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Leif J. Jönsson · Markku Saloheimo · Merja Penttilä

Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*

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Abstract A cDNA coding for laccase was isolated from the ligninolytic fungus *Trametes versicolor* by RNA-PCR. The cDNA corresponds to the gene *lcc1*, which encodes a laccase isoenzyme of 498 amino-acid residues preceded by a 22-residue signal peptide. The *lcc1* cDNA was cloned into the vector pHIL-D2 for expression in *Pichia pastoris* under the control of the *AOX1* promoter. Transformants were found to secrete active recombinant enzyme after induction with methanol. The use of growth medium buffered to pH 6.0 and control of pH during cultivation were found to be important, or even necessary, for obtaining activity in liquid cultures. The effect of exchanging the native secretion signal for the *Saccharomyces cerevisiae* α-factor pre-pro secretion signal was studied by cloning the portion encoding the mature enzyme into the vector pPIC9. The activity obtained for the construct encoding the native laccase signal sequence was found to be seven-fold higher than for the construct encoding the α-factor secretion signal. Utilisation of the *P. pastoris pep4* mutant strain SMD1168 was found to provide a two-fold higher level of activity compared with *P. pastoris* GS115.

Key words Laccase · *Trametes versicolor* · Heterologous expression · *Pichia pastoris*

Introduction

Laccases are produced by many plants and fungi, and have been ascribed diverse biological functions in different organisms (Reinhammar 1984). White-rot fungi are generally good producers of laccases, which are considered a part of their ligninolytic system. Laccases are multicopper enzymes belonging to the blue oxidases (Messerschmidt 1993), which also include ascorbate oxidases and ceruloplasmins.

Trametes (*Coriolus*, *Polyporus*) *versicolor* is one of the best studied white-rot fungi. *T. versicolor* and its laccase are of interest for biological bleaching of pulp (Eriksson et al. 1990; Bourbonnais et al. 1995), and for de-toxification of wastes (Bergbauer et al. 1991; Roy-Arcand and Archibald 1991; Lamar 1992). The laccase from *T. versicolor* has been intensively studied by biophysical methods (Reinhammar 1984). *T. versicolor* laccase is a secreted glycosylated enzyme with four copper ions and two disulphide bridges (Reinhammar 1984; Jönsson et al. 1995). *T. versicolor* secretes several isoforms of laccase. *Lcc1* is a laccase gene which has been isolated from a genomic library of *T. versicolor* (Jönsson et al. 1995). *Lcc1* (EMBL Data Library accession number X84683), which contains ten introns, has previously been known only from genomic DNA, and it has not been known whether the gene is expressed in *T. versicolor* or whether it is silent. *T. versicolor* also secretes a multitude of peroxidase isoenzymes, and several peroxidase genes have recently been characterised from strain PRL572 (e.g. Jönsson and Nyman 1994; Johansson and Nyman 1996).

Ligninolytic enzymes are generally difficult to over-express heterologously in active form. Detection of active recombinant laccase has been reported in the yeast *Saccharomyces cerevisiae* (Kojima et al. 1990), and in the filamentous fungi *Trichoderma reesei* (Saloheimo and Niku-Paavola 1991) and *Aspergillus oryzae* (Yaver et al. 1996). Here, we study expression of *lcc1* in the methylotrophic yeast *Pichia pastoris*. *P. pastoris* has several advantages for expression of foreign proteins compared with *S. cerevisiae* (Cregg et al. 1993), such as the use of the strong and regulated *AOX1* promoter. The filamentous fungi are generally good hosts for protein secretion, but are more time-consuming to work with compared with *P. pastoris*. To have a rapid system for heterologous laccase expression

L. J. Jönsson (✉)
Applied Microbiology, Lund Institute of Technology/
Lund University, P. O. Box 124, S-22100 Lund, Sweden
Fax: ++46-46-222 42 03
e-mail: leif.jonsson@tmb.lth.se

