Electronic Journal of Biotechnology 28 (2017) 7–13

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Combined strategies for improving production of a thermo-alkali stable laccase in *Pichia pastoris*

Jiayi Wang, Lei Lu *, Fujuan Feng

College of Life Sciences, Northeast Forestry University, Harbin 150040, China

ARTICLE INFO

Article history:
Received 17 February 2017
Accepted 17 April 2017
Available online 22 April 2017

Keywords:
Bacillus licheniformis
Dye decolorization
Enzyme production
Expression optimization
Green biocatalysts
Industrial processes
Multicopper enzyme
Site-directed mutagenesis
Stability
Yeast
Yields

ABSTRACT

Background: Laccases are copper-containing enzymes which have been used as green biocatalysts for many industrial processes. Although bacterial laccases have high stabilities which facilitate their application under harsh conditions, their activities and production yields are usually very low. In this work, we attempt to use a combinatorial strategy, including site-directed mutagenesis, codon and cultivation optimization, for improving the productivity of a thermo-alkali stable bacterial laccase in *Pichia pastoris*.

Results: A D500G mutant of *Bacillus licheniformis* LS04 laccase, which was constructed by site-directed mutagenesis, demonstrated 2.1-fold higher activity when expressed in *P. pastoris*. The D500G variant retained similar catalytic characteristics to the wild-type laccase, and could efficiently decolorize synthetic dyes at alkaline conditions. Various cultivation factors such as medium components, pH and temperature were investigated for their effects on laccase expression. After cultivation optimization, a laccase activity of 347 ± 7 U/L was finally achieved for D500G after 3 d of induction, which was about 9.3 times higher than that of wild-type enzyme. The protein yield under the optimized conditions was about 59 mg/L for D500G.

Conclusions: The productivity of the thermo-alkali stable laccase from *B. licheniformis* expressed in *P. pastoris* was significantly improved through the combination of site-directed mutagenesis and optimization of the cultivation process. The mutant enzyme retains good stability under high temperature and alkaline conditions, and is a good candidate for industrial application in dye decolorization.

1. Introduction

Laccases (EC 1.10.3.2) belong to the multicopper oxidase family, a group of enzymes that are widespread in plants, fungi and bacteria. These enzymes contain four copper ions in their active site, which can be classified into three types according to their unique spectroscopic features [1]. Laccases can oxidize a wide range of organic compounds coupled with the four-electron reduction of molecular oxygen to water. Attributing to their low substrate specificity, laccases have been used as green biocatalysts for many industrial processes [2]. Recently, bacterial laccases have attracted much more interests due to their high stabilities under harsh conditions. Nevertheless, their activities and production yields are usually too low to achieve the requirements of industrial applications [3].

Recombinant production of enzymes in easily cultivable hosts may overcome this obstacle through the use of strong promoters, multiple gene copies, efficient signal peptide and optimized cultivation conditions [4]. Moreover, protein engineering can be employed to improve enzyme properties or product titers by site-directed or random mutagenesis [5]. *Escherichia coli* is the most commonly used host for heterologous protein expression. Although laccases from different bacterial sources have been expressed in *E. coli*, the recombinant enzyme often forms insoluble aggregates which are difficult to refold and purify [3]. The methylotrophic yeast *Pichia pastoris* is another widely used expression system for heterologous protein production. It can be grown to high cell densities and produce high titers of recombinant protein, most of which are produced in a secreted form [6]. The secretion of properly folded proteins offers a way to avoid the need for high-cost and low-yielding cell disruption or refolding process, and therefore simplifies the downstream processing.

Previously, we have successfully expressed several thermostable laccases from *Bacillus* sp. in *P. pastoris* [7,8,9]. Since the foreign protein expression in *P. pastoris* is affected by a variety of factors at both genetic and culture level, there is still potential to increase productivity of these recombinant laccases [6,10]. In this work, combined strategies, including site-directed mutagenesis, codon and cultivation optimization, were used to improve the expression level of

* Corresponding author.
E-mail address: leilu82@yahoo.com (L. Lu).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.04.002

B. licheniformis laccase in *P. pastoris*. Biochemical characteristics and dye decolorization ability of the mutant enzyme were also investigated.

2. Materials and methods

2.1. Materials

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), reactive blue 19 (RB19), reactive black 5 (RB5), indigo carmine (IC) and acetosyringone (ACE) were all Sigma-Aldrich products (St. Louis, MO, USA). Fast Mutagenesis System was obtained from TransGen (Beijing, China). Gel Extraction Kit and Plasmid Mini Kit I were purchased from Omega Bio-Tek (Norcross, GA, USA). Restriction enzymes and T4 DNA ligase were purchased from NEB (Ipswich, MA, USA). Zeocin was purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were of analytical reagent grade.

2.2. Microorganisms, plasmids and media

E. coli Top10 competent cells (Tiangen, Beijing, China) were used for subcloning procedures and were grown in Low Salt LB medium. Expression vector pPICZαA and *P. pastoris* SMD1168H were products of Invitrogen (Carlsbad, CA).

The recombinant plasmid pPICZαA/lac, which contains the laccase gene from *B. licheniformis* LS04, was previously constructed and transformed into *P. pastoris* SMD1168H [7]. Yeast extract-peptone-dextrose (YPD), buffered glycerol-complex (BMGY) and buffered minimal methanol (BMM) media were prepared following the manual of the EasySelect *Pichia* Expression Kit (Invitrogen).

