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Optimization of the expression of a laccase gene from *Trametes versicolor* in *Pichia methanolica*

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Abstract A cDNA encoding for laccase (*Lcc1*) was isolated from the ligninolytic fungus *Trametes versicolor* by reverse transcriptase polymerase chain reaction. The *Lcc1* gene was subcloned into the *Pichia methanolica* expression vector pMETαA and transformed into the *P. methanolica* strains PMAD11 and PMAD16. The extracellular laccase activity of the PMAD11 recombinants was found to be 1.3-fold higher than that of the PMAD16 recombinants. The identity of the recombinant protein was further confirmed by immunodetection using the Western blot analysis. As expected, the molecular mass of the mature laccase was 64.0 kDa, similar to that of the native form. The effects of copper concentration, cultivation temperature, pH and methanol concentration in the BMMY on laccase expression were investigated. The laccase activity in the PMAD11 recombinant was up to 12.6 U ml^{-1} by optimization.

Introduction

Laccases belong to the group of enzymes called the blue copper proteins or blue copper oxidases (Messerschmidt 1993). Laccases are heterogeneous in their biochemical properties and molecular structures. Generally, laccases can be characterized by a molecular mass of around 60–80 kDa, a p*I* of 3–6, a glycosylation corresponding to 10–20% of the protein molecular mass and the existence of 1–4 isozymes (Thurston 1994).

Trametes (*Coriolus*, *polyporus*) *versicolor* is one of the best studied white rot fungi (Jönsson et al. 1997). The most active of the enzymes from *T. versicolor* is laccase (Roy-Arcand and Archibald 1991). The predominant isomers in liquid cultures of *T. versicolor* are *Lcc1* and *Lcc2* (Fahraeus and Reinhammar 1967). Laccases are very interesting tools for industrial applications, i.e. for biological bleaching in pulp and paper industries, for detoxification of recalcitrant biochemicals, for bioconversion of chemicals or for treatment of beverages in the agrochemical industry (Gianfreda et al. 1999).

The expression of active recombinant laccases has been reported in the filamentous fungus *Aspergillus oryzae* (Yaver et al. 1996) and the yeasts *Saccharomyces cerevisiae* (Kojima et al. 1990) and *Pichia pastoris* (Jönsson et al. 1997). The present study addresses the production of a recombinant laccase (*Lcc1*) in the *Pichia methanolica*. The filamentous fungi are generally good hosts for protein secretion, but are more time-consuming to work with than *P. methanolica*. Heterologous expression of laccases in the *P. methanolica* would be of special interest in generating large amounts of enzyme.

Materials and methods

Strains, media and vectors

T. versicolor was from Chinese Academy of Sciences

Escherichia coli strain JM109 was used in all DNA manipulations. *E. coli* was grown in the Luria–Bertani medium (tryptone 10 g l^{-1}, NaCl 10 g l^{-1}, yeast extract 5 g l^{-1}, agar 16 g l^{-1}).

The *P. methanolica* strains used for heterologous expression were PMAD11 (ade2–11) and PMAD16 (ade2–11 pep4$^{\Delta}$ prb1$^{\Delta}$). *P. methanolica* was grown in the

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YPAD medium (yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, dextrose 20 g l^{-1}, adenine 0.1 g l^{-1}, agar 20 g l^{-1}).

The vector pMETαA (Invitrogen) was used for expression in *P. methanolica*. The expression of inserts was controlled by the methanol-inducible AUG1 promoter. pMETαA possesses the α-secretion factor from *S. cerevisiae*.

Gene cloning/construction of vectors

RNA was isolated from *T. versicolor*. The fungus was grown in 250-ml baffled flasks containing 25 ml medium suitable for laccase (*Lcc1*) production at 25°C in a shaking incubator (150 rpm). After 13 days, mycelia were harvested. Isolation of total RNA was performed using TRIzol Reagent according to the manufacturer's instructions. The cDNA was synthesized from total RNA by reverse transcriptase polymerase chain reaction (RT-PCR) using the BcaBEST RT-PCR kit with oligo dT primers. The *Lcc1* cDNA without laccase signal peptide was PCR amplified using the upstream primer 5′–5′-TTGAATTCGCTATC GGGCCTGTGACCGACCT-3′ and the downstream primer 5′-TTGGATCCTTAGAGGTCGGATGAGTCAAGAG C-3′. The PCR conditions employed were: 94°C for 1 min (once), +94°C for 30 s, 51°C for 30 s, 72°C for 2 min (30 cycles), +72°C for 5 min (once). The purified PCR products were cloned into the pUC19 vector, sequenced and subsequently excised using *Eco*RI and *Bam*HI, followed by purification from an agarose gel and insertion into pMETαA, resulting in the vector pMETαA/*Lcc1*. After transformation into *E. coli* and isolation of plasmid DNA, the presence of the inserts was determined by both PCR and restriction enzyme digestion, followed by agarose gel electrophoresis. Sequence analysis was performed on pMETαA/*Lcc1* to ensure the integrity of the constructs.

