FINAL REPORT

Engineering Palm Peroxidases for Wastewater Treatment and Water Pollution Monitoring

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Abstract

Peroxidases are a large family of enzymes that typically catalyze an oxidation reaction utilizing peroxides such as hydrogen peroxide. Windmill palm peroxidase (WPP) is one of the most stable peroxidases, which possesses ultrahigh thermal stability (up to 90 °C) and extraordinary pH tolerance (pH 2 to 11). In this project, the peroxidase gene was cloned from palm tree leaves. Eleven mutations to the native palm peroxidase gene were designed based on the glycosylation sites of the native peroxidase and then created. The genes were transformed into yeast (*Pichia pastoris*) for expression. Of the eleven mutations, two yeast mutant strains produced a peroxidase that was highly active and stable. The engineered palm peroxidase has a His-Tag at the C-terminus of the amino acid sequence, which facilitates the palm peroxidase purification. The engineered peroxidase was purified and immobilized by Ni^{2+}-nitrilotriacetic acid (NTA) derivative functionalized carbon coated cobalt magnetic nanoparticles (Co/C NPs). We designed and manufactured two new custom-made bioreactors. Each bioreactor has a pH probe, a DO probe, a temperature probe, and a motor for automatic stirring. Utilization of the bioreactor for palm peroxidase expression is being undertaken.

Introduction

Peroxidases are a large family of enzymes that typically catalyze oxidation utilizing peroxides such as hydrogen peroxide. Horseradish peroxidase (HRP) is an enzyme widely used in water pollution monitoring and wastewater treatment. However, its poor stability has limited its applications, particularly for wastewater treatment. Many research efforts have been focused on searching for a HRP substitute. It has been demonstrated that palm peroxidase has exceptional stability, high activity and unique catalytic properties (Sakharov et al. 2001).

In this project, yeast (*Pichia pastoris*) was transformed with engineered genes to produce a novel palm peroxidase. Eleven mutations to the native palm peroxidase gene were designed based on the glycosylation sites of the native peroxidase and then created. Mutations at glycosylation sites determine the importance of the site for enzyme stability and activity. If the glycosylation site is deleted or edited, and the peroxidase functions normally, it can be assumed that the enzyme size can be made smaller through gene engineering with no loss of activity and possible increase in productivity. Another significance of enzyme size reduction is to improve wastewater treatment because enzymes small in size have high mass transfer and can improve wastewater treatment efficiency.

Objectives

1) To obtain an enzyme mutant with a smaller size than the native.
2) To design a suitable bioreactor for enhancing engineered palm peroxidase productivity.

Results and Discussion

The palm peroxidase gene sequence was synthesized according to the *P. pastoris* codon usage bias, which encodes an α-mating sequence at the N-terminus for extracellular expression and an extra amino acid sequence GGSGGSHHHHHH (His-Tag) at the C- or N-terminus for its purification and immobilization. The synthetic gene was constructed into pPink-HC vector (Invitrogen) and transformed to PichiaPink Strain (Invitrogen) to screen high expression strains.

Upon testing both terminals for use in the purification step of the engineered peroxidase, it was found that a His-Tag at the C-terminal of the enzyme yielded significantly higher purification results. Thus, the C-terminal His-Tag was utilized for the engineered peroxidase.

The engineered peroxidase was purified and immobilized by Ni^{2+}-nitrilotriacetic acid (NTA) derivative functionalized carbon coated cobalt magnetic nanoparticles (Co/C NPs). The Co/C core/shell magnetic NPs are commercially available in Turbobeads (Zurich, Switzerland) and Sigma-Aldrich (St. Louis, MO).

In general, enzyme can be covalently or non-covalently immobilized on magnetic NPs to fabricate nanobiocatalyst (Wang et al. 2009 and 2011; Lee et al. 2008; Dyal et al. 2003; Lee et al. 2009; Shukoor et al. 2008). Covalent attachment usually enables enzyme stabilization during catalysis; however, this often results in compromised enzyme activity due to the potential damage of the enzyme active center (Wang et al. 2011). Although absorption and entrapment-based non-covalent attachments could keep enzyme activity free, enzyme leakage will happen during recycling use because of the weak binding forces (Lee et al. 2009; Shukoor et al. 2008). Alternatively, affinity immobilization is an excellent methodology, since it can retain enzyme conformation and activity and minimize enzyme loss (Lee et al. 2009). Currently, His-tagged enzymes can be easily engineered and produced and can also be purified using traditional affinity chromatography or by using magnetic nanoparticles functionalized with metal-IDA/NTA (Wang et al. 2011). In the present study, strong magnetic Co/C NPs were simply functionalized with NTA derivative and were used to purify and immobilize the engineered peroxidase in one pot from the raw yeast fermentation culture medium.

Table 1. Primers used for site mutations at the glycosylation sites.

