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Expression of lignin peroxidase H2 from *Phanerochaete chrysosporium* by multi-copy recombinant *Pichia* strain

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Abstract

The *lip*H2 gene, encoding the expression of lignin peroxidase, was cloned from *Phanerochaete chrysosporium* BKM-F-1767 and expressed in *Pichia pastoris* X-33, a yeast. The cDNA of LiPH2 was generated from total RNA extracted from *P. chrysosporium* by PCR with primers that do not contain a *P. chrysosporium* lignin peroxidase secretion signal. The gene was then successfully inserted into the expression vector pPICZα, and resulted in the recombinant vector pPICZα-*lip*H2. The transformation was conducted in two ways. One was using the wild *Pichia pastoris* as the recipients, which results in the recombinant *P. pastoris* with single or low *lip*H2 gene copy. The second was using *P. pastoris* and single or low *lip*H2 gene copy as the recipients, which results in the recombinant *P. pastoris* with multi-copies of *lip*H2 genes. This study firstly expressed the gene *lip*H2 in *P. pastoris* and achieved the successful expression of the *lip*H2 depending upon the generation of a recombinant strain that contained multiple copies. The lignin peroxidase activity reached a maximum of 15 U/L after 12 h induction.

Key words: heterologous expression; lignin peroxidase; *Pichia pastoris*

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Introduction

White rot fungi are the only known organisms, which is capable of extensively degrading lignin to CO_2 and H_2O in pure culture. They can degrade many environmental pollutants well, and thus have great application potential in the environmental field. It has been tested in oxidative dechlorination of methoxychlor (Hirai *et al*., 2004), removal of estrogenic activity (Tamagawa *et al*., 2005, 2006, 2007), decolorization of coal humic acid (Kabe *et al*., 2005), treatment of industrial dye effluents (Wesenberg *et al*., 2003), oxidation of polycyclic aromatic hydrocarbons (Zheng and Obbard, 2002), decolorization of olive mill wastewaters (Sayadi and Ellouz, 1995), and so on.

Extracellular enzymes, especially lignin peroxidase (LiP) and manganese peroxidase (MnP), are the key to the degradative process of white rot fungi (Glenn *et al*., 1983; Tien and Kirk, 1983; Kuwahara *et al*., 1984). Some recent studies show that direct use of the enzyme can achieve significant fast degradation of pollutants and would represent a new research and development direction (Yu *et al*., 2006). Furthermore, this would be a prerequisite of using the enzyme in large scale fermentation.

However, the extracellular enzymes production process is hampered by several factors. The fungi secrete the enzymes as a secondary metabolite under nitrogen or other nutrient limited conditions. Their expression is difficult to regulate. The sensitivity of these fungi to high shear forces in the fermentor is also a problem (Kirk *et al*., 1978), which always cause the inactivation of these enzymes even in the absence of mycelia.

To utilize the extracellular enzyme of white rot fungus for degrading environmental pollutants, recombinant peroxidase production had been considered. MnP is quite specific for reducing substrates whereas LiP is relatively unspecific. The heterologous expression of LiP has been explored in various hosts. Particularly, the heterologous expression of LiPH2 (Nie *et al*., 1998) and LiPH8 (Doyle and Smith, 1996) in *Escherichia coli* enables rapid high-yield protein production; however, it is strongly limited by the low yield of *in vitro* folding which is necessary to incorporate the heme into the protein recovered from inclusion bodies. Heterologous expression in eukaryotic systems implies the *in vivo* incorporation of heme and some protein glycosylation. Heterologous expression of LiPH2 (Johnson *et al*., 1992) and LiPH8 (Johnson and Li, 1991) in a baculovirus expression system is attempted and low levels of active peroxidase is detected in the growth medium. In addition to the high cost and low enzyme production, enzymes with only partial activity (60% for LiPH8 and 77% for LiPH2) were obtained. However, there are very few studies on fungal hosts despite their advantages. The *Aspergillus niger* expression of LiPH8 (Aifa *et al*., 1999) has been reported, but its yields for

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ligninolytic peroxidase are comparatively low. Meanwhile, the *Pichia methanolica* expression of LiPH8 (Wang *et al*., 2004) has also been reported. So far, the successful expression of LiPH2 has not been reported even though it is a very important enzyme of white rot fungi.

