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ORIGINAL ARTICLE

Molecular cloning, expression and characterization of *poxa1b* gene from *Pleurotus ostreatus*

Mahnaz Mohtashami[1] · Jamshid Fooladi[2] · Aliakbar Haddad-Mashadrizeh[3,4] · Mohammadreza Housaindokht[5]

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Abstract

In recent decades, fungus laccases (p-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) have attracted the attention of researches due to their wide range of biotechnological and industrial applications. In the present study, we have cloned a gene encoding laccase (*poxa1b*) from *Pleurotus ostreatus* and then heterologously expressed in *Escherichia coli* BL21. The biochemical properties of POXA1b were characterized using ABTS as a typical substrate of laccases. Moreover, the in vitro oxidation of the benzo[a]pyrene was investigated in the presence or absence of ABTS. The codon-optimized *poxa1b* showed higher expression yields and efficiency in comparison with the wild-type ($p < 0.01$). The maximum activity of POXA1b (2075 UL-1) was observed after incubation at 50 °C for 0.5 h and the enzyme retained more than 85% of its initial activity after 2 h incubation at 25–45 °C. The optimum pH of the enzyme was pH4 and the enzyme was stable when being incubated at pH range from 2.5 to 4.5 for 2 h in the absence of ABTS, the enzyme oxidized a little amount of benzo[a]pyrene, whereas its oxidation enhanced following the ABTS addition. These findings indicate POXA1b of *P. ostreatus* as a promising candidate for further biotechnological approaches.

Keywords Laccases · POXA1b · Codon optimization · Cloning and expression · ABTS · Benzo[a]pyrene

Introduction

Laccases are blue multi-copper oxidases (EC 1.10.3.2) that catalyze the oxidation of a broad range of organic and inorganic substrates by coupling the reduction of O_2 to H_2O [1]. Laccases' reaction has certain features such as being cofactor independent, do not produce any toxic intermediates and produce water at the end of the reaction making them attractive candidates in "greening" chemical processes [2, 3]. In addition, using laccases capacity has raised interest in many industries including pulp delignification, textile dye bleaching and bioremediation, petrochemical, paper and food processing, due to their suitable properties to oxidize a wide range of substrates [4, 5]. However, the practical use of laccases in industrial contexts is faced with certain obstacles, such as the large scale production of enzymes. The production of laccases from native sources are not suitable to be used in industrial applications because it has inefficient yields and the purification procedure is expensive [4, 6]. Heterologous expression of laccases having similar properties, including high substrate specificity and desired stability as the ones from their native source can pave the way for their large scale production with more reasonable cost [4, 7].

Laccases are produced by means of a wide range of organisms such as bacteria, higher plants, insects and fungi. Prokaryotic sources of laccases display a higher thermal and alkaline pH stability in comparison to their eukaryotic counterparts [8]. However, the bacterial laccases display a low redox potential, which is a crucial factor impacting on substrate specificity and which prohibit the oxidation of high reduction potential substrates [9]. Fungi laccases were the center of attention of many researchers in the last few decades due to certain properties

✉ Aliakbar Haddad-Mashadrizeh
a.haddad@um.ac.ir

1 Department of Microbiology, Faculty of Biological Science, Alzahra University, Tehran, Iran

2 Department of Biotechnology, Faculty of Biological Science, Alzahra University, Tehran, Iran

3 Recombinant Proteins Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

4 Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

5 Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

such as high rodex potential and they exhibit an improved stability for application in harsh conditions of industrial fields [5, 10–12]. *Pleurotus ostreatus* (*P. ostreatus*), a white -rot basidiomycete, has been considered as the potential source of laccase enzymes during the last decade [13, 14]. *P. ostreatus* secretes different extracellular laccase isoenzymes such as phenol oxidase A1b (POXA1b), POXA2, and POXC. POXA1b is a high redox potential (+0.650 V) laccase that showed remarkable potential for industrial applications. POXA1b has noticeable properties like thermostability in a wide range of temperatures (25–65 °C) and its half time (t1/2) is nearly 3 h at 60 °C. It is also stable in the pH range of 3 to 9 and displays a considerable stability at pH 9 (30 days) [14, 15]. Furthermore, its high production yield at heterologous expression systems has put it as a proper candidate for large scale production of laccases [12, 16]. POXA1b has been reported to be successfully applied in different fields such as the synthesis of dyes [17], bioremediation [11, 18] and fruit juice clarification [19].

