Insertion of \textit{prpoD\_rpoS} fragment enhances expression of recombinant protein by \textit{dps} auto-inducible promoter in \textit{Escherichia coli}

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Abstract

Background Nowadays, recombinant therapeutic proteins have been widely produced and consumed. For the safety and effectiveness of the protein production, an auto-inducible expression vector is required to replace inducer interference, which is uneconomic and could be harmful. In this research, an auto-inducible expression plasmid, pCAD\textsubscript{2\_sod} (a pBR322 derivate plasmid), which was under \textit{dps} (RpoS-dependent gene) promoter control, was modified to provide RpoS at earlier phase. Hence, accumulates more target protein and resulting a new plasmid, pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS}. pCAD\textsubscript{2\_sod} had been constructed to automatically induces the expression of recombinant superoxide dismutase (SOD) from \textit{Staphylococcus equorum} (rMnSODSeq) in the stationary growth phase of \textit{Escherichia coli}. This work aimed to obtain pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS} and determine the expression level of rMnSODSeq on mRNA and protein level.

Method and results A synthetic \textit{rpoS} coding region under \textit{rpoD} promoter control (\textit{prpoD\_rpoS}) was inserted to pCAD\textsubscript{2\_sod} and generated pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS}. The rMnSODSeq (24.3 kDa) produced from pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS} was \textsim{} 1.5 fold higher at 37 °C and more intense at 43 °C compared to that from pCAD\textsubscript{2\_sod}, likewise shifted to earlier phase (after 1 h of incubation), as shown in the SDS-PAGE. The dismutase activity was also retained after zymography assay. The mRNA level from pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS} was determined by qPCR and gave quantification cycle (Cq) values of cDNA lowest among others. It made the relative quantification (RQ) of the mRNA expression towards \textit{rho} reference gene were high.

Conclusions The \textit{prpoD\_rpoS} insertion shifts and increases the rMnSODSeq production from stationary to exponential phase. The pCAD\textsubscript{2\_sod}\textsuperscript{\_rpoS} plasmid is potential for further recombinant protein productions.

Keywords Auto-inducible · RpoS · \textit{dps} promoter · Superoxide dismutase

Introduction

The usage of recombinant protein as therapeutic agent has annually increased approximately 35 per cent since 2001. It is a good economic development indication for biopharmaceutical company [1]. In 2018, there are 316 biopharmaceutical products consist of recombinant therapeutic protein and nucleic acid approved in USA and Europe [2]. The major challenges in recombinant protein production are to reduce the production cost, improve the productivity both in upstream and downstream, and obtain high titer while maintaining the quality of the recombinant protein products [1].

\textit{Escherichia coli} had attested its versatility and economic potential in production [3]. There is a wealth of knowledge and comprehensive tools for \textit{E. coli} systems, such as expression vectors, production strains, protein folding and fermentation technologies that are well tailored for industrial applications. Hence, with its recent advancements, the use of \textit{E. coli} has been a preferred choice and a workhorse not only for expression of non-glycosylated proteins in the biotech industry, but also a complex protein production,
bacterial N-linked glycosylation, novel strain engineering and creation of *E. coli* cell-free systems [4]. Varieties of promoters are used in the production of recombinant proteins. Some of the strong promoters are induced by isopropyl-β-D-thiogalacto-pyranoside (IPTG) (i.e. *tac, trc*, and *lac*), which however has been reported to the limitation of its toxicity for the host cells and also causing an ineffective production cost due to its expensiveness [1,4–6].

In the previous research, an auto-inducible expression plasmid, which derive of pBR322, with *dps* promoter (pCAD*sod*) had been constructed to express *sod* from *Staphylococcus equorum* encoding a Mn superoxide dismutase (*rMnSODSeq*) in stationary phase. The *dps* promoter in a high stability medium copy number plasmid, pCAD*sod*, had given a high level expression of *rMnSODSeq* when the host cells entered stationary phase as high as from pBM-sod (IPTG-inducible T7 promoter) [5]. This plasmid was continuously modified for its stability and performance by the addition of *cer* fragment and *dapD* coding sequence (pCAD₂*sod*), which later used in this research. By using an auto-inducible expression plasmid, the recombinant protein expression will be free of chemical impurity originated from the induction and offers a low-cost production system [5]. Some strategies have been developed to make an auto-induction expression system by modify the growth medium components [7], and using the Self-Inducible Expression (SILEX) system [6]. Other strategy that is offered by this study is by modify the presence of sigma factor needed by the promoter to earlier phase. Hence, promoter can be activated faster and the target protein accumulates more.