M. Saloheimo · M. Penttilä
VTT Biotechnology and Food Research,
P. O. Box 1500, FIN-02044 VTT, Finland

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would be of special interest for the generation of site-directed mutants. The amino-acid residues around the type-1 copper of *T. versicolor* laccase provide interesting targets for site-directed mutagenesis for studies of the effect on the reduction potential of the enzyme. Comparisons can be made with other type-1 copper proteins (Malmström 1994), such as azurin. Site-directed mutagenesis and the preparation of fusion proteins is of potential interest for engineering the enzyme to become more suitable for biotechnical applications. Heterologous expression of laccase in yeasts is also of interest for the production of larger quantities of a single ligninolytic enzyme and for the construction of yeast strains with novel properties, such as improved resistance against phenolic inhibitors.

Materials and methods

Preparation of RNA. RNA was isolated from *Trametes versicolor* PRL572 and SBUG1050. The fungus was grown in shake flasks at 27°C in synthetic medium (Fåhraeus and Reinhammar 1967). After 5 days both glucose (to 50 mM) and ferulic acid (to 0.2 mM) were added to the cultures. Additional ferulic acid (0.2 mM) was added after 6 days, the mycelium harvested later the same day, and RNA prepared (Chomczynski and Sacchi 1987).

RNA-PCR. Cloning of the *lcc1* cDNA from *T. versicolor* by RNA-PCR was made using the upstream primer 5′-GCTCTAGAATGGGCAGGTTCTCATCTCTCTG-3′ and the downstream primer 5′-GCGAATTCTTTGAACCGATTAGAGGTCGGATG-3′. Reverse transcription was carried out using the 'Expand' transcriptase followed by amplification with the Expand High Fidelity PCR System (Boehringer Mannheim) according to the following procedure: 94°C 150 s (once) + 94°C 30 s, 50°C 30 s, 72°C 100 s (10 cycles) + 94°C 30 s, 55°C 30 s, 72°C 150 s (10 cycles) + 94°C 30 s, 55°C 30 s, 72°C 4 min (10 cycles) + 72°C 7 min (once). PCR products were hydrolysed with *Xba*I and *Eco*RI and cloned into pBluescript II SK.

Strains and vectors for cloning and expression. Cloning procedures were performed using *E. coli* XL-1 Blue and DH5*α*. PCR products were cloned into pBluescript II SK (Stratagene). *P. pastoris* GS115 (*his4*) and SMD1168 (*his4, pep4)* were employed for heterologous expression. The vectors pHIL-D2 and pPIC9 (Invitrogen) were used for expression in *P. pastoris*.

Cloning into P. pastoris expression vectors. The laccase cDNA was transferred from pBluescript II SK by cutting with *Xba*I and *Eco*RI, blunt-ending with Klenow fragment, followed by purification from agarose gel, and insertion into blunt-ended pHIL-D2 linearised with *Eco*RI. For cloning into pPIC9, PCR-amplification of the laccase cDNA in pBluescript II SK was performed with the upstream primer 5′-CGGAATTCGCTATCGGGCCTGTGACCGACC-3′ and the downstream primer 5′-CGTCTAGATTCTTTGAACCGATTAGAGGTCG-3′. PCR was carried out using Vent DNA polymerase (New England Biolabs) according to the following scheme: 94°C 3 min (once) + 94°C 1 min, 55°C 1 min, 72°C 3 min (27 cycles) + 72°C 10 min (once). The PCR-products were hydrolysed with *Eco*RI and *Xba*I, inserted into pBluescript II SK, excised with *Eco*RI and *Not*I, purified from agarose gel, and inserted into pPIC9 digested with *Eco*RI and *Not*I. The laccase expression plasmids are displayed in Fig. 1.

DNA sequencing. DNA sequencing of PCR-products used for expression was carried out in both directions using primer walking. Sequenase (US Biochemicals) was used according to instructions from the manufacturer.

