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Review Article

An Overview of Culture Conditions for Recombinant Protein Expression in *Escherichia coli*

Iman Permana Maksum [1*], Firdausi Permata Ummu Latifah [1], Ahmad Nabiel [1], Niky Fuzi Lestari [1], Sriwidodo Sriwidodo [2], Mamlikatu Ilmi Azizah [1], Diandra Firdiani Utami [1]

[1] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang, 45363, Indonesia
[2] Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, 45363, Indonesia

Corresponding Author: Iman Permana Maksum, Professor, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang, 45363, Indonesia. Tel: +6282240839482, E-mail: iman.permana@unpad.ac.id

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Abstract
Escherichia coli is one of the most commonly used organisms for producing recombinant protein. The recognized cell genome and well-known cell factory of the bacteria make it an ideal heterologous system of preference for the production of recombinant proteins. Over recent years, this cell system has been modified to improve the production of therapeutic proteins, and several methodologies are now available. One of the scientists' most preferred strategies is maintaining cell survival by optimizing the culture conditions. This review summarises some experiments related to those culture conditions and discusses how they affect recombinant protein expression in *E. coli*.

Keywords: Recombinant protein, *Escherichia coli*, Culture expression parameters
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Introduction

Recently, recombinant proteins have become widely used in biomedical research as they have many clinical advantages. Indeed, recombinant therapeutic agents, diagnostics, drugs, and vaccines have been widely developed to meet the increasing demands and scientific developments. The first recombinant protein used in clinical practice was recombinant human insulin in 1982 to treat diabetes mellitus.[1] Since then, many scientists have developed numerous recombinant proteins to treat and diagnose several diseases. For example, our team is currently developing recombinant human epidermal growth factor as a therapeutic wound healing agent,[2,3] and recombinant proteins are being developed as a new vaccine candidate for SARS-CoV-2 by producing its spike protein subunit.[4] Therefore, the production of recombinant proteins is a valuable technique for clinical and biomedical research.

The production of recombinant proteins involves various methods to produce large quantities of protein using several host cells, including various prokaryotic systems, insect, yeast, mammalian, and plant cells.[5] The bacterial production of recombinant proteins is a commonly used technique, with *Escherichia coli* (*E. coli*) being the organism of choice due to its cost-effectiveness and rapid growth.[6] Also, it provides a simple expression system, toxin-free product, and well-characterized genomic database offering many advantages in constructing the gene of interest.[7,8]

Recombinant proteins can be expressed either intracellularly or extracellularly. During intracellular production, the protein is produced within the cell cytoplasm. However, inclusion bodies may form due to many microenvironment factors.[9] The formation of disulfide bonds in the *E. coli* cytoplasm reduces the chance of proteolytic degradation and can enhance protein yield. Co-expression of the target protein with chaperones and slowing the production rate by modifying cultivation conditions are two major strategies scientists employ to correct protein folding and increase protein solubility.[10,11] The solubility of the expressed protein can also be increased by supplementation of chemical chaperones and cofactors,[9] such as osmolytes (also called chemical chaperones), native chaperone inducers, and metal ions (such as magnesium and iron-sulphur ion).[12,13] However, the production of inclusion bodies may sometimes be beneficial, especially if the target protein can be easily refolded, so the expression conditions can be modified to favour the formation of inclusion bodies.

In contrast, the extracellular *E. coli* expression system involves protein translocation into the periplasm or culture medium by fusing the target protein with signal peptides such as PelB, OmpA, TorA, and PhoA.[14-17] Extracellular production guarantees protein folding since target proteins

are transported into the periplasm matrix and undergo disulfide bond formation. Since protein production in *E. coli* does not favour post-translational modifications, secreting target protein into the periplasm is preferred to produce a perfectly folded protein.[18] Insights into the protein translocation across bacterial membranes will ultimately produce recombinant proteins in the preferred bacterial compartments.[19] Recently, several strategies have been developed to increase extracellular protein production, such as supplementation of the culture medium with 1% Triton X-100 and 2% glycine,[20,21] as well as the use of several "leaky" strains such as *lpp* (Braun's lipoprotein) deletion strain and wall-less strains.[22,23] Other strategies caused little damage to the host cell membrane by co-expressing target proteins with phospholipase C from *Bacillus cereus*.[24-26] In addition, co-expression with lysis proteins such as bacteriocin release protein (BRP) and kill gene protein have lso been used to promote protein release into the culture medium.[27]

Several strategies regarding protein expression have been developed but are challenging in practice, with most problems caused by inappropriate culture conditions. Therefore, optimizing optimization the culture conditions is essential in recombinant protein production in *E. coli.* The optimum culture conditions include agitation, incubation temperature, pH, and growth medium.[9,11,28,29] This study provides recent experiments related on these issues and briefly rationalizes why they affect protein expression in *E. coli*.

Optimization of Various Factors Affecting Expression of Recombinant Protein in *E. coli*

Enhancing Solubility by Intracellular Expression

Intracellular protein expression in *E. coli* will lead to the formation of inclusion bodies due to hydrophobic interactions between misfolded or partially folded proteins that form aggregates, and inclusion bodies. Incorrect protein folding occurs due to an insufficient concentration of intracellular chaperones or an insufficient oxidative environment in the cytoplasm.[30,31]

Inclusion bodies are often undesirable because they have no biological activity, but they have several advantages. They account for 30% of the total cellular protein, are resistant to degradation, and are easily separated because they are different from other cellular components. In addition, the inclusion body has a secondary structure similar to the native structure. In general, inclusion bodies are homogeneous and composed of recombinant protein, the content of which can reach 90% of the total protein.[32,33] The main challenge with inclusion bodies is to return the misfolded recombinant protein to its native state so that it has biological activity. Several strategies can overcome these problems, such as using chaperone co-expression, adding maltose binding protein (MBP) protein fusion protein, and refolding by a freeze-thawing method and the glutathione redox system.

