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Overexpression and Characterization of a Laccase from Geobacillus Thermoglucosidasius

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OVEREXPRESSION AND CHARACTERIZATION OF A LACCASE FROM *GEOBACILLUS THERMOGLUCOSIDASIUS*

BY

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THESIS

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DEDICATION

I lovingly dedicate this work to my parents.

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ABSTRACT

OVEREXPRESSION AND CHARACTERIZATION OF A LACCASE FROM

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The use of enzymes as industrial oxidants has become popular due to their high substrate specificity and mild reaction conditions. Specifically, laccases are multi-copper oxidases that can oxidize a disparate range of organic substrates using oxygen and producing water as a byproduct without requirement for additional reactive compounds. Currently, all laccases used in industrial processes are fungal in origin. Although fungal laccases have high activities under near-ambient conditions, their use is limited at higher temperatures. Also, expression of fungal laccases in heterologous hosts is limited due to incorrect glycosylation. Bacterial laccases are much easier to express heterologously and are more active and stable at high temperatures, pH and salt concentrations.

Geobacillus is a genus of gram-positive thermophilic bacteria, many of which have been found to naturally secrete proteins at high levels. A novel laccase has been predicted to be present in multiple *Geobacillus* strains using comparative genomics. This laccase is

approximately half the size of those found in other gram-positives or fungi, making it a better candidate for lignocellulosic biomass degradation because of easier access to the substrate. In this work, we seek to isolate and characterize this laccase, and determine the types of substrates it can oxidize. We then want to compare the activity of our laccase with that of a fungal laccase at different temperatures.

A plasmid was successfully constructed for the overexpression of laccase in *Geobacillus thermoglucosidasius 95A1* and *Escherichia coli DH5α*. The novel laccase was isolated and purified from *E. coli*. The laccase was characterized by determining the activity for 5 substrates at a range of pHs and temperatures. Finally, the thermal stability of our laccase was compared with that from a fungal source, *Trametes versicolor*. Laccase from *G. thermoglucosidasius* demonstrated a 20-fold higher initial activity than *Trametes* laccase at 80°C, and was superior to the latter in terms of thermal stability and activity at high temperatures.

CHAPTER 1: INTRODUCTION

1.1 The future of liquid fuels

The supply of crude oil is finite and dwindling. According to the US Energy Information Administration, the world has enough crude oil to supply predicted demand for the next 25 years. However, variations between predicted and actual future demand may shorten the time we have left.[1] The world consumed over 89.4 million barrels of petroleum products per day in 2012, up from 84.7 million barrels per day in 2008.[2] These numbers are predicted to increase steadily due to the rising world population and higher rates of consumption in fast developing countries such as China. Out of the 89.4 million, the United States consumed 18.5 million barrels per day yet only produced 11.1 million barrels.[2] Although the supply of liquid transportation fuel produced in the US exceeds domestic demand, rising tensions in oil-producing countries and unpredictable price fluctuations are incentives for the United States to reduce our dependence on foreign petroleum products. Because the majority of liquid transportation fuels come from crude oil, there has to be a push for finding an alternative, domestic source.

For every 10 gallons of liquid transportation fuel from a petroleum source the United States consumes, only 1 gallon of biofuel from renewable resources is consumed. When compared to a country such as Brazil, which generates a net surplus of biofuels and consumes almost no gasoline from a petroleum source, it would appear that the United States is lagging behind in terms of becoming independent from fossil fuels.[2]

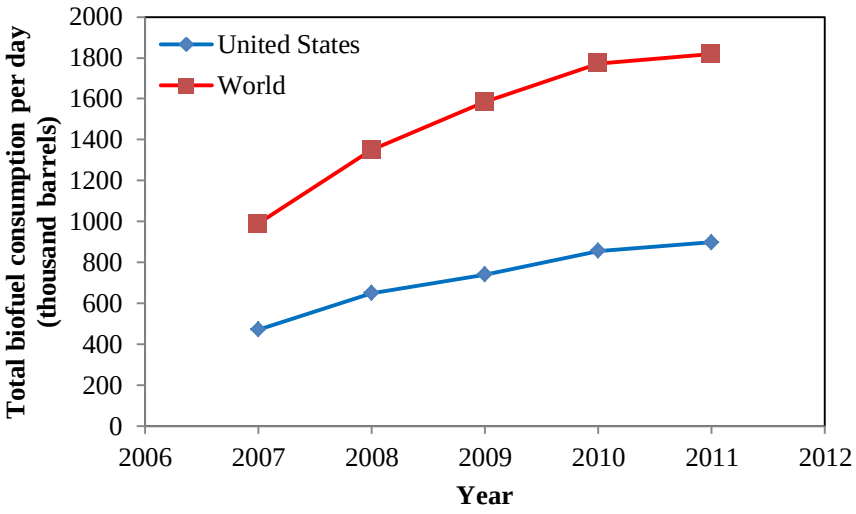

Figure 1. Per diem consumption of biofuels in the world and United States, adapted from data from the U.S. Energy Information Administration[2]

Government funded subsidies and research are leading to a shift from fossil fuels to renewable biofuels. In 2005, congress passed the Energy Policy Act, which was later supplemented with the Energy Independence and Security Act of 2007. These acts created a renewable fuel standard, which mandated the amounts of biofuels that are to be produced in future years.[3]

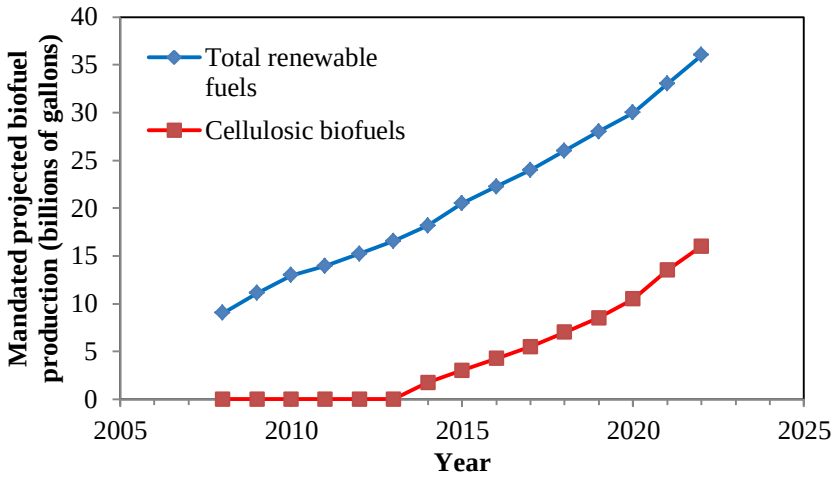

Figure 2. Projected US biofuel production, adapted from the Energy Independence and Security Act of 2007[3]

A "green movement" is sweeping the country as more and more people realize that we cannot forever rely on fossil fuels to satisfy our increasing need for energy. Raised awareness in both the private and public sectors has led to increased funding and research in the field of renewable energies and specifically, biofuels. Skyrocketing fuel costs, diminishing reserves and escalating tensions with oil-producing nations are causing a push away from fossil fuels and towards generating transportation fuels via renewable resources such as biomass. This increasing demand must be met with equal advances in the technology of synthesizing biofuels from biomass.

1.2 Deconstruction of lignocellulosic biomass

1.2.1 Thermochemical vs. biochemical methods

Most of the energy available to us on Earth comes from the sun. Plants naturally and efficiently convert this energy into lignocellulosic biomass. By using biomass to produce biofuels, we are indirectly taking advantage of the efficient conversion of light to chemical energy via plants. Lignocellulosic biomass generally refers to plant-based materials that are composed of three polymers- cellulose, hemicellulose and lignin. It is the most abundant renewable raw material for biofuel production. Cellulose is a straight chain polymer composed of multiple D-glucose molecules. It is found along with hemicellulose, a less-structured polymer of different kinds of sugar molecules such as pentose, arabinose and xylose, in the cell wall of plant cells. Filling in the spaces between the cellulose and hemicellulose polymers is lignin, a very complex polymer comprised of different aromatic alcohols. Unlike cellulose and hemicellulose, lignin is hydrophobic, does not have a conserved or repetitive structure, and presents the greatest obstacle for biochemical decomposition.[4,5]

One method of converting biomass to biofuels is pyrolysis. Pyrolysis involves burning the biomass at high temperatures (generally greater than 800°C), which converts the organic compounds in the biomass to smaller molecules. Some, such as hydrogen, carbon monoxide and methane are useful while others like carbon dioxide and nitrogen are not. This mixture is generally referred to as "syngas," which can be further converted to liquid hydrocarbons with the use of catalyst. One of the most common reactions, the Fischer-Tropsch reaction, converts hydrogen and carbon monoxide into a mixture of alkanes, alkenes and alcohols over a transition metal catalyst.[6] Thermochemical conversion of lignocellulosic biomass is relatively independent of lignin content. Reactors that were built to pyrolyze one type of biomass can usually pyrolyze a different type of biomass during an "off-season." Current facilities designed to produce synthesis gas from coal can also take advantage of biomass by blending coal with biomass with minimal changes to plant infrastructure.[4] However, pyrolysis involves decomposition at high temperatures and pressures, which can foul catalysts or in some cases, permanently damage them. Heating the biomass to high temperatures is also an energy intensive process, and although strides have been made in recycling heat from downstream processes, the entropy generated in the overall pyrolysis reactions hinders the thermodynamic efficiency of thermochemical conversion of biomass to biofuels.[4]

An alternative to thermochemical deconstruction, and the motivation behind this work, is biochemical decomposition of lignocellulosic biomass. The first step in this is size reduction of the biomass. The biomass must be ground up to small enough sizes to facilitate the effective mass transfer of reagent molecules to and through the biomass. Pretreatment with weak acids has been shown to increase the porosity of biomass, and can help increase the solubility of some sugars.[4,5] Exposing cellulose to the hydrolyzing enzymes is key for effective degradation.

Although biochemical decomposition does not require large amounts of heat energy like thermochemical decomposition, pretreatment can be expensive, often prohibitively so. The lignin content of the biomass also presents challenges. Access of the cellulose and hemicellulose to the hydrolyzing enzymes is blocked by lignin. Current research in this area is focusing on two paths of biochemical degradation- developing microbes that can convert constituent sugars in the plant wall into fuels such as ethanol, and searching and engineering enzymes that degrade lignin to allow access to cellulose and hemicellulose. There has been research using *Geobacillus* to accomplish the former.[7] However, the focus of this work is on the latter- the investigation of a novel laccase, some of which have lignin-degrading capabilities. Removing lignin from biomass will allow better access of hydrolyzing enzymes to the key sugars found in cellulose and hemicellulose, and will increase the cost efficiency of biochemical degradation.

1.2.2 Bottlenecks- deconstruction of lignin

Lignin serves as the backbone that holds the cellulose and hemicellulose together and gives plants their structure. The structure of lignin, shown here in figure 3, is extremely complex and will vary widely across plant species.[8]

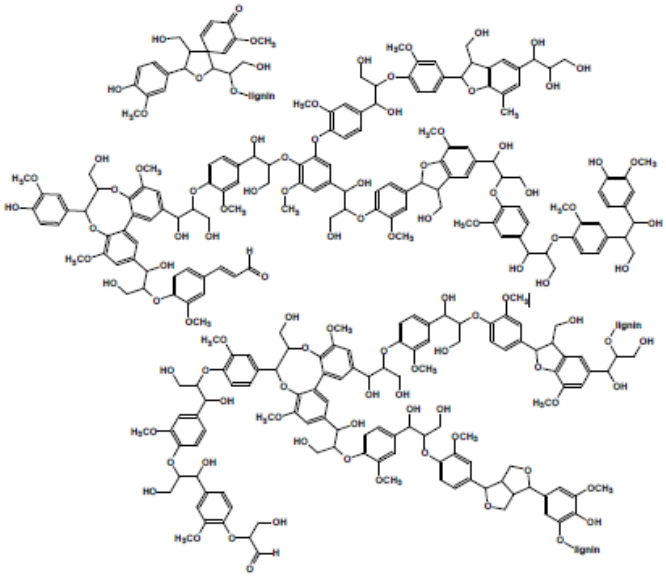

Figure 3. A sample structure of lignin from a softwood[8]

Lignin contains a large amount of aromatic compounds in contrast to cellulose and hemicellulose, which are not aromatic. According to Carroll and Somerville, a 5% increase in lignin content can lead to a 2-fold decrease in sugar yield.[4] Because of the complex and varied structure, it is difficult to find a single chemical modification that will work on all biomass sources. However, lignin is comprised of two main "building blocks"- guaiacol and syringol molecules linked via carbon-carbon and carbon-oxygen bonds.[8] Because lignin is tightly wound in gaps between cellulose and hemicellulose, mass transfer of hydrolyzing enzymes into the biomass can limit degradation. Thus, there is a need for an "all-purpose" enzyme that will degrade the guaiacol and syringol building blocks and allow access to the inner structure of the biomass. [9]

The pulp and paper industry currently use laccases to remove lignin from pulp and to improve general paper quality and texture.[9,10] Laccases have been proven to be effective oxidizers of organic materials, especially those containing aromatic compounds such as lignin.[5,9]

It should therefore be possible to pretreat biomass with laccases prior to enzymatic hydrolysis, or to use both hydrolytic and ligninalytic enzymes together to allow access to and degradation of the cellulosic matrix. This approach still faces multiple challenges. During the degradation of cellulosic biomass, compounds that are harmful to some microbes are produced.[4] The laccases would need to be stable in the presence of a variety of other compounds. The laccases should also be thermally stable. Higher temperatures will lead to faster decomposition rates. Lastly, the laccase macromolecule needs to be small enough to penetrate into the lignocellulosic matrix to react and degrade the aromatic compounds in lignin.

1.3 Laccases

1.3.1 Occurances of laccases in nature and their function

Laccases belong to the oxidase family of enzymes, and are related to the peroxidases, but use molecular oxygen as a co-substrate rather than hydrogen peroxide. They are widely dispersed in nature, occurring in plants, fungi and some bacteria. Laccases were first discovered and studied in *Rhus vernicifera*, the Japanese lacquer tree, in 1883, which is where the name laccase comes from. In this species, it was found to harden and darken the sap, which is linked to defense mechanisms and wound healing.[11] The role of plant laccases is not as defined as those from fungi or bacteria.[9,11] Plant laccases are generally extracellular proteins, and are more highly glycosylated. They have an optimum pH range between 5 and 7.5[12] It is generally agreed that laccases play a role in lignin polymerization and cell wall structure.[10]

Fungal laccases are the most widely studied. In contrast with plant laccases, most fungal laccases are responsible for lignin degradation. Most notable are the laccases of white-rot fungi, which use plants as hosts, and can consume degraded lignin by secreting laccase.[13] Fungal

laccases generally show the highest activity at pHs between 3.5 and 5.5.[12] Because the enzymatic degradation of lignin can release compounds that are toxic either to the fungus or the laccase, it is also thought that fungal laccases share a role in detoxification of these byproducts.[9] Although the sequences at the active sites of fungal laccases are conserved, the location of the enzyme within the cell and the degree of glycosylation varies widely between species.[10]

Bacterial laccases are the newest and least studied. Although not common throughout the bacterial domain, they are widespread within specific genera such as *Streptomyces*, *Azospirillum*, *Bacillus and Geobacillus*.[14] There are also limited occurrences in gram-negatives thought to be linked to providing the cell with copper resistance, since free copper is toxic to the cell.[15] As of yet, there is very limited use of bacterial laccases in industrial processes. Unlike fungal laccases, bacterial laccases are not glycosylated and maintain activity at higher pHs.[13,14] The most widely studied laccase from bacteria is CotA from *Bacillus subtilis*. CotA is present in the outer surface of the bacterial endospore, and is responsible for spore pigmentation and structure. A highly stable protein, it protects the spore from heat and UV light.[15] Although bacterial laccases share a similar overall secondary structure with fungal laccases, single amino acid substitutions change the structure and function within the 3 primary domains of the enzyme. Because endospores are capable of tolerating abnormally high temperatures without degradation, the thermal stability of their laccases are of interest to this work.