pCAD₂*sod* plasmid carries *dps* promoter which has been known as an RNA polymerase sigma factor subunit S (σ⁵, RpoS)-dependent [8, 9]. In *E. coli*, the σ⁵ or σ³⁸ controls the regulon of starvation, while other sigma factors evolved to respond different stressors [10]. Some of the genes induced as part of the RpoS response are truly RpoS specific, while others are also expressed by the vegetative sigma factor, RpoD (σ³⁰), under some specific growth conditions [11]. RpoD is an essential main vegetative sigma factor that controlled approximately more than 2000 genes in *E. coli* that is activated by high growth rate [12]. Modifying both sigma factors in one expression system could become a new invention in the field of study regarding recombinant protein overproduction in *E. coli*. Since RpoS was known to be positively regulated the expression of *dps* gene [13], its presence could automatically induce the activation of *dps* promoter in the constructed expression vector and make the recombinant protein production become more cost-effective. This research aimed to obtain synthetic gene encoding RpoS fused with *rpoD* promoter (*rpod_rpoS*) to be inserted into pCAD₂*sod*, generate a modified auto-inducible expression vector, pCAD₂⁺*sod*, and determine the expression of its recombinant gene, *rMnSODSeq*. By the end of the research, there are series of plasmids comprised of pCAD*sod*, pCAD₂⁺*sod*, and pCAD₂⁺*sod*.

**Materials and methods**

**Bacterial strain, plasmid and culture growth condition**

Bacteria *E. coli* strain TOP10 was used for cloning and gene expression. Plasmid pCAD₂*sod* is available at the Laboratory of Pharmaceutical Biotechnology, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia, while plasmid pUCrpoS were prepared by GenScript, USA with the gene of interest sequences were synthetically constructed. Selective Luria Bertani (LB) agar and liquid medium containing 100 μg ml⁻¹ of ampicillin was used for selection, bacterial growth, and protein production. A general growth condition was incubation at 37 °C and 150 rpm agitation, otherwise specified other.

**Plasmids constructions**

Two plasmids, pUCrpoS and pCAD₂⁺*sod*, were constructed and the diagram for the construction is displayed in Fig. 1. RpoS sequences consist of 330 amino acids was obtained from NCBI (Acc. Nr: WP_000081550.1) with preferred *E. coli* B codon usage optimization for its highly expression was performed using online program Optimizer [14]. Meanwhile, the terminator sequence of *rpoS* was predicted using online program ARNold [15]. The nucleotide sequences for *rpoD* promoter and its transcription initiation site was taken prior to the *rpoD* Open Reading Frame (ORF) from NCBI [Acc. Nr: NC_012971.2 (3079129..3080970)] which was analyzed using online program BPROM (Softberry inc.) and Fruitfly promoter prediction program [16]. Analyses were done altogether with optimization of the Translation Initiation Rates (TIR) by designing a synthetic Ribosome Binding Site (RBS) using Genome Compiler program (Genome Compiler Corp.). The *rpoS* mRNA secondary structure was analyzed using Kinefold [17]. Those complete fusion sequences were flanked by *HpaI* restriction site sequence in both 3′ and 5′ ends. The complete synthetic gene sequence carried *rpoS* ORF under *rpoD* promoter control (*rpod_rpoS*) was inserted into pUC57 plasmid, generated pUCrpoS (GenScript, USA).

The pCAD₂⁺*sod* plasmid was prepared by introducing the *rpod_rpoS* gene in the pUCrpoS into pCAD₂*sod* using QuickStep PCR cloning method [18] with modification. pCAD₂*sod* was derived from pCAD*sod* that carries *rMnSODSeq* ORF under *dps* promoter control [5]. Since
the *Hpa*I recognition site sequences were present in the pCAD$_2$*sod* plasmid in two places flanking *bla* coding gene, the QuickStep PCR cloning method was designed to carry *prpoD_rpoS* gene to be inserted into the *Hpa*I restriction enzyme recognition site near the *dps* promoter sequence. The modifications introduced on the QuickStep PCR cloning method were on the primer sequences and their molar ratios used to optimize the PCR result. The first step.

The pCAD$_2$*sod* plasmid was also carrying *cer* fragment and *dapD* coding gene (not discussed in this paper).

**Fig. 1** Illustration of Construction of Recombinant pCAD$_2^{+}$*sod* and mRNA secondary structure prediction of native and recombinant rpoS. A QuickStep cloning method was conducted by involving pUCrpoS and pCAD$_2$*sod*. The *prpoD_rpoS* fragment was amplified and inserted into pCAD$_2$*sod* to generate new plasmid, pCAD$_2^{+}$*sod*. B In the native rpoS mRNA secondary structure, with a high free-energy (~50.2 kcal/mol), loops formation caused the RBS and start codon were closed. C Meanwhile, the recombinant rpoS mRNA secondary structure with the highest free-energy less negative (~10.7 kcal/mol) gave an opened RBS and start codon. The in silico prediction was conducted using Kinefold [16]
of PCR to create megaprimers was conducted with following condition: one minute of pre-denaturation step at 95 °C, 30 cycles of a sequence of 10 s at 95 °C, 30 s at 65 °C, 40 s at 72 °C, and finally 2 min of post-elongation step at 72 °C. Each primer pair was used in separated PCR mixture with the same condition as mentioned. The PCR reaction mixture consisted of 2.5 µl of 2 mM dNTPs, 1.5 µl of 25 mM MgSO4, 0.5 µl of KOD-Plus-Neo (Toyobo), 2.5 µl of 10× PCR buffer for KOD-Plus-Neo, 2 ng of pUCrpoS as the template, and 10 µM for each primer pair of ForQS/Rev_rpoQ (Asym 1) or RevQS/For_rpoQ (Asym 2), with molar ratio of 10:1 and 25:1, respectively, after optimization. The volume was adjusted to 25 µl with nuclease-free water. After PCR product was confirmed to have an ssDNA form, it was purified and prepared to be used in the second PCR step as the megaprimer. The second step of PCR used the first step PCR product with following condition: 1 min of pre-denaturation step at 95 °C, 30 cycles of a sequence of 15 s at 95 °C, 1 min at 62 °C, 3 min at 72 °C, and finally 5 min of post-elongation step at 72 °C. The PCR reaction mixture consisted of 5 µl of 2 mM dNTPs, 3 µl of 25 mM MgSO4, 1 µl of KOD-Plus-Neo (Toyobo), 5 µl of 10× PCR buffer for KOD-Plus-Neo, 20 ng of pCAD2_sod as the template, and 200 ng of each megaprimer from previous step.