Co-expression of Chaperones

Protein folding is very important for protein structure and function.[34] Chaperones are proteins that can monitor non-native conformations, stabilize proteins and assist folding but are not part of the final native protein structure.[35] Molecular chaperones have several roles, namely, to help stabilize the conformation of non-native proteins, to assist the folding process to the native state, or to open proteins that fail to fold properly or extract proteins from their aggregates.[36] The co-expression of chaperones in *E. coli* increases the soluble fraction and decreases inclusion bodies. Nishihara et al., (2000) co-expressed Trigger Factor chaperones and GroEL/ES in *E. coli* to help protein folding and prevent the formation of recombinant protein inclusion bodies endostatin, ORP150 and lysozyme.[37] In addition, Maksum et al., (2020) investigated the effect of the co-expression of GroEL/ES and DnaK-DnaJ-GrpE on the expression of prethrombin-2 in *E. coli* ER2566, showing that co-expression with GroEL/ES increased the soluble fraction.[38]

Addition of MBP Fusion Protein

The addition of fusion protein can increase the solubility of the target protein; for example, MBP increases the solubility of the target protein through a transient interaction between MBP and the partially folded portion of the target protein. MBP prevents partially folded proteins from forming insoluble aggregates until spontaneous folding or folding mediated by endogenous chaperones occurs, thereby increasing the solubility of the target protein.[39] Previous studies have compared the protein solubility resulting from the expression of the *Thermus thermophiles* in *E. coli* BL21 (DE3) without and with the addition of MBP, indicating that MBP tag fusion increases the amount of soluble protein produced.[40]

Refolding with Freeze-thawing and Glutathione Redox System

A refolding step is required to obtain the active protein from the inclusion bodies. The reduced concentration of the denaturant during refolding will facilitate the folding of the dissolved protein. The freeze-thawing method is a soft dissolution approach for inclusion bodies that increases the recovery of soluble protein without destroying the native-like secondary structure. Adding a redox system during refolding in proteins with disulfide bridges will help form the correct disulfide bridges.[41,42] The addition of a redox system will provide a state resembling the redox environment for disulphide bridge formation *in vivo*.[43] A mixture of reduced glutathione (GSH) and oxidized glutathione (GSSG) is a widely used system for the refolding of various proteins.[44-46] Thus, adding a GSH/GSSG redox system during the refolding process will help form disulfide bridges in proteins that have disulfide bridges. Recombinant *hEGF* (human epidermal growth factor) was expressed as inclusion

bodies intracellularly in *E. coli* BL21 (DE3) using the CBD-Ssp fusion protein DnaB-hEGF. The CBD-SspDnaB-hEGF fusion protein was dissolved by the freeze-drying method, thawed, and refolded to obtain native *hEGF* protein with the addition of the GSH/GSSH redox system.[47] The study of recovery hEGF from bacterial IBs through freeze-thawing solubilization and glutathione-based oxidative refolding was also conducted. The refolded hEGF demonstrated heat-resistant properties, interacting with specific antibodies on ELISA, and stimulated keratinocyte proliferation, confirming that hEGF was correctly folded.[48]

Enhancing Solubility by Extracellular Expression

Extracellular expression of recombinant proteins in the periplasm or culture media can minimize proteolytic degradation and produce correctly folded proteins because they facilitate the formation of disulfide bonds to increase their solubility, stability, and biological activity and facilitate the isolation and purification process due to low concentrations of contaminants from host cell proteins.[49,50]

A signal peptide sequence is required to direct the secretion of recombinant proteins into the periplasmic space between the inner and outer membranes of gram-negative bacteria. Signal peptides are short proteins consisting of several amino acids and direct proteins out of the cytoplasm. The signal peptide is composed of a positively charged amino-terminal (N-region), a hydrophobic centre (H-region), and a polar cleavage region (C-region).[51] During translocation through the inner membrane into the periplasmic oxidizing environment, the signal peptide linked at the N-terminal cleaved, allowing the chain to fold, and facilitating disulfide bond formation and correct folding. The periplasmic expression has been widely produced invarious scFv antibodies.[46] The two most frequently used pathways for protein secretion are the Sec-dependent and TAT pathways which are thought to use SPase type I at the end of the translocation process. SPase I cleave non-lipoprotein translocated pre-proteins are post-translated and co-translated.[47]

Sec Dependant Pathway

The major components of the SecB-dependent pathway are the membrane translocon complex, SecYEG, the mobile cytosolic component, SecA ATPase nanomotor, and the SecB chaperon. SecB delivers the preprotein to SecA, and then the preprotein-SecA complex binds to the SecYEG translocon.[49] SecA undergoes a repeat cycle of ATP hydrolysis that requires energy for preprotein translocation through the SecYEG channel translocon. After nearly 80% of the preprotein has been synthesized, SPase I can cleave the signal peptide to produce a mature protein. The signal peptide is then degraded by signal peptide peptidase, a membrane-bound enzyme whose active site is in the periplasm that cleaves by the Ser-Lys duo mechanism.[50] Proteins are secreted through the Sec-dependent pathway, usually by post-translational translocation (Figure 1), where the protein crosses the inner membrane after being completely synthesizedby the ribosome.[51]

