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Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*

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A cDNA encoding a laccase enzyme was isolated from a *Trametes versicolor* cDNA library. The gene was subcloned into the *Pichia pastoris* expression vector pPIC3.5 and transformed into the *P. pastoris* strains KM71 and GS115. Laccase-secreting transformants were selected by their ability to oxidise the substrate ABTS. No difference in laccase activity was observed between culture supernatants from GS115 (proteolytic) and KM71 (nonproteolytic) strains. The presence of at least 200 μM copper was necessary for optimal laccase activity in the culture supernatants. During growth of *P. pastoris* on minimal medium the pH of the medium was reduced to <3.0. If alanine was added to the medium the pH reduction was not as pronounced and at alanine concentrations >0.6% w/v the pH was kept constant for >7 days. Cultures in which the pH was maintained by alanine metabolism produced higher levels of laccase activity than those grown in the absence of alanine. This study describes the development of a medium that allows convenient pH control of *P. pastoris* without the need for continuous neutralisation.
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Keywords: laccase; *Pichia pastoris*; heterologous expression

Introduction

Laccases are phenoloxidases that contain two to four copper atoms per molecule. Oxidation of phenolic substrates is accompanied by reduction of molecular oxygen to water. The substrate range of laccases is broad, and individual laccases differ in their substrate specificity. Nonphenolic compounds can also be attacked by laccases in the presence of a mediator such as the laccase substrate ABTS (2,2′-azinobis [3-6-sulphonate]) [2]. *Trametes versicolor* laccases can oxidise anthracene and benzo-[*a*]-pyrene in the presence of a fungal mediator [7]. Individual basidiomycetes produce more than one laccase isozyme; *T. versicolor* produces at least two and another *Trametes* species, *T. villosa,* is reported to produce five laccase isozymes [19]. There are a number of possible industrial applications for laccases including paper pulping/bleaching [3,18], bioremediation [16,17] and biosensors [8,14].

Industrial scale production of enzymes requires relatively high levels of expression in a producing organism that is suitable for use in large-scale fermentation. As a result the genes for many industrially important enzymes have been inserted into heterologous hosts such as *Aspergillus* and yeasts. A frequently used expression organism is the methylotropic yeast *Pichia pastoris* that can grow on methanol as a sole carbon and energy source. The methanol utilisation pathway is highly inducible; the first enzyme in the pathway, methanol oxidase, is undetectable in cells grown on glucose but comprises up to 35% of the total protein in methanol-grown cells [11]. A number of *P. pastoris* expression vectors have been constructed using the methanol oxidase promoter to drive expression of a large number of heterologous proteins. The list of heterologous proteins expressed numbers greater than 300, including prokaryotic, eukaryotic and viral proteins [5]. Two laccase genes from *T. versicolor* have been expressed in yeast expression systems, *lcc*1 in *P. pastoris* [9] and *lcc*2 in *Saccharomyces cerevisiae* [4]. A laccase from another white rot fungus, *Pycnoporus cinnabarinus*, has also recently been expressed in *P. pastoris* [10], indicating the suitability of the system for laccase expression.

There are a number of strategies used to optimise the level of heterologous protein expression. These include using multicopy transformants to increase gene dosage, prevention of proteolytic degradation of the expressed protein by using protease deficient mutants and by control of environmental parameters such as temperature and pH [13]. In particular, expression of the *T. versicolor* laccase *lcc*1 in *P. pastoris* was improved if the pH was periodically adjusted to pH 6.0 during fermentation [9].

Industrial scale *P. pastoris* fermentations are usually fed-batch processes in which pH, dissolved oxygen and nutrient availability are carefully controlled and extremely high cell densities (up to 450 g wet weight/l) can be achieved [12]. In laboratory-scale experiments, shake flasks are commonly used, or if pH control is required, bench-scale fermenters are used. In experiments where a number of different culture conditions are being examined pH control using small-scale fermenters may not be feasible due to the cost of purchasing the equipment. The alternative is to periodically neutralise shake-flask cultures but this does not maintain a truly constant pH.

In this study, a laccase cDNA isolated from a *T. versicolor* cDNA library was expressed in *P. pastoris*. The effect of copper availability on the activity of the expressed laccase was determined and a medium was designed in which the pH was maintained at a constant value without the need for continuous neutralisation.

