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1. Microbial recombinant protein: An epic from fundamental to future panorama of life science

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Abstract. Proteins are the large biomolecules that are required for the various biological processes to sustain life. It accelerating all biochemical reactions in the form of enzymes, building the structural frame of body by structural proteins, transmitting signal for performing prompt activity by body accessories, acting like soldier for defense mechanism and so on and so forth. Microbial proteins are presently acquiring much attention with rapid development of microbial technology that can make easily an aid in daily life in the form of enzymes, food supplements, vitamins, antibodies, antibiotics, vaccines and so many other formats. Microbial proteins are favored for large scale production of recombinant protein due to their high yields, economic feasibility, consistency, easy way of modification and optimization, constant supply due to absence of seasonal rise and fall, greater catalytic activity and so many other advantages over non-microbial proteins. Efficient strategies for recombinant protein production are acquiring increasing importance over traditional techniques that require high amount of high-quality proteins to grab the market.

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Such strategies include molecular biology techniques, as well as advancement in proteomic technologies and easy manipulation of the culture environment. In this chapter, study is mainly focused on basics and brief idea of microbial recombinant proteins with their application based types and classes, developmental strategies including genetic engineering and systems required for higher production of recombinant protein. Recent trends of recombinant proteins productions, their applications in different areas of modern science and their future perspective for strategies improvement are also reviewed and discussed.

Introduction

Proteins are the main structural constituents of life and synthesized by all living organisms as a vital part of their natural metabolism. Proteins in different forms play significant role for supporting the life vehicle keep moving till death and their activities are omnipresent throughout the body; such as enzymes that also known as biocatalyst of metabolic reactions, antibodies in immune responses, receptors for cell signaling and remaining most of them are structural proteins. The protein in two different ways can be manifested for manufacturing very useful items in the area of biopharmaceutical, enzyme and agricultural industries and that may be either in native form or recombinant form. In current time, the application of genetic engineering based recombinant protein (r-protein) is much more focused due to its easy availability with large quantity. In other hand, the native protein is directly obtained from microbial resources with a limited quantity making it less sophisticated as an ordinary application. Production of recombinant proteins involves isolation of promising genes from a source organism by implying different efficient isolation techniques followed by cloning of the appropriate gene into an expression vector under the command of an inducible promoter and thereafter efficient expression of that recombinant protein by optimizing several factors including favorable expression signals at transcription and translation levels, correct protein folding and pattern of cell growth [1].

The journey of recombinant protein in the area of microbial biotechnology was started when microbial fermentation industry began first large-scale anaerobic fermentation to produce acetone, butanol and citric acid like chemicals in the early 1900s. Later on, these microbial industries revolutionized in the area of medicine, diagnostics, detergents, textiles, leather, food, nutrition, paper, pulp, plastics and polymers. The black and white form of microbial biotechnology turned into color in the year of 1971 with the discovery of recombinant DNA by Berg, Cohen and Boyer in California. By 2002, over 155 approved pharmaceuticals and vaccines had

been developed by biopharmaceutical companies. Today, the Food and Drug Administration (FDA) list comprises more than 200 approved protein derivatives and pharmaceuticals which are continuously manufactured for daily uses [2].

Another breakthrough was achieved by researchers at worldwide level is the successful synthesis of therapeutic enzymes, used nowadays for preparation of present day medical drugs. Therapeutic enzymes have a wide range of specific uses: as oncolytics, thrombolytics, and anticoagulants or as replacements for metabolic deficiencies. Despite them, there is an emerging group of various enzymes of different function and especially proteolytic enzymes are broadly used as anti-inflammatory agents [3].

Most of the enzymes used now days in food processing industries are derived from recombinant microorganisms. With the help of new genetic techniques, enzyme manufacturers develop and manufacture enzymes with adequate quantity, improved properties and superior quality. Enzymes of microbial origin with difficulty to culture at laboratory or industrial conditions can be optimized for efficient synthesis by judicious selection of host microorganisms and construction of appropriate recombinant strains capable of competent production of enzymes free from other undesirable enzymes or microbial metabolites. Enzymes used in food processing are supplied as enzyme preparations. An enzyme preparation typically consists of the enzyme of interest and several additional substances viz. preservatives, diluents, and stabilizers. All these materials are projected to be of suitable purity consistent with current good manufacturing practice (cGMP) [4].Thus, recombinant proteins exhibited a broad range of applications in different industries whether it may be food, textile, medicine, dairy, or any other. With the encroachment of modern microbial biotechnology and protein engineering we came into the edge of success where we can easily introduce or modify the competence of the genes that are crucial for us to produce these novel proteins.

In this book chapter, we will explain about types and classes of microbial recombinant protein and their gradual development over the time from recombinant microorganisms. We will also discuss characteristics of the host microorganisms, strategy for construction of recombinant protein production, and recent trends in application of recombinant protein in the area of modern human society. We will also briefly illustrate the advanced technologies adjusted for enhanced production of r-protein and its future scope that could contribute a major development of biotechnology.

Microbial recombinant protein: Basics and brief idea

Amino acid is the building block of proteins and a standard protein molecule is made out of many thousands of amino acids. The protein made up of a complex 3-D shape and also a determining factor of most of the protein's function in the living body. Proteins are very essential of all the materials and that are the basic structural unit of all living organisms. The proteins are formed in the cells by the ribosome by a process called "Translation" in which an mRNA molecule (transcript from DNA found in nucleus) migrates from nucleus to Cytoplasm carrying the genetic code of the DNA and proteins are translated in the cytoplasm with the help of another protein molecule called Ribosome.

Recombinant protein is a protein whose code is carried by a **recombinant DNA** (rDNA). The term **recombinant DNA** means that consist of two different segments of DNA: a plasmid and a DNA of interest. Now-a-days, the application of bacteria for synthesizing **recombinant protein** is highly developed. This technique is often used to produce many important hormones and therapeutics for daily and medical uses. Using rDNA and introducing it to a plasmid of speedily reproducing bacteria facilitates the construction of recombinant protein. These recombinant proteins may be variety of types and that can be antigens, antibodies, hormones and enzymes.

Before introducing in the world of recombinant protein (rProtein) derived from microbial resources, we should be clear about the basics and principal of recombinant DNA technology and its implication in genetic engineering. A huge number of proteins are having a defined importance to make the present lifestyle mobile and as well as to make people happy for good health. But in most of the cases, either the naturally occurring proteins are very limited to access for common peoples or deficient for a part of human population because of genetic defects. Therefore there is an urgent requirement of synthesizing these mostly valuable naturally occurring and chemically derived proteins with the help of genetic engineering approach.