The second step PCR product was directly digested with DpnI (Thermo Scientific) and incubated at 37 °C. After 72 h of incubation, the PCR product was introduced into competent E. coli TOP10 using heat-shock method (42 °C; 90 s) and the transformants were selected on an LB agar plate containing ampicillin, overnight at 37 °C. The transformants which grew after incubation were characterized by isolating their plasmids. The characterizations included three different methods: PCR, restriction, and DNA sequencing analyses. In PCR characterization, the isolated plasmid was amplified using pair of primers (F_Cadmbl and R_Notlaq) [5] which differentiated the length of PCR product as larger if pCAD2_sod plasmid compared to the one from the original plasmid (no insertion). The isolated plasmid was also digested by HpaI (Thermo Scientific) restriction enzyme and the DNA fragment formed after digestion was visualized using agarose gel electrophoresis. The last characterization was DNA sequencing towards particular sequence in the isolated plasmid using primer F_Konf and R_Konf (Macrogen Inc., Singapore) to confirm the presence of prpoD_rpoS nucleotide sequences in the plasmid. The primers sequences are listed in Table 1.

**Overproduction and zymography assay of rMnSODSeq**

rMnSODSeq was overproduced in each of E. coli TOP10 harboring pCAD2_sod and pCAD2+sod as a fusion protein with 6×His-tag at its amino terminus. The recombinant bacteria were grown overnight in an Erlenmeyer flask containing LB broth at 37 °C on a rotary shaker at an agitation speed of 150 rpm and sub-cultured to a new LB broth with a starting OD600 around 0.05. The sub-cultured bacteria continued to be incubated at 37 °C on a rotary shaker at an agitation speed of 150 rpm. To study the auto-inducible expression of rMnSODSeq, the cells were harvested in some time points (after 1, 2, 3, 4, 5, 6, and 24 h of incubation). The protein expression was checked from 200 ml of culture on each point and cold-centrifuged (4 °C) for 15 min at 2851×g. The cell pellets were then resuspended using lysis equilibration wash (LEW) buffer pH 8 with 1 mM phenylmethylsulfonyl fluoride (PMSF), disrupted by ultra-sonication, and the protein contained in the supernatant (lysate) was characterized using Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) standard protocol as previously reported [19]. The characterizations were carried out in triplicate and each of the lysate put on the gels was in the same amount of total protein (1.5 ng) after Bradford assay. The intensity of the protein band was compared to an E. coli protein of 20 kDa band, which was constitutively produced in all conditions under study. The comparison of the bands was analyzed semi-quantitatively using ImageJ software [20]. The dismutase activity of rMnSODSeq was observed on a 10% native PAGE followed by negative staining zymography analysis with modifications [20]. The presence of a clear zone at the expected band after light exposure in the gel is an indication of rMnSODSeq dismutase activity.

rMnSODSeq was overproduced as previous experiment with slight differences on the growth temperature. At first, the recombinant E. coli was grown in an Erlenmeyer flask containing LB broth at 37 °C, and then switched to 43 °C after 2 h (OD600 0.1–0.3), 5 h (OD600 0.8–1.0), and 24 h (OD600 1.8–2.0) of incubation. The protein expression was checked from 200 ml of culture on each point and cold-centrifuged (4 °C) for 15 min at 2851×g. The cell pellets were then resuspended using lysis equilibration wash (LEW) buffer pH 8 with 1 mM phenylmethylsulfonyl fluoride (PMSF), disrupted by ultra-sonication, and the protein contained in the supernatant (lysate) was characterized using Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) standard protocol as previously reported [19]. The characterizations were carried out in triplicate and each of the lysate put on the gels was in the same amount of total protein (1.5 ng) after Bradford assay. The intensity of the protein band was compared to an E. coli protein of 20 kDa band, which was constitutively produced in all conditions under study. The comparison of the bands was analyzed semi-quantitatively using ImageJ software [20]. The dismutase activity of rMnSODSeq was observed on a 10% native PAGE followed by negative staining zymography analysis with modifications [20]. The presence of a clear zone at the expected band after light exposure in the gel is an indication of rMnSODSeq dismutase activity.

