

Review

Evolving Paradigms of Recombinant Protein Production in Pharmaceutical Industry: A Rigorous Review

Achuth Jayakrishnan ¹, Wan Rosalina Wan Rosli ², Ahmad Rashidi Mohd Tahir ², Fashli Syafiq Abd Razak ², Phei Er Kee ³, Hui Suan Ng ⁴, Yik-Ling Chew ⁵, Siew-Keah Lee ⁶, Mahenthiran Ramasamy ⁷, Ching Siang Tan ^{8,*} and Kai Bin Liew ^{2,*}

¹ Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore 641028, India; achuth.j@hicas.ac.in

² Faculty of Pharmacy, University of Cyberjaya, Persiaran Bestari, Cyberjaya 63000, Malaysia; rosalina@cyberjaya.edu.my (W.R.W.R.); rashidi@cyberjaya.edu.my (A.R.M.T.); fashli@cyberjaya.edu.my (F.S.A.R.)

³ Biorefinery and Bioprocessing Engineering Laboratory, Department of Chemical Engineering and Materials Science, Yuan Ze University, Chungli, Taoyuan 320, Taiwan; kee@cyberjaya.edu.my

⁴ UCSI-Cheras Low Carbon Innovation Hub Research Consortium, UCSI University, Kuala Lumpur 56000, Malaysia; grraceng@cyberjaya.edu.my

⁵ Faculty of Pharmaceutical Sciences, UCSI University, Kuala Lumpur 56000, Malaysia; chewyl@ucsiuniversity.edu.my

⁶ M. Kandiah Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Jalan Sungai Long, Bandar Sungai Long, Kajang 43000, Malaysia; leesiewkeah@utar.edu.my

⁷ Department of Microbiology, Dr. N.G.P. Arts and Science College, Coimbatore 641048, India; mahenthiran@drngpasc.ac.in

⁸ School of Pharmacy, KPJ Healthcare University, Lot PT 17010 PersiaranSeriemas, Kota Seriemas, Nilai 71800, Malaysia

* Correspondence: tcsiang@kpju.edu.my (C.S.T.); liewkaibin@cyberjaya.edu.my (K.B.L.)



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Abstract: Many beneficial proteins have limited natural availability, which often restricts their supply and thereby reduces their potential for therapeutic or industrial usage. The advent of recombinant DNA (rDNA) technology enables the utilization of different microbes as surrogate hosts to facilitate the production of these proteins. This microbial technology continues to evolve and integrate with modern innovations to develop more effective approaches for increasing the production of recombinant biopharmaceuticals. These strategies encompass fermentation technology, metabolic engineering, the deployment of strong promoters, novel vector elements such as inducers and enhancers, protein tags, secretion signals, synthetic biology, high-throughput devices for cloning, and process screening. This appraisal commences with a general overview regarding the manufacture of recombinant proteins by microbes and the production of biopharmaceuticals, their trends towards the development of biopharmaceuticals, and then discusses the approaches adopted for accomplishing this. The design of the upstream process, which also involves host selection, vector design, and promoter design, is a crucial component of production strategies. On the other hand, the downstream process focuses on extraction and purification techniques. Additionally, the review covers the most modern tools and resources, methods for overcoming low expression, the cost of producing biopharmaceuticals in microbes, and readily available recombinant protein products.

Keywords: recombinant protein; rDNA; biopharmaceuticals; expression; vector

1. Introduction

Drug discovery and development are long processes that involve billions in investments. In ancient times, traditional treatments were developed by chance and the accumulation of experience passed down from generation to generation on the use of natural resources such as plants and animal body parts without evidence-based scientific studies. The technique of extraction was often used to extract the ingredients from natural

plants believed to have healing effects [1]. Modern scientific drug discovery research has a long history and can be traced back to around the early 1900s. The trend of research has shifted from discovering small-molecule drugs to big macromolecules. The discovery of new medicine usually starts with the identification of a macromolecule, a dysfunctional signaling pathway, or a molecular mechanism apparently linked to a disease condition (pre-discovery stage) [2].

The human body produces thousands of proteins and enzymes for the normal physiological functions of human beings [3]. Some examples of these functions include the regulation of body temperature, blood glucose levels, cognitive functions, catalysis of biochemical reactions in the body, detoxification of foreign particles by the liver, and signaling agents. The absence or deficiency of these biological proteins due to aging or impairment of organs often leads to the development of severe pathological conditions such as mental disorders, diabetes, impaired blood clotting, and many others [4].

A common way to treat this deficiency is by replacing it by administering the missing proteins into the body so that the concentration of the required protein will reach a clinically significant level for routine biological function. These externally administered proteins can be produced *ex vivo* in biological systems [4]. The therapeutic proteins produced must be of high purity, be able to contribute to full clinical functionalities, have a simple and affordable industrial large-scale manufacturing process, and be free of hazardous contaminants.

The choice of recombinant hosts, procedures, equipment, facilities, and production strategies must be well-planned to ensure a high-quality product, as most of the recombinant proteins are in injectable form, directly affecting human life. Due to the rapid advancement in technology development, various recombinant products (peptides and proteins) are already on the market, with many more potential candidates undergoing clinical trials [5].

The focus of recombinant protein technology is not solely on the production of biosimilar products. Many discussions have focused on producing a more cell- or receptor-targeted recombinant protein with a reduced dose and fewer side effects, increasing potency and targeting efficiency. Such targets can be achieved by modifications in the amino acid sequence, generating fusions between therapeutic proteins and specific peptide ligands or antibodies that interact with particular cell receptors. By incorporating advanced biotechnology to engineer modifications in protein structure, the newer generation of recombinant protein products is more stable and has advantages over plain natural polypeptides [6].

2. Trends in Biopharmaceutical Proteins

The pharmaceutical industry has undergone a groundbreaking transformation with the advent of biopharmaceuticals, offering innovative treatment options for patients grappling with serious ailments. Among these, biopharmaceutical proteins have emerged as a particularly promising class of drugs due to their high specificity and effectiveness. However, their complex nature demands precise characterization of protein structure and activity to ensure safety and efficacy. This article delves into the latest trends in biopharmaceutical proteins and emphasizes the pivotal role of sensitive analytical technologies in their development. We also explore the diverse range of evolving analytical tools that cater to the needs of biopharmaceutical research and development (R&D), underscoring the importance of accurate protein characterization for protein therapeutics.

The study of complex biotherapeutics and their impurities requires advanced analytical technologies that can accurately determine their structure and function. Mass spectrometry, nuclear magnetic resonance spectroscopy, electronic microscopy, and X-ray crystallography are all commonly used techniques for analyzing protein structure. These methods can identify any changes in the protein's structure that may impact its effectiveness or toxicity. X-ray crystallography provides valuable insights into protein function and interactions by revealing their three-dimensional structures at high resolution. Additionally, chromatography, capillary electrophoresis, and immunoassays are crucial analytical tools for detecting and measuring impurities in biopharmaceutical proteins. Electronic

microscopy techniques provide information on the morphology, size, shape, and structure of the targeted compound. Overall, sensitive analytical technologies are crucial for ensuring the safety and efficacy of biopharmaceutical proteins.

Biopharmaceutical proteins require input from various fields, such as protein chemistry, bioinformatics, and structural biology. As the sector expands, so do the analytical tools available for research and development (R&D), which must cater to diverse and growing demands. Innovative solutions, such as mass spectrometry, have allowed the detection and measurement of numerous post-translational modifications in proteins [6]. As the demands of biopharmaceutical research and development continue to expand, there is an increasing need for innovative analytical techniques. One promising method includes the use of hydrogen-deuterium exchange mass spectrometry and native mass spectrometry as tools for studying protein dynamics and interactions. Additionally, novel technologies such as microfluidics, single-molecule imaging, and cryo-electron microscopy are also being developed to keep up with the evolving industry demands. These diverse and evolving analytical tools are essential for advancing the development of biopharmaceutical proteins [7].

