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Review

Cell-Free *Escherichia coli* Synthesis System Based on Crude Cell Extracts: Acquisition of Crude Extracts and Energy Regeneration

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Abstract: Cell-free synthetic biology is advancing with unprecedented control and design. The development of cell-free biosynthesis involves both pure enzyme and crude enzyme systems. The relatively cheap crude enzyme system is more suitable for the scientific research needs of ordinary laboratories. The key factor in giving full play to the advantages of the system is to obtain high-quality cell crude extract and its energy regeneration system, but there is no systematic report on the development history of these two aspects. Therefore, in this paper, the development history of the process of obtaining crude extract from cell-free biosynthesis was carried out based on Escherichia coli, which is widely used at present, and the energy regeneration system was briefly introduced. Finally, the challenges of current cell-free synthetic systems are discussed.

Keywords: cell-free biosynthesis; acquisition of crude extracts; approaches and processes; energy regeneration; history

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1. Introduction

Synthetic biology crosses multiple disciplines, including biology, chemistry, computing, mathematics, and physics. This rapidly expanding field is vital and relevant to the unprecedented situations currently facing Earth—climate change, environmental pollution, and the energy shortage crisis—as people seek to create competitive new drugs, nutritional products, biological energy, and so on, to replace current manufacturing methods [1]. Early synthetic biology systems based on living cells in vitro were limited by the narrow operating conditions of these microscopic hosts, including temperature, the concentration of small molecules, and the nature of the metabolites, which need adjusting to achieve a balance between normal growth and production. Consequently, the product yield and conversion rate were greatly restricted, and these living-cell-based systems were insufficient to support the high production of the target chemicals, thereby limiting the extension and application of synthetic biology [2], especially for some high-value-added products and expensive drugs. Cell-free synthetic biology (CFSB) systems—with high conversion rates, rapid design-build-test cycle, and open environments that are free from toxic proteins and metabolic mass transfer constraints—have been fully developed to address multiple challenges of earlier synthetic biology technologies.

CFSB systems separate cell growth from target production formation and exhibit several key advantages over in vivo synthesis [1,3,4], including (1) plug and play: mixed-match strategy makes the screening of pathway enzymes easier; (2) no metabolic mass transfer constraints because of the lack of cell membranes and walls; (3) avoids possible toxicity constraints caused from unnatural products and metabolic intermediates; (4) permits easy

manipulation of reaction conditions; (5) prevents microbial environmental pollution; and (6) open and simple reaction conditions that only need to meet the synthesis conditions of the product without considering cell growth conditions or sterility requirements. Two commonly used cell-free systems are based on nucleic acid templates and non-nucleic acid templates (Figure 1). For CFSB based on nucleic acids, two approaches can be used. One approach is to artificially add various RNA polymerases, aminoacyl tRNA, ribosomes, nucleotides, energy, cofactors, and templates for the transcription and translation process, thus simulating the intracellular environment in vitro to carry out the central dogma in vitro and synthesize the various required proteins. The other approach is to make a reaction system by taking crude extracts from cells. Various coenzymes, cofactors, and energy-generating substances in the cell can be obtained and maintain their normal functions externally. The addition of a nucleic acid template to these obtained substances results in the production of the target protein. This method is called cell-free protein synthesis (CFPS). If desired, the corresponding substrate can be added to catalyze the synthesis of the corresponding compound using the protein; this is termed cell-free metabolic engineering (CFME). For non-nucleic acid templates, two commonly used approaches are the purified enzyme system and the crude lysis system. For the purified enzyme system, two processes can be used identical to those of the CFSB-based nucleic acid templates, while the crude lysis system can be used for CFPS or CFME directly. The development of this engineering technology highlights the potential of industrial biotechnology and metabolic engineering in effectively expanding the range of traditional bioengineering models, and this technology continues to evolve with unprecedented levels of control and design freedom [5].

