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Review Article

Cell-Free Production of Recombinant Proteins

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Abstract: Cell-free production systems have gained much attention in the last decade due to a quest by researchers to develop more complex and potentially viable proteins, which are otherwise 'toxic' or 'difficult-to-express' for conventional cell-based systems. Conventional cell-based systems have presented scientist with several other limitations, thereby impeding not only the production but also, the optimization of already expressed proteins both on the bench and large scale. Novel recombinant proteins are believed to be the next generation of products that will transform many fields, especially for therapeutics and diagnostics in medicine. Gleaning from the massive impact of the growth and improvement of penicillin, it is not difficult to see the grave importance of optimizing present processes to be more time and cost efficient. Cell-free protein expression systems (CFPES) already show promising potentials in the development of a wide range of areas that they are already being applied to. The development of high-throughput screening processes, production of integral membrane proteins and difficult to express proteins, are a few key applications to watch out for. It's numerous advantages, such as flexibility, ease of manipulation, cost and time efficiency to mention a few, make it an even more exciting prospect over the long-standing and ever praised E. Coli and Chinese Hamster Ovary (CHO) whole expression systems. This review succinctly presents the concept of cell-free production as a choice system for recombinant protein, highlighting, many recent applications, advantages and prospects. It also sets out to describe methods of recombinant production using cell-free extracts, while still touching on its presenting limitations.

INTRODUCTION

Proteins are one of the most versatile and widely employed macromolecules in the pharmaceutical industry. Their ability to serve as either therapeutics themselves or as a drug target, due to their large presence and vital regulatory roles in the human body, is what makes proteins the major focus for drug development. The advent of genetic engineering and a variety of accompanying technologies in the last three decades, has been a major key to the advancement of therapeutic protein production by many biopharmaceutical companies.

Conventionally, through genetic transformations, cells can be engineered to improve the quality and efficacy of protein products [1]. These protein products are referred to as recombinant proteins (rP). These are proteins that have been amended/ engineered at the gene level (recombinant DNA) and expressed in a living system that supports its mRNA translation and post modifications. Recombinant technology has brought about great advancements in disease therapies. In 1982, the first therapeutic recombinant protein was produced, insulin [2]. Hundreds more are available now for treatment of a variety of illnesses. Recombinant proteins have also been used in the food/consumables industry in the form of novel enzymes that aid in the speedy processing of food raw materials and as cleaning agents in the detergent industry.

A variety of cell culture systems have been used over the years to express recombinant proteins and they include: Bacteria, Yeast, Fungi, Insect cells, Plants and Mammalian cells, in general terms [3]. Each of these cell culture systems have a wide range of genetically engineered cell lines, used to produce a variety of protein products, depending on the characteristics/features desired. Bacteria and yeast in the early years of recombinant protein production, were the most commonly used. Several small sized proteins, not needing extensive post-modifications, were produced using these systems, and today, therapeutic proteins made from them make up about 37% of the market, the second largest group after mammalian cells [2,4].

Yet, the world is increasingly being ravaged by diseases (cancer, autoimmune disorders and many infectious diseases) that seem to demand for proteins with even more complex features and physicochemical properties, not naturally found within the human system. Cancer for instance, is a disease that is yet to be fully elucidated, with a plethora of drugs that don't seem to have been able to effectively tackle the disease. In recent years, biopharmaceutical anticancer drugs such as onconase [5] and other therapeutics like the urokinase, a plasminogen activator have been found to have promising breakthrough

effects [6]. The synthesis and production of these unique proteins has been found to be toxic and very difficult to express even in mammalian cells. With further research, the production and scale up of consistent batches of these vital proteins have become feasible through the cell-free systems [5,6].

Cell-free protein synthesis, was first described by Nirenberg and Matthaei in 1961 [7] and has now become a point of attraction in the past decade, owing to its numerous foreseen advantages, such as flexibility and ease of manipulation. So far, it has been applied to various areas, all majorly with an overall aim for more efficient expression of proteins, beyond its previous engagement in the research and understanding of molecular basics [8]. Cell-free production expression systems (CFPES) for recombinant proteins is a very vital tool for the development of unique therapeutics that will yet save many from the plethora of diseases, still termed 'incurable'. With the advent of CFPES, high-throughput methods will also be developed that would revolutionize the way research is done now. Its role cannot be overemphasized in fully optimizing present production processes of conventional expression systems [9]. The massive impact of the full optimization of the penicillin production, is a pointer to the pertinence of having systems such as CFPES flourish.