Expression in P. pastoris and detection of laccase activity. P. pastoris was transformed by the spheroplast method (as described in the *Pichia* expression system manual, Invitrogen). Plasmid DNA was digested with *Sac*I or *Not*I prior to transformation. For all expression experiments, transformants with vector without any laccase insert were used as controls. Transformants were picked from RDB plates (all media prepared according to the *Pichia* expression system manual, Invitrogen) and transferred to plates with either glucose (MD) or methanol (MM) as a carbon source, for examination of methanol utilisation. Assays for laccase activity were carried out by using ABTS as the reducing substrate. The agar-plate assay was carried out on BMM or BMMY plates supplemented with 0.2 mM ABTS and 0.1 mM $CuSO_4$, and monitored by visual inspection. The spectrophotometric assay was performed directly with liquid culture medium, and consisted of 375 µl water, 125 µl 400 mM NaAc pH 5.2, 250 µl 1.6 mM ABTS, and 250 µl culture medium. The pH of the assay mixture was tested after addition of the sample to ensure a final pH in accordance with that of the assay buffer. The assay was monitored at 414 nm and 30°C with a Shimadzu UV-2101. *P. pastoris* was cultivated at 30°C in 50 ml of BMM or MM medium supplemented with 0.1 mM of $CuSO_4$ in baffled 250-ml shake flasks. The experiment investigating culture conditions suitable for expression (see Fig. 2) was carried out using one transformant of each type. When different transformants were directly compared with each other (see Fig. 3), three transformants of each type were tested in parallel to take into account variations among individual transformants.

Results

Introns are often not correctly spliced from genes expressed in a heterologous host. Therefore, *lcc1* cDNA was used for *P. pastoris* expression. The RNA-PCR gave a single product of the expected size (1585 bp) of the coding region of *lcc1*. This result was obtained with RNA from both *T. versicolor* PRL572 and SBUG1050. RNA-PCR products from PRL572 were selected for heterologous expression, since *lcc1* from PRL572 has been studied previously (Jönsson et al. 1995).

DNA sequencing of the clone used for heterologous expression revealed two nucleotides deviating from the chromosomal sequence of *lcc1* (Jönsson et al. 1995). The two nucleotide substitutions should generate two amino-acid substitutions, involving residues 377 (codon GAC instead of AAC, giving Asp instead of Asn) and 466 (codon CTC instead of TTC, giving Leu instead of Phe). The sequence of the PCR-product generated from the cDNA and cloned into pPIC9 did not show any further deviations.

The *lcc1* cDNA including the native signal sequence was cloned into the vector pHIL-D2 between the *AOX1* promoter and the transcription termination signal (Fig. 1 A). The resulting plasmid and the control (vector without insert) were cut with *Not*I, and transformed into *P. pastoris* GS115 spheroplasts. With plasmid digested with *Not*I, which cuts twice (at the 5′ *AOX1* and the 3′ *AOX1* regions), both Mut^+ and Mut^S transformants were generated. Mut^+ (methanol utilisation plus) and Mut^S (methanol utilisation slow) transformants were distinguished by the growth rate, which is higher for Mut^+ than for Mut^S on agar plates containing methanol as a carbon source (MM-plates). Mut^+ transformants are expected to have the transformed DNA inserted into the *AOX1* or *his4* loci of the