A growing trend in the pharmaceutical industry is the use of biopharmaceutical proteins, which are highly specific and effective. To achieve success in biopharmaceutical development, it is crucial to accurately characterize proteins. Complex and costly, the development of biopharmaceutical proteins requires extensive optimization and characterization of the protein structure. Nonetheless, the advantages offered by biopharmaceutical proteins make the effort worthwhile. Soon, the protein therapeutics market is predicted to grow significantly, with estimates reaching \$217.5 billion by 2025, according to a recent report by Grand View Research [8]. Biotechnological innovations and the skyrocketing incidence of chronic diseases are the primary drivers behind this projection. Moreover, pharmaceutical enterprises and biotech startups are teaming up more frequently in the quest to develop superior protein therapeutics. For instance, Amgen is collaborating with Generate Biomedicines to create protein therapeutics for clinical targets. These collaborations are essential for advancing the development of biopharmaceutical proteins and improving patient outcomes [8].

Driven by their high specificity and effectiveness in treating debilitating diseases, biopharmaceutical proteins are experiencing a surge in popularity. Understanding complex biotherapeutics and impurities is key, with sensitive analytical technologies taking center stage. Analytical tools are evolving to cater to the growing demands of biopharmaceutical R&D. Success in biopharmaceutical development hinges on precise protein characterization, leading to an increase in collaborative efforts between pharmaceutical companies and biotech startups. To advance the development and efficacy of biopharmaceutical proteins, it will be crucial to create novel analytical tools as the field of biopharmaceuticals continues to grow. This is vital for improving patient outcomes and navigating such an evolving landscape.

3. Recombinant Pharmaceutical Protein Production in Microbes

The biotechnology era was kickstarted by the successful expression of human insulin in *Escherichia coli* [9]. Since Humulin was approved by the FDA in 1982 as the first recombinant protein therapy, the technology has made great strides, with market share currently valued at \$400 billion [10]. Proteins are a versatile class of macromolecules with functions such as biochemical catalysts and molecule transporters, as well as forming receptors and structural molecules, making them a precious source of therapeutics [11]. Proteins also play a prominent role in the flow of genetic information as the major products and prime modulators of gene expression [12].

Recombinant DNA technology has unlocked the vast possibilities of producing existing and novel proteins through heterologous expression in both prokaryotic and eukaryotic hosts. It refers to the process of manufacturing biomolecules using genetically engineered cells. In essence, any protein can be expressed if the gene sequence is known. It is estimated

that humans have between 25,000 and 40,000 unique genes coded in their genomes [11]. Adding to that the infinite potential of engineering novel proteins, our arsenal for producing new therapeutics would continue to expand.

Recombinant protein production starts with the cloning of the target gene into a vector that would be inserted into the expression host, followed by upstream bioprocessing, and subsequently the downstream process to purify and formulate into end products [13]. Each phase has its own set of considerations to be optimized before embarking on industrial-scale production to ensure maximum efficiency in terms of costs, labor, and environmental impact [14].

3.1. Microbial Host Selection

Various hosts of prokaryote and eukaryote origin have been explored in the search for a viable expression of human proteins, including bacteria, yeasts, insect cells, mammalian cells, transgenic plants, and transgenic animals [15]. In any of the expression systems, two major areas of consideration are related to the manufacturing process and the expression host. In terms of manufacturing, important issues to ponder include bioprocess optimization and operation, production capability and capacity, cost, and regulatory requirements [16]. In host selection, the compatibility of the source gene and host, the properties of the expressed protein, and inherent host characteristics should be considered [16].

Determining the best host system that fits specific aims is regarded as a critical decision. Key differences among the expression hosts include protein yield and productivity, mechanisms for post-translational modifications, and most importantly, the capability to produce proteins that retain the desired features [14]. The main considerations for the expression of recombinant proteins are the vector and the bacterial strain [17]. A survey of the literature crowns *E. coli* as the most popular expression host, followed by the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* [18]. Microbial hosts, such as bacteria and yeasts, reign supreme due to extensive research in the area that enables fast development of recombinant organisms, efficient culture conditions, and a simple downstream process [19]. Prokaryotic systems are efficient workhorses for non-glycosylated proteins, but eukaryotic systems should be considered for proteins that require post-translational modifications (PTMs) [20].

3.1.1. *Escherichia coli*

Since the success of expressing human insulin, *E. coli* has been widely used to express recombinant products, such as therapeutic enzymes and proteins [21]. In 2012, a study reported that 30% of therapeutic products produced in heterologous systems originate from *E. coli*. Therefore, the current number is likely to be higher. This is largely due to its superior growth kinetics that shorten the time required to achieve high cell density, accessible and cost-effective media requirements for fermentation, and a swift bacterial transformation process that could take less than 10 min [22].

E. coli genetics and biology are thoroughly understood, easing the manipulation and development of recombinant clones [21]. However, challenges in using *E. coli* as a host include the lack of PTMs, the formation of inclusion bodies (IB), low expression levels or inactive proteins due to codon bias, and the presence of pyrogens [15]. Various strains have been developed to overcome these challenges, such as AD494 (derived from *E. coli* K-12), which has the capability to support disulfide bond formation in the proteins expressed in the cytoplasm, and Rosetta (derived from *E. coli* BL21), which addresses the low expression of proteins made up of *E. coli* rare codons [18].

Protein expression in *E. coli* can be in the form of secretory proteins, soluble proteins, and inclusion bodies (IBs) consisting of intracellular protein aggregates that are insoluble and inactive [21]. Ideally, proteins should be secreted extracellularly or into the periplasmic space, but as some strains are deficient in secretory mechanisms, IBs are formed instead [21]. Proteins in IBs need to be extracted out, solubilized, and refolded in separate processes, causing a loss of 75 to 85% of the recovered protein [17]. In an effort to overcome the

formation of IBs, fermentation can be set at lower temperatures, and the growth conditions can be manipulated by adding sugars and modifying the pH [16].

3.1.2. Yeasts

Yeasts such as *Saccharomyces cerevisiae*, *Pichia Pastoris*, and *Yarrowia lipolytica* have been developed as recombinant protein expression hosts to produce organic compounds such as sterols and aromatics, as well as therapeutic proteins including insulin and interferons [19]. Yeasts have surpassed prokaryotic systems as they are non-pathogenic, easy to scale up, and possess the capability to correctly fold proteins and perform PTMs [23].

The genome of the baker's yeast, *S. cerevisiae*, was the first complete eukaryotic sequence to be published [24]. A thorough understanding of its genome makes it a valuable host for the expression of recombinant proteins because genetic manipulations can be carried out with ease [15]. Proteins are also expressed at high levels extracellularly, which makes purification easier [21]. Nevertheless, high expression of recombinant protein causes intracellular accumulation that negatively impacts product yield and triggers cellular stress in yeasts [15]. There is still limited uptake of *S. cerevisiae* to produce therapeutic proteins because the products are unstable and have reduced efficiency due to the hypermannosylation of proteins [23].

3.1.3. Other Hosts

Food-grade and widely accepted as safe, *Lactococcus lactis* is the perfect bacterial host for generating recombinant proteins in the pharmaceutical industry for therapeutic applications. The ability of *Lactobacillus lactis* to secrete stable recombinant proteins into the growth medium with few proteases, resulting in a properly folded, full-length protein, its gram-positive nature, preventing the presence of contaminating endotoxins, and its rapid growth to high cell densities, which facilitate large-scale fermentation, represent a few of its most important features [25]. Similarly, *Bacillus subtilis* is a well-known expression host that offers advantages, including a high degree of genetic tractability, non-toxicity, an economic growing medium, secretion capabilities, and a vast availability of genetic tools like promoter systems, shuttle vectors, and signal peptides [26,27]. When considering yeast as hosts for recombinant protein production, in addition to *S. cerevisiae* and *Pichia pastoris*, several non-conventional yeast species like *Hansenula polymorpha*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis* have been developed as substitute hosts for the synthesis of heterologous proteins [28].

