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Comparison of laccase production levels in *Pichia pastoris* and *Cryptococcus* sp. S-2

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The heterologous expression of the laccase gene from *Trametes versicolor* and *Gaeumannomyces graminis* was evaluated in the yeasts *Pichia pastoris* and *Cryptococcus* sp. S-2. The expression levels of both laccase genes in *Cryptococcus* sp. S-2 were considerably higher than those in *P. pastoris*. The codon usage of *Cryptococcus* sp. S-2 as well as the GC content were similar to those of *T. versicolor* and *G. graminis*. These results suggest that using a host with a similar codon usage for the expressed gene may improve protein expression. The use of *Cryptococcus* sp. S-2 as a host may be advantageous for the heterologous expression of genes with high GC content. Moreover, this yeast provides the same advantages as *P. pastoris* for the production of recombinant proteins, such as growth on minimal medium, capacity for high-density growth during fermentation, and capability for post-translational modifications. Therefore, we propose that *Cryptococcus* sp. S-2 be used as an expression host to improve enzyme production levels when other hosts have not yielded good results.

[**Key words:** *Cryptococcus* sp. S-2; GC content; Heterologous expression; Laccase; *Pichia pastoris*]

Laccases (EC1.10.3.2) are multicopper enzymes that catalyze the oxidation of various phenolic compounds and aromatic amines with a concomitant reduction of molecular oxygen to water. These enzymes are widely distributed in nature and can be found in many plants, fungi, insects, and bacteria (1–3). Because of their wide substrate specificity, laccases have attracted broad interest for industrial and biotechnological applications, such as dye decolorization, paper pulp bleaching, juice and wine clarification, detoxification of environmental pollutants, use as biosensors, and organic synthesis (4).

White rot fungi are natural sources of laccases, but the amount of enzymes produced by these fungi is insufficient for industrial and biotechnological applications. A possible solution for improved enzyme production is the generation of recombinant laccases in heterologous hosts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Trichoderma reesei*, *Yarrowia lipolytica*, and *Aspergillus* sp. (5,6). However, laccase production levels in heterologous hosts have not always yielded good results (5).

Various approaches have been attempted to increase enzyme production, such as the use of strong promoters and multiple gene copies, codon optimization, altering of secretory signal sequences, and optimization of culture conditions; these were successful in some cases. Another choice for improving the enzyme production levels would be the use of basidiomycetes as an expression host. Laccase genes used in such studies were usually cloned from basidiomycetes with a typically high GC content, whereas the expression hosts were primarily ascomycetes with comparatively low GC content. Thus, the codon usage between these organisms tends to be different. It has been reported that differences in codon usage between foreign genes and the endogenous genes of expression hosts significantly affect the yield of recombinant proteins, and that codon optimization improves expression efficiency (7).

The yeast strain *Cryptococcus* sp. S-2, isolated in our laboratory, is a basidiomycete with a GC content of 67% (8). Recently, a host–vector system for this yeast was developed and proved to be an efficient tool for the production of recombinant proteins (9). Therefore, we attempted to use this basidiomycetous yeast as a laccase expression host.

We report here the heterologous expression of laccase genes with high GC content (one gene from *Trametes versicolor* and the other from *Gaeumannomyces graminis*, a filamentous ascomycete) in the basidiomycetous yeast *Cryptococcus* sp. S-2 and show the usefulness of *Cryptococcus* sp. S-2 for recombinant protein production.

MATERIALS AND METHODS

Organisms and culture conditions *T. versicolor* (NBRC 4930) was maintained on potato dextrose agar plates (Difco, Detroit, MI, USA) at 25°C. For RNA isolation, 7-mm agar plugs containing mycelia were inoculated into liquid medium comprising 0.5% malt extract, 1% peptone, 2% glucose, and 0.005% $CuSO_4$ (10), and were incubated for 10 days at 25°C on a rotary shaker (120 rpm). *G. graminis* (NBRC 5278) was maintained on potato dextrose agar plates at 25°C. For RNA isolation, mycelia were inoculated into LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH 7.0) supplemented with 400 μM $CuSO_4$ (11) and incubated for 4 days at 25°C on a rotary shaker (120 rpm). *P. pastoris* X33 (Invitrogen, Carlsbad, CA, USA) and *Cryptococcus* sp. S-2 U-5 (*ura5*) were used for the heterologous expression of laccase genes. The wild-type *Cryptococcus* sp. S-2 has been deposited in the International Patent Organism Depositary (accession number: FERM ABP-10961). *P. pastoris* was maintained on YPD (1% yeast extract,

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2% peptone, 2% glucose) agar plates at 30°C. *Cryptococcus* sp. S-2 U-5 (*ura5*) was maintained on YM (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% glucose) agar plates at 25°C.

Cloning of laccase genes Total RNA was isolated from mycelia using Isogen-LS (Nippon Gene, Tokyo, Japan). First strand cDNA was synthesized from total RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo(dT) primers.