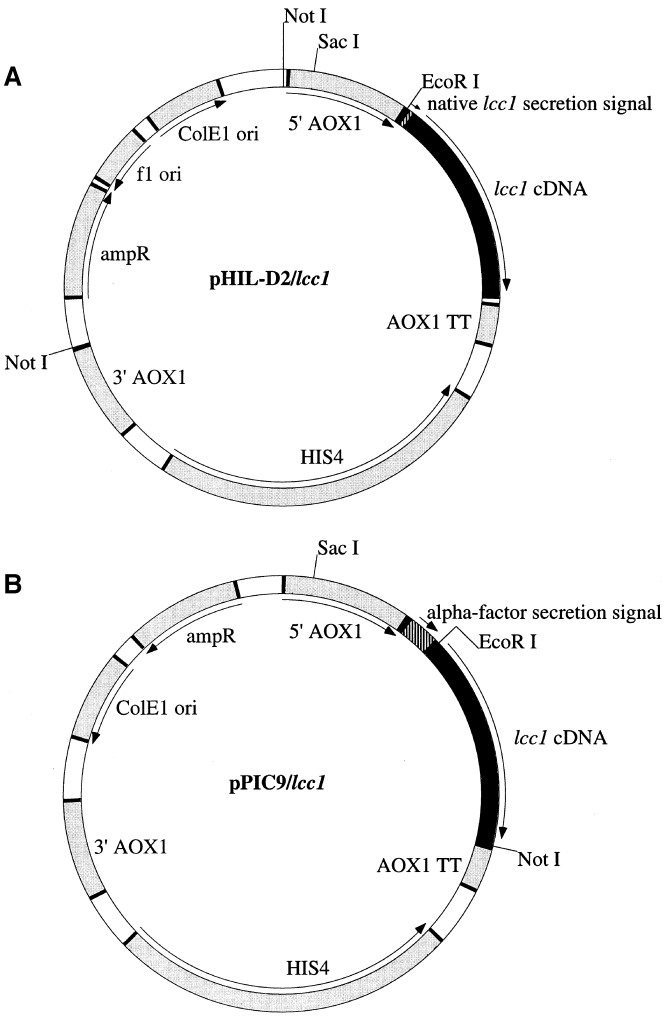

Fig. 1A, B Plasmids constructed for the expression of laccase in *P. pastoris*. The *lcc1* gene was cloned into the vector pHIL-D2 (**A**) together with its own signal sequence, and into the vector pPIC9 (**B**) after the α-factor secretion signal (after removal of the native *lcc1* secretion signal, as described in Materials and methods). The pHIL-D2/*lcc1* plasmid is 9.8 kbp and pPIC9/*lcc1* 9.5 kbp. *AOX1* is the alcohol oxidase gene from *P. pastoris*, and TT is the transcription termination fragment. *HIS4* is the *P. pastoris* gene encoding histidinol dehydrogenase. Other segments indicated are used for propagation in *E. coli*

