Fermentation Strategies for Improved Heterologous Expression of Laccase in *Pichia pastoris*

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Abstract: Improved expression of recombinant laccase by *Pichia pastoris* carrying the *lcc1* cDNA isolated from *Trametes versicolor* was achieved by optimization of the cultivation conditions in a fermentor equipped with a methanol sensor system. The results indicated that the activity obtained in fermentor cultivations was at least 7 times higher than in shake-flask cultures. Three different strategies for fermentor cultivations were compared: A (30°C, 1.0% methanol), B (20°C, 1.0% methanol), and C (20°C, 0.5% methanol). The laccase activity, particularly the specific activity, could be improved by decreasing the cultivation temperature. The mechanisms behind the temperature effect on the laccase activity may be ascribed to poor stability, release of more proteases from dead cells, and folding problems at higher temperature. The results showed that the methanol concentration had a marked effect on the production of active heterologous laccase. A fivefold higher volumetric laccase activity was obtained when the methanol concentration was kept at 0.5% instead of 1.0%. The detrimental effect of methanol on the production of recombinant laccase may be attributed to lower laccase stability, a higher proteolytic activity, and folding problems due to higher growth rate at 1.0% methanol. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **79**: 438–449, 2002.
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INTRODUCTION

Laccases are blue copper-containing phenol oxidases (EC 1.10.3.2) that are widely distributed in plants and certain fungi (Reinhammar, 1997). Laccases have been ascribed diverse biological functions in different organisms. They catalyze the oxidation of a variety of aromatic compounds, in particular phenols, with the concomitant reduction of molecular oxygen to water. White-rot fungi, such as *Trametes* (*Coriolus*, *Polyporus*) *versicolor*, are generally good producers of laccases. *T. versicolor* laccase is a secreted glycosylated enzyme with two disulphide bridges and four copper ions, one of which gives the protein its characteristic greenish-blue color (Reinhammar, 1984; Jönsson et al., 1995).

Recently, there has been great interest in laccase in relation to both the structure of the active site and the catalytic mode of action (Ducros et al., 1998) as well as the possibilities of using the enzyme for various applications. Potential applications include (i) delignification and biobleaching of pulp (Bourbonnais et al., 1997; Smith et al., 1997), (ii) detoxification of lignocellulose hydrolysates for ethanol production by yeast (Jönsson et al., 1998; Larsson et al., 1999), (iii) treatment of wastewater from industrial plants (Bergbauer et al., 1991), (iv) enzymatic removal of phenolic compounds in beverages (Cantarelli et al., 1989; Servili et al., 2000), (v) enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla et al., 2000), (vi) construction of biosensors (Ghindilis, 2000), and (vii) manufacture of new compounded materials by using laccase and lignin wastes (Huttermann et al., 2001). All these applications require large quantities of enzyme, which makes the expression of laccase in heterologous systems an important issue.

The methylotrophic yeast *Pichia pastoris* can be used to express recombinant proteins under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter *AOX1* (Cereghino and Cregg, 2000). *P. pastoris* has the potential for high expression levels (Buckholz and Gleeson, 1991; Romanos et al., 1992; Cregg et al., 1993), efficient secretion of extracellular protein, post-translational modifications such as glycosylation (Tschopp et al., 1987) and growth to high cell densities on defined minimal medium (Brierley et al.,

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1990). A possible advantage with *P. pastoris* compared to many filamentous fungi is that it does not produce cellulolytic enzymes and laccase produced in this host could, therefore, potentially be applied directly in the pulp and paper industry without any purification. In addition, molecular genetic methods for *P. pastoris* are rapid and well developed, and the organism is easy to cultivate in large scale.

Previously, the *lcc1* cDNA, isolated from the white-rot fungus *T. versicolor*, was expressed in *P. pastoris* and was found to provide active secreted laccase (Jönsson et al., 1997). However, the expression levels obtained in shake-flask cultures were too low to be convenient for biochemical characterization of the recombinant enzyme. The objective of this work was, therefore, to optimize the expression of recombinant laccase by *P. pastoris* by using controlled conditions in fermentor cultivations and to find new strategies to improve the laccase activity.

Regulation and induction of heterologous gene expression by methanol has been shown to be simple, easy to scale-up, and cost-effective for industrial fermentations (Cregg et al., 1993). However, high concentrations of methanol are toxic to *P. pastoris* because of the accumulation of formaldehyde and hydrogen peroxide inside the cells (Couderc and Baratti, 1980; Murray et al., 1989). Therefore, accurate regulation of the methanol concentration in *P. pastoris* cultures is necessary not only to maintain the induction of genes under the control of the *AOX1* promoter but also to prevent accumulation of methanol to levels that are toxic to the cells. To obtain high cell densities while keeping a low level of methanol, a fed-batch strategy is commonly used. In the past, several complex feeding schemes were devised by using the dissolved oxygen (DO) spike method (Stratton et al., 1998). However, the DO spike protocol has several drawbacks. For example, it is complicated to implement and may repeatedly expose the cells to non-inducing levels of methanol because it is designed to maintain the residual methanol concentration close to zero, which is not necessarily optimal for maximum protein production (Zhang et al., 2000). Moreover, Mut^s clones, which have a disrupted *AOX1* gene and exhibit a slow methanol utilization rate, consume methanol too slowly for the "oxygen spike" technique to be practical (Guarna et al., 1997). Similarly, this technique cannot be used when mixed feed of glycerol and methanol is used to increase protein productivity during fermentation. Alternatively, it is of course possible to monitor the methanol concentration using HPLC or GC, but these methods are expensive and not easily implemented on-line. Therefore, in this study, a methanol sensor system, consisting of a probe with a gas-permeable silicone rubber tubing connected to an air carrier line, was used to conveniently monitor and control the methanol concentration on-line (Guarna et al., 1997; Wagner et al., 1997).