The generalized model of conversion of DNA into mRNA by transcription and thereby alteration of RNA into protein (Structural, Signaling and with enzymatic activity) by translation is universal and that can achieved in vitro by implying rDNA technology (Figure1A). The early work in this field employed in bacteria. Specific types of bacteria contain small circular DNA molecules called plasmids (different from chromosomal DNA) have the ability to transmit to daughter cells by the way of reproductions called binary fission or cell division or conjugation. Scientists have learned the technique to introduce promisible proteins coding gene into

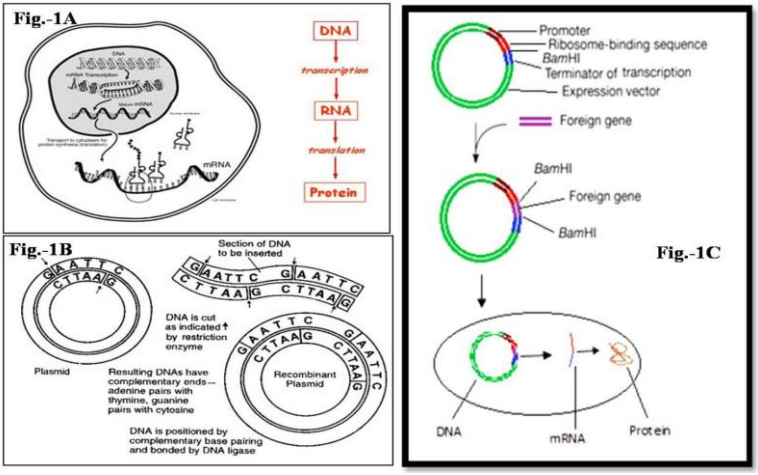


Figure 1. Basic recipes and principals of rDNA technology used for recombinant protein production. **Figure 1A.** A general workflow of synthesis of target protein in cell and which is also known as 'Central Dogma' theory proposed by Francis Crick, 1958. (Source: http://mcb.berkeley.edu/courses/mcb130L/Originals/Lecture_2.ppt). **Figure 1B.** A basic genetic engineering approach showing cutting and rejoining of plasmid by introducing foreign DNA with the help of molecular enzymes. (Source: http://www.icr.org/article/production-therapeutic-proteins-by-genetic-enginee/). **Figure 1C.** Expression of foreign protein in microbial cell. (Source: http://nptel.ac.in/courses/102103013/).

the plasmid and thereby multiplying the cell culture into suitable media to maximize the quantity of desirable genes (Figure1B) [5]. Then the enhanced quantity of the target gene is cloned into a specifically designed vector having typical transcription and translation machineries and that can be easily replicate into an efficient expression system (It may be bacteria, virus, insect, yeast cell, animal cell etc.) (Figure1C) [6].

Naturally occurring valuable and important proteins isolated from tissues or blood samples of target animals or plants are available in such minute quantities that the isolation inconsiderable amounts required highly expensive processing charges and that is not economically fitted concerning production. So there is a genuine need of an automated expression system by which target protein is genetically engineered for large-scale production and this is now successfully achieved for different types of recombinant proteins (Figure 2). The root of genetic engineering dealing with final production of rProtein is mainly based on the mind-blowing playing of one type of enzymes.

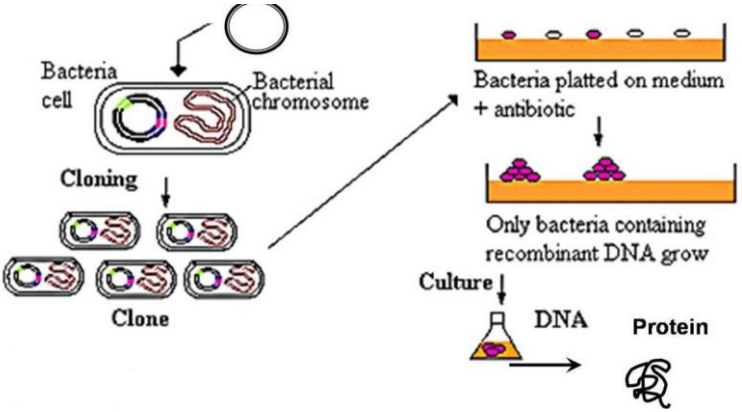


Figure 2. Typical protein expression workflows. (Source: http://www.accessexellence.org/AB/GG/plasmid.html).

Scientists have identified and isolated these enzymes (called restriction enzymes, or restriction endonucleases) which cut genes in very specific location. The target gene that codes for the desired therapeutic protein located on the chromosome of source microorganisms is isolated and cut out using appropriate restriction enzymes and used to cut out plasmid also. Thus, the two cutting ends of target gene will be linked up with open ends of the plasmid with the help of another enzyme called DNA ligase. These plasmids, now including the human gene, are reinserted into bacteria. These bacteria can be cultured, manufacturing identical bacteria carrying large quantities of the target gene along with the bacterial DNA. The protein is then isolated from the bacterial culture, purified, and processed accordingly for commercial and therapeutic use (Figure 1B and 1C).

Types and classes of microbial recombinant protein:

Production of recombinant proteins in microbial system is revolutionizing biochemistry [7]. The excellent benefit of recombinant DNA technology has been the production of microbial recombinant proteins used for human therapeutics such as hormones, growth factors and antibodies [26-36]. Microorganisms offer various advantages over the available sources such as easy handling, higher multiplication rate and high production yield. Microorganism produces a large number of valuable products like protein, nucleic acids, carbohydrates polymers (macromolecule) and smaller molecules [8]. A much larger number of recombinant proteins are used in the research

laboratory. These include both commercially available proteins (for example most of the enzymes used in the molecular biology laboratory), and those that are generated in the course of specific research projects. On the basis of size of recombinant protein, the systems used for manufacturing the protein can be divided in to two classes:

Table 1. Major classes of microbial recombinant protein are based on their applications and some important examples are listed here.

Recombinant proteins	Microbial source	References
Enzymes and related proteins		
Prochymosin *B. taurus*	*Bos taurus*	[4]
Esterase–lipase, Laccase, Pectinesterase and Phospholipase	*Aspergillus oryzae*	
Pullulanase	*Bacillus licheniformis*	
α-acetolactate decarboxylase, α-amylase and MaltogenicPullulanase	*Bacillus subtilis*, *Pseudomonas* and *Xuorescens BiovarI*	
Pectin lyase	*Trichoderma reesei*	
Glucose oxidase	*Aspergillus niger* and *A. oryzae*	
β-galactosidase	*A. oryzae*	[9]
α-amylase (*Bacillus amyloliquefaciens*)	*B. subtilis*	[10]
α-amylase (*Bacillus stearothermophilus*)	*Bacillus brevis*	[11]
Pepsinogen (swine)	*B. brevis*	
α-amylase (human)	*B. brevis*	[12]
Cellulase	*B. brevis*	[13]
Lipase A	*B. subtilis*	[14]
PHA depolymerase A (*Paucimonaslemoignei*)	*B. subtilis*	[15]
Dextransucrase (*Leuconostocmesenteroides*)	*B. megaterium*	[16]
Protein disulfide isomerase	*B. brevis*	[17]
Staphylokinase	*B. subtilis*	[18]
β-Galactosidase	*Escherichia coli*	[19]
β-galactosidase	*A. oryzae*	
Chloramphenicol acetyltransferase	*E. coli*	
Alkaline phosphatase	*E. coli*	[20]
Dihydrofolate reductase	*E. coli*	[21]

