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OPTIMIZATION OF CULTURE MEDIA FOR INDUSTRIAL CULTIVATION OF THE RECOMBINANT STRAIN *ESCHERICHIA COLI* BL21

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This study presents a comprehensive analysis of scientific literature published between 2019 and 2024, indexed in Web of Science and Scopus databases. The review focuses on identifying optimization strategies for culture media to enhance the industrial cultivation of Escherichia coli BL21 strain for the production of recombinant proteins. This strain is widely used in industry due to its lack of certain proteases, making it ideal for producing stable protein products. The research highlights key factors influencing protein expression and biomass growth, including carbon and nitrogen sources, trace elements, additional components, and pH levels. Altering these key factors can increase cell yield and product quality. The analysis revealed that optimizing the culture medium composition through the use of alternative carbon and nitrogen sources can significantly improve bacterial cell growth and impact the quantity and quality of the recombinant protein. Alcohols such as mannitol and glycerol, sugars like lactose, as well as sugar-containing by-products from the food industry can be used as alternative carbon sources (blackstrap molasses, corn-steep liquor and whey). Additionally, complex compounds like lignocellulose can be utilized. Many alternative carbon sources can also provide nitrogen. The use of alternative carbon and nitrogen sources, on the one hand, can reduce the cost of recombinant protein production and thus affect bioeconomy, but on the other hand, can influence metabolic pathways for the assimilation of other elements and alter the duration of growth phases, which is crucial for industrial microbial cultivation. Optimization of the culture medium has complex consequences, and this process should be considered holistically.

Key words: Escherichia coli BL21, media optimization, alternative carbon source, alternative nitrogen source, recombinant protein.

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ОПТИМІЗАЦІЯ ПОЖИВНИХ СЕРЕДОВИЩ ДЛЯ ПРОМИСЛОВОГО КУЛЬТИВУВАННЯ РЕКОМБІНАНТНОГО ШТАМУ *ESCHERICHIA COLI* BL21

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В роботі відображено комплексний аналіз даних наукової літератури, опублікованої в період між 2019 і 2024 роками, відображеної в виданнях, що індексуються в базах даних Web of Science та Scopus. Огляд літератури зосереджено на визначенні можливостей оптимізації поживного середовища для промислового культивування штаму Escherichia coli BL21 з метою отримання рекомбінантних білків. Вказаний штам широко використовується в промисловості. Відсутність в цьому штамі певних протеаз робить його ідеальним інструментом для отримання стабільних білкових продуктів. Дослідження звертає увагу на ключові фактори, що впливають на експресію білків та приріст біомаси, включаючи джерела карбону та нітрогену, мікроелементи та додаткові компоненти, а також значення pH поживного середовища. Результатом змін цих ключових факторів є підвищення кількості клітин та якості продукту. В результаті аналізу літератури встановлено, що оптимізація складу поживного середовища через використання альтернативних джерел карбону та нітрогену може значно підвищити врожай клітин штаму бактерії та впливати на якість та кількість рекомбінантного білку. Як альтернативні джерела карбону можуть бути використані спирти – манітол, гліцерин, цукри – лактоза, а також цукровмісні сполуки, що є відходами харчової промисловості (патока, кукурудзяний екстракт, сироватка). Крім того, можливе використання комплексних сполук – лігнінцелюлози. Часто альтернативні джерела карбону можуть слугувати і джерелами нітрогену. Використання альтернативних джерел карбону та нітрогену може, з одного боку, бути одним з біоекономічних факторів здешевлення виробництва рекомбінантних білків, а з іншого боку, може впливати на метаболічні шляхи засвоєння інших елементів, та змінювати тривалість фаз росту культури, що важливо за промислового культивування мікроорганізму. Оптимізація поживного середовища має комплексні наслідки, і саме так необхідно розглядати цей процес.

Ключові слова: *Escherichia coli BL21, оптимізація середовища, альтернативне джерело вуглецю, альтернативне джерело азоту, рекомбінантний білок.*

Introduction

Escherichia coli (E. coli) BL21 is a widely utilized bacterial strain for heterologous protein expression. It's a derivative of the *E. coli* B lineage and has been engineered to lack several proteases, enzymes that break down proteins. This characteristic is crucial for maintaining the stability of the target protein being produced. Due to its favorable characteristics, such as ease of cultivation, high growth rate, and the ability to express a broad spectrum of proteins, *E. coli* BL21 has become an indispensable tool in biotechnology.