Cultivation of *P. pastoris* in fermentors is generally advisable because several fermentation parameters, such as pH and dissolved oxygen, can be adjusted and controlled to achieve higher levels of the desired protein (Cregg et al., 1993). In this work, the effects of temperature and methanol concentration on the expression of laccase by a Mut^+ transformant of *P. pastoris* were investigated. Possible mechanisms behind the findings are discussed.

MATERIALS AND METHODS

Chemicals

All chemicals were of spectral or analytical grade. Unless otherwise stated, all chemicals used in the preparation of culture media were obtained from Merck Eurolab AB (Stockholm, Sweden). Distilled/deionized water (Millipore, Bedford, MA) was used in all the experiments.

Organism

The *Pichia pastoris* strain SMD 1168 (*his4*, *pep4*) was transformed with the *lcc1* laccase gene (Jönsson et al., 1997) under control of the *AOX1* promoter by using the vector pHIL-D2, which contains both the promoter and transcription terminator of the *AOX1* gene. A Mut^+ phenotype transformant was maintained on YPD agar plates [Yeast Extract Peptone Dextrose medium: 10 g/L yeast extract, 20 g/L peptone (Oxoid Ltd. Hampshire, UK), 20 g/L dextrose (BDH, Poole, UK), and 20 g/L agar].

Culture Media

(1) BMGY/BMMY/BMM medium (buffered complex glycerol medium, buffered complex methanol medium, and buffered minimal methanol medium): BMGY contained 10 g/L yeast extract, 20 g/L peptone, 100 m*M* potassium phosphate buffer (pH 6.0), 13.4 g/L yeast nitrogen base with ammonium sulfate without amino acids (Difco, Detroit, MI), 400 μg/L biotin (Sigma-Aldrich, Steinheim, Germany), and 10 mL/L glycerol. For BMMY, the glycerol was substituted by 5 mL/L methanol. For BMM, yeast extract and peptone were omitted.

(2) PTM_1 trace salts contained 6.0 g/L cupric sulfate · $5H_2O$, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate · H_2O, 0.2 g/L sodium molybdate · $2H_2O$, 0.02 g/L boric acid, 0.5 g/L cobalt chloride, 20.0 g/L zinc chloride, 65.0 g/L ferrous sulfate · $7H_2O$, 0.2 g/L biotin, and 5.0 mL/L sulfuric acid.

(3) Fermentation basal salt medium contained 26.7 mL/L phosphoric acid (85%, Sigma, St. Louis, MO), 1.0 g/L calcium chloride · 2 H_2O, 18.2 g/L potassium sulfate, 14.9 g/L magnesium sulfate · $7H_2O$, 4.13 g/L potassium hydroxide, 40 g/L glycerol, 4.4 mL/L PTM_1

trace metal salts, and 0.5 mL/L antifoam emulsion (Dow Corning Antifoam RD Emulsion, Dow Corning, Midland, MI).

(4) The methanol feed solution consisted of filter-sterilized methanol (100%, BDH) supplemented with 12 mL/L PTM_1 trace salts. A concentrated aqueous solution of ammonium hydroxide (28–30%, Sigma) was used to adjust the pH to 5.0 and to provide the yeast with a nitrogen source.

Preparation of Inocula

Cells were transferred from YPD agar plates into 250-mL baffled Erlenmeyer flasks containing 50 mL of BMGY medium and 0.1 m*M* $CuSO_4$, and grown at 30°C in a rotary water bath-shaking incubator (Infors AG, Bottmingen, Switzerland) at 250 rpm. The cells were harvested in log-phase growth and were used as inoculum for the shake-flask and fermentor cultivations.

Shake-Flask Cultivations

Shake-flask cultivations were performed in 1000-mL baffled flasks containing 100 mL BMM or BMMY medium supplemented with 0.1 m*M* $CuSO_4$ at 20 or 30°C in the rotary shaker (250 rpm). The inoculum was harvested aseptically by centrifugation at 3000 g for 5 min under sterile conditions at room temperature. The pellet was then resuspended in BMM or BMMY medium to an OD_{600} of 1.0, as measured with a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). An OD_{600} of 1.0 corresponds to a concentration of approximately 0.2 g (dry weight) per liter. Filter-sterilized 100% methanol was added to a final concentration of 0.5–2.0% daily to maintain induction. Samples were taken daily for spectrophotometric determination of cell growth and for assay of laccase activity.

Fermentor Cultivations

To scale up the expression of laccase in *P. pastoris*, a BioFlo III bench-top fermentor (New Brunswick Scientific Co. Inc., Edison, NJ) with a 2.5-L working volume water-jacketed glass vessel was used for batch and fed-batch fermentations. The fermentor was equipped with an Ingold pH electrode, an Ingold dissolved oxygen electrode (Mettler Toledo GmbH, Urdorf, Switzerland), and a methanol sensor system (Raven Biotech. Co., Vancouver, Canada) (Guarna et al., 1997) with a peristaltic pump (Model VS2-10R, Alitea AB, Stockholm, Sweden).

The fermentation of *P. pastoris* included two phases: first a growth phase on glycerol followed by an induction phase on methanol. All fermentations began with a batch growth phase in 1.0 L of basal salt medium containing 40 g/L glycerol as carbon source at 30°C to obtain high cell density. Before inoculation, the pH was adjusted to 5.0 with concentrated ammonium hydroxide, and 4.35 mL PTM_1 trace salts were supplemented aseptically. An inoculum (as described above) of 50 mL was added to the fermentor so that a final optical density of approximately 1.0 at 600 nm was obtained. The oxygen was supplied by using a constant flow of air (0.5 L/min) that was controlled by mass flow meters (type E6222A-2RH; Bronkhorst High-Tech, Ruurlo, The Netherlands). The agitation was set to be controlled automatically by the microprocessor of the fermentor in a range from a minimum of 500 rpm to a maximum of 1000 rpm to keep the dissolved oxygen (DO) level well over 20% of the saturation level. In addition to air, pure oxygen was supplied during the late period of batch cultivation on glycerol so that the DO was kept in the range of 20–30% of saturation. The pH of the medium was automatically maintained at 5.0 with ammonium hydroxide. The composition of the exhaust gas was continuously monitored by using a carbon dioxide and oxygen analysis system based on acoustic detection (model 1308; Brüel & Kjaer, Copenhagen, Denmark).

