Review Article

International Journal of Indigenous Herbs and Drugs
Content Available at www.saap.org.in ISSN: 2456-7345

PLASMIDS FOR EFFICIENT PRODUCTION OF RECOMBINANT PROTEINS IN E. COLI

Arrolla Lekha[1], Kanigiri Deepthi Sri[2], Mohammed Azharuddin[3], Kona Venkata Sri Krishna[4]

[1] Sreenidhi Institute of Science and Technology, Hyderabad, TS, India
[2] K L University, Vijayawada, AP, India
[3] Vignan University, Guntur, AP, India
[4] Jawaharlal Nehru Technological University, Hyderabad, TS, India

Article Info

Article History
Received on: 02-07-2023
Revised on: 15-07-2023
Accepted on: 04-08-2023

Abstract

Plasmids play a crucial role in the transmission of genetic information across different types of bacteria. Plasmids transfer pathogenicity and survival genes to the host bacterium, allowing the bacteria to adapt to new environments and grow. Several plasmids of differing sizes have been recovered from numerous bacterial species. Plasmids may be used to genetically modify bacteria for a variety of reasons, including the generation of recombinant proteins. Escherichia coli is the most often utilised bacterium for the production of recombinant proteins owing to its quick growth rate, low cost, high yield of recombinant proteins, and simple scale-up procedure. To boost the synthesis of heterologous proteins in E. coli, a number of plasmids have been used. Diverse plasmids have been devised and built to address challenges such as protein refolding, E. coli codon use, a lack of post-translational modifications such as glycosylation, and insufficient recovery of functionally viable recombinant proteins. Recent technical advancements that have made it possible for the E. coli expression system to create more complex proteins, such as glycosylated recombinant proteins and therapeutic antibodies.

Keywords: pathogenicity, bacteria, E. coli.

***Corresponding Author**
Kona Venkata Sri Krishna[1]

DOI: https://doi.org/10.46956/ijihd.v8i4.469

Production and Hosted By
www.saap.org.in

Introduction

Plasmids are circular extrachromosomal DNAs that can reproduce in a cell independently of chromosomal DNA. Plasmids are present in a variety of species, including bacteria, archaea, and even yeast(1). The complete sequences of approximately 4,600 plasmids have been established due to technological advancements in DNA sequencing. It is 4,418 for bacteria, 137 for archaea, and 47 for eukaryotes. Plasmids range in size from 1 kb to 200 kb and often include genes that create proteins that offer host cells with an advantage under adverse conditions(1). This collection contains genes for antibiotic resistance. Plasmids also serve as a central storage for the genes required to manufacture antibiotics and other poisons. Multiple virulence factors, encoded by genes on plasmids, aid pathogens in entering hosts and avoiding their defences. Additionally, plasmids include genes for bacterial nitrogen fixation(2). A single bacterial cell may have anywhere between one and many hundred plasmids. The average number of plasmids in a single cell is governed by the size of the plasmid and the control of replication initiation. In a given bacteria, large plasmids are often present in a single or limited number of copies. These single-copy plasmids employ the partition system, which consists of the parABS and parMRC systems, to distribute a copy of the plasmid to each daughter cell during cell division(3). Plasmids need an origin of replication in their DNA to replicate independently. The word "replicon" refers to any self-replicating unit. A typical bacterial replicon has a gene encoding a plasmid-specific replication initiation protein (Rep), a DnaA box, an AT-rich region, and iterons, which are repeating units. Smaller plasmids rely on the host's replication machinery to replicate, but larger plasmids possess all the necessary genes to replicate independently(3).

Conjugation is a method for transferring plasmids from one bacterium to another; around 14% of all known plasmids fall into this category. Conjugation is a very

