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Production of recombinant proteins by microbes and higher organisms

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ABSTRACT

Large proteins are usually expressed in a eukaryotic system 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*. However, this bacterium cannot express very large proteins. Also, for S–S rich proteins, and proteins that require post-translational modifications, *E. coli* is not the system of choice. The two most utilized yeasts are *Saccharomyces cerevisiae* and *Pichia pastoris*. Yeasts can produce high yields of proteins at low cost, proteins larger than 50 kD can be produced, signal sequences can be removed, and glycosylation can be carried out. The baculoviral system can carry out more complex post-translational modifications of proteins. The most popular system for producing recombinant mammalian glycosylated proteins is that of mammalian cells. Genetically modified animals secrete recombinant proteins in their milk, blood or urine. Similarly, transgenic plants such as *Arabidopsis thaliana* and others can generate many recombinant proteins.

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Contents

1. Introduction . . . 297
2. Enzyme production . . . 298
3. Systems for producing recombinant proteins . . . 298
 3.1. Bacteria . . . 299
  3.1.1. *E. coli* . . . 299
  3.1.2. *Bacillus* . . . 300
  3.1.3. Other bacteria . . . 300
 3.2. Yeasts . . . 300
 3.3. Filamentous fungi (molds) . . . 302
 3.4. Insect cells . . . 302
 3.5. Mammalian cells . . . 302
 3.6. Transgenic animals . . . 303
 3.7. Transgenic plants . . . 304
4. Conclusions . . . 304
References . . . 305

1. Introduction

Proteins, the building blocks of life, are synthesized by all living forms as part of their natural metabolism. Some proteins, such as enzymes, serve as biocatalysts and increase the rate of metabolic reactions, while others form the cytoskeleton. Proteins play a significant role in cell signaling, immune responses, cell adhesion,

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Seikagaku Corporation Exhibit 2110 - Page 1 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

and the cell cycle. They are commercially produced in industries with the aid of genetic engineering and protein engineering. Native and recombinant proteins benefit major sectors of the biopharmaceutical industry, the enzyme industry, and the agricultural industry. Products of these industries in turn augment the fields of medicine, diagnostics, food, nutrition, detergents, textiles, leather, paper, pulp, polymers and plastics. The first protein vaccine produced was the cow-pox vaccine by Jenner in 1796. The microbial fermentation industry was born in the early 1900s when the first large-scale anaerobic fermentations to manufacture chemicals such as acetone and butanol began, followed by the aerobic production of citric acid. Penicillin was discovered in 1927 but its development did not occur until the start of the 1940s, prior to the time that streptomycin was discovered. The first protein pharmaceutical produced was insulin by Banting and Best in 1922. The modern biotechnology era began in 1971 with the establishment of the Cetus Corporation in California about 1–2 years before the discovery of recombinant DNA by Berg, Cohen and Boyer in California. This was followed 5 years later by the start of Genentech, and then by other corporations such as Amgen and Biogen, etc.

By 2002, over 155 approved pharmaceuticals and vaccines had been developed by biopharmaceutical companies. Today, more than 200 approved peptide and protein pharmaceuticals are on the FDA list. Some of the recombinant protein pharmaceuticals produced are human insulin, albumin, human growth hormone (HGH), Factor VIII, and many more. Biopharmaceuticals have been instrumental in radically improving human health (Swartz, 1996): (i) diabetics no longer have to fear producing antibodies to animal insulin; (ii) children deficient in growth hormone no longer have to suffer from dwarfism or fear the risk of contracting Kreutzfeld–Jacob syndrome; (iii) children who have chronic granulomatous disease can lead a normal life by taking gamma interferon therapy; and (iv) patients undergoing cancer chemotherapy or radiation therapy can recover more quickly with fewer infections when they use granulocyte colony-stimulating factor (G-CSF). Many other examples of the conquest of disease could be mentioned.

2. Enzyme production

The enzyme industry flourished in the 1980s and 1990s when microbial enzymes came onto the scene. In the 1970s, most of the enzymes used were traditionally derived from plant and animal sources, which resulted in a low level of availability, high prices, and stunted growth of the enzyme industry. Microbial enzymes proved economically favorable since cultivation of microbes was much simpler and faster than that of plants and animals and the producing organisms could be easily manipulated genetically to produce desired qualities and quantities of enzymes. Some of the major industrial uses of enzymes in manufacturing include (1) *Escherichia coli* amidase to produce 6-aminopenicillanic acid (6-APA) at 40,000 tons/year; (2) *Streptomyces* xylose isomerase to isomerize D-glucose to D-fructose at 100,000 tons/year; and (3) *Pseudomonas chlorapis* nitrile hydratase to produce acrylamide from acrylonitrile at 30,000 tons/year (Jaeger et al., 2002). Amylases are produced at an annual rate of 95,000 tons per year. The total market for industrial enzymes reached $2 billion in 2000 and has risen to $2.5 billion today. The leading enzyme is protease which accounts for 57% of the market. Others include amylase, glucoamylase, xylose isomerase, lactase, lipase, cellulase, pullulanase and xylanase. The food and feed industries are the largest customers for industrial enzymes. Over half of the industrial enzymes are made by yeasts and molds, with bacteria producing about 30%. Animals provide 8% and plants 4%. Enzymes also play a key role in catalyzing reactions which lead to the microbial formation of antibiotics and other secondary metabolites.

Over the years, higher titers of enzymes were obtained using "brute force" mutagenesis and random screening of microorganisms. Recombinant DNA technology acted as a boon for the enzyme industry in the following ways (Falch, 1991): (i) plant and animal enzymes could be made by microbial fermentations, e.g., chymosin; (ii) enzymes from organisms difficult to grow or handle genetically were now produced by industrial organisms such as species of *Aspergillus* and *Trichoderma*, and *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Bacillus licheniformis* (e.g., thermophilic lipase was produced by *Aspergillus oryzae* and *Thermoanaerobacter* cyclodextrin glycosyl transferase by *Bacillus*); (iii) enzyme productivity was increased by the use of multiple gene copies, strong promoters and efficient signal sequences; (iv) production of a useful enzyme from a pathogenic or toxin-producing species could now be done in a safe host; and (v) protein engineering was employed to improve the stability, activity and/or specificity of an enzyme.