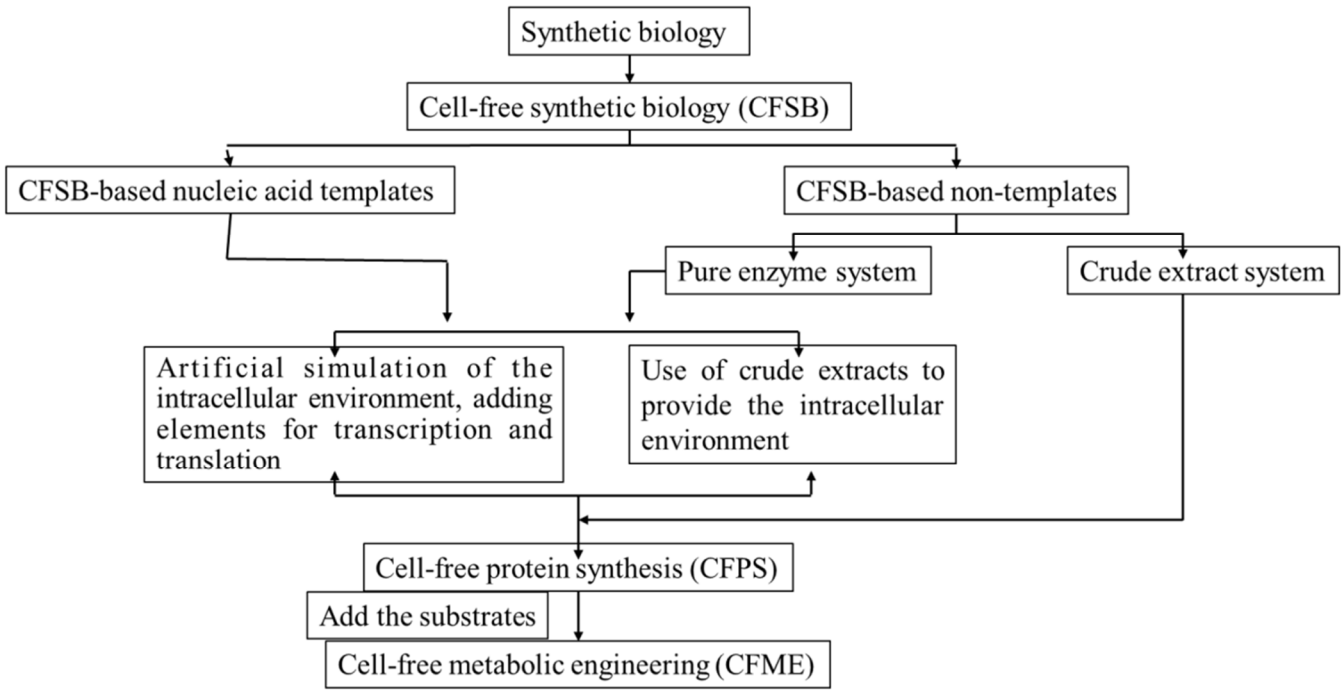

Figure 1. Cell-free systems based on nucleic acid templates and non-nucleic acid templates.

Purification systems have precise components and are often used for accurate monitoring and modeling to achieve rapid iterations of the design-build-test cycle, but the high cost of coenzyme additions and other factors limit further expansion of production and industrial application. Meanwhile, the application and development of the system based on crude extraction of cells effectively made up for this defect. Two key factors for the successful construction and efficient utilization of the system are the efficient acquisition of crude extracts from cells and the energy regeneration system. To date, there have been no systematic reports on this aspect of the technology, including the history and method process. Therefore, in this paper, the development history of the cell-free crude extraction

system of *E. coli* was reviewed, and the energy regeneration system of microbial cell crude extract was briefly summarized.

2. The Development of CFSB Based on Crude Cell Extracts

The crude-extracts-based CFSB system refers to the cleavage of cultured living cells under certain conditions to obtain active intracellular substances, which are subsequently used as the reaction liquid with templates or related enzymes. The substances include residual energy and cofactors, or the original intracellular metabolic pathways of energy and cofactors that can continue to function. CFSB based on crude cell extracts can be divided into CFPS and CFME. The development of CFPS began in 1958 when Zamecnik et al. demonstrated that peptide synthesis occurred on ribosomes and that ATP, GTP, tRNA, and so on were required [6]. Nirenberg and Matthaei then interpreted the genetic code and its function in protein synthesis in 1961. These findings laid the foundation for the study of genetic engineering and related molecular biology, and it also won the 1968 Nobel Prize in Physiology and Medicine [7]. As the great potential and advantages of this technique emerged, related research on cell-free synthetic biology was gradually conducted using crude cell extraction from various microorganisms, such as yeast [8], *Bacillus* [9], *Pseudomonas* [10], *Streptomyces* [11], and *Vibrio* [12]. The prototype of the crude-extracts-based CFME system was proposed in 1897 with ethanol fermentation by a non-living yeast lysate [13]. A pioneering study of CFME using the cell-free crude extraction system of *E. coli* was performed in 2010 when Bujara et al. synthesized dihydroxyacetone phosphate (DHAP) from glucose using an *E. coli* crude lysate [14]. However, the concept of CMFE was not actually proposed until the work of Swartz in 2012 [15]. Subsequently, under the promotion of Jewett's team at Northwestern University, USA, in-depth studies on CFME of a related pure enzyme system and a cell crude extract system were conducted, which promoted the rapid development of CFME [5]. Thus, it is evident that the study of synthetic biology using cell-free crude extracts has continuously developed from previous theories and discoveries (Figure 2).

