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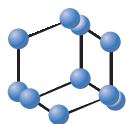
Boosting Auto-Induction of Recombinant Proteins in *Escherichia coli* with Glucose and Lactose Additives

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Abstract: *Background*: Auto-induction is a convenient way to produce recombinant proteins without inducer addition using *lac* operon-controlled *Escherichia coli* expression systems. Auto-induction can occur unintentionally using a complex culture medium prepared by mixing culture substrates. The differences in culture substrates sometimes lead to variations in the induction level.

Objectives: In this study, we investigated the feasibility of using glucose and lactose as boosters of auto-induction with a complex culture medium.

Methods: First, auto-induction levels were assessed by quantifying recombinant GFPuv expression under the control of the T7 *lac* promoter. Effectiveness of the additive-containing medium was examined using ovine angiotensinogen (*tac* promoter-based expression) and *Thermus thermophilus* manganese-catalase (T7 *lac* promoter-based expression).

Results: Auto-induced GFPuv expression was observed with the enzymatic protein digest Polypepton, but not with another digest tryptone. Regardless of the type of protein digest, supplementing Terrific Broth medium with glucose (at a final concentration of 2.9 g/L) and lactose (at a final concentration of 7.6 g/L) was successful in obtaining an induction level similar to that achieved with a commercially available auto-induction medium. The two recombinant proteins were produced in milligram quantity of purified protein per liter of culture.

Conclusion: The medium composition shown in this study would be practically useful for attaining reliable auto-induction for *E. coli*-based recombinant protein production.

1. INTRODUCTION

One of the popular methods for producing recombinant proteins in *Escherichia coli* cells is the *lac* operon-controlled expression system [1, 2]. Protein production using this expression system can be initiated by the addition of an inducer, isopropyl-β-D-thiogalactoside (IPTG). In addition, recombinant proteins under *lac* promoter control can also be produced without IPTG induction, where a small basal expression level leads to substantial expression of the target protein in the absence of inducers [3, 4]. This phenomenon was coined "auto-induction" by Studier [4], because the target protein can be produced automatically, without the need to monitor bacterial growth and add an inducer at the proper time. Hence, auto-induction has been proposed as a convenient and efficient method for recombinant protein production, from the analysis of individual proteins on a small laboratory scale to the production of many proteins on a large project scale [4].

Auto-induction is based on diauxic growth on lactose after the consumption of preferable carbon sources [2, 4-6]. Glucose is the preferred as a carbon source. If glucose is present in the growth medium, catabolite repression occurs,

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which prevents auto-induction. When glucose is depleted, lactose can be taken up and converted to allolactose, a natural inducer. F. William studier [4] systematically examined the factors that affect bacterial growth and auto-induction levels and reported the composition of an auto-inducing defined culture medium. The auto-induction strategy has been utilized for various purposes, including selenomethionine- labeling [7], isotope-labeling [6], increased protein production yield [8, 9], and increased glycosylation efficiency in *E. coli* host cells [10].

To simplify the culture medium composition, Li *et al*. [11] developed another defined auto-induction medium, which utilizes ammonium and phosphate salts, citric acid, ferric citrate, trace elements, and carbon sources (glucose, lactose, and glycerol); interestingly, amino acids and vitamins, two ingredients added by the researcher [4] in his medium, were shown to be dispensable in this study. Using three target proteins with different properties, this study optimized the carbon source concentrations in test tube and shake-flask cultures, and successfully produced various proteins using different T7 *lac* promoter-based expression systems [11]. The chosen combination of carbon sources was 2.9 g/L glucose, 7.6 g/L lactose, and 11.2 g/L glycerol.

As part of the protein "structurome" project of the thermophilic bacterium *Thermus thermophilus* [12, 13], we observed that some culture media lead to auto-induction, while others do not [14]. Out of 2,200 predicted *T. thermophilus* genes, 2,000 open reading frames were cloned into the T7 *lac* promoter-based expression plasmid and screened for expression and solubility. Approximately 60% of the target proteins were produced by auto-induction in the *E. coli* BL21(DE3) strain cultured in Luria-Bertani (LB) medium [14, 15]. Scalable production of recombinant *T. thermophilus* protein was performed by auto-induction, which contributed to structure-based functional studies [16-25]. Complex culture media, such as LB medium, can be easily prepared by mixing a few culture substrates, such as tryptone and yeast extract. However, the differences in culture substrates sometimes lead to variations in auto-induction levels, as observed by other researchers [3, 4]. Since glucose and glycerol work as primary carbon sources to support bacterial growth and lactose triggers the *lac* operon-controlled expression [4], we anticipated that the carbon source composition optimized by Li *et al*. [11] would be the key to the general applicability of an auto-induction medium.

In this study, we examined the effect of glucose and lactose additives on recombinant protein production by auto-induction and sought to determine the feasibility of using sugar additives to boost auto-induction in a complex culture medium. Three different proteins were utilized for this evaluation: GFPuv (T7 *lac* promoter-based expression), ovine angiotensinogen (oANG; *tac* promoter-based expression), and *T. thermophilus* manganese-catalase (MnCAT; T7 *lac* promoter-based expression). Glucose and lactose were added at the same concentrations as described previously [11].

2. MATERIALS AND METHODS

2.1. Construction of Expression Plasmid

The *GFPuv* gene was amplified from the pGFPuv vector (catalog number 632312, Clontech Laboratories, Mountain View, CA, USA) [26] using polymerase chain reaction (PCR). The sequences of the forward and reverse primers were 5′-AGGAGATATACATATGAGTAAAGGAGAAG-AACTTTTCACT-3′ and 5′-GTGGTGCTCGAGTGCTTT-GTAGAGCTCATCCATGC-3′, respectively. The DNA fragment from the pET-22b(+) vector (catalog number 69744-3, Novagen, Madison, WI, USA) was amplified by PCR. The sequences of the forward and reverse primers were 5′-GCACTCGAGCACCAC-3′ and 5′-CATATGT-ATATCTCCTTCTTAAAGTTAAAC-3′, respectively. The PCR product of the *GFPuv* gene was cloned into the linearized pET-22b vector using Gibson Assembly® Master Mix (New England Biolabs, Herts, UK). The resulting plasmid (pET22b-GFPuv-6His) was verified by sequencing. The encoded protein comprised 247 amino acid residues (27.9 kDa), which included GFPuv (M^1-K^{238}; GenBank ID: AAB06048) and a C-terminal extension (ALEHHHHHH).

