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RESEARCH

Expression of rMnSODSeq regulated by the auto-inducible *dps* promoter in *Escherichia coli*

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ABSTRACT

Recombinant protein production has been favored in *Escherichia coli* by using the T7 promoter, strongly regulated by IPTG, as a strategy for high transcriptional efficiency and to regulate protein overexpression. Unfortunately, IPTG is expensive and could be toxic to cells. In this setting, auto-inducible promoters emerged for recombinant protein production, as the *dps* promoter, which is growth phase-dependent and active in stationary phase. Previously, the plasmid pCAD-*sod* construct was generated, expressing the rMnSODSeq gene expression cassette under the dps promoter, and rMnSODSeq protein was expressed in *E. coli*. In this work, the rMnSODSeq overproduction using pCAD-sod under the regulation of *dps* promoter is characterized in *E. coli* TOP10. The influence of the incubation time and growth medium variables was analyzed. Protein yield was determined by SDS-PAGE assisted with ImageJ software, the protein was purified using a Ni^{2+}-NTA column, and its activity was confirmed using zymography. Lastly, plasmid stability in *E. coli* TOP10 grown in both Luria-Bertani (LB) and Terrific Broth (TB) media were also assessed. Optimized overproduction was attained in 200 mL culture of both LB and TB at 37 °C and 150 rpm for 24 h. Overproduction in TB provided higher cell densities, while SOD percentage of total protein in LB reached the highest values (48.70 ± 1.15 %). Protein purification yielded rMnSODSeq with electrophoretic purity above 90 % and zymography analysis confirmed that the protein showed dismutation activity. The use of dps auto-inducible promoter in pCAD-*sod* plasmid for rMnSODSeq overproduction in *E. coli* system is promising for further upscaling.

Keywords: auto-inducible *dps* promoter, *Escherichia coli*, pCAD-*sod*, rMnSODSeq

RESUMEN

Expresión de rMnSODSeq recombinante regulada con el promotor autoinducible *dps* en *Escherichia coli*. La producción de proteínas recombinantes ha favorecido el uso del promotor T7, fuertemente regulado por IPTG, con alta eficiencia transcripcional en *Escherichia coli* y para controlar la sobreexpresión de proteínas. Sin embargo, el alto costo del IPTG y su toxicidad celular han llevado a considerar otros sistemas, como los promotores autoinducibles. Uno de estos es el promotor dps, dependiente de la fase de crecimiento y activo en fase estacionaria. En este trabajo se caracterizó la sobreproducción de la proteína rMnSODSeq (23.47 kDa) a partir de la construcción plasmídica pCAD-sod, bajo el control del promotor *dps*, en *E. coli* TOP10. Además, se estudió el efecto de las variables Tiempo de incubación y Medio de cultivo, y se determinó el rendimiento de proteína mediante SDS-PAGE analizada con el software ImageJ. La proteína se purificó en una columna de Ni^{2+}-NTA, y la actividad se verificó mediante zimografía. También se evaluó la estabilidad del plásmido en *E. coli* TOP10 cultivado en medio Luria-Bertani (LB) y Terrific Broth (TB). Las condiciones óptimas de sobreproducción en 200 mL para ambos medios fueron: 37 °C, 150 rpm y 24 h. En TB se obtuvo una mayor densidad celular, mientras que en LB se alcanzó una mayor concentración de rMnSODSeq en proteínas totales (48.70 ± 1.15 %). La proteína se obtuvo con una pureza electroforética superior al 90 %. El análisis de zimografía confirmó la actividad de dismutación de la proteína. El uso del promotor auto-inducible dps en el plásmido pCAD-sod en *E. coli* TOP10 es prometedor para su escalado.

