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Research progress in the development of cell-free systems and applications in synthetic biology

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Abstract

Cell-free Protein Synthesis, or (CFPS), is an interesting innovation in synthetic biology that allows for precise and controlled manufacturing of proteins and biomolecules. This review explores the progress made in CFPS techniques, especially in lysate preparation, computational modelling, gene impairments, and system improvements. Advances in optimized ribosomes, synthetic genetic circuits, and codon usage have improved how big and how well CFPS systems can work. Also, the combination of microfluidics, new energy supply systems, and linear DNA templates has broadened the field of application of CFPS in bioprocess, targeted therapies and ecotoxicological assessment. However, other problems such as the depletion of resources, accumulation of byproducts and protease activity have ranked higher than these advancements, which calls for updating of the system and control of the process. This review also discusses the mRNA secondary structure elements, translation regulation elements and translation enhancing tools that have been employed to increase the efficiency of translation. The predicted growth of CFPS is predicated on its capacity to expand, incorporate biotechnological advancements and break biochemical barriers, hence making it a critical technology in the next generation of therapeutic as well as diagnostic interventions.

Keywords: Cell-free protein synthesis; Extract based systems; Cell-free systems; Codon optimization; Microfluidic technology

1. Introduction

The progressive nature of cell-free systems has provided impressive flexibility and control of biological systems in the domain of synthetic biology [1]. Connected to the concept of in vitro cellular models, cell-free systems allow engineers and researchers the capacity to bypass the complexities associated with cell metabolism and cell regulation as these constructs do not utilize real cells [2]. These systems also incorporate processes through biological means to produce proteins and other biomolecules in a controlled manner all of which have great potential as well as novel applications [3]. The goal of synthetic biology is to create customized new system and to modify natural biological systems. Cell free system is the framework that allows the researchers to study the biological processes without needing the actual living cell. It has benefited the study of a variety of genetic circuits, generation of therapeutic proteins and as well as the development of complex natural products [4].

Cell free systems play an important role in synthetic biology because this system has the capacity for fast iteration and precise control within specific constraints [5].

Cell free protein synthesis (CFPS) is the primary focus of the cell free systems and it has gained exceptional attention in research. Recent advancement in molecular biology, biochemistry, and genetic engineering has greatly enhanced its

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capabilities. Cell free systems use genetic template to synthesize proteins by using important cellular machinery like tRNA, enzymes and ribosomes that are involved in transcription and translation. Cell free systems remove the requirement of maintaining cell viability. Elimination of the living cell allows the researchers to focus on improving the manufacturing process [6][7]. Lysate obtained from wheat germ, Escherichia coli and mammalian cells provides several advantages like increases yield of protein and post transcriptional modification. The efficiency of cell free systems can be increased by customized ribosomes and synthetic genetic constructs that provides improvement in translation and flexibility of design [8].

Techniques like optimizing codons, predicting mRNA structure, and balancing regulatory elements are used to improve protein expression [9]. The process of modifying DNA sequences to align with the host organism's preferential codon usage is called codon optimization, which eventually enhances translation efficiency.

Similarly the regulatory elements can be targeted to optimize the expression levels such as RBS and promoters, enhancing translation efficiency through stabilized secondary structures modification [10]. One important development that has also assisted in the advancement of cell-free systems is the use of computational methods [11]. It can be noticed that there is a large group of methods which include bioinformatics tools as well as machine learning algorithms which allow for the forecasting of optimum reaction conditions, recognition of bottleneck, and detection of new system elements. Such approaches to the application of cell-free systems provide the construction of models of complex biochemical processes that guide the experimental design [12].

Extract-based systems, a significant group in CFPS, have shown impressive scalability and cost-efficiency. These systems depend on cell lysates that have necessary machinery for both transcription and translation. Advancements in energy regeneration and feeding techniques have addressed issues concerning resource depletion and reaction duration, rendering extract-based systems appropriate for use in industrial applications. Correspondingly, utilizing linear DNA templates presents a convenient option compared to plasmids, leading to reduced preparation cost and time. Though the linear DNA template is more prone to damage, the new stabilization techniques have made it more viable [13] [14]. Microfluidic platforms are becoming important instruments that improves cell free system. It enhances the efficiency of cell free system by reducing reaction time and allow for high crucial throughput screening. These technologies are helpful as several parameters can be tested concurrently, the reaction time can be controlled precisely and the effectiveness of resource can be increased [15]. The cell free systems has the capability to synthesize biomolecules and proteins on demand. This has led to the advancement in personalized medicine, enabling the synthesis of patient-specific therapeutics. The ability of a cell free system to be used in environmental monitoring, diagnostics and the production of biobased materials focuses attention on their versatility and impact in society [16].

Although the introduction of cell free systems is exceptional, there are still obstacles to overcome like byproduct formation, resource limitation and protein cleavage. These biochemical factors should be addressed by researchers. Cell-free systems resolve these problems by using specific energy sources, protease inhibitors, and practical methods to get rid of unwanted by-products such as certain wastes.

With the advancement of cell free systems, synthetic biology has achieved new progress and discoveries. This is because the researchers can control experiments better and make biological changes. In contrary to traditional vivo systems, cell free systems do not depend on living cell that allows the researchers to avoid problems related to cellular metabolism and regulation. Such ventures facilitate biomolecules and proteins synthesis within a biochemistry controlled setting and in doing so enables further exploration into clinical and biotechnological modifications for such biomolecules and proteins throughout the boundaries [3]. A field of synthetic biology aims to alter the currently existing biological systems and create new custom designed systems. The concept of cell-free systems is aligned with certain systems as it allows biological processes to be carried out without the constraints of cellular enclosures. The construction of genetic circuits including the synthesis of therapeutic proteins, as well as the creation of artificial biosystems with the help of these systems has been very convenient. Their ability to work within controlled limits makes possible precise control and quick responses suggesting that cell-free systems are critical components of current synthetic biology [17].