Table 1. Continued

Aspartic proteinase	*Rhizomucor miehei* and *A.oryzae*	[22]
Lipases	*Pseudomonas mendocina*, *Aspergillus sp*.	
β-glucanase	*Trichoderma reesei (longibrachiatum)*	
Hydrolases oxidoreductases	*Aspergillus niger*	[23]
Mn2+-oxidizing peroxidases	*DexPhanerochaete chrysosporium* and *Pleurotus eryngii*	
Cellulase	*A.oryzae, T. reesei (longibrachiatum)*	[24]
Lactases	*Streptococcus lactis*, *Kulyveromyces sp.* and *Escherichia coli*	[26]
Hormones and signal proteins		
Epidermal growth factor (human)	*B. brevis*	[27]
Epidermal growth factor (human)	*B. subtilis*	[28]
Epidermal growth factor (mouse)	*B. brevis*	[29]
Mouse/human chimeric Fab′	*B. brevis*	[30]
Interferon-α2 (human)	*B. subtilis*	[31]
Interleukin-2 (human)	*B. brevis*	[32]
Interleukin-6 (human)	*B. brevis*	[33]
Proinsulin	*B. subtilis*	[34]
β-gal-OmpF	*E. coli*	[35]
OmpF	*E. coli*	[36]
Antibody		
Fibrin specific single-chain antibody	*B. subtilis*	[37]
Antibiotic		
Penicillin G acylase	*B. subtilis*	[38]
Polymyxin B	*Bacillus polymyxa*	[39]
Erythromycin	*Streptomyces erythreus*	
Neomycin	*Streptomyces fradiae*	
Streptomycin	*Streptomyces griseus*	
Tetracycline	*Streptomyces rimosus*	
Rifamycin	*Streptomyces mediterranei*	
Other Proteins and Toxins		
Prochymosin	*Bos taurus*	[35]
Chymosin	*Klayveromyces lactis*, *A.niger* and *E. coli*	[25]

Table 1. Continued

Phytase Chymosin	*Aspergillus niger*	[3]
Streptavidin	*B. subtilis*	[36]
Thioredoxin (*Aliciclobacillusacidocaldarius*)	*B. subtilis*	[40]
Toxin A (*Clostridium difficile*)	*B. megaterium*	[41]
Cholera toxin B	*B. brevis*	[42]
c-Type cytochromes	*E. coli*	[43]
Amino Acids		
Arginine	*E. coli (*B &K12) and *Bacillus subtilis*	[1]
Leucine	*Anabaena sp.*	
Isoleucine	*Saccharomyces cerevisiae*	
Proline	*Pichia pastoris* and *Picrophilus torridus*	
Glycine	*Caenorhabditis elegans, Plasmodium falciparum*	

- **Prokaryotic system**

The easiest and quickest expression of proteins can be carried out in *E. coli*. This bacterium cannot express very large proteins, S-S rich proteins and proteins that require post translational modifications.

- **Eukaryotic system**

Yeasts are able to produce high yields of proteins at low cost. It can produce proteins larger than 50 kDa, signal sequence can be removed and Glycosylation can be carried out. The two most utilized yeasts are *Saccharomyces cerevisiae* and *Pichia pastoris*.

- **Classes of microbial recombinant protein**

There is no such well accepted classes designed for recombinant protein with microbial resources but on the basis of their applications the proteins can be categorized in some important classes like enzymes, antibodies, antibiotics and so on. Some of the important classes are described below.

- **Microbial enzymes**

Enzymes are made up of proteins. The manufacturing of enzymes for use as drugs is an important fact of today's pharmaceuticals. For example:

Protease: *Bacillus polymyxa*, Amylase:*Aspergillus oryzae*, Glucosidase::*Aspergillus niger*, species, Catalase:: *A. niger* etc (Table 1) [3-25].

- **Antibodies**

The increasing demand for recombinant antibodies as detection reagents in research, diagnostics, and therapy requires appropriate production systems. For example: Gram-positive bacteria are known to efficiently secrete recombinant proteins into the medium. Recently, a report showing the production of scFv and scFab fragments in *Bacillus megaterium* (Table 1) [36].

- **Antibiotics**

The antibiotics in current use for the treatment of various infectious diseases are obtained from various microbes. Studies on soil bacteria and fungi have shown that microorganisms are a rich source of structurally unique bioactive substances. For example- Penicillin acylase:: *penicillium.* The isopenicillin N synthetase ("CyClaSe") gene of *Cephalosporium acremonium* has been cloned in *Escherichia coli*. Cyclase gene of *Penicillium chrysogenum* and *Streptomyces clavuligerus* has also been cloned in *E. coli* system. The expandase/ hydroxylase gene of *C. acremonium* has been cloned in *E. coli* (Table 1) [37-38].

- **Microbial proteins**

Some important microbial proteins are also reported from different microbial resources and are enormously utilized at commercial level. Some of them are Protease, Amylase, laccase, xylanase, chymosin, growth hormone, trypsin, asparaginase etc (Table 1) [3, 26, 35-42].

- **Microbial vaccines**

Recombinant protein subunit vaccines are formulated using protein antigens those have been synthesized in heterologous host cells. Several host cells are available for this purpose, ranging from *Escherichia coli* to mammalian cell lines. For example- *Saccharomyces cerevisiae* has also been used in the manufacture of 11 approved vaccines against hepatitis B virus and one against human papillomavirus; in both cases, the recombinant protein forms highly immunogenic virus-like particles.

- **Food supplements**

Important recombinant protein extracts include β -carotene, astaxanthin, and C-phycocyanin (C-PC). The carotenoid β-carotene has a wide range of applications. It can be used as a food coloring agent, a source of pro-vitamin A. The carotenoid astaxanthin has potential applications in the food and feed industries.