By the 1990s, many enzymes were produced by recombinant techniques. In 1993, over 50% of the industrial enzyme market was provided by recombinant processes (Hodgson, 1994); sales were $140 million (Stroh, 1994). Plant phytase, produced in recombinant *Aspergillus niger* was used as a feed for 50% of all pigs in Holland. A 1000-fold increase in phytase production was achieved in *A. niger* by the use of recombinant technology (Van Hartingsveldt et al., 1993). Industrial lipases were cloned in *Humicola* and industrially produced by *A. oryzae*. They are used for laundry cleaning, inter-esterification of lipids and esterification of glucosides, producing glycolipids which have applications as biodegradable non-ionic surfactants for detergents, skin care products, contact lenses and as food emulsifiers. Mammalian chymosin was cloned and produced by *A. niger* or *E. coli* and recombinant chymosin was approved in the USA; its price was half that of natural calf chymosin. Over 60% of the enzymes used in the detergent, food and starch processing industries were recombinant products as far back as the mid-1990s (Cowan, 1996).

Today, with the aid of recombinant DNA technology and protein engineering, enzymes can be tailor-made to suit the requirements of the users or of the process. It is no longer necessary to settle for an enzyme's natural properties. Enzymes of superior quality have been obtained by protein engineering, specifically by site-directed mutagenesis. Single changes in amino acid sequences yielded changes in pH optimum, thermostability, feedback inhibition, carbon source inhibition, substrate specificity, Vmax, K_m and K_i. A new and important method for improving enzymes was directed evolution (also known as applied molecular evolution or directed molecular evolution) (Kuchner and Arnold, 1997; Arnold, 1998; Johannes and Zhao, 2006). Unlike site directed mutagenesis, this method of pooling and recombining parts of similar genes from different species or strains yields remarkable improvements in enzymes in a very short amount of time. The procedure actually mimics nature in that mutation, selection and recombination are used to evolve highly adapted proteins, but it is much faster than nature. The technique can be used to improve protein pharmaceuticals, small molecule pharmaceuticals, gene therapy, DNA vaccines, recombinant protein vaccines, viral vaccines and to evolve viruses. Proteins from directed evolution work were already on the market in 2000 (Tobin et al., 2000).

Many enzymes are used as therapeutic agents to treat gastrointestinal and rheumatic diseases, thromboses, cystic fibrosis, metabolic disease and cancer. Sales of therapeutic enzymes were $2.3 billion in 1996 while in 1998 markets for therapeutic enzymes were as follows (Stroh, 1999): Pulmozyme (DNase) for cystic fibrosis, acute myocardial infarction and ischemic stroke, $350 million; Ceredase® and Cerezyme® (r-DNA version) for Gaucher's disease, $387 million. By 2007, the market for Cerezyme® reached $1.1 billion. The therapeutic market is in addition to the industrial enzyme market discussed above.

3. Systems for producing recombinant proteins

By means of genetic engineering, desired proteins are massively generated to meet the copious demands of industry. Hence, most

Seikagaku Corporation Exhibit 2110 - Page 2 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

biopharmaceuticals produced today are recombinant. The first step to recombinant protein production is getting the desired DNA cloned; then the protein is amplified in the chosen expression system. There is a wide variety of protein expression systems available. Proteins can be expressed in cell cultures of bacteria, yeasts, molds, mammals, plants or insects, or via transgenic plants and animals. Protein quality, functionality, production speed and yield are the most important factors to consider when choosing the right expression system for recombinant protein production.

As of 2002, there were about 140 therapeutic proteins approved in Europe and the USA (Walsh, 2003). Non-glycosylated proteins are usually made in *E. coli* or yeasts and they constitute 40% of the therapeutic protein market. N-glycosylated proteins are usually made in mammalian cells which mimic human glycosylation. Chinese hamster ovary (CHO) cells provide about 50% of the therapeutic protein market but the process is very expensive and the glycoproteins made are not exactly the human type, and in some cases, they must be modified. Yeasts, molds and insect cells are generally unable to provide mammalian glycosylation. However, the popular methylotrophic yeast, *Pichia pastoris*, has been genetically engineered to produce a human type of glycosylation (see below).

3.1. Bacteria

3.1.1. E. coli

E. coli is one of the earliest and most widely used hosts for the production of heterologous proteins (Terpe, 2006). Advantages and disadvantages are shown in Table 1. These include rapid growth, rapid expression, ease of culture and high product yields (Swartz, 1996). It is used for massive production of many commercialized proteins. This system is excellent for functional expression of non-glycosylated proteins. *E. coli* genetics are far better understood than those of any other microorganism. Recent progress in the 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. Its genome can be quickly and precisely modified with ease, promotor control is not difficult, and plasmid copy number can be readily altered. This system also features alteration of metabolic carbon flow, avoidance of incorporation of amino acid analogs, formation of intracellular disulfide bonds, and reproducible performance with computer control. *E. coli* can accumulate recombinant proteins up to 80% of its dry weight and survives a variety of environmental conditions.

The *E. coli* system has some drawbacks, however, which have to be overcome for efficient expression of proteins. High cell densities result in toxicity due to acetate formation; however, this can be avoided by controlling the level of oxygen. Proteins which are produced as inclusion bodies are often inactive, insoluble and require refolding. In addition, there is a problem producing proteins with many disulfide bonds and refolding these proteins is extremely difficult. The *E. coli* system produces unmodified proteins without glycosylation which is the reason why some produced antibodies fail to recognize mammalian proteins (Jenkins and Curling, 1994). Surprisingly, the non-glycosylated human tPA produced in *E. coli* was fully active in vitro (Sarmientos et al., 1989). Despite the lack of the usual tPA glycosylation, the product had a four-fold longer half-life in plasma and a corresponding longer clearance rate in animals (Dartar et al., 1993). The amount produced was 5–10% of total *E. coli* protein.

Table 1
Characteristics of *E. coli* expression systems

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

To improve the *E. coli* process situation, the following measures have been taken: (i) use of different promoters to regulate expression; (ii) use of different host strains; (iii) co-expression of chaperones and/or foldases; (iv) lowering of temperature; (v) secretion of proteins into the periplasmic space or into the medium; (vi) reducing the rate of protein synthesis; (vii) changing the growth medium; (viii) addition of a fusion partner; (ix) expression of a fragment of the protein; and (x) in vitro denaturation and refolding of the protein (Swartz, 2001; Choi and Lee, 2004; Mergulhao et al., 2005; Shiloach and Fass, 2005; Maldonado et al., 2007; Chou, 2007; Wong et al., 2008).