2.3. Site-directed mutagenesis and codon optimization

The plasmid pPICZαA/lac was used as template for site-directed mutagenesis [7]. A mutant D500G was constructed by PCR using Fast Mutagenesis System according to the supplier's protocol. For creation of the variant, the following primers were used: D500G-F 5′-GAGCAC GAAGATTACGGTATGATGCGCCCTCT-3′ and D500G-R 5′-CCGTAATCTTC GTGCTCAAGGATGTGGCAGT-3′. The PCR products were digested by DMT enzyme and transformed into *E. coli* Top 10. The mutant plasmid was confirmed by DNA sequencing, and then transformed into *P. pastoris* SMD1168H as formerly described by Lu et al. [7].

The sequence of mutant D500G was further optimized based on the codon usage bias of *P. pastoris*. The codon-optimized gene containing the restriction sites *Eco*RI and *Xba*I was designed by OptimumGene and synthesized by Genscript (Nanjing, China). Then it was cloned into pPICZαA for expression in *P. pastoris*.

Homology modeling was performed using the SWISS-MODEL Server (http://swissmodel.expasy.org/). CotA protein from *B. subtilis* (PDB ID: 1W8E) that showed 65.87% identity to *B. licheniformis* laccase was used as template.

2.4. Enzyme assay

Laccase activity was measured at 30°C using ABTS as substrate [7]. The oxidation of ABTS (1 mM) were measured at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) in 0.1 M citrate–phosphate buffer (pH 4.6). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. All assays were carried out in triplicate.

2.5. Purification and characterization of the mutant

The positive transformants were grown in 25 mL BMGY medium at 30°C in a rotary incubator (200 rpm) until the OD_{600} was approximately 2.0–6.0. The cultures were centrifuged (3000 × g at 4°C for 5 min), and the cell pellets were diluted with BMM medium supplemented with 0.2 mM $CuSO_4$ and 0.8% (*w*/*v*) alanine to an OD_{600} of about 1.0. The flasks were cultivated at 30°C, 200 rpm, and 0.5% (*v*/*v*, final concentration) methanol was added daily to induce the production of recombinant laccase.

Purification of the mutant laccase was performed using ultrafiltration, anion-exchange chromatography, and gel filtration according to Chen et al. [9]. The optimal pH and temperature as well as pH and thermal stability of the mutant enzyme were determined as previously described by Lu et al. [7].

2.6. Dye decolorization

The mutant laccase was tested for its ability in decolorization of three synthetic dyes with different structures (reactive blue 19, reactive black 5 and indigo carmine). The concentration of dyes was 100, 40 and 25 mg/L for reactive blue 19 (RB19), reactive black 5 (RB5) and indigo carmine (IC), respectively. Decolorization was conducted with 20 U/L of laccase in the absence or presence of 0.1 mM acetosyringone (ACE) [7]. The molar ratio of RB19:RB5:IC:ACE was about 1.6:0.4:0.5:1. Control samples were run in parallel without laccase. All reactions were performed in triplicate.

2.7. Cultivation optimization

The effects of various cultivation factors that may influence the expression level of laccase were investigated, including carbon sources, nitrogen sources, copper concentration, methanol concentration, medium pH, surfactants addition as well as cultivation temperature. One milliliter cultures were withdrawn after 3 d of methanol induction, and were centrifuged at 10000 × g for 3 min. Then the cell pellets were dried to a constant weight at 105°C for determination of dry cell weight (g/L). The supernatants were collected for laccase activity assay. All measurements were conducted in triplicate.

3. Results

3.1. Site-directed mutagenesis and codon optimization

The result of multiple sequence alignments of *B. licheniformis* laccase and other laccases indicates that Asp500 is a relatively conserved residue, with a glycine at the corresponding position in other non-*Bacillus* laccases [7,11]. The variant D500G was constructed to investigate its effect on the expression level of *B. licheniformis* laccase in *P. pastoris*. The volumetric activity with ABTS as substrate reached 162 ± 10 U/L for D500G after 3 d of cultivation. Compared to the wild-type laccase produced under the same conditions (as mentioned in Section 2.5), replacing aspartic acid with glycine led to about 2.1-fold higher for laccase activity expressed in *P. pastoris*. The sequence of D500G mutant was further optimized for heterologous production in *P. pastoris*. According to the analysis of codon usage bias in *P. pastoris*, the native gene employs tandem rare codons that can reduce the efficiency of translation (Fig. S1). Total 433 nucleotides were substituted in the optimized sequence of D500G. The codon adaptation index (CAI) of the mutant gene was upgraded from 0.62 to 0.92, and the frequency of optimal codons was significantly increased after codon optimization (Fig. S2). Besides, the optimization process included modifying some negative cis-acting sites as well as breaking several Stem-Loop structures. However, there was little variation in laccase activity of the supernatant when the optimized D500G was expressed in *P. pastoris*, indicating codon optimization had no apparent improvement on the productivity.

Table 1
Kinetic parameters of the mutant and wild-type 04lac expressed in *P. pastoris*.

Enzyme	K_m (μM)	k_{cat} (s^{-1})	Specific activity (μmol min^{-1} mg^{-1})
04lac[a]	44.0 ± 2.8	5.6 ± 0.1	5.0 ± 0.1
D500G	58.2 ± 2.9	6.7 ± 0.1	5.9 ± 0.1

[a] From Lu et al. [7].