There is a broad range of hosts for heterogeneous production of fungi proteins. *Escherichia coli* (*E. coli*) is one of the excellent and most favorable protein expression systems for the production of heterologous proteins due to its desirable properties, including rapid growth, rapid expression, ease of culture and high production yield [20, 21]. There is a problem in using *E. coli* as a host to the heterogeneous expression of eukaryote proteins because of the significant differences in codon usage [22–25]. Codon optimization is required to improve the production of eukaryote protein in *E. coli* expression system [24, 26, 27]. The Codon optimization Performed by two alternative strategies, includes introducing a plasmid encoding tRNAs that are rare in *E. coli* or changing the scarce codon in the target gene in accordance with the codon preference characteristics of the *E. coli* without the alteration in amino acid sequence of the target protein [22, 25, 26, 28]. The aim of this research is to clone and express fungal laccases *poxa1b* gene from *P. ostreatus* in .*E. coli* following codon optimization. In addition, the activity and stability of recombinant POXA1b under different pH and temperature conditions were evaluated and its capability for oxidation of benzo[a]pyrene (BaP) was determined. To the best of our knowledge, this work is the first report on successful recombinant POXA1b production with unique features using codon optimization in *E. coli* and that it is an opportunity for large scale production of the enzyme.

Materials and methods

Chemicals, microorganisms, vectors

BaP and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Merk (Darmstadt, Germany). All reagents used were analytic grade and were purchased from Sigma. *E. coli* DH5a competent cells and *E. coli* BL21 (DE3) competent cells were employed as hosts for cloning and protein expression, respectively and obtained from PTCC. *E. coli* strains DH5a and BL21 (DE3) were cultured in Luria–Bertani (LB) agar medium (10 g/l bacto tryptone, 10 g/l NaCl, 5 g/l yeast extract). Selective medium was supplemented with 100 µg/ml of ampicillin. PET-22b(+) plasmid was used as a vector for *poxa1b* cloning and expression. Molecular biology techniques were prepared and performed using the standard protocols.

Codon optimization web servers

The codon optimization strategy employed in the present study is referred to as 'one amino acid-one codon'. In this method, the most preferred codon of the *E. coli* expression system for a given amino acid is utilized in the sequence of target [29]. The sequence of *P. ostreatus* was obtained from GeneBank (GenBank AJ005018). The optimizer web server (http://www.genoms.uvr.es) was used for rare codon detection. The *E. coli* rare codon analyzer2 (http://www.faculty.ucr.edu) was utilized for gene sequence optimization. The Gene script web server (http://www.genescript.com) was applied to analyze the designed sequence by Codon Adaptation Index (CAI).

Cloning and expression of the *poxa1b* gene

The sequence of codon optimized *poxa1b* and the native gene (1647-bp) were synthesized by Green Biosystems Company and cloned in Pet-22b(+) vector. The expected fragments were confirmed using digestion by *Xho*I and *Xba*I restriction enzyme and agarose gel electrophoresis. The POX1Ab/ Pet-22b(+) transformed into *E. coli* DH5α competent cells via the heat shock method. The cell components were mainly obtained by *E. coli* Calcium Chloride competent cell method. The LB medium containing 100 µg/ml of ampicillin was used to select the desired colons. The plasmid isolation was performed by a plasmid extraction kit (Thermo Fisher Scientific).

Recombinant Pet/*poxa1b* was transformed into *E. coli* BL21 (DE3) competent cells for protein overproduction. Strains of *E. coli* BL21 were grown in LB medium supplemented by 2 mg/ml of ampicillin and incubated at 37 °C for 18 h with 250 rpm shaking. At a culture broth OD_{600} of 0.4–0.7 recombinant protein expression was induced by supplementing with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 0.25 mM $CuSo_4$, as indicated by Guan et al. [30]. The cells were incubated in a shaking incubator (200 rpm) at 30 °C for 12 h. Moreover, to investigate the optimum condition of POXA1b expression in *E. coli* BL21 (DE3), the expression process was performed under different cultivation conditions such as different IPTG

concentration (0.5–2.5 mM), induction time (2–12 h) and temperature (20–45 °C). The cells were collected by centrifugation and then resuspended in cell resuspension buffer (pH 7.9) containing 500 mM NaCl, 20 mM Tris–HCl, 5 mM Imidazole. After sonication, the cell extracts were processed to be used in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and laccase analytical activity.

Electrophoresis analysis

The SDS-PAGE was used to confirm recombinant protein expression process and to determine molecular weight of the laccases enzyme in codon-optimized and native samples. The SDS-PAGE was carried out in a 12% polyacryl amide gel and the proteins were visualized by the staining of gel with Coomassie brilliant blue. The molecular weight of the expressed laccase was estimated by standard molecular weight markers (10–250 kDa, Bio-Rad).

