PISOLITHUS ARRHIZUS:

INSIGHTS TOWARD ITS LIFE IN A GEOTHERMAL ENVIRONMENT

A University Thesis Presented to the Faculty

of

California State University, East Bay

In Partial Fulfillment

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Master of Science in Biological Sciences

By

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Abstract

The ectomycorrhizal fungus genus *Pisolithus* is only found in two geothermal areas on Earth: New Zealand and Norris Geyser Basing (NGB), Yellowstone National Park (YNP). In NGB, YNP *Pisolithus arrhizus* (Scop.) Rauschert is growing without apparent mycorrhizal associations. This study provides some of the first insights into the life of *P. arrhizus* in a geothermal area. Nine genera of bacteria associated with the fruiting body of *P. arrhizus* were cultured and identified using the 16S rRNA gene: *Bacillus, Brevibacillus, Brevibacterium, Clostridium, Desulfotomaculum, Geobacillus, Nocardia, Paenibacillus,* and *Serratia*. Further insight into the life of *P. arrhizus* in NGB, YNP is provide through enzyme assays. A total of fifteen different enzymes involved in different energy pathways were assayed for activity. *P. arrhizus* proved challenging to work with, and yielded inconclusive results for most enzymes. Sulfite oxidase proved a model example of inconclusive results, while glucose-6-phosphate dehydrogenase and polyphenol oxidase (PPO) were both present. The PPO from *P. arrhizus* in NGB was active on every diphenol tested: catechol, 4-methylcatechol, L-DOPA and cholorgenic acid. Further, the PPO cannot be classified as a tyrosinase because no cresolase (monophenolase) activity was observed on tyrosine. Additionally, the PPO has a working pH range of ≤ 5.1 to ≥ 8.6.

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Approved: Date:

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Dr. Brian A. Perry — 5/15/2017

Dr. Monika Sommerhalter — 5/15/2017

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Table of Contents

List of Figures

List of Tables

List of Figures and Tables in the Appendices

Abbreviations

ADA – 4-amino-N,N-diethylaniline sulfate

ATPase - Adenosine 5'-Triphosphatase

BID – Bacterial IAA Degrader

BIP – Bacterial IAA Producer

BBE – Bacteroides Bile Esculin Agar

BRU – Brucella Blood Agar

BSA – Bovine Serum Albumin

CD – Catechol 1,2 Dioxygenase

CO - Catecholase

DPP – Dehydrated *Pisolithus* Powder

EcM - Ectomycorrhizal

EDTA – Ethylenediaminetetraacetic Acid

G6PDH – Glucose-6-Phosphate Dehydrogenase

IAA – Indole Acetic Acid

ITS – Internal Transcribed Spacer

L-DOPA - 3,4-Dihydroxy-L-Phenylalanine

LBA – Laked Blood Agar

LKV – Brucella Laked Blood Agar with Kanamycin Vancomycin

MHB – Mycorrhiza Helper Bactera

NA – Norris Annex

NGB – Norris Geyser Basin

NP – Norris Proper

PAGE – Polyacrylamide Gel Electrophoresis

PEA – Phenylethyl Alcohol Blood Agar

PGPR – Plant Growth Promoting Rhizobacteria

PCE – *Pisolithus* Crude Extract

PisoEA – *Pisolithus* Extract Agar

PisoEB – *Pisolithus* Extract Broth

PPO – Polyphenol Oxidase

PRAS – Pre-Reduced, Anaerobically Sterilized

PTSA – *Pisolithus* Tryptic Soy Agar

PTSB – *Pisolithus* Tryptic Soy Broth

RT – Room Temperature

SDS – Sodium Dodecyl Sulfate

SO – Sulfite Oxidase

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

TYR – Tryosinases

YNP – Yellowstone National Park

Chapter 1: Introduction

1.1. Field Site

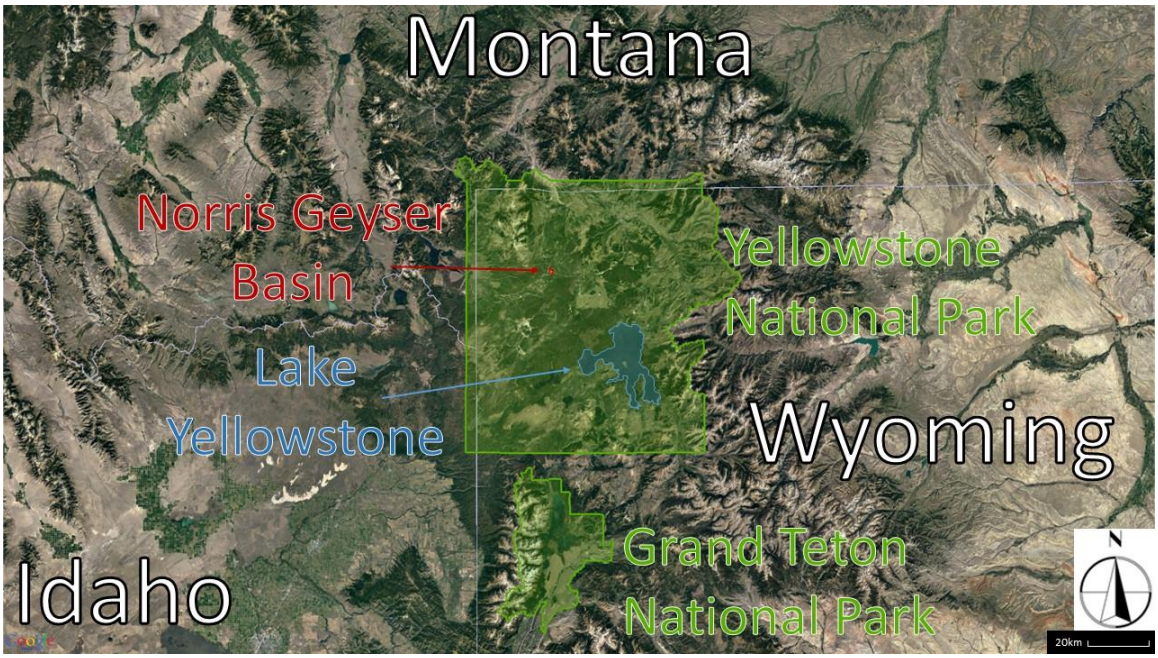


Figure 1: Yellowstone National Park – 20 km Scale

Established in 1872, Yellowstone National Park (YNP) is the heart of a 34,375 mi^2 intact temperate ecosystem which includes: two national parks, portions of five national forests, three national wildlife refuges, Bureau of Land Management holdings, as well as private and tribal lands across three states (Figure 1). The majority of the park rests upon volcanic bedrock above 7,500 feet (nps.gov/yell). There are 10,000 geothermal features including geysers, hot springs, mud pots, and steam vents distributed over 9,000 km^2 (McCleskey et al., 2016). Over the course of geologic time, the Yellowstone hotspot has caused geothermal activity throughout western North America.

Currently, the Yellowstone hotspot presents itself in the form of the Yellowstone Plateau Volcanic Field (Christiansen et al., 2002), a massive hydrothermal system with a convective heat discharge of ~6.1 GW (Ingebritsen et al., 2001).

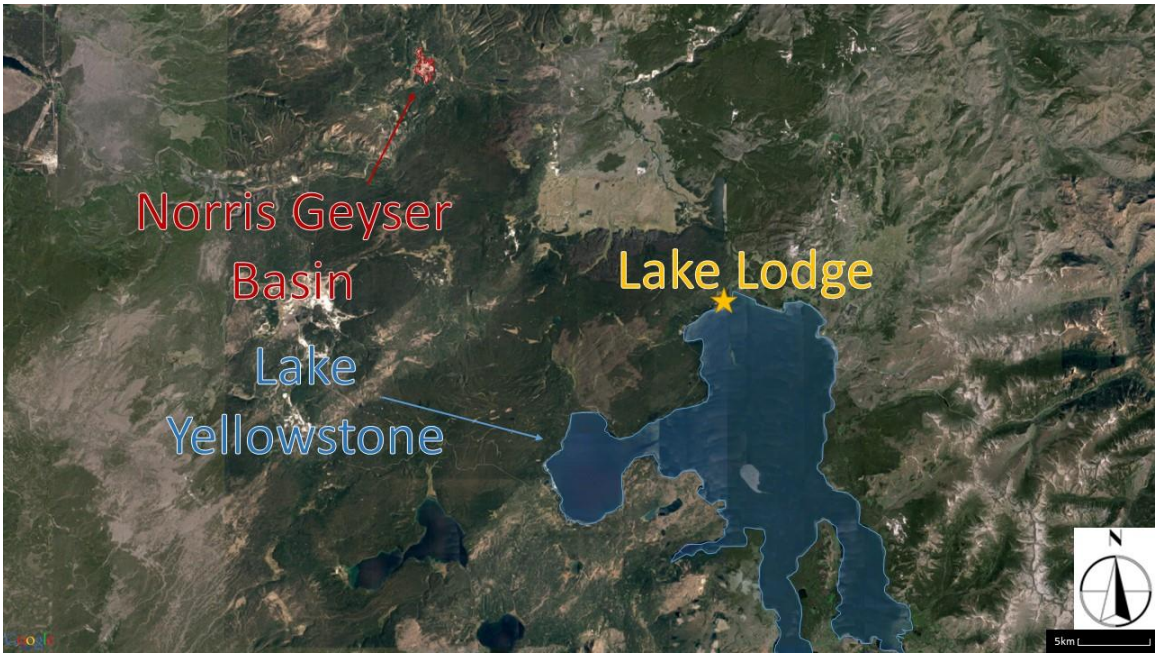


Figure 2: Lake Lodge to Norris Geyser Basin – 5 km Scale

The hydrothermal area of study, Norris Geyser Basin (NGB), is located ~20 miles northwest of Lake Yellowstone (Figure 2). NGB is composed of three main areas: Porcelain Basin, Back Basin, and One Hundred Springs Plain (Figure 3). NGB is the hottest and most dynamic hydrothermal system in YNP (https://www.nps.gov/yell/learn/photosmultimedia/norris-geyser-basin-tour.htm); the temperatures of the geysers, hot springs, and hydrothermally altered ground differ from year to year. Hydrothermal fluid flows over a vast area creating acid-sulfate soils (Neale

et al., 2016). A unique aspect of NGB is that 98% of the hydrothermal system drains into a lone creek, Tantalus Creek (Friedman, 2007).

Neale et al. (2016) mapped changing patterns of hydrothermal activity in NGB and found that large magnitude changes in hydrothermal activity were detected along the northeast edge of Porcelain Basin (aka Porcelain Terrace) (Figure 3). A north-trending fracture in Porcelain Terrace (PT) delineates an elevated zone of dynamic hydrothermal activity in NGB. Hydrothermal activity in PT creates surface temperatures that range from lows of 10-20°C to highs of 40-50°C. One of the sampling sites in this study, Norris Proper (NP), is located at the southern edge of PT (Figure 4).

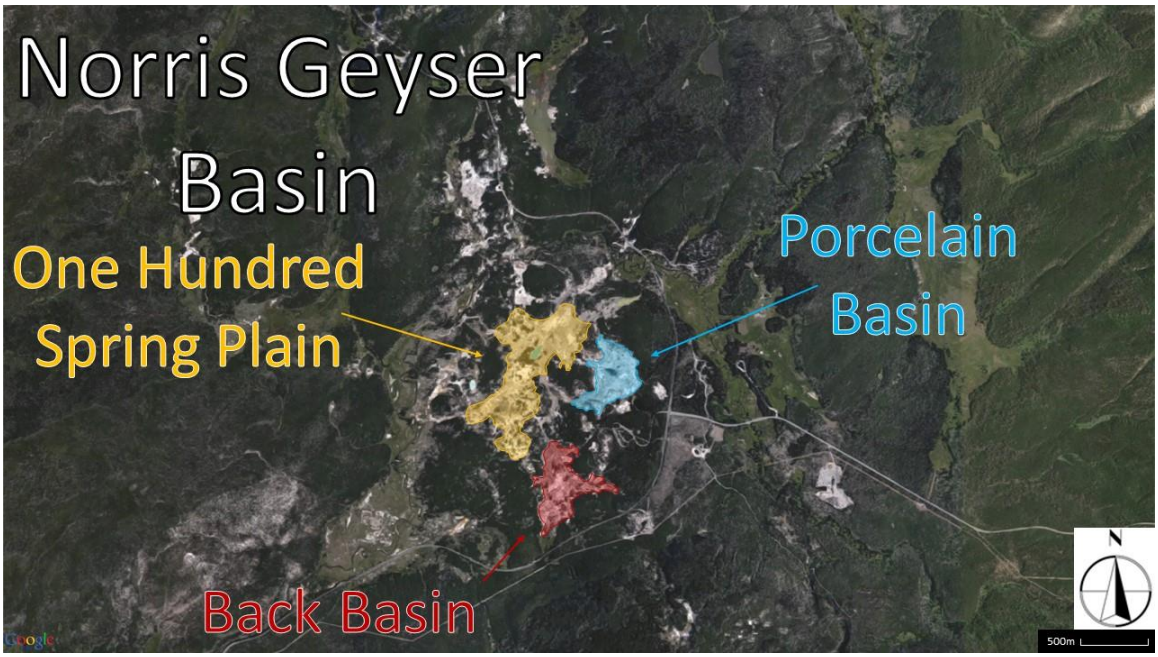


Figure 3: Norris Geyser Basin: Entire View – 500 m Scale

The other sampling site, Norris Annex (NA), is located in a lodgepole pine stand ~100 meters to the southeast of PT. NA is a ~1-hectare thermal area that is

noncontiguous with the remainder of NGB. While park visitors are not permitted to walk through either site, NA is conveniently located out of sight of the designated boardwalk system. NP is visible from the Porcelain Basin Boardwalk system (Figure 5, the black arrows indicate the photograph's vantage point).

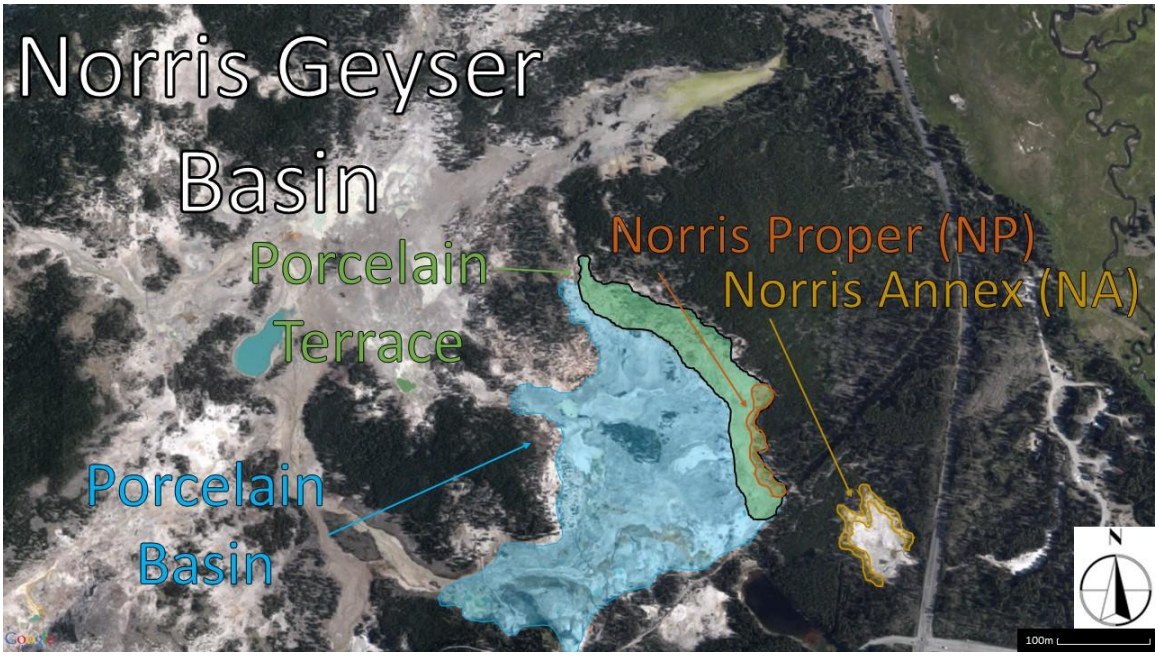


Figure 4: Norris Geyser Basin Site Map – 100 m Scale

Pisolithus arrhizus (Scop.) Rauschert grow from the tree line into the thermal area. Cullings (unpublished) identified the samples as *Pisolithus tinctorius* (Pers.) Coker & Couch using the internal transcribed spacer (ITS) from the 18s rRNA gene (96% match to NCBI accession KJ809558). I will refer to the samples as *P. arrhizus* rather than *P. tinctorius* because *Pisolithus tinctorius* is an illegitimate name has been replaced with *P. arrhizus*. The samples used in this research were collected exclusively where isolated trees were able to take root within the thermal area at NGB, YNP due the treacherous

nature of thermal areas. There are many sections where a thin silica crust covers boiling pools that are known to collapse and swallow any passerby careless enough to tempt fate. Figure 6 shows where *P. arrhizus* samples were collected in each site.

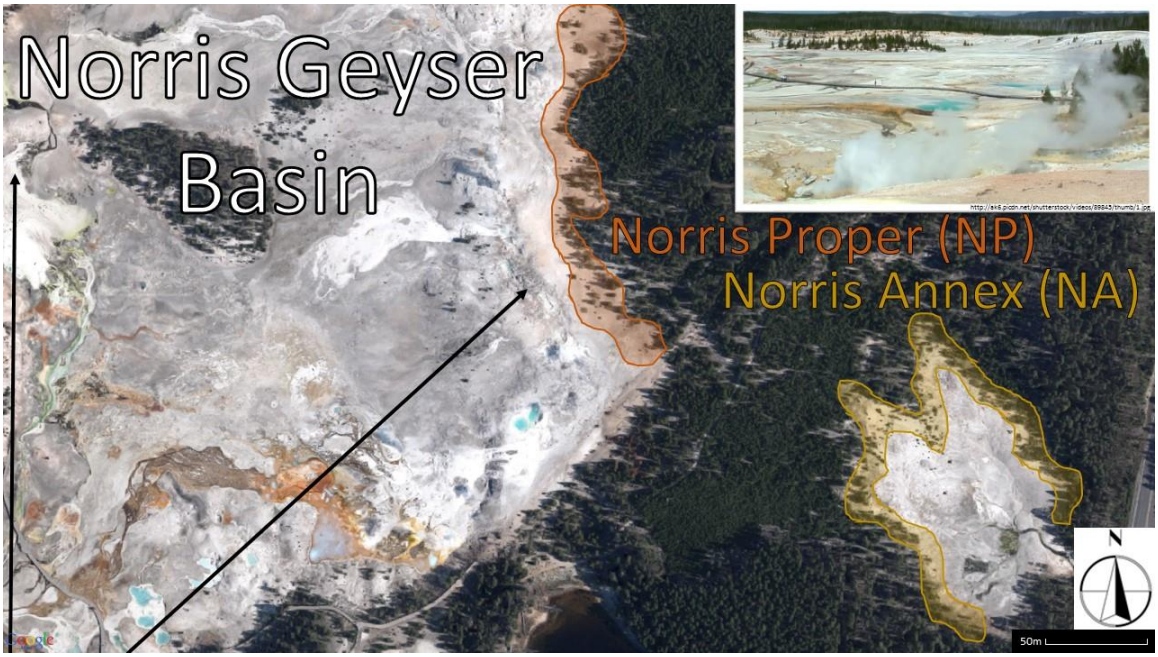


Figure 5: Norris Geyser Basin Site Map – 50 m Scale (black arrows indicate photo vantage point)

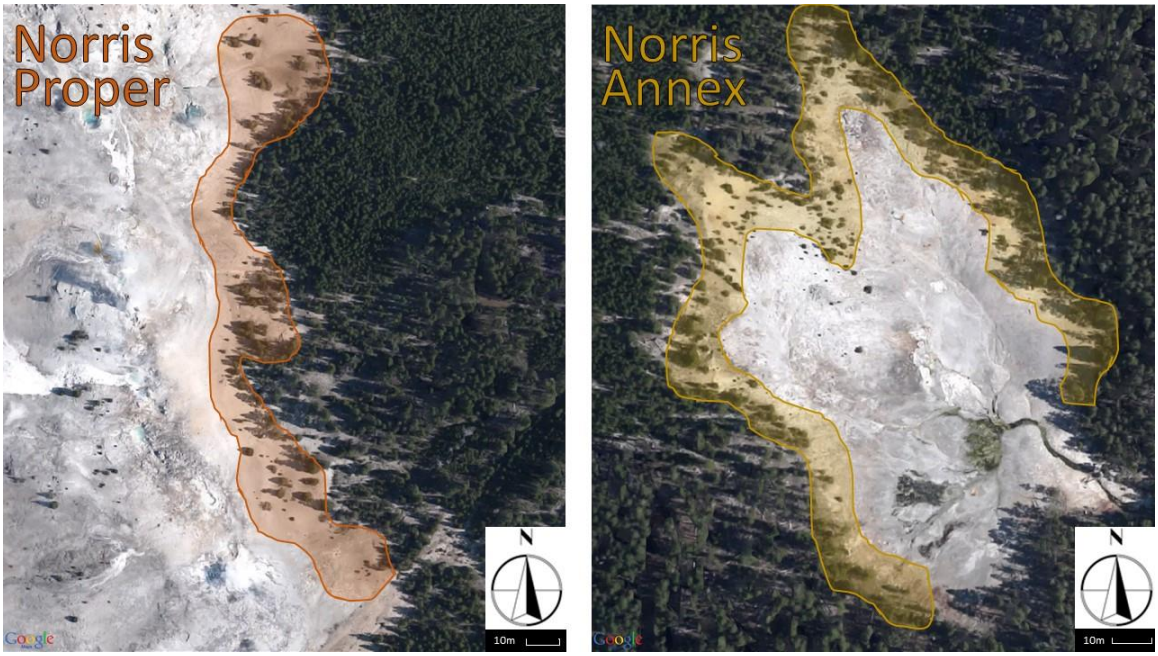


Figure 6 : Norris Proper and Norris Annex Site Maps – 10 m Scale

1.2. *Pisolithus*

Pisolithus is a genus containing ectomycorrhizal (EcM) fungi that are capable of growing across a range of tropical and temperate ecosystems; occurring naturally in 33 countries, including 38 of the 50 states (Marx, 1977). *Pisolithus* has been observed in a variety of habitats that encompass forests to mine tailings, and is well known to tolerate extreme conditions such as high temperature, low pH, drought, low soil fertility, and high heavy metal concentrations (Ho, 1987). EcM associations can form with a diversity of woody plants families including: Pinaceae, Mytaceae, Fagaceae, Mimosaceae, Dipterocarpaceae, and Cistaceae (Marx et al., 1976).

Martin et al. (2002) analyzed 148 *Pisolithus* collections from around the world with samples from natural vegetation and forest plantations. They identified at least 11, potentially 13, different phylogenetic species within *Pisolithus*. Nearly all *Pisolithus* species can be separated into two strong branches: lineage A and B. Lineage A associates with a wide range of vascular plants and contains the well-known *P. tinctorius* and *P. marmoratus* consortia. Lineage B contains *P. albus* and *P. microcarpus* and associates with eucalypts and acacias.

Pisolithus spp. have been identified growing in geothermal areas in only two locations on Earth: New Zealand and Yellowstone National Park, WY. Moyersoen & Beever (2004) observed three species of *Pisolithus* (*P. marmoratus, P. albus,* and *P.* species 10) in EcM associations with *Kunzea ericoides* var *microflora* in New Zealand geothermal areas. *Pisolithus* was not only present, but dominated the EcM community. Their observations are in stark contrast to those of Cullings and Makhija (2001) in Norris Geyser Basin, YNP. NGB *Pisolithus* fruiting bodies were observed in geothermal areas, but were not engaged in EcM associations. Cullings identified fruiting bodies in NGB as *Pisolithus tinctorius* (=*arrhizus*) using the internal transcribed spacer (ITS) sequence (unpublished data). *Pisolithus arrhizus* accounted for the majority of the above-ground (fruiting bodies) fungal diversity within the thermal areas, but surprisingly was not present in any *Pinus contorta* mycorrhizae in the thermal area or surrounding forest.

Cullings and Makhija (2001) compared organic matter and nutrient levels in the thermal areas to the surrounding forest. They found the thermal soils to have higher ammonium, calcium, and magnesium levels but lower pH, organic matter, phosphorus, potassium, aluminum, and total nitrogen, as compared to the forest soils. Both thermal

and forest soils had similar nitrate levels. See Table 1 below for the complete analysis. The soil temperature was recorded on two days in July under leaf litter in the shade of at least 3 trees. Thermal soils were found to have an elevated temperature of 37 ± 2.6°C as compared to forest soils with a temperature of 17 ± 1.5°C.

Table 1: Organic Matter and Nutrient Levels in Forest and Thermal Soils at Norris Geyser Basin

Soil	Conen (mean ± SE) of:								
	Organic Matter (%)	NH_4 (ppm)	NO_3 (ppm)	Ca (meg/liter)	Mg (meg/liter)	P (ppm)	K (ppm)	Total N (%)	Al (ppm)
Forest	6.72 ± 0.03	14 ± 0.7	0.70 ± 0.05	0.62 ± 0.70	0.24 ± 0.03	47 ± 6.2	180 ± 4.2	0.24 ± 0.02	8900 ± 370
Thermal	2.9 ± 0.6[a]	19 ± 1.3[b]	0.68 ± 0.13[c]	3.7 ± 1.8[b]	0.90 ± 0.30[d]	29 ± 8.8[c]	100 ± 13[b]	0.13 ± 0.05[d]	4700 ± 610[d]

[a] Significantly different from the forest soil value ($P < 0.001$), as determined by the Mann-Whitney U test.
[b] Significantly different from the forest soil value ($P < 0.05$), as determined by the Mann-Whitney U test.
[c] Not significantly different from the forest soil value, as determined by Mann-Whitney U test.
[d] Significantly different from the forest soil value ($P < 0.01$), as determined by Mann-Whitney U test.
*From Cullings & Makhija (2001) - Table 1

Pisolithus is well known to be tolerant of harsh conditions, including high temperature and low pH, thus it is not surprising to find *Pisolithus*, a thermophilic acidiophile, living around acid hot springs. Cullings and Makhija's discovery of *P. arrhizus* growing independent of EcM associations was unexpected. They proposed a few hypotheses with the most likely being that *P. arrhizus* may be acquiring carbon through saprophytic growth despite the significantly reduced levels of organic matter in the thermal soils, and the fact that *P. arrhizus* is not known to live saprophytically.

