Report America 2009

Paul Ruschitzka

Marshall Plan Foundation Scholarship

at

The University of Tennessee

Timetable: Marshall Plan Foundation Scholarship at The University of Tennessee

The Management Center Innsbruck (MCI) provided interested students at the end of September 2008 with information about the possibility of doing research work in the USA as a fellow of the Marshall Plan Foundation Austria.

On the 3rd November 2008 Research Associate Professor, Dr. John Sanseverino and Managing Director of The Center for Environmental Biotechnology (CEB) at The University of Tennessee (UT) gave me the permission to work as a research fellow at the Center of Environmental Biotechnology, and I could decide which research project I would like to participate in.
I chose to work on a bio-energy related topic “Marshlands as a Source of Lignocellulose-degrading Microorganisms”.

The Marshall Plan Foundation gave me the honor to be a withholder of one of their scholarships on the 20th of November 2008.

I performed research work at the Buchan Lab, University of Tennessee, according to the requirements of the Marshall Plan Foundation from the 2nd March to the 12th August 2009 and spent another four weeks in October 2009 in the USA to tighten relationships with scientists and friends from abroad.

The University of Tennessee

(http://www.utk.edu/)

The Center for Environmental Biotechnology (CEB), was established in 1986 to foster a multidisciplinary approach for training the next generation of environmental scientists and solving environmental problems through biotechnology. Over the past 22 years, the CEB has distinguished itself as a world leader in developing the interdisciplinary research field of Environmental Biotechnology. The CEB was first in the nation to create a graduate research training program that truly integrates the sciences and engineering disciplines needed to produce trained professionals in the field. This was accomplished through grass root efforts of UT and ORNL faculty and staff with conceptual support by department heads and administrators at UT.

The Buchan Lab

Dr. Alison Buchan Assistant Professor

Major Research Interests

> Our research is driven by a desire to understand the roles of microbes in natural environments. Major objectives include linking microbial taxa to critical ecosystem processes, exploring functional gene diversity and its relation to community structure and biogeochemical processes, and identifying novel enzymes and/or catabolic pathways.
>
> Much of our work focuses on the Roseobacter clade, an abundant, broadly distributed and biogeochemically relevant group of heterotrophic marine bacteria. Aspects of Roseobacter biology that are of particular interest to us include catabolism of plant-derived compounds, attachment and colonization of surfaces, and geographical distributions. In addition, we have recently begun to examine viruses that infect this important clade. Finally, we have initiated a project to explore the role of sensory mechanisms in defining rhizosphere microbial communities in terrestrial systems.

The project I worked on was funded by a grant from the Southeastern Sun Grant Center with funds provided by the United States Department of Transportation, Research and Innovative Technology Administration.

The whole report about the research work that I took part in can be found under the following link. http://sungrant.tennessee.edu/NR/rdonlyres/4F100631-7827-446E-9CF8-C1F871DBB8EA/1845/finalreport_Buchan2009.pdf

Identifying Novel Lignin and Lignocellullose Degrading Enzymes From Natural Decomposer Communities

A Final Report Submitted to

The Southeastern Sun Grant Center

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Project Period, December 1, 2007 – May 31, 2009 [Dates for Project Period]

April 30, 2007 [Original Date of Submission]

This project was funded by a grant from the Southeastern Sun Grant Center with funds provided by the United States Department of Transportation, Research and Innovative Technology Administration.

Abstract

Coastal salt marshes harbor microbial communities efficient in the breakdown of lignocellulose. In these highly productive ecosystems, dominated by the smooth cordgrass (*Spartina alterniflora*), plant material is efficiently degraded by indigenous microbial assemblages. These decomposer communities may serve as an untapped resource of microbes for the transformation of lignin-containing plant material and could be useful in the pre-treatment of cellulosic feedstock (i.e. *Panicum virgatum* [switchgrass]) for ethanol production. In order to evaluate this proposal, microbial populations from a well-characterized salt marsh system on Sapelo Island, GA, were used to establish mesocosms amended with switchgrass. Previous studies have shown variation in the microbial communities associated with *Spartina* in different stages of degradation, thus, microbes found on living *Spartina*, dead culms, and sediment-associated decaying plant material were used as inocula in separate mesocosms containing an artificial seawater medium and chopped switchgrass. During the 11-month enrichment, microbial communities were monitored using terminal-restriction fragment length polymorphism analysis. At the end of the enrichment period, switchgrass-amended mesocosms inoculated with living *Spartina* and sediment-associated microbial communities had a higher microbial diversity relative to their unamended counterparts. The converse was found for the microbial communities originally associated with dead culms. Isolated representatives were obtained and are being screened for enzymatic activities. Tracer studies with ^{13}C-labeled switchgrass will allow for the identification of active members of the community.

Acknowledgments

The experimental results presented in this final report are the efforts of a UTK Microbiology graduate student (Christopher Gulvik), a postdoctoral fellow of the Center for Environmental Biotechnology (Melanie DeClaudio) and a visiting undergraduate student from The Engineering, Environmental and Biotechnology Department of the Management Center Innsbruck of Austria (Paul Ruschitzka). In addition, we gratefully acknowledge the scientific expertise of Dr. David Finkelstein of the UTK Department of Earth and Planetary Sciences. Dr. Finkelstein volunteered his time and expertise in stable isotope analysis to assist us in the ^{13}C-labeling experiments. We are also grateful to Dr. Melissa Booth from the University of Georgia Marine Institute for collecting and providing us with the salt marsh grass samples and Dr. Janice Zale of the University of Tennessee Agricultural Institute for providing us with switchgrass seeds. Finally, we thank Burnette Crombie for establishing the switchgrass seedlings.

Support for this research was provided by a grant from the Southeastern Sun Grant Center with funds provided by the U.S. Department of Transportation Research and Innovative Technology Administration (DTOS59-07-G-00050). Cost-sharing for John Sanseverino's salary was provided by the University of Tennessee Center for Environmental Biotechnology.

Identifying putative members form an enriched lignocellulose degradation community

Bachelor Thesis II

Over-worked "Bachelorarbeit 2"

In order to obtain the academic degree
„Bachelor of Science in Engineering"

Course of study:
„Engineering, Environmental & Biotechnology**"**
Management Center Innsbruck

Assessor:
Dr. Christoph Griesbeck

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Declaration in lieu of oath

I hereby declare, under oath, that this bachelor thesis has been my independent work and has not been aided with any prohibited means. I declare, to the best of my knowledge and belief, that all passages taken from published and unpublished sources or documents have been reproduced whether as original, slightly changed or in thought, have been mentioned as such at the corresponding places of the thesis, by citation, where the extent of the original quotes is indicated.
The paper has not been submitted for evaluation to another examination authority or has been published in this form or another.

Axams, 20 / 01 / 2010 ________________________________

Acknowledgements

I would like to thank the Austrian Marshall Plan Foundation for allowing me to have the opportunity of doing research abroad at The University of Tennessee by choosing me for one of their scholarships, as well as the MCI for their great collaboration. Special appreciation goes out to my friends and family for their marvelous support during the time I was in America and when I came back to Austria. Special thanks to my girlfriend for being patient and helpful during the writing process and also to Chris for showing great encouragement with any questions I had. And thanks to all the other people I met at The University of Tennessee for teaching me so much.

Abstract

Current research is trying to invent new sources for energy in order to step back from fossil fuels. Bio-fuels are considered possible substitutes with convincing aspects, such as renewability and environmental friendliness. Particular attention is paid to not edible second generation plant material as feedstock. The lignocellulose structure of vascular plants is comprised of lignin, hemicelluloses, and cellulose. Lignin forms rigid structures with the other fractions, hence making their fermentable sugars hardly accessible for biological ethanol production. Existing pre-treatments to remove the lignin fraction are not efficient and require high amounts of chemicals. The research project, Identifying Novel Lignin and Lignocellulose Degrading Enzymes From Natural Decomposer Communities (A. Buchan et al., submitted 2007), aims at establishing an enzymatic pre-treatment to set the fermentable sugars free. This thesis, as a part of the project, describes the process of identifying representative organisms from a community that degrades lignocellulose plant material.

The salt marches system of Sapelo Island in Georgia, USA is known to be a very productive ecosystem, and therefore, inhabits a highly active microbial decomposer community. Samples of this community were taken and active decomposer organisms enriched by growing them in media that only contained lignocellulose material as a Carbon source. The plant material used is switchgrass, a potential feedstock for biological ethanol production. Alterations of the sample composition were monitored with terminal-restriction fragment length polymorphism (T-RFLP) community analysis. Only the bacterial community was monitored with T-RFLP. This is because fungal laccases are well studied, but not much is known about bacterial laccases thus far. Organisms were isolated in order to identify active community representatives. This was performed via comparing the T-RFLP results of the isolates with the ones from the community. Six putative representatives were sequenced and identified as three different organisms: *Pseudomonas putida GB-1, Pseudomonas fluorescens Pf0-1,* and *Lysinibacillus sphaericus C3-C41.*

Through this thesis a protocol has been provided for the identification of putative representatives from the enrichment community and can be applied for further isolates. Final proof for organisms to be involved in lignocellulose degradation will be provided by C^{13} labeling of switchgrass, but it is not possible by the procedure described in this thesis.

Kurzfassung

Um nicht mehr von fossilen Rohstoffen abhängig zu sein, versucht die Forschung neue Energiequellen zu erschließen. Als möglicher Ersatz werden Biokraftstoffe wegen ihrer Nachhaltigkeit sowie Umweltfreundlichkeit in Betracht gezogen. Als zu verwendenden Rohstoff liegt das Hauptaugenmerk auf Pflanzenmaterial der zweiten Generation. Dieses nich essbare vaskuläre Pflanzenmaterial besteht aus den drei Hauptkomponenten Lignin, Zellulose und Hemicellulose, die als lignozelluloser Pflanzenanteil zusammen gefasst werden können. Lignin bildet dabei starre Verknüpfungen zwischen den drei Bestandteilen, wodurch die Verarbeitung der fermentierbaren Zucker zu Biokraftstoffen nicht möglich ist. Derzeitige Vorbehandlungen, um die starre Ligninstruktur zu zersetzen, sind mit dem Einsatz großer Mengen an Chemikalien verbunden und überzeugen keineswegs durch Effektivität. Das Forschungsprojekt, Identifying Novel Lignin and Lignocellulose Degrading Enzymes From Natural Decomposer Communities (A. Buchan et al., submitted 2007), zielt darauf ab, eine neue enzymatisch bedingte Vorbehandlung zu entwerfen, um fermentierbare Zucker freizulegen. Als Teil der Forschung beschreibt diese Bachelorarbeit den Vorgang zur Identifizierung von Organismen aus einer microbiolgischen Gemeinschaft, die in der Lage sind, lignozelluloses Pflanzenmaterial zu zersetzen.

Das Küstengebiet von Sapelo Island in Georgia, USA, ist bekannt für sehr schnellen Umsatz an Pflanzenmaterial, da es eine äußerst aktive Gemeinschaft an Mikroorganismen beheimatet. Mikrobielle Proben dieser Gemeinschaft wurden entnommen. Die Lignozellulose zersetzenden Organismen wurden angereichert, da als Kohlenstoffquelle nur Pflanzenmaterial zur Verfügung stand. Bei diesem Material handelte es sich um Switchgrass, einen möglichen Rohstoff für die Erzeugung von Bioethanol. Veränderungen der mikrobiellen Zusammensetzung während der Anreicherung wurden durch Terminalen-Restriktions-Fragment-Längen-Polymorphismus (T-RFLP) überwacht. T-RFLP wurde nur auf Bakterien angewandt, da die Laccase Aktivität von Pilzen schon besser erforscht ist, als die der Bakterien.

Organismen aus der Anreicherungs Gemeinschaft wurden isoliert. Um diejenigen zu identifizieren, die an der Lignozellulose-Zersetzung beteiligt sind, wurden T-RFLP Resultate der einzelnen Organismen mit denen Resultaten der Gemeinschaft verglichen. Sechs mögliche Vertreter der Anreicherungs-Gemeinschaft wurden sequenziert und als drei verschiedene Organismen identifiziert: *Pseudomonas putida GB-1, Pseudomonas fluorescens Pf0-1,* und *Lysinibacillus sphaericus C3-C41*.

In diese Bachelorarbeit wurde ein Protokoll erstellt, das zur Identifizierung möglicher Vertreter einer mikrobiellen Gemeinschaft dient und für isolierte Organismen angewandt werden kann. Die eindeutige Bestimmung, ob ein Organismus an der Zersetzung von Lignozellulose beteiligt ist, kann durch markieren von Switchgrass mit C^{13} erfolgen. Das hier präsentierte Protokoll kann jedoch keinen Beweis dafür erbringen.

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1. Introduction

1.1 Statement of the problem

We are living in a fast changing world with many alterations and innovations in very short intervals. According to these circumstances, there is a steady increasing demand for energy in the form of fossil fuels, rising from about 12 million tons per day in 2008 to 16 million tons per day by 2030. The sector of transportation will record especially much higher consumptions. These are going to boost from 4 million tons per day to 6.4 million tons per day by 2030 (Luque et al., 2008).

The investigations, nowadays, are focusing on getting away from fossil fuels as our main energy source. Reasons include the fact that fossil fuels will not last forever (Demirbas, 2007), as well as concerns about their role in the green house gases (GHG) issue (Demirbas, 2007, Balat, 2009, Cherubini et al., 2009).

1.2 Current bio-fuel situation

According to the current state of science, there is no immediate way to make a total switch from fossil fuels to another energy source, although the variety of approaches in order to produce bio-fuels are high in number. The article (Luque et al., 2008) gives a good overview of the different attempts including a description of the methods used to receive the specific bio-fuels. Furthermore the article gives figures about recent achievements. Over 200 major fleets in the US are running on biodiesel, and it is available at over 1000 gas stations in Europe.