Yeast transformation and identification

Yeast transformation was performed as described by Choi and Kim (2002). pMETαA/*Lcc1* plasmid DNA was digested with *Pac*I prior to transformation into electrocompetent cells of *P. methanolica* PMAD11 and PMAD16, respectively. The vectors pMETαA without inserts were simultaneously transformed into *P. methanolica* PMAD11 and PMAD16 and used as controls. Transformant colonies were picked from minimal glucose (MD) plates and transferred to plates with either MD or minimal methanol (MM) as the carbon source for examination of methanol utilization. Some integrants were checked for the presence of the expression cassette in their genome by PCR using primer specific for the *Lcc1* cDNA.

Assay of laccase activity

Culture aliquots (1 ml) were collected daily, and cells were removed by centrifugation (10,000×*g* for 15 min). The laccase activity assay system contained 100 mM sodium acetate (pH 4.5), 0.5 mM ABTS (Sigma) and 100 μl culture supernatant appropriately diluted in a total volume of 1.0 ml. The reaction mixture was kept at 25°C for 5 min, then the change in absorbance at 420 nm was recorded with a spectrophotometer. One unit was defined as the amount of enzyme which oxidized 1 μmol ABTS per minute.

The agar plate assay, which allowed the selection of transformants secreting laccase, was carried out on MM histidine plates supplemented with 0.2 mM ABTS. The plates were incubated for 3 days at 30°C and checked for the development of a green colour.

Induction and expression of the foreign gene

The control and laccase-secreting transformants were inoculated into buffered dextrose complex medium (BMDY; yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, pH 6.0, 100 mM potassium phosphate buffer, yeast nitrogen base (YNB) 13.4 g l^{-1}, biotin 400 μg l^{-1}, dextrose 20 g l^{-1}) and incubated at 30°C in a shaking incubator (250 rpm). When the turbidity of the culture reached an optical density at 600 nm of ~4.0, the cells were harvested by centrifugation at 3,000×*g* for 5 min. The cells were resuspended in a buffered methanol complex medium (BMMY; yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, pH 6.0 100 mM potassium phosphate buffer, YNB 13.4 g l^{-1}, biotin 400 μg l^{-1}, methanol 5 ml l^{-1}). The flasks were incubated at 30°C, and 0.5% (v/v) methanol was added daily. Samples were taken at intervals for determination of optical density and laccase activity. After performing the above experiments, the transformant with the highest laccase-secreting ability was selected for the following experiments.

Western blot

P. methanolica transformants PMAD11 and PMAD16 were cultured in the BMMY medium. After 5 days of growth, the cultures were harvested by centrifugation. The supernatant was collected and dialysed against distilled water for 6 h at 8°C. The samples were subsequently lyophilized, dissolved in loading buffer and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Mini-Protean II, Bio-Rad). The proteins were electrotransferred to a poly (vinylidene difluoride) membrane using a Trans-Blot semi-dry cell (Bio-Rad). Detection of laccase on the membrane was performed using antibodies from a rabbit immunized with laccase from *T. versicolor* in combination with goat anti-rabbit Ig conjugated with alkaline phosphatase (Bio-Rad).

Optimization of laccase expression

To examine the effect of copper on laccase activity, $CuSO_4$ was added at various concentrations. The effects of cultivation temperature, pH and methanol concentration in the BMMY on laccase expression were also investigated.

The supernatant was collected and separated by SDS-PAGE at different induction time.

Results

Gene cloning/construction of vectors

The RT-PCR product, confirmed by agarose gel electrophoresis analysis, was subjected directly to cDNA sequence analysis. A 1,497-bp nucleotide sequence was obtained. Plasmid pMETαA/*Lcc1* was composed of the inducible promoter AUG1, the *S. cerevisiae* α-factor secretion signal upstream of the *Lcc1* cDNA sequence and a termination transcription signal. To study the expression of *Lcc1* gene in *P. methanolica*, we used the strains PMAD11 and PMAD16. For each construct, ~60–80 transformants were obtained after selection of recombinants on the MD plates.