A fundamental application of cell-free systems, CFPS (Cell-free protein synthesis), has been a central focus of research and development. While the idea originated many decades ago, recent advancement in biochemistry, engineering and molecular biology has considerably improved its potential. CFPS systems depend on key translation and transcription components derived from cells, including ribosomes, enzymes and tRNAs, to directly create proteins via genetic templates. The elimination of living cells removes the necessity for sustaining cell viability, enabling researchers to concentrate exclusively on enhancing the production process [18].

Advancements in CFPS are strongly linked to advancement in lysate preparation, computational modelling and genetic engineering. Lysates obtained from various sources like Escherichia coli, mammalian cells and wheat germ provide unique benefits, ranging from elevated protein yields to the inclusion of post-translational modifications. Synthetic genetic constructs and tailored ribosomes broaden the functions of cell-free systems by facilitating increased translation efficiency and flexible system design [8].

2. Biochemical techniques to design cell-free systems

2.1. Advanced Cell-Free System Components

New lysates: The use of innovative lysates in cell-free systems has become a valuable tool for synthesizing proteins and producing metabolites. Recent developments focus on improving these lysates to increase specificity and production, especially when dealing with secondary metabolites and complex proteins. A new lysate provides a great opportunity to utilize the metabolic abilities of various organisms. Eliminating the cellular environment enables the production of biological substances without the requirement of sustaining cell survival and allows metabolic engineers to investigate new chemical transformation systems [19].

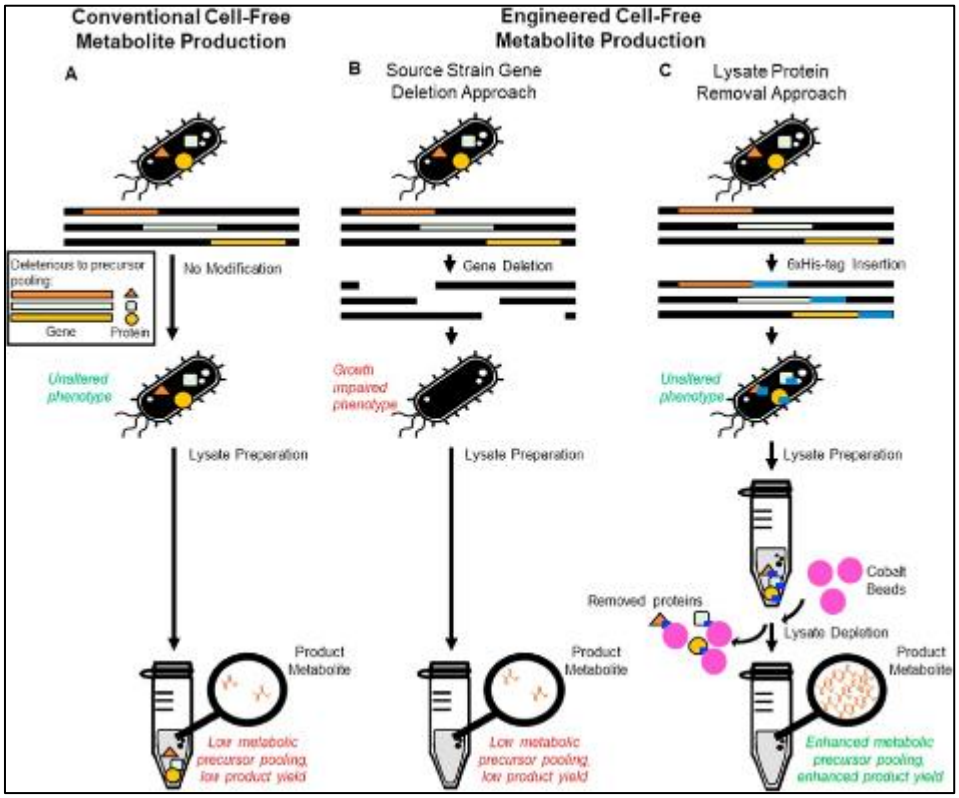

Figure 1 Overview of methods used for the preparation of Cell-free metabolic engineering lysates. A. The complex metabolism of *E. coli* lysates, used for producing cell-free metabolites, can hinder the yield of central metabolic precursors. B & C. Cell-free metabolic engineering (CFME) aims to simplify lysate complexity to better direct carbon flux and concentrate central metabolic precursors. B. The traditional CFME approach works by deleting specific genes to reduce the lysate complexity in source strain, this often leads to growth impairment or deadly outcomes because of the inability to remove essential genes. C. The innovative approach involves engineering source strains to naturally express recognition sequences, like 6xHis-tags, in target proteins. These proteins can then be removed from lysates through affinity purification, minimizing impact on source strain growth and enhancing the concentration of specific metabolic products [19]

The study rewired S. cerevisiae and *E. coli* cells to produce novel cell-free lysates. These lysates, which have pathway enzymes, increase product fluxes and streamline discovery techniques, allowing efficient screening pathway variations and enzymes [20]. Cell-free lysates from tobacco BY-2 cells are utilized as a robust screening tool for recombinant proteins in small 50-100 µL reactions and for producing proteins that are challenging to express in living cells because of their toxicity. Significantly, the BYL has active mitochondria that provide energy for protein production and are

capable of producing recombinant proteins at the rate of up to 3 mg per mL through Batch reactions of transcription-translation performed in vitro, which is approximately 15 times more efficient than other batch mode of eukaryotic cell-free systems [21].