As a matter of fact, the Tennessee public transportation is already run by biodiesel only. A global change from fossil fuel to bio-fuel in the sector of transportation is probably a desirable and possible first step on the way to independence from petrol resources. In regard to bio-fuels for mobilization, bio ethanol is so far the most promising substitute (Gray et al., 2006, Keshwani and Cheng, 2009).

1.3 Enzymatic pretreatment as solution

This thesis, based on research work at The University of Tennessee (USA), describes an attempt to pave the way for an enzymatic pretreatment of the biological feedstock, to replace parts of the amount of fossil fuels used with second generation bio-fuels. Second generation bio-fuels are produced from natural feedstocks that are not edible, and therefore do not cause a problem in the fuel versus food issue (Luque et al., 2008).

There is a wide variety of possible second generation plant material feed stocks, but one of the most efficient ones is switchgrass (*Panicum virgatum*) with an over 700% higher energy output

than input and advantages like water efficiency and being highly perennial (Rawe, 2008, Keshwani and Cheng, 2009). Therefore, switchgrass was chosen as the representative plant material of interest for this research study.

Current chemical pretreatments to make plant material fermentable, such as the AMMONIA FREEZE EXPLOSION (AFEX) pretreatment require high energy inputs as well as large amounts of chemicals (Holtzapple et al., 1991, Murnen et al., 2007).
An enzymatic pretreatment able to set free all the useful compounds for industrial ethanol conversion can be seen as a worldwide need. Therefore, no extreme conditions would be required, the use of chemicals and energy lowered, bio-fuels would become more competitive with fossil fuels, and we attribute something to improve the GHG issue and for a healthier environment.

1.4 Lignocelluloses conversion

Second generation plant material principally comprises of three compounds together known as lignocellulosic biomass. The fractions are cellulose, hemicelluloses, and lignin. Making these compounds fermentable is required for further processing like the production of ethanol. Therefore, the polymeric lignocellulosic biomass has to be cleaved into monomer structures, such as monosaccharide or aromatic monomers. Dehydrated ethanol will be received by distillation of the fermentation broth. (Chandel et al., 2007)
Discovering organisms and enzymes that are able to degrade the lignin fraction of biomass is the overall goal of this research work. If this can be accomplished a wide range of applications is opened and the production of bio-ethanol will be one large aspect.

1.4.1 Cellulose:

Cellulose is a polymer of glucose molecules in β 1,4-glcosidic linkages. Cellulose comprises 40-50% of plant material. For fermentation β- glucosidase is applied to break down cellulose into glucose monomers. (Chandel et al., 2007) (*Molecular Cell- Biology*, 5th Edition, CHAPTER 6.6, Plant Tissues, 232)

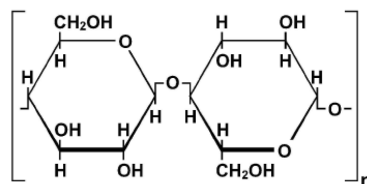

Figure 1 cellulose structure (Kogel-Knabner, 2002)

1.4.2 Hemicellulose:

Hemicellulose is a short and highly branched hetero-polymer comprised of the five- and six-carbon monosaccharides, D-xylose, D-glucose, D-galactose, D-mannose, and L-arabinose. About 20 to 30% of plant material is hemicelluloses. Together with pectin, it creates a network linking cellulose microfibrils.

To degrade hemicelluloses a mixture of many enzymes working together is required in order to receive fermentable five- and six-carbon monosaccharides. This hemicellulase mixture is required for non-acid pretreatments where the hemicelluloses fraction does not get removed in order to produce bio-ethanol. (Gray et al., 2006)(Chandel et al., 2007) (*Molecular Cell- Biology*, 5th Edition, CHAPTER 6.6, Plant Tissues, 232)

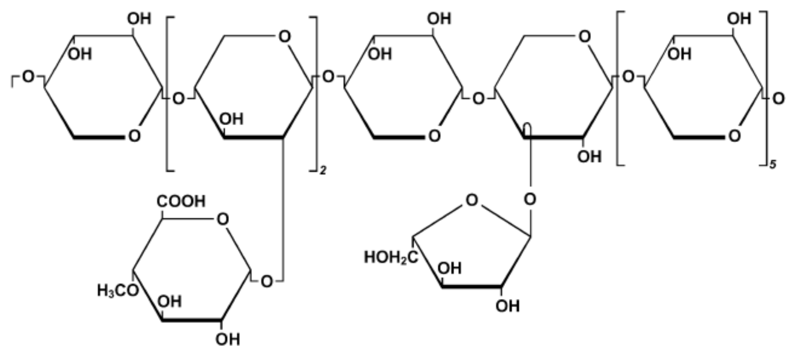

Figure 2 (Kogel-Knabner, 2002) example for representative hemicellulose unit

1.4.3 Lignin

Lignin supports plant cells against compression forces and microbial attacks by building a rigid structure in association with cellulose, hemicelluloses, and pectin.

It is the most abundant biopolymer in nature beside polysaccharides. Lignin comprises a structure of various phenyl propane units connected by ether or carbon-carbon linkages. The energy level in lignin is even higher than the one in carbohydrates, but as a big disadvantage, lignin blocks β-glucosidase from making contact with cellulose and interacts with β-glucosidases, which lowers their enzyme activity. The affinity of these, non-polar cellulose hydrolyzing enzymes, to lignin is about 13%.

The cellulase pretreatment is very expensive with costs up to 30 cents/gallon. Therefore, enzymes with lower affinity to lignin need to be engineered in order to receive higher yields for cellulose and hemicellulose degradation. The lignin-cellulase interaction will also be reduced if lignin is exposed to an enzymatic treatment itself, which can be performed with the use of laccases (Gray et al., 2006, Rawe, 2008, Higuchi, 1990, Kogel-Knabner, 2002)
(Chandel et al., 2007) (*Molecular Cell- Biology*, 5th Edition, CHAPTER 6.6, Plant Tissues, 232).

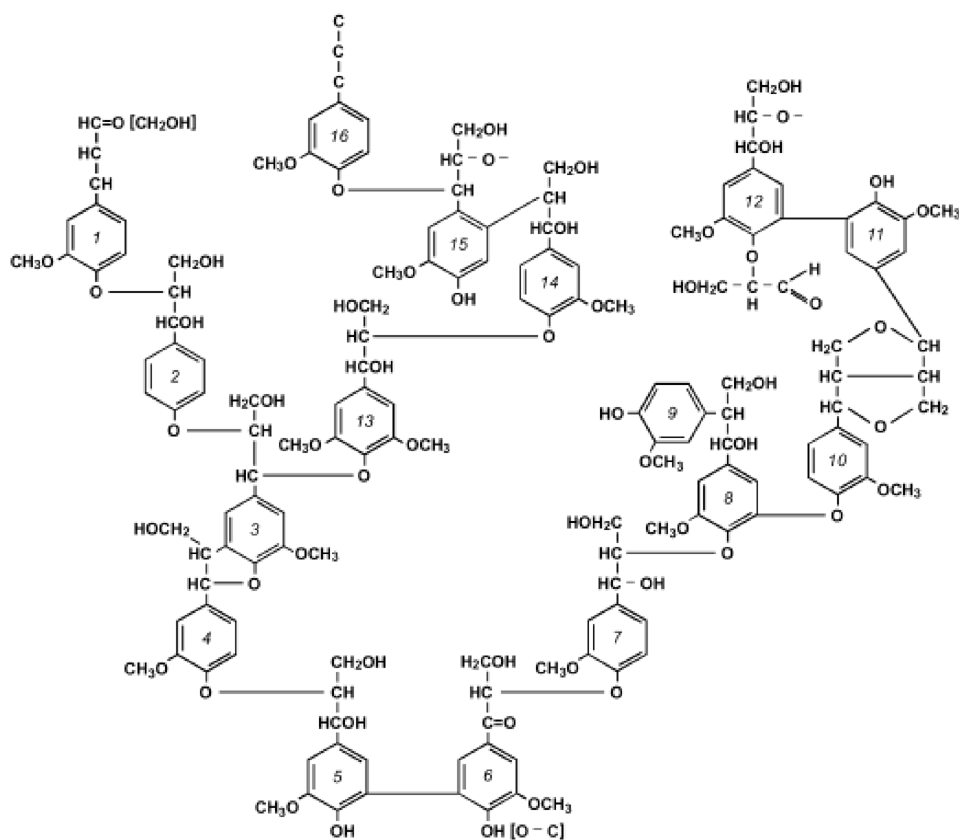

Figure 3 Lignin structure (Adler, 1977) (Kogel-Knabner, 2002)

1.4.4 Laccase

Laccase is a glycoprotein containing various oligosaccharide chains linked to a backbone consisting of about 500 amino-acids. Laccases are multi-copper containing oxidases, the four copper bindings have different accessibilities to the solvent and serve as electron donators on the active enzyme sites which also comprise oxygen.

Oxidase transfers four electrons to oxygen in order to form water without creating hydrogen peroxide. Laccase or lignin peroxidase is a relatively simple member of this group of enzymes. Laccases can catalyze the oxidation of various phenol and amine compounds, such as lignin, and also catalyzes the demethylation of lignin and methoxyphenol-acid.

(Yaropolov et al., 1994)

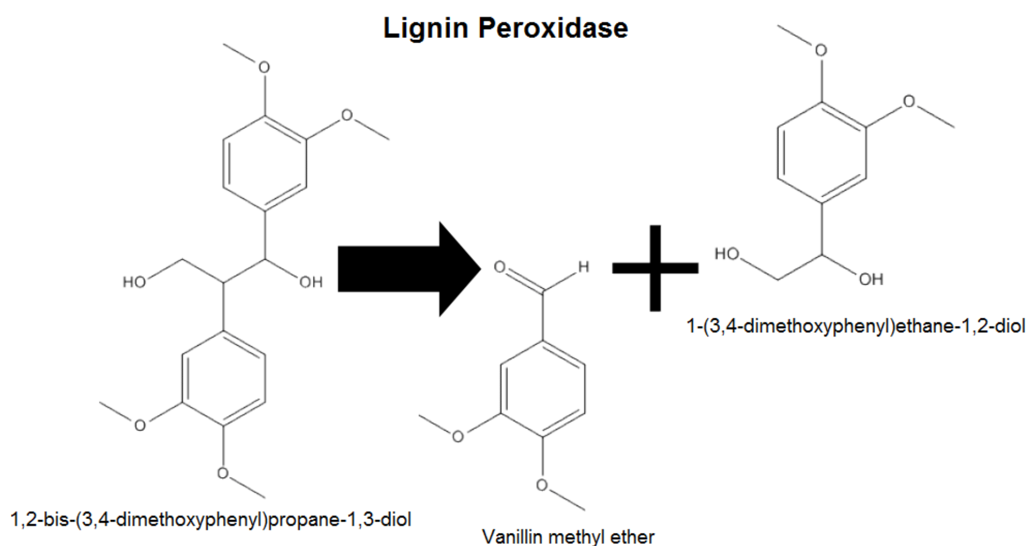

Figure 4 principle of Lignin Peroxidase, based on A. Buchan et al., submitted 2007)

Figure 4 shows the principle process of a typical lignin structure being reduced to subunits by lignin peroxidase. According to the abilities of laccase, these monomeric aromatic compounds could be demetylated and further processed.

Laccases are already used for various applications, such as polymer synthesis, textile dye bleaching, or removal of phenolics from wines. Only fungi laccases are used for industrial purposes because they are well studied already. Fungal laccases are extracellular whereas the working mechanism for bacterial laccases is still uncertain. For bacteria, CotA from the endospore coat of *Bacillus subtilis* is the best studied laccase so far, but their existence is also known for other bacteria, such as *Azospirillum lipoferum,* which forms laccases inside the cell (Sharma et al., 2007).

An attempt to find bacterial laccases was performed by comparing fungal laccase sequences with whole bacterial genomes. A variety of putative bacterial laccases was obtained that even show similar features according to protein folding (Alexandre and Zhulin, 2000).

1.5 Identifying Novel Lignin and Lignocellulose Degrading Enzymes From Natural Decomposer Communities

“This project was funded by a grant from the Southeastern Sun Grant Center with funds provided by the United States Department of Transportation, Research and Innovative Technology Administration.” (A. Buchan et al., submitted 2007)

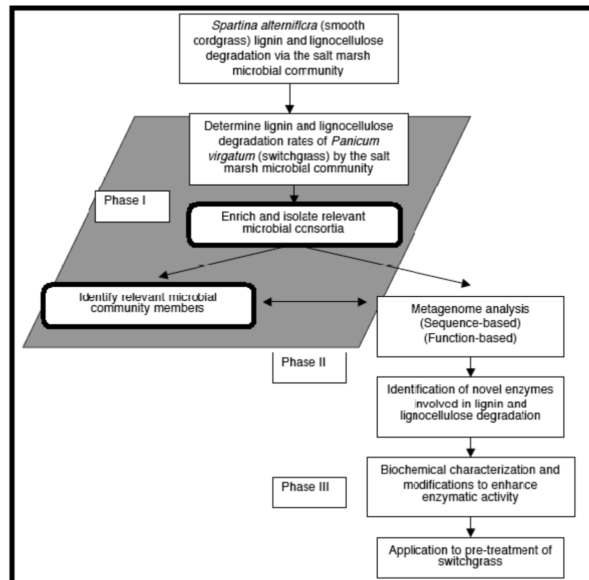

Figure 5 Long-term research strategy for identification and isolation of lignin and lignocellulose degrading enzymes (A. Buchan et al., submitted 2007)

Only a small part of the research strategy shown in figure 5 is described in this thesis.

- Enrich and isolate relevant microbial consortia
- Identify relevant microbial community members

1.5.1 Degradation community enrichment through mesocosms

The project, Identifying Novel Lignin and Lignocellullose Degrading Enzymes From Natural Decomposer Communities, is based on a PI’s research at the Microbiology Department and Center for Environmental Biotechnology at the University of Tennessee. The research work described in this bachelor thesis is constitutive to the eleven months of previously done research by PhD student Christopher Gulvik.

The aim is to enrich a microbiological community with the ability to degrade lignocellulose plant material.