Expression of *Lcc1* in *P. methanolica*

Initially, transformants were assayed for activity on agar plates containing ABTS, giving a green zone around the transformant secreting laccase. The assays of the plates showed stronger colour development for PMAD11 transformants compared to corresponding PMAD16 transformants. Coloured zones on assay plates were never observed for control transformants without the laccase gene. Subsequent experiments using liquid cultures also included controls with transformants generated using a vector without any insert. Activity was not detected in any of the control cultures. For both PMAD11 and PMAD16 transformants, laccase activity was found in the supernatant of the culture medium, indicating that *Lcc1* was secreted from yeast cells. Ten transformants from each strain were screened to examine for differences in laccase production, but all produced similar levels of laccase. For the PMAD16 transformants, the laccase activity reached a maximum of 3.17 U ml^{-1}, and the cell culture density reached an OD_{600} value of 12.1 after 5 days (Fig. 1). On the other hand, for the PMAD11 transformants, the laccase activity reached a maximum of 4.12 U ml^{-1}, and the cellular density reached an OD_{600} value of 15.6 after 5 days (Fig. 1). In addition, Fig. 1 shows that laccase production follows cellular growth. One of the PMAD11 transformants was selected to be used in subsequent experiments of optimization.

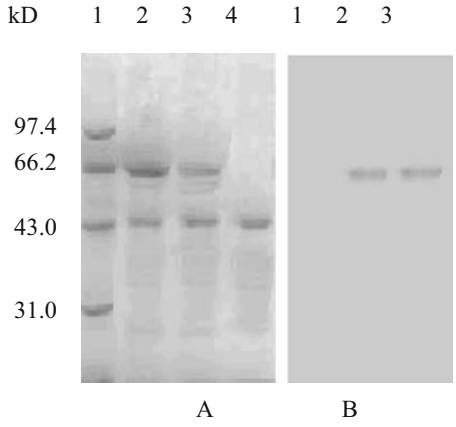

Fig. 2 SDS-PAGE gel (**a**) and Western blot (**b**) analysis of proteins secreted by the transformants PMAD11 and PMAD16. **a** *Lane1* low molecular weight markers, *lane2* PMAD11 (pMETαA/*Lcc1*), *lane 3* PMAD16 (pMETαA/*Lcc1*), *lane 4* PMAD 11 (pMETαA control). **b** *Lane 1* PMAD 11 (pMETαA control), *lane2* PMAD16 (pMETαA/*Lcc1*), *lane 3* PMAD11 (pMETαA/*Lcc1*)

Western blot

Productions of the recombinant laccase for the PMAD11 and PMAD16 were studied by electrophoresis on an SDS-PAGE (Fig. 2a). A clear band of about 64 kDa was observed, corresponding to the native form. The Western blot analysis showed a unique band corresponding to the 64-kDa protein (Fig. 2b), indicating that the productions were the recombinant laccase.

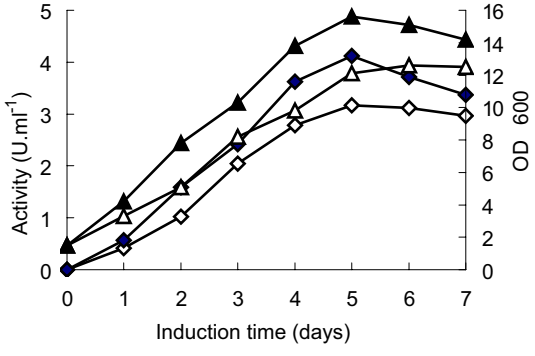

Fig. 1 Comparisons of laccase expression in the transformants PMAD11 and PMAD16. Activity of PMAD11 (◆), activity of PMAD16 (◇), OD_{600} of PMAD11 (▲) and OD_{600} of PMAD16 (△)

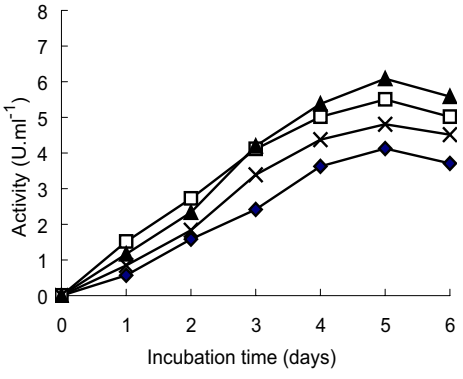

Fig. 3 Laccase activity level of the PMAD11 transformant incubated at 30°C in BMMY medium at different copper concentrations. The $CuSO_4$ concentrations evaluated were 0 mM (◆), 0.1 mM (□), 0.2 mM (▲) and 0.3 mM (×)