Customized ribosomes: Customized ribosomes are essential for improving cell-free protein synthesis (CFPS) by enhancing total protein production and integrating noncanonical amino acids (AAs). These systems use modified ribosomes to enhance the genetic code and improve protein function, leading to potential advancements in medicine and material science. The customization of ribosomes also enhances the production of membrane proteins by resolving problems with ribosome aggregation and stalling, essential for achieving increased yields. The incorporation of lipid membranes into the translation process may boost the binding of new chains, leading to increased protein production. [22]. The addition of new side chain groups of UNAAs can lead to proteins developing different chemical properties and forming distinct structures and functions of proteins. It offers new opportunities for protein engineering, presents a new perspective for biological research, synthetic biology, and biotherapy, and is now a pivotal emerging application in cutting-edge fields. Customized ribosomes enable the specific incorporation of non-canonical amino acids, expanding the potential functionalities of proteins. For example, the iSAT system has been fine-tuned to accurately incorporate p-azido-phenylalanine and p-acetyl-phenylalanine into proteins such as super folder green fluorescent protein. [23]. Through the utilization of orthogonal translation systems (OTSs) as a basis, UNAA incorporation techniques involve stop codon suppression, codon redistribution, frameshift suppression, and unnatural base pairs. [24]. By changing the anti-Shine–Dalgarno sequence of *E. coli* ribosomes, researchers were able to develop orthogonal ribosomes that selectively bind to engineered mRNAs containing complementary Shine–Dalgarno sequences, thus avoiding interference with regular housekeeping mRNAs. Alterations in the genetic makeup of the catalytic centre resulted in ribosome variants that showed enhanced compatibility with unnatural substrates such as β-amino acids, D-amino acids, dipeptides, and long-chain carbon amino acids. Additionally, by binding the two ribosomal subunits, ribosomes in bacteria can be manipulated without interfering with natural ribosomes. In this way ribosomes are engineered safely that would otherwise be harmful to a cell. [25].

Synthetic genetic frameworks: The development of constructing synthetic genetic frameworks and generating prototypes has become an innovative method in synthetic biology, particularly for rapid screening and improving genetic components. By avoiding the time-consuming procedures of conventional genetic engineering in organisms, this approach streamlines the development and enhancement of genetic structures. CFE systems have sped up the process of analyzing genetic components in different organisms, enabling fast prototyping in chloroplast biotechnology. These systems eliminates the necessity for transforming entire plants by allowing the in vitro testing and analysis of genetic components. A strong foundation for comprehensive examination of DNA sequences offered by the cell-free systems of chloroplast cells, allowing efficient characterization of components. A useful tool for upcoming chloroplast engineering projects is comprehensive analysis of 5′ and 3′UTRs in chloroplast gene expression for higher plants [26].

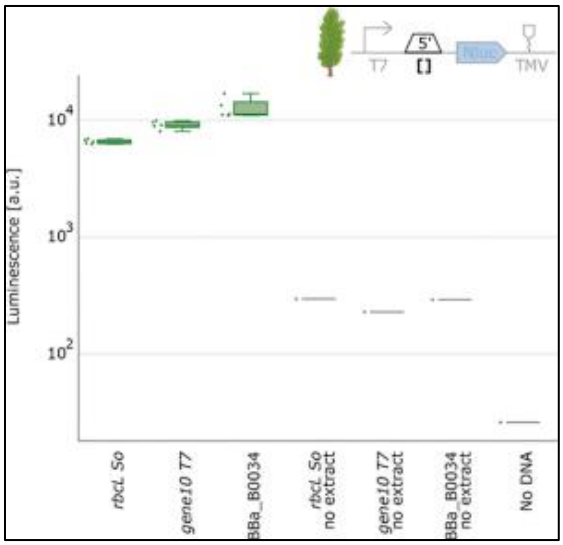

Figure 2 Poplar chloroplast cell-free extracts was used to perform the characterization of the 5′UTR. Different 5′UTRs measured NanoLuc luminescence signals from DNA templates. Negative controls were either without extract or DNA. Cell-free reactions, including T7 RNA polymerase, were prepared in a total volume of 10 µL, and NanoLuc activity was evaluated after 6 hours of incubation at 20°C (N = 5) [26]

The successful characterization of parts in poplar focuses on a significant key benefit of chloroplast cell-free systems. Because trees like poplar have slower growth and longer generation time, part characterization in vivo may take years.

2.1.1. Codon Optimization

Codon optimization modifies the genetic code to align with the preferences of the host organism and is crucial for increasing the effectiveness of cell-free protein synthesis. Protein production can be enhanced with the help of this method. Codon optimization involves aligning a gene's codon usage with the host organism's that results in improved protein synthesis. For instance, in a research project using the RED reporter gene, the usage of the most common codons in *E. coli* was increased that resulted in 20% increase in fluorescence intensity [27]. Contrary to the traditional methods, evolutionary algorithms have been designed to improve coding sequences for several organisms simultaneously that results in higher expression levels [28]. The efficiency of translation can be increased by this method because it selects the codons that reduces the possibility of restrictions in protein production.