High cell density fermentations of *E. coli* have resulted in dry cell contents of 20 to 175 g/l (Lee, 1996). The acetate production and toxicity problem can be solved by feeding glucose exponentially, and keeping the specific growth rate below that which brings on acetate production. In this way, yields as high as 5.5 g/L of α-consensus interferon in broth were attained (Fieshko, 1989). Growth in a long-term chemostat (219 generations under the low dilution rate of 0.05 h^{-1}) yielded an *E. coli* mutant that had an increased specific growth rate, increased biomass yields, shorter lag phase, less acetate production and increased resistance to stress (Weikert et al., 1997). This strain produced increased levels of secreted heterologous proteins (Weikert et al., 1998).

Heterologous proteins produced as inclusion bodies in *E. coli* are inactive, aggregated and insoluble, usually possessing non-native intra- and inter-molecular disulfide bonds and unusual free cysteines (Fischer et al., 1993). To obtain active protein, these bodies must be removed from the cell, the proteins solubilized by denaturants which unfold the proteins, and disulfide bonds must be eliminated using reducing agents. Refolding is accomplished by the removal of the denaturant and the reducing agent, followed by renaturation of the protein. Renaturation processes used include (i) air oxidation, (ii) the glutathione reoxidation system, and (iii) the mixed disulfides of protein-S-sulfonate and protein-S-glutathione system. Heterologous recombinant proteins can be made in biologically active soluble form at high levels when their genes are fused to the *E. coli* thioredoxin gene (LaVallie et al., 1993). Murine IL-2, human IL-3, murine IL-4, murine IL-5, human IL-6, human M1P-l alpha, human IL-11, human M-CSL, murine L1F, murine SF and human BMP-2 are produced at levels of 5–20% of total proteins as fusions in *E. coli* cytoplasm. Some fusions retain the thioredoxin properties of being released by osmotic shock or freeze/thaw methods, and high thermal stability. Thioredoxin is small (11 kD) and is normally produced at 40% of total cell protein in soluble form (Lunn et al., 1984). Another useful method of reducing the formation of inclusion bodies containing heterologous proteins is to lower the temperature of growth from 37 °C to 30 °C (Schein, 1989).

Higher yields are normally produced in the cytoplasm than in the periplasmic space. Cytoplasmic proteins can be exported to simplify purification and facilitate correct folding. This must be done with proteins containing disulfide bonds since the cytoplasm is too reducing an environment. To secrete these proteins into the periplasm, a fusion is made with a leader peptide at the N-terminus. To get the proteins out of the periplasm and into the medium, osmotic shock or cell wall permeabilization is used. To increase production, a promoter system (*lac*, *tac*, *trc*) is used. Promoter systems must be strong and tightly regulated so that they have a low-basal level of expression, easily transferable to other *E. coli* strains, and have a simple and inexpensive induction technique, independent of media ingredients.

Secretion of recombinant proteins by *E. coli* into the periplasm or into the medium has many advantages over intracellular production as inclusion bodies. It helps downstream processing, folding and in vivo stability, and allows the production of soluble, active proteins at a reduced processing cost (Mergulhao et al., 2005). High level excretion

Seikagaku Corporation Exhibit 2110 - Page 3 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

Table 2
Advantages of *Bacillus* expression systems

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
Efficient and cost effective recovery

has been obtained with the following heterologous proteins: PhoA (alkaline phosphatase) at 5.2 g/L into the periplasm; LFT (levan fructotransferase) at 4 g/L into the medium; hGCSF (human granulocyte colony-stimulatory factor) at 3.2 g/L into the periplasm; cellulose binding domain at 2.8 g/L into the periplasm; IGF-1 (insulin-like growth factor) at 2.5 g/L into the periplasm; cholera toxin B at 1 g/L into the medium (Mergulhao et al., 2005). As early as 1993, recombinant processes in *E. coli* were responsible for almost $5 billion worth of products, i.e., insulin, human growth hormone, α, β, γ-interferons and G-CSF (Swartz, 1996).

3.1.2. Bacillus

Other useful bacterial systems are those of the Gram-positive bacilli. These are mainly preferred for homologous expression of enzymes such as proteases (for detergents) and amylases (for starch and baking). Some advantages of using *Bacillus* systems are shown in Table 2. Some of these advantages are only present in industrial strains which are often unavailable to academic researchers. In addition, the genomes of *Bacillus subtilis* and *B. licheniformis* have been sequenced, and there is no production of harmful exotoxins or endotoxins. The secretion of the desired proteins into the fermentation medium results in easy downstream processing, eliminating the need for cell disruption or chemical processing techniques. This makes recovery relatively efficient and cost-effective. The species generally used for expression are *Bacillus megaterium*, *B. subtilis*, *B. licheniformis* and *Bacillus brevis*. They do not have lipopolysaccharide-containing outer membranes as do Gram-negative bacteria. Industrial strains of *B. subtilis* are high secretors and host strains used for successful expression of recombinant proteins are often deleted for genes *amy*E, *apr*E, *npr*E, *spo*IIAC, *srf*C and transformed via natural competence. *Bacillus* protein yields are as high as 3 g/L.

There is a problem with *B. subtilis* because of its production of many proteases which sometimes destroy the recombinant proteins. They include seven known proteases (He et al., 1991), five of which are extracellular:

(i) Subtilisin (*aprE* gene): major alkaline serine protease.
(ii) Neutral protease (*nprE*): major metalloprotease, contains Zn.
(iii) Minor serine protease (*epr*); inhibited by phenylmethanesulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA).
(iv) Bacillopeptidase F (*bpf*): another minor serine protease/esterase; inhibited by PMSF.
(v) Minor metalloesterase (*mpe*).
(vi) ISP-I (*isp-I*): major intracellular serine protease, requires Ca.
(vii) ISP-II (*isp-II*): minor intracellular serine protease.