Palabras clave: promotor auto-inducible *dps*, *Escherichia coli*, pCAD-*sod*, rMnSODSeq

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Introduction

Strategies for achieving highly efficient recombinant protein production have been continuously developed, due to the increasing demand for high quality protein products. In fact, nearly thirty percent of total biopharmaceuticals circulating in the market are produced using bacterial hosts [1], and *Escherichia coli* has been the most widely used so far. It is well known for its advantages of relatively fast growth and the higher cell densities achieved during fermentation. Moreover, there is a wide range of culture media choices, along with the abundance of information for its implementation [2]. But the expression system used is determinant in many scenarios for the success of the protein production strategy. Strong expression promoters as the T7 promoter system have been available to produce recombinant proteins for up to 50 % of total cell protein [2]. Particularly in this system, induction is attained by adding lactose or its analog, isopropyl β-D-1-thiogalactopyranoside (IPTG), to regulate protein expression.

However, the use of T7 promoter repressed by the *lac* operon requires IPTG as its inducer. Not only being expensive, IPTG may be toxic to cells by giving too much metabolic burden [3]. A possible solution is to replace this chemically regulated promoter by an auto-inducible promoter, as *dps*. This is a growth-dependent promoter that does not require the addition of a chemical inducer [4]. In a previous study, a low

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copy number plasmid construct named pCAD-sod was generated. It bears the rMnSODSeq gene expression cassette, from which the *Staphylococcus equorum* manganese superoxide dismutase rMnSODSeq is expressed as recombinant protein, fused to a histidine tag under the control of the *dps* promoter [5, 6]. rMnSODSeq was overproduced in *Escherichia coli* [6], while further implementations require the characterization of protein overproduction and purification in host strains and under culture conditions more suitable for scaling up purposes.

The role of culture medium in plasmid stability during the production process as source of nutrients for the host cell is one key aspect [7], together with protein stability for the given expression system. In this regard, the recombinant hybrid *Stahylococcus equorum* Mn superoxide dismutase from (rMnSODSeq) of the pCAD-*sod* construct was used as model protein. This is a 23.47-kDa enzyme exhibiting antioxidant activity and stable at high temperatures [8], advantageously supporting the analysis of temperature on culture parameters. Therefore, the aim of this work was to characterize the production of the rMnSODSeq protein production in *E. coli* TOP10 host cells, more suitable for production purposes, under the control of the *dps* auto-inducible promoter. It was carried out up to the purification process and integrated with plasmid stability analysis.

Materials and methods

Strains and materials

E. coli TOP10, recombinant *E. coli* TOP10/pCAD-*sod*, and *E. coli* BL21(DE3)/pJExpress414-*sod* clones were used (source of reagents and composition)[9]. All bacteria were cultured in Luria Bertani (LB) medium unless specified, at 37 °C and under 150 rpm. Cultures harbouring plasmids were further supplemented with 100 µg/mL Ampicillin (PT Bernofarm, Indonesia). Bacterial growth media used in this study were LB medium and Terrific Broth (TB) medium (source of reagents and composition). For protein purification, Ni^{2+}-NTA resin (Qiagen, country) was used.

Methods

Protein expression

E. coli TOP10/pCAD-sod clone was confirmed by migration and restriction analyses [9]. Cells were grown in selective LB medium at 37 °C under shaking at 150 rpm for 18 h. As much as 2.5 mL of the culture was then sub-cultured (5 %) to 50 mL fresh LB medium containing ampicillin, followed by incubation under the same condition for 24 h. Samples were collected after 1, 3, 6, 20, and 24 h to determine the optimum incubation time for the expression of rMnSODSeq. Cultures were centrifuged at 9600 × *g*, at 4 °C for 10 min. Then, the pellets were collected and resuspended in lysis buffer containing DTT and β-mercaptoethanol (20 mg/50 µL) and heated in boiling water for 10 min for cell lysis. Cell debris and insoluble proteins were separated from soluble substances by centrifugation at 9600 × *g*, 4 °C for 10 min. Supernatants were analyzed by 15 % SDS-PAGE to detect the 23.47 kDa protein band corresponding to rMnSODSeq [9].

The optimum incubation time obtained was upscaled into 200 mL culture volume, with similar procedures applied to LB and TB media for further characterization. Prior to cell harvest, 100 µL culture was collected for plasmid stability testing. The pellet was resuspended in 0.5 g/mL PMSF protease inhibitor solution in 1× LEW buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8). Cell suspensions were lysed by sonication with 60 % amplitude, 15:15 seconds on:off, for 20 min (tip or bath sonicator, firm, country), and centrifuged 9600 × *g* at 4 °C for 10 min. Supernatants containing total soluble proteins were collected and labelled as crude proteins.