Laccases enzyme assay

The total Protein content in the cell extracts of the codon-optimized sample and the native sample was measured using the Bradford assay based on bovine serum albumin standard curve. The enzyme activity in cell extracts was assayed by measuring the ABTS oxidation at 25 °C. The rate of ABTS oxidation was determined by monitoring the increase in A420 (ε 420 = 3600 LM^{-1} cm^{-1}), as reported previously [31]. The reaction mixture contained 1 mM ABTS, 0.1 M sodium acetate buffer (pH 5), 100 µl culture supernatants and incubated for 10 min. The absorbance values of the samples were read using a spectrophotometric plate reader at 420 nm against a satiable blank. One unit of laccase enzyme activity was expressed as the amount of the enzyme that oxidized 1 µmol of ABTS per minute and was determined after an assay time of 2 min using this formula [32]: $A\left(UL^{-1}\right) = \Delta EVt/0.036Vs$. In this equation U is the enzyme activity, ΔE is the enhancement in absorbance per minute, Vt is the cuvette total volume (ml), and Vs is the sample volume in the cuvette (ml).

Oxidation of polycyclic aromatic hydrocarbons (PAHs) by recombinant POXA1b

BaP as a typical substrate of laccases was assayed to evaluate PAH oxidation by codon-optimized POXA1b under the following conditions such as (1) heat denatured POXA1b (boiled at 100 °C for 20 min) [33] and BaP as control sample, (2) POXA1b and BaP, and (3) POXA1b, BaP and ABTS. All experiments were carried out in 40-ml tubes containing 5 ml reaction volume with POXA1b adjusted to 4 U/ml by diluting with Na-acetate buffer (pH 4.5). BaP was dissolved in acetone to give a 10 mM concentration and 35 µl of it added to the reaction mixture to a final concentration of 70 µM. The PAH bioavailability was increased by adding tween-80 to all tubes to a final concentration of 1%. ABTS was added to one experiment to a final concentration of 1 mM to determine the influence of it on oxidation of BaP. After incubation for 24 h at 30 °C, all experiments were centrifuged at 13,000×*g* for 10 min and the supernatants were then analyzed by high-performance liquid chromatography (HPLC). A HPLC system (Knauer, Bad Homburg, Germany) equipped with a dual λ absorbance detector and a Supercosil LC-PAH (5 µm, 25.0 cm × 4.6 mm ID) column was applied to separate the Bap. An analysis was performed using 40-min programed in an acetonitrile/ water gradient mode (0–5 min 40:60%, 5–30 min ramp to 0: 100% and 30–40 min hold at 0:100%) at a flow rate of 1.5 ml/min. The wavelength used to determine the concentration of BaP was 254 nm. The percentage of BaP oxidized was calculated from the area under the absorbance peak using this formula: [34] $[(C_i - C_f)/C_f] \times 100$. In this equation C_i and C_f are the concentration of BaP in the experiment and control respectively. All experiments including the controls were carried out in triplicate.

pH and thermal stability of laccases activity

The effect of temperature on the activity of codon-optimized laccase and its thermal stability were examined at various incubation temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65 °C) at pH 4 for 0.5, 2 and 10 h. The laccase activity as a function of pH and its pH stability were determined at the pH range of 3–10 (3–7, citrate phosphate buffer; 8, phosphate buffer; 9, bicarbonate buffer) at 25 °C for 0.5, 2, and 10 h [35]. Following the incubation of 100 µl culture supernatant containing codon-optimized enzyme in indicated conditions the enzyme activity was measured in the presence of the ABTS (1 mM) within 10 min as explained previously ("Laccases enzyme assay" section).

Statistical analysis

In this study, all experiments were done in triplicate and presented as the mean ± SD. The analysis of variance followed by a Tukey post-hoc test was conducted. Using the SPSS software (version 18.0). $p < 0.05$ the statistical analysis was considered significant.

Results

Optimizing the expression of *poxa1b* gene

The SDS-PAGE analysis of the cell extracts demonstrated that the cell culture medium induced with1Mm IPTG

showed an expression of POXA1b protein band in 57 kDa. No expression band was seen in non-induced experiment (Fig. 1). As shown in Fig. 1b the expression of codon-optimized POXA1b was higher in the presence of 1 mM IPTG, at 25 °C within 10 h compared with 1.5 mM IPTG, at 45 °C within 2 h respectively. In addition, the expression of POXA1 in codon-optimized sample was noticeable rather than the native sample (1 mM IPTG, at 25 °C within 10 h). Whereas, no difference was observed in the molecular weight of codon-optimized and the native enzyme indicating that the codon optimization was performed based on the bacterial host codon preference without any changes in amino acid sequences of the POXA1b protein. The laccase activity results demonstrated approximately threefold increase in the activity of POXA1B after codon optimization in comparison with the native enzyme in 25 °C (codon optimized enzyme: 1525 UL^{-1}; native enzyme :610 UL^{-1} ; $p < 0.01$).

