

# Heterologous Expression of *Trametes versicolor* Laccase in *Pichia pastoris* and *Aspergillus niger*

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## Abstract

Convenient expression systems for efficient heterologous production of different laccases are needed for their characterization and application. The laccase cDNAs *lcc1* and *lcc2* from *Trametes versicolor* were expressed in *Pichia pastoris* and *Aspergillus niger* under control of their respective glyceraldehyde-3-phosphate dehydrogenase promoters and with the native secretion signal directing catalytically active laccase to the medium. *P. pastoris* batch cultures in shake-flasks gave higher volumetric activity (1.3 U/L) and a better activity to biomass ratio with glucose than with glycerol or maltose as carbon source. Preliminary experiments with fed-batch cultures of *P. pastoris* in bioreactors yielded higher activity (2.8 U/L) than the shake-flask experiments, although the levels remained moderate and useful primarily for screening purposes. With *A. niger*, high levels of laccase (2700 U/L) were produced using a minimal medium containing sucrose and yeast extract. Recombinant laccase from *A. niger* harboring the *lcc2* cDNA was purified to homogeneity and it was found to be a 70-kDa homogeneous enzyme with biochemical and catalytic properties similar to those of native *T. versicolor* laccase A.

**Index Entries:** Laccase; heterologous expression; *Pichia pastoris*; *Aspergillus niger*.

## Introduction

Laccases are phenol-oxidizing enzymes that are of interest in several different applications (reviewed in ref. [1]). Possible applications include textile processing, detoxification of industrial effluents and pollutants, detoxification of lignocellulose hydrolysates in fuel ethanol production, utilization as an environmentally benign oxidant in the production of chemicals, delignification of pulp for paper manufacture, catalysis of grafting

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processes in the development of novel polymers, production of fiberboards, use in fuel cells, and utilization in biosensors for monitoring phenolic pollutants and drugs. The properties of different laccases show a great deal of divergence. Laccases with high-redox potential, such as the laccase from the white-rot fungus *Trametes (Polyporus, Coriolus) versicolor* (2), are required for oxidation of recalcitrant substrates. Considering the wide range of applications for laccases (1), there is a need for heterologous expression systems to screen mutated laccases for novel properties or for large-scale production of selected laccases.

Previous studies suggest that yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* are convenient systems for rapid expression of laccase genes. However, production levels in yeast have been quite low (up to approx 5 mg/L), whereas filamentous fungi in general have given 2–30 times higher levels (10–135 mg/L) (3). *P. pastoris* is easy to manipulate genetically, easy to use in conventional fed-batch fermentations, secretes low levels of native proteins, and capable of adding both O- and N-linked glycans to secreted proteins (4). Filamentous fungi, such as *A. niger*, have the ability to produce and secrete exceptionally large amounts of properly folded proteins with the correct cofactors incorporated and can produce proteins that contain O- and N-linked glycans without extensive hyperglycosylation (5–7).

It has been shown previously that production of laccase in *P. pastoris* using the *AOX1* promoter system is negatively affected by increasing methanol concentration (8). The *AOX1* promoter requires methanol for induction, which makes it difficult to use lower concentrations because that would negatively affect the mRNA expression level. Therefore, it should be of interest to consider the glyceraldehyde-3-phosphate dehydrogenase promoter system as an option for expression in *P. pastoris*, as well as in *A. niger*.

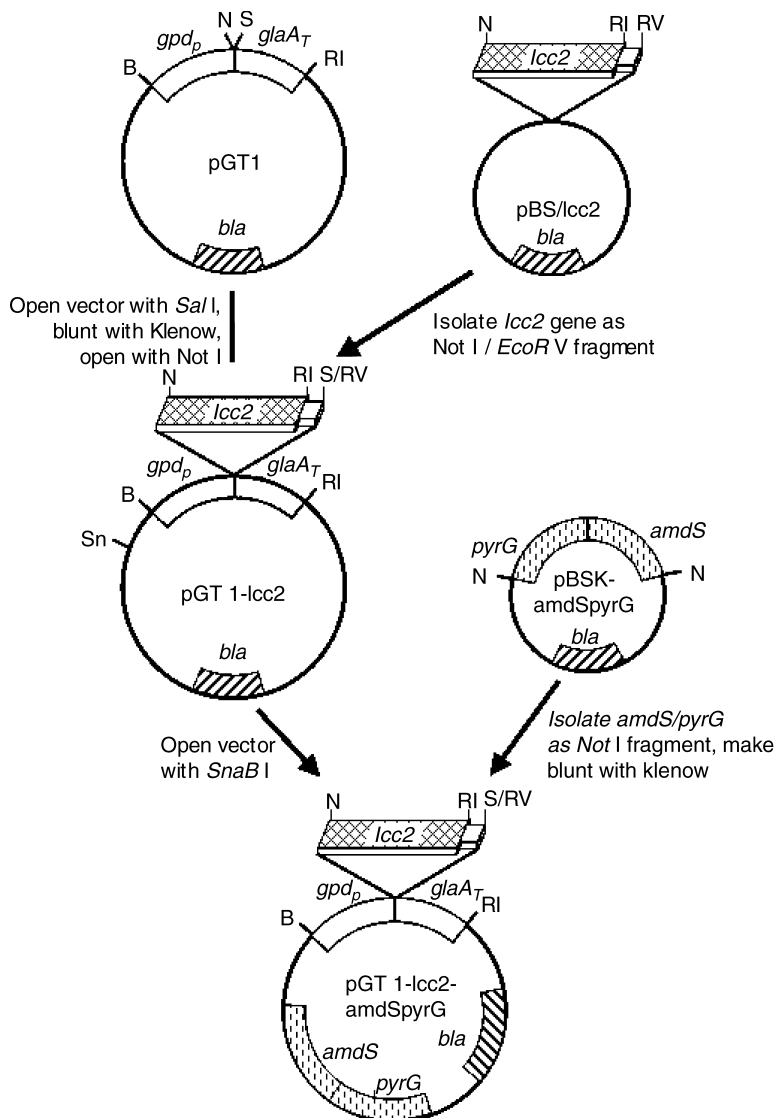
In this study, we have explored the potential in using *P. pastoris* for screening purposes and *A. niger* for production of selected laccases. The cDNAs *lcc1* and *lcc2* from *T. versicolor* were expressed in *P. pastoris* and *A. niger*, and the effects of different media and cultivation conditions on the laccase production levels were investigated. The recombinant laccase expressed in *A. niger* was purified to homogeneity and its biochemical and catalytic properties were compared to the well-characterized native *T. versicolor* laccases A (LccA) and B (LccB) (2).

## Methods

### *Microbial Strains and Recombinant DNA*

The *lcc1* and *lcc2* cDNA genes from *T. (Coriolus, Polyporus) versicolor* (9–11) were used in the construction of plasmids for expression of laccases in *P. pastoris* and *A. niger*.