After depletion of the glycerol, methanol containing 12 mL of PTM_1 trace salts per liter was fed to start the methanol fed-batch phase (the induction phase or production phase when the *AOX1* promoter is used). The methanol was the sole carbon source. Pure oxygen was supplemented to keep the DO level above 20% during the whole induction phase on methanol. The methanol concentration was monitored and regulated on-line by the methanol sensor system. Samples were withdrawn every 12 h for optical density measurement, gravimetric analysis of biomass, laccase activity assay, and determination of total soluble protein and gel electrophoresis analysis. Antifoam was injected manually as required throughout the fermentation. Three fermentation strategies (as described below) were investigated.

Fermentation Strategies

Three different fermentation strategies, denoted A, B, and C, were used. In strategy A, the temperature was maintained at 30°C throughout the whole cultivation. Methanol was fed stepwise from 0.1 to 0.5% (v/v) at the beginning of the induction phase for half a day to adapt the culture to growth on methanol. Then, the methanol concentration was increased to 1.0% and kept at this level. In strategy B, the methanol feeding was the same as in strategy A, but the temperature was decreased from 30 to 20°C in the beginning of the induction phase on methanol and maintained at 20°C throughout the induction phase. In strategy C, the temperature was adjusted as described in strategy B. Methanol was added to the fermentor to reach a final concentration of 0.5% immediately after the glycerol was consumed and maintained at 0.5% during the entire induction phase.

Analyses of Fermentation Results

Cell Mass and Growth Rate

The optical density (OD) of the samples was measured against distilled water at 600 nm with a spectrophotometer (U-1100; Hitachi Ltd., Tokyo, Japan) after appropriate dilution ensuring a value within the linear range (0.1–1.0). The biomass was determined gravimetrically (as dry weight) for each time point. To determine the cell dry weight (CDW), 2.5 mL of the cell suspension was filtered through predried and preweighed Supor-200 membrane filters with a pore size of 0.45 μm (Gelman Laboratory, Pall Co., MI). The filtered pellets were then washed with 7.5 mL double-deionized water (Millipore) and dried in a microwave oven (Whirlpool, Benton Harbor, MI) at a power setting of 350 W for 15 min. The maximum growth rate (μ_{max}) was determined from the slope of the logarithmic dry weight (ln [CDW]) obtained in the log-phase versus time.

Determination of Protein Concentration

The concentration of protein in cell-free samples was determined by using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL) following the manufacturer's instructions. Bovine serum albumin was used as the standard.

Effect of Temperature on the Stability of the Laccase

To determine the stability of laccase, both native laccase prepared and purified from *T. versicolor* (Fåhraeus and Reinhammar, 1967) and recombinant laccase in the crude culture broth of *P. pastoris* (strategy C) were assessed. The samples were first diluted with distilled water to an activity of around 9 U/mL. One-milliliter aliquots were then incubated in 1.5-mL tubes in water baths (Infors AG, Bottmingen, Switzerland) with 250 rpm shaking for 24 h at temperatures ranging from 10 to 30°C. After incubation, the laccase activity was measured spectrophotometrically at 414 nm.

Effect of Methanol Concentration on the Stability of the Laccase

The effect of the methanol concentration on the stability of recombinant laccase was assessed by incubation of 10 U of laccase from the supernatant of the fermentation (strategy C) mixed with deionized water and methanol. The native laccase from *T. versicolor* was used as control. Additional methanol was added to 0.5%, 1.0%, and 2.0% in a final volume of 1.0 mL in 1.5-mL tubes. The laccase samples were incubated at room temperature (≈23°C) for 24 h. The enzyme activity was measured spectrophotometrically at 414 nm as described below. The measurements were performed twice.

Laccase Activity Assay

Cell-free samples were obtained from the shake-flask cultivations and fermentations by centrifugation. Because BMMY contains interfering compounds, the supernatants from BMMY medium (for shake flask cultivations) were dialyzed before laccase activity assays. For dialysis of BMMY culture supernatants, Spectra/Pro membrane tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a molecular weight cutoff of 12,000–14,000 was used. The samples were dialyzed against deionized water at 8°C overnight.

The detection of laccase activity was performed with a spectrophotometer (U-2000, Hitachi, Tokyo, Japan) using a reaction medium containing 0.4 m*M* ABTS (2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), 50 m*M* acetate buffer (pH 5.2), supernatant from the fermentations after appropriate dilution in a total volume of 1.0 mL. The change in absorbance at 414 nm was recorded with the spectrophotometer for 5 min at 25°C. For calculating the activity, an extinction coefficient (ε) of 3.6×10^4 $M^{-1} \cdot cm^{-1}$ was used (Childs and Bardsley, 1975). Laccase activity was expressed in units per milliliter of sample. One unit was defined as the amount of enzyme, which forms 1 micromole ABTS radical cation per minute under the conditions of the assay. The specific activity was expressed as the laccase activity in the supernatant as a function of the total amount of protein (in units per milligram of protein).

Protease Activity Assay

Proteolytic activity in the *P. pastoris* cultures was assayed by using the QuantiCleave protease assay kit (Pierce). This assay is based on the use of succinylated casein in conjunction with trinitrobenzenesulfonic acid (TNBSA) (Hatakeyama et al., 1992; Bubnis and Ofner, 1992). Proteases will cleave peptide bonds in the succinylated casein and expose primary amines (predominantly α-amines). Thereafter, TNBSA will react with the primary amines to generate an orange-yellow color. The color change was quantitated at 450 nm by using a spectrophotometer (U-2000, Hitachi). The assay was performed at 37°C for 20 min at pH 5.0 (acetate buffer, 50 m*M*) or pH 8.5 (borate buffer, 50 m*M*).

SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed by using 10% (w/v) polyacrylamide gels under reducing and denaturing conditions with a Mini-PROTEAN II system (Bio-Rad, Hercules, CA). Fifteen-microliter supernatant was loaded on the gel together with 5 μL of sample buffer. The protein in the gel was stained with Coomassie Brilliant Blue R-250. A silver stain kit (Bio-Rad) was used to examine the time course of protein secretion. The molecular weight of the enzyme was estimated

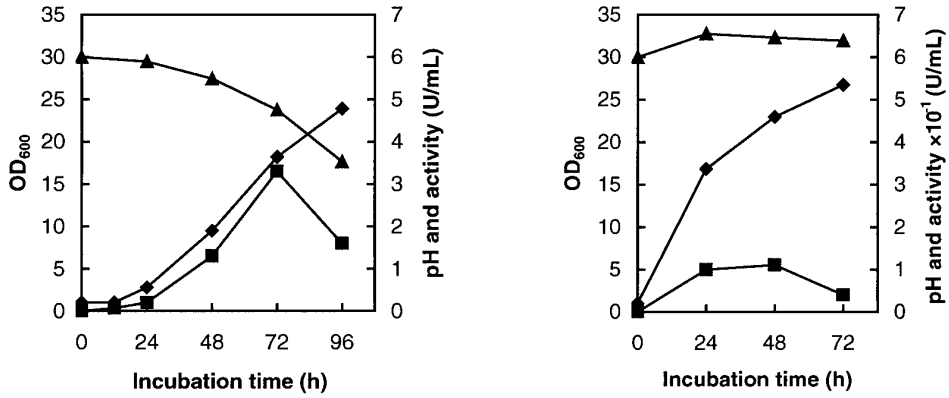

Figure 1. Time course of shake-flask cultivations in BMM (left) and BMMY (right) medium at 30°C. Methanol was added daily to a final concentration of 0.5%. The pH (▲), the OD_{600} (◆), and the laccase activity (■) are shown.

by using the low molecular weight marker (Pharmacia Biotech, Uppsala, Sweden) containing: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Native-PAGE Analysis

Native-PAGE analysis was performed by using a 4–15% gradient gel (Bio-Rad) under nondenaturing conditions (i.e., SDS and 2-mercaptoethanol were omitted in the sample buffer and the samples were not boiled). The culture supernatant was concentrated 10–12 times by using Centricon centrifugal filter devices with YM-10 membranes (Millipore). Two native PAGE gels containing the same samples were stained with Coomassie Brilliant Blue R-250 and ABTS, respectively. The ABTS staining was performed by incubating the gel in a solution containing 0.4 m*M* ABTS and 50 m*M* acetate buffer, pH 5, on a shaker for 15–30 min at room temperature. During incubation, the pH of the ABTS staining solution was checked with pH paper and adjusted to 5 with acetic acid to avoid alkaline pH.

RESULTS

Heterologous Laccase Expression in Shake-Flask Cultivations

Heterologous laccase expression was initially assessed in shake-flask cultivations with BMM and BMMY medium at 30°C (Fig. 1). The pH of the culture broth declined gradually. With BMM medium, the highest laccase activity was 3.3 U/mL, which was observed after 3 days of induction; after that it declined to 1.6 U/mL (Fig. 1). With BMMY medium, the highest activity observed was only 0.11 U/mL (Fig. 1). The BMMY medium contained compounds interfering with the ABTS laccase activity assay. Therefore, the supernatants from cultivations with BMMY medium were dialyzed before the assay was performed (as described in Materials and Methods).

Effect of Methanol on Laccase Expression in Shake-Flask Cultivations

Variation of methanol concentration between 0.5 and 2.0% caused much less difference in cell growth (μ_{max} was approximately 0.044–0.047 h^{-1}) than in laccase activity (2.0–11.5 U/mL) (Fig. 2). The methanol accumulated gradually after 1 day when the concentration was 1.0% or higher (data not shown). However, this did not affect the growth to any greater extent (Fig. 2). At the end of the cultivations, the cell density achieved by daily methanol addition to 1.0% exceeded that obtained by daily methanol addition to 0.5%, which means that growth was not inhibited by the 1.0% methanol protocol. The laccase activity increased with lower level of methanol (Fig. 2). The highest activity, 11.5 U/mL, was obtained at 20°C by using 0.5% methanol additions and was around fourfold higher than at 30°C (Fig. 1).

Stability of Recombinant Laccase

Effect of Temperature

The stability of recombinant laccase decreased with increasing temperature (Fig. 3). If the residual activity at 10°C was set to 100%, 88% and 42% of the activity remained after incubation for 24 h at 20°C and 30°C, respectively. The stability of native laccase showed a similar correlation to temperature (data not shown).

Effect of Methanol Concentration

The recombinant laccase activity decreased with increasing methanol concentration (Fig. 4). Compared to the sample with 0.5% methanol, 90% and 80% of the activity remained after 24 h at room temperature in the samples treated with 1.0% and 2.0% methanol, respec-

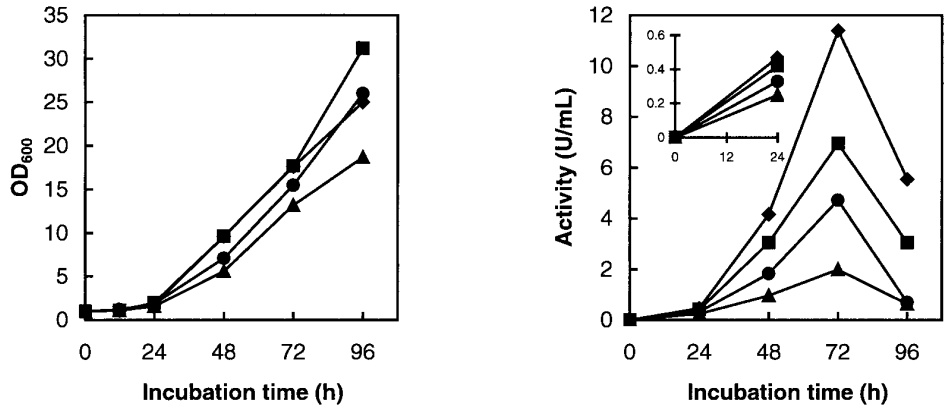

Figure 2. Effect of methanol concentration on laccase expression in shake-flask cultivations with BMM medium at 20°C. Methanol was added daily to a concentration of 0.5% (◆), 1.0% (■), 1.5% (●) and 2.0% (▲). The OD_{600} (left) and the laccase activity (right) are shown. The laccase activity during the first 24 h is indicated in the inset (right).

tively. In contrast, the activity of the native laccase was not negatively affected by methanol.