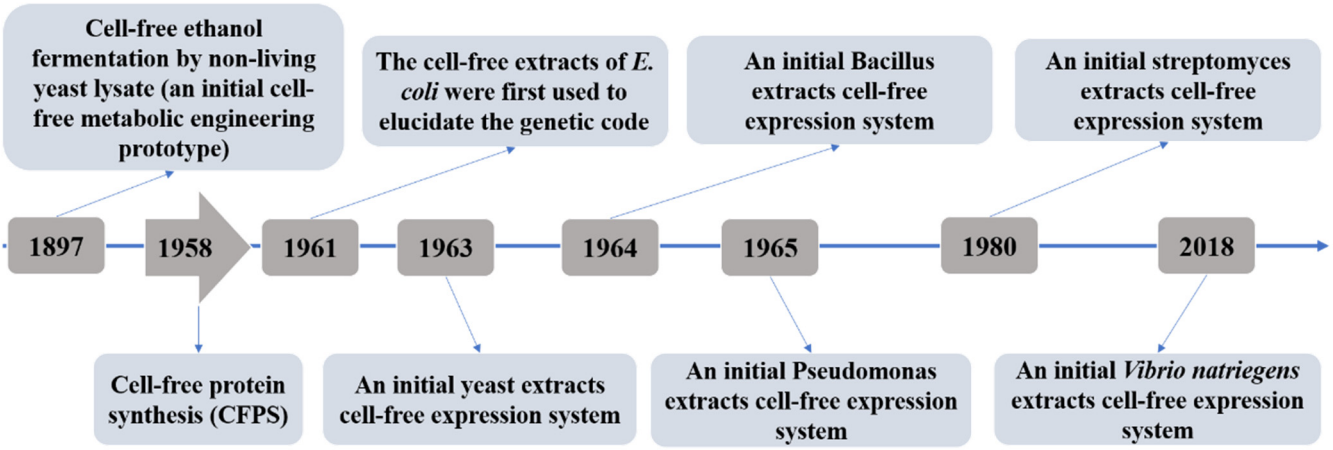

Figure 2. Study of synthetic biology using cell-free crude extracts.

3. Preparation of Crude Extracts from *E. coli* Cells

The key point of crude-extracts-based CFSB with CFPS and CFME is to maximize access to intracellular active substances, such as ATP, NAD, NADPH, ribosome, amino acids, NTPs, RNA polymerase, tRNA, elongation factor, substrate, and salt [1,16,17]. The basic acquisition process predominantly follows: cell collection (-) cell fragmentation (-) centrifugal collection (-) preculture (run-off reaction) (-) dialysis (-) crude extract (-)cell-free reaction. With the deepening of research, bacterial collection methods, cell lysis, operating temperature, centrifugal speed, crushing methods and parameters, preculture process, and other aspects are evolving iteratively all the time, and progress is being made towards a

more convenient and efficient development of CFSB research in related fields, and the main time course of development is shown in Figure 3.

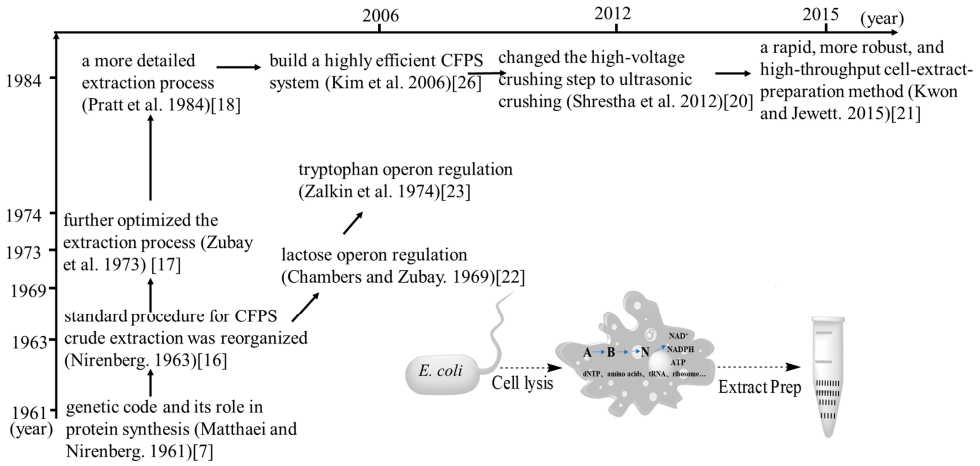

Figure 3. Development of CFSB research in related fields and the main time course of development.

In 1961, crude extracts of *E. coli* were used to reveal the genetic code and its role in protein synthesis [7]. At that time, the extraction process was simply reported, and it was only through alumina grinding and centrifugation at a low temperature (5 °C) that maximal activity of intracellular substances was retained. Thus, in 1963, the standard procedure for CFPS crude extraction was reorganized and reported by Nirenberg [16]. In this process, nuclease was introduced to remove residual intracellular templates (for the experiments that require the addition of templates for CFPS, which are more demanding). Then, to make the intracellular material more active, a process of preculture (run-off reaction) was included after obtaining the crude cell extracts. If subsequent ribosome removal experiments were needed, centrifugation could be performed at 105,000× *g* and 3 °C for 120 min, and the resulting extract can be rapidly frozen using liquid nitrogen with only a small loss of vitality during a six-month storage period. If kept at −20 °C, approximately 5% vitality is lost per week. In the subsequent studies on CFPS, most of the cell extraction processes were based on this protocol. Zubay et al. further optimized the extraction process by dividing the S-30 buffer (0.01 M tris-Ac, pH 8.2, 0.06 M potassium chloride, 0.014 M magnesium acetate, 0.001 M dithiothreitol) into buffer I and buffer II for cell washing and cell crushing, respectively [17]; this facilitated the acquisition of more challenging intracellular crude extracts. Pratt changed S-30 to S30, replacing potassium chloride with potassium acetate, possibly because acetate is more easily moved in and out of cells, and a more detailed extraction process for intracellular substances was developed [18], resulting in the attainment of 9.12 mg/mL proteins [19]. As shown in Table 1, many similarities can be found between the methods of the different research groups, including: (1) buffers added for the washing process or before crushing contain ions such as Mg^{2+}, NH^{4+}, or K^{+}, which simulate the intracellular environment as much as possible (in vivo, magnesium, potassium, and ammonium are used to balance the charge of the phosphate group and other anions), so as to retain the maximal activity of the intracellular proteins, energy, and cofactors; (2) mercaptoethanol was added to the crude extract to avoid the loss of enzyme activity and was operated at low temperatures where possible; (3) high pressure was used to break the cells, high eccentricity was used to separate unbroken cells, broken cell walls, and intermediate denatured proteins. Hypercentrifugation (30,000× *g*) was used to remove intracellular genomic DNA and prevent it from continuing to guide protein synthesis in the crude extract of broken cells, which would be a waste of resources; (4) the preculture process included a run-off reaction that was designed to release ribosomes and promote degradation of endogenous mRNA and DNA. The addition of substances such as phosphoenolpyruvate (PEP) in the middle of the process was used for energy regeneration