Different conditions such as temperature, IPTG concentration and induction duration with IPTG were investigated to optimize the codon –optimized POXA1b expression in *E. coli* BL21 (DE3) when OD_{600} of broth medium was 0.6. The influence of various temperatures (25–45 °C) on POXA1b expression was evaluated within 12 h incubation time in the presence of 1 mM IPTG. The enzyme activity was estimated against ABTS as a typical substrate of laccases. The appearance of the green color in reaction corresponds to the oxidation of ABTS by POXA1b (Fig. 2). The highest level of POXA1b activity was observed at 25 °C (1525 UL^{-1}) and an increase in temperature reduced the enzyme activity (Fig. 3). The effect of IPTG concentration on POXA1b protein expression was evaluated in the presence of different concentrations of IPTG (0.5, 1, 1.5, 2. 2.5 mM) at 30 °C and 12 h incubation time. As shown in Fig. 4, the maximum activity of POXA1b was detected in the presence of 1 mM IPTG (1539 UL^{-1}) and then falls when the IPTG concentration was increased from 1 mM. Moreover, the effect of induction duration on POXA1b activity was measured within the time intervals of 2–12 h at 30 °C in the presence of 1 mM IPTG. As depicted in Fig. 5, the enzyme activity was increased when induction duration elevated form 2 to 12 h.

The effect of temperature on POXA1b activity

The optimum activity of the POXA1b was observed at 50 °C, with the value activity of 2075 UL^{-1} after incubation for 0.5 h. As shown in Fig. 6, the enzyme activity of

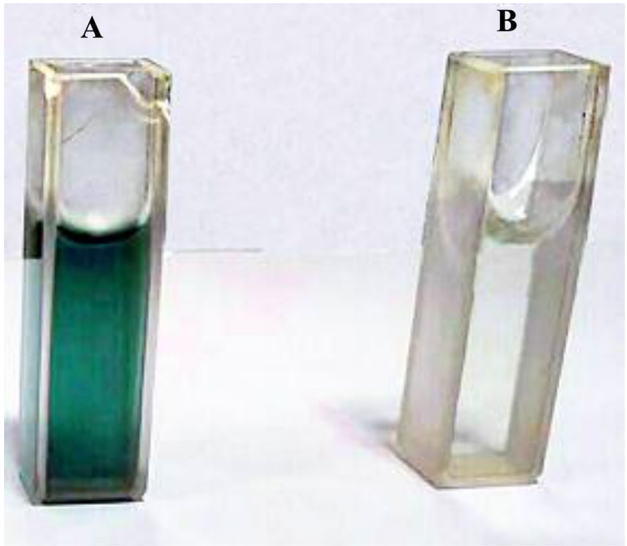

Fig. 2 The assay solution of POXA1b. **a** After adding ABTS; **b** before adding ABTS (negative control)

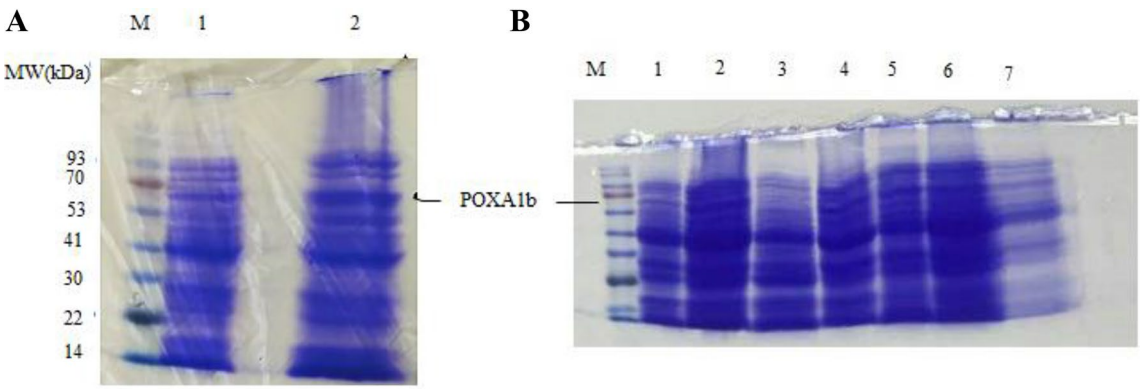

Fig. 1 The SDS-PAGE analysis of codon *optimized and the native POXA1b* expression in *E. coli*. **a** The Codon Optimized *POXA1b* expression in the culture broth $OD_{600}=0.4$ (lane 1) and the $OD_{600}=0.6$ (lane 2) in the presence of 1 mM of IPTG, at 25 °C within 10 h. **b** The expression of codon optimized-POXA1b in the presence of 1 mM of IPTG, at 25 °C within 2 h (lane 1) and 10 h (lane 2); its expression in the presence of 1 (lane 4) and 1.5 (lane 5) mM of IPTG, at 25 °C within 10 h; and its expression in the presence of 1 mM of IPTG, at temperatures of 25 (lane 6); and 45 °C (lane 7) within 10 h; and the expression of native POXA1b in *E. coli* in the presence of 1 mM IPTG and at a temperature of 25 °C within 10 h (lane 3); M is a size marker of protein (10–250 kDa)