Protein purification

Protein purification was performed by affinity chromatography in Ni^{2+}-NTA columns (firm, country). Optimization was conducted to determine optimum imidazole concentration as the eluent. Resin was saturated by 15 mM imidazole in 1× LEW buffer, followed by incubation of the crude protein supernatants in resin for 10 min. The flow-through was collected and the resin washed with 1× LEW buffer. For protein elution, gradient concentrations of imidazole (50, 100, 200, and 250 mM) were applied sequentially. Each fraction was collected and analyzed by 15 % SDS-PAGE to determine optimum imidazole concentration for purification. The target protein was purified with the established purification procedure.

Protein characterization

The yield and purity of rMnSODSeq were determined by SDS-PAGE followed by ImageJ version 1.50i software analysis [10]. Purity was expressed as ratio of target protein to total proteins, in percentage. Protein concentration was calculated by extrapolating Area Under Curves (AUCs) of samples to those of various concentrations of BSA as the standard. Calibration curve was generated using 0.3; 0.5; 1; 1.5; and 2 mg/mL BSA. As much as 5 µL were loaded for various concentrations of BSA, 3 µL for crude protein and 20 µL of sample were loaded for standard protein and each fraction. Protein concentration was then estimated as the amount of rMnSODSeq expressed in mg. Purified rMnSODSeq was used as the standard. *E. coli* BL21(DE3)/pJExpress-*sod* having rMnSODSeq gene expression cassette under the control of T7 promoter was used for comparison.

rMnSODSeq was further characterized by a dismutase activity test using zymography. A 10 % native-PAGE followed by incubation in fresh 1.23 mM NBT solution in 100 mM Potassium phosphate buffer, pH 7.4, was applied for this purpose. After washing, gel was soaked in 100 mM Potassium phosphate buffer, pH 7.4, containing 28 mM TEMED and 0.028 mM riboflavin, in dark for 15 min, followed by another washing. The gels were exposed to 18-watt fluorescence light for 10 min. Dismutase activity was detected by the formation of clear zone in the gel.

Plasmid stability testing

Plasmid stability was tested by processing bacterial cultures at the end of protein overproduction. Serial dilutions of a 100 µL sample were prepared, and diluted cell suspensions were seeded onto LB agar without

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antibiotic, followed by overnight incubation at 37 °C to obtain single colonies. As many as 250-300 colonies were replica-plated onto LB agar with and without ampicillin. After incubation for 18 h at 37 °C, the number of colonies were counted. The ratio between the number of colonies grown in LB agar with ampicillin and without ampicillin was calculated and expressed as percentage, representing plasmid stability.

Results

Protein expression

Prior to characterization of protein overexpression, *E. coli* clones were ckecked by migration and restriction analyses to confirm the plasmid identity. Results showed that *E. coli* TOP10/pCAD-*sod* and *E. coli* BL21(DE3)-pJExpress-*sod* clones were verified as bearing the right plasmid (Figure 1).

Subsequently, *E. coli* TOP10/pCAD-sod cells were cultured for rMnSODSeq overexpression, and characterized by varying the incubation time of 50 mL LB cultures. Sampling time were 1, 3, 6, 20, and 24 h of incubation, representing exponential and stationary phase of *E. coli* TOP10/pCAD-*sod* growth [10]. Up to 6.18 % of total proteins rMnSODSeq expression was achieved after 6 h. Results exhibited distinct rMnSODSeq expression, by changes in the intensity of the ~23.47 kDa band at 20 and 24 h, giving 16.48 and 25.27 % rMnSODSeq, respectively (Figure 2). Overall, the highest rMnSODSeq expression was reached at 24 h incubation, this optimum incubation time being set for further upscaling.