2.1.2. How it works

This procedure helps in improvement of expression level and in the incorporation of non-standard amino acids by regulating codon usage and making sure the availability of tRNA. Studies have shown that the synthesis of recombinant proteins, like human erythropoietin is increased considerably by altering certain codons [29].

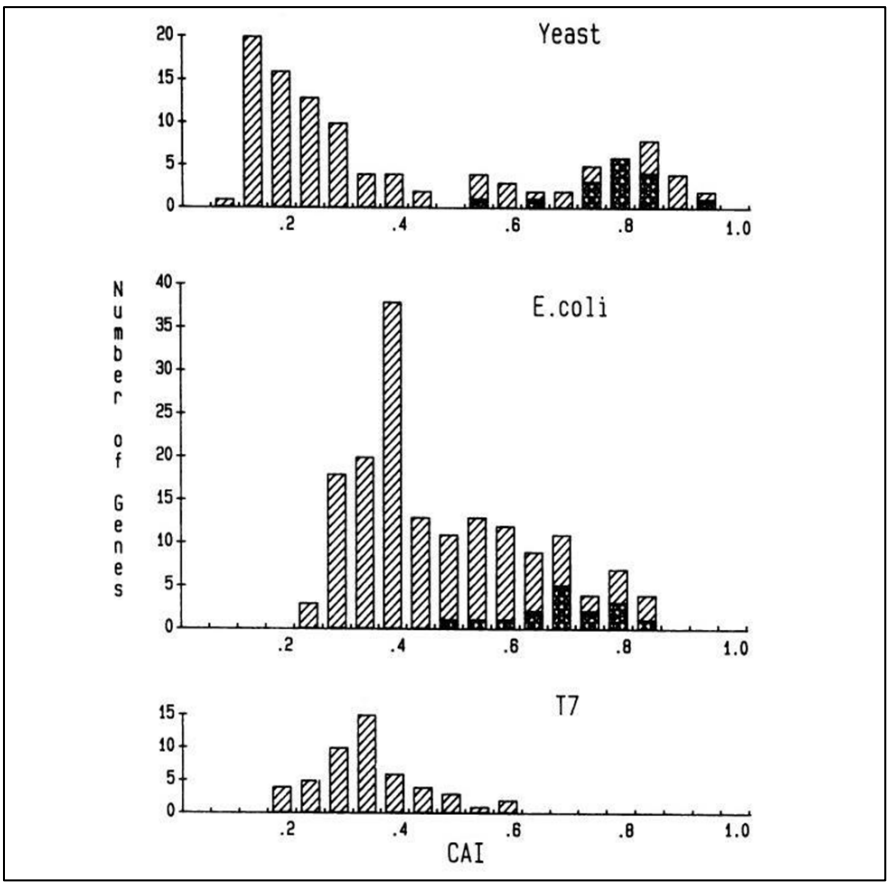

Figure 3 This graph shows the distribution of the Codon Adaptation Index value in three different organisms. (a) This graph interprets the broad distribution and low CAI value for yeast ribosomal protein genes, this means using the best codons isn't as critical for yeast. (b) CAI value of *E. coli* is higher and the graph is narrowly distributed, it shows that using the best codons is more important for *E. coli*. (c) The graph is more narrower and it shows that Using the best codons is extremely important for T7

Codon optimization can be achieved by selecting the most appropriate codon sequences. By this technique the gene expression in host organisms can be improved. The Codon Pair Bias (CPB) and Codon Adaptation Index (CAI) are the two important metrics used in this optimization process [30]. The CAI evaluates the alignment of a certain sequence of codons with the preferred codon used in the host organism that has a significant effect on the efficiency of protein synthesis. A superior CAI suggests a more suitable match, resulting in higher levels of expression.

However, CPB assesses how often particular codon pairs appear in a sequence, as some pairs can improve the efficiency of translation and lessen the chance of errors in protein production. These metrics help researchers direct the optimization process to discover codon sequences that maximize expression levels and maintain the functionality and stability of expressed proteins. [31]. Another approach would involve streamlining genetic codons by minimizing redundancy, which leads to enhanced translation efficiency. This is done by utilizing one tRNA per amino acid, thus simplifying the process of synthesis. [32]. In addition, eliminating natural tRNAs from the cellular lysates, increases the CFPS system's flexibility, allowing for the incorporation of artificial tRNAs aligned with preferred codons. [33] . Combinatorial engineering employs a high-throughput method to alter gene sequences to enhance protein expression in recombinant systems. By randomizing +2 and +3 codons, essential in protein synthesis initiation, significant variations in expression were observed across hundreds of clones, up to 70-fold differences. Slight modifications in the random codons sequence directly affected the levels of expression, proving that even small changes can lead to notable differences in protein production. Researchers can choose the best codon sequences from a wide variety of expression levels, thus improving the efficiency of gene expression. This technique shows consistent changes in gene expression based on codons in living organisms, proving its potential in vivo. From this we understand that indicates that this method is a flexible system for effective protein production, necessary for generating proteins with particular roles in different biological environments. [34]. Codon optimization allows for fine-tuning the levels of protein expression, creating a flexible method for generating proteins at specific amounts, which is essential for synthetic biology applications. [34].