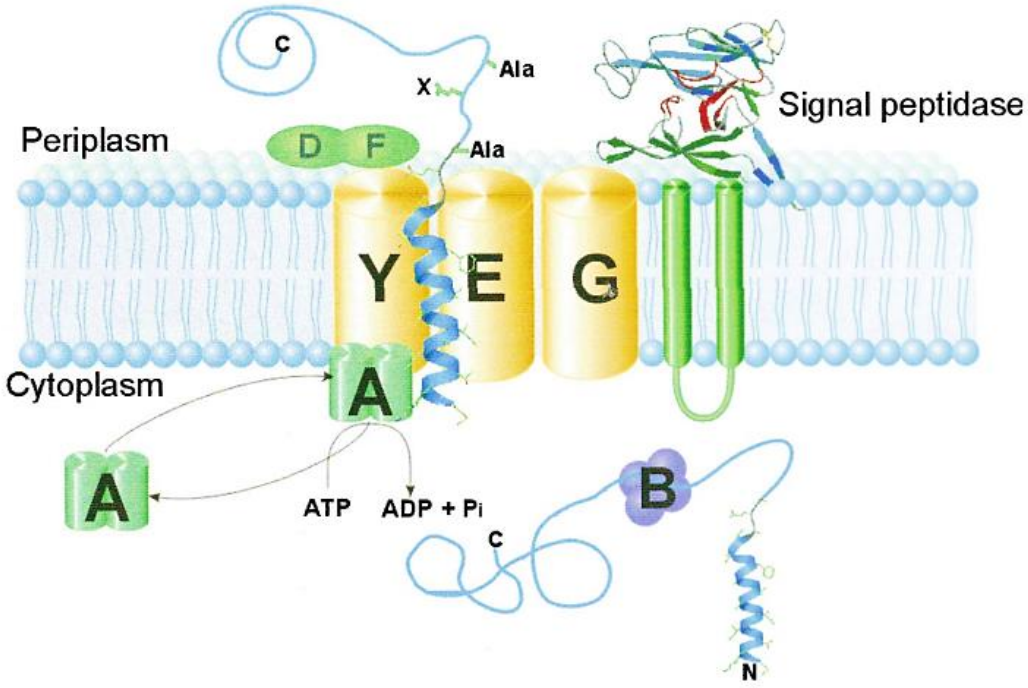

Figure 1. The Vast Majority of Bacterial Secretory Proteins are Exported Post-Translationally Across the Cytoplasmic Membrane Via the Sec-Dependent Pathway. The preprotein is targeted to the cytoplasmic membrane surface with the assistance of the export chaperone SecB. SecA, an ATPase, drives the preprotein chain across the membrane through the SecYEG channel, using the energy of ATP hydrolysis. Once the preprotein is translocated across the membrane, the signal peptide is cleaved off by the Type I signal peptidase.[51]

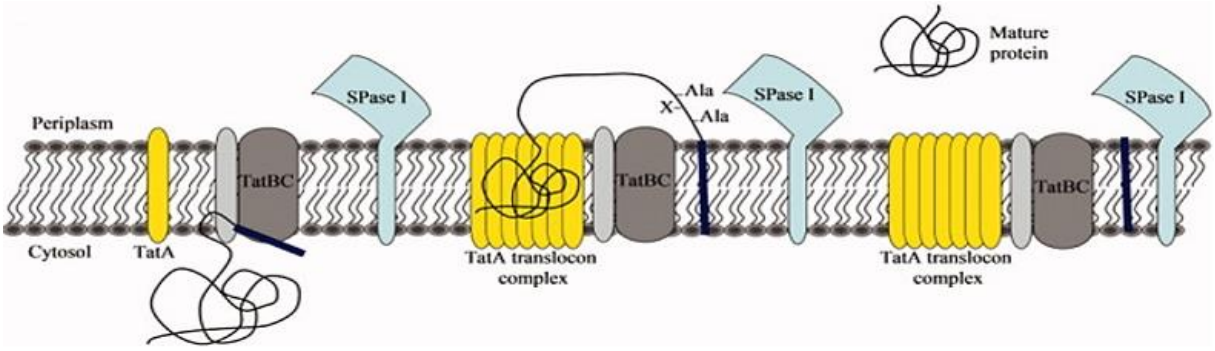

Figure 2. The Tat-Pathway is a Post-Translational Translocation Pathway Used for Secretion of Fully Folded Preproteins. The TatBC complex recognizes and binds the signal sequence of a Tat-dependent preprotein. This causes the recruitment of TatA and the formation of an appropriately sized TatA translocon. The fully-folded preprotein is then secreted into the periplasm, while the signal sequence remains in the membrane. The mature protein is released into the periplasm once the SPase I cleaves the signal peptide.[49]

TAT Pathway

The TAT pathway is a protein transport system that can export fully folded proteins, such as some enzymes containing redox cofactors. The *E. coli* TAT pathway includes three membrane proteins: TatA, TatB, and TatC. Protein secretion by the TAT pathway is targeted via a signal peptide containing an almost unchanged variant of the twin-arginine motif (Figure 2). The TatB then recognizes it, and TatC complexes on the membrane and when the signal peptide binds to TatBC, TatA recruitment occurs and forms a large channel. The signal peptide will remain in the membrane where SPase I cleaves the signal peptide to cause the release of mature protein into the periplasm.[49] The signal peptide for the TAT pathway is usually longer (up to 58 amino acids) than the signal peptide for the Sec pathway. It contains two Arg residues in the n-region adjacent to the h-region. Typically, an Arg or Lys residue in the c-region ensures that the preprotein is not translocated through the Sec pathway.[49]

Culture Conditions Affecting Recombinant Protein Expression

Agitation

Aerobic fermentation occurs in the presence of oxygen and tends to be more intense with a shorter duration than anaerobic fermentation. During aerobic fermentation, the low solubility of oxygen in water is a major problem limiting the oxygen availability. The oxygen transfer rate (OTR) can be increased to maintain the concentration of dissolved oxygen (DO), typically by increasing the aeration and/or agitation rate, offering a gas-liquid interfacial area, larger driving force, and longer residence of the gas bubbles in the liquid to maintain oxygen transfer into the bulk liquid. The effects of DO and periodic changes in pressure have also been reported, with an oscillating dissolved oxygen tension and pressure pulsation (an increased air/O_2 pressure) used to increase the OTR or DO level in the fermentation liquid to increase protein production.[28]