2.2. Medium Preparation

LB Broth Miller (LBM; catalog number 20068-75; Nacalai Tesque, Kyoto, Japan) was added to deionized water at a concentration of 25 g/L (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). Two types of LB media were prepared in this study: LB-1 medium contained 10 g/L Polypepton (catalog number 394-00115; Nihon Pharmaceutical, Tokyo, Japan), 5 g/L yeast extract D-3 (catalog number 390-00531; Nihon Pharmaceutical), and 10 g/L NaCl (catalog number 32320-05; Nacalai Tesque), whereas LB-2 medium contained 10 g/L tryptone (catalog number 35640-95; Nacalai Tesque), 5 g/L yeast extract D-3 (Nihon Pharmaceutical), and 10 g/L NaCl (Nacalai Tesque). Two types of Terrific Broth (TB) media were prepared in this study: TB-1 medium contained 12 g/L Polypepton (Nihon Pharmaceutical), 24 g/L yeast extract D-3 (Nihon Pharmaceutical), 11.7 g/L glycerol, 9.4 g/L K_2HPO_4, and 2.2 g/L KH_2PO_4, while TB-2 medium contained 12 g/L tryptone (Nacalai Tesque), 24 g/L yeast extract D-3 (Nihon Pharmaceutical), 11.7 g/L glycerol, 9.4 g/L K_2HPO_4, and 2.2 g/L KH_2PO_4. Six grams of Overnight Express™ Instant TB (ON) medium (catalog number 71491; Novagen) [5, 27] and 1.26 g glycerol were added to 100 mL of deionized water.

Deionized water was used to prepare the medium. All media except ON medium were adjusted to a pH of 7.2, using NaOH. No pH adjustment was performed to prepare the ON medium. To prepare additives for auto-induction, 2.55 g of D-(+)-glucose (Nacalai Tesque) was dissolved in 20 mL of deionized water, while 6.69 g lactose monohydrate (Wako Pure Chemicals, Osaka, Japan) was dissolved in 20 mL of deionized water. All media except ON medium and the two additives were autoclaved at 121°C for 15 min. The ON medium was autoclaved at 105°C for 5 min. All culture media were supplemented with 50 μg/mL ampicillin before use.

Three types of TB medium mimics were prepared to express recombinant MnCAT. TB-1 mimic 1 was prepared by supplementing LB-1 medium with four solutions (potassium phosphate buffer concentrate, glycerol, glucose, and lactose), which resulted in a final concentration of 9.1 g/L Polypepton, 4.6 g/L yeast extract D-3, 9.1 g/L NaCl, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 11.2 g/L glycerol, 2.9 g/L glucose, and 7.6 g/L lactose. TB-1 mimic 2 was prepared by supplementing LB-1 medium with two solutions (potassium phosphate buffer concentrate and lactose), yielding a final concentration of 9.6 g/L Polypepton, 4.8 g/L yeast extract D-3, 9.6 g/L NaCl, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, and 7.6 g/L lactose. The TB-2 mimic was prepared by supplementing LB-2 medium with the above four solutions to have a composition similar to that of the additive-containing TB-2 medium.

2.3. Expression of Recombinant GFPuv

To prepare the additive-containing media, 0.1 mL of glucose solution (127.5 g/L), 0.1 mL of lactose solution (334.5 g/L), and 0.2 mL of sterilized deionized were added to 4 mL of the respective four culture media (LB-1, LB-2, TB-1, and TB-2 media), which resulted in a final concentration of 2.9 g/L glucose and 7.6 g/L lactose. The media without additives were prepared by adding 0.4 mL of sterilized deionized to 4 mL of the four media, as well as the two control media (LBM and ON media).

E. coli BL21(DE3) cells were transformed with pET22b-GFPuv-6His to obtain transformant colonies on LBM agar plates with ampicillin. Ten randomly selected colonies were suspended in 2 mL of LBM medium supplemented with ampicillin. After the transformed cells were grown at 37°C to mid-log phase, the cells were preserved as glycerol stocks at -80°C. After the glycerol stock was added to ampicillin-containing LB medium, the cells were precultured at 37°C with shaking at 120 min^{-1} using a shaking water bath (Personal-11, TAITEC, Tokyo, Japan). A preculture aliquot (100 μL) was added to each culture medium (4.4 mL), prepared as described above. In a shaking water bath, the cells were grown in test tubes (n=3) at 37°C, with shaking at 120 min^{-1} for 6 h, followed by further culturing at 25°C, with shaking at 120 min^{-1} for 14 h. After cultivation, the optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories, Hercules, CA, USA) and regarded as the final OD_{600} of the culture medium. The culture solution was centrifuged to obtain the cell pellet and the supernatant. The pH of the supernatant was measured using a compact pH meter (Horiba, Kyoto, Japan) and was regarded as the final pH of the culture medium.

2.4. Quantification of Recombinant GFPuv

The harvested cell pellets (n=3) were suspended in phosphate-buffered saline (PBS) and diluted with PBS to obtain an OD_{600} of approximately 0.5, followed by OD_{600} quantification using a multimode microplate reader (Spark™ 10M, Tecan, Männedorf, Switzerland). To prepare total cell lysates, the cells were lysed on ice by sonication with an ultrasonic cell disruptor (Microson™ XL 2000, Misonix, Farmingdale, NY, USA). The total cell lysate was centrifuged to prepare soluble and insoluble fractions.

The relative GFPuv fluorescence of the soluble fractions was measured using a microplate reader at excitation and emission wavelengths of 360 and 535 nm, respectively. The "relative fluorescence per cell density" was calculated by dividing the fluorescence of the cell lysate supernatant by the OD_{600} of the cells subjected to cell lysis. The "relative total fluorescence" was calculated by multiplying the "relative fluorescence per cell density" by the final OD_{600}.