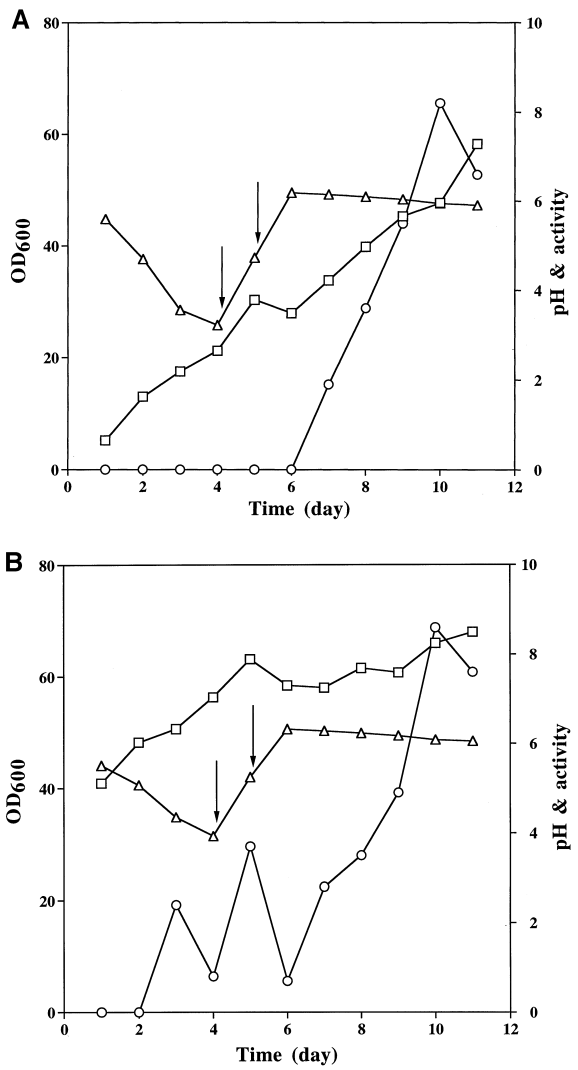

Fig. 2A, B The effect of pH on the expression of recombinant laccase in *P. pastoris* GS115. **A** shows a Mut^+ and **B** a Mut^S transformant, both from the construct with the native laccase signal sequence in pHIL-D2. The activity (○), the OD_{600} (□), and the pH (△) are indicated. After initial cultivation using glycerol as carbon source (BMG medium), cells were harvested by centrifugation and transferred to methanol medium (BMM) supplemented with $CuSO_4$. As recommended, a higher inoculum was used for the slow methanol-utilising transformant (Mut^S). Methanol (to 0.5%) was added daily. Activity is indicated in arbitrary units. *Arrows* indicate adjustment of pH

P. pastoris genome, and Mut^S transformants are expected to result from a gene replacement event causing removal of the *AOX1* coding region from the genome (*Pichia* expression system manual, Invitrogen), resulting in impaired ability to metabolise methanol. Initially, transformants were assayed for activity on agar plates containing ABTS, giving a coloured zone around transformants secreting laccase. Coloured zones on assay plates were never observed for control transformants not carrying the laccase gene. Subsequent experiments using liquid cultures also included controls with transformants generated by using a vector without any insert. Activity was never detected in any of the control cultures. A Mut^+ and a Mut^S transformant were used for examination of the culture conditions suitable for expression, as shown in Fig. 2A and B respectively. The laccase activity of both types of transformants was found to be positively affected by not allowing the pH of the cultures in buffered MM-medium (BMM) to drop too low during the growth. This result is supported by the fact that no activity was observed in corresponding cultures in which the same transformants were grown in unbuffered medium, and that no activity was observed in Mut^+ cultures even in the buffered (BMM) medium if pH was not raised during growth (cf. Fig. 2 A). Since the pH

