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Review

Additivities for Soluble Recombinant Protein Expression in Cytoplasm of *Escherichia coli*

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Abstract: Recombinant protein expression in *Escherichia coli* is a fundamental technique in molecular biology and biotechnology. This review provides a comprehensive overview of various additivities to enhance the expression levels of soluble recombinant proteins in *E. coli*. The discussion encompasses five key aspects. Inducer Optimization: strategies for optimizing the inducer concentration to enhance protein expression. Autoinduction system optimization: the examination of glucose, lactose, and glycerol optimization within autoinduction systems to improve protein production. Osmolytes and osmoprotectants: an analysis of the use of osmolytes and osmoprotectants, such as sorbitol and glycine-betaine, to overcome with ease osmotic stress and enhance protein solubility. Ethanol additives: the impact of ethanol on *E. coli* physiology and its potential to improve recombinant protein expression. Cofactors and metabolic precursors: insights into the addition of cofactors, such as pyridoxal phosphate, riboflavin, thiamine, and pyridoxine, and the utilization of metabolic precursors to enhance the corresponding protein expression. This review highlights both the successful strategies and challenges in recombinant protein expression and provides insights into potential future research directions. Understanding and optimizing these factors is crucial for the efficient production of recombinant proteins for various applications in biotechnology. Furthermore, based on the analyzed data, we propose a straightforward scheme to optimize the additives in the cultivation medium.

Keywords: *Escherichia coli*; protein expression; additives; cytoplasm

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1. Introduction

Recombinant protein expression has revolutionized the production of valuable proteins for various applications, ranging from therapeutic drugs to industrial enzymes. Among the numerous expression systems available, *Escherichia coli* remains a popular choice [1,2] due to its fast growth, well-characterized genetics, wide experimental experience and robustness in operation. However, one significant challenge in *E. coli* expression is the formation of insoluble aggregates called inclusion bodies, which hinder the recovery of functional, soluble proteins [3,4]. While the formation of inclusion bodies can simplify the purification of proteins, it does not guarantee that the process of in vitro refolding will result in high quantities of a biologically active product. Inclusion bodies are commonly formed when the overexpressed recombinant protein exceeds the host's folding capacity or encounters unfavorable conditions in the cell [4,5]. Traditionally, refolding these proteins from inclusion bodies has been a labor-intensive and low-yield process. Despite advances in in vitro refolding strategies, achieving high yields of correctly folded, soluble proteins from

inclusion bodies remains a complex task [6]. It is worth noting that reports of unsuccessful attempts at refolding are rare in the literature.

To address this challenge, researchers have explored various strategies to enhance the solubility and yield of recombinant proteins in *E. coli*. One promising approach involves the use of the co-expression of chaperones, such as heat shock proteins, which assist in the folding and stabilization of proteins [7,8]. Chaperones, such as DnaK, DnaJ and GroEL/ES, interact with the newly synthesized polypeptide chains, preventing misfolding and promoting correct folding, thus increasing the likelihood of obtaining soluble proteins [9]. In addition to chaperones, fusion tags have been employed to enhance protein solubility and facilitate purification [9]. These tags, such as maltose-binding protein (MBP), glutathione S-transferase (GST), or polyhistidine (His-tag), can improve protein stability, prevent aggregation, and provide affinity handles for purification techniques. The choice of fusion tag depends on the specific requirements of the protein and downstream applications. Another aspect of optimizing recombinant protein expression in *E. coli* involves codon optimization. *E. coli* has biased codon usage, and the use of codons rarely found in the host organism can lead to inefficient translation and protein misfolding. Codon optimization involves redesigning the DNA sequence of the target gene to incorporate the codons preferred by *E. coli*, thereby enhancing the translation efficiency and protein production. This approach can be used to improve protein solubility and yield in *E. coli* expression systems [10]. On the other hand, an increased translation rate can, on the contrary, lead to protein misfolding and insolubility [11]. A simple and rapid approach to reduce the number of inclusion bodies is to lower the temperature during induction. Typically, the temperature is lowered to 15–25 °C. There are also examples of successful cultivation at temperatures below 10 °C [12]. Special strains are developed for cultivation at reduced temperatures, such as *E. coli* ArcticExpress, which co-expresses the cold-adapted chaperonins Cpn10 and Cpn60 from the psychrophilic bacterium *Oleispira antarctica* [13].