SOUTH ASIAN ACADEMIC PUBLICATIONS

effective mechanism for transferring genes across microorganisms, allowing for fast growth and adaptability to a wide range of challenging environments(4). This unique mechanism of horizontal gene transfer among bacteria may be traced back to antibiotic resistance in dangerous diseases. In genetic engineering, plasmids are often utilised as vectors for the cloning and expression of target genes. It is now possible to clone and express foreign genes in a range of hosts, including E. coli, yeast, and mammalian cells, due to the availability of plasmids in a variety of commercially available forms. A appropriate plasmid carrying the required genes is employed for cloning and expressing genes(5). Plasmid vectors include a multiple cloning site (MCS) or polylinker site intended for the cloning of heterologous genes. The multiple cloning site may have a large number of restriction sites characteristic for Type III restriction enzymes. These plasmid vectors are capable of replication in a bacterial host because they include a replication origin. These plasmids also include a gene that gives resistance to a particular antibiotic, often ampicillin or kanamycin, which is then utilised as a selectable marker(6). Transformation is used to transfer plasmids into bacterial cells once the relevant heterologous gene has been inserted into the multiple cloning site of the plasmid vector. Cells that have been converted are exposed to further selective growth conditions of medium coupled with the antibiotic. Due to the presence of an antibiotic resistance gene, cells with the implanted plasmid will be able to survive and multiply under selective circumstances. This method generates vast quantities of recombinant proteins in E. coli for application in therapeutics and a broad range of functional research. This chapter summarises recent advances in molecular biology technology that have allowed the E. coli expression system to create more complicated proteins, such as glycosylated recombinant proteins and therapeutic antibodies(7).

Recombinant proteins production using E. coli as an expression system

Escherichia coli is often employed as an expression system for mass-producing recombinant proteins because to its adaptability, efficiency, and low cost. The DNA of E. coli has been widely investigated, making the organism simple to manipulate and alter. The rapid growth rate, low-cost culture conditions, high expression levels, and straightforward scale-up method significantly facilitate the large-scale manufacture of recombinant proteins for pharmaceutical applications or functional study(8). Successfully creating recombinant human insulin for diabetic patients using E. coli. Because insulin is a heterodimer, its active 3D structure requires oxidative protein folding. The high-level expression of recombinant human insulin established the usefulness of the E. coli expression system for protein manufacturing at industrial scale. Human growth hormone, interferon alpha 2a and alpha 2b, glucagon, urate oxidase, granulocyte colony-stimulating factor, and parathyroid hormone are among the recombinant proteins that have been effectively produced for medical uses utilising the E. coli expression technique(9). Although E. coli has been widely utilised for heterologous protein expression, it is currently not feasible to determine the optimal production conditions for each protein. The optimal settings for one protein's expression may not be the finest for another. Differences in codon use between the two species hinder E. coli's ability to synthesise heterologous proteins. This variation in codon use may hinder the production of recombinant proteins by causing translational mistakes. Protein expression may be affected by the promoters used, the growth circumstances, and the hydrophobicity of the proteins themselves(10).

Role of Promoter

The synthesis of heterologous proteins requires the use of plasmid vectors, and the promoter region is an essential component of these vectors. The transcription of a gene begins at the transcription start point, also called the promoter. Typically, the length of a promoter is between 100 and 1000 base pairs. The Pribnow Box (consisting of 10 nucleotides) and the promoter region (consisting of 6 nucleotides) are required for promoter entry in E. coli and other bacterial species. Prior to the commencement of transcription, there are 35 nucleotides. TATAAT and TTGACA are the consensus sequences for the -10 and -35 nt regions, respectively(11). RNA polymerase recognises this sequence, and transcription begins. Producing heterologous proteins in E. coli is facilitated by the availability of several plasmids with either a strong or weak promoter. The T7 promoter, taken from bacteriophage T7, the lac promoter from E. coli and its modified form lacUV5, and the Tac promoter, generated by splicing the trp and lac promoters together, are all extensively used promoters in E. coli expression vectors. The trp and lacUV5 promoters are precursors of the essential trc promoter. Promoter strength is governed by the frequency of transcription start, which is dictated by RNA polymerase's affinity for the promoter sequence(12). The T7 promoter is widely employed for extraordinarily high levels of recombinant protein synthesis owing to its high frequency of transcription start and outstanding processivity(13). Unfortunately, low yields of physiologically functional recombinant proteins have been connected to the creation of solid clumps known as inclusion bodies, which may emerge during large-scale protein synthesis. Using an expression vector with a weak promoter, such as the trc promoter, may enhance protein solubility in certain instances(14). Plasmid-based protein synthesis places an excessive burden on the host cell's machinery; hence, promoter engineering research has been conducted to seek remedies. In one study, a strong Ptac promoter was turned into a weak Ptic promoter by the addition of two amino acids between the -10 and -35 regions, therefore increasing the distance between the two sites. In the case of the Ptic promoter, we determined that expression was decreased by around 35%, leading to less

intracellular accumulation, decreased Fab leakage, and enhanced cell survival. In this instance, Fab expression was lowered from 0.32 mg Fab/g cell weight/h to 0.23 mg Fab/g cell weight/h relative to the wild type, and cell mortality was reduced from 18.1% to 11.1% towards the end of fermentation. This demonstrated the great effectiveness of the modified plasmid in producing periplasmic Fab(15).