If *P. arrhizus* is growing saprophytically, it is likely that *P. arrhizus* fruiting bodies would be found in the forest soils where there was significantly more organic matter. In this study, I aim to begin characterizing the energetics of *P. arrhizus* from

NGB, YNP through the identification of microbial symbionts and active enzymes, using various culture, molecular, and instrument-based techniques.

Chapter 2: Bacterial Culturing

2.1. Bacterial Culturing: Introduction

2.1.1. Genetic Identification with 16S rRNA Gene

Historically, the identification of unknown microbes was dependent upon the ability of the researcher to obtain a pure culture so a comparison of the morphology and phenotype could be made against published tables, reviewed by Clarridge III (2004). With the advent of phylogenetics in the 1980's, it became possible to determine the evolutionary relationships between bacteria (or any other life form) simply by comparing conserved regions of the genetic code (Woese, 1987). Ribosomal RNA genes are ideal for phylogenetic study because ribosomes are a universal cellular structure and are highly conserved due to their essential function, reviewed by (Clarridge III, 2004). The 16S rRNA gene is the most commonly used gene for eubacteria (Palys et al., 1997). The 16S rRNA gene from eubacteria can be compared with the 16S rRNA gene from archaea and the 18S rRNA gene from eukaryotes. Culturing is instrumental for the study of bacterial genetics, physiology, biochemistry, and behavior (Palleroni, 1997), yet the ability to detect microbes by genetic identity allows researchers to study unculturable communities (Gray & Head, 2001). Most microorganisms are considered to be viable but nonculturable (viable but are refractory to standard culture).

There are many factors to consider when trying to genetically identify a nonculturable microorganism. PCR primers can be designed for specific taxa, or be "universal" within a domain. It is important to note that no primer is truly "universal" and different primers will have varying specificities to 16S rRNA genes, reviewed in (Baker et al., 2003). PCR-amplified 16S rRNA libraries are frequently incomplete due to

the immense diversity of bacteria (Gans et al., 2005). In addition, DNA extraction is dependent upon cell lysis; in diverse environmental samples, bacterial cell wall composition varies and requires different treatment (Wintzingerode et al., 1997).

All regions of the 16S rRNA gene do not exhibit the same rate of mutation (Ueda et al., 1999) and the regions with a high rate of mutation vary between species. Bacteria from all major phyla can be differentiated by genus, species, and/or subspecies. The 16S rRNA gene has highly conserved regions, but is long enough and undergoes enough polymorphisms to yield statistically-significant data on evolutionary relationships, reviewed by (Clarridge III, 2004). The 16S rRNA gene is approximately 1,550-bp in length and can be used for genetic identification with a complete (Sacchi et al., 2002) or partial sequence (Kattar et al., 2000). Phylogenetic trees based on 16S data are similar to trees based on whole-genome sequences, although they are subject to more variation (Bansal & Meyer, 2002) and have less statistical support.

2.1.2. Genetic Identification of Cultured Bacteria

The identification of unknown bacteria was historically dependent upon their characterization using biochemical tests. This remains the preferred method in clinical studies where chemoheterotrophic bacteria respond well physiologically to media and growth conditions, yielding results which tend to be very consistent. The same cannot be stated for environmental bacteria. Often, environmental bacteria display a variety of physiological states due to stresses that have not been well-characterized. Thus, biochemical test results can be inconsistent and lead to incorrect identification.

The genetic identification of cultured microorganisms generates data that can be compared with databases that contain rapidly increasing numbers of nonculturable and uncultured-culturable microorganisms. This tool allows researchers identify bacterial communities rather than only the culturable isolates. However, a molecular identification is only as good as the database used to make that identification. When studying environmental bacteria, it is common to get a perfect 16S match to "Uncultured Clone," or a perfect match to two or more species in different genera. Additionally, there is no guarantee that the matching sequences do not contain sequencing errors. A combination of culture and molecular techniques can help to overcome the challenges of environmental bacteria. Here, I used culture and molecular-based techniques to identify the bacterial symbionts of *P. arrhizus*.

2.2 Bacterial Culturing: Methods and Materials

2.2.1 *Pisolithus* samples

Pisolithus sample collection was done at two sites in and adjacent to the Norris Geyser Basin at Yellowstone National Park during late summer. The sampling site in NGB was on the forest's edge in the easternmost corner of the thermal area; this site will be referred to as Norris Proper (NP). Sample collection was also done along the perimeter of a smaller thermal area residing a hundred meters east of NP; this site will be referred to as Norris Annex (NA). Samples were collected and immediately stored on dry ice. Samples had to be transported to from NGB to Lake Lodge, the location of the field lab and lodging (Figure 2) where they were stored on dry ice until transported to

California State University Easy Bay and/or NASA's Ames Research Center where they were stored in the freezer (-20 and -80°C), approximately 2-4 days.

2.2.2. Culture Media

Tryptic soy broth (TSB) and tryptic soy agar (TSA) (Difco, Inc., St. Louis, MO) were the primary types of media used for both aerobic and anaerobic culturing of chemoheterotrophic bacteria. Preparation of TSB and TSA differed depending on its intended use. TSB and TSA for aerobic culturing was prepared with no special considerations.

For anaerobic culturing, the stock media were in an Erlenmeyer flask that was directly transferred from the autoclave to the vacuum port of the anaerobic chamber (Bactron Anaerobic/Environmental Chamber by Sheldon Manufacturing, Cornelius, OR) before it had a chance to cool. A manual purge cycle of the vacuum port, rather than an auto-cycle, was used to prevent the media from boiling over with the loss of atmospheric pressure. Once the media were in the anaerobic chamber it was either poured into Petri dishes or left to cool depending upon if it was TSA or TSB, respectively.

Dehydrated *Pisolithus* powder (DPP) was used to supplement TSB and TSA, creating *Pisolithus* tryptic soy broth (PTSB) and *Pisolithus* tryptic soy agar (PTSA), respectively. DPP was also used as the sole nutrient in a *Pisolithus* Extract culture medium, both broth (PisoEB) and agar plates (PisoEA). To make DPP, *Pisolithus* was put in a drying oven at 46°C until at a constant weight, drying time ranged from a few hours to a few days depending upon if the sample was a fragment or whole fruiting body. Once completely dehydrated, the sample was ground with a mortar and pestle and passed

through a #30 sieve. DPP was added pinch by pinch to media at a concentration of 1 g/L with a magnetic stir bar on a hot plate. A three-speed cycling of the stir bar was used to get the DPP into solution: medium spinning where the vortex did not touch the bottom of the flask, fast spinning where the vortex did reach the bottom of the flask, and no spinning to allow the floating DPP to disperse. All DPP containing media were autoclaved prior to culturing.

Pre-Reduced Anaerobically Sterilized (PRAS) media plates were ordered from Anaerobe Systems (Morgan Hill, CA). PRAS combination package AS-424 was used to culture anaerobes in the field. It contained: Brucella Blood Agar Mono Plate (BRU), Phenylethyl Alcohol Blood Agar Mono Plate (PEA), Laked Blood Agar Mono Plate (LBA), and Bacteroides Bile Esculin Agar (BBE)/ Brucella Laked Blood Agar with Kanamycin Vancomycin (LKV) Biplate.

2.2.3 Culture Methods

Both fresh and frozen (-20°C and -80°C) *Pisolithus* samples were cut with a flame sterilized single-edged razor blade. A thin, 0.5-1 cm, wedge was sectioned from fresh samples and used for inoculation (Figure 7).

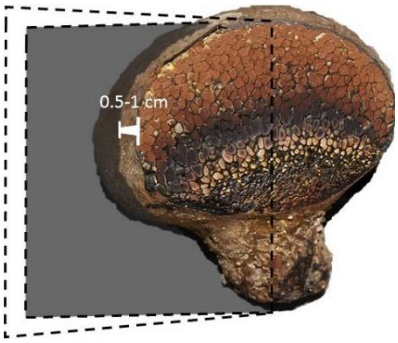


Figure 7: Fresh Pisolithus Section

Small pieces or shavings were cut or scraped off frozen samples when only a portion of the fruiting body was to be used in order to preserve the integrity of the sample. Tissue and bacteria from the outer peridium was included. Frozen samples that were to be completely consumed were allowed to defrost so a wedge could be sectioned. Due to the variability of internal microenvironments, a wedge was the only way to use a portion of

the fruiting body and be certain all possible microbial species would be transferred to the culture media.

Aerobic and anaerobic cultures were started by inoculating *Pisolithus* extract broth, PTSA, and PTSB. PRAS was also inoculated for anaerobic culturing. PTSB and PisoEB were inoculated and vortexed prior to incubation. Plates were streaked directly with a *Pisolithus* wedge as well as with inoculated and vortexed PisoEB prior to incubation. After two days of incubation, liquid cultures were transferred to plates for isolation and identification. Numerous subcultures were required to achieve pure cultures. All isolates were Gram stained.

2.2.4 Gram-staining

Gram stains were used to identify cellular morphologies and arrangements in an attempt to obtain pure cultures in preparation for DNA sequencing. Aerobic culture emulsions were air dried and heat fixed over a Bunsen burner. Anaerobic culture emulsions were air dried and methanol fixed in the anaerobic chamber (Bactron Anaerobic/Environmental Chamber by Sheldon Manufacturing, Cornelius, OR). All slides were subject to standard Gram staining procedure, viewed under 100x using immersion oil, and cellular morphology and arrangement were recorded.

2.2.5 DNA Purification

DNA from pure, 24 hr old cultures, was purified using a Qiagen DNeasy Blood & Tissue Kit. The procedure for Gram positive species was detailed on page 45 of the DNeasy Blood & Tissue Handbook (July 2006) Protocol: Pretreatment for Gram-Positive

Bacteria. The procedure for Gram-negative species was detailed on page 44 of the DNeasy Blood & Tissue Handbook (July 2006) Protocol: Pretreatment for Gram Negative Bacteria.

2.2.6 PCR

PCR was done on the 16S rRNA gene for all isolates using the 27F and 1492R primers and Platinum PCR SuperMix or Platinum Blue PCR SuperMix (Invitrogen; Carlsbad, CA). Thermocycling was performed on a ProFlex 3x32-well PCR System (Applied Biosystems; Foster City, CA). See appendix A for primer sequences, reaction mix, and thermocycling parameters.

2.2.7 Sequencing

Sanger sequencing (Sanger et al., 1977) was done out-of-house by Elim Biopharmaceuticals (Hayward, CA). Samples were prepared to contain 30-50 ng PCR product and 8 pmol B27F or 1492R primer in 15 µL total volume.

2.2.8. Sequence Clean-up

16S sequences were cleaned up using Chromas lite (Technelysium, South Brisbane, Australia). After the software-based automatic removal of low-quality sequence data, sequence ends were manually trimmed to further increase sequence quality.

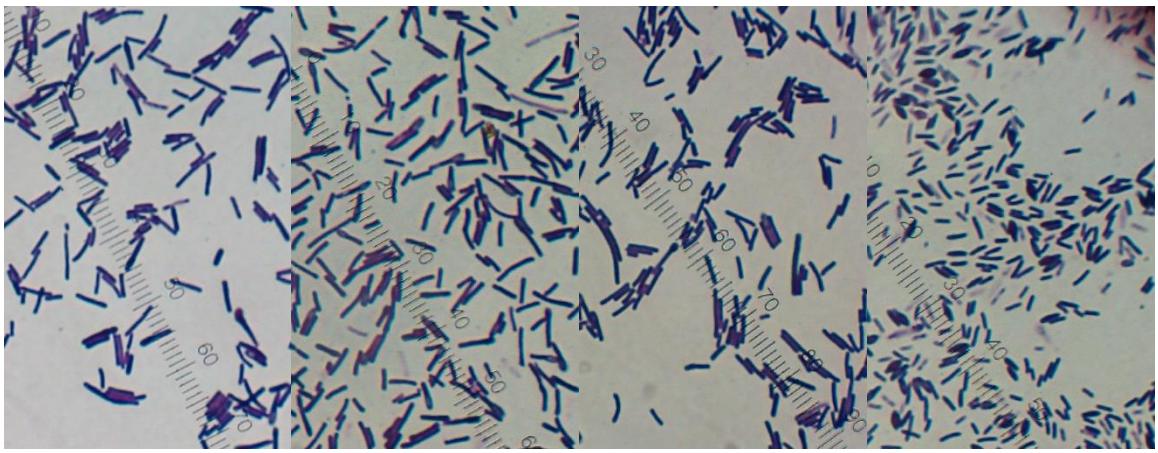
2.2.9. NCBI BLAST

Sequences were analyzed using NCBI Standard Nucleotide BLAST using the nucleotide collection database for highly similar sequences (megablast). Two metrics were used for species identification: best match as a function of score, and most hits. Results that were identified as "uncultured" were ignored, with the exception of one sequence that did not have any known hits.

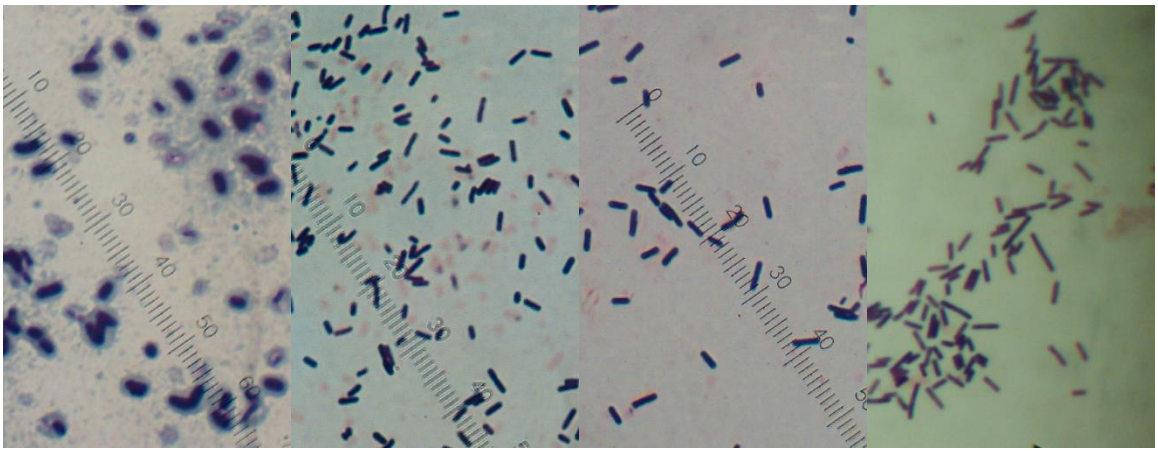
2.3. Bacterial Culturing: Results

2.3.1 Gram Stain

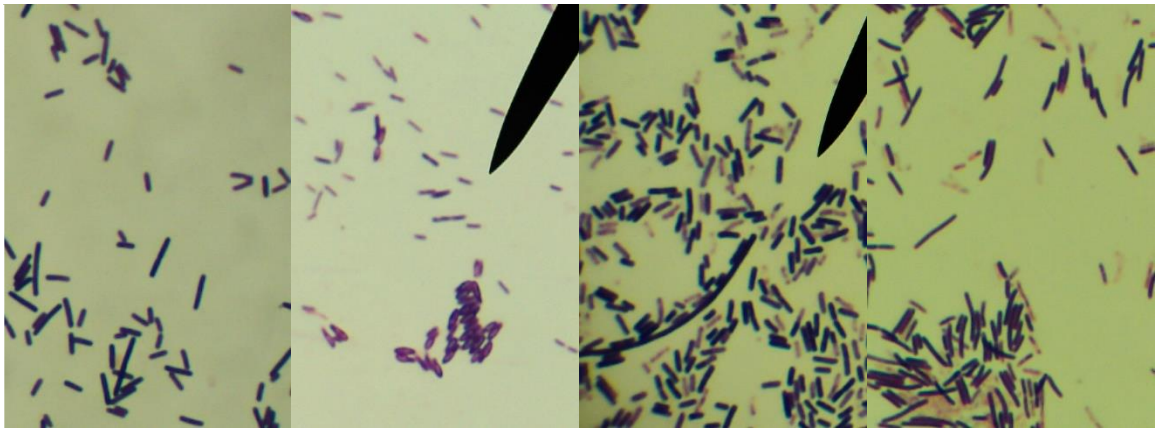
Every unique colony morphology was subcultured and Gram stained until a pure culture was obtained. Cultures were sorted by colony morphology, cellular morphology, and Gram reaction. Figure 8 shows a series of Gram stain images identified by sample code and genus. Genus identification is from 16S gene sequencing (section 2.3.2), the last picture in Figure 8 shows agarose gel electrophoresis of the 16S PCR product.



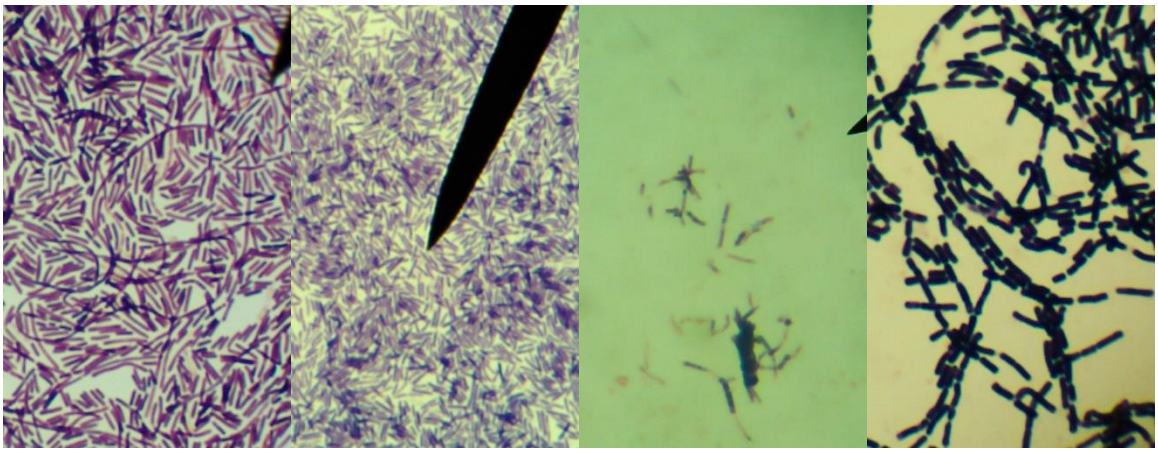
AC1A - *Geobacillus* AC1B - *Geobacillus* AC3A - *Geobacillus* AC3B – *Brevibacillus*



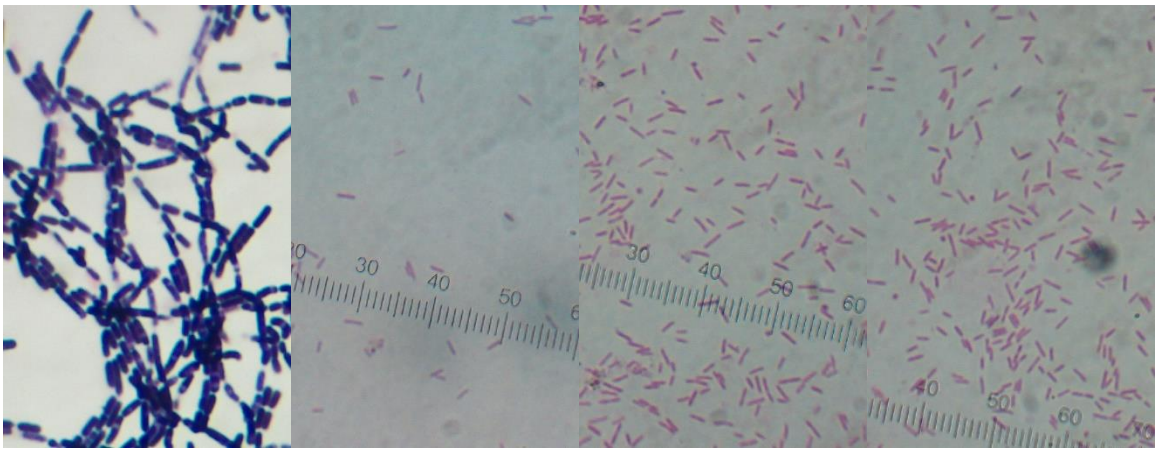
AI4A – *Brevibacterium* AI5A – *Bacillus* AI6A – Bacillus BX-A1A – *Bacillus*



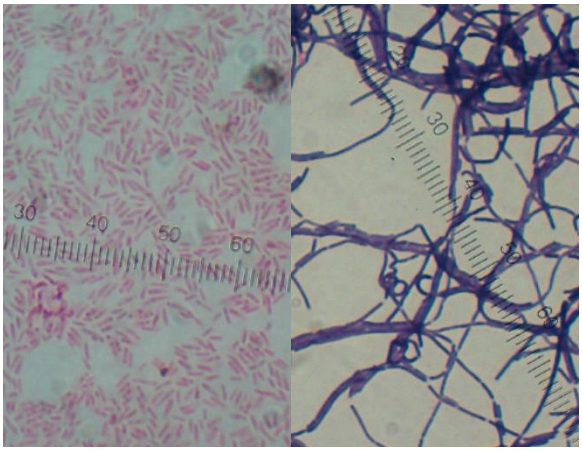
BX-A1B – *Bacillus* BX-A2 –*Bacillus* BX-A3 – *Paenibacillus* BX-B1 – *Paenibacillus*

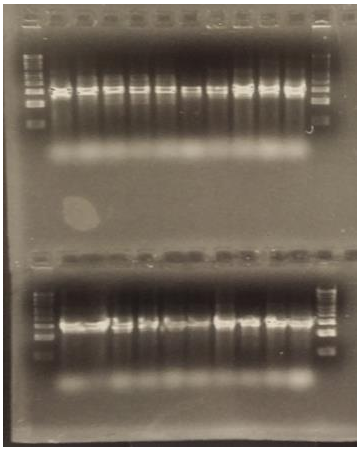


BX-C2 – *Bacillus* BX-F1A – *Paenibacillus* BX-K1 – *Nocardia* BX-N1 – *Bacillus*



BX-P1 - *Bacillus* NP3-Bru-2 – *Serratia* NP3-BRU-3 – *Clostridium* NP3-BRU-7 – *Desulfotomaculum*





NP3-PEA-1 – *Desulfotomaculum* V3D – Unknown 16S PCR Products on Agarose Gel

Figure 8: Gram Stains and 16S PCR Products on Agarose Gel

2.3.2 16S Identification of Cultured Bacterial Isolates

Table 2 below contains 16S species identification data for different bacterial isolates cultured in this study. Each isolate has two identifications: best match and most hits. The use of multiple identification strategies was necessary to increase confidence in the species ID because there are numerous examples where the partial 16S sequence resulted in a 100% match to multiple species or genera. While the "best match" is preferable as an identification method, the use of "most hits" in the NCBI database

provides the necessary supplemental information to know whether there were multiple perfect matches. Additionally, it gives insight into what species was more common. With multiple isolates being perfect match to multiple species or genera it was necessary to provide additional information. For the purpose of discussing the results, the "best match" will be used.