In this report, we aim to present concisely the role of CFPES as a choice system for an optimized production of rare and effective therapeutic proteins while throwing light on the conventional cell-based systems.

RECOMBINANT PROTEINS PRODUCTION/EXPRESSION

In a much broader perspective, proteins are final products of genes encoded in the deoxyribonucleic acid (DNA) of all living organisms, coding specific information about their function and structure. They are the most abundant biomolecule found in living things and they perform a wide variety of functions. Proteins function in very vital roles [10] such as, cell to cell communication, immune system response, regulators of metabolic reactions and even as catalyst to these reactions. They also perform structural functions, conferring rigidity and support to the cell wall and membrane structures. Proteins can also function as pigments in living organisms, such as the popular green fluorescent protein (GFP). The practicality of proteins can neither be underplayed nor over emphasized and hence, have been recognized by scientists/researchers over the years as a major tool for therapy and diagnostics in medicine especially, as well as a wide range of other disciplines.

To express a recombinant protein, the gene of interest is isolated, amplified, altered to suit the need, cloned, with the help of vectors, and special enzymes, which assist with the cutting and pasting of the genes and then the gene construct is put back in a system that supports the expression of the desired protein [11].

CONVENTIONAL PROTEIN EXPRESSION SYSTEMS

Bacteria (*E. Coli*)

E.Coli gained much of its status in the 1950's, in molecular biology research and due to vast knowledge that had been gained about them, they became the first handy tool for production of recombinant proteins. *E. Coli* known as the 'microbial factory' has been extensively employed in the production of a wide variety of recombinant protein, such as insulin [2]. It has been a system of choice for many years due to its easy handleability, cheap growth requirements and high expressions of product in its cytosol [12]. However, some of the limitations encountered using the *E. Coli- based* system include the formation of inclusion bodies in bacteria, as a result of clumping of heterologous proteins, stemming from factors, such as: high temperatures, speed and quantity of expression and even size of the protein [13]. Furthermore, their inability to effect post-translational modifications to proteins, the production of endotoxins and proteolytic enzymes that both contaminated and destroyed the product, affected their worth for production of more complex therapeutic proteins, like the antibodies. Especially because the downstream process among other things, became very laborious and costly [14-16].

Fungi

A wide variety of enzymes and other peptide and protein molecules have been successfully produced from fungi. Inhibitory peptides such as angiotensin-I converting enzyme has been produced from mushroom hydrolysates [17], enzymes such as beta-galactosidase which is significant for lactose intolerant treatments and the asparaginase enzyme for destruction of hematopoietic selective diseases from the [18]. High levels of secretions associated with the fungi decomposing lifestyle, as well as their shared characteristics with other eukaryotic cells (human cells) makes them very attractive options [19]. Yet, despite the vast use and very promising applications of the fungi-based systems, the high cost of downstream purification after production, is not sustainable [17,18].

Yeast

Two commonly used yeasts are *Saccharomyces Cerevisae* and *Pichia Pastoris*. Their ability as a eukaryotic organism to properly fold larger proteins and perform some post-translational activities such as the basic and most common glycosylation, while still having the flexibility and low-cost requirements of bacteria cells, is what has also made yeasts an attractive option [2,20]. However, their inability to correctly fold more complex proteins with a variety of other post-translational motifs and their susceptibility to virus also makes them very unreliable for production for many advanced therapeutic proteins [10].

Insects

Another promising host that has been considered by researchers is the use of insect cells for the production of recombinant proteins. Over the years, the possibility of using insect-cell based systems have been explored, while trying to prove its comparative advantages to mammalian cells [21]. Insect cells have been shown to effectively undergo post-translational modifications and expressed higher levels of protein when infected with the baculovirus expression (BVE) system. They are also more tolerant to waste product concentrations and have even been found to be easier to culture when compared to mammalian cells [22].