3.2. Enzyme characterization and dye decolorization

The purified mutant exhibited a slightly higher K_m and k_{cat} values than that of the wild-type (Table 1). The pH profile against ABTS revealed a slight shift toward more alkaline values after mutagenesis, with a change in pH optimum from 4.2 to 4.6 (Fig. 1a). Additionally, D500G displayed a higher stability at pH 9.0 than the wild-type enzyme during the first 3 d of incubation (Fig. 1b). Both D500G and wild-type 04lac showed an optimal temperature at 70°C (Fig. 2a). But a lower thermostability was found for the mutant, which demonstrated a half-life of inactivation at 70°C of about 1.8 h and retained about 29% of activity after 10 h at 60°C (Fig. 2b). The mutant and wild-type exhibited similar decolorization ability toward the tested dyes at pH 9.0. Indigo carmine (IC) can be gradually decolorized in the absence of mediator, with about 76% and 72% decolorization after 6 h for the wild-type and D500G, respectively (Fig. 3a). While no decolorization for reactive blue 19 (RB19) and reactive black 5 (RB5) was detected by laccase alone, the participation of acetosyringone (ACE) significantly improved the decolorization process. D500G could decolorize about 81% of RB5 and RB19 after 1 h, which was slightly lower than that of the wild-type laccase (Fig. 3b).

3.3. Cultivation optimization

Recombinant protein production in *P. pastoris* is greatly influenced by culture conditions such as medium components, pH and temperature. Therefore, we optimized these factors through one-factor-at-a-time experiments, and the optimized conditions were cumulative through the results shown in Fig. 4. The use of a multi-carbon substrate in addition to methanol is a widely accepted approach to improve protein yields in *P. pastoris* [12,13]. Various carbon sources except the control were added into BMM medium containing 0.2 mM $CuSO_4$ and 0.8% alanine. Addition of 0.5% sorbitol, mannitol or trehalose significantly improved both cell growth and laccase activity, while addition of lactose had little effect on laccase production (Fig. 4a). Maximal increase in laccase activity (57%) was observed when sorbitol was used as the co-substrate. The effects of a variety of nitrogen sources (1%) on expression of D500G are shown in Fig. 4b. Control without additional nitrogen supplementation was run in parallel. Yeast extract and peptone supported higher laccase activity and biomass, while the laccase activity was very low in urea or casamino acids containing medium. The addition of peptone resulted in approximately 32% increase in laccase activity in the extracellular medium (Fig. 4b). When different concentrations of alanine (from 0 to 2%) were added at the beginning of induction, a drastic increase in cell density and laccase activity was obtained. Laccase activity declined when alanine concentration exceeded 1.2% (Fig. 4c).

Cell growth was not obviously influenced by surfactants supplementation, while laccase expression level was inhibited in the presence of 0.01% SDS and NP-40. The addition of 0.1% Tween 20 or Tween 80 slightly increased laccase production (Fig. 4d). Copper is an essential cofactor for laccase which plays a crucial role in the proper function of laccase. Compared with the absence of added copper, the addition of 0.2 mM $CuSO_4$ in the medium brought a 1.6-fold higher laccase activity. Copper concentration varying from 0.2 to 1 mM was found to have no toxic effects on cell growth, however, high copper concentrations resulted in reduced laccase production (Fig. 4e). The influence of methanol concentration on recombinant laccase production was investigated in the range from 0.25 to 1.5%. As shown in Fig. 4f, the highest laccase activity reached 313 ± 11 U/L, by using 0.5% of methanol for induction. No inhibition effect on cell growth was observed when the concentration of methanol increased to 1.5%.

The medium pH and induction temperature are also very important for protein production. Laccase activity was slightly affected when the recombinant *P. pastoris* was grown in medium with intial pH from 5.5 to 6.5. Maximal laccase activity in the culture supernatants was detected at pH 6.5, and then it decreased with an increase of medium pH (Fig. 4g). Although lowering the temperature caused an increase in biomass, laccase expression level gradually decreased. The optimal induction temperature for laccase production was 28°C, followed by 30°C and 26°C (Fig. 4h). The optimized conditions for D500G expression were as follows: BMM medium (pH 6.5) was supplemented with 0.2 mM $CuSO_4$, 1.2% alanine, 0.5% sorbitol, 1% peptone and 0.1% Tween 20, 0.5% methanol was used for protein production, and the cultures were incubated at 28°C, 200 rpm. A laccase activity of 347 ± 7 U/L was finally achieved after 3 d of induction, which was about 9.3 times higher than the previously reported result of the wild-type [7]. About 59 mg of D500G could be obtained from 1 L culture under the optimized conditions.

4. Discussion

The Asp500 in *B. licheniformis* laccase is adjacent to Met501, which is the axial ligand of the T1 copper site (Fig. 5). It has been reported that substitution of this residue with glycine could facilitate the soluble expression of laccase in *E. coli* [11,14]. Therefore, we performed site-directed mutagenesis to investigate the possibility of enhancing the yield of *B. licheniformis* laccase in *P. pastoris*. The laccase activity for D500G expression was 2.1 times higher than the wild-type.

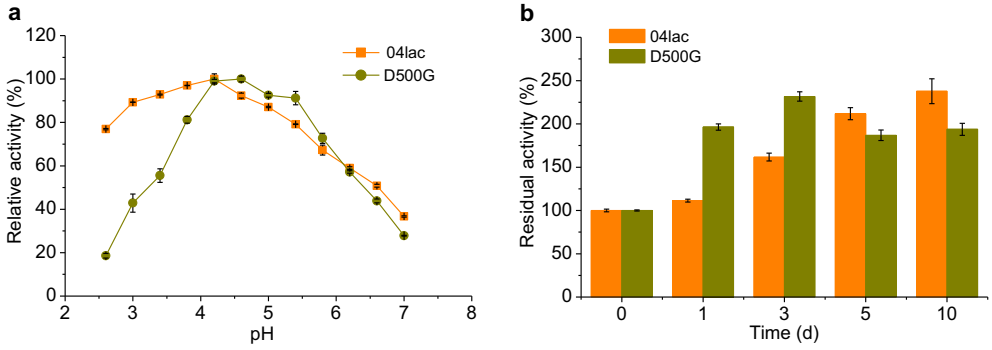

Fig. 1. Effect of pH on activity (a) and stability (b) of 04lac and D500G. (a) Laccase activity was measured with ABTS as substrate; (b) the stability was tested in 0.1 M Tris–HCl buffer (pH 9.0).