Total cell lysate and its soluble and insoluble fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie Brilliant Blue (CBB) staining.

To investigate the effect of glucose and lactose concentrations, the glucose concentration was varied from 0 to 5.8 g/L, while the lactose concentration was varied from 0 to 7.6 g/L, with TB-1 medium used as the auto-inducing medium. The cells were grown in test tubes (n=1, one tube per condition), as described above. Final OD_{600}, final pH, relative fluorescence per cell density of the cell lysate supernatant, and relative total fluorescence were obtained and visualized using a heatmap.

2.5. Expression of Recombinant oANG

The additive-containing media were prepared by adding 0.1 mL of glucose solution (128 g/L) and 0.3 mL of lactose solution (117 g/L) to 4 mL of the respective four culture media, resulting in final concentrations of 2.9 g/L glucose and 7.98 g/L lactose. Six media without additives were prepared as described above (Section 2.3).

E. coli BL21 cells were transformed with the pTAC-oANG-His plasmid, which enabled the production of His-tag-fused recombinant oANG [28]. Similar to the procedure described in Section 2.3, ten randomly selected colonies were used to prepare the glycerol stocks, followed by preculture. The preculture aliquots were then added to 4.4 mL culture medium each, which were prepared as described above. The cells were grown in test tubes (n=3) at 30°C with shaking at 120 min^{-1} for 20 h in a shaking water bath. After cultivation, the final OD_{600} and final pH of the medium supernatant were measured.

2.6. Quantification of Recombinant oANG

The harvested cell pellets (n=3) were suspended in PBS and diluted to an OD_{600} of approximately 4. To prepare total cell lysates, the cells were lysed on ice by sonication with an ultrasonic cell disruptor (UD-200, TOMY, Tokyo, Japan). Recombinant oANG was purified from the cleared cell lysate using His Mag Sepharose® Ni beads (GE Healthcare, Buckinghamshire, UK) and a magnetic separation rack (MagnaRack™, Invitrogen, Carlsbad, CA, USA). After washing with 20 m M Tris-HCl (pH 8.0), oANG was eluted

with 20 m M Tris-HCl and 0.5 M imidazole (pH 8.0). The eluted fractions were subjected to SDS-PAGE, followed by CBB staining.

Recombinant oANG was quantified using enzyme-linked immunosorbent assay (ELISA). One hundred micro litres of the eluted fractions and serial dilutions of oANG standard were aliquoted into appropriate wells in a 96-well microplate (high binding), and incubated at 4°C for 20 h to allow for immobilization. The oANG standard was prepared as described previously [28]. The resulting wells were incubated at 4°C for 1 day with a blocking buffer [PBS containing 4% (w/v) casein]. After washing with the buffer [PBS containing 0.05% (v/v) Tween-20], each well was incubated at room temperature for 2 h with 100 μL of rabbit anti-oANG antibody diluted in a ratio of 1:10,000 with Can Get Signal™ Solution 1 (NKB-101, Toyobo, Osaka, Japan). The washed wells were then treated at room temperature for 1 h with 100 μL of horseradish peroxidase-conjugated anti-rabbit antibody (Medical and Biological Laboratories, Nagoya, Japan), which was diluted in a ratio of 1:10,000 with Can Get Signal™ Solution 2 (NKB-101). Finally, 150 μL of 55 mM 3,3',5,5'-tetramethylbenzidine and 50 μL of hydrogen peroxide (used at a dilution of 1:3,000) were sequentially added to each well, and the microplate was incubated at room temperature in the dark for color development. The reaction was terminated by adding 100 μL of 2 M sulfuric acid. The absorbance at 450 nm was measured using a Spark™ 10M multimode microplate reader. The concentration of purified recombinant oANG was estimated from the linear region of the standard curve, ranging from 12.5 ng/mL to 1.25 μg/mL.

The "oANG amount per cell density" was calculated by dividing the amount of purified oANG by OD_{600}-based cell density. The "total oANG amount" was calculated by multiplying oANG amount per cell density by final OD_{600}.

2.7. Expression of Recombinant MnCAT

E. coli BL21(DE3) cells were transformed with an expression plasmid containing the *T. thermophilus MnCAT* gene (*ttha0122, Thermus thermophilus* HB8) cloned into the pET-11a plasmid (Novagen). The expression plasmid was obtained from RIKEN BioResource Center (Tsukuba, Japan; https://web.brc.riken.jp/en/). Ten randomly selected colonies were used to prepare glycerol stocks. Preculture was conducted as described in Section 2.3. For IPTG induction, a preculture aliquot (200 μL) was added to 10 mL of LBM medium. Cells were then incubated in a test tube at 37°C and a shaking speed of 120 min^{-1}. After the cells were grown to mid-log phase, IPTG was added to a final concentration of 0.2 mM, and the culture was maintained for another 3 h. Simultaneously, a non-induced cell culture was established in a test tube using 10 mL of LBM medium at 37°C and a shaking speed of 120 min^{-1} for 20 h. For auto-induction, a preculture aliquot (40 μL) was added to 2 mL of TB medium mimics. The cells were grown in test tubes at 37°C with shaking at 120 min^{-1} for 20 h using a shaking water bath.

The harvested cell pellets were dissolved in PBS and lysed using sonication (Microson™ XL 2000) to prepare the total cell lysate. The lysate was centrifuged to obtain the soluble and insoluble fractions. The soluble fraction was then heat-treated at 70°C for 10 min, followed by centrifugation to obtain the soluble and insoluble fractions after heat treatment. The obtained fractions were subjected to SDS-PAGE, followed by CBB staining.