Even though the production of recombinant proteins from insect cells have matured into a useful system for the industry, there are still a lot of drawbacks associated with insect cell cultures. The extensive review by Ikonomou and colleagues [22] showed that there are still lots of gaps in the knowledge for using insect cells. Insect cell cultures tend to use up lots of medium containing glucose and amino acid

supplements. The use of BVE systems to also promote translation is a limitation to the use of these cells, as within the systems of these viruses are proteases, that tend to destroy proteins produced under certain conditions [10]. These limitations, alongside the need to still find optimal ways for its gas exchange make the use of this system less appealing for industrial applications.

Green Algae and Plants

The use of chloroplast-containing cell-based systems have also not been far-fetched. From the exploration of algae cells to the use of fully-grown whole terrestrial plants and its parts, the advantages of using this system seems to outweigh many of the previously mentioned cell-based systems [23]. Green algae have been exploited for the production of both monoclonal antibodies [23] as well as seven other recombinant proteins as reported by Rasala and colleagues [24]. The production of therapeutic proteins from micro-algae specifically, *Chlamydomonas reinhardtii* is well established [25] and presents advantages of rapid scalability, minimum cost, proper folding and post-translational modification etc. Plants either cultured in growth media or fully in the soil have also been explored using either the leaves or whole plants to produce therapeutic proteins such as monoclonal antibodies and recombinant alpha-1 antitrypsin produced in *Nicotiana benthamiana*, some of which have undergone clinical trials as well [24,26]. The major fear, however, is the possibility of immunogenic reactions by the human body, due to disparities in the genetic make-up of humans and plants, specifically the plant-specific glycans molecules [23], which seem to have been addressed with transient expression strategies (that is, no incorporation of protein genome into plant host genome) [27].

Mammalian Cells (Chinese Hamster Ovary)

The mammalian cell system is the most vastly used protein production system and several novel proteins have been developed using these cells. High-throughput cell line developments have brought about highly optimized systems, that make production cost-efficient, scalable, robust and safe products [2]. Out of all the biopharmaceuticals approved in the past decade, 56% are made from mammalian cells [28]. Chinese Hamster Ovary (CHO) cell lines dominate the mammalian cell-based systems used. Despite the vast applications of these outstanding host organisms, the limitations still exist, many of which will be identified in subsequent sections.

Overall, the choice of expression systems for therapeutic protein, borders on factors such as [29]:

1. Difference in codon preference from one organism to another. This could greatly affect the expression of the recombinant product, hence organisms most suitable for their expression are either found or the organism is supplemented with tRNA needed. Alternatively, the coding sequence can be changed to use codons more frequently used by host organism [30].
2. Location of protein expressed, intracellular or extracellular? Proteins secreted outside the cell are easier to purify than those within. For cost-effective production, extracellular secretions are usually prioritized
3. Level of post-translational modifications needed is also a major factor to consider when expressing proteins, as prokaryotes are very limited in this aspect, except aided with the supplements as seen in a few reports and even when aided, it tends to cause conformational stress on the cells [31]
4. Stability of the protein of interest is also essential and this can be conferred by: determining the N-terminal acid of protein, co-expressing a molecular chaperone, or eliminating 'PEST' codons from the genetic sequences [32]

Essentially, the quality, functionality, speed of production and yield are the underlying factors behind all other factors considered when choosing the best method for expression of recombinant proteins [10].

CELL-FREE RECOMBINANT PROTEIN EXPRESSION SYSTEM (CFPES)

Cell-free protein synthesis (CFPS) was first described almost 6 decades ago by Nirenberg and Matthaei, as a tool for the *in-vitro* investigation of the de novo protein synthesis [7]. The cell-free synthesis of molecules was also employed in the discovery of antibiotics and the expression of a few chemical products. For a while, this system had been neglected, as the advent of genetic engineering, isotope-labelling and cell culture techniques made it possible for one to study the workings of cells *in vivo* [33].

In this period, native and recombinant proteins have been developed as powerful therapeutics from a variety of expression systems, as discussed above. Most of this development has been done *in-vivo* and even large manufacturing scale setups have been established over time by various biopharmaceuticals to produce this highly important treatments. A most prominent system employed over the last two decades in the development of many novel recombinant proteins is the CHO cell system, due to the numerous advantages it presents. However, production using the CHO cell system is extremely expensive, time-consuming, with obvious batch to batch inconsistencies [2] which scientists/researchers are working to overcome, while striving to optimize the overall efficiency of the system. The cell-free expression system is a powerful technology that has once again being revived in the past decade, as a promising system for the efficient and rapid production of complex recombinant proteins. The constant need to optimize present systems to achieve even higher production titers cannot be overemphasized. The advantages of an optimized system can be exemplified with the advancement observed with penicillin development.