to obtain a more vigorous crude extract; (5) finally, the inclusion of dialysis was to provide a more suitable storage fluid for the resulting extracts.

Table 1. Standard procedure for obtaining cell crude extract.

	[16]	[17]	[18]	[20]	[21]
buffer	standard buffer (0.01 M tris-Ac, pH 7.8, 0.06 M potassium chloride, 0.014 M magnesium acetate, 0.006 M 2-mercaptoethanol)	S-30 buffer (0.01 M tris-Ac, pH 8.2, 0.06 M potassium chloride, 0.014 M magnesium acetate, 0.001 M dithiothreitol)	buffer S30 (0.01 M Tris/acetic acid, pH 8.2; 0.014 M magnesium acetate; 0.06 M potassium acetate; 0.002 M dithiothreitol)	buffer A (0.01 M Tris; 0.014 M magnesium acetate; 0.06 M potassium glutamate; 0.001 M dithiothreitol)	buffer A (0.01 M tris-Ac, pH 8.2, 60 mM potassium glutamate, 0.014 M magnesium acetate, 0.002 M dithiothreitol)
ways	Alumina grinding method and high-pressure crushing method	High-pressure crushing method	High-pressure crushing method	High-pressure crushing method and ultrasonic crushing method	High-pressure crushing method and ultrasonic crushing method
preculture (run-off reaction)	Yes	Yes	Yes	No	Not essential
Energy regeneration	standard buffer (0.01 M tris-Ac, pH 7.8, 0.06 M potassium chloride, 0.014 M magnesium acetate, 0.006 M 2-mercaptoethanol)	S-30 buffer (0.01 M tris-Ac, pH 8.2, 0.06 M potassium chloride, 0.014 M magnesium acetate, 0.001 M dithiothreitol)	buffer S30 (0.01 M Tris/acetic acid, pH 8.2; 0.014 M magnesium acetate; 0.06 M potassium acetate; 0.002 M dithiothreitol)	-	-
dialysis	Yes	Yes	Yes	No	Not essential

Nirenberg [16], Zubay [17], and Pratt [18] each developed a process for their own research plans and achieved different results, yet their approaches provided an alternative 'standard protocol' for different research teams to follow (Supplementary Table S1). The classical mechanisms of lactose operon regulation [22] and tryptophan operon regulation [23] were revealed based on the methods of obtaining cell extracts published by Nirenberg. The standard procedure provided by Pratt took approximately 10.5 h to complete, without the time required for cell culture. While this time-consuming and labor-intensive system was acceptable in the laboratory, further optimization was necessary if it was to be used to promote the further development of CFBS systems for industrial use. Hence, Liu et al. optimized the number of times the collected cells were washed, the cell breakage rate at high pressure, the high centrifugation rate of 30,000× *g*, and the run-off reaction [19]. It turned out that (1) the number of times the collected bacteria were washed had a limited effect on the subsequent extraction; (2) when cells were broken with a high-pressure homogenizer, there was no problem with the excessive flow rate cleaving genomic DNA and causing difficulties in subsequent separation. Thus, the rate at which cells were broken with the homogenizer could be increased; (3) repeated centrifugation of the obtained intracellular lysates at 30,000× *g* caused loss of enzyme activity and, therefore, should not be excessive; and (4) the run-off reaction was not necessary for the process of polymer separation and mRNA degradation, and the dialysis time could be markedly reduced. The optimized total time was ultimately reduced to six hours. The activity of chloramphenicol acetyl transferase (CAT) in the crude extracts obtained via this optimized method was comparable to that of the CAT synthesized from the crude extracts obtained by the standard procedure of Pratt. In subsequent experiments with the scale-up of the non-cellular response experimental system, Zawada et al. combined their own experimental needs and added a run-off reaction (30 °C for 2.5 h) [24] on the basis of the process provided by Liu. This also provides evidence of how the extraction process of crude extracts from cells can be broken down and assembled. Kim et al. (1996) also optimized the standard process provided by Pratt to build a highly efficient CFPS system using *E. coli*. Buffer S30 was divided into buffer A (10 mM tris/acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM dithiothreitol, and 0.5 mL/L mercaptoethanol) and buffer B (buffer A without mercaptoethanol); buffer A was designed for washing and collecting cells, while buffer B was used for the subsequent reactions [25]. However, the high speed, preculture, dialysis, and other steps involved in this process were cumbersome, time-consuming, and costly. Therefore, this group continued to optimize the method and found that the processes of high rotation speed, preculture, and dialysis could be abolished. Instead, a simple centrifugation procedure at low speed

(12,000 RCF, 10 min) followed by a brief incubation period was sufficient to produce an active extract to support the CFPS reactions, and, significantly, the total reagent cost and processing time were reduced by 80% and 60%, respectively, compared with the current standard procedure for preparing S30 extract [26]. Although this was a breakthrough in time, the high-voltage equipment required for this process was beyond the reach of some laboratories; Shrestha et al., therefore, changed the high-voltage crushing step to ultrasonic crushing [20] (Supplementary Table S2).

