Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(4):1068-1074

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Heterologus Protein Expression in Different Host Systems

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ABSTRACT

The demand for recombinant proteins has steadily increased and the production of such recombinant proteins in heterologous systems has improved significantly in recent years. Most applications involve difficult to produce proteins including complex proteins and glycoproteins thus promoting the development and improvement of a wide range of production platforms. For the production of all recombinant proteins no individual system is optimal, so the diversity of platforms based on different host-vector systems has been developed. Here, we discuss the most commonly used production platforms based on the bacteria, several species of yeast, and a selection of mammalian cell lines production highlighting their unique advantages and disadvantages. The significance of mammalian host system like CHO and its unique advantages and disadvantages are highlighted in this review.

Keywords: protein expression, recombinant proteins, heterologous systems, host systems

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INTRODUCTION

Biotechnology-derived recombinant proteins are responsible for advances in medicine and industrial applications. The new class of drugs derived from recombinant proteins are extremely vital in the treatment of many ailments like cancer, genetic disorders, hormone deficiency and AIDS. The naturally derived biologicals are same or similar to molecules in living cells and hence more compatible with biological systems in comparison to chemical drugs. Industrial uses of enzymes in food, textile, detergent, leather, medicinal chemistry sectors are also increasing. The growing need of therapeutic and other applications of enzymes and proteins could only be met by heterologous synthesis of recombinant proteins [1].

A new generation of biological systems have evolved with the advancement of recombinant protein production by surpassing the challenges through optimising the existing expression systems [2]. Expression systems encompass the technologies including biological materials and the associated methods necessary for genetically modified organisms in the production of recombinant proteins and other products. They include vectors (usually plasmids) used to transfer genetic material into host cells as well as the source and transformed cells themselves. The vectors may be commercial products on their own and in some cases, such as those used for gene therapy or live viral vaccines[3].

Proteins expressed in the mammalian host cells (higher eukaryotes), *Escherichia coli*, (bacterial host cell, prokaryotes) and yeasts (lower eukaryotes) have gained FDA approval for the therapeutic application by using these expression systems. All the expression systems have their own advantages and disadvantages (Table-1). The application of these expression systems mainly depends on the following criteria :(a) The production cost (b) immunogenicity of the proteins (expressed protein from one host may be immunogenic in other system) and (c) the nature of protein (non-glycosylated or glycosylated). This article presents a review of most commonly used vector-host systems for recombinant protein expression and production.

Different host systems

Two well-known general categories of expression systems include Prokaryotic and eukaryotic systems among which the former are generally easier to handle and are satisfactory. However, there are serious restrictions in using prokaryotic cells for the production of eukaryotic proteins. Though many host systems are used in purified proteins (structure, enzymology, drug discovery) and protein therapeutics [1].

Escherichia coli:

Escherichia coli (E. coli) is the most widely employed host due to the extensive knowledge about its physiology, genetics and complete genomic sequence which greatly facilitates gene cloning and cultivation. The modified strains have been genetically manipulated so that they are generally regarded as safe for large-scale use in recombinant protein production [1] .

During the first step of expression screen, only a couple of *E. coli* strains like BL21(DE3) and some derivatives of the K-12 lineage are necessary [4] . The versatility of the system can be demonstrated by large-scale protein expression trials which have shown that <50% of bacterial proteins and <15% of non-bacterial proteins can be expressed in *E. coli* in a soluble form [5].

Majority of the proteins are strategically available as cytoplasmic, periplasmic and secretary proteins, when expressed in *E. coli*. Mammalian proteins when expressed in *E. coli* may often aggregate and form as inclusion bodies which is due to the lack of proper chaperone systems in *E. coli*. [6, 7, 8]. For industrial purposes, the inclusion body formation is also advantageous because of easy separation of these proteins from soluble proteins and are usually resistant to degradation by the proteolytic enzymes from the host [9, 10]. The natural folding and secretion process of immunoglobulins in eukaryotes are imitated in the *E. coli* periplasmic strategy. With the help of special signal sequences pelB [11] , phoA [12] and ompA [13] the protein for example the fragment antigen binding (Fab) region of an antigen can be directed towards and expressed in the periplasmic space where the more oxidising environment is present than cytoplasm. By the simple osmotic shock method the proteins can be extracted from the periplasm of *E. coli* .