Scale-Up of Recombinant Laccase Production in Fermentors

Strategy A

The end of the glycerol batch phase was indicated by a spike in the DO caused by the exhaustion of glycerol and by a decrease in CO_2 in the off-gas to a level close to that in the beginning of the fermentation. Within 24–26 h after inoculation, the cells had consumed all the glycerol and had reached 20–22 g/L cell dry weight, corresponding to an OD value at 600 nm of around 100–110. After the growth phase on glycerol finished, the induction phase was initiated by feeding methanol with three different fermentation strategies.

In strategy A, the fermentation was performed at 30°C with 1.0% (v/v) methanol. Methanol was the sole carbon source and was automatically kept at 1.0% by the methanol sensor system. After 4 days, the fermentation yielded 120 g of dry biomass per liter (Fig. 5A). The consumption of methanol was fast owing to the Mut^+ phenotype of the strain. The maximum specific growth rate (μ_{max}) calculated for logarithmic growth was 0.035 h^{-1}.

The highest laccase activity, 24 U/mL, was reached after 3.5 days at a cell dry weight of 100 g/L. This activity was about 7 times higher than in shake-flask cultures with BMM medium at 30°C (3.3 U/mL, Fig. 1). The total soluble extracellular protein concentration at the time of the highest volumetric activity was 550 mg/L, giving a specific laccase activity of only 43 U/mg protein. However, the highest specific activity obtained at an earlier time-point was 150 U/mg protein (Fig. 5A). Both laccase activity and total amount of extracellular

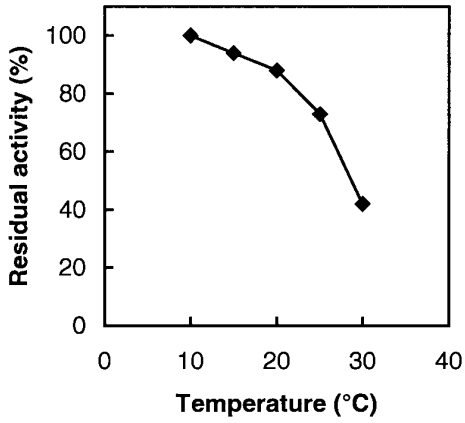

Figure 3. Stability of crude recombinant laccase at different temperatures. Laccase was incubated for 24 h in the culture medium after centrifugation. The residual activity of the 10°C sample was set to 100%.

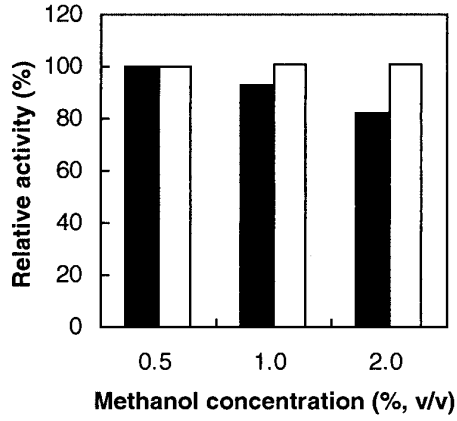

Figure 4. Laccase activity after incubation in different concentrations of methanol. The residual activity of the 0.5% sample was set to 100%. Recombinant laccase (■) and native laccase (□) are shown.