The efficiency of protein expression in *E. coli* BL21 is significantly influenced by the composition of the culture medium. Optimization of the culture medium composition can lead to a substantial increase in protein yield, improved quality, and reduced production costs.

A considerable body of research has been dedicated to the optimization of culture media for *E. coli* cultivation. These studies have demonstrated that the composition of the culture medium can significantly impact protein expression, including yield, solubility, activity, and complexity.

The aim of this study is to analyze scientific publications on the optimization of culture media for industrial cultivation of the recombinant strain *Escherichia coli* BL21, published between 2019 and 2024 in peer-reviewed journals indexed in the *Web of Science* and *Scopus* databases.

Material and methods

The search of scientific literature published between 2019 and 2024, indexed in *Web of Science* and *Scopus* databases for this review was conducted using the following primary keywords: «*Escherichia coli* BL21», «recombinant protein expression», «culture medium optimization», and «industrial cultivation» in Google scholar and Connected papers publication resources. Additionally, supplementary keywords such as «nutrient composition», «metabolic engineering» and «fermentation parameters» were used.

In order to select relevant publications, the following search strategies were used:

1) Phrase searching – keywords were enclosed in quotation marks to search for exact matches;

2) Use of Boolean operators AND, OR, and NOT to combine keywords and refine the search;

3) Use of the wildcard character (*) to substitute for one or more unknown characters within a keyword.

Results

Escherichia coli BL21 is a widely used bacterial strain for various biotechnological applications, including protein production and biofuel synthesis. Some of the key factors that influence protein expression in *E. coli* BL21 include carbon sources, nitrogen sources, trace elements, and pH. Studies have shown that supplementing defined media with yeast extract can reduce lag phase and increase biomass production (Shukla & Mishra, 2021). Optimization techniques like Plackett-Burman and Box-Behnken designs have been used to identify significant factors, such as yeast extract and mineral concentrations, leading to increased enzyme production (Duan et al., 2020). The choice of expression system, including promoter strength and plasmid copy number, significantly impacts recombinant protein production (Lozano Terol et al., 2021). Balancing these factors is essential to maximize soluble protein expression while minimizing metabolic burden. Optimized media and expression systems can result in higher protein yields, improved stability, and enhanced product specificity, which are crucial for industrial-scale production of recombinant proteins in *E. coli* BL21(Shahzadi et al., 2021).

Carbon sources. Manipulating carbon and nitrogen sources can increase recombinant protein production in *E. coli* BL21 (Lozano Terol et al., 2019). Traditionally, glucose and ammonium salts have been the primary carbon and nitrogen sources used for *E. coli* cultivation. However, exploring alternative carbon and nitrogen sources can offer several advantages, such as reduced cost, improved sustainability, and the potential to enhance recombinant protein production. One promising alternative carbon source is glycerol, a byproduct of the biodiesel industry. Glycerol has been shown to support robust growth of *E. coli* BL21 and can be utilized as a carbon source for the production of various recombinant proteins. Furthermore, the use of glycerol as a carbon source has been reported to improve the quality and yield of recombinant proteins in *E. coli.* (Lozano Terol et al., 2019)

Besides, Höhmann et al. (2024) investigated glycolate as a sole carbon source, observing that *E. coli* BL21 required extensive adaptation time but eventually reached growth rates comparable to other strains.

Lactose, another inexpensive and renewable carbon source, has also been explored as an alternative to glucose for *E. coli* BL21 cultivation. Lactose-based media can induce the *lac* operon, leading to improved recombinant protein production in *E. coli* BL21 strains engineered for lactose utilization. Moreover, combining lactose with other carbon sources, such as glucose or glycerol, can enhance bacterial growth rates and biomass production. Lactose, employed at a concentration of 10% (w/v), served as the carbon source to optimize recombinant truncated *SpA* expression in *E. coli*.