Twenty-five years after the discovery of penicillin by Sir Alexander Fleming and it's expansion by Edward Penley Abraham, the product titer and volumes of penicillin had increased exponentially. This rapid improvement in the production scale and sophistication of manufacturing techniques, greatly slashed the price of penicillin by an unbelievable 99.5%. Hence, many people were able to get treatments at affordable cost, thereby saving many lives [34]. Cell-free production as can be implied from the name, is the *in-vitro* expression/production of biomolecules (primarily, protein) without using the living cells. The extracts of the living cells (the expression system of choice) are instead used for production [35]. *Figure 1* shows graphically, the concept of the cell-free production system. The cell-free system was developed on the notion that cell integrity is not needed for the synthesis of proteins to occur.

Fig 1: Simplified illustration of the Cell-free production system [35]

EMPLOYING THE CELL-FREE PROTEIN EXPRESSION SYSTEM (CFPES)

In the development of the CFPES, cell lysates/free extracts are derived from a suitable cell, which has the biochemical capabilities to produce the protein of interest. That is, the origin of the protein, its nature and potential application should all be considered when choosing a system for the extraction of the lysates [36]. There are two major classes of the CFPES [37], and they are those derived from:

1. Prokaryotes, typically *E. coli,* as was first described to be used by Nirenberg and Matthaei in 1961 and*;*
2. Eukaryotes, which include rabbit reticulocytes, wheat germ cells, and even more recently CHO cells.

Over the years, a variety of studies have shown *E. coli* systems to produce even much higher protein titers than the eukaryotic systems. However, the quality of proteins expressed by *E. coli* as described by Gagoski *et al.* in 2016, were observed to have high tendency to aggregate, having only 10% of the proteins tested to be predominantly uniformed aggregates. They were also found to be associated with truncated pronounced species of proteins particularly larger than 70kDa [38].This report is supported by studies conducted by Hillebrecht and Chong earlier in 2008, who confirmed the inability of prokaryotic cell-free extract to synthesize an artificial fusion protein [39]. Furthermore, Gagoski and colleagues showed that, among the eukaryotes cells they tested, the wheat germ expression system proved to be the most productive [38].

E. coli expression systems are the oldest and most versatile system used due to its relatively cheap preparation and yet laudable expression of high protein yields. So much so that, Zawada and colleagues in 2011, successfully applied it to a manufacturing scale-up production of cytokine [40]. Other systems capable of eukaryotic type of posttranslational modifications have however, been explored in order to systematically combat the limitations associated with prokaryotic systems. Alternative cell-free expression systems used includes yeast, insects and plants [41].

PREPARATION OF CELL-FREE SYSTEMS

The preparation of a CFPES begins with the collection of the cell crude lysate, containing all the component needed for the translation process. Addition of co-factors, amino acids and a source of energy are essential supplements to the mix [33]. CFPE systems can be prepared using two major methods/approaches [35,41,42]:

1. **The Linked/Coupled CFPES/ 'lysate-based' CFPES**: This system is conducted by coupling the transcription and translation processes in one mixture. This is carried out by first transcribing the DNA encoding the protein of interest in a reaction vessel. The RNA polymerase could be added alongside the DNA to aid its transcription, if not already present in the kit. DNA could be either a linear/ circular plasmid DNA or linear PCR product (taking note of the pros and cons of whichever is chosen). Afterwards, the reaction mixture (cell lysate with supplements, described above) is then added to create a 'translation mix'. The soluble protein derived from the translation process can then be purified through a number of conventional downstream methods already available, such as centrifugation and the chromatographic separation using a charged column with affinity for His-tags. *Figure 2* shows a schematic description of the linked system.

