Advanced Recombinant Manganese Peroxidase for Biosynthesis of Lignin Bioproducts

Phase I Final Report

STTR Grant #: DE-SC0007503

Topic 17: Catalysis
Subtopic b: Chemical Catalysis of Lignin

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Executive Summary

The core purpose of this Phase I STTR was to evaluate the feasibility of a new method of producing a recombinant version of manganese peroxidase (MnP) enzyme. MnP is a potentially valuable enzyme for producing high value lignin products and also for industrial de-coloring operations such as biobleaching of pulp and color removal from textile dye effluents. This lignin-modifying enzyme is produced in small amounts by the native host, a white rot fungus. Previous work by Oregon State University developed a secreted recombinant version of the enzyme in the yeast *Pichia pastoris.* Unfortunately, the expression is barely moderate and the enzyme is heavily glycosylated, which inhibits purification. In this work, the gene for the enzyme is given a tag which targets production of the enzyme to the peroxisome. This is a promising approach since this location is also where heme and hydrogen peroxide are sequestered, which are both necessary cofactors for MnP.

More than ten recombinant strains were constructed, verified, and expressed in the *Pichia* system. Constitutive (GAP) and methanol-induced promoters (AOX) were tried for peroxisomal targeted, cytosolic, and secreted versions of MnP. Only the secreted strains showed activity. The amount of expression was not significantly changed. The degree of glycosylation was lessened using the AOX (methanol) promotoer, but the resulting enzyme was still not able to be purified using immobilized metal affinity chromatography.

Additional work beyond the scope of the defined Phase I project was undertaken to construct, verify, and express *Pichia* strains that mutated the MnP glycosylation sites to inhibit this process. These strains did not show significant activity. The cause is not known, but it is possible that these sites are important to the structure of the enzyme.

Also beyond the scope proposed for our Phase I STTR, the team collaborated with AbSci, a startup with a new *E. coli* based expression system focused on the production of antibodies and enzymes containing disulfide bonds and requiring folding/post-translational modification. With only limited time remaining in the Phase I schedule, a single construct was made to produce MnP with this system. The enzyme was produced in the soluble fraction of the cell lysate, but no activity was measured.

MnP from the existing recombinant source was used to act on lignin. The lignin was from a Kraft process and had a molecular weight of about 10,000 Da. Using 1000 Da dialysis membranes and UV-visible spectroscopy, no modification of either lignin was evident in the dialysate or the retentate. Assays using 2,6 dimethoxy phenol (DMP) as a substrate showed consistent activity throughout the project.

In summary, these results fell far short of our expectations. A Phase II proposal was not submitted. Possible reasons for the failure of peroxisomal targeting include destruction by native hydrogen peroxide, native proteases, or unforeseen causes. The AbSci system was only lighted tested and further work may yield a strain with active enzyme. The lack of evidence for lignin modification may be due to the techniques employed. NMR or GC-MS studies may reveal evidence of modification.

Identification and Significance of the Opportunity

Lignin is one of the most abundant polymers on earth and manganese peroxidase (MnP) is a primary enzyme by which lignin is recycled in the environment. MnP has potential in the biochemical processing of lignocellulosic materials, but only small amounts and concentrations of MnP can be produced with current biotechnologies.

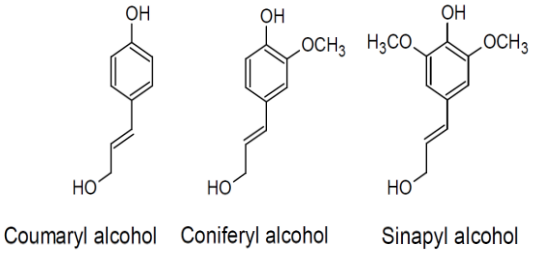

Figure 1. Aromatic C9 units of the lignin polymer

Lignin is fundamentally different than the cellulose and hemicellulose fractions of biomass: it is an amorphous poly-phenolic polymer formed when three types of phenylpropanoid monomers (coniferyl, sinapyl, and p-coumaryl alcohols, Figure 1) polymerize by enzyme mediated dehydrogenation reactions. The resulting three-dimensional lignin polymer (Figure 2) lacks the regularity found in other polymers such as cellulose and proteins (Dence and Lin, 1992), rendering it more resistant to biodegradation. The overall structure is complex and varies with biomass source and processing. Lignin lends rigidity to plant stems allowing them to withstand the forces of wind and gravity, and constitutes a protective barrier against microbial attack (Glasser et al., 2000).

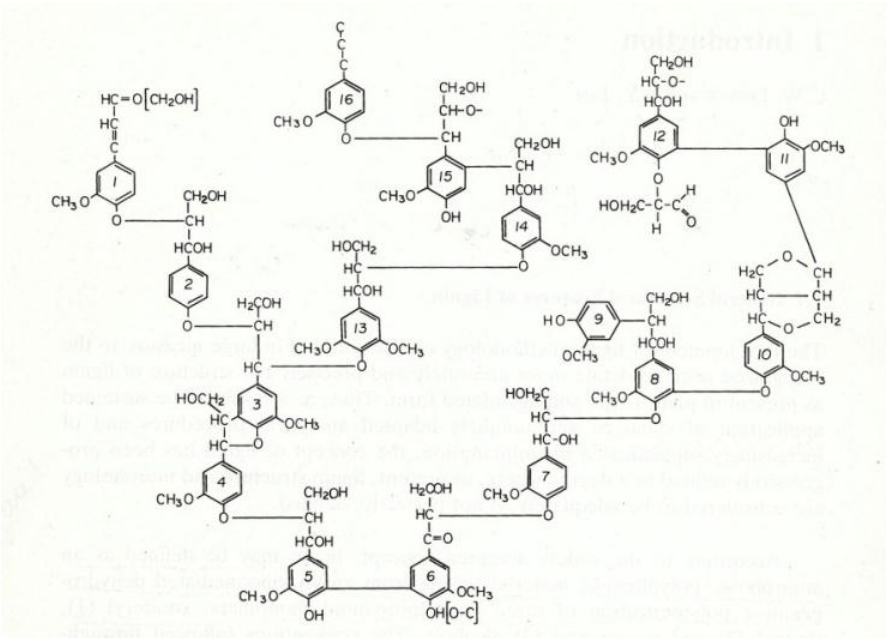

Figure 2. Model of spruce lignin (Adler, 1977)