**Quantitative real-time PCR (qPCR)**

Overnight culture of E. coli TOP10 harboring pCAD2_sod and pCAD2+sod was each sub-cultured to three new LB broths in Erlenmeyer flasks with ratio of 1:5. Each flask differentiates for the incubation times of 2 h (OD600 0.1–0.3), 5 h (OD600 0.8–1.0), and 24 h (OD600 1.8–2.0) at 37 °C on a rotary shaker at an agitation speed of 150 rpm with a starting OD600 around 0.05, represented E. coli growth phase of mid-exponential, late-exponential, and stationary phase, respectively. The cells were harvested by centrifugation and total RNA from 0.05 g of the cell pellets were extracted and purified using TRizol® reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration and purity were confirmed by measuring the ratio of absorbance...
at 260 nm/280 nm and 260 nm/230 nm using µDrop Multiscan GO Microplate spectrophotometer (Thermo Scientific) before and after treated with RNase-free DNase I (Thermo Scientific). One microgram of total RNA was reverse transcribed using random primer in the ReverTra Ace-α® (Toyobo) master mix of 20 µl total volume following manufacturer’s instructions.

The qPCR was performed on the CFX96 Thermal Cycler (BioRad) using SensiFAST SYBR NO-ROX master mix (Bioline). Each reaction mixture consisted of 10 µl SensiFAST SYBR NO-ROX master mix, 20 µM of each forward and reverse primer, and 1 µl of cDNA template in a total volume of 20 µl. Cycling was performed in following condition: 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at 60 °C. Melt curve analysis program (65 to 95 °C with an increment of 0.5 °C for 5 s) was added in the end of amplification program to verify the primer specificity. All qPCR assays were carried out using two technical replication and non-template control (NTC), as well as two independent cDNA syntheses. The amplification efficiency of each primer was determined using 2.5-fold dilution series of cDNA synthesized from the isolated RNA of *E. coli* TOP10 pCAD2+_sod in various growth phases. The efficiency was calculated as $E = 10^{(-1/B)}$ with $B$ was a linear regression slope in the $Y = Bx+A$ equation of exponential cDNA amounts towards the Cq values. Cq values were referred to the fractional PCR cycle at which the target was quantified in a given sample on the instrument, same as threshold cycle (Ct), crossing point (Cp), or a take-off point (Top) currently used in other literatures [21, 22]. In the meantime, relative quantification (RQ) was a comparative quantification that measures the relative

| Primer name | Primer sequences (5’→3’) | Efficienciesa | References |
|-------------|--------------------------|--------------|------------|
| RevQS       | AGG GTT ATT GTC TCA TGA GCG GGT TAA CCG ACT GAG AGG CAG CCG CAA ATA | 1.99 | This research |
| F_{Aan}     | CGG CGC GGT TAT GTG TTC CGC TAT TCT GGC TGT TAA CGC ACA GAA AAG GCC AGC CT | 2.05 | This research |
| F_{Konf}    | GGA TTT TCT TCG CTA TTC GCG ACC ACC ACC ACC | NAb | Pharmacetical biotechnology lab, ITB |
| Rev_{rpoQ}  | CAG ATG CAA CAG AAG AGG CCA GCC T | 2.05 | This research |
| F_{Konf}    | AAA CAG GCT GTG TTCT CAA GTG | NAb | Pharmacetical biotechnology lab, ITB |
| F_{rpoQ}    | CAG CCG GGT TAG TAG ATC TCA CCA ACC GTA AAG AAC ACA CCA CCG CCG CCG | 2.05 | This research |
| Rev_{rpoQ}  | CTG CTT CAT ATC GTC ATC ATC ATC GTA GGT GG | 2.05 | This research |
| F_{rpoQ}    | CAA CTT CGA CAA ACC TGA AAA | 1.97 | This research |
| Rev_{rpoQ}  | ACC GTT ACC ACC TTC CAT AC | 1.98 | This research |
| F_{recA}    | TTA AAC AGG CGG ACT ATC AG | 2.72 | This research |
| Rev_{recA}  | CTG CTT CTG CTA TCA GCT T | 2.72 | This research |
| F_{gyrA}    | GTG ACC CGT CGT ACT ATT TT | 2.72 | This research |
| Rev_{gyrA}  | GAT GAT CCG GTC GAT GTT CG | NAb | [32] |
| F_{cygG}    | TTG TCG GGG GTG GTG ATG TC | NAb | [32] |
| Rev_{cygG}  | ATG CCG TGA ACT GTG GGA TAA ACG | NAb | [32] |

*Calculated using $E = 10^{(-1/B)}$; $B$ = equation slope

*Not available
change in mRNA expression levels [23]. The Cq values of the target genes, rMnSODSeq and rpoS, were measured as well as the reference gene for each experiment. Analysis for changes in the gene expression was quantified by relative quantification (RQ) assay for both target genes, which was calculated through $2^{-\Delta\Delta Cq}$ method [24].