Materials and methods

Microbial strains and vectors

T. versicolor CU1 was maintained on malt extract agar plates stored at 4°C. *Escherichia coli* strains were maintained on LB agar containing the appropriate antibiotic and stock cultures were

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maintained in LB broth containing 40% v/v glycerol at −70°C. *P. pastoris* strains and the pPIC3.5 vector were obtained from Invitrogen (Groningen, The Netherlands) as part of the *P. pastoris* expression kit. *P. pastoris* cultures were routinely stored on yeast extract–dextrose-peptone medium (2% w/v yeast extract, 2% peptone, 2% dextrose) (YPD) slants at 4°C and stock cultures in glycerol at −70°C.

Cloning of the laccase cDNA

A Lambda Zap II *T. versicolor* CU1 cDNA library was constructed by Stratagene (Cambridge, UK) which contained 3.3×10^9 amplified recombinants per milliliter. A laccase cDNA sequence was amplified by PCR from *T. versicolor* 290 using the forward primer 5′-ATTGGCACGGCTTCTTCC and the reverse primer 5′-GATCTGGATGGAGTCGAC. The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and a laccase gene probe was prepared by excising the cloned fragment with *Apa*I/*Sac*I and labelling it with ^{32}P using the Prime-A-Gene kit (Promega). *E. coli* XL1-Blue cells were infected with the Lambda-Zap II *T. versicolor* CU1 cDNA library and plated at 5×10^4 PFU per 150-mm petri dish at 37°C. Plaque lifts were performed in duplicate onto nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the DNA was cross-linked to the membranes using a Stratalink UV cross-linking oven (Stratagene, La Jolla, CA). Hybridisation was done in 0.5 M sodium phosphate–5% SDS at 55°C. The membranes were washed sequentially in (a) 2× SSC at room temp, (b) 2× SSC–0.1% SDS at 55°C and (c) 2× SSC at room temperature, after which positive clones were identified by autoradiography. The laccase insert was excised *in vivo* in the pBluescript vector to create the pLAC1 plasmid and sequenced. The resulting sequence data was assembled using the DNASTAR software package (DNASTAR, Madison WI) and the BLAST algorithm [1] was used to search protein and DNA sequence databases for similar sequences. The sequence has been submitted to GENBANK (accession number AY049725).

Expression of the laccase gene in P. pastoris

The laccase cDNA was excised from the pLAC1 plasmid by digestion with *Hin*dIII and *Nar*I. The laccase fragment ends were filled in using Klenow polymerase to create a blunt-ended fragment suitable for ligation into the *Sna*B1 site of the *P. pastoris* expression vector pPIC3.5. The ligation was transformed into *E. coli* XL1-Blue cells and the resulting transformants screened for correctly orientated clones by digestion with appropriate restriction enzymes. One correctly orientated clone was selected and designated pJOC17. *P. pastoris* GS115 and KM71 (both His$^-$) cells were transformed with *Stu*1 linearised pJOC17. Digestion with *Stu*1 should result in integration of the linearised DNA at the *his* locus. Transformants were selected by plating them on minimal dextrose plates to detect the His$^+$ phenotype resulting from successful integration of the pJOC17 plasmid. His$^+$ transformants were screened for production of laccase by replica plating on minimal methanol plates containing 0.2 mM $CuSO_4$ and 0.1 mM ABTS. The plates were incubated inverted at room temperature and 100 ml of methanol was added to the lid of the plate each day to compensate for loss by evaporation. Laccase-producing transformants were readily identified by the presence of a green halo around the *Pichia* colonies.

Expression of laccase by P. pastoris in liquid culture

The *P. pastoris* transformants were grown overnight in yeast extract–peptone–glycerol (YPG) broth, which contained 2% w/v yeast extract, 2%w/v peptone and 2% w/v glycerol. Use of glycerol as an energy source prevented the possibility of catabolite repression by trace amounts of glucose. The culture medium for expression of the laccase was buffered minimal medium (BMM) containing 0.5% methanol, 0.1 M potassium phosphate, 33 g/l yeast nitrogen base and 100 g/l ammonium sulphate at pH 6.0. Copper as $CuSO_4$ was added at various concentrations and in some experiments D/L alanine was added as indicated in the text. Overnight cultures were centrifuged to harvest the cells and washed twice in quarter-strength Ringers solution to remove all traces of YPG medium before inoculation into BMM. Twenty-milliliter culture volumes in 100-ml baffled flasks were incubated at 22°C in a water bath with reciprocal agitation at 120 rpm. Samples were taken at intervals for determination of optical density and laccase activity. Methanol was added daily to a concentration of 1.0% to replace that consumed by the cells or lost by evaporation. Optical densities were measured at 600 nm. Laccase assays contained 100 mM sodium acetate pH 5.0, 0.5 mM ABTS and 100 μl of the culture supernatant in a total volume of 1 ml. Laccase activity was measured as the increase in absorbance at 420 nm and expressed as activity units per liter where 1 unit is 1 μmol ABTS oxidised per minute. The molar extinction coefficient for ABTS at 420 nm was taken to be 36,000 M^{-1} cm^{-1}.