Another straightforward method for influencing the expression levels of soluble protein forms is to introduce additives to the culture medium. These additives can modify the cellular environment and facilitate the correct folding of recombinant proteins in the cytoplasm, ultimately leading to an increased soluble protein expression. This article aims to provide an overview of the recent studies on the additives for the *E. coli* cultivation enhancing soluble recombinant protein expression in the cytoplasm. We will explore the role of different additives in promoting proper protein folding, preventing aggregation, and improving the overall efficiency of protein production in *E. coli*.

2. Inducer

Recombinant protein expression can be achieved through self-induction or by adding an inducer. Increasing the inducer concentration is expected to lead to higher expression yields, but increased expression levels can result in the formation of inclusion bodies (Figure 1A). Conversely, reducing the inducer concentration may lead to a decrease in the protein synthesis rate and a reduction in the number of inclusion bodies. Therefore, when expressing proteins prone to inclusion body formation, strategies aimed at both increasing and decreasing the inducer concentration should be considered.

Systems using lac-based promoters are the most potent and well-studied expression systems. Lac-based promoters operate using an "on" or "off" mechanism, which leads to challenges in adjusting the inducer concentration to reduce the enzyme synthesis rate [14]. IPTG is one of the most widely used and effective inducers [15]. Increasing the concentration of IPTG often leads to higher yields of active enzyme forms [16]. However, high concentrations of IPTG can inhibit the growth of *E. coli* [17–19]. Reducing the concentration of IPTG can also improve the yields of soluble protein expression. Reducing the IPTG concentration from 1.2 to 0.3 mM resulted in an increased yield of the soluble form of the recombinant bovine sex-determining region Y protein [20]. During the expression of the leptospiral protein, the highest yield of soluble proteins and the best cell growth were observed at the lowest investigated concentration of IPTG, which was 0.1 mM [21]. When

studying the influence of IPTG ranging from 0.25 to 1.25 mM, the maximum expression of the receptor activator of nuclear factor-κB was determined to be at 0.3 mM IPTG [22]. The study on the influence of IPTG concentration (0.25, 0.5, 1, or 2 mM) on the expression levels of the thioredoxin fusion with the epithelial cell adhesion molecule's extracellular domain showed that the highest yield of the protein was achieved with 0.5 mM of IPTG [23]. The synthesis rate of the yellow fluorescence protein was not dependent on the IPTG concentration, but reducing the IPTG concentration led to an increased delay before protein synthesis [24]. Reducing the IPTG concentration below 0.1 mM can be employed to increase the delay during induction. In some cases, optimizing the IPTG concentration during induction has little effect on protein expression levels [18,19,25–27].

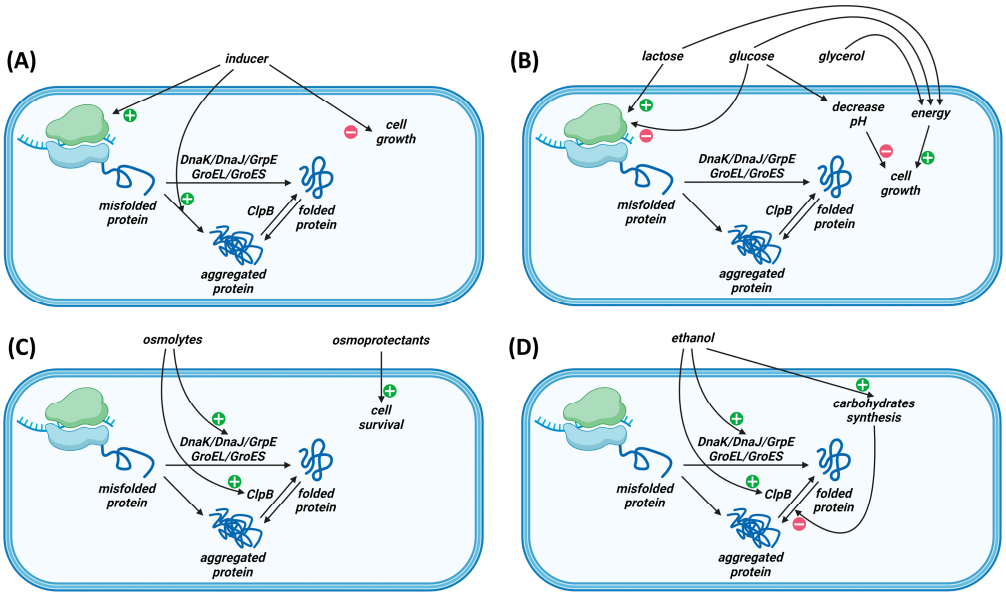

Figure 1. Possible mechanism of influence of the inducer (**A**), glucose/lactose/glycerol (**B**), osmolytes (**C**) and ethanol (**D**) on recombinant protein expression in *E. coli*.