Results

Construction of pCAD$_2^+$ _sod

The pCAD$_2^+$ _sod was constructed by QuickStep PCR cloning method. In our work, the ratio of the primers was modified and optimized to form an ssDNA carrying a fusion DNA fragment, prpoD_rpoS, which was subsequently used as megaprimer. The presence of prpoD_rpoS DNA fragment was showed by an amplification product using a FKonf and RKonf primer pair. Five, out of seven, isolated plasmids from the E. coli TOP10 transformants gave a PCR product of 2957 basepairs (bp) in size, showed that the DNA fragment (1127 bp) was inserted. Meanwhile, E. coli transformants that gave PCR product with smaller size (1830 bp) did not contain modified plasmid. The digestion of the isolated modified plasmid using HpaI restriction enzyme produced three DNA fragments i.e. the plasmid backbone, prpoD_rpoS fragment, and bla gene (3296, 1121, and 1009 bp, respectively) and the sequencing of the plasmid using a FKonf and RKonf Primer pair showed that the plasmid carried correct sequence and orientation of the prpoD_rpoS fragment. These data confirmed that pCAD$_2^+$ _sod was correctly constructed (Suppl.1).

Shift of rMnSODSeq production into exponential phase

The level of rMnSODSeq of 24.3 kDa (designated with * in the SDS-PAGE) produced from E. coli TOP10 pCAD$_2^+$ _sod after 24 h of incubation at 37 °C was higher about ~1.5 fold compared to that produced from E. coli TOP10 pCAD$_2$ _sod in the SDS-PAGE electrophoregram (Fig. 2A) after analyzed semi-quantitatively using ImageJ software [20] (Suppl. 2). The intensity of the protein band was based on a comparison to an E. coli protein of 20 kDa band (designated with → in Fig. 2), which was constitutively produced in all conditions under study. The comparison towards 20 kDa protein was conducted since the loaded lysate was the same in all well. The rMnSODSeq produced from E. coli TOP10 pCAD$_2^+$ _sod retained its dismutase activity demonstrated by the formation of clear zone on the zymogram (Fig. 2B). In the study of the role of RpoS during exponential phase, the rMnSODSeq protein band has been produced in the exponential phase from E. coli TOP10 pCAD$_2^+$ _sod after 1 h incubation, while the one from pCAD$_2$ _sod after 4 h incubation at 37 °C (Fig. 2C).

Effect of growth temperature on rMnSODSeq overproduction

As previously mentioned, a protein of 20 kDa was used in the comparison of protein band intensity. In the exponential phase (4 h) at 43 °C, the intensity of rMnSODSeq protein band produced from E. coli Top10 pCAD$_2^+$ _sod was more intense compared to that from E. coli Top10 pCAD$_2$ _sod and it was also more intense compared to the band produced at 37 °C. Meanwhile, the comparison of the rMnSODSeq protein band in the stationary phase (18 h) showed that the intensity of the protein band from both recombinant clones was quite similar. Though after comparing to the 20 kDa protein band, the intensity of protein bands at 43 °C tended to be more intense compared to that at 37 °C (Fig. 2D).

Quantitative real-time PCR (qPCR)

The Cq values trend of rMnSODSeq synthesis cDNA from E. coli TOP10 pCAD$_2^+$ _sod, in all phases, were found consistently lower to the one carried pCAD$_2$ _sod, therefore gave higher values of the relative quantification (RQ) as well (Fig. 3). RQ was defined to normalize the expression level of mRNA to have reliable qPCR assay by controlling the variations process [22]. Unfortunately, through the biological variations of the samples, the standard deviation (SD) of the RQ value was large among every experiment even though the enhancement of the data trend was consistent (Table 2). A huge differentiation on the RQ value between the duplication independent cDNA was also depending on the relative calculation towards the reference gene.