Various important advantages include potentially very high expression levels, low cost, simple culture conditions, rapid growth, scalable, simple transformation protocols, and any parameters can be altered to optimise expression. Few disadvantages include inefficient disulfide bond formation, poor folding of proteins in the cytoplasm (inc. bacterial proteins), inclusion body formation, *in vitro* refolding protocols may be inefficient and can negate advantages; codon usage is different to eukaryotes, minimal post-translational modifications and endotoxin production. To overcome the disadvantages newly developed engineered strains can help alleviate the problems with disulfide bond formation (Shuffle and Origami) and codon bias (Rossetta and CodonPlus ril/rp)[14].

Alternative Bacterial Expression system:

The gram positive anaerobic lactic acid bacterium is the emerging platform for the recombinant microbial cell factories as it is considered as the GRAS (generally recognized as safe) organism by the US FDA [15]. Along with the advantages and major economical application in fermentation and food industry scientists are considering the gram positive bacterium as the alternate source for the production of recombinant proteins as these organisms do not produce the endotoxins in its derivatives [15, 16]. *Bacillus subtilis* is one of the most considered alternative host system because of the presence of an excellent secretion system which simplifies the downstream process and secretes the properly folded proteins [17]. *Lactococcus lactis* is yet another promising alternative prokaryotic expression system. *L. lactis* is majorly used in the production of recombinant hypoallergen, biologically active murine interferon-gamma and considered as a new vaccine delivery system as it can target to mucosal level [18, 19, 20].

The strict aerobic gram negative *Pseudomonas* sps with its high GC content made it as an ideal host for the production of few restriction endonucleases and natural GC rich products [21, 22] . *Streptomyces* is the other important source for the production of antibiotic peptides, synthetases and drug modifying enzymes because of its low level extracellular proteases [22].

Yeast expression system:

The enormous increase in gaining interest for the yeast as expression system is due to the fact that it shares the properties of both prokaryotic and eukaryotic expression systems. The well characterized molecular and genetic system of yeasts allows easy gene manipulations, nuclear stable copy number and the availability of strong promoter helps to attain the higher cell densities. Yeasts are used in food and brewery industry from long time and are generally considered as GRAS because of absence of endotoxins in contrary to *E. coli* and Oncogenes or viral DNA in contrary to mammalian system. Yeast expression system can used for the structure and functional analysis of

membrane protein, because of the presence of identical secretory and signaling pathway with that of higher eukaryotes [23].

The non-methylotrophic genera such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* [24], *Kluyveromyces lactis* [25, 26], *Arxula adeninivorans* (*A. adeninivorans*) [27], *Yarrowia lipolytica* (*Y. lipolytica*) [28] and the methylotrophic genera such as *Pichia pastoris* [29, 30] *Ogataea (Hansenula) polymorpha* [31, 32, 33, 34] are mostly used yeasts strains for the heterologus protein production.

S. cerevisiae contains high copy stably-inherited plasmid and is considered as a prototype for almost 25 years because of its economic efficiency and safety considerations [35] . *S. cerevisiae* is an important cell factory as it is used for production of several large volume products including insulin and insulin analogs [36]. *P. pastoris* is used increasingly as the host cell of choice because of its ability to produce high yields of properly-folded proteins in exceptionally high density cultures including tetanus toxin, mouse epidermal growth factor, membrane proteins including human ABC transporters, aquaporins and tetraspanins [37]. The main advantage of using *S. pombe* for gene expression of matured heterologus proteins is due to the presence of well developed golgi apparatus and the presence of galactosyl transferase system [38].