In large-scale fermentation, such as bioreactors, the specific surface area (surface to volume ratio) is significantly decreased, so when the relative surface aeration for gas exchange decreases limits the process. Since oxygen has low solubility in liquids, a sufficient amount of oxygen is vital to achieving high cell density, particularly for large-scale fermentations. A limiting factor in aerobic fermentation occurs when the DO level is below the concentration of critical DO, thus affecting the cell growth rate. Higher gas flow rates and stirrer speeds increase the capacity for oxygen transfer. However, there is more risk of cell damage due to the increased shear stress. Therefore, selecting the appropriate agitation speed and shape of the impeller is essential to improve mixing and oxygen transfer.[28]

Agitation speed is vital for microbial growth and product synthesis; the microbes require oxygen throughout fermentation for both processes. Hence, dissolved oxygen concentration can affect microbial metabolism and product yield. The agitation speed significantly influences the dissolved oxygen concentration inside the fermenter.[52] Thus it has been optimized to enhance the acquisition of target recombinant proteins.

A previous study optimized optimized the agitation in the production of alkaline amylase by *B. subtilis*,[52] showing that at an agitation speed of 550 rpm, the maximum activity of recombinant alkaline amylase reached 342.5 U/ml at 72 h, 1.2- and 1.3-fold higher compared to growth at 450 and 650 rpm and the DCW of *B. subtilis* was highest (5.7 g/L), with the maximum specific growth rate of *B. subtilis*. A high agitation speed (650 rpm) supported faster *B. subtilis* growth in the early phases but the culture rapidly arrived at the stationary phase, indicating that although the high agitation speed supported rapid growth, the higher shear force was possibly harmful to the cells.[52]

The agitation was also optimized to produceα-IFN2b protein in *E. coli* Rosetta-gami2 DE3. It is difficult to achieve a high cell density in standard agitation due to the limited oxygen transfer, so more vigorous shaking conditions

were applied to facilitate sufficient oxygen transfer from the gas to the liquid phase in a 96-deep well plate to achieve high growth rates and increase α-IFN2b production.[53] Oxygen transfer, aeration, and agitation rates are significant in large-scale production.[54] The effect of the agitation rate was evaluated by incubating recombinant cultures with different agitation levels (100-300 rpm) in the presence of 2.5 vvm and 2.0 vvm air supply for b-xylosidase and xylanase enzyme, respectively, with the maximum expression of recombinant endoxylanase (6.58 U/ml in 21 min) observed at 200 rpm.[55]

Agitation and aeration are important parameters in the optimization of scale-up processing. Air is essential to maintain the aerobic environment for the production of enzymes, while suitable agitation is necessary for the uniform availability of nutrients and dissolved oxygen to the microorganisms. Agitation affects the microorganisms in several ways, such as cell structure damage, growth variation, morphology changes, and metabolite formation.[56,57] Many studies have reported the most favorable production of endoxylanase enzyme from *Paecilomyces sinclairii*, *Blakeslea trispora*, *Thermomyces lanuginosus*, and *Aspergillus niger* respectively, at 1.0 vvm aeration rate and 200 rpm agitation speed in bioreactors.[58] Another study reported the greatest production of recombinant xylanase enzyme on a large scale from *Pichia pastoris* at 170 rpm agitation speed and the highest production of recombinant xylanase enzyme in a bioreactor from *B. subtilis* was achieved with a 150 rpm agitation rate.[59] Dissolved oxygen in the medium impacts microbial growth, indirectly influencing the enzyme production rate.[60] The maximum production of recombinant endoxylanase enzymes attained with 20% and 25%, dissolved oxygen in the medium. Extreme oxygen concentrations can negatively impact microbial growth,[61] with a high concentration causing cell stress and toxicity for various cells. In the presence of a saturated oxygen level, many toxic compounds, like hydrogen peroxide and superoxide, are produced in the fermentation media,[62] which are toxic to the organism due to their higher reactivity than molecular oxygen. The rate of agitation, which is vital in laboratory research and large-scale production, is an aspect that could affect both cell growth and productivity.[55]

In 1990, Huang et al., investigated the influence of the agitation rate on the production of the enzyme, as well as the plasmid stability of recombinant *E. coli* immobilized immobilized in carrageenan gel. The entrapped recombinant cells supported enhanced enzyme production and plasmid stability compared to free cells at different agitation rates (50, 100, and 150 rpm). Moreover, a higher agitation rate (200 rpm) significantly reduced the stability of the plasmid both in the free and entrapped cells, consequently reducing enzyme production. Another study by Singh and Yadav confirmed the high production of L-aspartic acid (566 mg/g wet wt) from immobilized recombinant cells entrapped in sodium alginate (agitation rate 250 rpm) compared to free cells (154 mg/g wet wt). A lack of nutrients and oxygen at a low agitation rate would decrease the density of the immobilized cells.[63] In contrast, a higher agitation rate could increase enzyme secretion in immobilized cells due to the increased bacterial adhesion by entrapping the cells in the matrix.[64] Consequently, the cells have less metabolic energy and cellular activity required for protein production. The plasmid stability was 55% at 50 rpm, 49% at 100 rpm, 45.65% at 150 rpm, 44.39% at 200 rpm, and 37.78% at 250 rpm. The deliberate growth of the microbial cells at low agitation rates was due to the limited nutrient supply and resulted in extensive biofilm formation.[65]