Besides the aforementioned organisms, several other microorganisms have been emerging as promising hosts for recombinant protein production. The filamentous fungus *Myceliophthora thermophila* has altered the morphology of its cells, leading to lower viscosity during fermentation and consequently facilitating its scalability [29]. *Corynebacterium glutamicum* is a well-established host for industrial-level applications that has recently been associated with recombinant protein production with the advent of compatible expression constructs and secretion capabilities [30]. Microalgae are promising hosts for recombinant protein production, particularly due to their comparatively high growth rates and phototropic lifestyle, making them solar-powered and, therefore, potentially cost-effective. Both the single-cell green algae *Chlamydomonas reinhardtii* and the diatom *Phaeodactylum tricorutum* exhibit promising secretion levels and the ability to manufacture functional antibodies [31,32].

3.2. Vector and Promoter Systems

Plasmid vectors are typically used for the formation of recombinant clones. The anatomy of an expression vector typically consists of a replicon with one origin of replication and control elements, a promoter, an operator, multiple cloning sites, and a selection marker [17]. Commercially available vectors include the pET, pUC, and pQE vector series [22]. Important considerations when selecting the vector include the copy number and plasmid compatibility because they impact the organism and resulting protein yields [22].

Promoters are regulatory sequences that dictate whether a gene is expressed and are key elements to ensure the efficient expression of proteins [16]. Ideal promoters should have the capacity to ramp up production of recombinant products until 30% of the protein composition in the cell and should be easily controlled using inhibitors or activators to avoid toxicity [16]. Common promoter systems are taken from bacteria, such as lac, tac, and trc, and also from bacteriophages such as T7, T5, and SP6 systems [17].

Promoter sequences from *E. coli* are numerous because it is a preferred expression host. Optimum promoters should be strong, easily implantable into other *E. coli* hosts, have a simple and cost-effective induction mechanism, be unaffected by culture media components, and exhibit low expression in the absence of inducers [18]. Leaky expression due to the low level of lac promoter repressor protein LacI is circumvented by engineering the lacI gene into lacI^Q to increase expression by 10-fold [22]. Expression of the bacterial promoter's lac, tac, and trc is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and governed by catabolite repression [18]. Catabolite repression hampers expression in the presence of carbon sources such as glucose in the culture due to the lack of the promoter inducer, adenosine monophosphate (cAMP), which would only be produced in a low glucose environment [22]. To enable protein production in media containing glucose, the lacUV5 promoter was developed and integrated into the pUC plasmid series [17]. Both lac and lacUV5 are considered weak promoters, so stronger promoters such as tac and trc can be used to achieve high levels of total cell protein [18].

The T7 RNA polymerase (RNAP) is a widely used system that incorporates the target gene into a plasmid that contains the T7 RNAP promoter while having the T7 polymerase generated by λDE3 under the regulation of the lacUV5 promoter [22]. The T7 RNAP system has high activity and can generate up to 50% of the total protein in the cell [22]. However, the high transcription rate negatively impacts the host and may lead to cell death [17]. This leaky expression can be countered by increasing the glucose level in the medium, using lacI^Q, and including T7 lysozyme to inhibit T7 RNAP activity [17].

The yeast expression system can facilitate post-translation modification, protein folding and secretion, proper disulfide bond formation, higher protein yield, regulated gene expression, and reduced endotoxin contamination. Some of the most widely employed yeast-based promoter systems include GAL1, ADH2, TEF1, MET25/MET3, PGK1, and AOX1. GAL1 is a tightly regulated promoter, with induction facilitated through the presence of galactose, thereby allowing controlled and tunable expression of the targeted gene. These are the most frequently employed inducible promoters in *S. cerevisiae*. They exhibit very rigorous regulation; in the presence of glucose, they are substantially obstructed, whereas in the presence of galactose, their activation intensity is significantly enhanced [33]. pADH2 is derived from alcohol dehydrogenase II of *S. cerevisiae*, which is also a widely used inducible promoter. When glucose is present, pADH2 expression is strongly inhibited, and it begins to activate when glucose is depleted. The TEF1 is a constitutive promoter that exhibits a combination of regulatory elements, ensuring constant transcription in both fermentative and non-fermentative growth conditions. Similarly, the translation elongation factor EF-1 α (TEF1) has a promoter, ensuring approximately stable expression during all growth phases and in media containing different carbon sources [34]. The promoter MET25 elicits moderate levels of expression in complete media, whereas if methionine is absent, its activity is derepressed, leading to high levels of protein expression [35]. This system could be employed in the production of proteins with specific requirements related to sulfur-containing amino acids. The PGK1 (Phosphoglycerate Kinase 1) promoter is often used for constitutive and moderate expression. It can be suitable for proteins that do not require extremely high expression levels. The AOX1 promoter in *Pichia pastoris* is methanol-inducible, making it suitable for controlled expression. It is often employed when high expression levels are needed. Most of the expression vectors for *P. pastoris* use the inducible promoter from the alcohol oxidase gene (AOX1), which codes for the first enzyme in the methanol utilization pathway [36].

4. Upstream Process Development

The upstream process includes cell line development, clone selection, and bioprocessing. Fermentation requirements for *E. coli* are extensively documented, which makes it easily bred on a lab and industrial scale. It is necessary to confirm that the cells to be used in bioprocessing will produce proteins of the desired quality and quantity, as this would be the benchmark to measure the performance of commercial manufacturing.

Bioprocess development would commence by performing the fermentation on a small scale using test tubes and shaking flasks to confirm the optimum growth of the microbe before bioreactor fermentation [15]. Key areas to be considered when fine-tuning the bioreactor culture are optimization of media, fermentation system, and process parameters [15]. Media has been demonstrated to impact cell metabolism and protein yield as it influences cell transcription and translation [21]. Fermentation systems such as bioreactor tanks or single-use bioreactors also need a suitable mode to be chosen, such as batch, fed batch, or continuous fermentation, that would ensure maximum returns in terms of cost and efficiency [21]. Essential parameters that should be focused on to ensure sustainable production of recombinant proteins on a commercial scale include pH, temperature, dissolved oxygen, carbon dioxide concentration, aeration, agitation of the slurry, and the shear force that would result from it [16,37].

Numerous production processes have been developed as a result of yeast's acceptance as a desirable host for the synthesis of recombinant proteins. The primary factors influencing process performance in yeast include temperature, shear stress, pH, pressure, substrate gradient, and dissolved oxygen. Furthermore, there are several process intensification strategies that intend to improve performance and streamline resource management to address techno-economic constraints and augment industrial feasibility. The strategies encompass feed (growth rate controlled, mixed substrate, and intermittent feeding), oxygen (lowering growth rate, increasing total air pressure, and hypoxic conditions), continuous cultivation, lowering process temperature, lowering process pH, and strain selection and development [37].

5. Downstream Process Development

The downstream process is known as the process of purifying and obtaining the drug substance from a natural source. The main purpose of the downstream process is to obtain a drug product with desired purity, efficacy, and a reasonable cost per unit [38]. This phase is a critical step in ensuring that the drug candidate is free from any impurities and produced sufficiently from the extraction. Depending on the nature of the production, specific methods and techniques are required [39]. When the target biomolecule is produced extracellularly, the culture medium is concentrated before purification transpires. If the desired biomolecule is generated inside the cells, however, it is crucial to extract the cells, lyse them, and then remove any residual debris.

Additionally, a recombinant protein requires multiple purification steps and analytical methods to ensure that impurities such as cell proteins, nucleic acids, endotoxins, and viral particles are completely eliminated [38].

The downstream process involves several steps that are crucial for the purification and isolation of the protein of interest. The downstream process can be separated into three different stages: extraction, purification, and polishing (Figure 1). The extraction process involves the capture of the product of interest from the cells, followed by intermediate purification that removes the impurities using methods such as centrifugation, filtration, and chromatography. And it finally ends with polishing the materials from contaminants and impurities [15].