Advantages of this system are high expression levels, low cost, simple culture conditions, relatively rapid growth, scalable, choice of secreted or intracellular expression, efficient protein secretion and allows simple purification, extensive post-translational modification of proteins, efficient protein folding, N-glycosylation more like higher eukaryotes than with *S. cerevisiae*, and endotoxin-free protein production. Disadvantages include use of methanol as inducer is a safety (fire) hazard at scale, glycosylation is still different to mammalian cells [14]. Hypermannosylation which hinders the proper folding of heterologus protein there by raises the immunogenicity is a common feature in yeast [1]. Despite this, genetic advances have been made as highlighted by the development of a "humanised" *P. pastoris* strain capable of replicating the most essential glycosylation pathways found in mammalian cells and permitting the production of active recombinant protein like human erythropoietin [37].

Baculovirus-infected insect cells:

Expression of recombinant proteins in the baculovirus/insect cell expression system is employed because it enables post-translational protein modification and high yields of recombinant protein. The system is capable of facilitating the functional expression of many proteins either secreted or intracellularly located within infected insect cells. Advantages are good expression levels (esp. for intracellular proteins), relatively rapid growth, efficient protein folding, moderately scalable, extensive post-translational modification of proteins, glycosylation more like mammalian cells, enzymatically relatively easy deglycosylate proteins (good for structure determination), and endotoxin-free production [39]. Disadvantages include expensive culture media, large volumes of virus needed on scale-up, inefficient processing of pro-peptides in secretary pathway, glycosylation still different to mammalian cells [40], viral infection leads to cell lysis and potential degradation of expressed proteins [14].

Today Baculovirus technology has matured to the state in which it can be applied for numerous applications. Transduction of a plethora of mammalian cell types in addition to infection of insect cells established itself as a versatile gene delivery and protein expression platform for production of recombinant proteins [39].

Mammalian cells:

The approval of Chinese hamster ovary (CHO)-derived tissue plasminogen activator (tPA, Activase) in 1986 revolutionized medicine and raised the possibility of using mammalian cell culture for the manufacturing of protein therapeutic products. The development of hybridoma technology to produce mAb's by Kohler and Milstein (1975) [41] has revolutionised mammalian cell culture in large quantities in *in vitro*. Transient or stable transfection methods are used based on the purpose of the expressed protein. Transient expression by COS cells and stable transfection by the CHO cells are more frequently used mammalian cells [42, 43, 44] . CHO cells are capable of adapting and growing in suspension culture which is ideal for large scale culture, pose less risk as few human viruses are able to propagate in them, can grow in serum-free and chemically defined media which ensures reproducibility between different batches of cell culture, and allow post translational modifications to recombinant proteins which are compatible and bioactive in humans especially glycosylation of glycoproteins [45].

One of the most important factors for success of CHO cells is that they are quite adaptable and able to grow to very high densities in suspension cultures. Also isolation of cells deficient in DHFR enzyme leads to an effective means for selection of stable clones and amplification of genes [46] . The major advantages include good expression levels, moderately scalable, suspension-adapted cells facilitate scale-up, efficient protein folding, good for secreted proteins, excellent post-translational modifications, and endotoxin-free. Few disadvantages include expensive culture media, complex growth requirements[14].

Few problems with gene targeting of CHO cells have been observed. Targeting vector frequently copies target sequence on CHO genome and the truncated end of the vector sequence is extended for several kb beyond target homologous region on the vector and randomly integrated elsewhere on the genome. This gives rise to many pseudo-homologous recombinants with the same sequence on non-target and target loci. They can't be distinguished from true homologous recombinants by genomic PCR Chromosomal abnormalities that affect copy number and chromosome location of target loci accumulate in CHO cells[47].