A derivative of the BL21 (DE3) expression strain has been engineered by introducing a lacY1 deletion mutation. This modification enables the T7 promoter system to be finely controlled and responsive to varying concentrations of IPTG [28]. The *E. coli* Tuner (DE3) is a strain containing a lacZY deletion, which allows for the precise modulation of the induction level and, in certain instances, facilitates the production of soluble proteins. The number of studies using the Tuner (DE3) strain and optimizing the inducer concentration is limited. In the optimization of cyclomaltodextrinase expression, the IPTG concentration was varied from 5 µM to 1 mM, and the highest expression yield was observed at a concentration of 50 µM [28].

Another commonly used promoter is the pBAD promoter from the araBAD operon, which enables *E. coli* cells to transport and metabolize arabinose [29]. Systems utilizing the pBAD promoter are positively controlled, and low basal expression levels are expected, which is particularly important for toxic proteins [30]. When using the pBAD promoter, protein expression levels depend on the concentration of arabinose within two orders of magnitude [31]. This allows for the titration of protein expression levels over a wide range of arabinose concentrations. However, it is worth noting that at low arabinose concentrations, the system forms subpopulations of producing and non-producing cells [30,32,33]. This can lead to a situation where expression levels decrease not due to a reduction in the synthesis rate of the enzyme but due to a decrease in the number of producing cells. When using glucose as the carbon source and arabinose as the inducer, a catabolite repression effect is observed, with glucose having a stronger repression effect compared to glycerol [24]. The catabolite repression effect can be utilized to optimize the type and concentration of the carbon source in inducible systems and autoinduction systems (as discussed below).

3. Glucose, Lactose and Glycerol

Glucose is a readily available and widely used additive in *E. coli* cultivation. The addition of glucose to the culture medium often reduces the expression rate of recombinant proteins due to the effect known as catabolite repression [34]. The addition of 1% glucose can significantly reduce the inducibility of *E. coli* BL21 (DE3) cells [35,36]. Reducing the expression rate can lead to increased yields of protein expression in a soluble form. This is especially important when expressing highly toxic proteins, where even low levels of expression can lead to cell death before reaching the required cell density. By optimizing the initial glucose concentration, it was possible to increase the yield of recombinant interferon α-2b by more than 2-fold [37]. The optimal glucose concentration was found to be 2% (20 g/L). Further increases in concentration resulted in a significant reduction in the protein yield.

During the cultivation process, the utilization of glucose by cells leads to a production of unwanted byproducts such as lactate and acetate, which can inhibit cell growth and recombinant protein production. The addition of glucose can significantly decrease pH and halt cell growth, even when using buffering systems (Figure 1B). Adding 2% glucose in combination with 66 mM of phosphate buffer in an M9 medium leads to a decrease in pH and a reduction in cell growth [36]. Therefore, the use of high glucose concentrations is undesirable. The negative impact of pH reduction when using high glucose concentrations can be mitigated by adding succinate, fumarate, aspartate, or glutamate or by controlling the pH during cultivation in bioreactors.