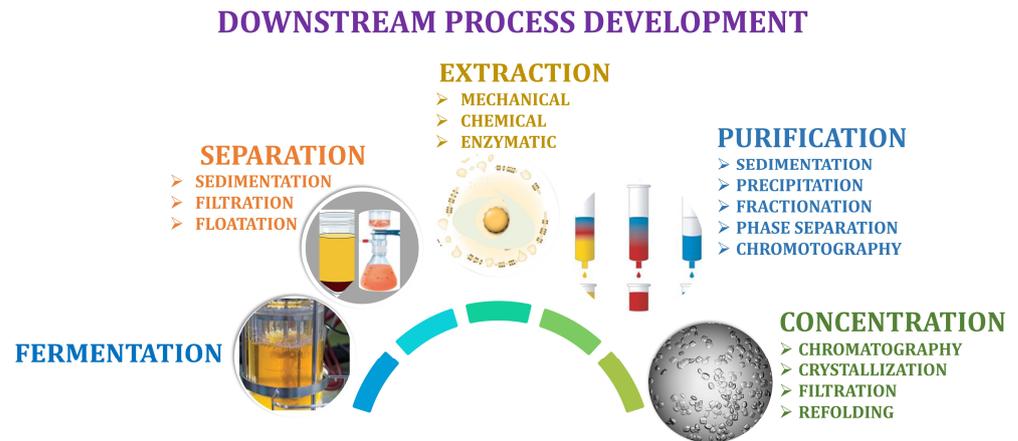


Figure 1. Flowchart of the purification of recombinant proteins.

5.1. Extraction

Typically, the desired recombinant protein is obtained through the fermentation of microbial cells. Depending on the requirements of the protein and the microbial system, various techniques can be used to separate the protein from the culture medium. Techniques such as centrifugation, sedimentation, flotation, microfiltration, and depth filtration are widely used for this purpose.

Once harvested, the microbial cells will lyse the cell wall to extract the protein content. The extraction of proteins can be achieved by three different mechanisms: mechanical, chemical, and enzymatic treatment. Mechanical methods such as bead milling, heat shock, osmotic shock, impingement, and high-pressure homogenization physically break up the cell wall to release the desired protein content. For bead milling, the cell wall is disrupted by mechanical agitation with the aid of glass, ceramic, or metal beads. The FastPrep[®] system, a technology that was introduced, combines multiple processes such as grinding, homogenization, and shear forces that slice the sample apart to expose the protein extract. This method is rapid and may reduce the risk of product contamination. Not only that, but this method also provides the ability to control the temperature, as the energy release may affect the sample and damage the protein [40]. Mechanical methods are known to be effective for large-scale production. However, some factors, such as cell type, sample volume, equipment availability, and the protein profile, need to be considered when using this method. Mechanical methods can be complicated, as they may release cell debris and cellular components into the extract, which then requires more steps to purify the protein. The force applied during the process might affect the protein by denaturing or degrading it [41].

The chemical methods involve the use of alkalis, organic solvents, or detergents. Alkalis can be used to recover intracellular products if the target molecule that is expressed is alkali-stable. Comparably, other organic solvents are employed for the same function, and these solvents possess the ability to remove phospholipids from the cell membrane, thereby reducing the membrane's integrity and resulting in cell lysis. Ionic detergents are also utilized in cell lysing procedures; they work by denaturing membrane proteins to facilitate their solubilization and membrane extraction [42].

Enzymatic methods such as lysozyme, glucanase, mannanase, and proteases provide an effective and selective approach for extracting the protein. Due to its chemical and biological instability, the protein is very sensitive and easily lost during production. For this reason, a high-efficiency process is required to reduce the loss of the protein. When using this method, the determination of the enzyme depends on the type of cell, its composition, and the desired protein. Since lysozyme acts on the β -(1–4) glycosidic links that are present in the peptidoglycan layer, it is more effective against gram-positive organisms than gram-negative ones [43]. Enzymatic degradation of the yeast cell wall is accomplished by combining the enzymatic activity of glucanases and proteases [44]. In an experiment

to extract protein content from Spent brewer's yeast, a combination of enzymatic and high-pressure homogenization methods was utilized. The process begins with the pre-treatment of the yeast with β -glucanase as the enzyme followed by lysing it through a homogenizer [45].

5.2. Purification

Protein purification is a critical step in the downstream process of recombinant proteins. The main objective of these steps is to ensure that the protein obtained from the extraction process is free from any impurities or contaminants. There are several methods available for the purification process; however, the choice is dependent on the specific protein and its properties. The process optimization will determine the purity, yield, and functionality of the recombinant protein. After extracting the protein from the microbes and before initiating the purification, a clarification process needs to be carried out first. The purpose of this process is to remove insoluble cell debris, precipitates, and large particles. The subjection of cellular lysate to centrifugation at a lower speed facilitates the removal of cellular debris alongside unbroken cells, while an enhanced speed enables the removal of ribosomal materials and other particulates [46]. Alternatively, ammonium sulfate or polyethylene glycol fractionation [47], phase partitioning [48], and membrane filtration techniques [49] can be applied to clarify the substrate.

Chromatography is a technique used to purify recombinant protein-based biopharmaceuticals. The commonly used chromatography techniques for purification purposes are affinity, ion exchange, hydrophobic interaction, and size exclusion or gel filtration chromatography. This step is important to obtain high-purity proteins without the host cells and other impurities [50]. Although the production of high-concentration proteins is desired in the upstream process, this will lead to a higher concentration of other host cell proteins (HCP). As a result, this will add a burden to the chromatography work because it will lead to a higher volume of chromatography resin and a higher buffer requirement. HCPs are the main source of impurities, and the HCPs of each process vary significantly from each other in their molecular mass, charge, hydrophobicity, and structure. Efforts need to be put into reducing unwanted HCP production. Cell lines producing a lower amount of HCP should be used during upstream process development to make the purification process easier [51].

Affinity chromatography (AC) is one of the most commonly used techniques for the purification of proteins, peptides, and viral vectors [52]. Some of the commonly used affinity tags are hexa histidine (His), glutathione S-transferase (GST), and maltose-binding protein (MBP) [53]. ProteinA chromatography is the method of choice to purify mAb [54]. However, the limitation of this method is the issue of leachability, with non-specific binding of host cell proteins, DNA, and other cell culture-derived impurities. Other chromatography methods are required to recover these impurities [55].

Purification of recombinant proteins often involves the use of ion exchange chromatography (IEC). IEC uses the concept of cation and anion exchange to remove unwanted impurities such as product variants, remaining HCP and DNA, media components, leached Protein A, endotoxins, and viruses [56]. Another study reports that cation exchange chromatography (CEX) is able to remove viruses during the manufacture of mAbs. Hydrophobic interaction chromatography (HIC) is a commonly used technique as a polishing step to purify recombinant proteins. The concept of HIC is that the binding of proteins to ligands occurs at high ionic strength, whereas the opposite occurs at low ionic strength, where the elution of proteins happens [57].

Size exclusion chromatography (SEC) or gel filtration chromatography is also used in protein purification. The principle of separation is based on molecular weight. SEC has been reported to be used to purify scFv and insulin-like growth factor receptors [58] and for aggregate removal and desalting [59].

Membrane-based chromatography, a chromatography with a specific ligand attached to microfiltration membrane pores, is a newer type of technique used for purification.

The separation occurs when the impurities present bind to the membrane at neutral to slightly basic pH and low conductivity. Parameters that affect the binding affinities include membrane size distribution, thickness, and flow distribution [60]. Recently, nanofibers have been used as membranes to improve the system affinity [52].

5.3. Product Concentration

The final stage of downstream processing entails concentrating the product and producing the final formulation, along with removing any remaining contaminants, charge variations, and misfolded isoforms of the target biomolecule. The steps involved here include hydrophobic interaction chromatography (HIC), simulated moving bed (SMB) chromatography, crystallization, single-pass and high-performance tangential flow filtration, continuous refolding, and cascade diafiltration. The recombinant proteins that exhibit subtle variations in their hydrophobic properties are purified through the HIC technique. It is the most widely used polishing method for monoclonal antibody purification, as the process often tends to easily retain the aggregates on it [61]. The SMB strategy is seldom adopted for purification of the recombinant protein as the method is utilized to separate biomolecules that exist as different enantiomers, which is very rare in biotechnology products [62]. Crystallization of the recombinant proteins is a way to obtain highly purified molecules as they are folded and arranged in a regular lattice pattern, thereby facilitating their ease of selection from the misfolded aggregates [63]. The formation of proteins as inclusion bodies mandates their refolding, since their purification would have been accomplished through denaturation strategies. The refolding of such proteins can be realized through dialysis against refolding buffer or through expanded bed chromatographic techniques [64]. The diafiltration method carried is out to achieve the removal of salts that are added during different stages of purification through a continuous mode operation strategy performed by using a cascade of membrane set-ups that are arranged in a countercurrent manner and is known as cascade diafiltration [65].