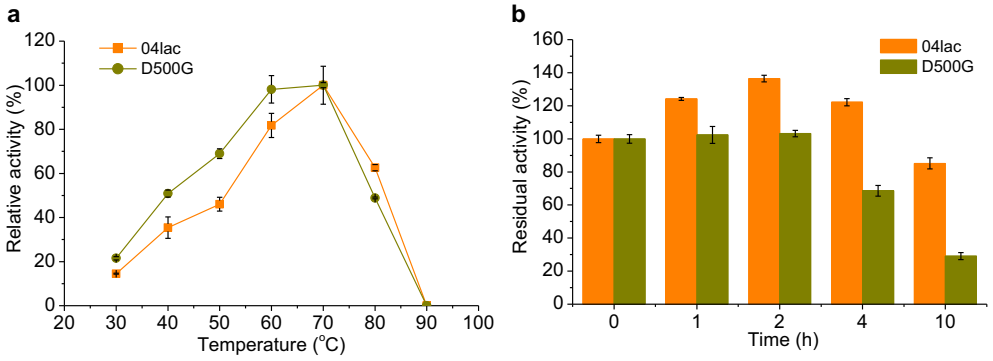

Fig. 2. Effect of temperature on activity (a) and stability (b) of 04lac and D500G. (a) Laccase activity was measured with ABTS as substrate; (b) the stability was tested at 60°C.

Considering there is a 1.2-fold increase in specific activity for the mutant (Table 1), the actual improvement in expression level is about 1.8-fold. While the D500G mutant of *B. licheniformis* DSM13 laccase showed a similar increase in K_m value, its specific activity was lower than that of the wild-type. The improvement in total activity of this D500G variant was mainly ascribed to a higher quantity of soluble protein expressed in *E. coli* [11]. Another D500G mutant of *Bacillus* sp. HR03 laccase displayed higher preference for ABTS than its parent type (54% decrease in K_m), and the 7.3-fold enhanced expression in *E. coli* was a result of an increase in both k_{cat} and expression level [14]. Since *P. pastoris* displays a general bias toward a subset of codons, this may affect the heterologous gene expression in *P. pastoris* [15]. Generally, a CAI above 0.8 is regarded as good, in terms of high gene expression level. Codon usage analysis indicated that the native gene employs tandem rare codons, which may reduce the efficiency of translation. Although the codon usage frequency was enhanced by synonymous codon substitution, no distinct change in laccase activity was found after 3 d of cultivation, suggesting codon usage bias had little effect on the expression of D500G in *P. pastoris*. In contrast, a 2.4-fold higher activity for glucose isomerase expressed in *P. pastoris* was obtained by codon optimization [16].

Since the wild-type 04lac is a thermo-alkali stable enzyme, we investigated the effects of mutagenesis on the stability of the enzyme. Overall, the D500G variant retained similar catalytic characteristics to the wild-type enzyme. However, a decrease in thermostability was found for D500G. The activity of wild-type laccase increased during the first 4 h-incubation at 60°C, whereas no evident thermal activation was observed for the D500G (Fig. 2b). Heat activation is commonly reported for thermostable bacterial laccases, many of them undergo activation after short incubation at elevated temperatures [17,18,19]. For example, activity of laccases from *Thermobaculum terrenum* and *Bacillus clausii* increased up to about 200% within 20–60 min of incubation at 70°C [17,18]. This phenomenon may be explained by the removal of possible inhibitory molecules from the enzyme active site or the formation of a more flexible and active conformation when the protein is incubated at increasing temperature [18,19]. Moreover, the D500G mutant had a lower half-life of inactivation at 70°C than the wild-type. This negative effect was not observed for the D500G mutant of *B. licheniformis* DSM13 laccase, which exhibited similar stability at 70°C to its wild-type [11]. According to previous report, the wild-type laccase from *B. licheniformis* DSM13 remained 43% of activity after 1 h incubation at 70°C [20]. Therefore, the D500G in the present study showed a slightly higher half-life at 70°C compared to the D500G mutant of *B. licheniformis* DSM13 laccase. In general, there is a trade-off between enzymatic activity and stability, introducing mutations for enhanced activity is normally achieved at the cost of thermostability [21]. Homology model analysis suggests that an Asp-to-Gly substitution reduced the number of hydrogen bonds formed at position 500, which may explain the decrease of protein thermostability (Fig. 5b and c).

Although the D500G variant could decolorize IC without mediator addition, the decolorizing activity was very low (less than 20% after 1 h). A significant improvement in decolorization occurred when ACE was used as mediator (Fig. 3). Similar results have been reported for the D500G mutant of *B. licheniformis* DSM13 laccase, which displayed poor activity toward IC and could almost completely decolorize it with violuric acid as mediator [11]. The decolorization efficiency of RB19 was much lower than IC. In the presence of mediator, only 35% and 42% decolorization were achieved after 1 h for the D500G mutants from *B. licheniformis* LS04 and *B. licheniformis* DSM13, respectively [11].