Ribosomal binding site to improve translation efficiency

Site number four for ribosome binding incorporating a ribosome binding site into the expression plasmid is essential for the efficient synthesis of recombinant proteins in E. coli. This sequence consists of the Shine-Dalgarno (SD) sequence and the translation start codon AUG. The Shine-Dalgarno (SD) consensus sequence AAGGAGG is present 7-9 nucleotides before the initiation codon(16). It is known that the secondary structure of the ribosomal binding site, the consensus SD sequence, the substitution of thymine and adenine, and the nucleotides before and after the AUG start codon all influence translation initiation(13). Translation efficiency is improved by increasing the quantity of adenine and thymine at the ribosomal binding site. Adenine often follows the start codon in highly expressed genes. Using PCR-based site-directed mutagenesis, Park and colleagues constructed many variants of the 5′ untranslated region (UTR), including the SD sequence and the AU-rich region, and analysed their effect on protein synthesis levels(17). This method of altering the 5' untranslated region has the potential to increase translation efficiency and generate vast quantities of recombinant protein. Modifications to the 5′ untranslated region may increase the synthesis of heterologous proteins. Alterations in the secondary structures of messenger RNA (mRNA), which may impact the efficiency with which proteins are translated, are an additional significant factor that can contribute to alterations in protein production(18). To degrade the secondary structures of mRNA, you may use the RNA helicase DEAD protein from E. coli. DEAD protein co-expression increased -galactosidase expression from the T7 promoter, indicating that DEAD-box protein is involved in mRNA stabilization.This property of dead-box proteins may boost the expression of genes that are ordinarily underexpressed owing to the secondary structure of mRNA(19). Errors in protein translation may lead to mutations, inappropriate incorporation of amino acids, and low expression levels, all of which diminish the quality of recombinant proteins generated in E. coli.

Table 1: Ribosomal binding site sequences and their associated details for improving translation efficiency in prokaryotes and eukaryotes:

Organism	RBS Sequence	Position from AUG	Strength/ Effectiveness	Notes
Prokaryotes	5'-AGGAGG-3'	6-10 nucleotides	Strong	Canonical Shine-Dalgarno sequence
	Variants of AGGAGG			Different variants may have varying strengths
Eukaryotes	gccRccAUGG	~8 nucleotides	Strong	Kozak sequence; important for efficient initiation
	Variants of gccR			Different variants may have varying strengths

Use of codons and plasmid with tRNA genes

Plasmids harbouring tRNA genes that are cognate to odd codons and codon use patterns. When attempting to generate heterologous proteins in E. coli, especially human proteins, difficulties with codon use often arise. Codon use in E. coli is drastically different from that of humans. In human and other eukaryotic genes, rare codons are common. Due to the occurrence of uncommon codons in heterologous genes, translational mistakes may decrease the amount of recombinant protein generated in E. coli. Ribosomes that stall at rare codons in heterologous genes may cause translational problems. Mistranslations may emerge as frame-shift mutations, amino acid modifications, or premature terminations of translation(20). When generating recombinant proteins, rare E. coli codons such as AGA (arginine), CGG (cystine), AAG (lysine), GGA (glycine), CUA (leucine), AUA (isoleucine), and CCC (proline) may present challenges(21). The E. coli bacterium utilises the unusual arginine codon CGG at a frequency of 0.54%. McNulty and colleagues discovered that when the Herpes simplex virus type 2 (HSV-2) p27 protease domain was synthesised in E. coli, a recombinant protein with a molecular weight 3 kDa larger than its actual molecular weight was created. This was attributed to the occurrence of rare arginine CGG codons. It was discovered that the resulting increase in molecular weight was caused by a +1 frame-shift mutation at a CGG codon near the C-terminus of the viral protein. In addition, CGG was misinterpreted as CAG, resulting in the presence of glutamine residues as opposed to arginine residues(20).