The ultrafiltration technique has been employed to purify protein-based drugs [66]. An ultrafiltration membrane is used to desalt the recombinant proteins [67]. Another study reported the use of filter aid (diatomaceous earth) coupled with crossflow ultrafiltration to eliminate unwanted contaminant proteins and DNA molecules [68]. A study focusing on the purification of conjugated vaccine products using ultrafiltration was also conducted. In this ultrafiltration process, a cascade of ultrafiltration steps is followed by a sterile filtration step. This approach was applied in the purification of influenza VLPs, achieving approximately 80% recoveries [69].

6. Strategies to Overcome Low or No Expression

The primary requirement for recombinant protein expression is to derive a high quantity of soluble products of the bacterial cell. The recombinant protein expression can be influenced by several factors, such as medium compositions, choice of expression hosts, fusion tags, rate of protein synthesis, point of induction, concentration of inducer, promoter strength, and copy number of the expression vector [70]. These factors often result in recombinant proteins being compartmentalized into insoluble inclusion bodies or lead to inefficient protein translocation as well as generating a metabolic burden on the host bacteria [71]. Several strategies can be employed to overcome the challenges associated with expression inadequacy.

The optimum medium composition plays a significant role in the soluble expression of the target protein. There are several media constituents that have a substantial influence, such as salts, peptone, and yeast. Furthermore, the addition of prosthetic groups or cofactors are essential to the proper folding and stability of the expressed proteins [72,73]. The production of recombinant L-asparaginase through effective nutrient combinations demonstrated the significance of medium optimization. The study was elaboratively devised to assess different nutrient constituents, such as carbohydrates, amino acids, and salts, that enhance L-asparaginase production, especially in their soluble forms [74]. Another

study reveals an 18.6-fold increase in the extracellular production of the recombinant protein and alkaline phosphatase upon inclusion of sucrose, glycine, and triton x in the growth medium [75]. Therefore, medium composition optimization plays a significant role in recombinant protein expression.

Amid the presence of various expression hosts at disposal for pharmaceutical protein production, *E. coli* stands out as the most widely researched organism. There are numerous *E. coli*-based expression hosts at the disposal for commercial purposes, each with its own distinct attributes. Some of these features include the facilitation of cytoplasmic disulfide bond formation, the expression of membrane proteins, the presence of proteins with rare codons, proteins with increased stability, and toxic protein expression capacity [76]. The microbial hosts other than *E. coli* are yeasts, which constitutes about 20% of the total biopharmaceutical protein share [77]. The yeast *Pichia pastoris* is extensively used for recombinant protein production due to the presence of several beneficial traits, such as the cultivation of high cell density in bioreactors, the occurrence of robust and regulated promoters, and the capacity to secrete large quantities of heterologous proteins [78]. Another widely and routinely used yeast is *Saccharomyces cerevisiae*, which can yield soluble cytosolic recombinant proteins, facilitate post-translational modifications for eukaryotic proteins, etc. [79]. Therefore, the appropriate selection of the host organism is of paramount significance for the proficient expression of the target protein.

The foremost factor that could influence the recovery of the desired protein is its soluble form, which could be facilitated through the incorporation of fusion tags in the desired gene of interest. Apart from imparting solubility functions, fusion tags could also assist during purification and detection. Moreover, recent developments have led to the creation of fusion tags with additional properties such as improvised yield generation and the promotion of proper folding in the desired protein [80]. However, the choice of the fusion tag should be exclusively based on the target protein, as their presence would have a larger influence on the protein's native state interaction, cellular localization, post-translational modifications, and solubility nature [81].

Insoluble recombinant proteins often tend to form inclusion bodies when they reach a certain concentration level. This scenario can be regulated by controlling the protein synthesis rate by monitoring the time of induction, temperature and duration of induction, inducer concentration, and other miscellaneous factors. The time of induction determines the density of cells present in the fermentation medium, which could facilitate the soluble expression of the recombinant proteins. The predominant studies have undertaken induction of the recombinant protein at the early mid-log phase; however, some reports also suggest induction during the late-log phase or sometimes even during the stationary phase. Furthermore, the duration of induction and the incubation temperature post-induction also play a prominent role in efficient recombinant protein production. The protein synthesis rate can be reduced through the maintenance of a lower post-induction temperature, which in turn prevents the formation of inclusion bodies [82]. Higher temperatures favor the aggregation reaction due to the high temperature dependence of the hydrophobic interactions involved in protein aggregation [83]. Some recombinant proteins necessitate prolonged induction duration for proper expression, and these conditions further demand incubation at a lower temperature post-induction. Nevertheless, the most appropriate combination of the post-induction temperature and duration depends on the protein being expressed and needs to be optimized accordingly.

The inducer concentration in the fermentation medium also plays a vital role in the expression of recombinant proteins. A lower concentration of the inducer may sometimes lead to inefficient induction, resulting in a lower recombinant protein yield. Conversely, excessive inducer concentration may cause reduced cell growth due to its toxic effects, which again results in a reduced yield [84]. Therefore, the concentration of the inducer is generally maintained above the critical concentration, i.e., the concentration below which the recombinant protein yield tends to become a function of the inducer concentration [85].

Thus, the inducer concentration also plays a crucial role in recombinant protein production and sufficient yield generation.

The strength of the promoter system employed plays a crucial role in regulating the metabolic burden within the host cell. The most routinely employed promoter systems are lac-derived ones, such as P_{T7lac} , P_{tac} , and P_{trc} , that work based on the negative regulation by lacI, wherein the expression is induced in the presence of lactose or its analogue molecule (isopropyl β -D-1-thiogalactopyranoside). The bacteriophage t7 RNA polymerase present in some *E. coli* strains, such as BL21 (DE3), transcribes the gene regulated by the P_{T7} promoter system, and this RNA polymerase is considered five times faster than that of the regular *E. coli* RNA polymerase. Hence, P_{T7} is regarded as one of the strong promoter systems. Likewise, P_{tac} and P_{trc} are deemed strong promoter systems. Therefore, the promoter system strength is also often a significant factor in recombinant protein production [86].

The origin of replication is another chief component that influences the cell metabolic burden, as it is accountable for maintaining the copy number of the expression vector [18]. Based on this, vectors are categorized into low copy numbers (10 copies/cell), as exhibited by p15A plasmids, while medium copy numbers (15–20 copies/cell) and high copy numbers (500–700 copies/cell) are observed in some of the pMB1 derivatives [86].

To overcome the low expression of recombinant proteins in yeast, well-characterized constitutive or inducible promoters with robust transcriptional activity have been developed. In *S. cerevisiae*, high-level expression of heterologous genes has often been regulated by the strong constitutive promoters of TEF1 and GPD (glyceraldehyde 3-phosphate dehydrogenase, also known as TDH3). The generation of misfolded proteins, which cluster within the cells and are unable to secrete effectively into the medium, has proven to be the main disadvantage of such constitutive systems, though. Therefore, the utility of inducible promoters becomes indispensable in such scenarios, which enables the regulation of gene expression through the concentration of the inducer molecule. *S. cerevisiae* utilizes GAL1 and GAL 10 promoters for such purposes. The methylotrophic yeast *P. pastoris* often relies on its substantial methanol-inducible promoter system, pAOX1, for such purposes. The yeast *Y. lipolytica* employs a strong XPR2 alkaline extracellular protease promoter pXPR2 system. The EYK1 promoter system, which can be activated by erythritol or erythrulose, is another mechanism used by the same organism to significantly increase the recombinant protein yield [87].