The strong and tightly regulated AOX1 promoter, which is fully induced upon the addition of methanol, is the most used promoter in

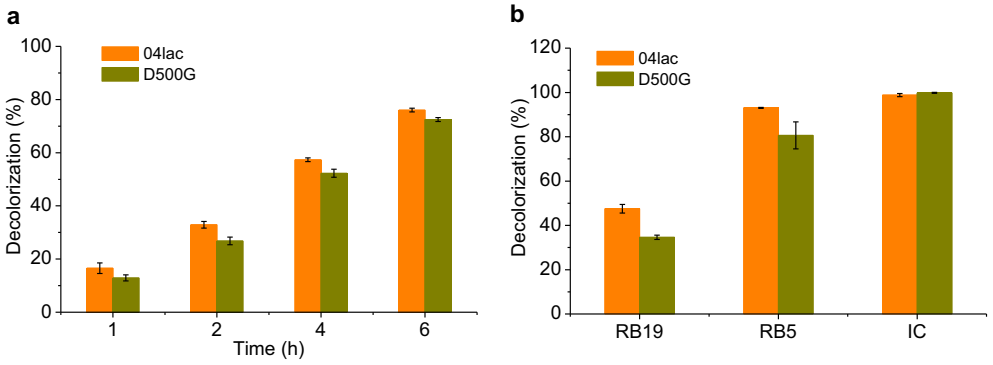

Fig. 3. Decolorization of synthetic dyes by 04lac and D500G. (a) Indigo carmine (IC) was decolorized without mediator; (b) reactive blue 19 (RB19), reactive black 5 (RB5) and IC were decolorized in the presence of 0.1 mM acetosyringone in 1 h.

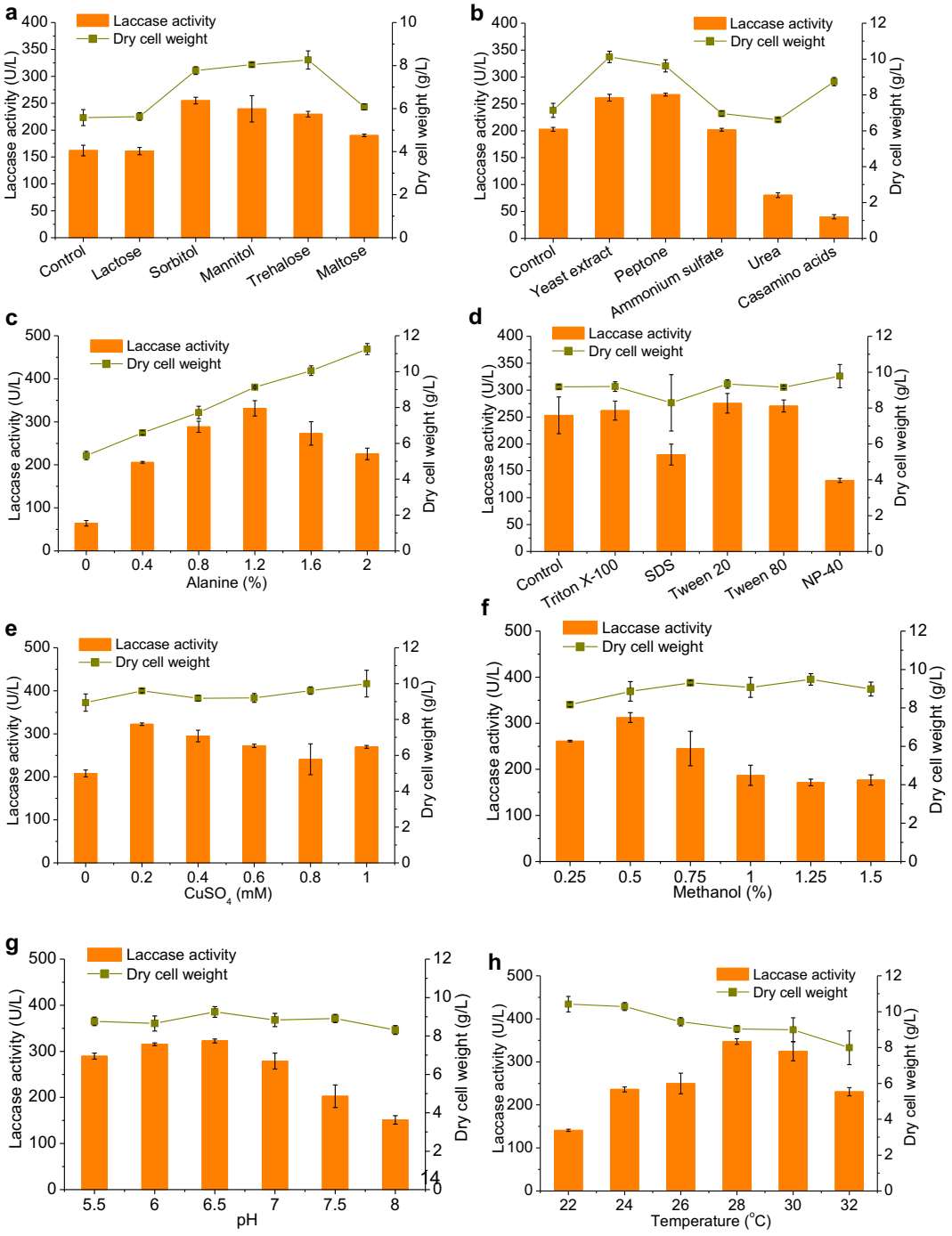

Fig. 4. Effects of various cultivation factors on D500G expression in *P. pastoris*. (a) carbon sources; (b) nitrogen sources; (c) alanine concentration; (d) surfactants; (e) copper concentration; (f) methanol concentration; (g) pH; (h) temperature.