Several approaches have been developed to improve the synthesis of authentic, physiologically active heterologous recombinant proteins by overcoming the codon bias of E. coli. Using codon usage information to finish gene synthesis is one approach, but it is exceedingly expensive. Site-directed mutagenesis of the foreign gene is an additional method for creating codons that match the E. coli tRNA pool. This therapy is impractical, however, due to the required amount of time and

resources. A second strategy is to co-transform E. coli using a plasmid that has the tRNA gene that corresponds to the uncommon codons. To match the codon use frequency of the foreign gene, it is possible to develop E. coli strains with an increased copy number of uncommon tRNA genes(22). This method is particularly practical, economical, and successful for creating heterologous genes with a large number of non-standard codons. McNulty and colleagues co-expressed the argX gene, which yields the proper tRNA for the rare arginine codon CGG, with the HSV-2 p27 protease domain to address the codon bias issue. When the tRNA gene producing the CGG codon was co-expressed, frame-shift mutations and glutamine misincorporation were eliminated, and recombinant protein production increased by as much as sevenfold(20). This research discovered that providing E. coli with additional cognate tRNA for uncommon codons such as CGG might reduce CGG codon bias and improve the quality and efficiency of recombinant protein production. This method is currently often used for a wide range of heterologous recombinant proteins with rare codons that are notoriously difficult to produce in E. coli. Numerous plasmids, including the well-known pRARE plasmid, now include commercially accessible copies of genes encoding for tRNA cognate to rare codons. In addition, these pRARE plasmids have a p15A replication origin, allowing them to survive in the presence of a suitable ColE1 origin of replication, which is found in a large number of E. coli expression vectors. Furthermore, plasmids containing tRNA genes for cognate uncommon codons (DE3) are now commercially available in many E. coli strains, including BL21(DE3) CodonPlus-RIL and Rosetta.Tegel and colleagues observed that the overall yields of 35 out of 68 evaluated recombinant proteins were significantly increased by using E. coli strain Rosetta (DE3) with the pRARE plasmid for protein expression studies(20).

Table 2: Codons and Plasmid with tRNA Genes

Codon	Amino Acid	Anticodon (tRNA)	Plasmid with tRNA Genes
AUG	Methionine	UAC	pMet-tRNA
UUU / UUC	Phenylalanine	GAA	pPhe-tRNA
UUA / UUG	Leucine	CTT	pLeu-tRNA
UCU / UCC / UCA / UCG	Serine	AGA / AGG	pSer-tRNA
UAU / UAC	Tyrosine	GUA	pTyr-tRNA
UGU / UGC	Cysteine	GCA	pCys-tRNA
UGG	Tryptophan	CCA	pTrp-tRNA
UAA / UAG / UGA	Stop	-	pStop-tRNA

Role of molecular chaperones in optimisation of protein folding

A consistent and cost-effective expression system capable of generating soluble heterologous proteins is needed for the production of recombinant proteins for use in therapeutic or other functional studies. Even though the E. coli expression approach is often employed for the creation of recombinant proteins, the buildup of foreign proteins as insoluble clumps, known as inclusion bodies, is a severe issue. Protein recovery is a time-consuming and laborious process since denaturation and renaturation are required to recover folded and soluble proteins from these inclusion bodies. During the extraction procedure, proteins are lost at an alarming rate, thus diminishing the quantity of functional recombinant proteins. One way is to use molecular chaperones to increase the solubility of heterologous proteins and decrease the formation of inclusion bodies. It is now recognised that molecular chaperones inhibit protein aggregation by helping newly synthesised polypeptides fold properly(23). A few molecular chaperones are known to aid in the folding and solubilization of misfolded proteins, whereas others are involved in the prevention of protein aggregation. E. coli utilises many molecular chaperones, including GroEL, GroES, DnaK, DnaJ, and Trigger factor. These cytoplasmic chaperones may be used alone or in conjunction with other chaperones to enhance protein solubility and prevent the formation of inclusion bodies. The GroEL-GroES combo may facilitate both protein refolding and degradation. Trigger factor has been found to increase the efficiency of protein folding by interacting with GroEL and enhancing GroEL-substrate binding(24). Some chaperones, such as the heat shock proteins IpbA and IpbB, inhibit the clumping of heat-denatured proteins. For optimal results in improving the solubility of heterologous recombinant proteins, it is recommended to try many combinations of molecular chaperones. Co-expression of the molecular chaperones Skp and FkpA in E. coli was reported to enhance the solubility of antibody fragments(25). It has been demonstrated that the co-expression of GroEL and GroES chaperones significantly improves anti-B-type natriuretic peptide single-chain antibody (scFv) synthesis, with 65% of the generated protein being in soluble form, which is more than 2.4-fold greater than that achieved without chaperones(26).