- **Bioactive compounds**

Actinomycetes are the potential microorganisms play a significant role in the production of antimicrobial agents and other industrially important bioactive compounds like drugs and natural pigments. Different molecular techniques such as genetic fingerprinting, meta-genomics, meta-proteomics, 16S r RNA, genus specific primers, RAPD, RFLP, Proteomics and bioinformatics tools are applied for discovering and characterizing the huge actinomycetes diversity holding the key importance of bioactive compounds [44]. A large number of antimicrobial compounds have been isolated from actinomycetes sp. while rare and novel species of this genus are expected to contain as yet undiscovered bioactive metabolites. Some of them are widely utilized as antimicrobial agents such as Abyssomicin (Antibacterial and antitumor) from *Verrucosispora* sp., Actinoflavoside (Antifungal) from *Streptomyces* sp., Analogs-metacycloprodigiosin (Anticancer) from *Saccharopolyspora* sp., Essramycin (anti-inflammatory) from *Streptomyces* sp. and so on and so forth. A number of more than 100 bioactive compounds not only reported from different species of actinomycetes but also around 64 novel bioactive compounds have been isolated from the marine actinobacteria which show higher antimicrobial and anticancer activities [45].

Development of microbial recombinant protein in support of genetic engineering approach

Like biochemical principles, genetic principles are universal. The study of microbial genetics has contributed much to what we know about the genetics of all organisms. Molecular genetics can be used to manipulate genes in order to alter the expression and production of microbial products, including the expression of novel recombinant proteins. The compounds can be synthesized by genetic manipulation from different micro-organisms; to enhance the production of those compounds that are isolated from plants or

animals, even up to 1000-fold for small metabolites can be increased. The recombinant DNA technology has dramatically broadened the spectrum of microbial genetic manipulations; many novel host systems have been explored to produce commercially important products like therapeutic proteins, antibiotics, small molecules, bio-similars etc.

This technology includes the use of restriction endonucleases, polymerases and DNA ligases as a mean to specifically cut and paste fragments of DNA is the main base. Foreign DNA fragments can be introduced into a vector molecule (a plasmid or a bacteriophage), which enables the DNA to replicate after introduction into a bacterial cell.

- **Molecular tools for genetic engineering of microorganisms**

A number of molecular tools are needed to manipulate microorganisms for the expressions of desired proteins (Figure 3) are as following:-

(1) Gene transfer methods to deliver the selected genes into desired hosts;
(2) Cloning vectors;
(3) Promoters to control the expression of the desired genes; and
(4) Selectable marker genes to identify recombinant microorganisms.

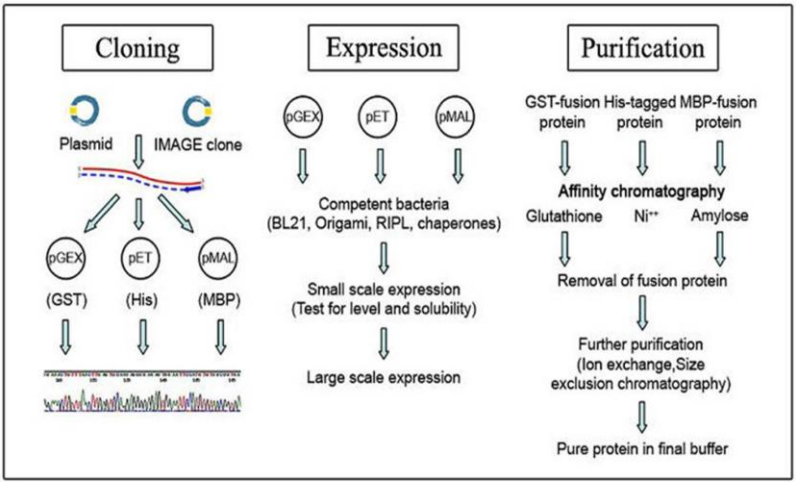


Figure 3. Three major steps for generating microbial recombinant protein are cloning, expression and purification (Source http://www.southampton.ac.uk/cruk/protein/index.page).

(1) Gene transfer methods

The gene transfer methods basically used to introduce DNA into desired microorganisms. *Transformation* is the most frequently used method. In *Transformation*, uptake of plasmid DNA by recipient microorganisms is accomplished when they are in a physiological stage of competence, which usually occurs at a specific growth stage. DNA uptake based on naturally occurring competence is usually inefficient. It can be induced by treating bacterial cells with chemicals to facilitate DNA uptake. The Table 2 describes the basically used gene transfer methods [46].

Table 2. Gene transfer methods used with several commercially important microorganisms.

Type of Organism	Industrial Applications	Gene Transfer Methods
Aspergillus	Food fermentations	Protoplast transformation, Electroporation and Biolistic transformation
Yeasts	Food and beverage fermentations	Protoplast transformation and Electroporation
Bacillus	Industrial enzymes, Antibiotics and Insecticides	Transformation of competent cells, Fine chemicals Protoplast, Transformation and Electroporation
Corynebacterium	Amino acids	Protoplast transformation, Conjugation and Electroporation
Escherichia coli	Therapeutic protein Biodegradable plastics	Transformation of competent cells, Electroporation
Lactic acid bacteria	Food fermentation and Organic acids	Protoplast transformation and Electroporation
Pseudomonas	Plant biocontrol agents And Bioremediation	Conjugation and Protoplast transformation
Streptomyces	Antibiotics, Antiparasitic agents, Anti-tumor and Herbicides	Electroporation and Conjugation

➢ **DNA cloning**

DNA cloning is the production of large number of identical DNA molecules from a single ancestral DNA molecule. The essential characteristic of DNA cloning is that the desired DNA fragments must be selectively amplified resulting in a large increase in copy number of selected DNA sequences. In practice, this involves multiple rounds of DNA replication catalyzed by DNA polymerase acting on one or more types of template DNA molecule. Essentially two different DNA cloning approaches are used: cell-based and cell free DNA cloning.

The basic steps in DNA cloning:

1) The workflow consisting of insertion of a foreign DNA fragment into a carrier DNA molecule (vector) to produce a recombinant DNA (r DNA).
2) The r DNA is then introduced into a host cell for multiplication and production of numerous copies of itself within the host (preferably bacteria).
3) After a large number of divisions and replications, colonies or clones of identical host cells are produced, carrying one or more copies of the r DNA.
4) The colony carrying the recombinant DNA of interest is then identified, isolated, analyzed, sub-cultured and maintained as a recombinant strain. [5].

(2) Cloning vectors

There are a large classes of vectors are available, but the choice of cloning vector to carry out genetic modifications depends on the choice of the gene transfer method, the desired outcome of the modification, and the application of the modified microorganism. The replicating vectors of high or low copy numbers are commonly used to express the desired genes in heterologous hosts for manufacturing expressed proteins. Cosmid and bacterial artificial chromosome vectors, which accept 100 kb large DNA fragments, are necessary when cloning a large piece of DNA into a heterologous host for manipulation and high-level metabolite production. List of some important vectors used in genetic modification are described in Table 3[47].

Table 3. Some recombinant DNA cloning vectors with their characteristics [48].