Previous research has shown that modifying the signal peptide, which guides the translocation of recombinant proteins across the cell membrane, can significantly improve the secretory production of these proteins in *E. coli* BL21. Continuous biomanufacturing processes utilizing *E. coli* often encounter a decline in productivity after approximately four to five days of cultivation, with the specific timeframe influenced by dilution rate. Glucose is a commonly employed carbon source for *E. coli* cultivation and is frequently paired with isopropyl β-D-1-thiogalactopyranoside (IPTG) for protein induction in these systems (Kittler et al., 2020). Khani & Bagheri (2020) proposed skimmed milk as an alternative to IPTG for inducing protein expression, reporting high levels of recombinant protein production and improved bacterial growth rates. Supplementation of critical amino acids (AAs) improves uptake rate of glycerol and lactose in wild type *E. coli* BL21(DE3) in defined medium. A feeding strategy of mixed glycerol-lactose feed along with supplement of critical AAs enhances recombinant production of pramlintide multimer. High cell density cultivation of *E. coli* using mixed glycerol-lactose feed and critical AAs supplement resulted in final cell density of 52.2 ± 0.90 g L-1 (Kumar et al., 2021)

Autoinduction using lactose as an inducer, combined with glycerol, glucose, and glycine as carbon sources, significantly enhanced nanobody expression compared to traditional LB medium and also reduce impurities and toxicity compared to IPTG (Rezaei et al., 2020). Surprisingly, acetate, typically considered detrimental, proved effective as a carbon source when coupled with yeast extract, resulting in high yields of a sweet protein (Leone et al., 2015). The choice of carbon and nitrogen sources greatly impacts protein production, with complex medium supplemented with glycerol showing promising results. Additionally, genetic manipulation of acetate metabolism, particularly the deletion of the *ackA* gene,

led to a fivefold increase in protein yield and reduced acetate accumulation (Lozano Terol et al., 2019). These findings highlight the importance of optimizing carbon sources and strain engineering to enhance recombinant protein production in *E. coli* BL21. Optimizing carbon sources, including glycerol, glucose, and lactose, can significantly enhance protein yield (Rezaei et al., 2020). The choice of expression system, including promoter strength and plasmid copy number, impacts protein production, with a balance needed to maximize soluble protein expression (Lozano Terol et al., 2021). While glucose-fed continuous cultivations show a productivity drop over time, glycerol-fed systems demonstrate a «Lazarus effect», recovering productivity after approximately 150 hours of induction (Kittler et al., 2020). This phenomenon may enable stabilization of continuous *E. coli* cultivation. Additionally, galactose utilization in *E. coli* BL21(DE3) might cause fluctuating productivity due to its weak induction properties (Li et al., 2021). In glucose and yeast extract combination the strain reached maximum viable cell count of 11.8 log CFU ml-1 and biomass yield of 5.25 g L-1 at the end of 24 hours. The next best combination with malic acid and yeast extract showed cell count of 9.25 log CFU ml-1 and biomass yield of 4.13 g L-1 at 24 h time. Mannitol was identified as an effective carbon source that could increase the production of CypA protein in *E.coli* BL21 recombinant strain cultivation. Interestingly, it was shown that glutamate can serve as an alternative for both carbon and nitrogen source for high production of recombinant proteins in *E. coli* BL21(Chiang et al., 2022). More complex compounds could be used as well like, for example, the study (Wang et al., 2021) successfully demonstrated the production of bovine and human α-casein proteins in *E. coli* using lignocellulosic sugars as the carbon source. This proof-of-concept is a promising starting point for producing high-value food or feed proteins from bulk residual biomass like lignocellulose, supporting a sustainable bioeconomy.

Nitrogen sources. While these studies focused on carbon sources, Nagappa et al. (2022) showed that common microbiology rich media (tryptone, peptone, yeast extract, and casamino acids) can effectively replace commercial amino acid sources in cell-free expression systems. However, amino acid composition of cell culture media affects trace metal tolerance and cholesterol synthesis in *E. coli* BL21 (Rawat et al., 2023). The change in amino acid composition affected not only the expected pathways related to cell cycle and amino acid response, but also had an unexpected impact on genes involved in zinc transport. Among potential nitrogen sources that could be used for recombinant strain *E. coli* BL21 are peptone, tryptone, cheese whey, corn steep liquor. The use of some byproducts such as blackstrap molasses, corn-steep liquor and cheese whey, as an alternative for carbon and nitrogen sources of medium, were found to enhance the cell growth. In the study (Carranza-Saavedra et al., 2021) deproteinized whey as a source of carbon and nitrogen provided the highest specific growth rate of recombinant *E. coli.* Deng et al. (2022) achieved high-level expression of nitrile hydratase in *E. coli* BL21 through systematic optimization of fermentation conditions.