The salt marches at the Georgia Costal Ecosystem deliver a microbiological community that is known for fast degradation of plant material. Isolation of this community and growing it with plant material as the only carbon source should cause an enrichment of only bacteria and fungi that participate in the lignocellulose decomposing process.

A mesocosm experiment is performed to enrich the degradation community. Terminal Restriction Fragment Length Polymorphism (T-RFLP) community analysis is applied to monitor the enrichment process. T-RFLP results show peaks indicating different organisms within a community as described in 3.7 T-RFLP.

Figure 6 mesocosms to enrich the degradation community

The mesocosms, as shown in figure 6, represent small systems that reflect processes in nature at a laboratory scale of about 250ml. No interaction between the environment and the mesocosms takes place besides a small exchange of air.

1.5.2 Microscopic analysis

The aim of microscopic analysis is to get an idea of the community composition inside the mesocosm, whether it is mainly fungi or bacteria and to which point the switchgrass, or *spartina,* particles have been degraded. Another hope is to see fungi or bacterial cells being attached to plant material and putatively degrading it.

1.5.3 Identification of members of the putative degrading community

The enrichment project is after eleven month T(11mo.) at a point where specific data about the involved organisms becomes inevitable. T-RFLP is performed for isolates with the assumption that results can be directly compared with data from the T(11mo.) T-RFLP. The goal is to find matching peaks to confirm that the isolate is a member of the enriched T(11mo.) mesocosm. Isolates that meet this requirement will be sequenced and identified. In the best case these organisms hold laccase activity.
The protocol can be separated into four main steps:
<u>Isolation of organisms</u>
<u>Selection of isolate representatives</u>
<u>T-RFLP of isolates</u>
<u>Isolate identification and screening for laccase activity</u>

Isolation of organisms

Isolates have to be cultivated from the enrichment community after T(11mo.) where lignocellulose degrading organisms should be very abundant. Only bacteria will be chosen for T-RFLP because the community analysis has so far only been done for bacteria, which means only bacterial isolates can be compared with the T(11mo.) T-RFLP data

Selection of isolate representatives

The aim is to create a sensible selection out of all isolates that will be further processed. There is no previous knowledge about these isolates. DNA extracted from the isolates and amplified through PCR is required to perform T-RFLP.
The decision whether T-RFLP is applied for an isolate or not depends on how the DNA extraction kit works and on the results of spectrometric analysis to determine the concentration of extracted DNA. Another criterion is whether isolates are fungi or bacteria, which is shown by the results of the PCR amplification using a bacterial and a fungal set of primers for all isolates.

T-RFLP of isolates

The final selection for T-RFLP has to comprise of 15 isolate DNA samples. This is due to the sequencing instrument for T-RFLP consisting of a 16 capillary array, which means that 15 samples and one negative control will be simultaneously injected and analyzed.
The T-RFLP chart of each isolate has to be compared with the T(11mo.) T-RFLP charts. Members of the putative degradation community might be found if isolates appear to be cut by the *CofI* restriction enzyme at the same number of base pairs (bp) as the community.

Isolate identification and screening for laccase activity

Samples that show T-RFLP peaks at the same number of base pairs as the community analysis will be identified by sequencing. This sequenced bacterial DNA fragment can be compared with the database and might match with a bacterial phylum or a specific organism. The screening for laccase activity will be performed by comparing sequences of putative laccases with the whole genome of the sequenced organisms.

2 Material

2.1 Environmental samples

- Switchgrass (*Panicum virgatum*)
- Putative Microbiological degrading communities form salt march at the Georgia Costal Ecosystem (GCE) affiliated with *Spartina alterniflora*.
    - Living plant material
    - Surface sediments
    - Dead culms

2.2 Media

- Basal medium: 1.5% Sea salts, 66μM K_2HPO4, 4mM NH_4CL, 250 mM Tri-HCL, pH7.5
- YTSS- agar plates: 0.4% Yeast extract, 0.25 % Tryptone, 2% Sea salts, and 1.8% Agar
- V8- agar plates 2% Campbell's Co. V8 , 2% Agar , 1.5% Sea Salts (pH ends up being 4-5)

2.3 Reagents

Product	**Company**	**Catalog #**
Taq Polymerase High Fidelity 10x High Fidelity PCR Buffer $MgSO_4$	Invitrogen	11304-029
dNTP Solution Set	New England BioLabs	N0446S
1 kb DNA Ladder	New England BioLabs	N3232S
Ethidium Bromide	Sigma	E8751-5
AGAROSE	Fisher	BP1356-500
Hi-Di Formamide	Applied Biosystems	4311320C
1200 LIZ Size Standard	Applied Biosystems	4375252C
*Cfo*I restriction enzyme	New England Biolabs	R0139
TAE 50X Buffer	Fisher	BP1332-4
Ammonium acetate	Fisher	BP326-500
Glycogen	Ambion	AM9510
Ethyl Alcohol (EtOH)	Decon	S93232

Primers
Fungal ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') ITS4A (5' CGCCGTTACTGGGGCAATCCCTG 3')
Bacterial FAM-8F (FAM 5' AGAGTTTGATCMTGGCTCAG 3', M is A or C) 27F (5' AGAGTTTGATCCTGGCTCAG 3') 1522R (5' AAGGAGGTGATCCANCCRCA 3', N is A, T, C, or G, and R is A or C)

2.4 Kits

- MoBio Laboratories, Inc. UltraClean™ Soil DNA Isolation Kit Version 03252005
  Elution buffer (S5 solution is 10 mM Tris at pH 8)

- QIAquick Gel Extraction Kit using a microcentrifuge
  Elution Buffer (EB buffer is 10mM Tris pH 8-8.5)

- QIAquick PCR Purification Kit Protocol
  Elution Buffer(EB buffer is 10mM Tris pH 8-8.5)

2.5 Instruments

- Tabletop Shaker Eppendorf
- Microscope Nikon Eclipse TE 2000 – U
- Microcentrifuge Eppendorf Centrifuge 5415 D
- Spectrophotometer NanoDrop ND-1000
- Vortex machines Fisher Scientific
- PCR cycler BioRad DNA Engine
- Cold centrifuge Eppendorf
- Gel electrophoresis BioRad
- Sequencing instrument ABI PRISM® Genetic Analyzer 3100
- Software:
  - ABI GeneScan
  - BioEdit

3 Methods

3.1 Microscopic analysis

Microscopic analysis is performed with a phase contrast inverted light-optical microscope connected to a computer with a maximum resolution of 1000 times on the screen.

3.2 Isolation of organisms

Fungi are assumed to be cultivated on V8-agar plates which are based on V8 juice that should to be a good growth media for fungi due to many supplements and a relatively low pH between 4 and 5.
Bacteria are cultivated on a media based on yeast, tryptone, and sea salt (YTSS).
Every incubation step is performed under laminar flow conditions with a minimal risk of cross contamination.
For isolation always one single colony is taken and transferred to a new plate. The incubation parameters are 3 days at ~22°C for fungi as well as for bacteria.

3.3 Kits

3.3.1 MO BIO Laboratories, Inc. UltraClean™ Soil DNA Isolation Kit protocol.

The purpose of this kit is to isolate DNA from organisms.
The cells break open by the impact of SDS and a mechanical treatment of vortexing a mixture of cells and solution with beads. An inhibitor removal solution is added for a better PCR performance. Centrifugation forms pellets of cell debris and unwanted material, and the supernatant containing the DNA can be removed.
A salt solution is added to the DNA supernatant that makes it bind to a silica spin filter. The elution of DNA from the spin filter is caused by solution S5 that does not contain any salts.

Manual UltraClean™ Soil DNA Isolation Kit Version 03252005

3.3.2 QIAquick Gel Extraction Kit

Using a micro centrifuge
The QIAquick Gel extraction kit is used to purify DNA from gel after gel electrophoresis.

This extraction protocol requires the specific mass of gel in each tube to calculate the amount of needed buffer solutions. Therefore, the mass of the empty tubes is measured, as well as the mass of the tube including the DNA containing gel slices.
Incubating the gel slices with a buffer at high temperature will solubilize the agarose gel completely. The DNA will bind to a QIAquick column and can be eluted from the column with EB buffer.

QIAquick Gel extraction kit handbook 07/2002

3.3.3 QIAquick PCR Purification Kit Protocol

Using a micro centrifuge
This kit can be used to purify single or double-stranded DNA fragments from PCR. The DNA fragment sizes can range from 100 bases to 10 kilo bases.
A buffer mixed with the PCR sample causes the DNA to stick to the QIAquick column while other PCR products are discarded as flow-through during centrifugation. Another washing step is applied before the purified DNA fragments are eluted with EB buffer or water (pH 7-8.5).

QIAquick PCR Purification Kit handbook 11/2006

3.4 PCR

Polymerase chain reaction is a technique to amplify DNA if a sequence of at least 18 to 20 base pairs on the end of each DNA strand is known. Denaturing the DNA by applying high temperature up to 95°C will form sDNA. Specific primers of the known sequences can bind to the sDNA at a certain temperature depending on the bases of the primer. This is called annealing. Polymerase will cause deoxynucleotides dNTDs to elongate the newly built strand starting at the 5' ends of the primers. The temperature and time settings are very specific for each step and determine the quality of the PCR.
The wanted DNA sequence is amplified exponentially with the number of denaturing, annealing and elongation circles.
(*Molecular Cell- Biology*, 5th Edition, CHAPTER 9.3, Characterizing and Using Cloned DNA Fragments, 374)

3.4.1 Platinum^R Taq DNA Polymerase High Fidelity

The Platinum^R *Thermus aquaticus (Taq)* DNA Polymerase High Fidelity is an enzyme mixture of *Taq* DNA Polymerase, Platinum^R *Taq* Antibody and *Pyrococcus species GB-D* Polymerase. The Platinum^R *Taq* Antibody builds a complex with *Taq* DNA polymerase at room temperature which inhibits the polymerase activity. This makes excellent preparation of the PCR sample at room temperature possible.
The initial denaturation step at 94°C will restore polymerase activity again.
Pyrococcus species GB-D Polymerase possesses a proofreading ability of 3' to 5' exonuclease activity increasing the fidelity approximately six times over the activity of *Taq* DNA polymerase alone.

Hi-Fi Taq PCR Recipe	
vol.(ul):	
2.00	50mM MgSO4
5.00	10x rxn buffer
1.00	dNTP mix (10mM ea)
1.00	forward primer (XuM)
1.00	reverse primer (XuM)
0.25	HF Taq polymerase (5U/ul)
2.50	DNA template (1ng/ul PCR rxn vol.)
37.25	dH2O
1	[ENTER] desired # tubes
50	[ENTER] total rxn volume (ul)
20.00	[ENTER] DNA stock conc. (ng/ul) of least conc. Sample (#1)
47.50	mix/tube before DNA added (ul)
2.50	DNA/tube if added last (ul)

Table 1 Hi-Fi *Taq* PCR Recipe

Table 1 shows the composition of a PCR mixture with a total reaction volume of 50µl and a DNA stock concentration of 20 ng/µl. The number of tubes, the reaction volume, and the DNA stock concentration can by varied on the Hi-Fi *Taq* PCR Recipe excel sheet, and the compound volumes will be calculated. The primer concentration for genomic DNA and cDNA is 0.2 µM. (invitrogen^{TM} Platinum^R *Taq* DNA Polymerase High Fidelity user's manual)

The reagents for PCR are usually bought at high concentration levels and are eluted to small stacks of the required end concentration. This lowers the risk for the original reagent to be contaminated and reduces errors according to concentration issues.

PCR is prepared by combining the volumes shown on Table 1 in following order.
If there is more than one sample to amplify, a master mix (MM) will be prepared: dH2O, MgSO4, 10x rxn buffer, dNTP mix, forward primer, reverse primer, and Taq Polymerase. All agents are on ice during the entire process.

MM is homogenized and transferred to each PCR tube. After the DNA is added, the tubes are briefly vortexed and spun down for 1 second to eliminate any bubbles and cause the whole volume to move to the bottom tube.

PCR settings for fungi

Initial denaturing at 94°C for 2.5 minutes for is followed by 35 cycles at 94°C for 15 seconds denaturing, 53°C for 30 seconds annealing, 72°C for 90 seconds elongation, and a final extension step of 72°C for 10 minutes.
For fungal DNA, the ascomycete-specific primers ITS1F (forward) and ITS4A (reverse) are used that amplify a sequence of about 600 base pairs within the rRNA operon.

PCR settings for bacteria:

Initial denaturing at 95°C for 3 minutes for is followed by 30 cycles at 95°C for 60 seconds denaturing, 60°C for 60 seconds annealing, 72°C for 90 seconds elongation, and a final extension step of 72°C for 10 minutes.
The bacterial primers, 27F (forward) and 1522R (reverse), amplify about 1500 base pairs of the 16S rRNA gene.
Instead of 27F, FAM-8F is used for T-RFLP purposes. FAM-8F is fluorescent labeled with FAM (carboxyfluorescein) on the 5' end.

3.5 Gel electrophoresis

Gel electrophoresis can be used as a powerful separation method for DNA or proteins.
DNA is separated according to the size of the fragments. For small fragments up to about 2000 nucleotides, the gel exists of polyacrylamide, for larger fragments an agarose gel is used. The matrix of the gel will cause the DNA to not diffuse. Therefore fragments of the same size will move with the exact same speed. The mechanism behind the separation is that DNA is strongly negatively charged at conditions of a neutral pH. DNA strands will move towards the cathode when an electric field is applied to the gel. Small fragments move faster than large fragments. The number of nucleotides can be determined by comparing with a suitable leader. Ethidium bromide is used to make DNA visible under UV light by intercalating between base pairs and causing a fluorescent dye at exactly the position where the DNA is located.
(*Molecular Cell- Biology*, 5th Edition, CHAPTER 9.3, Characterizing and Using Cloned DNA Fragments, 371)

For gel electrophoresis the gel is a mixture of 1% agarose with a 1x TAE Buffer. The gel is sitting in 1x TAE buffer as an electrical conductor. 50x TAE buffer is comprised of 242g Tris, 100mL of 0.5M EDTA pH 8.0, 57.1mL Glacial Acetic Acid, with MQ water added to a total volume of 1000ml and a final pH of 7.8.