Incubation Temperature

Imperfect folding during recombinant protein production results in accumulating and aggregating insoluble inclusion bodies (IBs).[66] The recombinant protein produced in *E. coli* is commonly secreted into the cytoplasm, periplasm, and growth medium (extracellular fraction). The expression inside the cytoplasm is favoured since production yields are habitually high. Protein folding in the cytoplasm can be improved at low temperatures[11] using cold-inducible promoters to reduce the possibility of protein aggregation.[66] The protein expression in *E. coli* at low temperatures can increase protein solubility, which is more complex to be expressed than soluble proteins. The proper folding of recently transcribed proteins is determined by slower protein expression rates and favoured by the reduced cellular protein concentration. Hence, lowering the incubation temperature is the preferred approach to reduce the protein synthesis rate. Aggregation occurs at higher temperatures due to the temperature reliance on hydrophobic interactions.[9]

Furthermore, protein expression at low temperatures can increase the possibility of accurate folding patterns and higher protein stability before the temperature dependence of hydrophobic interactions. It has been reported that the expression related to the toxic phenotype observed at 37 °C is restrained at low temperatures. The enhanced expression level and protein activity at lower temperatures are related to the increased expression of the folding helper chaperone proteins in *E. coli.* Moreover, growth at a lower temperature, such as in a range of 15-23 °C can also reduce the degradation of the expressed protein.[67]

Temperature plays a vital role in producing soluble recombinant protein, and generally, inclusion body formation can be prevented by decreasing the rate of protein synthesis by lowering the post-induction temperature. High temperatures can enhance cell growth but disrupt protein expression as higher growth rates lead to plasmid loss and stimulate misplaced expression vectors, particularly for plasmid expression. Overall, higher temperatures favour aggregation

reactions because of the strong temperature reliance on hydrophobic interactions.[68] Each expressed protein has a different optimum temperature depending on its characteristics. For example, the expression of recombinant flounder growth hormone (FGH) is maximal at 25-30 °C,[11] whereas the optimum temperature for recombinant HSPA6 protein expression is 37 °C.[69] This highlights the need to optimize the temperature for each recombinant target protein to increase expression.

An increase in temperature decreases cell viability because, at high temperatures, the activation of the specificity factor will produce proteins that help in folding to support cell survival (Figure 3A). However, this also causes cells to survive rather than produce growth-supporting functional proteins which, in turn, will increase the number of viable but non-culturable cells (VBNC). These VBNC will then experience death or lysis due to the accumulation of free radical species (ROS) as the result of the high temperature-induced formation of IBs due to the misfolding of catalase and superoxidase. Subsequently, ROS accumulation leads to mutations in the *katE* and *sodA* genes, catalase, and superoxidase coding genes that act as antioxidants, eventually causing oxidative stress. Also, long-term extremly high temperatures will trigger the aggregation and denaturation of functional proteins in cells.[70] Therefore, recombinant protein expression production above 37 °C in *E. coli* will yield less product because of its low viability.

Low temperatures (below room temperature) are not recommended for expressing recombinant proteins in *E. coli* unless for particular special strains that can survive and grow at low temperatures. Human prethrombin-2 was expressed at 12 °C using the *E. coli* BL21 (DE3) Arctic Express expression system.[8] At low temperatures (Figure 3B), cell growth slows down, accompanied by changes in cellular physiology, such as decreased membrane fluidity and stabilization of the secondary structure of nucleic acids, which leads to a decrease in the efficiency of RNA transcription, translation, and degradation.[71] However, it has been shown that the temperature drop is not significantly below the optimum temperature of *E. coli* growth (below 37 °C) and above room temperature, providing a benefit for the target protein in the form of dissolved protein because when the expression temperature is lowered, the production slows down. Hence there is more time for appropriate protein folding.[72]