Trace elements and supplements. To ensure efficient cultivation and maximize the yields of desired products, researchers have explored various strategies to optimize the growth conditions and medium composition for *E. coli* BL21. Specifically, the supplementation of trace elements and other key nutrients has been identified as a critical factor in supporting the growth and productivity of *E. coli* BL21 cultures (Basiony et al., 2022). These micronutrients play essential roles in cellular metabolism, enzyme activity, and overall physiological function. Specifically, trace elements such as iron, magnesium, calcium, and zinc serve as cofactors for various enzymes, while vitamins like thiamine, riboflavin, and biotin act as co-enzymes, co-substrates, and regulators of metabolic pathways (Ge et al., 2023).

The appropriate selection and supplementation of these micronutrients can have a significant impact on the growth, viability, and productivity of *E. coli* BL21. For instance, insufficient levels of iron can lead to reduced cell growth and impaired respiration, as the metal is a critical component of enzymes involved in electron transport and energy production. On the other hand, optimizing the concentration of iron and other trace elements in the medium has been shown to enhance cell viability and improve the secretion of recombinant proteins, as these micronutrients provide the necessary support for efficient growth (Corless et al., 2020). Interestingly, the unintentional introduction of trace elements into the media lends further credence to this idea. Thus, trace impurities in the reagents used to prepare M9 minimal medium affected physiological activities of *E. coli*, such as cell growth,

substrate consumption, and byproduct formation (Soma et al., 2023).

One prominent study has demonstrated the potential of an integrated modeling approach to rationally optimize the bioprocess conditions for *E. coli* BL21 cultivation (Yeoh et al., 2020). The researchers developed a comprehensive model that coupled the kinetics of the cell factory with the computational fluid dynamics of the bioreactor, allowing them to capture the spatiotemporal distributions of bioproduction. Through this model-driven approach, the researchers were able to perform full-factorial predictions to identify the optimal operating conditions that yielded a bioconversion efficiency of 94% when using ferulic acid as the precursor, which represents one of the highest reported values for recombinant *E. coli* (Yeoh et al., 2020). In addition to these model-based optimization strategies, recent studies have also explored the role of specific trace elements and supplements in enhancing the viability and productivity of *E. coli* BL21 cultures. The study (Sapavatu & Kakkerla, 2023) found that a rich media combined with the trace element zinc sulfate was effective for achieving high cell density growth and high expression of the target protein (CRM197) in recombinant *E. coli*. Vitamins as well as metals could be used for media optimization. Addition of plant extracts and liposomal vitamin K1 can stimulate protein synthesis (Motronenko et al., 2020). Most defined media formulations for *E. coli* cultivation already include a basic vitamin mix (often referred to as vitamin B complex) to support general growth and metabolism. However, the specific impact of individual vitamins on recombinant protein yield and quality in *E. coli* BL21 is still needed to be studied.

Besides nutrient availability, pH and buffering capacity also play a role in cell and product yields. Optimal pH conditions are essential for enzyme activity, protein folding, and overall cellular homeostasis. Deviations from the optimal pH can lead to reduced growth rates, decreased protein yield, and even cell death. The ideal pH range for *E. coli* BL21 is typically between 6.8 and 7.2. Augmenting the buffering capacity of M9 minimal medium resulted in approximately a twofold enhancement of heterologously expressed protein yield in *E. coli* BL21(DE3) cells (Azatian et al., 2019). The protein yield was correlated with the ability of the medium to resist changes in pH over time, with the most buffered media producing the highest yields.

Conclusions

This study demonstrates the critical role of culture medium optimization in enhancing recombinant protein production in *E. coli* BL21. The possible carbon sources for media optimization could be lactose, glycerol and mixture of those two. The supplement of cas-aminoacids as nitrogen source and protein inducers could have valuable effect in industrial environment. Besides, an important thing is to control pH level and buffer capacity. The findings provide valuable insights for developing efficient both academic and industrial applications, while also highlighting certain limitations that must be considered.

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