S.no.	Type	Vector	Features
1.	Plasmid (*E. coli*)	pBR322	Carries genes for Tetracycline and Ampicillin resistance.
2.	Plasmid (yeast-*E. coli*hybrid)	pYe(CEN3)41	Multiplies in *E. coli* or yeast cells.
3.	Cosmid (artificially constructed *E. coli* plasmid carrying lambda cos site)	pJC720	Can be packaged in lambda phage particles for efficient introduction into bacteria; replicates as a plasmid; useful for cloning large DNA inserts.
4.	YAC (yeast artificial chromosome)	pYAC	Carries gene for ampicillin resistance; multiplies in *Saccharomyces cerevisiae*.
5.	BAC (bacterial artificial chromosome)	pBAC108L	Modified F plasmid that can carry 100–300 kb fragments; has a *cosN*site and a chloramphenicol resistance marker.
6.	Plasmid	Ti	Maize plasmid.

(3) Promoters

A *promoter* is a segment of DNA that regulates the expression of the gene under its control. It is important to choose an appropriate promoter for the expression of the target genes for desired timing and level of expression [46]. Promoter is the most critical component of an expression vector since it controls the very first stage of gene expression and also regulates the rate of transcription. An expression vector should carry a strong promoter so that highest possible rate of gene expression could be achieved. Regulation of promoter is another important factor to be considered during construction of an expression [48]. Most commonly used promoters types used for *E. coli* expression are listed in Table 4. The promoter can be classified in to two main groups-

Constitutive promoter - Constitutive promoters are continuously active.
Inducible promoters - inducible promoters become activated only when certain conditions, such as the presence of an inducer, are met.

Table 4. Most frequently used promoters for an *E.coli* expression vector.

Promoter	Function
lac promoter	It regulates transcription of lac z gene coding for β-galactosidase. It can be induced by Isopropylthiogalactoside (IPTG)
trp promoter	It regulates transcription of a cluster genes involved in tryptophan biosynthesis.It is repressed by tryptophan and easily induced by 3- β-indoleacrylic acid
tac promoter	It is hybrid of trp and lac promoter but is stronger than either of them.It is induced by IPTG
λP_L promoter	It's a very strong promoter responsible for transcription of λDNA molecule in *E.coli*. It is repressed by product product of λcI gene called λ repressor.

(4) Selectable marker genes

Selectable marker genes encode proteins conferring resistance to antibiotics. This is an important part of cloning vectors and is required for identification of transformed cells. The number of transformed cells is very less than non-transformed cells so this selection is very necessary. These transformed cells are identified using a toxic concentration of the selection agent to inhibit the growth of the non-transformed cells [46]. Identification of cells containing the vector molecule requires the presence of suitable marker gene on the vector molecule where the expression provides a means of identifying cells containing it. Two most popular marker genes are–

(a) Antibiotics resistance gene

A host cell strain is selected that is sensitive to a particular antibiotic. The corresponding vector has been engineered to contain a gene which confers resistance to a series of commonly available antibiotics (Table 5).

(b) Color substance developing genes

These genes produce enzymes which give specific color in the presence of particular substances. For example lacZ gene encodes β-galactosidase, an enzyme that splits lactose into glucose and galactose. β-galactosidase also acts on a colorless substance Xgal and develops blue colour [48].

Table 5. Examples of some important antibiotics commonly used as selectable markers.

Antibiotic	Mode of action
Ampicillin	Inhibits the synthesis of the gram – negative cell wall and it acts as a competitive inhibitor of the enzyme transpeptidase.
Tetracycline	Binds with the 30S subunit of the ribosome and inhibits translation.
Chloramphenicol	Binds to the ribosomal 50S subunit and inhibits translation.
Kanamycin	Binds to the ribosomal component and inhibit translation.
Bleomycin	Bind to DNA and cause strand break.
Hygromycin	Inhibits translation by interfering with ribosome translocation and it acts against both prokaryotes and eukaryotes.

➢ Expression systems:

The heterologous proteins production involves suitable expression system. Prokaryotic and eukaryotic systems are the two general categories of expression systems. There is no universal expression system for heterologous protein production. All expression systems have some advantages as well as some disadvantages that should be considered in selecting which one to use (Table 6).

Table 6. Prokaryotic and eukaryotic systems used for different types of proteins expressions [47].

System	Advantages	Drawbacks	Stage of development
Prokaryotic			
Escherichia coli	High yield, large choice of genetic elements	No post-translational modifications, secretion difficult	Production
Bacillus	Secretion lowprotease, surface display	No post-translational modifications	Production / Development
Caulobacter crescentus	Easy purification Secretion	No post-translational modifications	Research/ Development
Lactobacillus zeae	Adapted to temperature sensitive products	No post-translational modifications	Development
Exotic hosts (cold bacteria)	-	Restricted to specific applications	Research

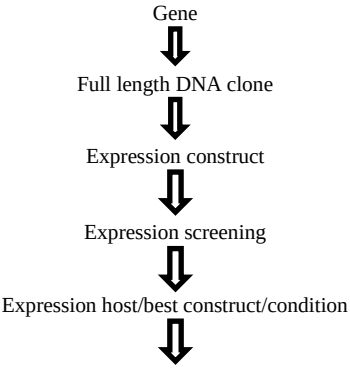
Table 6. Continued

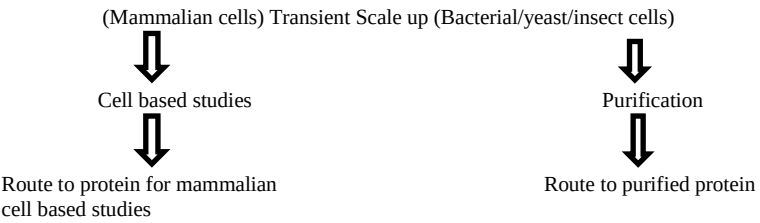
Eukaryotic			
Mammalian cells	Secretion suitable for complex molecules	Additives, low yield	Production
Insect cells	High yield, simple media, Viral safety	Glycosylation profile	Production / Development
Vegetal	Biomass secretion, viral safety	Glycosylation profile	Production / Development
Yeast,	Biomass secretion	Glycosylation profile	Production
Trypanosome	Growing capacity in extreme condition/waste material	Genetics still needs to explored	Research / Development
Transgenic animals	Mammalian- like Glycosylation, suitable for complex molecule	Genetic still needs to explored, time consuming, restricted to high added-value products	Research / Development

Systems for producing recombinant microbial proteins:

In eukaryotic system large proteins are usually expressed while smaller ones are expressed in prokaryotic systems. For proteins that require glycosylation, mammalian cells, fungi or the baculovirus system is chosen. The least expensive, easiest and quickest expression of proteins can be carried out in *Escherichia coli*.