Table 2: Species Identification from 16S rRNA gene Sequences

Sample Code	Primer	NCBI BLAST: Species ID Best Match (Number of Hits) Score [a]	Identities for Best Match	NCBI BLAST: Species ID Most Hits (Number of Hits) Score [b]	Identities for Most Hits	Culture Environment
AC1A	B27F	*Geobacillus toebii (39) 1467*	794/794 100%	*Geobacillus toebii (39) 1467*	794/794 100%	Aerobic
AC1B	B27F	*Geobacillus toebii (39) 1471*	795/796 99%	*Geobacillus toebii (39) 1471*	795/796 99%	Aerobic
AC3A	B27F	*Geobacillus toebii (39) 1463*	792/792 100%	*Geobacillus toebii (39) 1463*	792/792 100%	Aerobic
AC3B	B27F	*Brevibacillus borstelensis (65) 1735*	939/939 100%	*Brevibacillus borstelensis (65) 1735*	939/939 100%	Aerobic
AI4A	B27F	*Brevibacterium halotolerans (6) 1567*	848/848 100%	*Bacillus mojavensis (24) 1567*	848/848 100%	Aerobic
AI5A	B27F	*Brevibacterium halotolerans (11) 1718*	930/930 100%	*Bacillus subtilis (26) 1718*	930/930 100%	Aerobic
AI6A	B27F	*Bacillus subtilis* (21) 1825	991/992 99%	*Bacillus mojavensis (27) 1825*	991/992 99%	Aerobic

Sample Code	Primer	NCBI BLAST: Species ID Best Match (Number of Hits) Score [a]	Identities for Best Match	NCBI BLAST: Species ID Most Hits (Number of Hits) Score [b]	Identities for Most Hits	Culture Environment
BX-A1A-2B	B27F	*Bacillus foraminis* (1) 1533	830/830 100%	*Bacillus horneckiae* (14) 1515	827/830 99%	Aerobic
BX-A1A-2B	1492R	*Bacillus foraminis* (1) 1557	843/843 100%	*Bacillus kochii* (25) 1535	839/843 99%	Aerobic
BX-A1A-3	B27F	*Bacillus foraminis* (3) 1533	830/830 100%	*Bacillus horneckiae* (14) 1515	827/830 99%	Aerobic
BX-A1A-3	1492R	*Bacillus foraminis* (1) 1430	774/774 100%	*Bacillus kochii* (24) 1408	770/774 99%	Aerobic
BX-A1B-2B	B27F	*Bacillus foraminis* (3) 1476	799/799 100%	*Bacillus horneckiae* (14) 1458	796/799 99%	Aerobic
BX-A1B-2B	1492R	*Bacillus foraminis* (1) 1520	823/823 100%	*Bacillus kochii* (29) 1498	819/823 99%	Aerobic
BX-A1B-4	B27F	*Bacillus foraminis* (3) 1472	797/797 100%	*Bacillus horneckiae* (14) 1454	794/797 99%	Aerobic
BX-A1B-4	1492R	*Bacillus foraminis* (1) 1478	800/800 100%	*Bacillus kochii* (23) 1456	796/800 99%	Aerobic
BX-A2-2B	1492R	*Paenibacillus chibensis* (7) 1417	775/779 99%	*Paenibacillus lautus* (27) 1369	766/779 98%	Aerobic
BX-A2-2E	B27F	*Paenibacillus chibensis* (9) 1066	620/641 97%	*Paenibacillus chibensis* (9) 1066	620/641 97%	Aerobic
BX-A2-2E	1492R	*Paenibacillus chibensis* (7) 1544	849/857 99%	*Paenibacillus lautus* (23) 1489	840/857 98%	Aerobic

Sample Code	Primer	NCBI BLAST: Species ID Best Match (Number of Hits) Score [a]	Identities for Best Match	NCBI BLAST: Species ID Most Hits (Number of Hits) Score [b]	Identities for Most Hits	Culture Environment
BX-A2C-2B	B27F	*Bacillus foraminis* (3) 1539	833/833 100%	*Bacillus horneckiae* (14) 1520	830/833 99%	Aerobic
BX-A2C-2B	1492R	*Bacillus foraminis* (1) 1517	821/821 100%	*Bacillus kochii* (29) 1495	817/821 100%	Aerobic
BX-A3-4	B27F	*Paenibacillus pabuli (3) 1389*	759/762 99%	*Paenibacillus tundra (9) 1387*	758/761 99%	Aerobic
BX-A3-4	1492R	*Paenibacillus taichungensis (17) 1037*	631/666 95%	*Paenibacillus pabuli (25) 1031*	630/666 95%	Aerobic
BX-B1-2B02	B27F	*Paenibacillus chibensis* (9) 1079	621/639 97%	*Paenibacillus chibensis* (9) 1079	621/639 97%	Aerobic
BX-B1-2B02	1492R	*Paenibacillus chibensis* (8) 590	321/322 99%	*Paenibacillus cookii* (9) 571	317/322 98%	Aerobic
BX-C2-3	B27F	*Bacillus sporo-thermodurans* (11) 1062	601/614 98%	*Bacillus oleronius* (39) 1035	597/615 97%	Aerobic
BX-C2-3	1492R	*Bacillus sporo-thermodurans* (14) 1574	863/868 99%	*Bacillus oleronius* (33) 1496	849/868 98%	Aerobic
BX-C2A-2A	B27F	*Paenibacillus chibensis (9) 1146*	633/640 99%	*Paenibacillus chibensis (9) 1146*	633/640 99%	Aerobic
BX-C2A-2A	1492R	*Paenibacillus chibensis (7) 1524*	835/840 99%	*Paenibacillus lautus (29) 1471*	825/840 98%	Aerobic
BX-C2B	B27F	*Paenibacillus chibensis (9) 1146*	635/642 99%	*Paenibacillus chibensis (9) 1146*	635/642 99%	Aerobic
BX-C2B	1492R	*Paenibacillus chibensis (7) 1515*	829/833 99%	*Paenibacillus lautus (26) 1469*	822/836 98%	Aerobic

Sample Code	Primer	NCBI BLAST: Species ID Best Match (Number of Hits) Score [a]	Identities for Best Match	NCBI BLAST: Species ID Most Hits (Number of Hits) Score [b]	Identities for Most Hits	Culture Environment
BX-F1A-3	B27F	*Paenibacillus lautus (41) 1574*	737/737 100%	*Paenibacillus lautus (41) 1574*	737/737 100%	Aerobic
BX-F1A-3	1492R	*Paenibacillus lautus (51) 1574*	854/854 99%	*Paenibacillus lautus (51) 1574*	854/854 99%	Aerobic
BX-J1-2A	B27F	*Bacillus niacin (7) 1267*	710/721 98%	*Bacillus drentensis (8) 1256*	708/721 98%	Aerobic
BX-J1-2A	1492R	*Bacillus drentensis (8) 1546*	841/843 99%	*Bacillus niacin (10) 1541*	840/843 99%	Aerobic
BX-J1-2B	B27F	*Paenibacillus tundra (9) 1395*	759/761 99%	*Paenibacillus pabuli (9) 1395*	760/762 99%	Aerobic
BX-J1-2B	1492R	*Paenibacillus taichungensis (17) 1085*	639/665 96%	*Paenibacillus pabuli (25) 1079*	638/665 96%	Aerobic
BX-K1-2A	B27F	*Nocardia casuarinae (2) 845*	520/552 94%	*Nocardia veterana (22) 837*	519/552 94%	Aerobic
BX-K1-2A	1492R	*Nocardia aobensis (8) 1502*	845/860 98%	*Nocardia farcinica (18) 1452*	834/858 97%	Aerobic
BX-N1-4	1492R	*Bacillus cereus (68) 1482*	802/802 100%	*Bacillus thuringiensis (13) 1482*	802/802 100%	Aerobic
BX-N1-4	B27F	*Bacillus thuringiensis (11) 1633*	889/891 99%	*Bacillus cereus (75) 1633*	888/890 99%	Aerobic
BX-P1-4	B27F	*Bacillus cereus (68) 1539*	833/833 100%	*Bacillus thuringiensis (13) 1539*	833/833 100%	Aerobic
BX-P1-4	1492R	*Bacillus thuringiensis (12) 1580*	857/858 99%	*Bacillus cereus (67) 1580*	858/859 99%	Aerobic

Sample Code	Primer	NCBI BLAST: Species ID Best Match (Number of Hits) Score [a]	Identities for Best Match	NCBI BLAST: Species ID Most Hits (Number of Hits) Score [b]	Identities for Most Hits	Culture Environment
NP3-BRU-1	B27F	*Clostridium subterminale (13) 1637*	892/895 99%	*Clostridium subterminale (13) 1637*	892/895 99%	Anaerobic
NP3-BRU-2	B27F	*Serratia marcescens* (42) 1648	892/892 100%	*Serratia marcescens* (42) 1648	892/892 100%	Anaerobic
NP3-BRU-3	B27F	*Clostridium celerecrescens* (9) 1729	946/950 99%	*Desulfotomaculum sp.* (10) 1740	947/949 99%	Anaerobic
NP3-BRU-3PP	B27F	*Desulfotomaculum guttoideum* (8) 1668	905/906 99%	*Clostridium celerecrescens* (11) 1664	906/908 99%	Anaerobic
NP3-BRU-4	B27F	*Clostridium subterminale (7) 1277*	700/704 99%	*Clostridium subterminale (7) 1277*	700/704 99%	Anaerobic
NP3-BRU-5	B27F	*Clostridium celerecrescens* (11) 1362	737/737 100%	*Clostridium celerecrescens* (11) 1362	737/737 100%	Anaerobic
NP3-BRU-6	B27F	*Clostridium subterminale (8) 1363*	749/754 99%	*Clostridium subterminale (8) 1363*	749/754 99%	Anaerobic
NP3-BRU-7	B27F	*Desulfotomaculum guttoideum* (8) 1321	715/715 100%	*Clostridium celerecrescens* (11) 1321	715/715 100%	Anaerobic
NP3-PEA-1	B27F	*Desulfotomaculum guttoideum* (6) 1687	917/919 99%	*Clostridium celerecrescens* (10) 1687	918/920 99%	Anaerobic
V3D	B27F	*Uncultured Clone (135) 1404*	772/777 99%	*Uncultured Clone (135) 1404*	772/777 99%	Aerobic

[a] – Information in the first gray block represents the "Best Match" from NCBI using megablast
[b] – Information in the second white block represents the "Most Hits" from NCBI using megablast
See Appendix B for 16S gene sequences

Of the 35 isolates sequenced, 17 were sequenced with the forward primer only and 18 were sequenced with both the forward and reverse primers. Only 1 of the 18 isolates that were sequenced with forward and reverse primers gave a conflicting "best match" identification and had enough overlapping sequence to merge the sequences into a more complete 16S gene sequence, BX-J1-2A. Additional primer design and sequencing could have provided complete gene sequences for better identification of all isolates. This was not performed for several reasons: genus level identification is adequate for assessing the possible functional roles of bacterial symbionts, even with more complete 16S gene sequences it is probable that the "best match" would be to uncultured members of the same genus, and this was the most cost effective method of sequencing.

A total of 9 known genera (*Bacillus, Brevibacillus, Brevibacterium, Clostridium, Desulfotomaculum, Geobacillus, Nocardia, Paenibacillus,* and *Serratia*) and 1 unknown isolate was cultured and identified. The majority, 21 of 35 isolates, fell into two families in the order *Bacillales: Bacillaceae (Bacillus* – 10 isolates and *Geobacillus* – 3 isolates) and *Paenibacillaceae (Brevibacillus* – 1 isolate and *Paenibacillus* – 7 isolates). These taxa are aerobic (or facultatively anaerobic), rod-shaped, endospore forming, Gram-positive organisms, which were traditionally classified as *Bacillus* spp. (Shida et. al., 1997). The phylogenetic movement starting in the 1990s sparked a reorganization of the *Bacillus* group into many new genera including: Amphibacillus, *Alicyclobacillus, Aneurinibacillus, Bacillus, Brevibacillus, Gracilibacillus, Geobacillus, Filobacillus, Jeotgalibacillus, Marinibacillus, Paenibacillus, Salibacillus, Virgibacillus, Ureibacillus* (Xu & Cote, 2003). While these new genera were separated on the basis of genetic

differences, a partial (or complete) 16S rRNA gene sequence is often not sufficient to assign taxonomic identity at the species level for an unknown isolate, as Table 2 demonstrates.

Bacillus, Geobacillus, Brevibacillus, and *Paenibacillus* are all are aerobic or facultatively anaerobic (Goto et al., 2000; Zeigler, 2014; Panda et al., 2014; and Shida et al., 1997). All isolates from these genera were obligate aerobes in this study. Members of these genera are all found in soil. *Bacillus* is the most commonly cultured genus of this group and is considered ubiquitous (Goto et al, 2000).

In a review by Zeigler (2014), *Geobacillus* spp. were described as obligate thermophiles, 35-80 °C, yet have been isolated from nearly all reaches of Earth including all 7 continents, high altitude soils, cold deep sea sediments, marine and terrestrial hot springs, cools soils, and hot compost. Despite *Geobacillus'* inability to grow in most of the environments, it is unprecedently common. *Geobacillus* is known to utilize plant matter and hydrocarbons. Poli et al. (2006) isolated a strain of *G. toebii* that was able to degrade hydrocarbons in the presences of numerous heavy metals. In this study *G. toebii* was isolated from *Pisolithus* which contains copious hydrocarbons and heavy metals (data not shown).

Brevibacillus spp. are Gram-positive, but can also be Gram-variable (Panda et al., 2014). Member of this genus have been found in rocks, dust, aquatic environments, as well as the guts of insects and mammals. Panda et al. also report *B. borstelensis*, isolated from *Pisolithus* in this study, having a thermostable peptidase.

Paenibacillus spp. are known to be more fastidious in their growth habits than *Bacillus* spp. (Gardener, 2004), a contributing factor to *Bacillus* being cultured more

frequently. Like B*acillus*, *Paenibacillus* are common soil bacteria, however some *Paenibacillus* spp. have been reported to fix nitrogen (Coelho et al., 2003). None of the species identified in this study are known to be nitrogen fixers, but they have been studied less than the *Paenibacillus* spp. typically associated with agricultural rhizospheres. *Paenibacillus* spp. are typically characterized biochemically by the extracellular enzymes that hydrolyze alginate, chondroitin, chitin, curdlan, and others (Shida et al., 1997).

Two isolates from the genus *Brevibacterium* may have been cultured, but these sequences also had a perfect match to multiple *Bacillus* spp., 848/848 and 930/930 identities respectively. This further demonstrates the challenges of identifying environmental samples and the limitations of our databases. Environmental samples frequently have limited to no published data describing them because research funding is centered around clinical and agricultural applications. *Brevibacterium* are pleomorphic (Alves et al., 2002) Gram-positive bacteria that lack mycolic acid, but contain *meso*-diaminopimelic acid in the cell wall (Cai & Collins, 1994). Members of this genus are obligate aerobes that live in high salt environments (Collins, 2006). *Brevibacterium* have been isolated from skin, dairy, marine fish, and sea water.

Eight of the 35 isolates are from the order *Clostridiales*. Of the 8 bacteria in this group, 5 have a "best match" to *Clostridium*, with the other 3 identifying as *Desulfotomaculum*. The *Desulfotomaculum* isolates could be *Clostridium* with identities being: 905/906 vs 906/908, 715/715 vs 715/715, and 917/919 vs 918/920 for NP3-BRU-3PP, NP3-BRU-7, and NP3-PEA-1, respectively. All isolates were obligate anaerobes.

Clostridium spp. are pleomorphic Gram-positive rods and cocci that decolorize easily and appear Gram-negative (Public Health England, 2015). Aerotolerance can vary between obligate anaerobic to aerotolerant anaerobic. Some members of *Clostridium* produce endospores while others do not, the isolates cultured in this study produced spores. *Clostridium* spp. are saccharolytic and proteolytic, and usually catalase negative. Tagu and Martin (1995) putatively identified a sphingomyelinase from *Eucalyptus globulus-Pisolithus tinctorius* (=*P. arrhizus*) ectomycorrhiza from *Clostridium*.

Desulfotomaculum spp. are sulfur-reducing bacteria capable of forming heat-resistant endospores. These *Clostridia*-like bacteria are most abundant in dry and oxic conditions, or conditions that shift from oxic to anoxic (Widdel, 2006). In rice paddy studies, *Desulfotomaculum* is one of the most abundant bulk soil associated sulfur-reducers, as compared to plant associated sulfur-reducers (Stubner & Meuser, 2000). This distribution in bulk soil versus rhizosphere is likely attributed to *Desulfotomaculum*'s broad nutritional range compared to non-spore-forming sulfur-reducing bacteria. *Desulfotomaculum* can us H_2, alcohols, fatty acids, other aliphatic monocarboxylic or dicarboxylic acids, alanine, hexoses, or phenyl-substituted organic acids as electron donors (Widdel, 2006).

A single *Norcardia* isolate was cultured. Members of *Nocardia* are non-motile, rod to coccoid shaped, obligately aerobic *Actinomycetes* that are most closely related to the genus *Rhodococcus.* *Nocardia* may also be characterized by *meso*-diaminopimelic acid, arabinose and galactose in the cell wall (Chun & Goodfellow, 1995). Their mycolic acids contain 46-60 carbon atoms (Roth et al., 2003). The principal phospholipids are diphosphatidylglycerol, phosphatidylethanol amine, phosphatidylinositol, and

phosphatidylinositol mannosides (Chun & Goodfellow, 1995). *Nocardia* is most commonly associated with soil and animal/human infection (Gordon & Mihm, 1962).

Serratia are rod shaped, Gram-negative, facultative anaerobes from the family *Enterobacteraceae*. They are widely distributed and are commonly isolated from mammals, plants, soil, water, and hospitals. Some *Serratia* spp. produce a non-diffusible red pigment called prodigiosin (Grimont & Grimont, 2006). The strain of *Serratia marcescens* isolated in this study did not produce prodigiosin. Prodigiosin-positive strains are toxic to protozoa and more common in healthy environments. *Serratia marcescens* is believed to play a role in mineralizing organic iron, as well as dissolving gold and copper. *Serratia marcescens* is known to produce five enzymes with chitinolytic activity. They also produce extracellular gelatinase, lecithinase, and DNase (Grimont & Grimont, 2006).

In this study, species identification was not always possible, but did not impede the identification of the bacterial symbionts. Genus level identification is sufficient to characterize the "players" in the system and provide evidence to support preliminary hypotheses describing the system as a whole. To obtain an accurate species identification using molecular techniques, whole genome sequencing (or sequencing at multiple loci) should be employed. Beyond species identification, whole genome sequencing would provide a means to understand this system from a genomics perspective. This genetic data could provide insight into the genes present in this system.

In the case of this research where all the bacterial species sequenced were cultured, knowing the genetic potential of the system would allow the researcher to test the function of that potential. Whole genome sequencing was not a viable option for

every isolate cultured, so a different approach using enzyme assays was taken to further characterize *P. arrhizus* and its bacterial symbionts.

Chapter 3: Enzymatic Analysis

3.1. Enzymatic Analysis: Introduction

3.1.1. Enzyme Assays

To begin to characterize biochemical activity within the *P. arrhizus* fruiting bodies, I conducted tests for a variety of enzymatic activities. The origin of the enzymatic activity may be bacterial or fungal. I hope to gain insight into what is going on metabolically within the fruiting bodies when existing under extreme conditions. Because the energy source for *P. arrhizus* in NGB, YNP is unknown, a "shot-in-the-dark" approach to enzyme assay selection is used in this research.

Fifteen different enzymes representing an array of metabolic processes were assayed. Acetate kinase phosphorylates acetate for the production of acetyl-CoA, an essential intermediate in many metabolic pathways (Ingram-Smith et al., 2006). Adenosine 5'-Triphosphatase hydrolyzes ATP to ADP, releasing energy that is used to catalyze other reactions in the cell (de Meis, L., 2001). ATP sulfurylase produces ATP and sulfate from APS and PPi (Kramer & Cypionka, 1989). Aldolase is an important enzyme in sugar metabolism, reversibly converting fructose-1,6-biphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Siebers & Schonheit, 2005). Cytochrome p450 reductase transfers electrons from NADPH to cytochrome p450 (Varadarajan et al., 2010). Glucose-6-phosphate dehydrogenase catalyzes the first step of the pentose phosphate pathways, creating NADPH (Cappellini & Fiorelli, 2008). Glyceraldehyde-3-phosphate dehydrogenase is an essential enzyme in glycolysis (Sirover, 1999). Malate synthase is an essential part of the glyoxylate cycle (Kunze et al., 2002). NADPH-FMN oxidoreductase uses NADPH and FMN as substrates to make

reduced flavin mononucleotide, $FMNH_2$ (Jablonski & DeLuca, 1978). Polyphenol oxidase is oxidizes phenols to quinones (Bravo & Osorio, 2016). Pyruvate decarboxylase catalyzes the decarboxylation of pyruvic acid producing acetaldehyde and carbon dioxide (Gounaris et al., 1971). Pyruvate dehydrogenase is the first enzyme in the pyruvate dehydrogenase complex, which ultimately produces acetyl-CoA (Wieland, 1983). Rubisco is the initial CO_2 fixing enzyme in photosynthesis (Spreitzer & Salvucci, 2002). Sulfite oxidase catalyzes the oxidation of sulfite to sulfate, essential for the oxidative degradation of cysteine and methionine (Kisker et al., 1997).

This chapter will discuss the challenges of working with *P. arrhizus* for enzyme assays, but will focus on one enzyme, polyphenol oxidase.

3.1.2. Polyphenol Oxidases

Polyphenol oxidase (PPO) is a ubiquitous copper-containing oxidoreductase found in most species including bacteria, plants, animals, and fungi (Flurkey & Inlow, 2008; Mayer, A. M. 2006). Molecular oxygen is used by some PPOs to catalyze two reactions: *ortho*-hydroxylation of monophenols to *o*-diphenols (cresolase/monophenolase activity; EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (catecholase/diphenolase activity; EC 1.10.3.1) (Figure 9) (Bravo & Osorio, 2016; Marusek et al., 2006). All enzymes that catalyze the latter reaction are classified as PPOs (Flurkey & Inlow, 2008). Tyrosinases (TYRs) possess both cresolase and catecholase (CO) activity. Self-polymerization of the *o*-quinone products results in the formation of high molecular weight black/brown pigments known as melanins (Marusek et al., 2006).

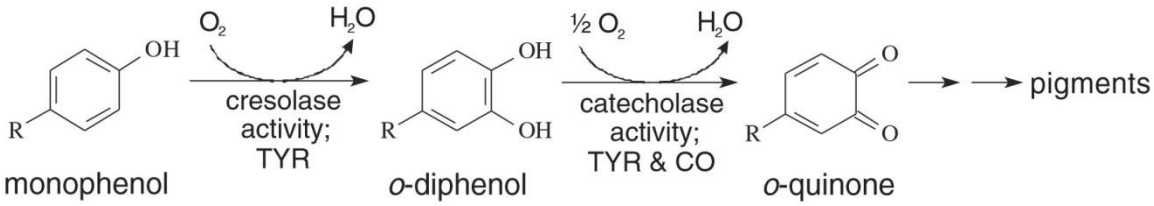


Figure 9: Polyphenol oxidase nomenclature and reaction (Image from Marusek et al., 2006)

These melanins are the cause of browning in produce, thus making PPOs of great economic concern (Marusek et al., 2006). Plants are believed to upregulate PPO in damaged tissue to create a melanin scab in response to invasion by insects and microorganisms (Flurkey & Inlow, 2008). Induction of fungal PPO has been researched significantly less, but PPO is still active in melanin development (Mayer, 2006). Beyond the UV protection that melanin provides (Jacobson, 2000), there is evidence that fungal melanins contribute in defense and resistance to stressors, increase pathogenicity, and function in the formation and stability of spores (Flurkey & Inlow, 2008). In the mushroom *Agariucus bisporus,* Soler-Rivas et al. (2001) found that TYR (PPO) activation caused a browning of the cap in response to infection with the bacterium *Pseudomonas tolaasii.* The same effect was observed when the mushroom was treated with the tolaasin toxin. A study by Cutler and Swatek (1969) with 29 *Basidiobolus* isolates showed that all 9 human isolates produced a black pigment after 48 hours at 30°C, while all the environmental isolates either produced a pink pigment or no pigment at all. While this example is dated, PPO is one of the longest studied enzymes; a review by Mayer (2006) notes that the first publication came out 120 years ago: Bertrand 1896. Earlier, Mayer and Harel (1979) reviewed a study where

mutants of *Penicillium* that lacked a functional PPO were infertile, but were able to recover their fertility with the addition of two PPOs from wild-type *Penicillium*.

PPOs are universal enzymes across diverse genera with various functions. In plants, PPOs are believed to be located in the chloroplasts. Fungal PPOs appear most regularly as a cytoplasmic enzyme; reviewed in (Mayer, 2006). However, the fungus *Amylomyces rouxii* has been shown to produce an extracellular TYR (Montiel et al., 2004). The extensively-studied *Basidiomycete* mushroom *Agaricus bisporus* possess cell wall-associated TYR; reviewed in (Rast et al., 2003). On a genetic level, *A. bisporus* possess six distinct PPO isoforms (Mauracher et al., 2014). In vivo, many PPOs are believed to operate as monomers, but some fungal PPOs have been shown to function as tetramers or other small oligomers; reviewed in (Halaoui et al., 2006).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) can characterize PPO subunits by molecular mass because SDS dissociates and unfolds oligomers and subunits, respectively (Flurkey & Inlow, 2008). Since the majority of PPOs, up to 95%, exist in a latent form (Van Gelder et al., 1997) PPO needs to be activated in order to perform enzymatic analysis. SDS is an anionic detergent that acts as a PPO activator. SDS-PAGE PPO activity stains are a useful tool to characterize PPO MW, isoforms, substrate specificity, and optimal conditions (Cheema & Sommerhalter, 2015).

Phenols are aromatic hydrocarbon rings with hydroxyl groups. Monophenols have one hydroxyl group, while diphenols have two adjacent hydroxyl groups. Catechol is the simplest diphenol, and 4-methylcatechol is a methylated variation of catechol. Chlorogenic acid is an intermediate in lignin biosynthesis (Boerjan et al., 2003). Tyrosine is a

monophenol variation of L-DOPA. These are the five phenolic compounds used in this research for the characterization of PPO from *P. arrhizus* (Figure 10).

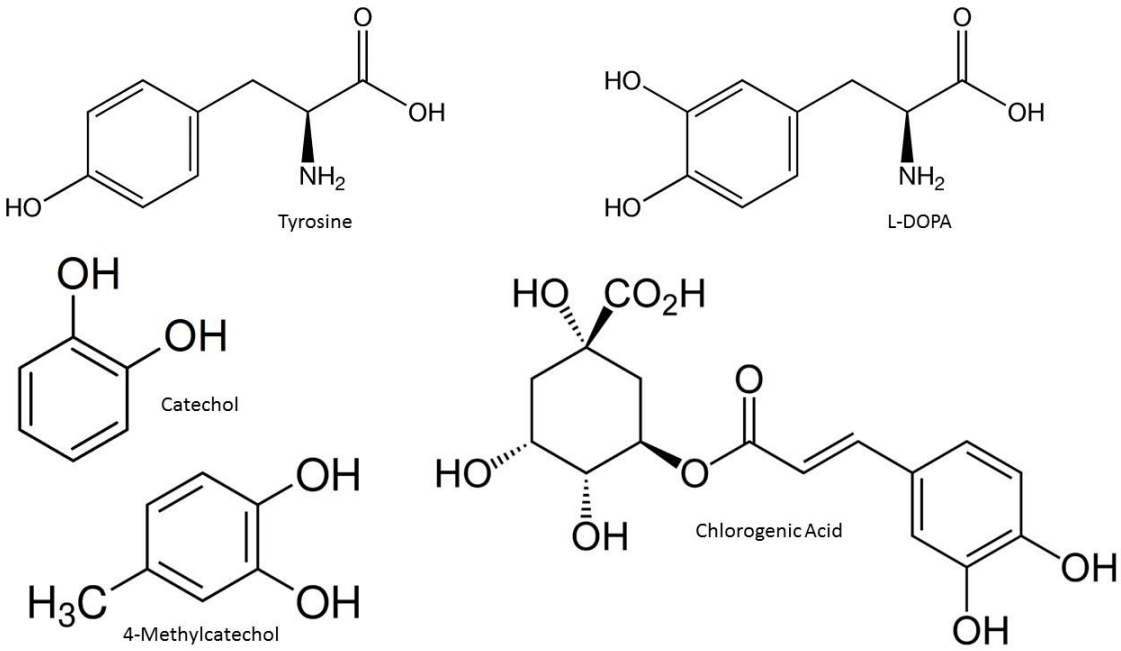


Figure 10 Phenolic Compounds Used in this Study

3.2. Enzymatic Analysis: Methods and Materials

3.2.1. Determination of *Pisolithus* "Self-Reactivity"

In order to determine if the *Pisolithus* crude extract (PCE) was stable enough to be used in qualitative and quantitative enzyme assays, a "self-reactivity" assay was conducted. Prior to preparation of the crude extract, the spectrophotometer was prepared for use and blanked with distilled H_2O. The PCE was prepared in a previously frozen (-20°C) mortar and pestle on a bed of ice at room temperature (RT). The extract

concentration was not quantitatively measured; a ~1 cm^3 piece of frozen *Pisolithus* was cut with a flame sterilized straight razor and immediately placed in the frozen mortar and crushed with the frozen pestle. The primary consideration was to keep the *Pisolithus* sample frozen throughout extract preparation to get a spectrophotometric reading of how the extract reacted when *Pisolithus*' cellular components were free to "self-react."