7. Economics of Microbial Production of Biopharmaceuticals

The biopharmaceutical industry is a multibillion-dollar enterprise encompassing research, development, manufacturing, and marketing sectors. Especially when the past two years were engulfed in the worst pandemic of over a century, the biopharmaceutical industry played a pivotal role in swiftly controlling the situation through both COVID-19 vaccine development as well as other therapeutics.

However, a closer look at the problems ailing the industry for the past few decades will shed light on its declining productivity within the R&D division, thereby leading to increased product costs [88]. The economics of any drug development should take into consideration the costs involved in R&D, clinical trials, process development, and manufacturing activities, along with the durations, resources involved, and success rates. The development of new biopharmaceutical products involves exploratory discovery research to identify effective lead molecules with potential applications. Amongst numerous molecules, the one exhibiting prominent action is selected for further product development. Thereafter, this potential candidate molecule undergoes pre-clinical studies to establish its safety and effectiveness. Following this, the developer must seek approval from the regulatory authorities to commence clinical studies on human patients. Upon successful completion, the data pertaining to all the safety, efficacy, and dosage collated through pre-clinical and clinical studies are utilized to obtain market entry approval from the regulatory authorities. Therefore, the economics of the final product include the process development cost, clinical manufacturing cost, process performance qualification cost, and clinical trial

cost per phase [89]. Each of these stages in biopharmaceutical development plays a significant role in the proficient and cost-effective production of the protein, which is critical to sustaining its economic viability. Thus, it is imperative to note that the price of the pharmaceutical proteins does not reflect the cost of goods (CoG) employed for production but is rather a reflection of the fiscal obligations associated with R&D, patent limitations, clinical trials, marketing, and the return of these investments [90].

At every stage of biopharmaceutical development, efficient and cost-effective protein production is critically important to maintaining the economic viability of both the product and the company developing it. This is often evident from their cost in comparison to industrial-use recombinant proteins, wherein the former has a retail price of a billion dollars per kg, while the latter often costs around tens of dollars for the same quantity. The biopharmaceuticals manufactured through microbial hosts are expected to be of considerably lower prices compared to the other modes of production. However, the pharmaceutical companies are inclined to spend nearly a higher proportion on their product marketing, such that sometimes it even amounts to half or equal to that of the product's R&D cost [91]. Additionally, the manufacturers also incur liability costs at around 2% of their total revenue [92]. In the year 2016, the highest biopharmaceutical sales were attributed to adalimumab (sold as Humira pen), which constituted sales of about 16.5 billion USD.

Data retrieved from Le Merie and Fierce Pharma financial report suggest total global sales of over 343 billion US dollars for the year 2021. This includes recombinant monoclonal antibody sales to the tune of 217.3 billion USD, other recombinant protein proportions are about 53.6 billion USD; COVID vaccines comprise around 54.5 billion USD; 11.6 billion USD of biosimilars; and 6.8 billion USD of nucleic acid and engineered cell based. The top-selling biopharmaceutical product for the year 2021 was Comirnaty, the mRNA-based COVID-19 vaccine sold by Pfizer & BioNTech for about 36.8 billion USD, which is followed by Humira (adalimumab), sold by AbbVie & Eisai for about 21.2 billion USD (PhRMA Annual Membership Survey, 2018).

The revenue estimates for recombinant proteins in the global market for the period from 2023 to 2035 are expected to be about 9 billion USD. The market has already generated revenue of around 2 billion USD in the year 2023. This growth is driven by the ever-increasing demand for biopharmaceuticals due to the consistent and rising prevalence of chronic diseases worldwide (Recombinant Proteins Market Analysis by Host Cell, 2023). According to Coherent market insights recombinant market analysis published on January 2023, the recombinant protein market was globally valued at 2808.4 million USD in the year 2022 and is projected to grow at 11.5% during the forecast period of 2022–2023 (Recombinant protein market analysis by Coherent market insights, 2023). As per the bioengineered protein drugs global market report 2023, the growth is forecasted to range from about 349.20 billion USD in 2022 to 379.36 billion USD in 2023 and is expected to reach 514.7 billion USD in 2027 at a growth rate of 7.9%.

8. Latest Technologies in Recombinant Products

Recombinant DNA technology is playing a vital role in improving health conditions by developing new vaccines and pharmaceuticals. Treatment strategies are also enhanced through the development of diagnostic kits, monitoring devices, and new therapeutic approaches. The most recent developments in protein engineering techniques have transformed the possibility for drug developers and manufacturers to precisely customize and maximize the essential functional features of target proteins while maintaining and, in some cases, enhancing their safety, efficacy, or both. Several protein-engineering approaches are currently utilized to achieve enhanced production yield and purity, improvised targeting capability, increased circulating half-life, and novel therapeutic modes of action [93]. The growth of genetic engineering, along with our growing knowledge of cell physiology and stress, is crucial to this advancement. Genetic resources and methods have changed significantly since the advent of genetic engineering, moving from a limited number of DNA modification methods and scarce genetic materials (plasmids, genome sequences, etc.) to

much more controlled, widespread, and high-throughput gene manipulation methods and widespread commercial applications. Custom recombinant proteins can be produced using this diversified set of synthetic promoters, fusion partners, signal sequences, and strains in conjunction with bioprocess development and downstream procedures. The widely used cell-based systems in biopharma for the manufacture of proteins include bacterial, yeast, and mammalian cell lines.

The limitations of the conventional techniques used for producing recombinant proteins have been resolved by recent technological advancements. The formation of endotoxins is a key obstacle in the *Escherichia coli* expression system; however, recently, an endotoxin-free strain of *E. coli* has been discovered. The development of breakthrough technologies in *E. coli* systems allowed for the synthesis of difficult-to-express sophisticated products such as full-length glycosylated monoclonal antibodies in considerable quantities, tiny peptides, and antibody fragments [21]. Numerous new strategies for producing recombinant proteins in microorganisms have been developed and are currently under investigation. The most significant ones, such as genome-scale metabolic engineering, co-expression of chaperones and folding catalysts, advanced promoter and expression systems, ribosome engineering, continuous fermentation processes, synthetic biology, and CRISPR-Cas9-based gene editing, are discussed below.

The potential for producing recombinant proteins in bacteria has been significantly improved owing to the CRISPR Cas 9 technology. Difficult-to-express proteins, autolytic proteins, membrane proteins, and antimicrobial peptides can be produced by changing the T7 RNAP ribosomal binding site sequences in a genome and generating a BL21(DE3) variant host strain library with different T7 RNAP ribosomal binding site sequences with improvised translation levels. Such a system has shown a 298-fold increase in productivity compared to the parent strain [94].

The use of double promoter expression systems is a potential strategy for enhancing the production of heterologous proteins. Two approaches could be adopted for this method: (i) double promoter systems that operate sequentially, and (ii) double promoter systems that operate concurrently. Among these metabolic design techniques, increasing the transcriptional activity with two promoters activated under analogous conditions inside the production domain or extending the expression time with two promoters active under different conditions can be implemented independently of the host [95].

Molecular chaperones are often co-expressed with the recombinant proteins to support the proper folding of proteins [96]. Heat shock proteins (Hsps) are a significant group of molecular chaperones involved in stress resistance that are typically employed as expression partners to collaborate and process unfolded or aggregated proteins [22]. The application of mRNA engineering techniques can facilitate the coupling of translation and folding activity with spatial constraints to stimulate the generation of functionally active soluble proteins. Such a strategy is a cost-effective way to increase the prospect of solubilizing recombinant proteins that are prone to aggregation, giving conventional chaperone systems more strength and minimizing their dependency on ineffective posttranslational processes [97].