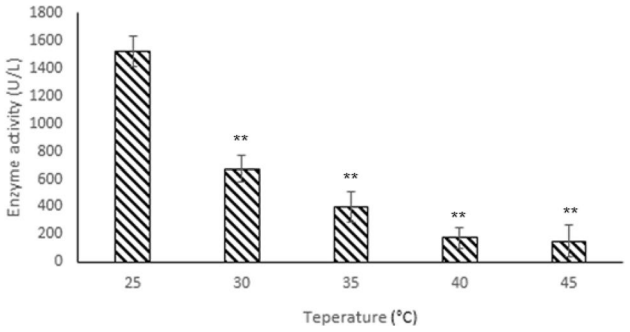

Fig. 3 The effect of temperature on *POXA1b* production in LB medium. The cells were incubated at a temperature range from 25 to 45 °C for 12 h in the presence of 1 mM of *IPTG*. **p < 0.001 versus control (25 °C). The statistical analysis was performed by ANOVA

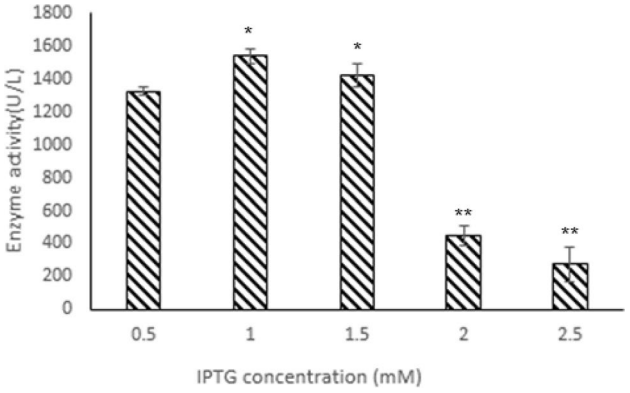

Fig. 4 The effect of *IPTG* concentration on *POXA1b* production in LB medium. The cells were incubated at 30 °C for 12 h in the presence of different concentrations of *IPTG* (0.5–2.5 mM). *p < 0.001 and **p < 0.001 versus control (0.5 mM). The statistical analysis was performed by ANOVA

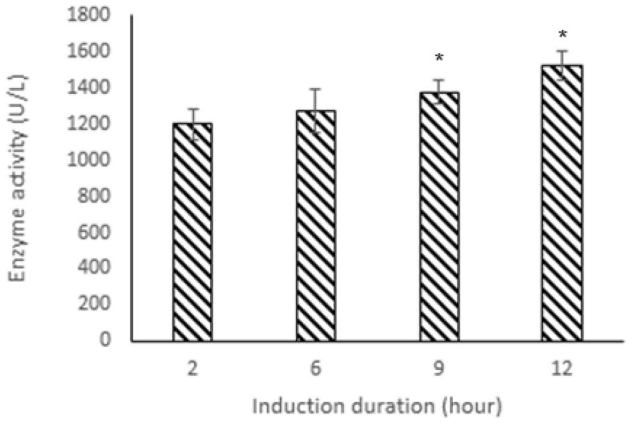

Fig. 5 The effect of induction time with *IPTG* on *POXA1b* production in LB medium. The cells were incubated with 1 mM of *IPTG* at 30 °C for a different time interval (2–12 h). *p < 0.05 versus control (2 h). The statistical analysis was performed by ANOVA

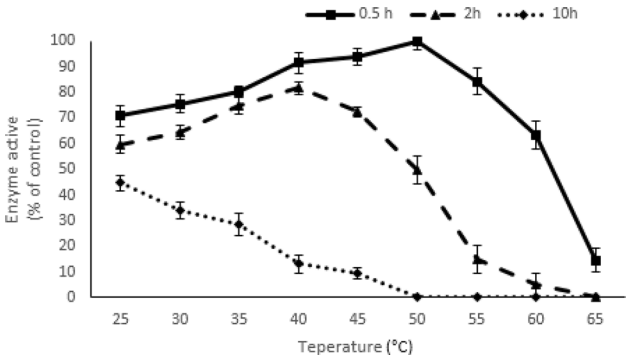

Fig. 6 The effect of temperature on *POXA1b* enzyme activity against ABTS as typical substrate of laccases. The temperature profiles of enzyme were analyzed in the temperature range from 25 to 65 °C and pH 4 within 0.5, 2, 10 h incubation time the activity presented as % of the highest enzyme activity

POXA1b was increased between 25 and 50 °C and then was decreased when temperature increased to 65 °C. As POXA1b was incubated at different temperatures for 2 h, all of the enzyme relative activities were markedly reduced compared to 0.5 h incubation. The enzyme retained its thermostability after 2 h incubation at 25–45 °C. The activity of POXA1b reached 70% of maximum activity, when incubated at 50 °C for 2 h. Within the 10 h of incubation, this laccase retained 40% of its activity at 25 °C. The enzyme activity at 30–65 °C, though being incubated for 10 h, was less than 40% of the initial activity.