2.8. Purification and Activation of Recombinant MnCAT

The preculture aliquot (2 mL) was added to 0.5 L of TB-1 medium supplemented with 2.9 g/L glucose and 7.6 g/L lactose. The cells were grown for 20 h at 37°C using a culture system, aerated at 0.5 volume of air per unit volume of medium per min, and agitated at 500 rpm with a magnetic stirrer bar in a 2 L glass bottle [28]. After lysis of the harvested cells, recombinant oANG was purified using heat-treatment (70°C, 15 min) and successive steps using the stirred batch adsorption method with TOYOPEARL® DEAE-650M resin (Tosoh, Tokyo, Japan) and the chromatography on HiTrap® Q HP column (1 mL, GE Healthcare) and Superdex® 200 10/300 GL column (GE Healthcare). The final preparation was dissolved in 20 m M Tris-HCl and 0.3 M NaCl (pH 7.0).

According to a previous report [29], recombinant MnCAT was converted to an active holo-form by incubation with manganese (Mn) ions at high temperatures. MnCAT preparation was desalted on an NAP-10 column (GE Healthcare) equilibrated with 20 mM 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0). The MnCAT concentration was determined using the molecular extinction coefficient at 280 nm (30,337 $M^{-1}.cm^{-1}$), calculated according to the formula provided by Kuramitsu *et al*. [30]. MnCAT (0.5 mM) was incubated with 80 mM $MnCl_2$ in 20 mM MOSP buffer (pH 7.0) at 85°C for 12 h using a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories). In addition, MnCAT (0.5 mM) was incubated under the same conditions, except that no $MnCl_2$ was added to the solution, which yielded an apo-form of MnCAT. After centrifugation to remove the debris, the cleared supernatant was subjected to molecular sieve chromatography on a Superdex® 200 10/300 GL column equilibrated with 20 m M Tris-HCl and 0.3 M NaCl (pH 7.0). The MnCAT preparation was desalted against 20 mM MOPS buffer (pH 7.0) using an NAP-10 column.

The apo-form and holo-form of MnCAT (20 nM each) were incubated with 24 mM H_2O_2 at room temperature in 50 mM potassium phosphate buffer (pH 7.0). The decomposition was monitored at an absorbance of 240 nm (ε_{240}=43.6 $M^{-1}.cm^{-1}$) [31] using a spectrophotometer (U-3310, Hitachi, Tokyo, Japan).

2.9. Statistics

The results are expressed as the mean ± standard deviation, wherever applicable. Statistical analysis and figure preparation were conducted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1. Effect of Glucose and Lactose Additives on the Expression of Recombinant GFPuv

GFPuv was chosen as the reporter protein for ease of evaluation of auto-induction levels. GFPuv is a tailored green fluorescent protein that can be expressed in a recombinant form in *E. coli* cells and quantified by measuring its fluorescence intensity upon excitation with ultraviolet light [26]. The *GFPuv* gene was introduced under the control of the T7 *lac* promoter. When expressed in *E. coli* cells at 37°C without IPTG induction, most of the recombinant GFPuv were accumulated in the insoluble fraction. When expressed in two phases (6 h at 37°C and 14 h at 25°C), a higher amount of soluble GFPuv was observed, which allowed for the estimation of auto-induction levels using this reporter protein.

The culture outcomes are shown in Figure **1A**. The four test media (without additive supplementation) provided final OD$_{600}$ values ranging from 5.4 to 8.3 (Figure **1A**, *white bar*). The final cell densities obtained with LB-1 and TB-1 media reached a level similar to that of ON, while those of LB-2 and TB-2 were greater than that of ON. Additive supplementation of LB media (LB-1 and LB-2) resulted in a significant decrease in cell density and final pH value. Additive supplementation of TB-1 increased the cell density, while no significant change in cell density was observed in the case of TB-2 with additives. The pH drops in the case of TB media were smaller than those in the case of LB media.

The fluorescence data without additive supplementation showed that GFPuv was produced with the Polypepton-based culture media (LB-1 and TB-1), but not with the tryptone-based media (LB-2 and TB-2) (Figure **1B**). These results are consistent with the SDS-PAGE data (Figure **2A** and **2B**). As observed in previous studies [3, 4], different medium compositions utilizing different culture substrates altered auto-induction levels.

When grown with additives, no significant fluorescence was detected in either LB-1 or LB-2 media (Figure **1B**), which is consistent with the absence of GFPuv in the SDS-PAGE gel (Figure **2C**). Soluble and fluorescent GFPuv were produced with additive-containing TB-1 and TB-2 media (Figure **1B**, *gray bar*), an observation supported by the SDS-PAGE analysis (Figure **2D**, *LaneS*). The fluorescence levels per cell density, as well as the total fluorescence levels, were comparable to those of ON (Figure **1B** and **1C**). Regardless of the type of enzymatic protein digest, supplementing TB medium with glucose (2.9 g/L) and lactose (7.6 g/L) was successful in achieving the levels of auto-induction observed in ON, a commercially available auto-induction medium.

Next, we varied the glucose and lactose concentrations, to examine their effect on recombinant GFPuv auto-induction with TB-1. A higher glucose concentration resulted in a higher OD$_{600}$ and lower final pH of the

