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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

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ABSTRACT

In this study, protease-deficient *Pichia pastoris* strain SMD1168H was selected for heterologous expression of the CotA laccase from *Bacillus subtilis*. Four non-repressing carbon sources were individually applied to facilitate the recombinant laccase production. An up to 76-fold increase in laccase activity was achieved through sorbitol addition in combination with pH adjustment. Recombinant CotA (rCotA) demonstrated a remarkable stability under alkaline conditions, the enzyme retained 637% and 94.37% of its initial activity after 10-day incubation at pH 9.0 and 10.0, respectively. The rCotA laccase showed an outstanding thermostability and exhibited higher tolerance towards organic solvents than the spore laccase. The K_m and k_{cat} values of rCotA laccase for ABTS were of 146.4 ± 2.7 μM and $14.4 \pm 0.1\ s^{-1}$ and for SGZ of 12.7 ± 2.6 μM and $6.9 \pm 0.6\ s^{-1}$, respectively. A repeated-batch decolorization experiment was carried out at pH 10.0, 40 °C to evaluate the capacity of rCotA for repetitive decolorization of indigo carmine under alkaline conditions. During the first six repeated cycles, more than 95% decolorization was observed in 10 min and total color was removed within one hour. The decolorization efficiency stayed above 90% after twenty decolorization cycles.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are members of multi-copper oxidase (MCO) groups. These enzymes catalyze the oxidation of substrates with the concomitant reduction of oxygen to water. Laccases have a nonspecific oxidation capacity; they can catalyze a wide range of reactions including cross-linking of phenolic compounds, degradation of polymers, and ring cleavage of aromatic compounds [1]. Owing to their low substrate specificity, laccases have attracted considerable interest in various fields, including pulp bleaching, industrial effluent treatment and biosensors, etc. [2–4]. Laccases are widely distributed in plants, fungi, bacteria and insects. Although numerous laccases, especially microbial laccases have been well characterized, their applications in industrial processes have so far been limited. Since most industrial processes may require harsh conditions, such as high temperatures, extremely acidic/alkaline pHs and high ionic strengths, laccases usually lose their activities under these conditions [5]. Therefore, it is necessary to develop robust laccases that show high resistance to these adverse conditions.

Bacillus strains form endospores to resist harsh conditions. The dormant cells are surrounded by a multi-protein coat consisting of around 60 types of proteins, which participate in spore resistance, morphogenesis and enzymatic functions [6]. One of the spore coat proteins, named CotA, has been verified to exhibit laccase activity [7]. Moreover, CotA laccase is able to maintain high activity under elevated temperatures, alkaline conditions and other tough environments. The remarkable properties of CotA laccase make the enzyme a potential catalyst for industrial applications. To date, the CotA laccases of different *Bacillus* species have been used in biofuel cell applications, dye effluent treatment, and lignocellulose surface functionalization in laboratory-scale [8–11]. Nevertheless, it is challenging to achieve large-scale production of CotA laccase for commercial use, as the spore-bound nature of CotA severely handicaps the preparation/purification of the enzyme. Investigators attempted to tackle the problems by expressing CotA laccase in *Escherichia coli* [12–14]. However, the majority of the recombinant CotA laccases were expressed as inclusion bodies and the expression level was unsatisfactory to meet industrial demands. In contrast, the eukaryotic host *Pichia pastoris* has a more

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sophisticated post-translational modification system and has been successfully used to express high yields of recombinant proteins through high-cell-density fermentation [15], which is suitable for the large-scale production of CotA laccase.

Previously, a *Bacillus subtilis* strain LS02 was isolated from forest soil by our research group and the spore laccase from the strain was well characterized to be an alkali resistant, thermostable, organic solvent tolerant enzyme with outstanding potentials in dye decolorization [16]. To furthermore enlarge the production of LS02 CotA laccase for practical application, *P. pastoris* was selected as the expression host for heterologous expression of the recombinant laccase. Non-repressing carbon sources were used to enhance the production of rCotA laccase. The biochemical properties of rCotA laccase were characterized. Also, the capacity of rCotA laccase for repetitive dye decolorization under alkaline conditions was evaluated.

2. Materials and methods

2.1. Strains, plasmids and reagents

B. subtilis strain LS02 is stored in the China General Microbiological Culture Collection Center (CGMCC No. 4261). The sequence of *CotA* gene from LS02 has been deposited in GenBank (Accession No: GU972587). *E. coli* strains Top10 and JM109 were supplied by Tiangen (Beijing, China) and Takara (Dalian, China), respectively. *P. pastoris* strain SMD1168H and expression vector pPICZαB were purchased from Invitrogen (Carlsbad, CA, USA). PCR reagents, restriction enzymes, pMD18-T vector and T4 DNA ligase were from Takara (Dalian, China). DNA manipulation kits were supplied by Omega (Norcross, GA, USA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), syringaldazine (SGZ), indigo carmine and acetosyringone were from Sigma–Aldrich (St. Louis, MO, USA). Other reagents were of analytical grade.