Primer	Sequence
GM-1-F	GACCTTCAGATTGGTTTTTACCAAACTTCCTGTCCAACTGCTG
GM-1-R	CAGCAGTTGGACAGGAAGTTTGGTAAAAACCAATCTGAAGGTC
GM-2-F	GTCGCTGCCGCATTTGCCCAAAATTCTGGTATTGCACCTG
GM-2-R	CAGGTGCAATACCAGAATTTTGGGCAAATGCGGCAGCGAC
GM-3-F	GTCTTGTTGGATTCTACAGCACAAAATACCGCTGAGAAGGACGC
GM-3-R	GCGTCCTTCTCAGCGGTATTTTGTGCTGTAGAATCCAACAAGAC
GM-4-F	CTGCTAACCTTGCCGGTCAAATCACCTACCAGGTCCCTAG
GM-4-R	CTAGGGACCTGGTAGGTGATTTGACCGGCAAGGTTAGCAG
GM-5-F	CACAAATTCCATCACCTTTGTTTCAGGCTACTCAGCTTATCAACAG
GM-5-R	CTGTTGATAAGCTGAGTAGCCTGAAACAAAGGTGATGGAATTTGTG
GM-6-F	CAGCTTATCAACAGTTTCGCTCAAAAGACTTTGACAGCCGATGAG
GM-6-R	CTCATCGGCTGTCAAAGTCTTTTGAGCGAAACTGTTGATAAGCTG
GM-7-F	CAAACAGATTGTACAACTTCCAGTCAACTAGTGGAATTGACCCAAC
GM-7-R	GTTGGGTCAATTCCACTAGTTGACTGGAAGTTGTACAATCTGTTTG
GM-8-F	CTTAGAAACACATGCCCAGCTCAGTCTACTAGATTCACTCCTATTAC
GM-8-R	GTAATAGGAGTGAATCTAGTAGACTGAGCTGGGCATGTGTTTCTAAG
GM-9-F	GCCTTGGTTACCGAAGCACAGCTTTCCGCAGCTGTTAAAGC
GM-9-R	GCTTTAACAGCTGCGGAAAGCTGTGCTTCGGTAACCAAGGC
GM-10-F	GCTGTTAAAGCTAACGCCATGCAGTTGACTGCATGGGCTTCC
GM-10-R	GGAAGCCCATGCAGTCAACTGCATGGCGTTAGCTTTAACAGC
GM-11-F	CTCAAGGAGAGATCAGAACTCAGTGTTCCGTCGTCAACTCAGG
GM-11-R	CCTGAGTTGACGACGGAACACTGAGTTCTGATCTCTCCTTGAG

Eleven pairs of specific primers (Table 1) were designed for glycosylation site mutation. Each mutant vector sequence was verified by gene sequencing. After transformation of the vector into the yeast, expression was induced by adding 0.5% methanol to the culture medium for all eleven strains. Small amounts of enzyme were produced from the eleven mutants to serve as a baseline. The enzymatic activity of the eleven mutants was observed to select the mutation(s) that would yield a comparatively higher activity. Of the eleven, two strains produced an enzyme that was highly active and stable (GM-3, GM-7).

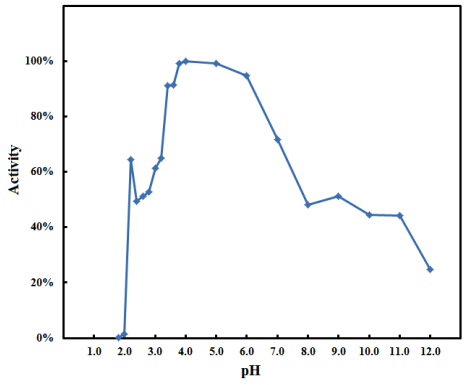

Figure 1. pH stability of engineered peroxidase.

The pH stability is important for engineered peroxidase for wastewater treatment. As shown in Figure 1, the engineered peroxidase was treated at different pHs (ranging from 2 to12) for 1 h and was determined if the enzyme activity was compromised after treatment. The result showed that the engineered peroxidase can remain above 50% activity from pH 3 to 9, which is better than the HRP pH stability (pH 5 to 8).

The next step was to express large quantities of peroxidase using these two mutants. A rudimentary bioreactor was designed for this purpose (Fig. 2). This design was tested but yielded poor results. The monitoring of pH and O_2 levels, which is very important to maintaining a stable environment for the yeast growth and peroxidase expression, was nonexistent. With this design, the parameters were unable to be optimized, leading to a lower output than anticipated. Due to the lack of productivity with the initial bioreactor, we have designed and custom made a new bioreactor (Fig. 3). The new bioreactor (Fig. 3) is being utilized for the scaled-up expression. As shown in Figure 3, two identical bioreactors were designed and produced to facilitate the optimization of the enzyme expression parameters. Each bioreactor contains a pH probe, DO probe, temperature probe, and a motor for automatic stirring. Due to some parts of the bioreactor being unable to be autoclaved, whole bioreactor sterilization is currently being undertaken.

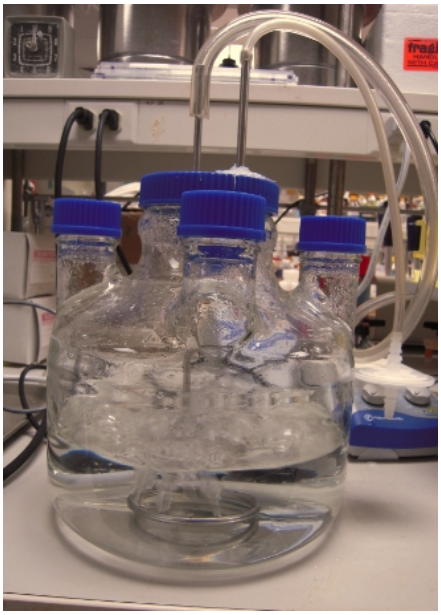

Figure 2. 5-L rudimentary bioreactor with air input.

Notable Achievements

- Designed and manufactured two new custom-made bioreactors.
- Designed and created eleven mutants—two mutants (GM-3, GM-7) can produce active and stable peroxidase.
- Trained one undergraduate student.
- Trained one post-doctoral fellow. The post-doctoral fellow has been offered a new post as an associate professor.

Figure 3. 3-L custom-made bioreactor with pH, O_2, and temperature probes.

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