The first two enzymes account for 96–98% of the extracellular protease activity. Other research groups have reported six to eight extracellular proteases. Wu et al. (1991) removed six and only 0.32% activity remained. Growth in the presence of 2 mM PMSF eliminated all the protease activity. A *B. subtilis* strain has been developed for genetic engineering which is deficient in eight extracellular proteases (Murashima et al., 2002). Care has to be taken with regard to excessive growth rates and aeration. Production of extracellular human alpha interferon by *B. subtilis* is repressed by high growth rate and by excess oxygen (Meyer and Fiechter, 1985).

An exoprotease-deficient *B. licheniformis* host strain has been specifically tailored for heterologous gene expression. It is asporogenous and gives high extracellular expression levels with minimal loss of product due to proteolytic cleavage subsequent to secretion. To obtain a more genetically stable system after transformation and to increase production levels, the α-amylase gene has also been removed. A comparison of host organisms was made for production of interleukin-3 (van Leen et al., 1991) among *E. coli*, *B. licheniformis*, *S. cerevisiae*, *K. lactis* and C127 mammalian cells. The best system was reported to be *B. licheniformis*.

B. brevis is also used to express heterologous genes due to its much lower protease activity and production of a proteinase inhibitor (Udaka and Yamagata, 1994). Human epidermal growth factor was produced in *B. brevis* at a level of 3 g/L (Ebisu et al., 1992).

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

3.1.3. Other bacteria

An improved Gram-negative host for recombinant protein production has been developed using *Ralstonia eutropha* (Barnard et al., 2004.) The system appears superior to *E. coli* with respect to inclusion body formation. 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*. The *Pf*enex system using *Pseudomonas fluorescens* has yielded 4 g/L of trimeric TNF-alpha (Squires and Lucy, 2008). *Staphylococcus carnosus* can produce 2 g/L of secreted mammalian protein whereas the level made by *Streptomyces lividans* is 0.2 g/L (Hansson et al., 2002).

3.2. Yeasts

Yeasts, the single-celled eukaryotic fungal organisms, are often used to produce recombinant proteins that are not produced well in *E. coli* because of problems dealing with folding or the need for glycosylation. The major advantages of yeast expression systems are listed in Table 3. The yeast strains are genetically well characterized and are known to perform many posttranslational modifications. They are easier and less expensive to work with than insect or mammalian cells, and are easily adapted to fermentation processes. The two most utilized yeast strains are *S. cerevisiae* and the methylotrophic yeast *P. pastoris*. Various yeast species have proven to be extremely 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.

S. cerevisiae offers certain advantages over bacteria as a cloning host (Gellison et al., 1992). (i) It has a long history of use in industrial fermentation. (ii) It can secrete heterologous proteins into the

Table 3
Advantages of yeast expression systems

High yield
Stable production strains
Durability
Cost effective
High density growth
High productivity
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

Seikagaku Corporation Exhibit 2110 - Page 4 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

extracellular broth when proper signal sequences have been attached to the structural genes. (iii) It carries out glycosylation of proteins. However, glycosylation by *S. cerevisiae* is often unacceptable for mammalian proteins because the O-linked oligosaccharides contain only mannose whereas higher eukaryotic proteins have sialylated O-linked chains. Furthermore, the yeast over-glycosylates N-linked sites leading to reduction in both activity and receptor-binding, and may cause immunological problems. Products on the market which are made in *S. cerevisiae* are insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor.

Almost all excreted eukaryotic polypeptides are glycosylated. Glycosylation is species-, tissue- and cell-type-specific (Parekh, 1989). In some cases, a normally glycosylated protein is active without the carbohydrate moiety and can be made in bacteria. This is the case with γ-interferon (Rinderknecht et al., 1984). In cases where glycosylation is necessary for stability or proper folding (e.g., erythropoietin and human chorionic gonadotropin), this can often be provided by recombinant yeast, mold, insect or mammalian cells. Mammalian secreted proteins are glycosylated with D-mannose sugars covalently bound to asparagine-linked N-acetyl-D-glucosamine molecules. Fungal enzymes which are excreted often show the same type of glycosylation (Elbein and Molyneux, 1985), although additional carbohydrates linked to the oxygen of serine or threonine sometimes are present in fungal proteins (Nunberg et al., 1984).

The glycosylation of a protein can be different depending on factors such as the medium in which the cells are grown. The glycosylation influences 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 (Warren, 1990). That glycosylation increases the stability of proteins, is shown by cloning genes encoding bacterial non-glycosylated proteins in yeast. The yeast versions were glycosylated and more stable (Dixon, 1991). Glycosylation also affects pharmacokinetics (residence time in vivo) (Jenkins and Curling, 1994). Examples of stability enhancement are the protection against proteolytic attack by terminal sialic acid on erythropoietin (EPO) (Goldwasser et al., 1974), Tissue Plasminogen Activator (TPA) (Wittwer and Howard, 1990) and interferons (Cantell et al., 1992). With regard to activity, human EPO is 1000-fold more active *in vivo* than its desialylated form but they both have similar *in vitro* activities (Yamaguchi et al., 1991). Glycosylation occurs through (i) an N-glycosidic bond to the R-group of an asparagine residue in a sequence Asn-X-Ser/Thr; or (ii) an O-glycosidic bond to the R-group of serine, threonine, hydroxproline or hydroxylysine. However, these amino acids may only be partially glycosylated or unglycosylated leading to the problem of heterogeneity. In the future, cloned glycosyl transferases will be used to ensure homogeneity ("glycosylation engineering").

Methylotrophic yeasts have become very attractive as hosts for the industrial production of recombinant proteins since the promoters controlling the expression of these genes are among the strongest and most strictly regulated yeast promoters. The cells themselves can be grown rapidly to high densities, and the level of product expression can be regulated by simple manipulation of the medium. Methylotrophic yeasts can be grown to a density as high as 130 g/L (Gellison et al., 1992). The four known genera of methylotrophic yeast (*Hansenula*, *Pichia*, *Candida*, and *Torulopsis*) share a common metabolic pathway that enables them to use methanol as a sole carbon source. In a transcriptionally regulated response to methanol induction, several of the enzymes are rapidly synthesized at high levels.