For the expression in *P. pastoris*, the *lcc1* and *lcc2* cDNA genes were inserted into the vectors pGAPZ A and pGAPZ B, respectively. The expression cassette of the pGAPZ vectors includes the glyceraldehyde-3-phosphate



**Fig. 1.** Construction of plasmids for expression of laccase in *A. niger*. B, *Bam*H I; N, *Not* I; RI, *EcoR* I; RV, *EcoR* V; S, *Sal* I; Sn, *SnaB* I. pGT1-*lcc1*-*amdS*-*pyrG* was made similarly.

dehydrogenase gene (*GAP*) promoter region and the transcriptional terminator of the alcohol oxidase 1 gene (*AOX1*). The pGAPZ vectors are designed for constitutive expression in *P. pastoris* (Invitrogen, Carlsbad, CA). *P. pastoris* SMD1168, a *his4 pep4* strain, was transformed by electroporation.

Figure 1 summarizes the construction of plasmids for the expression in *A. niger* D15 (obtained from TNO, Zeist, The Netherlands). Insertion of the *lcc1* and *lcc2* cDNA genes into the plasmid pGT1, which harbors the *Aspergillus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and glucoamylase (*gla*) terminator from pSPORT (12), generated pGT1-*lcc1*

and pGT1-*lcc2*, respectively. Insertion of an *amdSpyrG* fragment taken from pBSKII(+)-*amdSpyrG* generated pGT1-*lcc1-amdSpyrG* and pGT1-*lcc2-amdSpyrG*, respectively (Fig. 1). *A. niger* spheroplasts were formed using Novozyme 234 (Sigma, St. Louis, MO) and transformed in accordance with the method of Punt and van den Hondel (13).

### *Selection of Laccase-Expressing Transformants*

*P. pastoris* transformants that survived a concentration of 0.1 mg/mL zeocin were spread on BMG agar plates (Invitrogen) containing glucose instead of glycerol, 0.1 mM CuSO<sub>4</sub> and 0.2 mM ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. The color development was followed at room temperature (approx 22°C). Laccase-expressing transformants were selected and incubated in 50 mL buffered minimal glycerol (BMG) medium with 0.1 mM CuSO<sub>4</sub>. An addition of 0.3 mL of a 0.6 M solution of potassium phosphate (pH 6.0) and 0.2 mL of a 0.4 M solution of NaOH was made daily. For selection of top laccase producers, samples were taken daily to determine the laccase activity (see Analyses of Samples).

*A. niger* transformants containing the *lcc1* and *lcc2* cDNA constructs as well as a negative control strain obtained from a transformation of *A. niger* D15 with the pBSKII(+)-*amdSpyrG* plasmid (Fig. 1) were plated onto agar plates containing 5% glucose, 0.2% amino acid pool without tyrosine or phenylalanine (200 mg each of adenine, uracil, tryptophan, arginine, methionine, and histidine; 300 mg of lysine; 600 mg of leucine; 2000 mg of threonine; and 65 mg of inositol), 0.5% yeast extract, 0.1 mM CuSO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% ABTS, 0.1% 1000× trace elements [the 1000X trace element solution contained (per 100 mL deionized water) 2.2 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.1 g H<sub>3</sub>BO<sub>3</sub>, 0.5 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.17 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.16 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.15 g Na<sub>2</sub>MoO<sub>4</sub>·2 H<sub>2</sub>O, 5 g ethylene diamine tetraacetic acid (EDTA), and the pH was 6.5], and 1× AspA with nitrate [50X AspA with nitrate contained (per 500 mL deionized water): 150 g NaNO<sub>3</sub>, 13 g KCl, 38 g KH<sub>2</sub>PO<sub>4</sub>]. The plates were incubated at 30°C for 48 h and the color development was followed. Laccase-expressing *A. niger* transformants were transferred to 50 mL medium containing 0.5% yeast extract, 1% glucose, 0.1% casamino acids (Difco, BD, Franklin Lakes, NJ), 2% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1X trace element solution, and 1X AspA with nitrate by inoculation with spores to a concentration of 1 × 10<sup>6</sup> spores/mL (experimental series 1). The cultures were then grown for 48, 72, or 144 h. The mycelia were harvested by filtering through Mira cloth (Calbiochem, San Diego, CA) and the culture supernatants were collected to determine the laccase activity.

### *Shake-Flask Cultivations of P. pastoris*

A selected laccase-producing transformant (designated *P. pastoris/lcc2*) was incubated in BMG medium and also in the same medium but with glucose or maltose instead of glycerol. The initial concentration of carbon source was 0.5–3%. The concentration of YNB (Yeast Nitrogen Base w/o amino acids, a component of BMG medium), was varied in the range 1–3%. In addition, the medium contained 0.4% histidine, 1 mM CuSO<sub>4</sub>, and

0.8% alanine. The cultivation was allowed to continue for 7 d at 20°C and samples were taken daily to determine the laccase activity.

### *Parallel Fed-Batch Cultivations of P. pastoris in Multibioreactor*

#### Preparation of Inoculum

A colony of *P. pastoris/lcc2* containing pGAPZ/*lcc2* was transferred from a yeast peptone dextrose (YPD) agar plate into a baffled flask containing 200 mL of yeast peptone dextrose (YPD) (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) and incubated at 30°C with shaking (200 rpm in a G25 orbital shaker, New Brunswick Scientific, Edison, NJ) for 20 h until an OD<sub>600</sub> (optical density at 600 nm) of greater than 3 was reached.

#### Batch Phase

A Sixfors multibioreactor system (Infors, Bottmingen-Basel, Switzerland) equipped with three parallel temperature-controlled 500 mL bioreactors with sensors for pH and pO<sub>2</sub> was used. Each bioreactor contained 180-mL basal salt medium (Invitrogen) not including glycerol but supplemented with 4% glucose, 2 g/L histidine, 4.35 mL/L PTM trace salt solution (Invitrogen), and 0.2 mM CuSO<sub>4</sub>. The pH of the medium was adjusted to 5.0 with 28% ammonium hydroxide before the inoculum (3 mL) was added to a final OD<sub>600</sub> of 1. The bioreactors were maintained at a temperature of 20°C and at an agitation rate of 1000 rpm. Air was introduced into the bioreactor at a rate of 30 L/h and the pH was automatically maintained at 5.0 by the addition of a 2.8% ammonium hydroxide solution. The batch phase was allowed to continue until the glucose was depleted, which occurred after approx 40 h. Thereafter, the fed-batch phase was initiated.

#### Fed-Batch Phase

The medium that was fed into the bioreactor was the same as in the batch phase, except that different concentrations of glucose were used. Bioreactors A–C were fed with glucose at a rate of 0.06, 0.17, and 0.43 g/h, respectively. Samples were taken daily and centrifuged in a Mini Spin microcentrifuge (Eppendorf, Hamburg, Germany) at maximum speed for 15 min. The supernatants were transferred to fresh tubes, which were snap-frozen in liquid nitrogen. Samples were analyzed with respect to laccase activity, protein concentration, protease activity, OD<sub>600</sub>, wet cell weight (WCW), and glucose concentration (*see Analyses of Samples*).