2.1.3. mRNA Structure Prediction and Optimization

The secondary structure of messenger RNA plays a vital role in the biosynthesis of proteins. Its detrimental effect on translation may decrease the production of protein by obstructing or avoiding the initiation and movement of ribosomes on the mRNA, emerging as a crucial factor in regulating gene expression. The formation of secondary structures may be predicted by various algorithms by calculating the minimal free energy of RNA sequences or carry out the inverse method of deriving an RNA sequence for a particular structure. The significant impact of modified RNA sequences to avoid secondary structures on gene expression has been shown by multiple recent studies. In the research conducted by Studer and Joseph [35], the authors modified numerous mRNA sequences to regulate the presence and strength of secondary structures close to translation initiation sites. The inverse correlation between the strength of the structures and the ease with which mRNA associates with the ribosomes was observed. Furthermore, sequences lacking secondary structures were more likely to form stable initiation complexes, which are a determinant of translation efficiency, since they bonded with the 30S ribosomal subunit more quickly in *Escherichia coli*. Similar findings were obtained by studying the expression of human interferon-α and human interleukin-10. Translation and heterologous expression of both proteins were effectively improved 10-fold with the introduction of silent mutations that exposed the start codon from secondary structures. Similarly, research demonstrated that the L1 gene from human papillomavirus type 16 was modified to prevent the formation of secondary structures when expressed in Saccharomyces cerevisiae, resulting in a 4 fold increase in expression compared to the wild type. The initial method for enhancing the secondary structure of mRNA sequences was introduced using a rapid correlated MFE estimation technique. Even though the estimation algorithm wasn't designed for maximum precision, the outcomes are closely related to those obtained from precise techniques like RNAfold, facilitating faster calculation of synonymous genes with improved structures. Overall, the tests show an average improvement of >40% in MFE, based on RNAfold measurements. In addition to allowing the optimization of mRNA sequences alone, this method can be used with other factors influencing gene expression, including codon usage, harmonization and GC content, to optimize the in silico engineering of recombinant genes for heterologous expression [36]

In cell-free systems, the modification of mRNA sequences can considerably increase protein production. When coupled with RNase E-deficient extracts, modifications such as adding a T7 terminator sequence to mRNA substantially increase protein production in cell-free systems leading to increased yield and greater stability of the synthesized proteins. As demonstrated by producing 2.6 mg/mL of protein over 15 hours, incorporating a stem-loop structure at the 3' end of mRNA shields it from degradation, allowing for longer translation periods. [37]. Additionally, by altering translation initiation using an eIF2α-S52A modified CHO cell lysate, the protein synthesis rate can be enhanced. Furthermore, the costs of cell-free protein synthesis are decreased by the integration of T7 RNA polymerase into the CHO cell lysate by stable and transient transfection. [38].

2.1.4. Regulatory Element optimization

Promoters and enhancers are elements that regulate gene expression based on internal and external signals [39]. Strategically placing regulatory elements like operators and promoters can effectively tune gene expression in cell-free systems. The positioning of these components about the promoter has a substantial effect on transcription levels [40]. For example, in E.coli, transcription is significantly reduced when TetO is placed within the initial 13 base pairs

following the PT7 promoter. Conversely, transcriptional activity is enhanced when TetO is positioned directly in front of the promoter. This shows that precise positioning can either hinder or enhance gene expression, depending on the intended effect [41]. Researchers can modify the interaction dynamics of these elements to adjust the levels of target gene expression, resulting in enhanced efficiency and control of protein synthesis. Another approach includes utilizing modified forms of RNAPT7 in E.coli with reduced activity, allowing for improved regulation of gene expression [42]. Tandem and/or monocistronic genes may be constructed in expression vectors under the regulation of a T7 promoter. Modifying gene expressions in T7 systems can be accomplished by using synthetic promoters or mutated forms of RNAPT7. Progresses in lysate preparation techniques have enhanced gene expression by endogenous promoters, potentially removing the necessity for T7 systems [43] [44]. This method modifies gene expression to meet certain needs, reducing problems from excessive expression and ensuring efficient protein production for proper function. Moreover, creating custom synthetic promoters for specific purposes offers another way to control gene expression. These promoters are able to adapt to different cellular environments or signals, providing a flexible approach to regulating gene activity, ultimately resulting in enhanced protein production. The organization of genes in operons also impacts levels of expression. Although the placement of genes in operons controlled by the T7 promoter doesn't influence transcript levels, the arrangement of genes in terms of translation context can influence the expression of individual genes. Therefore, optimizing gene sequence can improve whole functionality and yield [45].

The selection of the correct ribosome binding site (RBS) sequences is crucial to enhancing translation efficiency in cell-free protein synthesis systems. [46]. The efficiency of translation is directly impacted by the strength of the binding sites of ribosomes, as a robust RBS increases ribosome attachment to mRNA and improves protein production. On the other hand, a weakened RBS strength may decrease translation efficiency, hindering the production of proteins. As RBS strength is an essential factor, other variables—such as molecular crowding, mRNA secondary structure [47], Translation efficiency can also be influenced by factors such as mRNA secondary structure, molecular crowding, and GC content based on coding sequence can impact translation efficiency alongside RBS strength, which can complicate predictions that rely only on RBS strength. Moreover, the translation efficiency can be affected by the presence of infrequent codons in mRNA. While enhancing codon usage can enhance protein production, it may also lead to misfolding and decrease the functionality of proteins. Therefore, it is important to carefully consider RBS selection in addition to codon optimization in order to achieve optimal protein yield and correct protein folding. Interestingly, the effectiveness of RBS in cell-free systems often aligns with its effectiveness in living systems, indicating that findings from cell-free experiments can help anticipate how genetic constructs will behave in organisms. [48]