All data shown are representative of triplicate results.

Results

Cloning of the laccase cDNA

The DNA fragment cloned into pBluescript is 2506 nucleotides in length; a 1557 nucleotide open reading frame (ORF) from nucleotides 692–2249 translates to a 519-amino-acid protein of which the first 20 amino acids form a secretion signal peptide. Comparison of the nucleotide sequence with the nucleotide sequence databases showed that the sequence is 95% identical to the *lcc*1 laccase from *T. versicolor* [9]. Digestion of the plasmid with *Nar*1 and *Hin*dIII released a 1722-bp fragment, which contained the entire laccase ORF together with a 3′ untranslated region. Ligation of this fragment into the *Pichia* expression vector pPIC3.5 produced a 9.63-kb plasmid designated pJOC17. This

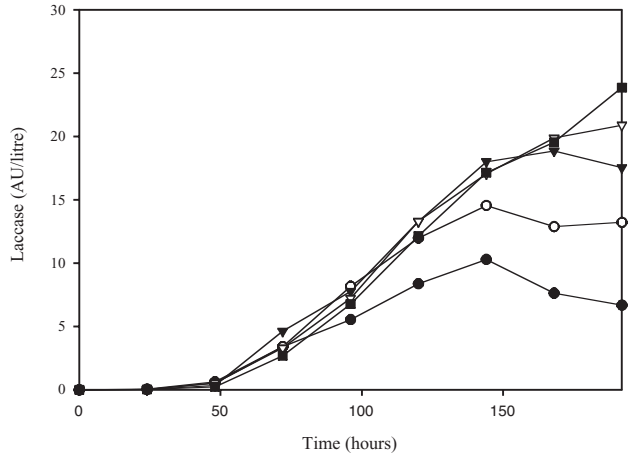

Figure 1 Laccase production by *P. pastoris* LP8 transformants in buffered minimal medium (●) and buffered minimal medium with 0.2% (○), 0.4% (▼), 0.6% (▽) and 0.8% (■) D/L-alanine.

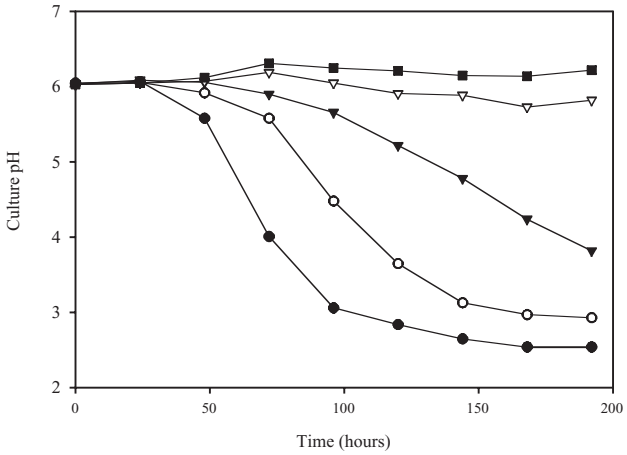

Figure 2 Culture pH of *P. pastoris* LP8 grown in buffered minimal medium (●) and buffered minimal medium with 0.2% (○), 0.4% (▼), 0.6% (▽) and 0.8% (■) D/L-alanine.

plasmid was linearised with *Stu*1 and transformed into *P. pastoris* by electroporation. His^+ transformants were obtained at the relatively low frequency of $<100/\mu g$ DNA. Approximately 37% of the His^+ KM71 transformants and 65% of the GS115 transformants produced green haloes when spotted onto minimal medium containing ABTS.