Protein overproduction was studied in 200 mL culture of either LB and TB culture media, grown at 37 °C, 150 rpm for 24. A higher cell weight was attained in TB culture grown in TB medium, up to 2.55 ± 0.06 g (Table). However, SDS-PAGE analysis revealed that rMnSODSeq ratio to total protein was higher in cells grown in LB medium, reaching 48.70 ± 1.15 % (Table). The amount of total SOD was about two-fold higher in *E. coli* TOP10/pCAD-*sod* cultured in TB medium, as compared to that of LB medium (Figure 3).

Protein purification

Purification was conducted by affinity chromatography using Ni^{2+}-NTA column, since the recombinant rMnSODSeq produced was fused with a histidine tag. Characterization was done by varying imidazole concentrations as eluent. Data shown confirmed that good binding of the protein of interest occurred in the column, since no ~23,47 kDa band was visible in the flow-through (Figure 4). It was also shown that rMnSODSeq was eluted at 200 and 250 mM imidazole, giving a ~23.47 kDa band parallel to the standard rMnSODSeq with more than 90 % purity in those fractions (Figure 4). Hence, 200 and 250 mM were chosen as the optimum imidazole concentrations for protein elution. Afterwards, the rMnSODSeq obtained was purified from crude protein with the best purification conditions.

Protein characterization

Once purified, the rMnSODSeq protein yield and purity were analyzed and compared from from LB and TB and culture media. It was shown that LB and TB media gave equivalent results, with 45.89 and 47.05 mg/L rMnSODSeq, respectively, obtained from 200 mM and 250 mM imidazole-eluted fractions, with 90-100 % purity based on SDS-PAGE analysis (Table).

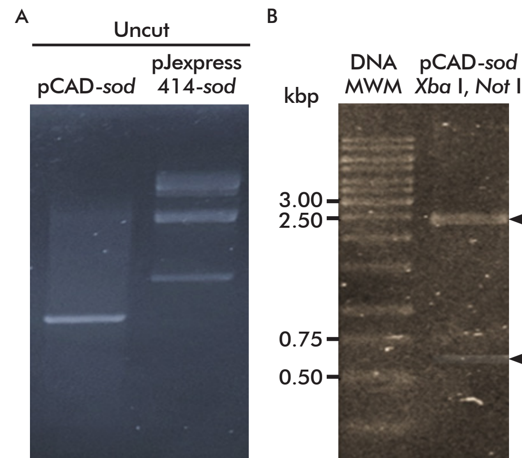

Figure 1. Migration and restriction analyses of pCAD-sod confirmed *E. coli* TOP10/pCAD-*sod* clone. A) Migration analysis of pCAD-*sod* (3176 bp) and pJExpress414-*sod* (4610 bp). B) Restriction analysis of pCAD-sod using *Xba* I and *Not* I, expected to cut out sod expression cassette (634 bp) from pCAD (2541 bp). Target bands are pointed by arrowheads. DNA MWM corresponds to the GeneRuler 1 kb DNA Ladder (Fermentas, Canada).

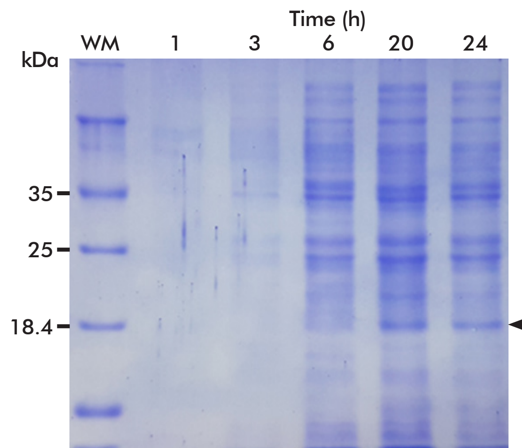

Figure 2. Crude proteins expression of *E. coli* TOP10/pCAD-*sod* grown in 50-mL LB cultures at 1, 3, 6, 20 and 24 h of incubation. rMnSODSeq was expressed distinctively at the 20 and 24 h. The arrowhead indicates the target protein bands (23.47 kDa). WM: Pierce™ Unstained Protein MW Marker (Thermo Scientific, California).