1.3.2 General structure and reaction mechanisms

Although there are significant differences within domains between laccases from difference sources, there is a general conserved overall structure between all laccases. Glycosylation in fungal laccases is thought to be responsible for secretion, increased activity and thermal stability, whereas bacterial laccases lack glycosylation.[13] Most laccases contain 4 copper

atoms at the catalytic center of the enzyme, and are held in place by histidine-rich amino acid regions. Mutated laccases in which histidines were substituted for other amino acids showed a significant reduction in activity.[15] The copper atoms are responsible for electron exchange and consequently, enzymatic activity. There are three "types" of copper, designated T1 to T3. Oxidation of the substrate occurs at the T1 copper site, and the reduction of molecular oxygen to water occurs at the T2 and T3 coppers.[13,16]

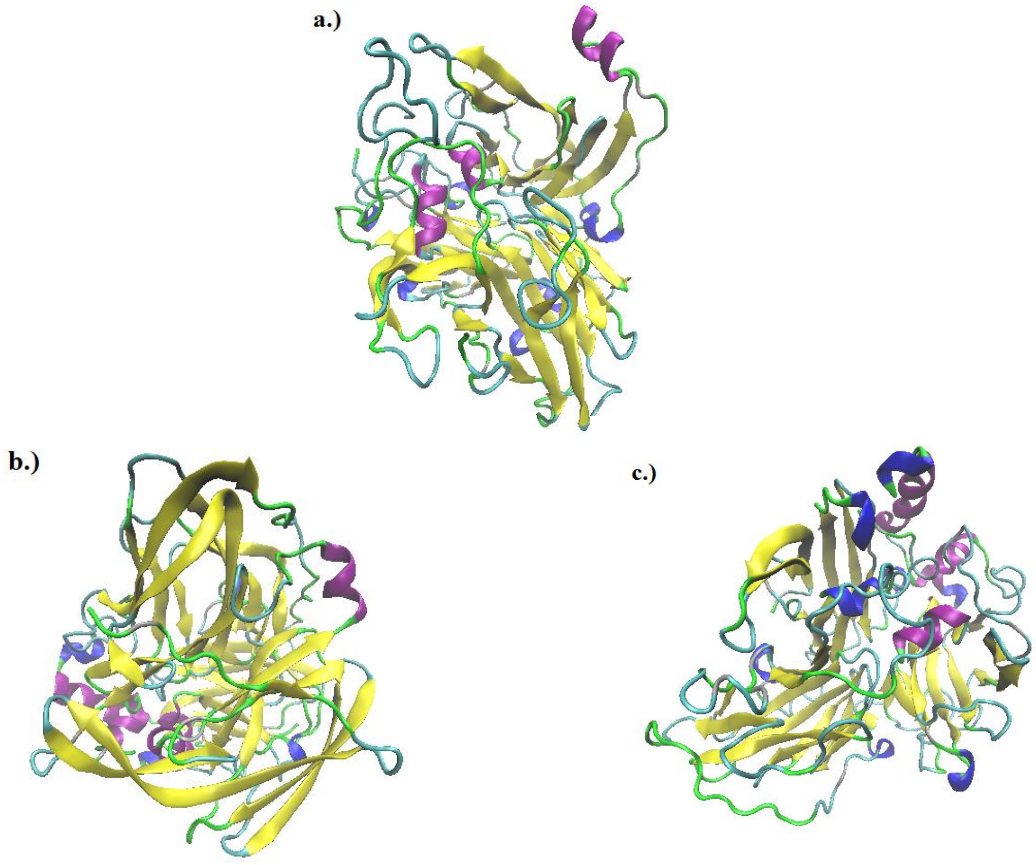

Figure 4. Ribbon diagrams for naturally occurring laccases from different sources. a.) CotA from Bacillus subtilis[17]. b.) CueO from E. Coli[18]. c.) laccase from Trametes versicolor[19]. α-helices are shown in purple, 3,10 helices are in blue, extended β-sheets are in yellow, bridge β-sheets are in silver, turns are in cyan and coils are in green.

Bacterial laccases are the focus of this work, and the CotA laccase isolated from *Bacillus subtilis,* shown in Figure 4, is a model bacterial laccase.[15] Most laccases contain three distinct domains, although some recent work suggests that some bacterial laccases have only two domains.[16] Starting at the N-terminal end, the first domain contains an 8-stranded β-barrel and a coiled subdomain that connects the first and second domain with hydrogen bonding. Note that this coil appears in bacterial but not fungal laccases, and may contribute to the thermal stability of bacterial laccases.[15] The second domain is a 12-strand β-barrel, whose signature component is an extended loop that connects the second and third domains. The third domain contains the T1 copper site and is distinguished by an α-helix extended outward from the enzymatic center. This α-helix forms a "lid," and may control substrate access to the active site. The interface connecting the first and second domains gives the CotA laccase a more compact configuration than the CueO or fungal laccase, and is hypothesized to contribute to its thermal stability.[15]

Most laccases oxidize substrates using the same general reaction mechanism. Laccases have the ability to join monomers to create polymeric compounds, cleave aromatic ring compounds, and degrade polymers.[14,15] This study is interested in lignin degradation, and thus the last two classes of reactions. The oxidation of phenolic compounds follows a slightly different reaction pathway than non-phenolic ones. For phenolic compounds, the substrate must find the active site of the enzyme where the T1 copper is located. A single electron is transferred from the substrate to the T1 copper, forming a free radical. This unstable radical can either undergo further oxidation by the laccase by the removal of another electron, or it can react with solvent molecules or other substrate molecules in a non-enzymatic reaction. The single electron at the T1 copper is transferred through the interior of the enzyme to the T2 and T3 coppers. It is here that the electron is transferred to molecular oxygen, which is reduced to water. [13]

Laccase can also oxidize both phenolic and non-phenolic compounds through a slightly different pathway with the help of a redox mediator molecule.[13,14,15] ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] is a model compound for this purpose. The mediator acts as an "electron shuttle," which transports the electron from an ABTS molecule to the T1 copper, which forms the ABTS radical. The ABTS radical, which is very reactive, can oxidize other substrate molecules. The transfer of the electron from the T1 copper to the T2 and T3 coppers follows the same path as laccase reacting with a phenolic substrate described above. This has been proven in the pulp and paper industry, where ABTS can diffuse into lignocellulosic biomass, disrupting lignin structure and allowing for further oxidation of aromatic monolignols by laccase.[13]

It is important to note that the T2 and T3 coppers do not participate in the single electron transfer from the substrate to the copper atoms. Rather, the T2 and T3 coppers can only reduce molecular oxygen to water. This is because the T2 and T3 coppers are "buried" in the interior of the laccase, whereas the T1 copper is near the surface of a relatively shallow pocket that comprises the active site. Large substrate molecules can interact with the T1 pocket near the active site but steric hindrance prevents interaction with the T2 and T3 coppers. Oxygen is the only substrate molecule small enough to diffuse through the laccase to the T2 and T3 coppers where it is reduced.[13]

1.3.3 Industrial uses

Laccases have numerous industrial applications across a wide range of disciplines. Currently, all laccases in use by industry are from a fungal source.[20] The most relevant application to this work is the degradation of lignin in biomass in pulp and paper industrial streams.[10,21] This is accomplished through the use of redox mediators, the mechanism of which is

described in the previous section. Because the enzyme is too large to penetrate into lignocellulosic biomass, mediators such as ABTS are used to diffuse into the biomass and disrupt the lignin structure. This significantly increases the rate of degradation as opposed to using laccase without a mediator.[10] In addition to using laccase directly, there are some applications in which laccase-expressing organisms are used for biomass fermentation for ethanol production. Strains of yeast that were expressing laccase from a fungal source were shown to produce a higher yield of ethanol compared to those not expressing laccase.[10]

The second most common industrial application is dye decolorization. Textile dye effluents present an environmental pollution issue as some wastes need to be decolorized prior to disposal. Laccase has been shown to effectively decolorize commercial dyes such as congo red, crystal violet and bromophenol blue under slightly alkaline conditions.[10,20] Industrial effluents can contain other compounds that need to be oxidized prior to disposal. Enzymatic oxidation via laccase is safer and more efficient than chemical means of oxidation as less harmful byproducts are produced.[22]

There are many specialized uses of laccase in addition to lignin removal from biomass and wastewater decolorization. Laccases that degrade the phenolic compounds in lignin should also, in theory, degrade phenolic compounds found in pollutants such as oil spills. However, this is an untested application, and laccase use in an open environment may need additional stabilizing chemicals.[10] On the same note, laccases can be used as biosensors, detecting phenolic compounds in pollutants.[20] Finally, some more unique applications of laccase include the removal of phenolic compounds from white grapes for wine making, distinguishing between morphine and codeine for medical assays after injection, and as a herbicide degrader using ABTS as a redox mediator.[10]

1.3.4 Bacterial vs. fungal laccases

Although fungal laccases are most widely used in industry, the focus of this work is the advantages of using bacterial laccases. In their natural environment, both fungal and bacterial laccases show activity towards degrading lignocellulosic biomass. However, while fungal laccases are usually secreted, bacterial laccases are intracellular enzymes involved in sporulation or copper atom mitigation. Bacterial laccases are generally less active than fungal laccases under optimum conditions.[20] Current research suggests low enzyme yield in bacteria compared to fungi, and obtaining laccase from endospores is virtually impossible.[23] However, bacterial laccases offer significant advantages over fungal laccases. A coiled subdomain connecting the first and second domains is unique to bacterial laccases, and is hypothesized to contribute to the higher thermal stability of bacterial over fungal laccases.[17] Bacterial laccases are also more stable at alkaline pHs and higher salt and metal concentrations compared to fungal laccases.[14] The latter is of interest since the activity of some laccases is directly correlated with copper concentration in solution.[24] Lastly, the compact structure of bacterial laccases may allow for better diffusion into lignocellulosic biomass, whereas fungal laccases require mediators for lignocellulosic degradation.[17] The reasons listed above justify the further investigation of bacterial laccases.

1.4 Geobacillus thermoglucosidasius

1.4.1 Overview

Geobacillus, or "*earth*" *Bacillus*, is a genus of gram-positive, thermophilic bacteria. Strains of *Geobacillus* have been isolated from hot environments such as soil, hot springs and petroleum wells worldwide.[25] Until 1997, *Geobacillus* was classified under the genus of *Bacillus*. *Geobacillus thermoglucosidasius*, a species of *Geobacillus*, was isolated from soil in Japan in 1983.[26] Its name is a combination of *therme*, meaning heat, and *glucosidasius*, meaning the

utilization of starch as a food source. It is a rod-shaped, gram positive bacterium approximately 3.0 μm long and 0.9 μm in diameter. *G. thermoglucosidasius* is capable of both aerobic and anaerobic metabolism, and can form one bacterial endospore per cell upon cell stress.[26] The endospores are resistant to heat, UV light, and solvents, allowing them to carry on cellular DNA and reproduce cells should the environment become favorable to cell growth. *Geobacillus* is capable of growing at temperatures between 37°C and 75°C, with optimum growth between 55°C and 65°C. This is abnormally high for most bacteria.[25] The stability of the bacterium at high temperature leads researchers to believe that enzymes produced by the cell are also thermostable.

1.4.2 Current research

Enzymes isolated from *Geobacillus* are of interest to researchers due to retained activity at high temperatures. It is confirmed that *Geobacillus* can thrive in hot environments because its enzymes are stable at high temperatures.[27,28] AUT-01, a strain of *G. thermoglucosidasius*, has been shown to express acrylamidase, an enzyme capable of degrading acrylamide and acrylamide-containing compounds. AUT-01 is capable of using acrylamide as a carbon/nitrogen source, which lends itself to bioremediation applications due to its toxicity and presence in land pollution and even some foods.[27] Heterologous expression of a pyruvate decarboxylase in *Geobacillus* has also been investigated. Pyruvate decarboxylase, along with alcohol dehydrogenase, allow *Geobacillus* to ferment 5- and 6-carbon sugars for ethanol production.[29] The discovery of *Geobacillus* in the soils of oil producing fields has lead to the investigation of their potential use for bioremediation and biodegradation of crude oil.[30]

Research in the field of thermostable enzymes is driving the investigation of their potential use in industry. The production of thermostable enzymes using these bacteria has

significant industrial applications, such as the fermentation of sugars, and lignin and biomass degradation. Whereas enzymes isolated from other sources may have limitations such as activity at higher temperatures, this work aims to show that enzymes from *Geobacillus* are stable and active at temperatures as high as 80°C. Several US patents have been issued to researchers demonstrating the thermal stability of enzymes from *Geobacillus*, some of which are shown here in Table 1. The need for enzymes active at higher and higher temperatures is leading researchers to investigate thermophilic organisms such as *Geobacillus.*

Table 1. U.S. patents issued for enzymes from *Geobacillus*[31*]

Enzyme	Application	Patent #
α-arabinofuranosidase	deligifying wood pulp	US05434071[32]
acetate kinase	glycolysis/metabolism	US05610045[33]
α-amylase	hydrolysis of starch	US05824532[34]
DNA polymerase	DNA replication & construction	US05747298[35]
Neutral Protease	Protein degradation	US06103512[36]
Polynucleotide phosphorylase	RNA degradation	US04331762[37]
Pyruvate kinase	glycolysis	US04331762[37]
Xylanase	deligifying wood pulp	US05434071[32]
Xylosidase	wood pulp degradation	US05489526[38]

*Adapted from the Bacillus Genetic Stock Center Catalog

1.4.3 Laccases from *Geobacillus*

Enzymes from *Geobacillus* have proven to be stable and active at higher temperatures, which make them of interest to industrial applications. Specifically, this work is interested in laccases from *Geobacillus* strains for the degradation of lignocellulosic biomass. It is predicted that a novel laccase from *Geobacillus* will demonstrate activity at high temperatures, much like those listed in table 1. The completely annotated genome is only known for three *Geobacillus*

strains, however, the National Center for Biotechnology Information (NCBI) has predicted a laccase in the gDNA of *Geobacillus thermoglucosidasius* C56-YS93.[39] Specifically, the NCBI predicts a "multi-copper polyphenol oxidoreductase," with a protein ID of AEH 48673.1.[39] Furthermore, the Basic Local Alignment Search Tool (BLAST) provided by the NCBI can perform sequential alignments in other *Geobacillus* strains, which can be used to predict the presence of laccases in strains that may not be annotated.[40] The BLAST results are shown in table 2. The identity is the measure of the sequence "similarity" between the query sequence and the search results.

Table 2. Laccase-like sequences predicted in *Geobacillus* strains using BLAST[40]

Strain	Length (Amino Acids)	Identity	Accession No.
G. thermoglucosidasius (C56-YS93)	272	N/A*	WP 003251898.1
G. sp. Y4.1MC1	272	99%	WP 013401304.1
G. sp. WCH70	272	81%	WP 015863368.1
G. caldoxylosilyticus	274	77%	WP 017434715.1
G. thermocatenulatus	274	61%	WP 025949772.1
G. thermodenitrificans	274	61%	WP 029760424.1
G. sp. C56-T3	274	60%	WP 013145836.1
G. sp. G11MC16	274	60%	WP 008878671.1

*The laccase sequence from *Geobacillus C56-YS93* was used as the query for BLAST.

The presence of genes with a similar sequence to that of laccase in *G. thermoglucosidasius* C56-YS93 suggests that laccase may be a significant enzyme across the genus. In addition to *Geobacillus*, there are numerous *Bacillus* strains that share a somewhat similar gene identity to that of laccase from *Geobacillus*, albeit at lower than 60%.[40] Currently, there does not exist any crystal structures or characterization of laccases from *Geobacillus*. However, the CotA laccase from *B. subtilis* has been well characterized, and can be used as a model bacterial laccase. Since *Geobacillus* grows a higher temperatures than *Bacillus,* it is

predicted that laccase from *Geobacillus* will share a somewhat similar overall structure and function to that from *B. subtilis*, although with greater stability and activity at higher temperatures. For the purpose of this work, we will focus on the characterization of the predicted laccase from *G. thermoglucosidasius C56-YS93*.

1.5 Summary

The increasing worldwide demand for liquid transportation fuels needs to be met with equal advances in the technology of synthesizing renewable biofuels from lignocellulosic biomass. Enzymatic degradation can be advantageous to thermochemical degradation, although enzyme cost and lignin content of biomass are two of the biggest obstacles. Laccases are widely used in industry, and are proven effective degraders of the aromatic compounds that comprise lignin. Currently, industrial laccases are fungal, and have limited activity at higher temperatures, pHs and salt concentrations. Additionally, larger enzyme size prevents penetration into the lignocellulosic matrix, and requires the use of redox mediators. Laccases have been predicted in several *Geobacillus* strains, which are gram-positive thermophilic bacteria. Other enzymes from *Geobacillus* have demonstrated superior thermal stability over their fungal counterparts. This work seeks to isolate and characterize the predicted laccase from *Geobacillus thermoglucosidasius* C56-YS93. The types of substrates this laccase can oxidize will be determined, as will maintained activity at high temperatures compared to a fungal laccase.

CHAPTER 2: EXPERIMENTAL SECTION

2.1 Strains, plasmids and chemicals

All strains used in this work are summarized in Table 3.

Table 3. Bacterial strains and their use(s)

Strain	Source	Use	Growth Media (solid/liquid)
E. coli DH5α	Coli Genetic Stock Center	Molecular cloning/laccase expression	LB/LB agar
G. thermoglucosidasius 95A1	Bacillus Genetic Stock Center	Laccase expression	TBAB/TGP
G. thermoglucosidasius C56		Source of gDNA- template for inserts	

The pNW33N shuttle vector was obtained from the Bacillus Genetic Stock Center. pSE380 was purchased from Invitrogen. Plasmid maps for pNW33N and pSE380 are shown in figure 5.

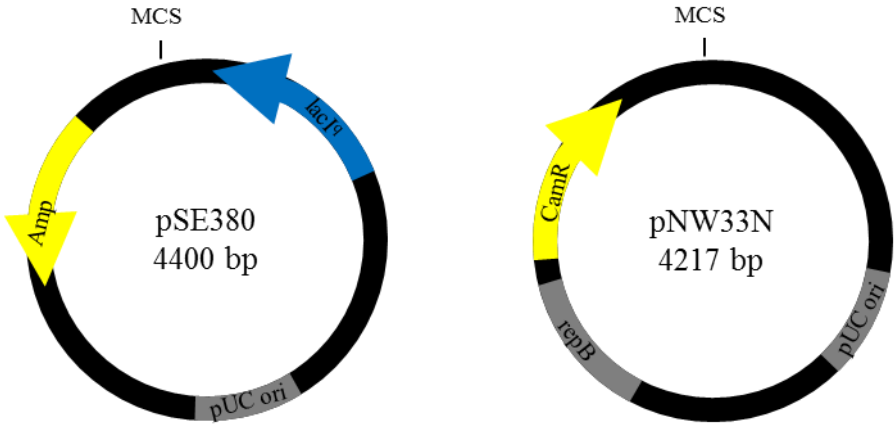

Figure 5. Plasmid maps for pNW33N and pSE380. pNW33N has an antibiotic resistance marker for chloramphenicol in both *E. coli* and *Geobacillus*, and pSE380 has an antibiotic resistance marker for ampicillin in *E. coli*. Both vectors contain multiple cloning sites (MCS).