For expression in *P. pastoris*, a laccase gene was amplified from cDNA using Takara LA Taq® DNA polymerase (Takara Bio, Shiga, Japan) and the PP-TVF and PP-TVR primers for *T. versicolor LAC5* (GenBank accession no. AB539566) and the PP-GGF and PP-GGR primers for *G. graminis LAC2* (11). All primers used in this study are listed in Table 1. Purified PCR products were inserted into a pCR®2.1-TOPO® vector (Invitrogen) and verified by sequencing. *Eco*RI/*Not*I-digested fragments were introduced into the *Eco*RI/*Not*I site of the pPICZ vector (Invitrogen).

We used the expression vectors, pCsUX and pCsUX-S, for the expression of laccase in *Cryptococcus* sp. S-2. The pCsUX vector contains the xylanase promoter and terminator as well as the URA5 gene, with the promoter and terminator as selection markers (9). The pCsUX-S vector contains the xylanase secretion signal sequencein addition to the elements found in pCsUX. All genes or gene fragments originate from *Cryptococcus* sp. S-2.

For the construction of the laccase genes with native secretion signal sequences, laccase genes were amplified from cDNA using KOD plus DNA polymerase (Toyobo, Osaka, Japan) and the CS-TVF-1 and CS-TVR-1 primers for *T. versicolor LAC5* and the CS-GGF-1 and CS-GGR-1 primers for *G. graminis LAC2*. The purified products were inserted into the pCsUX vector using the In-Fusion™ Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA, USA). For the construction of these genes with xylanase secretion signal sequences, theCS-TVF-2 and CS-TVR-2 primers were used for the *T. versicolor* laccase gene and the CS-GGF-2 and CS-GGR-2 primers for the *G. graminis* laccase gene; PCR products were inserted into the pCsUX-S vector.

Transformation of *P. pastoris* Competent *P. pastoris* X33 cells were prepared for electroporation according to the manufacturer's instructions. To improve the transformation efficiency, the cells were pretreated with 10 mM dithiothreitol (12). The final concentration of the competent cells was approximately 2.5×10^{10} cells/ml. Eighty microliters of the competent cells were mixed with 8 μg of *Pme*I-digested linearized DNA and transferred to a prechilled 0.2-cm electroporation cuvette. The cells were electroporated (1.5 kV, 25 μF, and 200 Ω) using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). The electroporated cells were immediately diluted with 1 ml of 1 M sorbitol, transferred to culture tubes, and incubated for 1 h at 30°C. Then, an equal volume of YPD medium was added, and the cells were incubated at 30°C for an additional hour with shaking. After incubation, the cells were spread onto YPD selection plates containing 1 M sorbitol, 500 μg/ml Zeocin™ (Invitrogen), and 2% agar. Transformants were transferred to the new YPD selection plates, and subsequently, laccase-producing transformants were selected using MM [1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, and 0.5% methanol] plates containing 0.2 mM 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) and 0.4 mM $CuSO_4$.

Transformation of *Cryptococcus* sp. S-2 Competent *Cryptococcus* sp. S-2 U-5 cells were prepared according to Masaki et al. (9). The final concentration of these competent cells was approximately 2×10^{10} cells/ml. Fifty microliters of the competent cells were mixed with 5 μg of *Sse*8387I-digested linearized DNA and were then transferred to a prechilled 0.2-cm electroporation cuvette. The cells were subjected to an electric pulse (0.47 kV, 25 μF, and ∞) using the Gene Pulser. The electroporated cells were immediately diluted with 1 ml of buffer [270 mM sucrose, 1 mM $MgCl_2$, 10 mM Tris−HCl (pH7.0)] and spread onto YNB-ura selection plates containing 2% glucose, 0.67% YNB without amino acids -ura dropout supplements (BD Biosciences, Franklin Lakes, NJ, USA), and 2% agar. The plates were incubated for 1 week at 25°C. Transformants were transferred to the new YNB-ura selection plates, and subsequently, laccase-producing transformants were selected using YNB-ura plates containing 0.2 mM ABTS and 0.4 mM $CuSO_4$.

Expression of laccase genes in *P. pastoris* (shake flask culture) The laccase-secreting yeasts were precultured in 40 ml of BMGY medium [1.34% YNB, 100 mM potassium phosphate (pH 6.0), 1% yeast extract, 2% peptone, and 1% glycerol] at 30°C on a rotary shaker until the OD_{600} reached 2.0−6.0. The cells were collected by centrifugation at 4000 ×*g* and resuspended in 40 ml of BMMY medium [1.34% YNB, 100 mM potassium phosphate (pH 6.0), 1% yeast extract, 2% peptone, and 0.5% methanol] containing 0.4 mM $CuSO_4$ to an OD_{600} of 1.0. The cultures were then incubated at 30°C on a rotary shaker for 5 days. Methanol (0.5%) was added to the medium daily.