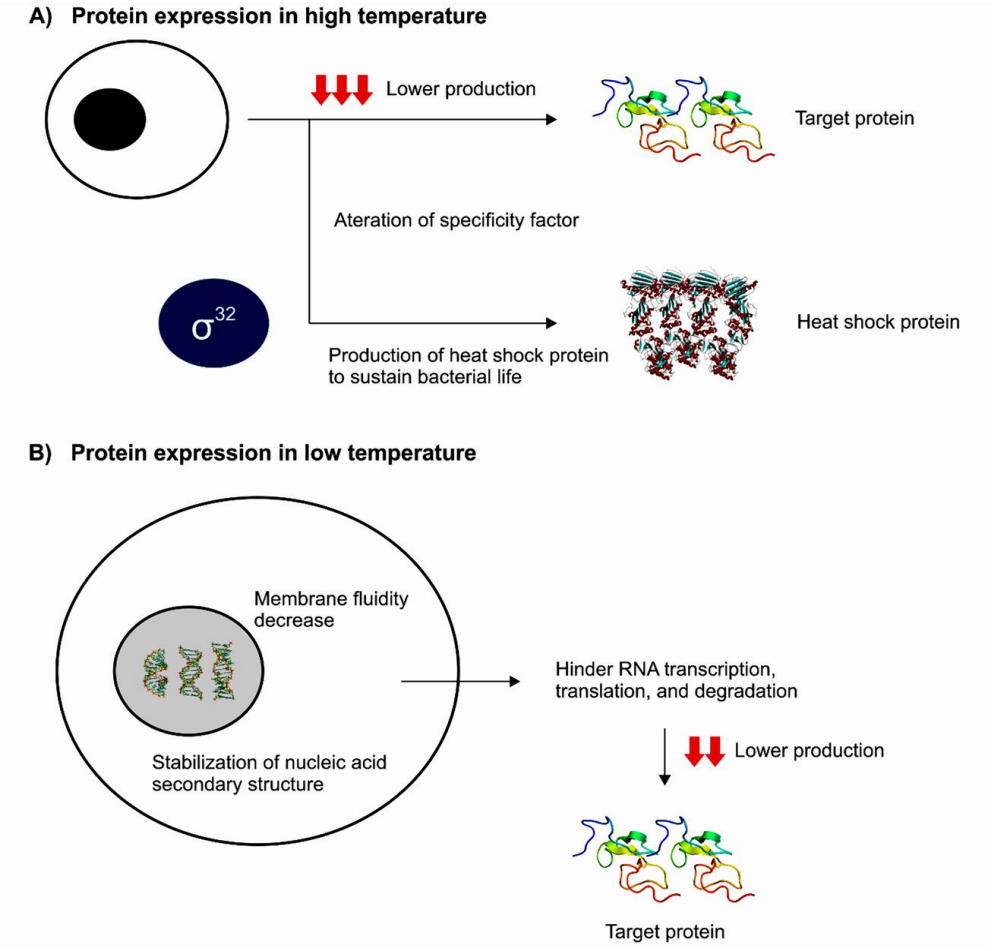

Figure 3. Effect of Incubation Temperature on Recombinant Protein Expression. **A)** High temperature activates sigma-32 which alter RNA transcription to express heat shock-related protein. **B)** Change of cellular physiology in low temperature affect efficiency in RNA transcription, translation and degradation which lead to low target protein expression.

Table 1. Summary of Recent *E. coli* Medium Composition in Protein Expression

No.	Protein Expressed	*E. coli* Strain	Medium	Medium composition	Reported by
1	hEGF	BL21 (DE3)	Unnamed	- Fermentation medium 12 L scale: 12 g/L sucrose, 3 g/L glycerol, 7.5 g/L yeast extract, 16.1 g/L $Na_2HPO_4.12H_2O$, 4.18 g/L KH_2PO_4, 0.56 g/L $Mg_2SO_4.7H_2O$, 0.56 g/L NaCl, 0.71 g/L $NaNO_3$, - Feeding medium contained: 426 g/L glycerol, 85 g/L tryptone, 43 g/L yeast extract, and 5.3 g/L $Mg_2SO_4.7H_2O$.	[76]
2	Hepcidin	BL21 (DE3)	2x YT medium	- 16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl	[77]
3	hEGF	ER2566 (DE3)	LB medium	- 10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl	[78]
4	Pig Liver Esterase (γPLE)	*E. coli* Origami™ (DE3)	LBrich, TB, mineral medium 1, mineral medium 2, complex medium 1, complex medium 2, feed medium 1, feed medium 2.	- LBrich medium according to Neubauer et al. (2007): 10 g/L yeast extract,20 g/L tryptone, 5 g/L NaCl, with or without 10% of a potassium phosphate buffer (0.17 M KH_2PO_4; 0,72 M K_2HPO_4, pH 7.5), and with or without 2 mM glucose; - TB medium: 24 g/L yeast extract, 12 g/L tryptone,4 mL/L glycerol, 100 mM potassium phosphate buffer (pH 7.5); - Mineral medium 1 according to Riesenberg (1991) was modified: 13.5 g/L KH_2PO_4 (),4 g/L $(NH_4)_2HPO_4$ (),1.7 g/L citric acid, 1.4g/L $MgSO_4.7H_2O$, 10 mL/L trace element solution, (20 g/L glucose, or 4 mL/L glycerol) - Mineral medium 2 according to Cutayar and Poillon (1989) was modified: 20 g/L KH_2PO_4,17.2 g/L $K_2HPO_4.3H_2O$,12 g/L $Na_2HPO_4.2H_2O$,0.34 g/L $(NH_4)_2SO_4$, 23.4 g/L yeast extract,0.4 g/L $MgSO_4.7H_2O$,10 mL/L trace element solution,20g/L glucose, or 4 mL/L glycerol -Complex medium 1 with glycerol or glucose: Tryptone (7 g/L), NaCl (5 g/L), peptone (3 g/L), yeast extract (10 g/L), $K_2HPO_4.3H_2O$ (2.5 g/L), 30 mM glycerol, or 5 g/L glucose - Complex medium 2 with glucose according to Neubauer et al., (2007) was modified: 30 g/L tryptone,5 g/L NaCl, 20 g/L yeast extract, 2.0 g/L Na_2SO_4, 2.5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 1 g/L $(NH_4)_2$-H-citrate, 14.6 g/L K_2HPO_4, 3.6 g/L $NaH_2PO_4.2H_2O$, 2 g/L $MgSO_4$, 100 mg/L thiamine, 2 mM glucose - Feed medium 1: 17 g tryptone, 5 g NaCl, 3 g peptone, 10 g yeast extract, 2.5 g $K_2HPO_4.3H_2O$ in 250 mL A. dest. and 25% glycerol - Feed medium 2: 20 g tryptone, 5 g NaCl, 10 g yeast extract, 5 g MgSO4, 10 mM glucose in 250 mL aquadest	[79]
5	hEGF	DH5α	M9 medium and modified M9	- M9 medium: 4g/L glucose, 15.12g/L $Na_2HPO_4.12H_2O$, 3g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.5 g/L $MgSO_4.7H_2O$, 0.011g/L $CaCl_2$, and mass concentration of 1% vitamin B_1 0.2 mL/L - modified M9: 10 g/L glucose, 2.27 g/L $Na_2HPO_4.12H_2O$, 0.45g/L KH_2PO_4, 0.5g/L NaCl, 1 g/L NH_4Cl, 0.5g/L $MgSO_4.7H_2O$, 0.011g/L $CaCl_2$, mass concentration of 1% vitamin B_1, 0.2ml/L and 0.4ml/L trace elements solution.	[80]
6	P64K	K12 GC366	Minimum Medium	- Fermentation medium 1,5 L scale: 10 g/L casein hydrolysate, 10 g/L tryptone, 10 g/L glucose, 0.015 g/L $CaCl_2{\cdot}2H_2O$, 0.246 g/L $MgSO_4{\cdot}7H_2O$	[81]
7	SlyD	BL21(DE3), NiCo21(DE3), and NiCo22(DE3)	Unnamed	- Fermentation medium: 20 g/L animal-free soy peptone, 10 g/L yeast extract, 10 g/L NaCl, 30 g/L glycerol, 0.75 g/L KH_2PO_4, 0.75 g/L Na_2HPO_4, 0.70 g/L NH_4Cl, 0.085 g/L K_2SO_4, 0.62 g/L $MgSO_4.7H_2O$, 0.0125 g/L Fe(III) citrate, 0.015 g/L $MnCl_2.7H_2O$, 0.0013 g/L $Zn(CH_3COO)_2.2H_2O$, 0.0025 g/L H_3BO_3, 0.0025 g/L $Na_2MoO_4.2H_2O$, 0.0025 g/L $CoCl_2.6H_2O$, 0.0015 g/L $CuCl_2.2H_2O$, 0.0014 g/L Na_2EDTA	[82]
8	Xylonic acid	BL21 (DE3)	Super optimal Broth (SOB)	- 20 g/L of tryptone, 5 g/L of yeast extract, 0.5 g/L of NaCl, 0.186 g/L of KCl, 2.4 g/L of $MgSO_4$	[68]
9	ADH and FDH	BL21 (DE3)	Complex medium	- 20 g/L peptone, 20 g/L yeast extract, 30 g/L glucose, 13.0 g/L KH_2PO_4, 10 g/L K_2HPO_4, 5g/L NaCl, 2 g/L $(NH_4)_2SO_4$,1g/L $MgSO_4{\cdot}7H_2O$, and 0.2 g/L NH_4Cl (pH 6.8)	[83]
10	IGF-1	W3110	Unnamed	- 55.7 mM ammonium sulfate, 13.9 mM sodium monobasic phosphate, 21.9 mM potassium dibasic phosphate, 5 mM sodium citrate, 29.6 mM potassium chloride, 14.7 mM magnesium sulfate, 1.11% NZ amine AS, 1.11% yeast extract, 0.11% glucose, 0.002% ferric chloride,	[84]
11	Mouse endostatin	YK537	Unnamed	- Fermenter medium 10 L scale: 1% yeast, 2% polypeptone, 0.5% casamino acids, 0.2% $MgSO_4$, 0.2% NH_4Cl, 0.2% $(NH_4)2SO_4$, 0.1% NaCl, 0.6% glucose	[85]
12	Cas 9	BL21 (DE3) and BL21 (DE3) Rosetta	Defined medium	- medium components per L: 10.0 g/L glucose,13.3 g/L KH_2PO_4,4.0 g/L $(NH_4)2HPO_4$,1.2 g/L $MgSO_4.7H_2O$,1.7 g/L citric acid, 14.1 mg/L EDTA,100.8 mg/L Fe(III)	[86]