An appropriate amount of a 6xloading buffer is added to the sample before loading it onto the gel. The fluorescent dye is added by letting the gel sit in ethidium bromide for ~30 minutes after the separation by electrophoresis has been performed.
When an amplified fragment size between 10 kilo bases and 500 bases needs to be determined, 5 µl of 1 kilo base (1kb) DNA leader are used.

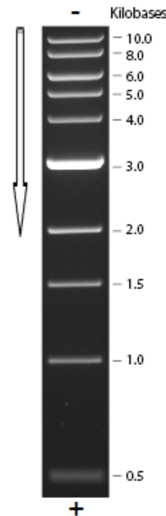

Figure 7 1kb DNA leader

3.6 Restriction enzymes

Restriction enzymes are endonucleases produced by bacteria to protect them from foreign incoming DNA by cutting it. The cleavage is performed at restriction enzyme-specific restriction sites. These sites are mostly palindromic sequences of four to eight base pairs. Cutting them can create either sticky or blunt ends. Sticky ends show an overlap of the 5’ or the 3’ end of the DNA string.
A modification enzyme is active in bacteria to add methyl groups at possible restriction sites so the restriction enzyme will not cut the DNA of the bacteria.
(*Molecular Cell- Biology*, 5th Edition, Chapter 9.3, Characterizing and Using Cloned DNA Fragments, 161-162)

3.6.1 DNA Digestion by *Cfo*I

The restriction enzyme *Cfo*I comes from *Clostridium formicoaceticum* and is a representative of type II restriction endonucleases. It cuts the 4 base pair restriction site 5’ GCGC 3’ creating sticky ends with a 5’ overlap 5’ GCG_C 3’. The temperature at which *Cfo*I is active is 37°C. Theoretically a 4 base pair restriction enzyme cuts once every 256 base pairs and, is therefore, not as selective as enzymes with a 6 or 8 base pair restriction site.

3.7 T-RFLP

Terminal - Restriction Fragment Length Polymorphism (T-RFLP) is a high-throughput fingerprint method to receive phylogenetic information about microbiological communities or isolates. It works for fungal ribosomal genes, as well as for bacterial and archaeal 16S rRNA genes. PCR is performed, which multiplies certain ITS regions of fungi and 16S rRNA gene regions for bacteria and archaea. One of the primers used is labeled with a fluorescent dye, which is necessary to identify and cut out the amplified sequences after gel electrophoresis. A gel purification step is required before fractioning the DNA with a restriction enzyme that has four base-pair recognition sites. Only the size of the terminal restriction fragments with the fluorescent dye will be determined by the sequencing equipment giving the exact number of base pairs where the restriction enzyme cut the DNA string (Liu et al., 1997, Schutte et al., 2008).

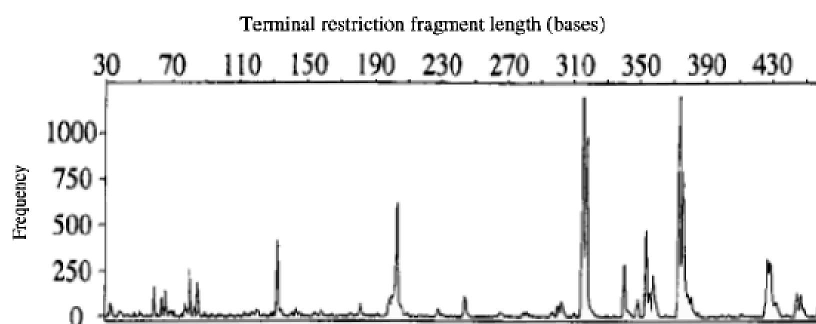

Figure 8 sample data for T-RFLP from (Liu et al., 1997)

Figure 8 is a possible T-RFLP map of community analyses. Each peak means that the amplified DNA has been cut by the restriction enzyme at the shown number of base pairs. The sequence where the restriction enzyme was active is known and very specific. Also the start of the sample sequence is known, which is determined by the fluorescent PCR primer. This data can be used for assumptions about phylogenetic heritage of the analyzed organisms. The most popular use of T-RFLP is to compare microbial communities or check for alterations within communities at certain time points.

The frequency at the y-axis of the figure can be seen as a percentage of fluorescence for one peak compared to the entire fluorescence of the sample.

3.7.1 Protocol for T-RFLP using ABI PRISM® Genetic Analyzer 3100

- PCR is performed for DNA amplification using a 5'end-FAM-labelled primer.
- The gel for PCR purification is poured and stored in a 1x TAE buffer.
  PCR fragments of interest are excised from the gel and appropriate gel slices are combined in 2ml tubes. When cutting out the bands no fluorescent glow should be left at the gel but the slices must not be too large. No additional gel should be transferred to the tube beside the band. Nitril gloves have to be worn during the whole process because the gel was previously stained in ethidium bromide.
- Gel slices are processed with Qiagen Gel Extraction Kit.
- DNA concentration in purified samples is measured via NanoDrop-1000 (at least 5-6ng/ul per sample required).
- Restriction digests are performed in 1.5 ml eppendorf tubes containing 120ng DNA for environmental samples or 10-15ng DNA for clones; best results are generated from total digestion volumes of no more than 50ul.
- Digestion at 37°C for 3.5hrs.
- Digested DNA is processed for overnight ethanol precipitation at -20°C.
  Ethanol precipitation is used to remove salts from DNA and cause the precipitation of DNA fragments.
  Add these reagents to the 1.5 ml eppendorf tubes containing digested DNA:
  - 90µl sterile milliQ water
  - 10µl ammonium acetate
  - 1µl glycogen
  - 300µl cold EtOH (gently invert to mix)
- The ethanol precipitated samples are cold-centrifuged (16K x g, 15min., 4°C) in batches of 12 to ensure pellets remain intact while decanting supernatant. Supernatants are poured off along the side of tube opposite the pellet. This is based on how the tubes were arranged in the centrifuge.
  The tubes are inverted on a paper towel to get rid of the remaining liquid.
- Invisible pellets are dried in a Speed-Vac at 35°C for 25minutes. Vacuum is applied to a centrifuge to keep the pellet down but evaporate all liquid compounds off the tube. Vacuum must not be turned on before centrifugation, or the DNA pellets will be sucked out of the tube.
  Dried pellets can be stored at -20°C for up to 2 weeks.
- Formamide and internal standard master mix is prepared for all of the samples and the negative control; the calculation is done including one extra volume.
- DNA pellets are dissolved in the master mix by gently pipetting up and down the sides of tube. A quick vortex and quick spin step are applied to the sample.

Samples are heated for 5min at 95°C and immediately cooled on ice for at least 30seconds and centrifuged for one second.

- Samples are transfer to a 96-well-plate. The plate is briefly centrifuged.
- Setup for sequencing instrument parameters is done.
- T-RFLP sequencing run is started.

Recipe based on A. Buchan et al., submitted 2007)

3.8 Sequencing Sanger method

Sequencing a template DNA with the Sanger method requires known primers, polymerase, deoxyribonucleotide triphosphates (dNTPs), and dideoxyribonucleoside triphosphates ddNTPs. Four reactions are prepared for each of them containing a small amount of ddNTPs from one of the four bases: adenine, thymine, guanine, or cytosine. Starting from the primers, polymerase will add different dNTPs and ddNTPs depending on the template DNA. Only in about 1% of the cases when a certain dNTP is added it will be the ddNTP instead. This will stop the polymerase activity and add a certain fluorescent dye to the end of the sequence. The color of the fluorescence is different for the ddNTPs depending on the base.
Gel electrophoresis of the four reactions will result in a certain order of different fluorescent dyes with each color indicating one of the bases, as shown in figure 9.
(*Molecular Cell- Biology*, 5th Edition, Chapter 9.3, Characterizing and Using Cloned DNA Fragments, 373)

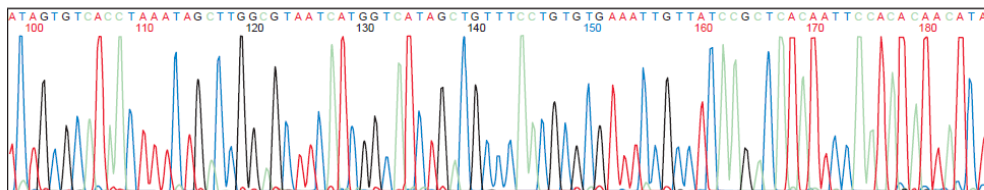

Figure 9 sequencing results Sanger method (*Molecular Cell- Biology*, 5th Edition, Chapter 9.3, Characterizing and Using Cloned DNA Fragments, 373)

3.9 NanoDrop ND-1000 Spectrophotometer

Spectrophotometry is based on the ability of the sample to absorb light at different wavelengths. The NanoDrop ND-1000 Spectrophotometer is used to measure DNA concentrations requiring only 2 µl of the sample and no cuvette. The sample is directly placed onto the spectrophotometer, as shown in figure 8. DNA absorbs light at a wavelength of 260 nm an optical density of 1.0 (OD_{260} 1.0) is equivalent a DNA concentration of 50 ng/µl.
To determine the purity of the sample, the absorbance ratio 260nm/280nm and 260nm/230nm is calculated. Proteins absorb at 280nm and certain carbohydrates at 230nm.
For pure DNA, the 260/280 ratio should be 1.8 and the 260/230 ratio about 2.

Figure 10: NanoDrop Spectrophotometer ND-1000

(Thermo Scientific NanoDrop 1000 Spectrophotometer v3.7 User's Manual)

Before applying the DNA samples, a blank is measured using the solution with which the DNA is dissolved in. The result of the blank is used as reference with a DNA concentration of zero. The lens and sample holder of the spectrophotometer are cleaned after every measurement, and after the last sample the blank is applied again to monitor changes of the zero reference.

4. Results

These results show the progress of the attempt to identify novel lignin and lignocellullose degrading enzymes from natural decomposer communities.

4.1 Mesocosms as a starting point

The mesocosm experiment is performed to create communities enriched with lignocellulose degrading organisms as described in 1.5.1.

Six 500ml baffled flasks were labeled with Mesocosm- A, B, C, D, E, F.
The three communities affiliated with the different parts of Spartina alterniflora were chopped up and each of them was added to two different 500ml baffled flasks.

Living plant material: pieces of ~2cm, 17g added to flasks A and D

Dead culms: pieces of ~2cm, 7.5g added to flasks B and E

Surface sediments: pieces of ~2.5cm3, 50g added to flasks C and F

100ml of Basal medium were added to each flask. Mesocosms A, B, and C additionally contain 4g of 2cm Switchgrass pieces. The 500ml baffled flasks were covered with aluminum foil as a light protection and were placed on a tabletop shaker at 100 rpm at a temperature of ~22°C. After enrichment for 7 months a tenth of each community was transferred to fresh basal media, with another 4g of Switchgrass for A, B and C. All conditions were kept the same.
Samples were taken after an incubation of T(0mo.), T(5mo.), and T(11mo.) to check for alteration of the communities by the controlling tool Terminal Restriction Fragment Length Polymorphism. The T-RFLP analysis has so far only been applied for bacterial communities. Freezer stocks of the community DNA have been made for every mesocosm after zero, five and eleven months of incubation. They are stored at -80°C.

The abundance of microorganisms within these 6 communities has dramatically changed. Many organisms were extinguished and others have become more dominant. Therefore, the changes alternate between all of the 6 mesocosms, there is not a certain organism that seems to be ubiquitous within the six samples as far as T-RFLP results can tell us. It was monitored though that the mesocosms A, C and E showed higher microbial diversity than D, F and B (A. Buchan et al., submitted 2007).

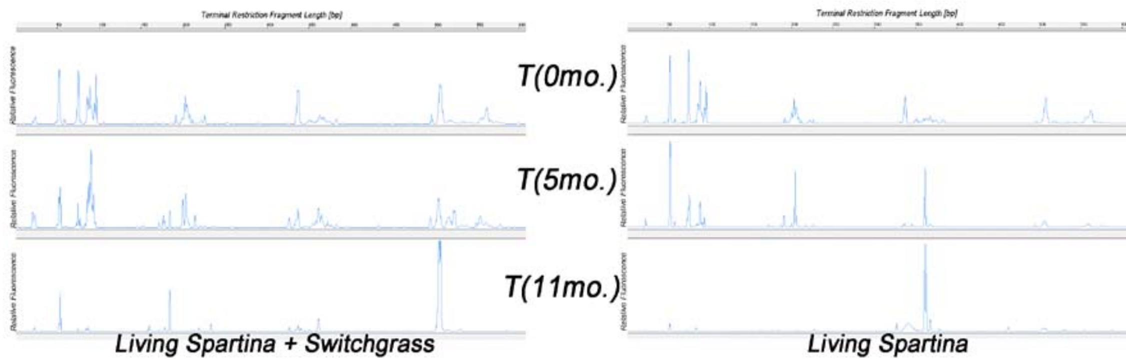

Figure 11 T-RFLP results for mesocosm A and D (A. Buchan et al., submitted 2007)

Figure 11 shows some of the alterations of mesocosms A on the left and D on the right side after time. T(0mo.) is the start point of the enrichment process T(5mo.) after five- and T(11mo.) after eleven month. The x-axis shows the number of base pairs where the *Cfo*I restriction enzyme cut the DNA string and the y-axis shows the relative fluorescence.
Peaks are becoming fewer and therefore more abundant, but small peaks can be of great importance by indicating a certain microorganism.
The fact that the dominant bacterial organisms are different from one to another mesocosm comparing all 11mo samples suggests that the degradation of lignocellulose material is done by a composer community rather than one single organism.

4.2 Microscopic community analysis

As mentioned in 1.5.2 (Microscopic analysis), the aim is to find visible indications for laccase activity in the mesocosms.