Optimizing glucose concentration is particularly important when using autoinduction systems. The autoinduction system is based on the ability of certain compounds to prevent the induction of the target protein by lactose. The lactose metabolism triggers induction after the depletion of glucose reserves. Glycerol can be used as an additional carbon source, which can be metabolized concurrently with lactose after glucose depletion [38] and has a significantly lower ability to suppress the use of other carbon sources by cells [39]. Glycerol also leads to less acidification of the pH compared to glucose. Therefore, a combination of glucose, glycerol and lactose is often used in autoinduction. The use of the autoinduction system allows cells to grow to a high density before induction is initiated. The use of the autoinduction system can lead to higher yields compared to IPTG induction. When using the ZYM-20052 autoinduction medium (2.5% glycerol, 0.05% glucose and 0.2% lactose), the yield of the soluble form of one of the three studied proteins was significantly higher than when using the nutrient-rich Dynamite medium with IPTG induction [40]. Media such as 5052, containing 0.5% glycerol, 0.05% glucose, and 0.2% lactose, facilitate the autoinduction of a wide range of proteins under various growth conditions [36]. Optimizing the components of the autoinduction medium can significantly increase the yield of the target protein. This optimization becomes particularly important when changing aeration conditions because high levels of aeration can lead to a reduced expression when using the autoinduction system [41]. It appears that increasing the lactose concentration to ~0.5% and the glycerol concentration to ~0.8% may be necessary under high aeration conditions [42]. High concentrations of glycerol and lactose can also enhance expression yields through osmotic shock (see below). The autoinduction system can also be employed with the pBAD promoter in BL21-AI cells [36]. Instead of 0.2% lactose, 0.05% L-arabinose can be used as the starting concentration for optimization in this case.

4. Osmolytes and Osmoprotectants

The most extensively studied prokaryotic protein disaggregation system in cytoplasm consists of heat shock proteins DnaK/DnaJ/GrpE, GroEL/ES, and ClpB [43–45]. Under osmotic shock conditions, the expression of heat shock proteins increases [46]. Due to the enhanced synthesis of chaperone proteins, one can anticipate an increase in the expression levels of soluble recombinant proteins under osmotic shock conditions (Figure 1C). One of the adaptation mechanisms of non-halophilic bacteria to high salt concentrations is the accumulation of osmoprotectants or compatible solutes, which prevents water loss due

to osmotic pressure [47]. Compatible solutes are organic compounds that can accumulate within the cell and do not harm the biochemical processes inside [48]. During the cultivation of *E. coli* in high osmolarity media, high levels of glycine-betaine uptake have been observed [49]. Additionally, it was found that the addition of glycine-betaine and proline-betaine stimulated the growth of *E. coli* cells under osmotic stress conditions [50]. The high energetic cost of producing osmoprotectants necessitates their addition to the culture medium [51]. For recombinant protein expression in *E. coli*, NaCl and sorbitol are commonly used for inducing osmotic stress, with glycine-betaine being the most frequently used osmoprotectant.

Numerous studies provide compelling evidence for the efficacy of osmotic shock in increasing the expression levels of poorly soluble enzymes in *E. coli*. In Oganesyan's work, six out of nine proteins showed improved expression in an LB medium with the addition of 0.5 M of NaCl together with 1 mM of betaine [52]. The addition of 660 mM of sorbitol and 2.5 mM of betaine resulted in a 2.4-fold increase in the yield of dimethylallyl pyrophosphate:5′-AMP dimethylallyltransferase at 37 °C, whereas the addition of 1000 mM of sorbitol with betaine at 25 °C resulted in a 6.5-fold increase [53]. An amount of 500 mM of sorbitol without betaine increased the soluble forms of three out of eight enzymes by approximately 1.5 to 2 times [54]. Co-expression with chaperones and supplementation with 0.5 M of sorbitol increased the yields of the transforming growth factor beta 3 [55]. Further enhancement was achieved with 1 M of trehalose, while other osmolytes like ethylene glycol, arginine hydrochloride, and sucrose did not increase the soluble protein yield. In addition to NaCl and sorbitol, high concentrations of glycerol and arginine are often used for osmotic shock. The addition of 0.4% glycerol during induction increased the yields of human phenylalanine hydroxylase wild-type and mutant enzymes [56]. The optimal concentration of sorbitol during the expression of the diphtheria toxin variant with its N-terminus fused to a SUMO tag was 200 mM [57]. Adding 0.3 M of sorbitol or 0.2 M of arginine increased the yield of soluble proteins [20]. The addition of 2% glycerol or 0.2 M of sorbitol increased the yield of active cholesterol oxidase [58]. Supplements of 0.5 M of sorbitol and 0.2 M of arginine in culture media resulted in an increased number of active inclusion bodies of GFP [59]. In some cases, the addition of osmolytes and osmoprotectants has been ineffective in promoting soluble protein expression. For instance, it was not possible to express aminotransferase from *Sphingopyxis* sp. MTA144 in a soluble form with the addition of 2.5 mM of betaine or 600 mM of sorbitol [27]. Furthermore, the addition of betaine did not lead to increased expression levels of porphyrinogen IX oxidase [60]. In certain instances, such as in the case of the human serotonin transporter, the addition of 1 M of sorbitol and 250 mM of betaine even resulted in a decrease in the yield of the membrane protein [61]. The impact of sorbitol, arginine, trehalose, and NaCl additives on the yield of the soluble flagellin of Salmonella enterica serovar Enteritidis was investigated. The maximum yield of soluble proteins was observed when 200 mM of sorbitol was added. A comparable result was observed with the addition of 100 mM and 250 mM of arginine [62].