Microorganisms have evolved a variety of enzymes for degrading the different components of lignocellulosic material, including cellulose (cellulases), hemicellulose (xylanases), and lignin (ligninolytic enzymes), to effectively recycle plant biomass into the environment as CO_2 and H_2O. The ligninolytic enzymes include lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases. These enzymes attack lignin directly, and thereby are essential to the degradation and cycling of carbon from plant biomass. Microorganisms degrade plant biomass in a series of biochemical reactions that yield materials and energy for cell growth. One of the early steps of this process is the enzyme-catalyzed removal or separation of the lignin polymer from the plant matrix. Manganese peroxidase (MnP) appears to be the most effective lignin-degrading enzyme commonly produced and secreted by microorganisms. Lignin removal renders the cellulose and hemicelluloses susceptible to degradation by cellulases and xylanases, respectively. As a glucose polymer, cellulose is often the most desirable component of plant biomass from the standpoint of both microbial growth and paper manufacture. The lignin is highly aromatic in nature and may be the best portion of the feedstock for chemical synthesis. Lignin is enzymatically converted to phenolics and other aromatics by MnP. These aromatics, as well as other wood derived chemical products, can be further acted on by MnP to produce an array of different products (Hofrichter, 2002, Figure 3).

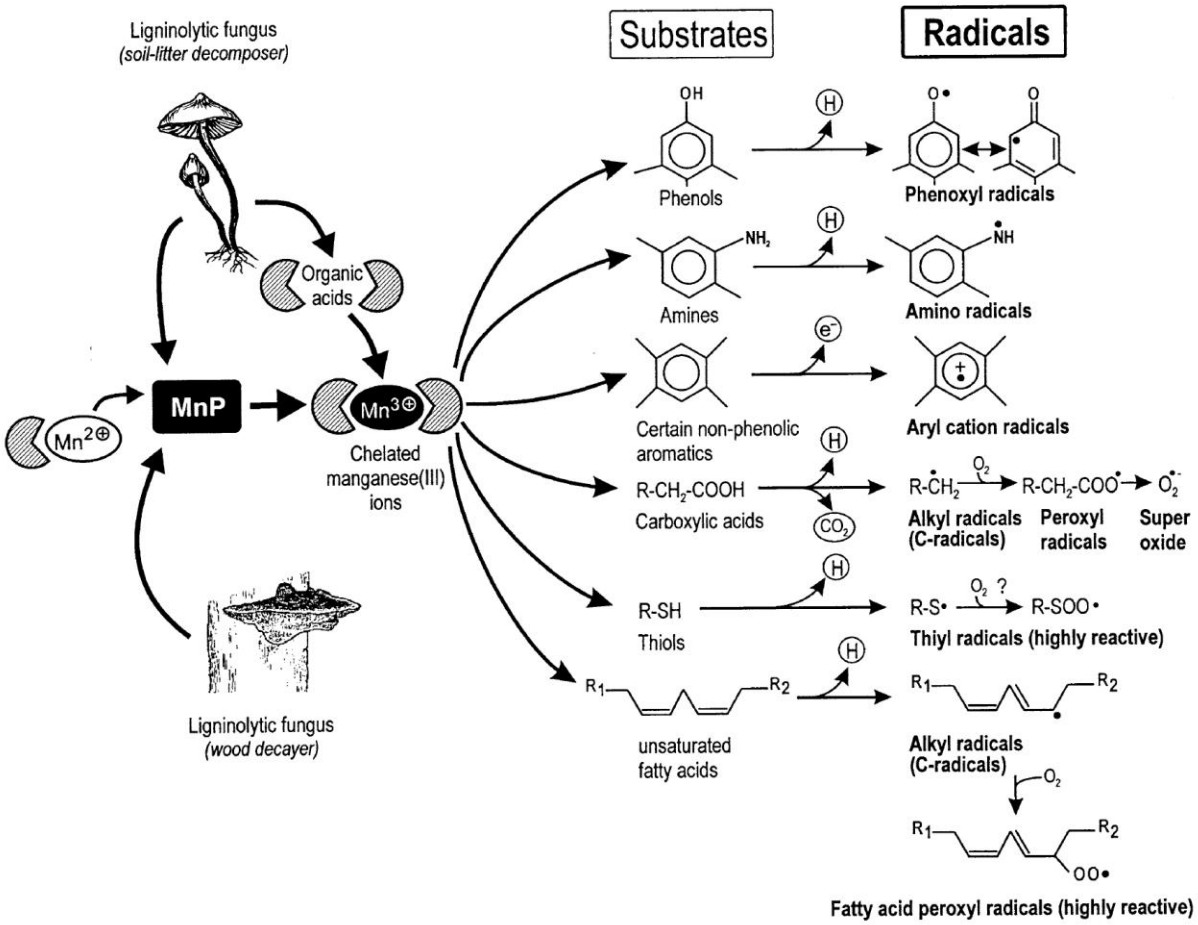

Figure 3.MnP mediated transformations of lignin (Hofrichter, 2002)

Biological processes for fuel and chemical production are often biomimetic in nature, relying on natural microorganisms and enzymes for their inspiration. Considerable effort has been invested in the discovery and development of improved cellulases for converting cellulose to sugars, and in fermentation strains and strategies for converting these sugars to ethanol. In contrast, lignin modification processes typically entail high pressure and temperature physiochemical processes with little contribution from natural processes. This is due to a lack of understanding of the mechanisms of enzymatic lignin degradation, as well as difficulty in producing purified heme peroxidases (MnP and lignin peroxidase) at practical concentrations in industrial scale fermentations. While cellulase costs are a major factor in overall estimates for cellulosic ethanol economics, MnP has an important difference that results in much lower anticipated MnP loading and cost required for biomass (lignin) processing. Cellulases must physically contact the solid substrate (cellulose) to perform their catalytic activity and therefore reuse of the enzymes is difficult. The catalytic moiety in MnP transformation of lignin is Mn^{3+}. MnP oxidizes the Mn, which then diffuses to the solid lignin site for reaction. The MnP can be physically distant from the lignin, immobilized onto a carrier. In this approach, the MnP will go through many catalytic cycles, re-oxidizing the diffusing, chelated Mn.