Targeting a high and stable yield of LiPH2 (GenBank no. X15599), the present study addresses the construction of recombinant *Pichia pastoris* carrying lignin peroxidase (LiPH2) gene and investigates the expression of the LiPH2 gene. This study has built the foundation for further research and application.

1 Materials and methods

1.1 Strains, media, plasmids, and culturing conditions

The *P. chrysosporium* BKM-F-1767 (ATCC no. 24725) was cultured in the way similar to that by Li *et al*. (1994), with dextrin as carbon source. *Escherichia coli* DH5α, used for plasmid construction and propagation, was cultured in a low-salt LB (Luria-Bertani) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0). *Pichia pastoris* X-33, a wild-type *Pichia* strain purchased from Invitrogen, USA, used for expression of LiP, was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose).

pUCm-T vector (Takara, Japan) was used for cloning PCR fragments and pPICZαA vector (Invitrogen, USA), and was used for protein expression in *P. pastoris*. pPICZαA contains the tightly regulated AOX1 promoter and the *Saccharomyces cerevisiae* α-factor secretion signal located immediately upstream of the multiple cloning site. The vector contains the zeocin resistance gene for positive selection in *E. coli* and *P. pastoris*.

1.2 Gene cloning and construction of the expression vector

Phanerochaete chrysosporium was cultured for 8 d, then the mycelia were harvested. RNA was extracted using Trizol reagent following the manufacturer's instructions. RT-PCR was performed with an upstream primer of 5'-cggaattcccgaacctcgacaagcg-3' and a downstream primer of 5'-gctctagagttgggggacggcgg-3', using the total RNA as the template. The primers were designed with inserted *Eco*RI and *Xba*I restriction sites (underlined) permitting directional cloning of the amplified DNA in frame with the α factor leader sequence in the pPICZαA expression vector. The amplification was carried out under the following conditions: the first step was at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 52°C for 1 min, and 72°C for 2 min, and the final extension was carried out at 72°C for 10 min. The sequence correctness of the amplified fragment was confirmed by DNA sequencing. The amplified fragment was first subcloned into pUCm-T vector and resulted in the recombinant vector pUCm-T-*lip*H2. The pUCm-T-*lip*H2 was digested with *Eco*RI and *Xba*I to generate a 1050 bp fragment which was subsequently introduced into the pPICZαA expression vector using *Eco*RI and *Xba*I sites. Following transformation into *E. coli* DH5α, one recombinant plasmid nominated as pPICZαA-*lip*H2 was selected on a LB agar plate containing 25 μg/mL zeocin. The insertion was checked by restriction analysis and DNA sequencing.

1.3 Transformation of *P. pastoris* and screening of recombinant colonies

Plasmid pPICZαA-*lip*H2 was digested with *Sac*I and integrated into pretreated *P. pastoris* X-33 by electroporation using a Gene pulser apparatus (Biorad, Hercules, USA) according to Invitrogen instructions. Transformants of *P. pastoris* containing LiPH2 DNA were selected on the basis of zeocin resistance using YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 mol/L sorbitol) agar plates containing 100, 500, and 1000 μg/mL zeocin, respectively. Zeocin-resistant colonies were replicaplated onto minimal methanol (MM) plates containing 1.34% yeast nitrogen base, 0.5% methanol, (4×10^{-5})% biotin, and minimal dextrose (MD) plates (the same composition as MM but with 2% dextrose instead of methanol) to determine methanol-utilizing phenotypes. After 3–4 d of incubation, the Mut^+ phenotypes grew normally on both MM and MD whereas the Mut^- phenotypes grew very slowly on MM plates. Selecting the Mut^+ transformants and grow them in 20 mL of buffered glycerol complex medium (BMGY) in 250 mL flasks at 30°C and 250 r/min. The insertion was checked by PCR using the two universal primers 5'AOX1 (sequence: 5'-GACTGGTTCCAATTGACAAGC-3') and 3'AOX1 (sequences: 5'-GCAAATGGCATTCTGACATCC-3'), and DNA sequencing.