The enhancement of soluble enzyme expression levels through the addition of osmolytes may not solely be attributed to the activation of chaperone expression. Osmolytes such as sorbitol, glycerol, and trehalose are often used as protein stabilizers [59,63]. They have the ability to inhibit the unfolding of native conformations into unfolded/incorrectly folded forms through a mechanism similar to that of other polyatomic alcohols [64].

5. Ethanol

The addition of ethanol to the cultivation medium is one of the approaches used to increase the expression levels of soluble protein forms. The introduction of ethanol to bacteria results in significant physiological changes, such as a protein and ion leakage from membranes or an increased membrane permeability [65,66]. Proteomic analysis shows that the addition of ethanol leads to an increase in the quantity of heat shock proteins [67] (Figure 1D). Numerous proteins related to carbohydrate synthesis and transport show a significant increase in expression when exposed to ethanol [68,69]. Carbohydrates are known

as stabilizers for most proteins, and altering their levels can lead to increased expression yields in a soluble form. Transcriptomic analysis indicates the presence of ethanol-induced oxidative stress, leading to hypoxia and a reduced aerobic metabolism [69,70]. This results in a slowdown of macromolecule biosynthesis, which, in turn, reduces the quantity of misfolded proteins.

The number of studies with successful applications of ethanol additives to obtain soluble protein forms is significantly lower compared to the number of studies using osmolytes and osmoprotectors. Typically, 3% ethanol is added to the cultivation medium. The addition of ethanol led to a 2-fold increase in the yield of Ranibizumab [71]. Adding 3% ethanol resulted in increased expression levels of four out of six examined proteins [72,73]. The addition of 3% ethanol during the cultivation of the fusion protein preS2-S′-b-galactosidase increased cultivation yields during induction at 30 and 42 °C. In contrast, induction at 37 °C with 3% ethanol did not change the cultivation yields [74]. The addition of ethanol at 37 °C did not significantly affect the yield of CT26-poly-neoepitopes, whereas at 22 °C, the addition of 2% ethanol resulted in increased expression yields. [75]. The addition of 3% ethanol at 20 °C resulted in an increase in the total quantity of the infectious hematopoietic necrosis virus nucleoprotein in both the soluble and insoluble fractions [76]. The addition of 3% ethanol did not alter the expression level of Protoporphyrinogen IX Oxidase [60].

6. Cofactors

Various enzymes with prosthetic groups often require sufficient quantities of corresponding cofactors or their precursors. Their addition can increase the yield of such enzymes [77]. One of the problems limiting the use of many cofactors as additives in expression is their high cost. In such cases, a metabolic precursor of the cofactor can be used.

During the expression of recombinant human hemoglobin, the addition of hemin resulted in an increase in the amount of soluble proteins [78]. Positive effects on the expression of heme-containing proteins may be achieved by adding thiamine and δ-aminolevulinic acid. During the expression of recombinant Cytochrome P450 1B1, the addition of thiamine did not lead to an increase in expression levels, whereas the addition of δ-aminolevulinic acid at concentrations of up to 1 mM resulted in a significant increase in the protein yield [79]. Thiamine supplements can be particularly important when cultivating strains of *E. coli* derived from *E. coli* K12, as they may be deficient in enzymes involved in thiamine anabolism [80,81].

Adding 1 μM of riboflavin 2 h before induction during the expression of FAD-containing protoporphyrinogen oxidase increased the amount of the recovered enzyme by approximately 4-fold [82]. The positive effect on enzyme expression upon the addition of riboflavin in *E. coli* may be related to the presence of a riboflavin transmembrane import system (YpaA protein in *E. coli*) and an endogenous riboflavin biosynthesis pathway [83–85]. Notably, *E. coli* BL21 (derived from *E. coli* B) is more prone to accumulate riboflavin than *E. coli* MG1655 (similar to *E. coli* K-12) [86].