### *Media Optimization for A. niger Transformants Using Shake-Flask Cultures*

#### Experimental Series 2 and 3

To determine optimal medium composition for laccase production, spores of the *A. niger* transformant expressing the highest level of laccase (designated *A. niger/lcc2*) were used to inoculate different media (Table 1)

Table 1  
Media Used for Optimization of Laccase Production by *A. niger*

| Ingredient (g/L)                                | Media composition |                   |                   |                   |                   |                   |                   |
|---|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|   | A                 | B                 | C                 | D                 | E                 | F                 | G                 |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 45                |                   |                   |                   |                   |                   | 3 <sup>a</sup>    |
| KH <sub>2</sub> PO <sub>4</sub>                 | 23                | 1.5 <sup>b</sup>  | 1.5 <sup>b</sup>  |                   | 1.5 <sup>b</sup>  |                   |                   |
| FeSO <sub>4</sub> ·H <sub>2</sub> O             | 0.1               |                   |                   |                   |                   |                   |                   |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O            | 7                 | 0.49 <sup>c</sup> | 0.49 <sup>c</sup> | 0.49 <sup>c</sup> | 0.49 <sup>c</sup> | 0.49 <sup>c</sup> | 0.98 <sup>d</sup> |
| Sucrose   | 50                |                   | 50                |                   |                   |                   | 100               |
| Urea  | 11                |                   |                   |                   |                   |                   |                   |
| Yeast extract                                   | 5                 |                   | 5                 |                   | 5                 | 5                 |                   |
| Glucose   |                   | 10                |                   | 100               | 10                |                   |                   |
| 1000X trace elements                            |                   | 1 mL              | 1 mL              | 1 mL              | 1 mL              | 1 mL              | 2 mL              |
| NaNO <sub>3</sub>                               |                   | 6 <sup>b</sup>    | 6 <sup>b</sup>    | 5.95              | 6 <sup>b</sup>    | 6 <sup>b</sup>    | 12 <sup>a</sup>   |
| KCl   |                   | 0.52 <sup>b</sup> | 0.52 <sup>b</sup> | 0.52              | 0.52 <sup>b</sup> | 0.52 <sup>b</sup> | 1.04 <sup>a</sup> |
| NaH <sub>2</sub> PO <sub>4</sub>                |                   |                   |                   | 35.60             |                   |                   |                   |
| Initial pH                                      | 4, 5, 6           | 4, 5, 6           | 4, 5, 6           | 5                 | 5                 | 5                 | 5                 |

<sup>a</sup>As 40 mL 50X AspA+N salts.

<sup>b</sup>As 20 mL 50X AspA+N salts.

<sup>c</sup>As 2 mL of 1 M.

<sup>d</sup>As 4 mL of 1 M.

to a final concentration of  $1 \times 10^6$  spores/mL. The inoculum was generated by resuspending spores in a sterile 0.9% NaCl solution. The spore suspension was stored at 4°C until use. The cultures were incubated in a volume of 100 mL at 30°C with shaking (200 rpm). The performance of the transformant in media A–D (experimental series 2) (Table 1) was studied for 4 d. In the experiments with media E–G (experimental series 3) (Table 1), the cultivation was allowed to continue for 10 d.

#### Experimental Series 4

The media optimization was continued by investigating the effect of the addition of 0.5% yeast extract to the medium, as well as the use of a preculture. The 20-mL precultures were all inoculated to a final concentration of  $5 \times 10^6$  spores/mL using the *A. niger/lcc2* transformant. The precultures were incubated at 30°C for 36 h and then transferred to 80-mL fresh media.

#### Experimental Series 5

Additional experiments were carried out at pH 4.0–6.0 and at 25°C and 30°C. The laccase activity was measured daily. The results of these and earlier experiments were used to plan the following experiment.

#### Experimental Series 6

The production of laccase by the transformant *A. niger/lcc2* was followed over a time period of 10 d in cultures with three different media in a volume of 250 mL. The first medium consisted of a minimal salt medium

(0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.6%  $\text{NaNO}_3$ , 0.05%  $\text{KCl}$ , 0.15%  $\text{KH}_2\text{PO}_4$ , 1x trace elements) supplemented with 1% glucose and 0.5% yeast extract. The second medium was a double-strength minimal salt medium (0.1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2%  $\text{NaNO}_3$ , 0.1%  $\text{KCl}$ , 0.3%  $\text{KH}_2\text{PO}_4$ , 2x trace elements) with 10% sucrose. The third medium was the same as the second but supplemented with 0.5% yeast extract. The pH of the first two media at the time of inoculation was 5.0, whereas the pH of the third was 6.0.

#### Purification of Heterologously Expressed Laccase From *A. niger*

The transformant *A. niger/lcc2* was cultivated in 250 mL of previously optimized medium (0.1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2%  $\text{NaNO}_3$ , 0.1%  $\text{KCl}$ , 0.3%  $\text{KH}_2\text{PO}_4$ , 2x trace elements) with 10% sucrose and 0.5% yeast extract, and with the pH adjusted to 6.0. The culture was inoculated to  $1 \times 10^6$  spores/mL and incubated at 30°C and 200 rpm for 11 d. The extracellular fraction was harvested by filtration. Ammonium sulfate precipitation was carried out at 70% saturation (at pH 4.2). The harvested protein precipitate was resuspended in 20 mM imidazole buffer, pH 7.0, and then snap-frozen in liquid  $\text{N}_2$  in aliquots. The aliquots were freeze-dried and stored at -70°C. They were then resuspended in a 20 mM solution of potassium phosphate (pH 6.0) and dialyzed against the same buffer before purification.

The first purification step was done by loading the dialyzed sample onto a DEAE Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden), pre-equilibrated with 100 mM phosphate buffer (pH 6.0). A NaCl gradient from 0 to 500 mM was applied to the column, and the laccase started eluting at 40 mM NaCl. Fractions collected between 40 and 125 mM NaCl were pooled and dialyzed against 20 mM 3-(*N*-morpholino)propanesulfonic acid) MOPS buffer, pH 7.2, overnight.

The second purification step was done by loading the dialyzed DEAE fractions onto a HiTrap Q FF column (Amersham Biosciences) equilibrated with 20 mM MOPS, pH 7.2. The protein was eluted with a 0–500 mM NaCl gradient. The laccase eluted from 150 mM NaCl. Fractions collected between 150 and 250 mM NaCl were pooled and dialyzed overnight against 10 mM MOPS, pH 6.5.

The dialyzed protein solution was concentrated 15 times, using 5-kDa cutoff spin columns (Millipore, Bedford, MA). This preparation was used for kinetic analysis and comparison with two different forms of the native protein, laccase A (LccA) and laccase B (LccB), isolated from *T. versicolor* (2,14).