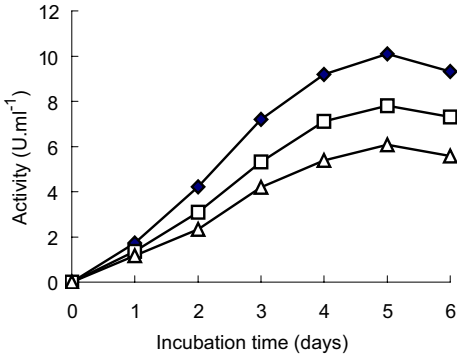

Fig. 4 The effect of temperature on laccase activity of the transformant PMAD11 in BMMY medium containing 0.2 mM $CuSO_4$. The temperatures evaluated were 20°C (♦), 25°C (□) and 30°C (△)

Effect of copper concentration in the BMMY on laccase activity

The PMAD11 transformant with the highest laccase-secreting ability was selected for examining the effect of copper concentration on laccase activity (Fig. 3). If copper was absent in the medium, the laccase activity level was very low. A copper concentration of 0.2 mM led to the highest laccase activity (6.08 U ml^{-1}). When the copper concentration increased to 0.3 mM, the activity began to decrease slightly.

Effect of cultivation temperature on laccase expression

The PMAD11 transformant was used to study the effect of temperature on laccase expression (Fig. 4). The cultures were first grown at 30°C in the BMDY medium and then transferred to the BMMY medium containing 0.2 mM $CuSO_4$ at 20, 25 and 30°C, respectively. The culture supernatants were assayed daily for 6 consecutive days. The optimal secreting time of laccase was 5 days. The production of active enzyme is favoured by lower cultivation temperature. Comparing the highest values during this period, the cultures kept at 20°C provided about 1.7-fold more activity than those kept at 30°C.

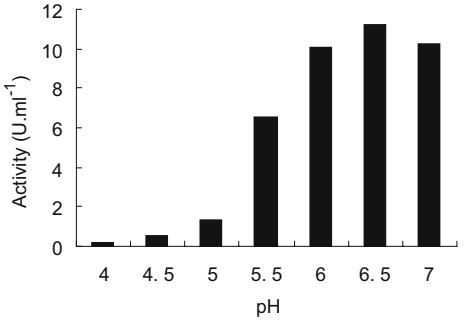

Fig. 5 Laccase activity level of the PMAD11 transformant incubated at 20°C in BMMY medium containing 0.2 mM $CuSO_4$ at different pH values

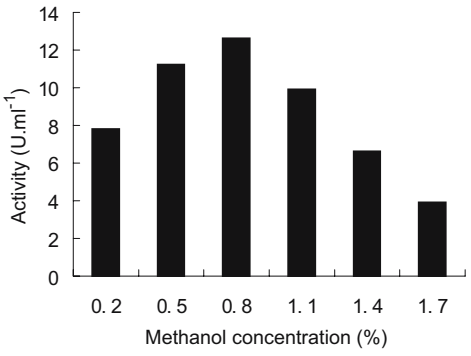

Fig. 6 Laccase activity level of the PMAD11 transformant incubated at 20°C in BMMY medium (pH6.5) containing 0.2 mM $CuSO_4$ at different methanol concentrations

Effect of different pH values in the BMMY on laccase expression

To study the effect of pH in the BMMY on laccase expression, we incubated the PMAD11 transformant at 20°C in a BMMY medium containing 0.2 mM $CuSO_4$ at different pH. The cultures were prepared with initial pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, and the culture supernatants were assayed at the fifth day. Laccase production was optimal at pH 6.5, while very little laccase activity was produced at pHs 4.0, 4.5 and 5.0 (Fig. 5).

Effect of methanol concentration in the BMMY on laccase expression

The PMAD11 transformant was also used to study the effect of methanol concentration on laccase expression in the optimal cultures mentioned above (Fig. 6). The culture supernatants were assayed at the fifth day. With increasing methanol concentration, the laccase activity was enhanced. The methanol concentration of 0.8% (v/v) resulted in the highest laccase activity (12.6 U ml^{-1}). When the methanol concentration increased to 1.1% (v/v), the activity began to decrease slightly.