The addition of cofactors can increase the yield of activity not only by increasing solubility, but also by increasing the specific enzymatic activity. The addition of 0.02 mM of pyridoxal phosphate (pyridoxine-5-phosphate) increased the yield of active glutamate decarboxylase by 2–2.5 times and simultaneously double increased in glutamate decarboxylase specific activity [87]. Pyridoxine can be taken up by *E. coli* cells and used for the synthesis of pyridoxal phosphate [88]. The addition of pyridoxine at concentrations above 0.05 mM allows for an almost 1.8-fold increase in the yield of active glutamate decarboxylase and a 1.5-fold increase in the specific activity of the enzyme [89]. The addition of pyridoxine also resulted in a 2.8-fold increase in the stability of glutamate decarboxylase.

Vitamin additives or their precursors cannot always increase the yield of the active soluble form of a protein. For example, pyridoxal phosphate, which is a co-enzyme of aminotransferase FumI, has no effect when added to the medium at a concentration of 1 mM [27]. Additionally, adding 0.1 M of FAD, FMN, and riboflavin during cultivation did not lead to increased expression yields of human D-amino acid oxidase [90].

7. Optimization

Based on this analysis of existing approaches to enhance expression levels, we propose a simple and versatile scheme for optimizing additives in the culture medium to increase the yield of soluble protein forms (Figure 2). The first step involves optimizing the inducer concentration for induction systems or the ratios of lactose/glucose/glycerol components for autoinduction systems. The literature's data indicate that the optimal inducer concentration significantly varies depending on the target protein. Therefore, we suggest optimizing within a wide range of inducer concentrations and narrowing it down in case of success. For IPTG induction, concentrations can be optimized in the range of 0.1 to 1.0 mM. When using autoinduction systems, a starting point could be a medium like 5052 supplemented with 0.5% glycerol, 0.05% glucose, and 0.2% lactose. To reduce the number of experiments, it is advisable to simultaneously vary each component within a wide range as part of parallel optimization. For instance, in a series of nine parallel experiments, test additions of 0.2%, 0.5%, and 1.0% glycerol, 0.02%, 0.05%, and 0.1% glucose, and 0.1%, 0.2%, and 0.5% lactose while keeping the other components constant.