Laccase production by P. pastoris transformants

Laccase-producing transformants of *P. pastoris* grown in buffered minimal medium produced measurable concentrations of laccase after incubation periods of at least 24–48 h. Control strains transformed with pPIC3.5 did not produce any laccase activity. There was no significant difference in laccase production between the protease-positive strain GS115 and the protease-negative strain KM71 (data not shown). Ten transformants from each strain were screened to examine for differences in laccase production, but all produced similar levels of laccase. One of the GS115 transformants (LP8) was selected at random for use in subsequent experiments.

The effect of alanine on laccase production

During preliminary studies on laccase production by the transformants it was observed that the pH of cultures decreased rapidly to less than 3.0. While examining the effect of different carbon sources on methanol induction we observed that metabolism of the amino acid alanine stabilised the pH of the cultures during fermentation. The effect of adding different concentrations of alanine to BMM on the culture pH and laccase production by *P. pastoris* LP8 is shown in Figures 1 and 2. The drop in pH was

Table 1 pH, laccase concentration, biomass and laccase specific activity after 192 h fermentation

Alanine concentration (% w/v)	pH	Laccase concentration (AU/l)	Cell dry weight (g/l)	Specific activity (AU/g dry weight)
0	25	6.7	16.9	0.4
0.2	2.9	13.3	16.0	0.8
0.4	3.8	17.5	19.5	0.9
0.6	5.8	20.9	20.0	1.0
0.8	6.1	23.9	22.3	1.1

Table 2 Laccase production and biomass yield for LP8 cultures grown at different pH values in BMM with 0.8% D/L-alanine

pH	Laccase (U/l)	Biomass (g/l)	Laccase/biomass (U/g biomass)
4.5	5.7	17.5	0.32
5.0	10.6	17.9	0.59
5.5	13.4	21.3	0.63
6.0	15.8	16.9	0.94
6.5	14.6	15.8	0.92
7.0	5.0	12.5	0.4

greatest in the absence of alanine and was inversely proportional to the concentration of alanine added. In cultures with 0.6% w/v alanine or higher, the pH was maintained between 5 and 6 for up to 192 h. The amount of laccase activity increased in proportion to the alanine concentration; the activity in medium with 0.8% alanine was approximately two to three times greater than in cultures without alanine. In cultures grown without alanine laccase production levelled off earlier, in contrast to cultures with added alanine where the increase in laccase activity continued to the end of the fermentation. Addition of alanine to BMM medium also increased the biomass yield in the fermentations by approximately 30%. The laccase activity as a function of biomass increased from 0.4 to 1.1 AU/g dry weight when 0.8% D/L-alanine was present, so increased production of laccase was not a result of increased biomass (Table 1). Alanine was added as D/L-alanine; however, a subsequent experiment revealed that only the L-isomer was effective in maintaining culture pH (data not shown).

Laccase production at different pH values in the presence of alanine

In order to separate the effect of pH from any specific effect of alanine, cultures were set up with initial pH values of 4.5, 5.0, 5.6, 6.0, 6.5 and 7.0, with each culture containing 0.8% alanine. Laccase production was optimal at pH 6.0–6.5, while very little laccase activity was produced at pH 4.5 or pH 7.0 (Table 2). The amount of laccase produced per unit biomass followed a similar trend. The pH control produced by alanine was noticeably less successful when the pH values at inoculation were less than pH 5.5; at pH 6.0, 6.5

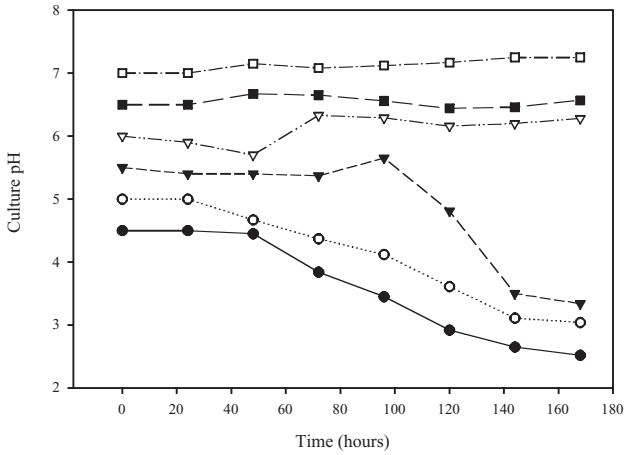

Figure 3 Culture pH of *P. pastoris* LP8 grown in buffered minimal medium with 0.8% alanine with the initial pH adjusted to 4.5 (●), 5.0 (○), 5.5 (▼), 6.0 (▽), 6.5(■) and 7.0 (□).