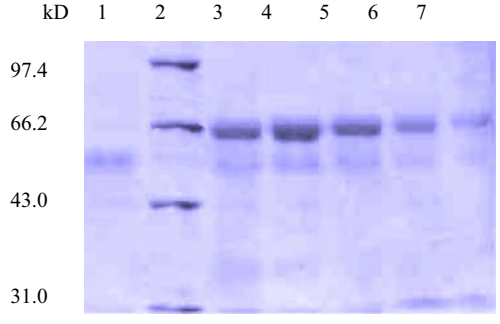

Fig. 7 SDS-PAGE analysis of laccase production by PMAD11 at different induction days. *Lane 1* negative control (5d); *lane 2* low molecular weight protein marker; *lanes3–7* 6d, 5d, 4d, 3d and 2d

SDS-PAGE analysis of laccase production

The PMAD11 transformant was induced in the optimal cultures mentioned above. The supernatant was collected and separated by SDS-PAGE at different induction time (Fig. 7). The optimal secreting time of laccase was 5 days.

Discussion

In this study, the cDNA cloned from *T. versicolor* was expressed in *P. methanolica* PMAD11 and PMAD16. We detected the functional forms of heterologous laccase in the cultures. Laccases are copper-containing enzymes and contain two to four copper atoms per enzyme. A previous study in this laboratory demonstrated the importance of copper availability to the activity of laccase enzymes from *T. versicolor*. Copper ions did not influence the degree of protein synthesis, but the activity of the laccase was directly proportional to the concentration of copper in the growth medium. The correlation between copper availability and laccase activity is probably due to the enzymes for copper requirement.

The temperature effect is of particular interest since it has a marked effect on the production of active heterologous laccase. It has been previously reported that a lower cultivation temperature improves the production of heterologous protein in *P. pastoris* system (Cassland and Jönsson 1999). In the present study, we identified the temperature as an important parameter for the optimization of the production of heterologous laccase in yeast systems, in general.

The laccase activity of PMAD11 transformant was found to be positively affected by the pH in BMMY. It is evident from the results presented here that low pH (below 5.5) is detrimental for the production of active laccase. One of the possible explanations is the susceptibility to proteases under acidic conditions.

In addition, the methanol concentration in the BMMY is also an important parameter for optimization of the production of heterologous laccase. In this study, the methanol concentration of 0.8% (v/v) improved heterologous laccase activity compared to 1.1%. It is shown that high methanol concentration is also detrimental to the laccase expression, although this expression system can utilize methanol as its sole carbon and energy source.

In conclusion, we have characterized the heterologous laccase by Western blot and found a heterogeneity similar to that expected, thus suggesting no occurrence of hyperglycosylation. We have also demonstrated a number of factors affecting the levels of laccase activity, such as the copper concentration, cultivation temperature, pH values and methanol concentration in the BMMY. Although the laccase expression level is 12.6 U ml^{-1} using PMAD11 transformant, laccase production could be further improved using certain methods. The fermentor cultivations and selection for multiple integration events would be two of the methods to enhance expression levels. Recombinant laccase will be improved further by using transformant in fermentor and will be used to test this enzyme for industrial applications.

References

Cassland P, Jönsson LJ (1999) Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl Microbiol Biotechnol 52:393–400

Choi J, Kim EE (2002) Expression of the active human and duck hepatitis B virus polymerases in heterologous system of *Pichia methanolica*. Antivir Res 55:279–290

Fahraeus G, Reinhammar B (1967) Large scale production and purification of laccase from the fungus *Polyporus versicolor* and some properties of laccase. Acta Chem Scand 21:2367–2378

Gianfreda L, Xu F and Bollag JM (1999) Laccase: a useful group of oxidoreductive enzymes. Biores J 3:1–25

Jönsson L, Saloheimo M, Penttila M (1997) Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. Curr Genet 32:425–430

Kojima Y, Tsukuda Y, Kawai Y, Tsukamoto A, Sugiura J, Sakaino M, Kita Y (1990) Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 265:15224–15230

Messerschmidt A (1993) Blue copper oxidases. Adv Inorg Chem 40:121–185

Roy-Arcand L, Archibald FS (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor*. Enzyme Microb Technol 13:194–203

Thurston CF (1994) The structure and function of fungal laccase. Microbiology 140:19–26

Yaver DS, Xu F, Golightly EJ, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dallboge H (1996) Purification, characterization, molecular cloning and expression of two laccases from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62:834–841