Journal of Bioscience and Bioengineering
VOL. 122 No. 6, 716–721, 2016

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Heterologous expression of a new manganese-dependent peroxidase gene from *Peniophora incarnata* KUC8836 and its ability to remove anthracene in *Saccharomyces cerevisiae*

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Received 22 December 2015; accepted 9 June 2016
Available online 25 June 2016

The white rot fungus *Peniophora incarnata* KUC8836 has received an attention as the greatest degrader of polycyclic aromatic hydrocarbons (PAHs), which are hazardous xenobiotics and recalcitrant pollutants. To characterize the mechanisms through which MnP degrades PAHs, heterologous expression of manganese-dependent peroxidase (MnP) gene *pimp1* was performed in *Saccharomyces cerevisiae* BY4741 via the pGEM-T Easy vector, resulting in the recombinant plasmid pESC-URA/*pimp1* containing the MnP signal peptide. MnP was significantly secreted into the culture medium with galactose as an active protein with higher efficiency (3.58 U mL^{-1}) by transformants than by the wild-type *S. cerevisiae*. The recombinant MnP protein was shown to have a molecular weight of 44 kDa by western blotting analysis. With regard to enhancing the bioremediation of PAHs in the environment, anthracene was effectively degraded by the MnP encoded by *pimp1*, with a degradation rate of 6.5% when Tween 80 was added. In addition, the MnP activity of the transformant exhibited the highest efficiency (2.49 U mL^{-1}) during the degradation. These results show that *pimp1* might be useful for biodegradation and gene expression technologies at a transcriptional level, and genetic approaches can be improved by incorporating the highly ligninolytic gene *pimp1* and the fungus *P. incarnata* KUC8836.

[**Key words:** Anthracene; Biodegradation; Heterologous expression; Manganese-dependent peroxidase; *Peniophora incarnata* KUC8836; *Saccharomyces cerevisiae*]

White rot fungi exhibit considerable potential for the biodegradation of various recalcitrant pollutants in the environment the ligninolytic enzymes extracellularly produced by white rot fungi are particularly promising for the degradation of xenobiotics due to their low specificity for substrates (1,2). There are two types of ligninolytic enzymes: phenol oxidases and peroxidases (3). The peroxidases are heme-containing enzymes and are classified into two different types based on their very different substrate spectra (4). One type is manganese-dependent peroxidase (MnP), and the other is lignin peroxidase (LiP). MnP is mainly secreted by ligninolytic enzymes from white rot fungi during lignin degradation. MnP has a manganese-binding site, which consists of several acidic amino acid residues and one heme group (5). Additionally, Mn^{2+} is the best reducing substrate, contributing a single electron to compound I of MnP (4). This physiological chelator is considered to indirectly degrade lignin and xenobiotic compounds (5).

Since the discovery of MnP, interest in this enzyme has greatly increased due to its potential applications in biopulping, biobleaching and bioremediation (6). MnPs are promising enzymes and an eco-friendly alternative to the conventional physicochemical processes presently employed for various uses such as in the textile, pharmaceutical, and food industries (7). In vitro experiments performed using MnP from *Irpex lacteus* demonstrated that this enzyme is capable of degrading the representative polycyclic aromatic hydrocarbons (PAHs) (8). The major degradation products of anthracene were identified, and the results revealed a new role of MnP in PAH degradation by *I. lacteus*, suggesting a previously unknown pathway for anthracene degradation. One white rot fungus, *Peniophora incarnata* KUC8836, has also been recently regarded as a remarkable degrader of xenobiotics, including PAHs (9). In addition, *P. incarnata* KUC8836 demonstrated enhancement of degradation rate of anthracene up to 95% by induction of its MnP activity as Tween 80 was supplemented (10). MnP has great potential for promising applications in biotechnology and industry. Several studies have investigated the homologous expression of peroxidases in ligninolytic fungi in submerged culture (11). In addition, the MnP gene was heterologously expressed in *Aspergillus niger* for the degradation of PAH in soil (12).

In a previous study, the gene encoding manganese-dependent peroxidase (MnP, EC 1.11.1.13) was newly identified in *P. incarnata* KUC8836 during the degradation of anthracene and was named *pimp1* (Genbank accession no. KJ622361). To develop an advanced strategy for bioremediation, a novel MnP gene from *P. incarnata* KUC8836 was used for heterologous expression in *Saccharomyces cerevisiae*, and its characteristics were examined for the degradation of xenobiotics in this study. It is anticipated that the new MnP

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.06.006

gene from the novel white rot fungus will greatly promote the precise elucidation of the biological mechanism of MnP with heterologous expression.