1.4 Generation of multi-copy recombinants

Pichia pastoris is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology. The recombinant *Pichia* strain which had been confirmed with the integration of *lip*H2 was used as a recipient in this transformation experiment for generating the multi-copy recombinants. The electroporation of *Pichia* was done as described in Section 1.3. Multi-copy recombinant colonies were selected from YPDS plates containing 1000 μg/mL zeocin, while the YPDS plates containing 100, 200, 400, and 800 μg/mL zeocin were used as controls. The multi-copy recombinant was also checked by PCR.

1.5 Production of recombinant LiPH2

Cultivation of *P. pastoris* transformant included two phases: a growth phase on glycerol, and an induction phase on methanol. First, *P. pastoris* Mut^+ strains were grown in a 50-mL buffered complex medium containing glycerol (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB, (4×10^{-5})% biotin, 1% glycerol) in 250 mL flasks at 30°C and 250 r/min for approximately 18 h. Second, the 50 mL culture was centrifuged at 3000 $\times g$ for 5 min for harvesting the recombinant cells, and then the cells were resuspended in a 30-mL buffered methanol-complex medium (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB, (4×10^{-5})% biotin, 0.5% methanol)

in 250 mL flask which was incubated at 30°C, 250 r/min. During the induction phase, 100% (*V/V*) ethanol was added continuously to keep its concentration at 0.5%. Mut$^+$ strain carrying only the integrated pPICZαA vector without *lip*H2 gene insert was used as control.

Culture of yeast was centrifuged at 10000 ×*g* for 1 min, and the cell-free supernatant was subjected to further analysis. Sodium dodecyl sulfate-PAGE (SDS-PAGE) was employed to evaluate the molecular weights of the expressed proteins. The stacking and separation gels contained 5% and 12% polyacrylamide, respectively. The proteins were visualized after staining with Coomassie Brilliant Blue R-250 (0.1%). The lignin peroxidase activity was determined according to the method of Tien and Kirk (1988).

2 Results and discussion

2.1 Cloning and sequence analysis of *P. chrysosporium* lignin peroxidase isozyme *lip*H2 gene

Sequencing results showed that the amplified fragment, LiPH2 cDNA, which is exactly the same as previously described (de Boer, 1987), encodes a protein with 350 amino acids and does not contain the 21-aa putative signal peptide. The vector pUCm-T-*lip*H2 is shown in Fig. 1.

2.2 Vector construction for LiP expression in *P. pastoris* and screening of recombinant colonies

Plasmid pPICZαA-*lip*H2 has an inducible promoter AOX1, *S. cerevisiae* α-factor secretion signal in the upstream region of the LiPH2 cDNA sequence (Fig. 2), and a termination transcription signal. In the first transformation experiment, only four recombinants, namely X-33A, X-33B, X-33C, and X-33D, were obtained from the YPDS selecting plates containing 100 μg/mL zeocin. All of them confirmed integration in the AOX1 locus of the sequence encoding LiPH2 by PCR (Fig. 3). No recombinants were generated on the YPDS plates containing 500 or 1000 μg/mL zeocin. One recombinant (X-33A) was then used as a recipient in the following transformation experiments for the generation of multi-copy recombinants. A large number of recombinants grew on the YPDS plates containing 100, 200, 400, and 800 μg/mL zeocin whereas approximately 50–100 transformants were obtained from the YPDS plates containing 1000 μg/mL zeocin.

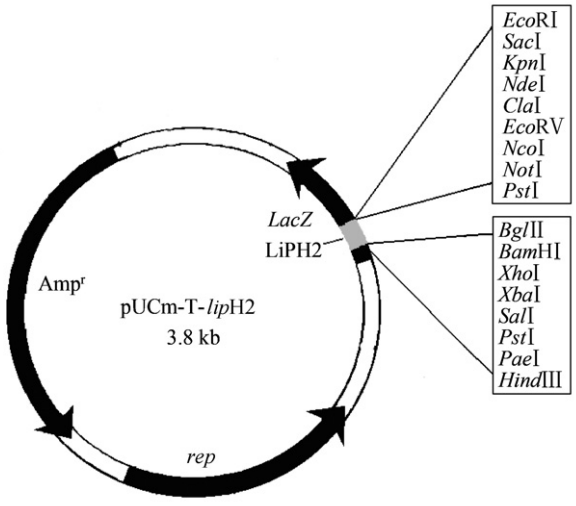

Fig. 1 Vector constructed for the cloning of LiPH2 cDNA in *E .coli* as well as excision by *Eco*RI and *Xba*I.