heterologous protein production by *P. pastoris*. However, it can be severely repressed by common substrates like glucose and glycerol [6]. Mixed feeding of non-repressing carbon sources and methanol is an alternative to ensure good cell growth while inducing the expression of foreign protein. Several carbon sources such as sorbitol, mannitol, trehalose, and alanine have been reported to be used as co-substrates for increasing product titers [8,12,13]. All the tested carbon sources demonstrated a positive effect in increasing biomass and laccase activity except lactose, and sorbitol supported the highest increase in laccase productivity (Fig. 4a). Sorbitol co-feeding during the induction phase can not only decrease cell specific oxygen uptake and heat production but also increase the induction level of AOX1 promoter [22]. The other benefits of sorbitol co-feeding strategy include enhancement of cell viability and decrease of proteolytic activity [23]. Despite alanine is less frequently used in mixed feeding strategy than sorbitol, it is very useful in expression of foreign proteins that are sensitive to acidic conditions. Alanine can serve as both carbon and nitrogen source for *P. pastoris*, which favors cell growth, and thus increases the production yield. Furthermore, the metabolism of alanine has positive buffering effects and can maintain the pH of medium for a long period of time [8,13,24]. As the laccase from *B. licheniformis* is not stable in acidic environment [7], alanine addition greatly improved the expression level of the D500G mutant (Fig. 4c). Similarly, alanine supplemented medium was found to be more

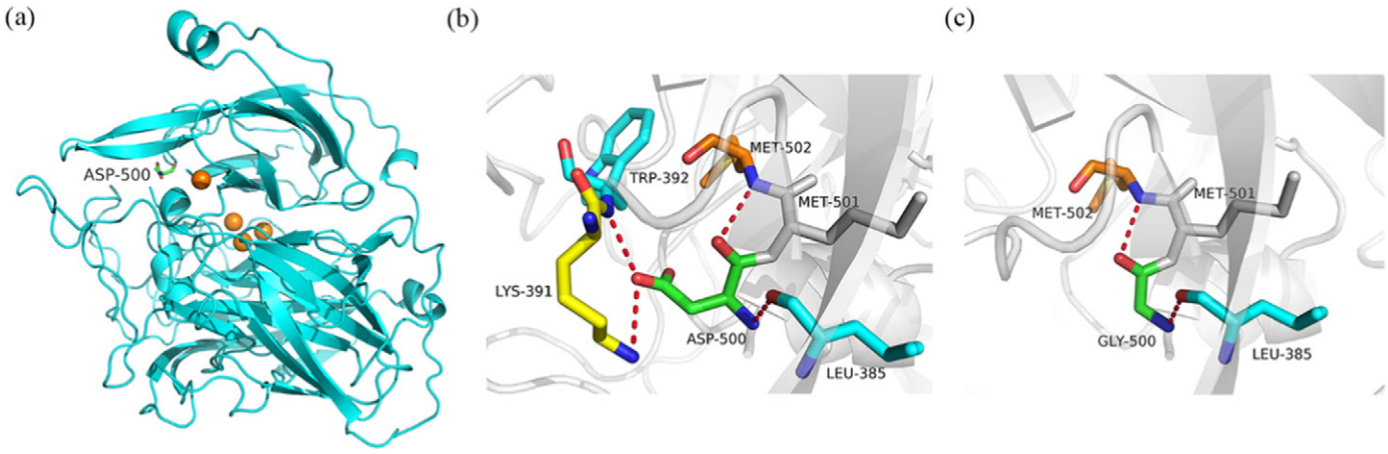

Fig. 5. Homology model of the wild type laccase and D500G mutant. (a) Asp500 is shown in green. The copper atoms are highlighted in orange; Hydrogen bonds before (b) and after (c) mutation are shown as red dashes.

efficient in producing shrimp trypsinogen by *P. pastoris*, which may be ascribed to improved cell viability and greater stability of the recombinant protein [13]. High concentration of alanine might create an imbalance between the protein synthesis and protein folding and secretion, and thus resulted in protein accumulation and proteolysis [25]. Therefore, a decline in laccase expression level was observed when the alanine concentration exceeded 1.2% (Fig. 4c).

Nitrogen limitation may lead to an increase in protease activity. Supplementation of amino acid-rich compounds, such as yeast extract or casamino acids, has been shown to have positive effects on protein production [12,26]. These supplements may inhibit protease activity by acting as alternative and competing substrates and repress protease induction caused by nitrogen starvation [27]. Although addition of casamino acids facilitated the growth of *P. pastoris*, it negatively affected the laccase production (Fig. 4b). This was possibly due to the instability of laccase in casamino acids supplemented culture or the inhibition effect of casamino acids on laccase expression, as there was little increase in laccase activity after the first day of induction.

Typically, an appropriate copper concentration in the medium is required for the production of active laccase in heterologous hosts [28,29]. In our previous study, laccase activity is nearly undetectable (<5 U/L) when the wild-type *B. licheniformis* laccase was expressed in *P. pastoris* without copper supplementation. By contrast, a significantly higher activity for D500G (208 ± 8 U/L) was obtained when the recombinant *P. pastoris* was grown in the absence of exogenously added copper (Fig. 4e). Apart from improving laccase catalytic activity, mutagenesis of laccase gene may result in increase of correctly folded enzyme. Furthermore, copper concentration of the medium can be slightly increased after optimization, as medium constituents like peptone carry trace amounts of copper. During the induction phase, methanol is fed as inducer and carbon source to support the production of foreign protein. The excess methanol in the medium can, however, have adverse effect on cell growth and production yield. This is due to the accumulation of formaldehyde and hydrogen peroxide, which are oxidized products of methanol by alcohol oxidase [10,30].