At present, the rapid iteration of the design-build-test cycle for crude-extracts-based CFSB is still in the laboratory application stage. Most of the reaction systems are small, and the requirements are less. Therefore, it is necessary to establish a rapid, more robust, and high-throughput cell-extract-preparation method for the laboratory application stage. Consequently, Jewett's team conducted a follow-up study on the method provided by Shrestha (Supplementary Table S2) and obtained 40.7 mg/mL of protein [21]. As shown in Table 1, the time-consuming and labor-intensive run-off reaction step could be omitted, but otherwise, the process was essentially the same, with a few changes in the middle parameters, such as eccentricity and centrifugation time. These parameters might be tunable to serve their purpose, as evidenced by Jewett's team in a follow-up study for 2′3-butanediol [27] and limonene [28]. The basic process was the same as that in Supplementary Table S2, except that the cells were broken under high pressure and were then centrifuged at 30,000× g and 4 °C for 30 min. In our previous study, the modular co-catalysis cell-free system was first used for pinene production. Crude extracts with high activity were acquired by reference to the extraction method reported by Jewett et al., and the highest yield of pinene (104.7 mg/L/h) was obtained [29].

To obtain high-quality and high-vitality crude cell extracts, researchers not only optimized the extraction process but also conducted related studies in other aspects. ATP is an important energy source to obtain in crude cell extracts, and there is a lot of phosphatase in the periplasmic space of cells, which is responsible for approximately 40% of ATP hydrolysis. Therefore, Kang et al. prepared the protoplasm system, removing the phosphatase and then breaking the cells to extract the intracellular material, which increased protein production by approximately 30% [30]. To the same purpose, Kim et al. prevented hydrolysis of approximately 30% ATP and increased the yield by 40% simply through adding phosphate and glucose to the growth medium to inhibit the expression of alkaline phosphatase and hexose phosphatase [31]. In addition, it was also possible to simplify cell growth [32,33] and use high-density fermentation [19] to increase protein content from the perspective of strain culture or use freeze-drying technology to extend the storage time of the crude extracts with high activity [34].

E. coli is the most widely used bacteria for CFSB. Continuous development and modification of the CFSB process with this bacterial species have provided suggestions and the basis for the development of CFSB systems with other microorganisms, such as yeast, *Streptomyces*, *Bacillus*, *V. natriegens*, and *Pseudomonas*.

4. Energy Regeneration

Compared with a cell-free pure enzyme system, the cell-free crude extraction system has received considerable interest because it can use some existing energy and cofactors in lysates for protein synthesis at a low cost. The quality (activity) of the crude cell extract is key to the efficiency of the subsequent product synthesis. Among these existing factors, ATP is critical to maintaining the vitality of the crude extract and is also one of the predominant factors needed for the termination of protein synthesis in the absence of cellular responses [35]. Therefore, preculture (run-off reaction) was historically always involved in the process of obtaining crude extracts of cells, and the so-called second energy substance was added in the middle of the process to perform energy regeneration and maximize the vitality of the crude extracts of cells. Other measures can also be used. Energy regeneration in cell-free systems is usually obtained through the cleavage of phosphate bonds. However, following additional research and expanded knowledge,

some later energy regeneration systems were developed that do not require the breakage of phosphate bonds.

4.1. Phosphate Bond Breakage to Provide Energy

In early ATP regeneration systems, PEP, acetylphosphoric acid, and phosphocreatine with high-energy phosphate bonds were commonly used to produce ATP in cell extracts by binding enzymes such as pyruvate kinase, acetylkinase, or creatine kinase. In early versions of the standard process for extracting crude extract from *E. coli* cells [16–18], PEP was the most commonly used secondary energy source and was included in the run-off reaction after obtaining crude extract. Subsequent trials revealed that PEP-based ATP regeneration systems were approximately twice as effective as those based on phosphocreatine [36]. However, one drawback of PEP for energy regeneration is the need for auxiliary pyruvate kinase, which increases costs. To address this limitation, Ryabova et al. converted acetyl phosphate to replace PEP for energy for the first time in an in vitro translation study using *E. coli* in 1995 [37]. Although the decomposition of acetylphosphoric acid requires the action of acetylkinase, this enzyme is always present in the bacteria, which effectively reduces the cost, and compared with the energy supply of the PEP system, the activity of the obtained crude extract of the cell is not weakened. Moreover, glucose 6-phosphate (G-6-P) has also been used as a secondary energy source due to its high-energy phosphate bond. Swartz proposed another scheme to replace PEP with G-6-P as the second energy substance, and although the early protein synthesis was less efficient than with PEP, the generation of the final product was up to 30% more efficient. The reason for this may be that ATP is produced over a longer period of time [25].