In the calculation of amplification efficiency (E) for each reference gene candidate, recA and rho gene gave an E value of 1.98 and 1.97, respectively; while gyrA gene gave an E value of 2.72, which was higher than the acceptable amplification efficiencies in range of 1.85–2.05 [23] (Table 1). The Cq data trend of recA was inconsistent in the stationary phase among three different samples, in contrast to the Cq value of rho. Despite the consistent Cq of gyrA in various growth phase, too high efficiency showed that the use of gyrA as reference gene needed more optimization in the applied experimental condition. Thus, rho was chosen as the reference gene in this study (Suppl. 3). Using $2^{-\Delta\Delta Cq}$ method, the expression of rMnSODSeq relative to rho gene from E. coli TOP10 pCAD$_2^+$ _sod showed that the gene was expressed in 752.2: 3206.8; and 21,334.8 fold towards the non-recombinant one as well as higher compared to the one harboring pCAD$_2$ _sod in mid-exponential, late-exponential, and stationary phase, respectively (Table 2).
Fig. 2 rMnSODSeq expression profile in *E. coli* TOP10. **A** The expression of rMnSODSeq (*), 24.3 kDa, from pCAD2+/sod after 24 h at 37 °C showed ~1.5 times more intense protein band compared to rMnSODSeq from pCAD2−/sod, while the non recombinant cells showed no expression of the recombinant protein. The intensity of the band was compared to a reference *E. coli* protein band of 20 kDa (←) using ImageJ [20]. **B** Zymography analysis after Native-PAGE running gels showed that the dismutase activity of rMnSODSeq from both different recombinant clones was retained. **C** In earlier hours of incubation (1–6 h) at the exponential phase, the rMnSODSeq protein bands from pCAD2+/sod had appeared since the first hour of incubation, while from pCAD2−/sod it was seen after 4 h of incubation. **D** The rMnSODSeq protein bands after incubation at 43 °C from pCAD2+/sod showed a more intense band compared to that from pCAD2−/sod in the mid-exponential phase (4 h). Comparison of the rMnSODSeq protein band in the stationary phase (18 h) showed that the intensity of the protein bands from both recombinant clones were quite similar. Those bands were tended to be denser compared to the bands at 37 °C when referred to another *E. coli* protein band of 20 kDa as reference (→). M was protein marker, P was pure rMnSODSeq, NR was non-recombinant
RpoD (σ\textsuperscript{70}) and RpoS (σ\textsuperscript{S}) are both sigma factor subunits of RNA polymerase that are able to be effectively competed [11]. RpoD is known to be a constitutive sigma factor of RNA polymerase, which works in the early phase of bacterial growth [29], while RpoS is an alternative sigma factor produced by many Gram-negative bacteria and primarily controls genes which are expressed in stationary phase in response to nutrient deprivation [30]. Promoter motifs recognized by housekeeping σ\textsuperscript{70} are TTGACA and TATAAT in −35 and −10 regions, respectively [31]. These were basically identical to −35 (TTGACA) and −10 [CTATA(A/C) T] consensus sequences of σ\textsuperscript{S}-dependent genes [8]. Nevertheless, even though the recognition of the promoter has similar efficiency in vitro by both holoenzyme of RNA polymerase associated with σ\textsuperscript{S} (Eσ\textsuperscript{S}) and σ\textsuperscript{70} (Eσ\textsuperscript{70}), a large amount of data has clearly shown that the presence of a C residue immediately upstream of the −10 region favors promoter recognition by Eσ\textsuperscript{S} [11, 32, 33], −10 region sequence of dps promoter in the backbone of the pCAD\textsubscript{2}+\_sod plasmid was TGCTATA [5]. This made the expression of the coding sequence under dps promoter control on the plasmid was more dependent on Er\textsuperscript{5}, since a promoter consist of C at the −13 position was known to give result in strongest expression using σ\textsuperscript{S} [34]. Naturally, the dps promoter is downregulated during exponential phase when cells are not actively being exposed to oxidative stress (i.e. H\textsubscript{2}O\textsubscript{2}), which induced OxyR to activates Er\textsuperscript{70}. Another downregulation mechanisms also involve nucleoid-associated proteins, such as factor for inversion stimulation (Fis) and histone-like nucleoid structuring (H-NS), which binds to the dps promoter and blocks transcription by Er\textsuperscript{70} in exponentially growing cells [35–37]. Meanwhile, in the presence of σ\textsuperscript{S} in the stationary phase, the consensus sequences of histonelike integration host factor (IHF) within the dps promoter has mediated its induction and promotes the transcription [36]. In this research, the existence of prpoD\_rpoS fragment on the expression system has brought a new feature which enable recombinant RpoS to be presented in the exponential phase, instead of naturally works at the stationary phase of growth. Despite its presence in the exponentially growing cells which has been known to be highly regulated, the alteration of initial sequence of the recombinant rpoS coding sequence will reduce the involvement of some of the negative regulators. This condition made the use of RpoS was more reliable than RpoD in this research.

The addition of the prpoD\_rpoS fragment was intended to provide recombinant RpoS, which hypothetically shifts the expression of rMnSODSeq coding region in the exponential phase, by affecting the activity of the stationary phase dps promoter in the overproduction process. The production of RpoS is highly regulated at the transcriptional, translational, and posttranslational levels [38]. The transcriptional level could be set aside, since the native rpoS promoter was

Discussion

In our previous work, we constructed a recombinant plasmid, pCAD\textsubscript{2}sod, where the expression of rMnSODSeq gene was regulated under dps promoter. Hence, the use of auto-inducible dps promoter in a medium copy number plasmid with good stability in E. coli expression system offers high level of expression of rMnSODSeq at the stationary phase [5]. In stationary phase, cells enter a period of no growth since the metabolism-linked genes are turned off [25], though it provides high cell densities for increased product formation. However, low growth rates and protease activity brought on by depleted nutrient levels can reduce the yield of foreign protein in stationary phase [26].