A further purification step was carried out by loading the HiTrap Q-purified protein onto a MonoP HR 5/20 column (Amersham Biosciences). The column was equilibrated with 20 mM MOPS buffer, pH 7.2, and a pH gradient from 7.2 to 3.0 was applied using 10% PolyBuffer 74 (Amersham Biosciences), pH 3.0, as eluent. A peak was visible for fractions eluting at pH 3.0–4.0 and the relevant fractions were neutralized by adding 100  $\mu\text{L}$  of 1 M Tris-HCl per 2 mL fraction and pooled. The protein solution was dialyzed

against 10 mM phosphate buffer, pH 6.0, and concentrated using the 5-kDa spin columns.

### *Analyses of Samples*

#### Laccase Activity

Assays were performed as described previously (9). One unit was defined as the amount of laccase that forms 1  $\mu\text{mol}$  of ABTS radical cation ( $\epsilon = 3.6 \times 10^4/\text{M}/\text{cm}$  at 414 nm [15]). To avoid assay interference by medium components, the laccase activity in samples from *P. pastoris* cultures was measured after purification of the samples using Microspin G-25 columns (Amersham Biosciences, Sweden) equilibrated with 10 mM phosphate buffer (pH 6.0).

#### Protein Concentration

The protein concentrations in samples from *P. pastoris* cultures were determined using Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine-serum albumin as the standard. The protein concentrations in samples from *A. niger* cultures were estimated using the Lowry method (16). During the purification of laccase from *A. niger*, the protein concentration was determined using the BCA protein assay reagent kit (Pierce, IL).

#### Optical Density

The OD of the samples was measured against distilled water at 600 nm with a ultraviolet-1601PC spectrophotometer (Shimadzu, Kyoto, Japan) after appropriate dilution.

#### Wet Cell Weight

The WCW was determined by centrifugation of the samples at maximum speed with a Mini Spin centrifuge (Eppendorf, Germany) for 15 min in preweighed test tubes and subsequent removal of the supernatant.

#### Glucose Concentration

The glucose concentration was determined using a Glucometer Elite XL (Bayer, Leverkusen, Germany) after appropriate dilution in artificial liquor containing (per liter of deionized water) 8.65 mg NaCl, 176.4 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 182.9 mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 201.3 mg KCl, and NaOH to a pH of 7.4.

#### Electrophoresis

Estimation of the purity, size, and pI of the enzyme was obtained by using the PhastGel system (Amersham Biosciences), with a 4–15% gradient gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an isoelectric focusing (IEF) 3–9 gel for IEF. Two identical IEF gels were run at the same time and one was stained with Coomassie brilliant blue. The other was used for zymogram analysis and was immersed for 5 min in a solution of 0.4 mM ABTS in 50 mM NaAc, pH 5.0.



### Glycosylation

To determine the level of glycosylation of the recombinant laccase and the native LccA sample, deglycosylation was carried out using *N*-glycosidase F (Roche, Mannheim, Germany). First, 8  $\mu\text{g}$  of laccase (in 5  $\mu\text{L}$  of a solution of 1% SDS, 1%  $\beta$ -mercaptoethanol, and 20 mM sodium phosphate [pH 8.6]) was denatured by boiling for 3 min. The denatured sample was subsequently deglycosylated in a final volume of 20  $\mu\text{L}$  containing 1% Nonidet P-40, 0.5% EDTA, 1%  $\beta$ -mercaptoethanol, 25 mM sodium phosphate buffer (pH 7.2), 0.25% SDS, and 5 U of the deglycosylation enzyme. The reactions were incubated at 37°C for 2 h, and 4  $\mu\text{L}$  was loaded onto a 12% homogeneous SDS-PAGE gel using the PhastGel system. Control reactions without *N*-glycosidase F addition were done in parallel.

### Kinetic Analysis

The  $K_M$  values of LccA, LccB, and the recombinant laccase purified from *A. niger* were determined using the substrate 2,4,5-trimethoxybenzyl alcohol and were performed as described elsewhere (32).

### Protease Activity

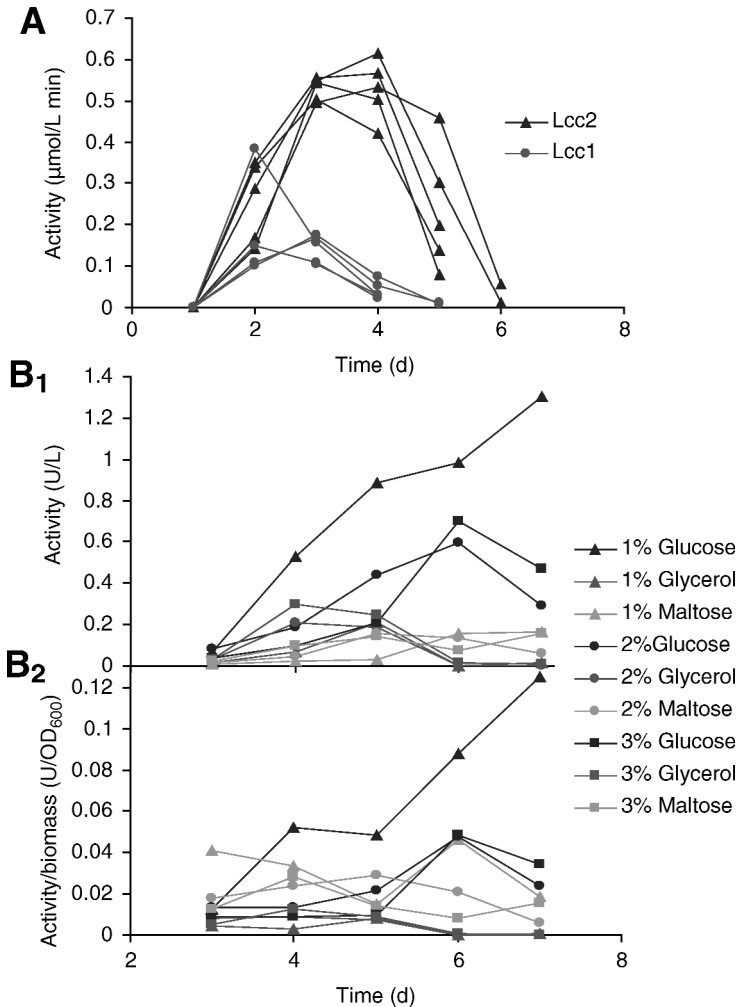
The protease activity was determined using the QuantiCleave fluorescent protease assay kit (Pierce, IL), which is based on proteolytic digestion of a fluorescein thiocarbamoyl-casein conjugate and measurement of the fluorescence at 538 nm. The proteolytic activity at pH 5.0 was assayed using an LS 55 luminescence spectrometer (PerkinElmer, Wellesley, MA).