The crude-extracts-based CFSB system of *E. coli* was initially the most widely used system and has since been replicated with numerous other bacteria. Consequently, the process of breaking phosphate bonds to provide energy has also been imitated. However, the cleavage of phosphate bonds leads to the accumulation of phosphate and alters the pH, which affects the continuous synthesis of subsequent proteins. Swartz et al. mitigated this side effect by shortening the time for ATP regeneration using PEP in CFPS studies.

4.2. Non-Phosphate-Bond Breaks to Provide Energy

Pyruvate can be catalyzed to acetylphosphoric acid by pyruvate oxidase (E.C.1.2.3.3) from species of the genera *Lactobacillus* or *Pediococcus*; this process generates ATP and reduces phosphate accumulation. Therefore, as the continuous accumulation of phosphate would affect the continuous synthesis rate of proteins, Swartz proposed to replace PEP with pyruvate as the second energy substance [35,38]. Similarly, since pyruvate can be used as a secondary energy source for ATP, G-6-P has also been successfully used for ATP regeneration during glycolysis, which implies that the intermediate products of the glycolysis pathway can be used for ATP regeneration. Therefore, in a follow-up study, the scheme of ATP regeneration using G-3-P was proposed by Kelwich [39].

4.3. Others

In addition to the energy regeneration mentioned above, there are other ways to increase the energy supply. For example, Jewett et al. found that compared with the acetate solution, the glutamate solution was more conducive to the interaction between protein and nucleic acid. Added glutamate can directly enter the TCA cycle to form α-ketoglutaric acid, thereby increasing ATP regeneration [38]. Using this method, the CAT protein was synthesized via a glutamate-phosphate/nucleoside monophosphate system, and a high yield of 12,000 mg/L was obtained in 2 h [40].

Research on ATP regeneration to increase the production of cell-free protein synthesis is time-consuming and labor-intensive. Therefore, the research of designing and utilizing the advantage of free assembly of the system to realize the direct energy supply has been developed. For example, the Entner–Doudoroff Pathway (ED) can use a molecule of glucose to produce one ATP molecule, while the EMP pathway (Embden–Meyerhaf–

Parnas Pathway) can generate two ATPs, thereby permitting balanced utilization of ATP metabolism [1].

Energy is required for the synthesis of CFPS. In addition to intracellular residual energy, cofactors, and other substances, the crude extract also contains a series of metabolic pathways from the original host cells, which can still perform relevant metabolic activities to generate ATP in vitro. In studies of cell-free systems with less demand for energy, the capacity regeneration system can be completely ignored. For example, Khattak reported that for ethanol synthesis using the crude-extracts-based CFME of yeast [41], the concentration of ATP and NAD in the crude extract was sufficient to meet experimental needs, and the reaction could, therefore, be performed directly after obtaining the crude extract of the yeast. In our studies on the crude-extracts-based CFME of *E. coli* for pinene [29] and chlorogenic acid [42], energy regeneration steps were also ignored, and good results were still achieved.

5. Conclusions

The CFBS system has become an important tool in the next generation of the biological manufacturing industry. The high activity of the crude extracts can reduce or avoid the need to include expensive coenzymes, energy, and other substances in the cell-free reaction solution, enabling the system to be utilized in many mainstream laboratories. *E. coli* is the most widely used strain in the cell-free field at present. Research on cell-free biosynthesis with other microbes, such as yeast, *Streptomyces*, *Bacillus*, *V. natriegens*, and *Pseudomonas*, is continuously developing, and good progress has been made in different fields. Optimizing the extraction method of crude extracts makes the process more efficient. In addition, solving the problems encountered in the energy regeneration system means the energy supply becomes more economical. However, the cell-free crude extract biosynthesis system is still in its infancy, and numerous issues remain in crude extract acquisition and energy regeneration. One issue is the low concentration of obtained enzymes. At present, the highest enzyme content obtained in crude cell extracts is 40 mg/mL [21], but some proteins have intracellular local concentrations of 200 mg/mL [5]. A second issue is the increased dispersal of heterologous proteins after heterologous expression. Heterologous proteins can be aggregated by organelles or signal guidance to increase unit protein concentration. However, expressed heterologous proteins were dispersed, which reduced the concentration of the unit enzyme and the catalytic activity of the enzyme. Poor enzyme stability is another issue of the cell-free crude extracts. Although the activity of crude extracts can be markedly improved through continuous optimization of the extraction method and energy regeneration system, the stability of enzymes in crude extracts becomes another limiting factor. Finally, in addition to the various energy and cofactors required by the crude extracts of cells, the crude extracts also retain their own metabolic pathways, which may be conducive to energy regeneration or may continue to consume energy, and the effects of these metabolic pathways on acellular synthesis need to be studied. In summary, further research on CFSB is needed, and strategies for the immobilization of enzymes (intracellular or extracellular) to increase the stability and local content of enzymes are recommended.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10061122/s1, Table S1: Standard process of *E. coli*; Table S2: Extraction methods of crude extracts from *E. coli* (optimization).