LB (Luria-Bertani) media powder from Difco laboratories was purchased from VWR, and was used as the primary media to grow *E. coli* strains. *Geobacillus* strains were grown in TGP media, which consisted of 0.5% sodium chloride, 1.7% tryptone, 0.3% soytone, 0.25% K_2HPO_4, 0.4% sodium pyruvate and 0.4% glycerol. Tryptone and soytone were from Difco laboratories, USP grade K_2HPO_4 was purchased from Fisher Scientific, sodium pyruvate was from Alfa Aesar, and glycerol and sodium chloride were both purchased from Amresco. 1.5% agar, which was purchased from Difco laboratories, was used to solidify the LB media for use in petri dishes. Tryptose blood agar base (TBAB) was also from Difco laboratories and was used directly for plating *Geobacillus*. USP grade ampicillin from Amresco and chloramphenicol from Cellgro were used for antibiotic resistance screening of colonies. Isopropyl β-D-1-thiogalactopyranoside (IPTG) purchased from Sigma life sciences was used to turn on IPTG inducible promoters.

Trizma (Tris) base, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), proteinase K from *Tritirachium album*, hexadecyltrimethylammonium bromide (CTAB), 24:1 chloroform/isoamyl alcohol, 25:24:1 phenol/chloroform/isoamyl alcohol, isopropanol, potassium chloride, piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), sorbitol, copper chloride dehydrate, bromophenol blue, β-mercaptoethanol, glycine, citric acid, 2-(N-morpholine)-ethanesulfonic acid (MES), guaiacol and 2,2'-azine-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was all purchased from Sigma life sciences. 2,6-dimethoxyphenol, 4-methoxybenzyl alcohol, and 3,4-dimethoxybenzyl alcohol (veratryl alcohol) were purchased from A Aldrich chemistry.

Ethidium bromide, manganese (II) chloride tetrahydrate, calcium chloride dehydrate and sodium phosphate were purchased from Amresco. Agarose, dimethyl sulfoxide (DMSO) and

imidazole were obtained from Fisher Scientific. Miscellaneous chemicals also used in this work include ethanol from Macron fine chemicals, acetic acid from Sigma-Aldrich, sodium hydroxide and sodium phosphate dibasic from VWR, and mannitol from Alfa Aesar.

All media, buffers and all other solutions used can be found in their appropriate sections below, or in the compiled tables in Appendix C.

2.2 Genomic DNA extraction from *Geobacillus C56-YS93*

The extraction of gDNA from C56 was performed using the protocol from *Current Protocols in Molecular Biology*.[42] 5 mL of *Geobacillus C56* was incubated overnight at 60°C in TGP media. Because *Geobacillus* is gram-positive, 1 mg/mL of lysozyme was supplemented after resuspension of the cell pellet to aid in cell wall disruption. The final gDNA pellet was resuspended in 100 μL of TE buffer (Tris/EDTA buffer), which consisted of 10 mM TrisCl and 1mM EDTA. The gDNA of *Geobacillus* C56 was needed as a template for PCR amplification of P_{recA}, $P_{\beta glu}$ and laccase.

2.3 DNA amplification by Polymerase Chain Reaction (PCR)

All amplifications were performed using a BioRad T100 Thermal Cycler. Taq DNA Polymerase was GoTaq Green MasterMix purchased from Promega (Ref. # M712C). Custom oglionucleotide primers were ordered from Integrated DNA Technologies (IDT). All primers were suspended in autoclaved water upon arrival to a stock concentration of 30 μM. A list of primers used in this work is shown in table 4.

Table 4. Primers used for Polymerase Chain Reaction

Insert	Sequence (5' to 3')	Enzyme	T_m (°C)ǂǂ
P_{recA}	GTAGAGCTCCACGTTCCCGCTTCTGTTTT	SacI	63.5
P_{recA}	CTATCTAGAACTCCTCCTTTTTCTTGACT	XbaI	55.7
laccaseǂ	TGATCTAGAATGCACCACCACCACCATCATTTAGACATTTTTCAACAAGCGGGA	XbaI	68.2
laccaseǂ	ATTGCATGCTTAATGATGATGATGATGATGTTCCCCCTTCCTGCCGATAAAC	SphI	67.3
gfpmut3	GCTGCATGCAGGAGGAGTTACGTAATGCGTAAAGGAGAAGAACT	SphI	67.2
gfpmut3	AACAAGCTTTTATTATTTGTATAGTTCATCCATGCC	HindIII	57.9
laccase	TGAGAATTCAGGAGGAGTTACGTATATGTTAGACATTTTTCAACAAGC	EcoRI	63.0
laccase	GTTGAGCTCTCATTCCCCCTTCCTGCCGATAAACG	SacI	66.4
$P_{\beta glu}$	ATAGAGCTCCGATAAACGCGAAGAAGGTG	SacI	61.4
$P_{\beta glu}$	ATATCTAGAATGAACCTCCTTTATGTTCG	XBaI	56.6

ǂDenotes the addition of 6 Histidine residues at both 5' and 3' ends

ǂǂMelting temperature calculated by Integrated DNA Technologies (IDT)

The PCR reaction mixture consisted of 25 µL of Green MasterMix, 22 µL of autoclaved water, 1 µL each of forward and reverse primers, and 1 µL of template DNA. PCR conditions were dependent on the insert undergoing amplification, and are shown in table 5. Denaturation, annealing and extension steps were repeated 33 times before progressing to the final extension.

Table 5. PCR conditions for amplification of inserts

Step	P_{recA}	$P_{\beta glu}$	laccase	gfpmut3
Initial Denaturation	95°C for 2 minutes			
Denaturation	95°C for 30 seconds			
Annealing	50.7°C for 30 s	51.6°C for 30 s	62.3°C for 30 s	52.9°C for 30 s
Extension	73.0°C for 30 s	73.0°C for 30 s	73.0°C for 50 s	73.0°C for 55 s
Final Extension	73.0°C for 5 minutes			
Hold	4.0°C			

2.4 PCR cleanup and DNA concentrator

Following PCR, amplified DNA fragments were purified using the DNA Clean & Concentrator kit from Zymo Research (Cat. No. D4004). The purpose of this was to remove unwanted polymerase, primers and nucleotides from the reaction mixture prior to the digestion of

the inserts with restriction enzymes. Each starting 50 μL reaction mixture was eluted with 42.5 μL of water such that an adequate amount of DNA was present for restriction digest. The amount of elution buffer could be tuned to concentrate dilute aliquots of DNA when necessary.

2.5 DNA digest with restriction endonucleases

All restriction endonucleases (restriction enzymes) and their respective buffers were purchased from New England Biolabs. SacI, XbaI, SphI, EcoRI and HindIII all demonstrated 100% activity in NE Buffer #4. Total reaction volume was 50 μL, and contained 42.5 μL or DNA (insert or vector), 5 μL of 10x buffer, 1 μL of each appropriate enzyme, and 0.5 μL bovine syrum albumin (BSA). Reaction mixtures were incubated at 37°C for 3 hours, and were either used immediately, incubated at 4°C overnight or stored at 20°C. DNA digests that were to be left overnight at 4°C in the thermal cycler were first heated to 65°C for 25 minutes to deactivate the enzyme to prevent nonspecific digestion.

For insertion into pNW33N, P_{recA} and $P_{\beta glu}$ were digested with SacI and XbaI, laccase was digested with XbaI and SphI, and gfpmut3 was digested with SphI and HindIII. pNW33N was also digested with the respective enzymes for each insert. Laccase for insertion into pSE380 was digested with EcoRI and SacI.

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA molecules based on size. DNA gels were run with 1% agarose dissolved in Tris/acetate/EDTA (TAE) buffer, which consisted of 40 mM Tris acetate and 2 mM Na_2EDTA. Gels were stained with 0.5 μg/mL ethidium bromide for DNA band visualization, and were run using the PowerPac Basic unit from BioRad. Gels were run for approximately 1 hour, or until sufficient band separation was achieved. DNA was

run alongside a 1 kilobase ladder, purchased from New England Biolabs, and each sample was mixed with an appropriate amount of 6x gel loading dye, also from New England Biolabs. DNA to be visualized was run at a total volume of 9 μL, and DNA to be recovered for further use was run in deeper wells with a volume of 50 μL. Image capturing was performed with a UVP Benchtop 2UV BioDoc-It Imaging System at 302 nm for visualization or 365 nm for visualization and recovery.

2.7 DNA recovery from agarose gels

DNA was recovered from agarose gel following electrophoresis using the Zymoclean Gel DNA Recovery Kit (Cat. No. D4002), the protocol for which can be found in Appendix 1. To ensure adequate concentrations for the following ligation step, inserts were eluted from the spin columns with 6 μL of elution buffer, and vectors were eluted with 8 μL. Concentrated inserts and vectors were either used immediately or stored at 20°C.

2.8 Ligation

Inserts and vectors were ligated using supplies from Promega. A total reaction mixture of 10 μL was used, and contained 5 μL of 2x Rapid Ligation Buffer, 2.7 μL of insert, 1.3 μL of vector, and 1 μL of T4 DNA Ligase (Ref. # M180B). Multiple ligations were run in parallel. One reaction mixture was incubated at 24°C for 1 hour in the thermal cycler, and was then used immediately for transformation. The other ligation was left at room temperature on the benchtop overnight, which was used for transformation the next day if the transformation from the first ligation was unsuccessful.

2.9 Preparation of chemically competent *E. Coli*

Chemical competence was induced in wild-type DH5α (*E. Coli*) using the Inoue method given in *Molecular Cloning* with a few modifications where necessary.[43] Inoue transformation buffer (ITB) was prepared using 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, and 10 mM PIPES in water. Aliquots were kept at -20°C or used immediately. Liquid cultures of DH5α were grown overnight at room temperature to an OD of approximately 0.6. OD was measured using an Ultraspec 10 cell density meter from Amersham Biosciences. Cells were centrifuged at 4000 rpm at 4°C, and the cell pellet was washed three times in cold ITB. DMSO was added to a final concentration of 7.5%, and competent cells were either used immediately, or stored in aliquots at -80°C.

2.10 Heat shock transformation of *E. Coli*

Chemically competent *E. Coli* were transformed using the protocol in *Molecular Cloning*.[43] 50 µL of competent cells were mixed with 10 µL of ligation mixture or 1 µL of purified plasmid DNA. The mixture was placed on ice for 30 minutes, transferred to a 42°C waterbath for 90 seconds, and then placed back on ice for 5 minutes. 1 mL of LB was added to the competent cells and DNA, and the cells were recovered for 1 hour at 37°C. 200 µL of the recovered cells were spread on a plate with the appropriate antibiotic. Transformation plates were incubated overnight and were checked for colonies the following day. Plates were incubated for another day if no colonies were present.

2.11 Bacterial culture and plasmid DNA isolation

Transformed colonies were swirled into a 5 mL liquid culture with appropriate antibiotic concentration, shown here in table 6.

Table 6. Working antibiotic concentrations for different vectors and strains

strain	parent vector	antibiotic	working concentration
E. Coli DH5α	pNW33N	chloramphenicol	20-30 μg/mL
E. Coli DH5α	pSE380	ampicillin	100 μg/mL
Geobacillus 95A1	pNW33N	chloramphenicol	5-7 μg/mL

The Zymo Research ZR Plasmid Miniprep- Classic kit (Cat. No. D4016) was used to isolate plasmid DNA from transformed strains. The manufacture's protocol can be found in Appendix 1. DNA was eluted with 30 μL instead of the suggested 50 μL to ensure high DNA concentration.

2.12 Preparation of electrocompetent *Geobacillus 95A1*

Electrocompetence was induced in the wild type *Geobacillus 95A1* strain for introduction of foreign plasmids. 50 mL of *Geobacillus* culture was grown in TGP at 52°C from a freezer stock until the OD reached 2.0. The cells were spun down at 4,000 rpm, and were re-suspended and washed three times in cold electroporation buffer, which consisted of 0.5 M mannitol, 0.5 M sorbitol and 10% glycerol. Cells were then re-suspended in 1 mL of buffer and were used immediately or stored at -80°C.

2.13 Electroporation of *Geobacillus* 95A1

Electroporation was used to transform *Geobacillus* 95A1 with constructed plasmids. Transformations were performed using a BioRad Gene Pulser Xcell and BioRad PC Module. 1 mm, sterile cuvettes were purchased from VWR and were chilled at -20°C prior to use. 60 μL of electrocompetent cells were mixed with 2 μL of purified plasmid DNA. The mixture was then loaded into an electroporation cuvette and was pulsed. Electroporation settings used were 2.5 kV, 10 μF and 600 Ω, with an exponentially decaying pulse. Cells were immediately suspended in 1 mL of pre-warmed TGP, and were recovered at 52°C for 1 hour. 200 μL of recovered cells were

plated on TBAB with the appropriate antibiotic. Plates were incubated overnight at 60 °C and were checked for transformants the following day.

2.14 Cell line freezer stocks

A library of all transformed strains was created, and was stored at -80°C until strains were needed. E. Coli strains were grown for 5 to 6 hours in LB with appropriate antibiotic. Freezer stocks consisted of 50% liquid cell culture and 25% glycerol in water. For *Geobacillus* strains, cells were grown on plates with antibiotic overnight. A large "scoop" of colonies were suspended in 10% glycerol and stored at -80°C.

2.15 Colony PCR

Colonies appearing on overnight transformation plates were screened for the appropriate constructed plasmid using colony PCR. A 25 μL total reaction volume was used, and consisted of 12.5 μL of Green MasterMix, 10 μL autoclaved water, and 1 μL each of forward and reverse primers. A single colony was then suspended in the reaction mixture. PCR conditions are similar to those found in table 4. The initial denaturation was lengthened to 7 minutes to ensure cell lysis. The annealing temperature was 5°C below the lowest primer melting temperature. The extension time was 1 minute per kilobase of total inserts to be amplified. 7.5 μL of the reaction mixture was then run on an agarose gel. Transformants were deemed positive or negative based on the visualization of amplified inserts.

2.16 Laccase extraction from transformed cell lines

Intracellular laccase was obtained from transformed cell lines via sonication. The general protocol was adapted from that found in *Molecular Cloning*, although with a few differences.[44] 6 liters of *E. Coli* or *Geobacillus* producing laccase strains were grown overnight in the

appropriate chloramphenicol concentration. Cultures were spun down at 4°C for 15 minutes at 4,000 rpm. Cell pellets were re-suspended in sonication buffer, which consisted of 50 mM Tris-Cl, 500 mM NaCl, and 15% glycerol. The sonicator used in this work was purchased from Qsonica Sonicators (Pt. No. Q700), and a microtip was used during sonication (Pt. No. 4420). For *Geobacillus*, lysozyme was added to a final concentration of 1 mg/mL to aid in cell wall degradation. For both strains, PMSF was added to a final concentration to 1 mM to inhibit protease activity. EDTA was not added as a protease inhibitor because of potential interference with downstream metal affinity chromatography. Samples were kept on ice during sonication to further inhibit protease activity. Cells were sonicated for 10 minutes, which consisted of 30 second sonication intervals followed by 30 second cooling intervals. Sonicated cells were then spun down for 45 minutes at 15,000 rpm to precipitate cell debris. The resulting supernatant was used as a crude cell extract.

2.17 Laccase purification

Laccase was purified from cell extract using immobilized metal ion chromatography. HisPur Ni-NTA columns were purchased from Thermoscientific (Prod. No. 88229). The manufacturer's suggested protocol was followed, and can be found in appendix 1. 10 mM imidazole in PBS was used for equilibration buffer, 25 mM imidazole in PBS was used for wash buffer, and 250 mM imidazole in PBS was used for elution buffer. After elution, $CuCl_2$ was added to a final concentration of 1 mM, and was stored at 4°C overnight. Laccase was then transferred to 1 mL aliquots and stored at -20°C until needed. Columns were regenerated using 20 mM MES buffer, pH 5.0 with 0.1 M NaCl, and were stored as a 50% slurry in 20% ethanol.

2.18 SDS-PAGE and gel staining

BioRad Mini-PROTEAN TGX precast gels (Cat. No. 456-1094) were purchased for running SDS-PAGE gels. 2x Laemmli sample buffer contained 65.8 mM Tris-Cl, 2.1% SDS, 26.3% glycerol and 0.01% bromophenol blue. Total sample volume was 40 µL, and contained 20 µL of purified protein, 19 µL of 2x Laemmli sample buffer and 1 µL of 2-Mercaptoethanol. The mixture was heated to 95°C for 5 minutes to denature the protein. Samples were run alongside Benchmark His-tagged Protein Standard (Ref. No. LC5606). Protein gel running buffer was composed of 2.5 mM Tris base, 19.2 mM glycine and 0.1% SDS, pH 8.3. Protein samples were run for 30 minutes at a constant voltage of 200 volts in a BioRad Mini-PROTEAN Tetra System.

The gel was removed and stained using Novex in-gel stain by Life Technologies (Cat. No. LC6030). The gel was placed in a fixing solution composed of 50% ethanol and 10% acetic acid in water for 1 hour. The gel was washed twice with ultrapure water, and was then stained for 1 hour. Following staining, the gel was washed with 20 mM phosphate buffer, pH 7.8 and was then visualized at 302 nm.

2.19 Laccase activity assays

Laccase activity was tested using 5 different substrates: guaiacol, ABTS, 2,6-dimethoxyphenol, 4-methoxybenzyl alcohol and veratryl alcohol. The reaction mixture consisted of 800 µL of 150 mM citrate-phosphate buffer (varying pHs), 100 µL of 10x substrate for a final concentration of 5 mM, and 100 µL of purified enzyme. The reaction kinetics were tested from pH 3.0 to 7.0, and at temperatures from 50°C to 80°C. Reaction progress was monitored spectrophotometrically using a Genesys 10uv spectrophotometer by Thermo Scientific in 1 cm cuvettes supplied by VWR (Ref. No. 97000-586). Substrates, along with their appropriate wavelengths and molar absorptivities are shown in table 7.