Expression of laccase genes in *Cryptococcus* sp. S-2 (shake flask culture) The laccase-secreting yeasts were precultured in 5 ml of YM medium for 2 days at 25°C with shaking. The cells were collected by centrifugation at 4000 ×*g* and were resuspended in 40 ml of BMXY medium [1.34% YNB, 100 mM potassium phosphate (pH 6.0), 1% yeast extract, 2% peptone, and 3% or 6% xylose] containing 0.4 mM $CuSO_4$ to an OD_{600} of 0.1. The cultures were then incubated at 25°C on a rotary shaker for 4 days.

Laccase production by *Cryptococcus* sp. S-2 in high cell density fermentation Transformants into which *T. versicolor LAC5* with a native signal sequence or *G. graminis LAC2* with a xylanase signal sequence had been introduced were selected for expression in high cell density fermentation. Transformants into which *T. versicolor LAC5* with a xylanase signal sequence had been introduced were also used in high cell density fermentation, but the production level was less than that achieved with the native signal sequence (data not shown).

Fermentation was performed according to the *Pichia* fermentation process guidelines provided by Invitrogen, with slight modifications. Compositions of fermentation basal salts medium and PTM_1 trace salts were as described in these guidelines, except that biotin in PTM_1 trace salts was substituted for thiamine-HCl to facilitate yeast growth. Fermentation was executed using a 2-l fermenter (Able, Tokyo, Japan) with an additional feed pump, water cooler, and computer for automatic control.

Transformants were precultured in YM medium for 3 days at 25°C on a rotary shaker. Inoculums (100 ml) were added to 1000 ml of fermentation basal salt medium with PTM_1 trace salts (pH 5.0 adjusted with 28% ammonium hydroxide), which contained 4% xylose instead of glycerol as a carbon source, because the xylanase promoter is strongly induced by xylose. Cultures were then incubated at 25°C until xylose was exhausted. After exhaustion of xylose, 50% xylose solution with PTM_1 trace salts was continuously supplied through the feed pump. pH was maintained at 5.0 by adding 14% ammonium hydroxide, which also provided a nitrogen source for the culture. During the xylose fed-batch phase, dissolved oxygen (DO) was maintained above 20% by controlling the xylose feed rate. In case of *G. graminis* laccase production, 0.4 mM $CuSO_4$ was added to the basal medium and 1.2 mM $CuSO_4$ was added to the xylose feeding solution. The production level of *G. graminis* laccase was so high that additional $CuSO_4$ was needed, whereas for *T. versicolor* laccase production, sufficient $CuSO_4$ was present in the basal medium and xylose feeding solution.

Laccase activity assay Laccase activity in the culture supernatant was determined using ABTS as the substrate. The reaction mixture contained 1.7 ml of 100 mM acetate buffer (pH 5.0), 200 μl of 10 mM ABTS, and 100 μl of enzyme solution. After incubation for 20 min at 30°C, the reaction was stopped by adding an equal volume of 5% trichloroacetic acid (13). The formation of cation radicals was detected by measuring the increase in absorbance at 420 nm ($\varepsilon 420 = 36{,}000\ M^{-1}CM^{-1}$). One

TABLE 1. Primers used in this study.

Oligonucleotide	Sequence
1. Primers for cloning of laccase gene from *T. versicolor* (for *P. pastoris*)	
PP-TVF	GCGAATTCACTATGACTGGGCTCCGTCTTCTTCC
PP-TVR	GAGCGGCCGCTGTTCACTGGTCGTCAGCGTCGAGGGC
2. Primers for cloning of laccase gene from *G. graminis* (for *P. pastoris*)	
PP-GGF	GCGAATTCAACATGGTTGCTCACCACTCTTTCA
PP-GGR	GAGCGGCCGCGCTTAGATGCCCGAGTCCTCGAGCTT
3. Primers for cloning of laccase gene from *T. versicolor* (for *Cryptococcus* sp. S-2)	
CS-TVF-1	CTTTGACCGCGACGCGATGACTGGGCTCCGTCTTCTTCCTTCCT
CS-TVR-1	AATTCACTAGTACGCGTCACTGGTCGTCAGCGTCGAGGGCGTC
CS-TVF-2	ACCCCCATCGCAATTGAGAAGCGTGGGATCGGGCCCGTGCTTGACCTTACCATCTCCA
CS-TVR-2	GGGACGCTTCTCAATTTCACTGGTCGTCAGCGTCGAGGGCGTCAT
4. Primers for cloning of laccase gene from *G. graminis* (for *Cryptococcus* sp. S-2)	
CS-GGF-1	CTTGCTTTGACCGCGATGGTTGCTCACCACTCTTT
CS-GGR-1	CCCATTCCTGAGCGTTTAGATGCCCGAGTCCTCGA
CS-GGF-2	ACCCCCATCGCAATTGAGAAGCGTTCTCCTTTCGGGGAGCTCCAGCAGAGGGCCGAGCC
CS-GGR-2	GGGACGCTTCTCAATTTTAGATGCCCGAGTCCTCGAGCTTGATGT