harvested media (Figure **3A** and **B**). Relatively high fluorescence levels were obtained with the combination of glucose and lactose (Figure **3C** and **D**).
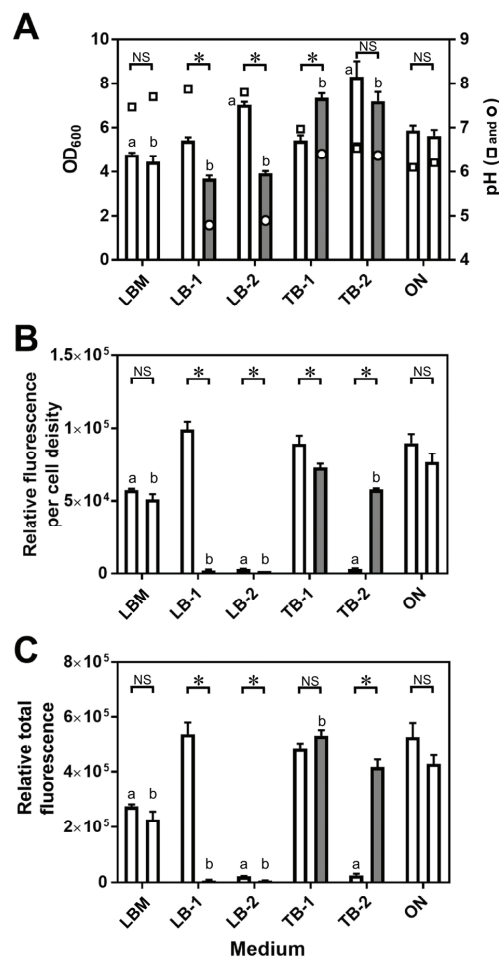
Figure 1. Effect of additives on recombinant GFPuv production using auto-induction. (**A**) Final OD$_{600}$ and final pH of the culture media. OD$_{600}$, grown without additives (*white bar*); OD$_{600}$, grown with additives (*gray bar*); pH, grown without additives (*open square*); pH, grown with additives (*open circle*). (**B**) Relative fluorescence per cell density. (**C**) Relative total fluorescence. The fluorescence values obtained without (*white bar*) or with (*gray bar*) additives are shown in the panels. Transformed *E. coli* cells were grown with six different culture media *without* additives, the results for which are shown on the *left* of each medium condition. Independently, four cultures *with* additives and two control cultures *without* additives were carried out, the results for which are shown on the *right* of each condition. The two independent cultures of each condition were subjected to statistical analysis using a Student's t-test: *, *P*<0.05; NS, not significant. One-way analysis of variance followed by Dunnett's test was performed for comparison of culture media. a, *P*<0.05 compared to ON medium (*left*); b, *P*<0.05 compared to ON medium (*right*). Error bar indicates the standard deviation value. Statistical significance was identified in pH results for all conditions except TB-2 medium (not shown for clarity). **Abbreviations:** LB, Luria-Bertani; LBM, LB Broth Miller; OD$_{600}$, optical density at 600 nm; ON, Overnight Express™ Instant TB; TB, Terrific Broth.
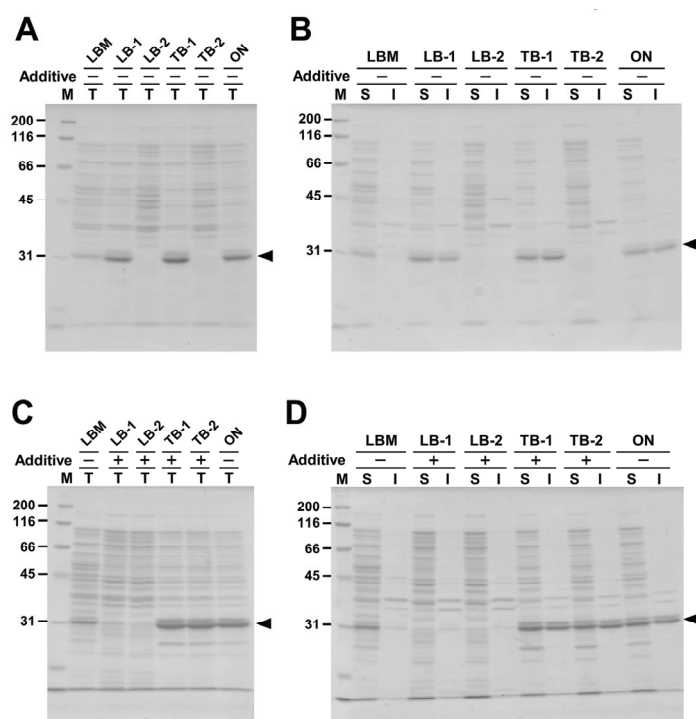
Figure 2. Comparison of recombinant GFPuv expression (**A**) and solubility (**B**) in the *absence* of additives with the expression (**C**) and solubility (**D**) in the *presence* of additives. The total cell lysate (T), soluble fraction (S), and insoluble fraction (I) of GFPuv-expressing recombinant *E. coli* cells were subjected to SDS-PAGE followed by CBB staining. The culture media used and the absence (-) or presence (+) of additives have been indicated *above*. Lane M, molecular marker. Molecular weights of the marker proteins are shown on the *left*. The arrowhead on the *right* represents the size of GFPuv. CBB, Coomassie Brilliant Blue; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).
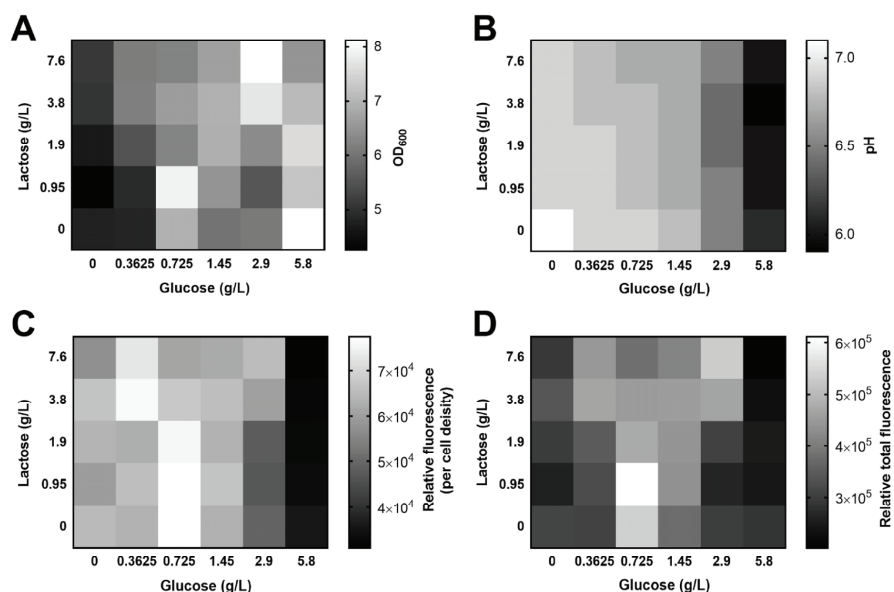
Figure 3. Effect of glucose and lactose concentrations on recombinant GFPuv production using TB-1 medium-based auto-induction. OD_{600} (**A**), pH (**B**), relative fluorescence per cell density of the cell lysate supernatant (**C**), and relative total fluorescence (**D**) were visualized using a heatmap. The values are indicated by the range of white (maximum) and black (minimum) intensities. OD_{600}, optical density at 600 nm. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Effect of Glucose and Lactose Additives on Recombinant oANG Auto-Induction