## Results and Discussion

The expression of two isoenzymes of laccase from *T. versicolor* in *P. pastoris* and *A. niger* was investigated. In both cases, the expression was under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, the *GAP* promoter of *P. pastoris* (17) and the *gpd* promoter of *Aspergillus* (18). The *GAP* promoter gives constitutive expression, although its strength varies depending on the carbon source (17). This is the first report of a laccase expressed in *P. pastoris* with the *GAP* promoter, as previous attempts have been made using the *AOX1* promoter.

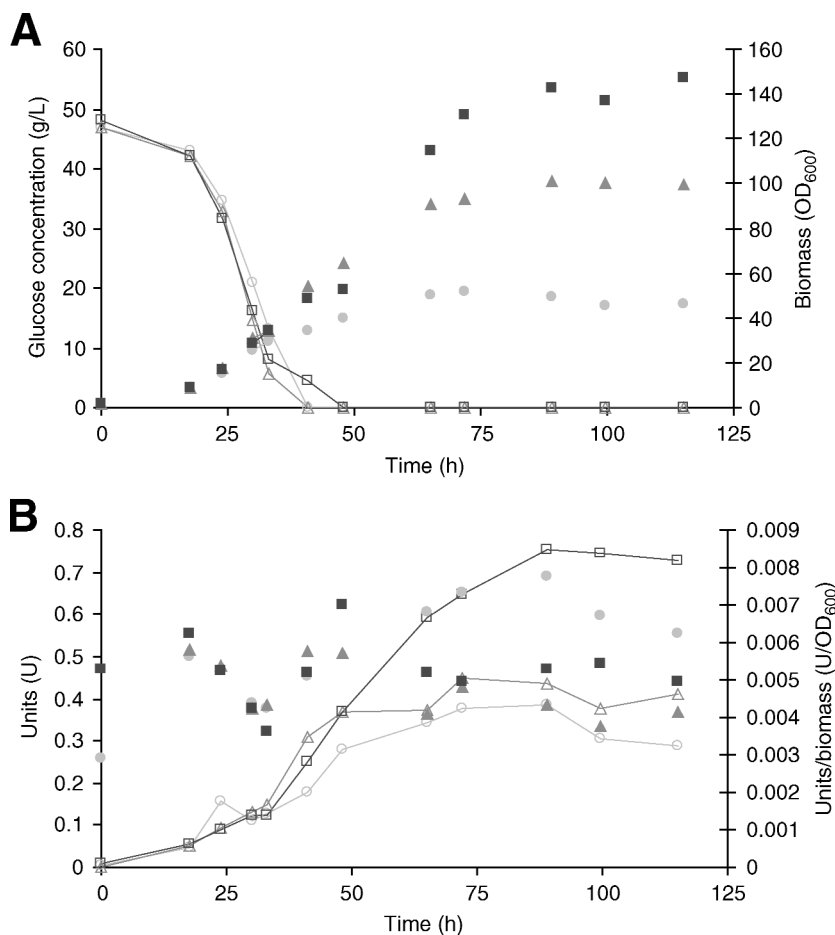
Analysis of shake-flask cultures revealed that *P. pastoris* transformants with the *lcc2* gene gave approx three to four times higher activity than transformants with the *lcc1* gene (Fig. 2A). The transformant giving the highest activity (*P. pastoris/lcc2*) (approx 0.6 U/L) (Fig. 2A) was chosen for further studies. The choice of laccase gene for expression appears to have a great impact on the levels of activity reached.

Shake-flask cultures of *P. pastoris/lcc2*, in which the type and concentration of carbon source were varied, gave higher laccase activity (1.3 U/L) with glucose than with glycerol (0.7 U/L) or maltose (0.6 U/L) (Fig. 2B). Cells grown on glycerol showed higher growth rate than cells grown on glucose, which in turn showed higher growth rate than cells grown on



**Fig. 2.** Shake-flask cultures of *P. pastoris*. **(A)** Comparison of *lcc1* and *lcc2* transformants, **(B<sub>1</sub>)** volumetric activity with *P. pastoris/lcc2*, **(B<sub>2</sub>)** ratio of activity to biomass with *P. pastoris/lcc2*.

maltose. The cultures reached an OD<sub>600</sub> of 27 (glycerol), 20 (glucose), and 10 (maltose). Previous results suggest that a slow growth rate is usually better for reaching high-laccase activity (8), but the maltose cultures did not follow that trend. The ratio of activity to biomass was higher with glucose than with maltose or glycerol (Fig. 2B). This result shows that the laccase activity depends on more than just the growth rate. Growth on different carbon sources results in distinct patterns of intracellular proteins (19), which in turn may affect the production of heterologous proteins. For laccase production, a glucose concentration of 1% was better than 2–3% (Fig. 2B). When even lower glucose concentrations were studied, 0.5% glucose gave about the same activity to biomass ratio as 1% glucose (not shown). The activities reached with the *GAP* promoter can be compared



**Fig. 3.** Bioreactor cultures of *P. pastoris/lcc2*. **(A)** Glucose concentration and biomass, **(B)** units ( $\mu\text{mol}/\text{min}$ ) and units/biomass. Filled symbols show biomass and units/biomass [fermentor A (●), fermentor B (▲), and fermentor C (■)]. Open symbols show glucose concentration and units.

with the expression of the same gene but with the *AOX1* promoter, which gave an activity of 0.35 U/L in the X-33 strain.

The influence of different feeding rates of glucose on the selected *P. pastoris/lcc2* transformant was investigated in parallel fed-batch cultures using a multibioreactor system (Fig. 3). The batch phase lasted for about 40 h (Fig. 3A) and resulted in a biomass concentration of 40–60 g/L. In the fed-batch phase, glucose was added at different rates for 82 h and the biomass concentrations in the bioreactors started to vary (Fig. 3A). The biomass concentration in fermentor A did not increase that much in the fed-batch phase, whereas fermentors B and C reached biomass concentrations of 80–90 g/L and 130–140 g/L, respectively. Fermentor C reached the highest volumetric activity (2.8 U/L), whereas fermentors B and A reached 1.8 and 1.3 U/L, respectively. The activity increased until approx 90 h, and

thereafter the level was constant or decreased slightly (Fig. 3B). The ratio of activity to biomass was slightly higher in fermentor A (Fig. 3B), but the relatively low values compared to the shake-flask cultures (Fig. 2B) indicate that further improvements should be possible. The specific activity (not shown) increased for about 50 h and subsequently leveled off.

Compared to a number of reports documenting high-level expression of foreign genes in *P. pastoris* (4,20), the concentration of secreted protein in this study (0.1 g/L) was satisfactory. When laccases from *Pleurotus sajor-caju* (21) and *Pycnoporus cinnabarinus* (22) were expressed in *P. pastoris* using the *AOX1* system, the protein concentrations were 0.11 and 0.008 g/L, respectively. Although the total protein concentration in our study was at the same level or higher, the specific activity was much lower than in those studies (21,22). The use of controlled conditions in a bioreactor instead of using shake-flasks generally improves heterologous protein production (20). The laccase production reported in the current study could probably be enhanced further by optimization of medium and cultivation conditions.