Manganese peroxidase (MnP)

MnP was first discovered and cloned from the white rot fungus (Figure 4) *Phanerochaete chrysosporium* (Glenn et al., 1986; Pease et al., 1989), and has subsequently been found in virtually all lignin degrading fungi (Kirk and Farrell, 1987). The overwhelming importance of MnP in lignin catalysis is further supported by the observation that Mn content is highly correlated with litter decomposition in temperate and boreal forests. This enzyme is typically produced by white-rot fungi during secondary metabolism. MnP is a heme peroxidase that catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}. Heme is a tightly held cofactor to the enzyme and is typically a heterocyclic organic compound that is coordinated to a central metal atom (usually iron)(Figure 5). Organic acids chelate Mn^{3+} to create the diffusible oxidants that attack lignin structures (Kirk and Cullen, 1998). There are also less well understood reactions involving organic acids and redox mediators that appear to be active against lignin-like structures in the absence of H_2O_2 (Hofrichter et al., 1998; Harazono et al., 2003). MnP is a 46 kDa acidic glycoprotein that contains one molecule of heme, substrate binding sites for peroxide and Mn^{2+}, and two calcium ions that confer thermal stability to the active sites (Sutherland and Aust, 1996). Elimination of the glycosylation site via site-directed mutagenesis or enzymatic deglycosylation do not affect the catalytic activity or stability of the enzyme (Zhang et al., 2009; Nie et al., 1999).

Figure 4.White rot fungus Phanerochaetechrysosporium. Top: Growing on wood chips. Bottom: Magnified view of the (photo from the USDOE Joint Genome Institute website)

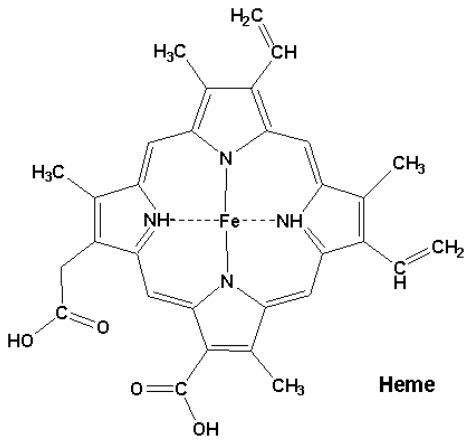

Figure 5. Heme, the catalytic center of the enzyme MnP (Mathews et al, 2000)

Production of manganese peroxidase (MnP)

The concentrations of MnP produced naturally by white-rot fungi during secondary metabolism are typically very low (5 mg/L), and the culture supernatant often contains an array of enzymes, some of which may damage desirable components of the plant biomass. White-rot fungi commonly grow as mycelia, and do not perform well in high cell density, stirred-tank reactors typical of industrial fermentations, although considerable progress has been made by screening large numbers of isolates for strains with desirable characteristics and by using novel production strategies (Herpoel et al., 1999; 41 mg/L MnP). Another approach to increase enzyme titers is to place the *mnp* gene under control of an alternative promoter in the original host (homologous expression), which allows production under primary metabolism (Mayfield et al., 1994; 9 mg/L MnP; Tsukihara et al., 2006; 46 mg/L MnP).

Another alternative for producing MnP is to clone the gene encoding MnP synthesis (*mnp*) from the white-rot fungus, place it under the control of a strong promoter, and insert it in the DNA of an alternative host more suitable for "traditional" batch and fed-batch industrial fermentations (heterologous expression). By using a host species with no natural cellulase or ligninolytic enzyme activity, contaminating enzymes potentially detrimental to cellulose are avoided. Manganese peroxidase (*mnp1*) encoding genes from *P. chrysosporium* have been expressed in *E. coli*, but only inactive inclusion bodies resulted (Whitman et al., 1995). Recombinant MnP (rMnP) was successfully produced in the baculovirus expression system (Pease et al., 1991, 5 mg/L), the filamentous fungi *Aspergillus oryzae* (Stewart et al., 1996; 5 mg/L rMnP) and *A. niger* (Conesa et al., 2000; 100 mg/L rMnP). In Dr. Kelly's previous work, the methylotrophic yeast *P. pastoris* (Gu et al., 2003; Jiang et al., 2008a, 25 mg/L rMnP; Yee et al., 2011, 30 mg/L) has also been utilized with some success. In all of these heterologous expression systems, maximum rMnP production requires very high concentrations of exogenous heme, ranging from 0.1g/L heme in *P. pastoris* to 5 g/L hemoglobin in *A. niger*. The results of these studies indicate the central role of heme availability during maturation of heme-containing proteins. All of these expression systems, including those employing white-rot fungi, produce far less MnP than the 1-10 g/L titers typical for enzymes of commercial relevance. For applications involving the conversion of agricultural and forestry residues to bio-products, it is most certainly necessary for the enzymes to be inexpensive.

The innovative approach:
Peroxisomal targeting of recombinant manganese peroxidase in *P. pastoris*

Peroxisomes are organelles found in all eukaryotic cells, and function as compartments for hydrogen peroxide producing oxidases involved in fatty acid and methanol metabolism. Peroxisomal matrix proteins are nuclear encoded, synthesized on free ribosomes, and imported post-translationally

(Girzalsky et al., 2009). Peroxisomes can import totally processed proteins containing co-factors, and these proteins are not glycosylated during uptake. Within the peroxisome, proteins are protected from proteolytic degradation. The most common peroxisome targeting signal (PTS) is the tripeptide SKL (PTS1), although there are other conserved variants located at the C-terminus of proteins.The specific PTS for alcohol oxidase in *P. pastoris* is LARF (Waterham et al., 1997).

This approach may alleviate problems with folding, heme availability, and hyperglycosylation during translation and secretion of rMnP that are currently encountered in heterologous production systems as well as reducing host protease activity against rMnP or any harmful effects of rMnP to the host cells (Wang et al., 2009).

In addition to producing more rMnP per cell, the peroxisome targeting technology facilitates higher titers compared to the secreted approach due to the ease of cell/broth separations. Cells can be easily separated from broth via centrifugation, filtration, or even settling; thereby concentrating the MnP. Using the peroxisomal targeting approach, we believe that this team can construct a recombinant strain of *Pichia pastoris* that will be capable of producing industrially useful quantities of manganese peroxidase for the synthesis of biochemicals from lignin.

Potential Applications and Benefits

A new commercial source of the lignin-catalyst MnP at reduced cost would increase the efficiency of chemical production from lignin, and provide a new source of phenolics and aromatic chemicals that is competitive with petroleum and coal-based options. Inexpensive MnP may also be an enabling technology for other manufacturing processes based on wood and plant residues. Biomass based technologies, such as paper manufacture and biofuels production, do not actually employ the lignin component in the manufacturing process other than for its energy value. This lignin could provide an additional revenue stream if converted to industrially useful organic chemicals, rendering all these bioprocesses more cost effective in their overall economics.