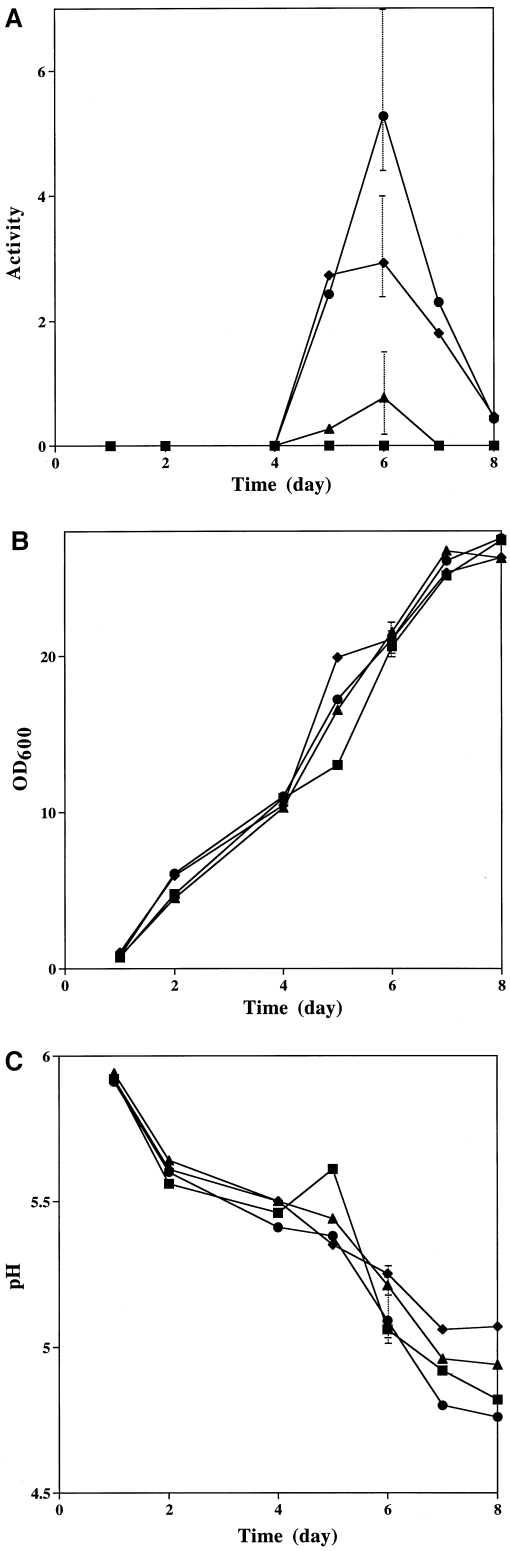

Fig. 3A–C Comparisons of laccase expression using the native and the α-factor secretion signal in *P. pastoris* GS115 (*his4*) and SMD1168 (*his4*, *pep4*). All transformants are Mut$^+$. Activity (**A**), OD_{600} (**B**), and pH (**C**) are indicated for the following constructs: vector without insert in SMD1168 (■), α-factor secretion signal in SMD1168 (▲), native secretion signal in SMD1168 (●), and native secretion signal in GS115 (◆). The mean value for the three transformants of each type is shown (the limits of variation are indicated for the day of maximum activity). Addition of methanol and adjustment of pH was done daily

of the Mut$^+$ cultures dropped more rapidly (Fig. 2), the need for control of pH was more important for the Mut$^+$ transformant. The final activity obtained with the Mut$^+$ and the Mut^S transformants studied was about the same (Fig. 2), but several transformants of each type would have to be studied to make a general conclusion regarding the expression level.

The *lcc1* cDNA without the native signal sequence was joined with the α-factor secretion signal sequence of pPIC9 between the *AOX1* promoter and the transcription termination signal (Fig. 1 B). The resulting plasmid was cut with *Sac*I, and transformed into *P. pastoris* GS115 and SMD1168 spheroplasts. *Not*I could not be used in this case, since pPIC9 contains only one *Not*I site (in the polylinker). For comparison, a vector without an insert and the pHIL-D2 construct encoding the native *lcc1* signal peptide were also cut with *Sac*I prior to transformation into *P. pastoris* GS115 and SMD1168. With a plasmid linearised with *Sac*I, which cuts the plasmid once within the 5′ *AOX1* region, Mut$^+$ transformants were generated.

Twelve Mut$^+$ transformants, all generated by cutting either pHIL-D2- or pPIC9-based plasmids with *Sac*I and transformed into either GS115 or SMD1168 as host strains, were used for the experiment shown in Fig. 3. The variation in peak activity among the transformants of each type never reached the range of the other types (Fig. 3 A). When comparing the OD_{600} and pH (Fig. 3 B, C), transformants of different types overlap. This indicates that the variations in activity, but not in OD_{600} and pH, are significant. The *pep4* mutant SMD1168 gave a two-fold higher activity than the regular expression strain, GS115. Assays of liquid cultures showed sevenfold higher laccase activity for the construct with a native secretion signal than for the construct with the α-factor secretion signal. These results are supported by the observation, using the agar-plate assay, of stronger colour development for SMD1168 transformants compared with corresponding GS115 transformants, and for the construct encoding the native secretion signal compared with the construct encoding the α-factor secretion signal.