Plasmid pCAD\textsubscript{2}sod used in this research carried cer fragment and dapD coding sequence for its stability and performance (not discussed in this paper) and equipped with RpoS-dependent dps promoter to express rMnSODSeq in the stationary phase. The presence of RpoS in exponential phase by default is low, since its translation is shut off even if its transcription occurs, and during normal conditions the RpoS is rapidly degraded [11]. The changes in RpoS level during growth phase play an important role in providing differential expression of RpoS-dependent gene expression [27]. The use of dps promoter in achieving high level protein production in E. coli has been shown by involving H\textsubscript{2}O\textsubscript{2} induction [28]. By inclusion of additional RpoS from the pCAD\textsubscript{2}+\_sod plasmid at the exponential growth phase in this work, it was as expected that activation of dps promoter and improvement of the auto-inducible production of rMnSODSeq occurred at earlier phase.
During exponential phase, RpoS is rapidly degraded at the posttranslational level by the ClpXP protease after delivery by RssB, an adaptor protein [38]. However, the anti-adaptor proteins (IraP, IraD, IraM, and IraL) which are made under specific stress or starvation conditions, will block RpoS degradation through direct interactions with RssB. The RssB-anti-adaptor complex is unable to bind and deliver RpoS to ClpXP, thereby stabilizing RpoS [41, 42]. The stress or starvation conditions can be applied in another work for pCAD\textsubscript{2}\_sod performance optimization. In our work, the production of additional recombinant RpoS from the pCAD\textsubscript{2}\_sod plasmid in the system switched the expression of rMnSODSeq coding region to exponential phase through the earlier activation of stationary dps promoter. To study the role of RpoS in the current expression system, the overproduction incubation temperature was switched from 37 to 43 °C after the culture reach the mid-exponential phase. The temperature was maintained at 37 °C from the beginning to allow the activation of the rpoD promoter, while 43 °C was chosen to prevent recombinant rpoS mRNA degradation through the inactivation of endonuclease RNase E at the exponential phase. Battesti et al. described several RpoS regulons regarding stress inductions and each of its effector genes [11]. Stresses related to dps are include DNA damage by UV, high temperature, low pH, high pH, and oxidative stress; but only the first two stresses were related to the regulation level of mRNA stability, translation, and degradation of RpoS. Related to heat-shock, in a high temperature at 43.5 °C, the level of RpoS protein was found increase 20 fold associated with the inactivation of the essential endonuclease RNase E along with a detection of threefold increasing in rpoS mRNA levels as well [43]. This research result showed that higher incubation temperature affected the expression of rMnSODSeq in both exponential and stationary phase (Fig. 2D). It reports the role of synthetic rpoS coding region under rpoD promoter regulation in pCAD\textsubscript{2}\_sod on the shift of gene expression from stationary to exponential phase and the expression level of gene product.

The expression profile of rMnSODSeq mRNA in different growth phases of E. coli harboring each of the plasmid (pCAD\textsubscript{2}\_sod and pCAD\textsubscript{2}\_sod) described the effect of the prpoD\_rpoS insertion in the expression system. rMnSODSeq cDNA Cq from pCAD\textsubscript{2}\_sod was tended to be lower compared to that from pCAD\textsubscript{2}\_sod and non-recombinant.

### Table 2. rMnSODSeq and rpoS Cq and RQ values

| Sample E. coli TOP10 | Mid-exponential (2 h) | Late-exponential (5 h) | Stationary (24 h) |
|---------------------|----------------------|-----------------------|------------------|
|                     | Cq\textsuperscript{a} | RQ\textsuperscript{b} | Cq\textsuperscript{a} | RQ\textsuperscript{b} | Cq\textsuperscript{a} | RQ\textsuperscript{b} |
| rMnSODSeq           |                      |                       |                  |
| Non-recombinant     | 28.22 ± 1.16         | 1.0                   | 26.82 ± 0.99     | 1.0                | 26.02 ± 1.11 | 1.0            |
| pCAD\textsubscript{2}\_sod | 19.24 ± 2.83         | 250.5 ± 179.4         | 17.56 ± 1.03     | 226.7 ± 86.7       | 19.03 ± 5.31 | 443.2 ± 102.3 |
| pCAD\textsubscript{2}\_sod | 18.01 ± 1.20         | 752.2 ± 364.4         | 16.57 ± 1.79     | 3206.8 ± 1870.0    | 17.77 ± 2.17 | 21,334.8 ± 18,981.3 |
| rpoS                |                      |                       |                  |
| Non-recombinant     | 24.61 ± 5.01         | 1.0                   | 22.19 ± 3.39     | 1.0                | 23.37 ± 2.17 | 1.0            |
| pCAD\textsubscript{2}\_sod | 21.91 ± 0.62         | 15.6 ± 21.7           | 23.56 ± 1.59     | 0.1 ± 0.1          | 26.46 ± 3.05 | 0.4 ± 0.2      |
| pCAD\textsubscript{2}\_sod | 21.63 ± 3.67         | 7.4 ± 8.1             | 21.42 ± 0.27     | 4.2 ± 1.6          | 23.76 ± 2.08 | 43.0 ± 15.8   |

\textsuperscript{a}Cq mean was measured from two independent cDNAs

\textsuperscript{b}Quantified using 2−ΔΔCq method; relative to rho reference gene