1µl of each mesocosm was placed on microscope slides homogenized with a drop of distillated water. Two samples on one microscope slide were covered with a cover slip each. No heat fixation was applied. Rough adjustment of the microscope was performed at 40x magnification which equaled a 400x magnification on the computer screen. Pictures were taken at a thousand-fold total magnification with a drop of immersion oil on top of the lens of the microscope.

A thousand-fold magnification means that bacteria can be seen as small dots with a diameter some were around the size of 1 millimeter. The resolution in figures 12 and 13 is less then 1000x which only applied for the computer screen at the University of Tennessee. The scale has been deleted when formatting the pictures.

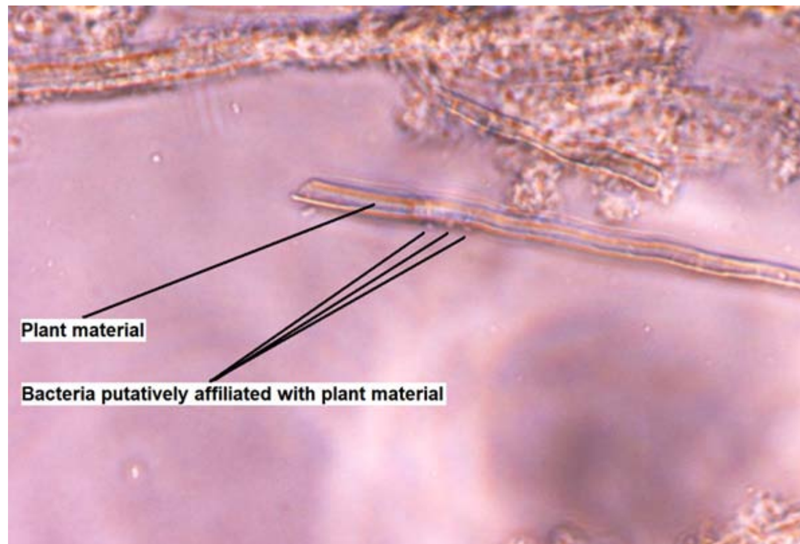

Figure 12 microscopy mesocosm A bacteria

Figure 12 shows organisms in the size of bacteria that seem to be attached to strings of plant material in mesocosm A.

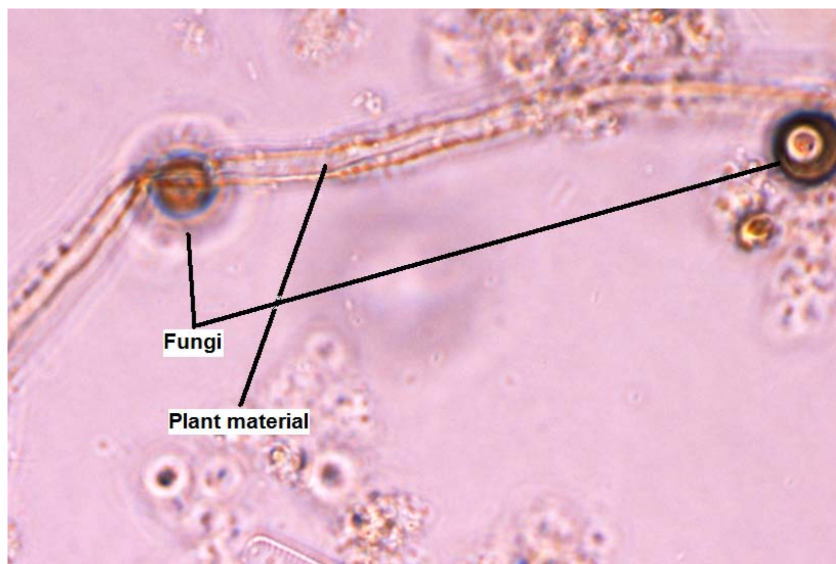

Figure 13 microscopy mesocosm A fungi

Figure 13 is shows putative fungi affiliated with plant material in mesocosm A

Putative bacteria and fungi were found under the microscope and even seemed to be attached to the plant material. The size of plant material was visibly smaller since it was exposed to the microbial community. Fungi were only found in samples A, B, and C, which are the mesocosms where switchgrass has been added.

4.3 Identification of members of the putative degrading community

Based on the research described in 4.1 (Mesocosms as a starting point) and according to the findings achieved in 4.2 (Microscopic community analysis), an enriched lignocellulose degrading community is available for further investigations. It comprises isolation and identification of organisms from the degrading community, as well as scanning them for laccase activity.

4.3.1 Isolation of organisms

6 plates of YTSS-agar and 6 plates of V8-agar were labeled with mesocosm A to F.
100 µl of each mesocosm A to F were incubated for about three days at ~22°C to the equally labeled plates. This incubation created a variety of 25 fungi and bacteria. They were transferred to separate YTSS or V8-agar plates labeled pRu 1 to 25 and the letter of the mesocosm from which the organism originally came.
The first separation caused the growth of 24 organisms that appeared to be different. They were incubated to new plates with the final labeling pRu 1 to 24, the mesocosm number and letter of the first separation, the date, and the name of the media.
Three more isolations were performed as described in “3.2 Isolation of organisms”.
One colony of each final isolate was transferred to one of four partitioned plates and frozen at a temperature of -80°C.

24 organisms were isolated that were different in appearance of their colonies or smell.
16 of these were supposed to be bacteria growing on YTSS. Eight of them were putative fungi growing on V8 agar.

YTSS agar for bacteria			V8 agar for fungi		
Isolate #	Mesocosm	smell	Isolate #	Mesocosm	smell
1	A1	*	17	A14	*
2	A2	*	18	B16	*
3	A3	**	19	B17	0
4	B4	*	20	C19	0
5	B4	0	21	D9	0
6	B5	*	22	D22	0
7	B5	0	23	E23	*
8	C6	*	24	F25	*
9	D7	0			
10	D8	*			
11	D9	*			
12	D10	*			
13	E11	*			
14	F12	**			
15	F13	0			
16	A1	*			

Table 2 Isolates of mesocosms T(11mo.)

Table 2 shows from which mesocosm each isolate came. The number behind the letter counts through all the initial organisms found. At the first separation the colonies on the A1, B4 and B5 plate turned out to be two different organisms due to appearance. Some of the organisms transferred to the V8 agar for initial separation did not grow at all. The scale for smell was rated subjectively for later identification of the organism. “0” stands for no smell “*”for bad and “**” for very bad smell.

Figure 14: 24 isolates freezer stocks.

Figure 14 shows all of the 24 isolates being transferred to 4 partitioned YTSS plates, which were frozen at -80°C. All isolates grew on YTSS, which indicated the low abundance of fungi beyond them. One organism on the plate on top formed spores and therefore was detected to be a fungus still growing on YTSS. The freezer stocks are made to re-cultivate the organism in case interesting findings are achieved later.

4.3.2 Selection of isolate representatives

In this chapter, PCR was performed for the extracted and quantified DNA from isolates. The results determined which 15 isolates were further processed for T-RFLP.

24 DNA extractions were performed using all the colonies from each of the final isolate plates. The extraction is done according to the UltraClean^{TM} Soil DNA Isolation Kit protocol. Alternating from the protocol, the centrifugation step 7 was performed twice as long to pellet down all cell debris, soil beads, and humic acids. DNA was exposed to the S5 solution for one minute before centrifugation at step 20 in order to detach all DNA from the spin filter.
The DNA extraction worked well for almost every organism. Isolate # 8 appeared to be extremely gluey and could neither be separated from cell debris after centrifugation nor be processed through the silica membrane at the very end of the extraction protocol. Isolate # 23 was by accident contaminated with isolate # 24; therefore, only half of the DNA from # 24 could be recovered.

21 tubes remain with DNA dissolved in 50µl of Mo Bio elution buffer S5 after the DNA extraction process. S5 does not contain EDTA to inhibit the function of DNase, therefore, DNA has to be stored at - 20°C.

The concentration of the extracted DNA was measured by NanoDrop ND-1000, as described in 3.9 (NanoDrop ND-1000 Spectrophotometer).

Test type: Nucleic Acid

Sample ID	ng/ul	A260	A280	260/280	260/230
P1 (A)	96.89	1.938	1.008	1.92	0.73
P2 (A)	14.60	0.292	0.243	1.20	0.35
P3 (A)	74.42	1.488	0.758	1.96	0.91
P4 (B)	76.03	1.521	0.792	1.92	0.57
P5 (B)	29.38	0.588	0.307	1.91	0.35
P6 (B)	37.90	0.758	0.484	1.57	0.46
P7 (B)	11.97	0.239	0.116	2.06	0.20
P9 (C)	43.09	0.862	0.539	1.60	0.56
P10 (D)	24.72	0.494	0.262	1.89	0.39
P11 (D)	173.57	3.471	1.982	1.75	1.07
P12 (D)	16.55	0.331	0.426	0.78	0.10
P13 (E)	31.46	0.629	0.874	0.72	0.11
P14 (F)	167.19	3.344	1.722	1.94	1.10
P15 (F)	97.62	1.952	1.065	1.83	1.03
P16 (A)	20.80	0.416	0.246	1.69	0.26
P17 (A)	74.02	1.480	0.769	1.92	1.56
P18 (B)	7.02	0.140	0.049	2.89	0.16
P19 (B)	8.24	0.165	0.068	2.42	0.16
P20 (C)	8.96	0.179	0.107	1.67	0.41
P21 (D)	2.09	0.042	0.023	1.80	0.13
P22 (D)	4.80	0.096	0.024	4.03	0.21
P23 (E)	73.58	1.472	0.770	1.91	1.80
P24 (F)	14.29	0.286	0.171	1.68	0.24
S5 controll	-3.16	-0.063	-0.064	0.99	0.84

Table 3 NanoDrop spectrophotometer DNA concentration for selection of isolates

Table 3 reflects the DNA concentration of the samples after extraction with the Mo Bio kit.
The samples were labeled P1 to P24 which equals isolate# 1 to 24.
The total DNA concentration should preferably be higher than 20 ng/µl. That is the concentration used for calculation of the PCR mixture. Isolates P18 to P22 have a DNA concentration below 10ng/µl. P12 and P13 show low 260/280 ratio which is an indication for the presence of proteins in the case of DNase, which mean, that no results can be expected for PCR amplification.

PCR was performed once with the fungi specific primers, ITS1F and ITS4A, and once with the bacterial primers, 27F and 1522R, as described in 3.4.1 (Platinum^R Taq DNA Polymerase High Fidelity)

Hi-Fi Taq PCR Recipe	
vol.(ul):	
20.80	50mM MgSO4
52.00	10x rxn buffer
10.40	dNTP mix (10mM ea)
10.40	forward primer (XuM)
10.40	reverse primer (XuM)
2.60	HF Taq polymerase (5U/ul)
26.00	DNA template (1ng/ul PCR rxn vol.)
387.40	dH2O
26	[ENTER] desired # tubes
20	[ENTER] total rxn volume (ul)
20.00	[ENTER] DNA stock conc. (ng/ul) of least conc. Sample (#1)
19.00	mix/tube before DNA added (ul)
1.00	DNA/tube if added last (ul)

Table 4 Hi-Fi Taq PCR Recipe for Isolate selection

The recipe of table 4 was prepared two times, once for each set of primers. A total number of 26 tubes included 23 samples one negative and one positive control, as well as an extra volume of master mix. The results of the PCR were visualized by gel electrophoresis. 6 µl DNA were loaded on 250ml 1% agarose gel, as described in 3.5 (Gel electrophoresis). Bands should appear at 600 or 1500 base pairs, therefore, the 1kb DNA leader was added to the gel as well. The running conditions were 120V for 70 minutes. Afterward the gel was post stained in ethidium bromide for 30 minutes before an image of the brands was taken under UV light.

Lane #	Upper half 1500 bp	Lower half 600 bp
1	-	-
2	1 kb leader	1 kb leader
3	-	-
4	-	-
5	P1	P1
6	P2	P2
7	P3	P3
8	P4	P4
9	P5	P5
10	P6	P6
12	P7	P7
13	P9	P9
14	P10	P10
15	P12	P12
16	P13	P13
17	P14	P14
18	P15	P15
19	P16	P16
20	P17	P17
21	P18	P18
22	P19	P19
23	P20	P20
24	P21	P21
25	P22	P22
26	P23	P23
27	P24	P24
28	-	-
29	Neg. control bacteria	Neg. control fungi
30	Pos. control bacteria	Pos. control fungi

Table 5: Capitation of gel electrophoresis for isolate selection

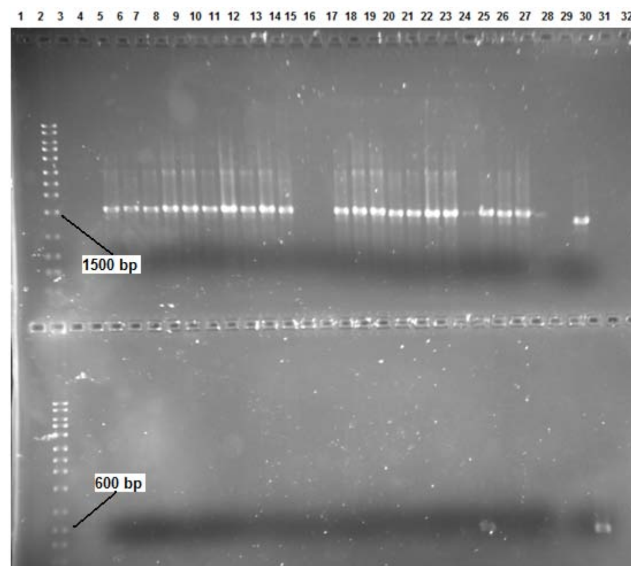

Figure 15: Gel electrophoresis for isolate selection

Figure 16 shows the results of amplifying the extracted isolate DNA by PCR. All positive and negative controls worked meaning every bacterial and fungal amplified DNA could be detected on this gel.