- **Typical protein expression workflows:**





Prokaryotic expression systems

E. coli is by far the most widely employed host, provided post translational modifications of the product are not essential. It combines high growth rates along with ability to express high levels of heterologous proteins. Strains used for recombinant production have been genetically manipulated to that they are generally regarded as safe for large scale fermentation. Purification has been greatly simplified by recombinant fusion proteins which can be affinity purified, example glutathione-S-transferase and maltose binding fusion protein.

Eukaryotic expression system

In prokaryotic expression systems, most protein products of cloned eukaryotic genes become insoluble aggregates called inclusion bodies and are very difficult to recover as functional proteins. Another problem is that prokaryotes do not carry out the same kinds of post translational modification such as glycosylation, phosphorylation as eukaryotes do. This can affect a protein's activity or stability or its response to antibodies. Advantage of eukaryotic expression system includes very high levels of expression and the disadvantage is that eukaryotic cells do grow slower than prokaryotic cells. By genetic engineering; desired proteins are massively generated to meet the high demands of industry. Some important expression systems are as follows.

1) Bacteria

1.1) *E. coli*

Due to rapid growth, rapid expression, ease of culture and high product yields *E. coli* is one of the earliest and most widely used hosts for the production of heterologous proteins with a number of diverse characteristics (Table 7). For the production of many commercialized

proteins this system is mainly used. Due to its understandable genetics this system is very good for expression of non-glycosylated protein. Its genome can be quickly and precisely modified, promoter control is not tough and plasmid copy number can be readily altered. Fundamental understanding of transcription, translation, and protein folding in *E. coli*, together with the availability of improved genetic tools, is making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. *E. coli* bacteria are able to accumulate recombinant proteins up to 80% of its dry weight and survive in different environmental conditions.

Table 7. Characteristics of *E. coli* expression system.

Advantages	**Disadvantages**
1. Rapid expression. 2. High yields. 3. Ease of culture and genome modifications. 4. Inexpensive. 5. Mass production is fast and cost effective.	1. Proteins with disulfide bonds difficult to express. 2. Produce unglycosylated proteins. 3. Proteins produced with endotoxins. 4. Acetate formation resulting in cell toxicity. 5. Proteins produced as inclusion bodies, are inactive; require refolding.

1.2) Bacillus

The Gram-positive bacillus is one of the useful bacterial systems. They mainly preferred for homologous expression of enzymes such as proteases (for detergents) and amylases (for starch and baking).
Advantages of Bacillus expression system:

- Strong secretion with no involvement of intracellular inclusion bodies
- Ease of manipulation
- Genetically well characterized systems
- Highly developed transformation and gene replacement technologies
- Superior growth characteristics
- Metabolically robust
- Generally recognized as safe (GRAS status) by US FDA (United States Food and Drug Administration)
- Efficient and cost effective recovery

Heterologous proteins are successfully expressed in Bacillus systems include interleukin-3EGF and esterase from Pseudomonas. Homologous proteins include *Bacillus stearothermophilus* xylanase, naproxenesterase, amylases and various proteases.

1.3) Other bacteria

Ralstonia eutropha is used to develop an improved Gram-negative host for recombinant protein production. In case of inclusion bodies formation this system appear superior to *E. coli*. Organophosphohydrolase, a protein prone to inclusion body formation with a production of less than 100 mg/L in *E. coli*, was produced at 10 g/L in *R. eutropha*. *Staphylococcus carnosus* can produce 2 g/L of secreted mammalian protein whereas the level made by *Streptomyces lividans* is 0.2 g/L. The Pfenex system using *Pseudomonas fluorescens* has yielded 4 g/L of trimeric TNF-alpha.

2) Yeasts

Yeast the single celled eukaryotic organism is used to produce recombinant proteins that are not well developed in *E. coli* because of glycosylation. Yeast is easy in handling; less expensive and very well performs the post translational modifications. The two most utilized yeast strains are *S. cerevisiae* and the methylotrophic yeast *P. pastoris*. Various yeast species are extreme useful for expression and analysis of recombinant eukaryotic proteins. For example, *A. niger* glucose oxidase can be produced by *S. cerevisiae* at 9 g/L. Almost all excreted eukaryotic polypeptides are glycosylated. Glycosylation is species-, tissue- and cell-type-specific [46]. The glycosylation mainly affects the reaction kinetics (if the protein is an enzyme), solubility, serum half-life, thermal stability, in vivo activity, immunogenicity and receptor binding. With regard to peptides, galactosylated enkephalins are 1000–10,000 times more active than the peptide alone. Pharmacokinetics are also affected by Glycosylation. Examples of stability enhancement are the protection against proteolytic attack by terminal sialic acid on erythropoietin (EPO) [49].

Advantages of yeast expression systems are: High yield, Stable production strains, Suitability for production of isotopically-labeled protein, rapid growth in chemically defined media, product processing similar to mammalian cells, can handle S–S rich proteins, can assist protein folding, can glycosylate proteins, durability, cost effective, high density growth and high productivity.

3) Filamentous fungi (molds)

The most attractive host of this group is Filamentous fungi such as *A. niger* because of their ability to secrete high levels of bioactive proteins

with post-translational processing such as glycosylation. The titer of a genetically-engineered bovine chymosin-producing strain of *Aspergillus awamori* was improved 500% by conventional mutagenesis and screening. Humanized immunoglobulin full length antibodies were produced and secreted by *A. niger*. For yield improvement the strategies which is used is strong homologous promoters, which increase gene copy number, gene fusions with a gene encoding a naturally well-secreted protein, protease-deficient host strains, and screening for high titers following random mutagenesis. The production of heterologous protein by filamentous fungi is sometimes severely hampered by fungal proteases. *Aspergillus nidulans* contains about 80 protease genes [50].

Advantages of baculovirus infected insect cell expression system:

1) Proper protein folding
2) High expression levels
3) Easy scale up
4) Post translational modifications
5) Flexibility of protein size
6) Efficient cleavage of signal peptides
7) Multiple genes expressed simultaneously

4) Insect cells

Insect cells have more complex posttranslational modifications than fungi. The most commonly used vector system for recombinant protein expression in insects is the baculovirus. Baculovirus which is the most widely used is nuclear polyhedrosis virus (*Autographa californica*) which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily in vitro. The usual host is the fall armyworm (*Spodoptera frugiperda*) in suspension culture. The virus contains a gene encoding the protein polyhedron which is made at very high levels normally and is not necessary for virus replication.

The baculovirus-assisted insect cell expression offers many advantages, among them the primitives are:

- Eukaryotic posttranslational modifications without complication, including phosphorylation, N- and O-glycosylation, correct signal peptide cleavage, proper proteolytic processing, acylation, palmitylation, myristylation, amidation, carboxymethylation, and prenylation.
- Proper protein folding and S–S bond formation, unlike the reducing environment of *E. coli* cytoplasm.
- High expression levels: There are some disadvantages of insect cell systems but some of which can be overcome.