When *P. pastoris* was used to express and determine the size of laccases from *T. versicolor* (8), *P. cinnabarinus* (22), *Fomes lignosus* (23), and *P. sajor-caju* (21), the recombinant laccases were found to be between 5% and 36% larger than the native enzymes, strongly indicating hyperglycosylation. However, no reports show that hyperglycosylation affects the activity of the enzyme produced.

The use of a proteinase A mutant (*pep4*) strain (SMD1168) has been reported to be beneficial for the production of secreted recombinant proteins (24), and that has also been observed for laccase (9). In this study, the protease level in the fermentation medium was around 0.6 µg/mL (with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin as the standard) after 17 h and was fairly constant throughout the whole fermentation, never reaching a level greater than 1 µg/mL. It has been shown that cells grown on glycerol have lower levels of total protease activity compared with cells grown on methanol as the sole carbon source (25). This could be a problem when laccase is expressed with the *AOX1* promoter and is one of the reasons why alternative carbon sources are of interest to study.

Very few transformants were obtained for *A. niger*. The transformation with pGT1-*lcc1-amdSpirG* and pGT1-*lcc2-amdSpirG* gave rise to nine and seven transformants, respectively. Of these 16 transformants, 11 showed color development on ABTS plates. No development of color was observed with the control transformed with pBSKII(+)-*amdSpirG*.

To select a top laccase producer, transformants were grown in shake-flasks (experimental series 1). The results obtained showed that most of the activity was lost by 144 h, with the relative activities at 48 and 72 h being very similar (data not shown). Again, the control transformant showed no activity. Specific activities of laccase in the supernatants were calculated indicating that *lcc2* gave higher specific activity than *lcc1*, although the

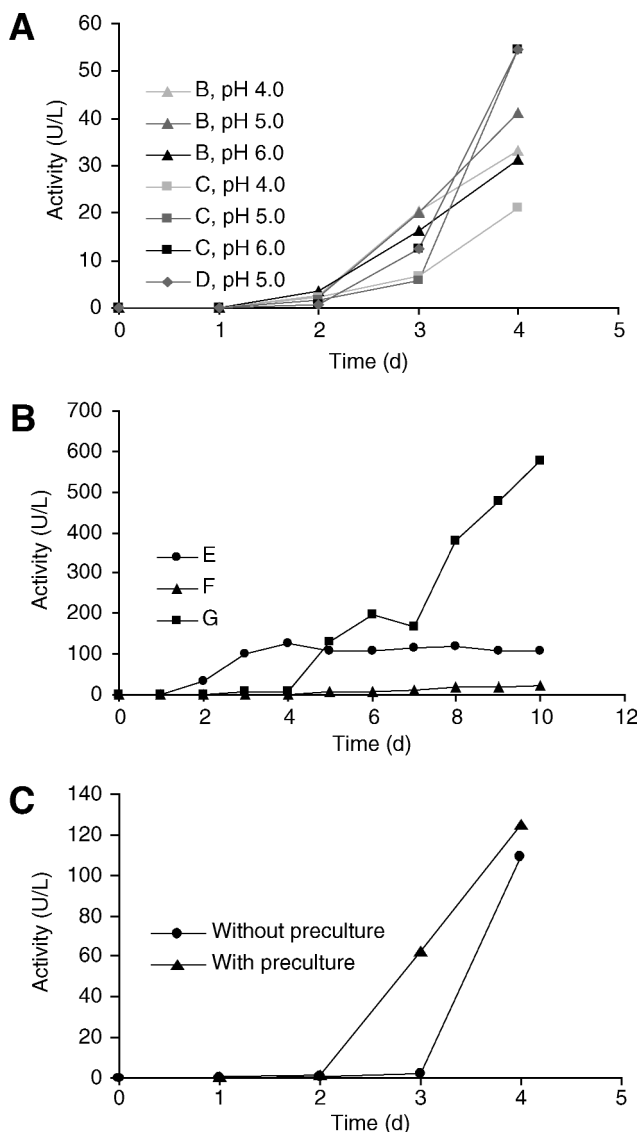


Fig. 4. Media optimization for *A. niger/lcc2* in (A) experimental series 2 with media A–D, (B) experimental series 3 with media E–G, and (C) experimental series 4 with and without preculture.

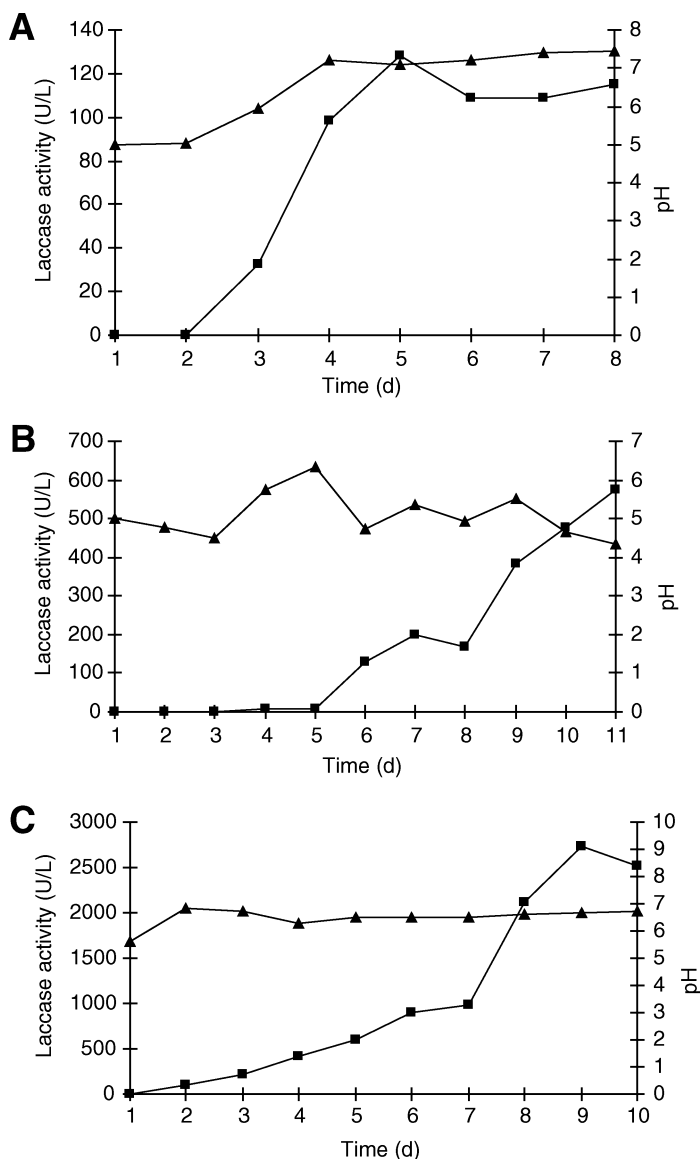
activity also varied between *lcc2* transformants (data not shown). The average volumetric and specific activity was almost 10 times higher for the *lcc2* transformants in comparison to the *lcc1* transformants. An *lcc2* transformant (*A. niger/lcc2*) was selected to be used in shake-flask analysis for media optimization.