The DNA amplified with bacterial primers 27F and 1522R are shown on the upper half of figure 15 and appear as bands with the size of 1500 bp. Fungal DNA amplified with ITS1F and ITS4A

shows up as bands at the 600 base pair mark. P12 and P13 were not amplified by PCR at all, and the isolate P24 shows a weaker band than the other bacterial organisms.
P21 seems to be the only fungal organism of all isolations but was contaminated with bacteria considering it shows a band at 1500 bp as well. The fungal positive control does not show up very strong either.
15 isolates were chosen for T-RFLP according to the results of PCR, DNA extraction, and spectrophotometry meaning 9 isolates were dismissed from the selection for various reasons. The DNA of organism P8 could not be extracted by the UltraClean™ Soil DNA Isolation Kit. P12 and P13 did not show bands on the gel after PCR amplification. Fungi cannot be used for this T-RFLP, therefore, P21 was not accepted. P23 was contaminated with P24 which showed bad bands at gel electrophoresis. In order to obtain 15 samples, P19, P20, and P22 were excluded as well. These samples also showed low DNA concentrations after DNA extraction.

4.3.3 T-RFLP of isolates

T-RFLP was performed for 15 bacteria isolates with the purpose to obtain results that match with the data from the T(11mo.) mesocosm T-RFLP. This would be an indication for the successful isolation of organisms originating from the T(11mo.) enrichment community. Identifying these organisms would be the next step required.

The DNA extract of the 15 isolates was newly labeled to eliminate confusion, as shown in table 6.

New label	Former label	mesocosm
s1	P1	A
s2	P2	A
s3	P3	A
s4	P4	B
s5	P5	B
s6	P6	B
s7	P7	B
s8	P9	C
s9	P10	D
s10	P11	D
s11	P14	F
s12	P15	F
s13	P16	A
s14	P17	A
s15	P18	B

Table 6: labeling for T-RFLP

T-RFLP was performed following the protocol as described in 3.7 (T-RFLP).

At a first step the DNA extract of the isolates was amplified by PCR according to 2.3.1 (Platinum^R Taq DNA Polymerase High Fidelity).

Hi-Fi Taq PCR Recipe	
vol.(ul):	
39.60	50mM MgSO4
99.00	10x rxn buffer
19.80	dNTP mix (10mM ea)
19.80	forward primer (XuM)
19.80	reverse primer (XuM)
4.95	HF Taq polymerase (5U/ul)
49.50	DNA template (1ng/ul PCR rxn vol.)
737.55	dH2O
33	[ENTER] desired # tubes
30	[ENTER] total rxn volume (ul)
20.00	[ENTER] DNA stock conc. (ng/ul) of least conc. Sample (#1)
28.50	mix/tube before DNA added (ul)
1.50	DNA/tube if added last (ul)

Table 7 PCR recipe for T-RFLP

60 µl of PCR amplified DNA are required for T-RFLP for each isolate sample, therefore, two PCR tubes were used for each DNA sample s1 to s15 with a reaction volume of 30 µl each. The fluorescent labeled forward primer FAM-8F and the reverse primer 1522R were used. As soon as the fluorescent primer was added, the samples were, as far as possible, not exposed to light in order to keep the signal strong for T-RFLP.
PCR was performed to amplify the 1500 base pair sequence of the bacteria 16s rRNA gene.
A 250 ml 1% agarose gel for electrophoresis was poured and stored in 1x TAE buffer.
The 30 µl PCR products were mixed with a 5 µl 6x loading buffer and 32 µl of this mixture were added to each lane. The same samples were placed next to each other skipping one lane between the next different samples to avoid cross contamination.
The gel electrophoresis was performed at 120 V for 70 minutes.

Post staining of the gel was done for 30 minutes in ethidium bromide.
No picture of the gel was taken to prevent the fluorescent labeled DNA from additional exposure to UV light. The bands were cut out under UV light. The purpose was to obtain only the fluorescent labeled 1.5 kilo base sequences that represent the 16S rRNA gene in order to digest only that size fragment for T-RFLP. Each pair of slices originating from the same sample DNA was put together in one 2ml tube.

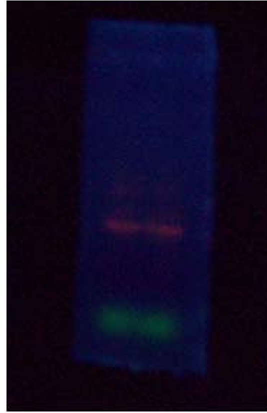

Figure 16 T-RFLP gel

Figure 18 shows 1500 base pair PCR product as red bands. The green bands at a smaller base pair size are most likely primer binding to themselves.

The QIAquick Gel extraction kit was used to purify the DNA from the gel, hence the mass of the DNA gel was measured and the amount of buffer calculated.

Sample # s	Empty tube [mg]	Tube + Gel [mg]	Gel [mg]	QG buffer [µl]	Isopropanol [µl]
1	1503.7	1732.0	228.3	684.9	228.3
2	1502.4	1695.3	192.9	578.7	192.9
3	1503.5	1704.7	201.2	603.6	201.2
4	1497.3	1668.4	171.1	513.3	171.1
5	1500.2	1689.2	189.0	567.0	189.0
6	1511.1	1687.7	176.6	529.8	176.6
7	1512.7	1701.7	189.0	567.0	189.0
8	1510.5	1760.5	250.0	750.0	250.0
9	1500.8	1706.0	205.2	615.6	205.2
10	1498.3	1695.1	196.8	590.4	196.8
11	1501.2	1730.7	229.5	688.5	229.5
12	1505.8	1731.0	225.2	675.6	225.2
13	1508.3	1741.5	233.2	699.6	233.2
14	1494.7	1661.5	166.8	500.4	166.8
15	1482.7	1687.6	204.9	614.7	204.9

Table 8: Calculation of buffer for QIAquick gel extraction

The gel extraction was performed as described by the QIAquick Gel extraction kit handbook 07/2002. Some variations were made such as step 3. When the gel was dissolved in the QG buffer, the solution was only vortexed once after 6 minutes instead of every 2 to 3 minutes. About 100 µl isopropanol were added to each sample although a sequence of 1500 base pairs did not require isopropanol in order to show better yields. The DNA elution from the QIAquick membrane at step 13 was performed with 40 and 35 µl EB Buffer after allowing it to sit on the membrane for 5 minutes. The two volumes were combined in the same tube. Spectrophotometry was performed as described in 3.9 (NanoDrop ND-1000 Spectrophotometer), to receive the DNA concentration.

To perform a proper digestion of the DNA sequences, the total volume of each sample had to be 30µl with a total of 10 ng DNA.

Sample #	DNA [ng/µl]	Dilution	DNA [µl]	Enzyme[µl]	10x Buffer[µl]	H_20 [µl]
s1	18.78	1/2	1.06	1	3	24.94
s2	6.09	1	1.64	1	3	24.36
s3	21.2	1/3	1.42	1	3	24.58
s4	18.2	1/2	1.10	1	3	24.90
s5	11.12	1/2	1.80	1	3	24.20
s6	14.03	1/2	1.43	1	3	24.57
s7	20.41	1/3	1.47	1	3	24.53
s8	17.76	1/2	1.13	1	3	24.87
s9	18.84	1/2	1.06	1	3	24.94
s10	3.23	1	3.10	1	3	22.90
s11	14.88	1/2	1.34	1	3	24.66
s12	14.81	1/2	1.35	1	3	24.65
s13	21.37	1/3	1.40	1	3	24.60
s14	15.96	1/2	1.25	1	3	24.75
s15	18.6	1/2	1.08	1	3	24.92

Table 9 preparation for DNA digestion

Table 9 shows the results of spectrophotometry and the required volumes to prepare a DNA digestion. High DNA concentrations were diluted. Therefore, no volume below 1µl of DNA was required for the recipe to keep the accuracy high.

The digestion was prepared by combining distilled water and the sample DNA first, then the 10xBuffer and finally the enzyme. The samples were immediately incubated for 3.5 hours at 37°C as soon as *Cfo*I was added. After the digestion, samples were immediately put on ice to inactivate the restriction enzyme.

Ethanol precipitation was performed after digestion according to 3.7 T-RFLP**.** The mixture was 3x gently inverted before overnight precipitation at -20°C.

Next, the samples were exposed to centrifugation at 3°C and 16k x g for 20 minutes. The supernatant was carefully poured away and further processed with Speed-Vac. 17 volumes of master mix were prepared with 215.9µl formamide and 5.1µl LIZ1200 which equals a volume of 13µl for each sample.

The method was completed as described in 3.7 T-RFLP

	1	2	3	4	5	...	12
A			s1	s2			
B			s3	s4			
C			s5	s6			
D			s7	s8			
E			s9	s10			
F			s11	s12			
G			s13	s14			
H			s15	blank			

Table 10 loading of the 96-well-plate

Table 10 gives information for the T-RFLP run about the rows used on the 96-well-plate. The ABI PRISM® Genetic Analyzer 3100 can only perform 16-capillary runs. That means the samples have to be in either rows 1/2, 3/4, 5/6, 7/8, 9/10, or 11/12. Otherwise, would require double the costs. One GeneScan run with 16 samples cost 48$.

GeneScan 3100 instrument settings:

module setup:		
	run temp.	60°C
	capillary fill volume	184 steps
	current tolerance	100uA
	run current	100uA
	voltage tolerance	0.6kV
	prerun voltage	15kV
	prerun time	180sec
	injection voltage	1kV
	injection time	35sec (this varies depending on LIZ internal standard and FAM intensities)
	run voltage	8kV
	#steps	10nk
	voltage step interval	60sec
	data delay time	1sec
	run time (est.4.5sec/bp)*	**3750sec/650bp fragments**, 5000sec/1000bp, **7000sec/1500bp**
		**run times are extended to account for occasional detection shifts per capillary*

Table 11 settings for GeneScan 3100

Table 11 shows the parameters that were inserted to the ABI PRISM® Genetic Analyzer 3100 in order to run the T-RFLP.

	Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area
	1B, 1	18.93	186.80	280	6442
s1	1B, 2	19.57	201.65	1622	31335
	1B, 3	20.44	221.59	61	1057
	2B, 1	19.54	199.15	60	1102
	2B, 2	19.76	204.11	239	5135
s2	2B, 3	20.42	219.20	8420	102935
	2B, 4	20.45	219.88	8337	106041
	2B, 5	35.91	572.67	693	17609
	3B, 1	18.90	186.93	168	3684
	3B, 2	19.53	201.51	8383	96991
s3	3B, 3	19.54	201.87	8340	102906
	3B, 4	20.44	222.53	58	1037
	3B, 5	34.43	556.33	83	1122
	3B, 6	34.50	558.00	161	3414
	4B, 1	19.53	199.82	68	1123
s4	4B, 2	33.74	527.71	61	797
	4B, 3	33.77	528.39	65	1292
	4B, 4	35.53	569.98	3922	101280
s5	5B, 1	34.53	570.31	575	11128
	5B, 2	34.59	571.96	212	2907
s6	6B, 1	33.02	530.19	54	1523
	6B, 2	34.64	570.68	4643	131909
s7	7B, 1	34.17	570.26	3504	92743
	8B, 1	32.14	529.47	52	834
	8B, 2	32.17	530.10	51	807
s8	8B, 3	33.70	571.02	5137	135063
	8B, 4	40.31	756.37	76	2038
s9	9B, 1	19.00	198.05	68	1093
	9B, 2	33.34	570.92	3625	99584
	10B, 1	18.40	187.27	140	3177
	10B, 2	18.99	201.74	8408	96412
	10B, 3	19.02	202.32	8339	103630
s10	10B, 4	25.59	369.77	189	3400
	10B, 5	32.45	556.81	426	6193
	10B, 6	32.50	558.33	754	15437
	10B, 7	41.62	836.70	188	4883
	11B, 1	18.91	201.82	8129	145026
s11	11B, 2	32.07	556.42	61	738
	11B, 3	32.12	557.93	110	2320
	13B, 1	18.87	200.46	77	1383
s13	13B, 2	20.27	235.40	7766	188440
	13B, 3	32.42	567.30	169	4977
	14B, 1	18.27	187.19	95	2168
	14B, 2	18.86	201.86	8339	83448
s14	14B, 3	18.88	202.25	8319	96790
	14B, 4	32.13	556.54	112	1669
	14B, 5	32.18	558.11	163	3118
	15B, 1	18.87	196.27	71	1153
s15	15B, 2	32.88	570.33	4341	115019
	15B, 3	38.78	754.43	69	1670

Table 12 T-RFLP data of isolates

Table 12 shows the results of the isolate T-RFLP. The longer fragments are cut by *Cfo*I, it takes longer for the peaks to appear. Peak size and peak area give information about how much fluorescent labeled DNA is detected. Peaks that are smaller than 1% of the total peak height or area of a sample are not taken into account. Isolate s12 is missing in table 10 because the internal standard did not show up well enough.

.

The isolate peaks were compared with the peaks of the T(11mo.) community T-RFLP. Peaks with the same terminal restriction fragment length, as present in T(11mo.) community, are found at 185, 203 and 234 base pairs. Six of the isolate samples show one or more of these restriction sites.