Table. Output parameters of rMnSODSeq purification of *E. coli* TOP10/pCAD-*sod* in LB and TB media

Culture medium	Purity (%)	rMnSODSeq concentration (mg/L)	Total rMnSODSeq (mg/L)	rMnSODSeq recovery (%)
LB	100	22.22 ± 7.34	45.89	61.80 ± 0.97
	96.41 ± 2.99 %	23.67 ± 8.15		49.33 ±16.75
TB	100	20.25 ± 3.81	47.05	50.66 ± 1.34
	97.67 ± 2.02 %	26.80 ± 6.93		55.13 ± 10.19

Unfortunately, impurities of 45.0 and 66.2 kDa protein were still present (Figure 5). rMnSODSeq amount in purified sample relative to initial rMnSODSeq in crude protein ranged from 49.33 + 16.75% to 61.8 + 0.97% (Table). These results were comparable to *E. coli* BL21(DE3)/pJExpress414-*sod* bearing an rMnSODSeq gene expression cassette regulated by T7 promoter using IPTG induction in LB medium, giving 50.75 %.

The purified rMnSODSeq was then characterized by zymography for dismutase activity, detected as a clear zone formed in the electrophoregram. Activity testing was performed for crude and purified proteins samples from both LB and TB media. A 23.47 kDa protein band parallel to standard rMnSODSeq exhibited dismutase activity, showing no differences of dismutase activity between proteins produced by culture grown in LB and TB media (Figure 6).

pCAD-sod stability testing

Plasmid stability testing showed that *E. coli* TOP10/pCAD-*sod* culture grown in LB and TB media gave plasmid retention time of 100 % and 99.88 + 0.21 % for LB and TB media, respectively. Plasmid retention was evidenced after 24 h incubation, covering the entire overproduction period.

Discussion

This research characterized optimal conditions for rMnSODSeq overexpression in *E. coli* TOP10/pCAD-sod under the control of the auto-inducible *dps* promoter. This system advantageously supports growth phase-regulated gene expression instead of those requiring inducer addition. Incubation time and growth medium were analyzed, together with cell weight, the amount of rMnSODSeq produced and plasmid stability. The protein was purified using Ni^{2+}-NTA resin.

The *dps* promoter function relies on the expression of the Dps protein, which plays a role in chromosome condensation during stationary phase under stress, and its expression depends on RNA polymerase containing σ^{38} subunit in early stationary phase and σ^{70} in exponential phase [11]. Other bacterial proteins as Fis and H-NS selectively repress the *dps* promoter, preventing transcription initiation by σ^{70} but not σ^{38}, hence transcription by the promoter is activated in early stationary phase [12]. The activated promoter can be induced for high protein expression by adding H_2O_2, as previously shown for β-galactosidase overexpression, but with problems in protein insolubility [13]. Those limitations were further improved in another study, but protein levels varied attending to the given protein expressed [14].

Considering this background and taking advantage of our previous study with the pCAD-*sod* construct [6], it was studied the optimal conditions for rMnSODSeq overproduction using pCAD-*sod* under the regulation of *dps* promoter in *E. coli* TOP10 cells. Analysis was performed until post-purification and plasmid stability was investigated in 200 mL cultures.

The optimization of culture kinetics showed that a 25-kDa protein band was observed starting at 6 h, which corresponds to the early stationary phase [15]. As expected due to the dps promoter used, the highest intensity of the target protein was observed between

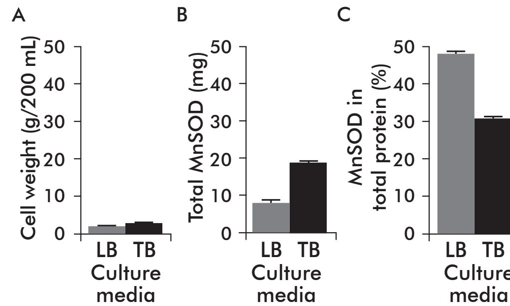

Figure 3. Output parameters of rMnSODSeq overproduction of *E. coli* TOP10/pCAD-*sod* in LB and TB media. A) Cell weight. B) Total MnSOD activity. C) MnSOD in total protein. All data were in triplicates. MnSOD: rMnSODSeq protein.