				citrate,4.5 mg/L thiamine HCl,2.5 mg/L $CoCl_2.6H_2O$, 15.0 mg/L $MnCl_2.4H_2O$,1.5 mg/L $CuCl_2.2H_2O$,3.0 mg H_3BO_3,2.1 mg/L $Na_2MoO_4.2H_2O$, and 33.8 mg/L $Zn(CH_3COO)_2.2H_2O$.	
13	Human insulin	*Escherichia coli* 20 IBA 1	GMS Medium	Not described	[87]
14	Human serotonin transporter	BL21 CodonPlus (DE3) RP, C41 (DE3), C43 (DE3) and Lemo21 (DE3)	ZY-based complex autoinduction medium	- 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl	[88]
15	GST, GFP and CYP	BL21 (DE3)	BYT, BYT-glycerol, YTA medium	- Buffered YT medium (BYT): 5 g/L yeast extract, 10 g/L tryptone, 9 g/L $Na_2HPO_4.12H_2O$ and 1 g/L KH_2PO_4 - BYT-glycerol medium: 10 g/L glycerol, and BYT-glucose medium contained 10 g/L glucose. - YTA medium: 10 g/L yeast extract, 10 g/L tryptone, 33 g/L $(NH_4)_2SO_4$, 4.5 g/L $Na_2HPO_4.12H_2O$ and 0.5 g/L KH_2PO_4.	[89]

Growth Medium

Recombinant protein production requires nutrients for bacterial growth There is restricted control of the growth parameters to prevent negative impacts, such as the changes in pH, substrate depletion, and dissolved oxygen concentration, as well as the accumulation of inhibitory materials from several different metabolic pathways. These changes are not advantageous for producing perfectly folded and insoluble proteins. Suitable and well-organised protein folding requires specific helpers, such as cofactors in the growth media, usually metal ions, that significantly increase soluble protein's folding rate and yield.[67]

Fine-tuning the culture conditions and culture medium is necessary and can be achieved by optimizing or varying the composition of the culture medium. The composition of amino acids, yeast extract, and salts may enhance the protein yield.[73] A minimal medium consisting of a carbon source, such as glucose (which plays a role both as a carbon source and an energy source) and salts that supply nitrogen, phosphorus, and trace metals, is the standard medium for *E. coli* to proliferate. Nevertheless, *E. coli* grow faster in a rich medium that supplies the cells with nucleotide precursors, amino acids, vitamins, and other metabolites that support cell growth and protein synthesis.[74] However, sugar metabolism is regulated hierarchically in a medium containing more than a single carbon source. The expression of metabolic genes for the respective sugars is regulated through catabolite expression, where the sugar in the upper hierarchy represses gene expression for lower sugar metabolism.[75]

Table 1 details several culture media routinely used for recombinant protein expression. Various media, like LB, TB, and 2YT, can be used to optimize the protein concentration by adding some salt-related buffer to maintain the pH for optimal bacterial growth. The addition of cofactors or prosthetic groups is crucial for protein stability, appropriate folding, and protein solubility to avoid the formation of IBs.