After initially grinding the *Pisolithus* in the frozen mortar and pestle, 1mL of distilled H_2O was added as a diluent, the mixture was ground again, and a 1.5 µL sample of PCE were added to the pedestal of the Nanodrop 2000/2000c (ThermoFisher Scientific, Waltham, MA). Absorbance measurements were recorded at the following wavelengths: 212, 265, 340, 412, 550, and 660 nm. These six wavelengths represent the detectable spectra for sixteen enzyme assays that were attempted for this research.

A total of 3 mL of distilled H_2O was added milliliter by milliliter with absorbance measurements following each addition. After 3 mL of distilled H_2O were added, 7-8 absorbance readings were taken with the sample on ice. The mortar was then removed from the bed of ice and stored at RT for an additional 10-20 minutes of absorbance measurements at all six wavelengths. The outside of the mortar was warmed for a few seconds with hands between some of the later readings to ensure PCE self-reactivity was measured at room temperature and above.

3.2.2. Enzyme Assays for Specific Metabolic Pathways

To characterize the metabolism of *Pisolithus* and its microbial symbionts, a total fifteen enzyme assays were selected from Sigma Aldrich: acetate kinase, adenosine 5'-triphosphatase (ATPase), adenosine 5'-triphosphate sulfurylase, aldolase, cytochorome

p450 reductase, glucose-6-phosphate dehydrogenase (G6PDH), glyceraldehyde-3-phosphate dehydrogenase, malate synthase, NAD-pyrophosphorylase, NADPH-FMN oxidoreductase, polyphenol oxidase (PPO), pyruvate decarboxylase, pyruvate dehydrogenase, D-rubulose-1,5 diphosphate decarboxylase, and sulfite oxidase (SO). See Appendix C for links to enzyme assay protocols and references. All sixteen enzyme assays were performed using PCE directly as enzyme. The amount of PCE varied depending on the volume of enzyme specified by the protocol, but PCE was added directly to the reaction mix at the specified volume. This specified volume is arbitrary because PCE contained an unknown assortment of enzymes in an unknown concentration with potentially unknown inhibitors.

Protocols that did not have a limiting reagent (i.e. any reagent that was too expensive to purchase large enough quantities for numerous runs) were assayed first. The four assays that did not have a limiting reagent were: acetate kinase, ATPase, PPO, and sulfite oxidase. These were attempted first to determine if assay sensitivities were high enough to detect the presence of enzymes in PCE. Unknown factors, such as where energy originated in the system, what molecules and pathways were involved in energy transfer, what enzymes were present and in what concentrations, what inhibitors were present, and were present enzymes of fungal or bacterial origin, made it difficult to predict if an enzyme assay should be expected to yield a positive or negative result. Because of these unknown factors, these four protocols had PCE and reagent concentrations modified, as well as additional control conditions added over numerous trials.

3.2.3. Glucose-6-Phosphate Dehydrogenase Spectrophotometric Assay

A continuous spectrophotometric rate determination assay for sulfite oxidase was used to determine if any G6PDH was present in *Pisolithus* samples. A 1.5 mL reaction cocktail without enzyme was prepared for each sample. Microcentrifuge tubes were incubated on a dry bath (model 2001FS, ThermoFisher, Scientific Waltham, MA) at 25°C. PCE was added to the reaction cocktail and A_{340nm} was recorded immediately using a Nanodrop 2000/2000c (ThermoFisher Scientific, Waltham, MA) for ~10 minutes.

This method was modified from a procedure published by Sigma Aldrich (Enzymatic Assay of Glucose-6-Phosphate Dehydrogenase (EC 1.1.1.49)) referencing "Noltmann, E.A., Gubler, C.J., & Kuby, S.A. (1961). *J. Biol. Chem.* 236, 1225-1230." See Appendix D for reaction cocktail and reagent composition.

3.2.4. Sulfite Oxidase Spectrophotometric Assay

A continuous spectrophotometric rate determination assay for sulfite oxidase was used to determine if any SO was present in *Pisolithus* samples. A 1.5 mL reaction cocktail without enzyme was prepared for each sample in microcentrifuge tubes and incubated at room temperature. Sample composition differed in each run and are detailed in each data set. The variables that were manipulated were sulfite concentration, cytochrome c presence, and observation length. PCE was added to the reaction cocktail and A_{550nm} was recorded immediately using a Nanodrop 2000/2000c (ThermoFisher Scientific, Waltham, MA). The ΔA_{550nm} was recorded for various time periods depending on the run, a typical length run was 30-60 minutes. Sulfate has a maximum

absorbance at 550 nm, thus an increase in A_{550nm} represents the production of sulfate and can be interpreted as a positive result for the presence of SO.

This method was modified from a procedure published by Sigma Aldrich (Enzymatic Assay of Sulfite Oxidase (EC 1.8.3.1)) referencing "MacLeod, R.M., Farkas, W., Fridovich, I. & Handler, P. (1961). *J. Biol. Chem*. 236, 1841." See Appendix E for reaction cocktail and reagent composition.

3.2.5. Polyphenol Oxidase Spectrophotometric Assay

A continuous spectrophotometric rate determination assay for polyphenol oxidase activity was used to determine if any PPO was present in *Pisolithus* samples. This method was used as a qualitative measure of PPO activity prior to the SDS-PAGE PPO activity stain.

A 1.5 mL reaction cocktail without enzyme was prepared for each sample. Microcentrifuge tubes were incubated on a dry bath (model 2001FS, ThermoFisher, Scientific Waltham, MA) at 30°C, and cuvettes were incubated at room temperature. Immediately before use, a 10% (w/v) PCE was made by grinding a small piece (~200mg) of *Pisolithus* with a mortar and pestle in 50 mM potassium phosphate buffer pH 7.5 at 30°C. A test, blank, and control conditions were used. The test contained substrate (10 mM chlorogenic acid) and PCE, the blank contained only substrate, and the control contained only *Pisolithus* extract. Spectrophotometric measurements were taken using a Nanodrop 2000/2000c (ThermoFisher Scientific, Waltham, MA). Measurements were taken using both the pedestal and the cuvette functions. When using the pedestal, a P2 micropipette was used to transfer 1.5 µL of reaction cocktail from the microcentrifuge

tube to the pedestal. A fresh 1.5 µL sample was used at each reading. The pedestal function was preferred for several reasons: microcentrifuge tubes were continuously incubated in the dry bath; a single batch of PCE could be used for test, blank, and control conditions; and test, blank, and control conditions were run simultaneously which eliminated extraneous variables such as time from extract preparation to spectrophotometric reading. Baseline correction at 750 nm was used to normalize data. Results were exported and graphed in Excel. The total ΔA_{265nm} over the duration of the experiment was calculated as:

(average last 5 data points) – (average of the first 5 data points) = total ΔA_{265nm}

This method was modified from a procedure published by Sigma Aldrich (Enzymatic Assay of Phenolase (EC 1.10.3.1)) referencing "Potato phenolases" (Patil et al., 1965). See Appendix F for reaction cocktail and reagent composition.

3.2.6 Statistical Comparison of Two Independent Slopes

A t-test comparing the slopes of two independent regression lines was used to determine the statistical significance of individual runs using Excel. The equations are detailed in Table 3 below.

Table 3: T-test to Compare the Slopes of Independent Regression Lines	
Analysis of Individual Runs	
Variable	Excel Formula
n	=COUNT(x)
b	=SLOPE(y,x)
s_{y-x}	=STEYX(y,x)
s_x	=STDEV(x)
s_b	$=s_{y-x}/(s_x*SQRT(n-1))$
Comparison of Two Slopes	
s_{b1-b2}	$=SQRT(s_{b1}\text{^}2+s_{b2}\text{^}2)$
t	$=(b_1-b_2)/(s_{b1-b2})$
df	$=(n_1+n_2-4)$
alpha	0.05
p-value	=TDIST(|t|,df,2)
t-crit	=TINV(α,df)
sig	=IF(p<α,"Yes","No")

3.2.7. Determination of PPO Units/mg *P. arrhizus*

Determining the units/mg enzyme was impossible because the PPO was assayed in a crude extract where the amount of enzyme was not quantified. Instead units/mg *P. arrhizus* was used as a measure of enzyme activity. The decrease in A_{265nm} was obtained using the maximum linear rate for both test and blank. Maximum linear rate is defined here as the greatest slope of a trendline that can be generated by five consecutive data points. The following equation was used for quantification of PPO activity.

$$\text{Units/mg } \textit{Pisolithus} = \frac{(\Delta A_{265nm}/\text{min Test} - \Delta A_{265nm}/\text{min Blank})}{(0.01)\ (\text{mg } \textit{Pisolithus}/\text{Reaction Mix})}$$

0.01 = The change in A_{265nm}/min per unit PPO at pH 7.5 at 30°C in a 1.5 mL reaction mix

Unit Definition: One unit will produce a ΔA_{265nm} of 0.01 per minute at pH 7.5 at 30°C, using cholorgenic acid as substrate in a 1.5 mL reaction mix.

This equation and unit definition were modified from "Enzymatic Assay of Phenolase (EC 1.10.3.1)" (Sigma Aldrich, St. Louis, MO). The original equation and unit definition calculated U/mg PPO using purified PPO in a 3mL reaction.

3.2.8. Determination of Total Protein Concentration

Total protein concentration was determined using a Coomassie (Bradford) Protein Assay Kit with a Bovine Serum Albumin (BSA) standard (Fisher Scientific PI23200). The binding of Coomassie dye to protein causes an immediate color change from brown to blue; the unbound brown dye has an absorbance maximum of 465 nm, while the bound blue dye has an absorbance maximum of 595 nm.

Total protein concentration was estimated from the BSA standard curve using the dilution scheme for a 1-25 µg/mL working range. Dilution scheme and standard curve are provided in Appendix G.

A mortar and pestle was used to grind previously frozen *Pisolithus* with the original water content in diluent at a concentration of 100 mg/mL. Serial dilutions were used to create a range of working concentrations: 1:500, 1:600, 1:700. 1:800, 1:900, and 1:1000. Protein concentrations were measured using the cuvette function on a Nanodrop 2000c (ThermoFisher Scientific Waltham, MA) at 595 nm.

3.2.9. Preparation of Crude Enzyme Extract for SDS-PAGE PPO Activity Stain

Samples were removed from the freezer (-20°C or -80°C) and ~300 mg was cut with a new razor in a sterile Petri dish on a frozen ceramic cutting board and immediately returned to the freezer. Removing a few hundred milligrams of *Pisolithus* form a frozen fruiting body with a razor was possible, however to preserve the integrity of the sample it had to be done fast to prevent thawing. The rapid sectioning of frozen fruiting bodies prevented one from carefully selecting specific regions of the fruiting body, therefore, *Pisolithus* samples were sectioned by longitudinally shaving pieces from the side of the fruiting body. The ~300 mg sample was weighed and immediately transferred to a mortar where it was ground dry. Buffer (100mM sodium phosphate, pH 7.2) was added to create a concentration of 200 mg *Pisolithus* per mL of buffer (~1.5 mL), and ground with mortar and pestle until dissolved. A P1000 with a wide bore pipette tip was used to transfer the crude extract into a microcentrifuge tube. Samples were spun at 13,000 rpm for 10 minutes at 4°C. The polyphenol oxidase (PPO) remained in the supernatant, and was transferred to a clean microcentrifuge tube.

3.2.10. SDS-PAGE

SDS-PAGE was used to sort total protein by molecular weight with an electric field in preparation for a PPO activity stain. Novex® WedgeWell 4-20 % Tris-Glycine Mini Gels (Firsher Scientific XP04200BOX) were loaded with 40 µL of 1:1 crude extract supernatant: loading dye, or 10 µL of Life Technologies SeeBlue Pre-Stained Standard (Fisher Scientific LC5625). Electrophoresis was run at 125 V for 2-3 hours in a Novex®

EI9001-XCELL Mini Cell gel box (ThermoFisher) with a Bio-Rad PowerPac 200 power source (BioRad, Hercules, CA).

See Appendix H for loading dye and running buffer composition.

3.2.11. SDS-PAGE PPO Activity Stain

Gel cassettes were opened so the gel was adhered to only half of the cassette. A single-edged razor blade was used to cut gel into strips containing the protein standard and a single run of each sample. Gel strips were equilibrated by immersion for 5+ minutes in 20 mL of 100 mM sodium phosphate buffer either at pH 7.2 or a specified pH when studying the effect of pH on PPO activity. After equilibration, 5 mL of substrate solution was added to the equilibration buffer and gels were incubated for 10+ minutes, followed by a wash with buffer. Washed gels were stained with a 25 mM 4-amino-N,N-diethylaniline sulfate (ADA) in 10 mM HCl solution until bands appeared indicating PPO activity (see Appendix H for run specific conditions). Gels were imaged on a Bio-Rad ChemiDoc XRS+ with Image Lab Software using the colorimetric protocol. Occasionally, a second substrate bath and staining cycle was necessary.

PPO was characterized by number of isoforms, substrate specificity, pH tolerance, and whether monophenolase activity occurred. Substrate specificity was assessed using a variety of diphenols including chlorogenic acid, catechol, 4-methylcatechol, and 3,4-dihydroxy-L-phenylalanine (L-DOPA). It was possible to use catechol and 4-methylcatechol at 100 mM concentrations, but chlorogenic acid and L-DOPA were less soluble. Chlorogenic acid and L-DOPA were used at 33 mM and 8-20 mM, respectively. To determine if the PPO from *Pisolithus* could be classified as a tyrosinase (i.e. cresolase

and catecholase activity) tyrosine was used as a substrate. Tyrosine was used as a sole substrate and in combination with very low, 0.1 mM, concentrations of diphenols to help initiate a reaction. A positive control of 33 mM chlorogenic acid or 100 mM 4-methylcatechol was used.

3.3 Enzymatic Analysis: Results

3.3.1. Assessment of *P. arrhizus* Self-Reactivity

The scatterplots of independent self-reactivity tests (Figures 11-16) show some variation between individual absorbance readings, but the overall trend of individual runs does not display noticeable increases or decreases in absorbance. Trendlines represent a moving average based on the previous 4 data points. A moving average was chosen over linear regression because the self-reactivity tests were used to assess how stable *Pisolithus* crude extract (PCE) was over time. While linear regression is useful for determining the rate of change in absorbency when there is a trend, it does not account for changes in trends. Because PCE is made by grinding every component of the fruiting body without purification, there was concern that released molecules would react with enzymes to make new products that would in turn react with different enzymes potentially causing a significant increase in absorbency before a significant decrease.

During the first few minutes of the self-reactivity test, a significant decrease in absorbance can be observed in some of the runs. This change in absorbency occurred while the extract was still being prepared on ice and each reading represents a different concentration of PCE. One mL of distilled H_2O was added before each of the first three

readings. After the first three readings, the PCE was at the final working concentration and stayed the same for the remainder of the assay.

The next 7-8 data points represent PCE self-reactivity at ~4°C where the mortar was still on the bed of ice. These data points can be observed in the scatterplot where the time between individual reads decreases around 5 minutes. After 7-8 readings, the mortar was removed for the ice and allowed to warm up. Readings continued for another 10-20 minutes. It is important to note that any enzyme assay using PCE as enzyme would be using PCE that is at room temperature, so PCE self-reactivity should be analyzed from ~10 minutes onward.

There is a noticeable difference in absorbance between individual reads from a single run, however PCE does not produce an observable trend in absorbance change over the course of an enzyme assay at 212, 265, 340, 412, 550, or 660 nm. The variability between individual reads within an individual run may be caused by three factors: 1.) Since PCE was micropipetted directly from the pestle it was prepared in, there was likely variability in the samples. This variability comes from at least two sources; not all of the *Pisolithus* was able to be ground into solution as there were small spongy pieces that remained and sampling error from micropipetting PCE from different locations in the pestle. 2.) Baseline correction was not used. 3.) The PCE may have been too concentrated in some runs, as absorbances of around 3.5 were recorded.