### Notes
- The mid-exponential phase (2 h)
- The late-exponential phase (5 h)
- The stationary phase (24 h)
Analysis using GraphPad Prism 9 (GraphPad Software, San Diego, California USA, www.graphpad.com) was showed a statistically significant value (P < 0.05) of the Cq of rMnSODSeq cDNA from pCAD2_+_sod towards non-recombinant in mid- and late-exponential phase. Nevertheless, the RQ values were varied as it were affected by reference gene used in the calculation of the mRNA relative expression. A reference gene is critical in normalizing cellular mRNA data. It should be stably expressed and appropriate for normalization under the applied experimental conditions [22]. For transcriptional study in E. coli, the choice of reliable reference genes has not been systematically validated [44].

cysG was found as one of reliable novel reference gene for transcription analysis in recombinant protein producing E. coli [44]. However, the use of cysG in this research was found unsuitable, since all the Cq values in various amounts of cDNA were approximating the NTC Cq values (unpublished data). It showed that there were no cysG mRNA expression detected. This finding most probably caused by the state of cysG expression in the conducted experimental condition was not suitable, despite the fact that cysG was found as the least stable gene during stress condition compared to rssA and hcaT gene [45]. Reference gene for this experiment ideally the one that stably expressed throughout E. coli growth phase upon the applied experimental design. Unfortunately, there was lack of information regarding E. coli reference gene that suitable with this experimental design. Thus, functional categories of genes such as DNA replication and transcription had become a consideration in the selection of reference gene candidates, i.e. recA and gyrA (DNA replication) and rho (transcription). Those genes were considered to have most stable expression across different conditions tested in other bacteria, i.e. Klebsiella pneumoniae [46].

The qPCR results indicated that the insertion of prpoD_rpoS into the plasmid has affected the shift and enhancement of rMnSODSeq expression in E. coli. The expression of rMnSODSeq in the mRNA level was in favor to its protein profile in correspondingly host growth curve. Nevertheless, despite the differentiation of the regulation between recombinant RpoS under rpoD promoter control to the native has enhanced the rMnSODSeq RQ value, which also showed in the protein band on the SDS-PAGE, the RQ value of rpoS mRNA was found inconsistent (Fig. 3). Since the primer used in this study to quantify the rpoS not specifically quantified the recombinant one, there was presumably another mechanism that causing the rMnSODSeq RQ enhancement. To ensure the factors that associated to the rMnSODSeq expression enhancement, further study needs to be conducted, i.e. microarray or transcriptome profiling [47–49].

The cells growth curves between the two recombinant E. coli host cells were also found to be slightly different (Fig. 3C). The one harboring pCAD2_+_sod plasmid was seen to have better growth on the exponential phase, since the cell density based on the OD600 value was found higher compared to the one harboring pCAD2_+ sod at the same time point. E. coli TOP10 pCAD2_+_sod carried prpoD_rpoS fragment and allowed the RpoS to be produced in the exponential phase has increased the possibility of expression of some RpoS-dependent genes during rapid growth. RpoS-dependent genes expression leads to a general stress resistance of cells [11, 50]. On account of RpoS regulates a large set of exponential phase specific genes, it was supporting the idea that RpoS is important in actively growing cells [51].

The translation of rpoS mRNA will produce RpoS protein that activates dps promoter. However, since this research approach in study through heat environmental changes that affected the rpoS mRNA stability has not firm, it still needs further experiment to be conducted. It was recognized that there are complex regulations towards recombinant RpoS unusual presence, especially because in nature the cells produce the native RpoS itself. The RpoS protein were not quantified in this research because it still need more observations to assess the differentiation of the native and recombinant RpoS, in order to proclaimed the recombinant RpoS works as expected. The insertion of prpoD_rpoS fragment in this research showed that its role in the indirectly expression of recombinant protein was fairly effective in providing a constitutive expression system, which would eliminate the dependency towards inducer agent. It will simplify the fermentation process. The modification on RpoS unusual existence or its competition with RpoD will influence E. coli protein expression mechanisms. The acquired feature of this system can be used to produce recombinant protein that is toxic to host cell, since the protein can be produced more in the earlier growth phase.

This work concluded that insertion of a coding sequence of rpoS gene which expression directed by rpoD promoter shifts the rMnSODSeq overproduction from stationary to exponential phase and increase its level of production in E. coli, either in the mRNA or protein level. This E. coli expression system is potential for further recombinant protein productions. To have an overall study on the gene expression profile of the recombinant and non-recombinant E. coli host using microarray or transcriptome profiling would be an advantage to clarify the role of RpoS through rMnSODSeq expression enhancement. The study on other recombinant protein model substituted rMnSODSeq ORF in the modified plasmid is our future plan to give additional insight upon the behavior of the constructed expression system to another recombinant protein profile.

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Author contributions DM performed experiments, analyzed the data, and wrote the paper. SNS supervised and analyzed the data. CR supervised and analyzed the data, and wrote the paper.

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Declarations

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

Ethical approval The ethical approval is not applicable, since it did not use animal nor human participants.

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