The oxidative power of MnP also enables biobleaching of pulp (Sasaki et al., 2001) and the decolorization of certain textile dyes to minimize environmental issues associated with these effluents (Lopez et al., 2004).

Lignin is the only renewable, high volume source of aromatics currently available. Depolymerization of the aromatic structure could result in simple BTX aromatics immediately useful in current synthetic petrochemical processes (Figure 6). Alternatively, a plethora of more complex aromatics may result, some of which may be useful and difficult to synthesize by conventional petrochemical routes. The free radical reactions catalyzed by MnP in the environment probably yield these simple aromatic compounds during the course of lignin deconstruction, and MnP catalyses of lignin could become a core processing technology in a future bio-based economy.

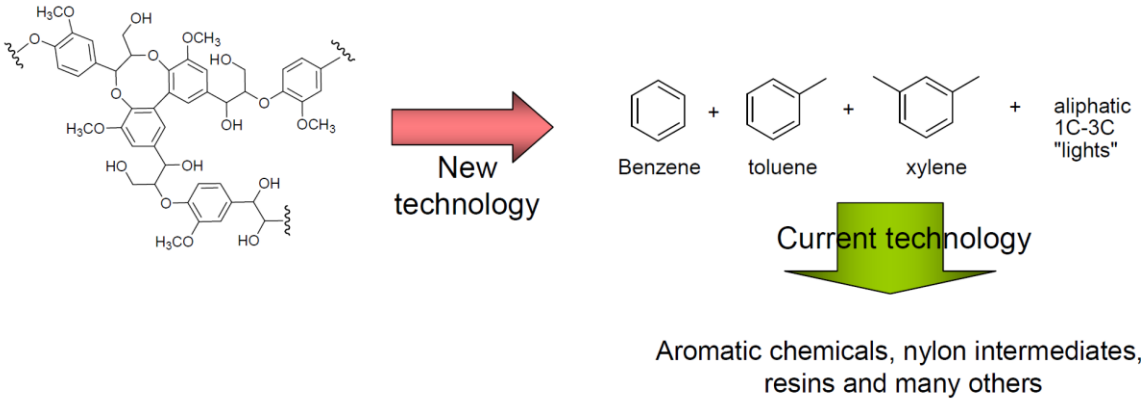

Figure 6. BTX Aromatics from lignin (Holladay et al., 2007)

Review of Technical Objectives

Technical Objective 1: Create recombinant strains of the yeast *Pichia pastoris* that produce manganese peroxidase in the peroxisome of the cell at a concentration of 0.5 grams of rMnP/L of fermentation broth. (>10X improvement from current strain).

Not accomplished.

Targeting of rMnP in recombinant P. pastoris. Previous work at Oregon State University created a recombinant *Pichia pastoris* (yeast) strain that produces rMnP by cloning the MnP gene from the native host *Phanerocheate chrysosporium* (fungus). This strain secretes active MnP into the culture, but enzyme titers remained too low for commercial application, and hyperglycosylation interferes with downstream purification methods. By targeting the production MnP to the peroxisome of the yeast cells, it was anticipated that enzyme titers would increase due to greater availability of heme which is required for folding and functionality. In addition, glycosylation would be eliminated since this occurs as part of the secretion process. Additional strains were also constructed in an attempt to achieve higher enzyme production by the yeast, as indicated below (Table I).

Table 1. Recombinant *P. pastoris* strains created during this project.

rMnP Targeted Location	**GAP promoter (pGAPZ)**	**AOX1 promoter (pPIC3.5K)**
Secreted	P#1 (existing)	MM/2-1
Intracellular	GIZ	MM/4-1
Peroxisome (SKI signal)	PSZ	PSP
Peroxisome (LARF signal)	GLF-1-S	BOXD1

Recombinant *P. pastoris* strains were created by using PCR (polymerase chain reaction) to change the signal sequence on the MnP and add appropriate stop codons and a histidine tag for antibody detection, and then cloning the resulting gene into plasmid pGAPZ (GAP constituitive promoter) or pPIC3.5K (AOX1 methanol inducible promoter). Following transformation into *E. coli*, strains containing the new plasmid vectors were isolated, and the plasmids extracted and the *mnp1* gene sequenced for verification of the desired contruct. The expression vectors were then electroporated into *P. pastoris* strain SMD and/or GS115. To verify insertion of the expression vector the genomic DNA was extracted from these recombinant strains, and the *mnp1* gene PCR amplified and sequenced. MnP production was determined by shake-flask cultivation using glucose and/or methanol as the growth substrate. For these experiments a new shake flask medium was developed using MES buffer to maintain the culture pH at 6 to protect the recombinant rMnP from proteolytic degradation which occurs at lower pH values.

Results of these experiments using MnP activity assays and SDS-PAGE and Western blots with anti-HIS antibodies for rMnP detection indicated that rMnP was produced at detectable levels only when it was targeted for secretion (strains P#1 and MM/2-1). Final culture enzyme concentrations (50 mg/L) were far below the target concentration (1g/L). These results are summarized in Figure 7.

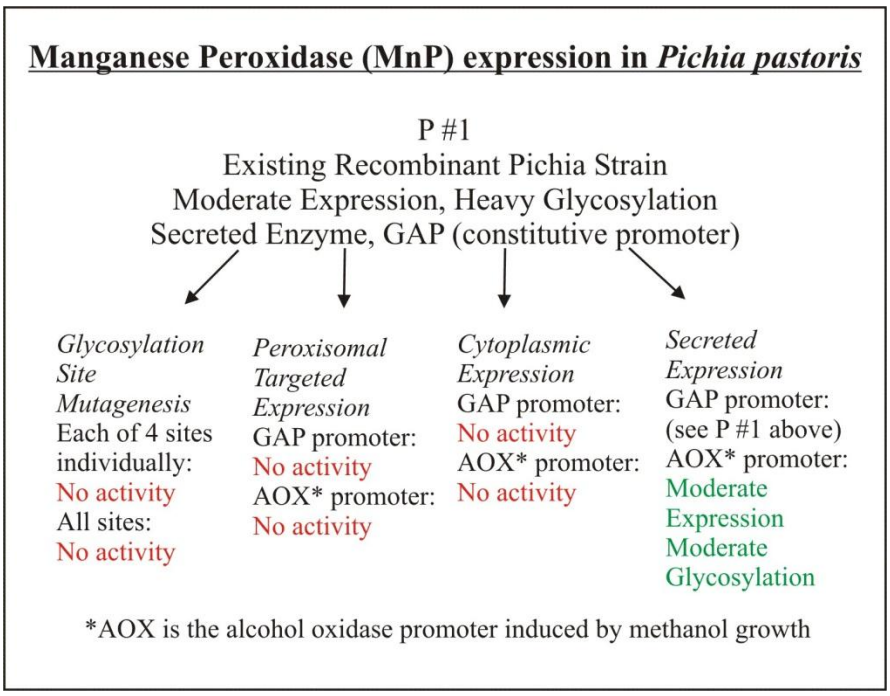

Figure 7. Recombinant Pichia strains for MnP production.