5) Mammalian cells

For the production of proteins requiring post-translational modifications, mammalian expression systems are often used. Mammalian cell cultures are particularly useful because the proteins are often made in a properly folded and glycosylated form, thus eliminating the need to renature them. For addition of fatty acid chains and for phosphorylating tyrosine, threonine and serine hydroxyl groups of eukaryotic cells are also useful. Mammalian cells have very high productivity rate and they can produce the protein up to 20–60 pg/cell/day. Genes for the glycosylated fertility hormones, human chorionic gonadotropin, and human luteinizing hormone have been cloned and expressed in mammalian cells. Normally antibodies were produced in mammalian cell culture at levels of 0.7–1.4 g/L. but nowadays higher values have been reported recently. For example, monoclonal antibody production in NSO animal cells reached over 2.5 g/l in fed-batch processes. Animal-free, protein-free and even chemically-defined media with good support of production have been developed. Mammalian cell cultures are particularly useful because the proteins are often made in a properly folded and glycosylated form, that's we don't need to renature them. Eukaryotic cells are also useful for addition of fatty acid chains and for phosphorylating tyrosine, threonine and serine hydroxyl groups.

6) Transgenic animals

Generally transgenic animals are being used for production of recombinant proteins in milk, egg white, blood, urine, seminal plasma and silk worm cocoons. Transgenic animals such as goat, mice, cow, pig, rabbit, and sheep are being developed as production systems; some aquatic animals are also being utilized. In the mammary glands of transgenic animals foreign proteins can be produced. Dairy animals produce 1 to 14 g/L of heterologous protein in milk every day for the 305 day lactation cycle each year. Human hemoglobin is produced in pigs at 40 g/L. The negative points in production of proteins by transgenic animals are the length of time needed to assess production level. This takes 3.5 months in mice, 15 months in pigs, 28 months in sheep and 32 months in cows. The cost of upkeep of cows under good agricultural practices is $10,000 per cow per year. Nowadays scientists are trying to use protozoa such as trypanosomes, in place of transgenic animals, to produce recombinant proteins such as vaccines, lymphokines etc. The production of transgenic trypanosomes expressing heterologous proteins has several advantages over transgenic animals. Some are as follows-

- Stable and precisely targeted integration into the genome by homologous recombination.
- A choice of integration into several defined sites, allowing expression of multi-subunit complexes.
- Easy maintenance of cells in a semi-defined system.
- Medium and growth to high densities (N2×107 ml−1).

7) Transgenic plants

For production of recombinant protein, use of plants, comparatively to live animals and animal cell cultures, is much safer and less expensive, requires less time, and is superior in terms of storage and distribution issues. In terms of cost, protein complexity, storage and distribution plant expression systems are believed to be even better than microbes. Transgenic plants can be produced in two ways:

- Insert the desired gene into a virus that is normally found in plants, such as the tobacco mosaic virus (TMV)in the tobacco plant, cucumber mosaic virus(CMV).
- The second way is to insert the desired gene directly into the plant DNA. Potential disadvantages of transgenic plants include possible contamination with pesticides, herbicides, and toxic plant metabolites.

Advantages of transgenic plants as protein expression systems are as follows:

➢ Easy and cheap scale up ➢ Cost effective ➢ High level of accumulation of proteins in plant tissues ➢ Relatively simple and cheap protein purification	➢ Proper folding and assembly of protein complexes ➢ Can produce complex proteins ➢ Low risk of contamination with animal and pathogens ➢ Post translational modifications.

Recent trends of recombinant protein and their applications in the area of microbial biotechnology:

Since the beginning of the modern biotechnology era in the late 70s, *Escherichia coli* was proved to be only one best weapon extensively used

for protein overexpression due to its extreme ability to multiply rapidly under wide environmental conditions with some well accepted expression mechanism. But as the time is flowing, the optimization of recombinant protein expression in *E. coli* has been experimented in several ways on trial basis by modifying different parameters such as expression vectors, host strains, media composition, and growth temperature [51]. Recently with some advancement of host modifications, a number of successful experiments come into the lime light of scientific studies where replacement of codons within a heterologous gene in the expression host (codon optimization), nucleotide sequence manipulation at the translational initiation region are promising [52] and most significantly the conservative method to generate stable cell lines comprises the transfection of a plasmid containing a promoter which drives the expression of a gene of interest and an antibiotic marker for selection [53]. Here we are focusing some most promising innovative strategies in the way of advancement of synthesizing recombinant protein from microbial resources:

- **Generation of stable cell lines to maximize the multiplication of recombinant proteins:**

First most useful approach is random integration of a plasmid-based vector. The conventional expression vector consist a promoter, a gene of interest, a polyadenylation signal (pA) and a selection marker (e.g., neomycin) transfected into cells integrates randomly into the host cell genome (Figure 4A). Second is random integration of a plasmid-based expression vector flanked by "chromatin modifiers" (CM) that shield the expression vectors from the effects of the chromatin results better expression and stability of the transgene compared with first one (Figure 4B). Third important approach is targeted integration of an expression vector into a chromatin permissive region (hot spot) by using the techniques of recombinase-mediated cassette exchange or somatic homologous recombination (Figure 4C). And the fourth most effective approach is Random integration of a 200 kb BAC containing an expression vector with an open chromatin locus (e.g., Rosa26 locus) and there by transfection into the cells and random integration into the host cell genome (Figure 4D). Several copies of the BAC-based vector can be co-integrated, thus resulting in high and stable expression levels of the transgene.

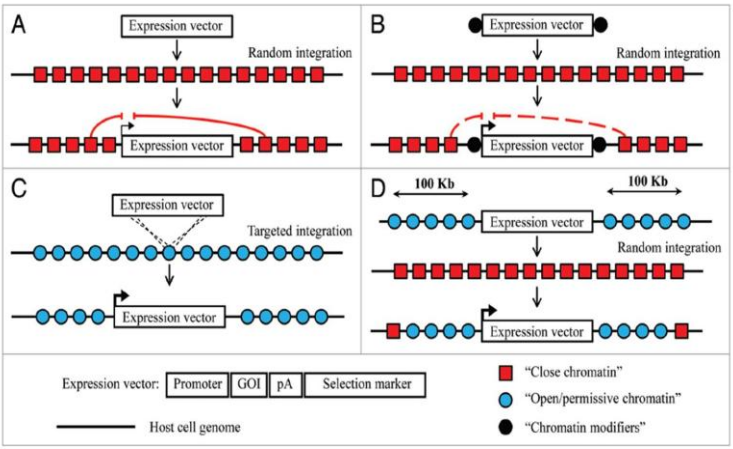


Figure 4. Four major strategies for generating stable cell lines to maximize expression of recombinant proteins (Source http://www.ncbi.nlm.nih.gov/pubmed/23680894).