ANG is a macromolecular precursor of angiotensin that regulates blood pressure and electrolyte balance [32]. ANG is specifically cleaved by the aspartic protease renin to initiate the angiotensin-processing cascade [32]. We have previously reported *E. coli*-based production of recombinant oANG with ON medium [28]. This recombinant protein was expressed from an IPTG-inducible *tac* promoter [28], which is a hybrid of the *lac* and *trp* promoters [1]. To replace the ON medium with the "self-made" auto-induction medium, we examined the effect of additives on recombinant oANG production.

Recombinant oANG was produced using the four media, with or without additives (2.9 g/L glucose and 7.98 g/L lactose). The four media (without additive supplementation) had final OD_{600} values ranging from 3.5 to 4.5, and cell density levels similar to that of ON (Figure **4A**). Additive supplementation of LB media (LB-1 and LB-2) led to a decrease in the final cell density and final pH, whereas such a decrease was not observed with TB-1 and TB-2 (Figure **4A**).

The amount of oANG obtained using affinity-based purification was evaluated using ELISA and SDS-PAGE. In accordance with a previous report [28], recombinant oANG was obtained with ON (Figures **4B** and **5**). Two independent cultures with ON showed a difference in the oANG amounts (Figure **4B**), which could possibly be attributed to changes in the viability of the cultivated *E. coli* cells. With or without additive supplementation, LB medium was not effective for oANG production (Figure **4B**). However, oANG was produced with TB-1 alone, TB-1 with additives, and TB-2 with additives (Figure **4B**), which is consistent with the SDS-PAGE data (Figure **5**). The total production amounts were comparable to those obtained with ON (Figure **4C**). These results indicate that TB media supplemented with glucose and lactose can function as an alternative auto-inducing medium in a *tac* promoter-based *E. coli* expression system.

For scalable auto-induction, batch cultivation was performed in a bioreactor [28] with additive-supplemented TB-1. Recombinant oANG (4.5 mg) was obtained using two steps of column chromatography from 0.5 L culture [33]. Enzymatic analyses showed that oANG preparations work as a renin substrate [33, 34], and the Michaelis constant and turnover number with human renin are similar to those of recombinant oANG expressed in mammalian cells [33].

### 3.3. Effect of Glucose and Lactose Additives on Recombinant MnCAT Auto-Induction

An extremely thermophilic bacterium, *T. thermophilus*, possesses a protein known as MnCAT, which functions as an H_2O_2 scavenger [35]. Native MnCAT was purified by heat treatment, followed by several purification steps [36]. In this study, we investigated the effectiveness of additives on the production of *T. thermophilus* MnCAT designed to be expressed from the T7 *lac* promoter in *E. coli* cells.
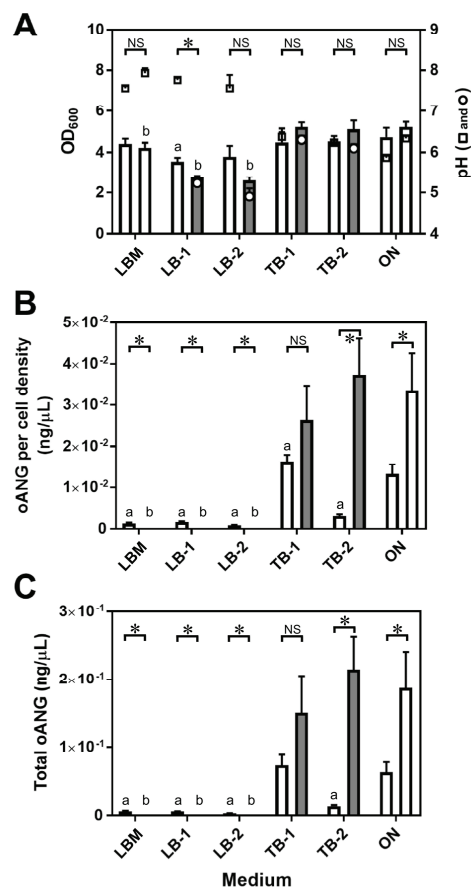
Figure 4. Effect of additives on recombinant oANG production using auto-induction. (**A**) Final OD_{600} and final pH of the culture medium. OD_{600}, grown without the additives (*white bar*); OD_{600}, grown with the additives (*gray bar*); pH, grown without the additives (*open square*); pH, grown with the additives (*open circle*). (**B**) oANG amount per cell density. (**C**) Total oANG amount. The amounts obtained without (*white bar*) or with (*gray bar*) additives are shown in the panels. The cultivation design, as well as, graphical representation of results are the same as in the case of GFPuv. The details of statistics analysis are same as those described in the legend for Figure **1**. Statistical significance was identified in pH results for all conditions except TB-1 and TB-2 media (not shown). LB, Luria-Bertani; LBM, LB Broth Miller; oANG, ovine angiotensinogen; OD_{600}, optical density at 600 nm; ON, Overnight Express™ Instant TB; TB, Terrific Broth.