Filamentous fungus:

The multicellular filamentous fungus *Aspergillus* is attaining interest for the heterologus protein production with its detailed knowledge about the genetics, strain optimization features and high capacity for the secretary protein production in industrial scale fermentation[48,22]. *A. niger* and *A. oryzae* are the most commonly used strains among the *Aspergilus* sps [49,50]. The inducible system is the choice for the production of proteins while expressing in the filamentous fungus when the recombinant proteins are toxic in nature. PglaA from *A. niger* [51] and PalcA from *A. nidulans* [52] are the commonly used inducible promoters for the expression of recombinant proteins. Solid state culture and gene fusion methods are used in filamentous fungus to improve the heterologus protein production [53].

Cell-free protein Production:

Cell-free protein synthesis has emerged as a powerful technology platform to satisfy the growing demand for simple and efficient protein production without the use of living organisms. While used for decades as a foundational research tool for understanding transcription and translation, recent advances have made possible cost-effective micro scale to manufacturing scale synthesis of complex proteins. These advances have inspired new applications in the synthesis of protein libraries for functional genomics and structural biology, the production of personalized medicines, and the expression of virus-like particles [54].

Extracts from the cells which are engaged in high protein production are commonly used such as rabbit reticulocytes (RRL), wheat germ (WGE), *E. coli* (ECE) and insects cell (such as SF9 or SF21) lysates and are commercially available now which can be scalable to quantities [54]. The advantages with the cell free expression systems are protein synthesis conditions can be manipulated, one can readily incorporate non-amino acids and can use PCR products as template amenable to simple high throughput approaches. Some of the disadvantages include limited post-translational modifications in absence of canine pancreatic microsomes and expensive at scale. Its practical applications are seen in production of purified protein (structure, enzymology, drug discovery), in-vitro expression cloning, isotopic labelling of proteins for NMR, incorporation of non-natural amino acids [14]. The recent development in advancement of cell free expression system was made to encounter the proteolysis problem by using the PURExpress® (NEB#E6800) from NEB which consists only the highly purified components (https://www.neb.com/products/e6800-purexpress-*in vitro*-protein-synthesis-kit).

Transgenic plants:

Transgenic plants as an expression system for the production of recombinant proteins were developed with the discovery of natural gene transfer mechanism which was used by the plant pathogenic bacterium (*Agrobacterium tumefaciens*) to introduce the genetic material into the genome of plants during the infection process [55] . In the transgenic plants the recombinant proteins are often expressed by integration of vector DNA with the host by the stable transformation using the natural method by *Agrobacterium*-mediated transformation or by the biolistic method(particle gun method) [56, 57, 58] or by infecting plants with recombinant viruses which express the transgene during their replication in host [59, 60]. *Nicotiana tabacum* and tomato [61] , banana [62], rice [63], potato[57] and wheat [64] are found to be ideal plants for the recombinant or heterologus proteins production. Cauliflower mosaic virus (CaMV), a constitutive promoter has been most widely used in plants for the recombinant protein expression . Temporal or spatial expression of the genes is required to avoid the harmfulness. Inducible promoter like RD29A gene promoter from *Arabidopsis* for stress tolerance, Tissue specific promoters like GluB1 from rice[65] , patatin from potato[66] , fruit specific promoters like ACC oxidase gene from apple[65] and seed-specific promoter arc5-I from common bean (*Phaseolus vulgare*) [67] are few examples for the recombinant protein production in specific manner. The expression cassette of E8 fruit ripening specific promoter when combined with the heat shock protein(HSP) from *Arabidopsis* produced increased accumulation of recombinant proteins [68].

The high potential of transgenic plants is seen in the field of bioreactors. The major application in relation to pharmaceutical industry is in production of antibodies and vaccines. Several special characteristics of vegetables have been found to be extremely applicable in recombinant protein production. The use of *Agrobacterium rhizogens* and *Chlamydomonas reinhardtii* in production of fully active antibodies are few of the examples for use of transgenic plants. Major advantages of using such plant based host system include compatibility, simple, well

defined and industry compliant culture media and strong viral safety component. Few of the drawbacks include restricted use in terms of variety of molecules which can be developed [2].