Inducer Concentration

An inducer plays a pivotal role in protein expression by switching on promotors that regulate protein expression. Thus, the inducer concentration is critical regardless of the promotor mode of action (positive/negative regulation). The concentration should be slightly above the threshold at which the promotor is activated.[90] IPTG is a commonly used inducer because of its high expression rate, and it is an allolactose that that *E. coli* cannot metabolize. However, in particular cases, IPTG usage offers drawbacks such as the metabolic burden on the cell due to the exacerbation of haloalkane.[91] The expression of heterologous proteins sometimes becomes an issue when the protein expressed is toxic to the host,[90,91] which the high rate of IPTG expression can exacerbate. Moreover, the lac operon is prone to the leaky expression;[9,91,92] therefore, IPTG usage should be reconsidered.

The rhamnose promotor is considered a tighter promotor than lactose. Hence, it has a relatively lower expression rate. Despite this disadvantage, the rhamnose promotor is suitable for producing toxic heterologous proteins.[92] Alternatively, skimmed milk as a natural inducer has been reported, which may give less substrate exacerbation burden on metabolism, helping to prevent overconsumption of the metabolic precursor caused by the metabolic burden.[91,93] An attempt to lower the IPTG threshold in cell culture has also been reported, with the *lac* operon expression system modified by adding a modified promotor, P*tac*L, resulting in the (*lac*O)-target gene-P*tac*L-*lac*I system. Using a random synthetic library, mutant P*tac*L4 was obtained, requiring less IPTG (from 500 to 20 μM).[94] These alternatives give insight into overcoming IPTG drawbacks while providing cost-effective expression methods.

Obtaining Optimal Conditions

Each parameter of the bacterial culture influences the protein yield differently. Therefore, protein expression should be optimized one factor at a time to determine the optimal conditions. However, this traditional optimization method is inconvenient when the expression needs to be scaled up as the number of parameters or factors will also

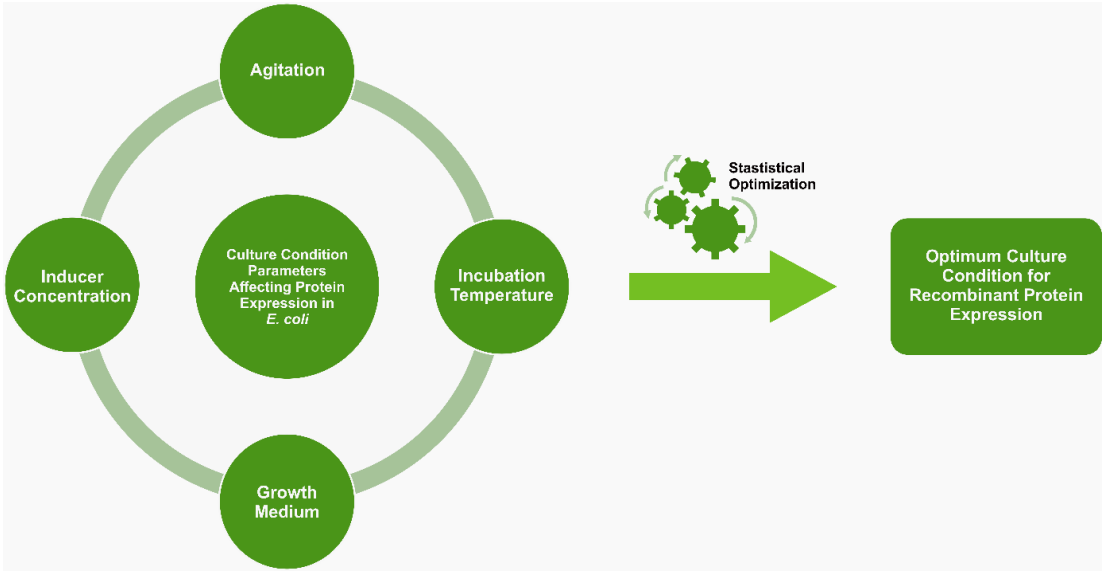

Figure 4. Overview of Culture Condition Parameters Affecting Recombinant Protein Expression. Parameters optimization using statistical design of experiment (DoE) helps to obtain optimum culture condition efficiently.

increase. An optimization process with statistical analysis would save time and resources while maintaining data reliability, such as factorial design and response surface methodology (RSM).[90] RSM experimental designs are beneficial when handling more than two factors that need to be optimized. Gutiérrez-González et al., (2019) optimized the culture conditions (post-induction temperature, post-induction time, and IPTG concentration) for the expression of three different proteins using a Box-Behnken RSM experimental design.[95] Therefore, a statistical approach helps determine the optimum conditions for recombinant protein expression (Figure 4).

Conclusion

No perfect expression system and conditions work well with all recombinant proteins, as every protein has different issues. Therefore, the production of each recombinant protein should be optimized to achieve a high yield. This study may enhance the knowledge and help scientists develop new strategies for eukaryotic protein production in a prokaryotic host, especially the *E. coli* expression system, which is of future value.

Authors' Contributions

IPM: Conceptualization, project administration, supervision, funding acquisition; FPUL: Methodology, writing - original draft; AN: Conceptualization, writing - original draft; NFL: Writing - original draft; SS: Methodology, formal analysis, conceptualization; MIA: Writing - original draft and revision, review and editing; DFU: Writing - revision.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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