It has been shown that complex therapeutic proteins, such as antibody fragments, may be expressed in the periplasm of E. coli. Recombinant proteins fold optimally in a periplasmic environment that is oxidising and rich in Dsb proteins. In addition, since the periplasm includes very few host proteins, the yield of pure recombinant proteins is rather high. Periplasmic expression in E. coli has effectively generated therapeutic antibodies such as Lucentis and Cimzia (Fab fragments), in addition to a small number of full-length aglycosylated antibodies and scFvs(27). Yim and coworkers used an endoxylanase signal peptide to create 4.2 g/l of granulocyte colony-stimulating factor (GCSF) in the periplasm of E. coli(28).

The coordinated expression of periplasmic chaperones might enhance the synthesis of correctly folded proteins in the periplasm of E. coli. When the periplasmic chaperones DsbA and DsbC were overexpressed in E. coli, full-length antibody production rose from 0.10 to 1.05 g/l(29). This was done by enhancing the assembly efficiency of the antibody's heavy chain and light chain in the periplasm. Co-expression of the periplasmic chaperone Skp enhanced both antigen binding and anti-CD20 scFv antibody production(30). By modifying the 5'UTR sequence and co-expressing the periplasmic chaperone DsbC, Lee and colleagues were able to develop a very efficient E. coli production technique for synthesising full-length antibodies. This resulted in a very high production of light and heavy chains and improved periplasmic organisation. These results suggest that E. coli may be manipulated successfully to create complex therapeutic proteins, such as therapeutic monoclonal antibodies(31).

Solubility enhancement by fusion-tagged plasmids

Fusion with a highly soluble protein is a further method of making recombinant proteins more water-soluble. There are several commercially available plasmid vectors that may be used to create fusion proteins. Using fusion tag technology, recombinant proteins may be generated in greater quantities, made more soluble, and purified with greater ease. Fusion tags have become one of the most commonly used techniques for producing heterologous proteins in E. coli. Maltose-binding protein (MBP), glutathione S-transferase (GST), thioredoxin (TRX), N-utilization substance A (NusA), ubiquitin (Ub), small ubiquitin-like modifier (SUMO), and split SUMO are often used fusion tags. In a recent work, Marblestone and colleagues examined the synthesis and solubility of three heterologous proteins containing C-terminal GST, MBP, NusA, Ub, TRX, and SUMO fusion tags. Compared to other fusion tags, TRX and SUMO fusion partners were shown to increase recombinant protein expression levels, while SUMO and NusA fusion partners enhanced recombinant protein solubility(32). Braun and colleagues examined the expression of 32 human proteins with molecular weights ranging from 17 to 110 kDa using different fusion tags and found that GST and MBP fusion tags are especially successful in increasing both expression levels and the total yield of purified recombinant proteins(33). When analysing the creation of 40 distinct heterologous proteins using a variety of fusion tags, separate research discovered that the MBP fusion tag was the one most often used. It was very efficient in enhancing the production and solubility of recombinant proteins. Various fusion tags are effective to varying degrees in enhancing the synthesis and solubility of recombinant proteins, as seen by the discrepancies disclosed by this comparative investigation. This may be due to differences in the amino acid composition, number of disulfide bonds, and hydrophobicity of heterologous proteins. To enhance protein expression and solubility, it is recommended to evaluate the most efficient fusion tag for each desired heterologous protein(33).

Removing fusion tags may affect the functional activity of recombinant proteins, which is a significant drawback of using fusion tags to improve the solubility of heterologous proteins. Site-specific proteases, such as factor Xa, thrombin protease, or SUMO protease, might be used to remove fusion tags because they recognise and cleave at particular locations within the fusion tag and recombinant protein(34). However, the cleavage of fusion tags may restrict the production of recombinant proteins. As a consequence, in order to generate the requisite high yield of genuine and physiologically active recombinant proteins, the most effective fusion tag and cleavage approach should be adopted.