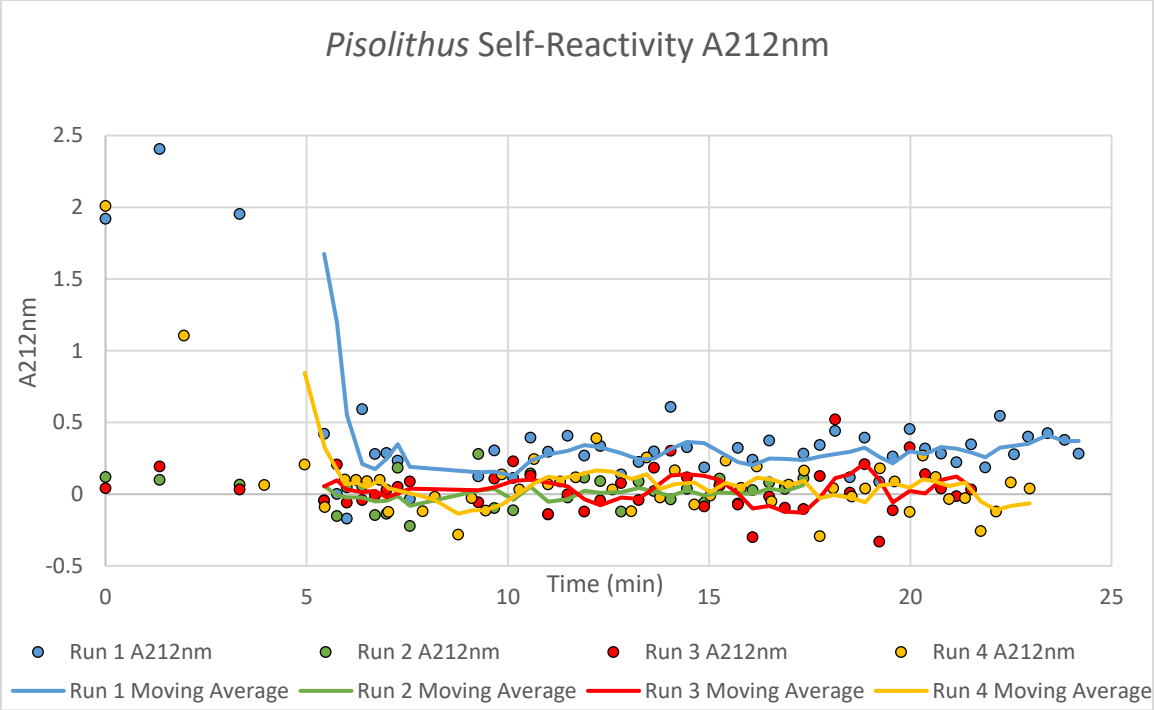


Figure 11: Pisolithus Self-Reactivity Graph: A212nm

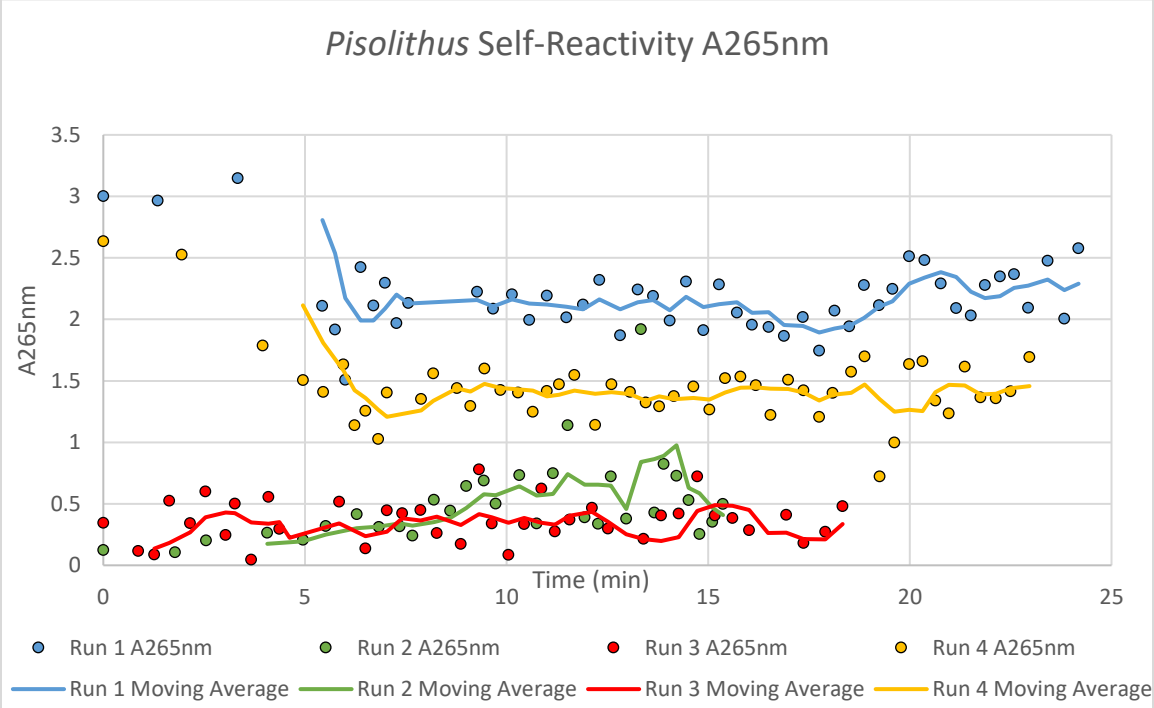


Figure 12: Pisolithus Self-Reactivity Graph: A265nm

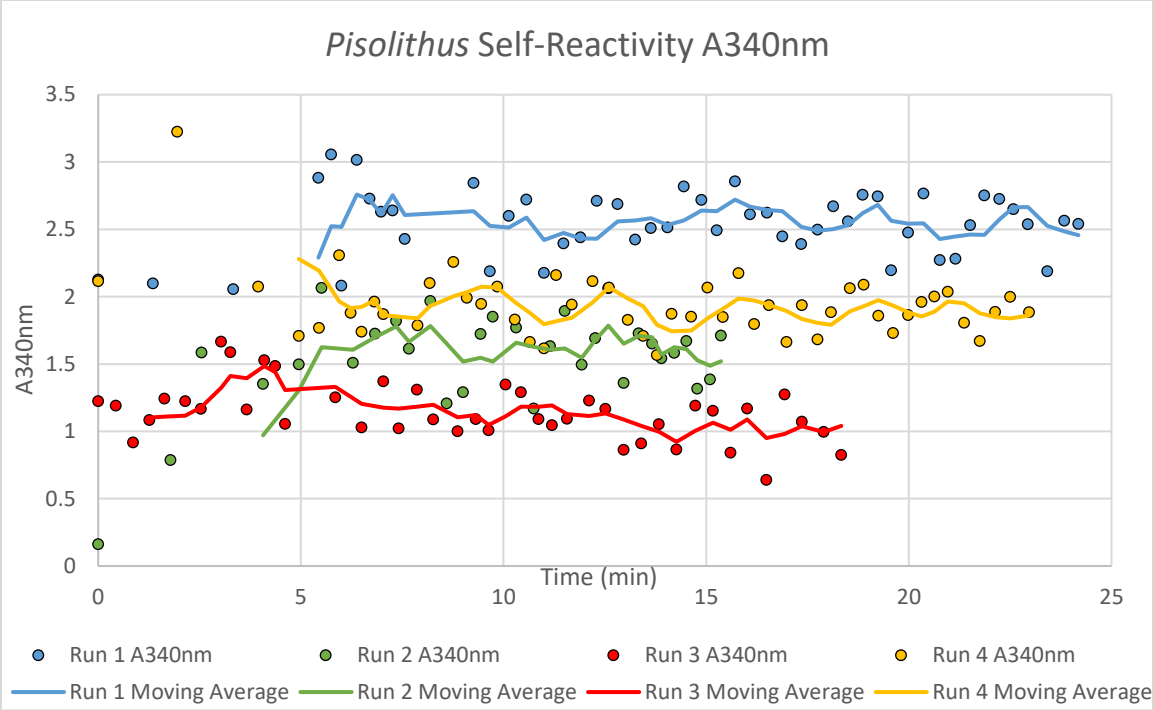


Figure 13: Pisolithus Self-Reactivity Graph: A340nm

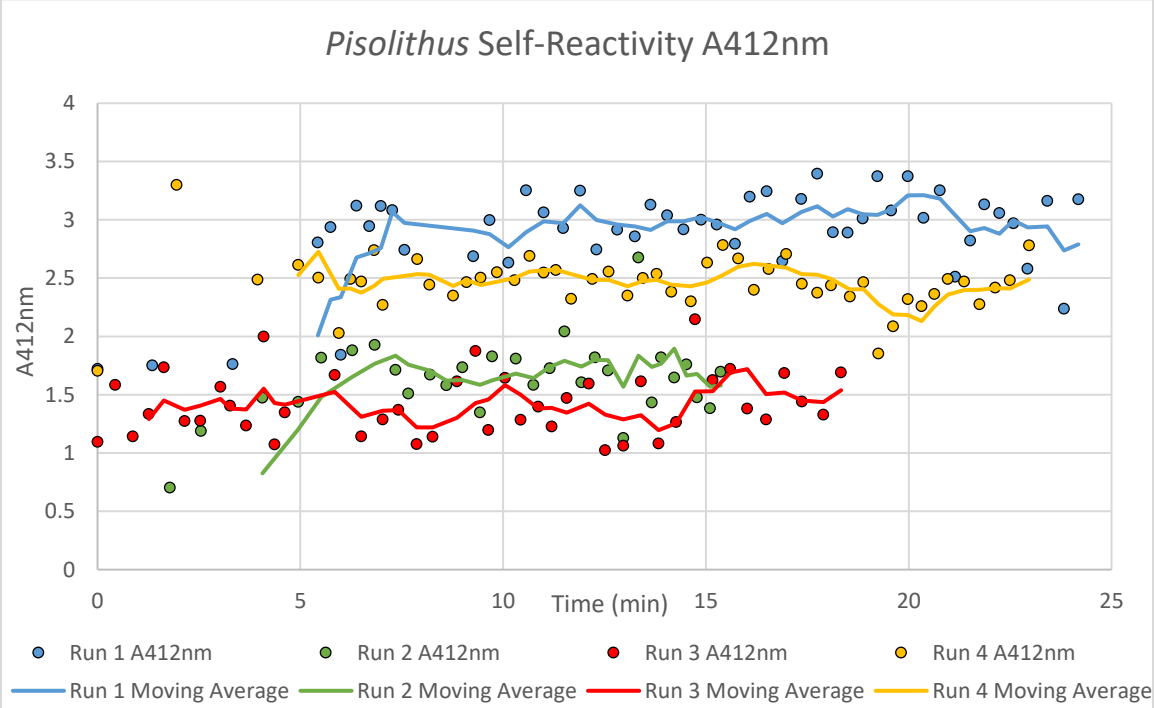


Figure 14: Pisolithus Self-Reactivity Graph: A412nm

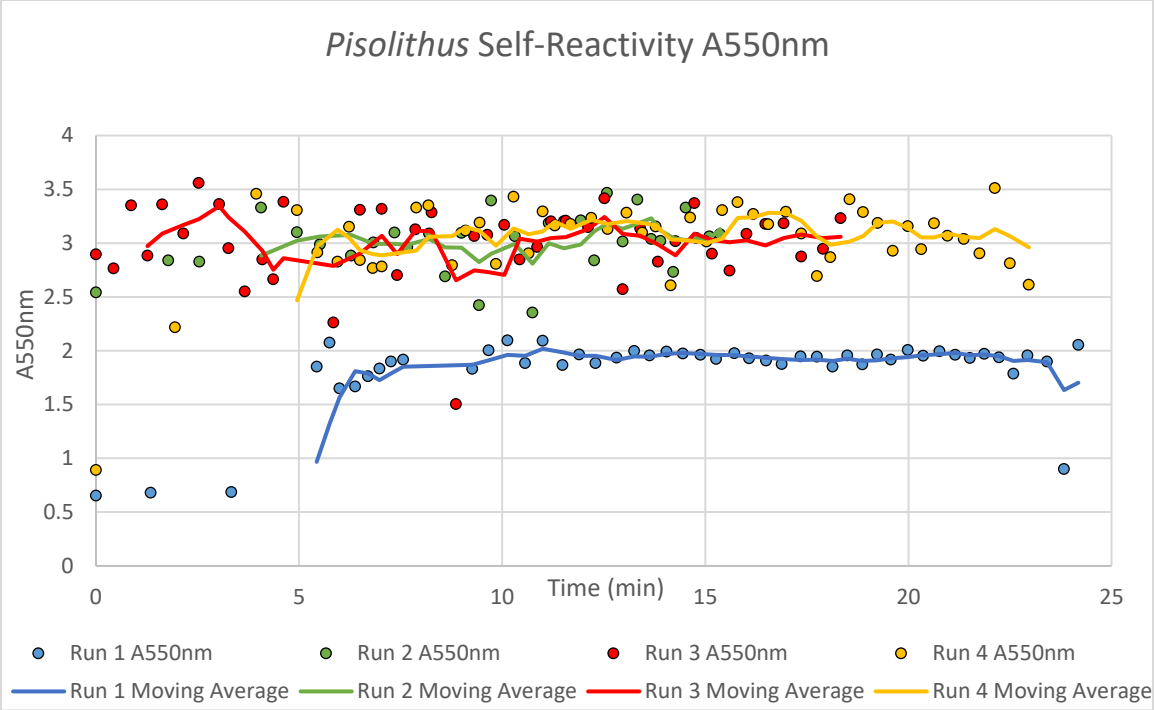


Figure 15: Pisolithus Self-Reactivity Graph: A550nm

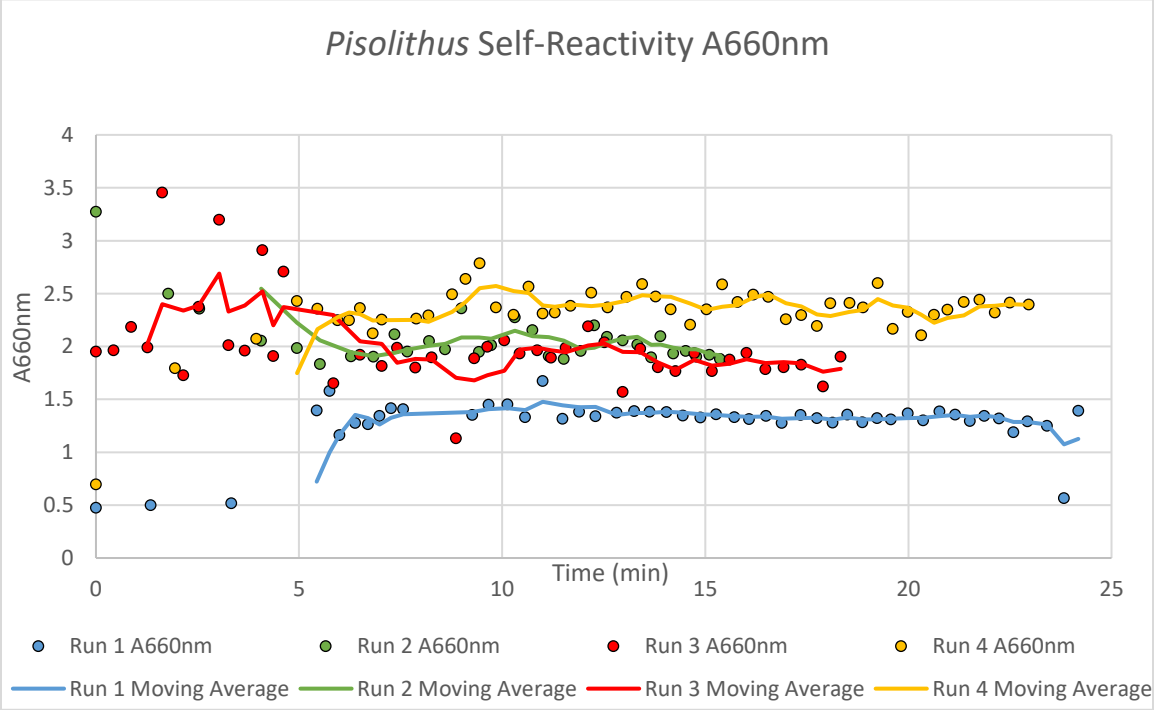


Figure 16: Pisolithus Self-Reactivity Graph: A660nm

I concluded that PCE was stable enough to be used directly as enzyme in an enzyme assay. There were obvious concerns using PCE as enzyme including low enzyme concentration, inhibitors, and the large quantity of pigment that earned *P. arrhizus* the common name of "Dye Ball." Due to the lack of an obvious energy source in this system, it was decided that a "shot-in-the-dark approach" to enzyme assay selection should be used. That apparent lack of energy source (no apparent ectomycorrhizal association) for *P. arrhizus* led to the hypothesis that bacteria were serving key functional roles in energy acquisition. Further, the fact that these *P. arrhizus* samples were growing in NGB, YNP, a unique thermal area, the list of possible energy sources and pathways was significantly longer. The possibility of PCE producing a false-negative in an enzyme assay was out-weighed by the insight a true-positive would give to understanding this system. Using PCE as directly as enzyme was significantly less time consuming than the purification of individual enzymes and allowed for the rapid testing of numerous energy pathways. Another reason for not purifying individual enzymes for assay was the fact that the enzymes would likely be of bacterial origin, potentially from a novel species and almost certainly from a species where the MW and properties of that enzyme were not known. Additionally, if an essential enzyme was of bacterial origin it is possible there would be functional redundancy across diverse bacterial lineages.

3.3.2. Enzyme Assay for Glucose-6-Phosphate Dehydrogenase

The G6PDH assay had blank and test conditions. The blank did not display any change in absorbance over the course of the experiment, while the test condition

increased in A340nm for the duration of the experiment, ~9 min (Figure 17). Regression analysis comparing two independent slopes show this is a statistically significant difference, p=0.008 (see section 3.2.6 for complete description of statistics). An increase in A_{340nm} indicates G6PDH activity, and the test condition had a maximum increase of 0.335 Abs at 340 nm. This indicates that G6PDH is present and active. This result was expected because *Pisolithus* is known to possess G6PDH (Goodfellow & Priest, 2012).

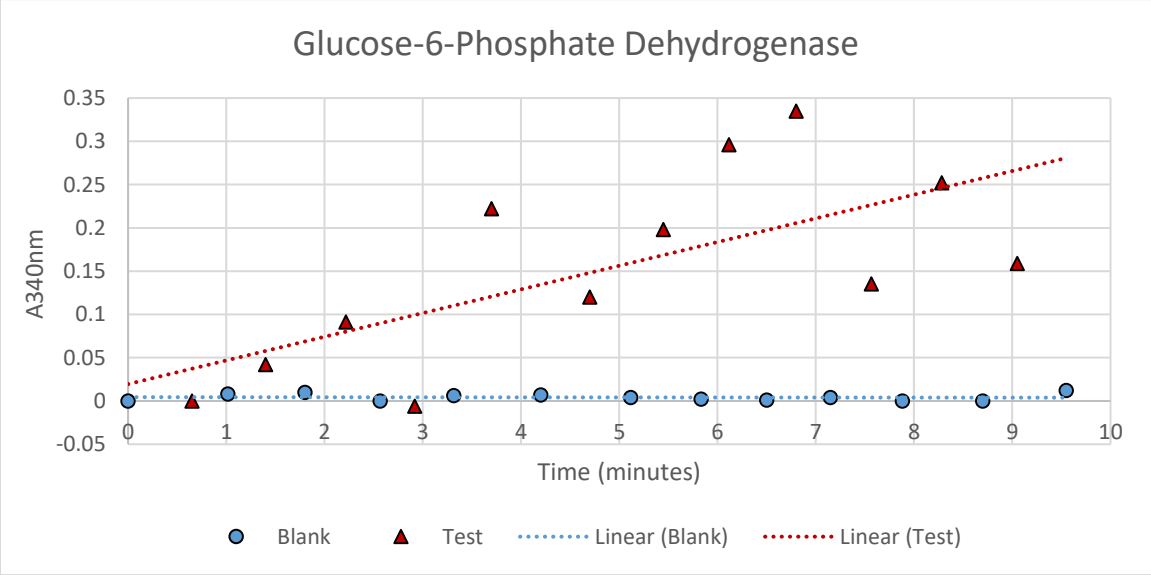


Figure 17: Glucose-6-Phosphate Dehydrogenase Assay Graph

3.3.3. Sulfite Oxidase Spectrophotometric Assay – Evidence of SO Activity

The SO assay gave inconsistent and inconclusive results. Initial test results (data not shown) appeared to have a marked increase in A_{550nm} indicating a positive result while the blank did not change. Further investigation revealed inconsistent patterns in the data where reactions were occurring when not expected and no reaction occurred when

expected. This assay was attempted twenty-four times and gave different results on almost every run. Variations of the protocol (Sigma Aldrich, St. Louis, MO) were attempted using different concentrations of sulfite and excluding cytochrome c.

Figure 18 shows a run where all conditions contain PCE and Tris HCl. The conditions that include the substrate sulfite were prepared at 100x concentrations. The purpose of markedly higher sulfite concentrations was to provide more substrate for a longer observation period. The four conditions include: 1.) Only PCE in Tris HCl (red) 2.) PCE in Tris HCl with cytochrome c (blue) 3.) PCE in Tris HCl and 100x sulfite (green) and 4.) PCE in Tris HCl with cytochrome c and 100x sulfite (yellow). The results suggest that the addition of cytochrome c is responsible for any increase in A_{550nm} regardless of the presence of sulfite. While both conditions lacking cytochrome c displayed a reduced increase in A_{550nm}, surprisingly the condition lacking sulfite had a greater increase in A_{550nm}.

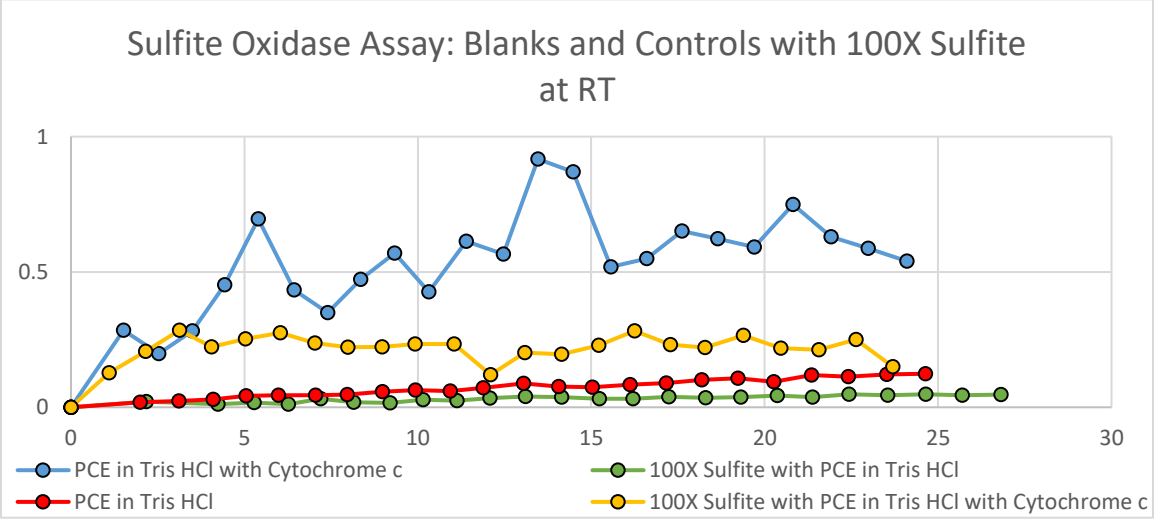


Figure 18: Sulfite Oxidase Assay: Blanks and Controls with 100x Sulfite at RT

These observations led me to three hypotheses since PCE was being used directly as enzyme in place of purified SO. First, it may not be necessary to add cytochrome c to the reaction cocktail because *Pisolithus* is eukaryotic and the PCE contains cytochrome c. Second, because PCE in Tris HCl without added sulfite had a greater increase in A_{550nm} than PCE in Tris HCl with 100x sulfite there may be sulfite already in PCE, and thus the *Pisolithus* fruiting bodies. Third, the slower rate of increase in A_{550nm} with 100x sulfite may be due to substrate inhibition. To test these hypotheses conducted more trials, without cytochrome c, testing the effect of different concentrations of sulfite.

Figures 19 and 20 are line graphs testing the effect of sulfite concentration on SO activity using the protocol dictated volume of enzyme (PCE) and a dilute concentration of PCE, respectively. Since SO was not purified and quantified from PCE this is qualitative data. The color scale in the graphs goes from dark green (no sulfite) to dark red (100x sulfite). Both runs display an increase in A_{550nm} throughout the observation period. As expected if SO is present, the more concentrated PCE produced a greater ΔA_{550nm} than dilute PCE.

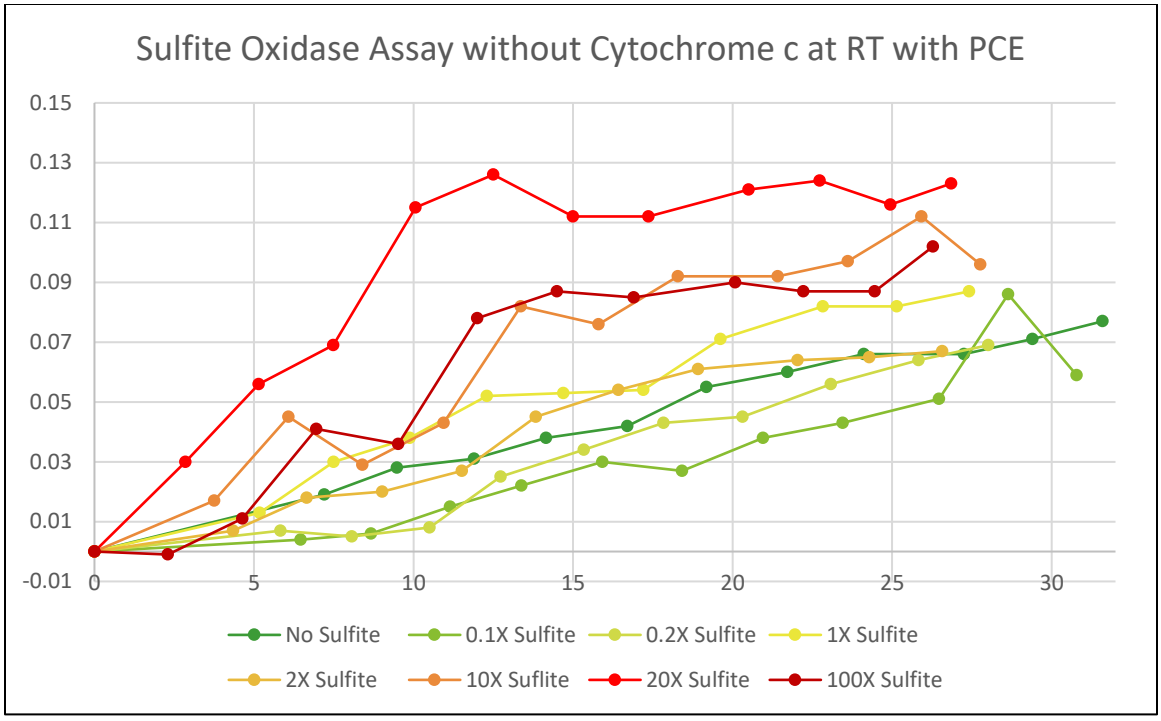


Figure 19: Sulfite Oxidase Assay without Cytochrome c at RT with PCE

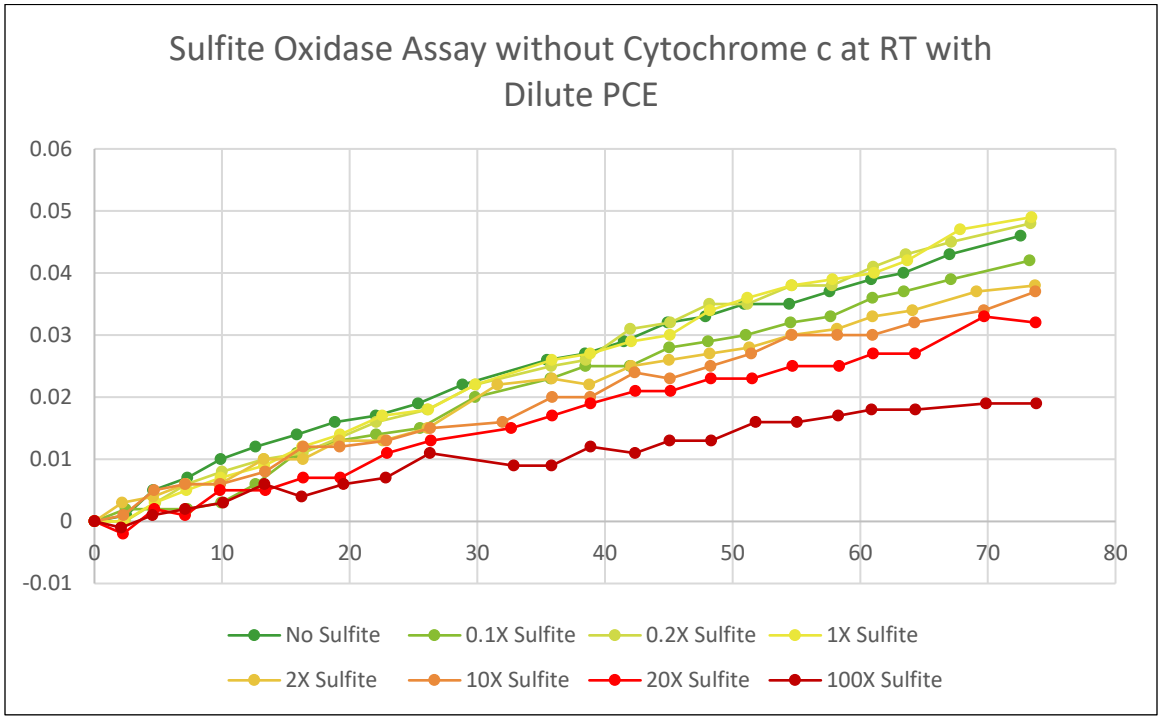


Figure 20: Sulfite Oxidase Assay without Cytochrome c at RT with Dilute PCE

Conversely, the sulfite concentration did not affect the rate of ΔA_{550nm} as expected. The sulfite concentration that displayed the greatest ΔA_{550nm} differed based on the concentration of PCE. With the more concentrated PCE the greatest ΔA_{550nm} was observed with more concentrated sulfite, and the smallest ΔA_{550nm} was observed with less concentrated sulfite, while the dilute PCE displayed the greatest ΔA_{550nm} with 1x sulfite and the smallest ΔA_{550nm} with 100x sulfite. Another unexpected inconsistency is the three highest concentrations of sulfite with more concentrated PCE plateauing, while the less concentrated sulfite solutions did not plateau. Additionally, none of the sulfite concentrations with dilute PCE plateaued.

Since these results were not consistently reproducible, it is difficult to draw meaningful conclusions, yet there some aspects that should be discussed. The discrepancy in ΔA_{550nm} rate between runs using the same sulfite concentrations but different PCE concentrations may be attributed to substrate inhibition. With dilute PCE 1x sulfite has the most efficient reaction with 100x sulfite having the slowest reaction, exactly what would be expected with substrate inhibition. With more concentrated PCE, the greatest ΔA_{550nm} is observed with the higher sulfite concentrations, but there is a lag phase at the beginning of the observation period. Here, 100x sulfite has the least ΔA_{550nm} for the first few minutes. Substrate inhibition may be responsible for this lag. It is possible that with the more concentrated PCE had enough SO present to reduce the sulfite concentration enough to allow the substrate inhibition to lessen after the first few minutes. If so, it makes sense that the higher sulfite concentrations had the greatest ΔA_{550nm}.

Alternatively, these results could represent a false positive due to some unforeseen reaction happening because PCE is being used instead of purified SO. If the *P. arrhizus* fruiting body does not possess sulfite, as hypothesized, then this is a false positive. The no sulfite condition produced a ΔA_{550nm} that is equivalent to the greatest ΔA_{550nm} in the dilute PCE trial. This would also invite numerous questions as to how sulfite concentration has a negative and positive affect on this unknown reaction.

The mixed results of this SO assay exemplify the challenges of characterizing an environmental biological sample. Enzyme assays are typically for stable samples where the presence of the enzyme and its properties are previously known, usually with a clinicial or purified samples, not exploratory science as is the case with this research. This SO and the other enzyme assays that produced inconclusive or negative results do not necessarily mean those enzymes are not present and active, rather it means there is no evidence to support or deny their presence. Many unknown factors are at play when using a crude extract, prepared by physically destroying all the membranes in a complete biological sample, in place of purified enzyme.

3.3.4. Polyphenol Oxidase Spectrophotometric Assay – Evidence of PPO Activity

A spectrophotometric assay was used to determine if PPO was present and quantifiable. *P. arrhizus* samples from both Norris Annex (NA1cr) and Norris Proper (NP1cr) were assayed in triplicate with test, blank, and control conditions. The initial and final absorbance measurement used to determine change in absorbance were the average of the first five reading and the final five readings, respectively. Averages were

used to account for variability between individual measurements so trends in ΔA_{265nm} could be observed. PPO activity is observed as a decrease in A_{265nm}.

In all NA1cr and NP1cr test conditions a decrease in A_{265nm} was observed (Table 4). NA1cr blank (substrate & no PCE) did not change, 0.000abs, and NP1cr blank had a negligible decrease of 0.003abs. Control conditions (PCE & no substrate) displayed similar results to the blanks: NA1cr had a negligible increase of 0.002 abs and NP1cr had no change, 0.000 abs. Both NA1cr and NP1cr test conditions (substrate & PCE) displayed a significant decrease in absorbance: 0.067 and 0.090 (see Table 5 for statistics), respectively.

Table 4: PPO Spectrophotometric Assay - Total ΔA_{265nm}

Sample	ΔA_{265nm} Blank	ΔA_{265nm} Control	ΔA_{265nm} Test
NA1cr Run 1	0.001	0.002	-0.082
NA1cr Run 2	-0.001	0.007	-0.055
NA1cr Run 3	-0.001	-0.003	-0.061
NA1cr Average	**0.000**	**0.002**	**-0.067**
NP1cr Run 1	-0.002	-0.001	-0.051
NP1cr Run 2	-0.004	-0.008	-0.149
NP1cr Run 3	-0.005	0.001	-0.141
NP1cr Average	**-0.003**	**-0.003**	**-0.114**
Combined Average	**-0.002**	**0.000**	**-0.090**

Figures 21 & 22 are scatterplots showing ΔA_{265nm} for NA1cr and NP1cr, respectively. Both scatterplots contain data for three runs using blank, control, and test conditions. All blank and control conditions have visibly flat trendlines, except for NA1cr run 2 control, and NP1cr run 1 control, which display a slight increase in A_{265nm}. All test conditions show a significant decrease in A_{265nm} over the duration of the assay.

A statistical analysis comparing the slopes of blank, control, and test conditions shows the observed decrease in test absorbance is statistically significant as compared to both blank and control conditions. Additionally, there is not a statistically significant difference between the blank and control in five of the six runs (Table 5). NA1cr run 2 is the only run where there is a statistically significant difference between the blank and control, but investigation of this difference shows the control had a small increase in A_{265nm} where the test had a large decrease.

Table 5: PPO Assay: Comparison of Two Slopes

Slopes Compared	Sample	Run	P-Value	Significant
Test & Blank	NA1cr	1	$1.34421 * 10^{-12}$	Yes
Test & Blank	NA1cr	2	$1.22282 * 10^{-15}$	Yes
Test & Blank	NA1cr	3	$1.0596 * 10^{-16}$	Yes
Test & Blank	NP1cr	1	$4.74585 * 10^{-07}$	Yes
Test & Blank	NP1cr	2	$1.14248 * 10^{-09}$	Yes
Test & Blank	NP1cr	3	$2.84844 * 10^{-09}$	Yes
Test & Control	NA1cr	1	$1.49 * 10^{-12}$	Yes
Test & Control	NA1cr	2	$6.44151 * 10^{-16}$	Yes
Test & Control	NA1cr	3	$1.43107 * 10^{-14}$	Yes
Test & Control	NP1cr	1	$1.09605 * 10^{-06}$	Yes
Test & Control	NP1cr	2	$3.01017 * 10^{-08}$	Yes
Test & Control	NP1cr	3	$2.60405 * 10^{-09}$	Yes
Blank & Control	NA1cr	1	0.330796839	No
Blank & Control	NA1cr	2	0.006570948	Yes
Blank & Control	NA1cr	3	0.749547647	No
Blank & Control	NP1cr	1	0.207680445	No
Blank & Control	NP1cr	2	0.873514176	No
Blank & Control	NP1cr	3	0.290441381	No

*See Section 3.2.6 for complete description of statistics. Significance calculated as a t-test based regression analysis comparing two independent slopes.