Most of the MnCAT proteins were insoluble when expressed by IPTG induction in the LBM medium (Figure **6A**). Soluble and heat-stable recombinant MnCAT was obtained with TB-1 mimic 1 (Figure **6B**, *Lane HS*). No significant soluble MnCAT was obtained with TB-1 mimic 2 (Figure **6B**, *Lane S*), which was prepared by supplementing LB-1 with lactose as the sole carbon source. This result indicates that the three carbon sources (glycerol, glucose, and lactose) are effective in harvesting soluble MnCAT. Moreover, heat-stable MnCAT was obtained using a TB-2 mimic (Figure **6C**). Auto-induction was realized again with the addition of the TB medium.
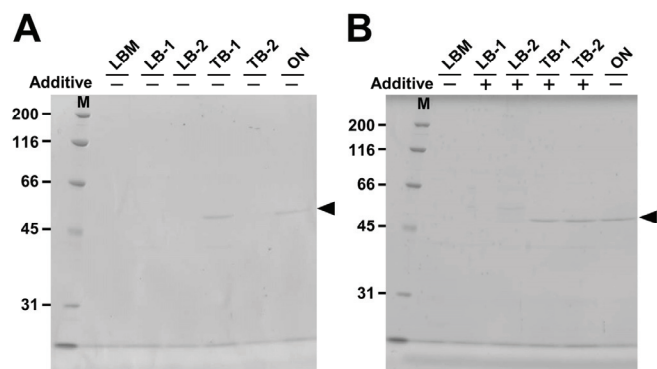
Figure 5. Protein production analysis of recombinant oANG obtained by culture in the absence (**A**) or presence (**B**) of additives. Purified oANG proteins were subjected to SDS-PAGE followed by CBB staining. The culture media and the absence (-) or presence (+) of additives are indicated *above*. Lane M, molecular marker. The molecular weights of the marker proteins are shown on the *left*. The arrowhead on the *right* represents the size of oANG. CBB, Coomassie Brilliant Blue; oANG, ovine angiotensinogen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

We then performed batch cultivation in a bioreactor to produce MnCAT with additive-supplemented TB-1. The apo-form of MnCAT was purified to homogeneity using heat treatment followed by three chromatographic steps (Figure **6D**). The production yield was 2.6 mg of purified protein from 0.5 L culture. After *in vitro* activation with Mn ions, the holo-form showed H_2O_2 scavenging activity (Figure **6E**).

4. DISCUSSION

This study aimed to investigate the feasibility of using glucose and lactose additives as a booster for auto-induction with a complex culture medium. We prepared four kinds of complex media by combining two medium compositions (LB and TB media) and two protein digest products (Polypepton and tryptone). Expression trials of three different proteins showed that auto-induction was successfully achieved with additive-containing TB media, regardless of the protein digest (Figure **1B**, **4B** and **6**). Notably, the protein production yield of TB-2 was greatly enhanced by additive supplementation (Figure **1B** and **4B**).
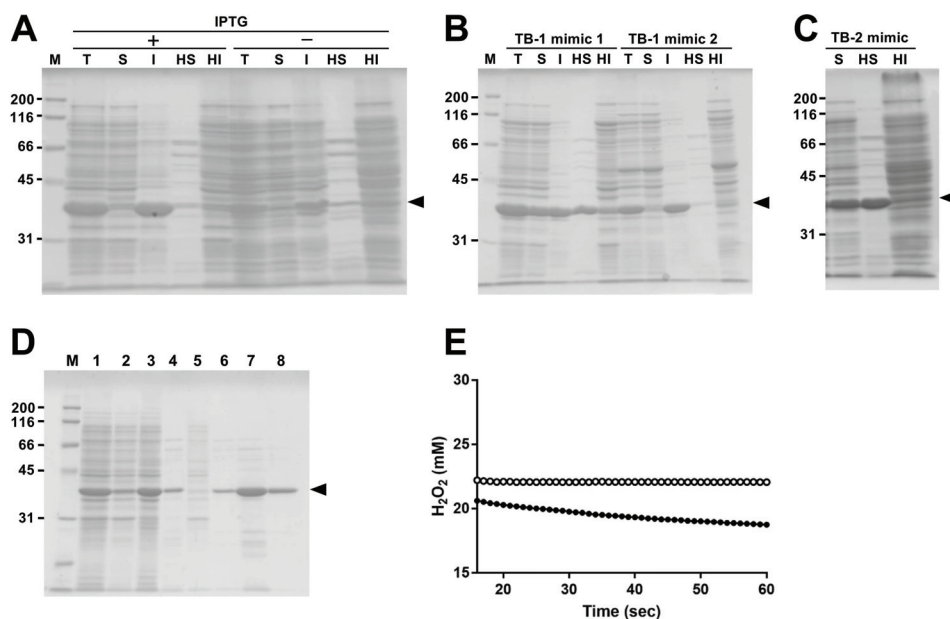
Figure 6. Production of recombinant MnCAT. (**A**) Protein production in the presence (+) or absence (-) of IPTG inducer. Transformed *E. coli* cells were cultured in LBM medium with either IPTG induction (final concentration 0.2 mM) for 3h or no IPTG induction for 20 h. (**B**) Protein production in TB-1 mimic 1 and 2 or (**C**) TB-2 mimic. TB-1 mimic 1 was prepared by supplementing LB-1 medium with potassium phosphate buffer concentrate, glycerol, glucose, and lactose. TB-1 mimic 2 was prepared by supplementing LB-1 medium with potassium phosphate buffer concentrate and lactose. TB-2 mimic is LB-2 medium containing potassium phosphate buffer concentrate and the three carbon sources. Lane M, molecular marker; T, total cell lysate; S, soluble fraction; I, insoluble fraction; HS, soluble fraction post heat treatment; HI, insoluble fraction post heat treatment. The molecular weights of the marker proteins are shown on the *left*. The arrowhead represents the size of MnCAT. (**D**) SDS-PAGE analysis of recombinant MnCAT preparations. The preparations after each purification step were subjected to SDS-PAGE followed by CBB staining. Lane M, molecular weight marker; lane 1, total cell lysate; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, soluble fraction post heat treatment; lane 5, insoluble fraction post heat treatment; lane 6, bound fraction after TOYOPEARL DEAE-650M; lane 7, after HiTrap® Q HP column chromatography; lane 8, after Superdex® 200 10/300 GL column chromatography. (**E**) H_2O_2 scavenging activity of the MnCAT preparations. Decomposition of the apo-form (*open circle*) and holo-form (*closed circle*) was monitored. The results obtained from 16 sec to 60 sec are shown here. CBB, Coomassie Brilliant Blue; IPTG, isopropyl-β-D-thiogalactoside; LBM, LB Broth Miller; MnCAT, manganese catalase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TB, Terrific Broth.