2. **The 'PURE' CFPES**: This is a kit marketed by New England Biolabs, newly developed to optimize the production of proteins from cell-free systems. It is an improvement on the lysate-based method because it is void of any molecules or metabolites not being used in the translation process, hence termed 'pure'. The system is also made up of purified recombinant entities/factors involved in the translation. The purification of proteins from this system, is slightly different, as the mix is first ultra-filtered to remove the large ribosomal content, then the other components of the system can then be captured with a chromatographic column, since they are all His-tagged. In this way, the protein of interest is free from any tags that may interfere with its proper function, especially as a therapeutic. The PURE method has shown great promise for the production of recombinant proteins, as many unnatural amino acids can be added to the system to develop artificial recombinant proteins [43].

A third but not common method mentioned by Katzen, Chang and Kudlicki in 2005, is the '**uncoupled' CFPES** method [36]. This is simply the translation of any given external RNA template, achieved by adding a crude cell lysate equipped with the translational apparatus, such as the enzymes, tRNA and factors. This is a classic in-vitro translation mechanism that doesn't yield as much as the two other systems mentioned above but could be used for quick experiments. The modes of all three reactions just discussed above is the '**batch**' mode reaction. Despite the amazing improvements on product titers witnessed with the cell-free system. These reactions are limited in the sense that the yields are still below desired standard for possible large-scale production. They also exhibit short life spans in production process due to speedy depletion of phosphate-energy sources, even in the absence of protein synthesis. The possible presence of endonucleases also affects the productivity of the cell-free system [33, 37].

Hence, a new mode of production was proposed by Spirin and coworkers in 1988, the **continuous** cell-free translation system. His method simply described the continuous addition of feeding buffer containing the essentials for the continuous synthesis of proteins and a simultaneous removal of the polypeptide product. This description is analogous to the perfusion mode designs we have now for the cell-based culture systems. Spirin's continuous mode did show great improvement in the production titers of the proteins expressed [44].

As the scientific world advances into the use of more high-throughput, energy saving methods, the continuous mode is not as appealing as it should have been, because of its laborious process. Other softer modes such as semi-continuous processes were developed, which showed even greater titers, yet it wasn't still practical to the development of user-friendly methods and was also a more expensive approach. Hence, the research has been intensified on the batch mode methods with significant improvements witnessed, with introduction of highly efficient bilayer diffusion system developed from extracts of the wheat germ cells, capable of satisfying the high-throughput approach [45].

More recently, many of the cell-free expression systems are being optimized in the laboratory by engineering the cells to be void of the nuclease producing machineries, before using the extracts. This has greatly contributed to improving the overall yield of proteins that could have been otherwise destroyed by a variety of these degrading enzymes upon lysing of the cells, especially in prokaryotes [33, 42].

PRACTICAL APPLICATIONS OF CELL-FREE EXPRESSION SYSTEMS

The use of CFPE systems have been proven to show very outstanding results in a variety of applications, using a number of the methods highlighted above. This section seeks to emphasize the practical applications of cell-free expression systems through a number of published materials, in a variety of fields but especially in the development of recombinant proteins, the focus of this review (Table 1).

- CFPE systems can and have been used for diagnostics and research to study phenomenon, such as the de novo synthesis of infectious viral particles. The work by Klein, Polyak and Lingappa in 2004, were able to demonstrate that 80% of hepatitis C virus (HCV) was assembled into HCV capsids, using rabbit reticulocyte lysate and wheat germ cell extracts [46].
- The cell-free system is also advancing the field of proteomics and biotech as it moves towards miniaturization of process methods and the development of high-through put techniques such as protein arrays (protein chips), and the use of microplate wells [47], even as we drive towards personalized medicine.

As regards development of recombinant proteins from cell-free lysate, a few of the several published works include:

- Expression of 'difficult to express' proteins have also been rapidly developed using extracts from blood cells [47]
- Development of monoclonal antibodies using CHO-based cell extracts has been reported by Martin and colleagues in 2017. Tran and colleagues in 2018, also used CHO lysate to produce recombinant streptokinase [48,49].
- CFPE systems are being considered as a promising option for the large-scale production of integral membrane proteins, a most abundant drug target, even though the reports on its use for their production is still quite limited [50]

Wheat germ CFS have also been employed in the production of malaria proteins, which could be outstanding drug candidates for the treatment of a disease killing many in sub-Saharan Africa [51].