MATERIALS AND METHODS

Cloning of MnP genomic DNA The full-length of MnP cDNA was previously ascertained from *P. incarnata* KUC8836 (KACC53476). This cDNA was recognized as *pimp1* (Genbank accession No. KJ622361), and it was amplified by PCR using the specific primers, pimp1- EcoRI and pimp1-SpeI (Table 1, restriction sites for EcoRI and SpeI were incorporated into upstream and downstream primers, respectively). PCR was performed using the full-length cDNA in one cycle of incubation at 94°C for 5 min, followed by 30 cycles of amplification were at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension step at 72°C for 5 min was then performed. Then the PCR fragment of *pimp1* was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and the MnP genomic DNA of the transformant was confirmed by PCR amplification using the primers, T7 and SP6 (Table 1). PCR was performed using the transformant in one cycle of incubation at 95°C for 10 min, followed by 30 cycles of amplification were at 95°C for 30 s, 56°C for 30 s, and 72°C for 80 s. A final extension step at 72°C for 5 min was then performed. Finally, the transformation of *pimp1* was sequenced (Macrogen, Seoul, Korea).

Heterologous expression of *pimp1* in *S. cerevisiae* BY4741 The PCR product cloned with a pGEM-T Easy vector was purified and digested with EcoRI (New England Biolabs Inc., Ipswich, MA, USA) and SpeI (New England Biolabs Inc., Ipswich, MA, USA). This digested fragment was inserted into multiple cloning site 1 (MCS1) of the pESC-URA vector (Agilent Technologies, Stratagene, Santa Clara, CA, USA), resulting in the recombinant plasmid pESC-URA/*pimp1* containing the MnP native signal peptide (Fig. 1). The pESC-URA/*pimp1* and the control vector pESC-URA were transformed into *S. cerevisiae* BY4741. The yeast strains were grown overnight at 30°C (200 rpm) in YPD (1% yeast extract, 2% peptone, and 2% glucose). The culture was diluted to an OD of 0.2 and grown at 30°C (200 rpm) in YPD for 3 h. Then, the recombinant plasmid was transformed into yeast cultures. The transformed cells were plated onto SD-URA (0.77 g CSM-URA, 1.79 g YNB without ammonium sulfate, 5 g ammonium sulfate, 20 g glucose, and 1 L distilled water) agar plates for selection of the URA^+ transformants. Some URA^+ transformants were selected randomly and grown on the SD-URA agar plates at 30°C for 2 days and then inoculated onto the liquid culture.

Production of MnP in liquid media and assessment of MnP activity The transformants were primarily grown overnight at 30°C (200 rpm) using a conical tube (50 mL, SPL Lifesciences Co. Ltd., Pocheon, Korea) containing 10 mL of galactose-URA (0.77 g CSM-URA, 1.79 g YNB without ammonium sulfate, 5 g ammonium sulfate, 20 g raffinose, 10 g galactose, and 1 L distilled water) medium. Generally, 1% raffinose was added to the minimal medium containing 2% galactose to give the cells a better growth advantage without affecting the induction process, and glucose-free galactose (<0.01% glucose) was particularly useful to induce the transcription of genes (13). The culture was diluted to an OD of 0.2 and grown at 30°C (200 rpm) using Erlenmeyer flasks (100 mL) containing 20 mL of galactose-URA for 7 days. The activity of MnP was assayed using a UV–Vis spectrophotometer. The enzyme activities were measured every day in each medium of the respective transformant. After filtration of the yeast cells using a syringe filter (0.45 μm), the enzymatic activity of the crude supernatant was measured. To assess the MnP activity, 0.5 M sodium malonate (pH 4.5), 5 mM $MnSO_4$, 1 mM 2,6-dimethoxylphenol, and 1 mM H_2O_2 were used (9). One unit of ligninolytic enzyme activity produced 1 μmol of reaction product per min at room temperature; the activity was expressed in U mL^{-1} of cell free extract (14). The transformed yeast with the control vector pESC-URA was the negative control. All of the experiments were performed in triplicate.

Western blotting analysis The transformants were primarily grown overnight at 30°C (200 rpm) using conical tubes containing 10 mL of SD-URA medium. The culture was diluted to an OD of 0.2 and grown at 30°C (200 rpm) in galactose-URA for 6 h. The whole yeast cell extracts were prepared using a NaOH and trichloroacetic acid (TCA) extraction method (15). The cell pellet was resuspended with 100 μL of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA). The protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, USA). Then, the membranes were incubated with OctA-Probe (D-8) horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody was diluted 1:500, followed by incubation at room temperature for 1 h. The signal for MnP was detected according to the methods described by Miller–Fleming et al. (16).