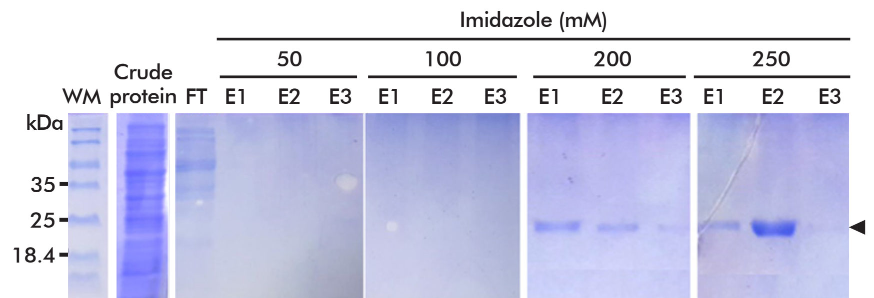

Figure 4. Optimization of imidazole concentration for rMnSODSeq using Ni^{2+}-NTA column chromatography. E: elution for each imidazole concentration, done in triplicates. Target protein bands are pointed by black arrow. FT: Flowthrough. The arrowhead indicates the target protein bands (23.47 kDa). WM: Pierce™ Unstained Protein MW Marker (Thermo Scientific, California).

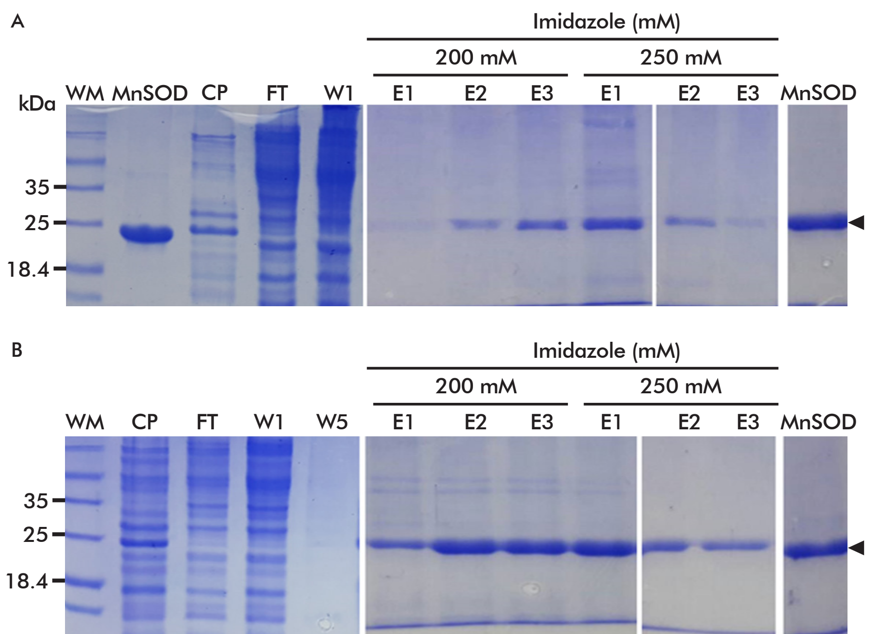

Figure 5. Purification of rMnSODSeq using Ni^{2+}-NTA column of *E. coli* TOP10/pCAD-*sod*. A) cultured in LB and TB medium. Elution using optimized imidazole concentration gave target protein with small amount of impurities. CP: crude protein. E: elution, elution for each imidazole concentration was done thrice. FT: Flowthrough. MnSOD: rMnSODSeq. W1, W5: washes 1 and 5, respectively. The arrowhead indicates the target protein bands (23.47 kDa). WM: Pierce™ Unstained Protein MW Marker (Thermo Scientific, California).

20 and 24 h of growth, with the highest amount of 25.27 % of total proteins reached at 24 h. Noteworthy, the upscaled culture gave an even higher amount of protein, accounting for 48 %.