- **Metabolic engineering strategies to enhance the flux through a pathway:**

There are various strategies used to improve the flux from a cellular intermediate to the desired product and these include enhancing pathways involving in the formation of rate limiting intermediates and pathways in addition to control the feedback and branched chain pathways by regulating switching on-off strategies. Genetic engineering approaches mostly focused on improving the first step of pathway i.e. transcription by improving vector design and subsequent enhancement of efficiency over time. With the help of strong promoters, the up regulated rates of transcription boost up the translational step for final over expression of recombinant protein. Moreover the supply of rate limiting factors in protein biosynthesis like ATP, amino acids and nucleotides act as critical precursors to influence pathway in positive way. Another critical impact is regulation of the flux through this pathway by minimizing the formation rates of end product incline sharply within a few hours post induction. Thus one way to increase recombinant protein synthesis would be to uncouple growth from product formation, therefore allowing the diversion of metabolic fluxes toward product formation. Moreover extracellular expression would significantly simplify downstream processing steps [54].

- **Strain engineering for improved expression of recombinant proteins in bacteria**

Genetic modifications can be performed into DNA in a specific manner within a specific cellular pathway known to be involved in protein biogenesis. Alternatively, in the case of poor expression with unknown cause, a library of random chromosomal gene fragments can be cloned and co-expressed with the target protein or, the entire genome may be incurred to random mutagenesis, subsequently isolate clones screening conferring increased protein production. Targeted strain engineering focuses on the imposing the mutations in DNA sequences known to affect protein synthesis, degradation, secretion or folding which can be classified with different approaches [51]:

- Acetylated protein production in *E. coli*.
- Engineering of mRNA stability and translational efficiency.
- Improving protein folding by chaperone co-expression.
- Expression of disulfide-bonded proteins.
- Glycoprotein production in *E. coli*.

- **High cell density cultivation approach for production of over expressed recombinant protein:**

High cell density cultivation (HCDC) is a powerful technique and offers an efficient means for the economical production of recombinant proteins and by which the annual market growth is expected to increase at a rate of 10-15% per annum. However, there are still some challenges associated with high cell density cultivation (HCDC) techniques. A number of approaches at various levels are available for increasing productivity in high cell density cultures which need strong information on genome, transcriptome and proteome levels for further progress in genetic engineering. Designing a suitable medium as well as nutrient strategy for supporting growth and the production phase is first and foremost step for easy availing of this technology. Optimizing physical conditions for enhancing mass and heat transfer and decreasing foam formation is a bottle neck in chemical engineering. Although, the effects of high cell density on *E. coli* metabolism has been studied, further investigations should be focused on understanding the fundamental cellular response of *E. coli* and other microorganisms to harsh conditions especially related to microbial recombinant protein production in high cell density cultures [55].

Future perspective of advancement of microbial recombinant protein and future prospective:

The emergence of recombinant DNA technology during the early 70's set a revolution in molecular biology. With the continuous improvement in recombinant DNA technology and protein engineering, recombinant protein has evolved as an important molecule that has been widely used in different industrial and therapeutical purposes. Their use and importance in research and in industry cannot be disputed. Microbial protein or enzymes are currently acquiring much attention with rapid development of enzyme technology. Microbial recombinant protein are preferred due to their economic feasibility, large production, consistency, easily product modification and optimization, continuous supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. There is no sign of deceleration in progress in the field of recombinant protein expression in microbial systems. Their establishment in the biotechnology toolkit was due to the ongoing efforts of researchers that continuously optimize well-known systems. This set of techniques was strengthened even further later on with the introduction of the polymerase chain reaction and allowed scientists to explore and understand essential life processes in an easy way. It also marked the birth of the modern biotech industry.

Making the transition to clinical production:

As development moves closer to commercialization, schedules, pricing, and other aspects of managing a supple chain become paramount. This transition from focusing on technical concerns to the context of the clinic and broader marketplace can be challenging. There are some points which can explain the future prospects of recombinant protein produced by microbes.

- The broadest future applications of microbial biotechnology are the production of pharmaceuticals, nutraceuticals by micro-organisms or bacteria that produce economically, clinically important products. Continuously we are generating new techniques which are being perfect to transfer human genes into cows, sheep, and goats to obtain many medically significant products from the milk of these animals. Development of diagnostics is to detect disease-causing organisms and monitor the safety of food and water quality.

- In future, investigators are trying to develop systems to identify pathogens which may be used as biological weapons by scoundrel nations or even terrorist groups in future.
- For the safety point of view researcher are trying to genetically modify bacteria which can emit a green fluorescent protein visible in ultraviolet light when they metabolize the explosive TNT leaking from land mines.
- Researchers imagine that one day when bacteria can be applied to an area of land with a crop duster and then they can be analyzed from a helicopter.
- Recombinant protein obtained from genetically modified microorganisms can be used a living sensor to detect any particular chemicals in soil, air or other inorganic or biological specimens. By using various biotechnological processes, genes can be added from other organisms that will confirm the ability to degrade toxicogenic chemicals such as toluene, commonly found in chemical and radiation waste sites.

Conclusion:

Microbes are omnipresent and have both beneficial and harmful effects on human health and environment but we are always trying to chase their deleterious impact by welcoming the advantageous entity from microbial world to sustain the life in better way. From the beginning of science, microbes have been utilized indirectly or directly to produce a numerous of primary and secondary products to benefit the mankind. With the arrival of genetic engineering, recombinant proteins took place in market resulting a drastically changes of development of the pharmaceutical industry. There after the concept of utilization of microbial genes and proteins with the help of rDNA technology appeared practically for huge production and now a day's microbial recombinant proteins are magnified each and every place of human world and some time confusing the terms whether they are 'micro' or 'macro' in terms of their applications. As discussed in this review, several approaches at different levels are available for increasing productivity of recombinant protein where we need not to upset about the initial little protein quantity from microbial resources. Information on genome, transcriptome and proteome levels is a great advantage for scientists and researchers to design and construct a well-engineered vector system or a well-adapted host for best over expression of a protein. A new technology platform that comes as a promising and driving major forces in the world in various fields microbial biotechnology called as 'synthetic

biology' and that is basically combination of all types of emerging technologies. This synthetic biology promises in futuristic way to make biological systems that perform in robotic manner as per human command, would be capable to solve chronic and complex acute diseases with the help of newly synthesized drugs and vaccines, can efficiently use the recombinant microbes for decreasing the effects of environmental pollution remarkably and development of recombinant microbial bioprocesses that can generate power significantly to solve the ever burning energy problem worldwide. The day is not so far when we can shut the door for 'bad' microbes totally until they get turned into 'good' microbes for taking part as a carrier for moving the life smoothly and they only come to us as a 'bless' not as a disease causing 'curse' to make a smile to each and every people in the world.

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