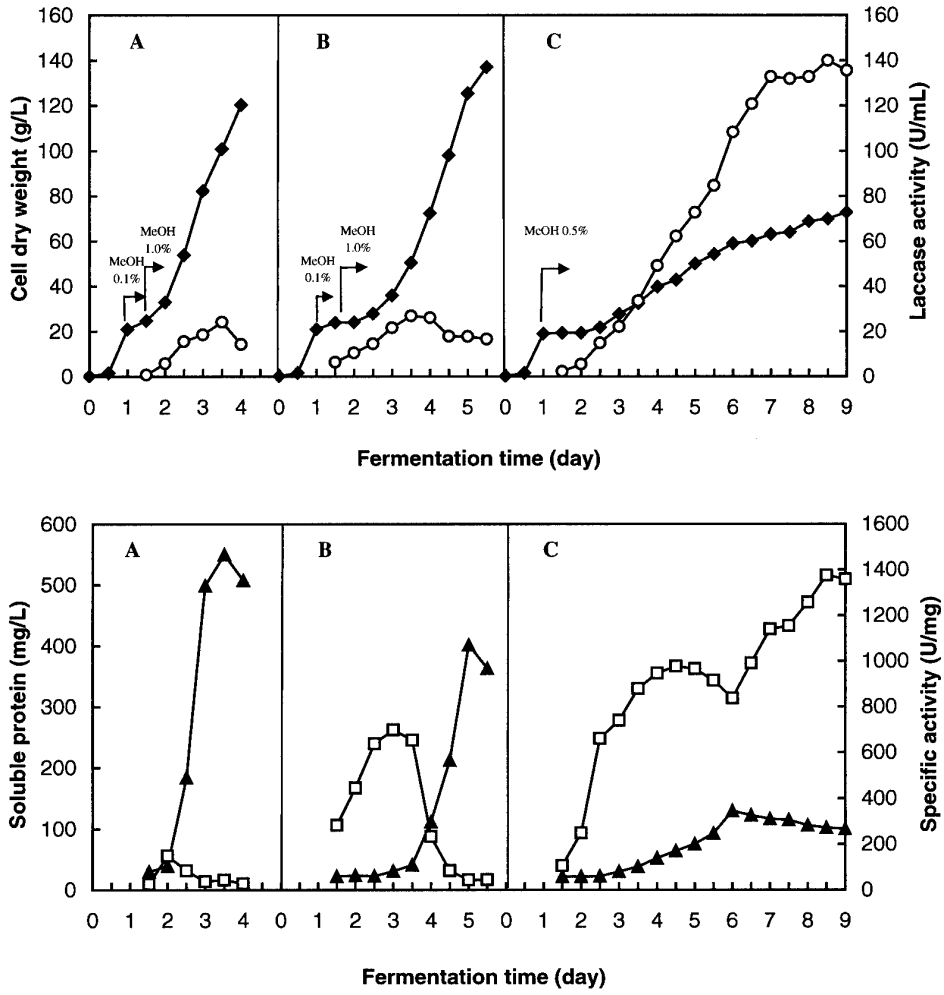

Figure 5. Fermentation of laccase-expressing *P. pastoris* using three different fermentation strategies A (30°C, 1%), B (20°C, 1%), and C (20°C, 0.5%). The cell dry weight (◆), the laccase activity (○), the total soluble protein (▲), and the specific activity (□) are indicated.

protein decreased significantly at the end of the fed-batch fermentation. The activity decreased even though the biomass increased further from 100 to 120 g/L.

Strategy B

In strategy B, 20°C and 1.0% methanol was used. The yeast still grew fast (Fig. 5B). However, compared to strategy A, the growth lag in the induction phase was 1 day longer when the temperature was decreased from 30 to 20°C. The growth rate after the lag phase reached a μ_{max} of 0.026 h^{-1}, which was slightly lower than in strategy A. The final biomass concentration was also beyond 100 g/L and the highest activity obtained (27 U/mL) was slightly higher than in strategy A (24 U/mL). The total soluble extracellular protein reached was 400 mg/L, giving a specific laccase activity of 44 U/mg protein at that time point. However, the highest specific activity obtained was 700 U/mg protein (Fig. 5B). The activity and amount of total protein decreased at the end of the fed-batch fermentation, as also observed for strategy A.

Although the difference in volumetric laccase activity between strategy A and B was slight, the level of total soluble protein at 20°C was lower than at 30°C. This contributed to the specific activity obtained at 20°C (700 U/mg protein), which was fivefold higher than at 30°C (150 U/mg protein). Therefore, it can be concluded that cultivation at 30°C was more beneficial for the growth of the yeast cells and the production of extracellular protein than cultivation at 20°C, but not for obtaining high laccase activity. In contrast, the laccase activity was improved by decreased cultivation temperature, particularly the specific activity.

Strategy C

In strategy C, 0.5% methanol was investigated at 20°C (Fig. 5C). The results showed that 0.5% methanol was

much better for producing active laccase than 1.0%. More than fivefold higher volumetric laccase activity (140 U/mL) was reached at 0.5% methanol compared to 1.0% (27 U/mL). The μ_{max} was only 0.012 h^{-1}, the lowest among the three fermentation strategies. The final dry cell weight reached only around 70 g/L. The total soluble protein concentration reached only 130 mg/L, and the highest specific laccase activity was 1380 U/mg protein. Furthermore, the results indicated that the total extracellular protein decreased slightly during the late period of the fermentation, as in the other fermentation strategies. The volumetric activity was at least 12-fold higher than in shake-flask cultures with BMM medium containing 0.5% methanol at 20°C (11.5 U/mL, Fig. 2).

Analyses of Culture Supernatants

SDS-PAGE Analysis

To determine the molecular weight of the recombinant laccase expressed by *P. pastoris*, SDS-PAGE was performed (Fig. 6). The M_r of the recombinant laccase (~67 kDa) was slightly higher than that of the native *T. versicolor* laccase, which is 64 kDa (Fåhraeus and Reinhammar, 1967). Figure 6 also indicates that a few other proteins were present in the *P. pastoris* cultures. For instance, bands around 30 and 43 kDa were found on the gel. Some protein bands, such as the ones around 43 kDa, were found also in the culture of the host strain SMD 1168 without any laccase gene inserted (data not shown).

To explain why the specific activity declined in the later phase of the fermentations, SDS-PAGE analysis of samples taken after different time points was performed. A more sensitive staining method, silver staining, was used. The amount of protein (0.5 μg) loaded on the gel was the same for all samples. The results indicate the appearance of many other proteins than laccase during the progress of the fermentations (not shown). The protein bands between 30 and 43 kDa were stronger at 30 than at 20°C, indicating that more protein was released into the culture medium at higher temperature. This result confirms the observation regarding the differences in total protein and specific activity between strategy A and B (Fig. 5A and B).

Zymogram Analysis

Zymogram analysis indicated that the molecular weight of the recombinant laccase was higher than that of native laccase (Fig. 7). Other protein bands were not associated with any activity. By using this novel zymogram staining technique, it was possible to identify and distinguish laccase from other proteins.

Proteolytic Activity Assay

Proteolytic activity was detected in the culture supernatants of all fermentations (Table I). The proteolytic activity decreased in the order strategy A, B, and C. The assay is generally performed at alkaline pH (8.5), but pH 5.0 was also tested because it was the pH of the fermentations. The proteolytic activity was higher at pH 5.0 than at pH 8.5, which indicates that most of the proteases, which were released by *P. pastoris* under the prevalent conditions, had acidic optimum.

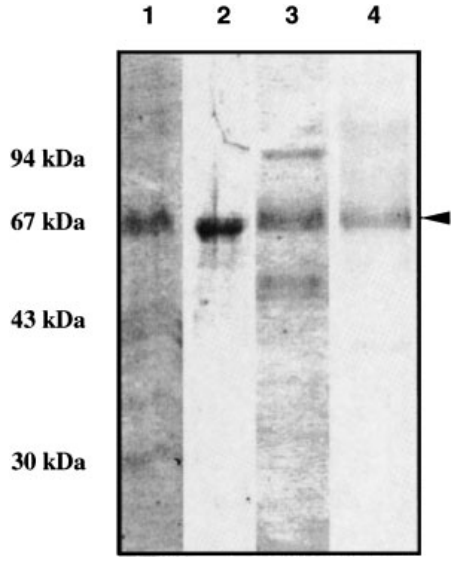

Figure 6. Proteins secreted by laccase-expressing *P. pastoris* analysed with SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Culture supernatants (15 μL) from strategy A after 3.5 days (protein concentration: 550 mg/L) (lane 1), from strategy B after 5.0 days (400 mg/L) (lane 3), and from strategy C after 6.0 days (130 mg/L) (lane 4) were loaded on the gel. Purified native laccase from *T. versicolor* was used for comparison (lane 2). The arrow indicates the recombinant laccase. The positions of the markers are shown to the left.