High-cell-density cultures in the fed-batch mode have traditionally been used for industrial heterologous protein synthesis. However, contemporary trends are moving towards continuous operation in the fed-batch mode, as evident from the recombinant lipase B production in *P. pastoris* under the constitutive promoter PGK. In fact, after six weeks, it was determined that continuous mode's lipase B production had been 5.8 times larger than that of the fed-batch operation [98]. Therefore, continuous biomanufacturing provides the opportunity for a time-independent process, increasing the time-space-yield of the recombinantly generated protein and further lowering production costs. The productivity of a continuous cultivation approach for recombinant inclusion body production in *E. coli* BL21(DE3) was shown to be significantly increased by a factor of 100 by using two stirred tank reactors to spatially segregate biomass formation from recombinant protein production [99]. Thus, continuous processing during upstream cultivation is a promising and

practical manufacturing strategy in biopharmaceutical production. Recent advancements and innovations in continuous processing have demonstrated notable improvements in perfusion feeding strategy, seeding production bioreactor, adapting available media, host cell engineering, and reaching high cell density [100].

As systems and synthetic biology have advanced, functional protein expression has undergone a significant change; specifically, data-driven systems biology has provided a knowledge-based framework on which to logically design, construct, and test biological entities in an increasingly complex manner [101]. This has made characterization of the bacterial host and recombinant proteins at the system level substantially easier, as the massive influx of high-throughput data on DNA, RNA, proteins, and metabolites has made it possible to visualize the overall transcriptional and translational process landscape [102,103]. The rapidly emerging technologies in DNA synthesis and assembling techniques have made the design and evaluation of biological systems more facile. The whole process was effective due to the availability of reliable computational tools and *in silico* models that streamlined extensive analysis and prediction of cellular networks [104,105]. Such novel strategies are expected to minimize the ambiguous effects of unidentified genetic components and reduce metabolic interference from native metabolic pathways while promoting more accurate regulation of cellular functions. This would contribute further to the enhancement of contemporary microbial cell factory efficiency. The construction of minimum genomes has similarities to the efforts to repurpose cells for specific tasks, such as genome recoding to speed up the production of synthetic polypeptides. This concept of minimal genomes and their potential implications in multiple areas of application, including the synthesis of heterologous proteins, were made tangible through the combination of synthetic and systems biology. The advantages of genome-reduced microbial strains exceeding their wild-type counterparts in terms of production of target proteins and desirable biomolecules have been emphasized by an increasing number of reports [106,107]. These reports suggest that in a target microbial cell, precisely envisioned genome reductions have constantly resulted in enhanced cellular properties such as genomic stability, growth rate, biomass, protein productivity, and genetic versatility (more receptive to genetic engineering).

Revolutionary Technologies for Recombinant Protein Production

A bioluminescent tagging technology based on peptides that allows for the monitoring of tagged proteins *in situ* and *in vitro*. In a continuous-culture parallel bioreactor system, the bioluminescent peptide tagging technology enables monitoring of recombinant proteins and offers an intriguing possibility to study the kinetics of recombinant protein generation in response to a variety of stimuli. This technology accurately counts recombinant bacteria from the mouse gastrointestinal tract, with numbers comparable to traditional plate counts and more sensitive than commercially available immunoassays [108]. Recombinant therapeutic proteins could be locally delivered to the gastrointestinal system via genetically modified microorganisms known as live biotherapeutic products (LBPs). The *in vivo* pharmacokinetics of the engineered fungi and the secreted biologic could be modulated by using one of three different tunable delivery strategies: (i) adjusting the oral dose of the engineered fungi; (ii) co-administering antibiotics; or (iii) regulating the titer of recombinant protein secretion [109].

An unconventional method for developing proteins that are challenging to generate is recombinant protein synthesis that takes place within the biofilms. An enhanced technique for producing recombinant proteins in biofilms shows prospects comparable to the traditional planktonic method, but with further benefits including reduced carbon source concentration for growth and reduced antibiotic requirements. This was established through the studies on the marine bacterium *Pseudoalteromonas haloplanktis* TAC125 from the Antarctic region employed to produce the fluorescent proteins GFP and mScarlet [110]. Cold atmospheric pressure plasma, or CAP, has been characterized as a unique approach that could be useful in enhancing the generation of recombinant proteins. By influencing cellular components and modulating the expression of specific stress genes, an essentially

stressful environment created with the non-lethal dosages of CAP could have a substantial effect on the synthesis of recombinant proteins. This was substantiated through real-time analysis of *Pichia pastoris* after CAP exposure, which demonstrated the increased production of recombinant enhanced green fluorescent protein (eGFP) as well as the other genes corresponding to the oxidative stress response [111].

An empirical approach for standardizing all the factors would require a lot of costly, time-consuming, multivariable examinations, which is seldom feasible. Machine learning methods are considered extremely promising alternatives, particularly in scenarios that frequently evolve and necessitate the ability to regularly adjust to new process conditions. This was illustrated by predicting the real-time optical density of *E. coli* during the generation of neurotrophin recombinant protein using a black-box machine learning (ML) approach based on recurrent neural networks (RNN) and long short-term memory (LSTM) neural networks [112].

9. Marketed Recombinant Microbial Products

The development of recombinant DNA technology for expression of the desired proteins in simple microbial systems such as *E. coli* has unlocked a completely new venture for the biotechnology industry. The first division within the biotechnology industry to grasp this upcoming and promising opportunity was the pharmaceutical sector. Humulin, a recombinant variant of human insulin developed by Eli Lilly & Co. (Indianapolis, IN, USA), is considered the first biopharmaceutical to find its way to the market after FDA approval in the year 1982 [113]. Followed by this, other natural proteins synthesized via the recombinant route received FDA approval in the 1980s. This included hormones, cytokines, and antibodies that were produced by different hosts. Among the recombinant alternatives that entered the market during this time period, six out of the nine were produced by microorganisms, and another five were from *E. coli*. Since then, the commercial trajectory of the recombinant proteins has been on a constant rise.

The success of insulin gene cloning and production has paved the way for new avenues within recombinant DNA technology to be explored. Different techniques were carried out to impart the desired attributes to the protein. For example, site-directed mutagenesis of the key amino acids in insulin protein was used to accomplish beneficial characteristics such as preferred pharmacokinetics, absorption level, peak, and duration of action. This has set the stage for the arrival of more products in the commercial market, such as aspart, detemir, glargine, glulisine, lispro, etc., [114].

Besides the pharmaceutical industry, recombinant proteins have also encroached upon other sectors, such as food and dairy, cosmetics, animal feed, and additive industries. These sectors too have several microbial recombinant proteins on their sleeves with various tailored applications. The several pharmaceutical products derived from bacteria, yeast, and other microorganisms are summarized in Table 1.

Table 1. Recombinant biopharmaceuticals manufactured with microbial hosts.

rDNA	Trade Name	Cell Factory	Application	Company	Reference
Insulin	Humulin	<i>E. coli</i>	Diabetes	Eli Lilly & Co., Indianapolis, IN, USA	[39]
Growth hormone	Protropin	<i>E. coli</i>	Pituitary dwarfism	Genentech, San Francisco, CA, USA	[39]
Interferon	Intron A	<i>E. coli</i>	Hairy cell leukemia	Schering-Plough Corporation, Kenilworth, NJ, USA	[39]
Hepatitis B Vaccine	Recombinax	<i>S. cerevisiae</i>	Hepatitis	Merck & Co., Rahway, NJ, USA	[39]
Interferon alpha 2a	Roferon A	<i>E. coli</i>	Chronic myeloid leukemia	Roche, Basel, Switzerland	[39]

Table 1. Cont.