3. Computational Methods:

Computational modelling provides a powerful tool for simulating cell-free systems [49]. Simulation refers to the process of creating virtual models that mimic the required biological, ecological and physical systems of actual real-world systems [50]. In case of Cell-free systems, simulation refers to predicting the behavior or outcome of biological processes in a cell-free environment. The analysis of complex interactions in a [51] cell-free environment can lead us to identify the rate-limiting steps, and enzyme kinetics through mathematical tools and enhancement of reaction conditions. Researchers have yet not explored the computational modelling for the enhancement of cell-free expression systems for lysate-based *E. coli*. However, PURE system for protein production was computationally modeled allowing 1000 reactions in a biochemical reaction network. This study indicates the complex dynamics of Cell-free Expressions, revealed the resilience and insensitivity of CFE correspond to fluctuations in kinetic parameters of central dogma. A mechanistic computational model independently revealed that inefficient ribosome usage and depletion of key initiation factors can obstruct CFE. Keeping in view this, researchers anticipate that computational modelling will enable the characterization of multiple components and will provide the constituents of CFE reactions [40].

Bioinformatics tools help optimize codon usage in DNA sequences to enhance translation efficiency and protein yield. Atsushi et al revealed the First comprehensive bioinformatics analysis of cell-free protein synthesis in 2009. Research shows the evaluation of 3066 human protein expression and their domains and analysis of their interlink with 39 physiochemical and structural properties of proteins in a cell-free system. Bioinformatic analysis revealed the 18 most important and impactful features that affect protein compatibility to cell-free expression. Features include length, hydrophobicity, pI, content of charged, nonpolar, and aromatic residues, cysteine content, solvent accessibility, presence of coiled coil, content of intrinsically disordered and structured (α-helix and β-sheet) sequence [52]**.** SynBiopython is an open-source library that provides tools for DNA sequence optimization, including codon usage tables that help in designing synthetic genes for better expression in cell-free systems [53].

Machine learning algorithms can adaptively optimize codon sequences based on experimental data, continuously improving the efficiency of protein synthesis in cell-free systems [54]. cell-free protein synthesis may be improved through a series of iterated high-throughput experiments guided by a machine-learning algorithm implementing a form

of evolutionary design of experiments (Evo-DoE). The desired experimental response, or evolutionary fitness, was defined as the yield of the target product, and new experimental conditions were discovered to have ~ 350% greater yield than the standard [55].

3.1. Microfluidic technology

Microfluidic Technology has revolutionized the field of synthetic biology and Cell-free systems by enabling the modular assembly of genetic circuits, design and fabrication of microfluidic devices and precise control of reaction conditions [56, 57]. This involves designing micro-fluidic devices by manipulating fluids at microliter to femtoliter, allowing us to process and handle a minimal amount in a controlled and continuous manner [58]. Small-scale microfluidic devices are more efficient and usually used for cell-free protein synthesis. These mostly include Microreactors, mixing units, and incubation chambers. The microreactors are designed to maintain steady-state conditions crucial for long-term protein synthesis [59]. For instance, T-junction is the most popular small-scale microfluidic device for droplet generation commonly used for cell-free protein synthesis [60]. T-junctions offer precise control over droplet size and formation for easy fabrication of droplets and can also be generated at higher frequencies. The droplet size produced by a T-junction system is influenced by factors such as flow velocity, fluid viscosity, and interfacial tension [61].

Another revolutionary type of microfluidic device is Co-flowing, developed by Ho et al. in 2015. They design co-flow glass microfluidic device to engineer artificial cells effectively [62]. These devices function by accurately managing the flow of multiple fluid streams through microscale channels, enabling the creation of intricate structures with high spatial resolution. In artificial cell fabrication, co-flowing microfluidic devices can encapsulate genetic material, proteins, and other biomolecules within a lipid bilayer membrane [63]. There are various techniques for developing, designing, and fabricating microfluidic devices. While photolithography is commonly used, alternative methods are also being explored. For example, Sasami and Sugenami have created a technique using consumer-grade laser cutters, simplifying and reducing the cost of device production. This innovation makes artificial cell production more accessible to a wider range of researchers [64].