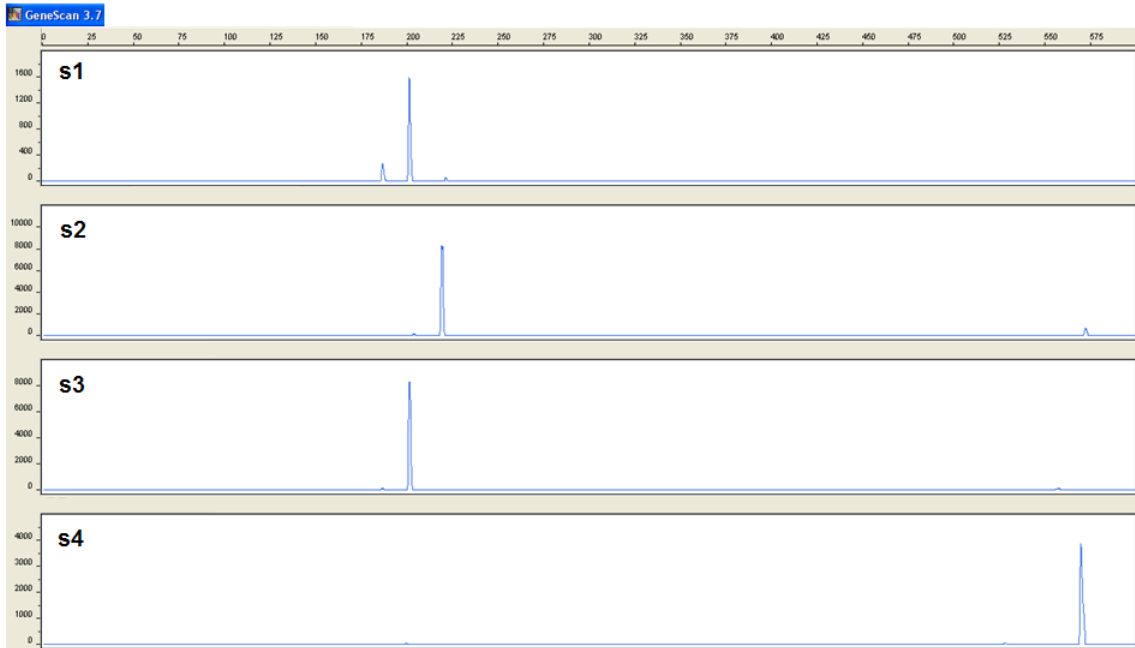

Figure 17 T-RFLP results s1 – s4

Figure 19 shows s1 and s3 having their dominant restriction site at ~ 203 base pairs but s2 and s4 are not cut at a base pair size of interest.

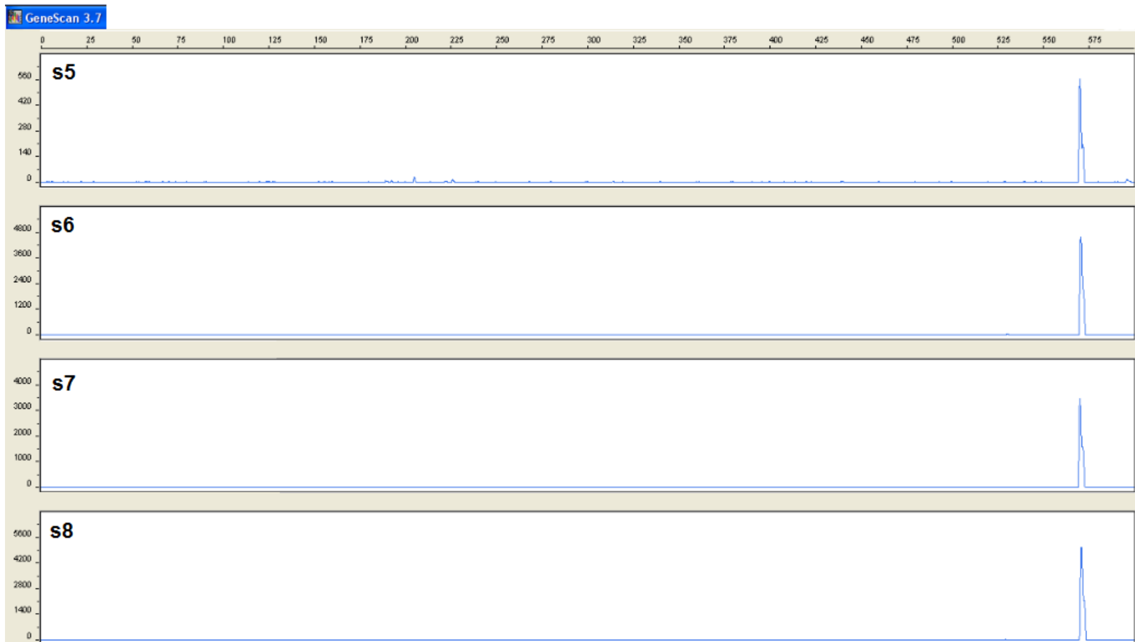

Figure 18 T-RFLP results s5 – s8

None of the isolates in figure 20 shows a cleavage at 185, 203, and 234 base pairs.

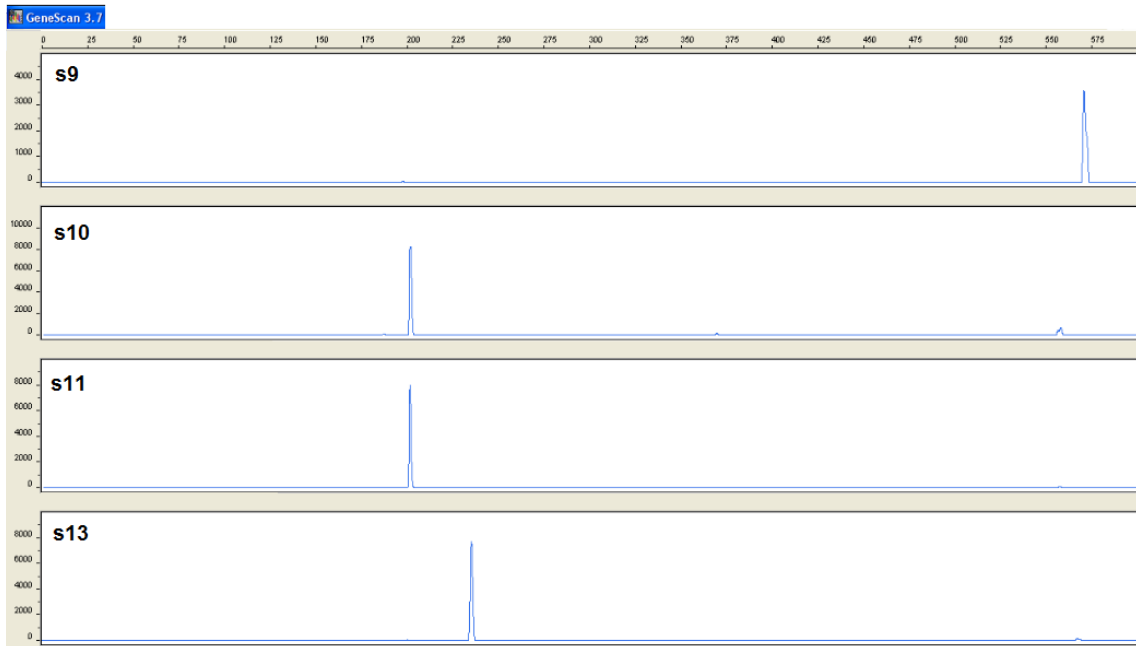

Figure 19 T-RFLP results s9 – s13

Figure 21 shows a peak around 203 base pairs for s10 and s11 and a dominant peak for s13 at ~234 base pairs.

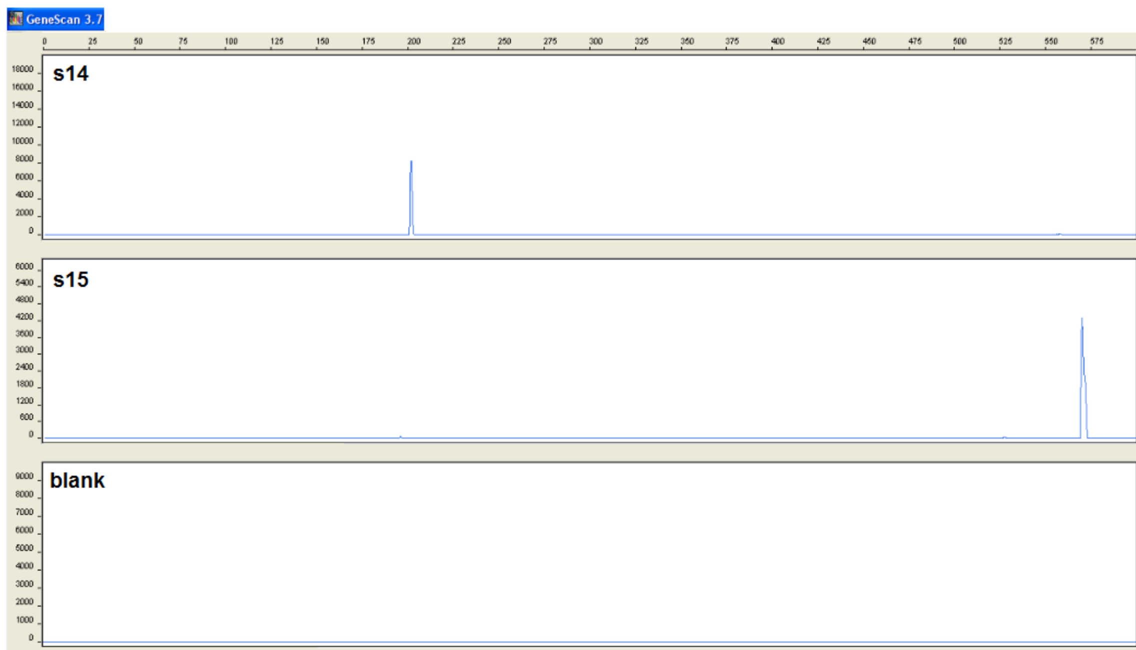

Figure 20 T-RFLP results s14, s15 and blank

Figure 22 shows that the master mix worked and was not contaminated because the blank shows up as a straight line. Isolate s14 could be a representative of the community showing its main peak at about 203 base pairs.

4.3.4 Isolate identification and screening for laccase activity

Isolates s1, s3, s10, s11, s13, and s14 were chosen to be sequenced and identified because they showed terminal restriction fragments at lengths of 185, 203 and 234 base pairs.

T-RFLP	Isolate plate	Mesocosm
s1	P1	A
s3	P3	A
s10	P11	D
s11	P14	F
s13	P16	A
s14	P17	A

Table 13 selection of isolates for sequencing depending on T-RFLP results

The selected isolates are representatives of the enrichment community of the certain T(11mo.) Mesocosm shown in table 13.

Hi-Fi Taq PCR Recipe	
vol.(ul):	
16.00	50mM $MgSO_4$
40.00	10x rxn buffer
8.00	dNTP mix (10mM ea)
8.00	forward primer (XuM)
8.00	reverse primer (XuM)
2.00	HF Taq polymerase (5U/ul)
20.00	DNA template (1ng/ul PCR rxn vol.)
298.00	dH_2O
8	[ENTER] desired # tubes
50	[ENTER] total rxn volume (ul)
20.00	[ENTER] DNA stock conc. (ng/ul) of least conc. Sample (#1)
47.50	mix/tube before DNA added (ul)
2.50	DNA/tube if added last (ul)

Table 14: PCR recipe for sequencing

The PCR was performed as described in Platinum^{R} Taq DNA Polymerase High Fidelity for the bacterial 16s rRNA gene. 27F was used as the forward primer and 1522R as reverse primer.

The set up for gel electrophoresis included a 50ml 1%agarose gel with 5µl DNA and 1µ 6x loading buffer for each sample row. The gel was run for 43 minutes at 100 Volts and post stained in ethidium bromide before a picture of the gel was taken.

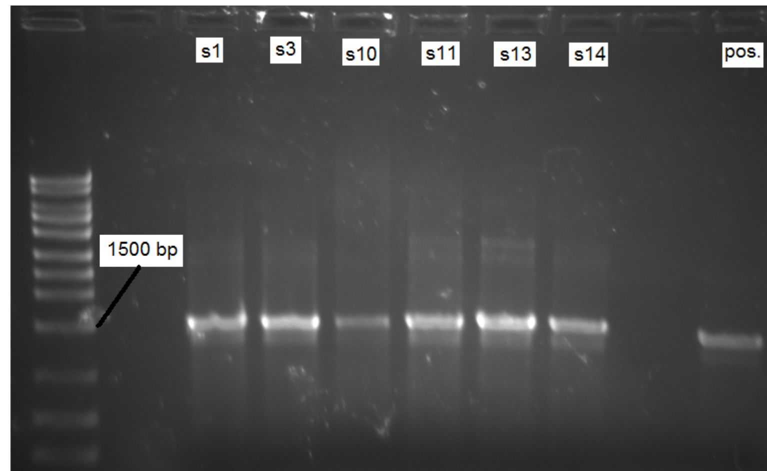

Figure 21 results of gel electrophoresis for sequencing

The leader on the very left side of figure 23 shows that a sequence of 1500 base pairs was amplified for each sample as well as for the positive control at the very right.

45 µl of PCR product from the 6 samples were purified as described in QIAquick PCR Purification Kit handbook 11/2006. The centrifugation of step 7 was performed for two minutes. 40 µl of elution buffer EB were added to the center of the QIAquick column for 3 minutes at step 9 before the purified DNA was eluted by centrifugation.

6 tubes containing purified DNA dissolved in 40µ EB buffer were further processed and sequenced by the Molecular Biology Resource Facility at The University of Tennessee.

The information obtained from sequencing, as described in 3.8 (Sequencing Sanger method), was the basis for identification of the organisms.

The sequencing was successful for all 6 samples. The number of identified bases varied from about 290 to 750. The results were accessible through the BioEdit Sequence Alignment Editor shown the same way as in figure 9.
The sequences were compared with the NCBI database using the tool blastn. The results are shown in table 15.
Isolates s1 and s3 had a sequence identified that is 100% identical with the organism *Pseudomonas putida GB-1*. s10, s11, s13, and s14 were calculated to be 99% similar with a certain organism according to NCBI.

Isolate	T-RF [bp]	Closest Relative in Genbank	Accession #	% ID
s1	185	*Pseudomonas putida GB-1*	NC_010322.1	100
s3	185	*Pseudomonas putida GB-1*	NC_010322.1	100
s10	185 & 203	*Pseudomonas putida GB-1*	NC_010322.1	99
s11	203	*Pseudomonas fluorescens Pf0-1*	NC_007492.1	99
s13	185 & 234	*Lysinibacillus sphaericus C3-41*	NC_010382.1	99
s14	203	*Pseudomonas fluorescens Pf0-1*	NC_007492.1	99

Table 15 identification of sequenced isolates

Three different organisms were identified: *Pseudomonas putida GB-1, Pseudomonas fluorescens Pf0-1, Lysinibacillus sphaericus C3-C41*.
Further investigations on their role in lignin degradation were performed.