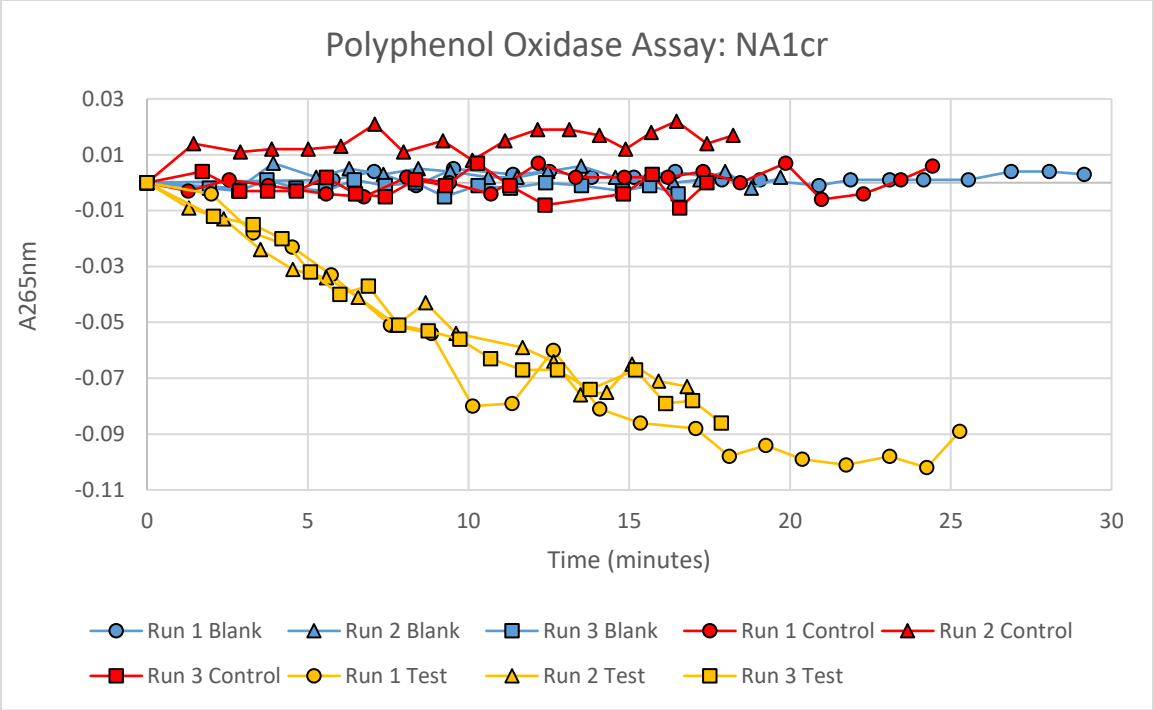


Figure 21: Polyphenol Oxidase Assay Graph: Norris Annex

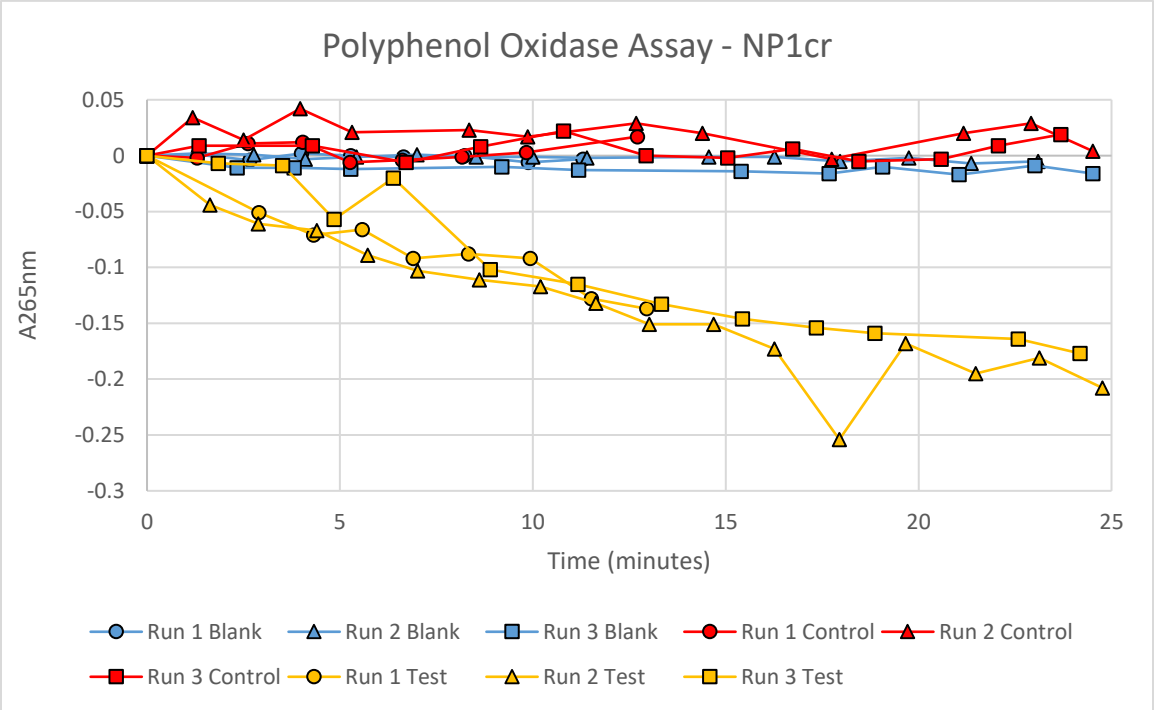


Figure 22: Polyphenol Oxidase Assay Graph: Norris Proper

3.3.5. Quantification of PPO Activity

To quantify the PPO activity of *Pisolithus* the maximum linear rate of ΔA_{265nm} was determined for test and blank conditions. Units of PPO per mg *Pisolithus* w calculated using the equation from "Enzymatic Assay of Phenolase (EC 1.10.3.1)" (Sigma Aldrich, St. Louis, MO).

PPO U/mg *Pisolithus* were relatively consistent between NA1cr and NP1cr runs with a range 0.15, and 0.10 U/mg, respectively. Average PPO U/mg *Pisolithus* were high in NP1cr (0.95 U/mg *Pisolithus)* than NA1cr (0.49 U/mg *Pisolithus*) (Table 6). Differences in average PPO activity could be reflective differences in developmental stage of fruiting bodies, sample quality at the time of collection, and/or limited sample size.

Table 6: Quantification of PPO per mg *P. arrhizus*

Sample	**Δ A_{265nm} per min Test**	**Δ A_{265nm} per min Blank**	***Pisolithus* (mg)**	**Units PPO per mg *Pisolithus***
NA1cr Run 1	0.0081	0.0003	1.50	0.52
NA1cr Run 2	0.0068	0.0008	1.50	0.40
NA1cr Run 3	0.0074	-0.0008	1.50	0.55
NA1cr Average	**0.00743**	**0.0001**	**1.50**	**0.49**
NP1cr Run 1	0.0126	-0.0007	1.50	0.89
NP1cr Run 2	0.0142	-0.0006	1.50	0.99
NP1cr Run 3	0.0136	-0.0008	1.50	0.96
NP1cr Average	**0.01347**	**-0.0007**	**1.50**	**0.95**

3.3.6. Bradford Assay

A Bradford assay was run to quantify the total protein content of *Pisolithus*. Two fruiting bodes were analyzed, NA1 and NP1. Both samples had to be assayed using the

1-25 mg/mL protocol, where serial dilutions of a stock 100mg/mL sample were assayed. Variability in protein concentration was observed between serial dilutions of the same fruiting body. As noted in the *Pisolithus* self-reactivity section, the *P. arrhizus* had a spongy tissue that was difficult to grind up, whereby small pieces of *P. arrhizus* were left in the stock 100mg/mL sample. When this sample was serially diluted, those pieces were inevitably were not diluted evenly. The total protein concentration was calculated for each of the six serial dilutions and averaged. NA1 had a protein concentration of 1.294% and NP1 had a protein concentration of 2.154%.

Table 7: Bradford Assay - Total Protein Quantification

Sample	**Dilution**	***Pisolithus* Concentration (mg/mL)**	**A595 nm**	**Diluted Sample Protein Concentration (µg/mL)**	***Pisolithus* Total Protein**
NA1	1:500	0.2	0.399	1.615	0.807 %
NA1	1:600	0.167	0.421	2.553	1.531 %
NA1	1:700	0.143	0.402	1.738	1.217 %
NA1	1:800	0.125	0.401	1.704	1.362 %
NA1	1:900	0.111	0.394	1.398	1.258 %
NA1	1:1000	0.1	0.398	1.549	1.549 %
NA1 Average					**1.294 %**
NP1	1:500	0.2	0.444	3.567	1.783 %
NP1	1:600	0.167	0.429	2.908	1.745 %
NP1	1:700	0.143	0.431	2.986	2.090 %
NP1	1:800	0.125	0.432	3.056	2.445 %
NP1	1:900	0.111	0.427	2.837	2.553 %
NP1	1:1000	0.1	0.415	2.31	2.310 %
NP1 Average					**2.154 %**

*Results for each dilution are the average of three spectrophotometric readings

These protein concentrations are on lower end of normal when compared to commercially- harvested edible mushrooms per the USDA. Normal protein

concentration in edible mushrooms ranges from 1.5 to 3.1% by fresh weight. See Appendix G for more information on the protein content of various edible mushrooms.

3.3.7. SDS-PAGE PPO Activity Stain

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) activity stain for polyphenol oxidase (PPO) was used to characterize the PPO from *Pisolithus*. At least seven isoforms were identified with catecholase activity with MWs ranging from < 49 kDa to > 62 kDa. The brown pigment ran off the gel with the single digit kDa markers. This brown pigment is likely melanin, the end product of PPO, and the lanes with the most pigment display the most PPO activity. Figure 23 shows two gels (Figures 23-26 have an orange box highlighting the bands of interest). Image A was taken on the Bio-Rad ChemiDoc XRS+ with Image Lab Software using chlorogenic acid as a substrate (left), the five lanes contain from left to right: (1.) Thermo Fisher SeeBlue Pre-stained Protein Standard, (2.) NP2, (3.) NP5, (4.) NP6, (5.) NP16. Image B is a color image of a gel to show how the SDS-PAGE PPO activity stain appears to the naked eye using 4-methylcatechol as a substrate (right).

In gel A, NP6 has at least seven distinct PPO isoforms, the highest molecular weight isoform is clearly visible in both NP5 and NP16. The most active PPO isoform in NP6, the second lowest MW observed, is also visible in NP2. While NP6 is the best sample to identify isoforms; NP2, NP5, and NP16 appear to have a few faint bands that match the intermediate MW isoforms of NP6. Additionally, NP6 and NP16 on gel B appear to have the full array of PPO isoforms.

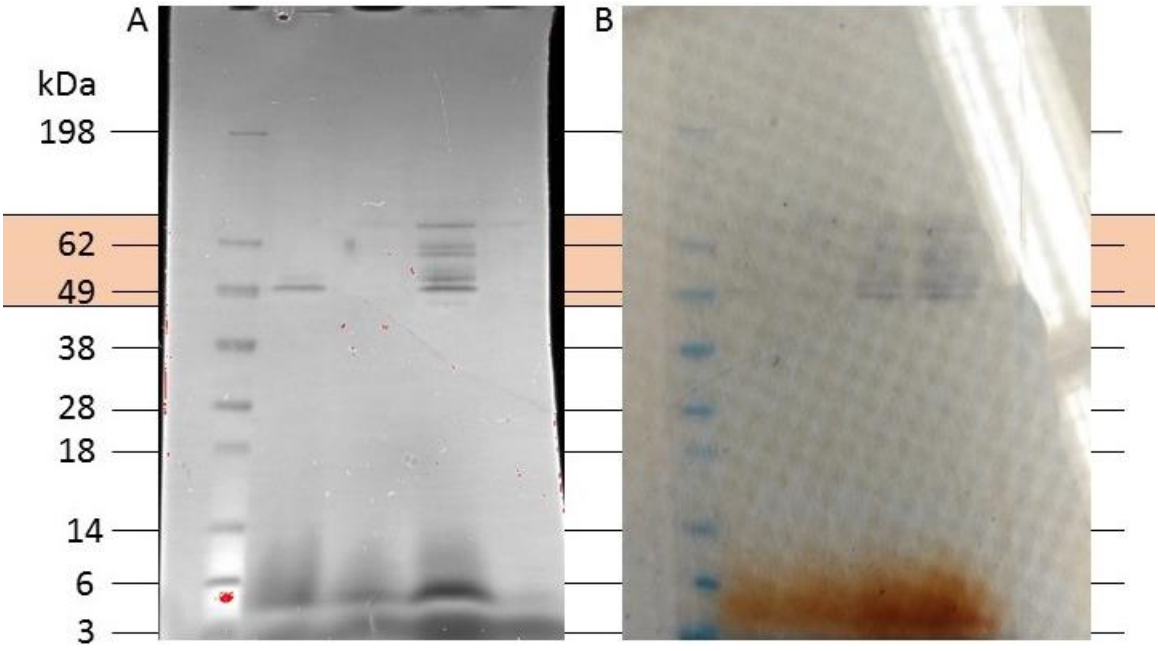


Figure 23: 33mM Chlorogenic Acid + 100mM 4-methylcatechol Activity Stain

* The faint orange box highlights bands associated with PPO activity

The *Pisolithus* samples were difficult to work with because they were immediately stored whole on dry ice and transferred to a -20°C or -80°C freezer to preserve the sample. The process of holding a fruiting body in place long enough to shave ~300mg of -80°C mushroom tissue that is ~90% inevitably caused some level of thaw. Over time, subsequent freeze-thaw cycles reduced PPO activity. This observation was made with the spectrophotometric assay and the SDS-PAGE PPO activity stain. The samples used in this assay were used for various other enzyme assays and had previously been subjected to limited freeze-thaw. Apparent lack of PPO activity in a sample could be the result of numerous micro freeze-thaw cycles. Working with these *P. arrhizus* fruiting bodies was further complicated by the logistics of sample collection at NGB, YNP, and a limited supply of fruiting bodies.

3.3.8. Determination of *P. arrhizus* PPO Substrate Specificity

After observing the presence of similar PPO isoforms in different samples, substrate specificity tests were run using a protein standard and two samples. All SDS-PAGE PPO activity stain gel images from this point on will have a protein standard in the leftmost lane, with NP6 and NP16 in the rightmost lanes.

In Figure 24, the gel using chlorogenic acid (same gel as Figure 23a) was assayed prior the other gels with all four *P. arrhizus* samples: NP2, NP5, NP6, and NP16. The gels assayed with L-DOPA, catechol, and 4-methylcatechol were assayed simultaneously using the same PCE supernatant for NP6 and NP16. A replicate gel was assayed at this time using chlorogenic acid as a substrate, but human error caused the assay to fail so the previous gel is shown here. Even though the chlororgenic acid gel was assayed prior to the other supbstrate gels, the concentration of PCE supernatant was the same in all runs.

The aforementioned reduction in NP6 PPO activity due to freeze-thaw can be observed between the former chlorogenic acid gel, and the later L-DOPA, catechol, and 4-methylcatechol gels. In contrast, NP16 appears to have increased PPO activity in the later runs, but further investigation may explain this increase in PPO activity. As noted earlier, there may be a correlation between the amount of low MW pigment at the bottom of the gel and PPO activity. The NP16 sample on the chlorogenic acid gel does not appear to have any pigment, yet the PPO activity is comparable to NP5, which has visible pigment.

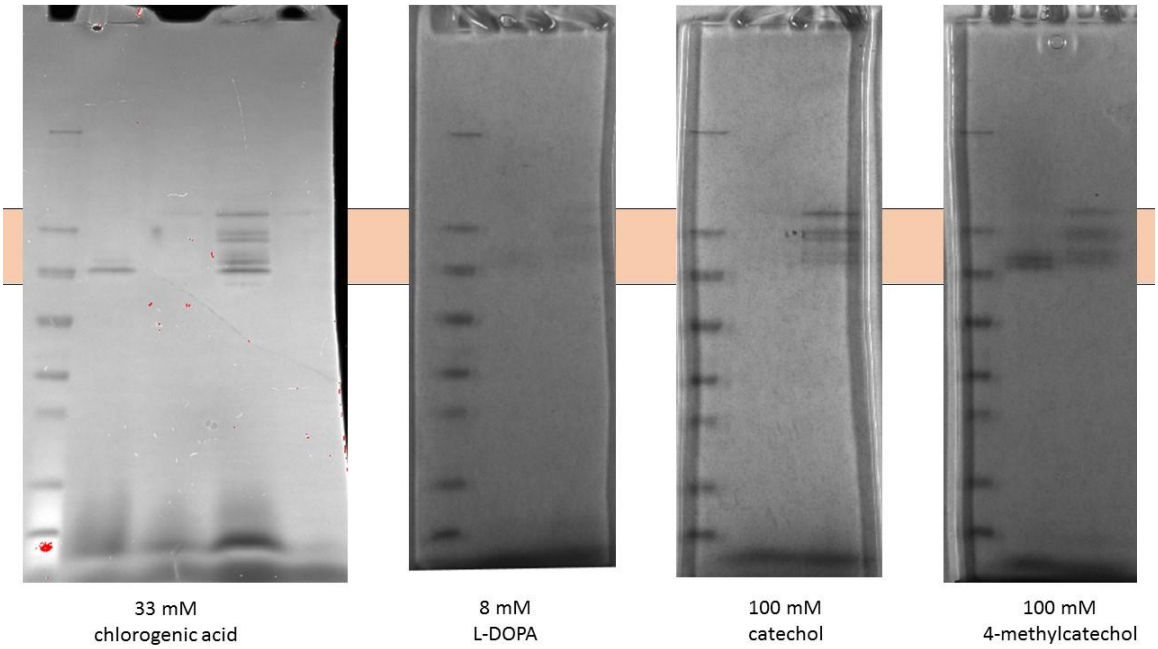


Figure 24: Substrate Specificity SDS-PAGE PPO Activity Stain

* The faint orange box highlights bands associated with PPO activity

PPO from *P. arrhizus* was able to catalyze a reaction using all four diphenols. Despite lower resolution and fainter bands on the L-DOPA, catechol, and 4-methylcatechol gels, all isoforms that were visible with chlorogenic acid are present. The high MW isoforms appear to be the most active on catechol, while the low MW isoforms appear to be more active on 4-methylcatechol. PPO from *Pisolthus* is able to catalyze a reaction with L-DOPA across all MWs. While the bands formed using L-DOPA are faint when compared to catechol and 4-methylcatechol, the concentration of L-DOPA was 12.5X less than both catechol and 4-methylcatechol. Making a quantitave comparison between *P. arrhizus* samples was difficult because of the innate difficulty of working with frozen *P. arrhizus*.

3.3.9. The Effect of pH on *P. arrhizus* PPO Activity

To determine how pH affects PPO from *P. arrhizus*, a series of 100 mM sodium phosphate buffers were made that sequentially increased by 0.7 pH from 5.1 to 8.6. These sodium phosphate buffers were used as the equilibration buffer the gels were incubated in before and after the addition of a 100 mM catechol solution. PPO activity was observed at all pH levels across all isoforms (Figure 25). Thus, it can be concluded that PPO from *P. arrhizus* has a functional pH range of ≤ pH 5.1 to ≥ pH 8.6. All gel images in Figure 26 have the greyscale inverted and bands indicating PPO activity are visible in the area between the two horizontal black lines highlighted in orange.

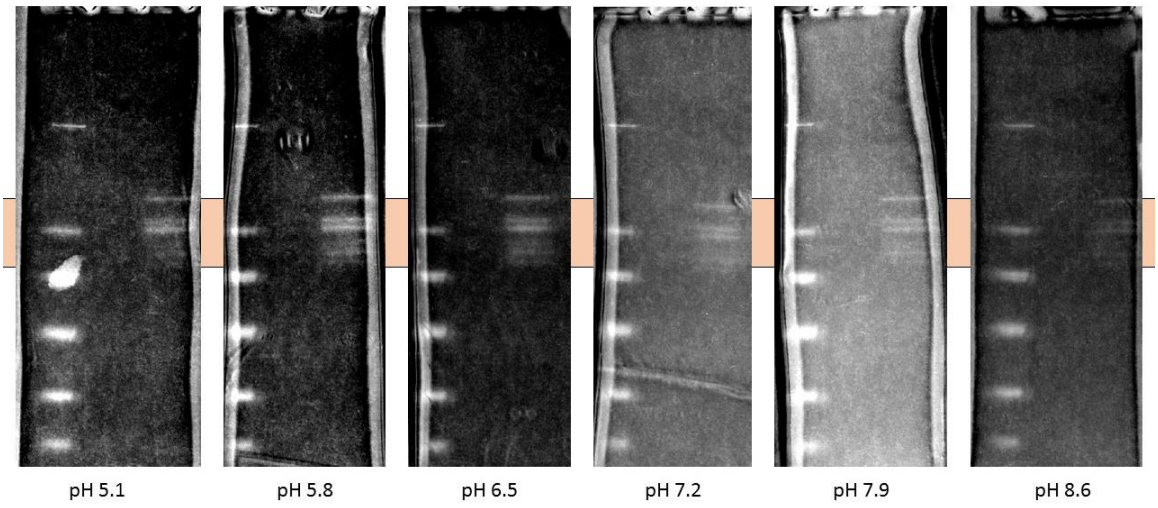


Figure 25: SDS-PAGE PPO Activity Stain for PPO pH Range

* The faint orange box highlights bands associated with PPO activity

3.3.10. Determination of *P. arrhizus* PPO Tyrosinase Activity

After establishing that *Pisolithus* possessed PPO, specifically catecholase activity, experiments were conducted to determine whether *Pisolithus* possess tyrosinase (TYR) activity (Figure 26). If any of the PPO isoforms identified using a diphenol substrate were a TYR, then the same band would be expected to form using a monophenol. Tyrosine was used as a substrate to test cresolase/TYR activity. Three preparations were used: tyrosine only, a 0.1 mM concentration of a diphenol, and a combination of tyrosine and 0.1 mM diphenol. The 0.1 mM diphenol solution, either containing 4-methylcatechol or chlorogenic acid, was used as a catalyst to "jump start" the TYR activity, but was in too low a concentration to cause a band to appear. No visible bands formed when using tyrosine as a substrate at either 8 or 20 mM regardless of the presence of a diphenol. The small dots on the gel (arrows) in the right three images are plastic artifacts from processing.

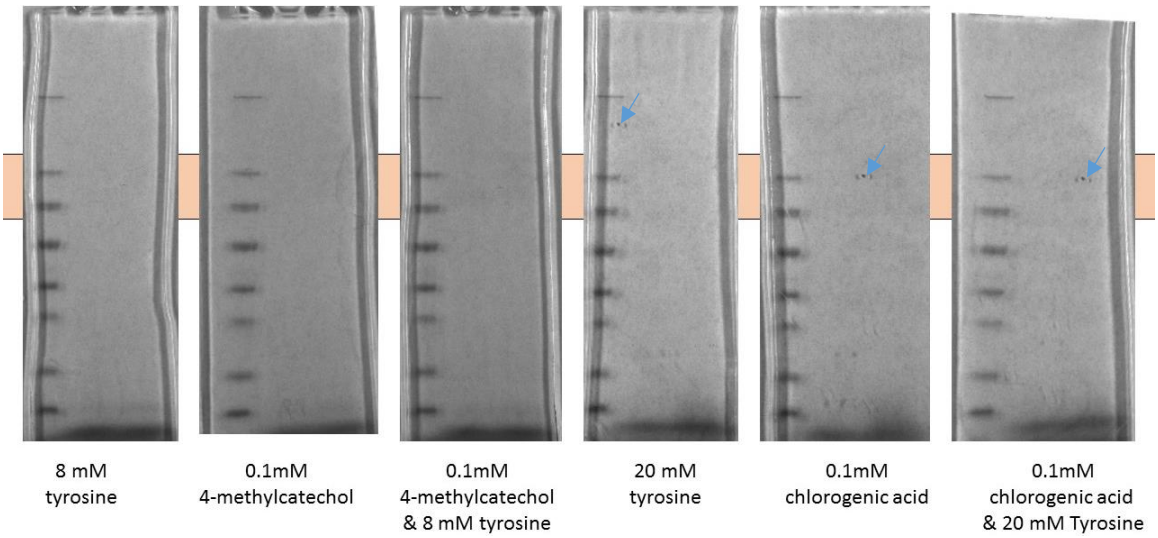


Figure 26: SDS-PAGE PPO Activity Stain for Cresolase Activity

* The faint orange box highlights bands associated with PPO activity

None of the PPO isoforms in *Pisolithus* are TYRs. Because the PPO from *P. arrhizus* produced observable bands with 8 mM L-DOPA (the diphenol variation of tyrosine), it is clear that *P. arrhizus* possess PPO with catecholase activity. If this PPO was a TYR (also possessing cresolase activity) an observable band would form with 8 mM tyrosine. There is no evidence to suggest the presence of any CO activity. This finding is in contrast to Gruhn and Miller (1991) who identified extracelluar and intracellular TYR from *P. tinctorius* (=*P. arrhizus*) grown in culture with and without added copper. The authors state: "The enzyme parameters changed slightly with storage, but the same trands were present." While it was observed that the longterm storage of the *P. arrhizus* samples in this study affected the PPO activity, the substrates used in Gruhn and Miller's work must be noted. Gruhn and Miller used *p*-cresol (a monophenol) and L-DOPA (a diphenol) not tyrosine to determine the presence of extracellular and intracellular "TYR," respectively. It is more accurate to state that Gruhn and Miller found intracellular PPO or CO activity, when using the terms as defined in this paper. The present study did not investigate the presence of extracellular TYR because the *Pisolithus* samples used were not cultured, and soil samples were not collected for this purpose. Thus, no conclusion can be made as the the presence of extracellular TYR from *Pisolithus*.

Chapter 4: Discussion

Nine genera of bacteria were cultured from *Pisolithus* samples that grow in the thermal soils of Norris Geyser Basin, YNP independent of any tree roots (Cullings & Makhija, 2001). Many of these bacteria are common soil microbes. These bacteria were cultured in aerobic and anaerobic conditions. It makes sense that both aerobic and anaerobic bacteria are present because there are zones of elevated O_2 as well as completely anaerobic zone inside *Pisolithus* (Appendix I). To further characterize *Pisolithus* from NGB, enzyme assays for various metabolic pathways were conducted. The samples proved challenging to work with and resulted in many inconclusive assays. Polyphenol oxidase from *Pisolithus* proved simpler to work with and was further characterized. Catecholase activity was detected using catechol, 4-methylcatechol, chlorogenic acid, and L-DOPA. No activity was observed on the monophenol tyrosine, indicating not TYR activity.