While *P. pastoris* is capable of growing well across a broad pH range (from 3.0 to 7.0), pH control is usually necessary for heterologous protein production. As it may affect both the product stability and proteolysis activity [27], therefore, the optimal pH value of the growth medium is largely depended on the target protein. For example, the highest enzyme activity for β-glucosidase from *Pichia etchellsii* expressed in *P. pastoris* was observed at pH 7.5 [12], whereas the maximal expression level for β-glucosidase from *Saccharomycopsis fibuligera* was obtained at pH 4.5 [30]. The low pH can severely decrease the expression level of both fungal and bacterial laccases [8,24]. Hence, maintaining the pH of culture medium in the range of 5.5 to 6.5 may be favorable for heterologous laccase production in *P. pastoris*. Most processes with *P. pastoris* are run at a temperature of 30°C or below, since high temperature impairs cell viability and results in folding problems and an increase in protease release [27]. Decreasing the induction temperature from 30°C to 22°C led to a 342% higher activity for leech hyaluronidase expressed in *P. pastoris* [31]. For the expression of D500G, a cultivation temperature below 28°C caused a remarkable reduction in laccase activity. Generally, decreasing temperature leads to a lower specific growth rate for *P. pastoris*, whereas no obvious change in cell growth was observed for *P. pastoris* expressing leech hyaluronidase when it was cultivated at 22–30°C [31]. In the present study, the biomass production was even enhanced at lower temperatures. Similar results have been reported for the production of *Rhizopus oryzae* lipase by *P. pastoris*, which may be partially explained by an increase in the average yield of substrate-into-biomass when lowering the temperature [32].

Conflict of interest

The authors declare that they have no conflict of interest.

Financial support

This work was supported by the National Natural Science Foundation of China (31200394) and the Fundamental Research Funds for the Central Universities (2572015EA02, DL13CA09).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2017.04.002.

References

[1] Forootanfar H, Faramarzi MA. Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications. Biotechnol Prog 2015;31:1443–63. http://dx.doi.org/10.1002/btpr.2173.
[2] Pezzella C, Guarino L, Piscitelli A. How to enjoy laccases. Cell Mol Life Sci 2015;72:923–40. http://dx.doi.org/10.1007/s00018-014-1823-9.
[3] Martins LO, Durão P, Brissos V, Lindley PF. Laccases of prokaryotic origin: Enzymes at the interface of protein science and protein technology. Cell Mol Life Sci 2015;72:911–22. http://dx.doi.org/10.1007/s00018-014-1822-x.
[4] Piscitelli A, Pezzella C, Giardina P, Faraco V, Giovanni S. Heterologous laccase production and its role in industrial applications. Bioeng Bugs 2010;1:252–62. http://dx.doi.org/10.4161/bbug.1.4.11438.
[5] Mate DM, Alcalde M. Laccase engineering: From rational design to directed evolution. Biotechnol Adv 2015;33:25–40. http://dx.doi.org/10.1016/j.biotechadv.2014.12.007.