Table 7. Wavelengths and molar absorptivities for laccase substrates

substrate	**wavelength (nm)**	**molar absorptivity ($M^{-1}cm^{-1}$)**
guaiacol[45]	465	48,000
ABTS[45]	436	29,300
2,6-dimethoxyphenol[46]	468	14,800
veratryl alcohol[46]	310	9,000
4-methoxybenzyl alcohol[46]	500	38,000

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Overview

The objective of this project was to overexpress and isolate the native laccase gene from *Geobacillus* C56-YS93 for the purpose of characterizing the novel enzyme. Many enzymes have been isolated from *Geobacillus* strains, and have demonstrated superb thermal stability and maintained activity at relatively high temperatures. Specifically, we were interested in the types of substrates the laccase from *Geobacillus* C56-YS93 could oxidize, and its stability over a range of pHs and temperatures. To accomplish this, we needed to construct a plasmid vector, containing the laccase gene and a strong promoter capable of replicating in both *E. Coli* and *Geobacillus*. The shuttle vector pNW33N was used as a parent plasmid for this purpose.[25] PCR was utilized to amplify two native promoters to C56 along with the laccase gene. 6 histidine residues were added to both the N- and C-termini of laccase for the purpose of using immobilized ion affinity chromatography for laccase purification. Gfpmut3 was added on the same operon as the promoter and laccase, and was used to easily confirm laccase expression in host *E. Coli*.

Upon confirmation of expression, it was necessary to determine an appropriate host for laccase expression and extraction. This was accomplished by constructing a new plasmid, one with an inducible promoter that could be tuned to turn expression on or off. This allowed us to determine whether or laccase expression was detrimental to host growth. Overexpression and extraction were performed using *E. Coli* as a host after the determination that laccase was not harmful to growth and that expression levels were higher in *E. Coli* than in *Geobacillus*.

Sonication was used to extract intracellular laccase from transformed E. Coli, and purification was performed using immobilized nickel ion chromatography. The quantification of laccase activity was then determined spectrophotometrically. Activity was tested on 5 different substrates at pHs ranging from 3.0 to 7.0, and at temperatures ranging from 50°C to 80°C. Finally, we wanted to demonstrate that our laccase maintained higher activity at higher temperatures than laccase isolated from *Trametes versicolor*, a fungus. We accomplished this by comparing the ability of both laccases to oxidize the substrate ABTS at 35°C and 80°C.

3.2 Construction of pTC08

pTC08 was the first plasmid vector constructed for the overexpression of laccase in *E. Coli* and *Geobacillus*. pNW33N was the parent plasmid used for the base of construction. The P_{recA} constitutive promoter was amplified with PCR using gDNA from C56 as a template, and the P_{recA} primers shown in table 4. The recA gene encodes an enzyme found across many strains of bacteria, and is responsible for repairing damaged DNA. It plays a key role in the SOS response of cells, which can be induced by exposing cells to heat or UV light. Although expression levels of recA depend on cell stress, cells maintain a basal level of recA protein crucial to DNA repair and cell survival, and thus we can consider P_{recA} to be a constitutive promoter.[47] Insertion of P_{recA} into pNW33N yielded pTC02.

Immediately following the P_{recA} insert was the laccase gene insert. Laccase was also amplified from C56 gDNA, and primers were designed such that 6 histidine residues were added to the N- and C-terminal ends of the laccase. The laccase primers are shown in table 4. This method of adding 6xHis tags is widely used for purification of recombinant proteins. The histidine residues chelate to the nickel resin in the chromatography column, and changing the

imidazole concentration in the eluent will change the binding affinity of the protein to the column.[48] pTC02 with the addition of laccase yielded pTC07.

Lastly, we wanted to add a reporter protein that would let us know whether or not laccase was being expressed. This reporter protein would have to be placed directly after laccase, on the same operon and under control of the same promoter (in this case, P_{recA}). The protein would also have to be thermally stable if the constructed plasmid was to be transformed into *Geobacillus*. A thermally stable version of green fluorescent protein (gfpmut3*) was chosen for this purpose. The gene for gfpmut3 was amplified from pKW1040, a plasmid constructed from another work which already contained gfpmut3* using the gfpmut3 primers in table 4. A synthetic ribosome binding site was added to the primers amplifying gfpmut3 to ensure translation.TC07 with gfpmut3* added after laccase was named pTC08. Figure 6 shows all amplified inserts run using agarose gel electrophoresis.

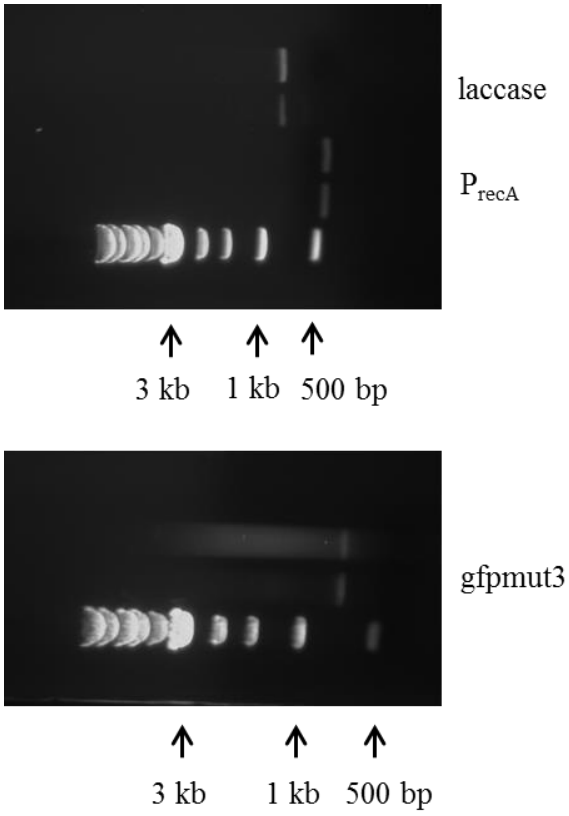

Figure 6. Amplified P_{recA} (465 bp), laccase (831 bp) and gfpmut3* (720 bp) run using agarose gel electrophesis

A plasmid map of pTC08 is shown in figure 7.

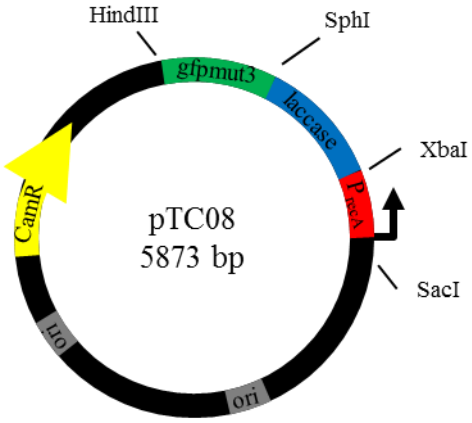

Figure 7. Plasmid map of pTC08 with inserts and enzyme cutting sites

pTC08 was constructed and cloned in *E. coli*. The resulting transformants did not show any evidence of GFP expression, although colony PCR did confirm the success of the transformation. Consequently, there was no reason to believe laccase was being expressed. Although the P_{recA} promoter is found in many bacterial species and has some basal level of expression, our version of the promoter was taken from *Geobacillus* C56. It was therefore thought that the *E. coli* could not recognize the promoter sequence, and thus translation (and consequently, transcription) was not occurring. For this reason, pTC08 was transformed into *Geobacillus* 95A1 to check for GFP expression.

Electroporation was used to transform 95A1 with pTC08. Colony PCR confirmed the successful transformation. Because GFP could not be visualized with the naked eye in *Geobacillus* colonies, a liquid culture was started, and sonication was used to obtain the cell lysate. There were no signs of GFP present in the cell lysate. It was concluded that the promoter was not turned on, or the expression levels were too low to visually detect the presence of GFP. Either way, the decision was made to construct as new plasmid for laccase overexpression containing a different inducible promoter. Although it may have been possible to increase expression levels with exposure to heat stress or UV light, this would be somewhat counterproductive, since this could potentially damage the cell, and the desired result of the cloning was healthy cells overexpressing laccase.

It should be noted that the construction of pTC08 was unusually challenging in E. Coli. In particular, the insertion of the laccase gene after the recA promoter to create pTC07 was more difficult than any other insertions. Although Xu et. al. have showed that laccases are present in *E. Coli* strains, laccases are not widely distributed in the species, so it was needed to check for

possible toxic effects of laccase expression on the host cell.[24] If laccase expression levels could be varied, the potential toxic effects of laccase expression on host growth could be investigated.

3.3 Construction of pTC06

pTC06 was constructed from pSE380, a commercially available plasmid containing an IPTG-inducible promoter and an ampicillin resistance marker. Laccase was amplified via PCR using the second set of laccase primers in table 4, and was inserted into pSE380. The plasmid map for pTC06 is shown in figure 8.

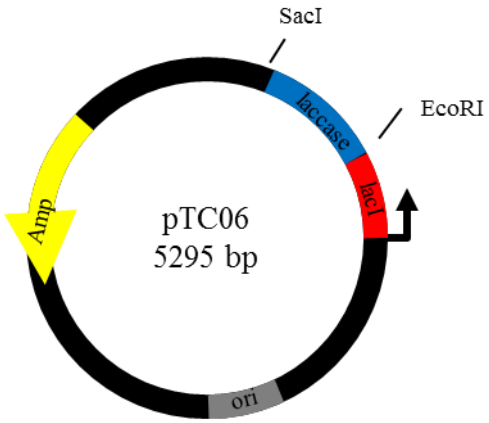

Figure 8. Plasmid map of pTC06 with inserts and enzyme cutting sites

pTC06 was successfully cloned in DH5α. Liquid cultures of DH5α were grown in LB with IPTG concentrations varying from 0 to 1 mM. The initial concentration of cells in the culture was controlled, and the OD of the cells was monitored every hour. Varying the levels of IPTG would turn the promoter on in different degrees, resulting in a cascade of laccase concentration across samples. If laccase was toxic to host growth, increased levels of IPTG would cause decreased growth rates. Two replicates of each concentration were run, and the results are shown in figure 9.

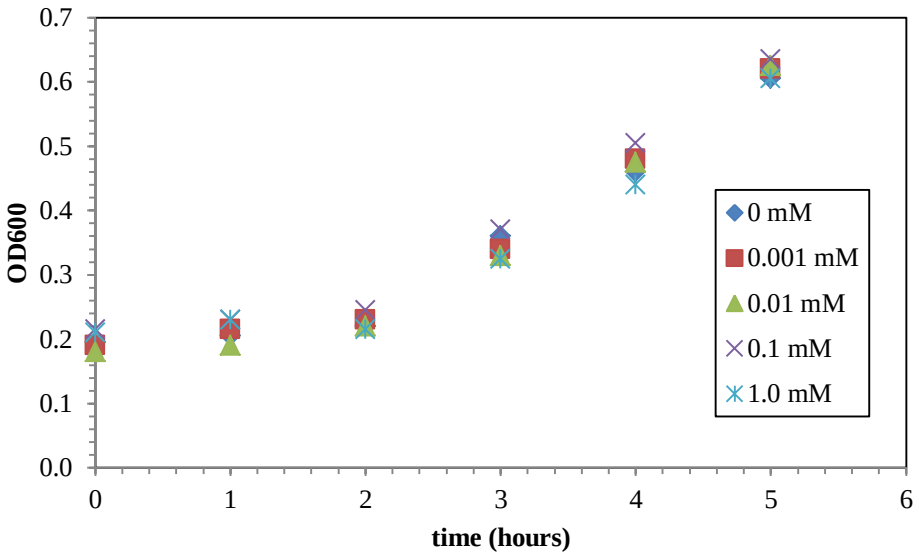

Figure 9. Effect of IPTG concentration on DH5α/pTC06 growth

There did not appear to be any correlation between IPTG concentration and the growth rate of DH5α/pTC06. From this, it was concluded that, 1.) the difficulty in cloning pTC08 in *E. coli* is not due to laccase expression hindering cell growth and 2.) assuming functionality is maintained, *E. coli* is an appropriate host for overexpression of laccase.

3.4 Construction of pTC13

Because there was no evidence of GFP or laccase expression under the control of the P_{recA} promoter, a new expression vector was created using a cellobiose-inducible promoter native to *Geobacillus* C56. This promoter was used by Bartosiak-Jentys et al. for studying the secretion of a glycosyl hydrolase from *G. thermoglcosidasius C56*.[49] In wild-type C56, this promoter turns expression of β-glucosidase on and off in the presence of cellobiose. β-glucosidase will break down cellobiose into its constituent glucose molecules, which the cells can then use as a carbon source. The aim of using this promoter was to turn laccase and GFP expression on and off to confirm whether or not the promoter was working. This would easily be confirmed with visual

GFP expression. The promoter, named $P_{\beta glu}$, was amplified from C56 gDNA using the $P_{\beta glu}$ primers in table 4, and inserted into pNW33N. This is shown in figure 10.

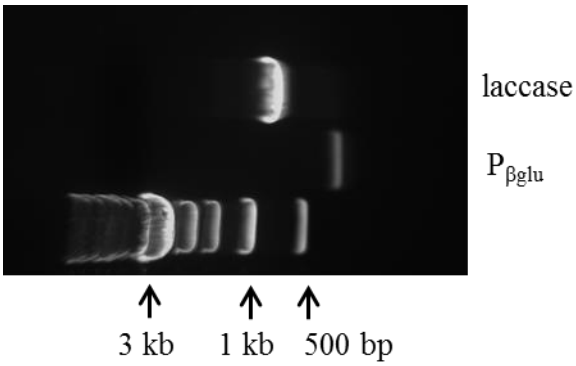

Figure 10. Amplified $P_{\beta glu}$ (269 bp) and laccase (831 bp) run using agarose gel electrophesis

The resulting plasmid was named pTC09. The rest of the plasmid was constructed in a similar fashion to that of pTC08. Laccase with the 6xHis tags was added after the $P_{\beta glu}$ promoter, which resulted in pTC12. Gfpmut3 was added after laccase to make the final construct, pTC13. A plasmid map of pTC13 is shown in figure 11. A plasmid table, containing all of the constructs used in this work is shown in table 8.

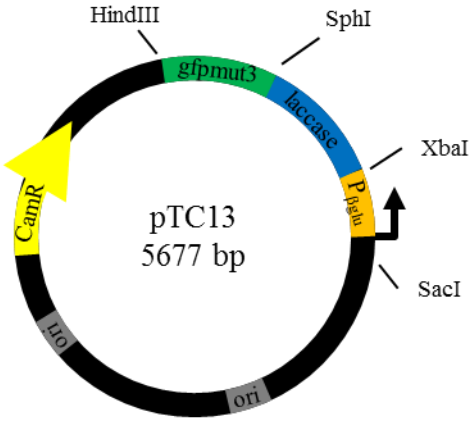

Figure 11. Plasmid map of pTC13 with inserts and enzyme cutting sites

Table 8. Plasmids constructed for the overexpression of laccase

Name	Parental vector	Resistance	Insert(s)
pNW33N	N/A	Cm	N/A
pSE380	N/A	Amp	N/A
pTC02	pNW33N	Cm	P_{recA}
pTC06	pSE380	Amp	laccase
pTC07	pNW33N	Cm	P_{recA} + laccase w/ 6xHis tags
pTC08	pNW33N	Cm	P_{recA} + laccase w/ 6xHis tags + gfpmut3*
pTC09	pNW33N	Cm	$P_{\beta glu}$
pTC12	pNW33N	Cm	$P_{\beta glu}$ + laccase w/6xHis tags
pTC13	pNW33N	Cm	$P_{\beta glu}$ + laccase w/6xHis tags + gfpmut3*

pTC13 was successfully cloned in *E. coli*. It should be noted that successful transformants grown on LB agar plates with chloramphenicol displayed signs of GFP expression, even in the absence of cellobiose. This is shown in figure 12, with DH5α/pTC13 on the right and wild type DH5α on the left. Cellobiose was not added to either plate.

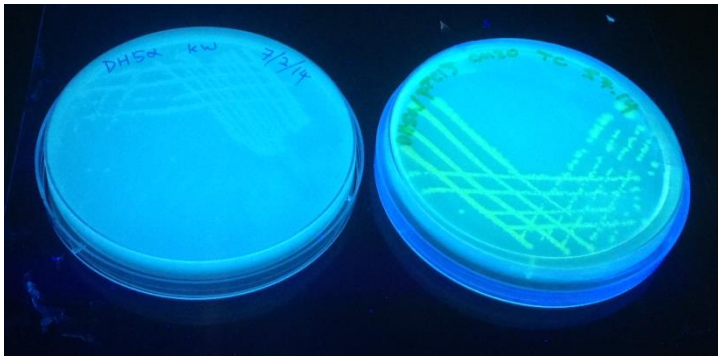

Figure 12. LB plates of wild-type DH5α (left) and DH5α/pTC13 (right)

Note the GFP expression in DH5α/pTC13 in the absence of the cellobiose inducer. Although it is unclear why the promoter is turned on in the absence of the inducer, there are a few possible explanations. The LB media used in this work is a complex, yet somewhat undefined mixture containing tryptone and yeast extract. Tryptone is the enzymatic digest of casein by the protease,

trypsin. It is a source of amino acids and peptides needed for cell growth.[50] The impurities of the tryptone can be quite high since it is a mixture of many different compounds. Some of these impurities may be carbohydrates. However, it is more likely that the yeast extract, which is an undefined mixture of amino acids, peptides and carbohydrates contains cellobiose or a molecule close in structure to cellobiose.[51] It is also possible that the LB contains a carbohydrate that, when enzymatically degraded by the cell for use as a nutrient, mimics the structure of cellobiose, thus turning on the inducible promoter. Although it is unclear how the promoter was being turned on, GFP (and therefore laccase) was being expressed, and it was decided that pTC13 would be the vector used for the overexpression and future extraction and purification of laccase.

3.5 Laccase detection and purification

Although there was evidence to suggest that laccase was being expressed in DH5α/pTC13, there was no way to guarantee that DH5α had the cellular machinery to correctly fold laccase from *Geobacillus*. Thus, it was not known if laccase would be functional. SDS-PAGE was used to explicitly detect the presence of intracellular laccase, along with using gel staining to take advantage of the histidine tags on the N- and C-terminus of the protein. After this, laccase functionality would be detected by enzymatic assay of the purified enzyme to conclude that DH5α/pTC13 was an appropriate expression host. If laccase obtained from DH5α/pTC13 was not functional, a different host, preferably *Geobacillus 95A1* would need to be used.