The effect of pH on POXA1b activity

As observed in Fig. 7, the POXA1b shows an increase of activity from pH 2.5 (1050 UL^{-1}) to maximum reaches of activity at pH 4.5 (1475 UL^{-1}) and then its activity decreased to the minimum at pH 8 (5 UL^{-1}), when incubated at 25 °C for 0.5 h. As to 2 h incubation, the enzyme activity was decreased in all the tested pH conditions compared to 0.5 h incubation. The relative activity at pHs 3.5–4.5 still gained 80% of the maximum value, even though the enzyme was incubated for 2 h. However, when POXA1b was held at pHs 2.5–8 for 10 h, all of the relative enzyme activities were less than 30% of the maximum value.

Oxidation of BaP by recombinant POXA1b

The ability of POXA1b to oxidize BaP was determined in reaction buffer alone and in the presence of ABTS. The HPLC analysis reveals biodegradation of BaP by POXA1b enzyme from *P. ostreatus* in the presence or absence of ABTS (Fig. 8). As shown in Fig. 9 in the absence of ABTS, 17% of BaP was oxidized by POXA1b at 30 °C. As

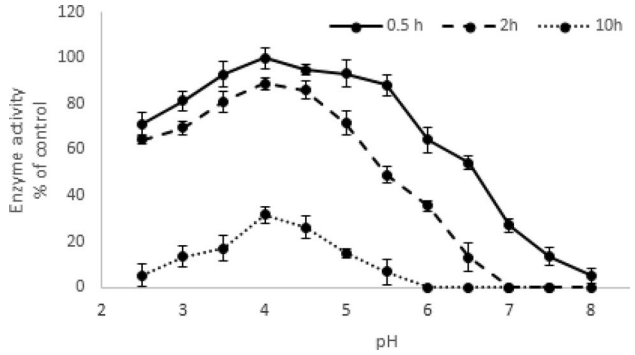

Fig. 7 The effect of pH on *POXA1b* enzyme activity against ABTS as a typical substrate of laccases. The pH profile of the enzyme was analysed in the pH ranging from 2.5 to 8 and at a temperature of 30 °C within 0.5, 2 and 10 h incubation time. The activity presented as % of the highest enzyme activity

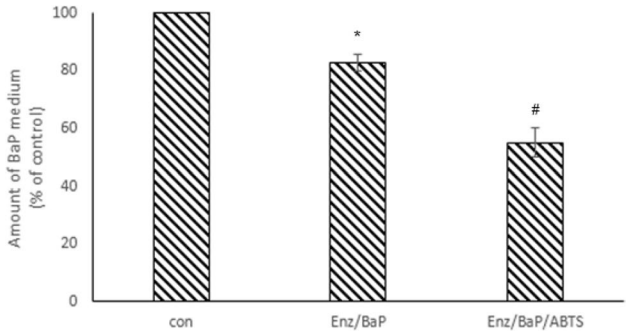

Fig. 9 The oxidation of BaP by *POXA1b* enzyme from *P. ostreatus* in the presence or absence of *ABTS*. The oxidation reaction was performed in Na-acetate buffer (pH 4.5) at 30 °C for 24 h. The values indicated % of the remained BaP in reaction media after 24 h of the enzyme reaction compared to the control (con). $^{*}p < 0.05$ versus control. $^{\#}p < 0.05$ versus Enz/BaP sample. The statistical analysis was performed by ANOVA

expected, the oxidation rate of PAHs increased in the presence of ABTS, thus removing 45% of BaP at 30 °C. These results indicate that the recombinant POXA1b is able to oxidize BaP and that some natural and synthetic mediators such as ABTS are required to enhance the in vitro substrate-oxidizing ability of this enzyme.

Discussion

Some properties such as high redox potential, stability in the wide range of pH and temperature and high production yields at heterologous expression have persuaded researchers to employ fungi laccases, such as POXA1b from *P. ostreatus*, for biotechnological and industrial applications [4, 5]. Due to the fact that all codons within the same codon family are not applied at a synonymous rate (codon bias) in heterologous protein expression in *E. coli*, it may be difficult to heterologously express eukaryotic genes in E. coli. Thus, to reach the maximum yield of recombinant protein optimization of rare codon in *E. coli the* expression system is necessary [22–24, 26, 27]. In this regard, *poxa1b* gene from *P. ostreatus* was optimized in accordance with the codon preference characteristics of the *E. coli* without alteration in the amino acid sequence and this was expressed in *E. coli* heterologously. The effect of such conditions as IPTG concentration, temperature and induction time was also evaluated in the enzyme expression. After the expression phase, its biochemical properties including molecular mass, enzymatic activity, activity dependence on pH and temperature were studied. We also investigated the oxidation of PAHs by expressed POXA1b.