Author Contributions: M.H. revised the paper, W.W. and T.G. referred to energy regeneration-related materials, X.L. referred to *E. coli*-related materials, and F.N. wrote the paper and referred to the rest of the materials. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Shi, T.; Han, P.; You, C.; Zhang, Y.H. An in vitro synthetic biology platform for emerging industrial biomanufacturing: Bottom-up pathway design. *Synth. Syst. Biotechnol.* **2018**, *3*, 186–195. [CrossRef] [PubMed]
2. Lim, H.J.; Kim, D.M. Cell-free metabolic engineering: Recent developments and future prospects. *Meth. Protoc.* **2019**, *2*, 33. [CrossRef] [PubMed]
3. Lu, Y. Cell-free synthetic biology: Engineering in an open world. *Synth. Syst. Biotechnol.* **2017**, *2*, 23–27. [CrossRef] [PubMed]
4. Levine, M.Z.; Gregorio, N.E.; Jewett, M.C.; Watts, K.R.; Oza, J.P. *Escherichia coil*-based cell-free protein synthesis: Protocols for a robust, flexible, and accessible platform technology. *J. Vis. Exp.* **2019**, *25*, 11. [CrossRef]
5. Dudley, Q.M.; Karim, A.S.; Jewett, M.C. Cell-free metabolic engineering: Biomanufacturing beyond the cell. *Biotechnol. J.* **2015**, *10*, 69–82. [CrossRef]
6. Hoagland, M.B.; Stephenson, M.L.; Scott, J.F.; Hecht, L.I.; Zamecnik, P.C. Soluble ribonucleic acid intermediate in protein synthesis. *J. Biol. Chem.* **1958**, *231*, 241–257. [CrossRef]
7. Matthaei, J.H.; Nirenberg, M.W. Characteristics and stabilization of dnaase-sensitive protein synthesis in *E. coli* extracts. *Proc. Natl. Acad. Sci. USA* **1961**, *47*, 1580–1588. [CrossRef]
8. So, A.G.; Davie, E.W. The incorporation of amino acids into protein in a cell-free system from yeast. *Biochemistry* **1963**, *2*, 132–136. [CrossRef]
9. Grünberger, D. The incorporation of amino acids-14C into proteins by ribosomes of *Bacillus cereus*. *Collect. Czech. Chem. Commun.* **1964**, *29*, 2400–2405. [CrossRef]
10. Shiio, T.; McFadden, B.A. Cell-free amino acid-incorporating system from *Pseudomonas indigofera*. *J. Bacterial.* **1965**, *90*, 978–983. [CrossRef]
11. O'Sullivan, J.; Abraham, E.P. The conversion of cephalosporins to 7′-alpha-methoxycephalosporins by cell-free extracts of *Streptomyces clavuligerus*. *Biochem. J.* **1980**, *186*, 613. [CrossRef] [PubMed]
12. Failmezger, J.; Scholz, S.; Blombach, B.; Siemann-Herzberg, M. Cell-free protein synthesis from fast-growing *Vibrio natriegens*. *Front. Microbiol.* **2018**, *9*, 1146. [CrossRef] [PubMed]
13. Buchner, E. Alkoholische gährung ohne hefezellen. *J. Inorg. Chem.* **1897**, *30*, 117–124. [CrossRef]
14. Bujara, M.; Schuemperli, M.; Billerbeek, S.; Heinemann, M.; Panke, S. Exploiting cell-free systems: Implementation and debugging of a aystem of biotransformations. *Biotechnol. Bioeng.* **2010**, *106*, 376–389. [CrossRef] [PubMed]
15. Swartz, J.R. Transforming biochemical engineering with cell-free biology. *AIChE J.* **2012**, *58*, 5–13. [CrossRef]
16. Nirenberg, M. Cell-free synthesis directed by messenger RNA. *Meth. Enzymol.* **1963**, *6*, 17–23. [CrossRef]
17. Zubay, G. In vitro synthesis of protein in microbial systems. *Annu. Rev. Genet.* **1973**, *7*, 267–287. [CrossRef]
18. Pratt, J.M. Coupled transcription-translation in prokaryotic cell-free sytems. *Transcrip. Transla. A Prac. App.* **1984**, 179–209. [CrossRef]
19. Liu, D.V.; Zawada, J.F.; Swartz, J.R. Streamlining *Escherichia coli* S30 extract preparation for economical cell-free protein synthesis. *Biotechnol. Progress.* **2005**, *21*, 460–465. [CrossRef]
20. Shrestha, P.; Holland, T.M.; Bundy, B.C. Streamlined extract preparation for *Escherichia coli*-based cell-free protein synthesis by sonication or bead vortex mixing. *Biotechniques* **2012**, *53*, 163–174. [CrossRef]
21. Kwon, Y.C.; Jewett, M.C. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci. Rep.* **2015**, *5*, 8663. [CrossRef] [PubMed]
22. Chambers, D.A.; Zubay, G. The stimulatory effect of cyclic adenosine 3′5′-monophosphate on DNA-directed synthesis of beta-galactosidase in a cell-free system. *Proc. Natl. Acad. Sci. USA* **1969**, *63*, 118–122. [CrossRef] [PubMed]
23. Zalkin, H.; Yanofsky, C.; Squires, C.L. Regulated in vitro synthesis of *Escherichia coli* tryptophan operon messenger ribonucleic acid and enzymes. *J. Biol. Chem.* **1974**, *249*, 465–475. [CrossRef]
24. Zawada, J.F.; Yin, G.; Steiner, A.R.; Yang, J.; Naresh, A.; Roy, S.M. Microscale to manufacturing scale-up of cell-free cytokine production—A new approach for shortening protein production development timelines. *Biotechnol. Bioeng.* **2011**, *108*, 1570–1578. [CrossRef]
25. Kim, D.M.; Swartz, J.R. Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis. *Biotechnol. Bioeng.* **2001**, *74*, 309–316. [CrossRef] [PubMed]
26. Kim, T.W.; Keum, J.W.; Oh, I.S.; Choi, C.Y.; Park, C.G.; Kim, D.M. Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. *J. Biotechnol.* **2006**, *126*, 554–561. [CrossRef]
27. Kay, J.E.; Jewett, M.C. Lysate of engineered *Escherichia coli* supports high-level conversion of glucose to 2,3-butanediol. *Metab. Eng.* **2015**, *32*, 133–142. [CrossRef]
28. Dudley, Q.M.; Nash, C.J.; Jewett, M.C. Cell-free biosynthesis of limonene using enzyme-enriched *Escherichia coli* lysates. *Synth Biol.* **2019**, *4*, ysz003. [CrossRef]
29. Niu, F.X.; Huang, Y.B.; Shen, Y.P.; Ji, L.N.; Liu, J.Z. Enhanced production of pinene by using a cell-free system with modular cocatalysis. *J. Agric. Food Chem.* **2020**, *68*, 2139–2145. [CrossRef]
30. Kang, S.H.; Oh, T.J.; Kim, R.G.; Kang, T.J.; Hwang, S.H.; Lee, E.Y. An efficient cell-free protein synthesis system using periplasmic phosphatase-removed S30 extract. *J. Microbiol. Methods* **2000**, *43*, 91–96. [CrossRef]
31. Kim, R.G.; Choi, C.Y. Expression-independent consumption of substrates in cell-free expression system from *Escherichia coli*. *J. Biotechnol.* **2000**, *84*, 27–32. [CrossRef]