The screening for laccase activity is based on the research described by (Alexandre and Zhulin, 2000) as mentioned in 1.4.4 (Laccase).

Putative bacterial laccases		
Species	Protein name _GenBank identification number eTiGR preliminary identification number	Similarity fungal laccase
Mycobacterium tuberculosum	Rv0846c_2916905	3e-36
Escherichia coli	PcoA_1073341	1e-29
Caulobacter crescentus	Contig_122e	6e-29
Pseudomonas syringae	CopA_116921	8e-28
Bordetella pertussis	Contig_449e	9e-28
Xanthomonas campestris	CopA_1073083	3e-26
Pseudomonas aeruginosa	Contig_52e	4e-26
Mycobacterium avium	Contig_982e	1e-22
Pseudomonas putida	CumA_4580028	2e-22
Rhodobacter capsulatus	3128288	1e-16
Yersinia pestis	Contig_768e	5e-15
Campylobacter jejuni	Contig_1e	3e-12
Escherichia coli	YacK_2506227	9e-09
Aquifex aeolicus	Sufl_2983586	1e-07

Table 16 putative bacterial laccases (modified from (Alexandre and Zhulin, 2000))

Table 16 shows different bacteria species that have sequences within their genome that are similar to laccase encoding sequences from fungi. The lower the digit for the similarity or e-value is the smaller are the differences between the compared sequences. The e-value is also based on the length of the compared sequences.

The organism *Pseudomonas putida GB-1* produces the multicooper oxidase CumA also shown in table 24 (Brouwers et al., 1999).
Rv0846c from *Mycobacterium tuberculosum* is according to figure 24 most similar to the fungal laccase. The protein sequence of Rv0846c was compared with the whole genome of *Lysinibacillus sphaericus C3-C41* and *Pseudomonas fluorescens Pf0-1* using tblastn.

Mycobacterium tuberculosum

PROBABLE OXIDASE

Protein name: **Rv0846c**

GenBank identification number: **2916905**

Compared with:

1. ***Lysinibacillus sphaericus* C3-41**

E-value =	3e-18	
Identities =	81/280	(28%)
Positives =	126/280	(45%)
Gaps =	37/280	(13%)

ref|YP_001696272.1| **copper resistance protein A precursor**
[Lysinibacillus sphaericus C3-41]

E-value =	3e-13	
Identities =	95/436	(21%)
Positives =	172/436	(39%)
Gaps =	67/436	(15%)

ref|YP_001698046.1| **blue copper oxidase cueO precursor**
[Lysinibacillus sphaericus C3-41]

2. ***Pseudomonas fluorescens Pf0-1***

E-value =	1e-40	
Identities =	109/308	(35%)
Positives =	157/308	(50%)
Gaps =	19/308	(6%)

ref|YP_349539.1| **copper-resistance protein CopA**
[Pseudomonas fluorescens Pf0-1]

Figure 22 results of screening for laccase activity

This screening showed that *Lysinibacillus sphaericus C3-C41* is not as similar to the possible oxidase site of *Mycobacterium tuberculosum* as *Pseudomonas fluorescens Pf0-1*. But proteins with related structures were found such as copper-resistance protein CopA and blue copper oxidase cueO precursor that indicates oxidase activity which is a requirement for lignin degradation as described in 1.4.4 (Laccase).

5 Discussion

Evidence for the enrichment of a lignocellulose degrading community through a mesocosm experiment was confirmed by microscopic analysis. This community was screened for organisms with laccase activity by creating a selection of representative isolates chosen via the comparison of T-RFLP data. Sequencing and identifying of these organisms indicated a successful isolation of *Pseudomonas putida GB-1*, an organism known for laccase similar activity and two other organisms that could possibly have laccase activity as well. There is no final prove that this isolates are abundant within the enriched mesocosms but it can be assumed due to the fact that they show T-RFLP peaks at the same number of base pairs as the community does, and their putative laccase activity.

5.1 Mesocosm

T-RFLP for the fungi community has not been done yet because the main interest is on bacterial decomposer organisms. With freezer stocks the T-RFLP for fungi community analysis can still be performed.

In the mesocosm experiment the only Carbon source for the growth of bacteria seems to be plant material. But according to the growth curve of bacteria there are also organisms dying throughout the enrichment process. Therefore dead bacteria cells can also play a role as Carbon source. A possible way to check on that will be Stable Isotope Probing (SIP). By labeling switchgrass with C^{13} isotopes the bacteria degrading plant material will uptake these isotopes and can be separated from the organism not affiliated in switchgrass degradation.

The set up for the mesocosm experiment is a closed system, and can hardly be compared with the open salt marches at the Georgian coast. This questions the reliability of the mesocosm experiment, whether the decomposing process is the same and as good as at the GCE.
The change of the pH inside the mesocosms also indicates alterations that would not be possible in nature, because the pH in the ecosystem remains almost constant.

5.2 Microscopic community analysis

These pictures do not prove that fungi or bacteria are actually degrading the plant material but they indicate some kind of interaction that we were hoping for. Most of the fungi and bacteria in our mesocosms are affiliated with the plant material and the size of the material is reduced after time which is the first indication for the enrichment of a lignocellulose degrading community. Samples for microscopy were taken randomly trying not to plug the pipette with any solids, so they might not totally represent the composition of each mesocosm.

5.3 Identification of members of the putative degrading community

The V8-agar medium did not fulfill its purpose as growth media for fungi because mainly bacteria grew on it. Furthermore YTSS and V8 media do not really represent the conditions in the mesocosms, hence organisms that grow very well on these agar-plates do not necessarily have to be abundant within the enrichment community. This is approved by the results according to 4.3.3 (T-RFLP of isolates). Most T-RFLP isolate samples show a dominant peak at 570 base pairs. This peak is not abundant at the community T-RFLP of the T(11mo.) mesocosms. One explanation for their appearance can be contamination by accident. But more likely these organisms were present in the original environmental sample and got suppressed during the enrichment process for a lignocellulose degrading community. YTSS or V8-agar, offering different carbon sources might cause their recultivation.
The reason why the T-RFLP of isolated organisms can show more than one peak is that a four base pair restriction enzyme is used that might cut a 1500 base pair sequence more than once.

According to the high number of existing Bacteria it is most likely that not only one organism is cut by *Cfo*I at a certain number of base pairs. A T-RFLP peak cannot be identified by identifying an organism that has the same terminal restriction fragment length. Even the selection of isolates sequenced in 4.3.4 (Isolate identification and screening for laccase activity) represents different organisms comprising an identical number of base pairs for the restriction fragment.

The identification of the organisms according to table 13 was not said to be 99% for samples s10, s11, s13, s14 but screening of the received data form sequencing and comparing it with misfits according to the NCBI results, implicated 100% matching with a certain organism.

Pseudomonas putida GB-1 is known to catalyze the oxidation of Mn^{2+} by encoding a protein that is homologous to multi-copper oxidase (laccase). This causes *Pseudomonas putida GB-1* quite likely to play a role in the lignin degrading community.
Scanning *Lysinibacillus sphaericus C3-C41* and *Pseudomonas fluorescens Pf0-1* for possible oxidase activity resulted in showing signs for possible laccase activity within these organisms.

6. Conclusion

The research work described in this thesis comprises a small aspect of the whole project Identifying Novel Lignin and Lignocellullose Degrading Enzymes From Natural Decomposer Communities, as shown in figure 5 (A. Buchan et al., submitted 2007). In this thesis the identification of putative members of an enriched lignocellulose degradation community was performed. It is very likely that the identified organism *Pseudomonas putida GB-1,* is a member of this community according to 5. (Discussion), but the whole procedure of enriching, isolating, and identifying organisms as described in 4. (Results) does not give final proof. One large challenge is the isolation of actual representatives of the lignocellulose degradation community. This is also the weak point of this research experiment according to 5.3 (Identification of members of the putative degrading community). However, this thesis describes a valid protocol for the identification of organisms from a community, based on the data of T-RFLP analysis.

6.1 Further Prospects

In January 2010 the enrichment of lignocellulose degrading organisms has been carried out for 22 months. Identifying isolates of this community using more selective growth media could result in new, interesting findings. Isolating representatives of the enriched community is also a necessary step in order to apply various assays to determine lignocellulase activity. For example, a laccase assay based on syringaldazine as a laccase substrate, was modified from (Lyons et al., 2003) and successfully performed for the laccase positive organism *Azospirillum lipoferum*.

Adding C^{13} labeled switchgrass to the mesocosms is an essential future prospect in order to identify community members with laccase activity as described in 5.1 (Mesocosm).

The entire project of Identifying Novel Lignin and Lignocellullose Degrading Enzymes From Natural Decomposer Communities is further processed as shown in figure 5 or described in A. Buchan et al., submitted 2007) to finally achieve an enzymatic pretreatment for the production of bio-fuels out of lignocellulose material. This sounds like a distant prospect but that will change as soon as the main organisms are identified that cause degradation of plant material inside the mesocosms.

References

Lodish – Berk – Matsudaira – Kaiser – Krieger – Scott – Zipursky – Darnell, Molecular Cell-Biology, W.H. Freeman & Company, 2003, 5th Edition

A. BUCHAN, J. SANSEVERINO, T. REYNOLDS (2009), Identifying Novel Lignin and Lignocellullose Degrading Enzymes From Natural Decomposer Communities, The Southeastern Sun Grant Center, Submitted 2007

A.K. Chandel, ES Chan, R. Rudravaram, M.L. Narasu, L.V. Rao, P. Ravindra, (2007) Economics and environmental impact of bioethanol production technologies: an appraisal, *Biotechnology and Molecular Biology Review* Volume: 2, Issue: 1, Pages: 14-32

ADLER, E. (1977) LIGNIN CHEMISTRY - PAST, PRESENT AND FUTURE. *Wood Science and Technology,* 11**,** 169-218.

ALEXANDRE, G. & ZHULIN, I. B. (2000) Laccases are widespread in bacteria. *Trends in Biotechnology,* 18**,** 41-42.

BALAT, M. (2009) Bioethanol as a Vehicular Fuel: A Critical Review. *Energy Sources Part a-Recovery Utilization and Environmental Effects,* 31**,** 1242-1255.

BROUWERS, G. J., DE VRIND, J. P. M., CORSTJENS, P., CORNELIS, P., BAYSSE, C. & DEJONG, E. (1999) cumA, a gene encoding a multicopper oxidase, is involved in Mn2+ oxidation in Pseudomonas putida GB-1. *Applied and Environmental Microbiology,* 65**,** 1762-1768.

CHERUBINI, F., BIRD, N. D., COWIE, A., JUNGMEIER, G., SCHLAMADINGER, B. & WOESS-GALLASCH, S. (2009) Energy- and greenhouse gas-based LCA of biofuel and bioenergy systems: Key issues, ranges and recommendations. *Resources Conservation and Recycling,* 53**,** 434-447.

DEMIRBAS, A. (2007) Progress and recent trends in biofuels. *Progress in Energy and Combustion Science,* 33**,** 1-18.

GRAY, K. A., ZHAO, L. S. & EMPTAGE, M. (2006) Bioethanol. *Current Opinion in Chemical Biology,* 10**,** 141-146.

HIGUCHI, T. (1990) LIGNIN BIOCHEMISTRY - BIOSYNTHESIS AND BIODEGRADATION. *Wood Science and Technology,* 24**,** 23-63.

HOLTZAPPLE, M. T., JUN, J. H., ASHOK, G., PATIBANDLA, S. L. & DALE, B. E. (1991) THE AMMONIA FREEZE EXPLOSION (AFEX) PROCESS - A PRACTICAL LIGNOCELLULOSE PRETREATMENT.

KESHWANI, D. R. & CHENG, J. J. (2009) Switchgrass for bioethanol and other value-added applications: A review. *Bioresource Technology,* 100**,** 1515-1523.

KOGEL-KNABNER, I. (2002) The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology & Biochemistry,* 34**,** 139-162.

LIU, W. T., MARSH, T. L., CHENG, H. & FORNEY, L. J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology,* 63**,** 4516-4522.

LUQUE, R., HERRERO-DAVILA, L., CAMPELO, J. M., CLARK, J. H., HIDALGO, J. M., LUNA, D., MARINAS, J. M. & ROMERO, A. A. (2008) Biofuels: a technological perspective. *Energy & Environmental Science,* 1**,** 542-564.

LYONS, J. I., NEWELL, S. Y., BUCHAN, A. & MORAN, M. A. (2003) Diversity of ascomycete laccase gene sequences in a southeastern US salt marsh. *Microbial Ecology,* 45**,** 270-281.

MURNEN, H. K., BALAN, V., CHUNDAWAT, S. P. S., BALS, B., SOUSA, L. D. & DALE, B. E. (2007) Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of Miscanthus x giganteus to fermentable sugars. *Biotechnology Progress,* 23**,** 846-850.

RAWE, B. (2008) Breakthroughs and Challenges in the Production of Cellulosic Ethanol. *MMG 445 Basic Biotechnology eJournal*.

SCHUTTE, U. M. E., ABDO, Z., BENT, S. J., SHYU, C., WILLIAMS, C. J., PIERSON, J. D. & FORNEY, L. J. (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Applied Microbiology and Biotechnology,* 80**,** 365-380.

SHARMA, P., GOEL, R. & CAPALASH, N. (2007) Bacterial laccases. *World Journal of Microbiology & Biotechnology,* 23**,** 823-832.

YAROPOLOV, A. I., SKOROBOGATKO, O. V., VARTANOV, S. S. & VARFOLOMEYEV, S. D. (1994) LACCASE - PROPERTIES, CATALYTIC MECHANISM, AND APPLICABILITY. *Applied Biochemistry and Biotechnology,* 49**,** 257-280.

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