DISCUSSION

Effects of pH

The decrease in laccase activity during the late period in shake-flask cultivation might be due to the low pH (Fig. 1). Previous results have indicated that low pH affects both recombinant (Jönsson et al., 1997) and native (Larsson et al., 2001) *T. versicolor* laccase

Table I. Proteolytic activity (arbitrary units) in the culture supernatants.

	Fermentation strategy		
Assay pH	A (30°C, 1%, 4.0 days)	B (20°C, 1%, 5.5 days)	C (20°C, 0.5%, 9.0 days)
pH 8.5	0.056	0.032	n.d.[a]
pH 5.0	0.30	0.27	0.21

[a]n.d.: not detectable.

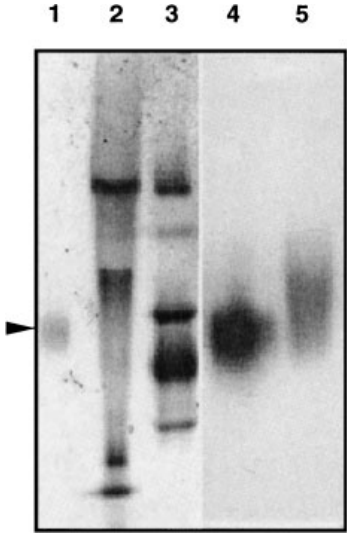

Figure 7. Native PAGE and zymogram analysis of laccase. Bands visualized by Coomassie Brilliant Blue R-250 staining (lanes 1, 2, and 3) and ABTS staining of active protein (lanes 4 and 5). Concentrated culture supernatants (15 μL) from strategy C after 8.5 days (activity was about 1400 U/mL) (lanes 2 and 5) were loaded on the gel. Purified native laccase from *T. versicolor* was used for comparison [lanes 1 (1.6 μg) and 4 (0.016 μg)]. Lane 3 contains marker proteins (30, 43, 67, and 94 kDa). The arrow indicates the native laccase.

negatively. Expression of other laccase isoforms in *P. pastoris*, for instance, laccase *LCCI* from *T. versicolor* (Gelo-pujic et al., 1999) and *lac1* from *Pycnoporus cinnabarinus* (Otterbein et al., 2000) has also been negatively affected by low pH.

Effects of Cultivation Temperature in the Induction Phase on Recombinant Laccase Expression

The temperature is an important parameter for optimization of laccase expression in yeast systems (Cassland and Jönsson, 1999). The activity of heterologous laccase expressed by *S. cerevisiae* cultivated in shake-flasks was found to be improved by lowering the temperature to approximately 19°C. The results also indicated that the laccase activity in *P. pastoris* shake-flask cultures was much higher at 16–19°C than at 28°C (Cassland and Jönsson, 1999), close to what is commonly used for heterologous expression in *P. pastoris*. The present study confirmed that cultivation at low temperature in shake flasks improved laccase activity (3.3 U/mL at 30°C, Fig. 1 and 11.5 U/mL at 20°C with 0.5% methanol additions, Fig. 2), and, in addition, showed that this was also the case under controlled conditions in a fermentor (Fig. 5).

P. pastoris is a widely used system for heterologous protein expression, but few results regarding production improvement by temperature optimization have been documented so far. Inan et al. (1999) showed that the temperature did not have any significant effect on the production of recombinant hookworm anticoagulant peptide (rAcAP-5) by *P. pastoris*. Li et al. (2001) showed that lower temperature increased the yield of recombinant herring antifreeze protein dramatically, which was attributed to improved protein folding as well as increased cell viability. Our results revealed that the cultivation temperature in the induction phase particularly influenced the specific laccase activity (Fig. 5). Together with the other observations, our results indicate that the effect of temperature on expression of heterologous proteins in the *P. pastoris* system is specific and due to the character of the expressed protein.

There may be several mechanisms behind the effect of fermentation temperature on laccase activity. First, poor thermostability of laccase may have led to lower activity in the 30°C cultures (Fig. 3). A second possible explanation is the action of proteases. Proteases can either be secreted or be released from dead cells. Both the Coomassie-stained and the silver-stained gels showed more low molecular weight protein bands in the culture medium at 30°C (strategy A) than at 20°C (strategy B). These low molecular weight protein bands could possibly be degradation products of proteins of higher molecular weight because protein bands were visible around 94 and 50 kDa at 20°C but not at 30°C (Fig. 6). Moreover, the protease assay confirmed that the proteolytic activity was higher in the samples from the fermentation performed at 30°C than at 20°C (Table I). Because the results presented in Table I were obtained by using the same incubation temperature (37°C) for all samples, the actual difference in proteolytic activity between the 20 and 30°C fermentation should be even greater because of a higher reaction rate at 30°C. Therefore, cultivation at 20°C decreases the degradation of secreted laccase compared to cultivation at 30°C. A third possibility is improper folding of the recombinant laccase at higher temperature. Cultivating the cells at lower temperature would slow the rate of protein synthesis as well as cell growth (μ_{max} was 0.026 and 0.035 h^{-1} at 20 and 30°C, respectively) and allow more time for the peptide chains to fold properly if folding is rate-limiting. Brierley et al. (1990) found that low growth rates could be desirable for production of certain recombinant proteins by Mut^s strains of *P. pastoris*. The fact that lower cultivation temperature may improve the production of heterologous protein in *E. coli* (Schein and Noteborn, 1988) has also been ascribed to aggregation problems at higher temperatures. In production of recombinant proteins using *E. coli*, lowering the cultivation temperature is routinely used for increasing the amount of properly folded protein.