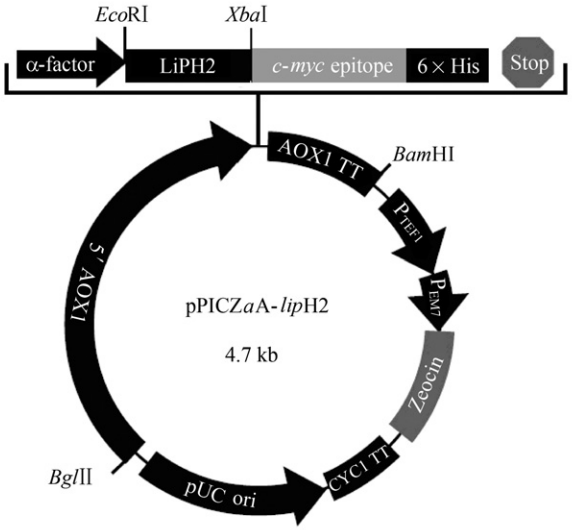

Fig. 2 Plasmids constructed for the expression of lignin peroxidase in *P. pastoris*. The LiPH2 cDNA was cloned into pPICZαA downstream of the native *S. cerevisiae* α-factor secretion signal. The C-terminal *myc* epitope tag permits the detection of the fusion protein by the anti-*myc* antibody or anti-*myc*-HRP antibody; the C-terminal polyhistidine tag permits the purification of the recombinant fusion protein on the metal-chelating resin; 5'*AOX1* and *AOX1 TT* are the promoter and transcription termination region of the alcohol oxidase gene from *P. pastoris*, respectively; *TEF1* is the promoter, particularly the transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae* that drives the expression of Zeocin resistance gene; EM7 is the constitutive promoter that drives the expression of the Zeocin resistance gene; Zeocin is the Zeocin resistance gene, and pUC ori allows replication and maintenance of the plasmid in *E. coli*.

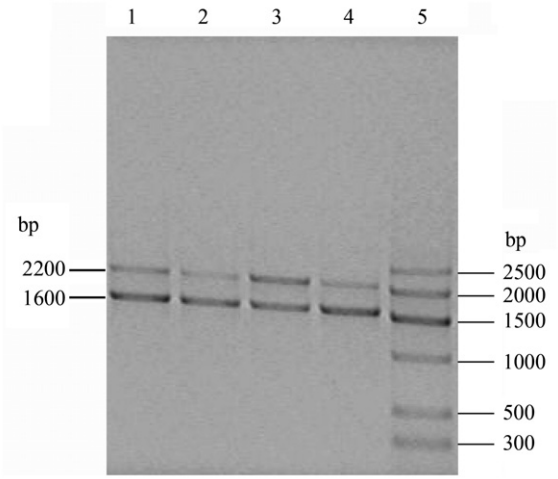

Fig. 3 Agarose gel electrophoresis of PCR products from genomic DNA of *Pichia pastoris* recombinant clones using primers 5'*AOX1*/3'*AOX1*. Lanes 1–4: recombinant clones (X-33A, X-33B, X-33C, and X-33D); lane 5: molecular weight markers. For Mut$^+$ integrants, there will be two bands. One will correspond to the size of *lip* gene, the other to the *AOX1* gene (approximately 2200 bp). Parent plasmids will produce the following sized PCR products. Adding the size of these products to the size of LiP to result in approximately 1600 bp.