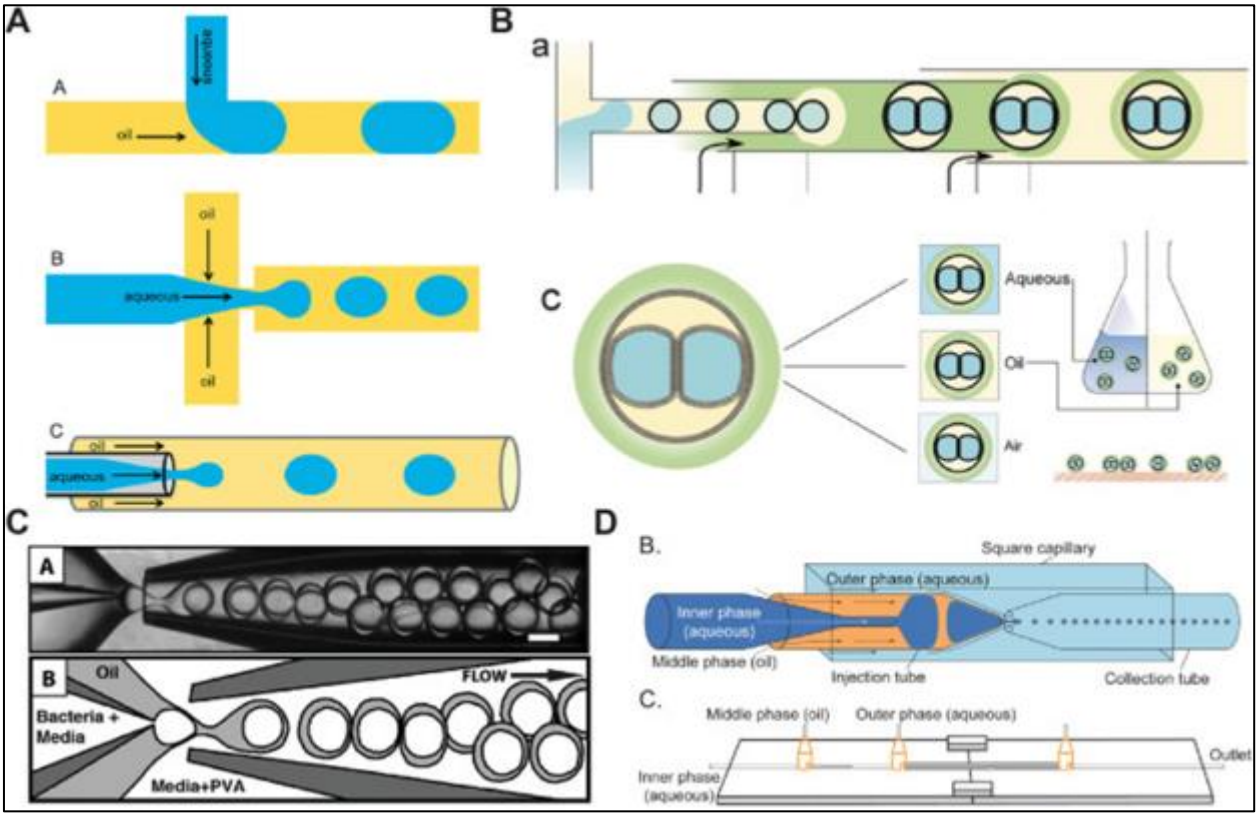

Figure 4 (A) various microfluidic drop maker geometries include T-junction, flow-focusing, and co-flowing designs. (B) Depicts the double coaxial microfluidic concept used for forming droplet interface bilayers (DIB) and encapsulating a droplet of oil containing DIBs. (C) Shows microscale double emulsions created with a flow-focusing glass-capillary microfluidic device, along with a schematic of the flow-focusing region and labeled liquid composition. (D) Provides a schematic of a co-flowing microfluidic device designed to generate double emulsions [64]

3.2. Biochemical Challenges and Limitations

Cell-free systems offer novel possibilities for biochemical uses, but they encounter considerable challenges and restrictions. These encompass concerns regarding enzyme stability, financial feasibility and reaction parameters of manufacturing methods. Grasping these difficulties is essential for progressing metabolic engineering and cell-free synthetic biochemistry [65].

Protein breakdown: Cell-free systems demonstrate a wide range of proteolytic activities in different organisms, suggesting their potential application in biotechnology. These samples can retain important enzyme activities in harsh environments, like elevated temperatures and fluctuating pH levels, which is essential for industrial operations [66]. The main source of proteolytic activity in cell extracts is mainly carried out by enzymes that are attached to cell envelope fragments from ruptured bacteria, instead of existing as freely floating exoenzymes [67]. The detection of protease activity in the supernatant post-centrifugation suggests that there is only limited amount of true free protease present in aquatic environments [68]. Protein levels in eukaryotic cells are kept in balance through the central regulatory module of protein degradation by the ubiquitin-proteasome system. A deviation from the optimal protein levels can damage cells, leading to the development of unhealthy tissues and a range of diseases such as cancer, neurodegenerative disorders, and cystic fibrosis in humans [69]. More precisely, within plants, the ubiquitin/26S proteasome system (UPS) has a significant influence in the regulation of protein degradation and plays a crucial role in various processes like development, immune response, and programmed cell death. The E3 ubiquitin ligase, in collaboration with E1 and E2 ligases, plays a crucial role in determining the substrate specificity of the UPS by identifying and labeling particular protein molecules for degradation with ubiquitin chains. The SCF complex, composed of Skp1/Cullin/F-box proteins, functions as an E3 ligase that identifies UPS substrates and mark them for ubiquitination through its F-box protein segment [70]. Protein-degrading PROTACs (Proteolysis-targeting chimaeras) are a promising technology that leverages the UPS to induce targeted protein degradation. A drawback of PROTACs is the unintended toxicity in normal cells due to off-target protein degradation. Spatial PROTACs have been created to minimize the off-target toxicity of PROTACs by allowing spatial control. Spatial PROTCs utilize cell-specific characteristics like membrane receptors and cellular environments, especially tumor microenvironments, or employ bioorthogonal chemical reactions for targeted protein degradation [71]. Using certain inhibitors can stop undesired proteolysis while expressing and purifying proteins. It is crucial to maintain protein integrity in cell-free systems by utilizing this method [72]

Resource Depletion: The sustainability and efficiency of the biochemical processes are remarkably affected by the limitations of energy sources and their depletion in cell-free system . Conventional energy sources such as phosphoenolpyruvate (PEP) often lead to brief reaction period due to instability and high costs. Although alternative energy sources have been developed but there are still obstructions to overcome in order to increase their efficiency. The efficiency of cell-free protein synthesis can be notably inhibited by the depletion of energy sources which results in extended reaction time and lower yield [73]. Building blocks deficiency in cell-free protein synthesis (CFPS) systems include translation initiation, tRNA supply, and resource competition are identified as critical bottleneck in cell-free protein syntheses, limiting protein titer and production volume compared to in vivo systems [74]. The production of purified enzymes is essential but often costly, affecting the overall productivity of cell-free systems. Moreover, maintaining the stability and functionality of enzymes is a significant challenge in biomanufacturing. Due to the enhanced stability of thermophilic enzymes at elevated temperatures, they have been identified as promising building blocks [75].