TABLE 1. Primers used for plasmid construction.

Primer	Nucleotide sequence
pimp1-EcoRI	5′-GAATTCATGTCCTTCACCACTCTTCTGTC-3′
pimp1-SpeI	5′-ACTAGTTTTGCCGAAGGGGGGA-3′
T7	5′-TAATACGACTCACTATAG-3′
SP6	5′-ATTTAGGTGACACTATAGAAT-3′

Degradation and analysis of anthracene The selected transformant was primarily grown overnight at 30°C (200 rpm) using a conical tube (50 mL) containing 10 mL of galactose-URA. The medium was prepared using Erlenmeyer flasks (100 mL) containing 50 mL of the indicated galactose-URA. In addition, 30 mg L^{-1} of anthracene dissolved in 1 mL of acetone was added to the medium, and a total of 0.5 g L^{-1} of Tween 80 was added to the liquid medium for the induction of MnP expression. After dilution of the culture to an OD of 0.2, the culture was incubated at 30°C (200 rpm) for 14 days. An uninoculated abiotic negative control flask; an inoculated biotic negative control, which was transformed yeast with the control vector pESC-URA; and a positive control flask containing the transformant without Tween 80 were treated with the same procedures. The liquid and solid parts of the fungal culture were extracted at the end of the incubation. Each sample was extracted three times with 50 mL of dichloromethane (Duksan, Korea) to obtain a final volume of 150 mL. Each extracted sample was concentrated to 5 mL using a vacuum rotary evaporator (Eyela N-1000 series, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The anthracene concentrations in the extracts were then determined by injecting 1 μL of the extract into a gas chromatography-mass spectrometer (GC–MS; Agilent technologies, Model 7890, Santa Clara, CA, USA). Separation was achieved using a DB-5 MS Ultra Inert column (0.25 mm in diameter and 25 m in length, film thickness of 0.25 μm). GC–MS analysis was conducted according to the method described by Lee et al. (14). The corresponding MnP activity was assessed every day during incubation. The methods used to assess the ligninolytic enzyme activity were described above.

Data analysis The degradation data were analyzed using the Statistical Analysis Systems (SAS 9.2, SAS Institute, Inc., Cary, NC, USA) package. A one-way analysis of variance (ANOVA) followed by Tukey's test was used to assess the significance of the different degradation values among the species (10). The mean values were compared using a significance level of $p < 0.05$.

RESULTS

Transformation of pimp1 in *S. cerevisiae* The pESC-URA vector was constructed for the expression of the *pimp1* cDNA in *S. cerevisiae* via the pGEM-T Easy vector (Fig. 1). All of the expression plasmids and the corresponding vectors without an insert were transformed into the yeast strain BY4741. The cloned pGEM-T Easy vector with *pimp1* and the recombinant plasmid pESC-URA/*pimp1* were confirmed with the restriction enzymes EcoRI and SpeI, respectively (Fig. S1). The insert was sequenced and matched the 1074 bp of *pimp1*. The URA^+ transformed cells were randomly selected and grown on the SD-URA agar plates at 30°C.

MnP production by the transformed yeast The MnP cDNA, *pimp1*, was under the yeast *GAL10* promoter. The MnP production was assayed in a liquid medium with different growth rates. The yeast strains in which the extracellular MnP production was driven by the *GAL10* promoter were grown in a raffinose medium with galactose to induce the promoter. Among the URA^+ transformed cells, three transformed cells were randomly selected and grown for MnP production. Among the three yeast transformants, the highest growth rates were demonstrated in transformant 1 for 10 days (Fig. S2). Transformant 1 also significantly produced MnP on the 2nd and 7th days of incubation (Fig. 2) and transformant 3 demonstrated the peak efficiency (1.92 U mL^{-1} of cell free extract) on the 5th day of incubation. Meanwhile transformant 3 showed the lowest MnP activity (0.68 U mL^{-1} of cell free extract) on the 7th day of incubation and MnP was not secreted form the control strain. Thus, the expression of MnP from the other genes, transformant 2 and transformant 3, resulted in induced MnP compared with the control strain, pEU-WT. Nevertheless, there was no significant difference in growth rate among the two transformants and the control strain; MnP was extracellularly expressed by the two transformants. The maximum MnP activity among the transformants was 3.58 U mL^{-1} of cell free extract in the transformant 1 culture. This transformant grew faster than the other transformants and the control strain carrying the empty