2.3 Expression of *lip*H2 in *P. pastoris*

Lignin peroxidase activity was detected in the supernatant of the multi-copy recombinants grown on buffered methanol-complex medium in shaken culture, indicating that LiPH2 was secreted by the multi-copy yeast cells. The lignin peroxidase activity reached a maximum of 15 U/L after 12 h (Fig. 4). However, lignin peroxidase activity was not found in the supernatant of the low-copy or single-copy culture medium (the culture medium of strains X-33A, X-33B, X-33C, and X-33D). For multi-copy recombinants, namely X-33mA and X-33mB, distinct bands corresponding to approximately 41 kDa on denaturing SDS-PAGE were observed after 24 h incubation (Fig. 5), while there was no band on denaturing SDS-PAGE for low-copy or single-copy recombinant strain X-33A and the control strain (X-33 control).

The vector pPICZαA contains myc epitope and the polyhistidine tag which contributes 2.5 kDa to the size of the lignin peroxidase protein. The molecular weight of native LiPH2 is 38.5 kDa. Therefore, the recombinant protein which was expressed by *P. pastoris* X-33 should be 41 kDa (38.5kDa+2.5kDa) as shown in Fig. 5. However, the fusion protein may diminish the activity of peroxidase. LiPH2 should be expressed without the C-terminal peptide in further research by containing a stop codon in its gene.

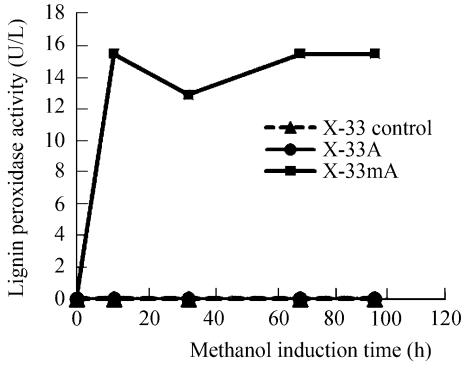

Fig. 4 Lignin peroxidase expression in *P. pastoris* according to X-33 control (recombinant constructed without lignin peroxidase gene insertion), X-33A (recombinant constructed with lignin peroxidase gene insertion), and X-33mA (recombinant constructed with multi-copy lignin peroxidase gene insertion) strains.

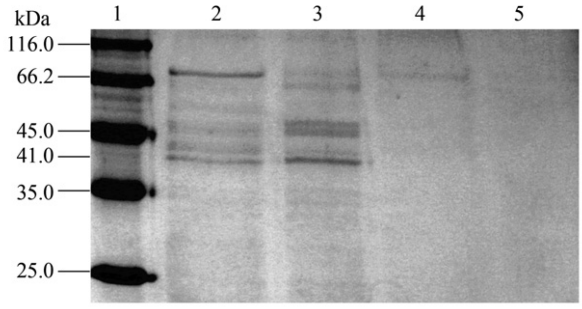

Fig. 5 SDS-PAGE analysis of proteins secreted by *P. pastoris*. Lane 1: low molecular weight markers; lane 2: X-33mA (recombinant constructed with multi-copy pPICZαA-*lip*H2); lane 3: X-33mB (another recombinant constructed with multi-copy pPICZαA-*lip*H2); lane 4: X-33A (recombinant constructed with pPICZαA-*lip*H2); lane 5: X-33 control (recombinant constructed with pPICZαA).

The activity of the recombinant protein with the fusion tag should be compared to the protein without it.

Only 2 multi-copy recombinants in this study were tested. Therefore, more multi-copy recombinants should be tested and the gene *lip*H2 should be optimized to improve the enzyme activity in the future.

In addition, temperature, pH, and O_2 are the key factors that affect lignin peroxidase production. The production of active enzymes can be improved by a low cultivation temperature, low pH, and adequate supply of O_2. Meanwhile, lignin peroxidase expression could be improved further using the X-33 multi-copy recombinant in the fermentor.

3 Conclusions

In this study, LiPH2 was expressed using the *P. pastoris* expression system. PCR and sequence analysis results indicate that the lignin peroxidase gene from *P. chrysosporium* was successfully cloned into *P. pastoris*. The lignin peroxidase activity was also presented by multi-copy recombinants. Moreover, the lignin peroxidase activity was continuously detected in the culture for 84 h. Lignin peroxidase was not detected in the culture of low-copy or single-copy recombinant. The results indicate that the multi-copy recombinant is more efficient in producing lignin peroxidase.

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