Intracellular laccase was obtained via sonication. DH5α/pTC13 was grown in LB media with a chloramphenicol concentration of 30 μg/mL for increased selection against cells without pTC13 and other contaminants. The cells were spun down and sonicated according to protocol found in chapter 2. The crude enzyme extract was run alongside extract obtained from wild-type

DH5α on an SDS-PAGE gel. The gel was then stained to visualize any histidine-tagged proteins. Protein bands were visualized at 302 nm, and the gel is shown here in figure 13.

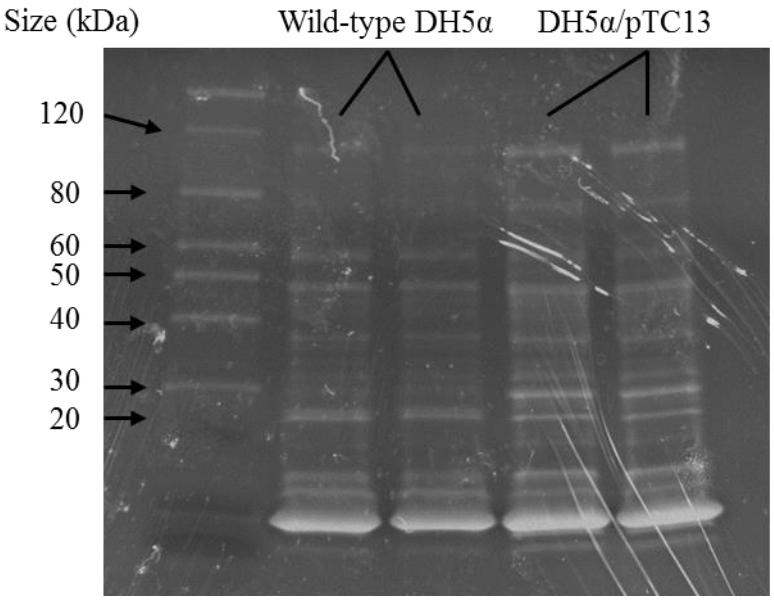

Figure 13. SDS-PAGE of wild-type DH5α and DH5α/pTC13 cell extracts

Note the presence of a band approximately 30 kDA (kilodaltons) in length. This is assumed to be our laccase, which is 29.8 kDa in length. The presence of a band in the DH5α/pTC13 extract but not the wild-type DH5α extract helps to confirm this, since the two extracts should be identical except for a band for laccase and (perhaps) GFP. It is important to note the abundance of noise in both samples. Theoretically, only proteins containing 6xHis tags would be visualized in the gel after staining. However, the crude cell extract contains a plethora of enzymes and proteins, some of which may contain extended lengths of histidine residues. According to the InVision His-tag In-gel Stain user guide, the stain is sensitive to proteins present in picomolar quantities.[52] Wells were loaded with 20 μL of cell extract instead of the recommended 5 μL to ensure bands would be visible. Decreasing the amount of extract in each well may lower the intensity of non-specific bands in the gel.

In case the laccase was not functional when expressed in *E. coli*, pTC13 was trasnsformed into *Geobacillus 95A1* via electroporation. 95A1/pTC13 was grown in parallel to wild-type 95A1. Cell extract was obtained using the procedure outlined in chapter 2. It is important to note here that GFP levels in the crude cell extract were much lower than that from DH5α/pTC13. A small amount of crude cell extract was purified using immobilized nickel ion chromatography. The purified laccase was run using SDS-PAGE followed by His-tag gel staining. The gel is shown in figure 14.

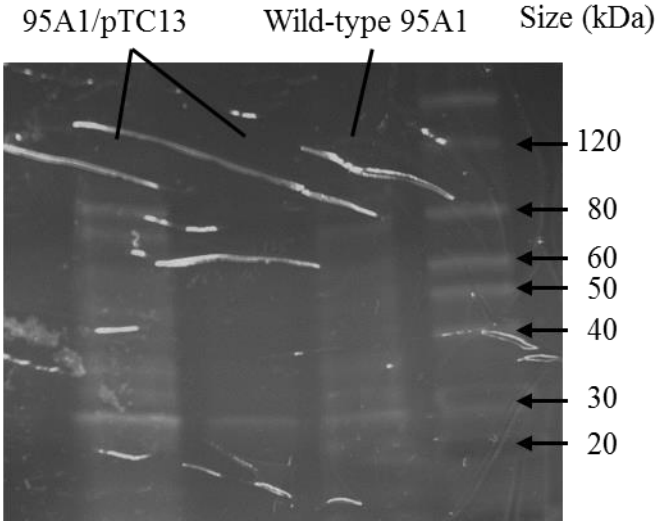

Figure 14. SDS-PAGE of laccase purified from 95A1/pTC13 and wild-type 95A1

It is interesting that the wild-type 95A1 sample seems to contain a band much like the 95A1/pTC13 sample approximately 30 kDA in size which is presumed to be the overexpressed laccase. This could be caused by a number of factors. *Geobacillus 95A1* is closely related to *Geobacillus C56*, which was used as the source of the gene coding for laccase. However, 95A1 has not yet been fully sequenced. It is therefore possible that wild-type 95A1 gDNA contains a gene for laccase as well. Because laccases are known for having histidine-rich segments of amino acids for the localization of copper atoms, it is also possible this laccase expressed from

wild-type 95A1 is being visualized by the His-tag gel stain. Also, because the crude cell extract from 95A1/pTC13 did not show very high levels of fluorescence, it is possible that the protein bands in figure 14 corresponding to 30 kDa lengths are not laccase, but another nonspecifically visualized protein. Because of the low GPF expression levels and somewhat inconclusive SDS-PAGE results, the decision was made to abandon *Geobacillus* as a laccase expression host, and to use DH5α/pTC13 to overexpress, isolate and characterize the laccase of interest. If laccase isolated from DH5α/pTC13 was not functional, a strain of *Bacillus* which is known to not contain any genes for laccase would be used as a host.

To obtain a sufficient amount of cell extract for large-scale laccase purification, 6 liters of DH5α/pTC13 was grown overnight in LB with 30 μg/mL chloramphenicol. Cell extract was obtained via sonication, and laccase was purified from the cell extract using chromatography. For the purpose of running quantitative activity assays, the purified laccase concentration needed to be estimated. The purified laccase was run alongside a His-tagged standard ladder of known concentration, and is shown in figure 15.

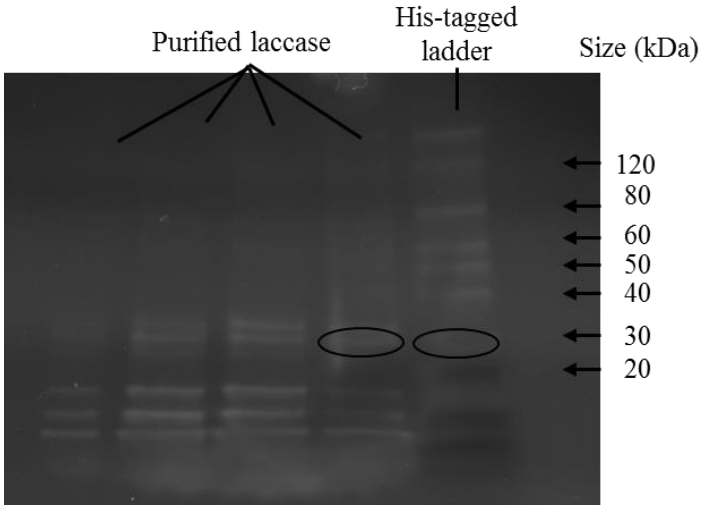

Figure 15. SDS-PAGE of purified laccase from DH5a/pTC13

ImageJ software was used to estimate the laccase concentration using the SDS-PAGE gel shown in figure 15.[53] By using this software, it was assumed that the intensity of the band was directly proportional to its concentration. Although Gassmann et al. warn against the accuracy of this assumption[54], the general protocol by Miller was followed to estimate the concentration for the sake of an "order of magnitude" approximation.[55] From a molecular standpoint, if each molecule emits a given number of photons upon excitation, then it is a fair assumption that the amount of photons emitted, or the band density, should be proportional to the concentration of emitting molecules. In this case, the band intensity is linearly proportional to the laccase concentration.

Analysis of the laccase and the ladder bands using the Image J software was performed. The ImageJ software reported an "integrated raw density" for each band of interest, which estimates the intensity of a selection, averaged over the selection. The software reported an integrated raw density of 136,776 for the 30 kDa band and 117,841 for the laccase band in figure 15. Assuming that integrated raw density is directly proportional to concentration, the purified laccase concentration is approximately 0.09 mg/mL. Although the exact concentration was not needed for the quantification of activity, it was needed for the comparison of laccase from *Geobacillus* to that from *Trametes versicolor*. A stock solution of laccase from *Trametes versicolor* at a concentration of 0.09 mg/mL was created for the purpose of comparing the thermal stability via liquid assays of both laccases.

3.6 Laccase characterization

To characterize the novel laccase from *G. thermoglucosidasius*, several different substrates were chosen based on assays performed with laccases in other works. One of the most closely studies bacterial laccase is the CotA laccase from *Bacillus subtilis*. Martins studied the oxidation of syringaldazine and ABTS by the laccase-containing spores of *Bacillus subtilis*.[56]

Similarly, Fatemeh et al. used guaiacol and ABTS, both aromatic and non-aromatic substrates to quantify CotA activity from *Bacillus subtilis* WPI.[45] Lastly, in characterizing CotA's ability to decolorize dyes, Guan et al. use ABTS, 2,6-dimethoxyphenol and syringaldazine for assay substrates.[20]

Although the model laccase from gram-positive bacteria is currently the CotA laccase, it was important to find a wide range of substrates for the characterization of the novel laccase from *Geobacillus*. Li et al. studied the CueO laccase from E. coli and its ability to oxidize ABTS.[24] In the paper by Margot et al., the ability of a bacterial laccase to oxidize the substrates ABTS, 2,6-dimethoxyphenol, syringaldazine and guaiacol was compared to a fungal laccase.[57] The oxidation of guaiacol using a novel laccase from *Peniophora sp*. was studied along with the treatment of pulp using the laccase with ABTS as a redox mediator by Shankar et al.[58] The ability of the laccase from *Trametes versicolor* to oxidize ABTS and 2,6-dimethoxyphenol was described in a paper by Johannes et al.[59] An extensive characterization of a novel polyphenol oxidase from Bovine Rumen was performed by Beloqui et al. In this work, they ranked the activity of the oxidase for 7 different substrates: syringaldazine, 2,6-dimethoxyphenol, veratryl alcohol, guaiacol, tetramethyl benzidine, 4-methoxybenzyl alcohol and ABTS.[46] For this work, the laccase from *G. thermoglucosidasius* was tested using 4 phenolic substrates (guaiacol, 2,6-dimethoxyphenol, veratryl alcohol and 4-methoxybenzyl alcohol) and 1 non-phenolic substrate (ABTS). It should be noted that syringaldazine was also chosen, yet was abandoned when great difficulty was experienced when attempting to dissolve in water or methanol.

Laccase activity was tested at pHs ranging from 3.0 to 7.0 and at temperatures from 50°C to 80°C. The reaction mixtures consisted of 800 μL of 150 mM citrate-phosphate buffer, and 100 μL of 10x substrate. The final substrate concentration of each mixture was 5 mM and was

independent of substrate choice. 100 µL of purified laccase was added last, upon which spectrophotometric readings started immediately. Reaction progress was monitored for 15 minutes with readings taken every 5 minutes. The initial rate of reaction was calculated by converting the change in absorbance to the change in concentration in the first 5 minutes by using the Beer-Lambert law shown here in equation 1.

$$A = \varepsilon l c \qquad \text{(Equation 1)}$$

A is the absorbance, ε is the molar absorptivity given in table 6, *l* is the path length (1 cm for our cuvettes) and c is the concentration. 1 unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate in 5 minutes. Activities are expressed in U/L. The activity of laccase as a function of temperature and pH for different substrates is shown in figures 16 to 20.

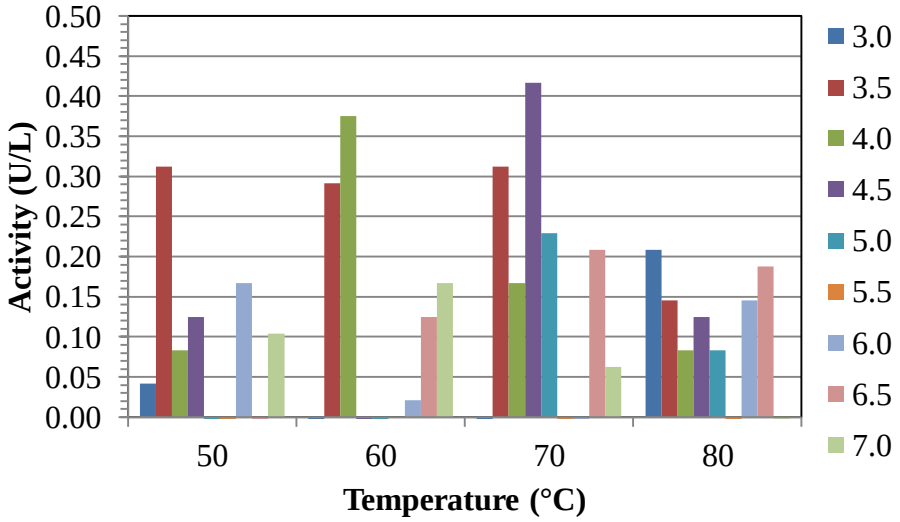

Figure 16. Activity of laccase as a function of temperature and pH with guaiacol as a substrate

The laccase-catalyzed oxidation of guaiacol was of particular interest to this work since the complex structure of lignin can be broken down into guaiacol and syringol building blocks.[8] High activity towards the guaiacol substrate would translate to potential uses for the laccase-catalyzed degradation of lignin in biomass. However, as seen in figure 16, there does not seem to be any significant trends in the data, nor is the activity for the guaiacol substrate very high. The highest activity observed was approximately 0.40 U/L at 70°C and a pH of 4.5. Activity at 80°C also seems to be relatively independent of pH.

Fatemeh et al. observed an activity of 2.28 U/L for the CotA laccase from *Bacillus subtilis* WPI using guaiacol as a substrate- nearly 7 times higher than the highest activity achieved by the *Geobacillus* laccase.[45] Activity from the *S. cyaneus* was approximately 25 times higher than that from *Geobacillus*, and laccase from *T. versicolor* was 375 times as active according to Margot et al.[57] However, the vast differences in enzyme activity is most likely due to enzyme concentration. Margot et al. concentrated the laccase from S. cyaneus 33 times via ultrafiltration, and the concentration of CotA laccase used by Fatemeh et al. is not made explicit.[57,45] More replicates are needed for determining the exact effects of pH and temperature on enzyme activity.

The ability of laccase to oxidize the non-phenolic substrate, ABTS, was also of importance to this work. ABTS, a redox mediator, can be coupled with laccases for the enzymatic degradation of lignocellulosic biomass if enzyme size prohibits diffusion into the matrix. Similar to guaiacol, a high activity for the ABTS substrate would suggest that laccase from *Geobacillus* may have lignin-degrading applications. Figure 17 shows the effect of temperature and pH on enzyme activity for the substrate ABTS.

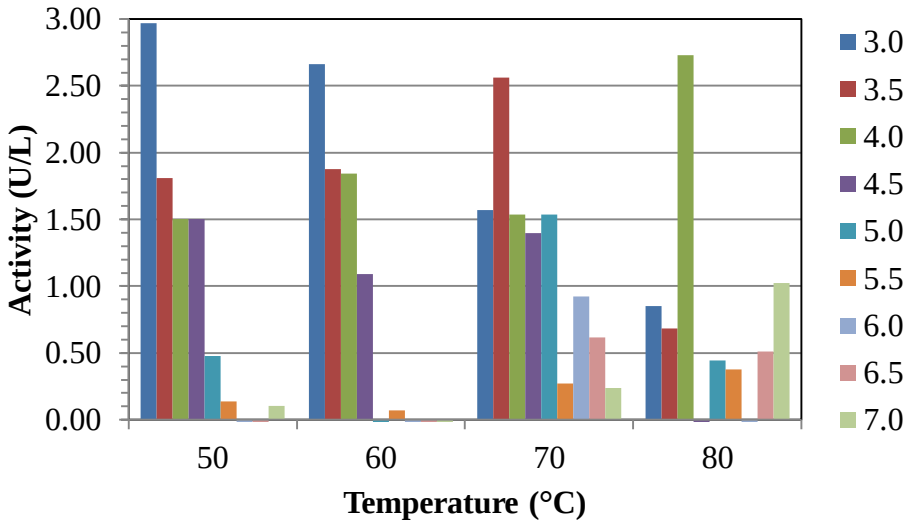

Figure 17. Activity of laccase as a function of temperature and pH with ABTS as a substrate

Unlike guaiacol, the trends in the ABTS activity data were more clear. The highest activity for the ABTS substrate was observed at lower pHs. The maximum activity for all temperatures was observed at pHs lower than 4.0, with activity dropping off at pH 4.5 or higher. The maximum activity was also much higher than that for guaiacol, 2.97 U/L versus 0.42 U/L, respectively.

For ABTS, Martins reported an activity of 1.28 U/mg of protein for the *Bacillus* CotA laccase.[56] Although the exact enzyme concentration for the assays aren't known, this is approximately 200 times higher than the activity for the laccase in this work. This is taking into account that the *Geobacillus* laccase assay used about 90 times as much enzyme than Martin's assay. The reason behind this large discrepancy is unknown. This data agrees better with experiments performed by Fatemeh et al., who reports an activity of 2.28 U/L- approximately 3.8 times higher than the laccase in this work.[45] Guan et al. report an activity approximately twice as high for ABTS than the highest activity for *Geobacillus* laccase.[20] Because of the clear trends in data, the relevance to lignin degradation applications and the higher activity than guaiacol,

ABTS was used further in this work for testing the thermal stability of the laccase from *Geobacillus*.