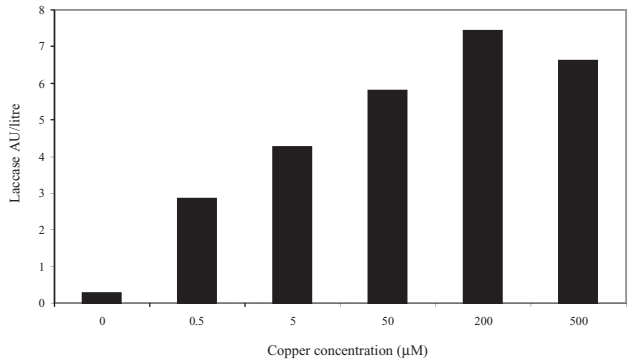

Figure 4 Laccase production by *P. pastoris* LP8 following 120 h growth in buffered minimal medium containing 0.8% alanine at a range of copper concentrations.

and 7.0 complete pH control was maintained for up to 144 h, while at pH 5.5 the pH decreased rapidly after approximately 100 h. At initial pH values lower than 5.5, the pH was not controlled very well; however, some pH control was present and the final pH of all cultures differed in proportion to the starting pH (Figure 3).

The effect of copper availability on laccase production

In BMM containing 0.8% alanine the level of laccase activity produced by *P. pastoris* LP8 was very low in the absence of copper. If copper was present in the medium, the laccase activity produced was in direct proportion to the amount of copper present. A copper concentration of 0.2 mM produced the highest level of laccase, increasing the copper concentration to 0.5 mM led to a slight reduction in the laccase levels (Figure 4). The biomass yield of the cultures was identical at all copper conditions so that the increase in laccase activity was not due to increased biomass (data not shown).

Discussion

A large number of heterologous proteins have been expressed in *P. pastoris* [5], including fungal laccases [9,10]. For any heterologous protein, expression conditions need to be optimised in order to achieve high levels of protein production [13]. An earlier study of laccase expression in *P. pastoris* and *S. cerevisiae* demonstrated the importance of pH and temperature on the level of laccase expression [9,4]. Laccases are copper-containing enzymes and contain two to four copper atoms per enzyme molecule. A previous study in this laboratory demonstrated the importance of copper availability to the activity of laccase enzymes from *T. versicolor* [6]. There have been no reports on the effect of copper on laccase activity in heterologous expression systems. In this study the effects of copper and pH on laccase production were examined and a growth medium designed in which pH control was achieved without the need for external pH control.

LP8 cultures grown in BMM expressed the laccase enzyme readily. However, it was obvious that the rapid decrease in pH during the fermentation was likely to be detrimental to laccase production, as was observed by Cassland and Jonsson [4]. During preliminary experiments in which the effect of different carbon sources on laccase production was examined, we observed that metabolism of alanine stabilised the culture pH. It is possible that metabolism of alanine released ammonia that neutralised acidic end products of methanol metabolism. When we applied the self-neutralising system to laccase-producing cultures there was a substantial increase the laccase activity in the culture supernatants and a lesser increase in the final biomass. The two- to three-fold increase in laccase activity outstripped the 30% increase in biomass indicating that the increased laccase activity was not simply due to increased biomass. It is likely that the increase in laccase activity resulted from greater enzyme stability in the pH-controlled cultures. The enhanced laccase activity appeared to result from the pH-stabilising effect, rather than from the presence of alanine per se as cultures grown at different pH values in medium containing a fixed concentration of alanine produced different levels of laccase (Table 2).

The importance of copper ions to the activity of laccase produced by *T. versicolor* has been demonstrated [6]. Copper ions did not influence the degree of protein synthesis but the activity of the laccase was directly proportional to the concentration of copper in the growth medium. The level of laccase expression from the AOX promoter should be the same in all cases. The correlation between copper availability and laccase activity is probably due to the requirement of the enzyme for copper [15].

In conclusion, this paper describes a novel medium for the growth of *P. pastoris* in which the pH is maintained at a constant level by simultaneous production of acidic and alkaline fermentation end products. The medium offers full pH control in shake-flask cultures without the need for continuous titration or periodic neutralisation of the culture. In routine laboratory fermentations, large numbers of flask cultures can be set up using this system without the need for expensive pH-control equipment. In this study, the medium has been used for heterologous expression of a laccase enzyme but it is likely that this system could be used for the expression of other heterologous proteins in *P. pastoris.*

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