Removal of glycosylation sites from mnp1.

Previous experiments indicated that the rMnP secreted by *P. pastoris* is hyperglycosylated (addition of carbohydrate, primarily mannose) resulting in a mass distribution of 50 to greater than 100 kDA, whereas the native host (*P. chrysosporium*) adds a much lesser amount of this carbohydrate. The result is that whereas the native enzyme can be purified by column chromatography, the rMnP binds irreversibly to the column. Sequence analysis indicates that the *mnp1* gene has 4 potential glycosylation sites (Asp-X-Ser/Thr; Figure 8). Experiments were conducted in this study to eliminate each of these glycosylation sites using site-directed mutagenesis to create rMnP more amenable to purification.

Each of the 4 potential glycosylation sites were mutated individually by changing the asparagine to threonine on the *mnp1* expression vector P#1 (Figure 8). Following mutagenesis, transformation into *E. coli*, plasmid extraction, and sequencing to verify effective mutagenesis, the expression vectors were electroporated into *P. pastoris*. DNA was extracted from the resulting yeast strains, and the mnp1 gene was PCR amplified and sequenced. Production and molecular mass of rMnP was determined in shake flask cultivation using MnP activity assays and SDS-PAGE and western blots with anti-HIS antibodies.

In all cases where mutagenesis was conducted, the final *P. pastoris* strains did not produce either intracellular or secreted rMnP. Whether this was due to the critical importance of these amino acids in the catalytic activity of MnP or was the result of a technical error in the recombinant DNA methodology remains open to question. Further control experiments are required to establish the efficacy of the mutagenesis and cloning experiments.

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1     GAA TTC ATC GCA ATG GCC TTC GGT TCT CTC CTC GCC TTC GTG GCT CTC GCC
55    GCC ATA ACT CGC GCC GCC CCG ACT GCG GAG TCT GCA GTC TGT CCA GAC GGT
109   ACC CGC GTC ACC AAC GCG GCG TGC TGC GCT TTC ATT CCG CTC GCA CAG GAT
163   TTG CAA GAG ACT CTG TTC CAG GGT GAC TGT GGC GAA GAT GCC CAC GAA GTC
217   ATC CGT CTG ACC TTC CAC GAC GCT ATT GCA ATC TCC CAG AGC CTA GGT CCT
271   CAG GCT GGC GGC GGT GCT GAC GGC TCC ATG CTG CAC TTC CCG ACA ATC GAG
325   CCC AAC TTC TCC GCC AAC AGC GGC ATC GAT GAC TCC GTC AAC AAC TTG CTT
379   CCC TTC ATG CAG AAA CAC GAC ACC ATC AGT GCC GCC GAT CTT GTA CAG TTC
433   GCC GGT GCG GTC GCG CTG AGC AAC TGC CCA GGT GCT CCT CGC CTC GAG TTC
487   ATG GCT GGA CGT CCG AAC ACT ACC ATC CCC GCA GTT GAG GGC CTC ATT CCT
541   GAG CCT CAA GAC AGC GTC ACC AAA ATC CTG CAG CGC TTC GAG GAC GCC GGC
595   AAC TTC TCG CCG TTC GAG GTC GTC TCG CTC CTG GCT TCA CAC ACC GTT GCT
649   CGT GCG GAC AAG GTC GAC GAG ACC ATC GAT GCT GCG CCC TTC GAC TCG ACA
703   CCC TTC ACC TTC GAC ACC CAG GTG TTC CTC GAG GTC CTG CTC AAG GGC ACA
757   GGC TTC CCG GGC TCG AAC AAC AAC ACC GGC GAG GTG ATG TCG CCG CTC CCA
811   CTC GGC AGC GGC AGC GAC ACG GGC GAG ATG CGC CTG CAG TCC GAC TTT GCG
865   CTC GCG CGC GAC GAG CGC ACG GCG TGC TTC TGG CAG TCG TTC GTC AAC GAG
919   CAG GAG TTC ATG GCG GCG AGC TTC AAG GCC GCG ATG GCG AAG CTT GCG ATC
973   CTC GGC CAC AGC CGC AGC AGC CTC ATT GAC TGC AGC GAC GTC GTC CCC GTC
1027  CCG AAG CCC GCC GTC AAC AAG CCC GCG ACG TTC CCC GCG ACG AAG GGC CCC
1081  AAG GAC CTC GAC ACG CTC ACG TGC AAG GCC CTC AAG TTC CCG ACG CTG ACC
1135  TCT GAC CCC GGT GCT ACC GAG ACC CTC ATC CCC CAC TGC TCC AAC GGC GGC
1189  ATG TCC TGC CCT GGT GTT CAG TTC GAT GGC CCT GCC TAA ACC ACT CAC CTC
      CGG CAA TGC ACC TTT AGT AGA TGT CGA TTC TCT AGA
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Figure 8. Location of coding regions for potential glycosylation sites (red) in the manganese peroxidase gene (mnp1).

Production of rMnP in fed-batch fermenters.

Previously we have produced secreted rMnP from recombinant *P. pastoris* in fed-batch fermentations with glucose as the growth substrate. In these studies, contamination with bacteria was often a problem. Although *P. pastoris* can grow at pH as low as 4, where yeast are more competitive than bacteria, the rMnP is not effectively maintained in the culture broth due to proteolytic degradation. This necessitates production at pH 6, where bacteria are competitive with yeast. In the current study, a new strategy was developed in which the pH was maintained at 5.5 during batch growth, followed by an increase in pH to 6 at the onset of the fed-batch phase. This was effective in decreasing the number of fermentor runs "lost" to bacterial contamination. Using this approach we could consistently produce 3,000 U/L secreted rMnP with *P. pastoris* P#1.