At the same time, protein overproduction in two types of medium showed that culture in LB yielded the higher rMnSODSeq amount, while TB culture produced higher cell weight, with higher total SOD activity. Significantly, TB contains higher amounts of yeast extract as the source of proteins, vitamins, minerals, and nucleic acids [16]. Extra yeast extract in TB as compared to LB promoted cell growth, as expected [17]. Besides, TB is buffered in slightly acidic pH (5.0), while LB is not buffered and with pH 7.0 [18]. Therefore, TB is somewhat expected to give higher rMnSODSeq production. Unfortunately, despite the increase in cell weight, the relative percentage of rMnSODSeq to total proteins was relatively low in TB as compared to LB. The rMnSODSeq ratio to total protein reached 48.70 ± 1.15 %, which was considered high for recombinant protein production in *E. coli* [19].

Purification and stability testing results demonstrated that production of rMnSODSeq in LB and TB media gave comparable rMnSODSeq purity and amount, along with the plasmid stability. Protein purity were 90-99 % and 100 % for LB and TB, respectively.

Particularly for recombinant SOD proteins, yields will depend on its production and purification processes [20]. There are other recombinant SOD available in the market from several companies, with purity of above 80 % and tp higher than 95 % [21]. Hence, the purity rMnSODSeq achieved in this study was considered acceptable [21]. Furthermore, the overproduction of rMnSODSeq by *E. coli* TOP10/pCAD-*sod* regulated by auto-inducible promoter under the optimal conditions found provided higher amounts of rMnSODSeq than those obtained in the *E. coli* BL21(DE3)/pJExpress414-sod system. That system is regulated by the strong T7 promoter and under IPTG induction. This shows that the dps promoter system is promising for upscaling rMnSODSeq production, particularly in the *E. coli* TOP 10 strain and for other proteins produced in *E. coli*.

Regarding the purification procedure, the Ni^{2+}-NTA column gave high purity of active target protein. Nevertheless, more sensitive analyses need to be conducted regarding more processative purification procedures, especially designed for high scale processing and increased purity (i.e., size exclusion column chromatography), to remove the smaller and larger impurities.

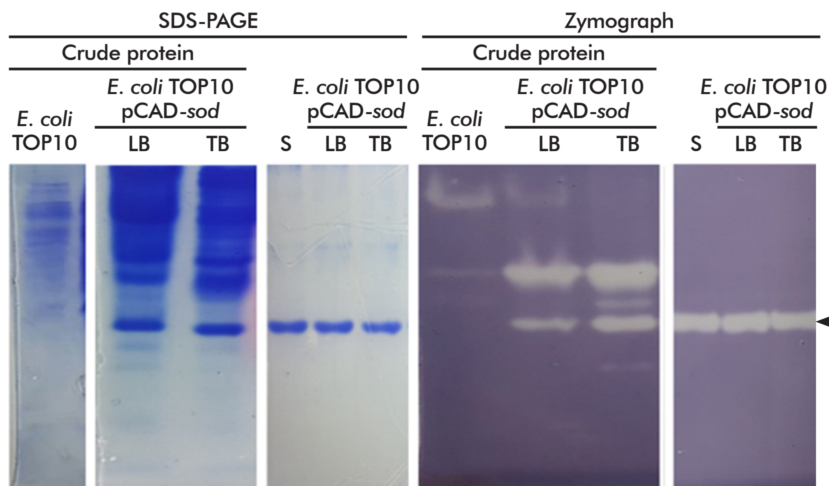

Figure 6. SDS-PAGE (15 %) and zymograph of rMnSODSeq produced by *E. coli* TOP10/pCAD-*sod*. rMnSODSeq produced exhibited dismutase activity. S: standard rMnSODSeq. The arrowhead indicates the target protein bands (23.47 kDa).

In summary, optimal conditions were established for recombinant rMnSODSeq overproduction in *E. coli* TOP10/pCAD-*sod*, at 37 °C with 150 rpm shaking for 24 h. Both LB and TB media gave comparable results regarding to protein yield, purity, and plasmid stability in 200 mL culture scale. These parameters are compatible with subsequent upscaling. Besides, the data will be beneficial for factorial design in the determination of which factor(s) actually affect rMnSODSeq production.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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