Laccase production in media A–D was studied during 4 d of cultivation (Fig. 4A). Medium A (high salts) was used by Balasubramaniam et al. (26) for the production of  $\beta$ -fructofuranosidase in *A. niger* NRRL 330.

Growth in medium A gave no extracellular laccase activity, and very low biomass concentration (4–5 g/L after 4 d) was obtained. Medium B gave extracellular laccase activity levels of 31–41 U/L depending on the pH. The biomass production was good (19 g/L at pH 6.0). At low pH, the biomass concentration was lower (only approx 10 g/L at pH 4.0). Medium C also gave extracellular laccase activity (21–55 U/L), with the activity levels increasing as the pH increased. The biomass concentration was 14–15 g/L regardless of pH. Medium D was used by Record et al. (27) for expression of the *P. cinnabarinus* laccase in *A. niger* D15 No. 26, similar to the host strain being used in our experiments. A maximum activity of approx 50 U/L was obtained (Fig. 4A), which is in agreement with the reported levels of *P. cinnabarinus* laccase activity of 50 U/L using the native secretion signal. The biomass production was low, only approx 6 g/L. As the laccase activity was still increasing at the end of the experiment, a longer time period was used in further studies (experimental series 3–6).

Laccase production in media E–G was studied over 10 d (Fig. 4B). Medium E showed excellent laccase activity. By d 4 of the experiment (to compare with media A–D), the activity levels were 128 U/L, which was the maximum level obtained in this medium. The glucose was spent by 48 h, which was the point at which the biomass peaked (6.1 g/L). The cells may have lysed during the next 2 d, as there was a small increase in extracellular laccase activity until 96 h (128 U/L), after which the activity dipped slightly and remained constant at approx 110–120 U/L until d 10. This may indicate that there is very little protease activity in the medium, as the laccase is not disappearing. *A. niger* D15 (the host organism) is a pH mutant which does not naturally acidify the medium, unlike like wild-type *Aspergillus*, and the acid proteases are not activated (28). Medium F showed very little activity, as well as very little biomass, which was to be expected as the only carbon source added was yeast extract. Medium G combined sucrose as a carbon source with double strength minimal medium. This medium gave by far the best laccase activity, which reached 576 U/L after 10 d (Fig. 4B). The results show that yeast extract has a positive effect on laccase activity when added to the minimal medium (medium B vs medium E). The lag phase before activity was seen could be reduced from 3–4 to 2 d. This phenomenon was investigated further in experimental series 4 using 2× minimal medium with 10% sucrose (medium G) with and without yeast extract, as well as using glucose in place of sucrose in this medium. The presence of yeast extract resulted in laccase production, with low levels seen after 48 h (data not shown). An attempt to reduce the lag phase of laccase activity even further was done by using precultures for inoculation of the shake-flasks. The lag phase was reduced from 3 to 2 d (Fig. 4C).

In experimental series 5, the effects of pH and temperature were determined through the cultivation of *A. niger/lcc2* in sucrose 2× minimal medium supplemented with yeast extract. Spores were inoculated into 50-mL medium with the pH adjusted to 4.0, 5.0, or 6.0 and the cultures were



**Fig. 5.** The production of laccase by the transformant *A. niger/lcc2* in (A) glucose minimal medium supplemented with yeast extract, (B) double-strength sucrose minimal medium, and (C) double-strength sucrose minimal medium supplemented with yeast extract. Activity (■) and pH (▲) are indicated.

grown at 25°C or 30°C. The best results were obtained with the pH 6 medium (data not shown).

Media composition and growth conditions for further studies were selected on basis of results from experimental series 1–5. In experimental series 6, the cultivation of *A. niger/lcc2* was carried out over a period of 10 d in glucose minimal medium supplemented with yeast extract. A rise in pH from 5.0 to 7.0 was seen during the cultivation (Fig. 5A). This should not

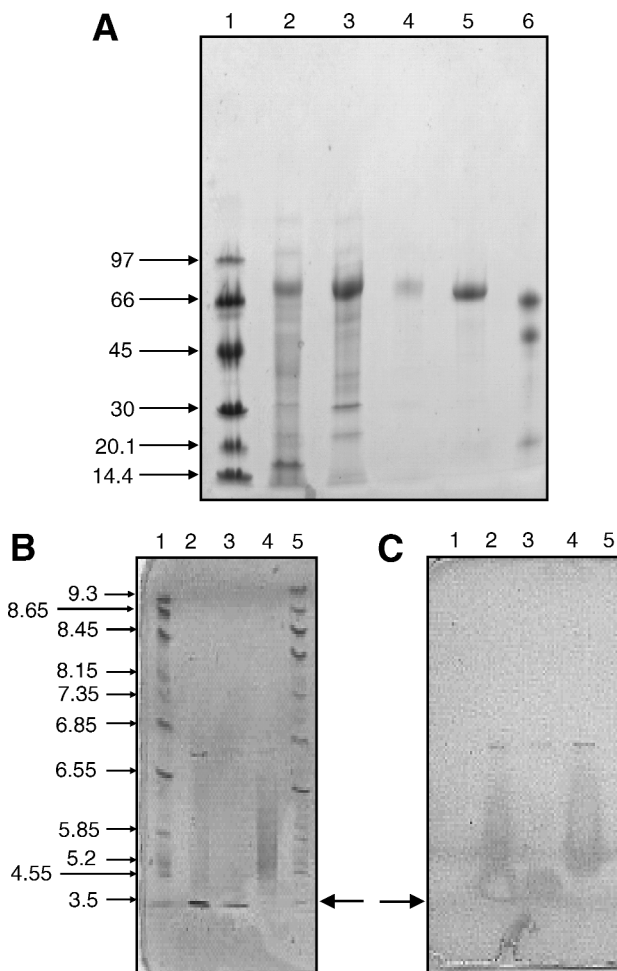
Table 2  
Purification of Recombinant Laccase From *A. niger/lcc2*

| Purification step               | Total volume (mL) | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---------------------------------|-------------------|--------------------|--------------------|--------------------------|-----------|---------------------|
| Resuspended freeze-dried sample | 5                 | 56.8               | 15.55              | 3.65                     | 100       | 1                   |
| DEAE fractions                  | 2.5               | 28.4               | 1.53               | 18.6                     | 50        | 5.1                 |
| HiTrap fractions                | 1.5               | 10.7               |                    |                          | 19        |                     |
| Mono P fractions                | 0.125             | 1                  | 0.04               | 25                       | 2         | 6.9                 |