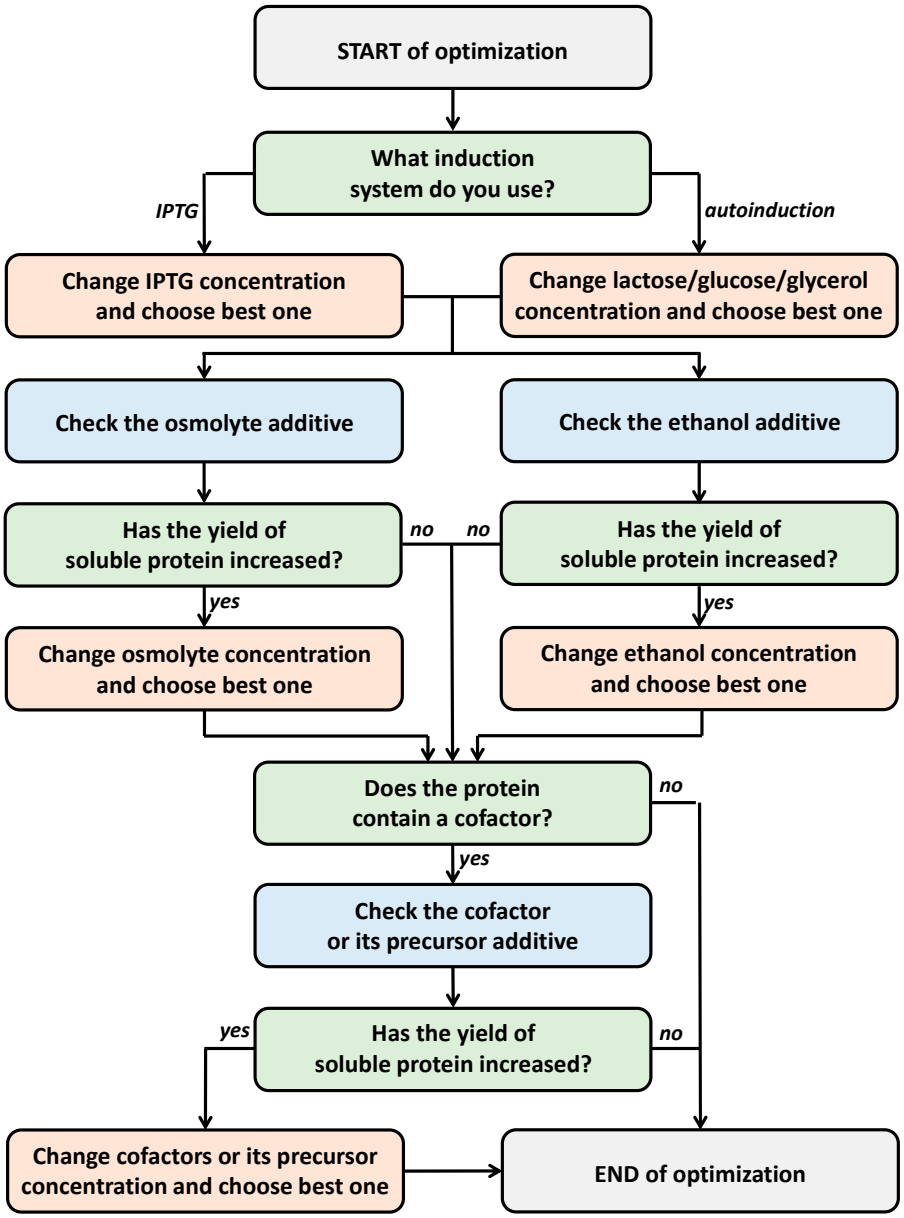

Figure 2. Scheme for optimizing additives.

The second step involves the parallel assessment of the effects of osmolytes/osmoprotectants and ethanol additives. For an osmolyte, we recommend using 0.5 M sorbitol, 0.5 M sodium chloride, or 2% glycerol. According to the literature, sorbitol is the most popular additive for conducting osmotic shock, but sodium chloride and glycerol are more cost-effective and readily available in laboratory practice. For an osmoprotectant, you can use a 2.5 mM betaine additive. In the absence of betaine, you can evaluate the impact of an osmolyte addition without an osmoprotectant. To assess the influence of ethanol, we propose using a 3% ethanol additive. If osmolyte/osmoprotectant or ethanol additives lead to increased expression levels, further optimization of the respective additive concentrations should be conducted.

In cases where the studied protein contains a cofactor, the optimization of the cofactor or its metabolic precursor additives can be considered. Such additives are often costly, so it is advisable to conduct this step in the final stages of optimization. If the addition of a cofactor or its precursor leads to increased expression yields, further optimization of the added quantities can be pursued. The proposed optimization scheme is simple and does not require a large number of experiments.

8. Conclusions and Future Perspectives

Enhancing the expression level of soluble recombinant proteins is an important task for molecular biology and biotechnology. Introducing additives in a culture medium is a technically straightforward method for optimizing the expression levels of recombinant proteins. Expression systems based on *E. coli* are among the most popular for obtaining recombinant proteins. This review provides a comprehensive overview of the use of additives to enhance the expression levels of recombinant proteins in *E. coli* in soluble forms.

The most popular and researched approaches include changing the inducer concentration and adding osmolytes. Based on studies [52,54], approximately half of the cases show increased expression levels of recombinant proteins in soluble forms under osmotic shock conditions. This effectiveness is comparable to the efficiency of a co-expression with chaperones [91].

The number of studies focusing on the impact of ethanol on the expression levels of recombinant proteins is significantly lower compared to osmolytes. It would be interesting to see research comparing the effectiveness of adding various osmolytes, ethanol, and other additives on the expression of a wide range of different proteins. Most studies focus on the impact of a single additive. It is unclear how a combination of additives will affect the expression levels of recombinant proteins.

Temperature is an important factor in optimizing the expression of recombinant proteins, and lowering it appears to increase the effectiveness of osmolyte and ethanol additives [53,74,75]. Predicting the impact of any additive in advance is not feasible; therefore, it is most practical to test additives that most commonly lead to increased expression levels of soluble enzyme forms.

It can be assumed that as the experimental knowledge base expands and our understanding of *E. coli* metabolism and regulation grows, researchers will continue to discover new additives that lead to increased yields of soluble enzymes. The rapidly advancing field of metabolic engineering may also aid in creating *E. coli* strains with metabolic pathways adapted for the highly efficient expression of recombinant proteins, including those requiring specific additives.

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