The major advantage of *Pichia* over *E. coli* is that the former is capable of producing disulfide bonds and glycosylation of proteins. This means that in cases where disulfides are necessary, *E. coli* might produce a misfolded protein, which is usually inactive or insoluble. Compared to other expression systems such as S2-cells from *Drosophila melanogaster* or Chinese Hamster Ovary (CH0) cells, *Pichia* usually gives much better yields. Cell lines from multicellular organisms usually require complex (rich) media, thereby increasing the cost of protein production process. Additionally, since *Pichia* can grow in media containing only one carbon source and one nitrogen source, it is suitable for isotopic labelling applications in e.g. protein NMR. An advantage of the methylotroph *P. pastoris*, as compared to other yeasts in making recombinant proteins, is its great ability to secrete proteins. Success has been achieved in genetically engineering the *P. pastoris* secretory pathway so that human type N-glycosylated proteins are produced (Choi et al., 2003). Among the advantages of methylotrophic yeasts over *S. cerevisiae* as a cloning host are the following: (i) higher protein productivity; (ii) avoidance of hyperglycosylation; (iii) growth in reasonably strong methanol solutions that would kill most other microorganisms, (iv) a system that is cheap to set up and maintain, and (v) integration of multicopies of foreign DNA into chromosomal DNA yielding stable transformants (Gellison et al., 1992).

Glycosylation is less extensive in *P. pastoris* than in *S. cerevisiae* (Dale et al., 1999) due to shorter chain lengths of N-linked high-mannose oligosaccharides, usually up to 20 residues compared to 50–150 residues in *S. cerevisiae*. *P. pastoris* also lacks α-1, 3-linked mannosyl transferase which produces α-1, 3-linked mannosyl terminal linkages in *S. cerevisiae* and causes a highly antigenic response in patients. Hirudin, a thrombin inhibitor from the medicinal leech, *Hirudo medicinalis* is now made by recombinant yeast (Sohn et al., 2001). Productivities of hirudin in different systems are shown in Table 4.

P. pastoris produces high levels of mammalian recombinant proteins in the extracellular medium. An insulin precursor was produced at 1.5 g/L (Wang et al., 2001). Other reports include 4 g/L of intracellular interleukin 2 as 30% of protein, 4 g/L of secreted human serum albumin (Cregg et al., 1993), 6 g/L of tumor necrosis factor (Dale et al., 1999) and other heterologous proteins (Macauly-Patrick et al., 2005), and 10 g/L of tumor necrosis factor (Sreekrishana et al., 1989). Production of serum albumin in *S. cerevisiae* amounted to 0.15 g/L whereas in *P. pastoris*, the titer was 10 g/L (Nevalainen et al., 2005). Gelatin has been produced in *P. pastoris*, at over 14 g/L (Werten et al., 1999). *P. pastoris* yielded 300 mg/l/day of recombinant human chitinase (Goodrick et al., 2001). Intracellular tetanus toxin fragment C was produced as 27% of protein with a titer of 12 g/L (Clare et al., 1991). Claims have been made that *P. pastoris* can make 20–30 g/l of recombinant proteins (Morrow, 2007).

There are however, some disadvantages of using *Pichia* as a host for heterologous expression. A number of proteins require chaperonins for proper folding. *Pichia* is unable to produce such proteins. A group led by Gerngross managed to create a strain that produces EPO in its normal human glycosylation form (Gerngross, 2004; Hamilton et al., 2006). This was achieved by exchanging the enzymes responsible for the yeast type of glycosylation, with the mammalian homologs. Thus, the altered glycosylation pattern allowed the protein to be fully functional in humans and since then, this human glycosylation of recombinant proteins made in the engineered *P. pastoris* has been shown with other human proteins.

Heterologous gene expression in another methylotroph *Hansenula polymorpha* yielded 1 g/L of intracellular hepatitis B S-antigen (50 gene copies/cell), 1.4 g/L of secreted glucoamylase (4 copies/cell), and

Table 4
Comparison of productivities of hirudin by recombinant hosts

Recombinant hosts	mg/L
BHK cells	0.05
Insect cells	0.40
Streptomyces lividans	0.25–0.5
Escherichia coli	200–300
Saccharomyces cerevisiae	40–500
Hansenula polymorpha	1500
Pichia pastoris	1500

Seikagaku Corporation Exhibit 2110 - Page 5 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

13.5 g/L of phytase. Secreted mammalian proteins can be made at 3 g/L by *K. lactis*.

3.3. Filamentous fungi (molds)

Filamentous fungi such as *A. niger* are attractive hosts for recombinant DNA technology because of their ability to secrete high levels of bioactive proteins with post-translational processing such as glycosylation. *A. niger* excretes 25 g/L of glucoamylase (Ward et al., 2006). Foreign genes can be incorporated via plasmids into chromosomes of the filamentous fungi where they integrate stably into the chromosome as tandem repeats providing superior long-term genetic stability. As many as 100 copies of a gene have been observed. Trichoderma reesei has been shown to glycosylate in a manner similar to that in mammalian cells (Salovouri et al., 1987).

The titer of a genetically-engineered bovine chymosin-producing strain of *Aspergillus awamori* was improved 500% by conventional mutagenesis and screening (Lamsa and Bloebaum, 1990). It was then increased from 250 mg/L to 1.1 g/L by nitrosoguanidine mutagenesis and selection for 2-deoxyglucose resistance (Dunn-Coleman et al., 1991, 1993). Transformants contained 5–10 integrated copies of the chymosin gene. Production of human lactoferrin by *A. awamori* via rDNA technology and classical strain improvement amounted to 2 g/L of extracellular protein (Ward et al., 1995). *A. niger* glucoamylase was made by *A. awamori* at 4.6 g/L. Humanized immunoglobulin full length antibodies were produced and secreted by *A. niger*. The monoclonal antibody Trastazumab was secreted at 0.9 g/L (Ward et al., 2004). Recombinant *A. oryzae* can produce 2 g/L of human lactoferrin (Ward et al., 1995) and 3.3 g/L of *Mucor* rennin (Christensen et al., 1988). *Fusarium* alkaline protease is produced by *Acremonium chrysogenum* at 4 g/L. Recombinant enzyme production has reached 35 g/L in *T. reesei* (Durand and Clanet, 1988). The fungus *Chrysosporium lucknowense* has been genetically converted into a non-filamentous, less viscous, low protease-producing strain that is capable of producing very high yields of heterologous proteins (Verdoes et al., 2007). Dyadic International Inc., the company responsible for the development of the *C. lucknowense* system, claims protein production levels of up to 100 g/L of protein.