A second methodology was implemented for production of rMnP from strains containing the *mnp1* gene cloned downstream of the methanol inducible promoter. This included a batch phase with glycerol, followed by fed-batch with methanol. *P. pastoris* MM/2-3 produced rMnP under this regime, but the rMnP titer remained similar to that observed for P#1 (Figure 9). Further improvements may be necessary to achieve full activity from this promoter.

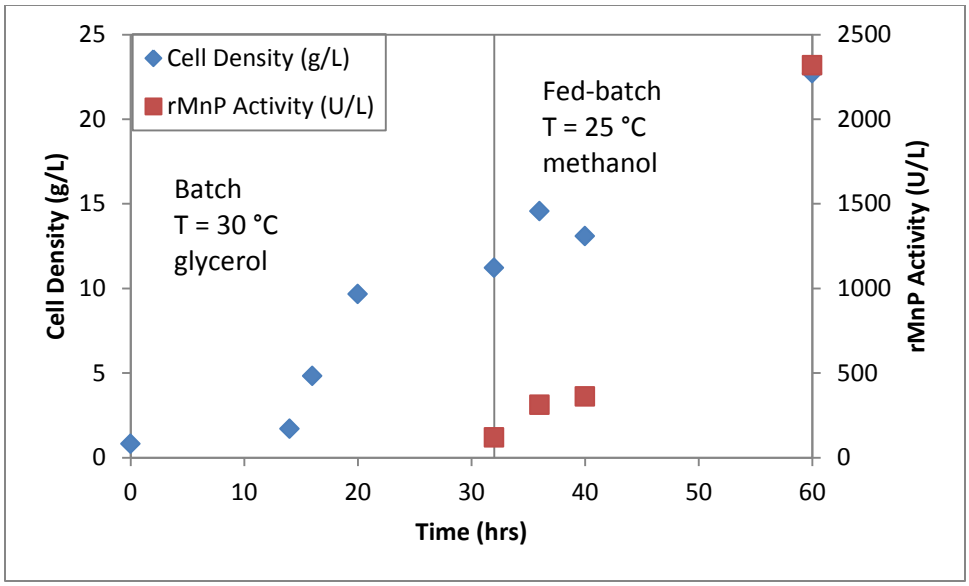

Figure 9. Production of secreted, methanol induced (AOX) rMnP in fed-batch fermentation.

An SDS PAGE gel image of the two functional strains is shown below (Figure 10).

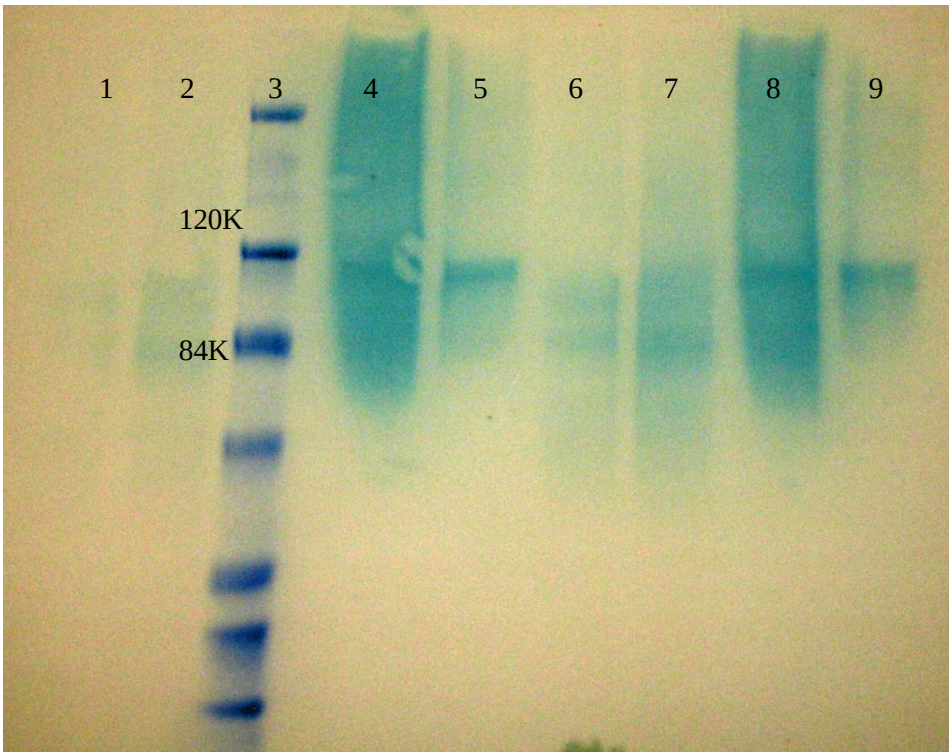

Figure 10. SDS PAGE gel of MnP Strains P#1 (GAP, secreted) and MM2-3 (AOX, secreted)

Table 2. Lane Identification for Figure 10.

Lane	Strain Induction
1	MM2-3 (AOX, cell pellet (5ug))
2	P#1 (GAP, cell pellet (5 ug))
3	Molecular weight Marker kDa (215, 120, 84, 60, 39, 28, 18)
4	P#1 (GAP, secreted) (3 ug)
5	MM2-3 (AOX, secreted) (3 ug)
6	MM2-3 (AOX, cell pellet) (20ug)
7	P#1 (GAP, cell pellet) (20 ug)
8	replicate of #4
9	replicate of #5

Comparing lanes 4 and 5, it appears that the AOX strain somewhat less glycosylated and net molecular weight is more consistent. Despite this improvement, the rMnP from this strain was not retained on a cobalt IMAC (immobilized metal affinity chromatography) column.

A third area of effort beyond the scope of the proposed project was undertaken to try to accomplish Technical Objective #1. Trillium Fiber Fuels and Oregon State University partnered with AbSci, a startup company in Portland, Oregon. AbSci has developed a proprietary *E. coli* expression platform that is well suited to MnP expression. Previous attempts to produce MnP in the productive *E.coli* microorganism have failed due to the formation of inclusion bodies (Whitwam et al., 1995). However, an active protein from this system could potentially be produced a high titers and without glycosylation since bacteria lack the apparatus to glycosylate. The specific advantages of AbSci's proprietary E. coli expression system include: 1) improved compatibility and solubility of components within the inducible coexpression system, 2) homogenously inducible expression of gene products that together form multimers, or other combinations of gene products (coexpression of two or more gene products), 3) improved control of gene product coexpression by independently titratible induction, 4) improved expression of gene product complexes and other difficult to express products such as multimeric products and products forming disulfide bonds, 5) streamlined optimization of gene product coexpression.