2,6-dimethoxyphenol (2,6-DMP) was another phenolic substrate tested in this work and many others. As can be seen in figure 18, the activity for 2,6-DMP was much higher than any other substrate.

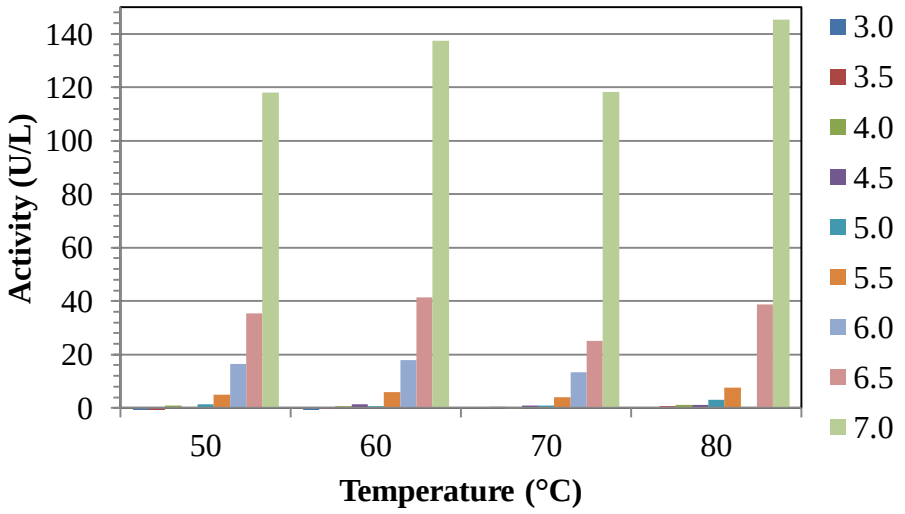

Figure 18. Activity of laccase as a function of temperature and pH with 2,6-dimethoxyphenol as a substrate

As with ABTS, there were clear trends in the data. Laccase activity was fairly independent of temperature, yet was strongly dependent on pH. Laccase demonstrated the highest activity for 2,6-DMP at a pH of 7.0, with activity sharply dropping at lower pHs. It is unknown if activity would be higher at pHs above 7.0. More assays utilizing a different buffer would be needed to test this. The maximum observed activity was 145 U/L at 80°C and a pH of 7.0.

Margot et al.report 25 U/L of enzyme activity for laccase from *S. cyaneus* at a pH of 3.5 and 60°C- approximately the same as that for *Geobacillus* laccase.[57] They also report 400 U/L of

activity for laccase from *T. versicolor* at a pH of 2.5 and 50°C- nearly 14 times that of *Geobacillus* laccase.[57] It is interesting that these two laccases have maximum activity at a relatively low pH while the *Geobacillus* laccase demonstrates higher activity at higher pHs. Loncar et al. found that laccase from *Bacillus amyloliquefaciens* had an activity of 235 U/L for 2,6-DMP, approximately 8 times higher than our laccase. Unfortunately, the ability of the CotA laccase to oxidize 2,6-DMP has not been well characterized, so laccase activity from *Geobacillus* must be compared to laccase activity from more distant species.

Veratryl alcohol and 4-methoxybenzyl alcohol, two more phenolic compounds, were tested as substrates via the paper by Beloqui et al.[46] The results are shown in figures 19 and 20.

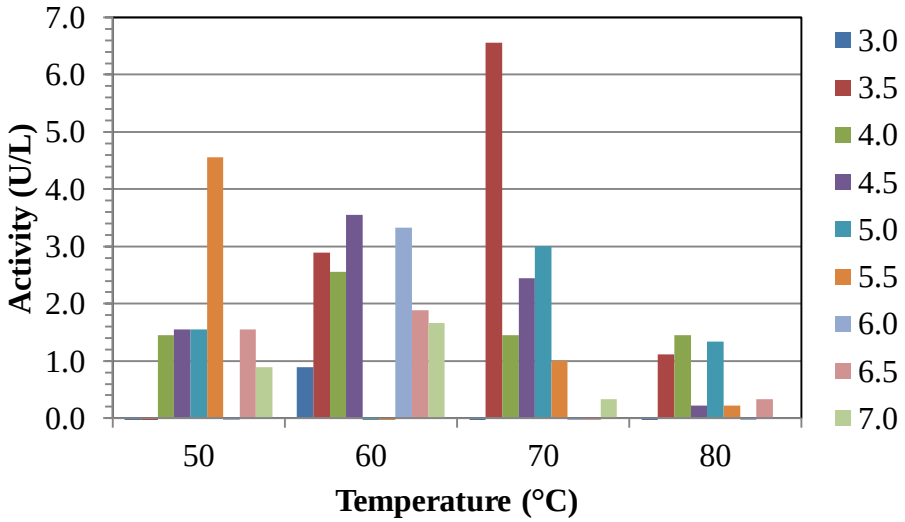

Figure 19. Activity of laccase as a function of temperature and pH with veratryl alcohol as a substrate

Similar to guaiacol, there are no clear trends in the data, although the highest activity seems to be at temperatures of 70°C and below and at pHs between 3.5 and 5.0. More replicates would be needed to determine whether or not a relationship exists between temperature and pH and enzyme activity. It is important to note, though, that the activity measured was notably higher for

veratryl alcohol than for guaiacol. It can be concluded, therefore, that laccase from *Geobacillus* has the capability to oxidize veratryl alcohol, although the optimum conditions are not well defined. Beloqui et al. characterize the ability of the novel laccase from bovine rumen to oxidize veratryl alcohol, and determing Michaelis-Menten parameters, although the activity is not given explicitly.[46]

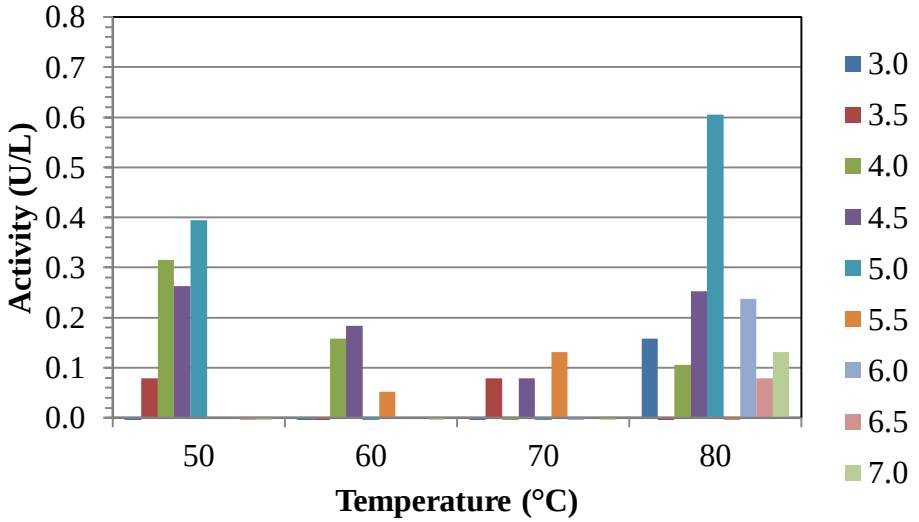

Figure 20. Activity of laccase as a function of temperature and pH with 4-methoxybenzyl alcohol as a substrate

Similar to the data obtained for guaiacol and veratryl alcohol, there is no clear relationship between temperature and pH and laccase activity. The highest activity seems to be at 80°C at pHs between 4.5 and 6.0. However, the level of activity is significantly less than that for ABTS, 2,6-DMP and even veratryl alcohol. There can therefore be no conclusion made as to whether or not laccase from *Geobacillus* has the ability to oxidize 4-methoxybenzyl alcohol. More replicates would be needed to determine the oxidation ability for this substrate.

The ability of laccase to oxidize a variety of compounds is ranked here: 2,6-DMP >> veratryl alcohol > ABTS > guaiacol > 4-methoxybenzyl alcohol. The data suggests that although

the optimum pH for the oxidation of each substrate is different, activity is, in most cases, fairly independent of temperature. If a relationship does exist between activity and temperature, more replicates for each set of temperatures and pHs would be needed to make any conclusions. Also, most activities were notably lower than those for other laccases for the same substrate. It is likely that, although purified, the laccase used in this work was not concentrated to the same extent that was found in other works.[57] The exact concentration of laccase in assays in other works is somewhat ambiguous. It is believed that increasing the concentration of laccase will greatly affect the activity observed, putting it more in line with activities in literature mentioned above. The seeming independence of activity from temperature, however, is of importance to this work. If laccase from *Geobacillus* is a spore coat protein like CotA from *Bacillus subtilis*, it is a fair assumption that stability and activity will be maintained over a wide range of temperatures.

Because bacterial endospores are known for their resiliency, and because laccase from *Geobacillus* may be a structural component of endospores, it is of interest to test the thermal stability of this novel laccase. The ability of the *Geobacillus* laccase to oxidize ABTS at 35°C and 80°C was tested against laccase from *T. versicolor* to determine the thermal stability and activity of the two enzymes. "Optimum" temperatures for the *T. versicolor* laccase range from 20°C to 50°C, so 35°C was used in this work as a low temperature at which to compare the two laccases.[60,61] 80°C was the upper limit for logistical purposes in terms of the handling of substrates, reaction mixtures, etc… However, it would be of great interest to test the *Geobacillus* laccase at temperatures above 80°C, given that spores from *Bacillus subtilis* have shown stability at temperatures above 90°C.[62] pH was held constant at 4.0, and each data point consisted of 5 replicates.

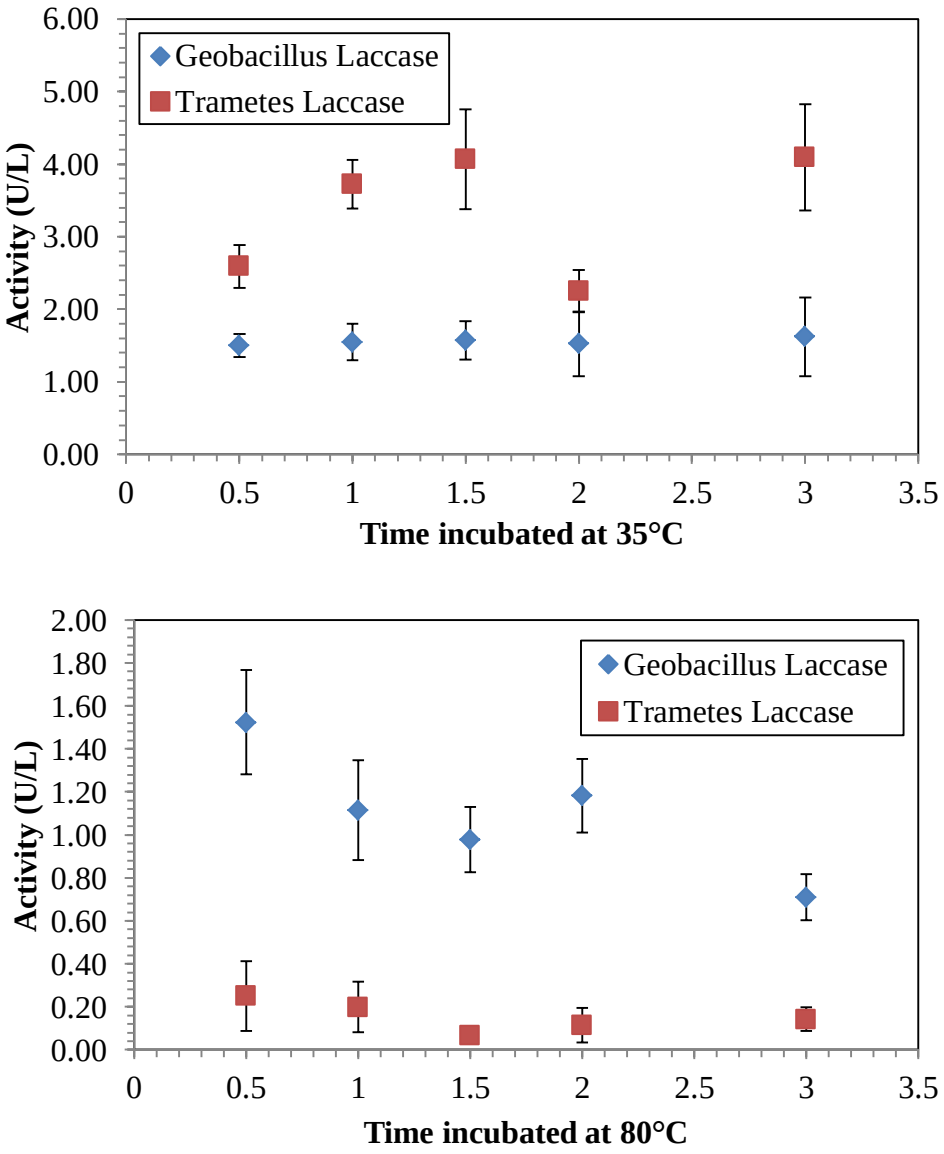

Figure 21. Laccase actvity of *Geobacillus* and *Trametes* laccase as a function of incubation time at 35°C and 80°C using ABTS as the substrate

At 35°C, the *Geobacillus* laccase activity is fairly independent of temperature, indicating the enzyme is thermally stable and active at this temperature. On average, the *T. versicolor* laccase is approximately 2 times as active under the same reaction conditions. This is to be expected- in general, bacterial laccases are not as active as fungal laccases.[20] Specifically, Margot et al. compare the activity of a bacterial laccase from *S. cyaneus* to the fungal laccase

from *T. versicolor*. The fungal laccase is 6.8, 400 and 30 times as active for the substrates ABTS, 2,6-DMP and guaiacol, respectively.[57] Taking this into account, the fact that *Geobacillus* laccase is only half as active as the *Trametes* laccase is quite good. At 35°C, the activity of the fungal laccase increases, and plateaus around 1.5 hours. The reason for this is unknown, as the reaction mixture was held at a constant temperature, even for the 30 minute readings. Because enzyme aliquots were stored at -20°C, it is possible that there is a "recovery" period for the enzyme to become fully active after thawing. Thus, the laccase from *T. versicolor* may be more sensitive to temperature than that from *Geobacillus*.

At 80°C, the *Geobacillus* laccase maintains greater activity than the *Trametes* laccase. The *Trametes* laccase activity is approximately 20-fold lower than when incubated at 35°C. Although the *Geobacillus* laccase starts with the same activity as when incubated at 35°C, the activity steadily decreases over time, indicating that the laccase may not be completely stable at 80°C. Martins observed that the CotA laccase had a half-life of 4 hours when oxidizing ABTS at 80°C.[56] This is similar to the laccase studied here, whose half-life is approximately 3 hours at 80°C. *Bacillus subtilis* grows optimally from 25 to 35°C, significantly lower than *Geobacillus*, whose optimum growth temperature is 60°C.[63] Martins also shows that the CotA laccase maintains significant activity even at 75°C. Even though the two bacteria grow at widely different temperatures, laccases from both appear to be thermally stable. This may further suggest that laccase from *Geobacillus* is an endospore coat protein because spores from both *Bacillus subtilis* and *Geobacillus* are thermally resilient. Nevertheless, laccase from *Geobacillus* demonstrates greater thermal stability and activity at higher temperatures than laccase from *Trametes versicolor*.

CHAPTER 4: CONCLUSIONS

Laccase from *G. thermoglucosidasius* C56-YS93 was successfully overexpressed in *E. coli* via the construction of pTC13 from the shuttle vector pNW33N. Although pTC13 was transformed into *G. thermoglucosidasius* 95A1, there were no detectable signs of overexpression. The construction of a second, IPTG inducible expression vector confirmed that *E. coli* would be a suitable host for laccase expression. IPTG levels were varied, and the expression level of laccase did not adversely affect host growth rate. Laccase was detected and extracted from DH5α/pTC13 cell extract. It was then successfully purified using immobilized nickel ion chromatography for the aim of characterization.

The novel laccase was characterized by its ability to oxidize 5 different substrates. Activity was tested at pHs from 3.0 to 7.0, and at temperatures from 50°C to 80°C. The ranking of activity for substrates tested is as follows: 2,6-DMP >> veratryl alcohol > ABTS > guaiacol > 4-methoxybenzyl alcohol. The thermal stability of the enzyme was demonstrated by comparing the ability to oxidize ABTS at 80°C. Laccase from *Geobacillus* showed a 20-fold higher initial activity than laccase from *T. versicolor* under the same conditions, and had a half-life of approximately 3 hours. Further testing is recommended to compare laccase from *Geobacillus* to the CotA spore coat laccase of *Bacillus subtilis* in terms of activity, substrate specificity and thermal stability. Whether or not the laccase studied in this work contributes to the endospore structure may help explain its maintained activity at high temperatures. Lastly, the crystal structure may also shed light onto the superior thermal stability of this bacterial laccase over comparable fungal laccases.