Fungal-bacterial and plant-bacterial interactions play an important role in fungal-plant symbioses. Mycorrhizas are metabolically, physically, and functionally influenced by bacteria (Frey-Klett & Garbaye, 2005). Mycorrhizal Helper Bacteria (MHB) belong to diverse genera including Proteobacteria: *Pseudomonas* (Founoune et al., 2002), *Burkholderia* (Poole et al., 2001), *Bradyrhizobium* (Xie et al., 1995); Firmicutes: *Bacillus* (von Alten et al., 1993) *Paenibacillus* (Poole et al., 2001); and Actinomycetes: *Rhodococcus* (Poole et al., 2001), and *Streptomyces* (Schrey et al., 2005) and have the ability to improve mycorrhizal root tip formation (Garbaye 1994). *Bacillus* and *Pseudomonas* species comprise a significant portion of MHB and plant growth promoting rhizobacteria (PGPR) that have been identified. It is likely that MHB and PGPR are the

same in many cases (Fitter & Garbaye, 1994). Numerous *Bacillus,* and *Paenibacillus* species were cultured from *Pisolithus* in this study. *Pseudomonas putida* was identified as the second most abundant bacterial symbiont of *Pisolithus,* behind *Sphingomonas* sp., using next generation sequencing (Cullings & Kaze, unpublished data). Additionally, *Nocardia*, a close relative to *Rhodococcus,* was cultured.

Brulé et al. (2001) found that the MHB *Pseudomonas fluorescens* was a strong inhibitor of the ectomycorrhizal fungus *Laccaria bicolor* in nutrient rich conditions, while it significantly stimulated *L. bicolor* growth under unfavorable conditions. These findings are in agreement with the leading hypothesis: MHB enhance presymbiotic survival and growth of mycorrhizal fungi (Frey-Klett et al., 1997), although it is not clear exactly how MHB enhance presymbiotic survival. NGB soils represent unfavorable conditions with low pH and low organic matter. The MHB associated with *P. arrhizus* in NGB, such as *Pseudomonas putida*, likely play a key role in the fungi's survival.

PGPR, such as the *Bacillus* spp. that I isolated, are known to aid in mycorrhiza formation either by direct or indirect mechanisms of action. Direct effects are classified as those that produce compounds or enzymes that promote plant growth, such as compounds that facilitate phosphate solubilization or the synthesis of plant growth hormones such as indole acetic acid (IAA). Indirect effects are those that include the production of effectors whose function, in turn, result in plant growth, such as antibiotics, cellulases, chitinases, and siderophores (Glick, 1995).

There are many known bacterial IAA producers (BIPs) including *Agrobacterium rhizogenes, Agrobacterium tumefaciens* (Lambrecht et al., 2000), *Bradyrhizobium japonicum* (Leveau & Lindow, 2005), *Erwinia herbicola* (Lambrecht et al., 2000),

Microbacterium sp., *Mycobacterium* sp. (Tsavkelova et al., 2007), *Pseudomonas putida,* (Leveau & Lindow, 2005), *Pseudomonas savastanoi* (Lambrecht et al., 2000), *Rhizobium* sp. and *Sphingomonas* sp. (Tsavkelova et al., 2007). BIPs and IAA in particular have been extensively studied (Lambrecht et al., 2000; Leveau & Lindow, 2005; Tsavkelova et al., 2007); what is not well understood is the process by which IAA is degraded by bacteria, specifically referred to as bacterial IAA degraders (BIDs). Leveau & Lindow (2005) discuss how a few studies have identified BIDs for the purpose of describing pathway intermediates, but their study focused on the ecological role of BIDs and thus falls short on describing fully the roles that these bacteria play in their hosts' metabolic processes. *Pseudomonas putida* strain 1290 was the BID used in their work.

Pseudomonas putida strain 1290 has the ability to utilize IAA as its sole energy, carbon, and nitrogen source. When *P. putida* 1290 was growing on IAA, the catechol 1,2 dioxygenase (CD) gene, *catR,* was found to be upregulated. The fact that the addition of catechol increased the oxygen uptake of *P. putida* 1290, and that a *catR* mutant of *P. putida* 1290 was unable to grow on IAA suggests that catechol is an intermediate in IAA catabolism. *P. putida* 1290 was also able to produced IAA with the addition of tryptophan to a growth medium (Leveau & Lindow, 2005). Catechol is the simplest diphenol, and was used as a substrate to characterize the PPO from *Pisolithus.*

The catechol 1,2 dioxygenase gene has been identified in other soil microorganisms including: *Acinetobacter calcoaceticus* (Patel et al., 1976), *Alcaligenes eutrophes, Arthrobacter crystallopoietes, Arthrobacter poscens* (Hou *et al.*, 1977), *Aspergillus niger* (Ninnekar & Vaidyanathan, 1981), *Brevibacterium flavum* (Hou et al., 1977), *Candida malosa* (Gomi & Horiguchi, 1988), *Corynebacterium* sp. (Hou et al.,

1977), *Frateuria* sp. (Aoki et al., 1984), *Pseudomonas aeruginosa*, *Pseudomonas oleovorans*, *Nocardia opaca* (Hou et al., 1977), *Rhizobium leguminosarum* (Chen & Lovell, 1990), and *Rhodococcus erythropolis* (Murakami et al., 1997). Leveau and Gerards (2008) identified 10 genes at the iac locus from *P. putida* strain 1290 that code for the genes necessary for IAA catabolism. They showed that 5 different species containing the iac locus, *P. putida* GB-1, *Burkholderia* sp. 383, *Sphingomonas wittichii* RW1, and *Rhodococcus* sp. RHA1, all grew on M9 minimal media with IAA as the sole carbon and energy source. Further, they confirmed there are members in the *Alpha-*, *Beta*, and *Gammaproteobacteria*, as well as the high G+C Gram-positive bacteria that utilize IAA. *Sphingomnas* and *Pseudomonas* are of the most relevance to this research, as they represent the two-most abundant genera in *P. arrhizus* at NGB, YNP.

Another study may help explain how *P. arrhizus* is surviving independent of mycorrhizal associations at NGB, YNP. Walker et al. (2005) found a photosynthetic endolithic community containing the single-celled red algae *Cyanidium* spp. (98-99% sequence identity to *Cyanidium caldarium* chloroplast rRNA genes) in NGB at YNP. The community was composed of 26% *Cyanidium* spp., and 37% *Mycobacterium* spp. (Figure 27 for complete community structure). Previously, *Mycobacterium* spp. had not been known to exceed 1% of the total community composition. An additional 11% of the community consisted of *Actinobacteria* other than *Mycobacterium,* for a total of 48% *Actinobacteria*. Together, *Cyanidium* spp. and the *Actinobacteria* constitute an astonishing 74% of this community.

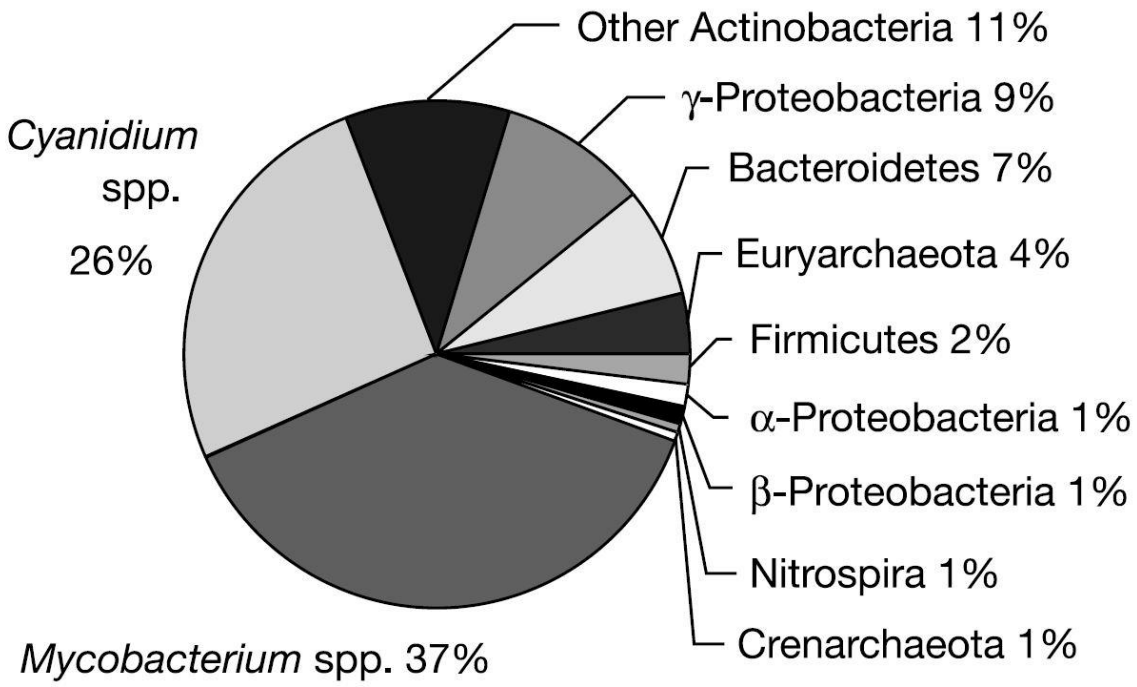


Figure 27: NGB Photosynthetic Endolithic Community Composition (from Walker et al., 2005)

In this study, the silica pebbles in the soil that was inadvertently collected with *P. arrhuzus* fruiting bodies appear to contain the same endolithic algae (Figure 28). It is highly probable this is the same endolithic community described by Walker et al. (2005) because both studies were conducted at Norris Geyser Basin, Yellowstone National Park. However, further research is needed to confirm the presence of *Cyanidium* spp. in the silica surrounding *P. arrhizus*.

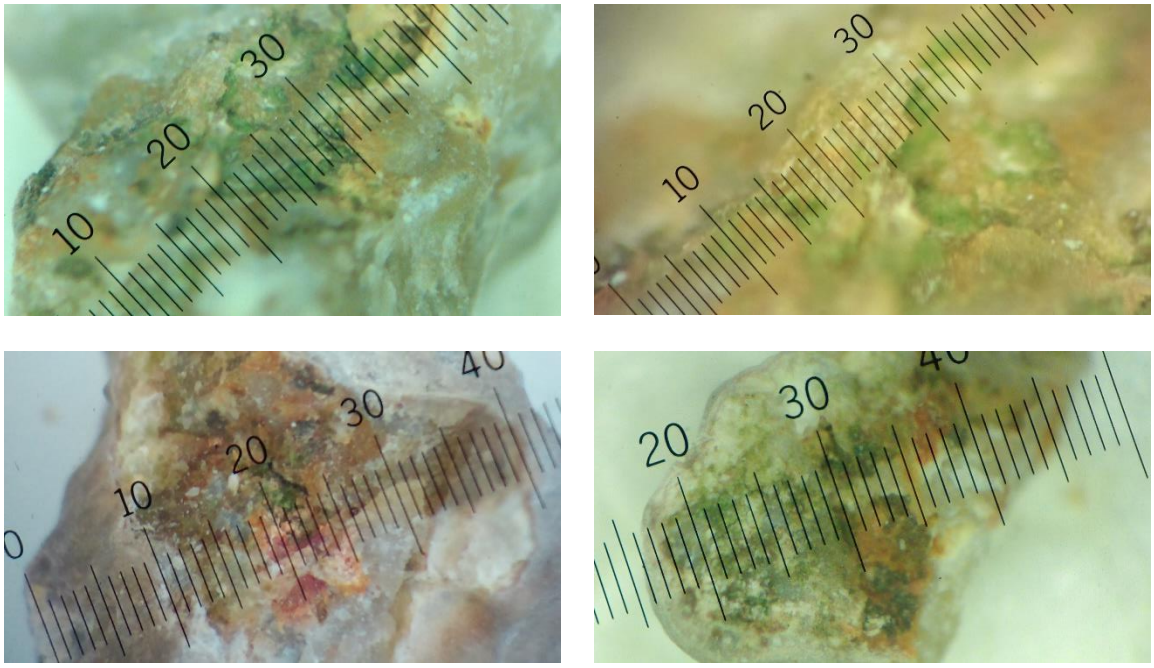


Figure 28: Photos of Silica Rocks Collected with P. arrhizus from NGB

It is possible that the non-mycorrhizal *P. arrhizus* in NGB are acquiring energy from the primary productivity of endolithic *Cyanidium* spp. Here I propose a possible energy cycle: Photosynthetic *Cyanidium* spp. are the primary producers in this thermal basin ecosystem lacking macro-plant life. The large *Mycobacterium* population could be a BIP population as Tsavkelova et al. (2007) describes. IAA production could stimulate *Cyanidium* spp. growth providing the *Mycobacterium* spp. with energy. The IAA would leach out of the silica where the *Pisolithus* and bacterial symbionts would use it is an energy, carbon, and nitrogen source.

Leveau & Lindow (2005) discuss how IAA could be considered an ideal food source for organisms that are able to catabolize it because of the C/N ratio, 8.6. If half of the carbon is used for energy production, the remaining C/N ratio is 4.3, close to the C/N

ratio of a typical bacterium, 3.6. Their research also showed that catechol 1,2 dioxygenase (CD) was critical for IAA metabolism, since catechol is an intermediate. While enzyme assays or genetic analysis are still needed to demonstrate the presence of CD, numerous species and close relatives of known CD possessing-microorganisms have been isolated or identified from *Pisolithus* spp. such as *Brevibacterium* spp., *Nocardia* sp., *Pseudomonas putida, Rhodococcus* spp., *Sphingamonas spp.* and an unidentified yeast. The abundance of BIDs in *Pisolithus* supports the hypothesis that IAA may be a carbon, energy, and nitrogen source for this system. The presence of all these organisms could represent functional redundancy.

If catechol is an intermediate of IAA catabolism for *Pisolithus arrhizus (or akin),* and this metabolism is occurring in *P. arrhizus* at NGB, the presence of PPO in *Pisolithus* spp. could be necessary to neutralize toxic phenols produced during the generation of energy. The product of PPO are *o*-quinones which self-polymerize into melanins. *Pisolithus* specimens from NA and NP have large quantities of high molecular weight brown/black pigments that exudate when sectioned (Figure 23b). Additionally, extreme variation of redox potential was observed in *Pisolithus* using O_2 probes *in situ* (unpublished data, see Appendix I). In the fruiting body of *P. arrhizus* from YNP, only a few millimeters separated completely anaerobic zones from zones containing greater than 2X ambient O_2. A possible explanation for the completely anaerobic zones is that CD and PPO activity utilize all available molecular oxygen in the process of energy generation and phenol detoxification/melanin production, respectively.

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Appendix A: PCR and Thermocycling

Table 8: PCR and Sequencing Primers		
Primer Name	**Sequence (5'-3')**	**Gene**
27F	AGA GTT TGA TCM TGG CTC AG	16S rRNA gene
1492R	CGG TTA CCT TGT TAC GAC TT	16S rRNA gene

Table 9: PCR Reaction Mix	
Reagent	**Amount**
Invitrogen Platinum PCR Super Mix High Fidelity	25 µL
16S Primers (10 mM 27F & 10 mM 1492R)	1 µL
Bacterial Genomic DNA	75-200 ng (3-10µL)

Table 10: PCR Thermocycling Parameters			
Temperature	**Duration**	**Cycles**	**Notes**
95°C	2:00	1	
95°C	0:30	9	Decrease annealing temperature by 1°C every cycle
61°C	0:30		
72°C	2:30		
95°C	0:30	25	
52°C	0:30		
72°C	2:30		
72°C	7:00	1	
4°C	∞	1	

Appendix B: 16S Sequences

>AC1A

AGTCGAGCGGACCGAACGGAAGCTTGCTTCTGTTCGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCGTAAGACCGGGA

TAACTCCGGGAAACCGGGGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGGTGGCTTTTGCTACCACTTACGGAT

GGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACACTG

GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCGACGCCGCGTGA

GCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAGAAGTACCGTTCGAATAGGGCGGTACGGTGACGGTACCTAACGA

GAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAG

GCGGTCCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGCG

GAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGC

GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGGTTT

>AC1B

TGCAGTCGAGCGGACCGAACGGAAGCTTGCTTCTGTTCGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCGTAAGACCG

GGATAACTCCGGGAAACCGGGGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGGTGGCTTTTGCTACCACTTACG

GATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACCGGCCACA

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>V3D

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Appendix C: Enzyme Assays

Table 11: Enzyme Assays	
Enzyme	**Sigma Aldrich Reference**
Acetate Kinase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/acetate_kinase.pdf
Adenosine 5'-Triphosphatase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/adenosine_5-triphosphatase_ph_7.pdf
Adenosine 5'-Triphosphate Sulfurylase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/adenosine_5-triphosphate_sulfurylase.pdf
Aldolase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/2/aldolase.pdf
Cytochorme p450 Reductase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/cytochromep450reductase.pdf
Glucose-6-Phosphate Dehydrogenase	http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-glucose-6-phosphate-dehydrogenase.html
Glyceraldehyde-3-Phosphate Dehydrogenase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/glyceralde3phosdehydrbaci.pdf
Malate Synthase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/malatesynthase.pdf
NAD-Pyrophosphorylase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/nadpyrophosphor.pdf
NADPH-FMN Oxidoreductase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/nadphfmnoxidoreductase.pdf
Polyphenol Oxidase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/2/phenolase.pdf
Pyruvate Decarboxylase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/pyruvate_decarboxylase.pdf
Pyruvate Dehydrogenase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/pyruvate_dehydrogenase.pdf
D-Rubulose-1,5, Diphosphate Decarboxylase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/dribuldiphosphcarb.pdf
Sulfite Oxidase	http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/sulfiteoxidase.pdf

Appendix D: Glucose-6-Phosphate Dehydrogenase Assay

Table 12: Glucose-6-Phosphate Dehydrogenase Assay Mixture (Per 1.5 mL Run)			
	Reagent	**Test**	**Blank**
A	Reaction Cocktail	1450 µL	1450 µL
B	Glycylglycine Buffer	0 µL	50 µL
F	*Pisolithus* Extract	50 µL	0 µL
	Total	**1500 µL**	**1500 µL**

Table 13: Glucose-6-Phosphate Dehydrogenase Reaction Cocktail Composition					
	Reagent	**Component**	**Diluent**	**Volume**	**Notes**
A	Distilled H_2O	dH_2O		21 mL	Mix and equilibrate to 25°C. Adjust pH to 7.4 with 1 M NaOH or 1 M HCl
B	250 mM Glycylglycine Buffer	Gly-Gly	dH_2O	5 mL	
C	Glucose-6-Phosphate	G-6-P	dH_2O	1 mL	
D	20 mM B-NADP Solution	B-NADP	dH_2O	1 mL	
E	300 mM Magnesium Chloride Solution	$MgCl_2$	dH_2O	1 mL	

Appendix E: Sulfite Oxidase Assay

Table 14: Sulfite Oxidase Assay Mixture (Per 1.5 mL Run)			
	Reagent	**Test**	**Blank**
A	Tris HCl Buffer	1385	1385
B	Sodium Sulfite Solution	15	0
C	Cytochrome c	50	50
D	Deionized Water	0	15
F	*Pisolithus* Extract	50	50
	Total	**1500 µL**	**1500 µL**

Table 15: Sulfite Oxidase Assay Reagent Composition					
	Reagent	**Component**	**Diluent**	**pH**	**Notes**
A	100 mM Tris HCl Buffer	Trizma Base	Distilled H_2O	8.5 @ 25°C	Adjust w/ 1 M HCl
B	33 mM Sodium Sulfite Solution	Sodium Sulfite	Reagent A		
C	2 mM Cytochrome c	Cytochrome c	Reagent A		
D	~0.3% (v/v) *Pisolithus* Extract	*Pisolithus*	Reagent A (~3mL)		Used ~1 cm^3 piece of *Pisolithus*

Appendix F: Polyphenol Oxidase Assay

Table 16: Polyphenol Oxidase Assay Mixture (Per 1.5 mL Run)				
	Reagent	**Test**	**Blank**	**Control**
A	Distilled H_2O	330 µL	335 µL	330 µL
B	KH_2PO_4 Buffer	1040 µL	1050 µL	1140 µL
C	Chlorogenic Acid	50 µL	50 µL	0
D	Ascorbic Acid	50 µL	50 µL	0
E	EDTA	15 µL	15 µL	15 µL
F	*Pisolithus* Extract	15 µL	0	15 µL
	Total	**1500 µL**	**1500 µL**	**1500 µL**

Table 17: Polyphenol Oxidase Assay Reagent Composition					
	Reagent	**Component**	**Diluent**	**pH**	**Notes**
A	50 mM Potassium Phosphate Buffer	KH_2PO_4	Distilled H_2O	7.5 @ 30°C	Adjust w/ 1 M KOH
B	10 mM Chlorogenic Acid	Chlorogenic Acid	Reagent A		
C	1 mM $_L$-Ascorbic Acid	$_L$-Ascorbic Acid	Reagent A		
D	100 mM EDTA	EDTA	Distilled H_2O		
E	10% (w/v) *Pisolithus* Extract (~200mg)	*Pisolithus*	Reagent A (~2mL)		Exactly 10% (w/v)