Discussion

Laccase from *T. versicolor* can be separated into two chromatographic fractions, A and B (Reinhammar 1984), which both contain several components, probably representing different isoforms of laccase. Nyman and co-workers were able to obtain distinct sequences by Edman degradation of these two fractions (Briving et al. 1980; Nyman, personal communication). More recently, two forms of *T. versicolor* laccase, I and II, have been analysed by Edman degradation by another group (Bourbonnais et al. 1995) working with another strain. From a comparison of data (Fig. 4) it is clear that laccase I and II correspond to data obtained from laccase B and A, respectively. In addition, a distinctly different *T. versicolor* laccase sequence was found in the analysis of the *lcc1* gene isolated from a

laccase A	GIGPV ADLTI TNAAV SPDGF SRQAV VVNGG
laccase B	AIGPV ASLVV ANAPV SPDGF LRDAI VVNGG
lcc1	AIGPV TDLTI SNADV SPDGF TRAAV LANGV

Fig. 4 Amino-terminal laccase sequences from *T. versicolor*. Included are Edman degradation data for laccase A and B (Nyman, personal communication) and the sequence deduced from the *lcc1* gene (Jönsson et al. 1995; present study). Unique residues are *underlined*. The amino-terminal portion of laccase II (Bourbonnais et al. 1995) is identical with laccase A. The only difference between the amino-termini of laccase I and laccase B is residue 30, which is Val in laccase I instead of Gly

genomic library (Jönsson et al. 1995), the one studied in this report. The fact that our RNA-PCR gave a single band of expected size for both *T. versicolor* strains analysed indicates that the *lcc1* gene is expressed and also conserved among different strains. Thus, *T. versicolor* expresses at least three distinctly different forms of laccase, the amino-termini of which are displayed in Fig. 4. By using a different set of primers for RNA-PCR, another laccase cDNA (*lcc2*) was isolated from *T. versicolor* and was found to encode an amino-terminal sequence identical with laccase A (Fig. 4).

Two nucleotides in the cDNA used for *Pichia* expression deviate from the known genomic sequence. The second substitution does not appear to be of much interest since the alignment of laccases from basidiomycete fungi shows that both Phe and Leu are present at this position (Jönsson et al. 1995). The first substitution is of more interest, since exchange for an Asp removes one of the six potential N-glycosylation sites. Whether the nucleotide differences represent natural variation or changes generated in the cDNA synthesis or PCR reaction is not known. Nevertheless, the laccase was successfully expressed and secreted in active form by *P. pastoris*.

Using low pH (pH 3) for protein production with *P. pastoris* is known to be possible, and sometimes even preferable (Scorer et al. 1993). It is however evident from the results presented here that low pH (below 4) is detrimental for the production of active laccase. One of the possible explanations is susceptibility to acidic proteases. The results showing higher activity when using the proteinase A negative strain SMD1168 suggest that proteolytic activity plays an important role in the expression of laccase in *P. pastoris*. The use of a *pep4* mutant strain has previously been reported to be beneficial for the production of a secreted recombinant protein, ghilanten, in *P. pastoris* (Brankamp et al. 1995). This is now confirmed in our study, with the advantage that the compared transformants are all Mut^+, whereas in the study of Brankamp et al. one of the compared strains was Mut^S and the other Mut^+.

The α-factor pre-pro secretion signal from *S. cerevisiae* has in several cases proved to be very useful for expression in *P. pastoris* (Cregg et al. 1993). Unfortunately, replacing the native signal sequence with the α-factor secretion signal resulted in lower activity in the present study. This might result, for example, from inefficient processing of the fusion protein or interference of the propeptide with the folding of laccase. It appears from this result that future attempts to raise the production level of laccase should primarily focus on other measures than fusions with the α-factor pre-pro region. To improve the stability of the recombinant laccase (cf. Fig. 3), the parameters used for cultivation should be carefully controlled. The expression level obtained so far is too low to be convenient for biochemical characterisation of the recombinant enzyme, but it is likely that the expression level can be enhanced further. Before making direct comparisons of the expression level with other host organisms, fermenter cultivations should be tested since these are generally advisable in order to achieve higher levels of expression using *P. pastoris* (Cregg et al. 1993). Selection for multiple integration events would be another way to enhance expression levels.

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