rDNA	Trade Name	Cell Factory	Application	Company	Reference
Granulocyte Colony Stimulating Factor	rG-CSF	<i>E. coli</i>	Chemotherapy-induced neutropenia	Amgen, Fairfield, NJ, USA	[93]
Granulocyte Macrophage Colony Stimulating Factor	rGM-CSF	<i>S. cerevisiae</i>	Chemotherapy-induced neutropenia	Sanofi, Paris, France	[93]
IFN-gamma protein	IFN-gamma protein	<i>E. coli</i>	Chronic granulomatous disease and Severe malignant osteopetrosis	Genentech, San Francisco, CA, USA	[115]
Pegylated interferon alpha-2a	Pegasys	<i>E. coli</i>	Hepatitis C	Genentech, San Francisco, CA, USA	[116]
Teriparatide	Forteo	<i>E. coli</i>	Osteoporosis	Eli Lilly & Co., Indianapolis, IN, USA	[117]
Somatotropin	Humatrope	<i>E. coli</i>	Growth hormone	Eli Lilly & Co., Indianapolis, IN, USA	[39]
Filgrastim	Neupogen	<i>E. coli</i>	Neutropenia	Amgen Inc., Fairfield, NJ, USA	[39]
PEG-Filgrastim	Neulasta	<i>E. coli</i>	Neutropenia	Amgen Inc., Fairfield, NJ, USA	[39]
rIL-2-diphtheria toxin	Ontak	<i>E. coli</i>	Cancer	Eisai Inc., Tokyo, Japan	[118]
Asparaginase	Erwinaze	<i>E. coli</i>	Cancer	EUSA Pharma, Hertford, UK	[119]
Glucarpidase	Voraxase	<i>E. coli</i>	Nephrology, Kidney failure	BTG International Inc., Conshohocken, PA, USA	[93]
Metreleptin	Myalept	<i>E. coli</i>	Lipodystrophy	Amylin Pharmaceutical, San Diego, CA, USA	[93]
Albiglutide	Tanzeum	<i>S. cerevisiae</i>	Diabetes	GlaxoSmithKline, GlaxoSmithKline, Middlesex, UK	[93]
Insulin glargine	Lantus	<i>E. coli</i>	Insulin receptor agonist	Sanofi-aventis, Paris, France	[39]
Endostatin	Endostar	<i>E. coli</i>	Lung cancer, Colorectal cancer	Simcere, Nanjing, China	[39]
Aldesleukin	Proleukin	<i>E. coli</i>	Metastatic renal cell carcinoma, metastatic melanoma	Novartis, Basel, Switzerland	[39]
Interferon alfa-2b	Intron-A	<i>E. coli</i>	Chronic hepatitis C, hairy cell leukemia, Behcet's disease etc.	Schering-Plough Corporation, Kenilworth, NJ, USA	[39]
Interferon gamma-1a	Actimmune	<i>E. coli</i>	Kidney cancer, sezary syndrome, mycosis fungoides	Genentech, San Francisco, CA, USA	[39]
Tasonermin	Beromun	<i>E. coli</i>	Soft tissue sarcoma	Boehringer Ingelheim, Ingelheim am Rhein, Shanghai, China	[39]
Granulocyte macrophage colony-stimulating factor	Molgramostim	<i>E. coli</i>	Myelodysplastic syndrome	Swiss Pharma Pvt Ltd., Gujarat, India	[39]
Nartograstim	Neu-up	<i>E. coli</i>	Solid tumor	Yakult Honsha, Tokyo, Japan	[39]
Palifermin	Kepivance	<i>E. coli</i>	Metastatic renal cell carcinoma, metastatic melanoma	Biovitrum, Stockholm, Sweden	[39]

Table 1. Cont.

rDNA	Trade Name	Cell Factory	Application	Company	Reference
Sargramostim	Leukine	<i>S. cerevisiae</i>	Acute myelocyticleukaemia	Bayer HealthCare Pharmaceuticals, Stockholm, Sweden	[39]
L1 capsid protein	Gardasil	<i>S. cerevisiae</i>	Papillomavirus vaccine	Merck & Co., Stockholm, Sweden	[120]
ORF2 protein	Hecolin	<i>E. coli</i>	Hepatitis E virus vaccine	Xiamen Innovax Biotech, Xiamen, China	[120]
2 fHbp variants	Trumenba	<i>E. coli</i>	<i>Neisseria meningitidis</i> serogrup B vaccine	Wyeth Pharmaceuticals, Inc., Collegeville, PA, USA	[120]
HBsAg + RTS chimera	Mosquirix	<i>S. cerevisiae</i>	Malaria vaccine	GlaxoSmithKline, Middlesex, UK	[120]
OspA & C chimeric	Vanguard crLyme	<i>E. coli</i>	<i>Borrelia burgdorferi</i> vaccine	Zoetis, Parsippany-Troy Hills, NJ, USA	[120]
Chimeric Protein Q	Letifend	<i>E. coli</i>	Leishmania vaccine	Zoetis, Parsippany-Troy Hills, NJ, USA	[120]
P45 env. antigen	Leucogen	<i>E. coli</i>	Feline leukemia virus vaccine	Kalbio Global Medika, Kalbio Global Medika, Bekasi Regency, Indonesia	[120]
CCE, mEq84, IdeE	Strangvac	<i>E. coli</i>	<i>Streptococcus equi</i> vaccine	Dechra Veterinary Products, Northwich, UK	[120]
Detemir Insulin	Levemir	<i>S. cerevisiae</i>	Diabetes	Novo Nordisk, Bagsvarerd, Denmark	[121]
Glucarpidase	Voraxaze	<i>E. coli</i>	Treatment of patients at risk of methotrexate toxicity	BTG Specialty Pharmaceuticals, Conshohocken, PA, USA	[93]
Parathyroid hormone	Natpara	<i>E. coli</i>	Treatment of hypoparathyroidism	Takeda Pharmaceuticals, Tokyo, Japan	[93]
Somatropin	Omnitrope	<i>E. coli</i>	Treatment of growth hormone deficiency	Sandoz, Basel, Switzerland	[122]
Semaglutide	Wegovy	<i>S. cerevisiae</i>	Weight loss and weight control	Novo Nordisk Novo Nordisk, Bagsvarerd, Denmark	[123]
Vosoritide	Voxzogo	<i>E. coli</i>	Achondroplasia	BioMarin Pharmaceutical, Novato, CA, USA	[124]
Liraglutide	Saxenda	<i>S. cerevisiae</i>	Obesity	Novo Nordisk, Bagsvarerd, Denmark	[125]
Nesiritide	Natrecor	<i>E. coli</i>	Acutely decompensated congestive heart failure	Johnson & Johnson, New Brunswick, NJ, USA	[126]
Anakinra	Kineret	<i>E. coli</i>	Rheumatoid arthritis	Swedish Orphan Biovitrum, Biovitrum, Stockholm, Sweden	[127]
Becaplermin	Regranex	<i>S. cerevisiae</i>	Lower-extremity diabetic neuropathic ulcers	Johnson & Johnson, New Brunswick, NJ, USA	[128]
Insulin	Insugen	<i>P. pastoris</i>	Diabetes therapy	Biocon, Karnataka, India	[129]

Table 1. Cont.

rDNA	Trade Name	Cell Factory	Application	Company	Reference
Ecallantide	Kalbitor	<i>P. pastoris</i>	Hereditary angioedema	Dyax, Lexington, MA, USA	[129]
Human serum albumin	Medway	<i>P. pastoris</i>	Blood volume expansion	Mitsubishi Tanabe Pharma, Osaka, Japan	[129]
IFN- α 2b	Shanferon	<i>P. pastoris</i>	Hepatitis C	Sanofi, Paris, France	[129]
Ocriplasmin	Jetrea	<i>P. pastoris</i>	Vitreomacular adhesion	ThromboGenics, Iselin, NJ, USA	[129]
HBV vaccine	Hepavax-Gene	<i>H. polymorpha</i>	Hepatitis B	Rhein Biotech, Maastricht, Netherlands	[129]
Pancrelipase	Creon	<i>Y. lipolytica</i>	Exocrine pancreatic insufficiency	Aptalis Pharma, Bridgewater, NJ, USA	[129]

10. Conclusions

Recombinant protein expression in microbial systems is advancing with no signs of slowing down. The development of numerous novel expression technologies, including promoters, modified hosts, secretion signals, and process optimization, has made it possible to create gram-scale amounts of proteins swiftly and efficiently. The ability to produce “high-yielding” and “cost-effective” therapeutic and nontherapeutic products that are crucial to industry is made possible by the effective implementation of innovative technologies. We have studied current advancements in recombinant protein expression made possible by implementing the latest developments like ribosome engineering, innovative excretion and secretion systems, synthetic biology, and systems engineering. The review has thoroughly considered the current approaches for producing recombinant proteins, their limitations, recent advancements that will enhance productivity, their financial repercussions, as well as the recombinant pharmaceuticals that are currently in the market.

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