Byproduct Build-up: In cell-free system, byproducts frequently build up because of concurrent metabolic routes. For example, as a byproduct of methyl monooxygenation, benzyl alcohol builds up during the breakdown of toluene, potentially obstructing the primary degradation route (Bordel et al., 2007). The build-up of byproducts can cause feedback inhibition, whereby elevated levels of metabolites hinder additional production. Incorporating separation techniques, like adsorbent resins, can significantly decrease byproduct levels, thus improving efficiency and overall yield of the process [76]. Using mechanistically based kinetic models can assist in forecasting and enhancing byproduct formation. These models offer revelations regarding metabolic constraints and inform modifications in processes[77]. In CFPS (cell-free protein synthesis), the removal of byproducts, especially inorganic phosphate, is essential because it can bind magnesium and hinder reactions. Methods to reduce phosphate buildup consist of adopting alternative energy sources, which can improve the duration and efficiency of reactions. This involves a semi-continuous reaction that facilitates the diffusion of small molecules between the dialysate and reaction mixture, enabling reaction to retain elevated NTP concentrations, lower levels of inorganic phosphate, and increased concentration of aspartic acid. The subsequent strategy aimed at enhancing *E. coli* CFPS has focused on initiating alternative energy routes that are independent of adding high-energy phosphate compounds to the reaction. This approach can be employed to prolong CFPS reaction duration and inhibit phosphate buildup. Instances of this comprise powering the reaction with polymeric carbohydrates and dextrins or a combined system that utilizes both glucose and creatine phosphate. Implementing one

of these methods might reduce phosphate production in yeast CFPS reactions, thereby prolonging the duration of the reaction. A further advantage would be eliminating reliance on the expensive secondary energy source, thereby enhancing the economic viability of yeast CFPS [78].

3.3. Future Research Directions

Purification methods also require creative modifications to allow for the rapid administration of cell-free synthesized proteins without relying on traditional large-scale purification equipment. Collectively, this groundbreaking research may ultimately surpass current products, leading to advanced therapeutic and diagnostic options. To genuinely harness this limitless potential, steadfast and intensified research dedication is essential. We may be on the brink of a future where next-generation healthcare solutions, driven by CFPS, are standard, making sophisticated healthcare widely available [79]. Translationally active CHO cell lysates used in cell-free systems offer a promising platform for synthesizing "hard-to-express" proteins. The technology enables the quick and simple synthesis of proteins. The adaptability of DNA template utilization presents a vast opportunity for rapid and straightforward screening methods. The mammalian basis of the system is extremely advantageous for generating a wide variety of target industrial proteins. Additionally, the system's open nature and the potential for automation and high-throughput protein synthesis provide a resource for streamlining prospect pharmaceutical pre-screening processes [80]. Recent studies have examined the strong quality control of CFPS. Emphasizing quality control will be essential for providing cell-free products for practical applications, particularly in areas like point-of-care diagnostics and on-demand bio-manufacturing. Moreover, the yields of GFP (green fluorescent protein) are frequently employed to assess the quality of CFPS, yet GFP production does not accurately represent real-world applications. For example, our latest research has evaluated a novel CFPS system utilizing various proteins of differing sizes, which include a peptide nanocage, GFP, and Cas9 [81]. The development of strong and versatile CFPS systems will enable the generation of a wide variety of proteins, differing in protein size, post-translational modifications, and folding. This encompasses peptides and proteins containing non-standard amino acids. Moreover, the significant decrease in the cost of CFPS systems and the capability to scale reactions will enable the integration of these systems into regular lab procedures. Ultimately, the capacity to easily store, distribute, and activate freeze-dried cell-free systems by merely adding water has created new possibilities for point-of-care diagnostics on demand bio- manufacturing. As CFPS remains relatively unfamiliar and underutilized in the community, enhancing educational kits and courses will raise awareness and visibility of the technology, fostering long-term growth in this area [82].

4. Conclusion

In conclusion, CFPS has however developed into being one of the most advanced and adaptable technologies in synthetic biology, certainly with more benefits than downsides when compared to cell based systems. The protein synthesis using CFPS has witnessed recent breakthroughs including better lysate formulation, codon optimizations and specially designed ribosomes to achieve protein synthesis in high yields and large amounts. In addition, the applications of microfluidics and computation have simplified the integration which lowers the cost and makes it suitable in many facets of industry and medicine.

In spite of these remarkable advancements, the effects of resource availability, byproducts and proteolysis still inhibit the efficacy of the CFPS systems. To overcome these biochemical barriers, there is need for further developments in improvements in systems, energy and removing byproducts. Also, it is necessary to improve the regulatory elements and mRNA structures to boost the efficiency of translation and total amount of protein produced.

Moving forward with the current trend, the prospects of CFPS seem to be significantly reshaping the fields of biotechnology, medicine, and diagnostics. As advances in science and discovery of new techniques that can help overcome hurdles are made, CFPS technologies will be more and more critical for personalized medicine, biomanufacturing and synthetic biology applications. The strengthening of quality controls together with the increase in the educational offer will also facilitate the fastest spread of CFPS technologies throughout the economy for new frontiers in biotechnology and health care services to be achieved.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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