The results of SDS-PAGE demonstrated that the codon-optimized POXA1b from *P. ostreatus* had monomeric structure in media and its molecular weight was 57 kDa (Fig. 1). Regarding this, our findings on molecular weight of codon-optimized POXA1b are consistent with what has

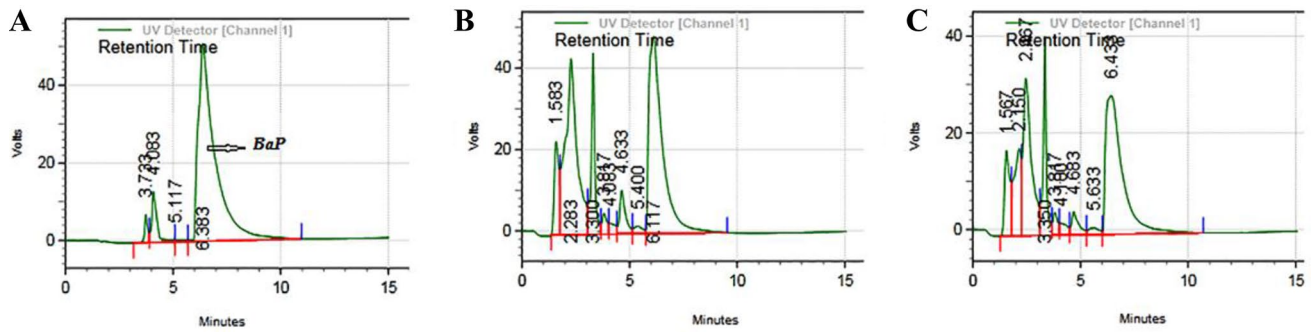

Fig. 8 HPLC chromatogram of the oxidation reaction of BaP by POXA1b: **a** boiled POXA1b and BaP as the control sample, **b** POXA1b and BaP, and **c** POXA1b, BaP and ABTS

been reported for the molecular weight of fungal laccases [36] and are especially in the basidiomycete fungus [37]. In *P. ostreatus*, the molecular mass of *LCC1* and the *LCC2* laccase was reported to be approximately the same as in our study (60 and 65 kDa respectively) [38]. In contrast, the purified laccase from *P. ostreatus* strain V-184 [38] and also the laccase isolated from *Trichoderma harzianum* [39], *Paraconiothyrium variabile* [40], and *Scytalidium thermophilum* [41] exhibit molecular weights greater than that found in our study (82, 79, 84, and 82 kDa) respectively .

The effect of rare codon on high-level expression of recombinant gene in *E. coli* has been analyzed by different investigators. We also evaluated the expression yield and efficiency of codon optimized synthetic gene of *poxa1b* in comparison with the wild-type. We observed that the codon optimization of the *poxa1b* gene markedly enhanced the expression levels of its protein, when compared with the expression level of the native *poxa1b* gene in *E. coli*. Regarding these, Nicola et al. have reported an increased expression of some eukaryotic genes in *E. coli* expression system following the codon optimization [22]. In addition, the high expression levels of codon optimized human *interleukin 11* in *E. coli* have been indicated by Montazeri-Najafabady et al. [24]. Moreover, codon optimization leads to an improved expression of recombinant proteins *VP1, VP2*, and *VP3* [27], *histone-like protein from Sulfolobus shibatae* (*Ssh10*), glutaredoxin-like protein from *methanobacterium thermoautotrophicum* (mtGrx) [23], and multistage candidate vaccine (FALVAC-1) [28] in *E. coli* expression system. Unlike these reports, Jung et al. have reported that the expression yield of synthetic gene of *CAL-B* in *E. coli is* increased by mutagenesis, but not by codon optimization [42].

Our results also demonstrated that the activity of the codon-optimized laccase was significantly higher than the wild-type enzyme. The laccase activity of codon-optimized *poxa1b* was higher than the activity reported for laccase gene expressed in *E. coli* without the codon optimized [43]. Although the enzyme activity of laccase isolated from *P. ostreatus* [44] and laccase gene obtained from *Bacillus licheniformis* [45] that is expressed in *E. coli* without codon optimization were higher of activity reported for codon-optimized POXA1b in our study: this may be attributed to the high production yield derived from the codon optimization that may decrease the correct folding of recombinant proteins. Our findings also demonstrated that the optimum conditions for POXA1b expression was observed in 1 mM IPTG concentration at 25 °C for 12 h (Figs. 3, 4, 5). It is previously attributed to higher rate of protein synthesis in a temperature more than 25 °C, which induces the miss-folding of protein due to the limited amount of molecular chaperons in the host cells and the toxic effects of IPTG in a higher concentration for bacterial host [46].