Polyphenol Oxidase Assay NA-1cr Run 1

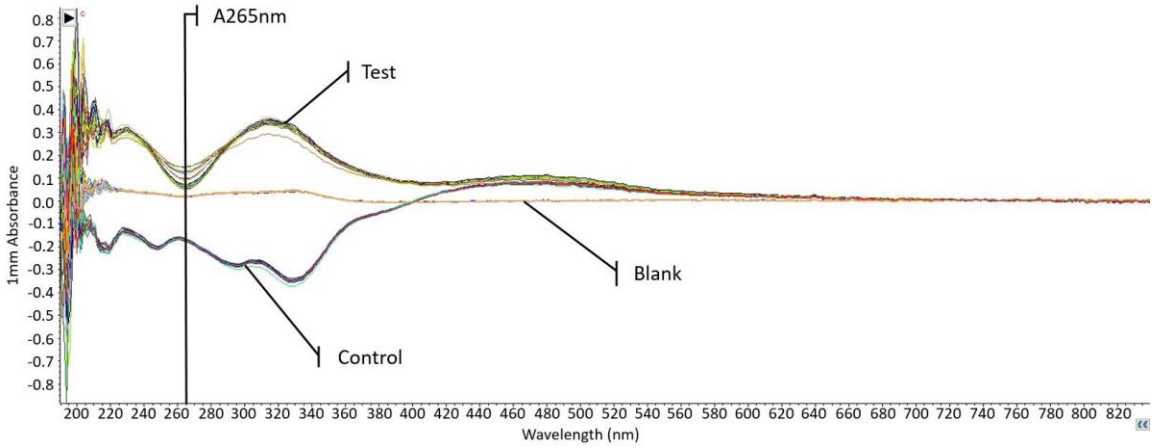

Figure 29: Polyphenol Oxidase Assay NA-1cr Run 1

Polyphenol Oxidase Assay NA-1cr Run 2

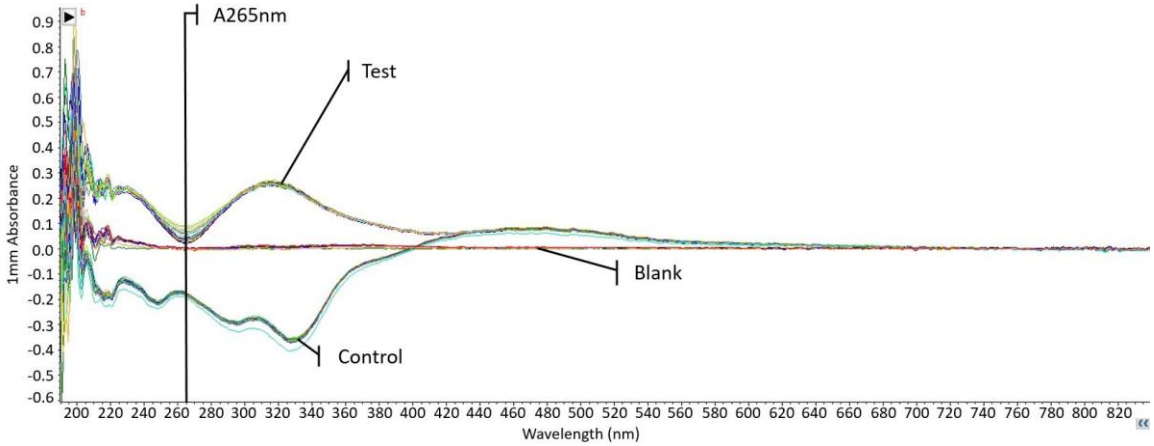

Figure 30: Polyphenol Oxidase Assay NA-1cr Run 2

Polyphenol Oxidase Assay NA1-cr Run 3

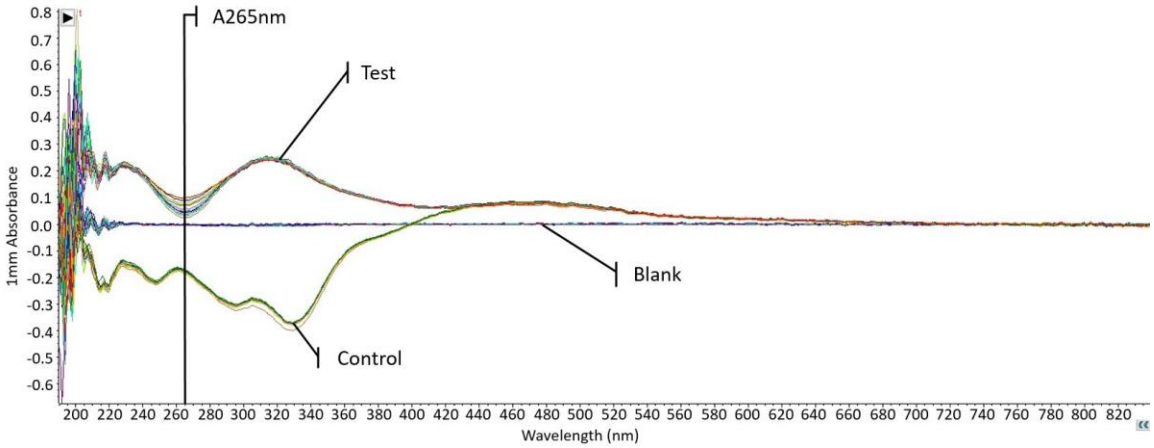

Figure 31: Polyphenol Oxidase Assay NA-1cr Run 3

Polyphenol Oxidase Assay NP1-cr Run 1

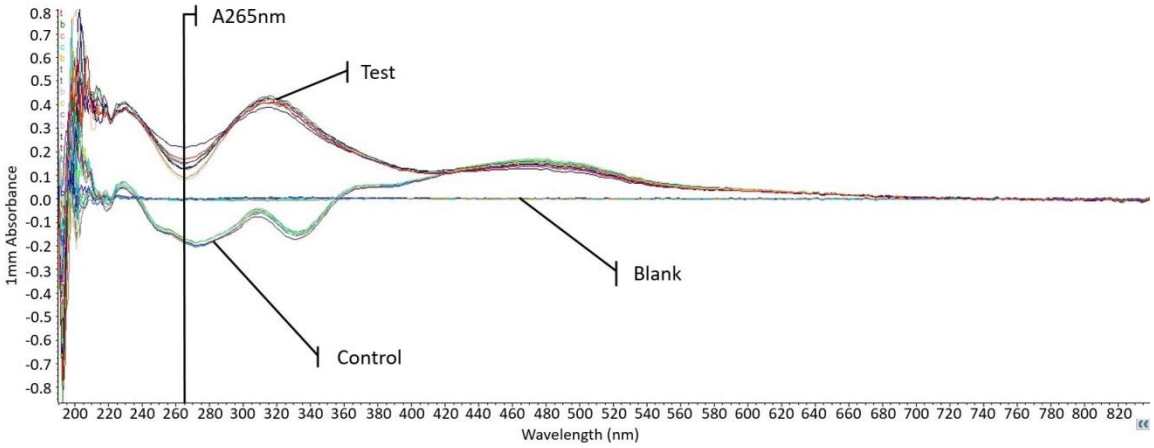

Figure 32: Polyphenol Oxidase Assay NP1-cr Run 1

Polyphenol Oxidase Assay NP-1cr Run 2

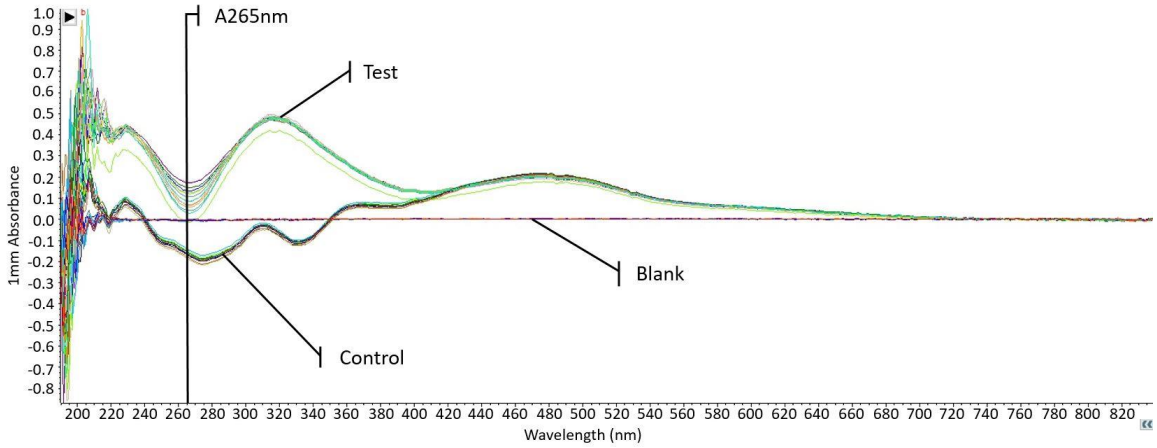

Figure 33: Polyphenol Oxidase Assay NP1-cr Run 2

Polyphenol Oxidase Assay NP-1cr Run 3

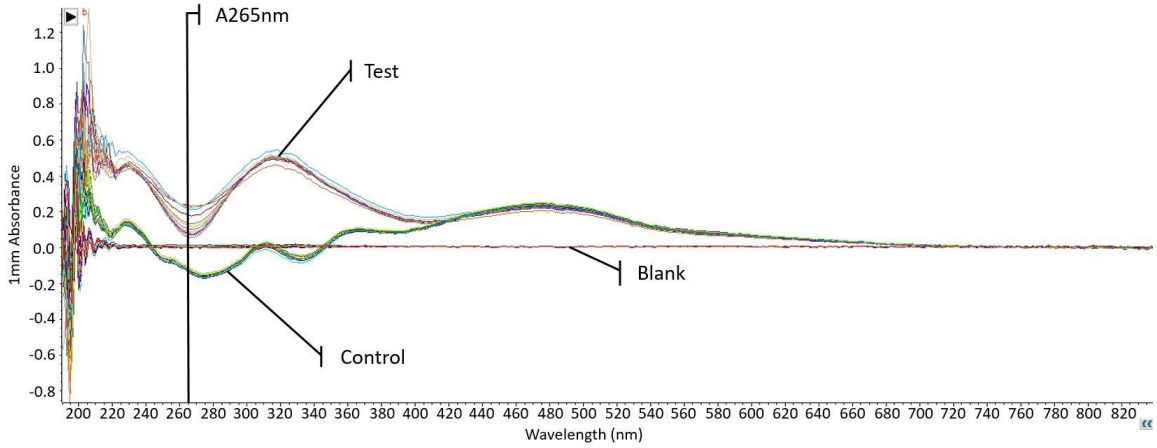

Figure 34: Polyphenol Oxidase Assay NP1-cr Run 3

Appendix G: Bradford Assay

Table 18: Dilution scheme for BSA standard ampules (2000 µg/mL) for a working protein range of 1-25 µg/mL			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	2370 µL	30 µL of Stock	25 µg/mL
B	4950 µL	50 µL of Stock	20 µg/mL
C	3970 µL	30 µL of Stock	15 µg/mL
D	2500 µL	2500 µL of vial B dilution	10 µg/mL
E	2000 µL	2000 µL of vial D dilution	5 µg/mL
F	1500 µL	1500 µL of vial E dilution	2.5 µg/mL
G	5000 µL	0	0 µg/mL = Blank

Bradford Standard Curve

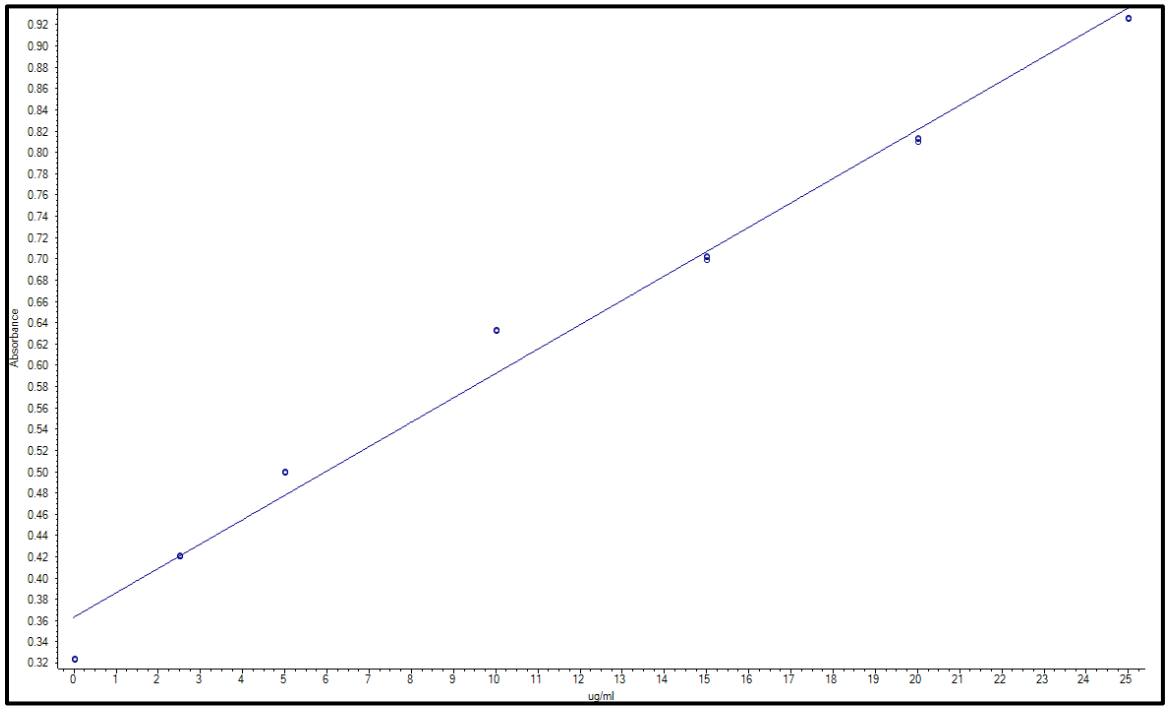

Figure 35: Bradford Standard Curve

Table 19: Protein Content of Various Edible Mushrooms - USDA Data	
Mushroom Type	Protein Content (percent)
Chanterelle	1.5%
Maitake	1.9%
Morel	3.1%
Portabella	3.1%
Shiitake	2.2%

Appendix H: SDS-PAGE PPO Activity Stain

Table 20: SDS-PAGE 2X Loading Dye		
Reagent	**Concentration**	**Volume**
Bromophenol Blue	0.4% (w/v)	0.75 mL
glycerol	100%	3 mL
Tris Buffer pH 6.8	1 M	1.25 mL
SDS	10% (w/v)	4.0 mL
Distilled water		1.0 mL
TOTAL		**10 mL**

Table 21: SDS-PAGE 1.0 M Tris-HCl buffer pH 6.8 (per 100mL) Running Buffer	
Reagent	**Mass/Volume**
Trizma base	12.1 g
Concentrated HCl	Adjust to pH 6.8
Distilled water	80 mL
TOTAL	Fill to 100mL with distilled H_2O

Table 22: SDS-PAGE PPO Activity Stain Conditions			
Experiment	**$NaPO_4$ Buffer pH**	**Substrate**	**Substrate Concentration**
Substrate Specificity	7.2	Chlorogenic acid	33 mM
Substrate Specificity	7.2	Catechol	100 mM
Substrate Specificity	7.2	4-methylcatechol	100 mM
Substrate Specificity	7.2	L-DOPA	8 & 20 mM
Effect of pH	5.1	Catechol	100 mM
Effect of pH	5.8	Catechol	100 mM
Effect of pH	6.5	Catechol	100 mM
Effect of pH	7.2	Catechol	100 mM
Effect of pH	7.9	Catechol	100 mM
Effect of pH	8.6	Catechol	100 mM
Monophenolase Test	7.2	Tyrosine	8 mM
Monophenolase Test	7.2	4-methylcatechol	0.1 mM
Monophenolase Test	7.2	Tyrosine and 4-methylcatechol	8 mM, 0.1 mM
Monophenolase Test	7.2	Tyrosine	20 mM
Monophenolase Test	7.2	Chlorogenic acid	0.1 mM
Monophenolase Test	7.2	Tyrosine and chlorogenic acid	20 mM, 0.1 mM

Appendix I: Supplemental Data

The data in this supplemental section are not fully presented, but were important in my thinking process. The FTIR and H_2S probe data are not reference in this thesis, but the O_2 probe data is referenced as unpublished data. These data were not presented and discussed in complete detail because they did not directly fit the topics discussed in this thesis.

Small granules from inside *Pisolithus* were identified as silica using FTIR.

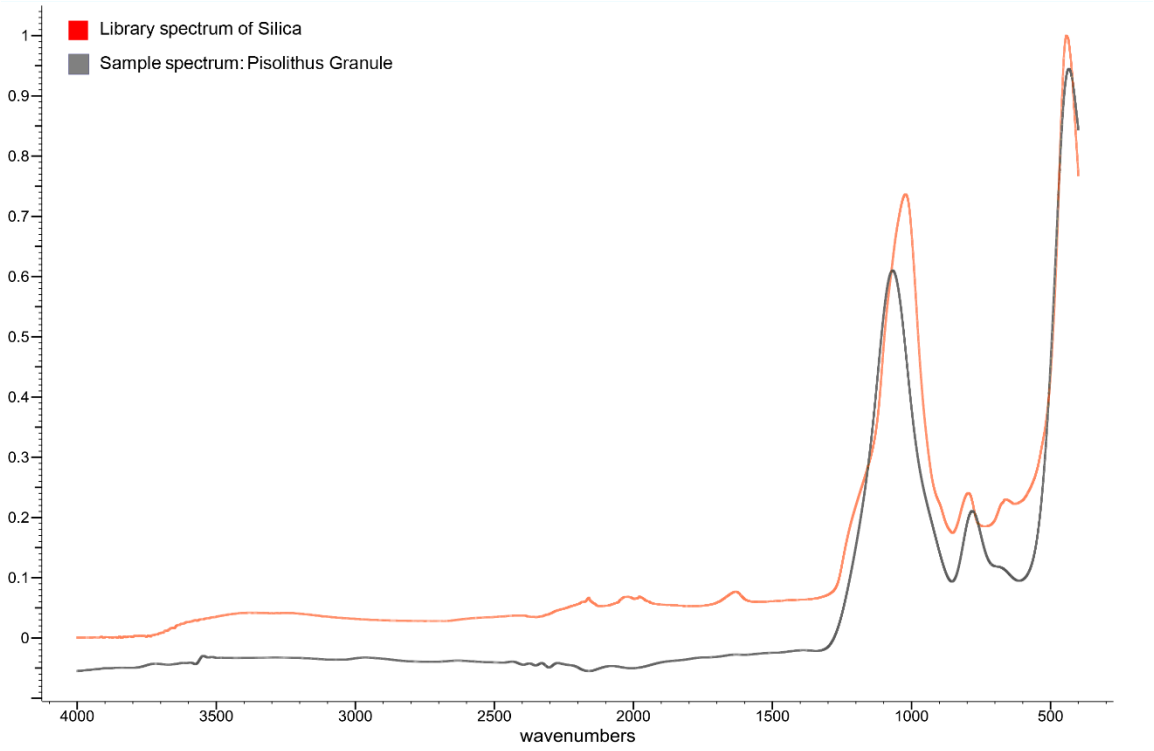

Figure 36: FTIR

Oxygen probes were used to investigate how O_2 levels change from the outside of *Pisolithus* to ~40 mm into *Pisolithus*. Readings begin at ambient O_2 levels and rapidly drop to completely anaerobic before increasing to as much as 4X ambient O_2 levels (Figures 37-39).

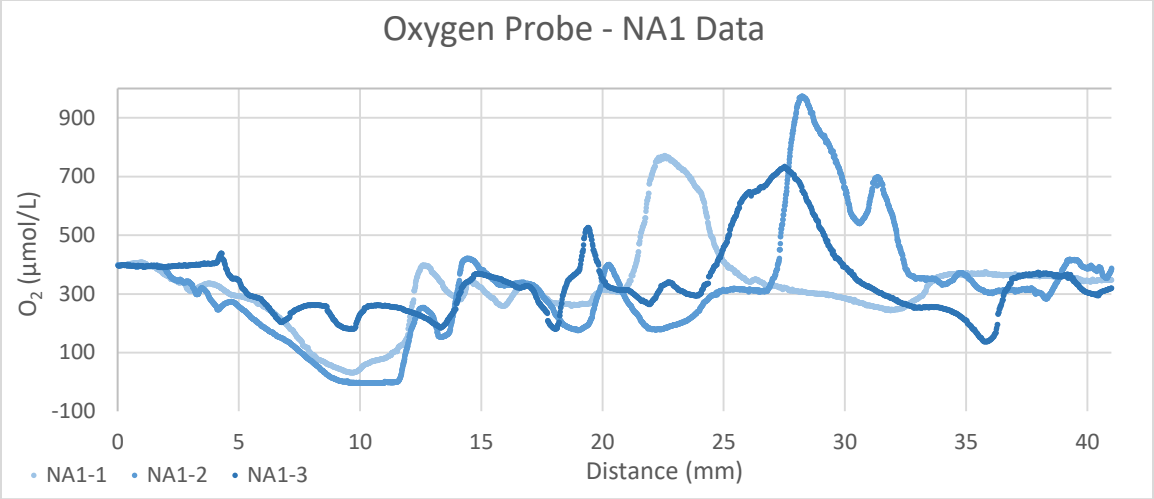

Figure 37: Oxygen Probe Graph: NA1-1, NA1-2, & NA1-3

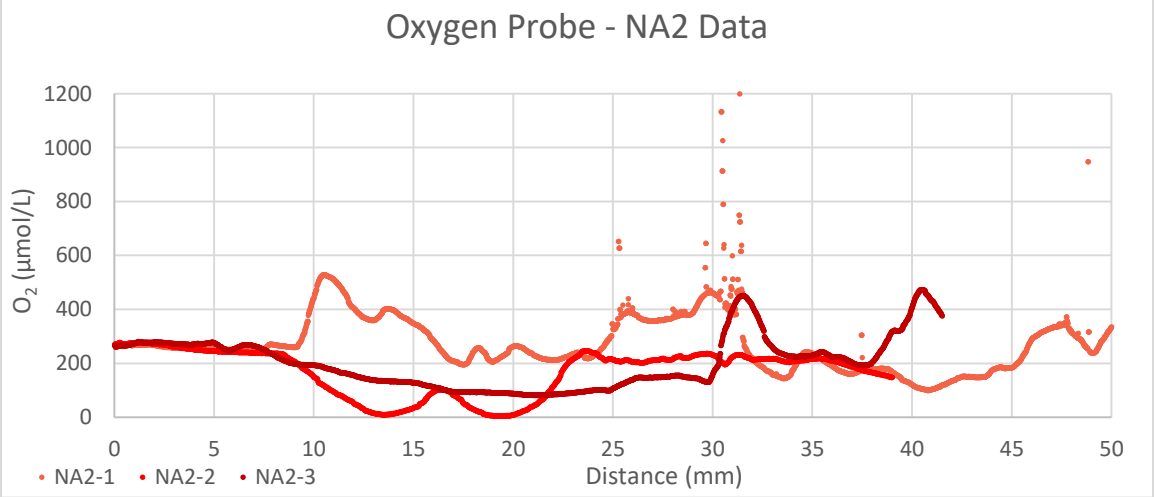

Figure 38: Oxygen Probe Graph: NA2-1, NA2-2, & NA2-3

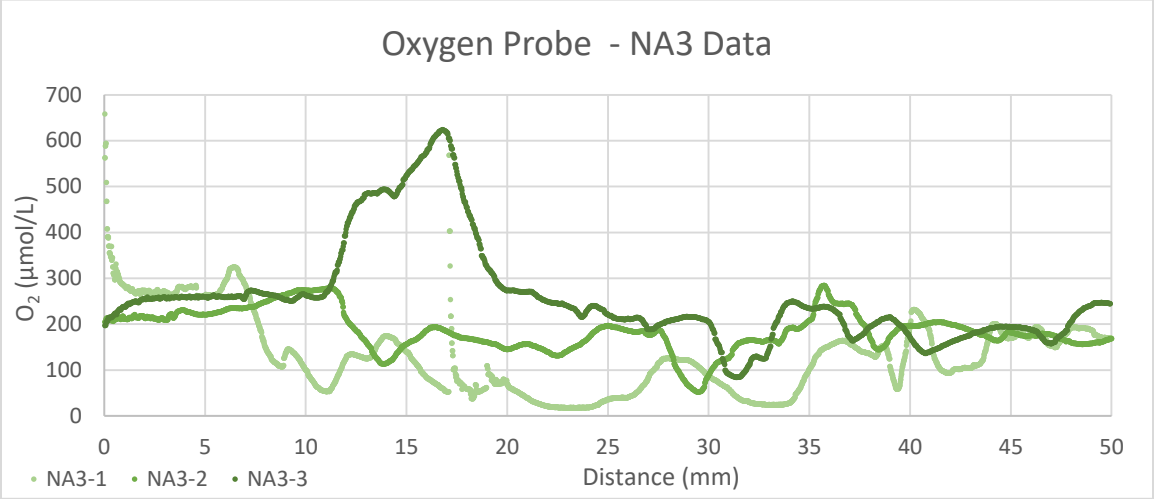

Figure 39: ***Oxygen Probe Graph: NA3-1, NA3-2, & NA3-3***

Hydrogen sulfide probes were used to show how H_2S levels change from the outside of *Pisolithus* to ~40 mm into *Pisolithus*. Readings begin at ambient H_2S levels (which are naturally high in NGB) and gradually decrease the deep the probe goes into *P. arrhizus*.

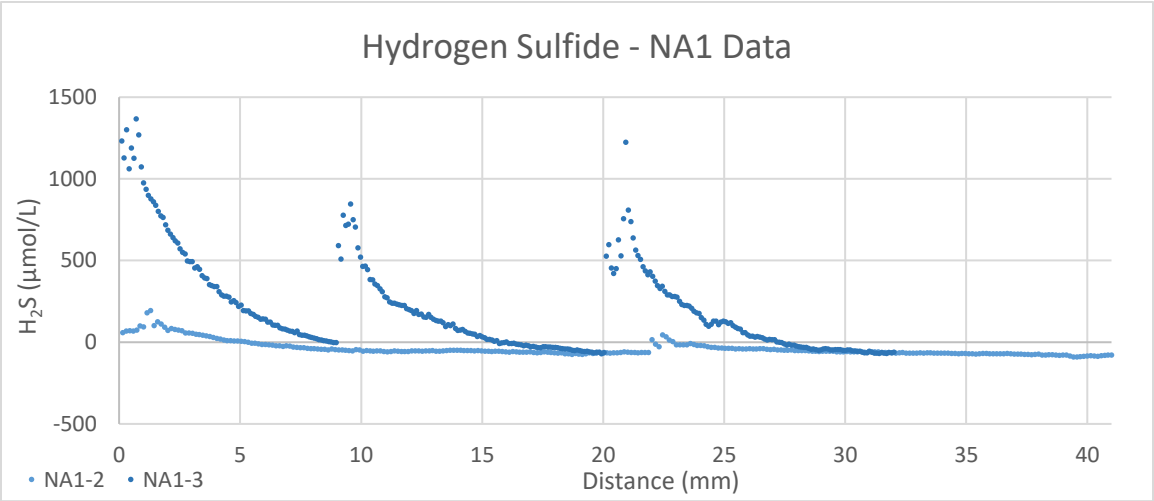

Figure 40: ***Hydrogen Sulfide Probe Graph: NA1-1, NA1-2, & NA1-3***

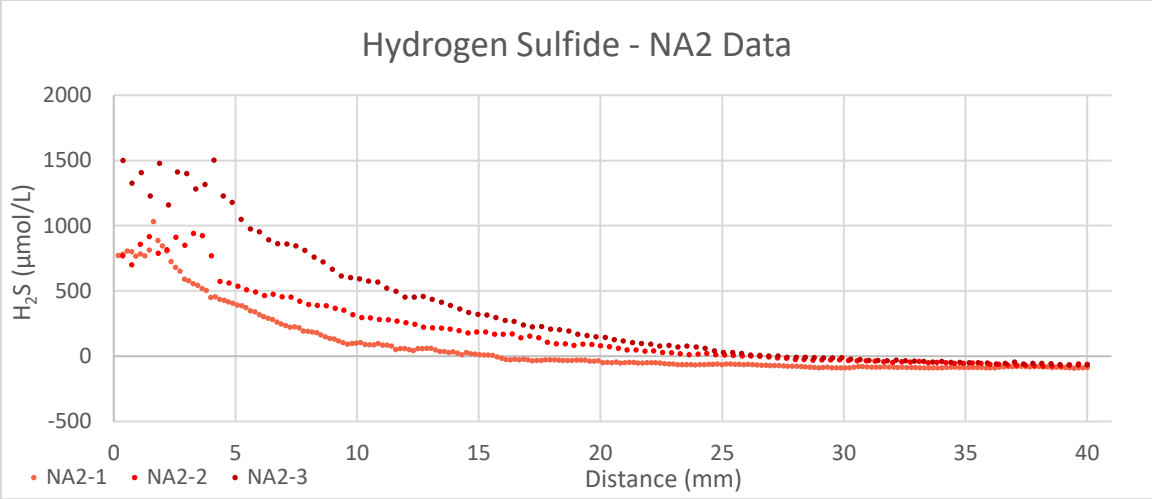

Figure 41: Hydrogen Sulfide Probe Graph: NA2-1, NA2-2, & NA2-3

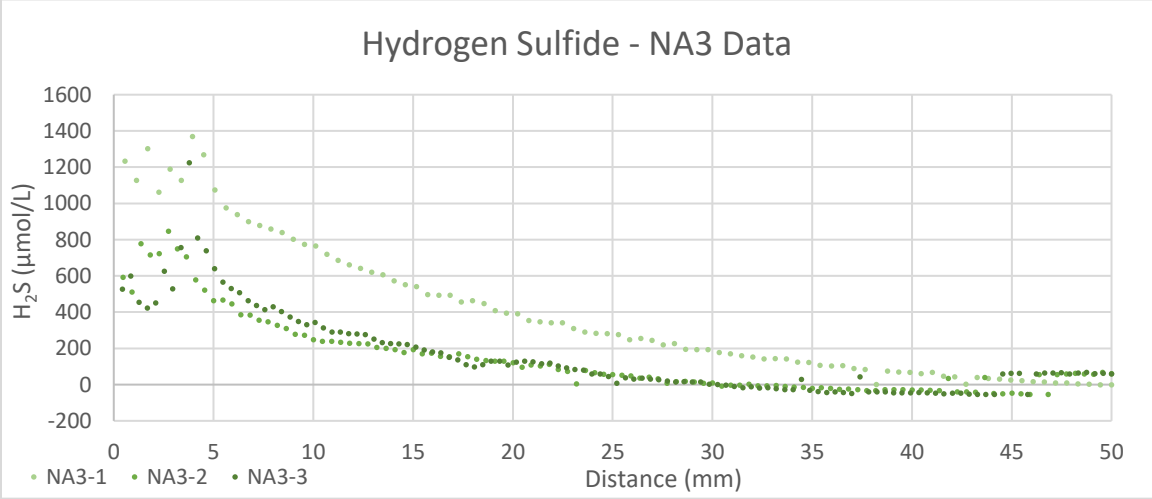

Figure 42: Hydrogen Sulfide Probe Graph: NA3-1, NA3-2, & NA3-3