A codon optimized version of the MnP gene was synthesized and expressed in the AbSci system. Although the work was intitated near the end of the Phase I project timeline, MnP was successfully expressed in this system. Unlike previous attempts with *E. coli*, the protein was found in the soluble portion of the cell lysate (Figure 11). Unfortunately, the protein has not shown activity thus far. Further experiments are underway to determine if the protein is properly formed and can be activated by simple post-processing.

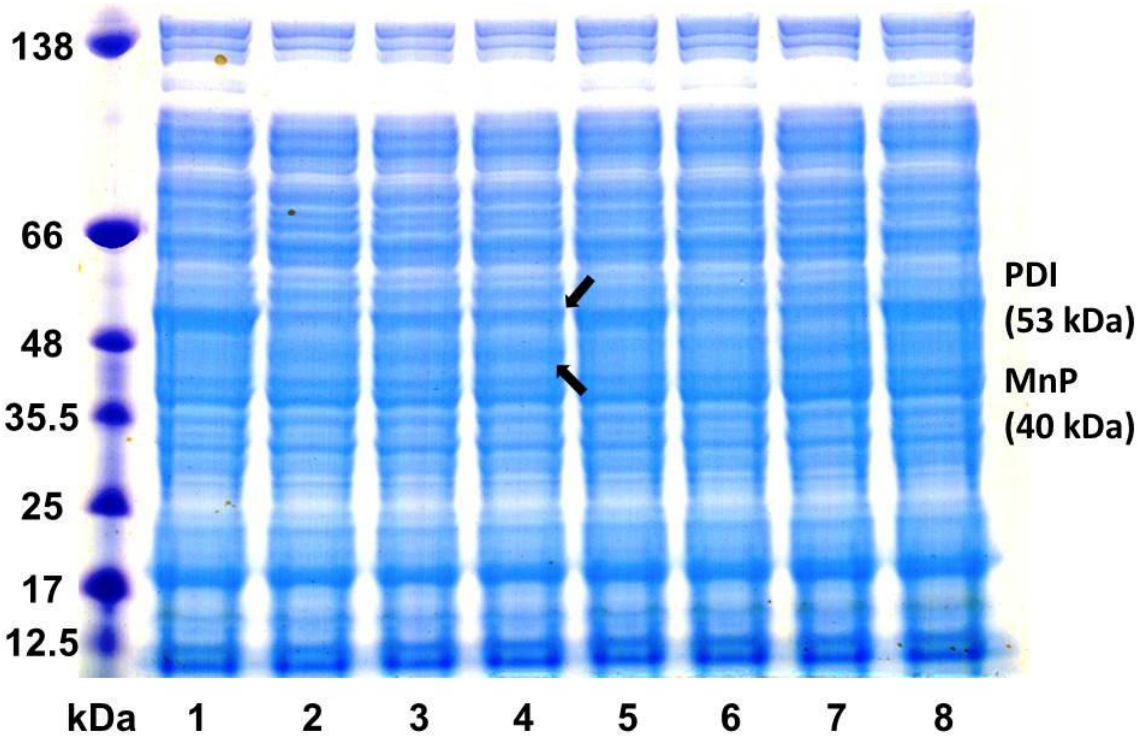

Figure 11. SDS PAGE gel of soluble MnP produced by AbSci E.coli.

Table 3. Lane Identification for Figure 11.

Lane	Strain Induction
1	Not induced
2	0.01% arabinose, 50 mM proprionate
3	0.01% arabinose, 25 mM proprionate
4	0.01% arabinose, 12.5mM proprionate
5	0.01% arabinose, 6.25mM proprionate
6	0.005% arabinose, 50 mM proprionate
7	0.005% arabinose, 5 mM proprionate
8	0.005% arabinose, 1 mM proprionate

Technical Objective 2:

Produce cell free extract (CFE) from the recombinant strain with activity greater than 300,000 units/liter (about 2 g/L).

Not accomplished. Since the strains created did not produce significant improvements in titer or purification success, the extracts were not significantly improved.

Technical Objective 3:

Create reaction products from the action of rMnP on lignin and analyze for phenolics and other simple aromatics with gas chromatography.

Not accomplished.

Lignin transformation experiments were conducted using secreted rMnP from the P#1 *P. pastoris* strain with specific activity of 300 U/l. Experiments focused on the possibility of producing low-molecular weight (e.g., MW<1000 Da) organic compounds from commercial Kraft alkali lignin (MW~10 kDa). The reaction mixture used was analogous to the MnP assay used to oxidize 2,6-DMP, and contained 0.4 mM $MnSO_4$, 50 mM sodium malonate (pH 4.5), 0.1g/l alkali lignin (Sigma Aldrich, MW~10 kDa), and rMnP enzyme. The mixture was activated by the addition of H_2O_2 to 0.1 mM at room temperature.

Prior work on lignin transformations by MnP in the Kelly lab at Oregon State University was done by Jeffrey Goby (Goby M.S. Thesis, 2009). One focus of that work was to detect the generation of phenolic compounds through the action of MnP on alkali lignin. They found that detection of phenolic transformations was difficult, despite using numerous assay methodologies (malonate-mediated oxidation, Folin, Pearl-Benson) and study of assay interferences. One complication is that the Mn^{3+}-malonate complex formed by MnP has absorbance at 270 nm that overlaps with the dominate absorbance band of phenolics and obscures detection of potential phenolic oxidation products.

For the current work, a 1-mL dialysis cell (Harvard Apparatus, 74-0216) with a cellulose acetate membrane (MWCO 1 kDa) was used to screen for potential scission of commercial alkali lignin (MW ~10 kDa) to form low molecular weight products (MW < 1 kDa) by action of MnP. Dialysis tests were performed on reaction mixtures with lignin and MnP-containing broth (300 U/l), with enzyme broth alone, and with lignin alone, each with or without activation by H_2O_2. Dialysis was allowed to progress for 20-24 hours against 30 mL of an exchange fluid containing 0.4 mM $MnSO_4$ and 50 mM sodium malonate (pH 4.5), i.e., the reaction mixture without enzyme, lignin, or H_2O_2. After dialysis, UV/vis spectra of the exchange fluid, or dialyzate, were taken in an effort to detect the presence of phenolic compounds, which absorb at 270 nm, that may have traversed the 1 kDa membrane. In the control case of lignin alone, almost no phenolic product was detected in the dialyzate after 24 hours. Hence, the unmodified alkali lignin contained almost no low-molecular weight compounds that could permeate the 1 kDa membrane. However, dialyzates from all of the various reaction mixtures containing MnP broth, with or without lignin, either activated or not activated, showed comparable UV/vis spectra. Thus, some compound or compounds that absorb at 270 nm in the enzyme-containing broth traversed the membrane. Either no lignin scission products were created, or their concentration was so low that they were not detectable. The Mn^{3+}-malonate complex created by MnP enzyme in the presence of Mn^{2+} and H_2O_2 absorbs at 270 nm (Wariishi, et al., 1992; Goby thesis, 2009) and can interfere with the detection of phenolic compounds, but this compound is highly reactive and short-lived, and absorbance at 270 nm was seen even in the absence of the H_2O_2 necessary to create it.