Despite the above successes, secreted yields of some heterologous proteins have been comparatively low in some cases. The strategies for yield improvement have included use of strong homologous promoters, increased 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. Such approaches have been effective with some target heterologous proteins but not with others. Hence, although there has been an improvement in the production of fungal proteins by recombinant DNA methods, there are usually transcription limitations (Verdoes et al., 1995). Although an increase in gene copies up to about five usually results in an equivalent increase in protein production, higher numbers of gene copies do not give equivalently high levels of protein. Since the level of mRNA correlates with the level of protein produced, transcription is the main problem. Studies on overproduction of glucoamylase in *A. niger* indicate the problem in transcription to be due to (i) the site of integration of the introduced gene copies and (ii) the available amount of *trans*-acting regulatory proteins. Also, heterologous protein production by filamentous fungi is sometimes severely hampered by fungal proteases. *Aspergillus nidulans* contains about 80 protease genes (Machida, 2002).

Table 5
Advantages of baculoviral infected insect cell expression system

Post translational modifications
Proper protein folding
High expression levels
Easy scale up
Safety
Flexibility of protein size
Efficient cleavage of signal peptides
Multiple genes expressed simultaneously

3.4. Insect cells

Insect cells (Table 5) are able to carry out more complex post-translational modifications than can be accomplished with fungi. They also have the best machinery for the folding of mammalian proteins and are therefore quite suitable for making soluble protein of mammalian origin (Agathos, 1991). The most commonly used vector system for recombinant protein expression in insects is the baculovirus. The most widely used baculovirus is the 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. A larval culture can be used which is much cheaper than a mammalian cell culture. Recombinant insect cell cultures have yielded over 200 proteins encoded by genes from viruses, bacteria, fungi, plants and animals (Knight, 1991). The baculovirus-assisted insect cell expression offers many advantages, as follows. (i) Eukaryotic post-translational modifications without complication, including phosphorylation, N- and O-glycosylation, correct signal peptide cleavage, proper proteolytic processing, acylation, palmitylation, myristylation, amidation, carboxymethylation, and prenylation (Luckow and Summers, 1988; Miller, 1988). (ii) Proper protein folding and S–S bond formation, unlike the reducing environment of *E. coli* cytoplasm. (iii) High expression levels. The virus contains a gene encoding the protein polyhedrin which is made at very high levels normally and is not necessary for virus replication. The gene to be cloned is placed under the strong control of the viral polyhedrin promoter, allowing expression of heterologous protein of up to 30% of cell protein. Production of recombinant proteins in the baculovirus expression vector system in insect cells reached 600 mg/L in 1988 (Maiorella and Harano, 1988). Recent information indicates that the baculovirus insect cell system can produce 11 g/L of recombinant protein (Morrow, 2007). (iv) Easy scale up with high-density suspension culture. (v) Safety; expression vectors are prepared from the baculovirus which can attack invertebrates but not vertebrates or plants, thus insuring safety. (vi) Lack of limit on protein size. (vii) Efficient cleavage of signal peptides. (viii) Simultaneous expression of multiple genes (Wilkinson and Cox, 1998).

Insect cell systems however, do have some shortcomings, some of which can be overcome. (i) Particular patterns of post-translational processing and expression must be empirically determined for each construct. (ii) Differences in proteins expressed by mammalian and baculovirus-infected insect cells. For example, inefficient secretion from insect cells may be circumvented by the addition of insect secretion signals (e.g., honeybee melittin sequence). (iii) Improperly folded proteins and proteins that occur as intracellular aggregates are sometimes formed, possibly due to expression late in the infection cycle. In such cases, harvesting cells at earlier times after infection may help. (iv) Low levels of expression. This can often be increased with optimization of time of expression and multiplicity of infection. (v) Incorrect glycosylation has been a problem with insect cells as hosts (Bisbee, 1993). The complete analysis of carbohydrate structures has been reported for a limited number of glycoproteins. Potential N-linked glycosylation sites are often either fully glycosylated or not glycosylated at all, as opposed to expression of various glycoforms that may occur in mammalian cells. Species-specific or tissue-specific modifications are unlikely to occur.

3.5. Mammalian cells

Mammalian expression systems are often used for production of proteins requiring mammalian post-translational modifications. The use of mammalian cell culture, chiefly immortalized Chinese hamster

Seikagaku Corporation Exhibit 2110 - Page 6 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

ovary (CHO) cells, began because of the need for erythropoietin (EPO) and tissue plasminogen activator (tPA) production in the early days of the biopharmaceutical effort, i.e., in the 1980s (Swartz, 1996). These glycosylated proteins could not be produced in *E. coli* at that time. CHO cells constitute the preferred system for producing monoclonal antibodies or recombinant proteins. Other cell types include (i) various mouse myelomas such as NS0 murine myeloma cells (Andersen and Krummen, 2002), (ii) SF-9, an insect cell line, (iii) baby hamster kidney (BHK) cells for production of cattle foot-and-mouth disease vaccine, (iv) green monkey kidney cells for polio vaccine (Wrotnowski, 1998) and (v) human cell lines such as human embryonic kidney (HEK) cells. NSO is a nonsecreting subclone of the NS-1 mouse melanoma cell line. In 1997, sales of biotherapeutics produced by cell culture were $3.25 billion whereas *E. coli* based biotherapeutics amounted to $2.85 billion (Langer, 1999). By 2006, production of therapeutic proteins by mammalian systems reached $20 billion (Griffin et al., 2007).

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. Eukaryotic cells are also useful for addition of fatty acid chains and for phosphorylating tyrosine, threonine and serine hydroxyl groups (Qiu, 1998). Mammalian cells have high productivity of 20–60 pg/cell/day. Human tPA was produced in CHO cells at 34 mg/L with an overall yield of 47%. Although production in *E. coli* was at a much higher level (460 mg/L), recovery was only 2.8% due to production as inclusion bodies and low renaturation yields (Dartar et al., 1993). Genes for the glycosylated fertility hormones, human chorionic gonadotropin, and human luteinizing hormone have been cloned and expressed in mammalian cells. Recombinant protein production in mammalian cells rose from 50 mg/L in 1986 to 4.7 g/L in 2004 mainly due to media improvements yielding increased growth (Aldridge, 2006). A titer of 2.5–3 g/L protein in 14 day CHO fed batch shake flask culture was achieved using $Fe_2(SeO_3)_3$ as ion carrier (Zhang et al., 2006). A number of mammalian processes are producing 3–5 g/L and, in some cases, protein titers have reached 10 g/L in industry (Ryll, 2008). A rather new system is that of a human cell line known as PER.C6 of Crucell Holland BV, which, in cooperation with DSM Biologics, was reported to produce 15 g/L (CocoMartin and Harmsen, 2008) and then later, 26 g/L of a monoclonal antibody (Jarvis, 2008).