Transgenic animals:

The modern state-of-art techniques in molecular biology field allowed researchers to express the recombinant proteins in specific organs or tissues by using the specially designed or modified vectors and gene transfer techniques [48]. Transgenic animals as model animals have gained popularity in last few decades due to their usage as models to study human disease, for example, as donors in xenotransplantation, to human patients and for production of biopharmaceutical products [69] . The production of recombinant proteins using transgenic animals is possible due to advancement in techniques of microinjection, embryonic stem cell manipulation and somatic nuclear transfer in recent years[69]. The microinjection of foreign DNA into the pronuclei of zygotes enabled generation of the first transgenic livestock, twenty years ago [70]. Expression of recombinant proteins is tissue specific which often restricted to certain tissues or organs for example the stimulation of mammary glands using regulatory elements derived from the milk protein genes[71]. Because of complexity of purification process these target protein production is rarely directed to other tissues.

Though with limited success, this host system is preferred whenever there is any protein of high pharmaceutical value need to be developed. The examples include production of recombinant clotting factor VIII production from mammary glands of transgenic sheep. The product expressed in the milk of transgenic goat, ATryn®, a recombinant form of human antithrombin III was the first to receive the approval from the European Commission (http://www.gtcbiotherapeutics.com/news.html). Several other proteins like mAbs, human recombinant α-1-proteinase inhibitor, human serum albumin etc., are in different stages of development. The major drawback includes cost and laborious and complex process[2].

Table 1: Comparison of various expression systems

Expression System	Immunogenicity	Glycosylation of Protein	Cost of Production	Capital Investment Required	Protein Yield	Contaminants
Bacteria	Yes	No	Low	Low	High	Endotoxin
Yeast	Yes	No	Low	Low	High	Low
Insect	Moderate	Yes	Moderate	Low	High	Low
Mammalian Cells	No	Yes	High	High	Low	Viruses, Oncogenes
Transgenic Plants	Moderate	Yes	Low	Low	High	Viruses, Oncogenes
Transgenic Animals	No	Yes	High	Low	High	Low

CONCLUSION

Protein production is the backbone of functional assays, biomarkers, structural and biophysical studies, mechanistic studies in vitro and in vivo, but also for therapeutic applications in pharmaceutical, biotech and academia. Over past few decades it has evolved into a mature discipline. Due to the increased importance of biopharmaceuticals and the growing demand for proteins used for structural and biophysical studies some of the factors have developed utmost importance including, the impact of genomics technologies on the analysis of large sets of structurally diverse proteins, and the increasing complexity of disease targets, the interest in innovative approaches for the expression, purification and characterisation of recombinant proteins [72].

Today mammalian system remains one of the most commonly used systems as compared to other host systems due to its advantages. It is very own to human cells, ultimate resource for production of extremely complex and high molecular weight proteins including multimeric antibodies. CHO cells lines are most commonly used cell lines in mammalian expression system for biopharma production due to quality yield and regulatory acceptance[2].

In spite of the advantages of CHO cells, industry is still relying on other expression systems. This is due to the lengthy developmental time and complexity in isolation of high expressing production cell line. Our lab is focusing on development of a novel CHO based expression system which reduces the cost, time and effort involved in the development of high expressing production cell line there by widening the use of CHO cell based expression system in bio-pharma industry.

Acknowledgements:

In the preparation of this review no sources of funding were used. I would like to thank Dr. Kiranmayee rao and Dr. Bhuvaneswari chodisetti for their valuable and fruitful discussions and comments. I would like to express my gratitude to Dr. Raj Kumar Kunaparaju and Dr. Sailaja Dorbha for the manuscript proofreading.

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