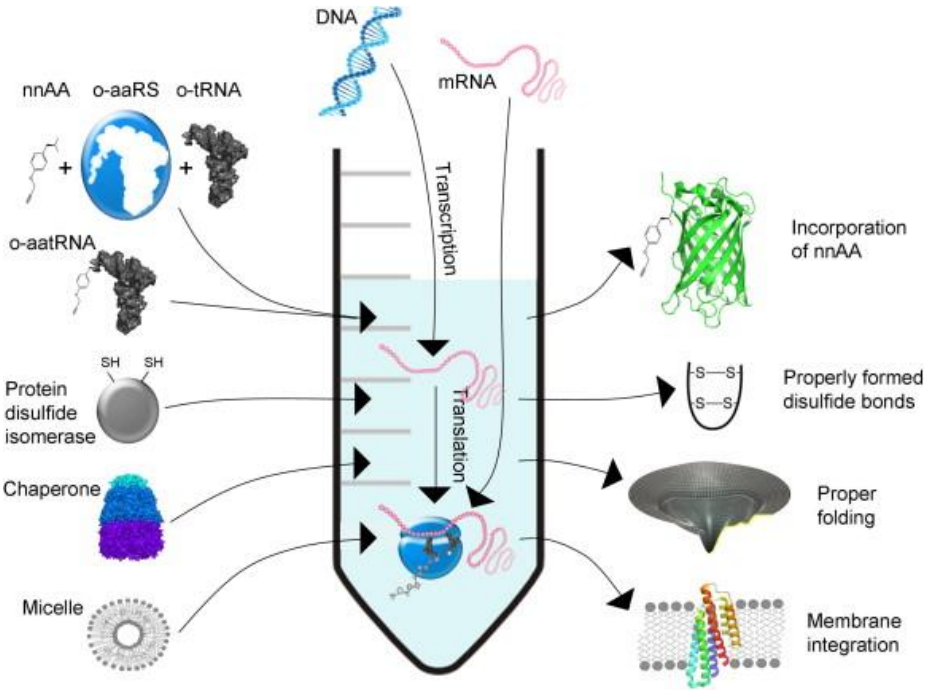

Fig 2: Schematic description of the coupled CFPE System [41]

Table 1: A brief list of the recent developments using the CFPES, summary of its advantages and few limitations

Recent Developments	Advantages	Limitations	References
Use of rabbit reticulocyte lysate and wheat germ cell extracts in diagnostic research of HCV	Gives a broad, less restrictive frame work for the study of many phenomenon		[46]
Synthesis of complex antibody formats specifically-IgG & single-chain variable and constant fragments fused (scFv-Fc), using a microsome containing CHO-based cell-free system (2017)	Opportunity to modify and specify conditions to produce otherwise highly-beneficial 'cell-toxic' protein therapeutics	Subsequent post-translational modifications can be optimized for	[52,53]
Production of enhanced-green fluorescent protein (EGFP) from *Streptomyces*-based CFPES (2017) and also from *Vibrio natriegens (2018)*	Faster, easier, cheaper and more robust recombinant protein production with a more flexible range of organisms to choose from	There is still room for optimization of the system	[54,55, 53,]
Cell-free approach used in the synthesis/manufacture of enzymes capable of producing vital chemicals such as limonene sustainably (2018)	Eliminates the need for extensive engineering of cell and microbial factories and extensive isotopic labeling and spectroscopy	There is still lack of proper regulation, limiting scale up processes	[56, 53]
Endotoxin-free production of crisantaspase; a protein therapeutic for the treatment of lymphoblastic leukemia (2019)	Elimination of endotoxins before protein expression; greatly reducing downstream processing time and cost		[57]

ADVANTAGES OF CELL-FREE SYSTEMS FOR PRODUCTION OF RECOMBINANT PROTEINS

Cell-free systems present a plethora of advantages, that have indeed been recognized by researchers across a wide range of disciplines in the past decade and are already being exploited, for the development of more complex and artificial recombinant proteins. Some of these advantages are:

- the ease of modifying conditions of the reactions to favor protein folding, glycosylation and chemical conjugation, giving room for flexibility of *in-vitro* protein synthesis to be open to automation and miniaturization [36].
- it provides a broad, less restrictive framework for the biochemical study of an interesting array of phenomenon, yet to be uncovered as seen with Nirenberg and Matthaei (1961) and for the production of toxic material, that would otherwise be impeded by the cell.
- elimination of large-scale manufacturing materials using up large amounts of costly, complicated media formulations
- Faster, easier and more efficient method for protein expression and downstream purification, as schematically described in *figure 3*. Since it does not entail the use of unpredictable transfection systems, cell culturing and extensive purification methods, that might otherwise result in protein denaturation, in the cell-based protein synthesis [35].
- Eliminates the need for NMR spectroscopy techniques and all of its limitations, such as the need for extensive isotope labelling, restrictions with amino acid scrambling and its overall costly expression procedures and many more [43]

CHALLENGES OF THE CFPE SYSTEM

In spite of the numerous advantages that the cell-free system presents the scientific community with, it is still limited in a few ways [53];

1. There is still a need for optimization of the CFPES technique in the post-translation modification of recombinant proteins
2. The system lacks proper regulation, and this is important for easy development into large scale processes and;
3. The life span of the processes is still very short-lived and pose a challenge for extended production of protein synthesis

FURTHER APPLICATIONS

Potential areas for application are rapidly expanding and they could include:

- In the study of RNA silencing, as a key regulatory tool and drug target
- Fusion with present technologies such as stem cell, 3D printing artificial intelligence, etc to develop even better cutting-edge tools [53]
- Employed in the large-scale production of membrane proteins and in the study of complex physiological responses [59].

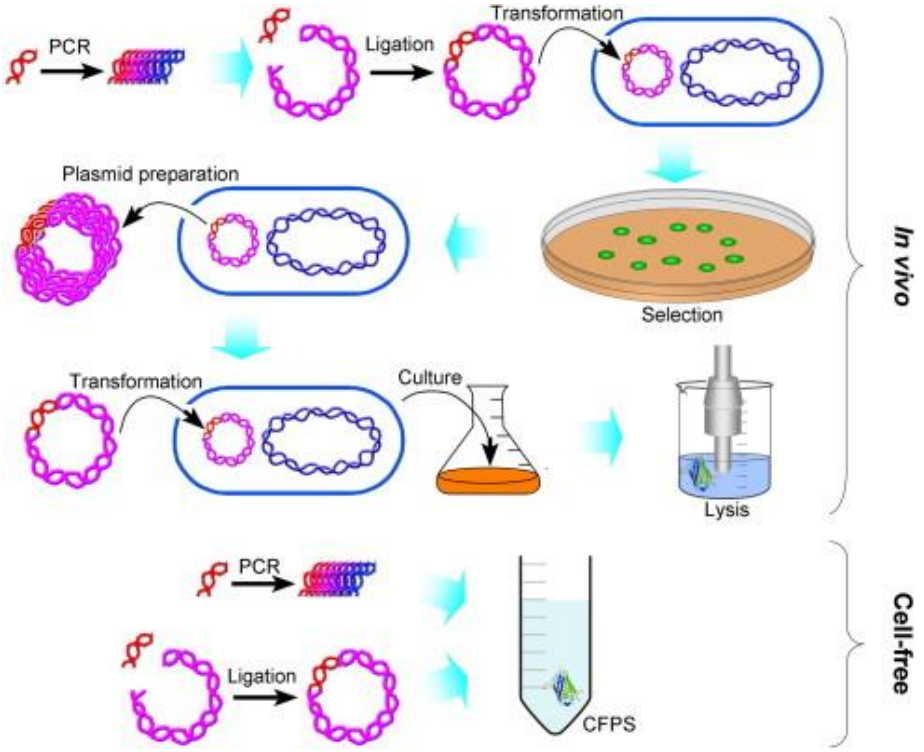

Fig 3: Schematic representation projecting the advantage of speed and ease associated with the CFPES in comparison to in-vivo cell cultures [41]

CONCLUSION

In this present time, researchers are quite focused on building high-throughput, efficient and user-friendly processes, that will maximize the energetics of a batch process and not the complications of a continuous or semi-continuous process. Cell-free production systems is indeed a promising prospect for the development of these fast, efficient processes. It promises to be the key to the full optimization of conventional based processes.

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CONFLICT OF INTEREST

Authors declare that no competing interests exist.

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