32. Kigawa, T.; Yabuki, T.; Matsuda, N.; Matsuda, T.; Nakajima, R.; Tanaka, A. Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression. *J. Struct. Funct. Genomics.* **2004**, *5*, 63–68. [CrossRef] [PubMed]
33. Yang, W.C.; Patel, K.G.; Wong, H.E.; Swartz, J.R. Simplifying and streamlining Escherichia coli-based cell-free protein synthesis. *Biotechnol. Prog.* **2012**, *28*, 413–420. [CrossRef] [PubMed]
34. Gregorio, N.E.; Kao, W.Y.; Williams, L.C.; Hight, C.M.; Patel, P.; Watts, K.R. Unlocking applications of cell-free biotechnology through enhanced shelf life and productivity of *E. coli* extracts. *ACS Synth. Biol.* **2020**, *9*, 766–778. [CrossRef]
35. Mortenson, L.E. Ferredoxin and ATP, requirements for nitrogen fixation in cell-free of *Clostridium pasteurianum*. *Proc. Natl. Acad. Sci. USA* **1964**, *52*, 272–279. [CrossRef]
36. Li, J.; Wang, H.; Kwon, Y.C.; Jewett, M.C. Establishing a high yielding streptomyces-based cell-free protein synthesis system. *Biotechnol. Bioeng.* **2017**, *114*, 1343–1353. [CrossRef]
37. Ryabova, L.A.; Vinokurov, L.M.; Shekhovtsova, E.A.; Alakhov, Y.B.; Spirin, A.S. Acetyl phosphatase as an energy source for bacterial cell-free translation systems. *Anal. Biochem.* **1995**, *226*, 184–186. [CrossRef]
38. Jewett, M.C.; Swartz, J.R. Mimicking the *Escherichia coli* cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnol. Bioeng.* **2004**, *86*, 19–26. [CrossRef]
39. Kelwick, R.; Webb, A.J.; MacDonald, J.T.; Freemont, P.S. Development of a *Bacillus subtilis* cell-free transcription-translation system for prototyping regulatory elements. *Metab. Eng.* **2016**, *38*, 370–381. [CrossRef]
40. Jewett, M.C.; Calhoun, K.A.; Voloshin, A.; Wuu, J.J.; Swartz, J.R. An integrated cell-free metabolic platform for protein production and synthetic biology. *Mol. Syst. Biol.* **2008**, *4*, 220. [CrossRef]
41. Khattak, W.A.; Ul-Islam, M.; Ullah, M.W.; Yu, B.; Khan, S.; Park, J.K. Yeast cell-free enzyme system for bio-ethanol production at elevated temperatures. *Process. Biochem.* **2014**, *49*, 357–364. [CrossRef]
42. Niu, F.X.; Yan, Z.B.; Huang, Y.B.; Liu, J.Z. Cell-free biosynthesis of chlorogenic acid using a mixture of chassis cell extracts and purified spy-cyclized enzymes. *J. Agric. Food Chem.* **2021**, *69*, 7938–7947. [CrossRef] [PubMed]