Table 1. Advantages and disadvantages of using IPTG induction and auto-induction for *lac* operon-based recombinant protein expression in *E. coli*.

Induction Methods	Advantages	Disadvantages
IPTG induction	● Expression can be strictly controlled by using an IPTG-inducible promoter [2]. ● Expression level can be controlled by IPTG concentration [2]. ● Toxic proteins can be expressed by suppressing basal expression through adding 0.5-1.0% glucose to the culture medium [1, 3].	● Induction timing is determined by monitoring bacterial growth, which requires tedious iterative monitoring steps. ● IPTG is a costly reagent.
Auto-induction	● Expression can be achieved without monitoring bacterial growth [2, 4]. ● High cell densities as well as a high production yield can be obtained [2, 4]. ● Basal expression can be suppressed by adding 0.05% glucose to the auto-inducing medium [4]. ● Parallel scalable screening of many target proteins is possible [4, 5].	● Medium composition sometimes leads to variability in the expression levels [3, 4]. ● Cultivation time is relatively long (*e.g.*, overnight culture).

The significance of the TB medium composition can be illustrated by comparing bacterial outcomes. Upon additive supplementation of LB-1 and LB-2, the final pH values became acidic (ca. pH 5), and the final OD_{600} values were lower than those obtained without additives (Figures **1A** and **4A**). This decrease in pH is consistent with a previous report [4]. As mentioned previously [4], the acids generated by glucose metabolism may exceed the limited buffering capacity of ZB media [4] and LB media (this study), resulting in such a pH drop and subsequent decreased growth. The auto-induction achieved in LB-1 was halted upon additive supplementation (Figure **1B**). This is partly because of catabolite repression by glucose, which might remain due to growth retardation. In contrast, the addition of TB-1 and TB-2 did not affect the final pH and OD_{600} (Figures **1A** and **4A**), which led to successful protein production (Figures **1B** and **4B**). TB-1 and TB-2 contained phosphate salts (ca. 54 mM K_2HPO_4 and 16 mM KH_2PO_4). The buffering capacity of phosphate may prevent significant reduction of the medium pH, thereby promoting growth until glucose is depleted to start auto-induction. Because TB media contain glycerol, our auto-inducing TB media included all three carbon sources proposed by Li *et al.* [11].

A common question regarding *the lac* operon-based *E. coli* expression system is which method of induction—IPTG induction or auto-induction—would be effective for obtaining the recombinant protein of interest. Table **1** summarizes the main advantages and disadvantages of using each induction method. In the "structurome" project [12], auto-induction was adopted as the first choice of expression screening, owing to the necessity of evaluating the expression levels and solubility of as many as 2,000 open reading frames simultaneously [14, 15]. In the screening, recombinant *E. coli* clones expressing each open reading frame were grown to saturation without measuring bacterial growth. Thus, auto-induction is allowed for efficient screening of a large quantity of clones. Additionally, IPTG induction was used as a complementary method to reevaluate the clones with unsuccessful outcomes after the first screening [15].

In our previous work [28], recombinant oANG was found in insoluble fractions when expressed at 37°C upon IPTG induction using a T7 *lac* promoter-based expression system. In contrast, a small amount of soluble oANG was detected by immunoblotting when expressed at 25°C upon IPTG induction [28]. Based on these results, we chose a *tac* promoter-based expression system. We observed that the soluble to insoluble ratio was approximately 1:1, when expressed at 37°C by IPTG induction and was detected *via* immunoblotting [28]. By optimizing the cultivation steps, we report that a home-made auto-induction medium was effective in producing recombinant oANG (Figures **4** and **5**). Recombinant MnCAT was successfully produced by auto-induction (Figure **6B-C**), whereas that produced by IPTG induction was mostly insoluble (Figure **6A**). However, the soluble-to-insoluble ratio achieved with a bioreactor (Figure **6D**, *Lanes 2* and *3*) was lower than that obtained using a test tube (Figure **6B**, *Lanes S* and *I*), even though an auto-induction medium with the same composition was used in both cultures. The production difference may be caused by the changes in the degree of aeration (*i.e.*, oxygen availability), which can influence cell growth and protein expression [5]. A suitable combination of induction methods and cultivation conditions should be attained by trial and error.

CONCLUSION

A combination of glucose and lactose additives with TB medium was found to be effective in attaining reliable auto-induction using a complex culture medium. Additive supplementation can overcome fluctuations in auto-induction levels that may be caused by the use of a complex medium. The effectiveness of the additive-containing medium was verified using a mammalian protein (oANG) and bacterial protein (*T. thermophilus* MnCAT). The medium composition described in this study would be practically useful for boosting auto-induction, and an expression trial with this medium could serve as one of the "what to do first" choices for *E. coli*-based recombinant protein production.

LIST OF ABBREVIATIONS

ANG	=	Angiotensinogen
CBB	=	Coomassie Brilliant Blue
ELISA	=	Enzyme-Linked Immunosorbent Assay

IPTG	=	Isopropyl-β-D-Thiogalactoside
LB	=	Luria-Bertani
LBM	=	LB Broth Miller
Mn	=	Manganese
MnCAT	=	Manganese Catalase
MOPS	=	3-morpholinopropanesulfonic Acid
oANG	=	Ovine ANG
OD_{600}	=	Optical Density at 600 nm
ON	=	Overnight Express™ Instant TB
PBS	=	Phosphate-Buffered Saline
PCR	=	Polymerase Chain Reaction
SDS-PAGE	=	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TB	=	Terrific Broth

AUTHORS' CONTRIBUTION

NT, IT, KT, and SY performed the experiments, analyzed the data, and wrote the manuscript. AS, MH, SO, TY, TN, and FS provided scientific and technical comments and wrote the paper. MH and AE performed statistical analyses. AE conceived, designed, and coordinated the study, and wrote the paper. All the authors have edited the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interests, financial or otherwise.

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