Many antibodies were produced in mammalian cell culture at levels of 0.7–1.4 g/L. However, 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 (Zhang and Robinson, 2005). Animal-free, protein-free and even chemically-defined media with good support of production have been developed. The Pfizer organization reported monoclonal antibody titers of 2.5–3.0 g/L in non-optimized shake flask experiments (Yu, 2006).

Mammalian systems do have some drawbacks as follows. (i) Poor secretion. Production of secreted foreign proteins by mammalian cells in the 1990s amounted to 1 to 10 mg/L with specific productivities of 0.1 to 1 pg/cell/day (Wurm and Bernard, 1999). The process duration was 5 to 10 days. Although higher titers have been reached, acceptable levels were 10–20 mg/L. (ii) Mammalian processes are expensive. The selling prices (per gram) of recombinant proteins were $375 for human insulin, $23,000 for tPA, $35,000 for human growth hormone, $384,000 for GM-CSF, $450,000 for G-CSF, and $840,000 for EPO. All except human insulin were made in mammalian cell cultures (Bisbee, 1993). The manufacturing of mammalian cell biopharmaceuticals in a fully validated plant requires 2 to 4 million dollars per year in costs of materials especially for media, 15 to 20 million dollars per year in manufacturing costs (including overhead, material and labor) and 40 to 60 million dollars to construct a facility of 25,000 ft^2 and to validate it. Added on to this is a huge cost for getting FDA approval, including proof of consistent performance, production of a bioactive product, and lack of contamination by viruses and DNA. Clinical trials and product approval requires at least 4–5 years at a cost of 60 to 100 million dollars (Bisbee, 1993). (iii) Mammalian cell processes also have a potential for product contamination by viruses (Bisbee, 1993).

3.6. Transgenic animals

Transgenic animals are being used for production of recombinant proteins in milk, egg white, blood, urine, seminal plasma and silk worm cocoons. Thus far, milk and urine seem to be best. Foreign proteins can be produced in the mammary glands of transgenic animals (Brem et al., 1993). Transgenic animals such as goats, mice, cows, pigs, rabbit, and sheep are being developed as production systems; some aquatic animals are also being utilized. Transgenic mice produce tPA and sheep ß-lactoglobulin and transgenic sheep produce human Factor IX in their milk. Transgenic sheep have been developed which produce milk containing 35 g/L of human α-1-antitrypsin, a serum glycoprotein approved in the U.S. for emphysema (Wright et al., 1991). tPA has been made in milk of transgenic goats at a level of 3 g/L (Glanz, 1992). Recombinant human protein C (an anticoagulant) is produced in the milk of transgenic pigs at the rate of 1 g/L/h (Velander et al., 1992). Cows produce 30 L of milk per day containing protein at 35 g/L; thus the total protein produced per day is 1 kg. Even if a recombinant protein was only made at 2 g/L, the annual production per cow would be 10 kg.

The amounts of milk produced by animals (L/year) are 8000 per cow, 1000 per goat, 300 per sheep and 8 per rabbit (Rudolph, 1997). Production titers were 14 g/L of anti-thrombin III in goat milk, 35 g/L of α-1-antitrypsin in sheep milk, and 8 g/L of α-glucosidase in rabbit milk; all genes were from humans. Transgenic expression of foreign milk proteins has yielded titers as high as 23 g/L although the usual figure is about 1 g/L. Transgenic sheep produce 5 g/L of recombinant fibrinogen for use as a tissue sealant and 0.4 g/L recombinant activated protein C, an anticoagulant used to treat deep-vein thrombosis (Dutton, 1996). Human hemoglobin is produced in pigs at 40 g/L. Transgenic expression of foreign non-milk proteins is usually much less than that of milk proteins. However, an exception is that of human α-1-antitrypsin in sheep as mentioned above (Wright et al., 1991). In most cases, the protein is as active as the native protein. Titers of human growth hormone in milk of mice are 4 g/L and that of anti-thrombin III is 2 g/L. Production in milk is more cost-effective than that in mammalian cell culture. Dairy animals produce 1 to 14 g/L of heterologous protein in milk everyday for the 305 day lactation cycle each year. Transgenic goats produce tPA with a glycosylation pattern different from that produced in cell culture and with a longer half life than native tPA. Transgenic animal products have been tested in human clinical trials and no adverse reactions or safety concerns were reported (McKown and Teutonico, 1999).

Human growth hormone has been produced in the urine of transgenic mice (Kerr et al., 1998) but only at 0.1–0.5 mg/L. One advantage of using the bladder as a bioreactor instead of the mammary gland is that animals can urinate earlier than they can lactate. Lactation requires 12 months for pigs, 14 months for sheep and goats, and 26 months for cattle, and lasts for 2 months for pigs, 6 months for sheep and goats, and 10 months for cattle. The periods between lactation cycles are 2–6 months. Under hormone treatment, a cow produces 10,000 L of milk per year compared to 6000 L of urine.

One of the negative points in production of proteins by transgenic animals is 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 (Chew, 1993). The cost of upkeep of cows under Good Agricultural Practices is $10,000 per cow per year.

The production of drugs in transgenic animals has been stalled by the demise of PPL Therapeutics of Scotland which, with the Roslin Institute, cloned Dolly, the sheep (Thayer, 2003). Their attempt to produce a lung drug in transgenic sheep for Bayer AG was stopped and the company was put up for sale.

Seikagaku Corporation Exhibit 2110 - Page 7 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

Scientists are trying to exploit 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. These include (i) stable and precisely targeted integration into the genome by homologous recombination, (ii) a choice of integration into several defined sites, allowing expression of multi-subunit complexes, and (iii) easy maintenance of cells in a semi-defined medium and growth to high densities ($>2\times10^7$ ml^{-1}).