[6] Ahmad M, Hirz M, Pichler H, Schwab H. Protein expression in *Pichia pastoris*: Recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 2014;98:5301–17. http://dx.doi.org/10.1007/s00253-014-5732-5.
[7] Lu L, Wang TN, Xu TF, Wang JY, Wang CL, Zhao M. Cloning and expression of thermo-alkali-stable laccase of *Bacillus licheniformis* in *Pichia pastoris* and its characterization. Bioresour Technol 2013;134:81–6. http://dx.doi.org/10.1016/j.biortech.2013.02.015.
[8] Wang TN, Lu L, Wang JY, Xu TF, Li J, Zhao M. Enhanced expression of an industry applicable CotA laccase from *Bacillus subtilis* in *Pichia pastoris* by non-repressing carbon sources together with pH adjustment: Recombinant enzyme characterization and dye decolorization. Process Biochem 2015;50:97–103. http://dx.doi.org/10.1016/j.procbio.2014.10.009.
[9] Chen B, Xu WQ, Pan XR, Lu L. A novel non-blue laccase from *Bacillus amyloliquefaciens*: Secretory expression and characterization. Int J Biol Macromol 2015;76:39–44. http://dx.doi.org/10.1016/j.ijbiomac.2015.02.019.
[10] Çalik P, Ata Ö, Güneş H, Massahi A, Boy E, Keskin A, et al. Recombinant protein production in *Pichia pastoris* under glyceraldehydes-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochem Eng J 2015;95:20–36. http://dx.doi.org/10.1016/j.bej.2014.12.003.
[11] Koschorreck K, Schmid RD, Urlacher VB. Improving the functional expression of a *Bacillus licheniformis* laccase by random and site-directed mutagenesis. BMC Biotechnol 2009;9:12. http://dx.doi.org/10.1186/1472-6750-9-12.
[12] Batra J, Beri D, Mishra S. Response surface methodology based optimization of β-glucosidase production from *Pichia pastoris*. Appl Biochem Biotechnol 2014;172: 380–93. http://dx.doi.org/10.1007/s12010-013-0519-1.
[13] Guerrero-Olazarán M, Escamilla-Treviño LL, Castillo-Galván M, Gallegos-López JA, Viader-Salvadó JM. Recombinant shrimp (*Litopenaeus vannamei*) trypsinogen production in *Pichia pastoris*. Biotechnol Prog 2009;25:1310–6. http://dx.doi.org/10.1002/btpr.197.
[14] Nasoohi N, Khajeh K, Mohammadian M, Ranjbar B. Enhancement of catalysis and functional expression of a bacterial laccase by single amino acid replacement. Int J Biol Macromol 2013;60:56–61. http://dx.doi.org/10.1016/j.ijbiomac.2013.05.011.
[15] Schutter KD, Lin YC, Tiels P, Hecke AV, Glinka S, Weber-Lehmann J, et al. Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat Biotechnol 2009;27:561–6. http://dx.doi.org/10.1038/nbt.1544.
[16] Ata Ö, Boy E, Güneş H, Çalık P. Codon optimization of *xylA* gene for recombinant glucose isomerase production in *Pichia pastoris* and fed-batch feeding strategies to fine-tune bioreactor performance. Bioprocess Biosyst Eng 2015;38:889–903. http://dx.doi.org/10.1007/s00449-014-1333-z.
[17] Brander S, Mikkelsen JD, Kepp KP. TtMCO: A highly thermostable laccase-like multicopper oxidase from the thermophilic *Thermobaculum terrenum*. J Mol Catal B: Enzym 2015;112:59–65. http://dx.doi.org/10.1016/j.molcatb.2014.12.002.
[18] Brander S, Mikkelsen JD, Kepp KP. Characterization of an alkali- and halide-resistant laccase expressed in *E coli*: CotA from *Bacillus clausii*. PLoS One 2014;9:e99402. http://dx.doi.org/10.1371/journal.pone.0099402.
[19] Kumar S, Jain KK, Rani S, Bhardwaj KN, Goel M, Kuhad RC. *In-vitro* refolding and characterization of recombinant laccase (CotA) from *Bacillus pumilus* MK001 and its potential for phenolics degradation. Mol Biotechnol 2016;58:789–800. http://dx.doi.org/10.1007/s12033-016-9978-2.
[20] Koschorreck K, Richter SM, Ene AB, Roduner E, Schmid RD, Urlacher VB. Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. Appl Microbiol Biotechnol 2008;79:217–24. http://dx.doi.org/10.1007/s00253-008-1417-2.
[21] Romero PA, Arnold FH. Exploring protein fitness landscapes by directed evolution. Nat Rev Mol Cell Bio 2009;10:866–76. http://dx.doi.org/10.1038/nrm2805.
[22] Niu H, Jost L, Pirlot N, Sassi H, Daukandt M, Rodriguez C, et al. A quantitative study of methanol/sorbitol co-feeding process of a *Pichia pastoris* Mut^+/pAOX1-lacZ strain. Microb Cell Fact 2013;12:33. http://dx.doi.org/10.1186/1475-2859-12-33.
[23] Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, et al. Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation. Bioresour Technol 2010;101:1318–23. http://dx.doi.org/10.1016/j.biortech.2009.09.025.
[24] O'Callaghan J, O'Brien MM, McClean K, Dobson ADW. Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. J Ind Microbiol Biotechnol 2002;29:55–9. http://dx.doi.org/10.1038/sj.jim.7000268.
[25] Puxbaum V, Mattanovich D, Gasser B. Quo vadis? The challenges of recombinant protein folding and secretion in *Pichia pastoris*. Appl Microbiol Biotechnol 2015; 99:2925–38. http://dx.doi.org/10.1007/s00253-015-6470-z.
[26] Joshi S, Satyanarayana T. Bioprocess for efficient production of recombinant *Pichia anomala* phytase and its applicability in dephytinizing chick feed and whole wheat flat Indian breads. J Ind Microbiol Biotechnol 2015;42:1389–400. http://dx.doi.org/10.1007/s10295-015-1670-1.
[27] Zhang Y, Liu R, Wu X. The proteolytic systems and heterologous proteins degradation in the methylotrophic yeast *Pichia pastoris*. Ann Microbiol 2007;57: 553–60. http://dx.doi.org/10.1007/BF03175354.
[28] Durão P, Chen Z, Fernandes AT, Hildebrandt P, Murgida DH, Todorovic S, et al. Copper incorporation into recombinant CotA laccase from *Bacillus subtilis*: Characterization of fully copper loaded enzymes. J Biol Inorg Chem 2008;13: 183–93. http://dx.doi.org/10.1007/s00775-007-0312-0.
[29] Hoshida H, Fujita T, Murata K, Kubo K, Akada R. Copper-dependent production of a *Pycnoporus coccineus* extracellular laccase in *Aspergillus oryzae* and *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 2005;69:1090–7. http://dx.doi.org/10.1271/bbb.69.1090.
[30] Ma Y, Liu X, Yin Y, Zou C, Wang W, Zou S, et al. Expression optimization and biochemical properties of two glycosyl hydrolase family 3 beta-glucosidase. J Biotechnol 2015;206:79–88. http://dx.doi.org/10.1016/j.jbiotec.2015.04.016.
[31] Berrios J, Flores MO, Díaz-Barrera A, Altamirano C, Martínez I, Cabrera Z. A comparative study of glycerol and sorbitol as co-substrates in methanol-induced cultures of *Pichia pastoris*: Temperature effect and scale-up simulation. J Ind Microbiol Biotechnol 2017;44:407–11. http://dx.doi.org/10.1007/s10295-016-1895-7.
[32] Kang Z, Zhang N, Zhang Y. Enhanced production of leech hyaluronidase by optimizing secretion and cultivation in *Pichia pastoris*. Appl Microbiol Biotechnol 2016;100:707–17. http://dx.doi.org/10.1007/s00253-015-7056-5.