Susceptibility to Acidic Proteases

One possible explanation for the decrease in laccase activity and total protein concentration in the later part

of the fermentations is susceptibility to acidic proteases. The pH was kept at 5.0 in the fermentor cultivations, and in the protease assay higher proteolytic activity was found at pH 5.0 than at pH 8.5 (Table I). A previous investigation indicated that the proteolytic activity played an important role for laccase expression by *P. pastoris* (Jönsson et al., 1997), because the protease deficient SMD 1168 strain performed better than GS115. The strain SMD 1168 is deficient in protease A and, therefore, also deficient in carboxypeptidase Y and protease B1, which are activated by protease A.

Although pH 5.0 is preferable for heterologous expressions of many proteins (Sreekrishna and Kropp, 1996; Stratton et al., 1998), the yield of recombinant protein has in some cases been improved by increasing the pH. For instance, production of human serum albumin (HSA) was improved more than threefold by using pH 5.85 instead of the generally used pH of 5.0 (Sreekrishna et al., 1990). Because pH 5.0 might reduce the level of active recombinant laccase due to susceptibility to acidic proteases as well as lower the enzyme stability (laccase was recently shown to be most stable at pH 6.0 and 7.0, Larsson et al., 2001), the optimal pH for the production of recombinant laccase needs to be investigated in more detail in the future.

Influence of Methanol Concentration on Recombinant Laccase Expression

The influence of two different methanol concentrations, 1.0% (v/v) and 0.5% (v/v) on recombinant laccase expression, was investigated at 20°C by comparing the feeding strategies B and C. A direct positive correlation between the amount of methanol consumed and the quantity of protein produced has been observed (Murray et al., 1989). In the present study, a positive correlation between total soluble protein and methanol consumption was evident. However, the activity of recombinant laccase did not correlate with the methanol concentration (Fig. 5B and C). The analyses showed that the concentration of native extracellular proteins increased with higher methanol concentration (Fig. 6).

The use of 1.0% methanol to induce the expression of foreign genes with *P. pastoris* has been recommended (Murray et al., 1989; Jiménez et al., 1997). Cell proliferation was not inhibited until the methanol concentration reached $>3\%$ (Murray et al., 1989). Our study also indicated that growth was not impaired with 1.0% methanol compared to 0.5%, and the cells grew twice as fast with 1.0% (μ_{max} 0.026 h^{-1}) as with 0.5% methanol (μ_{max} 0.012 h^{-1}) at 20°C (Fig. 5B and C). Similar observations were made for the shake-flask cultivations (Fig. 2).

Although 1.0% methanol was beneficial for the growth of the cell, the laccase activity was negatively affected. The lower laccase activity obtained may be partially due to lower enzyme stability in presence of increasing methanol concentration (Fig. 4). In addition, induction of the *AOX1* promoter with a lower methanol concentration would slow the rate of protein synthesis as well as the growth rate and allow more time for the peptide chains to fold properly. This effect may be similar to the temperature effect. Previously, Sreekrishna and Kropp (1996) concluded that longer fermentation times might be necessary to allow for accumulation of high levels of a slowly secreted protein in the broth. Furthermore, large amounts of methanol and a rapid metabolic rate when *P. pastoris* grows on 1.0% methanol may result in higher levels of formaldehyde and hydrogen peroxide, which could lead to the death of cells. This phenomenon occurs with another methylotrophic yeast, *Hansenula polymorpha* (Veenhuis et al., 1983). Thus, lower viability of cells in 1.0% methanol could lead to higher proteolytic activity (Table I) and release of more native proteins (Fig. 6). The possible effect of toxic products from methanol should also be kept in mind with regard to the effect of methanol on laccase stability in *P. pastoris* culture supernatant (Fig. 4), in particular because laccase was not negatively affected by up to 2% methanol in an aqueous solution without culture supernatant.

Gel Electrophoresis Analysis

The higher molecular weight of recombinant laccase compared with the native enzyme is likely due to glycosylation (Figs. 6 and 7). Compared to *S. cerevisiae*, *P. pastoris* is considered to be less likely to hyperglycosylate heterologous protein (Romanos et al., 1992). Previous studies showed that laccase expressed in *S. cerevisiae* was heterogeneous, and its M_r was much higher than that of the native enzyme (Cassland and Jönsson, 1999). Both *S. cerevisiae* and *P. pastoris* display N-linked glycosylation of the high-mannose type. Nevertheless, the length of the oligosaccharide chains added post-translationally to proteins in *P. pastoris* (average 8–14 mannose residues per side chain) is much shorter than in *S. cerevisiae* (50–150 mannose residues) (Tschopp et al., 1987; Grinna and Tschopp, 1989).

The other proteins appearing at 30°C might have been released from dead cells because of faster metabolic rate and/or comprise native secreted proteins from the host strain. Cell viability could be higher at lower temperature than 30°C (Li et al., 2001). Some additional protein bands were found around 30 and 43 kDa. Zymogram analysis with specific activity staining confirmed that the M_r of recombinant laccase was higher than the native, although some heterogeneity in electrophoretic mobility was apparent.

CONCLUSIONS

Laccase is a biotechnologically important enzyme, and it is of great interest to find simple and efficient systems for

heterologous expression. We have explored the use of advanced fermentation control and different fermentation strategies to optimize the expression of laccase in the methylotrophic yeast *P. pastoris*. This work is the first in which recombinant laccase expression by *P. pastoris* is investigated in detail under controlled conditions in fermentor, especially concerning the effect of the cultivation temperature and methanol concentration. Production of laccase in *P. pastoris* was found to be affected by several factors, including the cultivation temperature, the pH, the proteolytic activity, and the methanol concentration. It was possible to greatly enhance both the volumetric and specific laccase activity in *P. pastoris* cultures using carefully controlled conditions and a strategy based on low-temperature and low-methanol concentration.

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