The laccases stability is an important issue to use these in industries under harsh conditions such as high temperatures and/or acidic/basic pH. It was previously observed that poxA1 laccases show a maximum activity in the temperature ranges of 45–65 °C [47]. Our results illustrated that codon-optimized POXA1b has a maximum activity at 50 °C (Fig. 6) which is similar to the optimum temperature for laccases from *P. ostreatus* ARC280 [35], *P. ostreatus* HP-1 [48], *P. ostreatus* strain EM-1 [49] and *P. ostreatus* [50]. However, in another study the maximal activity for all laccases of *P. ostreatus* was reported in the temperature range of 30–40 °C [51]. Also in the other fungus the findings were controversial, so that the optimum activity for laccases from the mushroom *Lentinus tigrinus* [52], *Trametes* sp. HS-03 [53], *Hericium coralloides* [54] and *Trametes versicolor* IBL-04 [55] was seen at 60, 80, 40 and 40 °C respectively.

The codon-optimized POXA1b retained more than 85 and 50% of its initial activity in 25–45 °C and in the higher temperature of 60 °C for 120 min respectively, which approximately resembles to the thermal stability reported for laccase from *P. ostreatus* ARC280 (retained 100 to 80% of initial activity at temperature ranges of 30–50 °C for 120 min) [35]. The codon-optimized POXA1b shows a higher thermal stability compared to laccases from *P. ostreatus* strain EM-1 [49] and *Paraconiothyrium variabile* [35] which retained 22.6 and 50% of their activity after incubation at 50 °C for 60 min respectively. On the other hand, according to the half-life reported for typical fungal laccases (1 h at 70 °C and below 10 min at 80 °C) [39], the codon-optimized POXA1b shows low thermal stability at a higher temperature of 60 °C (loss of more than 90% of its initial activity in 30 min) which might be attributed to the lack of post-translational modifications in proteins expressed on *E. coli*. However, it was reported that the laccases do not need glycosylation to become functional [44, 56].

It is previously shown that the type of substrate affects the pH stability of laccases [57] so that, the maximum fungal laccase activity against non-phenolic and phenolic substrates was observed in the lower pH than 4 and in the pH range of 3 to 7 respectively [58, 59]. Considering this the codon-optimized POXA1b shows a maximum activity against the ABTS in pH4 and retained more than 80% of the initial activity within 120 min. In relation to this the maximum activity for laccases of *P. ostreatus* D1 [60], ARC280 [35], HP-1 [48] and *L. qinlingensis* laccase [61] was observed at pH 4, 3, 4.5 and 4.4 respectively. Moreover, as to the ABTS, the yellow laccase from *Leucoagaricus gongylophorus* [62], *Stropharia aeruginosa* [63], *Trametes hirsula* [64] and *Aspergillus niger* [65], and *Lentinus squarrosulus* [66] showed a maximum activity at pH 3, 3, 2.4, 2.2, and 4.5 respectively.

It is reported that the different species of white-rot fungi, including *Pleurotus* sp., generate laccases that have capacity

for degradation of PHAs such as BaP [67]. The rate of oxidation process by these enzymes can be enhanced through the incorporation of special compounds called mediators and which play a role as electron mediators [68]. In the present study, POXA1b from *P.ostreatus* could oxidize BaP and the addition of ABTS to the reaction mixture improved the catalytic oxidation process significantly (Figs. 8, 9). In agreement with our findings, Bhattacharya et al. presented similar results demonstrating enhancement in the BaP degradation rate by *P. ostreatus* in the presence of ABTS [69]. Similarly, several studies have been reported about the increase of BaP oxidation by laccase from *Trametes versicolor* following the addition of ABTS [70–72]. The mechanisms that underlie the role of ABTS in laccase-mediated oxidation reaction are not completely understood. However, Potthast et al. [39] reported some evidences implying the role of ABTS as a co-oxidant, which helps the enzyme to accomplish electron transfer process by transferring an electron to the enzyme [73].

Conclusions

In this study, we have successfully cloned and expressed *poxa1b* gene from *P. ostreatus in E. coli* after codon optimization. The yields of expression and efficiency of the enzyme activity were remarkably higher in the codon-optimized enzyme as compared to the native form. The biochemical properties of the codon-optimized POXA1b were characterized and found that they were stable at a temperature range from 25 to 45 °C as well as pH range from 2.5 to 4.5 for 2 h incubation. We also noticed that the codon-optimized POXA1b significantly oxidized BaP in the presence of ABTS. This study is the first report on successful recombinant POXA1b production with unique features using codon optimization in *E. coli* that it is an opportunity for a large scale production of the enzyme.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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