3.7. Transgenic plants

For recombinant protein production, use of plants, as compared to that of 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 fact, plant expression systems are believed to be even better than microbes in terms of cost, protein complexity, storage and distribution. The use of plants offers a number of advantages over other expression systems (Table 6). The low risk of contamination with animal pathogens includes viruses since no plant viruses have been found to be pathogenic to humans. Another advantage is that growth on an agricultural scale requires only water, minerals and sunlight, unlike mammalian cell cultivation which is an extremely delicate process, very expensive, requiring bioreactors that cost several hundred million dollars when production is scaled up to commercial levels.

Some added advantages of plant systems are glycosylation and targeting, compartmentalization and natural storage stability in certain organs. Simple proteins like interferons, and serum albumin were successfully expressed in plants between 1986 and 1990. However, proteins are often complex three-dimensional structures requiring the proper assembly of two or more subunits. Researchers demonstrated in 1989 and 1990 that plants were capable of expressing such proteins and assembling them in their active form when functional antibodies were successfully expressed in transgenic plants. Bacteria do not have this capacity. Transgenic plants have been used to produce valuable products such as β-D-glucuronidase (GUS), avidin, laccase and trypsin (Hood, 2002).

Transgenic plants can be produced in two ways. One way is to insert the desired gene into a virus that is normally found in plants, such as the tobacco mosaic virus in the tobacco plant. The other 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 (Fitzgerald, 2003).

Products with titers as high as 0.02–0.2% of dry cell weight have been achieved. Recombinant proteins have been produced in transgenic plants at levels as high as 14% of total tobacco soluble protein (phytase from *A. niger*) and 1% of canola seed weight (hirudin from *H. medicinalis*) (Kusnadi et al., 1997). Oilseed rape plants can produce enkephalin and a neuropeptide (Sterling, 1989). The peptide gene was inserted into the gene encoding the native storage protein by scientists at Plant Genetic Systems (Ghent, Belgium). By 1997, two products, avidin and GUS were ready for the market. GUS from *E. coli* was produced in corn at 0.7% of soluble seed protein. Active hepatitis B vaccine (hepatitis B surface antigen) was produced in transgenic tobacco plants. Despite these successes, commercial production of drugs in transgenic plants was slowed down by the closing down of the PPL Therapeutics (Thayer, 2003), as well as the exit of Monsanto corporation from this effort.

Table 6
Advantages of transgenic plants as protein expression systems

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

4. Conclusions

Microbes have been used to produce a myriad of primary and secondary products to benefit mankind for many decades. With the advent of genetic engineering, recombinant proteins entered the market, which radically changed the scenario of the pharmaceutical industry (Demain, 2004). Through the use of recombinant DNA, important genes, especially mammalian genes, could be amplified and cloned in foreign organisms. This provided a different approach to complex biological problem-solving. Many of the resultant biopharmaceuticals are produced using technologically advanced microbial and mammalian cell biosystems. These cell-based, protein manufacturing technologies offer many advantages, producing recombinant pharmaceutically important proteins which are safe and available in abundant supply.

Generally, proteins that are larger than 100 kD are expressed in a eukaryotic system while those smaller than 30 kD are expressed in a prokaryotic system. 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 *E. coli*. However, this bacterium cannot express very large proteins. Also, for S–S rich proteins, and proteins that require post-translational modifications, *E. coli* is not the system of choice, as it cannot carry out glycosylation and remove the S–S sequences. Sometimes, eukaryotic proteins can be toxic to bacteria. Yeasts are eukaryotes, have the advantage of growing to high cell densities and are thus suitable for making isotopically-labeled proteins for NMR. The two most utilized yeasts are *S. cerevisiae* and *P. pastoris*. Yeasts can produce high yields of proteins at low cost, proteins larger than 50 kD can be produced, signal sequences can be removed, and glycosylation can be carried out. Yeasts produce chaperonins to assist folding of certain proteins and can handle S–S rich proteins. The baculoviral system is a higher eukaryotic system than yeast and can carry out more complex post-translational modifications of proteins. It provides a better chance to obtain soluble protein when it is of mammalian origin, can express proteins larger than 50 kD and S–S rich proteins, can carry out glycosylation, removes signal sequences, has chaperonins for folding of proteins, is cheap and can produce high yields of proteins. The baculoviral system is however slow and time consuming and not as simple as yeasts. The most popular type of system for producing recombinant mammalian glycosylated proteins is that of mammalian cells. They can generate proteins larger than 50 kD, carry out authentic signal sequence removal, glycosylate and also have chaperonins. Some of the proteins expressed in mammalian systems are Factor VII, factor IX, γ-interferon, interleukin 2, human growth hormone, and tPA. However, selection of cell lines usually takes weeks and the cell culture is sustainable for only a limited time. Overall, 39% of recombinant proteins are made by *E. coli*, 35% by CHO cells, 15% by yeasts, 10% by other mammalian systems and 1% by other bacteria and other systems (Rader, 2008).

Genetically modified animals such as the cow, sheep, goat, and rabbit secrete recombinant proteins in their milk, blood or urine. Many useful biopharmaceuticals can be produced by transgenic animals such as vaccines, antibodies, and other biotherapeutics. Similarly, transgenic plants such as *Arabidopsis thaliana* and others can generate many recombinant proteins, e.g., vaccines, bioplastics, and biotherapeutics. Commercial development of transgenic animals and transgenic plants has been slow however, compared to the above systems.

Molecular biology has been the major driving force in biopharmaceutical research and the production of high levels of proteins. The biopharmaceutical industry is multifaceted, dealing with ribozymes, antisense molecules, monoclonal antibodies, genomics, proteomics,

Seikagaku Corporation Exhibit 2110 - Page 8 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

metabolomics, pharmacogenomics, combinatorial chemistry and biosynthesis, high throughput screening, bioinformatics, nanobiotechnology, gene therapy, tissue engineering and many other matters. Major impacts in the world have been made by genetic engineering which have changed the faces of pharmacology, medicine and industry. The next 50 years should feature major advances in (i) solving chronic and complex acute diseases by the production of new drugs and vaccines, (2) use of recombinant microbes to markedly decrease the effects of environmental pollution, and (iii) development of recombinant bioprocesses to solve the energy problem that the world faces today.

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Seikagaku Corporation Exhibit 2110 - Page 9 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

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Seikagaku Corporation Exhibit 2110 - Page 10 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023