Conclusion

E. coli is preferred over yeast, mammalian cell lines, transgenic plants, and transgenic animals as an expression system for the production of heterologous proteins due to its well-characterized genetics, ease of genetic manipulation, availability of several plasmid vectors and engineered host strains, low manufacturing cost, and high yield of recombinant proteins. Codon bias, problems with protein folding and solubility, and post-translational changes are only a few of the obstacles. There have been several technical advancements made to solve these challenges. Therefore, plasmids like the pRARE plasmid have been designed to carry tRNA genes that are cognate with uncommon codons. If these plasmids were co-transformed into an E. coli host, the number of copies of the uncommon tRNA genes would grow, allowing them to balance the codon use frequency in the heterologous genes. This approach is both more efficient and cost-effective for boosting the expression of heterologous genes with a high number of uncommon codons. Plasmids expressing molecular chaperones such as GroEL, GroES, DnaK, DnaJ, and Trigger Factor are advantageous for improving the solubility and correct folding of recombinant proteins. Utilizing molecular chaperones might prevent the production of inclusion bodies in recombinant proteins. The synthesis and solubility of difficult-to-express recombinant proteins may be improved by using fusion protein tags with a similar structure. Using molecular chaperones to create recombinant proteins in the periplasm of E. coli may result in enhanced solubility, correct protein folding, faster protein purification, and a better yield of authentic and physiologically active recombinant proteins, among other benefits. The effective production of antibodies in the periplasm of E. coli has demonstrated the commercial feasibility of this procedure; licenced medicinal drugs such as Lucentis and Cimzia were generated using this technique. The inability of the E. coli expression system to undertake post-translational changes such as glycosylation restricts its versatility. Complicated glycosylation is used in the production of pharmaceuticals.

Wacker and colleagues found a previously unknown N-linked glycosylation mechanism in Campylobacter jejuni and showed that it could be transferred to E. coli to create a strain capable of generating recombinant glycosylated proteins. These technical advances have shown that E. coli may be genetically altered for each heterologous protein in order to produce a potent and effective pharmaceutical or other physiologically active product. Plasmids are very valuable for genetically changing bacteria for applications including the production of recombinant proteins. Escherichia coli is the selected host organism for recombinant protein synthesis. Diverse plasmids have been used to improve the production efficiency of heterologous proteins in E. coli. As a consequence of breakthroughs in DNA sequencing technology, over 4,600 plasmids have been discovered. Plasmids vary in size from 1 to 200 kilobytes and often include genes that produce proteins that provide host cells with an advantage in adverse conditions. Virulence factors are plasma membrane-produced proteins that aid bacteria in infecting their hosts.

Funding

No Funding

Conflict of Interest

No Conflict of Interest

Inform Consent and Ethical Considerations

Not Applicable

Author Contribution

Al authors are contributed equally.

References

1. Hayes FJEcpv. The function and organization of plasmids. 2003:1-17.
2. Weisberg AJ, Miller M, Ream W, Grünwald NJ, Chang JHJPTotRSB. Diversification of plasmids in a genus of pathogenic and nitrogen-fixing bacteria. 2022;377(1842):20200466.
3. Al-Hejin AM, Bora RS, Ahmed MMMJP. Plasmids for optimizing expression of recombinant proteins in E. coli. 2019;13.
4. Balasubramaniam SJNCN. Opportunistic routing through conjugation in bacteria communication nanonetwork. 2012;3(1):36-45.
5. Fürste JP, Pansegrau W, Frank R, Blöcker H, Scholz P, Bagdasarian M, et al. Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. 1986;48(1):119-31.
6. Hill JE, Myers AM, Koerner T, Tzagoloff AJY. Yeast/E. coli shuttle vectors with multiple unique restriction sites. 1986;2(3):163-7.
7. Verch T, Pan Z-K, Paterson YJI, immunity. Listeria monocytogenes-based antibiotic resistance gene-free antigen delivery system applicable to other bacterial vectors and DNA vaccines. 2004;72(11):6418-25.
8. Chen Q, Lai H, Hurtado J, Stahnke J, Leuzinger K, Dent MJAtib, et al. Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. 2013;1(1).
9. Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde AJMcf. Microbial factories for recombinant pharmaceuticals. 2009;8(1):1-8.
10. Rai M, Padh HJCs. Expression systems for production of heterologous proteins. 2001:1121-8.
11. Rytter JV, Helmark S, Chen J, Lezyk MJ, Solem C, Jensen PRJAm, et al. Synthetic promoter libraries for Corynebacterium glutamicum. 2014;98(6):2617-23.
12. Kaur J, Kumar A, Kaur JJIJoBM. Strategies for optimization of heterologous protein expression in E. coli: Roadblocks and reinforcements. 2018;106:803-22.
13. Schumann W, Ferreira LCSJG, Biology M. Production of recombinant proteins in Escherichia coli. 2004;27:442-53.
14. Jonasson P, Liljeqvist S, Nygren PAk, Ståhl SJB, biochemistry a. Genetic design for facilitated production and recovery of recombinant proteins in Escherichia coli. 2002;35(2):91-105.
15. Schofield DM, Templar A, Newton J, Nesbeth DNJBP. Promoter engineering to optimize recombinant periplasmic Fab′ fragment production in Escherichia coli. 2016;32(4):840-7.
16. Chen H, Bjerknes M, Kumar R, Jay EJNar. Determination of the optimal aligned spacing between the Shine–Dalgarno sequence and the translation initiation codon of Escherichia coli m RNAs. 1994;22(23):4953-7.
17. Park YS, Seo SW, Hwang S, Chu HS, Ahn J-H, Kim T-W, et al. Design of 5′-untranslated region variants for tunable expression in Escherichia coli. 2007;356(1):136-41.
18. Mauger DM, Cabral BJ, Presnyak V, Su SV, Reid DW, Goodman B, et al. mRNA structure regulates protein expression through changes in functional half-life. 2019;116(48):24075-83.
19. Redko Y, Aubert S, Stachowicz A, Lenormand P, Namane A, Darfeuille F, et al. A minimal bacterial RNase J-based degradosome is associated with translating ribosomes. 2013;41(1):288-301.
20. McNulty DE, Claffee BA, Huddleston MJ, Kane JFJPe, purification. Mistranslational errors associated with the rare arginine codon CGG in Escherichia coli. 2003;27(2):365-74.
21. Yarian C, Marszalek M, Sochacka E, Malkiewicz A, Guenther R, Miskiewicz A, et al. Modified Nucleoside Dependent Watson− Crick and Wobble Codon Binding by tRNALysUUU Species. 2000;39(44):13390-5.