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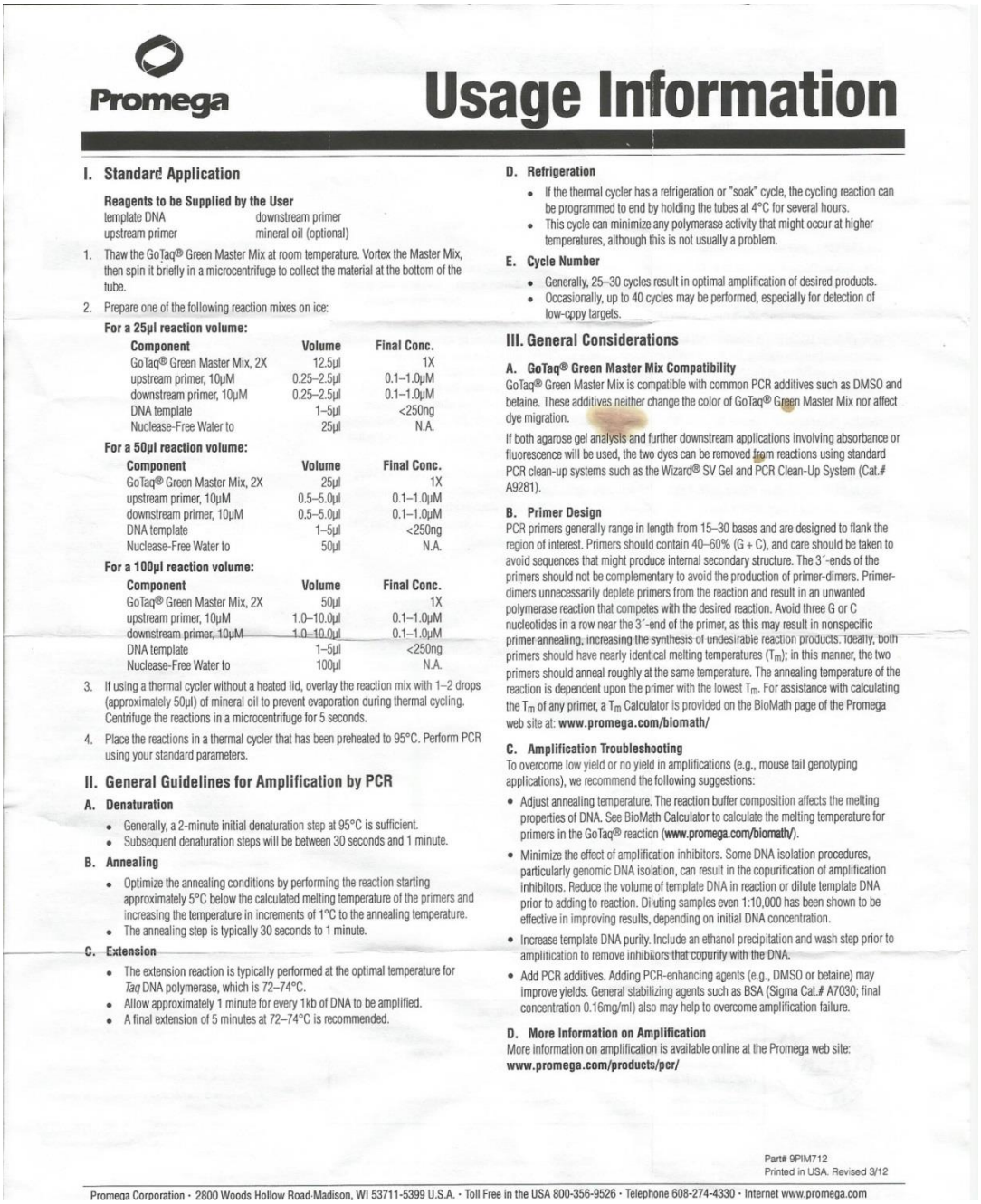
APPENDICES

APPENDIX A- MANUFACTURER'S PROTOCOLS

1. Go Taq Green Master Mix – Promega



Promega

Usage Information

I. Standard Application

Reagents to be Supplied by the User

template DNA	downstream primer
upstream primer	mineral oil (optional)

1. Thaw the GoTaq® Green Master Mix at room temperature. Vortex the Master Mix, then spin it briefly in a microcentrifuge to collect the material at the bottom of the tube.
2. Prepare one of the following reaction mixes on ice:

For a 25µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	12.5µl	1X
upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

For a 50µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	25µl	1X
upstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
downstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	50µl	N.A.

For a 100µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	50µl	1X
upstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
downstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	100µl	N.A.

3. If using a thermal cycler without a heated lid, overlay the reaction mix with 1–2 drops (approximately 50µl) of mineral oil to prevent evaporation during thermal cycling. Centrifuge the reactions in a microcentrifuge for 5 seconds.
4. Place the reactions in a thermal cycler that has been preheated to 95°C. Perform PCR using your standard parameters.

II. General Guidelines for Amplification by PCR

A. Denaturation

- Generally, a 2-minute initial denaturation step at 95°C is sufficient.
- Subsequent denaturation steps will be between 30 seconds and 1 minute.

B. Annealing

- Optimize the annealing conditions by performing the reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature in increments of 1°C to the annealing temperature.
- The annealing step is typically 30 seconds to 1 minute.

C. Extension

- The extension reaction is typically performed at the optimal temperature for *Taq* DNA polymerase, which is 72–74°C.
- Allow approximately 1 minute for every 1kb of DNA to be amplified.
- A final extension of 5 minutes at 72–74°C is recommended.

D. Refrigeration

- If the thermal cycler has a refrigeration or "soak" cycle, the cycling reaction can be programmed to end by holding the tubes at 4°C for several hours.
- This cycle can minimize any polymerase activity that might occur at higher temperatures, although this is not usually a problem.

E. Cycle Number

- Generally, 25–30 cycles result in optimal amplification of desired products.
- Occasionally, up to 40 cycles may be performed, especially for detection of low-copy targets.

III. General Considerations

A. GoTaq® Green Master Mix Compatibility

GoTaq® Green Master Mix is compatible with common PCR additives such as DMSO and betaine. These additives neither change the color of GoTaq® Green Master Mix nor affect dye migration.

If both agarose gel analysis and further downstream applications involving absorbance or fluorescence will be used, the two dyes can be removed from reactions using standard PCR clean-up systems such as the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281).

B. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C), and care should be taken to avoid sequences that might produce internal secondary structure. The 3´-ends of the primers should not be complementary to avoid the production of primer-dimers. Primer-dimers unnecessarily deplete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Avoid three G or C nucleotides in a row near the 3´-end of the primer, as this may result in nonspecific primer annealing, increasing the synthesis of undesirable reaction products. Ideally, both primers should have nearly identical melting temperatures (T_m); in this manner, the two primers should anneal roughly at the same temperature. The annealing temperature of the reaction is dependent upon the primer with the lowest T_m. For assistance with calculating the T_m of any primer, a T_m Calculator is provided on the BioMath page of the Promega web site at: **www.promega.com/biomath/**

C. Amplification Troubleshooting

To overcome low yield or no yield in amplifications (e.g., mouse tail genotyping applications), we recommend the following suggestions:

- Adjust annealing temperature. The reaction buffer composition affects the melting properties of DNA. See BioMath Calculator to calculate the melting temperature for primers in the GoTaq® reaction (**www.promega.com/biomath/**).
- Minimize the effect of amplification inhibitors. Some DNA isolation procedures, particularly genomic DNA isolation, can result in the copurification of amplification inhibitors. Reduce the volume of template DNA in reaction or dilute template DNA prior to adding to reaction. Diluting samples even 1:10,000 has been shown to be effective in improving results, depending on initial DNA concentration.
- Increase template DNA purity. Include an ethanol precipitation and wash step prior to amplification to remove inhibitors that copurify with the DNA.
- Add PCR additives. Adding PCR-enhancing agents (e.g., DMSO or betaine) may improve yields. General stabilizing agents such as BSA (Sigma Cat.# A7030; final concentration 0.16mg/ml) also may help to overcome amplification failure.

D. More Information on Amplification

More information on amplification is available online at the Promega web site: **www.promega.com/products/pcr/**

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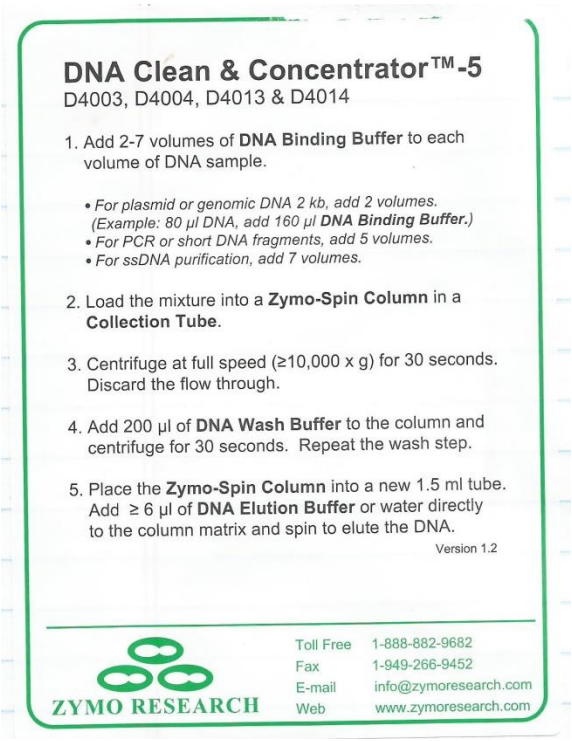
2. DNA Clean & Concentrator – Zymo Research



DNA Clean & Concentrator™-5
D4003, D4004, D4013 & D4014

1. Add 2-7 volumes of **DNA Binding Buffer** to each volume of DNA sample.

   - *For plasmid or genomic DNA 2 kb, add 2 volumes. (Example: 80 µl DNA, add 160 µl **DNA Binding Buffer.**)*
   - *For PCR or short DNA fragments, add 5 volumes.*
   - *For ssDNA purification, add 7 volumes.*

2. Load the mixture into a **Zymo-Spin Column** in a **Collection Tube**.
3. Centrifuge at full speed (≥10,000 x g) for 30 seconds. Discard the flow through.
4. Add 200 µl of **DNA Wash Buffer** to the column and centrifuge for 30 seconds. Repeat the wash step.
5. Place the **Zymo-Spin Column** into a new 1.5 ml tube. Add ≥ 6 µl of **DNA Elution Buffer** or water directly to the column matrix and spin to elute the DNA.

Version 1.2

ZYMO RESEARCH

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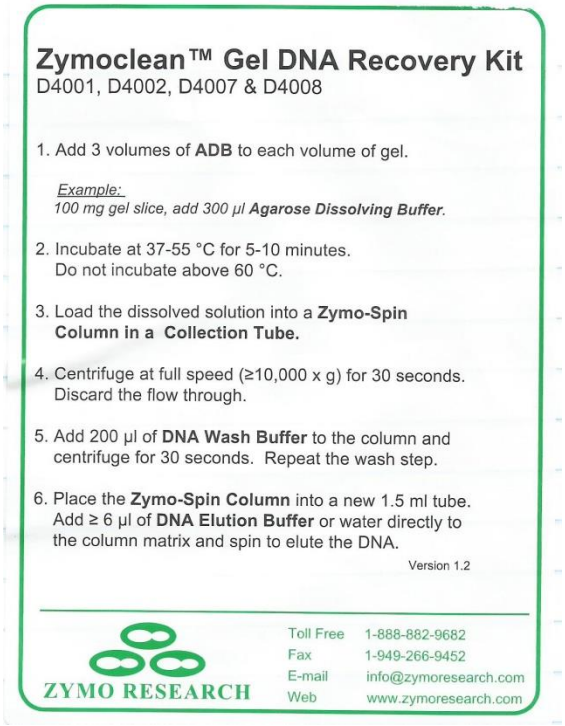
3. Zymoclean Gel DNA Recovery Kit – Zymo Research



Zymoclean™ Gel DNA Recovery Kit
D4001, D4002, D4007 & D4008

1. Add 3 volumes of **ADB** to each volume of gel.

   Example:
   *100 mg gel slice, add 300 µl **Agarose Dissolving Buffer**.*

2. Incubate at 37-55 °C for 5-10 minutes. Do not incubate above 60 °C.
3. Load the dissolved solution into a **Zymo-Spin Column in a Collection Tube.**
4. Centrifuge at full speed (≥10,000 x g) for 30 seconds. Discard the flow through.
5. Add 200 µl of **DNA Wash Buffer** to the column and centrifuge for 30 seconds. Repeat the wash step.
6. Place the **Zymo-Spin Column** into a new 1.5 ml tube. Add ≥ 6 µl of **DNA Elution Buffer** or water directly to the column matrix and spin to elute the DNA.

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4. LigaFast Rapid DNA Ligation System – Promega

Promega

Usage Information

I. Standard Application

A. Ligation of DNA

Material to Be Supplied by the User

- Nuclease-Free Water (Cat.# P1193)

We recommend starting with a 1:2 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. This ratio will vary with other types of vectors, for example, cDNA and genomic cloning vectors. The following example illustrates the conversion of molar ratios to mass ratios for a 3.0kb plasmid and a 0.5kb insert DNA fragment.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Example:

How much 0.5kb insert DNA should be added to a ligation in which 100ng of 3kb vector will be used? The desired vector:insert ratio will be 1:2.

$$\frac{100\text{ng vector} \times 0.5\text{kb insert}}{3\text{kb vector}} \times \frac{2}{1} = 33.3\text{ng insert}$$

The following ligation reaction of a 3kb vector and a 0.5kb insert DNA uses a 1:2 vector:insert ratio. Typical ligation reactions use 100–200ng of vector DNA.

1. Assemble the following reaction in a sterile microcentrifuge tube:

vector DNA	100ng
insert DNA	33ng
2X Rapid Ligation Buffer	5µl
T4 DNA Ligase (Weiss units)	3u
Nuclease-Free Water to final volume of	10µl

2. Incubate the reaction at room temperature for 5 minutes for cohesive-ended ligations, or 15 minutes for blunt-ended ligations.

Notes:

1. Ligation reactions performed using the 2X Rapid Ligation Buffer do not need to be cleaned up before transformation.
2. Concatamers may form as ligation products. The extent of concatamer formation depends on the vector:insert ratio, incubation temperature and incubation time (1). This should be taken into account when screening transformants.

II. Additional Information—T4 DNA Ligase

Molecular Weight: 68kDa (2).

Requirements: Mg^{2+}, ATP and DTT (2). The optimum concentration of Mg^{2+} is 0.5–1mM. Mn^{2+} may be substituted for Mg^{2+} but is only 25% as effective as Mg^{2+} (3).

Inhibition: 50% inhibition by greater than 150mM NaCl (activity measured at nicks) (2). Other inhibitors include 0.2M K^+, Cs^+, Li^+, NH_4^+ and 1mM spermine (3).

Inactivation: Heat at 70°C for 10 minutes (4).

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5 µL buffer
1.33 µL vector
2.67 µL insert
1 µL ligase

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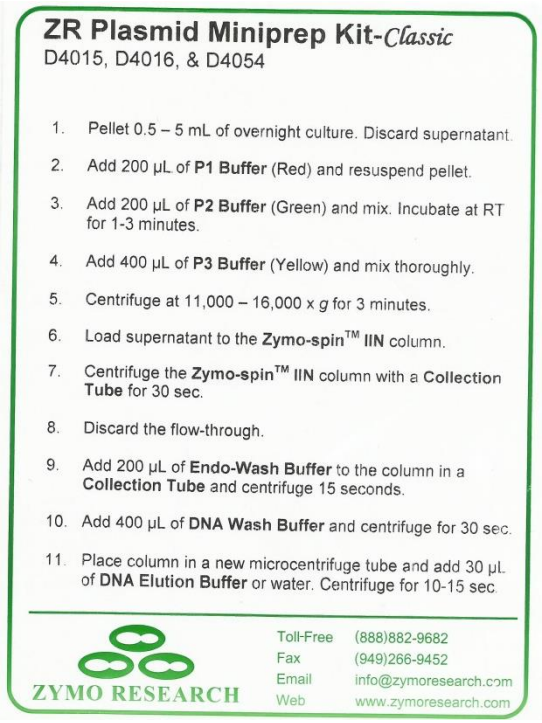
5. ZR Plasmid Miniprep Kit – Zymo Research



ZR Plasmid Miniprep Kit-*Classic*
D4015, D4016, & D4054

1. Pellet 0.5 – 5 mL of overnight culture. Discard supernatant.
2. Add 200 µL of **P1 Buffer** (Red) and resuspend pellet.
3. Add 200 µL of **P2 Buffer** (Green) and mix. Incubate at RT for 1-3 minutes.
4. Add 400 µL of **P3 Buffer** (Yellow) and mix thoroughly.
5. Centrifuge at 11,000 – 16,000 x *g* for 3 minutes.
6. Load supernatant to the **Zymo-spin™ IIN** column.
7. Centrifuge the **Zymo-spin™ IIN** column with a **Collection Tube** for 30 sec.
8. Discard the flow-through.
9. Add 200 µL of **Endo-Wash Buffer** to the column in a **Collection Tube** and centrifuge 15 seconds.
10. Add 400 µL of **DNA Wash Buffer** and centrifuge for 30 sec.
11. Place column in a new microcentrifuge tube and add 30 µL of **DNA Elution Buffer** or water. Centrifuge for 10-15 sec.

ZYMO RESEARCH

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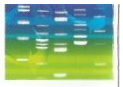
6. Mini-PROTEAN TGX Precast Gels – Bio-Rad

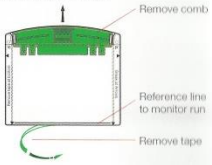
Mini-PROTEAN® TGX™ Precast Gels



Instructions for Using Mini-PROTEAN Precast Gels

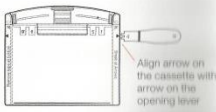
1. **Remove Comb:** Position thumb on indentation (middle of comb) and remove comb by pulling upward in one smooth motion.
2. **Remove Tape:** Pull the green tape gently to remove it from the bottom of the cassette.
3. **Assemble Mini-PROTEAN Tetra Cell:** Assemble the cassette into the running module of the Mini-PROTEAN Tetra system. Add running buffer to the inner and outer chambers. Use a syringe or a disposable transfer pipet to rinse the wells with running buffer.
4. **Run Gel:** Prepare the samples and load into the wells. If using Bio-Rad Precision Plus Protein™ standards, load 10 µl (5 µl for Precision Plus Protein™ WesternC™ standards). Run the gel until the dye front reaches the reference line. Refer to the instruction manual (#1658100) for more information on running conditions. At the completion of the run, disconnect the cell and remove the cassette.
5. **Open Cassette:** Align the arrow on the opening lever with the arrows marked on the cassette. Insert the lever between the cassette plates at the indicated locations and apply downward pressure to break the seal. Gently pull apart the two plates beginning from the top of the cassette.
6. **Remove Gel:** Gently remove the gel from the cassette.

