F1-1	0.05	0.01	0.20

Table 3. The growth curve average over 16 hours based on blank corrected in glucose and pectin at 32°C and the pectin/glucose growth ratio.

Discussion:

The data collected from the various experiments of the unknown pectinolytic organisms allowed for identification and partial characterization of the strains. From terrestrial environments, the predominant pectinolytic bacteria found in the gut of insects was *Bacillus licheniformis*. After researching this species, it was found that *Bacillus licheniformis* is mostly found in the soil, and is a gram-positive, cream colored, mesophilic bacterium. This species of bacteria is thought to contribute substantially to nutrient cycling due to the diversity of enzymes it produces. This would make sense as it was cultured readily in pectin media as well as glucose media and Luria Broth (EPA, 1997).

The growth curves of the unknown bacterial strains were found to be interesting as well (Figure 5). Unknown bacterial strain F1-1, *Geobacillus thermoglucosidasius,* had a high rate of growth in pectin minimal broth at 55°C at a 0.20 pectin/glucose growth ratio. This result is surprising due the strain simply being isolated from a flooded field. Even more surprising were the growth rates for the bacterial strains isolated at 4°C. The growth ratio of pectin/glucose for C1 was 0.30 and even greater was the growth rate ratio for C4-2 at 0.33. It would be interesting to study these strains isolated at 4°C in future projects.

Through 16s rRNA sequence analysis, a species of bacteria that matched the sequence of the unknown aquatic bacteria proved to be surprising. Unknown C4-1's sequence was matched to that of *Geobacillus thermoleovorans*, a thermophilic bacterium that has been found in hot springs in Malaysia. This species has drawn interest for its potential in biotechnology application as a source of thermostable enzymes (Sakaff et al. 2010). This bacteria readily grew in 55°C temperatures, and according to the literature, can thrive at temperatures as high as 68°C, showing great stability in its enzymes, a valuable trait (Sakaff et al. 2010).

The characterization of these microbes that grow well with pectin as their only energy source at high temperatures, allows researchers to isolate types of proteins that are able to function at high temperatures (such as 55°C). This can then lead to work in 'synthetic biology'; in which scientists engineer proteins, and microbes to synthesize biofuels using thermophilic fermentation, allowing biofuels to be made more efficiently and at a larger scale (Ghim et al. 2010). Through this study, many environmental bacterial strains were isolated that would be ample candidates for future investigations on their pectinolytic proteins, as their growth rates in pectin were comparable to that of in glucose.