Yeast Extract Peptone Dextrose Medium (YPD), Yeast Extract Peptone Dextrose Medium with sorbitol (YPDS), Buffered Glycerol-complex Medium (BMGY) and Buffered Minimal Methanol Medium (BMM) were prepared according to the manual of the Easyselect *Pichia* Expression Kit (Invitrogen). BMM medium containing 0.1 mM $CuSO_4$ (BMMC) was used as induction medium for recombinant laccase expression. To test the effects of non-repressing carbon sources on rCotA expression, 0.5% (w/v) mannitol/sorbitol/trehalose/alanine was initially added to BMMC medium to make BMMCM/BMMCS/BMMCT/BMMCA medium for induction use. The initial pH of BMGY, BMMC, BMMCM, BMMCS, BMMCT and BMMCA medium was 6.0 unless otherwise stated.

2.2. Recombinant expression vector construction

The *CotA* gene was amplified using the primers *CotA*-F (5′-GAACTGCAGGCACACTTGAAAAATTTG-3′) and *CotA*-R (5′-TCCCCGCGGTTATTTATGGGGATCAG-3′). The underlined sequences correspond to *Pst*I and *Sac*II restriction sites. The PCR amplification conditions were as follows: initial denaturation at 94 °C for 4 min followed by 30 cycles each consisting of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 2 min. The amplified DNA fragment was purified and inserted into a pMD18-T vector. Thereafter, the CotA-pMD18-T vector was sequentially digested by *Pst*I and *Sac*II and the resulting CotA fragment was subcloned into pPICZαB vector. Recombinant expression vector CotA-pPICZαB was transformed into *E. coli* Top10 cells and the positive transformants were confirmed by sequencing.

2.3. Transformation of Pichia pastoris and screening of recombinant clones

The recombinant expression vector CotA-pPICZαB was linearized with *Sac*I and was then transformed into *P. pastoris* SMD1168H through electroporation (Eppendorf Eporator, Hamburg, Germany). Positive transformants available on YPDS agar plates containing 100 mg/mL zeocin were further screened on BMMC agar plates supplemented with 0.1 mM ABTS. The colonies carrying larger green zones were inoculated into 30 mL BMGY media in 150 mL baffled flasks and incubated at 30 °C, 200 rpm until the OD_{600} reached 1.5–2.0. Then the cultures were centrifuged at 3000 × *g* and the cell pellets were resuspended to OD_{600} of 1.0 with 50 mL BMMC media. The cultures were cultivated in 250 mL baffled flasks at 30 °C, 200 rpm, with 0.5% (v/v) methanol being added every 24 h. The strain with highest laccase activity was named SMD1168H-CotA and stored for subsequent study.

2.4. Non-repressing carbon sources assisted cultivation of SMD1168H-CotA

A single colony of SMD1168H-CotA was inoculated in 100 mL BMGY medium in a 500 mL flask, and was cultivated at 30 °C, 200 rpm until the OD_{600} of the culture reached 2.0–6.0. The cells were harvested during the log phase and were suspended into 100 mL BMMCM/BMMCS/BMMCT/BMMCA induction media to an OD_{600} of 1.0. The induction was performed at 30 °C, 200 rpm. Every 24 h, 2 mL culture fluids were sampled for analysis and methanol was subsequently added to the cultures to a final concentration of 0.5% (v/v) for induction. Samples were transferred to pre-weighed centrifuge tubes for centrifugation (1300 rpm, 10 min). The supernatants were taken for determination of laccase activity and pH values. The tubes containing the pellets were dried to constant weight at 80 °C and then measured for cell dry weight. The BMMC medium was used as the control for rCotA production. All assays were performed in triplicate.

Another experiment was performed in parallel except that the initial pH of the induction media was buffered to 7.0 and the pH of the induction media was daily adjusted to 7.0 by 10% ammonium hydroxide. All assays were performed in triplicate.

2.5. Purification of the recombinant CotA

The culture supernatant was filtered through a 0.45 μm filter, and was treated with ammonium sulfate to 80% saturation at 0 °C. The precipitated protein was harvested by centrifugation at 10,000 × *g*, 4 °C for 30 min, and was dissolved in sterile deionized water. The enzyme solution was desalted and concentrated by 30-kDa Amicon ultra-15 centrifugal filter devices (Millipore, Bedford, MA, USA). Later, the sample was loaded on to a DEAE-Sepharose Fast Flow column (Dingguo ChangSheng Biotech Co. Ltd, Beijing, China) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5) at room temperature. The proteins were gradually eluted by NaCl gradient (50–1000 mM in equilibrating buffer). Fractions with highest laccase activity were pooled and used for biochemical analysis.

The culture supernatant and purified rCotA were subjected to SDS-PAGE (12% separation gel and 5% stacking gel). Blue Plus™ Protein Marker (14–100 kDa) (Transgen, Beijing, China) was used as molecular weight standard. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 and analyzed using Image Lab Software (Bio-rad, Richmond, CA, USA). The band of purified rCotA was excised from SDS-PAGE gel, digested with trypsin, and was subjected to a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) for analysis.