Preparing the Cassette



Running the Gel

Opening the Cassette



Sample Preparation

Instructions are provided for electrophoresis of Mini-PROTEAN TGX long shelf life precast gels using the Mini-PROTEAN Tetra cell system.

	Reagent	Reduced Sample	Non-reduced Sample
Prepare samples	Sample	5 µl	5 µl
	2x Laemmli sample buffer (catalog #161-0737)*	4.75 µl	5 µl
	β-mercaptoethanol** (catalog #161-0710)	0.25 µl	—
	Total volume	10 µl	10 µl
	Heat samples at 90–100°C for 5 min		
Prepare running buffer	Prepare 1x Laemmli SDS-PAGE running buffer by adding 100 ml 10x TGS running buffer (catalog #161-0732) to 900 ml deionized water.		
Load running buffer	Remove the comb and tape from the bottom of the gel as described on page 2 and assemble the the Mini-PROTEAN Tetra cell. Fill the upper (inner) buffer chamber of each core with 200 ml 1x TGS running buffer. Fill the lower (outer) buffer changer as indicated on the Running Conditions table.		
Load sample	Load the appropriate volume of your protein sample on the gel and run the gel.		

* Note: 4x Laemmli sample buffer also available (catalog # 161-0747). See manual for instructions on use.

** DTT may also be used as a reducing agent. If so, add DTT to a concentration of 100 mM in 2x Laemmli sample buffer. Mix the sample with 2x reducing Laemmli sample buffer at a 1:1 ratio.

Running Conditions for Standard and Rapid Protocols

	100 V Low Voltage	200 V Standard	300 V Rapid 1	400 V Rapid 2
Run time	85–95 min	30–40 min	15–20 min	10–15 min
Expected current (per gel)				
Initial:	15–20 mA	25–50 mA	55–75 mA	89–140 mA
Final:	5–10 mA	20–31 mA	45–70 mA	81–127 mA
Expected temperature	25°C	25–35°C	30–45°C	40–55°C
Lower buffer volume (for 2 gels)	550 ml	550 ml	800 ml	800 ml
Lower buffer volume (for 4 gels)	800 ml	800 ml	800 ml	800 ml

Note: 1. When running only 1–2 gels in the Mini-PROTEAN Tetra cell, do not leave the companion module in the tank.

2. Do not run different gel types (chemistries) or percentages at the same time.

For detailed instructions, refer to the Mini-PROTEAN Precast Gels Instruction Manual and Application Guide (bulletin 1658100), available at **www.bio-rad.com**, or contact Technical Support at lsg_techserv_us@bio-rad.com or at 1-800-424-6723.

BIO-RAD

7. HisPur Ni-NTA Purification Kit – Thermo Scientific

INSTRUCTIONS

Thermo SCIENTIFIC

HisPur™ Ni-NTA Purification Kit

88227 88228 88229 2206.1

Number	Description
88227	**HisPur Ni-NTA Purification Kit, 0.2mL**
	Kit Contents:
	HisPur Ni-NTA Spin Columns, 0.2mL resin bed, 25 each
	Phosphate-Buffered Saline (10X), 30mL, 200mM sodium phosphate, 3M sodium chloride, pH 7.4
	Imidazole (2M), 8mL, pH 7.4
	Collection Tubes, 80 each
	Column Plugs, 25 each
88228	**HisPur Ni-NTA Purification Kit, 1mL**
	Kit Contents:
	HisPur Ni-NTA Spin Columns, 1mL resin bed, 5 each
	Phosphate-Buffered Saline (10X), 30mL, 200mM sodium phosphate, 3M sodium chloride, pH 7.4
	Imidazole (2M), 8mL, pH 7.4
	Column Plugs, 5 each
88229	**HisPur Ni-NTA Purification Kit, 3mL**
	Kit Contents:
	HisPur Ni-NTA Spin Columns, 3mL resin bed, 5 each
	Phosphate-Buffered Saline (10X), 30mL, 200mM sodium phosphate, 3M sodium chloride, pH 7.4
	Imidazole (2M), 25mL, pH 7.4
	Column Plugs, 5 each

Binding Capacity: ≤ 60mg of a 28kDa 6xHis-tagged protein from a bacterial source per milliliter of settled resin

Resin: Crosslinked 6% agarose

Supplied: 50% slurry in 20% ethanol

Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific HisPur Ni-NTA Purification Kit enables effective immobilized metal affinity chromatography (IMAC) purification of polyhistidine-tagged proteins from a soluble protein extract. The Ni-NTA resin is composed of nickel-charged nitrilotriacetic acid (NTA) chelate immobilized onto 6% crosslinked agarose. The Ni-NTA resin is compatible with native or denaturing conditions and can be used in multiple formats, including conventional gravity-flow chromatography, spin column and FPLC. Ni-NTA resins are commonly chosen for His-tagged-protein purification because of the four metal-binding sites on the chelate, which allow for high-binding capacity and low metal ion leaching.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

Important Product Information

- Protein yield and purity are dependent upon the expression level, conformation and solubility characteristics of the recombinant fusion protein. Therefore, it is important to optimize these parameters before attempting a large-scale purification. For best results, perform a small-scale test to estimate the expression level and determine the solubility of each His-tagged protein.
- Optimization of the lysis procedure is critical for maximizing protein yield. Some methods for protein extraction include using commercially available detergent-based reagents, such as Thermo Scientific B-PER Bacterial Protein Extraction Reagent with Enzymes (Product No. 90078), and mechanical methods, such as freeze/thaw cycles, sonication or French press. Add EDTA-free protease inhibitors, such as Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (Product No. 78441), to protect proteins from degradation.
- Avoid using protease inhibitors or other additives that contain chelators, such as EDTA, or strong reducing agents, such as DTT or β-mercaptoethanol, which will disrupt the function of the nickel resin.
- When using the Thermo Scientific Coomassie Plus (Bradford) Assay (Product No. 23238) or Thermo Scientific Pierce 660 nm Protein Assay (Product No. 22660) to monitor protein concentration in the elution fractions, dilute the samples at least 1:2 before performing the protein assay.

Additional Materials Required

- Regeneration MES Buffer: 20mM 2-(*N*-morpholine)-ethanesulfonic acid, 0.1M sodium chloride; pH 5.0

Material Preparation

Depending on the specific protein, buffers might require some optimization. Use the table below to make buffers with different imidazole concentrations. Adjust the total volume depending on the resin volume used. For most proteins, the following imidazole concentrations are recommended:

- Equilibration Buffer: 10mM imidazole
- Wash Buffer: 25mM imidazole
- Elution Buffer: 250mM imidazole

Imidazole Final Conc. (mM)	10X PBS* (mL)	2M Imidazole (µL)	Water (mL)
Equilibration → 10	1	50	8.95
Wash → 25	1	125	8.875
40	1	200	8.8
60	1	300	8.7
75	1	375	8.625
150	1	750	8.25
200	1	1,000	8
Elution → 250	1	1,250	7.75
500	1	2,500	6.5

*Phosphate-buffered saline.

Procedure for Spin Purification of His-Tagged Proteins

Note: The total volume of the 0.2, 1 and 3mL columns are 1, 8 and 22mL, respectively. If a sample volume is greater than the column, perform multiple applications and centrifugations until the entire sample has been processed. Be careful not to exceed the resin's binding capacity. The HisPur Ni-NTA Spin Columns also may be used for gravity-flow purifications.

1. Equilibrate column(s) to working temperature. Perform purifications at room temperature or at 4°C.
2. Prepare sample by mixing protein extract with Equilibration Buffer so the total volume equals two resin-bed volumes.
3. Remove the bottom tab from the HisPur Ni-NTA Spin Column by gently twisting. Place column into a centrifuge tube.

   Note: Use 1.5, 15 or 50mL centrifuge tubes for the 0.2, 1 and 3mL spin columns, respectively.
4. Centrifuge column at 700 × *g* for 2 minutes to remove storage buffer.

5. Equilibrate column with two resin-bed volumes of Equilibration Buffer. Allow buffer to enter the resin bed.
6. Centrifuge column at 700 × *g* for 2 minutes to remove buffer.
7. Add the prepared protein extract to the column and allow it to enter the resin bed.

   Note: For maximal binding, the sample can be incubated for 30 minutes at room temperature or 4°C on an end-over-end rocking platform.
8. Centrifuge column at 700 × *g* for 2 minutes and collect the flow-through in a centrifuge tube.
9. Wash resin with two resin-bed volumes of Wash Buffer. Centrifuge at 700 × *g* for 2 minutes and collect fraction in a centrifuge tube. Repeat this step two more times collecting each fraction in a separate centrifuge tube.

   Note: If desired, perform additional washes. Monitor washes by measuring their absorbance at 280nm.
10. Elute His-tagged proteins from the resin by adding one resin-bed volume of Elution Buffer. Centrifuge at 700 × *g* for 2 minutes. Repeat this step two more times, collecting each fraction in a separate tube.

    Note: If performing gravity-flow add two resin-bed volumes of Elution Buffer to achieve proper flow characteristics.
11. Monitor protein elution by measuring the absorbance of the fractions at 280nm or by Coomassie Plus (Bradford) Assay Reagent (Product No. 23238). The eluted protein can be directly analyzed by SDS-PAGE.

    Note: To remove imidazole for downstream applications, use gel filtration (e.g., Thermo Scientific Zeba Spin Desalting Columns) or dialysis (e.g., Thermo Scientific Slide-A-Lyzer Dialysis Cassettes).

Procedure for Ni-NTA Resin Regeneration

The Ni-NTA resin may be used at least five times without affecting protein yield or purity. Between each use, perform the procedure as described below to remove residual imidazole and any nonspecifically adsorbed protein. To prevent cross-contamination of samples, designate a given column to one specific fusion protein.

1. Wash resin with 10 resin-bed volumes of MES Buffer.
2. Wash resin with 10 resin-bed volumes of ultrapure water.
3. Store resin as a 50% slurry in 20% ethanol.

Troubleshooting

Problem	Possible Cause	Solution
Low protein yield	Poor expression of soluble protein	Optimize expression conditions
	His-tagged protein forms inclusion bodies	Alter growth conditions to minimize inclusion body formation and maximize soluble protein yield
		Solubilize inclusion bodies and perform the purification with a compatible denaturant (e.g., Thermo Scientific Inclusion Body Solubilization Reagent, Product No. 78115)
	Insufficient cell lysis and extraction	Optimize cell lysis protocol
	His tag is absent	Verify the sequence or perform an ELISA or Western blot using an antibody against the His tag
	His tag is inaccessible using native conditions	See the Additional Information Section for denaturing conditions
	His-tagged protein has a low affinity to the column	Optimize the Equilibration or Wash buffer by decreasing the concentration of imidazole (see Table in the Material Preparation Section)
Poor protein purity	Insufficient column washing	Wash column additional times or modify the imidazole concentration (see Table in Material Preparation Section)
Slow column flow	Column is overloaded	Apply less protein extract to the column and make sure the extract is not too viscous or contaminated with cell debris

APPENDIX B - SEQUENCES

Sequences were obtained from the National Center for Biotechnology Information (NCBI). It should be noted that the sequence for the $P_{\beta glu}$ cellobiose-inducible promoter could not be located- the start and end sequence was obtained via the primers constructed by Bartosiak-Jentys et al.[49]

P_{recA} from C56 gDNA:

cacgttcccgcttctgttttagcaaccgacggagcggtaagtgaacgctgcgcgaagctgttggcggaaa
atgtccgcgcgctttgcggcgcagatattggcattagctttacaggcgtggcaggaccggatccgcttga
aggaaagtcggtcggtaccgtatatattggcatttccacttctgaaaatgaaacagctgtccacgccctc
gcgttatccggcccgcgtgatgcaatccgcacccgcactgctaagtacggctgctccattatattaaaaa
aattggcagctgcatgtttgtagctgctctcttcctcttgcctcccctcactgcttttttcctcgcttaag
gagaatttttctattcccgccatccaaaaaacgaatgaacgttcgatttttttcttggcaaccgccttaa
aaaacggtatagtataagtagtcaagaaaaaggaggagtttgtta

This is the sequence upstream of the start codon of the recA gene with the part highlighted the gene before it. PrecA was inserted into pNW33N to make pTC02.

Laccase from C56 gDNA:

atgttagaca tttttcaaca agcgggagaa gagatgctgt tgttgcacgg ccgccgttcg
tttcctaatc ttgtcgccgg atttacgacg aaacacggag gcgtcagcaa aggagcgttt
gcgacgttca atttagggct gcatgttggc gatgaggttt cttctgtttg ccgcaatcgg
cagcggctgg cggatctgct ccaatttccg ctggaacaat gggtatgttg cgaacaaata
cacgatgccc gcatcgaaaa ggtgacaagc agccaaagcg ggaaaggagc ggcggattac
aagtcagcca ttgctgggac ggacggcttg tatacaaaag aagctggatt gctgcttgcc
ttatgtttcg ctgattgcgt accgctttat tttatggcgc cgaaacatgg catgatcggt
cttgctcatg ccggctggcg gggaacggta aaaaacattg cgggagaaat gattcacctt
tggcatgagc gtgaacatat tcctttggat gacatatatg tggcgatcgg tccggcgatt
ggcgcttgct gctatatcgt cgatgaccgc gtcatcacgt acgttgattg cattttggat
ggtgaacagg ctccctataa gcaagtgagc ataggacaat atgcccttga tttaaaagag
ctgaataaag tattactgat acaggcagga gttcgtgaag aacatattga tatttccggg
tattgtacga gttgcgctga ttatttgttt ttttcccatc gtcgcgatca aggaaaaaca
ggaagaatga tggcgtttat cggcaggaag ggggaatga

This is the sequence coding for the laccase in the gDNA of *Geobacillus* C56. It was inserted after the P_{recA} promoter in pTC02 to make pTC07, and after the $P_{\beta glu}$ promoter in pTC09 to make pTC12. The start and stop codons are highlighted.

Gfpmut3 from pKW1040:

Atgcgtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatg
ggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttat
ttgcactactggaaaactacctgttccatggccaacacttgtcactactttcggttatggtgttcaatgc
tttgcgagatacccagatcatatgaaacagcatgacttttcaagagtgccatgcccgaaggttatgtac
aggaaagaactatatctttcaaagatgacgggaactacaagacacgtgctgaagtcaagtttgaaggtga
taccccttgttaatagaatcgagttaaaaggtattgattttaaagaagatggaaacattcttggacacaaa
ttggaatacaactataactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagcta
acttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccattatcaacaaaatactcc
aattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccctttcgaaagat
cccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctgggattacacatggcatgg
atgaactatacaaataataa

This is the sequence for the gfpmut3 gene. It was inserted after laccase in pTC07 and pTC12 to construct pTC08 and pTC13, respectively. The start and stop codons are highlighted.

APPENDIX C- SOLUTION AND MEDIA TABLES

Growth Media

Name	Use	Composition	Sterile?[1]
LB	Growth of E. coli strains in suspension	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl	A
LB Agar	Growth of E. coli strains on plates	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar	A
TGP	Growth of Geobacillus strains in suspension	0.5% NaCl, 1.7% tryptone, 0.3% soytone, 0.25% K_2HPO_4, 0.4% sodium pyruvate, 0.4% glycerol	A[2]
TBAB	Growth of Geobacillus strains on plates	10 g/L tryptose, 3 g/L beef extract, 5 g/L NaCl, 15 g/L Agar	A

1. A = autoclave, F = filter sterilize, N/A = no sterilization needed
2. NaCl, tryptone, soytone and K_2HPO_4 are dissolved in water and autoclaved. Filter-sterilized sodium pyruvate and glycerol are then added to complete the media

Solutions and Buffers

Name	Use	Composition	Sterile?
TE buffer	gDNA extraction	10 mM Tris-Cl, 1 mM EDTA	N/A
1x TAE buffer	Running buffer for DNA gel electrophoresis	40 mM Tris-acetate, 2 mM Na2EDTA	N/A
ITB	Making E. coli chemically competent	55 mM MnCl2, 15 mM CaCl2, 250 mM KCl, 10 mM PIPES	F
Electroporation buffer	Making Geobacillus 95A1 electrocompetent	0.5 M mannitol, 0.5 M sorbitol, 10% glycerol	F
Sonication buffer	Cell wash buffer for extracting intracellular protein	50 mM Tris-Cl, 500 mM NaCl, 15% glycerol	F
2x Laemmli sample buffer	Protein sample buffer for SDS-PAGE	65.8 mM Tris-Cl, 2.1% SDS, 26.3 % glycerol, 0.01% bromophenol blue	N/A
1x SDS-PAGE running buffer	Running buffer for SDS-PAGE	2.5 mM Tris base, 19.2 mM glycine, 0.1% SDS, pH 8.3	N/A
Fixing solution	Fixing solution for in-gel His-tag staining	50% ethanol, 10% acetic acid in water	N/A
Protein staining gel buffer	Buffer for in-gel His-tag staining	20 mM phosphate buffer, pH 7.8	N/A
Laccase assay buffer	Buffer used for quantification of laccase activity	150 mM citrate-phosphate buffer (varying pHs)	N/A

Immobilized Nickel Affinity Chromatography Buffers

Name	Composition
Equilibration buffer	10 mM imidazole in 1x PBS
Wash buffer	25 mM imidazole in 1x PBS
Elution buffer	250 mM imidazole in 1x PBS
Regeneration buffer	20 mM MES buffer, 0.1 M NaCl, pH 5.0
Storage buffer	20% ethanol in water