Appendix A. Cell lysis protocol

P. Pastoris Lysis:

1) To P. pastoris cells, resuspend in 5 mL 50 mM MES buffer pH 6.0 and add 1 mm glass beads to half the volume. Add DTT to 1mM.
2) Vortex for 2 minutes then place on ice for 5 minutes. Repeat vortexing four times.
3) Sonify cell suspension on power setting 2, 50% duty cycle for 2 minutes on ice. Repeat four times.
4) Clarify lystate by centrifugation on max (11,500 rpms) for 45 minutes at 4°C.
5) Remove cell free extract (CFE) and perform Bradford protein quantification, rMnP activity assay and Western Blot Analysis.

E. coli Lysis:

1) Freeze thaw cycle four times to help aid in lysis.
2) Add 1 mL of 50mM MES buffer pH6.0 to E. coli cell pellet. Add DTT to 1mM.
3) Sonify cell suspension on power setting 2, 50% duty cycle for 2 minutes on ice. Repeat four times.
4) Clarify lystate by centrifugation on max (11,500 rpms) for 45 minutes at 4°C.
5) Remove cell free extract (CFE) and perform Bradford protein quantification, rMnP activity assay and Western Blot Analysis.

Appendix B. MnP Assay Procedure

MnP assay

Specific activity of MnP was measured by monitoring the oxidation of 2,6-dimthoxyphenol (26-DMP) (Wariishi, et al.) using a Beckman DU-64 UV/VIS spectrophotometer. Assay reaction mixtures contained 0.4 mM $MnSO_4$, 50 mM sodium malonate (pH 4.5-5.5), 0.1 mM 2-6-DMP, and MnP enzyme. The extinction coefficient for the orange-brown product, 2,2',6,6'-tetramethoxydibenzo-1,1'-diquinone, is 49,500 $m^{-1}cm^{-1}$ at 469 nm. Activity is expressed as micromoles of product formed per minute (units) per liter (U/l). Samples of culture broth or cell-free extract were centrifuged at 4000*g* for 30-40 minutes to pellet yeast cells and heme particles, and the resultant supernatant containing soluble MnP was added to the reaction mixture. Reaction was initiated by addition of H_2O_2 to a concentration of 0.1 mM, and absorbance at 469 nM was measured after 1 min. When cell free extract of P. pastoris or E. coli was added to the reaction mixture containing sodium malonate at pH 4.5, a protein precipitate formed that clouded the reaction mixture and obscured the assay. To remedy this complication, sodium malonate at pH 5.0 was used in the reaction mixture, and the precipitate did not form. The change in pH did not affect rate of 2,6-DMP oxidation.

MnP assay of cell-free extracts

The assay for MnP activity in cell-free extracts (CFE) of P. pastoris or E. coli was complicated by reactions of H_2O_2 that competed with its participation in the creation of the Mn^{3+}-malonate complex by MnP. Figure 12 shows the progress of 2,6-DMP oxidation in the assay reaction mixture using a concentrated P#1 broth , E.coli CFE, or both the P#1 broth and CFE as the enzyme source. The P#1 broth contained ~900 U/l rMnP, and 2,6-DMP oxidized rapidly. The CFE of an AbSci E.coli CFE showed no rMnP activity; this is representative of all cell-free extracts of P. pastoris and AbSci E.coli intracellular or peroxisomal-targeted strains developed for this work. When the active P#1 supernatant was combined with an AbSci CFE for the assay, initial oxidation of 2,6-DMP was rapid, but slowed quickly to zero 30-60s after initiation with H_2O_2 to 0.1 mM (at time zero). When additional H_2O_2 (to 0.1 mM again) was added 3 minutes after starting the assay, 2,6-DMP again oxidized rapidly at first, then slowed and stopped (Figure 12). The critical learning from Figure 12 is that the assay would have detected rMnP in CFE if it had been present, despite the H_2O_2 scavenging effect of compounds in the CFE.

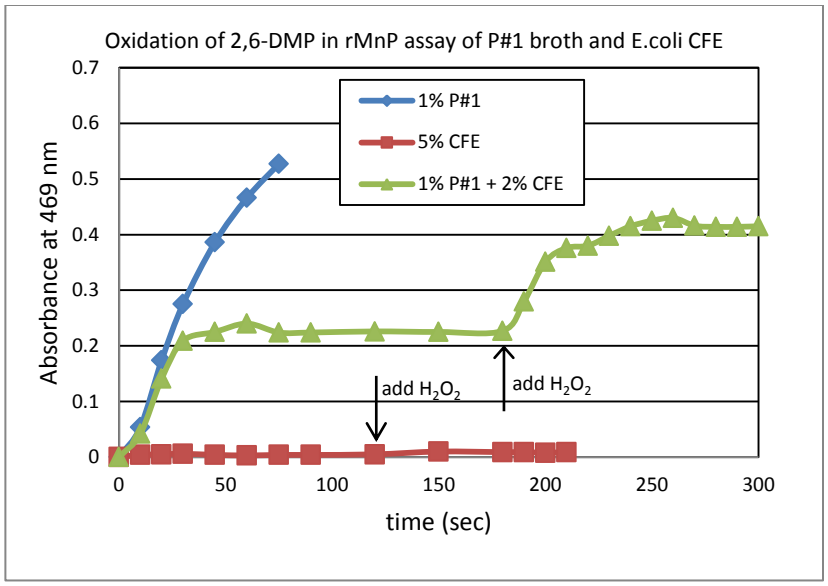

Figure 12. Progress of MnP assay for 1% P#1 broth, 5% E.coli CFE, and 1% P#1 supernatant plus 2% E.coli CFE. Additional H_2O_2 to 0.1 mM was added where indicated.