be a problem as it has been previously shown that laccase from *T. versicolor* has best stability between pH 6.0 and 7.0 (29). The activity increased rapidly between the second and the third day after inoculation to approx 100 U/L and then stayed at that level (Fig. 5A). A higher activity was reached with sucrose 2× minimal medium (Fig. 5B). In that case the activity appeared much later (d 6) and the highest activity was observed after 11 d. The pH dropped to 4.0, which is suboptimal for native laccase, with 70% of the activity remaining in comparison to pH 6.0–7.0 (29). In sucrose 2× minimal medium with yeast extract, the pH was stable at pH 6.0–7.0 during the whole period. The highest activity was reached after 9 d and reached approx 2700 U/L (Fig. 5C). In comparison to sucrose 2× minimal medium, the activity appeared sooner (d 2–3) in sucrose 2× minimal medium with yeast extract. The activity was similar to that determined in the work of Record et al. (27), who expressed a *P. cinnabarinus* laccase in *A. niger* using a similar strain but replaced the native laccase signal peptide with the 24-amino-acid-residue glucoamylase (*gla*) preprosequence from *A. niger*, as the production of laccase with the native signal peptide was quite low. In this study, we demonstrate that it is possible to express high levels of laccase with the native secretion signal. Recombinant Lcc2 protein was purified from the culture medium of *A. niger/lcc2* in three steps (Table 2). The purification was not optimized further regarding the yield, as enough enzyme was obtained to characterize the recombinant protein.

Recombinant Lcc2 from different stages of purification were run on an SDS-PAGE gel, with LccA and LccB preparations from *T. versicolor* as controls. Molecular weight determination indicated that the recombinant Lcc2 was similar to LccA rather than to LccB (Fig. 6A). This was to be expected, as the *lcc2* gene has been associated with the LccA form of the protein in the native host (10). The calibration indicated that the recombinant Lcc2 is 74 kDa vs 68 kDa of LccA. IEF was also carried out, using LccA and LccB as controls (Fig. 6B). The pI of the recombinant Lcc2 protein and LccA appeared identical and very acidic, at about 3.5. LccB showed a different pattern indicating a higher pI and the presence of several forms in the preparation (Fig. 6B). The expected pI ranges of preparations of

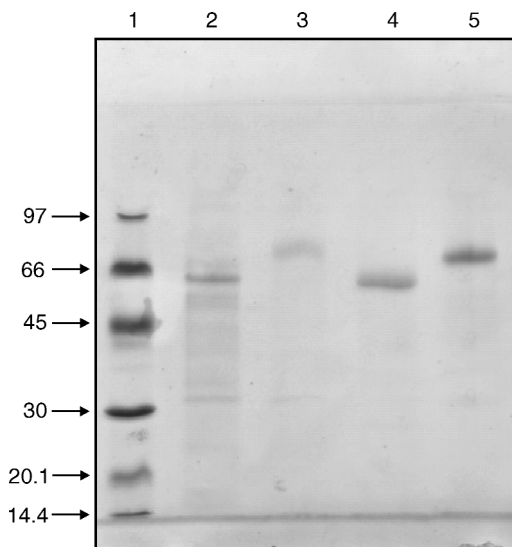




**Fig. 6.** (A) SDS-PAGE analysis: lane 1, marker proteins (sizes in kDa indicated to the left); lanes 2–4, recombinant Lcc2 protein from *A. niger/lcc2* after  $(\text{NH}_4)_2\text{SO}_4$  precipitation (lane 2), purification on HiTrap Q (lane 3), and purification on MonoP (lane 4); lanes 5 and 6, native LccA (lane 5) and LccB (lane 6). (B) IEF analysis: lanes 1 and 5, marker proteins (pI indicated to the left); lane 2, recombinant Lcc2 protein from *A. niger/lcc2*; lane 3, native LccA; lane 4, native LccB. (C) Zymogram analysis: lanes 1 and 5, marker proteins; lane 2, native LccA; lane 3, recombinant Lcc2 protein from *A. niger/lcc2* (position indicated by arrow); lane 4, native LccB.

LccA and LccB are 3.07–3.27 and 4.64–6.76, respectively (2). LccB may contain at least ten components. Zymogram analysis confirmed that the recombinant laccase was similar to LccA, whereas most of the LccB activity was found around pH 5.0 (Fig. 6C).

As can be seen in Fig. 7, both recombinant Lcc2 protein and native LccA have similar carbohydrate contents. The calculated carbohydrate content of recombinant Lcc2 protein is 16%, whereas LccA shows a carbohydrate content of 11%. Previous estimates of the carbohydrate content



**Fig. 7.** Deglycosylation of recombinant Lcc2 protein from *A. niger/lcc2* and native LccA. Lane 1, marker proteins (sizes in kDa indicated on left); lane 2, deglycosylated recombinant Lcc2; lane 3, untreated recombinant Lcc2; lane 4, deglycosylated LccA; lane 5, untreated LccA.

of LccA range from 10% to 14% (2). The molecular weight of the unglycosylated laccase as deduced from the amino-acid sequence is 53 kDa (10). The recombinant laccase appeared to be homogeneous. Laccase from *P. cinnabarinus* expressed in *A. niger* gave rise to a 70-kDa enzyme, which was the same as for the native protein (27). Laccase from *Trametes villosa* expressed in *Aspergillus oryzae* showed 0.5% and 10% glycosylation for the native and the recombinant enzyme, respectively (30). Recombinant laccase from *Myceliophthora thermophila* expressed in *A. oryzae* also showed a higher degree of glycosylation (31).

The  $K_M$  values were determined using 2,4,5-trimethoxybenzyl alcohol (Hong et al., in this volume) as the substrate. For LccA and the recombinant Lcc2 protein, substrate concentrations of 1–60 mM were tested, whereas for LccB the maximum concentration was increased to 90 mM, as the rate continued to increase at 60 mM. The results suggest that the  $K_M$  of LccA and recombinant laccase are similar, whereas the  $K_M$  of LccB is considerably higher.

## Conclusions

Production of catalytically active laccase from the *T. versicolor* cDNAs *lcc1* and *lcc2* under control of glyceraldehyde-3-phosphate dehydrogenase promoters was achieved in both *P. pastoris* and *A. niger*. Expression of laccase in *P. pastoris* using the *GAP* system gave better results with glucose than with glycerol or maltose as carbon source. The activity obtained with *P. pastoris* was considerably lower than for *A. niger*, but the *P. pastoris* system

may still be of interest for screening studies owing to convenience and speed. With *A. niger*, high laccase activity levels (2700 U/L using ABTS as reducing substrate) were obtained with the native secretion signal by using a medium containing sucrose and yeast extract.

Recombinant laccase produced by *A. niger* D15 transformed with the *lcc2* cDNA was purified to homogeneity, and its biochemical and catalytical properties were found to be similar to those of native laccase A from *T. versicolor*. This is an important finding for the applicability of *A. niger* for heterologous production of enzymes, as it is an indication that the activity and characteristics of enzymes produced in this manner can be correlated to the native enzymes.

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