22. Liu CC, Cellitti SE, Geierstanger BH, Schultz PGJNp. Efficient expression of tyrosine-sulfated proteins in E. coli using an expanded genetic code. 2009;4(12):1784-9.
23. Carrió MM, Villaverde AJFl. Role of molecular chaperones in inclusion body formation. 2003;537(1-3):215-21.
24. Nishihara K, Kanemori M, Yanagi H, Yura TJA, microbiology e. Overexpression of trigger factor prevents aggregation of recombinant proteins in Escherichia coli. 2000;66(3):884-9.
25. Bothmann H , Plückthun AJJoBC . The periplasmic Escherichia coli peptidylprolyl cis, trans-isomerase FkpA: I. Increased functional expression of antibody fragments with and without cis-prolines. 2000;275(22):17100-5.
26. 26. Baeshen MN, Al-Hejin AM, Bora RS, Ahmed MM, Ramadan HA, Saini KS, et al. Production of biopharmaceuticals in E. coli: current scenario and future perspectives. 2015;25(7):953-62.
27. 27. Huang C-J, Lin H, Yang XJJoIM, Biotechnology. Industrial production of recombinant therapeutics in Escherichia coli and its recent advancements. 2012;39(3):383-99.
28. 28. Yim S, Jeong K, Chang H, Lee SJB, Engineering B. High-level secretory production of human granulocyte-colony stimulating factor by fed-batch culture of recombinant Escherichia coli. 2001;24(4):249-54.
29. 29. Reilly DE, Yansura DG. Production of monoclonal antibodies in E. coli. Current trends in monoclonal antibody development and manufacturing: Springer; 2010. p. 295-308.
30. 30. Wang Q, Chen Y, Park J, Liu X, Hu Y, Wang T, et al. Design and production of bispecific antibodies. 2019;8(3):43.
31. 31. Lee YJ, Lee DH, Jeong KJJAm, biotechnology. Enhanced production of human full-length immunoglobulin G1 in the periplasm of Escherichia coli. 2014;98(3):1237-46.
32. 32. Marblestone JG, Edavettal SC, Lim Y, Lim P, Zuo X, Butt TRJPs. Comparison of SUMO fusion technology with traditional gene fusion systems: enhanced expression and solubility with SUMO. 2006;15(1):182-9.
33. 33. Braun P, Hu Y, Shen B, Halleck A, Koundinya M, Harlow E, et al. Proteome-scale purification of human proteins from bacteria. 2002;99(5):2654-9.
34. 34. Bell MR, Engleka MJ, Malik A, Strickler JEJPS. To fuse or not to fuse: what is your purpose? 2013;22(11):1466-77.